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GLA1 - Schlemm’s canal and beyond, novel targets for glaucoma

Revisiting the Role of Fibronectin for Trabecular Aquous Humor Outflow

Tamm, E.R., Eggentonser, S., Fuchofoer, R., Herrnberger, L.
University of Regensburg, Institute of Human Anatomy, Regensburg, Germany

Between the cells of Schlemm’s canal and those of the juxtaglomerular tissue (JCT), there is a lose extracellular matrix (ECM) containing fibrillar elements. A major part of this ECM is the glycoprotein fibronectin (FN) that is deposited in the developing trabecular meshwork (TM) already in fetal life. FN forms integrin-based contacts with adjacent cells and maintains integrin-based signaling. In addition, the FN matrix forms a scaffold that is essential for the deposition and maintenance of other ECM components including collagens. Since the fibrillar ECM of the JCT thickens in primary open angle glaucoma, the argument has been put forward that it is involved in maintenance of JCT outflow resistance. To test this hypothesis and to learn about the role of the fibrillar ECM in aqueous humor outflow pathways, we generated CAGG-Cre-ER(T/Flh/) mice with a tamoxifen-induced conditional deletion of FN at birth (PO). In tamoxifen-treated CAGG-Cre-ER(T/Flh/) mice, the expression of FN mRNA was reduced to 16% of the levels seen in control eyes (p < 0.01). In addition, there was a marked and significant decrease in the expression of mRNA for collagen-I (p < 0.01), collagen IV (p < 0.05) and elastin (p < 0.005). The immunoreactivity of FN in the JCT was markedly decreased as were the amounts of fibrillar ECM components seen by transmission electron microscopy. Otherwise, there were no obvious differences in ECM structure between FN-deleted mice and control littermates. At the age of 5–6 weeks, FN-deleted mice had an IOP of 15.4 ± 0.6 mmHg (mean ± SEM), as compared to 15.8 ± 0.4 mmHg in control littermates, a difference that was not statistically significant (n = 56). Next, we crossed CAGG-Cre-ER(T/Flh/) mice with beta1A-CGI mice overexpressing connective tissue growth factor (CTGF) in the eye to cause a dramatic increase in FN and formed the JCT outflow pathways and optic nerve axonal dam-

Neuronal Control of Intracranial Pressure through Innervation of the Schlemm’s Canal

Kuhnt, K.1, Clark, G.1, Kokini, A.2, de Vries, M.1,1, John, S.1,2

1The Jackson Laboratory, Bar Harbor, United States, 2Howard Hughes Medical Institute, Bar Harbor, United States

The nervous system is important in controlling intracranial pressure (ICP) regulation, however the precise control mechanisms are unclear. IOP results from a resistance to aqueous humor drainage (AHD) from the eye through the trabecular meshwork (TM) and Schlemm’s canal (SC). The SC inner wall is the last barrier to AHD drainage, and a critical determinant of IOP. Pressure in the episcleral veins draining the SC is also key to IOP control. We hypothesized that neurons innervating the SC inner wall, TM regions close to the SC and the limbal veins, sense and respond to pressure changes to maintain normal IOP. Using wholemount anterior segments of eyes, modern techniques and fluorescent reporter mice we have mapped neurons innervating the AHD drainage structures and limbal veins. This method allows us to study the innervation of the en-
tire mouse limbs in three dimensions without resorting to use of multiple tissue sections. This makes the registration and quantification of the neuronal termini in the context of the limbal vessels and the AQH drainage structures more accurate. We have identified nerve terminals within the SC inner wall, the SC associated veins and TM that are sympathetic, parasympathetic, and nitric in nature. These terminals are bare nerve endings with varicosities and thus show the characteristics of junctional and non synaptic neuronal transmission. These are also terminal with a club-like shape which are likely to be sensory in nature. These terminals are within micron scale distances from the SC inner wall and TM. The density of the various classes of nerve terminal in SC/TM region all around the limbus varies. Together, these nerve termini could sense and respond to IOP stimuli. In addition, we found that the innervation of the developing SC initiates around the time of SC lumen formation and inner wall differentiation. This suggests innervation likely correlates with the onset of outflow and its regulation. In summary, this study provides critical new information regarding the neural innervation of the AQH drainage structures and will be foundational in determining the mechanistic basis of neuronal control of IOP.

**Structure and Function of the Porcine Distal Outflow Tract**

Waxman, S., Chao, W., Dang, Y., Hong, Y., Shah, P., Esfandiari, H., Lathrop, K., Watkins, S., Watson, A., Loewen, R., Loewen, N.

*University of Pittsburgh, Pittsburgh, United States*

**Purpose:** To correlate structure, outflow function and outflow tract vessel diameter changes induced by nitric oxide (NO).

**Methods:** In a porcine anterior segment perfusion model, the effects of a nitric oxide donor (100 µM DETA-N0) on outflow function were compared to controls (n=8 per group) with trabecular meshwork (TM) and after circumferential ab interno trabeculotomy (AIT). Outflow structures were assessed with spectral domain optical coherence tomography (SD-OCT) before and after NO, or an NO synthase inhibitor (100 µM L-NAME) and the vasodilator, A.C.1, Kong, G.X.Y., Widdowson, P.

*University of Cambridge, John van Geest Centre for Brain Repair, Cambridge, United Kingdom, Babraham Research Campus, Querletu Ltd, Cambridge, United Kingdom*

Previous studies have demonstrated that intravitreal delivery of brain-derived neurotrophic factor (BDNF) by injection of recombinant protein or gene therapy can alleviate retinal ganglion cell (RGC) loss after optic nerve injury. BDNF gene therapy can improve RGC survival in experimental models of glaucoma, the leading cause of irreversible blindness worldwide. However, the therapeutic efficacy of BDNF supplementation alone is time limited due at least in part to BDNF receptor downregulation. TrkB downregulation has been reported in many neurological diseases including glaucoma, potentially limiting the effect of sustained or repeated BDNF delivery.

We characterized a novel gene therapy (AV2 TrkB-BA-MBD-NF) that not only increases BDNF production but also improves long term neuroprotective signaling by increasing expression of the BDNF receptor (TrkB) within the inner retina. This approach led to significant and sustained elevation of survival signaling pathways ERK and AKT within RGCs over 6 months and avoided downregulation. We validated the neuroprotective efficacy of AV2 TrkB-BA-MBDNF in a mouse model of optic nerve injury, where it outperformed conventional AV2 BDNF therapy, before showing powerful neuroprotection of RGCs and axons in a rat model of chronic intraocular pressure (IOP) elevation. We also showed that there were no adverse effects of using the vector on retinal structure or function as assessed by histology and electroretinography in young or aged animals. Further studies are underway to explore the potential of this vector as a candidate for progression into clinical studies to protect RGCs in patients with glaucoma and progressive visual loss despite conventional IOL-lowering treatment.

**Neuroregeneration in Glaucoma**

Crowston, J.

*University of Melbourne, Melbourne, Australia*

Improvement in visual function can be observed in a subgroup of individuals following IOP lowering, suggesting that injured non-functional RGCs have the capacity for recovery. We have developed an acute IOP injury to investigate functional recovery, how this may be modified and the characteristics of sick RGCs.

**Gene Therapy for Neuroprotection in Glaucoma**

Martin, K.R., Osborne, A., Tasneem, K., Lalana, S., Barber, A.C., Kong, G.X.Y., Widdowson, P.

**University of Cambridge, John van Geest Centre for Brain Repair, Cambridge, United Kingdom, Babraham Research Campus, Querletu Ltd, Cambridge, United Kingdom**

**AKT-dependent and Independent Pathways Mediate Pten Deletion-induced Cns Axon Regeneration**

Huang, H., Wang, Q., Sun, Y., Hu, Y.

*Stanford University, Palo Alto, United States*

Axon injury is a frequent consequence of trauma and axonopathy is a common early feature of CNS degenerative diseases causing lifelong neurological defects. Injuries of CNS axons result in loss of vital functions because CNS axons fail to regenerate in adult mammals. Growth failure is due to the diminished intrinsic regenerative capacity of mature neurons and the inhibitory environment of the adult CNS. Neutralizing extracellular inhibitor molecules genetically or pharmacologically yields only limited regeneration and functional recovery, highlighting the critical importance of neuron-intrinsic factors. Using mouse optic nerve crush as a CNS axon injury model, Pten was previously identified as a prominent intrinsic inhibitor of CNS axon regeneration. Pten acts as a brake for phosphatidylinositol-3 kinase (PI3K), deletion of which is thought to activate the PI3K-AKT-mTOR complex 1 (mTORC1) pathway. Here we have conducted an extensive molecular dissection of the cross-regulating mechanisms in axon regeneration that involve the downstream effectors of PI3K, AKT, mTORC1 and GSK3β. By exploiting the anatomical and technical advantages of retinal ganglion cells (RGCs) and the crushed optic nerve as an in vivo CNS axon injury model, we show that blocking either all three isoforms of AKT together or their downstream effectors, mTORC1 and GSK3β, significantly reduces axon regeneration in PTEN KO mice, suggesting the necessity of AKT-mTORC1/GSK3β in axon regeneration. However, that mTORC1/S6K1-mediated feedback inhibition prevents potent AKT activation, and combining Pten deletion with AKT overexpression or GSK3β inhibition achieves significantly more potent axon regeneration, consistent with partial activation of AKT in Pten KO mice. This result also suggests a key permissive signal from an unidentified AKT-independent pathway downstream of Pten deletion is required for stimulating the neuron-intrinsic growth machinery. Further studies into elucidating the AKT-independent pathway, this complex neuron-intrinsic balancing mechanism involving neces-

**Glaucoma**

**GLA2 - Neuroprotection and neuroregeneration for glaucoma treatment**

**Treatment with P38 Inhibitor Birb 796 is Neuroprotective in Models of Glaucoma**

Lambert, W., Yao, V., Ghose, P., Carlson, B., Calkins, D.

Vanderbilt Medical Center, Vanderbilt Eye Institute, Nashville, United States

Glaucoma is a group of optic neuropathies associated with aging and sensitivity to intracocular pressure (IOP). Early progression in glaucoma involves dysfunction of retinal ganglion cell (RGC) axons, which comprise the optic nerve. Anterograde transport deficits along RGC axons to central visual structures preclude outright degeneration. Preserving RGC axonal structure and function would therefore be beneficial and could abate subsequent progression. We assessed the neuroprotective effect of the p38 inhibitor, Birb 796 (BIRB-796) in an acute glaucoma model (microbead occlusion) in rats and squirrel monkeys (SMs). Animals received Birb 796 (3% drug solution) via daily topical corneal drops for 4 weeks (rats) or 29 weeks (SMs). Microbead occlusion elevated IOP 33% in rats and 42% in SMs; Birb 796 treatment did not affect IOP in any cohort. In vehicle-treated rats, elevated IOP reduced anterograde axonal transport to the superior colliculus, the most distal site in the optic projection in rodents, by 47% (p < 0.0001, n = 7); Birb 796 treatment prevented this reduction (p = 0.06, n = 8). The microbead-induced saline ratio of total axons in optic nerves from the vehicle group was reduced ~13% compared to a ratio of unity (p = 0.004, n = 8). Birb treatment resulted in axon counts comparable for the two eyes (p = 0.91), yielding a ratio that is significantly different from the vehicle group (p = 0.003). In SMs, microbead occlusion appeared to reduce anterograde transport to the superior colliculus and lateral geniculate nucleus in all SM groups (naive, vehicle and Birb). Birb 796 treatment did not alter phosphorylated p38 MAPK levels in the retina, but did reduce levels of downstream p38 MAPK targets phosphorylated Hsp27 (p = 0.04) and phosphorylated MAPKAP2 (p = 0.04, n = 3 - 4 eyes per group). Treatment with Birb 796 also reduced levels of Alzheimer’s precursor protein (APP; p < 0.02) in the optic nerve head while increasing levels of brain derived neurotrophic factor (BDNF; p < 0.03). Our data suggest that the p38 MAPK pathway plays a role in glaucomatous neurodegeneration. Birb 796 treatment attenuated axonal transport deficits and axon loss in rats, reduced activation of p38 MAPK target proteins in SM retinas, and modified expression of proteins believed involved in glaucoma pathology in the SM optic projection.
Omideneag Isopropyl (DE-117): The Next Generation Drug to Treat Glaucoma

Sharif, N., DE-117 Study & Project Team

Santen Incorporated, Global Ophthalmology R & D, Emeryville, United States

Omideneag Isopropyl (OMDI; DE-117) is a novel pro-drug which hydrolyzes to release its active free acid form (omideneag, OMD) into the aqueous humor (AQH) upon topical ocular (t.o.) instillation. OMD is a specific, non-prostanoid, EP2 receptor (EP2R) agonist that binds with a high affinity (Kᵢ = 3.6 nM) and selectively (1,1889-fold) to the EP2R to increase intracellular cAMP (EC₅₀ = 8.3 nM).

High levels of OMD (10 nM/ml) were observed in AQH of rabbits after t.o. dosing with OMDI (0.0001-0.01%), potently and efficaciously lowered and controlled intraocular pressure (IOP) in ocular normotensive rabbits (OMDI at 0.001-0.03%), dogs (OMDI at 0.0006%) and monkeys (OMDI at 0.0001% to 0.01%), and also in ocular hypertensive (OHT) monkeys (OMDI at 0.01%). No tachyphylaxis of IOP-lowering was observed after repeated t.o. dosing with OMDI. Additional IOP-lowering to OMDI was observed with timolol, brimonidine and brinzolamide. OMDI lowered IOP by increasing uveoscleral (UVS) and conventional / trabecular meshwork (TM) outflow.

In the present study, we investigated the pharmacological effects of H₂S-releasing compounds in mammalian ocular tissues associated with the regulation of aqueous humor dynamics and intraocular pressure (IOP) under in vitro, ex vivo and in vivo conditions. In isolated porcine and bovine iris-ciliary bodies, sodium hydrosulfide (NaH₂S), sodium sulfide (Na₂S), L-cysteine (0.1-10 µM) and L-cystine (0.1-10 µM) potentiated IOP lowering, synergistically and/or additively. In isolated porcine iris, a response was blocked by AOA and glibenclamide. In normotensive albino rabbits, GYY4137, AP67 and AP72 relaxed pre-contracted smooth muscles, lower IOP in a normotensive animal and increase aqueous humor outflow facility. Furthermore, these compounds can attenuate the release of excitatory amino acid neurotransmitters in the retina. Based on their demonstrated pharmacological actions in the eye, we conclude that H₂S-releasing compounds have a great potential to serve as a new class of agents for the treatment of glaucoma.

The Discovery and SAR of Rhopressa, the First FDA-Approved ROCK Inhibitor for the Treatment of Glaucoma, Containing the NCE Netarsudil

DeLong, M.

Aerie Pharmaceuticals and Duke University, Chemistry, Durham, United States

Kinase inhibitors, particularly inhibitors of rho kinases (ROCK inhibitors) have been known to effectively and markedly lower intraocular pressure in animal models of disease, but none had shown the proper biologic and physicochemical properties to be effective once-a-day treatments for glaucoma. Rhopressa, containing the New Chemical Entity (NCE) from our laboratories netarsudil, possessed the requisite properties in both animal models and in human testing and was approved for sale in the USA by the FDA in December of 2017. ROCK inhibitors are thought to act via increasing aqueous humor outflow through the trabecular outflow pathway, rather than the mechanisms utilized for traditional glaucoma drugs. The precursor of netarsudil was found by screening a library of GRK kinases, and developing an SAR over multiple generations on compounds, including AR-12286.

Hydrogen Sulfide-releasing Compounds: Potential Role in Glaucoma Pharmacotherapy

Ohio, S.¹, Niie-Mbye, Y.F.², Opere, C.³, Robinson, J.¹, Mitchell-Bush, L.¹, Whitman, M.¹

¹Texas Southern University, Pharmaceutical Sciences, Houston, United States, ²Creighton University, Pharmacy Sciences, Omaha, United States, ³University of Exeter, Institute of Medical and Clinical Science, Exeter, United Kingdom

Hydrogen sulphide (H₂S) is a gas that has been reported to possess pharmacological actions in several mammalian tissues and organs. In the present study, we investigated the pharmacological effects of H₂S-releasing compounds in mammalian ocular tissues associated with the regulation of aqueous humor dynamics and IOP.

In isolated porcine and bovine iris-ciliary bodies, sodium hydrosulfide (NaH₂S), sodium sulfide (Na₂S), L-cysteine (0.1-10 µM) and L-cystine (0.1-10 µM) caused a concentration-dependent increase in IOP-lowering, which was blocked by AOA and glibenclamide. In normotensive albino rabbits, GYY4137, AP67 and AP72 relaxed pre-contracted smooth muscles, lower IOP in a normotensive animal and increased ocular blood flow. Furthermore, these compounds can attenuate the release of excitatory amino acid neurotransmitters in the retina. Based on their demonstrated pharmacological actions in the eye, we conclude that H₂S-releasing compounds have a great potential to serve as a new class of agents for the treatment of glaucoma.
Mayhem in the IPL? Retinal ganglion cell degeneration in experimental glaucoma and mitochondrial neuropathy

Morgan, J.
Cardiff University, Cardiff, United Kingdom

The loss of retinal connectivity is a hallmark pathological event in many retinal diseases. In glaucoma and some optic neuropathies, it manifests as the pruning of retinal ganglion cell dendrites with a loss of synaptic connectivity within the inner plexiform layer. These changes have been observed in all models of experimental glaucoma but without a consistent specificity for any single retinal ganglion cell. Critically, dendritic degeneration and synaptic pruning occur prior to the onset of retinal ganglion cell loss and therefore present a therapeutic opportunity for the reversal of retinal ganglion cell damage and the recovery of retinal function. Preventing such neuronal loss is one of the major challenges in the treatment of glaucoma.

Mayhem in the IPL - Retinal ganglion cell degeneration in experimental glaucoma and mitochondrial neuropathy

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Biomarkers or Endpoints in Glaucoma

Cordeiro, M. P.1,2,3
1Imperial College London, London, United Kingdom, 2UCL Institute of Ophthalmology, London, United Kingdom, 3Western Eye Hospital, ICORD Clinical Trials Unit, London, United Kingdom

Clinical trials in glaucoma have historically used IOP as an endpoint - based on the fact that all treatments currently target IOP, the major modifiable risk factor. However, the recognition that IOP control itself may not be enough, and the emergence of neuroprotection as a potential therapeutic strategy, has led to a need for new measures of trial success. Several potential endpoints exist including autoimmune and genetic biomarkers, brain and retina imaging, clinical signatures of RGC disease and structural and functional endpoints. These will be covered in this talk including DARC - detection of apoptosing cells.

Glaucoma

The Waist of the Nerve Fiber Layer at the Optic Nerve Head (PIMD-2Pi), a Potential Morphometric Estimate of Glaucoma Progress

Söderberg, P.1, Sandberg-Melin, C.1,2
1Uppsala University, Gullstrands Lab, Ophthalmology, Dept. of Neurosciences, Uppsala, Sweden, 2Göteborg Regional Hospital, Ophthalmology, Göteborg, Sweden

Purpose: To estimate different sources of variability in measurements of pigment epithelium central limit-Inner Limit of the retina-Minimal Distance, averaged over 2-Pi radians (PIMD-2Pi) and to elucidate their impact on measurement strategy and precision of clinical measurements.

Methods: In total, 33 early to moderate stage glaucoma cases were included. 3D-volumes representing the optic nerve head (ONH) captured with Topcon OCT-2000 were transferred to Matlab for measurement of the waist of the nerve fiber layer in the ONH. One eye of each case was examined at two occasions (2 different days) with-in one month. At each occasion, 3D-volumes were recorded in triplicates. Each volume was segmented semi-automatically 3 times. Variance components in estimates of PIMD-2Pi were estimated with ANOVA assuming a hierarchical model. Consequences of measurement design were analyzed assuming a linear first order loss rate of 5% of baseline PIMD-2Pi per year and alpha = 0.05, power = 0.8.

Results: Clmy(0.05) for PIMD-2Pi was estimated to 220 780 my-m (df = 32). The variance for subjects, occasions, volumes and segmentations, respectively, was estimated to 1300, 20, 30, 40 my-m2.

Considering, the same subject followed over time, with 2 visits per year, a 0.05 change from baseline can be detected in 12 months. Comparison of averages of within subject change over time between independent groups allows, detection of a difference of 0.05 change from baseline with approximately 10 subjects per group. Compari-son of a measurement at a single visit to a normal database and cross-sectional comparison of PIMD-2Pi between independent groups are inefficient due to a large variation of PIMD-2Pi among subjects.

Conclusions: Comparison of measurements of PIMD-2Pi over time, within subject allows detection of average loss of ganglion cell axons earlier than with currently clinically applied methods for quantitative measurement of glaucoma.

GLAG - Metabolic dysfunction/ bioenergetics in glaucoma

Targeting Neuronal Mitochondria for Neuroprotection in Glaucoma

Williams, P

Karolinska Institutet, Department of Clinical Neuroscience, Section of Ophthalmology and Vision, Stockholm, Sweden

Background: Age, genetics, and high intraocular pressure (IOP) are major risk factors for glaucoma. Currently only treatments to allevi-ate high IOP have been taken to the clinic. These therapies do not treat the neurodegenerative component of glaucoma and neuropro-ective strategies are of great therapeutic need. Emerging research suggests that a systemic vulnerability to mitochondrial abnormali-ties exists in glaucoma patients. Such systemic susceptibility is ex-pected to increase glaucoma susceptibility with age. However, the role of mitochondrial health in glaucoma is yet to be fully elucidated.

Methods: RNA-seq was used to elucidate the very early mechanisms of retinal ganglion cell (RGC) degeneration in the DBA/2J mouse model of glaucoma. We assessed mito-chondrial and metabolic function in RGCs from DBA/2J mice. Using these data we established pharmacological and gene therapy protocols to treat glaucoma in DBA/2J mice, and as-sessed end-stage histological outcomes in addition to visu-al function, metabolic outcomes, and transcriptomic changes.

Results: Mitochondrial health declines with age and is exacerbated by periods of high IOP. This is coupled with a marked age-related reduction in retinal NAD+ levels. Restoring NAD+ (via nicotinamide treatment, Nmnat1 gene therapy, and/or the addition of WLD671 enzyme in NAD+ production) protects RGCs long-term and this pro-tection is increased by giving a low dose of nicotinamide. This is the first instance of a successful gene therapy in a complex age-related disease. Thus targeting neuronal metabolic decline and neuronal mitochondria may offer safe, neuroprotective treatments for glau-coma and other age-related neurodegenerations.

Mitochondrial Efficiency: The Holy Grail of Glaucoma Resistance?

Lascaratos, G.

King’s College Hospital, London, United Kingdom

Glaucomatous optic neuropathy, an important neurodegenerative condition and the commonest optic neuropathy in humans, is the leading cause of irreversible blindness worldwide. Its prevalence and incidence increase exponentially with ageing and raised intraocular pressure (IOP). Using glaucomatous optic neuropathy as an exemplar for neurodegeneration, this study investigates putative factors impacting resistance to neurodegeneration. Systemic mitochondrial dysfunction, oxidative stress and vascular parameters were compared from isolated lymphocytes, whole blood and urine sam-ples between 30 patients who have not developed the neuropathy despite being exposed for many years to very high IOP (‘resistant’), 30 fast deteriorating glaucoma patients despite having low IOP (‘susceptible’), and 30 age-similar controls. We found that ‘resis-tant’ individuals showed significantly higher rates of ADP phos-phorylation by mitochondrial respiratory complexes I, II, IV, in complex I as compared to controls. ADP phosphorylation rates were similar in controls and glaucoma patients. While it has been known for some years that mitochondrial dysfunction is implicated in neurodegeneration, this study provides a fresh perspective to the field of neurodegeneration by providing, for the first time, evidence that systemic mitochondrial efficiency above normal healthy levels is associated with an enhanced ability to withstand optic nerve injury. These results demonstrate the importance of cellular bioenerget-ics in glaucomatous disease progression, with potential relevance for other neurodegenerative disorders, and raise the possibility for new therapeutic targets in the field of neurodegeneration.
Cupping of the optic nerve is associated with remodelling of the extracellular matrix (ECM) and fibrosis in the lamina cribrosa (LC). Classically, the features of tissue fibrosis/scarring include myofibroblasts, ECM and fibrosis in the lamina cribrosa (LC). Cupping of the optic nerve is associated with remodelling of the LC. The lamina cribrosa is centrally involved in the pathogenesis of glaucoma.

In normal circumstances, the lamina cribrosa is a highly vascularized, avascular matrix that provides support for the optic nerve head. In the presence of disease, the lamina cribrosa becomes thinned, fibrosed, and remodeled. This remodelling is a key feature of glaucomatous optic neuropathy. Changes in the lamina cribrosa have been implicated in the pathogenesis of glaucoma.

The glaucomatous microenvironment is hypoxic, demonstrated by increased hypoxia inducible factor in the optic nerve head. LOXL1 ‘silencing’ via DNA methylation is observed in systemic diseases such as Cysts Laxa and bladder cancer. We believe methylation may regulate LOXL1 expression in pseudoxfoliation glaucoma. Axonal Metabolic Rescue in Glaucoma

Inman, D.M., Harun-Or-Rashid, M.

Northeast Ohio Medical University, Pharmaceutical Sciences, Rootstown, United States

Metabolic decline occurs with aging and contributes to neurodegeneration, including in the context of glaucoma. We documented significant activation of adenosine monophosphate-activated protein kinase (AMPK), indicative of insufficient energy, in pre-glaucomatous mouse retina and optic nerve. AMPK activation was accompanied by significant decline of glucose and monocarboxylate transporters, suggesting a mismatch between energy availability and utilization. Placing pre-glaucomatous mice on a ketogenic diet reversed the monocarboxylate transporter decline, an increase in mitochondrial number and oxidative phosphorylation, and utilization. Placing pre-glaucomatous mice on a ketogenic diet may represent a therapeutic option for glaucoma cupping.

The Hypoxic Microenvironment: How it Influences LOXL1 Expression in Pseudoxfoliation Glaucoma

Greene, A.1, Elvers, S.1, McDonnell, F.1, Irnaten, M.1, Dervan, E.1, O’Brien, C.1, Wallace, D.1

1Clinical Research Centre, School of Medicine, Dublin, Ireland, 2Duke University Eye Center, Ophthalmology, Durham, NC, United States, 3Clinical Research Centre, Dublin, Ireland, Mater Misericordiae University Hospital, Ophthalmology, Dublin, Ireland

Pseudoxfoliation glaucoma (PXG) is the most common cause of secondary open angle glaucoma. Its aetiology remains largely unknown. Lysyl oxidase-like 1 (LOXL1) is an enzyme encoded by the LOXL1 gene which catalyses collagen and elastin crosslinking. GWAS studies have identified 2 Single Nucleotide Polymorphisms (SNPs) in LOXL1, which increase the likelihood of developing pseudoxfoliation glaucoma. These SNPs are present in 50-80% of the normal population, suggesting environmental and epigenetic factors may be involved in disease development. The glaucomatous microenvironment is hypoxic, demonstrated by increased hypoxia inducible factor in the optic nerve head. LOXL1 ‘silencing’ via DNA methylation is observed in systemic diseases such as Cysts Laxa and bladder cancer. We believe methylation may regulate LOXL1 expression in pseudoxfoliation glaucoma, and that the hypoxic glaucomatous environment initiates this process. In our lab we have previously demonstrated increased global DNA methylation in glaucomatous lamina cribrosa cells. The aim of this study was to investigate LOXL1 levels and global methylation in PXG vs. cataract (CAT) patients as controls. We wished to investigate if culture of CAT Human Tenon Fibroblasts (HTFs) under hypoxic conditions (1% O2), which mimics the hypoxia of the lamina cribrosa, affected LOXL1 expression in the HTFs. Treatment of cataract controls with hypoxia causes changes in global DNA methylation in glaucomatous lamina cribrosa cells. The new insights gained will hopefully help guide candidate biomarker development.

The Hypoxia Microenvironment: How it Influences LOXL1 Expression in Pseudoxfoliation Glaucoma

Greene, A.1, Elvers, S.1, McDonnell, F.1, Irnaten, M.1, Dervan, E.1, O’Brien, C.1, Wallace, D.1

1Clinical Research Centre, School of Medicine, Dublin, Ireland, 2Duke University Eye Center, Ophthalmology, Durham, NC, United States, 3Clinical Research Centre, Dublin, Ireland, Mater Misericordiae University Hospital, Ophthalmology, Dublin, Ireland

The new insights gained will hopefully help guide candidate biomarker development. We documented significant activation of adenosine monophosphate-activated protein kinase (AMPK), indicative of insufficient energy, in pre-glaucomatous mouse retina and optic nerve. AMPK activation was accompanied by significant decline of glucose and monocarboxylate transporters, suggesting a mismatch between energy availability and utilization. Placing pre-glaucomatous mice on a ketogenic diet reversed the monocarboxylate transporter decline, an increase in mitochondrial number and oxidative phosphorylation, and utilization. Placing pre-glaucomatous mice on a ketogenic diet may represent a therapeutic option for glaucoma cupping.

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Glaucoma

Genomic locus modulating corneal thickness in the mouse identifies POU6F2, a potential risk of developing glaucoma

Geiser, E. 1, King, R. 1, Streubing, F.L. 1, Li, Y. 1, Wang, J. 1, Baille, J. C. 1, Wiggs, J. L. 1

1Emory, Atlanta, United States, 2Case Western Reserve University, Population and Quantitative Health Sciences, Cleveland, United States, 3Howard Medical School, Ophthalmology, Boston, United States

Central corneal thickness (CCT) is one of the most heritable ocular traits and it also plays a role in primary open angle glaucoma (POAG). The present study uses the BXD Recombinant Inbred strain to identify novel quantitative trait loci (QTLs) modulating CCT in the mouse with the potential of identifying a molecular link between CCT and the risk of developing POAG. The BXD RI strain set was used to define mammalian genomic loci modulating CCT, with a total of 818 corneas measured from 61 BXD strains (between 60-100 days of age). The mice were anesthetized and the eyes were positioned in front of the lens of the Micron IV Image-Guided OCT system or the Bioptron OCT system. CCT data for each strain was averaged and used to identify quantitative trait loci (QTLs) modulating this phenotype. The candidate genes and genomic loci identified in the mouse were then directly compared with the summary data from a human primary open-angle glaucoma genome wide association study (NEIGHBORHOOD) to determine if any genomic elements modulating mouse CCT are also risk factors for POAG. This analysis revealed one significant QTL on Chr 13 and a suggestive QTL on Chr 7. The significant locus on Chr 13 (13 to 19 Mb) was examined further to define candidate genes modulating this eye phenotype. For the Chr 13 QTL in the mouse, only one gene in the region (Pou6f2) contained nonsynonymous SNPs. The strongest signals on Chr 13 (13 to 19 Mb) were distributed over 2 chromosomes in the human: Chr 1 and Chr 7. These genomics loci were examined in the NEIGHBORHOOD for human glaucoma to determine if they are potential risk factors for human glaucoma identified using meta-data from human GWAS. The top 50 hits all resided within one gene (POU6F2), with the highest significance level of \( p > 10^{-13} \) for SNP rs76315987. POU6F2 is found in retinal ganglion cells and in corneal limbal stem cells. To test the effect of POU6F2 on CCT we examined the corneas of Pou6f2- null mice and the corneas were thinner than those of wild-type littermates. In addition, these Pou6f2 RGCs are some of the first ganglion cells to die in the DBA/2 model of glaucoma. Using a mouse genetic reference panel, we identified a transcript correlation, Pou6f2, that modulates CCT in the mouse. POU6F2 is also found in a subset of retinal ganglion cells and these RGCs are sensitive to injury. These data identify POU6F2 as a factor contributing to the correlation between CCT and glaucoma risk.

Mitochondrial Genetics and Primary Open Angle Glaucoma

Willoughby, C.E. 1, Vallabh, N. 1, Lane, B. 1, Cridle, D. 1, Choudhary, A. 1, Cheeseeman, R. 1, Simpson, D. 1

1Ulster University, Biomedical Sciences Research Institute, Coleraine, United Kingdom, 2University of Liverpool, Department of Eye and Vision Science, Liverpool, United Kingdom, 3University of Liverpool, Cellular and Molecular Physiology, Liverpool, United Kingdom, 4Royal Liverpool and Broadgreen University Hospitals NHS Trust, St Paul’s Eye Unit, Liverpool, United Kingdom, 5Queen’s University Belfast, Centre for Experimental Medicine, Belfast, United Kingdom

The mitochondrial genome is organized as a circular, double-stranded DNA molecule. To investigate this hypothesis, we examined the mitochondrial genome of mouse (16,600 base pairs encoding for only 37 genes). Mitochondrial DNA (mtDNA) can replicate independently of nuclear DNA and mtDNA lacks the protective histones and efficient DNA repair system associated with the nuclear genome. Mitochondrial mutations can be inherited through the maternal line or acquired throughout life (somatic mutations). Mitochondria generate and are a target of reactive oxygen species (ROS) and can contribute to their own accelerated mutational rate during periods of local oxidative stress. In humans there are usually 100-1000 separate copies of mtDNA present in each cell and several mtDNA variants can exist within a cell or tissue; this is termed heteroplasmy. These mtDNA mutations can coexist at a low frequency with wildtype mtDNAs, making it challenging to identify all mutations in the mitochondrial genome. New DNA sequencing methods termed massively parallel sequencing provide a powerful tool for identifying these variants. To identify candidate genes with enhanced sensitivity for the mitochondrial genome and provide adequate sequencing depth to identify heteroplasmic positions with enhanced sensitivity.

The aim of this study was to determine whether mutations in mtDNA play a role in primary open angle glaucoma (POAG) using massively parallel sequencing of the mitochondrial genome. The mtDNA from 100 POAG patients was amplified with enhanced sensitivity for the mitochondrial genome and provide adequate sequencing depth to identify heteroplasmic positions with enhanced sensitivity. For the mitochondrial genome and provide adequate sequencing depth to identify heteroplasmic positions with enhanced sensitivity.

Increased Retinal APBB2 and β-amyloid in Eyes with an APBB2 Risk Allele Associated with POAG

van der Heide, C.J. 1,2, Flamme-Wiese, M. 1, Khor, C.C. 3,4

1University of Iowa, Department of Ophthalmology and Visual Sciences, Carver College of Medicine, Iowa City, United States, 2University of Iowa, Department of Molecular Physiology and Biophysics, Carver College of Medicine, Iowa City, United States, 3Singapore Genome Center, Singapore, Singapore, 4Singapore Eye Research Institute, Singapore, Singapore, 5Institute for Translational Genomics and Population Sciences, Departments of Pediatrics and Medicine, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, United States, 6University of California, Department of Ophthalmology, San Diego, United States, 7Duke University Eye Center, Department of Ophthalmology, Durham, United States, 8Duke University Medical Center, Department of Medicine, Durham, United States

Purpose: Glaucoma is the most common cause of irreversible vision loss in the world and has a strong genetic basis. Recently, a large genome wide association study identified a significant association between a single nucleotide polymorphism (rs59892895) in the APBB2 gene (amyloid beta A4 precursor protein-binding, family B, member 2) and primary open angle glaucoma (POAG). The risk allele is present in 25% of individuals of African ancestry and not present in Caucasians or Asians. APBB2 has previously been associated with Alzheimer’s disease and functions in processing amyloid precursor protein into β-amyloid, a neurotoxic peptide. We hypothesized that the APBB2 risk allele confers risk for glaucoma by increasing APBB2 production and promoting toxic β-amyloid formation in retinal ganglion cells.

Methods: Donor eyes from African Americans with the APBB2 risk allele (n=2) and without the risk allele (n=2) were identified from a collection of eyes genotyped for the APBB2 risk allele rs59892895 using a real-time quantitative PCR assay. APBB2 and β-amyloid protein localization was assessed in retinal tissue sections from these eyes using immunohistochemistry. APBB2 and β-amyloid protein levels were qualitatively compared between African eyes with the APBB2 risk allele (n = 2) and without the APBB2 risk allele (n = 2). Donor eyes from an additional four age-matched Caucasian controls (without the APBB2 risk allele) were also analyzed. None of the eye donors had a history of glaucoma.

Results: APBB2 immunoreactivity was observed diffusely throughout all layers of the neural retina and was more prominent in eyes with the APBB2 risk allele than in eyes without the risk allele. β-amyloid immunoreactivity was localized to photoreceptor outer segments in eyes of both APBB2 risk allele carriers and in deeper retinal layers of the neural retina ganglion cell layer was only observed in risk allele carriers.

Conclusions: APBB2 expression is increased in the retinas of individuals with the APBB2 risk allele compared to those without the risk allele. Risk allele carriers also have prominent β-amyloid deposition in the retinal ganglion cell layer. Our results provide support for β-amyloid neurotoxicity as a potential mechanism of disease for glaucoma mediated by APBB2.

Increased Retinal APBB2 and β-amyloid in Eyes with an APBB2 Risk Allele Associated with POAG

Quantitative Traits and GWAS Unravelling the Genetics of Primary Open Angle Glaucoma

Burdon, K. International Glaucoma Genetics Consortium

Menzies Institute for Medical Research, University of Tasmania, Hobart, Australia

The last decade has seen a rapid acceleration in the discovery of risk loci for POAG from the early genome-wide association studies (GWAS) in distinct populations to recent analyses of very large publication data. The early studies used very modest numbers of patients but uncovered what are now well replicated loci. The expansion of this work into larger cohorts and additional populations predictably identified more novel risk loci and the very recent analyses combining the traditional clinic based cohorts with large population based biobanks has further enhanced our genetic understanding of POAG. A large body of work from the International Glaucoma Genetics Consortium (IGGCC) has mapped hundreds of loci influencing relevant glaucoma endophenotypes such as intraocular pressure and various disc parameters and shown that many of these loci are also associated with POAG. This approach has provided substantial insight into the mechanism of disease, showing which components of the disease process each risk locus influences. As the number of confirmed POAG loci continues to grow the potential utility of genomic risk scores to enhance prediction of disease risk has begun to be realised. The power and collaborative spirit of the IGGGC is undoubtedly a major success story and has kept ocular genetics at the forefront of gene discovery for complex disease.

ORAL PRESENTATIONS

Glaucoma
Willoughby, C.1, Lester, K.2

Pathway
Identifies Enrichment of the RhoA Signalling Pathway in POAG pathogenesis.

in RNA-seq is a powerful tool to investigate genome-wide gene expression profile was determined by microarray analysis or microscopy. RNA was extracted from the optic nerve heads and the microarray analyses of N15/N14 revealed that myelin at the MTZ, constitutively express higher levels of a gene associated with myelin phagocytosis, and increase expression of this gene in two mouse experimental glaucoma models. To determine whether the MTZ astrocytes might constitutively phagocytose myelin and increase this activity in animal models of glaucoma, astrocyte phagocytosis of myelin was assessed in cultured astrocytes and in mouse and monkey animal models through analyses of molecular markers as well as electron microscopy. In order to determine whether the myelin in the retrolaminar optic nerve might have unusually high turnover, optic nerves of metabolically labeled mice were analyzed by nanoscale secondary ion mass spectrometry (nanoSIMS). Cultured astrocytes were shown capable of phagocytosing and degrading small amounts of myelin. However, when the amount of myelin was increased, the cultured astrocytes continued to phagocytose but did not fully degrade all the internalized myelin, and developed morphologically diverse lipid-filled spherical organelles including lipid droplets. In mouse experimental glaucoma models, MTZ astrocytes had significant increases in similar spherical organelles and lipid droplets. Structural intermediate segments of myelin phagocytosis by astrocytes, similar to those observed during a developmental optic nerve shortening in frogs, were observed in the retrolaminar O Nh of both control mice and monkeys. In addition, myelin segments in the MTZ of mice were significantly shorter than myelin segments elsewhere in the optic nerve. Finally, nanoSIMS analyses of N15/N14 revealed that myelin at the MTZ experiences higher metabolic turnover than other myelin in the optic nerve head. Together, these data demonstrate that retrolaminar ONH astrocytes constitutively phagocytose myelin, and that they have altered myelin phagocytosis in experimental glaucoma models.

In previous work, we had demonstrated that optic nerve head (ONH) astrocytes constitutively phagocytose axonal material, including retinal ganglion cell (RGC) mitochondria, and that the astrocytes in the retrolaminar ONH, at the Myelination Transition Zone (MTZ), constitutively express higher levels of a gene associated with myelin phagocytosis, and increase expression of this gene in two mouse experimental glaucoma models. To determine whether the MTZ astrocytes might constitutively phagocytose myelin and increase this activity in animal models of glaucoma, astrocyte phagocytosis of myelin was assessed in cultured astrocytes and in mouse and monkey animal models through analyses of molecular markers as well as electron microscopy. In order to determine whether the myelin in the retrolaminar optic nerve might have unusually high turnover, optic nerves of metabolically labeled mice were analyzed by nanoscale secondary ion mass spectrometry (nanoSIMS). Cultured astrocytes were shown capable of phagocytosing and degrading small amounts of myelin. However, when the amount of myelin was increased, the cultured astrocytes continued to phagocytose but did not fully degrade all the internalized myelin, and developed morphologically diverse lipid-filled spherical organelles including lipid droplets. In mouse experimental glaucoma models, MTZ astrocytes had significant increases in similar spherical organelles and lipid droplets. Structural intermediate segments of myelin phagocytosis by astrocytes, similar to those observed during a developmental optic nerve shortening in frogs, were observed in the retrolaminar ONH of both control mice and monkeys. In addition, myelin segments in the MTZ of mice were significantly shorter than myelin segments elsewhere in the optic nerve. Finally, nanoSIMS analyses of N15/N14 revealed that myelin at the MTZ experiences higher metabolic turnover than other myelin in the optic nerve head. Together, these data demonstrate that retrolaminar ONH astrocytes constitutively phagocytose myelin, and that they have altered myelin phagocytosis in experimental glaucoma models.

Tehran, S.

ORAL PRESENTATIONS

University of Liverpool, Department of Eye, Lane, B.

2, Sheridan, C.2

-GB - Biomechanics and Astrocyte Mechanobiology in Glaucoma

Optic Nerve Astrocytes

Jakobs, T.1, Zhu, Y.1, Wang, R.1,2, Sun, D.1, Glaucoma

Schepens Eye Research Institute, Mass. Eye and Ear, Harvard Medical School, Boston, United States, 1First Affiliated Hospital of Xian Jiaotong University, Xian, China

In the initial segment of the optic nerve, the retinal ganglion cell axons are unmethylated until they have cleared the lamina cribrosa. In this region, the axons are in direct contact to astrocytes. This is the case whether or not the eye contains a true lamina cribrosa or, as in small rodents, only the astrocyte meshwork without collagenous plates. As in other parts of the brain, the astrocytes of the optic nerve react to injury by becoming reactive. In this study, we characterized astrocyte reactivity in the optic nerve head in detail. We used three models of optic nerve damage to induce reactivity. First, optic nerve crush as a model of a severe injury that affects both ganglion cell axons and the surrounding glia; second, a chronic microbead-induced increase of the intraocular pressure; and finally a transient increase of the intraocular pressure to 30 mmHg by direct cannulation of the anterior chamber. Morphological changes in the astrocytes were studied by confocal- and electron microscopy. RNA was extracted from the optic nerve heads and the gene expression profile was determined by microarray analysis or RNA-sequencing, followed by pathway analysis. In all three models, astrocytes showed morphological signs of reactivity. Unsurprisingly, these were most pronounced after optic nerve crush, where the astrocytes retracted most of their processes, and least pronounced after a transient elevation of intraocular pressure where the remodeling involved only the smaller branches. However, after chronic elevation of intraocular pressure we observed the growth of new processes that grew into axon bundles along the longitudinal axis of the nerve. A similar growth of longitudinal processes was also observed in 6-8 month old DBA/2J mice, a common model of hereditary glaucoma. In transmission electron microscopy, these longitudinal processes often contained degenerating mitochondria. Using injections of mitotracker dye into the superior colliculus, we show that the mitochondria contained in the astrocyte processes are of axonal origin. RNA-sequencing of the optic nerve head tissue showed 361 differentially expressed genes (in comparison to 1606 after nerve crush and 20 after transient elevation of the intraocular pressure). The pathways most consistently up-regulated in reactive astrocytes include STAT3, TGFβ1, and the ERBB family of receptors.
addition, we show that ANXA4 stabilizes membrane dynamics to facilitate cell repair in a calcium dependent manner. These observations suggest that pathologically relevant levels of biomechanical strain can directly compromise membrane integrity to increase cell permeability.

GLA9 - Pseudoexfoliation Glaucoma Update

Link between the Genetic and Functional Analyses in Pseudoexfoliation Syndrome/ Pseudoexfoliation Glaucoma

Pasutto, E.1, Berner, D.1, Zenkel, M.1, Reis, A.T., Aung, T., Khor, C.C.2, Schlötzer-Schrehardt, U.3, International PEX Consortium

1Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany, 2Friedrich-Alexander-Universität Erlangen-Nürnberg, Ophthalmology, Erlangen, Germany, 3Friedrich-Alexander-Universität Erlangen-Nürnberg, Human Genetics Institute, Erlangen, Germany, 4Singapore National Eye Centre, Glaucoma Department, Singapore, 5Genomics Institute of Singapore, Human Genomics Institute, Singapore, Singapore

Pseudoexfoliation (PEX) syndrome is an age related systemic disorder characterized by deposition of abnormally crosslinked, extracellular material in various ocular tissues. It often results in PEX glaucoma (PEXG), the most common identifiable form of secondary glaucoma, accounting for 25-75% of open angle glaucoma. To date several large, genome wide association studies (GWAS), based on a large international collaborative effort, have revealed seven associated loci. Although these loci are still not sufficient to explain the complete heritability of PEX, their identification has provided significant biological insight into the disease process by implicating distinct biological pathways including calcium signaling and pro teaseome function. Among them, LOKL1 is the major genetic and pathophysiologic risk factor for this complex disease. However, all associated LOKL1 risk variants identified to date, show frequent occurrence in healthy subjects and significant allele reversal between cases in different ethnic groups analyzed. Preliminary results of functional characterization for one of the protective, rare variant, p.R1407Q, which is located in the catalytic domain of LOKL1 show the effect to stabilize the extra-cellular matrix by upregulating matrix components such as elastin and fibrillin. On the other side, the common, protective variant n.7173049 which resides within a regulatory region appears to influence other genes than LOKL1 which could be involved in PEX disorder. These findings highlight the necessity of further characterization of these protective variants at the LOKL1 locus to understand the role of LOKL1 in PEX process. In parallel, transcriptome analysis on eye tissues from PEX patients and control subjects could help in understanding how all the seven genes identified so far and those not yet identified are related to disease.

Autophagic and Microtubule Dysfunction in Exfoliation Glaucoma

Bernstein, A.1, Boumil, E.1, Ritch, R.3, Wolosin, J.M.2, Ridl, M.2

1SUNY Upstate Medical University, Syracuse, United States, 2New York Eye and Ear Infirmary of Mount Sinai, New York, United States, 3Icahn School of Medicine at Mount Sinai, New York, United States

Exfoliation Syndrome is characterized by a buildup of proteinaceous exfoliation material (XFM) on the lens and outflow pathway in the anterior segment of the eye. Using tenon fibroblasts (TF) from Exfoliation Glaucoma (XFG) patients we have previously demonstrated that these cells display cellular impairments resembling those observed in age-related neurodegenerative diseases with pathology stemming from misfolded, aggregated proteins (aggregopaties). XFG-TF displayed slowed autophagic flux and impaired lysosomal positioning after starvation-induced catabolism, suggesting compromised degradation systems. This corresponded with an accumulation of nonfunctioning mitochondria. Since cellular degradation systems and mitochondrial function are coordinately regulated by the cytoskeleton, we tested if microtubule dynamics were altered in XFG cells. XFG-TF were cultured for 24 h in glass bottom dishes in complete media and transfected with human EB1-mCherry-GFP. Images were acquired on a Zeiss LSM510 scanning confocal microscope and analyzed by u-track multiple particle tracking software in “MT plus-ends” mode (utsouthwestern.edu/labs/danuser/software). In XFG-TF “dynamicity” (total plus end movement) and speed (μm/minute) of microtubule growth were 1.5-fold (p < 0.005) and 1.4-fold higher (p < 0.05), respectively, compared to age-matched PDAG and no Glaucoma controls. These data demonstrate that the microtubules in XFG-TF display an unstable “hyperdynamic” phenotype, similar to those in other diseases such as ALS and Alzheimer’s. Cargo vesicles such as lysosomes are coordinated through microtubule-associated complexes. Instability (hyperdynamic) at the plus end of microtubules is often associated with hyperacetylation at the minus end. This causes vesicle transport deficiencies. By Western blot we found a 2.04-fold greater overall acetylation p=0.5 of XFG-TF compared to control. Forced hyperacetylation by inhibition of the HDAC6 enzyme with 8 nM Tubacin in control PDAG cells produced XFG cellular phenotypes suggesting that dysfunctional microtubules may be a root cause of XFG cellular dysfunction. Targeting microtubule stability or acetylation in XFG could be a method of improving degradation systems and promoting subsequent clearance of XFM protein aggregates.

Potential Roles of Aqueous miRNAs in Exfoliation Glaucoma

Liu, Y.1,2, Drewry, M.1, Challa, P.1, Kuchtey, J.1, Navarro, I.1, Helwa, I.1, Stamer, W.D.1, Kuchtey, R.W.2

1Augusta University, Cellular Biology and Anatomy, Augusta, United States, 2Augusta University, Center for Biotechnology & Genomic Medicine, Augusta, United States, 3Augusta University, James & Jean Culver Vision Discovery Institute, Augusta, United States, 4Duke University Medical Center, Ophthalmology, Durham, United States, 5Vanderbilt Medical Center, Vanderbilt Eye Institute, Nashville, United States

Exfoliation glaucoma (XFG) has been linked to decreased conventional outflow of aqueous humor (AH). To better understand the molecular changes in the AH content under such conditions, we analyzed the miRNA profiles of AH samples from patients with XFG compared to non-glaucoma controls. Individual AH samples were collected from XFG patients and age-matched controls during surgical procedure. After RNA extraction, the miRNA profiles were individually determined in 12 XFG and 11 control samples. We identified 295 and 195 miRNAs in the XFG and control samples, respectively. Our differential expression analysis identified 5 significant miRNAs (mir-122-5p, mir-1144-3p, mir-320a, mir-322e, and mir-630) between XFG and controls. While none of these miRNAs have been previously linked to glaucoma, mir-122-5p may target three glaucoma-associated genes: OPTN, TMC1D, and TGF-β1. Pathway analysis revealed that these miRNAs are involved in potential glaucoma pathways, including focal adhesion, tight junctions, and TGF-β signaling. Comparison of the miRNA profile in AH to unrelated human serum (n = 12) exposed potential relationships between these two fluids, although they were not significantly correlated. In summary, we have successfully profiled the miRNA expression without amplification in individual human AH samples and identified several WFG-associated miRNAs. These miRNAs may play a role in pathways previously implicated in glaucoma and act as biomarkers for disease progression.

Reprogramming Glaucoma: An Insight into Epigenomic Glaucoma

Wallace, D.1,2

1School of Medicine, University College Dublin, Mater Misericordiae, Dublin, Ireland

Glaucoma is a common cause of blindness, which affects approximately 60 million people worldwide. Pseudoexfoliation (PXG) syn drome is currently the single most important identifiable cause for developing open angle glaucoma (PXFG). It is an age related general disorder of the extracellular matrix characterized by the production and progressive accumulation of fibrillar material in ocular tissue. PXF syndrome is a complex, multifactorial disease involving a combination of both genetic and non-genetic factors in its pathogenesis. Single nucleotide polymorphisms in the loxl2 oxidase-like 1 (LOXL2) gene have been identified as a major genetic risk factor for PXF syndrome and PXFG. This strong association has been uniformly replicated in all geographic populations studied to date, confirming an approximately 20-fold risk for disease. Surprisingly, they also occur in 50-80% of the normal population indicating that many other factors must contribute to disease pathogenesis and progression. Possible contributory factors may include oxidative stress, hypoxia as well as high levels of pro-fibrotic transforming growth factor beta 1 (TGFβ1). Given that tissue hypoxia can trigger epigenetic modifications such as DNA methylation, we hypothesize that the expression of LOXL2 may also be controlled (down-regulated) via methylation in PXFG such as reported in Cutsis Lax, bladder cancer and in aged human skin fibroblasts. We investigated this hypothesis using human tenon fibrinoblasts (HTFs) isolated from PXFG patients. Results show that expression of LOXL2 is decreased in PXFG HTFs compared to cataract controls and is associated with an increase in global methylation levels. A hypoxic [1% O2] environment altered global methylation levels and expression of LOXL2 in HTFs from cataract controls. LOXL2 expression could be restored (increased) by treatment with methylation inhibitors (5-aza-deoxycytidine). Furthermore, we have previously demonstrated that methylation can regulate TGFβ1 expression in lamina cribrosa and trabecular meshwork cells from primary open angle glaucoma donors. These data also demonstrated that hypoxia alters methylation levels and subsequently the expression of Ras Protein Activator-Like 1 (RAF1), which has anti-fibrotic properties. Moreover, 5-aza-deoxycytidine reversed the increased expression of TGFβ1 and the decreased expression of RAF1. These findings suggest the potential use of chromatin modifying intervention in PXFG.

ORAL PRESENTATIONS

Glucoma
Exfoliation glaucoma is a common type of secondary open-angle glaucoma linked to single nucleotide polymorphisms in the lysyl oxidase-like 1 (LOXL1) gene. Genetic studies have identified a putative mechanism by which non-coding variants in LOXL1 can result in reduced tissue expression levels of Loxl1 protein in multiple tissues, including the optic nerve head. The purpose of this study was to determine the effect of Loxl1 down-regulation on optic nerve head astrocytes (ONHA). Knockdown of Loxl1 expression in primary adult rat ONHA cultures was achieved by transfection of rat Loxl2 gene-specific siRNA. For comparison, reactive astrocytosis was induced experimentally by ONHA exposure to a humidified hyperbaric (20 - 25 mm Hg) atmosphere of 95% Air/5% CO2 for 20 h or to mechanical strain (10% static stretch) in a FlexCell® (FlexCell International) chamber. Quantitative PCR, immunoblotting and immunocytochemistry were performed using previously validated primers and antibodies. Loxl1 knockdown in ONHA elicited a statistically significant increase in GFAP immunoreactivity (n = 5, P < 0.001) concomitant with an increase in expression of voltage-gated calcium channel subunits (2.7-fold increase in Ca2a, P < 0.01; 1.8-fold increase in Ca2b1, P < 0.01) suggestive of reactive astrocytosis. At the same time, expression levels of the ECM proteins elastin, fibulin-2 and fibulin-4 were significantly decreased, suggestive of cellular elastopathy. Notably, induction of reactive astrocytosis by means of exposure to hyperbaric pressure and mechanical strain resulted in almost identical molecular signatures. In conclusion, LOXL1 variants associated with exfoliation glaucoma may sensitize the optic nerve head to the deleterious consequences of elevated IOP by inducing reactive astrocytosis and cellular elastopathy, thereby initiating the clinical progression of exfoliation glaucoma. Our data tentatively suggest a conserved mechanism underlying reactive astrocytosis in exfoliation glaucoma and other types of open angle glaucoma without LOXL1 variants.
Hallmark remodeling events required for formation of mature lens fiber cells include the elimination of cellular organelles to form the lens organelle-free zone (OFZ) and the regulated expression of critical fiber cell-specific proteins. However, despite the importance of these events for mature lens structure and lens transparency, the mechanisms governing the elimination of organelles and the expression of critical lens fiber structural proteins remain to be fully elucidated. The lens is an avascular tissue that obtains oxygen by diffusion resulting in a hypoxic gradient from the lens surface (1% oxygen) to the lens core (>0.1% oxygen). This gradient results in a hypoxic microenvironment in the region of the lens where the forming fiber cells are just beginning to eliminate their organelles to form the lens OFZ and to express critical lens fiber cell structural proteins required for lens transparency. Based these characteristics of the lens, we hypothesized that hypoxia could be required for the elimination of organelles to form the lens OFZ and for expression of critical lens fiber cell proteins. To test this hypothesis, we compared the elimination of organelles and the expression of critical lens fiber cell proteins in day 10 embryonic chick lenses exposed to hypoxia or normoxia. We also examined the role of the master regulator of the hypoxic response, transcription factor HIF1a in organelle elimination and expression of critical lens fiber cell proteins. Finally, we examined the requirement for hypoxia in the expression of the mitophagy protein BNIP3L and the requirement for BNIP3L in elimination of organelles and expression of critical lens fiber cell proteins. Western blotting was also performed using lens epithelial explants from these mice, to assess for changes in intracellular signalling. Results: At postnatal day 21, lenses from the resultant transgenic progeny of mice overexpressing bioactive TGFβ1 specifically in the lens that develop ASC, were crossed to Nox4-deficient mice. The eyes of the resultant progeny were grossly examined for the presence/absence of cataract, collected and processed for evaluation. Eye sections were Periodic-Acid Schiff stained, and immunofluorescence was used to assess for changes in EM/T/PARK markers. Western blotting was also performed using lens epithelial explants from these mice, to assess for changes in intracellular signalling. Results: At postnatal day 21, lenses from the resultant transgenic progeny of mice overexpressing bioactive TGFβ1, deficient for Nox4, were found to be transparent, and did not present anterior subcapsular cataracts as seen in the TGFβ overexpressing lines. Interest- ingly, while these lenses remained transparent, histology and immuno- staining revealed the presence of plaques that were devoid of any notable EMT markers, in contrast to the TGFβ overexpressing mice. The resultant plaques in the Nox4 deficient lines overexpress TGFβ, lost their epithelial morphology, however did not express the myofibroblast marker alpha-smooth muscle actin. In- terestingly, these plaques exhibited increased Prox1 nuclear local- ization. Western blotting of untreated lens explants from deficient and transgenic mice, also revealed elevated pSmad2/3 and pERK1/2 signalling. Taken together, these findings now provide a platform allowing us to de- lineate whether Nox4 is directly interacting with Smad2/3 or ERK1/2 or through an independent signalling intermediate.
While transforming growth factor beta (TGFβ) signaling drives this process, the mechanisms that activate the TGFβ pathway post-cataract surgery (PCS) are not well understood as many investigations into PCO either induce EMT with exogenous active TGFβ or study the process in vivo after EMT has commenced. As our prior investigations have revealed that it takes 48-72 hours for canonical TGFβ signaling to initiate PCs in a mouse cataract surgery model, we took an unbiased approach to determine how LECs respond to cataract surgery prior to the onset of TGFβ signaling. This analysis revealed that the LEC transcriptome is massively altered by 24 hours PCS which is one full day prior to the first detection of canonical TGFβ signaling. The differentially expressed genes included those important for lens biology, and fibrotic markers such as fibronectin and tenasin C. However, the most dramatic changes were in the expression of genes regulating the innate immune response with the top three altered genes (CCK1, S10Qa9 and CSF3) exhibiting greater than 1000 fold upregulation. Immunolocalization revealed that CCK1, S10Qa9, CSF3, COX-2, CCL2, LCN2 and HMObx protein levels upregulate in LECs between 1 hour and 6 hours PCS, and peak at 24 hours PCS, while their levels sharply attenuate by 3 days PCS. This massive upregulation of known inflammatory mediators precedes the infiltration of neutrophils into the eye at 18 hours PCS, the upregulation of canonical TGFβ signaling at 48 hours PCS and the infiltration of macrophages at 3 days PCS. These data demonstrate that LECs are immediately following lens injury which could drive post-surgical flare, and potentially pathogenesis of other ocular inflammatory diseases. As inflammation is a known initiator of TGFβ mediated fibrosis in other tissues, these data suggest that inflammation may be a major player in the onset of lens-associated fibrotic disease PCS.

The Human Capsular Bag Model of Posterior Capsule Opacification
Wormstone, M.
University of East Anglia, School of Biological Sciences, Norwich, United Kingdom

The human capsular bag model has served as a valuable tool to understand the biological processes governing PCO and has been used to evaluate clinical products including IOLs. To enhance the value of the model for evaluation and development of IOLs the system naturally has evolved with time. The first iteration, secured (anterior face up) the capsular bag containing an IOL to a dish using entomological pins at the equator. While this served useful for assessing the use of the IOL as a drug delivery system, it failed to allow interaction between the IOL and capsule soon used in experiments. To improve interaction between the IOL and capsule a modification was introduced, such that the bag was secured to the dish with the anterior capsule down. When the periphery was secured, this lead to good interaction between the optic edge and the capsule. Further modifications were made that replaced foetal calf serum with human serum and the application of TGFβ was also introduced to further mimic biological events associated with PCO. The original model was adapted to maintain integrity of the capsular bag. To achieve this the capsular bag was retained in association with the ciliary body secured to a dish with the bag containing an IOL to be suspended by the zonules over the lens of the ring. This allows a natural interaction between the IOL and capsular bag to occur. Culture conditions for these preparations involved either serum-free or human serum supplemented medium as the basic culture medium for the experimental duration. However, in patients, the elevation of proteins in the aqueous humour following surgery is transient, such that levels peak in the first week then decline to baseline. With this in mind, a protocol was developed that allows observation of the initial drive generated by elevated protein (serum and TGFβ) followed by reduced levels and ultimately a baseline i.e. serum-free medium. It was clear that these culture conditions can reflect clinical events associated with PCO, such that growth (proliferation/migration), matrix deposition, matrix contraction and EMT were significantly enhanced relative to serum-free controls. This latest iteration of the capsular bag model will, therefore, serve as a valuable and improved tool to evaluate performance characteristics of different IOLs. The human capsular bag model has been established that performing sham cataract surgery in canine cadaver lenses successfully recapitulates events observed during PCS in humans, in which cataract is regulated intracellularly by different signaling pathways including the canonical Smad3/7, as well as the non-canonical ERK/MAPK-pathways. These pathways are thought to potentially crossstalk and together lead to a number of different EMT features, including loss of epithelial phenotype, myofibroblast cell differentiation, extracellular matrix (ECM) accumulation and even altered redox signaling. Earlier studies have shown that these TGFβ-mediated pathways can be regulated by many different means, such as by other ocular growth factors (BMPs, EGF, FGF), ECM proteins and their modulators, and intracellular molecules, as members of the Sprouty family, that negatively regulate the Smad2/3- and ERK1/2-signaling pathways. Here we present some of our current work using mammalian models, examining the different modes of regulating the molecular mechanisms that drive EMT in the lens. We hope that a better understanding of these processes will lead to more effective means of controlling this aberrant lens cell behavior, and subsequently lead to the development of novel non-invasive theapeutics for human cataract.

Applying the Capsule Lens to Further the Understanding of Posterter Capsule Opacification
Chandler, H.
The Ohio State University, Columbus, United States

Cataract is the most common cause of treatable visual impairment in both dogs and humans. Phacoemulsification cataract extraction with intraocular lens (IOL) implantation is the most frequently performed ophthalmic surgical procedure in veterinary and human medicine, with a success rate of greater than 95% in both species. Between 80-100% of dogs that undergo phacoemulsification cataract surgery, develop posterior capsule opacification (PCO) within one year postoperatively and the underlying pathophysiology is similar to PCO in humans. Thus, canine PCO is a highly relevant model for human post-operative capsular opacification. We have successfully established that performing sham cataract surgery in canine cadaver lenses successfully recapitulates events observed during PCO for humans. Following surgery, our ex vivo cultures of canine lens capsules demonstrate lens epithelial cell proliferation and migration across the posterior capsule within 14 days and express markers associated with epithelial-mesenchymal transition and fibrosis including e-smooth muscle actin, lumican, slug, snail, and Akt. Using our canine PCO model, we have been able to further evaluate the disease mechanism and potential treatment options. Here, we present examples of PCO prevention strategies including targeting specific molecular signaling cascades, using altered phacoemulsification techniques, and modifying IOLs to regulate the cellular response. Further, due to the similarities between the human and canine lens, treatments demonstrating promise in the laboratory setting can be taken into the clinical setting using canine patients undergoing routine cataract surgery.

Regulation of TGFβ-mediated Pathways Leading to Lens Epithelial to Mesenchymal Transition: Mammalian Models for Understanding Fibrotic Cataract Pathology
Lovicu, F.J.1 2
Anatomy & Histology, The University of Sydney, Sydney, Australia, Save Sight Institute, Sydney, Australia

Different forms of human fibrotic cataract, such as anterior sub- capsular cataract (ASC) and posterior capsule opacification (PCO) result from an epithelial to mesenchymal transition (EMT) of lens epithelial cells. This key pathological mechanism is thought to be primarily driven by transforming growth factor-beta (TGFβ), not only in cataract, but in many other fibrotic pathologies. Stimulation of lens epithelial cells by TGFβ leading to EMT and subsequent cataract is regulated intracellularly by different signaling pathways including the canonical Smad3/7, as well as the non-canonical ERK/MAPK-pathways. These pathways are thought to potentially crossstalk and together lead to a number of different EMT features, including loss of epithelial phenotype, myofibroblast cell differentiation, extracellular matrix (ECM) accumulation and even altered redox signaling. Earlier studies have shown that these TGFβ-mediated pathways can be regulated by many different means, such as by other ocular growth factors (BMPs, EGF, FGF), ECM proteins and their modulators, and intracellular molecules, as members of the Sprouty family, that negatively regulate the Smad2/3- and ERK1/2-signaling pathways. Here we present some of our current work using mammalian models, examining the different modes of regulating the molecular mechanisms that drive EMT in the lens. We hope that a better understanding of these processes will lead to more effective means of controlling this aberrant lens cell behavior, and subsequently lead to the development of novel non-invasive theapeutics for human cataract.
Morphological Comparison of in vivo and in vitro Developed Posterior Capsule Opacification in Human Donor Eyes

D’Antin, J.C.1,2, Ribeiro Koch, C.1,3, Tresserra, F.1, Barraquer, R.1,2,3, Michael, R.1,3

1Institut Universitari Barraquer, Universitat Autònoma de Barcelon- na, Investigación, Barcelona, Spain, 2Centro de Oftalmología Bar- raquer, Investigación, Barcelona, Spain, 3Universidade Federal do Sul- do Piauí, São Paulo, Brazil, 2Universitat Universitàries, Department of Pathology, Barcelona, Spain, 3Universitat Internacional de Cata- lunya, Barcelona, Spain.

Purpose: Our purpose was to observe and compare how re- sidual human lens epithelial cells (LECs) developed after cat- aract surgery and if cell growth is always unorganized or if there is a tendency to reorganize and reform the lens.

Methods: Twenty-four human donor corneas were obtained, sam- ples were extracted and separated in to three different groups; IOL capsules (n=12); extracted capsular bags containing IOLs with varying degrees of Soemmering’s ring (SR) formation. Cultured capsules (n=6); samples in which emptied capsular bags had been cultured for 1 month. Intact lenses (n=6); lenses extracted from donor globes, 3 older samples with and 3 younger without IOLs.

Conclusions: The formation of Soemmering’s ring is a result of the interaction between the capsule and the IOL. The SR formation is more pronounced in older lenses. Vimentin was expressed similarly in all intact lenses, but more opaque but cells were much more organized and a clear dis- tinction between LECs and lens fiber cells could be observed. These differences were also observed in the cultured capsules. Vimentin stained all cells intensely except for in the largest growth. More opaque, and consisted of uneven and unorganized globular cells, and a growth thickness below the capsule of up to 100µm. Formation of AnkB is maintained at relatively high levels in the mature mouse lens. Importantly, AnkG cKO mouse lenses revealed a phe- notype starting from embryonic day E15.5, with progressive and extensive degeneration evident in P10 lenses. AnkG deficiency impaired lens growth, shape, and epithelial phenotype including shortening of epithelial length and height, disruption of polarity, lateral membrane assemblies, cell-cell junctions, membrane or- ganization of various transport proteins and the spectrin/actin cytoskeleton. Moreover, AnkG deficient lenses exhibited impair- ments in epithelial cell proliferation and survival. On the other hand, AnkB cKO mouse lenses did not reveal developmental ab- normalities. AnkB cKO mouse lenses however, showed a precipi- tuous decrease in weight between P12 to P16 stages and developed mature nuclei. As well, AnkB null lenses exhibited extensive disruption in fiber cell shape and organization with abnormalities in the lens cell and socket membrane interdigitations. Taken together, these results illustrate distinct and vital roles for AnkG and AnkB in lens morphogenesis, growth, shape, architecture and function. Supported by the funding source from the NIH (RO1EY025096).

Lens

Ankyrin-B and Ankyrin-G Play Distinct and Crucial Roles in Lens Morphogenesis, Cytorearchitecture and Function

Rao, V., Maddala, R.

Duke University School of Medicine, Ophthalmology, Durham, Unit- ed States.

Ocular lens development, growth and function depend on sequen- tial and highly orchestrated events involving membrane cytoskel- etal organization and cell-cell interactions. These events include the establishment of an epithelial cell phenotype, proliferation, survival and differentiation of lens epithelial cells into fiber cells, and maintenance of fiber cell cytoarchitecture. Despite continued efforts, our understanding of the molecular and cellular mecha- nisms which regulate and maintain the epithelial phenotype and fiber cytoarchitecture of lens is incomplete. Ankyrins are scat- folding proteins that link the spectrin-actin cytoskeleton to various membrane integral proteins and are involved in the regulation of multiple cellular activities. To determine the roles of ankyrin-G (AnkG) and ankyrin-B (AnkB) in lens morphogenesis, architecture and function, we developed and characterized AnkG and AnkB con- ditional knockout mouse models using the loxP-Cre recombination approach. AnkG and AnkB exhibit distinct distribution profiles in the lens with AnkB exhibiting abundant distribution in fiber cells while AnkG reveals discrete distribution to the epithelium. AnkG also exhibits developmental downregulation while the expres- sion of AnkB is maintained at relatively high levels in the mature mouse lens. Importantly, AnkG ckO mouse lenses revealed a phe- notype starting from embryonic day E15.5, with progressive and extensive degeneration evident in P10 lenses. AnkG deficiency impaired lens growth, shape, and epithelial phenotype including shortening of epithelial length and height, disruption of polarity, lateral membrane assemblies, cell-cell junctions, membrane or- ganization of various transport proteins and the spectrin/actin cytoskeleton. Moreover, AnkG deficient lenses exhibited impair- ments in epithelial cell proliferation and survival. On the other hand, AnkB cKO mouse lenses did not reveal developmental ab- normalities. AnkB cKO mouse lenses however, showed a precipi- tuous decrease in weight between P12 to P16 stages and developed mature nuclei. As well, AnkB null lenses exhibited extensive disruption in fiber cell shape and organization with abnormalities in the lens cell and socket membrane interdigitations. Taken together, these results illustrate distinct and vital roles for AnkG and AnkB in lens morphogenesis, growth, shape, architecture and function. Supported by the funding source from the NIH (RO1EY025096).

The Epithelial Template for Coordinated Growth of the Eye Lens

Kalligerakis, A.1,2, Obara, B.1,2, Jarrin, M.1,3, Pal, R.1, Wu, J.1,2, Saumeter, C.1,2, Girkin, J.1,2, Uwineza, A.1,2, Quinlan, R.1,3

1Durham University, Department of Biosciences, Durham, United Kingdom, 2Biophysical Sciences Institute, Durham, United Kingdom, 3Durham University, Department of Computer Science, Durham, United Kingdom, 4Durham University, Department of Chemistry, Durham, United Kingdom, 5Durham University, Department of Engineering, Durham, United Kingdom, 6Okinawa Institute of Science and Technology, Okinawa, Japan.

The origin of all lens cells can be traced back to the lens epithelium. To ensure coordinated, physiological lifelong growth, it is evident that the lens epithelium must be a highly regulated and strictly maintained cell population. Dramatic changes in epithelial cell behav- iour have been shown to have direct effects on lens growth and organisation, as evidenced by the relationship between the epithe- lium proliferation index and deposition of fibre cells during devel- opment, by djdt inactivation studies, and by exposure to ionising radiation. These evidence the importance of studies on the lens epithelium from a macroscopic, cell population standpoint; here we will demonstrate the application of a three-dimensional data capturing process to analyse the maturation and ageing events in the mouse eye lens. Using confocal microscopy and in silico recon- struction techniques, we have recorded the mouse lens epithelium organisation at different life stages, and have developed a distri- bution-based analysis algorithm to characterise long-term cell behav- iour.

Fine Tuning of Ocular FGF Activity Regulates Differentiation and Collective Movement of Lens Fibre Cells

Sugiyama, Y.1,2, McAvoy, J.2, Masai, I.1

1Okinawa Institute of Science and Technology, Okinawa, Japan, 2Save Sight Institute, The University of Sydney, Sydney, Australia.

Formation of a spherical ocular lens requires collective migration of lens fibre cells. After differentiation at the lens equator, the basal tips of lens fibre cells migrate toward the posterior pole of the lens, and simultaneously, their apical tips migrate toward the anterior pole. The movements of the tips are coordinated, so that lens fibre cells gradually increase cell height, forming a convex curve between the anterior and posterior poles. The tips of fibre cells that differ- entiated at the lens equator at the same time move in a similar direction and at the same speed, so that they maintain circular lens symmetry. Because a lens must fit within the ocular space, fibre cell migration might be regulated by the surrounding environment. It has been known that FGF concentration forms a gradient in the eye that specifies the lens equator as the location of fibre cell differentiation. However, FGF’s role in fibre cell migration has remained unknown. We speculated that lens fibre cells develop actin-based membrane protrusions to locate fibres in the posterior of the mouse lens. Importantly, AnkG cKO mouse lenses revealed a phenotypic abnormality related to the fibres at the equator: matrix protrusions were observed in fibres posterior to this location. This suggests that fibres newly differentiated at the equator are static and that active migration is induced during the following phase of fibre maturation. We then found that the basal membranes of equatorial fibres developed radial actin bundles and extended filopodia-like membrane protrusions in lenses that were isolated from eye cups and cultured with serum-free medium. FGF applied to medium blocked actin bundle and protrusion forma- tion. This indicates that reduced FGF concentration is required to promote extension of membrane protrusions. We propose a two-phase model in which FGF concentration is highest at the lens equator to induce fibre differentiation, and is reduced posteriorly to activate fibre migration. Change of FGF concentration appears to control mobility of fibre basal membranes by modulating actin dynamics.

Capsases Processing of Bfsp1 Produces C-terminal Fragments Containing Lipid Binding Domains

Jarrin, M.1,2, Uwineza, A.1, Freitag-Pohl, S.1, Brown, A.1, Tap- odi, A.1, Quinlan, R.1

1Durham University, Biosciences, Durham, United Kingdom, 2Durham University, Chemistry, Durham, United Kingdom, 3Durham University, Proteinomics, Durham, United Kingdom, 4University of Pécs, Medical School, Pécs, Hungary.

Beaded filament structural protein 1 (Bfsp1) co-assembles to form filaments that closely associate with a range of biomolecules to contribute to the optical properties of the lens. The C-terminal do- main of Bfsp1 is of particular interest as it contains mutations that are involved in the cataract development. To understand the mech- anistic details of how Bfsp1 causes cataract, we need to determine the functional role of the C-terminal region of Bfsp1 in the lens. A bioinformatics analysis of the C-terminal sequence of human
Lens

LEN4 - Lens differentiation, regeneration and cataract treatment

Mouse Lens Lacking SUMO1 Is Prone to Stress-Induced Apoptosis through Activation of both Extrinsic and Intrinsic Death Pathways

Liu, D.1, Liu, Y.1, Liu, F.1, Xie, J.1, Yang, L.1, Luo, Z.1, Wang, L.1, Fu, J.-L.1, Xiao, Y.1, Jiang, J.-W.1, Nie, Q.1, Gou, X.1, Chen, Z.1, Sun, Q.1, Qing, W.1, Gong, L.1, Zhang, L.1, Tang, X.1, Liu, Y.1, Nguyen, Q.D.1

1State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, China, 2Stanford University, Byers Eye Institute, Palo Alto, United Nations Interim Administration Mission in Kosovo

Purpose: Sumoylation is now established as one of the key regulatory protein modifications in eukaryotic cell. It regulates chromatin modification, transcription, mRNA repair, macromolecular assembly, protein homeostasis, trafficking, signal transduction, cell differentiation and stem cell development. It also acts as a molecular mechanism mediating global changes at the cellular and organism levels when stress conditions such as heat shock or oxidative stress occur. In the eye, sumoylation plays a key role in retina development, and it has caused effects on corneal dystrophy. Our recent studies revealed that sumoylation is necessary to activate the p38 MAPK. Moreover, sumoylation may play an important role in regulating lens differentiation. Here we show that sumoylation plays a key role in stress response.

Methods: SUMO1(-/-) knockout mice were established with cas9 Technology. Absence of SUMO1 was confirmed by RT-PCR and Western blot analysis. Adult mouse lenses from wild type and SUMO1 knockout mice were dissected out and irradiated with UVA for 60 minutes after 24-hour culture in vitro to exclude damaged lens. RNA-seq, qRT-PCR, Western blot analysis and immunocytochemistry were used to analyze the differential gene expression in both control and irradiated wild type and SUMO1 knockout lenses. Co-IP was used to determine the protein-protein interaction.

Results: After UVA irradiation, SUMO1(-/-) lenses develop cataract in 4 hours during the post-irradiation culture, while the wild type lenses remain transparent up to 10 hours. At the molecular level, caspase-3 in the SUMO1(-/-) lens was completely activated after 2-hour culture post-UVA irradiation while in the wild type lens majority of the caspase-3 exists in pro-caspase-3 and partial processed caspase-3. Co-IP revealed that caspase-3 and SUMO1 are sumoylated in the wild type animal but not in the SUMO1(-/-) lens. UVA irradiation also significantly activates p53, and upregulates caspase-8 but down regulates Bcl-2 in the SUMO1(-/-) lens.

Conclusion: SUMO1 regulates caspase-3 activation. Under stress condition, lack of SUMO1 greatly promotes activation of Caspase-3, and both the extrinsic and intrinsic pathways are initiated. (Supported by grants from National Natural Science Foundation of China, 81507824, 81770910, 81500738, 81500707, and 81700821 as well as the Fundamental Funds from the State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University).

The CREB Responsible Element Binding Protein (CREF) Regulates Lens Differentiation through Various Sets of Downstream Genes

Ling, W.1, Zhang, L.1, Gao, M.1, Gong, X.1, Xiang, J.1, Xiao, Y.1, Chen, Z.1, Gong, L.1, Fu, F.1, Luo, Z.1, Fu, J.1, Qing, W.1, Liu, M.1, Nguyen, Q.D.1, Li, D.1

Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, China, 2Huazhong University of Science and Technology, Wuhan, China, 3Byers Eye Institute, Stanford University School of Medicine, Watson Court, Palo Alto, CA, United States

Purpose: CREB is a general transcription factor whose functions are activated through phosphorylation at S133 by protein kinase A (PKA) and other kinases. CREB has been shown to play an essential role in promot- ing cell proliferation, neuronal survival and the synaptic plasticity associated with long-term memory. However, its functions in cell differentiation, especially lens differentiation has not been well characterized. In the present study using mouse model and cultured cells as model systems, we first examined the develop- omental expression patterns of the CREB in the mouse lenses with Edl05.1 to Ed10.5, and analyzed its role in promoting lens cell differentiation. Subsequently, we determined the down- stream genes of CREB which are involving the lens differentiation.

Methods: RNA-seq, qRT-PCR, Western blot analysis and immunocytochemistry were used to analyze expression patterns of the CREB downstream genes. Co-immunoprecipitation were used to deter- mine protein-protein interaction. Over-expressing and knock-down were used to analyze the effect of genes on lens differentiation.

Results: CREB was highly expressed in the corneal lens from the very beginning (ED 10.5) to adult mouse eye lens. Wild type CREB but not its S133A mutant promotes cell differentiation. Such func- tions are closely associated with its regulation of expression of various downstream genes which include transcription factors and differentiation associated genes. First, wild type CREB significant- ly mediated about 1743 (up:761, down:982) downstream genes expression during differentiation. Among which, over 100 genes have been shown to be closely related to differentiation. Second, CREB promotes lens cell differentiation by down-regulate Wnt7a and Pacl expression while up-regulate Wnt7b and Id17 expression.

Conclusion: CREB is an important transcription factor for lens de- velopment. (Supported by grants from National Natural Science Foundation of China, 81507824, 81770910, 81500738, 81500707, and 81700821 as well as the Fundamental Funds from the State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University).

Modulation of ERK1/2 in Lens by Spreds

Wazin, F.1,2, Susanto, A.1, Lovicu, F.1,3

1Bosch Institute, The University of Sydney, Anatomy and Histology, Sydney, Australia, 2Save Sight Institute, The University of Sydney, Sydney, Australia

The transparent and refractive properties of the ocular lens are de- pendent on its precise cellular architecture. The ERK/MAPK-signal- ling pathway plays a crucial role in regulating lens cell proliferation and differentiation, that is, in turn, is regulated by inhibitory molecules including the Spred family of proteins. We hypothesised that Spred protein phosphatases might balance the MAPK pathway and differ- entiation, required for maintenance of normal lens structure and growth. Embryonic and postnatal lens tissue of mutant mice either over-ex- pressing Spreds, or deficient for Spreds, were compared to control lenses tissue. Ocular tissues were collected at various developmen- tal ages and processed for histology, and sections were exam- ined after PAS staining and immunolabeling. The lens phenotype was characterized in these different mouse models, comparing lens size, fibre cell length, epithelial cell numbers and expression during differentiation. Among which, over 100 genes have been shown to be closely related to differentiation. Second, CREB promotes lens cell differentiation by down-regulate Wnt7a and Pacl expression while up-regulate Wnt7b and Id17 expression.

Conclusion: CREB is an important transcription factor for lens de- velopment. (Supported by grants from National Natural Science Foundation of China, 81507824, 81770910, 81500738, 81500707, and 81700821 as well as the Fundamental Funds from the State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University).

Tropomyosin 3.1 is a Target to Prevent Lens Epithelial Cell Enzymal Transition during Cataract Formation

Parreno, J., Fowler, V.

The Scrips Research Institute, Molecular Medicine, La Jolla, United States

Cataracts are any opacity in the lens that results in cloudy vision and are the most common cause of vision loss after the age of 40. One cause of cataracts is transdifferentiation of epithelial cells, situ- ated at the anterior region, into myofibroblasts through epitheli- al-to-mesenchymal transition (EMT). Myofibroblasts have elevated expression for contractile (α-smooth muscle actin; αSMA) and ma- trix (collagens)-like molecules ultimately resulting in lens opacification and light occlusion. Reorganization of cortical actin into stress fibers is an early step required for EMT and preventing ac- tive stress fiber formation can impede EMT. However, specifically targeting cataract formation with an EMT network has proven to be difficult. Targeting tropomyosin (Tpm) isoforms may be a route to perturb specific actin networks and prevent lens EMT. Tpms bind along F-actin and coordinate the in- teraction of other actin binding (nucleating, capping, cross-linking, severing) proteins with actin. There are over 40 different Tpm iso- forms and unique combinations of Tpms differ in terms of protein expression and isoform abundance in specific cells and tissues. The Scrips Research Institute has identified Tpm3.1 as a potential target to prevent lens EMT. Tpm3.1 is found in the epithelium, inner lens epithelium, and inner cell membrane of the lens. However, overexpression of Tpm3.1 in lens epithelium results in a phenotype associated with high myopia. These results suggest that specific isoforms of Tpms may be a potential target to prevent cataract formation.
actin stress fibers in another cell type (osteosarcoma). Therefore, we investigated Tpm1.3 as a target to prevent lens EMT. Immuno- cytochemistry reveals that Tpm1.3 associates with stress fibers in TGFβ2-treated lens epithelial cells. Additionally, the expression of Tpm1.3 is higher in the cells that have stress fibers containing αSMA. Pharmacological inhibition using Tpm1.3 inhibitor, TR100, prevents TGFβ2-induced expression of αSMA and collagen-I in lens epithelial cells. Using whole lenses ex vivo, we show that Tpm1.3 knockout mouse lens epithelial cells are resistant to TGFβ2-in-duced stress fiber formation and maintain cortical actin organization. Likewise, treatment of αSMA-GFP reporter mouse lenses with TR100 prevents TGFβ2-induced enhancement of αSMA expression.

Pilot studies on primate lenses show that TR100 can also prevent EMT in primate epithelial cells, suggesting broad species effective-ness. Thus, targeting Tpm1.3 is a means to prevent lens EMT and is a potential therapeutic target against cataracts.

Investigating the Initial Molecular Mechanisms of Human Cataract Formation Using Light-focusing Micro-lenses Derived from Pluripotent Stem Cells

O’Connor, M.1, 2, Umala Dewi, C.2, Kabir, M.H.2, Murphy, P.1, Ho, H.2, 1

1 Western Sydney University, Medical Sciences Research Group, Campbelltown, Australia; 2 Western Sydney University, School of Medicine, Campbelltown, Australia; *Victor Chang Cardiac Re- search Institute, Darlinghurst, Australia; "University of New South Wales, St. Vincent's Clinical School, Sydney, Australia

Cataract is a leading cause of blindness worldwide. Currently, vision can only be restored by surgically replacing the cataractous lens with an artificial intraocular lens. This procedure has sever-

Lens - Target identification and management of PCO

Open Bag IOLs and VEGF Inhibition in the Management of PCO

Eldred, J.1, Spalten, D.2, Wormstone, M.3

1 University of East Anglia, Norwich, United Kingdom; 2 King Edward VII Hospital, London, United Kingdom

Posterior capsule opacification (PCO) causes secondary visual acuity loss in a significant number of patients following cataract sur-

gery. Open bag IOL designs permit separation of the anterior capsular bag (AC) and posterior capsule (PC), reducing PCO incidence by limiting growth factor availability relative to traditional closed- bag IOL designs. The current study aimed to determine if vascu-
lar endothelial growth factor (VEGF) signalling was involved in this phenomenon and served as a target for PCO management. Three variants of human in vitro match-paired capsular bag models were employed:

1) Suspended capsular bag models derived from the bag-zonul-lar-ciliary complex, where a capsulorrhesis and lens ex- traction is performed with the capsular bag attached to a silicone ring, were used to assess open-bag IOLs (Anew Zephyr™) in comparison with closed-bag IOLs (Alcon Acrysol®).
2) Cell growth with reduced growth factor availability was evalu-
ated with a non-suspended model, in which the capsular bag was isolated from the ciliary body and secured to a culture dish with pins. Capsular bags were then cultured in either 1.5mL or 6mL of media or exposed to the pan-VEGF inhibitor, Axitinib. (3) Non-suspended capsular bag models were adapted by creating radial incisions in the AC which were then fold-
ed back and secured to a dish (a fully open bag model). Cell growth was observed in all models by phase-contrast microscopy and quantified with image analysis software. Growth factor levels, including VEGF, were employed. Fully open capsular bags implanted with Anew Zephyr™ IOLs relative to those im-

planted with Alcon Acrysol® IOLs. Cell growth was reduced in the non-suspended capsular bags cultured in 6mL SF medium com-
pared to 1.5mL. This was associated with reduced growth factor levels, including VEGF. Fully open capsular bags maintained in 1.5 mL SF media presented a further restriction on growth onto the PC, which was amplified with greater culture volume. Fully open capsular bags exposed to 5% human serum exhibited rap-

cidal growth across the posterior lens capsule, EMT and capsular wrinkling associated with PCO.

Resveratrol Inhibits Fibrotic Events Associated with PCO

Smith, A., Eldred, J., Wormstone, M.

University of East Anglia, Norwich, United Kingdom

Posterior capsule opacification (PCO) is a common complica-
tion that follows cataract surgery. PCO is a fibrotic disease in which the pro-fibrotic cytokine, transforming growth factor beta (TGFβ), is heavily implicated. Lens epithelial cell growth across the posterior lens capsule, epithelial to mesenchymal transi-
tion (EMT) and capsular wrinkling are events associated with PCO currently, there is no effective agent with which to prevent these events and thus development of PCO. Resveratrol (RESV) is a naturally occurring compound that is reported to possess anti-fibrotic properties which could be utilised to aid prevention of PCO. Here, we use the human capsular bag model to investi-
gate the ability of RESV to prevent cell growth across the posteri-
or lens capsule, EMT and capsular wrinkling associated with PCO. Human lens capsular bags were produced by culturing cataract surgery and treated with 10 ng/mL TGFβ2 and/or 30 µM RESV and cultured for 7 days. Conditions were refreshed at day 4. Cell growth across the posterior lens capsule was assessed daily by phase-contrast microscopy and quantified with image analy-
sis software. EMT was assessed by immunocytochemistry of the trans-differentiation marker, αSMA at experimental end-point (day 7). Capsular wrinkling was assessed at experimental end-point by phase-contrast microscopy and quantified with image analysis software. Real-time PCR was employed to assess gene expression. TGFβ2 treatment did not impact cell growth across the posterior or lens capsule compared to untreated controls at experimental models end-point, but did induce significant expression of αSMA and capsular wrinkling compared to untreated controls. Treatment with RESV significantly inhibited cell growth across the poste-
rior lens capsule, but did not alter αSMA expression or capsular wrinkling compared to untreated control capsular bags. However,
RESV treatment in the presence of TGFβ2 significantly inhibited cell growth across the posterior lens capsule, o5α expression and capsular wrinkling compared to capsular bags treated with TGFβ2 alone. RESV also significantly reduced the expression of genes known to be regulated by TGFβ signaling, including MMP1. Resveratrol significantly impaired events associated with PCO in addition to reducing expression of genes involved in this disease. Therefore, resveratrol has demonstrated potential as a therapeutic agent in prevention of PCO.

Pharmacological PCO Prophylaxis - Using the IOL as a Drug Delivery Device
Faculty of Medicine, UMI Munich, Ophthalmology, Munich, Germany

Purpose: The following was to compare drugs proposed for PCO prevention to find more suitable options. Drug delivery is still an issue, as a sufficient concentration must be reached in the eye over a certain period of time. We therefore evaluated different methods to modify the IOL as a drug delivery device.

Methods: FHL-124 was used to determine cell proliferation and toxicity after exposure to the drug using a dye reduction test (XTT). Prescreening preserved substances were soaked into an IOL by a hypersaturated coating solution and hypercritical fluid impregnation, coated to the IOL using PLGA. Those IOLs were tested for their effect on PCO in an anterior-segment model and the human ex vivo capsular bag model. Toxicity on a corneal endothelial cell line (CEC-SV40) was determined. Release kinetics of the substances from the IOL was measured.

Results: The substances inhibited cell growth at the following EC50: caffeic acid phenethyl ester 1.6 ± 0.9 mM, disulfiram 359 ± 33 mM, metrotrexate 980.5 ± 29.7 mM, rapamycin 70.2 ± 14.0 µM, and retinoic acid 1.1 ± 0.12 mM. Long-term inhibitory effects on PCO were estimated to be stable, reaching 40 million in 20 hours. At present, the only method to treat a cataract is by surgical intervention, in which the clouded lens is removed and replaced with a new artificial intraocular lens (IOL) and thus result in restoring a high-quality vision. Currently to manufacture an IOL either a moulding or lathing technique is used, which are associated with some drawbacks and prototype development require a considerable amount of time and may delay the life cycle for new IOL development. Herein, we developed a new fabrication tool for prototyping and development of intraocular lens and devices, using 3D printing technology. We produced a series of new material formulations and established a new fabrication method for the 3D printing of IOLs. The 3D printed IOLs were characterised for morphology, composition, thermal behaviour and biocompatibility. All the formulation resulted in highly transparent, foldable and biocompatible IOL. Moreover, the 3D printed IOLs were also implanted in the human lens capsular bag model using standard injection method. This new fabrication tool could potentially be used for a wide range of different applications and could be used to reduce the cost by eliminating the need for the extensive machining process.

The Role of αvβ8-integrin in Posterior Capsular Opacification (PCO) Pathogenesis
Shihab, M., Wang, Y., Duncan, M., The University of Delaware, Department of Biological Sciences, Newark, United States

Posterior capsular opacification, the major post cataract surgery (PCS) complication has both fibrotic and regenerative features. Fibrotic PCO is driven by activated transforming growth factor β (TGFβ) signaling. However, the mechanisms that activate the TGFβ pathway in IOL capsular tissue is not well understood. Previously we found that αvβ8-integrins are critical for fibrotic PCO pathogenesis. However, the identity of the specific β subunit that heterodimerizes with αv-integrin to drive fibrotic PCO is not known. Thus, we tested the role of β5, β6, and β8 integrins in PCO pathogenesis as all the β subunits upregulate by 48 hours PCS in a mouse cataract surgery model. To test this, homozygous β5 and β6-integrin null mice were subjected to lens fiber cell removal and samples were collected at different time points PCS. However, no changes in fibrotic response were detected in either β5- or β6-integrin null lenses compared to control lenses as measured by the epithelial mesenchymal transition (EMT) marker- a smooth muscle actin (αSMA) ICL. In contrast, mice conditionally lacking β8 integrin from the lens via the action of MLR1Cre (β8ITG cKO), had a block in lens fibrotic response as measured by the lack of upregulation of the common fibrotic markers αSMA, fibronectin and tenascin C suggesting that αvβ8 integrin is the main αv-integrin heterodimer mediating fibrotic PCO. β8ITG cKO lens epithelial cells (LECs) also proliferate less than control LECs by 48 hours PCS. Canonical TGFβ signaling is attenuated in β8ITG cKO LECs at 5 days PCS compared to controls suggesting that this is the underlying cause for the loss of fibrosis in β8ITG cKO mice. Notably, the αvβ8-integrin co-factor, MMP14 can activate latent TGFβ in other systems, and our recent RNAseq showed that MMP14 mRNA levels upregulate 6 fold by 48 hours PCS, the time when TGFβ signaling is first detected in LECs PCS. Immunofluorescent analysis revealed that MMP14 protein starts to deposit around LECs by 48 hours PCS. αvβ8-integrin mediated MMP14 deposition increases as PCO progresses. In contrast, MMP14 deposition is attenuated in β8ITG cKO LECs at 5 days PCS suggesting that αvβ8-integrin and MMP14 may collaborate to drive fibrotic PCO. A αvβ8-integrin is a “druggable” target, the main aim of this study suggests effective therapeutics to prevent fibrotic PCO.

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EGF Potentiates TGF-beta-Induced Epithelial-mesenchymal Transition in Lens by Enhancing EGF Receptor Signaling
Shiu, D.1,2, Lovicu, F.1
1Bouch Institute, The University of Sydney, Anatomy and Histology, Camperdown, Australia, 2Sight Science Institute, The University of Syd- ney, Clinical Ophthalmology and Eye Health, Sydney, Australia

The ocular lens is bathed by a cocktail of growth factors, such as fibroblast growth factor (FGF), bone morphogenetic factor (BMP), insulin-like growth factor (IGF), epidermal growth factor (EGF) and transforming growth factor-beta (TGFβ) that differentially influ- ence cellular behavior. While many of these promote normal behavior, such as EGF, TGFβ is unique in that it also is a potent inducer of lens cell pathology, namely, epithelial-mesenchymal transition (EMT) leading to cataract formation. The present study seeks to decipher how growth factors, such as EGF, impact on TGFβ-induced EMT in the lens. Lens epithelial cells (LECs) in explants prepared from 21-day-old Wistar rats were treated with either 200 pg/ml TGFβ2, 5 ng/ml EGF, or a combination of these, with or without 2-hour pre-treatment with PD153035 (EGFR inhibitor), U0126 (MEK inhibitor) or SI34 (Smad3 inhibitor). Co-treatment with TGFβ2 and EGF resulted in a more pronounced morphological elongation and transdifferentiation of LECs into myofibroblastic cells, with higher protein expression levels of mesenchymal markers (αSMA and tropomyosin) compared with TGFβ2 alone. Addition of EGF to a less potent dose of TGFβ2 (50 pg/ml) induced LECs to undergo EMT similar to treatment with a standard dose of TGFβ2 at 200 pg/ ml within 5 days culture. EGF alone did not induce EMT in LECs. Co-treatment with EGF and TGFβ2 also upregulated unique phosporylation profiles of Smad2/3, ERK1/2 and EGF-signaling that differed to treatment with either growth factor alone. Inhibition of EGF-signaling using PD153035 blocked the EMT response induced by co-treatment with EGF and TGFβ2. Taken together, our data demonstrate that EGF can potentiate TGFβ2 activity to enhance EMT in LECs, further highlighting the importance of EGFR-signaling in cataract formation. By directly blocking EGFR signaling, the activ- ity of both EGF and TGFβ2 can be simultaneously reduced, thereby serving as a potential target for cataract prevention.

AQP0 C-terminal End Truncation Is Critical for Establishing the Refractive Index Gradient in the Lens to Prevent Spherical Aberration
Vardaraj, K., Kumari, S., Stany Brook University, Physiology and Biophysics, Stony Brook, United States

Purpose: Investigate the importance of AQP0 post-translational C-terminal cleavage in establishing lens refractive index gradient. Methods: C-terminal cleavage of AQP0 was characterized in mouse lenses using postnatal day 10 (P10) and adult. Predominant form of C-terminal cleaved AQP0 was determined using SDS-PAGE and immunoblotting with extra-cellular loop anti- body, based on the abundance of the protein in the fiber cells. A genetically engineered Knock-in (KI) mouse model expressing only C-terminal cleaved AQP0 in the fiber cells was developed. Results: C-terminal cleaved form of AQP0 was detected as early as in P10 and in adult lenses. KI lens containing this C-terminal cleaved form of AQP0 (1-246 AA) remained transparent without but cataract under heterozygous condition. However, homozyg- ou KI mouse lens was transparent only until P15 and cataract
development ensued. Both heterozygous and homozygous Ki mice lenses could not establish proper refractive index gradient which resulted in spherical aberration. While spherical aberration occurred as late as six months in adult heterozygous mouse lenses, it was obvious as early as age P15 in homozygous Ki lenses.

**Conclusions:** Data collected using the novel mouse model suggest that gradient loss of C-terminal end of AQP0 which occur at inner cortex and outer nuclear regions of the lens during fiber cell maturation is a programmed post-translational modification directed toward adjusting the refractive index gradient of the constantly growing lens to prevent spherical aberration.

**Lens Channels Regulate Intracellular Hydrostatic Pressure**

White, T.W.,1, Chen, Y.,1, Gao, J.,1, Li, L., Sellitto, C.,1, Mathias, R.T.,1, Donaldson, P.J.2

1Stony Brook University, Stony Brook, United States, 2University of Auckland, Auckland, New Zealand

Lenses maintain an intracellular hydrostatic pressure gradient to drive a flow of intracellular fluid from central fiber cells to surface epithelial cells. The central pressure is normally ~340 mm Hg, while the surface pressure is maintained near 0 mm Hg by a feedback control system that relies on the mechanosensitive channels TRPV4 to sense negative pressure changes, and TRPV1 to sense negative pressure changes in the surface epithelium. The lens is attached to the muscles of the ciliary body by the zonules of Zinn. Contraction, or relaxation, of the ciliary muscle transmits mechanical tension to the lens through the zonules, which could theoretically affect the intracellular hydrostatic pressure gradient. The purpose of this study was to determine if mechanical tension generated by the ciliary muscle regulated the lens intracellular hydrostatic pressure within the intact eye. We measured surface intracellular hydrostatic pressures in mouse lenses while applying tropicamide to relax, or pilocarpine to contract the ciliary muscle. Tropicamide caused a decrease in lens surface pressure that was dependent on intact zonules and could be blocked by inhibition of TRPV4 with HC-067047. Pilocarpine caused a decrease in lens surface pressure that was dependent on intact zonules and could be blocked by inhibition of TRPV1 with A889425, or genetic deletion of the p110α catalytic subunit of PI3K. These results show that the feedback control system for hydrostatic pressure in lens surface cells can be regulated by mechanical tension exerted by the ciliary muscle through the zonules of Zinn. Modulation of the gradient of intracellular hydrostatic pressure in the lens would alter the absolute water content and potentially influence the gradient of refractive index. Supported by NIH grant EY026911.

**Cataract-linked Connexin50 Mutations Cause Misfolding and ER Stress: Approaches to Treatment**

Beyer, E.,1, Leiva, O.,1, Minogue, P.,1, Berthoud, V.1

University of Chicago, Pediatrics, Chicago, United States, 1University of Chicago, Chicago, United States

Mutations of the lens fiber gap junction proteins (connexins, Cx) have been linked to congenital cataracts. Mice Cx50D47A and human Cx50D47N are non-functional connexin mutants that cause dominantly-inherited cataracts. When expressed in cultured cells, both mutants exhibit impaired cellular trafficking and gap junction plaque formation. Lenses of Cx50D47A mice have cataracts, reduced size, drastically decreased levels of Cx50, and less severe reductions of Cx46. The PERK-dependent pathway of the ER response to misfolded proteins is activated, and these lenses have impaired differentiation with retained cellular organelles. Since treatments that enhance protein folding improve trafficking and plaque formation by Cx50D47N and other mutant connexins in vitro, and they are successful therapeutics for other diseases caused by misfolded proteins, we tested the efficacy of the chemi-cal chaperone, 4-phenylbutyric acid (4-PBA) in cultured cells and mice expressing Cx50D47A. 4-PBA treatment increased the formation of Cx50D47A-containing plaques at appositional membranes of tran-siently transfected HeLa cells. Heterozygous Cx50D47A mice were treated with 4-PBA by addition to the drinking water and parenter-al injection of pregnant mice (starting 10 days after pairing of males and females) and their pups. Lenses from 1-month-old mice were examined by darkfield illumination and immunofluorescence microscopy. Protein levels were determined by immunoblotting. Cxaract size and density were not detectably different between the control and the 4-PBA-treated groups. Lens size was not increased following treatment. Levels of Cx46 and Cx50 were significantly increased in 4-PBA-treated lenses compared with saline-treated lenses. Immunofluorescence showed an increased abundance of Cx46 immunoreactivity and puncta. The ratio of phosphorylated to total EIF2α was not altered, and levels of organelar proteins were not significantly reduced, suggesting that the ER response to misfolded proteins and differentiation were not changed. Thus, treatment with 4-PBA improved critical pathological issues in these mice (flow connexin and gap junction abundance), but the magnitude of this recovery (especially for Cx50) was inadequate to impact the reduced size or the opacification of Cx50D47A lenses.

**Functional Organization of AQP0-regulated Membrane Specializations in Lenses Fiber Cells**

Lo, W.-K.,1 Biswas, S.,1 Brako, L.,1 Vorontsova, I.,1 Shilling, T.,1 Hail, J.,1, Nakazawa, Y.,1 Oka, M.,1 Funakoshi-Tago, M.,1, Tamura, H.,1, Takehama, M.1

1Keio University, Faculty of Pharmacy, Tokyo, Japan, 2Yokohama University of Pharmacy, Kanagawa, Japan

Aquaporin 0 (AQP0) is the most abundant membrane protein in lens fiber cells. Although AQP0 is a member of the AQP fami-ly, it has limited water permeability compared with other mem-bers. AQP0 may also have cell adhesion-related functions, but the evidence is still limited. AQP0 contains six transmembrane domains, located intracellularly at both the N- and C-terminal re-gions, resulting in three extracellular loops called A-loop, C-loop, and D-loop and two intracellular loops called B-loop and D-loop. To elucidate the cell adhesion system of AQP0, the region of AQP0 required for cell adhesion was investigated in this study. Using the GST pulldown assay, AQP0 could bind to itself via the C-loop extracellular domain. We also found that 19 Pro and 19 Pro in C-loop region were important amino acids for cell adhesion. However, mu-tation of the C-loop in AQP0 did not affect its water permeability. AQP0 is known to bind lipids in the opposing membrane. Our data suggest that this cell-to-cell adhesion occurs not only in the AQP0/ lipids, but also via AQP0/AQP0 interaction through the C-loop do-main. Mutations in the C-loop amino acids did not affect the wa-ter permeability of AQP0 but did affect its cell adhesion function. Overall, these results support the hypothesis that AQP0 has two functions: cell adhesion and water permeability. Importantly, these dual functions of AQP0 were found to operate independently, which is necessary to maintain lens transparency.

**LEN7 - Aggregation of crystallin and cataract**

The Mutation Studies for Isomerization Hot Spots of Asparagine Residues in Lens αA-Crystallin

Takata, T., Fujiji, N.

Institute for Integrated Radiation and Nuclear Science, Kyoto Uni-versity, Kumatori-cho, Japan

**Purpose:** Recent studies indicated that spontaneous isomer-i-zation and deamidation increased in lens proteins during ag-ing. In this study, we applied mass spectrometry equipped with liquid chromatography (LC-MS/MS) to screen hotspot of isomerization on αA-crystallin (αA-Crys) in aged lenses. The contributions of isomerization at each position were evaluat-ed using a series of mutant αA-Crys. One of mutants, Asp151→Asn151 was utilized to screen factors for inducing sponta-neous reaction intermediate during deamidation/isomerization.
Methods: Each aged lens aggregate fraction was digested with trypsin. All of digestion fragments were applied to LC-MS/MS analysis to identify isomerization hot spots of Asp residues. Asp residues in the hotspot of αA-Crys were replaced by other amino acid using site-directed mutagenesis and recombinantly expressed by E. coli. Those proteins were compared with recombinant wild type αA-Crys. Heatened Asp151 + Asn151 mutant was used for sequential enzymatic digestion, and applied into LC-MS/MS to evaluate the modifications. Results: The results indicated the predominant isomerization of Asp 58 and Asp 151 of αA-Crys in aged lens. Isomerization of glutamate residue proximal to Asp was also confirmed. The substitution of Asp 58 or Asp 151 into other amino acid altered the stability and the specific chaperone function of αA-Crys. Asp151 + Asn151 mutants of αA-crystallin showed very quick deamidation/isomerization to Asp on four Asp isomers. Those formations were very dependent on pH. The major product from in vitro experiment was L-Asp, that was not identical the data from in vivo.

Conclusions: Isomerization hotspots were identified in αA-Crys. The Asp58 was isomerized age-dependently, while Asp151 was isomerized shortly after birth. The contribution from isomerization was different. The Asp151 contributed the flexibility of N-terminal extension of αA-crystallin and aggregate formation, therefore, would have a role for senile cataract formation in aged lens. The Asp151 > Asn151 mutants of αA-crystallin would be useful tool for investigating the spontaneous isomerization of protein. The difference of the ratio of isomers between in vivo and in vitro experiment implied the presence of specific mechanisms for race-
Loss of Fgfr2 function in early mouse lens development results in apoptosis and defective fiber cell differentiation, but simultaneous lacking both FGFR2 and PTEN. Genes in this category include downregulated in FGFR2-alone and upregulated in PTEN-alone genes highly expressed in lens development (according to the systems outcomes. A comparison of FGFR2-regulated genes with can mine complex data sets to reveal important, novel insights sion of 789 genes. Of these 1,448 genes, only 592 (40.9%) remain genes identified three transcription factors (TFs): NKX6-1, EVX2

### Lens Metabolites and Cataractogenesis

**Gong, X., Xia, C.-H.**

University of California at Berkeley, School of Optometry and Vi- sion Science Program, Berkeley, California, United States

The ocular lens relies on intercellular gap junction channels to transport small metabolites (MW < 1200 dalton), ions and water to maintain its transparency. Gap junction channels are mainly composed of connexin 46, encoded by the Gja3 gene, and connexin 50, encoded by the Gja8 gene. The Gja8 knockout (KO) mice develop nuclear cataracts with different severities depending on the strain backgrounds. In this study, metabolic profiling was conducted on lens- es of Gja8 KO and wild type (WT) mice in three different backgrounds including C57BL/6J (B6), 129SvJae (129) and mixed B6-129. Gja3 KO resulted in lens metabolite level changes, and significant differences were observed among different strains. Comparing the global biochemical profiles between KO and WT lenses, several key metabolic differences were revealed. 1) Gja3 KO resulted in reduced lens glutathione (GSH) levels. GSH is believed to be transported through gap junction channels. GSH reduction was observed in KO lenses of all strains. Oxidized glutathione (GSSG) levels were similar between KO and WT. Glutathione is synthesized from cysteine, which is the limiting component in glutathione synthesis. Cysteine levels and other metabolites derived from cysteine were reduced in KO lenses. Taurine and hy- potaurine, which have antioxidant properties, were also decreased in KO lenses. Taurine levels in B6 and mixed B/129/129 strains were higher than in 129 strain KO lenses. This might provide a clue to the antioxidant capacity in those strains with moderate nuclear cataracts. 2) We have further found that methionine was decreased in KO lens- es. A decline in homocysteine and methionine, coupled with a lack of decline in cystathionine may be consistent with more homocyste- in inhibition being utilized in the cystine synthesis transulfuration pathway. 3) KO resulted in reduced levels of glycolysis intermedi- ares. However, glucose uptake and utilization were different among various strains. Sugar alcohols remained relatively consistent despite varying glucose levels in different strains. 4) The sphingolipid levels were affected by the KO differently in var- ious strains. In conclusion, in Gja3 KO lenses, we observed changes of antioxidant levels, altered energy metabolism, as represented by the glycolysis pathway intermediates, and strain-dependent effect of the sphingolipids and phospholipids. These metabolic data will provide new testable hypotheses for examining the roles of gap junction communications in cataract formation.

### Systems biology of the Lens: iSyTE 2.0 Integrated Web Resource Tool for Lens Research

**Lachke, S.**

University of Delaware, Department of Biological Sciences and Cen- ter for Bioinformatics and Computational Biology, Newark, United States

The vertebrate lens has been investigated using cellular, biochem- ical and molecular genetics-based approaches. These efforts, in- volving targeted gene knockouts or knockdowns, have led to major advances in our understanding of the basic biology of lens devel- opment and homeostasis, and have provided key insights into the pathological basis of cataracts. However, the following challenges remain for lens research: (1) the identification of new genes linked to lens development and its associated defects such as cataract is not straightforward, and (2) a global picture of the interactions be- tween the numerous lens regulators to lens biology and pathology is not clear. Here, we describe the development and application of a systems-level web resource tool to address these challenges. In the recent past, genome-level approaches such as high-throughput transcript profiling (transcriptomics) has become increasingly fea- sible due to technologies such as microarrays and RNA sequencing. Their application to interrogate specific eye tissues and cell types holds high promise to impact ocular gene discovery. However, global gene expression profiling has brought with it new challeng- es, such as parsing through the large amounts of data to prioritize select candidates for further analysis. Toward this goal, we have developed and updated the web-resource tool called iSyTE 2.0 integrated Systems Tool for Eye gene discovery (https://research. bioinformatics.udel.edu/iSyTE/). iSyTE 2.0 is based on innovative processing and presentation of whole genome expression datasets for the lens. This integrated approach allows iSyTE 2.0 to effectively predict genes relevant to lens biology. As a proof of principle, iSyTE has greatly expedited gene discovery in the lens, leading to the identification of several new cataract associated genes (Tfrd7, Pmr3, Sep15, Mafg/k), and has contributed to the under- standing of many other important regulatory pathways (e.g. Sp1, n-Myc, etc.). Presently, we are working toward conversion of the wealth of molecular functional data in the lens literature into an in- teractive resource to derive and visualize “evidence-based” GRNs, defined as a circuit map of the regulator-target interactions, which will be discussed. It is anticipated that investigation of these new genes and understudied pathways will advance the etiology of pe- diatric cataracts, and the integration of the lens GRNs into iSyTE will increase its efficacy for ocular disease gene discovery.
Optics of the Lens and Changes with Ageing

Pierscionek, B.1, Hoshino, M.2, Yagi, N.3, Uesugi, K.2, Vorontsova, I.1, Hall, J.1, Regini, L.1
1Nottingham Trent University, School of Science and Technology, Nottingham, United Kingdom, 2Japan Synchrotron Radiation Research Institute (SPring-8), - Kouto, Sayo, Hyogo - Japan, 3UC Irvine, Irvine, CA, United States

The major function of the eye lens, to transmit and refract light, is linked to the distribution and concentration of the structural proteins. It is also synchronised with lenticular biomechanics. The advancement in imaging capacity of the lens is possible because of the presence of severe cataract and b) the dynamics of accommodation. Understanding this maintenance of optical function is necessary if implant lenses with improved image quality, accommodative capacity and adaptation to the eye as it ages, are to be created. Changes in the GRIN with age are an important gauge of certain physiological processes, such as growth linking lens stretching forces, epithelial cell proliferation, and is a result of age-related changes in the optomechanical properties of the lens. While the lens is generally treated as a linear elastic solid, its true behavior is far more complex. Accurate descriptions of these behaviors will give significant insights into the interrelationships between lens cellular architecture, physiology, and biomechanical properties.

Effects of Age-related Changes of Lens Physiological Optics on Overall Vision

Pan, X.1, Vaghefi, E.1, Lee, A.L.1, Donaldson, P.J.2, White, T.W.2
1University of Auckland, School of Optometry and Vision Science, Auckland, New Zealand, 2University of Auckland, School of Medical Science, Auckland, New Zealand, 3Stonybrook University, School of Medicine, NY, United States

Purpose: Vision quality is dependent on the physiological optics of the ocular lens, which in turn depends on its surface geometry and gradient of refractive index (GRIN). In this study, we aim to develop an anatomically and physiologically accurate models of the human eye that include all its optical surfaces and refractive indices, in order to evaluate the effect lens aging has on overall vision quality. Method: 57 participants ranging in age from 18 ~ 86 years old

Actin and Lens Biomechanics

Fowler, V., Cheng, C., Parreno, J.

The Scrigs Research Institute, Department of Molecular Medicine, La Jolla, United States

While the cytoskeleton confers mechanical properties on cells, the interplay between cytoskeletal organization and tissue mechanics may be difficult to assess or muted due to stress shielding from the extracellular matrix. The lens is an ideal tissue to study the relationship between organ function and cell structure. The optical function in focusing light is tied to its biomechanical properties and the lens is almost completely cellular. Actin filaments (F-actin) form diverse architectural networks in cells and tissues that impart cell shape, mechanical stiffness and resilience. F-actin networks are assembled by specialized cross-linking proteins to create tightly packed parallel filament bundles (fimbrin, fascin), loosely spaced parallel filaments (alpha-actinin), and mixed networks with randomly oriented filaments (spectrin), or branched dendritic arrays of filaments (Arp2/3). Tropomyosin (Tpm) bind along the length of F-actin, stabilizing filaments and regulating their interactions with F-actin cross-linking proteins. Here, we studied the function of Tpm in a mouse lens with reduced levels of the major lens fiber Tpm, Tpm3.5. Tpm3.5-depleted mouse lens membranes remain transparent, but are softer and less mechanically resilient, unable to resume their normal shape after release from load. Fiber cell shapes and membrane protrusions are unaffected, but immunostaining for F-actin-binding proteins reveals that Tpm3.5 depletion results in the formation of parallel filament bundles (fimbrin, fascin), loosely spaced parallel filaments (alpha-actinin), and mixed networks with randomly oriented filaments (spectrin), or branched dendritic arrays of filaments (Arp2/3). While incubation of lenses in the presence of GSK (50 mM) an activator of the mechanosensitive kinase TRPV4 caused a decrease in hydrostatic surface pressure that was similar to seen previously in the mouse lens. In LRT experiments Ouabain caused a swelling of the overall lens shape and increase in refractive index particularly at the lens core, both of which contributed to an increase in optical power and a change in the degree of spherical aberration. Using a dual experimental approach on one species of lens we have directly linked the distribution and concentration of the structural proteins to changes in the degree of spherical aberration. Using a dual experimental approach on one species of lens we have directly linked the distribution and concentration of the structural proteins to changes in the degree of spherical aberration. Using a dual experimental approach on one species of lens we have directly linked the distribution and concentration of the structural proteins to changes in the degree of spherical aberration. Using a dual experimental approach on one species of lens we have directly linked the distribution and concentration of the structural proteins to changes in the degree of spherical aberration. Using a dual experimental approach on one species of lens we have directly linked the distribution and concentration of the structural proteins to changes in the degree of spherical aberration. Using a dual experimental approach on one species of lens we have directly linked the distribution and concentration of the structural proteins to changes in the degree of spherical aberration. Using a dual experimental approach on one species of lens we have directly linked the distribution and concentration of the structural proteins to changes in the degree of spherical aberration.
Acylation of Lens Proteins Improves the Chaperone Activity of α-crystallin

Phosphorylation of αA-crystallin on S/T148 residue was inversely one activity of αA-crystallin was tested using those same constructs. One activity of αA-crystallin was tested using those same constructs. An activity of αA-crystallin was tested using those same constructs.

Peptide Chaperones: Effect of D-Amino Acids

Sharma, K.1, Phadte, A.1, Puttur, S.1

1University of Michigan School of Medicine, Ophthalmology and Biochemistry, Columbia, United States, 2University of Michigan School of Medicine, Ophthalmology, Columbia, United States

Previously we reported chaperone-like activity in a 19-mer peptide composed of L-amino acids representing the chaperone site in αA-crystallin and called it αA-mini-chaperone (J. Biol. Chem. 275, 3767-3771). Recently we showed that incorporation of a cell-permeating sequence to the chaperone peptide enhanced the cellular uptake and preserved the chaperone activity (Adv. Biosys. 2018, 2[1], 1700095). However, it is likely that the cell penetrating peptide or the αA-mini-chaperone will have a short in vivo half life because of its α-helical composition and susceptibility to proteolytic cleavage. This study was undertaken to investigate whether peptides having both D- and L- amino acids display chaperone-like activity. A new peptide (LDP-1), based on the active cell penetrating chaperone peptide but having both D- and L- amino acids was synthesized and tested for chaperone-like activity using unfolding alcohol dehydrogenase (ADH) assay. Far-UV CD profile of LDP-1 peptides was also recorded to determine the secondary structure of the new peptide. The results showed that LDP-1 has 20-25% greater chaperone-like activity compared to the αA-mini-chaperone. However, the αA-mini-chaperone that shows predominant β-sheet structure during far-UV CD analysis, the LDP-1 showed no characteristic CD profile. Therefore, the present study results show that propensity to form β-sheet is not required to possess chaperone-like activity in a peptide and incorporation of D-amino acids to the sequence of chaperone peptide does not diminish the chaperone-like activity. Thus, it is possible to develop peptide chaperone that would resist proteolytic cleavage and will have the potential to become a therapeutic agent of longer half-life.

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ORAL PRESENTATIONS
Lens
Phosphorylation of AlphaA-crystallin on T148 Directly Controls its Chaperone Activity and its Protective Function

Fort, P.1, Ruescas, A.1, Phadte, A.1, Sharma, K.1, Schey, K.1

1University of Michigan - Kellogg Eye Center, Ann Arbor, United States, 2University of Miami, Miami, United States

Numerous post-translational modifications have been reported as targetting alpha-crystallins during aging and diseases, some of which have been shown to significantly affect their chaperone function. This study focused on the regulation of the chaperone activity and protective function of αA-crystallin by its phosphorylation on S/T148, a site recently shown to be highly affected by diabetes.

Ocular tissues from diabetic animals and human donors, as well as cell culture systems, were used to assess the regulation and downstream effect of T148 phosphorylation on neuronal and glial cell functions. Retinal neurons and Müller glial cells were transfected with plasmids encoding either wild-type (WT), phosphorylatable (1484D), or non-phosphorylatable mutants (1484A) of αA-crystallin on the 1484 residue. Markers of the unfolded protein response were analyzed under normal or metabolic stress conditions. Additionally transfected Müller cells were also analyzed for activation and expression of inflammatory molecules, while the protective potential of the conditioned media from those same cells were tested on retinal neurons. Finally, the impact of this phosphorylation on the chaperone activity of αA-crystallin was tested using those same constructs. Phosphorylation of αA-crystallin on S/T148 residue was inversely proportional to ER stress under diabetic condition. Overexpression of the αA-crystallin phosphomimetic mutant protects neurons and to a lesser extent glia from metabolic stress conditions, while the non-phosphorylatable mutant does not (p < 0.01). αA-crystallin and its phosphorylation specifically regulates the PERR-associated branch of ER stress in neurons, but not in glia. Alternatively, in glia,

ENOL10 - Chemical modifications and peptides of crystallins in lens

Ray Tracing Confirms the Optical Properties of the Bovine Are Actively Maintained by the Lens Microcirculation System

Qiuy, C.1, Heilman, B.1, Donaldson, P.1, Vaghefi, E.2

1University of Auckland, Department of Physiology, School of Medical Sciences, Auckland, New Zealand, 2University of Miami, Miami, United States

Using a variety of MRI modalities and optical modelling software we have shown that the lens geometry and the gradient of refractive index (GRIN), which together determine the optical properties of the ocular lens, are actively maintained by the lens microcirculation system.[1] Based on this series of experiments we have proposed that monitoring the optical properties of lenses could serve as a good indicator at the whole tissue level of the underlying state of the cellular physiology of the lens. To investigate this hypothesis we have developed a novel laser ray tracing (LRT) system and subsequent image processing protocols that enable us to measure lens geometry and GRIN of isolated bovine lenses maintained in organ culture.[2] We then used this system to monitor the time course of changes to lens geometry, GRIN and power in bovine lenses organ cultured in the absence and presence of reagents known to alter the cellular physiology of the lens. Lenses cultured in Artificial Aqueous Humor (AAH) for up to 4 hours remained a constant power, geometry and GRIN over time. In contrast, lenses incubated in a high concentration of K+ (AAH-High K+), which inhibits the microcirculation system by depleting the lens potential, exhibited a rounded in lens geometry but a decrease in GRIN, which resulted in a small but significant increase in overall power. A similar effect on lens geometry, GRIN and power was observed in lenses perfused with 100% CO2, which by altering lens pH closes gap junctions in the outer cortex of the lens. In contrast, incubating lenses in ouabain (1mM), to block the Na/KATPase that drives the lens microcirculation, caused a more pronounced rounding of lenses, a shape increase in GRIN that was more prominent in the lens core relative to the outer cortex, and a substantial increase in overall power. Using LRT to directly measure lens optics we have confirmed earlier MRI experiments that show the microcirculation system actively controls the optical properties of the lens. Furthermore, we have shown that inhibiting different components (lens potential, gap junctional conductance and Na+ pump current) differentially affects lens geometry and GRIN the components that determine lens power. [1] Vaghefi et al., Invest Ophthalmol Vis Sci. 56:7195-208., 2015 [2] Qiuy, et al., Biomed. Opt. Express, 8:4947-4964, 2017.
a-α-crystallin phosphorylation on S/T148 regulates the activation and expression of inflammatory molecules. Interestingly, in vitro assays further demonstrated that mutation of the T148 was specifically associated with alterations of the chaperone activity of a-α-crystallin. Our results strongly support our hypothesis that phosphorylation on T148 controls the protective function of a-α-crystallin. They also show that this phosphorylate while in general regulating the chaperone activity of the protein, regulates different mechanisms in different cell types. Indeed it directly regulates neuronal survival through regulation of ER stress, while it regulates glial cells expression and secretion of inflammatory and protective factors.

Neuroprotective Potential of Peptain-1 for Glaucoma.
Stankiewska, D.L.1, Nam, M.H.-I., Nahomi, R.B.2, Chaphalkar, R.M.1, Krishnamoorthy, R.R.1, Nagara, R.H.1
1UNT Health Science Center, Pharmacology and Neuroscience, NTHI, Fort Worth, United States, 2University of Colorado School of Medicine, Department of Ophthalmology, Aurora, United States
Purpose: To determine if Peptain-1, the cell permeable core peptide derived from small heat shock protein αB-crystallin could inhibit retinal ganglion cell (RGC) death in rodent models of glaucoma. Methods: Primary rat RGCs and rat adult retinal explants were exposed to either normoxic or hypoxic conditions in the presence of Peptain-1 or scrambled peptide (12.5 µg/ml), following which RGC survival was assessed. Brown Norway rats were i.p. injected in one eye using the Morrison’s method, while the corresponding contralateral eye served as the controls. The rats were intraperitoneally (i.p.) 3h injected 10 µg of Peptain-1 three times per week for five weeks. Retrogradely labeled RGCs were counted in retinal flat mounts and axon counts were performed following standard optic nerve Immunohistochemical analysis of cytochrome c oxi- dase complex 6/2 (Cox6B2) was carried out in the retinas. Ischemia reperfusion (IR) injury was carried out in C57BL/6 mice by elevating the IOP to 80 mmHg for 30 min, followed by rapid reperfusion. Peptain-1 (1µg) was given three times per week immediately after the procedure and then once daily for 14 days post IR injury and surviving RGC counts were performed following Brn3a staining. In separate experiments, mice were i.p. injected with 5 µg of Peptain-1 /Cy7 to determine its ability to cross the blood retinal barrier. Results: Peptain-1 treatment decreased by (60%) hypoxia-induced primary RGC death compared to cells treated with a scrambled peptide (p< 0.001). Peptain-1 also significantly (p< 0.001) enhanced RGC survival (3.2 fold) in hypoxic retinas compared to untreated controls. Intraperitoneal injections of Peptain-1 significantly inhibited RGC death (p< 0.05) and decreased axonal loss (p< 0.02) in the Morrison’s model of glaucoma in rats following five weeks of IOP elevation and showed increased Cox6B2 levels, compared to those treated with the scrambled peptide. Peptain-1 treatment enhanced RGC survival by 50% (p< 0.01) after IR injury, compared to those treated with the scrambled peptide. Peptain-1/Cy7 was detected in the retina following its i.p. injection. Conclusions: The i.p injected Peptain-1 exhibits soma-, axo- and mito-protective properties, which could facilitate neuroprotection against insult that cause RGC death in rodents. Peptain-1 has the ability to penetrate the blood-retinal barrier indicating its potential to be developed as a neuroprotective agent for glaucoma.

The Crystal Structure of the Disulﬁde-linked α-Crystallin Dimer Provides Insight into an Aggregation-prone Oxidation Product Associated with Catarrhous Lenses
Grosas, A.B.1, Thorn, D.C.1, Mabbitt, P.D.1,2, Ray, N.J.1, Jackson, C.J.1,2, Carver, J.A.1
1Australian National University, Canberra, Australia, 2University of Dundee, Dundee, United Kingdom
α-Crystallin (CRYA) is a soluble structural protein present in high concentrations within the lens and conventionally is considered to be monomeric protein. It is largely responsible for maintaining the lenticular hydration, hence contributes to lens rigidness apart from its other roles such as chaperone activity of the protein, regulates different mechanisms in different cell types. Indeed it directly regulates neuronal survival through regulation of ER stress, while it regulates glial cells expression and secretion of inflammatory and protective factors.

COS1 - Advances in biomaterial technology and cell based therapies for corneal regeneration
Lagali, N.1, Xeroudaki, M.2, Thangavelu, M.1, Fagerholm, P.1, Raffat, M.1,3
1Linköping University, Linköping, Sweden, 2Linköping University, Ophthalmology, Linköping, Sweden, 3Linköping University, Biomedical Engineering, Linköping, Sweden, 4LinkCare Life Science AB, Linköping, Sweden
Due to a severe shortage in human donor tissue for transplantation and due to the high standards required to maintain tissue banks and access to donor corneas, alternatives are sought to meet the high global demand for implantable corneal tissue. Moreover, patient safety and predictability of the postoperative outcome after tissue implantation is paramount. To address these demands, a new implantable corneal stromal extracellular matrix has been developed. This double-crosslinked bioengineered porcine construct (BPDC0) has been engineered from high purity collagen to withstand corneal surgery, wound healing, and long term implantation. The BPDC0 is guaranteed to be completely free of all cellular components, thereby avoiding immunologic rejection. The stromal response in several models and clinically indicates that the material is biocompatible, stable, and retains its transparency post-implantation. Moreover, the corneal thickness and refractive properties can be tuned by suitable choice of biomaterial size and shape, in order to achieve the best possible visual outcomes in cases of corneal stromal disease such as scarring, dystrophy and advanced keratoconus. A new intra-stromal implantation procedure additionally provides a minimally-invasive surgery for implantation of the BPDC0 that avoids more invasive lamellar or penetrating keratoplasties, also avoiding suturing and long-term postoperative immunosuppression.

Making Corneas from Corneas
Ahearn, M.1
Trinity College Dublin, Trinity Centre for Bioengineering, Dublin, Ireland
Continuous advances in biomaterials have allowed a new generation of artificial corneas and tissue engineered corneal scaffolds to be developed. One issue with many of these biomaterials is that they lack many of the matrix proteins, signalling molecules and biochemical cues found in real corneal tissue particularly in the stroma. These extracellular matrix components can play a vital role in modulating how cells interact with the biomaterial and their presence has been shown to assist in tissue regeneration. For these reasons we have been examining the use of corneal extracellular matrix as a biomaterial for developing scaffolds suitable for corneal regeneration. One approach to doing this is to decellularize porcine corneal tissue for use as a scaffold. This approach has the advantage of providing an extracellular matrix composition, cell type and the collagen fibril architecture of the stroma. Different decellularization techniques have been explored as have different methods of incorporating human cells into the scaffolds. An alternative approach for generating scaffolds suitable for corneal regeneration is to fabricate hydrogels using corneal derived extracellular matrix. The physical and biochemical properties of these hydrogels have been characterized and the response of cells embedded into these hydrogels has been examined. The final approach that has been investigated is to incorporate corneal extracellular matrix into eletrop spun polymer fibers. This method allows scaffolds to be manufactured that contain the matrix molecules while also mimicking the native corneal fibril structure. The advantages and limitations of each approach shall be discussed and future applications shall be explored.

Initial Experience with Acellular Porcine Corneal APC Deep Anterior Lamellar Keratoplasty
Balidis, M.
Ophthalmic Eye Institute, Thessaloniki, Greece
Corneal blindness is a major cause of vision loss, estimated to affect over 10 million people worldwide. Once impaired through clouding or shape change, the best treatment option for restoring vision is corneal transplantation using full or partial thickness cadaveric grafts. However, donor corneas are globally limited and face rejection and graft failure, similar to other transplantable organs. Thus, there is a need for viable alternatives to donor corneas in order to increase supply, reduce rejection, and to minimize variability in tissue quality. To address this, researchers have developed new materials and strategies to tissue engineer or partial thickness corneal grafts in order to repair, regenerate, or replace the diseased cornea. A corneal porcine corneal (APC) was first transplanted in China in 2005 and replanted to replace infected anterior corneas in 17 patients with fungal corneal infections. Safety and efficacy of APC demonstrated in human keratoplasty for a follow-up period of up to 36 months. Epithelialization occurred in 43 APC grafts. Furthermore, most porcine corneal infections. Safety and efficacy of APC demonstrated in human keratoplasty for a follow-up period of up to 36 months. Epithelialization occurred in 43 APC grafts. Furthermore, most porcine corneal infections. Safety and efficacy of APC demonstrated in human keratoplasty for a follow-up period of up to 36 months. Epithelialization occurred in 43 APC grafts.
The Role of Substrate Curvature on Corneal Stromal Cell Behavior

Gouveia, R., Connon, C.
Newcastle University. Institute of Genetic Medicine, Newcastle-upon-Tyne, United Kingdom

Substrate topography is an important regulator of cell phenotype. However, despite its potential relevance, not much is known about the impact of millimeter-scaled (tissue-size) surface geometries on cell behavior. This is especially important in the cornea, where curvature is fundamental for the tissue’s function. To this purpose, we have developed and characterized novel micro-topographical templates that allowed exploring the impact of curved surface geometries on the migration, proliferation, and organization of human corneal stromal cells. Interestingly, these curved templates induced cells to migrate and organize in very precise orientations, and to subsequently produce large amounts of stromal-characteristic extracellular matrix (i.e., comprising highly aligned collagen fibrils in the well-defined, three-dimensional micro-architecture of the native organ). This demonstrated that curvature was sufficient to induce the alignment of human corneal stromal cells, and consequently, promote the bio-fabrication of stromal tissue equivalents with cornea-like shape and composition. Compared to tissues produced in vitro using traditional planar templates, curved tissues were shown to better reproduce the optical, structural, mechanical, and compositional characteristics of the native cornea. Importantly, such bottom-up bio-fabrication strategy assured these curved tissues were solely comprised by human-derived stroma cells and matrix, and the scaffolds and regained expression of keratocyte marker and high risk of rejection side-effects. Mesenchymal stem cells (MSCs), being multipotent and immunomodulatory in nature, appear to provide better alternatives. To explore the feasibility, here we used in vitro cultured human MSCs derived from dental pulp (DPSC), adipose (ADSC), and umbilical cord (UC-MSC) tissues. In the vitro cultured murine fibroblasts (NIH-3T3) and primary human limbal/corneal epithelial cells were used as positive and controls, respectively. Source-dependent variations were observed in the expression of mesenchymal stem cell markers (CD90, CD105, and Vimentin) and immunomodulatory molecules (TGFβ, and TGF-β), through immunofluorescence, western blotting, and immunomodulatory approaches that may be used to prevent graft rejection in high-risk corneal transplantation will be introduced.

Corneal Substitutes from Decellularized Porcine Tissues

Fernández-Pérez, J.1,2, Ahearn, M.1,2
1Trinity College Dublin, Trinity Center for Bioengineering, Dublin, Ireland, 2Trinity College Dublin, Department of Mechanical and Manufacturing Engineering, Dublin, Ireland

To overcome donor shortage for corneal transplantation, alternatives based on tissue engineering and cell therapy are being researched. Biomaterials used as corneal substitutes need to be transparent, allow for endogenous cell repopulation and have enough mechanical strength to be sutured. A promising biomaterial is decellularized tissue, as it maintains most of the microstructure within the tissue and matches native mechanical strength. In the present study, decellularized porcine corneas were repopulated with human, and positive and negative controls, respectively. Source-dependent variations were observed in the expression of mesenchymal stem cell markers (CD90, CD105, and Vimentin) and immunomodulatory molecules (TGFβ, and TGF-β), through immunofluorescence, western blotting, and immunomodulatory approaches that may be used to prevent graft rejection in high-risk corneal transplantation will be introduced.

Corneal Topography or a Failed Previous Graft. These conditions frequently lead to increased blood and lymphatic vessel infiltration to the recipient graft bed and some pre-existing high immunological risk. As outcomes for lower risk cornea transplant recipients, such as those who require a transplant as a result of keratoconus, improves the field is shifting its focus to improve outcomes for more complex cases. In this presentation we will discuss the therapeutic potential of MSC to promote corneal transplantation survival in both low- and high-risk pre-clinical models and its pathway to potential clinical translation.

CORNEAL AND OCULAR SURFACE

Mesenchymal Stem Cells for Corneal Epithelial Regeneration: Source Dependent Variations and Status of Transdifferentiation

Shukla, S.1, Naik, G.2, Kacham, S.3, Parcha, S.R.1, Chauhan, B.1, Sangwan, V.S.1,2,4

Scheepens Eye Research Institute, Mass. Eye and Ear; Harvard Medical School; Boston, United States, 1L. V. Prasad Eye Institute, Prof. Brian Holden Eye Research Centre, SSRI Stem Cell Laboratory, Hyderabad, India, 1National Institute of Technology, Warangal, Biotechnology, Warangal, India, 2Taj Kohli Cornea Institute, L V Prasad Eye Institute, Hyderabad, India

Substrate topography is an important regulator of cell phenotype. However, despite its potential relevance, not much is known about the impact of millimeter-scaled (tissue-size) surface geometries on cell behavior. This is especially important in the cornea, where curvature is fundamental for the tissue’s function. To this purpose, we have developed and characterized novel micro-topographical templates that allowed exploring the impact of curved surface geometries on the migration, proliferation, and organization of human corneal stromal cells. Interestingly, these curved templates induced cells to migrate and organize in very precise orientations, and to subsequently produce large amounts of stromal-characteristic extracellular matrix (i.e., comprising highly aligned collagen fibrils in the well-defined, three-dimensional micro-architecture of the native organ). This demonstrated that curvature was sufficient to induce the alignment of human corneal stromal cells, and consequently, promote the bio-fabrication of stromal tissue equivalents with cornea-like shape and composition. Compared to tissues produced in vitro using traditional planar templates, curved tissues were shown to better reproduce the optical, structural, mechanical, and compositional characteristics of the native cornea. Importantly, such bottom-up bio-fabrication strategy assured these curved tissues were solely comprised by human-derived stroma cells and matrix, and the scaffolds and regained expression of keratocyte marker and high risk of rejection side-effects. Mesenchymal stem cells (MSCs), being multipotent and immunomodulatory in nature, appear to provide better alternatives. To explore the feasibility, here we used in vitro cultured human MSCs derived from dental pulp (DPSC), adipose (ADSC), and umbilical cord (UC-MSC) tissues. In the vitro cultured murine fibroblasts (NIH-3T3) and primary human limbal/corneal epithelial cells were used as positive and negative controls, respectively. Source-dependent variations were observed in the expression of mesenchymal stem cell markers (CD90, CD105, and Vimentin) and immunomodulatory molecules (TGFβ, and TGF-β), through immunofluorescence, western blotting, and immunomodulatory approaches that may be used to prevent graft rejection in high-risk corneal transplantation will be introduced.

 COS2 - Corneal transplantation and immunomodulation

The Taming of the Shrew? Modulation of the Immune Response in Penetrating Keratoplasty. From the Past to the Future

Player, U. VISCORST Study Group
Charité, University Medicine Berlin, Berlin, Germany

Corneal transplantation remains the most frequently performed transplantation procedure in man. Even when the need for full thickness transplantation has eased over the last few years due to advances in surgery techniques, there are still many patients requiring full thickness transplantation particularly in the high-risk setting. Although keratoplasty is also considered as the most successful transplantation procedure, several studies indicate that the long-term survival of corneal grafts is significantly lower than that of transplantations performed in the past. Despite the immune privilege enjoyed by the cornea and anterior segment of the eye, immunological graft rejection is a major limitation to corneal transplantation. This presentation will provide an update on immunomodulatory agents most commonly used for high-risk corneal transplantation. Benefits and risks will be discussed and novel immunomodulatory approaches that may be used to prevent graft rejection in high-risk corneal transplantation will be introduced.

Mesenchymal Stem Cell Therapy to Promote Corneal Allograft Survival - From Bench to Bedside

Ritter, T.1,2, Lohan, P.1,2, Treacy, O.3, Murphy, N.1, Lynch, K.1, Chen, R.1, Marcos, M.1, Griffin, M.1,2, Ryan, A.1,2
1National University of Ireland Galway, Discipline of Medicine, Galway, Ireland, 2National University of Ireland Galway, Centre for Pharmacology and Therapeutics, Galway, Ireland

Corneal transplantation has been a target for mesenchymal stromal/stem cell (MSC) therapy for several years with a number of publications highlighting the therapeutic potential and mechanism of action of these cells. It is widely known that MSC posses potent anti-inflammatory capabilities and are able to modulate the activity of a wide array of immune cells including T cells, B cells, dendritic cells and macrophages. This modulation occurs through both cell-cell contact and the paracrine release of soluble factors such as nitric oxide and prostaglandin E2. High risk corneal transplant recipients suffer from a much higher rate of rejection than the average patient. Factors which place patients at high risk of rejection include corneal ulcers, bullous keratopathy or a failed previous graft. These conditions frequently lead to increased blood and lymphatic vessel infiltration to the recipient graft bed and some pre-existing high immunological risk. As outcomes for lower risk cornea transplant recipients, such as those who require a transplant as a result of keratoconus, improves the field is shifting its focus to improve outcomes for more complex cases. In this presentation we will discuss the therapeutic potential of MSC to promote corneal transplantation survival in both low- and high-risk pre-clinical models and its pathway to potential clinical translation.
It Gets Nerves to Control Ocular Surface inflammation

Ferrari, G.
San Raffaele Scientific Institute, Ophthalmology, Milano, Italy

Ocular surface inflammation is an area of critical and unmet medical need. Most of the available treatments aim to block inflammatory cell infiltration, inhibit specific pro-inflammatory cytokines, or to induce an anti-inflammatory milieu by means of biological medications. The most densely innervated tissue in the body, the cornea depends on proper nerve function for adequate wound healing. We previously showed that acute denervation induces inflammation and angiogenesis in the cornea. On the other side, inflammation in the cornea invariably impairs corneal nerve activity and/or morphology. Among the many nerve-released substances, Substance P is particularly interesting as its expression is significantly increased in clinically inflamed human eyes. Specific inhibition of the Neurokinin 1 Receptor Antagonist-the main receptor for Substance P-significantly inhibited inflammation and angiogenesis in the cornea. In addition, inflammation on the ocular surface is rapidly transmitted to the brain, possibly through release of Substance P by trigeminal nerves. Controlling the activity of specific neurotransmitters is an attractive new option for the treatment of ocular surface inflammation.

Sub-anticoagulant Dose Heparin is a Potential Therapy for Inflammation and Ocular Surface Disease in Dry Eye and oGVHD

Jain, S.1,2, Mun, C.1,2
University of Illinois at Chicago, Ophthalmology, Chicago, United States

Purpose: We have reported that neutrophil extracellular traps (NETs) can contribute to ocular surface pathologies in patients with Dry Eye and ocular graft-versus-host disease (oGVHD). Our purpose is to investigate whether disrupting NETs with heparin has therapeutic potential in oGVHD.

Methods: We performed in vitro experiments using cell lines (corneal epithelial cells and conjunctival fibroblasts) in scratch wound models, and in vivo experiments using murine corneal epithelial models, and compared the effects of NETs with heparinized NETs. Heparin eye drops (100 IU/mL) twice a day were given to a patient to treat severely symptomatic oGVHD.

Results: Incubation of NETs (generated from isolated peripheral blood human neutrophils) with sub-anticoagulant dose of Heparin (100 IU/mL) disrupted NETs as evidenced by the loss of Sytox green stained extracellular DNA strands and reduced levels of histone-associated DNA fragments (which represent intact NETs). Heparin 100 IU/mL dismantled NETs in mucocellular aggregates collected from oGVHD patients. Sub-anticoagulant dose of Heparin (100 IU/mL) did not delay epithelial wound closure whereas anti-inflammatory doses of heparin (1,000 IU/mL and 10,000 IU/mL) showed significant dose dependent delay in epithelial wound closure. LOH assay was performed using supernatant to determine cytotoxicity caused by NETs compared to RPMI culture media whereas significant cytotoxicity was observed with heparin 1,000 IU/mL and 10,000 IU/mL. NETs delayed epithelial healing; promoted fibroblast proliferation and myofibroblast transformation and enhance allogeneic T-cell proliferation. Dismantling with sub-anticoagulant dose heparin abrogates NET-induced epithelial, fibroblast and T-cell effects, Heparin eye drops (100 IU/mL) twice a day were given to a patient with oGVHD who was severely symptomatic despite topical therapy. After one month of heparin therapy, symptoms, as determined by OSDI, reduced from 67.5 to 19.4. Corneal staining decreased from 6/15 to 3/15 and conjunctival staining decreased from 4/6 to 1/6.

Conclusion: Sub-anticoagulant dose heparin (100 IU/mL) eye drop treatment has therapeutic potential in Dry Eye and oGVHD. Our data makes a case for controlled clinical trials to investigate the efficacy of heparin eye drops to prevent or treat Dry Eye and oGVHD.

Novel Approaches to Control Immunopathogenesis Associated with herpes Stromal Keratitis (HSK)

Sivas, S.
Wayne State University School of Medicine, Detroit, United States

Herpes Stromal Keratitis (HSK) is a chronic immunoinflammatory condition that develops in the corneal stroma in response to re- current corneal infection with herpes simplex virus-1 (HSV-1). Clinical manifestations of HSK involve the development of opacification and neovascularization in infected corneas. Mouse model has long been understood to pathogenesis of HSK, so that novel therapeutic approaches could be developed to ameliorate the severity of HSK. Our lab focus is to understand the cellular and molecular events involved in the pathogenesis of HSK. At cellular level, we recently demonstrated that IL-2 and IL-2 antibody complex treatment given prior to corneal HSV-1 infection resulted in an inhibition of inflammation. This study also showed the development of severe HSK lesions in a mouse model. An increased number of Foxp3 Treg in draining lymph nodes of HSV-1 infected mice reduced the influx of CD4 T cells in HSV-1 infected corneas. However, expansion of 1Treg during the progression of HSK lesions failed to reduce the severity of HSK. These results showed that increasing Foxp3 Treg cells in secondary lymphoid tissue during T cell priming stage in infected mice is more efficacious in controlling the severity of HSK. At molecular level, we documented the role of neurokinin-1 receptor (NK1R) in regulating the pathogenesis of HSK. NK1R is the higher affinity receptor of Substance P (SP) neuropeptide. Our results showed that administration of NK1R antagonist, spantide I, into HSV-1 infected corneas during the clinical disease period reduced the severity of HSK. However, NK1R knockout mice in comparison to B6 mice developed more severe HSK. Interestingly, an increased level of SP was detected in uninfected corneal (cornea and conjunctiva) tissue of NK1R−/− than control B6 mice. This was associated with mast cell degranulation and the massive accumulation of inflammatory immune cells near the limbal region of uninfected corneas of NK1R−/−, but not PPTA−/− mice. These results suggest NK1R independent action of SP could also promote inflammation at the ocular surface.

Together, our results suggest that a better understanding of the cellular and molecular events in HSV-1 infected corneas may lead to the development of novel immunomodulatory therapeutics to mitigate vision loss associated with HSK.

KLF4 and TGF-β Superfamily Crosstalk in Corneal Epithelial Homeostasis

Swamynathan, S.K., Tiwari, A., Alexander, N., Gnanial, J., Swamynathan, S.
University of Pittsburgh, Ophthalmology, Pittsburgh, United States

Previously, we reported that Krüppel-like factor-4 (KLF4), one of the most highly expressed transcription factors in the mouse corneal epithelial (CE) cells, maintains CE homeostasis by regulating their proliferation, and differentiation. KLF4 maintains CE identity by suppressing the expression of mesenchymal-specific genes, and promotes epithelial-specific genes. As both TGF-β and BMP-4 are known to induce epithelial mesenchymal transition (EMT), here we test the hypothesis that KLF4 contributes to CE homeostasis by modulating the expression of TGF-β and BMP-4. We ablated KLF4 gene in a spatiotemporally regulated manner within the adult CE by feeding ternary transgenic KLF4−/− (Klf4Δ/ΔCE; Krt12rtTA/rtTA; Tet-O-Cre) mice with doxycycline-chow for a month. Age and sex matched littermates fed with normal chow served as wild type (WT) controls. Expression of TGF-β1, TGF-β2, TGF-β1, TGF-β2, BMPA, SMAD2/3, SMAD1/5 and SMAD4 in WT and KLF4−/− corneas was examined by Q-PCR and immunofluorescent staining. KLF4−/− cells displayed elevated expression of TGF-β1 and TGF-β2, TGF-β1, TGF-β2, BMPA, SMAD2/3, SMAD1/5 and SMAD4 in WT and KLF4−/− corneas was examined by Q-PCR and immunofluorescent staining. KLF4−/− cells displayed elevated expression of TGF-β1 and TGF-β2, TGF-β1, TGF-β2, BMPA, SMAD2/3, SMAD1/5 and SMAD4 in WT and KLF4−/− corneas was examined by Q-PCR and immunofluorescent staining.
Cornea and Ocular Surface

COSA - Advances in corneal crosslinking

Corneal Cross-linking Solutions: A Better Way to Strengthen the Cornea for Keratoconus?

Paik, D.1, Zaybitiskaya, M.1, Jayossy, C.1, Chen, C.1, Takaoa, A.1, Myers, K.1, Suh, L.1, Trokel, S.1
1Columbia University, Ophthalmology, New York, United States
2Columbia University, Mechanical Engineering, New York, United States

Purpose: Topical therapeutic tissue cross-linking solutions offer an alternative method for tissue strengthening that can avoid the use of UV irradiation and painful epithelial debridement. Studies are being undertaken in order to identify conditions and delivery methods suitable for patient care use.

Methods: Corneal therapeutic tissue cross-linking (ctXL) using topical solutions containing sodium hydroxymethylglycinate (SMG) at 40 or 80mM was carried out on Dutch-belted rabbits [n=8] using 1.1% hydroxypropyl methylcellulose (HPMC) [viscosity < 15 CP]. SMG eyedrops (right eye) and vehicle control (left eye) were applied to the corneal surface 1-3 times/day for up to 3 months. In-termittent bolus dosing (every 15 min for 1 or 3.5 hours followed by 2 hours) was also carried out on two animals. The animals were evaluated in real-time using ultrasound pachymetry, applanation tonometry, confocal microscopy (HRT-3-RCM), corneal topography, and fluorescent epithelial staining. Post-mortem cross-linking effects were evaluated by mechanical inflation testing and thermal denaturation temperature, and routine histology was also included.

Results: The higher 80mM concentration caused some irritati-on and drying of the eye within 3 months. Of 2 animals, the 40mM group showed corneal stromal changes in 3 of 4 rabbits. The 40mM group showed corneal thickness, corneal water content, and corneal epithelial cell density were decreased (but not statistically significant) in one of 4 rabbits. None of the changes were noted in the 80mM group, and the higher 80mM concentration caused some irritati-on and drying of the eye within 3 months. Of 2 animals, the 40mM group showed corneal stromal changes in 3 of 4 rabbits. The 40mM group showed corneal thickness, corneal water content, and corneal epithelial cell density were decreased (but not statistically significant) in one of 4 rabbits. None of the changes were noted in the 80mM group.

Non Linear Corneal Collagen Cross Linking (NLO CXL)

Jester, J.1, Bradford, S.1, Mikula, E.1, Brown, D.1, Kim, S.W.2,3, Pearlman, E.1, Juhasz, T.1
1UC Irvine, Ophthalmology and Biomedical Engineering, Irvine, United States
2UC Irvine, Ophthalmology, Irvine, United States
3Yonsei University, Ophthalmology, Wonju, Korea, Republic of Korea

Purpose: Mechanical stiffening of the cornea through corneal crosslinking (CXL) using ultraviolet light and riboflavin to generate covalent bonds between collagen molecules has been established as a treatment for corneal ectatic disorders. However, the need for photosensitizers is a disadvantage of this approach. To address this issue, we have evaluated a non-linear optical (NLO) femtosecond laser approach to produce highly localized, tissue-specific photochemical crosslinks in the cornea that can be achieved at different depths and geometric patterns. Using a laser delivery device with a variable numerical aperture objective, we have previously established that mechanical stiffening of the cornea equivalent to that achieved with 3mW UVA light for 30 min can be achieved using highly focused, 760 nm femtosecond laser light generated by a 76 MHz output. This femtosecond laser is centered at 150 kHz. Measuring blue collagen autofluorescence (CAF) generated following collagen CXL, we have established that 300 nJ/pulse energy and <45 mW total power produces a linear increase in corneal CXL that is dependent on the number of laser pulses/µm spot. Equivalent CAF to that of UVA was achieved at 10 pulses/spot, at 150 KHz and 300 nJ/pulse. Using this method of corneal stiffening, finite element modeling of the effect on corneal topography indicates that 1-2 dipters of refractive flattening could be achieved following 1-4 minutes of NLO CXL treatment. Preliminary study of the antiinfectious effects of NLO CXL showed excellent killing of Pseudomonas aeruginosa plated onto agar plates using 10 pulses/spot at 300 nJ/pulse compared to 1 hour of 3mW UVA. Overall, these data suggest that NLO CXL may have important clinical applications for not only treating corneal ectasias, but also for treating refractive errors and infectious keratitis. Supported in part by NIH Grant EY024600 and Research to Prevent Blindness, Inc. Unrestricted Grant.

In vitro Effectiveness of Photodynamic Antimicrobial Chemical Therapy with TONS504 for Eradication of Acanthamoeba

Chikama, T.1, Pertwi, Y.D.1, Sueoka, K.1, Ko, J.A.1, Kuchi, Y.1, Onodera, M.2, Sakaguchi, T.3
1Hiroshima University, Ophthalmology and Visual Science, Hiroshi-ma, Japan
2Hiroshima University Hospital, Clinical Support, Hiroshima, Japan
3Hiroshima University, Virology, Hiroshima, Japan

Purpose: Microbial keratitis is a potential cause of corneal blindness. Here we investigated the effectiveness of photodynamic antimicrobial chemotherapy (PACT) with the cationic chlorin, genipin, to reduce keratitis caused by Acanthamoeba, a cause of corneal infection and blindness. Methods: Acanthamoeba castellanii (ATCC 30370) was subje cted to PACT with TONS504 (Porphyran Lab, Okayama, Japan) and a light-emitting diode (LED) device (SSC, Kyoto, Japan) that provides light at a single wavelength (660 nm). Acanthamoeba was allowed to grow above coverslips in a 24-well plate and was then exposed to TONS504 at various concentrations (0 to 10 mg/L, for trophozoite) and (10 to 20 mg/L, for cyst) irradiated at a light energy of 10-60 J/cm2, and incubated at 27°C for 3 h. The effectiveness of TONS504-PACT against Acanthamoeba was evaluated by determination of cell viability and by im-
munofluorescence staining for apoptotic and necrotic cells. The Effect of Medium Composition and Substrate Curvature on the Behavior of Keratocorpus-derived Corneal Stromal Cells

Song, A., Gouveia, R., Cannon, C.1
Institute of Genetic Medicine, Newcastle University, International Centre for Life, Newcastle upon Tyne, United Kingdom

The corneal stroma is comprised by highly-aligned collagen fibrils densely arranged in well-organized, orthogonally-oriented layers. This structure is crucial for maintaining the tissue’s mechanical and optical properties, and its loss compromises corneal function and integrity. Keratocorpus is a corneal ectasia characterized by the progressive thinning and deformation of the stroma, leading to severe astigmatism and decreased vision. It is likely the main cause for corneal transplantation in the developed world, its etiology is still unknown. Consequently, new and accurate models are presently required to study the disease’s onset and progression. However, the difficult culture of keratocorpus-derived corneal stromal cells (kCSCs) in serum-free conditions represents an important limitation for developing such models. Previously, we have shown that low-glucose medium and retinoic acid supplement significantly improved the viability and proliferation of healthy corneal stromal cells (hCSCs) in vitro. Moreover, we showed that substrate curvature alone was sufficient to induce cell and extra-cellular matrix (ECM) alignment. In the present study, we now aim to explore the influence of medium composition and substrate curvature on the behavior of kCSCs. To this purpose, cells were seeded on bespoke round-bottom vials and cultured for two months in low-glucose and retinoic acid-supplemented serum-free medium (LG+RA) and compared to high-glucose (HG) and serum-containing (FBS) media conditions. Cells on flat-bottom surfaces in corresponding media formulations were grown as planar controls. Cell and ECM alignment was evaluated throughout the culture period by phase-contrast and atomic force microscopy, respectively. Interestsingly, our results showed that LG+RA increased kCSC and hCSC proliferation over that in HG medium by 57% and 84%, respectively. Moreover, both cell types were able to attach, proliferate, and migrate circumferentially when cultured on curved substrates, with 56% of kCSCs and 62% of hCSCs assuming a highly-aligned stratified organization and depositing ECM in orthogonal arrangements similar to those of the native corneal stroma. This data thus represents a substantial advancement for the creation of new and sophisticated corneal stroma equivalents via bottom-up tissue bio-fabrication, solely comprised by kCSCs and their produced ECM. Such tissues will constitute important models to study the basis of keratocorpus in vitro.

COS6 - Genetics of keratocorpus

Genetic and Functional Studies of Keratocorpus

Chakravarti, S.1, Jun, A.S.2, Willoughby, C.1, Daoud, Y.3, Sobreira, N.1, Hui, N.1, Wohler, E.1,4
1NYU Langone Health, Department of Ophthalmology, New York, United States, 2Johns Hopkins University, Department of Ophthalmology, Baltimore, United States, 3Ulster University, Department of Ophthalmology, Coleraine, United Kingdom, 4Johns Hopkins University, Institute of Genetic Medicine, Baltimore, United States, 5NYU Langone Health, Center for Human Genetics and Genomics, New York, United States

Keratocorpus (KC) is a progressive, corneal thinning disease that is affected by multiple genes and environmental factors. To elucidate underlying genes and biological processes that contribute to keratocorpus, we have undertaken whole exome sequence analysis, transcriptomic and cell culture studies. We recruited patients with non-syndromic forms of keratocorpus with or without a positive family history. We obtained whole exome sequence (WES) on a cohort of 130 isolated cases from Baltimore and a second cohort of Northern Irish families. The Baltimore cohort comprised of 37 female, ranging in age from 13-80 years and of European (62%), African American (23.5%), Hispanic (4.2%), Asian (8.2%) and other (2.1%) ancestry. The Northern Irish cohort consists of 13 families, each with 2-3 affected family members. SNV coding alleles were considered pathogenic when these were nonsense, highly conserved missense (Phyloto score ≥ 4), or changes altering splice junctions and frame-shifting INDELs. We used non-Finnish Europeans from 1000Genomes as controls to determine frequency. Transcriptomic (RNA-seq) analyses were performed on discarded keratocorpus corneal tissues and donor corneas obtained from Lions Eye Institute for Transplant and Research, Tampa, FL. Cell culture models were established using stromal cells from donor and keratocorpus corneas. Findings from our WES data will be presented and discussed. The RNA-Seq study detected 22,507-36,946 transcripts per cornea with 170 transcripts commonly changed in all KC. Cultured keratocorpus stromal cells showed aggravated stress response with a functional block in ECM and structural proteins. The cell culture and transcriptomic data is being used to prioritize exonic genetic variants. Selected candidates gene are being tested for LOF using our stroma cell culture model.

New Advances in Genetics of Syndromic and Non-syndromic Keratocorpus

Bykhovskaya, V., Rabinowitz, Y.S.
Cedars-Sinai Medical Center, Board of Governors Regenerative Medicine Institute and Department of Surgery, Los Angeles, United States

Keratocorpus (KC) is a bilateral progressive corneal thinning and ectasia. The exact cause of KC is unknown with genetic factors playing a major role in the development of the disease. KC is most commonly seen in its non-syndromic form; however, it has also been frequently identified in patients with multi-systemic diseases such as Down syndrome, Ehlers-Danlos syndrome (EDS), among others. Such co-occurrence suggests possible involvement of overlapping genetic defects. Initially such defects were identified in COL5A1 gene which carries heterogeneous nonsense, frameshift or splice-site mutations in EDS patients. Interestingly, a potentially pathogenic rare variant in COL5A1 gene was identified in a large family with a was Alport-like renal abnormalities and KC. Defects of the cornea in classic Alport syndrome (also called Hereditary Nephritis) patients are usually limited to corneal opacity, corneal erosions, and posterior polymorphous corneal dystrophy and have been exclusively associated with mutations in genes coding for COL4A5 and COL4A6. In this family carrying COL5A1 gene variant, KC is the only observed corneal phenotype. On rare but well-documented occasions simultaneous presentation of Tuberous Sclerosis Complex (TSC) and KC phenotypes has been reported. TSC syndrome alters cellular proliferation and differentiation, resulting in hamartomas - benign tumors of various organs, most notably the brain. KC patients with TSC syndrome have overlapping clinical features. The presence of pathogenic TSC mutations could suggest possible involvement of overlapping genetic defects. In this study we compared KC patients with TSC and EDS to KC patients with no other systemic abnormalities. TSC mutations were mutually exclusive and overlapping clinical features. The presence of pathogenic TSC mutations could suggest possible involvement of overlapping genetic defects. In this study we compared KC patients with TSC and EDS to KC patients with no other systemic abnormalities. TSC mutations were mutually exclusive and overlapping clinical features.
The Integrated Stress Response in Keratoconus

Foster, J.1, Shinde, V.2, Soberman, U.1, Sathe, G.1, Liu, S.1, Wan, J.1, Qian, J.1, Daoud, Y.1, Pandey, A.1, Jun, A.1, Chakravarti, S.1

1 Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore, United States, 2 NYU Langone Health, Department of Ophthalmology, New York, United States, 3 Manipal Academy of Higher Education, Mangalore, Karnataka, India

Purpose: Keratoconus (KC) is a multifactorial disease where progressive thinning and weakening of the cornea leads to loss of visual acuity. Although the underlying etiology is poorly understood, a major endpoint is a dysfunctional stromal connective tissue matrix. Using multiple individual KC corneas, we determined that matrix production by keratocytes is severely impaired due to an altered stress response program.

Methods: KC and donor (DN) stromal keratocytes were cultured in low glucose serum-free medium containing insulin, selenium and transferrin. Fibronectin, collagens and proteins related to their chaperone, processing and export, matrix metalloproteinase, and transferrin. Fibronectin, collagens and proteins related to their chaperone, processing and export, matrix metalloproteinase, and transferrin. Fibronectin, collagens and proteins related to their chaperone, processing and export, matrix metalloproteinase, and transferrin. Fibronectin, collagens and proteins related to their chaperone, processing and export, matrix metalloproteinase, and transferrin.

Results: DN and KC keratocytes proliferated at a similar rate. However, immunoblotting of selected ECM proteins and global proteomics showed decreased fibronectin, collagens, PCOLCE, ADAMTS2, BMP1, HSP47, other structural and cytoskeletal proteins and their chaperones, processing and export proteins. In addition, collagen fibrillation was also decreased.

Conclusion: The profound decrease in structural proteins in cultured KC corneas and donor keratocytes is paralleled by a decrease in their chaperone, processing and export, matrix metalloproteinase, and transferrin. Fibronectin, collagens and proteins related to their chaperone, processing and export, matrix metalloproteinase, and transferrin. Fibronectin, collagens and proteins related to their chaperone, processing and export, matrix metalloproteinase, and transferrin.

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diet high in calcium, phosphate and lactose. We hypothesize that correcting Vit D deficiency in select patients with primary corneal disorders such as diabetic keratopathy will improve their ophthal-
mic outcomes.

**Corneal Epithelial Regeneration after Biosynthetic Corneal Implantation**

Griffith, M.

Université de Montréal, Ophthalmology, Montréal, Canada

We have reported the stimulation of epithelial growth over cell-free biosynthetic corneal implants made from chemically-cross-linked recombinant human collagen or collagen analogs based on mi-
metic peptides, both in clinical trials and in animal models. Recent results show that the collagen-based biomaterials of the implants stimulate in-growing epithelium to produce extracellular vesicles that appear to stimulate regeneration of the stromas and extracel-
lular matrix of the neo-corneas. We show that the incorporation of a phospholipid polymer into the implants suppressed inflamm-
ation in the regenerated neo-corneas of alkali-burned mini-pig models. Effects of inflammation suppression on the regenerating epithelium will be discussed.

**An Improved Treatment Strategy for Acute Ocular Disease Using Dry-processed Human Amnion**

Hopkinson, A.1,2, Sidey, L.1, Bitchfield, E.1,3, Mariti, N.1, McIntosh, O.1, Allen, C.1

1University of Nottingham, Department of Ophthalmology, Not-
ingham, United Kingdom, 2NuVision Biotherapies Limited, Notting-
ham, United Kingdom

**Purpose:** Timely and effective intervention in ocular surface disease is essential for preserving functional vision. Amnion has consider-
able potential utility as a corneal surface (OS) disease. Unfortunately, amnion is conventionally stored frozen which com-
promises tissue quality, and precludes in clinic application. We pro-
pose a novel delicately dehydrated human amnion with enhanced wound-healing function, and a new potential suture-free application strategy for improved treatment of acute corneal surface (OS) disease.

**Methods:** Cryopreserved amnion (CPAM) and amnion delicately dried, with or without freeze or heat drying, using low temper-
ature vacuum evaporation (VDAM licensed to NuVision® as Omni-
gen®), both manufactured in our laboratories, were prepared as a removable biological dressing (patch) to assess wound healing properties: i) in vitro (cell proliferation, viability, cytokotyes, scratch test); and ii) in vivo using an acute chemical injury pre-clinical

**Cornea and Ocular Surface**

**ORAL PRESENTATIONS**

**In vitro Modeling of Aniridia-related Pax6 Haploinsufficiency by the Use of CRISPR/Cas9 on Limbal Epithelial Stem Cells and Rescue by Recombinant Pax6 Protein**

Roux, L.1, Pettit, I.1, Ferrigno, O.2, Aberdam, D.1

1INSERM U976, Paris, France, 2INSERM U976, Paris, France

Haploinsufficiency of Pax6 in humans is the main cause of con-
genital aniridia, a rare eye disease characterized by iris hypoplasia and reduced visual acuity. Patients have also progression dis-
eries including cataract, glaucoma and corneal abnormalities mak-
ing their condition very challenging to manage. Aniridia-related keratopathy (ARK), caused by a combination of factors including limbal stem-cell deficiency, impaired healing response, abnormal differentiation, and infiltration of conjunctival cells onto the cor-
neal surface, affects up to 95% of patients. It usually begins in the first decade of life resulting in recurrent corneal erosions, sub-ep-
thelial fibrosis with corneal decompensation and opacification. Unfortunately, current treatment options for aniridia-related disease are currently limited. Although animal models partially reca-
pitulate this disease, there is no in vitro cellular model of AKT

**Human Stem Cells as a Powerful Tool for Cornea Surface Renewal**

Skottnan, H.

University of Tampere, Faculty of Medicine and Life Sciences, Bio-
medtech Institute, Tampere, Finland

Conal blindness affects millions of people worldwide, and there is a constant shortage of high quality donor tissue. Engineering a corneal transplant that could effectively regenerate the corne-
al epithelium and stroma is an important goal. Differentiation of limbal epithelial stem cells from human pluripotent stem cells (hPSC-LESC) provides new tools for studying epithelial renewal. In addition, human adipose stem cells (hASCs) provide an appealing cell source for corneal stromal regeneration due to their capaci-
ty to differentiate into a variety of cell types within the limbal micro-

**COS8 - Corneal cell-based therapy - Where are we now?**

**Stem Cell Tracking, Loss and Recovery in the Corneal Epithelium**

Shalom-Feuerstein, R.

Technion - Israel Institute of Technology, Haifa, Israel

The corneal epithelium serves as an excellent model for stem cell (SC) research. Nevertheless, fundamental feature of SC that regenerate the corneal epithelium remain under debate or un-
known. For example, SC location, prevalence and self-renewal mechanisms were under debate. Consequently, the methods that involve SC failure in pathology are not well. Recently, we established a multi-color “Confetti” lineage tracing system that proved that the murine limbus is the major site of bona fide SCs. Additionally, KIS-GFP transgene labeled the limbal SC/boundary compartment. In agreement, KIS-GFP+ basal lim-
bal epithelial cells expressed SC markers and were located at the margin site of corneal regeneration, as evident by lineage tracing. Interestingly however, surgical depletion of the limbal epithelium and KIS-GFP+ SC pool was restored by corneal committed cells which underwent centrifugal migration and dedifferentiation into bona fide SCs. The recovered corneas were transparent for many months, displayed normal marker expression and consistent dyn-
amic of SC regeneration. By contrast, damage to the limbal stro-
mal niche abolished K15-GFP recovery, and led typical limbal SC deficiency phenotype. Altogether, this study reconciles major in-
consistencies in the field and suggests that the while limbus is the major site of SCs, the cornea has an extremely efficient mechanism of self-repair, even from exhaustive SC loss.
Pre-Clinical and clinical evaluation of a regenerative bioengineered corneal implant as an alternative to human donor cornea for treatment of advanced keratoconus
Rafat, M.1,2, Lagali, N.

In this study, we evaluated the safety and efficacy of a novel bioengineered corneal implant (LinkCor®) in advanced keratoconus patients using a suture-free femtosecond-assisted intrastromal surgery. The implant was placed in the pocket and the cornea was covered by a bandage lens after the surgery. Topographic and refractive properties of the corneas were determined preoperatively and 6 months postoperatively using slit lamp biomicroscopic evaluation, uncorrected distance visual acuity (UDVA) and corrected distance visual acuity (CDVA) measurements in LogMAR, and Scheimpflug-based anterior segment tomography (Pentacam).

Methods: Highly transparent bioengineered corneal implants (LinkCor®) were developed using type I collagen and a cross-linking system and tested in vitro for physical and biological properties followed by successful implantation in minipig corneas for 6 months. The bioengineered implants were also tested in 10 advanced keratoconus patients using a suture-free femtosecond-assisted intrastromal surgery. All human subjects had thin corneas and were candidates for penetrating keratoplasty. An intrastromal pocket was created in each patient’s cornea using femtosecond laser. Bioengineered implants were placed in the pocket and the cornea was covered by a bandage lens after the surgery. Topographic and refractive properties of the corneas were determined preoperatively and 6 months postoperatively using slit lamp biomicroscopic evaluation, uncorrected distance visual acuity (UDVA) and corrected distance visual acuity (CDVA) measurements in LogMAR, and Scheimpflug-based anterior segment tomography (Pentacam).

Results: Mechanical strength and elasticity, transparency, resistance to aqueous humor, bacterial, and steroid tests of the bioengineered corneal implants (LinkCor®) exceeded threshold requirements for testing in animal models and for human use. Corneal hydrogels supported epithelial cell growth in vitro and remained quiescent, transparent, and stable after 6 months of intrastromal implantation in pigs and human corneas. Intrastromal transplantation preserved tissue structure, subbasal nerves, epithelial, endothelial, and kerocyte cells.

Conclusions: 6 months postoperatively in pigs and human suggest that LinkCor® bioengineered corneal implants remained clear and stable in pigs and in human models and had an overall satisfactory impact on patients’ corneas topographically, refractively, and vision parameters. Longer term studies in more patients will be needed to confirm these results although these early results suggest that LinkCor® can be used as a potential alternative to human donor transplantation eliminating the need for penetrating keratoplasty (PKP).

Key Words: Cornea, keratoconus, bioengineered corneal implants, keratoplasty, femtosecond laser

Importance of Limbal Tissue Stiffness in Wound Repair
Gouveia, R.M.3, Lepert, G.J., Gupta, S.1,4, Mohan, R.R.1,4, Pa- terson, C.1, Cannon, C.J.1

Newcastle University, Newcastle-upon-Tyne, United Kingdom, ‘Imperial College London, Blackett Laboratory, London, United King- dom, ‘Harry S. Truman Memorial Veterans Hospital, Columbia, United States, ‘University of Missouri, College of Veterinary Med- icine, Columbia, United States

Whilst the control of stem cell differentiation using substrates of differing compliance has been extensively explored in vitro, the signifi- cance of this mechanism at a physiological level is not known. Thus, we set out to explore this novel concept within the context of ocular surface biology. We hypothesized that the biomechani- cal properties of the corneal surface, beneath its limiting epithe- lium, play a fundamental role in the tissue’s homeostasis (i.e., by controlling epithelial cell proliferation and differentiation). Us- ing non-contact high-resolution Brillouin spectroscopy microscopy we showed that the matrix comprising the corneal outer edge (lim- bus) has significantly lower bulk modulus compared to that of the central cornea and that this difference is precisely delimitated in the organism. In addition, the areas of the limbus with distinctly softer properties were shown to be associated with limbal epithelial stem cell (LESC) residence. The importance of these differences, in tissue stiffness, was further demonstrated through biomechanical modu- lation of the corneal surface to affect LESC phenotype using a novel pharmacological approach. Specifically, collagenase-softerned cen- tral corneal tissues were capable of supporting the re-epithelial- ization of corneal epithelial cells expressing limbus-characteristic markers both in vivo and ex vivo. Moreover, we demonstrated that treating the corneal limbus with collagenase effectively restored the tissues’ capacity to support LESCs following alkali burn. To- gether, these results confirm that stiffness plays a fundamental role in directing the behavior of corneal epithelial cells on the corneal surface. Thus, we have shown that the phenotype of cor- neal epithelial cells can be predictably modulated in vivo and ex vivo through the creation of a suitable biomechanical niche in both healthy and wounded tissues.

The Role of Nerve Growth Factor in Maintaining the Limbal Stem Cell Proliferative Capacity, Colony-Forming Efficiency and Phenotype
Ghareeb, A., Kolli, S., Bojic, S., Figureido, F., Lako, M.

University of Newcastle, Institute of Genetic Medicine, Newcastle Upon Tyne, United Kingdom

Nerve growth factor (NGF) has demonstrated great benefit in the treatment of neurotrophic corneal ulcers and potential effec- tiveness in the treatment of several other ocular conditions. There is evidence for several modes of action in promoting corneal heal- ing, however only indirect evidence exists for NGF’s effects on limbal stem cells (LSCs). Understanding the role of NGF in LSC biology will improve our understanding of the LSC niche and the development of stem cell-based therapeutics. We study the changes in cell signalling which take place upon LSC differenti- ation and the effects of NGF-blocking on primary human LSCL Primary human limbal cultures derived from cadaveric corneal- ical bases were cultured on a layer of murine 3T3 fibroblasts for 40 days to allow differentiation. Differential expression of sig- nalling proteins between days 10 and 40 was measured by pro- tein microarray, with subsequent bioinformatic enrichment and network analysis. Expression of NGF and its receptor TrkA and p75NTR was also measured by Western Blot. The effect of addi- tion of anti-NGF antibody on cell morphology, colony-forming ef- ficiency and gene expression of putative differentiation markers was measured. Flow cytometry-based assays of proliferation and apoptosis were also used to study the effect of NGF blocking. Of 248 signalling proteins studied, NGF-receptor p75NTR underwent the greatest fold-change in expression between the stem cell and differentiated phenotypes, with expression decreasing 2.77-fold upon differentiation. Bioinformatic analysis shows that NGF signal- ling exists at the top of a hierarchical network, controlling the cell cycle, toll-like receptors, senescence regulatory pathways and VEGF signalling. Expression of NGF and TrkA also decreases upon differen- tiation. Primary human limbal cultures grown in the presence of an- ti-NGF antibodies show significant decreases in colony-forming ef- ficiency (p=0.039), proliferation (p=0.042) and expression of putative stem cell markers ABCG2 and C/EBPβ (p<0.03, p=0.04, respectively). NGF acts to maintain the limbal stem cell phenotype as eviden- ted by promoting proliferation, colony-forming efficiency and the expression of putative markers of the limbal stem cell phenotype. NGF is possibly a central paracrine signalling factor in the response to corneal epithelial injury. By improving the in vitro culture of limbal stem cells, NGF addition may improve the efficacy of limbal stem cell transplants.

Sustained and Widespread Gene Delivery to the Corneal Epithelium via in situ Transduction of Limbal Epithelial Stem Cells
Lasche, M.1, Kampik, D.1, Kawakami, S.1, Robinson, M.1, Larkin, F.1, Smith, A.1, Ali, R.1

1 UCL Institute of Ophthalmology, London, United Kingdom, 1Kyoto Prefectural University of Medicine, Department of Ophthalmology, Kyoto, Japan, 1Moorsfield Eye Hospital, London, United Kingdom, 1NIHR Biomedical Research Centre at Moorfields Eye Hospital NHS Foundation Trust and UCL Institute of Ophthalmology, London, United Kingdom

Efficient and sustained gene therapy of the corneal epithelium is challenging due to the tissues high rate of cellular turnover and its innate functionality to exclude foreign material. To date only a very limited gene transfer to the epithelium lasting <6 weeks has been shown to be aimed to achieve long-term gene expression following sustained gene therapy of the epithelium by targeting the limbal epithelial stem cells (LESCs) with both AAV and lentiviral vectors. Any genomic change mediated upon LESCs will be inherited by all daughter cells and, potentially, the tissue as a whole over time. We first investigated gene supplementation of murine (C57Bl/6J) LESCs via injection of integrating lentiviral vector (encoding GFP) around the entire circumference of the basal corneal junction using an ultrafine glass needle. This approach resulted in sub- stantial epithelial transgene expression forming circumferential streak-like patterns that were sustained in for ≥1 year in ~27% of injected eyes; a result highly indicative of LESC transduction. At its maximum extent transgene expression covered ~26% of the corneal surface. Having noted the ability of AAV to disseminate across Bowman’s membrane our second approach aimed to mediate genomic re- combination within LESCs via intrastromal injection of an AAV vector encoding CRE recombinase in the AAV-CRE mouse. This approach resulted in LESC transduction that was both more effective and more reproducible. Streaks of recombined epi- thelium sustained for ≥ 4 months formed in all injected eyes and at maximum extent covered 80% of the corneal surface. Within the same model system, we also assessed the capaci- ty of AAV to transduce LESCs via an early postnatal systemic de- livered. Following intraperitoneal injection of AAV-CRE at P0 re- combined cells were noted in all cellular layers of the cornea but were particularly notable in the stroma. In the epithelial streaks of recombined cells formed in all injected eyes and were sus- tained for ≥ 4 months in 92%. Maximum coverage was 10% in 12 months. These results constitute the first convincing demonstration of a poten- tially lifelong gene therapy of the corneal epithelium, a technique of potential utility is the treatment of inherited epithelial dystrophies via either sustained gene supplementation or transient genome editing.
AAV Gene Therapy in the Anterior Eye Prevents and Reverses MPS1 Corneal Disease

Myiadera, K.1, Conates, L.3, Carkin, K.1, O’Donnell, P.2, Bagel, J.1, Llanza, T.1, Spector, C.1, Song, L.1, Gilger, B.1, Hirsch, M.2
1University of Pennsylvania School of Veterinary Medicine, Philadelphia, United States, 2University of North Carolina, Chapel Hill, United States, 3University of Michigan, Ann Arbor, United States

Mucopolysaccharidosis1 (MPS1) is an autosomal recessive lysosomal storage disease resulting in severe phenotypes including neurocognitive defects, cardiac disease, and visual impairment due to corneal clouding. MPS1 is caused by mutations in the gene encoding alpha-L-iduronidase (IDUA), a ubiquitous enzyme that catalyzes the hydrolysis of glycosaminoglycans. Hematopoietic stem cell transplantation (HSCT) has proven successful at providing IDUA replacement therapy resulting in increased mental development and prolonged lifespan, sometimes by decades. However, HSCT fails to prevent the MPS1-associated vision loss necessitating investigation of an adeno-associated virus (AAV) IDUA gene addition strategy to address this deficiency of cell therapy. Initially, using patient cells and ex vivo corneal a chimeric capsid was identified as enhanced for inter-species corneal transduction following intrascleral injection. Then, packaged with an optimized IDUA cassette, safety and efficacy was determined in the most relevant disease model, MPS1 canine (N=10). In pre- and post-symptomatic MPS1 canine corneas, intrastromal AAV vector injection were well tolerated across a 150-fold dose range. Post-symptomatic dogs demonstrated corneal clearing as early as 1 week post-injection which progressed to complete clarity that was maintained for over a year. Pre-symptomatic canine corneas as early as week 2 after injection required daily instillation of AAV vector, whereas significantly lower doses were sufficient for prevention of vision loss. Post-mortem histological analyses demonstrate correction of multiple disease indicators that correlated to the presence of IDUA. The results from ten MPS1 canine corneas demonstrate that in all cases a single injection of AAV-IDUA is safe and effective for treating MPS1 corneal vision loss. Although initially envisioned for MPS1 patients that have received HSCT, this strategy is applicable to all MPS1 patients, provides a strategy for other lysosomal corneal diseases, and demonstrates successful long-term corneal gene therapy at an extremely low vector dose.

Development of a Point-of-Injury Gel Bandage for Ocular Trauma

Washington, M.1, Yu, J.1, Fedorchak, M.1,2,3
1University of Pittsburgh, Ophthalmology, Pittsburgh, United States, 2University of Pittsburgh, Bioengineering, Pittsburgh, United States, 3University of Pittsburgh, Clinical and Translational Science, Pittsburgh, United States

Ocular trauma is incredibly common, affecting nearly 2.5 million people each year in the United States, and is the leading cause of monocular blindness. Studies suggest that proper treatment and protection of traumatic ocular injuries at the point-of-injury are critical to preserving the form and function of the eye and its supporting adnexa. Among these primary needs are wound protection and support, infection prophylaxis, and control of inflammation. The purpose of this study was to test the hypothesis that an injectable, drug-eluting, thermoresponsive gel matrix can be utilized for comprehensive ocular trauma treatment. Our group utilized a poly(N-isopropylacrylamide-co-ethyl acrylate) (pNIPAAm-co-EA) copolymer gel matrix with absorbed corticosteroid (dexamethasone (DEX)) and interwoven natural polysaccharides. Using this material we have demonstrated that in vitro release of DEX is sustained linearly over 6 h, rheological properties similar to peripheral orbital fat can be obtained through incorporation of interwoven polysaccharides, and adequate axial volume filling, hydration, and open-globe stabilization is achievable. The microstructural composition and purity of pNIPAAm-co-EA copolymers were confirmed via FT-IR and DSC, and gelation from water to gel. Thermoresponsive gels utilized in this study were prepared via hydration of purified pNIPAAm-co-EA copolymers in nanopure water. Thermal and rheological properties of pNIPAAm-co-EA thermoresponsive gels were evaluated using UV-Vis, DSC, and parallel plate rheometry. An ex vivo porcine eye model was utilized to assess intraocular pressure stabilization efficacy of various formulations for full thickness open-globe laceration. Clinically relevant ocular irritation and wound healing assays including bovine corneal opacity and permeability (BCOP) test and corneal epithelial cell scratch wound assay provided initial demonstration of ocular compatibility and peribital wound healing functionality.

Drug Delivery for Corneal Cystinosis

Jimenez, J.1, Washington, M.A.2, Nischal, K.K.1,2,3, Fedorchak, M.V.1,2,3
1University of Pittsburgh, Bioengineering, Pittsburgh, United States, 2University of Pittsburgh School of Medicine, Ophthalmology, Pittsburgh, United States, 3Children’s Hospital of Pittsburgh, Pittsburgh, United States

Cystinotic corneal cysts in patients with Cystinosis are treated by hourly administration of topical cysteamine eye drops. The eye drop formulation requires a high concentration of cysteamine per drop to account for its instability as it is easily oxidized to inactive cysteamine. The strict dosing regimen and high concentration of drug per drop make this treatment inconvenient and painful for patients leading to almost universal non-compliance. A suitable controlled release formulation may help decrease the number of hourly eye drops and prolong the effect of treatment. The purpose of this study was to develop and test a controlled release formulation that provides cysteamine therapy in a single eye drop. We hypothesize that this formulation will address the issues of high concentration and frequency of administration of traditional cysteamine eyedrops. Our group has developed a thermo-responsive, poly (N-isopropylacrylamide) (pNIPAAm)-based gel drop that contains cysteamine-loaded Poly(lactic-co-glycolic) acid (PLGA) microspheres. This study describes the development of a cysteamine microsphere formulation optimized using several emulsion-based techniques. To achieve clinically relevant drug levels, microsphere formulations were fully characterized in vitro for stability, cytotoxicity, and release of cysteamine. The candidate formulation produced release kinetics for up to seven days with a burst release on the first day. The release profile within the first day was also determined. To determine the achievement of clinically relevant drug levels, future studies involve in-vitro efficacy using cystosinosis knockout fibroblasts, along with in-vivo drug release a biodistribution model. Acknowledgements: Cystinosis Research Foundation, National Institutes of Health P30EYO80988, Interdisciplinary Visions Sciences 5T32EY017271-09, and Eye and Ear Foundation of Pittsburgh, PA. Unrestricted Grant from Research to Prevent Blindness New York, NY

Localized AAV-PEDF Gene Therapy for Corneal Neovascularization in vivo

Mohan, R.
University of Missouri, Ophthalmology, Columbia, United States

Corneal neovascularization (CNV) is a major cause of global blindness. We hypothesized that localized AAV-Pigment Epithelium Derived Factor (PEDF) gene therapy would eliminate CNV in rabbits in vivo by restoring critical balance between pro- and anti-angiogenic factors and causing apoptosis in neovessels via Fas-Fas ligand signaling. New Zealand White rabbits were used. Topical alkali (1N NaOH) application for 1min on the central cornea produced CNV. PEDF gene therapy was administered into rabbit stroma in viscovault viscoelastic (lтр) (0.55; 5x10^5 pg/ml) using defined techniques: (a) 30min after alkali-burn (b) 3day after alkali-burn or (c) 1day prior to alkali-burn. Siltlamp biomicroscopy, H&E staining, stereomicroscope and immunofluorescence determined differential changes in CNV, vessel number, length and area, keratocyte density, apoptosis, and overall ocular health. Immunoblotting and qPCR quantified PEDF and Fas ligand expressions. Imaging data was analyzed with NIH Image and Adobe Photoshop. Localized AAV-PEDF gene transfer into keratocytes significantly increased PEDF levels in rabbit corneas in vivo[4 fold, p<0.01]. AAV-PEDF therapy given eyes exhibited dramatically reduced vasculature, vessel density, vessel-size (length and thickness) in rabbit cornea. AAV-PEDF therapy given 30min after injury showed 86-89% (p<0.001), 3days after injury exhibited 63-69% (p<0.001), and 1day prior to injury 95% (p<0.001) in morphometric analysis. A remarkably less and small-diameter blood vessel in PEDF-delivered corneas than the no-PEDF controls were detected by H&E and immunofluorescence. Detection of 2.5 fold increased Fas-ligand (p<0.05) in PEDF-delivered rabbit corneas suggested that PEDF-over-expression increases Fas-ligand significantly. Detection of double-labeled lectin+ and TUNEL+ cells in vessels suggested CNV resolution via apoptosis. Quantification of pro- and anti-angiogenic factors is underway. Localized and tissue-targeted AAV-PEDF gene therapy has potential to cure corneal neovascularization in vivo. Selective apoptosis in neovessels via Fas-Fas ligand pathway is the likely mechanism. Additional safety studies are warranted.
shown that mutations causing PPCD induce MET in the corneal endothelium, and this convergent pathogenetic mechanism leads to the failure of the endothelial barrier and disease.

**Advances in the Genetics of Corneal Dystrophies: A Convergent Pathogenic Mechanism for PPCD**

**Hardcastle, A.**

UCL Institute of Ophthalmology, London, United Kingdom

Since publication of the ICSD classification of corneal dystrophies—edition 2, we have made significant advances in our understanding of the genetic causes of corneal dystrophies. A new mutation in TGFBI was identified that causes a spectrum of phenotypes, including Epithelial Basement Membrane Dystrophy. We also discovered that mutations in COL17A1 cause Epithelial Recurrent Erosion Dystrophy. ZEB1 mutations leading to haploinsufficiency cause Posterior Polymorphic Corneal Dystrophy (PPCD3). Using whole genome sequencing, we recently identified non-coding mutations in a conserved region of the OVOL2 promoter as the cause of PPCD1. We also mapped a new locus for PPCD, PPCD4, on chr8q, and using retrieved cell lines, and guide RNAs in vivo and ex vivo, led to increased expression of TRAF-6 and IRAK-1 proteins. MiR-146a overexpression suppressed TRAF-6 and IRAK-1, whereas its inhibition led to the increase of TRAF-6 and IRAK-1. Moreover, miR-146a plays a critical role in LESC maintenance by regulating Notch signaling, supported by increased expression of K15 by miR-146a overexpression. Under inflammatory conditions, miR-146a may suppress inflammatory mediators through NF-kB pathway. Additionally, its downregulation of Numb, which is a miR-146a direct target and inflammation marker, may also contribute to its anti-inflammatory effects.

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**COS11 - Dry eye disease**

**Purslow, C.**

Keele University Science & Innovation Park, Newcastle-Under-Lyme, United Kingdom

Last year saw the publication of the second major scientific review on dry eye from the international group, the Tear Film and Ocular Surface (TFOS) Society, coming ten years after the first. This presentation will review the key highlights from the report, with emphasis on the changes insights into pathophysiology and management strategies, and also concluding with the recommendations form the report for direction in dry eye research.

**Sex, Gender and Hormone Effects on Dry Eye. The Role of Oestrogen**

Golebiowski, B.

School Of Optometry and Vision Science, University of New South Wales, UNSW Sydney, Australia

An important feature of dry eye disease is that it is more common in women than in men, as highlighted in a recent extensive review of dry eye. Female sex (biological representation) and gender (self-representation as a woman) are both risk factors for development of dry eye disease.
Substantial sex-related differences have been identified in the structure and physiology of ocular surface tissues. The higher prevalence of dry eye in women is largely attributed to the influence of hormones including the sex hormones, glucocorticoids, hypothalamic-pituitary-gland hormones, growth hormones, and hormones regulating thyroid function and glucose metabolism. Androgens appear to have a positive effect on tear film production, whereas the nature of oestrogen influence is less clear.

Our clinical work has recently shown that circulating levels of oestrogen impact meibomian gland function in postmenopausal women and in women treated with aromatase inhibitors. Hormonal variations during the menstrual cycle are also associated with changes in dry eye symptoms. Sex and gender differences in pain perception, sensitivity and tolerance, and associated bio-psycho-social factors, play a prominent role in dry eye symptoms and diagnosis. Gender has an important influence on differences in health-seeking behaviour, access to care and service utilisation and thus consequences for dry eye diagnosis and management.

The prevalence of dry eye disease between women and men arises from specific effects of sex and hormones on regulation of ocular surface tissues and tear film production, and from the sex- and gender-specific modulation of pain and symptoms, and care-seeking behaviour, and the inter-relationships between these factors. Further research is required to advance the understanding of the nature, extent, and mechanisms of sex, endocrine and gender effects on the ocular surface in health and disease.

Targeting Inflammation in Dry Eye Disease

Ni Garbhan, J.1, Pilson, Q.2, Smith, S.3, Connolly, S.1,2, Cryan, S.A.1, Murphy, C.2,3
1Royal College of Surgeons in Ireland, Ophthalmology and Molecular and Cellular Therapeutics, Dublin, Ireland, 2Royal Victoria Eye and Ear Hospital, Ophthalmology, Dublin, Ireland, 3Royal College of Surgeons in Ireland, School of Pharmacy, Dublin, Ireland

SS is a systemic autoimmune disorder characterized by dry eyes and dry mouth, secondary to reduced exocrine function of both the lacrimal and salivary glands. Extra glandular complications, appearing 5 to 10 years after initial diagnosis, occur in 20 - 40% of patients. At present there are few effective therapies for SS or diagnostic tests that allow identification of patients who will go on to develop further complications. It has been suggested that inflammation and exocrine gland dysfunction in SS leading to DED involves a complicated interplay between cytokine networks, innate immune cells and their mediators. Recent studies suggest that alterations in miR expression may contribute to the initiation and progression of pSS, although a functional link to pathogenic cytokine production has yet to be established. Our initial studies in peripheral immune cells from SS patients examining the expression levels of two well-characterised miRs with opposing regulatory roles in innate immunity have demonstrated enhanced expression of the pro-inflammatory miR-155 and significantly reduced levels of the IL-10 pro-miR, miR-21. These alterations resulted in reduced pro-inflammatory cytokine production as a result of altered expression of miR-regulated genes. These studies also identified novel miRs that are differentially expressed in peripheral immune cells that distinguish between patients with low or severe systemic disease activity, which could aid in patient stratification and targeted therapeutics.

As no study to date has focussed on the ocular surface we optimised the isolation of miR from primary human conjunctival epithelial cells (CECs) by expression cytokology and performed a miR and mRNA screen. We found several differentially expressed miRs and genes in pSS patients when compared to controls. Validation of one of these novel miRs and its predicted gene target confirmed that miR-744-Sp expression is significantly increased in CECs from pSS patients, whilst its predicted gene Pnelli3, a known negative regulator of type I IFN, was significantly reduced. Furthermore manipulation of miR-744-Sp expression using a mimic or antagonist resulted in reduced and increased expression of Pnelli3 respectively. The next phase of study will determine the potential of chitosan-based particles to act as anti-viral vectors for drug delivery and ultimately develop and optimise an idealised strategy for effective and targeted delivery of miR modulating agents.

Protection by a Metalloporphyrin Superoxide Dismutase Mimetic against Signs of Dry-eye Disease

Hakkarainen, J.J.1, Thapa, R.1, Žininauskaitė, A., Ghosh, A.K.1,2,3,4, Tenhunen, A.1,2,4, Raguaskaus, S.,2, Roessler, A.E.1, Kalesnykas, G.,4 Koulen, P.P.,5 Kaja, S.,1,4,5
1Experimetics Ltd, Research and Development Division, Kaapio, Finland, 2Loyola University Chicago, Graduate Program in Neuroscience, Health Sciences Division, Maywood, IL, United States, 3University of Missouri - Kansas City, Departments of Ophthalmology and Biomedical Sciences, Vision Research Center, School of Medicine, Kansas City, MO, United States, 4Loyola University Chicago, Department of Ophthalmology and Molecular Pharmacology and Therapeutics, Stritch School of Medicine, Maywood, IL, United States

Dry-eye disease (DED) is a multifactorial disease of the tear glands and ocular surface. Increased tissue levels of reactive oxygen species and oxidative stress are common hallmarks of DED and contribute to the associated ocular surface damage. Metalloporphyrin superoxide dismutase (SOD) mimetics are potent antioxidants with a well-documented safety profile. We hypothesized that the metalloporphyrin SOD mimetic Manganese(Ill)-5,10,15,20-tetrakis(N-methylpyridinium-2-yl) porphyrin pentachloride (MnTM-2-PyP) can protect corneal epithelial cells from oxidative stress-induced damage in vitro and in vivo. In vitro, human corneal epithelial cells (HCE-T) were exposed either to tert-butylihydroperoxide (tBHP) to induce oxidative stress or hyperosmolar conditions. Cells were treated with MnTM-2-PyP or vehicle. MnTM-2-PyP permeability across HCE-T cells was assessed using a xenysal system. In vivo, MnTM-2-PyP (0.1% w/v in saline) was delivered topically in the Siccalsystem™, a desiccating stress / scopolamine model for DED.

MnTM-2-PyP protected HCE-T cells in a dose-dependent manner according to tBHP-induced oxidative stress as determined by shifting the IC50 for tBHP from 317 µM (vehicle) to 595 µM (0.001% w/v), 2.5 mM (0.0005% w/v) and 3.4 mM (0.05% w/v) in the resazurin cell viability assay. In contrast, MnTM-2-PyP did not protect HCE-T cells from hyperosmolar insult. Its permeability coefficient across a barrier of HCE-T cells was 1.1 ± 0.1 × 10-6 cm·s-1 in saline and the mass balance was 62 ± 1%, suggesting low permeability but significant cellular uptake of MnTM-2-PyP. In vivo, dosing with MnTM-2-PyP resulted in a statistically significant reduction of corneal fluorescence staining (n = 10, P < 0.05) with similar efficacy as the 0.5% ophthalmic cyspine emulsion (Restasyl®). Furthermore, MnTM-2-PyP treatment resulted in a significant reduction in leukocyte infiltration into lacrimal glands. We did not observe any protective effect against loss of conjunctival goblet cells. Notably, MnTM-2-PyP did not produce ocular toxicity when administered topically. Our data suggest that MnTM-2-PyP, a prototypic synthetic metalloporphyrin compound with potent catalytic antioxidant activity, can improve signs of DED in vivo by reducing oxidative stress in corneal epithelial cells.

Acknowledgements

This study was sponsored by Experimetics Ltd. and K&P Scientific LLC. Support from the Dr. John P. and Therese E. Mulcahy Endowed Professorship in Ophthalmology (SK) is gratefully acknowledged.
**Ellipsometry of Human Tears**

**Glasgow, B.**

UCLA-Jules Stein Eye Institute, Ophthalmology, Pathology and Laboratory Medicine, Los Angeles, United States

Prior studies estimate the tear film lipid layer thickness in vivo ranges from 15 to 160 nm by interference techniques such as reflectometry. One study using a homemade ellipsometer measured the thickness of the superficial tear film in human subjects as 150±7 nm. Data including refractive index were fit with an assumed initial thickness of 150 nm. Advances in metrology for thin film coating and software development allow more precise measurements on substrates on a subnanometer scale. Polarization and phase changes are measured using varying angles of incidence and/or multiple wavelengths to spatially map tear film thickness variation. Biochemical information is revealed to fit for refractive indices separately determined for the ambient and substrate layers. Here, the steady state lipid layer thickness of the normal human tear film was mapped with ellipsometry. An in vitro approach obviates difficulties associated with eye movement, environmental variation and dynamic changes in the tear film. Tears were collected and pooled from 30 normal subjects in three lots. The polarization and phase shift parameters, $\psi$ and $\Delta$, of each lot were measured under controlled conditions in an imaging ellipsometer. $\psi$ and $\Delta$ were simultaneously fit to a global minimum for nulling conditions by least squares analysis. To determine the resolving abilities of the instrument in a fluid medium, calibration was performed on a monolayer of oleic acid over water. The thickness of the monolayer was determined in multiple experiments (range 1.1-1.6 nm) with an expected thickness of 1.2 nm. Measurement of the tear film revealed a heterogeneous surface layer with a model for the refractive index of 1.49 (published range 1.46-1.53 for melibian lipids). The lipid layer mean thickness was calculated from global minimum fit for psi and delta as 2.8 nm. Notably, the film was characterized by islands that fit a thickness up to 197 nm with a refractive index of 1.49. The data confirm that the superficial film on tears is lipid. In vitro metrology studies suggest a broader range of thickness than previously reported by other techniques. The broad range appears to be created by small raised islands of lipid in the superficial tear film.

**IMM1 - Cross-talk of innate immunity in the retina: Mononuclear phagocytes and RPE control retinal inflammation**

**Rashid, K.1, Wolf, A.1, Nebel, C.1, Langmann, T.1,2**

1University of Cologne, Laboratory for Experimental Immunology of the Eye, Cologne, Germany, 2University of Cologne, Center for Molecular Medicine (CMMC), Cologne, Germany

A chronic pro-inflammatory environment is a hallmark of retinal degenerative diseases and neurological disorders that affect vision. Inflammatory responses during retinal pathophysiology are orchestrated by microglial cells which constitute the resident immune cell population. Following activation, microglial cells lose their ramified protrusions, proliferate and rapidly migrate to the damaged areas and resolve tissue damage. However, sustained presence of tissue stress primes microglia to become overreactive and results in the excessive production of pro-inflammatory mediators that favor retinal degenerative changes. Consequently, interventions aimed at overriding microglial pro-inflammatory and pro-oxidative properties may attenuate photoreceptor demise and preserve retinal integrity. Here, we present evidence that retinal microglia critically influence ocular neoangiogenesis and trigger RPE inflammation activity during experimental retinal pathologies. We further highlight the positive effects of the sugar compound polysialic acid and the cytokine interferon beta (IFN-β) in modulating microgliosis and discuss their plausible mechanisms of action.

**Microglia-RPE Interactions and Immunotherapies for Retinal Degenerations**


University of Bonn, Ophthalmology, Bonn, Germany

Age-related macular degeneration (AMD) is the most common cause of blindness in all developed countries, and an effective treatment for the atrophic disease manifestation is still lacking. The NLRP3 inflammasome is activated in the retinal pigment epithelium (RPE) in atrophic AMD and may contribute to disease pathogenesis. We have identified lipofuscin-mediated photooxidative damage to lysosomal membranes as a mechanism of NLRP3 inflammasome activation in the RPE. Novel selective NLRP3 inhibitors are efficacious in preventing inflammasome activation and secondary cell death in RPE cells and thus represent a promising therapeutic strategy for clinical evaluation in atrophic AMD.

**ORAL PRESENTATIONS**

**Cornea and Ocular Surface**

**ORAL PRESENTATIONS**

**Ocular Immunology**
Complement Regulation at the Retina-chorial In- terface
Xu, H., Chen, M.
Queen’s University Belfast / Wellcome-Wolfson Institute for Experi- mental Medicine, Belfast, United Kingdom

Retina, as an immune privileged tissue, is separated from the sys- temic immune system. The neuronal retina is, however, protect- ed by its own innate immune system from external and internal pathogens/insults. Retinal innate immunity is attained by microglia and the complement system. Dysregulated complement activation is known to contribute to various retinal diseases including age-re- lated macular degeneration (AMD), uveoretinitis, and diabetic ret- inopathy. Retinal cells, including retinal pigment epithelial (RPE) cells, microglia and neurons produce various complement com- ponents and regulators. Under normal physiological conditions, retinal cells express high levels of complement regulators such as factor H, C1INH, CD46 and CD59. Whereas under inflammatory or oxidative conditions, the expression of the regulators is reduced and the expression of complement activators such as C1q, factor B, and C3 is increased in retinal cells. This presentation will discuss the role of the complement system in maintaining subretinal immune privilege, and the contribution of the dysregulated complement system in diseases involving the retina-choroidal interface, such as AMD and uveoretinitis.

The Role Of Akt2/FN1-CRYBA1 Pathway in Neutrophil Regulation during Age-related Macular Degeneration (AMD)
Ghosh, S.1, Shang, P.1, Yazdankhah, M.1, Bhutto, I.1, Hose, S.1, Lydia, G.2, Zigler, S.1, Sinha, D.1,2
1University of Pittsburgh School of Medicine, Department of Ophthal- mology, Pittsburgh, United States, 2Wimber Institute, Johns Hopkins University School of Medicine, Department of Ophthalmol- ogy, Baltimore, United States

AMD is a leading cause of blindness in the elderly. The atrophic (dry) form of the disease is more prevalent, but there is no treat- ment available for atrophic AMD at the present time. The impor- tance of microglia as immuno-modulatory cells in AMD has been established. However, while other pro-inflammatory cell types, like neutrophils, have been shown to be important in various inflam- matory diseases, a role for neutrophils in AMD remains uncertain. We have established a genetically engineered mouse model, the CRYBA1 KO (global knockout of CRYBA1, gene encoding for βA3/ A1-crystallin), which is associated with atrophic AMD- like pheno- type with increased inflammation in the retina. We have previously shown that lipocalin 2 (LCN-2), an adipokine known to play an im- portant role in innate immunity, also plays a pivotal role in activat- ing the inflammatory response in AMD. These inflammatory chang- es were also associated with increased microglial and Müller cell activation in the retina of the CRYBA1 KO mice. The present study evaluates the role of neutrophils in AMD progression and tries to determine the underlying signaling cascade involved in neutrophil regulation during AMD. Our data shows significant neutrophil infil- tration in the sub-macular choroid and retina of human AMD sam- ples compared to age-matched controls. Interestingly, the neutro- phils in the control retina do not express LCN-2, but the infiltrating neutrophils in the AMD patient samples show elevated expression of LCN-2. Moreover, we have also found that there is increased ex- pression of the neutrophil adhesion marker, ICAM-1 in both human AMD patients and CRYBA1 KO retina. RNAseq and western analyses reveals that the RPE/EC complex from CRYBA1 KO as well as human atrophic AMD patients expresses high levels of neutrophil-regulat- ing factors like IFNA, CXCL1, IFNY. Our results show that neutrophil migration during disease progression is regulated by AKT2-depen- dent NFκB/IRF3/IFNγ pathway. These findings suggest a possible role of the AKT2-dependent pathway in AMD, which could be of immense importance in understanding the para to chronic inflam- matory transition in the disease and thereby could provide nov- el therapeutic strategies. The authors would like to acknowledge funding support from Department of Defense, University of Pittsburgh School of Medicine, BrightFocus Foundation and RPB/ IRRF Catalyst Award for Innovative Research Approaches for AMD research.

Ghosh, S.1, Shang, P.1, Yazdankhah, M.1, Bhutto, I.1, Hose, S.1, Lydia, G.2, Zigler, S.1, Sinha, D.1,2
1University of Pittsburgh School of Medicine, Department of Ophthal- mology, Pittsburgh, United States, 2Wimber Institute, Johns Hopkins University School of Medicine, Department of Ophthalmol- ogy, Baltimore, United States

IMM2 - Microbiota & Eye diseases
Gut Microbiota and the Gut-retina Axis Influence Pathological Choroidal Neovascularization
Sapieha, P.1, Wilson, A.1, Sennabau, F.2, Andriessen, E.1
1University of Montreal, Montreal, Canada, 2Institut de la Vision-IN- SERMA, U 968, Unité Mixte de Recherche S 968, Paris, France

Pathological choroidal angiogenesis. Our study ultimately provides evidence for gut dysbiosis and the gut-retina axis in CNV evolution.

Gut Microbiota and the Gut-retina Axis Influence Pathological Choroidal Neovascularization
Sapieha, P.1, Wilson, A.1, Sennabau, F.2, Andriessen, E.1
1University of Montreal, Montreal, Canada, 2Institut de la Vision-IN- SERMA, U 968, Unité Mixte de Recherche S 968, Paris, France

Epidemiological data suggests that in men, overall abdominal obe- sity is the second most important environmental risk factor after smoking for progression to late-stage neovascular (NV) AMD. To date, the mechanisms that underscore this observation remain poorly defined. In the current study, we uncoupled weight gain from confounding factors and draw a link between gut dysbiosis and choroidal neovascularisation (CNV). Using mouse models of NV AMD, microbiota transplants and other paradigms that mod- ify the gut microbiome, we demonstrate that gut dysbiosis leads to heightened intestinal permeability and chronic low-grade inflam- mation characteristic of inflammaging that ultimately exacerbates pathologic choroidal angiogenesis. Our study ultimately provides evidence for gut dysbiosis and the gut-retina axis in CNV evolution.

Sapieha, P.1, Wilson, A.1, Sennabau, F.2, Andriessen, E.1
1University of Montreal, Montreal, Canada, 2Institut de la Vision-IN- SERMA, U 968, Unité Mixte de Recherche S 968, Paris, France

The Use of Predatory Prokaryotes to Control Human Ocular Pathogens
Shanks, R.1, Romanowski, E.1, Kadouri, D.2
1University of Pittsburgh, Ophthalmology, Pittsburgh, United States, 2Rutgers University, Oral Biology, Newark, United States

Antibiotic resistant microorganisms are an increasing cause for problem in hospitals around the world and a serious concern for ocular infections. In an attempt to find innovative approaches to eradicate antibiotic resistant bacteria, we tested whether predato- ry bacteria could successfully influence the viability of ocular iso- lates of Pseudomonas aeruginosans Serratia marcescens in vivo. Using our ocular surface occupancy model it was determined that predatory bacteria Bacillus amyloliquefaciens, strains HD100 and 109J, and Pseudomonas aeruginosans, strainARL-13, were able to accelerate the clearance of P. aeruginosans from the ocular surface 7 days after application, but did not affect the clearance of S. marcescens. Using an endophthalmitis model, we determined the impact of predato- ry bacteria on P. aeruginosans, S. marcescens, and methicillin resis- tant Staphylococcus aureus proliferation in the posterior segment. Prolinflammatory cytokines and pathogen numbers were evaluat- ed. These efficacy data build upon previous studies indicating that predatory bacteria are non-toxic to the ocular surface and suggest that predatory microbes can be developed as alternative therapy for eye infections when antibiotics fail.

Identification of Bacillus megaterium as the Major Etiology of Early Age-related Macular Degeneration
Wei, L.
Zhongshan Ophthalmic Center, Sun Yat-Sen University, Guangzhou, China

Age-related macular degeneration (AMD) is the leading cause of irreversible vision loss in the elderly worldwide. Despite identifica- tion of multiple genetic factors associated with AMD, the environ- mental factors triggering the damaging local inflammation in AMD remain unclear. Here, using quantitative PCR, negative staining transmission electron microscopy, and high-throughput sequenc- ing technologies, we find that resident microbiota inhabits the intraocular cavities in all eyes. A disease-specific signature of the microbial community differentiates several intraocular diseases in- cluding AMD. Importantly, we find that an AMD specific bacterium Bacillus megaterium induces activation of complement and retinal cell death in vitro and in vivo. Our study identifies bacterial infec- tion as the major etiology of early AMD, and provides a novel di- rection for the diagnosis, treatment, and prevention of the leading blinding disease in late life.

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Wei, L.
Zhongshan Ophthalmic Center, Sun Yat-Sen University, Guangzhou, China

Age-related macular degeneration (AMD) is the leading cause of irreversible vision loss in the elderly worldwide. Despite identifica- tion of multiple genetic factors associated with AMD, the environ- mental factors triggering the damaging local inflammation in AMD remain unclear. Here, using quantitative PCR, negative staining transmission electron microscopy, and high-throughput sequenc- ing technologies, we find that resident microbiota inhabits the intraocular cavities in all eyes. A disease-specific signature of the microbial community differentiates several intraocular diseases in- cluding AMD. Importantly, we find that an AMD specific bacterium Bacillus megaterium induces activation of complement and retinal cell death in vitro and in vivo. Our study identifies bacterial infec- tion as the major etiology of early AMD, and provides a novel di- rection for the diagnosis, treatment, and prevention of the leading blinding disease in late life.
domains of different proteins are highly homologous subtle differences allow for distinct signalling pathways to be activated. Recently one of the TIR adapters has also been shown to function in the enzymatic cleavage of NAD+, establishing the TIR adapter ‘SARM’ as a metabolic regulatory enzyme. Given the importance of control of metabolic pathways both for the provision of sufficient energy for visual function and due to metabolites capacity to influence the inflammatory arm of the innate immune system, this is of particular importance for retinal health. In this talk I will demonstrate how our data indicates that many TIR family proteins are abundantly expressed both in donor AMD eyes and in models of retinal degenerative disease, and that activation of alternative TIR containing receptors can have either protective or detrimental roles in models of both neovascular and dry AMD.

Infammosome in RPE and AMD

Efstathiou, N.1, Kosmidou, C.1, Hoang, M.1, Notomi, S.1, Hi rano, M.2, Takahashi, K.2, Olsen, T.3, Morizane, Y.4, Vavvas, D.1, 3

1Harvard Medical School/Massachusetts Eye and Ear Infirmary, Ophthalmology, Boston, United States, 2Okayama University Grad uate School of Medicine, Ophthalmology, Okayama, Japan, 3Mayo Clinic, Ophthalmology, Rochester, United States

Geographic atrophy is a multi-factorial disease with numerous molecular and environmental stressors implicated in its pathogene sis. We reported previously that nucleoside reverse transcriptase inhibitors (NRTIs), used extensively in patients to treat HIV and hepatitis B, were able to prevent RPE degeneration due to one such GA-related stressor, Alu RNA, by inhibiting the NLRP3 inflammasome in a manner separable from their reverse transcriptase in hibition activity. Numerous other RPE stressors implicated in AMD are also thought to act via NLRP3. Therefore, we sought to determine whether NRTIs also prevent RPE death due to these agents. We administered RPE stressors to mice via subretinal injection in mice receiving systemic or local NRTI treatment, RPE integrity was monitored by fundus imaging and RPE flat mount analysis. We found that NRTI treatment in doses equivalent to those used to treat viral infections in humans, was able to prevent inflammasome activation and RPE degeneration due to: amyloid beta [1–40], fer rioncinemic citrate, cigarette smoke extract, paraquat, A2E, and sodium iodate. Collectively, this study broad-spectrum RPE protective activity of NRTIs, and highlights this drug class as putative translational candidates for preventing RPE death.

Nucleoside Analogs Prevent RPE Degeneration due to Multiple AMD Stimuli

Gelfand, B.1, Hirahara, S.1, Kim, Y.1, Fowler, B.2, Hirano, Y.1, Banerjee, D.1, Fukuhara, J.1, Yasuma, R.1, Yasuma, T.1, Fukuda, S.1, Kerur, N.1, Ambati, J.1

1University of Virginia, Center for Advanced Vision Science, Department of Ophthalmology, Charlottesville, United States, 2University of Kentucky, Ophthalmology and Visual Sciences, Lexington, United States

Age-related macular degeneration (AMD) is the leading cause of blindness in the Western world and is characterized by degener ation of the retinal pigment epithelium (RPE). RPE degeneration leads to photoreceptor dysfunction, death and vision loss, while this degenerative process remains obscure leading to a shortfall in effective therapies. The NLRP3 inflammasome is a critical compo nent of the innate immune response and it has been associated with AMD, although conflicting data has been presented regarding its role, with one study proposing that activation of NLRP3 in immune cells is protective in AMD progression, and another suggesting that the non-immune RPE cells contain NLRP3 and its ac tivation is detrimental. Given the conflicting data in the literature, acknowledging that key biological reagents can be a major source of error, and in line with recent NIH requirements for authentica tion of key biological resources we examined with validated re sources whether NLRP3 protein is expressed in the non-immune RPE and if it plays a role in AMD. Rigorous validation suggested the following:

1. Most NLRP3 antibodies used in AMD research are not specific, casting doubt in published results reporting NLRP3 expression in human RPE. Contrary to prior work, we demonstrate that Dicer1 Knockout and Alu RNA induction do not induce NLRP3 in RPE. By utilizing established human RPE cell lines, human primary RPE cells, human induced pluripotent stem cell (hiPSC)-derived RPE from pa tients with overactive NLRP3 syndrome (Chronic infallible neurolog ic cutaneous and articulare, CINCA syndrome) and ex vivo RPE cells from AMD patients, this study provides evidence that NLRP3 is not expressed in RPE under basal, stimulated or diseased conditions. Collectively these data question the interpretation of prior studies that concluded that NLRP3 exists in RPE and/or that is a mediator of RPE dysfunction in AMD.

2. Knockout and Alu RNA induction do not induce NLRP3 in RPE. By rigorously validating our data, we demonstrate that NLRP3 is not expressed in human induced pluripotent stem cell (hiPSC)-derived RPE from patients with overactive NLRP3 syndrome (Chronic infallible neurologic cutaneous and articulare, CINCA syndrome) and ex vivo RPE cells from AMD patients, this study provides evidence that NLRP3 is not expressed in RPE under basal, stimulated or diseased conditions. Collectively these data question the interpretation of prior studies that concluded that NLRP3 exists in RPE and/or that is a mediator of RPE dysfunction in AMD.

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MM4 - Novel topics in immune homeostasis and ocular surface inflammatory disease

Immune Mediated Conjunctival Scarring: Current Understanding of the Aldehyde Dehydrogenase/ Retinoic Acid Autocrine and Paracrine Regulation of Fibrosis

Dart, J.1,2, Abraham, D.3, Norman, J.1, Saban, D.4, Calder, V.1, Daniels, J.1, Parnasse, J.4, Ong, H.S.1,2, Rauz, S.2

1University of Birmingham, Institute of Inflammation and Ageing, Birmingham, United Kingdom

Conjunctival scarring is associated with severe inflammatory eye diseases including allergic eye disease (AED). Severe morbidity results from the effects of the scarring in ocular mucous membrane pemphigoid (OMMP), trachoma, and Stevens-Johnson syndrome. Our group has found that aldehyde dehydrogenase (ALDH1A1) is upregulated in the conjunctiva of cultured OMMP patient fibro blast. Application of the ALDH metabolite, retinoic acid (RA), to normal human conjunctival fibroblasts in vitro induced a diseased phenotype (increased collagen production, decreased matrix contraction & proliferation, and altered actin organization). Conversely, application of the ALDH inhibitors, disulfiram and DEAB to OMMP fibroblasts in vitro restored their functionality to that of normal controls. ALDH1 was also upregulated in the conjunctiva of the mouse model of AED, used as a surrogate for OMMP, and in which topical application of disulfiram decreased both inflammation and fibrosis in vivo. Furthermore, using this model, Saban’s group have shown that induction of fibrosis is initiated by dendritic cells (DCs) through an ALDH/RA paracrine effect on fibroblast retinoid X receptors (RXRs): DC depletion and ALDH inhibition reduced fibrosis & RKR stimulation induced fibrosis. These data suggest that progressive scarring in OMMP results from ALDH/RA fibroblast autoregulation, that the ALDH subfamily has a central role in in mune-mediated ocular mucosal scarring, and that ALDH inhibition with disulfiram is a potential, and readily translatable, anti-fibrotic therapy. To establish whether these findings can be extended to additional scarring disorders the in vitro effects of ALDH inhibition on collagen production by fibroblasts from other diseases, are being studied. Although ALDH inhibition is effective in vivo in mouse AED and in our OMMP fibroblast model, the molecular mecha nisms underlying the pro-fibrotic effects of ALDH/RARa remain to be elucidated. Currently OMMP fibroblasts are being used to deter mine the mechanism by which ALDH/RARa regulates fibroblast metab olish, in particular whether dysregulated metabolism is driven by TGFβ signaling and/or by direct effects on cellular energy metab olish. Understanding the mechanism by which ALDH/RARa medi ates extracellular matrix production in OMMP fibroblasts is integral to the development of disulfiram as an anti-fibrotic drug. A clinical programme is in progress to develop topical ALDH inhibition as an anti-fibrotic therapy.
biont in Autoinflammatory Disease

St. Leger, A.1,2, Raychaudhuri, K.3, Almagrabi, F.1, Fuss, I.1, Goldbach-Mansky, R.1, Bishop, R.1, Mattapallil, M.3, Caspi, R.1

1University of Pittsburgh, Ophthalmology, Pittsburgh, United States; 2University of Pittsburgh, Immunology, Pittsburgh, United States; 3National Eye Institute, National Institutes of Health, Immunoregulation Section, Bethesda, United States, *National Institute of Allergy and Infectious Diseases, Clinical Immunology and Microbiology, Bethesda, United States, *National Eye Institute, National Institutes of Health, Consult Services Section, Bethesda, United States.

Introduction: We recently demonstrated that the mouse ocular surface harbors a resident commensal flora and identified Corynebacterium mastitidis (C. mast) as commensal that tunes mucosal immunity at the ocular surface by eliciting production of IL-17 from conjunctival γδ T cells. C. mast is also known to colonize humans. Muckle-Wells Syndrome (MWS) is one of several human autoinflammatory diseases known as Cryopyrin Associated Periodic Syndromes (CAPS). All are caused by gain-of-function mutations in the NLRP3 inflammasome gene, resulting in overproduction of IL-1, a strong stimulator of IL-17. Patients experience neutrophilic dermatitis, arthritis and severe recurrent conjunctivitis. We hypothesized that an aberrant immune response to commensal microbes at the ocular surface may be connected to the ocular disease in these patients.

Methods: We used a mouse model generated by a knock (KI) of the human mutated NLRP3 inflammasome gene, cloned from a MWS patient. The mice, initially negative for C. mast, were ocularly colonized with the bacterium. In vivo and in vitro responses to the commensal were assessed by changes in transcriptome, production of IL-1 and IL-17, neutrophil infiltration and clinical appearance. We also measured the reinduction of the transcriptome, production of IL-1 and IL-17, neutrophil infiltration and contact hypersensitivity were ocularly colonized with the bacterium.

Results: We found that mouse models replicating corneal injury and inflammation to evaluate light aversion behavior. Genetic mouse strains, surgical lesions and pharmacological conditions were employed to dissect the relative contributions of image based vision (rod and cone photoreceptors) and non-image based light perception (melanopsin-containing intrinsically photosensitive retinal ganglion cells, ipRGCs), and for free fibers to assess corneal sensitivity.

Methods: We use methods to identify the role of light aversion that is characteristic of CAPS disorders. Our results may have implications for clinical treatment of ocular inflammation in these patients.

No Gain, No Pain: A Potential Role of Melanopsin in Corneal Trigeminal Neurons

Parikh, S., Nusinowitz, S., Gorin, M.B., Matynia, A.

Jules Stein Eye, UCLA, Los Angeles, United States

Conclusion: We propose that overproduction of IL-1 and IL-17 at the ocular surface may be connected to the ocular disease in these patients.
Harnessing Activation of the "Innate Repair Receptor" (IRR) to Prevent Inflammatory Pathology in Diabetic Retina

Stitt, A.
Queen's University Belfast, Centre for Experimental Medicine, Belfast, United Kingdom

While diabetic retinopathy has been commonly considered as a disease of the retinal microvasculature, there is strong evidence that dysfunction is more widespread and that altered crosstalk between cells in the retino-vascular unit is critical for pathogenesis. This is evident by progressive activation of endothelium, glia and resident immune cells in diabetes and also at the late stages where retinal inflammation and ischemia occurs. This review lecture will assess the dysfunction of the neurovascu- lar unit in diabetic retinopathy and focus specifically on the role of the innate repair receptor (IRR). This heterodimeric receptor complex consists of CD131 (also known as the $\beta$ common receptor, $\beta CR$) and the erythropoietin receptor (EPOR) which is selectively expressed in the retina. We have previously demonstrated that VEGF leads to phos-phorylation of occludin at S490 promoting occludin ubiquiti- nation and endocytosis of the junctional complex. We now aim to elucidate the role of this phospho-site in blood-retinal barrier (BRB) permeability and visual function in vivo. Mice with transgenic Wt human occludin (WtOCC$^{+/+}$) under a CAG promoter followed by a floxed stop to allow conditioned expression, were crossed with mice expressing Tie2-Cre to obtain endothelial restricted expression. Or transgenic mice were crossed with endogenous occludin floxed (Occ$^{+/-}$) to obtain vascular restriction and deletion of endogenous occludin after tamoxifen injection. At 8 weeks, BRB permeability to Texas red-70 kDa dextran and FITC-BSA was assessed 36h after intravitreal VEGF injection. Retinal layer thickness was assessed in vivo using spectral domain optical coherence tomography (OCT). Diabetes was induced by streptozotocin injection and visual acuity and contrast sensitivity were determined. Changes were quantified using the optokinetic response using the Optometry system. Conditional expression of S490OCC$^-/-$ under the Tie2-Cre promoter led to a decrease in BRB permeability induced by VEGF, when compared to Tie2-Cre mice suggesting that overexpressing the occludin mutant leads to a protective effect against VEGF-induced permeability. In order to explore the direct effect of the occludin mutation, conditional expression of S490OCC$^-/-$ was compared to WtOCC$^{+/+}$ on the Occ$^{+/-}$background. When endogenous occludin is conditionally deleted the effects of S490OCC$^-/-$ become more profound when compared to WtOCC$^{+/+}$, as expression of the mutant reduces both the increase in VEGF-induced permeability as measured by solute flux and edema formation as determined by OCT measures of retinal thickness. Finally, the contribution of vascular permeability to visual function in diabetes was tested by conditional expression of the S490OCC$^-/-$ mutant under the Tie2 promoter. Endothelial expression of the occludin point mu-tant that promotes the BRB, prevented the reduction in visual acuity and contrast sensitivity induced by diabetes at 4 months. Our results show that occludin phosphorylation contributes a ma-jor role in VEGF-induced retinal permeability and edema in vivo. Importantly, protection of barrier properties can positively impact visual function in diabetes.

Conditional Endothelial Expression of Pro-barrier Occludin Mutant Preserves Visual Function in Diabetess

Antonetti, D., Goncalves, A., Lin, C.-M., Sheskey, S., Keil, J.
University of Michigan - Kellogg Eye Center, Ophthalmology, Ann Arbor, United States

We have previously demonstrated that VEGF leads to phosphorylation of occludin at S490 promoting occludin ubiquiti- nation and endocytosis of the junctional complex. We now aim to elucidate the role of this phospho-site in blood-retinal barrier (BRB) permeability and visual function in vivo. Mice with transgenic Wt human occludin (WtOCC$^{+/+}$) under a CAG promoter followed by a floxed stop to allow conditioned expression, were crossed with mice expressing Tie2-Cre to obtain endothelial restricted expression. Or transgenic mice were crossed with endogenous occludin floxed (Occ$^{+/-}$) to obtain vascular restriction and deletion of endogenous occludin after tamoxifen injection at P3. BRB permeability to texas red-70 kDa dextran and FITC-BSA was assessed 36h after intravitreal VEGF injection. Retinal layer thickness was assessed in vivo using spectral domain optical coherence tomography (OCT). Diabetes was induced by streptozotocin injection and visual acuity and contrast sensitivity were determined. Changes were quantified using the optokinetic response using the Optometry system. Conditional expression of S490OCC$^-/-$ under the Tie2-Cre promoter led to a decrease in BRB permeability induced by VEGF, when compared to Tie2-Cre mice suggesting that overexpressing the occludin mutant leads to a protective effect against VEGF-induced permeability. In order to explore the direct effect of the occludin mutation, conditional expression of S490OCC$^-/-$ was compared to WtOCC$^{+/+}$ on the Occ$^{+/-}$background. When endogenous occludin is conditionally deleted the effects of S490OCC$^-/-$ become more profound when compared to WtOCC$^{+/+}$, as expression of the mutant reduces both the increase in VEGF-induced permeability as measured by solute flux and edema formation as determined by OCT measures of retinal thickness. Finally, the contribution of vascular permeability to visual function in diabetes was tested by conditional expression of the S490OCC$^-/-$ mutant under the Tie2 promoter. Endothelial expression of the occludin point mu-tant that promotes the BRB, prevented the reduction in visual acuity and contrast sensitivity induced by diabetes at 4 months. Our results show that occludin phosphorylation contributes a ma-jor role in VEGF-induced retinal permeability and edema in vivo. Importantly, protection of barrier properties can positively impact visual function in diabetes.

Novel Insights into the Role of VEGF and NRP1 in Ocular Neangiogenesis

Farrant, A.
UCL Institute of Ophthalmology, London, United Kingdom

Ocular neovascularization (ONV) is associated with inflammation and edema-causing vascular hyperpermeability, which are all pathological features of sight-threatening human diseases, such as diabetic retinopathy and age-related macular degeneration. The vascular endothelial growth factor A (VEGF) is a key mediator of both ONV and excessive vascular permeability, with myeloid cells widely considered an important source of VEGF. Here, we show that myeloid cells contribute to ONV in a VEGF-independent fash-ion. Moreover, we provide novel insights on the molecular mech-anisms that distinguish VEGF-induced vascular hyperpermeability from other VEGF functions, which also include vascular and neural homeostasis. Our findings therefore suggest novel molecular tar-gets for therapeutic strategies aimed at more effectively treating edema without compromising long-term eye health.

Characterization of Monocyte Derived Macrophages in Diabetic Retinopathy


1Institut de la Vision, Sorbonne Université, INSERM, CNRS, Paris, France, 2Hôpital Lariboisière, Université Paris 7 - Sorbonne Par-is-Cité, Department of Ophthalmology, Paris, France, 3Trinity Col-lege Dublin, Department of Clinical Medicine, School of Medicine, School of Biochemistry and Immunology, Trinity Biomedical Scien-tifics Institute, Dublin, Ireland, 4Institute of Ophthalmology, Conde de Valenciana Foundation, Mexico City, Mexico, 7Centro Atención In-tegral de Paciente con Diabetes, Instituto Nacional de Ciencia Médi-cas y Nutrición Salvador Zubirán, Mexico City, Mexico, 5Universidad Nacional Autónoma de México, Faculty of Medicine, Department of Biochemistry, Mexico City, Mexico

Background: Diabetic retinopathy (DR) is the most common cause of irreversible blindness in the working age population. Besides hyperglycemia, dyslipidemia is a disregarded risk factor of DR development. DR patients have elevated intraocular levels of inflammatory cytokines and VEGF. Inflammatory macrophages (M$\phi$s), mostly derived from circulating monocytes (Mos) account for most of the cytokine production. We hypothesized that monocyte ex-posure to systemic hyperglycemia and/or dyslipidemia may prone their differentiation into $\text{M}^{\phi}$s with vascular remodelling capacities. Materials and methods: Mos were isolated from control and DR pa-tients and allowed to differentiate into $\text{M}^{\phi}$s for 18h. Donor control Mos were exposed to high glucose concentration (HG) or palmitic acid (PA). Cytokines were quantified by qPCR and multiplex analysis. Results: Mos from DR patients exhibited elevated levels of VEGF, CCL2 and IL-1$\beta$ compared to control donors. HG culture condition only slightly increased the production of inflammatory cytokines. In contrast, PA-treated Mos exhibited exacerbated production of inflam-matory cytokines (CCL2, IL-1$\beta$, IL-6, IL-8, TNF-$\alpha$) but not of VEGF. Conclusion: We demonstrated that similar to Mos from DR pa-tients, PA-treated but not HG-treated Mos differentiate into $\text{M}^{\phi}$s. This data suggests that dyslipidemia may participate to the chronic inflammation in DR patients and lead to vascular remodelling of the retina.

Ocular Immunology
Background: The objectives of our study were to develop and validate an assessment algorithm for referral from ophthalmologists of appropriate acute anterior uveitis (AAU) patients to rheumatologists that will aid the early diagnosis of Spondyloarthritis (SpA) and to evaluate the quality of life and 5 year clinical outcomes in this form of AAU.

Methods: All consecutive patients attending the emergency department of a local ophthalmology hospital with AAU, but who did not have a known diagnosis of SpA, were eligible to participate in this study. Patients with any other known cause of AAU were excluded. Two independent cohorts were enrolled. A test algorithm and Dublin Uveitis Evaluation Tool (DUET) algorithm (revised form of test algorithm) were used in these cohorts to identify patients as SpA suspects and non-SpA controls, respectively. The same test algorithm and DUET algorithm were analyzed using different biological and pathological routes through protein regulation, with potential therapeutic targets for uveitis.

Results: Study phase-1: algorithm development cohort (n=101): After rheumatologic evaluation of the entire cohort, 41.6% (n=42) had undiagnosed SpA. Our test algorithm was not validated in these cohorts to identify patients as SpA suspects and non-SpA controls, respectively. The same test algorithm and DUET algorithm were used in these cohorts to identify patients as SpA suspects and non-SpA controls, respectively. The same test algorithm and DUET algorithm were analyzed using different biological and pathological routes through protein regulation, with potential therapeutic targets for uveitis. After rheumatologic evaluation of the entire cohort, 41.6% (n=42) had undiagnosed SpA. Our test algorithm was not validated in these cohorts to identify patients as SpA suspects and non-SpA controls, respectively. The same test algorithm and DUET algorithm were analyzed using different biological and pathological routes through protein regulation, with potential therapeutic targets for uveitis.

Conclusion: After rheumatologic evaluation of the entire cohort, 41.6% (n=42) had undiagnosed SpA. Our test algorithm was not validated in these cohorts to identify patients as SpA suspects and non-SpA controls, respectively. The same test algorithm and DUET algorithm were analyzed using different biological and pathological routes through protein regulation, with potential therapeutic targets for uveitis. After rheumatologic evaluation of the entire cohort, 41.6% (n=42) had undiagnosed SpA. Our test algorithm was not validated in these cohorts to identify patients as SpA suspects and non-SpA controls, respectively. The same test algorithm and DUET algorithm were analyzed using different biological and pathological routes through protein regulation, with potential therapeutic targets for uveitis.
of IL-1β, inflammasome genes (Nlrp3, Casp1, Casp9, and Pyd) and chemokine genes (Cxc12, Cxcl1, Cxcl20) via qPCR and in situ hybridisation. Results: Elevated levels of IL-1β were detected primarily in outer retinal (IBA1+) microglial cells following photo-oxidative damage (P<0.05). This was accompanied by an increased expression (P<0.05) of inflammasome genes (Nlrp3, Casp1, Casp9) and chemokine genes (Cxc12, Cxcl1 and Cxcl10). Intravitreal injection of IL-1β sRNA significantly suppressed IL-1β retinal expression (P<0.05). Treatment with either the siRNA or IgG reduced Cxcl2 expression in Müller cells (P<0.05), Cxcl2 expression in RPE cells (P<0.05) and Cxcl10 expression (IgG only, P<0.05). Inhibition of IL-1β resulted in reduced microglia/macrophage recruitment into the outer retina (P<0.05), as well as a decrease in photoreceptor cell death (P<0.05).

Conclusion: Microglia-derived IL-1β promotes the expression of chemokines by Müller and RPE cells in retinal degenerations. The use of IL-1β inhibitors to manage detrimental microglia/macrophage activity could be beneficial in reducing consequent photoreceptor damage in retinal degenerations such as AMD. Strategies to manage IL-1β production through inflammasome activation may also be a useful therapeutic avenue for retinal degenerations.

Humoral Immunity in AMD: Predictive Value and Possible Role in Disease Progression

Xue, C.1,2, Morohoshi, K.1, Patel, N.1,3, Chong, V.4, Bird, A.1, Oho, S.1

1University of British Columbia, Department of Ophthalmology & Visual Sciences, Vancouver, Canada, 2Tokyo Medical and Dental University, Department of Ophthalmology, Tokyo, Japan, 3University College of London, London, United Kingdom, 4University of Oxford, Oxford Eye Hospital, Oxford, United Kingdom, 5University of British Columbia, Vancouver, Canada

The immune system has been strongly implicated as a contributor to the pathogenesis of AMD. Recent findings indicate that immunological factors in patients with a possible autoimmune response, are involved in the pathogenesis of age-related macular degeneration (AMD). To characterize additional biomarkers for the disease and further explore disease mechanisms, a new antigen microarray was used to screen 66 antigens of serum samples from patients with AMD and healthy controls. These antigens included ocular proteins expressed in the retina and choroid, constituents of drusen, immune molecules such as complement factors, and infectious disease antigens. The pathogenic role of an antibody associated with disease progression was also investigated. Sera from the AMD groups contained higher levels of IgG and IgM antibodies to various ocular antigens and immune molecules, respectively, when compared to the normal group. Anti-complement C4 IgG (odds ratio, OR=16.4) and anti-cytomegalovirus IgM (OR=13.0) were the best correlated with the development of dry AMD, and anti-apolipoprotein E IgG (OR=14.3) and anti-complement factor H IgM (OR=6.5) were the most reliable biomarkers for progression from dry to wet AMD. Moreover, IgGs purified from sera of AMD patients contained high reactivity to glutathione synthetase (GS) and inhibited activity of GS in a dose dependent manner. Ocular expression of GS decreased with age in mice, suggesting that the accumulation of glutamate, which has strong neurotoxicity, contributes to retinal degeneration. Our data demonstrate that patient seroreactivities to specific ocular antigens might be used as biomarkers for AMD.

Subretinal Mononuclear Phagocyte-derived IL-1β Induces Rod Loss and Cone Segment Degeneration

Charles-Messance, H., Eandi, C., Senslaub, F., Guillonneau, X.

Sorbonne Université, Institut de la Vision, Paris, France

In geographic atrophy (GA), one of the late forms of Age-Related Macular Degeneration (AMD), an extending atrophic zone forms, characterized by the loss of the retinal pigment epithelium and the degeneration of photoreceptors (PRs). Subretinal mononuclear phagocytes (MPs) invariably accumulate in the transitional zone (TZ) of atrophic zones (AZ). We here demonstrated that subretinal MPs produced elevated levels of IL-1β which is responsible for MP-induced rod loss and cone segment degeneration observed in the TZ of GA patients. MP-derived IL-1β impairs Müller glial cells, glutamate recycling, recapture and cystine transport, and subsequently leads to an extracellular increase in glutamate leading to rod degeneration. Inhibiting glutamate receptors or supplementing IL-1β treated retina with cystine is sufficient to protect rods from IL-1β-induced neurotoxicity. Our results will help to explore new perspectives to treat pathologies associated with subretinal inflammation such as late AMD.

Inflammation is considered an important contributor in the development of several diseases and neonatal conditions. Particularly, the pro-inflammatory cytokine interleukin-1β (IL-1β) and its receptor (IL-1R) play key roles in the pathophysiology of numerous debilitating diseases associated with acute exacerbations. This is clearly demonstrated for inflammatory bowel disease, various dermatitis, rheumatoid arthritis, as well as atherosclerosis and degenerative neurologic disorders. Meanwhile in retinopathy of prematurity (ROP), IL-1β amplifies proinflammatory factor production, retinal and choroidal microvascular degeneration and ensued neurotoxicity. Because IL-1β represents a dominant target for early therapeutic intervention, we designed and evaluated the efficacy of a novel small peptide modulator of IL-1R, namely 101.10 (sequence: rytve- la) in different models. First, we demonstrated that 101.10 is a potent and specific antagonist of IL-1β, with negative allosteric modulating properties, through a mechanism which spared the NF-κB pathway. Second, in a rat pup model of hypoxic-ischemic brain injury, where IL-1 and IL-1R expression is increased, we showed that 101.10 preserved microvascular density, parenchymal integrity, and brain mass. Third, in ROP model, early post-natal inhibition of IL-1R actions using 101.10 preserved retinal vasculature, choroidal thickness, decreased subretinal hypoxia, and prevented RPE/photoreceptor death, which resulted in life-long improved visual function. Fourth, in inflammation-induced murine models of preterm birth, 101.10 administered to the dam potently suppressed inflammation and preterm birth, and preserved retinovascular growth, choroidal integrity, resulting in improved retinal function. Together these findings suggest 101.10 as a potential therapeutic agent to prevent detrimental effects of IL-1β in ischemic retinopathies.

Inflammation is considered an important contributor in the development of several diseases and neonatal conditions. Particularly, the pro-inflammatory cytokine interleukin-1β (IL-1β) and its receptor (IL-1R) play key roles in the pathophysiology of numerous debilitating diseases associated with acute exacerbations. This is clearly demonstrated for inflammatory bowel disease, various dermatitis, rheumatoid arthritis, as well as atherosclerosis and degenerative neurologic disorders. Meanwhile in retinopathy of prematurity (ROP), IL-1β amplifies proinflammatory factor production, retinal and choroidal microvascular degeneration and ensued neurotoxicity. Because IL-1β represents a dominant target for early therapeutic intervention, we designed and evaluated the efficacy of a novel small peptide modulator of IL-1R, namely 101.10 (sequence: rytve-la) in different models. First, we demonstrated that 101.10 is a potent and specific antagonist of IL-1β, with negative allosteric modulating properties, through a mechanism which spared the NF-κB pathway. Second, in a rat pup model of hypoxic-ischemic brain injury, where IL-1 and IL-1R expression is increased, we showed that 101.10 preserved microvascular density, parenchymal integrity, and brain mass. Third, in ROP model, early post-natal inhibition of IL-1R actions using 101.10 preserved retinal vasculature, choroidal thickness, decreased subretinal hypoxia, and prevented RPE/photoreceptor death, which resulted in life-long improved visual function. Fourth, in inflammation-induced murine models of preterm birth, 101.10 administered to the dam potently suppressed inflammation and preterm birth, and preserved retinovascular growth, choroidal integrity, resulting in improved retinal function. Together these findings suggest 101.10 as a potential therapeutic agent to prevent detrimental effects of IL-1β in ischemic retinopathies.
Anatomy, Augusta, United States, Medical Colleges of Georgia, Oral Biology and Cellular Biology and reduction of responses to natural noise stimuli, photopic b-wave troretinogram analysis after 6 month diabetes showed significant of 12/15-LO in neuronal injury in DR by generating a new mouse HG-activated endothelial cells suggesting that endothelial rather major 12/15-LO-derived eicosanoids (12- and 15- HETEs). Myelop projection of retinal endothelial versus leukocytic12/15-LO in DR. LC-MS HETE induced leukocyte adhesion, permeability and inflammatory ER stress and permeability). While, treatment of HRECs with 15- retinal endothelial cells (HRECs) with high glucose (HG, 30mM DR and retina of diabetic mice showed significant increase in levels LC/MS lipidomic screening of vitreous samples from patients with leukostasis, retinal hyperpermeability and neovascularization. These changes were significantly improved in Ins2+/−/12/15-LO −/−. Our findings indicate that 12/15-LO is implicated in the neurovascular dysfunction and hence is a therapeutic target for neurovascular protection in DR.

Chemokines in Diabetic Retinopathy: The Good, the Bad and the Ugly
Struyf, S.1, Mohammad, G.2, De Hertogh, G.2, Van Damme, J.1, Abu El-Asrar, A.M.2,3
1Laboratory of Molecular Immunology, Rega Institute, KU Leuven, Belgium, 2King Saud University, Department of Ophthalmology, College of Medicine, Riyadh, Saudi Arabia, 3KU Leuven, Laboratory of Histochemistry and Cytochemistry, Leuven, Belgium, 4Rega Institute for Medical Research, University of Leuven, Leuven, Belgium, 5King Abdulaziz University Hospital, Riyadh, Saudi Arabia, 6Dr. Nasser Al-Rashid Research Chair in Ophthalmology, Riyadh, Saudi Arabia

Chemokine levels in the vitreous fluid of patients with proliferative diabetic retinopathy (PDR) are divergent from those of healthy controls. During disease onset, the inflammatory chemokines CCL8 and CCL2 attract leukocytes. Later, CCL12 may recruit endothelial cell precursor cells and fibrocytes, which are involved in establishment of neovessels and in formation of fibrovascular membranes. The myofibroblasts producing extracellular matrix molecules deposited in the fibrovascular membranes of the diabetic patients can originate from endothelial (precursor) cells via endoMT, from fibrocytes or from leukocyte-like precursors. Concurrently, also angiostatic chemokines (CXCL4, CXCL41 and CXCL10) are upregulated during the active disease stage, probably in an attempt to counteract the stimulatory effects (angiogenesis and increased vascular permeability) of VEGF. Finally, the fibrosis-inducing chemokines CCL2 and CXCL10 might play a role in the later stages of the patho. We have demonstrated that CXL41, a most potent angios TGF-β signaling, is a key regulator of fibrotic processes. These results demonstrate that angiogenic and anti-angiogenic chemokines cooperate to modify the angiogenic balance in diabetic retinopathy.

Osteoprotegerin is a Novel Biomarker of Proliferative Diabetic Retinopathy
Abu El-Azar, A.4, 5, Struyf, S.6, Mohammad, G.2, De Hertogh, G.2, Danne, J.1, Abu El-Asrar, A.M.2,6
1Laboratory of Molecular Immunology, Rega Institute, KU Leuven, Belgium, 2King Saud University, Department of Ophthalmology, College of Medicine, Riyadh, Saudi Arabia, 3KU Leuven, Laboratory of Histochemistry and Cytochemistry, Leuven, Belgium, 4Rega Institute for Medical Research, University of Leuven, Leuven, Belgium, 5King Abdulaziz University Hospital, Riyadh, Saudi Arabia, 6Dr. Nasser Al-Rashid Research Chair in Ophthalmology, Riyadh, Saudi Arabia

Osteoprotegerin (OPG) is a novel regulator of endo- barot, but not VEGF, MCP-1/CCL2 or thrombin induced upregulation of OPG in HREC. OPG induced ERK1/2 and Akt phosphorylation in HREC and stimulated their migration. OPG potentiated the angiogenic effect of VEGF in the in vivo matrigel plug assay. Conclusions: These results suggest that OPG is involved in PDR angiogenesis.

Involvement of Helper T Cell Immunity Balance in Diabetic Retinopathy
Taguchi, M.1,2, Nishio, Y.1, Inada, M.3, Harimoto, K.1, Karasawa, Y.1, Ito, M.1, Takeuchi, M.1
1National Defense Medical College, Ophthalmology, Tokorozawa, Japan, 2National Defense Medical College, Ophthalmology, Tokora- zawa, Japan, 3National Defense Medical College, Developmental Anatomy, Tokorozawa, Japan

Background: Diabetic retinopathy (DR) is one of the most important complications of diabetes mellitus. The main causes of DR are vascular damages in retinal blood vessels induced by hyperglycemia, oxidative stress, chronic inflammation, and immune imbalance. IL-17 is known as an angiogenic factor, that promo- tes development of microvessel structures. We have recently re- ported that vitreous levels of IL-4, IL-17A, IL-22, IL-31, and TNFs in proliferative diabetic retinopathy (PDR) patients were higher than the respective levels in serum, and that vitreous levels of these cytokines were higher in PDR than other diseases. These results suggest that Th2 cells producing IL-4 and IL-31 and Th17 cells producing IL-17A and IL-22 are involved in the development of DR. In this study, we examined whether reinforcement of Th2- and Th17-cell mediated immune responses progress DR. Method: Heterozygous Ins2+/− mice (Akita) develop pronounced and sustained hyperglycemia, high levels of albuminuria, and some histopathological changes in the retina. However, histopathological changes of DR leading to proliferation in basal membrane have not been observed in Akita mice, nor in other animal models of DR. We cross- bred Akita mice (Ins2+/−) with interferon gamma knock out (GKO) mice (Ifng−/−) for two generations to give birth to the mice with the genotype of Ins2+/−/Ifng−/− (Akita x GKO). Wild type, GKO, Akita, or Akita x GKO mice were immunized with OVA (5μg /0.1ml) emul- sified with Montanide ISA 51 at various sampling time-points in the 12-week-old. At 8-week-old, fundus photography, fluorescein angiography(FA), and leukostasis analysis using retinal flat mounts were performed. Then, expression of CD3, CD4, CD8, and MHC-II was measured by quantitative PCR. For statistical analysis, JMP Ver. 13 was used and the significant difference was set at <0.05.

Result: Leukostasis was significantly increased in Akita x GKO mice compared with the other three groups (p < 0.05, respec- tively). Expression of VEGF, ICAM-1, GATA-3 in Akita x GKO mice was significantly increased (p < 0.05) in the retina and spleen. Ret- inal exudative lesions corresponding to vascular leakages by FA was observed only in Akita x GKO mice. In addition, Akita x GKO mice presented constricted retinal vessels and neovascularization. Conclusion: These results demonstrated that activation of Th2- and Th17-cell mediated immune responses are involved in progression of DR.

Pro-inflammatory role of 12/15-Lipoxygenase-derived Eicosanoids in Diabetic Retinopathy
Al-Shabrawey, M.1, Ibrahim, A.1, Tawfik, A.1, Elmasry, K.1, Saul, A.1, Smith, S.1
1Augusta University, Dental and Medical Colleges of Georgia, Oral Biology, Anatomy, Vision Discovery Institute and Ophthalmology, Augusta, United States, 2Augusta University, Dental and Medical Colleges of Georgia, Oral Biology and Vision Discovery Institute, Augusta, United States, 3Augusta University, Dental and Medical Colleges of Georgia, Oral Biology and Cellular Biology and Anatomy, Augusta, United States, 4Augusta University, Medical College of Georgia, Vision Discovery Institute and Ophthalmology, Augusta, United States, 5Augusta University, Medical College of Georgia, Cellular Biology and Anatomy, Vision Discovery Institute and Ophthalmology, Augusta, United States

The pathogenesis of neurovascular dysfunction in diabetic retinopathy (DR) is highly complex and includes several factors that contribute to retinal cell death and hyperpermeability. However, the role of 12/15-lipoxygenase (LO)-derived eicosanoids remains unclear. We recently investigated the role of 12/15-LO in leukostasis, retinal hyperpermeability and neovascularization. There were significant increases in retinal expression and activity of 12/15-LO by diabetes in both human and experimental mice. LC/MS lipidomic screening of vitreous samples from patients with DR and retina of diabetic mice showed significant increase in levels of 12/15-LO metabolites (12- and 15-hydroxyeicosatetraenoic acids or HETEs) compared to non-diabetic groups. Treatment of human retinal endothelial cells (HRECs) with high glucose (HG, 30mm D-glucose) induced significant increases in 12/15-LO expression and activity. Inhibition or deletion of 12/15-LO attenuated the inflammatory response in retina of diabetic mice as shown by signifi- cant reduction in inflammatory markers (ICAM-1, VCAM-1, CD45, ER stress and permeability). While, treatment of HRECs with 15- HETE induced leukocyte adhesion, permeability and inflammatory cytokines, 12/15-LO inhibition or silencing prevented HG-induced endothelial inflammatory response. We also studied the contribu- tion of retinal endothelial versus leukocyte12/15-LO in DR. LC-MS screening of eicosanoids in plasma of 6-month streptozotocin-in- duced diabetic mice showed no significant changes in levels of major 12/15-LO-derived eicosanoids (12- and 15- HETEs). Myeloperoxidase assay of leukostasis, showed that leukocytes from 12/15- LO KO and wild type mice display a similar increase in adhesion to HG-activated endothelial cells suggesting that endothelial rather leukocyte 12/15-LO is implicated in DR. We also studied the role of 12/15-LO in neuronal injury in DR by generating a new mouse model of diabetes that lacks 12/15-LO (Ins2+/−/12/15-LO −/−). Electrotetrogram analysis after 6 month diabetes showed significant reduction of responses to natural noise stimuli, photopic b-wave amplitudes, and positive scotopic threshold at different flash in-
VEGF-B Protects Muller Cells under Hypoxic and Oxidative Conditions

Lorian-Salvador, M., Lechner, J., Augustine, J., Mei, C., Xu, H.

Welcome-Wolfson Institute of Experimental Medicine, School of Medicine, Dentistry and Biomedical Sciences, Queen's University Belfast, Belfast, United Kingdom

Muller cells play an important role in retinal pathophysiology. Muller cell-derived vascular endothelial growth factor (VEGF) is critically involved in retinal cell survival and function, although pathological levels of VEGF can induce the breakdown of the blood retinal barrier leading to macular oedema. There are different isoforms of VEGF, VEGF-A, VEGF-B, VEGF-C and VEGF-D, among which the role of Muller cell-derived VEGF-A in retinal health and disease has been studied extensively. Whereas, the role of other VEGF isoforms in retinal pathophysiology remains poorly defined. In this study we examined the VEGF expression profile in primary murine Muller cells and in an immortalized Muller cell line GMm1C1 and further examined the role of VEGF in Muller cell activation and function. Gla fibroblastic retinal protein (GFRAP), water and ions channels AQP4 and Kir4.1 and GLAST and Glutamine synthetase (GS) expression were measured by RT-PCR and Western Blot. Using real-time RT-PCR and ELISA, we found that Muller cells express higher levels of VEGF-B among the different VEGF members, along with its receptor VEGFR2 and the co-receptor NRP1 under normal culture conditions. Blocking VEGF-B using a neutralizing antibody did not affect the viability, functionality or induce glialization in Muller cells. Hypoxia and oxidative stress (H/NES) significantly altered the expression of VEGF-B and its receptor in GMm1C1 cells. Blocking VEGF-B and/or VEGFR1 and NRP1 compromise cell survival under hypoxic or oxidative stress conditions by increasing apoptosis. VEGF-B neutralization decreased Kir4.1 and AQP4 expression under hypoxic and oxidative conditions, suggesting a role of VEGF-B in water and ion maintenance. Furthermore, the addition of recombinant VEGF-B effectively restored normal expression of GS under hypoxic conditions, along with a significant decrease of GLAST mRNA after its neutralization, indicating a possible protective effect of glutamate clearance exerted by this growth factor. Our results suggest that VEGF-B plays an important role in Muller cell survival and function under hypoxic or oxidative conditions.

Caballero B, Sherman SJ, Falk T. Parkinsons Dis. 2017;2017:4263795

How Can We Manipulate Antigen Presentation in Order to Treat Retinal Diseases

Willermain, F.

Université Libre de Bruxelles, Ophthalmology CHU StPierre, Bruxelles, Belgium

The immune system is dedicated to the recognition and elimination of biological danger. While its innate arm harnesses receptors that recognize conserved pathogen patterns, the adaptive component has more recently evolved and can basically discriminate between self and non self. This highly specific system is based on the presentation of peptides by MHC class II molecules on antigen presenting cells to lymphocytes that will mount a specific immune response against the antigen. The adaptive immune system is thus very efficient to fight against pathogens, but is also unfortunately at the basis of autoimmunity, where the immune response targets self protein instead of foreign antigen. Interestingly, retinal MHC class II expression is a key feature of immune mediated retinal disease, such as uveitis, but occur also during degenerative or vascular disease, suggesting that local autoimmunity is also involved in those pathology. In this talk, the mouse model of non infectious uveitis will be used as a paradigm to study the role of MHC class II expression during retinal disease. We will first review data on the characterization of retinal cell type expressing MHC class II during uveitis. Next, we will discuss what has been proposed in terms of antigen presentation modulation in uveitis, and used transcriptomics results to propose alternative strategies.

IMM9 - The role of antigen presentation and autoimmunity in retinal diseases

Diversity in Autoimmunity against Retinal Antigens in Retinal Degeneration

Adamus, G.

Oregon Health & Science University, Portland, United States

Retinal degenerative diseases, such as retinitis pigmentosa (RP), age related retinal degeneration (AMD), and paraneoplastic and non-paraneoplastic autoimmune retinopathies are characterized by the progressive loss of rod photoreceptors followed by loss of cones. Recent findings support the concept that the immune/autoimmune mechanisms may play an important role in the degenerative process. During degeneration sequestered antigens can be released, which may trigger the immune responses to free molecules. In different forms of retinal degeneration overproduction of cellular debris resulting from photoreceptor death leads to subretinal infiltration of microglia/macrophages. Activated microglial cells phagocytose photoreceptor debris thus limit subsequent inflammation but they can also have an adverse role in initiating inflammation in the retina. The mouse and rat studies showed that anti-rtital-veinautoantibodies and T cells were generated during retinal disease in response to the increased presence of antigenic material released from dying photoreceptors. Passive transfer of dystrophic anti-rtital-vein antibodies enhanced disease progression by disrupting the BRB, attracting blood macrophages into the retina, and augmenting apoptotic photoreceptor death. These findings likely link anti-rtital-vein autoantibodies to activated macrophages entry and their possible role in neurodegeneration. Increased expression of pro-inflammatory cytokines and chemokines, activation of microglia, and photoreceptor apoptosis can predispose to the secondary autoimmunity in hereditary retinopathy, which is evident by the presence of anti-rtital-vein autoantibodies specific to proteins essential for phototransduction (rhodopsin, rod transducin, recoverin, PDE6) and glycolysis. They persist over the evolution of retinal degeneration and could perpetuate the condition. Altogether, autoimmunity could be responsible for the progression of photoreceptor cell death initiated by some other processes.

Non-infectious Uveitis: The Question of Autoimmunity

Forrester, J.V., Kuffova, L., Dick, A.D.

1University of Aberdeen, Aberdeen, United Kingdom, 2University of Bristol, Bristol, United Kingdom

The pathogenesis of uveitis is complex and multifactorial. Traditionally, uveitis is considered to be caused by infection or to be “non-infectious” / “undifferentiated”. Here, we offer a reappraisal of the gap between anterior vs posterior uveitis in the context of (a) the blood-retinal barrier and (b) its relation to autoimmunity, auto-inflammation, and infection. We propose that infection may be implicated directly or indirectly in many, if not most, forms of noninfectious or undifferentiated uveitis.

Difficulties of Diagnosis for Ocular Sarcoidosis in Japanese Unprecedented Ageing Society

Takayama, K., Harimoto, K., Sato, T., Kanda, T., Takeuchi, M.

National Defense Medical College, Department of Ophthalmology, Tokorozawa, Japan

The distribution of age at diagnosis in ocular sarcoidosis has shifted towards the older age groups in developed countries. In systemic sarcoidosis, age-related differences in the clinical presentation, which reflect the therapeutic strategies, were reported. Although the age-related difference in the clinical presentation have not been reported, we evaluate the age-related changes in the clinical features of ocular sarcoidosis. Clinical records of 100 consecutive Japanese patients from April 2010 to March 2016 in National Defense Medical College which were initially diagnosed with ocular sarcoidosis by International Workshop on Ocular Sarcoidosis criteria were retrospectively reviewed. They were classified into two main groups: retired (males 45 years; 50 patients) and working (< 65 years; 50 patients) groups. All patients received ophthalmic examination confirmed the presence of 7 intraocular signs and 4 laboratory examinations, defined by the criteria. In the retired group, significantly fewer uveitis signs (2.8 ± 1.5 and 3.6 ± 1.5, P = 0.0034) and laboratory results (1.5 ± 1.2 and 2.0 ± 1.2, P = 0.023) were detected, in the working group; statistical differences were found between the groups regarding the frequencies of mutton-fat kerato-prelatic precipitates (40% and 64%, P = 0.012), vitreous opacities (60% and 78%, P = 0.0059), bilateral inflammation (64% and 80%, P = 0.012), and bilateral hilar lymphadenopathy between the groups (52% and 78%, P < 0.001). Multiple linear regression analysis showed negative correlations between age and number of detected uveitis signs (r = -0.36, P = 0.001) and laboratory results (r = -0.20, P = 0.023). Our results indicated that the characteristic uveitis signs and laboratory results had a lower frequency in the elder patients compared with the younger patients. Prognosis or possible ocular sarcoidosis in the international criteria would increase because an aging society advances in developed countries.
tibodies, reduced retinal neovascularization and vaso-obliterration in OIR. Furthermore, pro-angiogenic and pro-inflammatory factors such as vascular endothelial growth factor (VEGF) and tumor necrosis factor (TNF) were also reduced in the retina of OIR mice treated with the CD8 depletion antibody but not with the CD4 depletion antibody. To further study CD8 T cells, Rag-1 KO mice which lack this cell population were evaluated, and were found to be protected from OIR-induced retinal vasculopathy and inflammation. To confirm that these effects were CD8 T cell-dependent, CD8 T cells were adoptively transferred into Rag-1 KO mice with OIR partially reconstituted CD8 T cells in lymphoid compartments and CD8 T cells infiltrated into the retina. Flow cytometry of lymphoid organs revealed that CD8 T cells are increased in lymphoid organs of OIR mice in the early phase of the disease and infiltrate the retina as the development of neovascularization is established. Conclusions: CD8 T cells promote the development of neovascularization in ischemic retinopathy by augmenting the inflammatory response in the retina.

**Mechanotransduction Drives the Activation of Retinal Müller Cells, Astrocytes and Microglia**

Lakk, M., Redmon, S.N., Yarishkin, O., Baumann, J.M., Krajci, D.

The University of Iowa School of Medicine, John A. Moran Eye Institute, Ophthalmology and Visual Sciences, Salt Lake City, United States

**Purpose:** It is not clear whether neuroinflammation in glaucomatous ONH or diabetic retinopathy is a secondary consequence of neuronal damage or whether retinal Müller cells (MC), microglia and astrocytes are directly activated by mechanical stressors such as pressure and swelling. To address this, we investigated the relative expression, localization and function of key mechanotransducing Ca2+-permeable ion channels in retinal glia subjected to pressure, swelling and/or strain stimuli. Methods: Cells, microglia and astrocytes from C57Bl/6 and transgenic GFAP:GCaMP5, CRALBP:GCaMP5, Iba1:GCaMP5 and TRPV4-GFP cells/retinas. Relative transcript levels were determined in magnetoseparated control, stretch-stimulated and/or glaucomatous/diabetic glia; protein levels were determined by immunoblots and IHC. Channel activation and calcium signaling in situ were studied in GCaMP-expressing retinal ganglion cell (RGC) cell lines. Results: Müller cells, microglia and astrocytes show changes in immune-related pathways, such as the complement cascade, NO- like receptor signaling, and toll-like receptor signaling, occur prior to retinal ganglion cell loss. Genes in these pathways are expressed in microglia, microglia, and monocytes/macrophages. Here, we focus on complement component C3 that were differentially sensitive to TRPV4 and piozo channel inhibitors and siRNAs. Vanilloid TRP channel agonists, pressure and swelling stimulus increased the frequency and amplitude of Ca transients in elevated Ca levels in Müller cells, microglia and astrocytes. TRPV4-dependent Ca increases in astrocytes were sensitive to antagonists of P2X receptor agonists. Finally, Piezo1 and TRP channels appear to transduce different facets of the mechanical (pressure, swelling, strain) response. Conclusion: Our results suggest that glial and cytokine release observed in glaucomatous ONH or diabetic retinopathy might be facilitated by calcium-dependent processes downstream from mechanotransducing ion channels expressed in MCs, microglia and astrocytes. The protective and neuroprotective actions of mechanical stress stimuli may be a common mechanism of neuroinflammation.

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**Inhibition of Early Immune Responses Exacerbate Retinal Ganglion Cell Loss in a Mouse Model of Ocular Hypertension**

Harder, J.1, Williams, P.2, Libby, R.1, Howell, G.1, John, S.1

1The Jackson Laboratory, Bar Harbor, United States; 2Karolinska Institute, Stockholm, Sweden; 3University of Rochester Medical School, Rochester, United States

**Purpose:** Recent findings indicate an important role of interleukin-33 (IL-33) signaling through its ST2 receptor in the pathophysiology of CNS diseases. However, it is currently unknown if IL-33/ST2 signaling is detrimental or beneficial for neuronal survival. Since expression of IL-33 is highly abundant in the optic nerve and glaucoma is the most common optic neuropathy disorder, we sought to determine the function of IL-33/ST2 receptor signaling in the glaucomatous optic neuropathy. Methods: Localization of ST2 was determined by immunostaining of human glaucomatous and healthy optic nerve head (ONH). Expression of IL-33, as well as soluble (sST2) and membrane-bound (ST2L) ST2 receptor expression, was analyzed by RT-PCR. The function of IL-33 signaling was investigated in vitro using an IL-33/LPS stimulation assay on microglia-like BV2 cells followed by analysis of secreted molecules. Results: rFTCP confirmed the presence of IL-33 in all examined ONH. sST2 expression is found almost 3 fold lower in glaucoma- tous ONHs, whereas the ST2 receptor shows an 8 fold increase. Double-immunostaining of ST2 and Iba1, a microglia/macrophage marker, confirmed expression of ST2 on microglia in the ONH. Stimulation of BV2 cells with IL-33 with and without LPS significantly increased expression of TNF-α (p<0.01), IL-4 (p<0.01) and IL- 10 (p<0.01) as well as decreased nitrite levels when compared to controls or LPS stimulation alone (both p<0.05). Decreased nitrite levels upon IL-18 levels could be detected. Transcript levels of sST2 are significantly decreased (p<0.05) upon IL-33/LPS stimulation and lead to an IL-33 dose-dependent increase of ST2L. Conclusion: These data indicate that in the glaucomatous ONH ST2 expression is altered in favor of the active, membrane bound, iso- form. In microglia-like BV2 cells secretion of both pro and anti-in- flammatory mediators, is affected upon IL-33 stimulation. Based on the large increase of IL-4 and 10 production and the reduction of nitrite levels, IL-33 seems to skew microglia towards an M2-like phenotype to promote anti-inflammatory and repair.

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**ORAL PRESENTATIONS**

**Ocular Immunology**

**IL-33/ST2 Signaling in the Glaucomatous Optic Nerve Head**

Kuehn, M.1,2, Gramlich, O.1,2

1University of Iowa, Ophthalmology and Visual Sciences, Iowa City, United States; 2Iowa City Veterans Affairs Medical Center, Center for the Prevention and Treatment of Vision Loss, Iowa City, United States

**Purpose:** Age-related glaucoma affects millions of people worldwide and is often associated with a chronic insult caused by ocular hyperten- sion. Based on evidence from human glaucoma and experimental models of disease, the response to ocular hypertension in the retina and optic nerve is complex. A large number of genes and co-ordinated activity between various types of cells appears to be an important component of the early pathogenic response to an ocu- lar hypertensive insult. To dissect this complexity, we used DDBA/2J mice that develop retinal ganglion cell specific cell death due to naturally occurring ocular hypertension. We have performed gene profiling on retina and optic nerve head tissues and specific cell types in these cell types. mRNA expression profiles show changes in immune-related pathways, such as the comple- ment cascade, NOD-like receptor signaling, and Toll-like receptor signaling, occur prior to retinal ganglion cell loss. Genes in these pathways are expressed in microglia, microglia, and monocytes/macrophages. Here, we focus on complement component C3 that were differentially sensitive to TRPV4 and piezo channel inhibitors and siRNAs. Vanilloid TRP channel agonists, pressure and swelling stimulus increased the frequency and amplitude of Ca transients in elevated Ca levels in Müller cells, microglia and astrocytes. TRPV4-dependent Ca increases in astrocytes were sensitive to antagonists of P2X receptor agonists. Finally, Piezo1 and TRP channels appear to transduce different facets of the mechanical (pressure, swelling, strain) response. Conclusion: Our results suggest that glial and cytokine release observed in glaucomatous ONH or diabetic retinopathy might be facilitated by calcium-dependent processes downstream from mechanotransducing ion channels expressed in MCs, microglia and astrocytes. The protective and neuroprotective actions of mechanical stress stimuli may be a common mechanism of neuroinflammation.

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**A Novel Clinically-relevant Optic Nerve Injury Model in Large Animals**


Wenzhou Eye Hospital, Wenzhou Medical University, Wenzhou, China

There are several groundbreaking discoveries to protect the retinal ganglion cells and promote optic nerve regeneration after optic nerve injuries. However, most of the studies were based on rodents. Here we developed a new large animal optic neuroinflammation model. This TON large animal model can offer a good opportunity to help bridge the gap between achievements of optic nerve repair in rodent and their clinical application in our TON patients.
Retinal vein occlusion (RVO) is a frequent cause of vision loss in the elderly population. There are two distinct types of RVO: branch retinal vein occlusion (BRVO) and central retinal vein occlusion (CRVO). Both of these two types can be non-ischemic or ischemic, the latter having a worse prognosis in terms of vision and potential complications. Both forms lead to neuroinflammation and may lead to atrophy of the inner retinal layers, mainly affecting the ganglion cells (RGC) and the retinal nerve fiber layer (RNFL). Previously established experimental BRVO in mice mimics the ischemic form in humans and showed activated microglia in the hypoxic area. In this study we investigated the consequences of microglia mediated neuroinflammation on RGC survival after ischemic BRVO in mice. Microglia cells were pharmacologically depleted by PLX5622, a small molecule inhibitor for the intracellular tyrosine kinase of the CSF-1 receptor (CSF1R). C3r1gfp/+ mice, specifically expressing green fluorescent protein (GFP) on microglia/macrophages, were fed with PLX5622 (incorporated into the mouse chow) for two weeks before laser-induced BRVO. One group of mice was continuously treated with PLX5622 for three more weeks, while a second group was switched to normal diet directly after BRVO induction (microglia recovery group). A third group received only laser-induced BRVO, without pre- and/or post-BRVO PLX5622-treatment. Depletion and changes in activation were observed in vivo by blue laser scanning laser ophthalmoscopy. Brn3a (retinal ganglion cells) and Iba1 (microglia/macrophages) histology of the whole mounted retinas showed an increased RGC survival in microglia depleted mice compared to the groups with non-depleted or recovered microglia cells. In addition, OCT scans revealed a delayed retinal atrophy in microglia depleted mice after BRVO. In summary, microglia depletion seems to be protective for RGC’s on ischemic branch retinal vein occlusion.

**Cell Type-specific Complement Expression in the Retina**

Pauly, D.1,2, Schäfer, N., Hauck, S.M.2, Grosche, A.1

1 University of Regensburg, Experimental Ophthalmology, Regensburg, Germany, 2 Helmholtz Center Munich, Research Unit Protein Science, Neuherberg, Germany, 3 Ludwig-Maximilians-Universität Munich, Department of Physiological Genomics, Munich, Germany

**Background:** Retinal complement activity has so far been related to microglia or to the influence of the systemic, liver-derived complement components. Here we describe for the first time a liver-independent retinal complement system in mouse and human retina by cell type-specific complement expression analysis.

**Methods:** The major five retinal cell types were isolated by MACS sorting from murine and human retinas. We analysed cell type-specific expression levels of complement components and complement activation products by Western blotting and qRT-PCR in cell populations and primary porcine RPE cells. Patch-clamp recordings and Ca2+ imaging revealed that insertion of a lytic pore-forming channel into retinal cell membranes increases intracellular Ca2+ concentrations.

**Results:** We found that microglia/macrophages and Müller cells expressed very high levels of complement components and activators. In contrast, neurons and RPE cells showed low expression of complement components and activators. Our findings suggest that activation of the classical complement pathway, but not the alternative pathway, plays a major role in retinal inflammation and degeneration.

**Conclusion:** Microglia/macrophages and Müller cells are the main sources of complement components and activators in the retina. This suggests that complement activation is a key factor in retinal degeneration and that targeting complement in the retina may be a promising therapeutic strategy.
Effect of the Colony Stimulating Factor-1 Receptor inhibitor, PLX5622, on the Disease Course of Experimental Choroidal Neovascularization in Mice
Kokona, D., Schwarzer, P., Ebner, A., Zinkernagel, M.S.

Activation of retinal microglia and infiltration of monocytes into the retina has been observed in choroidal neovascularization (CNV). Here, we employed the highly selective colony stimulating factor-1 receptor (CSF-1R) inhibitor, PLX5622, to deplete microglia and to investigate the effect of microglia depletion on the disease course of choroidal neovascularization (CNV) using C57BL6/J and C57.Gg-Tg(Csf1r-EgfP)Hume1 mouse. Mice had ad libitum access to chow containing PLX5622 or to standard rodent diet (control group), for the whole duration of the experiment. CNV was induced 1 week after the initiation of the PLX5622 diet by applying 3 laser spots around the optic nerve head of each eye. Mice were imaged in vivo with fluorescein angiography and/or auto fluorescence imaging at baseline, 1 week after the initiation of the PLX5622 diet, and at days 3, 7 and 14 after the induction of CNV. Animals were euthanized at day 14 and their eyes were prepared for retinal and retinal pigment epithelium (RPE)-choroidal wholemounts, using antibodies against microglia/macrophages and isocitrate-B4 for vascularule staining. Flow cytometry was performed in mouse eyespots 3 and 7 days after laser using markers for microglia and macrophages, and in peripheral blood and spleen. Immuno-histochimical studies revealed that PLX5622 effectively depleted microglia from the mouse retina and led to a significantly reduced CNV area (approximately 2-fold sensory CNV). These results confirm previous findings that cone OS optical length changes were observed between the IS/OS and RPE. In addition, the intensity of the IS/OS and COST were monitored, as well as other changes in axial reflectivity profile. For bright stimuli, most cones showed consistent and reproducible positive changes in OS phase in response to the stimulus flash, consistent with OS optical elongation. In addition, reflectivity of IS/OS was typically observed to decrease after stimulation. A number of paradoxical observations were made as well. Reflectivity of IS/OS was observed sometimes to increase after stimulation, and reflectivity of COST was observed to increase in some cones and decrease in others. In addition, a variety of other scattering changes were observed between the IS/OS and RPE. These results confirm previous findings that cone OS optical length changes in response to visible stimuli, but suggest that the sign of length change may depend upon stimulus strength and that a variety of other changes occur in the reflectance of the cone, which suggest a diversity of possible optical biomarkers for photoreceptor function.

Assessing Retinal Structure in Patients with X-linked Cone Opsin Mutations Using Adaptive Optics Scanning Light Ophthalmoscopy
Patterson, E.1, Kaltiozis, A.2,3, Kasliani, M.2,4, Gardner, J.2,3, Nitz, J.2, Hardcastle, A.2,3, Nitz, M.5, Michaelides, M.2,3, Carroll, J.2,3

1Medical College of Wisconsin, Department of Ophthalmology & Visual Science, Milwaukee, United States, 2University College London, UCL Institute of Ophthalmology, London, UK, 3Mayo Clinic, Jacksonville, FL, 4University of Washington, Department of Ophthalmology, Seattle, United States, 5Medical College of Wisconsin, Milwaukee, United States

Functioning cone photoreceptors are essential for most daily visual tasks, such as those that involve central fixation, bright lighting, and discrimination of fine spatial details or colour. Mutations that affect structural and/or functional integrity of the cone photoreceptors can arise due to various mechanisms: from recombination between long- and medium-wavelength sensitive opsins genes (OPN1LW and OPN1MW) respectively, to upregulated deletion of the Locus Control Region. Although opsins gene defects have traditionally been associated with colour vision deficiencies, the heterogeneity of both structural and functional effects is now well appreciated. Phenotypes range from simple dichromacy (commonly referred to as colour blindness) to progressive degeneration of cones, potentially causing devastating vision loss. Even among patients with the same mutation, variation in the specific sequence of the array, the expression level of correctly spliced cone opsin, and the ratio of functional to non-functional cone photoreceptors can affect the severity of the disease. High resolution Adaptive Optics Scanning Light Ophthalmoscopy (AOSLO) has emerged as a powerful tool for assessing photoreceptor integrity, as it enables cellular scale resolution in the living eye. Here we explore the relationship between genotype and phenotype across subjects with mutations affecting the OPN1LW/OPN1MW gene array. Forty-three patients with mutations affecting one or more OPN1LW/OPN1MW genes, with a range of colour vision phenotypes, were recruited for imaging. Using both confocal and non-confocal split-detection AOSLO, we acquired and analysed en face retinal images in 28 of 39 patients. Remnant cone inner segment structure was present at the fovea in all but one patient, despite variably disrupted waveguiding. A patient expressing the UVV4a 3 haplotype in the only gene in the array had cone inner segment structure in one imaging session, which had degenerated at a later session: this demonstrates the utility of AOSLO in monitoring disease progression and assessing therapeutic potentially. Finally, we present evidence for a correlation between the ratio of functional to non-functional cones in the retina and axial length and discuss the implications with relation to developmental mechanisms.
OCT Angiography and Cone Photoreceptor Imaging in Geographic Atrophy

Duncan, J.1, Qin, J.1, Rinella, N.1, Zhang, Q.2, Zhou, H.2, Delvene, D.1, Roorda, A.1, Porco, T.1, Wang, R.K.1,2, Schwartz, D.1

1University of California, San Francisco, Ophthalmology, San Francisco, United States, 2University of Washington, Bioengineering, Seattle, United States.

Purpose: To compare cone spacing and choriocapillaris (CC) perfusion adjacent to geographic atrophy (GA) in patients with age-related macular degeneration (AMD) and age-similar normal eyes.

Methods: Subjects were imaged using adaptive optics scanning laser ophthalmoscopy (AOSLO), fundus autofluorescence (FAF), and swept-source optical coherence tomography angiography (SSOCTA). The GA border was identified using FAF images; CC flow void was analyzed in 1° regions extending from the GA border. A grader masked to CC perfusion selected regions of interest (ROIs) with unambiguous cone mosaics in AOSLO images. At each ROI cone spacing and CC flow void were converted to Z-scores (standard deviations from the mean of 9 normal eyes aged 50-75 years for cone spacing, and 60 normal eyes aged 51-88 years for CC flow void).

Results: Excluding regions of GA and drusen, CC perfusion was significantly greater than in 2-age-similar normal eyes (exact permutation test, P = 0.036). CC flow void was negatively correlated with distance from the GA margin (r = -0.35, 95% CI 0.14 to 0.12). Increased cone spacing was significantly correlated with CC flow void (r = 0.38, 95% CI 0.14 to 0.68). Cone spacing was increased in 36% of ROIs, while CC flow void was increased in 96% of ROIs.

Conclusions: In eyes with GA due to AMD, CC hypoperfusion was significantly correlated with cone spacing. This is relevant for potential diagnostics of diseases that perturb the healthy structure of the eye fundus. Relatedly, we studied how light incidence onto the retina at oblique angles is absorbed by cone photoreceptors as determined by the Stiles-Crawford effect of the first kind with a characteristic directionality pattern that describes the drop-off in efficiency with increasing angle of incidence with numerical values that depend on the method of analysis used. We report on the experimental validation and compare it to theoretical simulations of photoreceptor mosaic imaging under different conditions, as well as the possible limitations of the model. The numerical simulations show that photoreceptor waveguiding may play a lesser role than commonly assumed. We also discuss how the light acceptance is determined by absorption in the visual pigments of the outer segments and how nonguided light may again play an essential role. Likewise, we show examples of refractive index enhanced structures that emulate the retina photoreceptor mosaic with potential applications as angular filters for retinal prostheses and advanced models of the human eye.

Ocular Imaging & Psychophysics

Understanding Retinal Directionality in Imaging and in Vision - Seeing beyond Waveguiding

Vohsken, B.1, Qaysi, S.1, Thomas, P.S.1, Keegan, D.1

1University College Dublin, School of Physics, Dublin, Ireland, 2Mater Misericordiae University Hospital, Dublin, Ireland.

We have developed objective methods using adaptive optics fundus photography and scanning laser ophthalmoscopy to examine directional light scattering from the retina. This is relevant for potential diagnostics of diseases that perturb the healthy structure of the eye fundus. Relatedly, we studied how light incidence onto the retina at oblique angles is absorbed by cone photoreceptors as determined by the Stiles-Crawford effect of the first kind with a characteristic directionality pattern that describes the drop-off in efficiency with increasing angle of incidence with numerical values that depend on the method of analysis used. We report on the experimental validation and compare it to theoretical simulations of photoreceptor mosaic imaging under different conditions, as well as the possible limitations of the model. The numerical simulations show that photoreceptor waveguiding may play a lesser role than commonly assumed. We also discuss how the light acceptance is determined by absorption in the visual pigments of the outer segments and how nonguided light may again play an essential role. Likewise, we show examples of refractive index enhanced structures that emulate the retina photoreceptor mosaic with potential applications as angular filters for retinal prostheses and advanced models of the human eye. Our findings suggest that both vision and high-resolution photoreceptor mosaic imaging needs renewed analysis to better account for also nonguided components of the light. We discuss future goals of our research expanding on these findings and how this may ultimately impact on our clinical studies of retinal disease.

Cone Photoreceptor Structure in RPE65-Associated Leber Congenital Amauarism

Kalitzeos, A.1, Kumaran, N.1, Georgiou, M.1, Singh, N.1, Kane, T.1, Kasilian, M.1, Dubra, A.1, Carroll, J.1, Michaelides, M.1

1UCL, Institute of Ophthalmology, London, United Kingdom, 2Stanford University/Biers Eye Institute, Palo Alto, United States, 3Medical College of Wisconsin Eye Institute, Milwaukee, United States.

RPE65-associated LCA (RPE65-LCA) is a severe early onset retinal dystrophy characterised by progressive rod cone photoreceptor loss. Past studies in RPE65-LCA have sought to examine remnant cone structure by means of OCT but the limited transverse resolution does not allow differentiation between red rods and cones. Adaptive Optics (AO) imaging uniquely affords an in vivo cellular resolution in vivo, thus providing the ability to directly track cone photoreceptors in RPE65-LCA. The purpose of this study is to report cone photoreceptor structure in molecularly confirmed patients with RPE65-LCA. Patients were recruited as part of an on-going prospective natural history study. Image sequences of the photoreceptor mosaic were acquired using a custom AO Scanning Light Ophthalmoscope (AOSLO-U) covering the fovea and the 8 degrees visual field perimerital retina in the superior meridian relative to it. Cone outer segments (confocal) and inner segments (split-detection) were resolved and recorded. Cone densities and mean inter-cell distances were derived and compared to respective figures from unaffected retinas. Despite RPE65-LCA being arguably one of the most intensively studied and well-understood among all rare molecular forms of human inherited retinopathies, this is the first study that presents a representative patient cohort and objectively quantifies the remnant cone structure. Given that protection against cone photoreceptor degeneration is a key goal of therapeutic intervention to thereby slow/prevent loss of central vision, in vivo quantification of cone photoreceptor structure will be invaluable.

IMA2 - Imaging and understanding retinal blood flow and vasculature disease

Quantifying Retinal Microvasculature with AOSLO vs. OCTA

Elsner, A.1, Burns, S.1, Arthur, E.1, Sapoznik, K.1, Papay, J.1, Muller, M.2

1Indiana University, School of Optometry, Bloomington, United States, 2Acion Imaging, LLC, Bloomington, United States.

Diabetic retinopathy (DR) and diabetic macular edema (DME) are common causes of visual impairment in working age adults, often under-detected or classified as less severe than shown by the retinal microvasculature. Advanced imaging techniques include both reflectance information and mapping of the motion of blood cells: adaptive optics scanning laser ophthalmoscopy (AOSLO) and OCT angiography (OCTA). Lesions such as tangential capillaries, which demonstrate epithelial cell migration and tubule formation, and other vessel remodelling are among key target lesions. The two methods have underlying quantitative differences and capabilities for detection of features. A key underlying difference between AOSLO and OCTA is the significantly higher lateral resolution of AOSLO (2 microns), but higher axial resolution of OCTA (<10 microns) compared with AOSLO (>40 microns). A second difference is the field of view, in which AOSLO is usually limited to an isoplanatic patch of a few deg visual angle, but often 10x larger in OCTA, depending upon instrumentation. A third difference is the particle motion seen only in OCTA videos. We used the Indiana AOSLO and the Spectralis OCT2 (Heidelberg) Four types of outcome measures are compared for factors that influence measurements:

1) The FAZ;
2) the capillary gap: distance from an arteriole or venule to the nearest capillaries, which provides similar information but is not limited to the central macula; and
3) visibility of the capillaries, and
4) capillary density. OCTA does not visualize vessel walls, but AOSLO has wall/lumen measures and ghost capillaries. The FAZ is seen for normal subjects and diabetic patients on both AOSLO and OCTA, but with differences as to specific capillaries seen, particularly in diabetic retinopathy. For both AOSLO and OCTA, the capillary gap is visualized worse outside the fovea. OCTA for pairs of arterioles and venules in 20 young, normal subjects, the width of the peripapillary capillary free zone (67.2±23.5 µm) was the perivenule capillary-free zone (14.4±9.2 µm). For 3 diabetic subjects, the distance from the arteriole lumen to the nearest capillaries for AOSLO was 59.0±21.0, 50.0±17.0, and 55.0±15.0 µm and for OCTA was 53.0±16.0, 51.0±20.0, and 52.0±15.0 µm, respectively.

Capillary density should not differ in simulated computations, but the larger pixels in OCTA lead to digitization errors in both capillary density and capillary tangents.

Alterations in Retinal Oxygen Delivery, Metabolism and Extraction Fraction due to Ischemia

Shahidi, M.

University of Southern California, Los Angeles, United States.

The retinal tissue requires an adequate supply of oxygen and nutrients to maintain normal function. Insufficient blood flow leads to hypoxia, which triggers development of vision-threatening pathologies in many retinal ischemic diseases. Hypoxia can also impair other retinal oxygen metabolism (MO2) (i.e., the oxygen is consumed), thus disabling cells to generate energy that is needed to maintain tissue viability and perform visual processing. MO2 is regulated by the balance of inner retinal oxygen delivery (DO2) (i.e., the rate oxygen is delivered into the retinal circulation) and oxygen extraction...
traction fraction (OEF) [the ratio of oxygen metabolism to delivery]. We investigated alterations in these key oxygen-metabolic metrics and the regulation of MO2 in a rodent model of retinal ischemia. We utilized our multimodal imaging technique for assessment of MO2, DO2, and OEF by measurements of retinal vascular oxygen content and blood flow in rats under partial or complete bilateral common carotid occlusion. MO2 and DO2 were reduced, whereas OEF was elevated under complete occlusion compared to partial occlusion. MO2 was relatively maintained under partial occlusion, but was reduced and equal to DO2 under complete occlusion. The findings help gain a better understanding of the pathophysiology of retinal ischemic conditions.

Insights Into the Retinal Vasculature from OCT Angiography in Normal Eyes and in Diabetic Retinopathy

Fawzi, A., Nesper, P.

Northwestern University, Ophthalmology, Chicago, United States

OCT Angiography provides 3D high resolution imaging of the retinal vasculature. Using this technology we have been able to re-construct the connectivity of the retinal capillary networks in 3D in healthy subjects. In diabetic eyes, we found that the retinal vascular capillary networks were differentially affected in the different stages of severity of diabetic retinopathy, providing novel insights into the pathophysiology of this vascular disease. Using statistical models, we identified a set of OCT angiography parameters which are effective at differentiating the stages of retinopathy. We will discuss these issues and their implications for the application of this technology in screening of patients.

Static and Dynamic Changes of Retinal Microvasculature in Multiple Sclerosis

Houston, S.1, Theofyllaktopoulos, V.1, Nunez Do Rio, J.M.2, Greenwood, J.1, Dubis, A.1

1University College London, Institute of Ophthalmology, London, United Kingdom, 2NIHR Biomedical Research Centre at Moorfields Eye Hospital and UCL Institute of Ophthalmology, London, United Kingdom

Purpose: Multiple sclerosis (MS) is a chronic inflammatory neurodegenerative disease. MRI studies have indicated altered brain perfusion in both the relapsing remitting (RRMS) and secondary progressive (SPMS) phases of MS, suggesting a vascular component to the disease. Due to the anatomical and physiological similarities of retinal and cerebral microvascularity, the retina may be used to investigate vascular changes in the brain and to provide insight into the link between vascular function and neurodegeneration in MS. The aim of this study was to explore retinal microvascular abnormalities in MS using adaptive optics imaging. Methods: Sixteen patients with MS (10 RRMS, 6 SPMS) and 8 controls were imaged using a custom built adaptive optics scanning light ophthalmoscope (AOSLO) with confocal and split detection imaging. AOSLO images of the retina and microvasculature were graded by 2 masked graders for the presence/absence of hairpin loops, intraluminal erythrocyte aggregates and inner retinal cysts (IRC). IRC were further classified according to their size. The speed of erythrocyte aggregates in 2 locations in MS patients (n = 6) and controls (n = 4) was measured using the Image/Manual Tracking Plug-in. The erythrocyte occupancy of retinal vessels was also assessed. Results: It was found that erythrocyte aggregates were significantly slower in MS patients (0.57±0.19mm/s) than in controls (0.89±0.24mm/s) (p = 0.005). For microvascular grading, graders had a fair agreement on hairpin loops (k = 0.368 [95% CI: 0.198, 0.538]) and good agreement for IRC (k = 0.627 [95% CI: 0.537, 0.717]). There was a significantly higher number of abnormalities in SPMS patients than controls (p = 0.02). Following IRC classification, it was found that the largest class of cysts were only found in MS patients. There was no significant difference in the number of small or medium sized cysts between control and MS patients. Conclusions: We found a significantly higher number of microvascular abnormalities in SPMS patients, which could be the result of long standing inflammation resulting in further degeneration. This is supported by decreased erythrocyte aggregate speed in MS patients when compared to controls. Avascular areas were shown to have a lower speed of erythrocyte aggregates in MS patients. This work was funded by the MS Society and NIHR Moorfields Biomedical Research Centre.

In vivo Imaging of Retinal Neovascularization Using Self-quenched ICG-based Imaging Agents

Feenstra, D.1, Denk, N.2, Jayagopal, A.1

1F. Hoffmann-La Roche, Ltd., Ophthalmology, preD, Basel, Switzerland, 2F. Hoffmann-La Roche, Ltd., Pharmaceutical Sciences, preD, Basel, Switzerland

New diagnostic tools capable of detecting vascular abnormalities early in retinal disease progression could help to identify patients who will respond to treatments and prevent or reduce unnecessary treatment burden in non-responders. The purpose of this study was to develop a novel imaging agent capable of detecting neovascularization in vivo. The membrane glycoprotein endoglin has low expression in resting endothelial cells (ECs) but is upregulated in proliferating ECs, making it an ideal target for imaging agents aimed at detecting neovascularization. In this study, we conjugated an endoglin specific antibody to the FDA-approved dye indocyanine green (ICG) to produce a covalently coupled endoglin-ICG product with self-quenching capacity due to non-covalent interactions between the dye and hydrophobic residues. However, upon binding to endoglin it is internalized with subsequent endosomal dissociation of the dye from the antibody which relieves quenching and allows for visualization of proliferating ECs. UV-vis spectrosocopy demonstrated that endoglin-ICG had an absorption peak at 700-800 nm, similar to the native dye. However, fluorescence spectroscopy showed that there were no observable peaks between 200-800 nm, confirming the self-quenching capacity of the conjugates. Upon dilution in 1% SDS, endoglin-ICG showed a significant increase in fluorescence compared to dilution in PBS, demonstrating that the self-quenching capacity could be abrogated under denaturing conditions. To determine whether endoglin-ICG could detect neovascularization in vivo the laser-choroidal neovascularization (CNV) mouse model was used. Mice were injected intravenously with 100 µg of endoglin-ICG 6 days after laser injury. ICG-ICG and unbound ICG were used as controls. 24 hours after administration of imaging probes, OCT imaging and ICG angiography were performed. Analysis, endoglin-ICG showed remarkable specificity for identifying areas of CNV. We found that the ICG signal intensity of endoglin-ICG was increased signal-to-background ratio (Mean Fluorescence Intensity: 2.8±1.08) compared to unbound ICG (MF=1.0±1.0) and ICG-ICG (MF=1.4±0.2) groups (n=6 mice/group, ANOVA, p<0.05) Non-invasive imaging techniques such as fluorescein and ICG angiography are routinely used in clinics to visualize leakage and disease progression in patients with retinal disease. This study further builds on these techniques to create a targeted imaging agent capable of visualizing neovascularization in vivo.

IIMA3 - Vision science with Virtual Reality

How Immersive Technologies Will Improve the Health and Education of a City

Maj-Williams, M.1,2

1University of Leeds, Leeds, United Kingdom, 2Bradford Institute for Health Research, Bradford, United Kingdom, 3Norwegian Centre for Vision, Kongsvinger, Norway

The ‘Born in Bradford’ study aims to improve the health and education of people living in Bradford (the 6th largest English city, population 293,717). The number of children dying in Bradford was almost double the national average in 2003 and levels of childhood illness are high, with poor educational outcomes and chronic unemployment facing school leavers. The BiB longitudinal cohort (www.borningbradford.nhs.uk) consists of >1,3500+ children and we are capturing all of the routine health and educational data available for these children, and using these data to implement interventions to help children identified with difficulties within Bradford. The routine data collection is supplemented by detailed investigations of the mother and child’s physical health and cognitive function. The mother and child’s physical health was tracked throughout pregnancy, birth and the early years to identify factors that impact on a child’s outcomes (in physical health, mental health, educational attainment and ultimately social mobility). Our cognitive assessments include rich measures of the child’s sensorimotor abilities and offline cognitive function (e.g. working memory and executive function abilities). The detailed cognitive investigations are conducted in discrete ‘sprints’, with the first sprint taking place when the children started school (4 to 5 years of age) and the second (current) sprint capturing the children when they enter the English primary school Year 4 (8 to 9 years of age). The first sprint included a comprehensive vision screening and we are about to embark on a detailed ophthalmic evaluation of all of the children within the cohort. This talk will present an overview of some of the findings from ‘Born in Bradford’ and discuss how the data might shed light on the aetiology of myopia, reveal the genetic and environmental risk factors for ophthalmic abnormality and document the impact of visual deficits on development and attainment in school subjects (such as reading). The talk will specifically focus on the emergence of immersive technologies within the children’s life time and consider the potential problems that such technologies might create at school with regard to learning and attainment. We are optimistic that the development of immersive technologies have the potential to improve the health and education of children across the city.

Distance Perception in VR

Hibbard, P.1, Hornsey, R.2

1University of Essex, Psychology, Colchester, United Kingdom, 2University of Kent, Canterbury, United Kingdom

The real size of an image, and its binocular disparity, both reduce as the distance to the object increases. In order to accurately perceive the size and shape of an object from retinal information, it is therefore necessary to know its distance. Observers typically show under-constancy, such that the apparent size and depth both tend to reduce with increasing distance. We assessed the degree of size and depth constancy for objects presented in consumer virtual reality, as a measure of the accuracy of 3D space in these devi- ces. Participants were presented with an ellipsoid in an Oculus Rift and HTC Vive, at distances between 40 and 100 cm at eye-height. Using the headsets’ controllers, their task was to alter the height and width of the ellipsoid to set its size, and the depth to set its shape, to match these against a hand-held tennis-ball. Results sho-
Simulating the Optics of the Human Eye with Ray-tracing

Lian, T.1, MacKenzie, K.2, Wandell, B.1

1Stanford University, Electrical Engineering, Stanford, United States, 2Facebook Reality Labs, Redmond, United States, 3Stanford University, Psychology, Stanford, United States

Background: Display technology design can benefit from a quantitative understanding of how changes in display parameters impact the human visual system. Vision scientists have developed many precise calculations and facts that characterize critical steps in vision, particularly at the first stages of light encoding. ISETBIO is an open-source implementation that aims to provide these computations. The initial implementation modeled image formation for distant or planar scenes. Here, we extend ISETBIO by using computer graphics and ray-tracing to model how spherical, three-dimensional scenes are transformed by human optics to the retinal irradiance.

Methods: Given a synthetic 3D scene, we trace rays using PBRIT (Physically Based Ray-Tracer) through an optical model, and obtain the spectral illuminance arriving at the retina. The optical model specifies surface parameters and wavelength-dependent index of refraction. We illustrate using the Navarro eye, which models the surfaces of the cornea, lens, and retina and matches their curvature, size, and asphericity to anatomical data. The methods can implement other eye models, including those with biconic surfaces. The simulation accounts for the chromatic dispersion of light in different ocular media, as well as the effects of accommodation and pupill size.

Results: To validate the model, we compare the retinal images generated from the simulation with experimental measurements. The sharpness of the computed retinal image matches statistical models in the literature. Further, the longitudinal chromatic aberration in our renderings closely matches experimental data.

Conclusion: The ability to model the spectral retinal irradiance from a 3D scene allows us to analyze the efficacy of different types of displays. This ability may also prove useful for understanding the demands on latency minimization and prediction. We will also describe some of the experiments from our lab using immersive VR and discuss how these allow us to investigate aspects of 3D perception that cannot be addressed using static observers.

IM4A - Advanced vision testing and imaging of ocular mechanics

How Well is the Crystalline Lens or IOL Attached to the Eye? The Lens and IOL Wobbling Effect

Taberner, J.1, Artal, P.2

1Anglia Ruskin University, Vision and Eye Research Unit, Cambridge, United Kingdom, 2Universidad de Murcia, Laboratorio de Optica, Murcia, Spain

The perception of vision is an extremely dynamic process. The eyes move continuously to keep objects of attention at the focus, and, after each ocular movement, the crystalline lens, or potentially an IOL, oscillates or ‘wobbles’ inside the eye due to the elastic nature of the zonular fibers (the lens/IOL wobbling effect). This effect can be observed with simple instrumentation (slit lamps) only if the oscillations are very large. Otherwise, the high frequency and the amplitude of the oscillations cannot be detected with the ‘naked’ eye. Currently, there are no commercial instruments to perform a quantitative and accurate detection (and follow-up) of lens/IOL wobbling.

In this talk, I will review my experiences in the development of advanced devices for measurement of lens wobbling using high-speed cameras. We have used Purkinje images (corneal refraction and lens/IOLs focusing) to precisely capture the movement of the ciliary muscle and IOLs. In parallel, we have characterized the wobbling of IOLs before and after the ciliary muscle is paralyzed (Taberner et al., Scientific Reports, 2016). Interestingly, when the muscle activity is paralyzed and subjects are at an advanced age (70+ years), they showed an attenuated wobbling effect compared to normal vision. This suggested the possibility to use lens wobbling parameters as an indirect measure of the ciliary muscle activity. In this context, I will also review an experiment (Taberner ro et al, ARVO 2016) where wobbling was measured at near and distant vision conditions in young and presbyopic subjects. Lens wobbling always increased at near conditions because of the zonular fiber relaxation. However, it did not increase more in younger than in presbyopic suggesting again that the ciliary muscle function is better preserved with aging. In summary, research in the lens wobbling effect can provide a much better understanding of the structural and visual problems affecting the crystalline lens and the ciliary muscle, and also which IOLs are better for people after cataract surgery. New recent devices can be used to facilitate better management and follow-up of the phenomena.
(2) OCT viborgraphy, in which the cornea is stimulated with 50-350 Hz acoustic vibrations, and (3) Air puff train pulse OCT viborgraphy, in which the cornea is stimulated with trains of air pulses.

In the first method, Scheimpflug, spectral or swept source OCT have been used, and the measured maximum deformation amplitudes is hundreds of microns. a. In the last two, phase sensitive swept source OCT is used, with micron corneal deformation amplitudes, and fundamental resonance frequencies in the range of 80-120 Hz. While these imaging methods allow measurement of dynamic corneal deformation parameters (i.e. deformation amplitude, to maximum deformation and recovery, deformation velocity, spatial indentation, corneal resonance frequency) those are only partially related to mechanical properties, and are, in general, also partially related to mechanical parameters such as corneal elasticity and viscosity-related parameters (i.e. Prony constants). Examples of application of this methodology include the assessment of standard (Ribofovin-UVA corneal) and new methods (Rose Bengal-green light) corneal cross-linking.

Visualization Function in Later Life: Physiological Aging vs. Chronological Aging

Katie, G.1, Hogg, D.R.2, Chakravarty, P.U.1, Anderson, P.R.2

1Queens University Belfast, Centre for Public Health, Belfast, United Kingdom, 2Ulster University, Department of Vision Sciences, Coleraine, United Kingdom

The rate of decline with ageing across a wide range of visual functions was investigated. We conducted these tests in a study sample that was subject to rigorous recruitment inclusion and exclusion criteria, and this sample was drawn from a large epidemiological study of Ageing (NICOLA). We found that distance visual acuity (DVA), low luminance visual acuity (LLVA), low luminance deficit (LLD), near visual acuity (NVA), contrast sensitivity was preserved to 70 years in the general population.

We assess how peripheral optical errors affect vision with the aim to design better peripheral corrections to (1) reduce the ocular growth in myopia and (2) improve the remaining vision for individuals with large central visual field loss. Compared to central vision, peripheral visual function depends on a complex interaction of optical and neural limitations. The optical aberrations, both chromatic and monochromatic, are considerably larger in the periphery and the neural sampling density is lower. Hence, visual acuity and contrast sensitivity of peripheral vision is lowered.

Peripheral Vision: Optical and Neural Factors

Lundström, L., Venkataraman, A.P., Romaschenko, D., Padogradgians, P., Winter, S., Unso, P.

Royal Institute of Technology (KTH), Applied Physics, Stockholm, Sweden

Infrared pulsed (IR) light is perceived as visible with a cor-responding effect of the wavelength of the excitation radiation. The explanation to this phenomenon is a 2-pho-ton absorption process. I will describe several of our experiments using this new modality of vision in the IR. We developed an instrument based on a set of scanning mirrors to deliver visual stimuli to the retina using IR pulsed light. Utilizing a femtosecond laser (435-fs pulse width) emitting at 1043 nm we measured the visual acuity in the IR and compared with that in the visible at 543 nm using a continuous wave He-Ne laser. After testing 6 subjects, although with some inter-subject variability, the visual acuity was similar in the visible and the IR. On the other hand, we tested the sensitivity to the IR by 2P absorption of the different types of photoreceptor cells responsible for the color vision. In particular, we tested the S-cones and the M-cones sensitivity to the IR by 2P absorption. With feedback present, gain to optical blur was significantly larger than to simulated blur. Without feedback, gain was reduced. In the first two experimental conditions were repeated in a second experiment where there was no feedback from light vergence or blur. Results: With feedback present, gain to optical blur was signi-ficantly larger than to simulated blur. Without feedback, eight subjects accommodated effectively to optical blur (gain = 0.50 +/-0.28), but the dynamic accommodation response to simulated blur was reduced (gain = 0.17 +/-0.03).

Conclusions: Accommodation responds to the convergence or divergence of light rays that form out-of-focus images, not simply to blur. Methods: Nine subjects viewed a monochromatic Maltese cross target in an adaptive-optics system that included a deformable mirror and aberrometer. Astigmatism and higher-order aberrations were recorded and removed at 20 Hz. In the first of two experiments, typical blur feedback was available to the subject from oscillations of accommodation, whereas in the second experiment, there was no feedback. Both experiments included two identical experimental conditions in which accommodation was driven either by optical blur from real out-of-focus images, or by computer-generated simulated blur. In the optical blur condition, the deformable mirror moved the target sinusoidally towards and away from the eye (-3D & -10D at 0.05, 0.1, 0.2, & 0.4 Hz, producing sinusoidal changes in both the vergence of light & blur & clearing of the out-of-focus image. Oscillations of accommodation provided trial-and-error feedback from both light vergence & blur. In the simulated blur condition, the target was always imaged accurately on the retina, while the image of the Maltese cross blur from sinusoidal changes in focus. To provide blur feedback, the target was blurred by an amount that depended on the subject’s accommodative error. Thus correct accommodation reduced blur while incorrect accommodation increased blur. Finally, the two experimental conditions were repeated in a second experiment where there was no feedback from light vergence or blur.
Comparison of Manual versus Computer Aided Photoreceptor Detection Methods in Usher Syndrome

Theodoulakopoulos, V.1, Houston, S.1, Mistlos, A.1, Davidson, B.2, Bergles, C.E., Moosajee, M.1, Dubis, A.1

1University College London, Institute of Ophthalmology, London, United Kingdom, 2University College London, Institute of Healthcare Engineering, London, United Kingdom

Purpose: Photoreceptor quantification is important for tracking the subtle changes associated with retinal degeneration. Several machine learning methods have been developed for both confocal and non-confocal photoreceptor imaging with AOSLO. However, these methods have been developed for specific diseases and their transferability is unknown. In preparation for treatment trials for retinitis pigmentosa (RP) associated with Usher syndrome, we sought to quantify reliability of photoreceptor identification using manual and automated methods.

Methods: Ten patients (age range 18-56 years); 5 with type 1 Usher syndrome (MYO7A), 5 with type 2 Usher syndrome (USH2A) and 2 with non-syndromic RP (USH2A), were imaged using a custom-built AOSLO. Twenty-seven pairs of simultaneous parfoveal confocal and split detection images, and 100 independent split detection images were selected to compare modalities and identification methods. Images were cropped to 100µm x 100µm. Two graders of differing experience compared modalities and identification methods. Images were acquired parafoveal confocal and split detection images, -mology, London, United Kingdom, -mology, London, United Kingdom, -mology, London, United Kingdom, -mology, London, United Kingdom.

Results: The manual grading detected 62-314 photoreceptors per image, while the automated grading found the average (range) of 0.32% (16.4 to 20.8%) for confocal and 0.33% (15.99 to 29.59%) for split detection. The expert grader on average 10.16% (8.04 to 26.19%) more cones on confocal and 11.86% (2.9 to 38.36%) on split detection than the novice. The expert grader found similar numbers of cones in both modalities (363 ± 360) vs the novice grader (328 ± 312). Both algorithms found considerably fewer photoreceptors than the manual grading. Algorithm 1 - 35% (range 13-69%) and algorithm 2 - 52% (range 11-93%). Visual inspection of automated methods confirmed poor cone identification.

Conclusions: Contrary to previous attempts, in these images there was better reproducibility of manual measures for confocal vs split detection. This is likely due to proximity to the fovea making identification in confocal easier than split detection. Significant losses in the machine learning methods confirm that algorithms need to be retrained on new diseases or at least on new photoreceptor appearances. Acknowledgments: NIHR Biomedical Resource Centre, Welcome Trust and EPSRC Doctoral Training Programme.

Evolution of Drusenoid Lesions in Rhesus Macaques as Seen on Multimodal Imaging and Histology

Yiu, G.1, Chung, S.1, Molhoff, L.2, Tieu, E.2, Cunefare, D.2, Farni, S.2, Roberts, J.1, Thomasy, S.1

University of California, Davis, Ophthalmology & Vision Sciences, Davis, United States, 2University of California, Davis, Sacramento, United States, 3Duke University, Biomedical Engineering, Durham, United States, 4California National Primate Research Center, Davis, United States, 5University of California, Davis, School of Veterinary Medicine, Davis, United States

Nonhuman primates are the only mammals to possess a true macula similar to humans, and spontaneously develop drusenoid deposits that are characteristic of age-related macular degeneration (AMD). In this study, we employ high-resolution in vivo ocular imaging to study the structural and imaging characteristics of drusenoid lesions and their progression in aged rhesus macaques, and ex vivo analysis of histological sections. Of 65 animals surveyed by ophthalmic examination and dilated fundoscopy, we identified drusenoid lesions in 20 animals (30.7%) and monitored their progression over 2 years using spectral domain optical coherence tomography (SD-OCT) and fundus autofluorescence (FAF), followed by histological analysis in a subset of eyes. Seeded segmentation of SD-OCT images were used to determine the average thickness of retinal layers across the central 6mm macular region, the volume of the retinal pigment epithelium (RPE)+drusen complex, and height of individual drusen lesions. Analysis of demographic and disease status revealed RPE+drusen complex, RPE+drusen complex, and height of individual drusen lesions. Analysis of demographic and disease status revealed -52% (range 11-93%). Visual inspection revealed membranous debris and features resembling basal laminar deposits in the soft drusen, as well as disruption of the photoreceptor layer, with lamellar deposits in the outer plexiform layer. Together, these results refine our understanding of drusenoid lesions as an important animal model for human AMD.

Stimulus-evoked Intrinsic Reflectance of Cone Photoreceptors: A Biomarker of Cone Function

Morgan, J.I.W.

University of Pennsylvania, Ophthalmology, Philadelphia, United States

Retinal imaging with adaptive optics (AO) has enabled noninvasive visualization of cone structure both in health and disease. Despite this, studies that assess cone function using AO remain sparse. Adaptive optics scanning light ophthalmoscope (AOSLO) images of the human cone mosaic have shown that visible light stimuli induce changes in infrared (IR) reflectivity. Here, we will discuss three experiments to establish stimulus-evoked intrinsic reflectance (dubbed the ‘reflectance response’) as an objective biomarker for cone function. For all experiments, we image the human cone mosaic using IR reflectance confocal AOSLO and determine its ratio of stimulus to baseline reflectance. Then, we will describe results from a recent set of experiments aimed at elucidating the spatial characteristics of color appearance and color detection. I will conclude by framing these results in relation to contemporary models of neural circuitry mediating color and spatial vision.

StIMaS - Probing the retina and vision at a single photoreceptor level, biomarkers, and multiphoton imaging

Sabesan, R.

University of Washington, Ophthalmology, Seattle, United States

The visual system reconstructs fine spatial detail and rich color experience from a paucity of wavelength and intensity signals originating in the cone mosaic. The role of the early visual system in decoding these elementary features of seeing remains mysterious. The trichromatic cone mosaic is organized in an interleaved fashion. As a consequence, at any given spatial location, the photoreceptors have different spatial frequencies available. This confounds a color essentially colorblind to a local scale. A comparison between cones of different types is thus essential for color to be retrieved in an image independent of intensity. Our studies are geared towards defining the neural substrates that govern such cone-opponent computations, including its spatial frequency characteristics, dependence on cone type, the local spectral arrangements, and the associated visual pathways. We use a combination of adaptive optics monochromatic aberration correction, high speed eye-tracking and chromatic aberration correction to allow light stimuli of multiple wavelengths to be targeted on individual cones. Consequently, visual perception upon control- lid activation of a single or a group of cones can be addressed in a living human yielding psychophysical measurements ultimately limited by the cone mosaic and downstream retinal and cortical circuitry. I will briefly describe the technological advances that enable such recordings. Then, I will describe results from a recent set of experiments aimed at outlining the spatial characteristics of color appearance and color detection. I will conclude by framing these results in relation to contemporary models of neural circuitry mediating color and spatial vision.
Functional retinal imaging with Optical Coherence Tomography

Zawadzki, R.J.1,2

1UC Davis EyePod Laboratory Dept. of Cell Biology and Human Anatomy, University of California Davis, Davis, United States, 2Vision Science and Advanced Retinal Imaging Laboratory (VSR) UC Davis Eye Center, University of California Davis, Sacramento, United States

Recent advances in the development of retinal Optical Coherence Tomography instrumentation and image processing allowed several successful demonstrations of light-induced functional responses of the retina. Several recent observations of functional retinal imaging in our laboratory on experimental animals will be presented. The physiological model explaining our current understanding of origins and kinetics of the measured signals, based on Starling osmo-elastic model of H2O transport, will be provided. I will also review some of the results presented by other groups with a focus on assessing the feasibility of our current model of light-induced changes in photoreceptors Outer Segment osmolarity and resulting swelling, to explain these observations. Implications of current results and possible future applications of OCT-based photoreceptor opthalmology for clinical and basic science (animal) studies will be presented as well.

Ocular Imaging & Psychophysics

ORAL PRESENTATIONS

RPE1 - Non-VEGF mechanisms in angiogenesis

IQGAP1 Regulates VEGF-induced Choroidal Endothelial Cell Migration through Rac1

Wang, H.1, Ushio-Fukai, M.2, Sacks, D.3, Hartnett, M.E.4

1University of Utah, Salt Lake City, United States, 2Augusta University Medicine, Augusta, United States, 3National Institute of Health, Bethesda, United States, 4University of Utah, Salt Lake City, United States

Two GTPases, Rac1 and Rap1a, have different roles in regulating reactive oxygen species (ROS) generated in RPE and choroidal endothelial cells (CECs) in CEC migration and choroidal neovascularization (CNV), as seen in age-related macular degeneration (AMD). Multidomain IQGAP1 coordinates signaling cascades to enable biologic events. We tested the hypothesis that IQGAP1 was important in CEC migration and invasive CNV. Primary human CECs treated with control PBS or VEGF (20 ng/ml) were assayed for: 1) Rac1 activity by immunoprecipitation (IP) with anti-GTP-Rac1 antibody and western blot of total Rac1, 2) co-IP of IQGAP1 and GTP-Rac1, and 3) phosphorylation (p) of IQGAP1 measured by co-IP of IQGAP1 and p-Serine. To determine if p-IQGAP1 was necessary for VEGF-mediated GTP-Rac1 and CEC migration, VEGF-induced p-VEGFR2 and GTP-Rac1, and CEC migration were measured in CECs co-transfected with IQGAP1 siRNA and plasmid DNA expressing either wild type IQGAP1 or IQGAP1 with serine 1441/1443 mutation into alanine. To inhibit binding of Rac1GTP to IQGAP1, CECs were transfected with a mutant Rac1 binding domain on IQGAP1 (IQGAP1/GDm) or wild type IQGAP1 (IQ/cont) and treated with VEGF or PBS. Iqgap1+/+ and Iqgap1-/− mice treated with laser or left untreated were assayed for total and p-IQGAP1 in lysates of RPE/choroids or labeled for IQGAP1 in sections. Statistics were with ANOVA and included 6 samples/condition. IQGAP1 was expressed in retinal sections of laser-induced CNV, but laser-induced CNV was increased in Iqgap1−/−mice compared to Iqgap1+/+ mice. Laser reduced total IQGAP1 but not p-IQGAP1. CECs transfected with GDm had less VEGF-induced Rac1GTP and Rac1/ IQGAP1 interactions compared to Iq/cont. CECs transfected with IQGAP1 siRNA had less VEGF-induced p-IQGAP1 and CEC migration compared to control. Constitutively active Rap1a inhibited Rac1 activation and Rac1/IQGAP1 interactions in association with increased VEGF-induced Rap1/IQGAP1 interactions. RPE/choroids from laser treated Iqgap1−/− mice had greater p-VEGFR2 compared to wild type. Our data support the hypothesis that VEGF-induced p-IQGAP1 was necessary for CEC migration and that active Rap1a, which interferes with NADPH oxidase generated ROS, inhibited these steps. Rac1GTP binding of GD in IQGAP1 is necessary for VEGF-induced activation of Rac1.

Negative Regulators of Angiogenesis and Exudative AMD

Sheibani, N.1, Sorenson, C.2

1University of Wisconsin School of Medicine and Public Health, Madison, United States, 2University of Wisconsin School of Medicine and Public Health, Pediatrics, Madison, United States

Age-related macular degeneration (AMD) is characterized by a progressive degeneration of the macula, and severe vision loss. The visual deficit initially arises from retinal degeneration (dry or atrophic AMD) and is often complicated by the secondary effects of choroidal neovascularization (CNV; wet or exudative AMD). The global occurrence of AMD is expected to double in the next decade due to aging of the population. Currently, VEGF-A antagonists are standard of care in the treatment of exudative AMD, and are a valuable additional treatment strategy in several other vascular retinal diseases. Thus, illustrating the therapeutic utility of blocking VEGF, a potent driver of angiogenesis. However, VEGF-A antagonists significantly improve vision in only 30% of patients and 20% of treated patients still progress to legal blindness. The severity of AMD in affected humans increases with age, with extensive degeneration of the retina. Furthermore, safety concerns are emerging regarding the long-term blockade of VEGF-A, which is constitutively high in the normal human retina and glomeruli. Thus, novel CNV treatments are highly desirable and represent an unmet medical need. Here I will discuss our integrated approach that will expand existing therapeutic options. In contrast to existing agents that target inhibition of a proangiogenic pathway mediated by VEGF, we have evaluated restoring the loss of endogenous angiogenesis suppressor pathways mediated by thrombospondin-1 (TSP1) and pigment epithelium-derived factor (PEDF) and their peptide mimetics. We have determined the physiological roles of these molecules in ocular vascular homeostasis and have evaluated the cell autonomous impact of these molecules in various ocular vascular cell types. Our results provide strong support for use of these targets individually or in combination with other therapeutics for attenuation of neovascularization.
Non-canonical Tyrosine Kinase Receptor Signaling Pathways

Boulton, M. 1, Qi, X. 1, Da Silva, J. 1, Mitter, S. 1, Godoy, J. 1, Grant, M. 2

1University of Alabama at Birmingham, Birmingham, United States
2University of Alabama at Birmingham, Ophthalmology and Visual Sciences, Birmingham, United States

The classic perception of receptor tyrosine kinases (RTKs) is that, upon activation by ligand binding, kinase-dependent phosphorylation leads to a signal transduction cascade culminating in transcriptional regulation. However, there is a growing body of evidence that trafficking of RTKs makes a critical contribution to their cellular function and provides alternative non-canonical signaling pathways which include targeting of RTKs to the nucleus and cell junctions. To date over 20 distinct RTKs (e.g. FGFR1, 2, 3; VEGFR1, 2; IGFR1; and Tie1) have been reported to traffic to the nucleus either as an intact receptor or a cleaved intracellular domain. These RTKs can chaperone transcription factors or other proteins such as ENSDs or cavelin-1 to the nucleus, serve directly as transcription regulators or regulate transcriptional activity. The classic view is that VEGFR2 regulates endothelial function and survival via a number of different canonical signaling pathways including Ras/MAPK, Src, PI3K and NOS while VEGFR1 signaling is more complex because it has only very weak kinase activity. There is now increasing evidence that VEGF-driven vascular permeability and neovascularization is highly dependent upon the targeted subcellular translocation of VEGFRs in endothelial cells and that the relative levels of specific VEGFRs within subcellular compartments dictates vascular permeability and angiogenic outcome. This is supported by the following observations:

1) VEGFRs are associated with adherens junctions and regulate vascular permeability;
2) VEGFRs translocate to the nucleus where they have the capacity to bind transcription factors and regulate gene expression;
3) Levels of VEGFRs are transient in subcellular compartments and their relative levels determine endothelial phenotype (e.g. high nuclear VEGFR2:VEGFR1 ratio is associated with angiogenesis while low nuclear VEGFR2:VEGFR1 ratio is associated with vascular quiescence; and
4) inhibition of intracellular VEGFR signaling abrogates the angiogenic response.

The mechanism by which VEGFRs translocate to the nucleus is unclear as they lack nuclear localization signals and alternative strategies for nuclear import/export include endocytic adaptor proteins and SUMOylation. The targeted trafficking of VEGFRs to, and maintenance of the VEGFR levels in, specific intracellular compartments represents an important non-canonical, and largely unexplored, pathway in the regulation of ocular angiogenesis.

MicroRNA-145 Regulates Endothelial Cell Function and Pathologic Angiogenesis by Suppression of Tmod3 in a Mouse Model of Proliferative Retinopathy

1Boston Children’s Hospital/Harvard Medical School, Ophthalmology and Visual Science, Boston, United States
2Boston University School of Medicine, Department of Ophthalmology, Boston, United States

Pathologic ocular angiogenesis commonly causes blindness in several eye diseases including proliferative retinopathy (PR). It is critical to define factors dysregulated in retinas with pathologic neovessels in order to develop effective targeted therapeutics. MicroRNAs (miRNAs) are a group of small regulatory RNAs that mediate many biological processes including angiogenesis. Our previous study in a miRNA-expression array identified several miRNAs, including miR-145, that were differentially expressed in the retinas isolated from a mouse model of oxygen-induced proliferative retinopathy (OIR). Here we investigated the specific role of miR-145 in regulating vascular endothelial cell (EC) and pathologic ocular angiogenesis in the mouse OIR model. Expression of miR-145 was significantly upregulated (~2-fold increase) in OIR mouse retinas compared with the room air controls. Intravitreal injection of synthetic miR-145 inhibitors drastically decreased levels of pathologic NV in OIR mice by ~50%. Moreover, cultured human retinal ECs treated with miR-145 mimics showed substantially increased EC angiogenic function including proliferation, migration, and tubular formation; whereas miR-145 inhibitors attenuated EC angiogenic function in vitro. Tropomodulin3 (Tmod3), an actin-capping protein, was identified as a miR-145 target in EC and validated by luciferase reporter assays. Expression of Tmod3 was downregulated in OIR retinas and miR-145 mimics induced human retinal ECs. Furthermore, treatment with miR-145 mimic in ECs led to cell shape elongation and change of the cytoskeletal architecture. Overall, our findings indicate that miR-145 is a novel regulator of pathologic ocular NV through regulation of Tmod3-dependent cytoskeleton dynamics and architecture. Modulation of miR-145 may serve as a potential therapeutic approach to develop treatments for PR and relevant vascular eye diseases.

RPE - RPE-Choroid pathologic immune processes: Molecules

The Membrane Attack Complex (MAC): A Smoking Gun in Age-related Macular Degeneration

Mullins, R., Stone, E., Tucker, B.
Institute for Vision Research, The University of Iowa, Ophthalmology and Visual Sciences, Iowa City, Iowa, United States

Human eyes with early age-related macular degeneration (AMD) are characterized by choriodal capillaris dropout and significantly increased abundance of the membrane attack complex (MAC), which is deposited on choriodapillaris vessels. Endothelial cells from the choroid are susceptible to MAC-mediated lysis in vitro, suggesting that MAC may be a major cause of choroidal endothelial cell death in vivo. Moreover, a common polymorphism (Y402H) in the complement factor H gene that is associated with increased risk of AMD is correlated with increased MAC formation in the choroid of human eyes. Data for the role of MAC in AMD will be discussed in this presentation, as well as strategies for protecting the choriodapillaris from complement-mediated lysis and for replacing these cells when they have been lost due to MAC injury.

Aryl Hydrocarbon Receptor Activity Modulates RPE and Choroidal Cell Health

Malek, G.
Duke University, Durham, United States

The aryl hydrocarbon receptor (AhR) is a ligand activated transcription factor, initially discovered for its role in regulating xenobiotic metabolism. However recently, extensive evidence has emerged supporting a multi-faceted role for AhR, modulating physiological pathways important in cell health and disease. We have discovered that the AhR plays a role in ocular aging and the pathogenesis of age-related macular degeneration (AMD), the leading cause of vision loss in the elderly. In retinal pigment epithelial (RPE) cells isolated from human donors, AhR activity is measurably decreased with a decrease in expression of factors associated with aberrant neovascularization and inflammation. Thrombocytopenic retinal or choroidal neovascularization is not seen in mice in the absence of AhR, experimentally induced choroidal neovascularization is exacerbated. Importantly, activation of the AhR receptor ameliorates laser-induced neovascularization in mice. These results argue in favor of a potential therapeutic role for the activated AhR pathway in regulating aspects of both dry and wet AMD, through its positive modifying effects on extracellular matrix remodeling, inflammation and angiogenesis.

The Role of Factor H and its Transcripts in AMD

Clark, S., Manchester AMD Research Group
University of Manchester, Faculty of Medicine, Biology and Health, Manchester, United Kingdom

The recent revolution in age-related macular degeneration (AMD) genetics has demonstrated that genetic alterations affecting the alternative pathway of the complement cascade have a major influence on AMD risk. One of the two most important genetic loci is on chromosome 1 and contains genes encoding complement factor H (FH) and the factor H related proteins (FHR proteins). In macular tissue, especially Bruch’s membrane, relatively high levels of a truncated splice variant of FH called factor H-like protein 2 (FHL-2) are present. Genetic variations in the CHF gene that encodes both the negative complement regulators FH and FHL-1 alter the amounts, or by altering their protein sequences, the functions of these proteins. In particular, a very common Y402H polymorphism affects the ability of FH-1 to localise to Bruch’s membrane and the inner choroid because it alters the proteins ability to bind heparan sulphate in these structures. Furthermore, genetic variations in the genes of the FHR proteins, including whole deletions of individual genes, also moderate levels of disease risk given their role as positive regulators of complement activation. The presence and/or absence of the FHR proteins alter the fine balance of complement pathways and proteins that ultimately act on the back of the eye. Finally, the lack of permeability of Bruch’s membrane to soluble complement proteins essentially creates two semi-independent compartments with respect to complement activation and regulation. Complement proteins synthesised locally on either side of Bruch’s membrane (or on the choroidal side perhaps derived from the circulation) predominately remain on their side of origin. This has implications for targeting specific complement components as a strategy for treating AMD.
Aberrant BMAL1 Dependent Claudin-5 Cycling Induces Geographic Atrophy

Hudson, N.1, Celkova, L.1, Fahey, E.1, Ozaki, E.1, Doyle, S.1, Campbell, M.1
1Smurfit Institute of Genetics, Trinity College Dublin, Dublin, Ireland
2School of Medicine, Trinity College Dublin, Dublin, Ireland

Photoreceptor outer segment (POS) phagocytosis and renewal occurs daily upon light onset. However, circadian rhythm involvement in retinal function has still not been fully elucidated. In this study we examined the circadian clock components role in regulating inner blood retina barrier (iBRB) function that we show is directly related to replenishment of shed POS.

For in vivo analyses wild-type C57Bl/6J mice (10-12 weeks) were sacrificed at 8AM or 8PM (corresponding to 12 h lights off: 12 h lights on respectively). Retinal protein and mRNA was extracted and tight junction and circadian clock components analysed by western blotting and qRT-PCR, respectively. For verification that the response was circadian and not diurnal, further cohorts of C57Bl/6J mice were housed in 24 h of darkness or an inverted light cycle for three weeks prior to sacrifice and retinal protein and transcript analysed.

In order to characterise iBRB integrity, phenotypic permeability changes were assessed using fundus fluorescein angiography (FFA) and dynamic contrast enhanced magnetic resonance imaging (DCE-MRI). Immunohistochemical analysis of claudin-5 expression as well as biotin extravasation was also undertaken. Serum shock experiments were carried out to re-establish circadian rhythms in vitro in primary human retinal microvascular endothelial cells. We found claudin-5 cycling throughout the day in the retinal vascular smooth muscle in a circadian, rather than diurnal, manner with lower expression at 8PM compared to 8AM, both at protein and transcript levels. These changes phenotypically led to more permeable retinal vessels in the evening compared to the morning as observed by FFA and DCE-MRI analyses. Circadian regulated changes in retinal vascular permeability was not evident in BMAL1−/−/Tie2Cre mice, where the clock gene BMAL1 was lacking in endothelial cells, directly implicating BMAL1 function in claudin-5 expression changes. C57Bl/6J mice exposed to a high fat diet in tandem with persistent claudin-5 suppression developed rapid onset of a geographic atrophy (GA) like phenotype. Our results suggest the iBRB is a highly dynamic structure playing a critical role in replenishing POS. Circadian regulation of claudin-5 facilitates exchange of material between the blood and neural retina.

A new therapeutic target for treating GA and suggests GA has a major microvascular component to its pathophysiology.

C-reactive Protein in AMD

Molins, B.1
Hospital Clinic-IDIBAPS, Barcelona, Spain

Age-related macular degeneration (AMD) is the leading cause of central vision loss among the elderly population in developed countries and an increasing global burden. The major risk factor is aging, compounded by other environmental factors and association with genetic variants for risk of progression. Although the etiology of AMD is not yet clearly understood, several pathogenic pathways have been proposed, including dysfunction of the retinal pigment epithelium, inflammation, and oxidative stress. The identification of AMD susceptibility genes encoding complement component regulatory activity. However, FH from AMD patients carrying the His402 polymorphism -associated with increased risk of AMD- shows an inhibitory dissociation mechanism, could be a promising target for AMD.

Subretinal Implantation of a Bioengineered Embryonic Stem Cell-Derived Retinal Pigment Epithelium Monolayer in Dry Age Related Macular Degeneration

1Keck School of Medicine, University of Southern California, Los Angeles, United States, 2Center for Stem Cell Biology and Engineering, University of California, Santa Barbara, United States, 3Retai Vision Associates Medical Group, Los Angeles, United States, 4Aristes Biotherapeutics, Fremont, United States

Non-neovascular AMD (NAMD) and associated geographic atrophy (GA) is a leading cause of vision loss, and treatment options are lacking. As a possible intervention, a bioengineered retinal pigment epithelial (RPE) monolayer was developed consisting of a monolayer of pluripotent stem cell-derived RPE cells on a synthetic scaffold. Human embryonic stem cells (hESC) are differentiated to an RPE fate, and RPE are then cultured to maturity on vitronectin-coated ultrathin parylene membranes, which have diffusion properties similar to the native Bruch’s membrane. A prospective, open-label, interventional, FDA cleared, phase I/IIa study is underway to assess the safety and efficacy of the bioengineered subretinal implant in 2 cohorts of subjects with vision loss from advanced NAMD and GA. Surgical delivery involved pars plana vitrectomy, subretinal dissection and implantation using a custom made insertion device. The primary outcome measure is a safety at 1 year post-implant. Secondary endpoints are assessed by EDRS visual acuity, microperimetry based fixation testing and optical coherence tomography (OCT). An analysis of the clinical trial will be presented at the meeting. Support for this program has been obtained from the California Institute of Regenerative Medicine, the William K. Bowes Foundation and from Santen Pharmaceutical Co, Ltd.

Endothelial Colony Forming Cell Modulation of Choroidal Angiogenesis

McKown, S., Canning, P., Bertelli, P., McNutt, S., McLaughlin, K., Medina, R., Stitt, A.
Queens University Belfast, Centre for Experimental Medicine, Belfast, United Kingdom

Purpose: Progressive atrophy of the choroidocapillaris is a feature of age-related macular degeneration (AMD). Enhancing repair of the choroidocapillaris could improve declining oxygenation of the outer retina and slow or reverse disease progression. Endothelial colony forming cells (ECFCs) are well-characterised progenitors with proven vascular reparative potential in the ischaemic retina. ECFC interaction with the choroidal vasculature is unknown, thus this study aims to investigate the potential of ECFCs to modulate the choroidal vasculature.
Methods: Choroidal explants were established from C57BL/6J mice sacrificed at different ages (postnatal (P) days 8, 10, 11.13), grown and imaged for 5 days (N=16). ECFCs were isolated from cord blood and characterised morphologically and by the presence of surface antigens, such as CD34 and CD105. Choroidal explants from P8 mice (N=18) in the presence/absence of ECFCs were established. Since ECFCs, in vivo, need to function in hypoxic conditions, a novel hypoxia-responsive miRNA termed miR-X was identified via microRNA array analysis of ECFCs. miR-X over-expressing ECFCs were incorporated into the choroidal explant model to assess its potential in improving the pro-angiogenic effect elicited by the cells. Human antigen-specific CD31 antibody differentiated between the human ECFCs and the murine choroidal sprouts by immunocytochemistry.

Results: Choroid explants from P8 mice sprouted faster and had a larger mean sprouting distance on day 5 than mice at P11 and P13 (p<0.001). Furthermore, P8 explants grown in co-culture with ECFCs had significantly increased sprouting distance vs. control explants (p<0.001). ECFC-conditioned media did not evoke this response in choroidal explants. ECFC-mediated pro-angiogenic effect was further enhanced when cells over-expressed miR-X, vs. no cell, non-transfected cells, and control-miRNA transfected cells (p<0.0001). ECFCs integrated with the choroidal vasculature, with ECFC containing vascular sprouts exhibiting increased branching and filopodia.

Conclusions: P8 explants sprout faster and of greater length than explants from older mice. ECFCs enhanced vascular network formation in choroidal explants, whilst miR-X over-expression, further enhanced the angiogenic response, thus demonstrating that these cells have potential to enhance repair of the atrophic choroidal vasculature. Future research effort is exploring their vasoprotective properties in vivo.

RPE Exosomes in Health and Disease

Bowes Rickman, C.1, Klingeborn, M.2, Skiba, N.2, Stamer, D.1

"Duke University Medical Center, Ophthalmology and Cell Biology, Durham, United States, "Duke University Medical Center, Ophthalmology and Biomedical Engineering, Durham, United States

Interest in utilizing 30-150 nanometer sized exosomes and other extracellular vesicles (EVs) as biomarkers of disease has increased exponentially in recent years. Exosomes have several unique features that define ideal biomarkers:

(i) A lipid bilayer provides protection for their cargo;
(ii) tissue-, cell-, or disease-specific proteins and nucleic acids as cargo; and
(iii) hardness enabling a wide range of methods for isolation and enrichment from body fluids.

To identify biomarkers for retinal disease, we isolated exosomes from the retinal pigmented epithelium (RPE), which forms the outer blood-retinal barrier of the eye. The RPE is a highly polarized epithelium, leading to the directional secretion of proteins, lipoprotein particles and EVs. We performed a mass spectrometry-based proteomic analysis of apically and basolaterally RPE-derived EVs by simultaneously profiling hundreds of proteins in exosome preparations of increasing purity. This approach, termed ProteoCorrelation Profiling (PCP), permits the analysis of any sub- or extracellular components/complexes that can be enriched by fractionation but not purified to homogeneity. In parallel, EV size distribution and concentration were determined using nanoparticle tracking analysis (ZetaView). Basolaterally released exosomes from RPE cells enter the systemic circulation via the choroidal and thus transmembrane RPE-specific exosomal proteins represent targets for immunolocalization of RPE-derived exosomes from blood. We used several different approaches to isolate RPE-derived EVs from blood including a new high-throughput acoustofluidics-based approach and immunoisolation. In conclusion, these data serve as a foundation for comparative studies aimed at elucidating the molecular pathophysiology of retinal diseases and to help identify potential therapeutic targets and systemic RNA and protein biomarkers to monitor and stratify disease.

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A Common Pathway Regulates Endosome Biogenesis and Exosome Secretion in the RPE

Germer, C., Rathnasamy, G., Tan, L.X., La Cunza, N., Lakkaraju, A.

University of California, Ophthalmology, San Francisco, United States

Abnormally enlarged early endosomes and increased secretion of exosomes are pathological features of neurodegenerative diseases, yet insight into the mechanisms and consequences underlying these phenomena remain elusive. Our recent studies have identified ceramide as a common regulator of early endosome expansion and exosome secretion in the.retinal pigmented epithelium (RPE). We observed swollen apical early endosomes in the RPE of aged human donors and in the pigmented Ab24 mouse model of Stargardt, early-onset macular degeneration. Using high-resolution live-cell imaging, our data show that age-related and pathological accumulation of lipofuscin bioretinoids increases ceramide at the apical surface of the RPE, which promotes inward budding and homotypic fusion of early endosomes. These enlarged endosomes internalize the complement protein C3 into the RPE, resulting in the intracellular generation of C3a fragments. The same mechanism - ceramide-induced inward budding - also drives increased formation of intraluminal vesicles in endosomes, which are subsequently released as exosomes upon fusion of the limiting endosome membrane with the plasma membrane. Current studies are focused on understanding the physiological and pathological consequences of RPE-derived exosomes.

Innate/Inflammatory Cross Talk Between Macrophages (Mps) and RPE Cells Are Mediated by Exosomes Secreted by RPE Cells: Proposal of New Trait for the Pathogenesis of Age-related Macular Degeneration (AMD)

Murakami, R.1, Ueno, M.1, Yamawaki, T.1, Ito, E.1, Kinoshita, S.1, Sotozono, C.1, Hamuro, J.1

1Kyoto Prefectural University of Medicine, Ophthalmology, Kyoto, Japan, 2Department of Frontier Medical Science and Technology for Ophthalmology, Kyoto, Japan

Introduction: The pathogenesis of AMD is aggravated by chronic inflammation. Intact RPE cells down-regulate the production of TNF-α by choroid infiltrating Mps, whereas degenerated RPE cells by oxidative stress were devoid of this regulatory function. Subsequently, locally produced TNF-α induces the production of pro-inflammatory cytokines, IL-6, IL-8, MCP-1 and angiogenic factor VEGF by RPE cells (Yamawaki et al, 2016). This implies that innate/inflammatory cross talk between Mps and RPE cells may be the indispensable trait for AMD pathogenesis.

Purpose: To elucidate the signal to trigger pro-regulated TNF-α production in innate/inflammatory cross talk between Mps and RPE cells.

Method: Mps cell line RAW 264.7 was co-cultured with primary RPE cells taken from C57BL/6J mice. MCP-1, IL-6, VEGF, and TNF-α in the culture supernatants (CSs) were quantified by ELISA. The expression profiles of complement-associated genes, TNF-α, and angiogenesis-associated genes (VEGF and PEDF) were analyzed by quantitative real-time PCR. For the preparation of exosomes (Exo), CSs were harvested after 24 hours in culture. RAW 264.7 with primary RPE cells, then Exo in each CSs were purified by either EV second-step or ultracentrifugation. IL-6, MCP-1, TNF-α and VEGF produced by RPE cells exposed 24 hrs to semi-purified Exo were quantified. The incorporation of the Exo either into RPE cells or RAW 264.7 was histologically quantified using Qdot 655 streptavidin conjugated biotyped Exo. Results: Elevated levels of CD63 positive Exo in co-cultures were constantly detected by western blot or FACS analysis. The produced Exo in co-culture CSs purified on EV column were incorporated solely into RAW264.7, but not into RPE cells. The ultracentrifugation semi-purified Exo, but not the Exo depleted residual CSs enhanced the secretion of MCP-1 and IL-6 in co-culture of Mps and RPE cells, while the enhancement of VEGF are similarly detected by the Exo deprived residual CSs. Most remarkable elevation was observed in TNF-α production by RAW264.7 in a dose-dependent manner even in the absence of RPE cells. The down-regulated TNF-α production by RAW264.7 in the presence of RPE cells was not reconstituted by the addition of Exo even in the co-culture. Conclusion: Exosome displays a critical role in the triggering of vis- a-vis inflammatory cytokines cycle through the elevation of TNF-α production by Mps.
Recessive Stargardt disease (STGD1) is an inherited macular degeneration caused by mutations in ABCA4, a membrane protein thought to be exclusively expressed in photoreceptor outer-segment disc. Loss of ABCA4 results in retinal pigment epithelial (RPE) deposition of birefringent lipofuscin and late-onset retinal degeneration. Preliminary studies showed that ABCA4 is additionaly expressed in the RPE. Here, we sought to further investigate the expression and function of ABCA4 in RPE cells. ABCA4 transcript was evaluated by RNaseQ in situ hybridization assay in human donor eyes, in human fetal (hf) RPE cultured cells, in wild-type (WT) and Abca4^-/- mouse eyes. ABCA4 protein was evaluated, by immunoblotting, and immunofluorescence in WT and Abca4^-/- mice, and also in Mertk^-/- mice which do not phagocyte photoreceptor outer segments, and in hrRPE cells which never contact photoreceptor cells. ABCA4 co-localization with endo-lysosomal markers was assessed by immunohistochemistry. ABCA4 was functionally evaluated in a transgenic mouse line that expresses ABCA4 in the RPE cells, but not in neural retina. Ocular birefringence were measured by high performance liquid chromatography. Retinal morphology, lipofuscin, and autofluorescence were assessed by light, electron, and confocal microscopy, respectively. By in situ hybridization of human and WT mouse eyes, ABCA4 mRNA was clearly visualized in photoreceptors and RPE cells. ABCA4 mRNA was also detected in hrRPE cultured cells. Notably, ABCA4 mRNA was absent in Abca4^-/- eyes. ABCA4 protein expression was confirmed in Mertk^-/- RPE and hrRPE cells by immunohistochemistry. By confocal microscopy, ABCA4 showed an intracellular membrane distribution and co-localized with endo-lysosomal structures in human and murine RPE cells. Compared to Abca4^-/- mice, transgenic mice expressing ABCA4 in RPE only (on the Abca4^-/- background) showed less birefringent lipofuscin accumulation and greater photoreceptor cells preservation. Taken together, these data suggest an active role for ABCA4-expressed in the RPE cells. Here, ABCA4 performs a similar function as in photoreceptor outer segments: ATP-dependent translocation of anionic inner membrane leaflet phospholipid phosphatidylserine (PS) from retinal dehde released during photoreceptor outer segments. These findings introduce a novel, cell-autonomous pathway in the pathophysiology of STGD1 with important therapeutic implications.

The RPE pigmemt epithelium (RPE) mediates complex functions essential for photoreceptor physiology and processes retinal vision. Thanks to its strategic location between neurosensory retina and choriocapillaries as well as the highly specialized external organization of its cells, the RPE also controls the chemical composition of the subretinal and choroidal extracellular spaces. As such, in complex with photoreceptors (PRs), interphotoreceptor matrix and chorioid, the RPE aids at maintaining a homeostatically stable environment vital to the functional responses to light. Thus, mutations in some of the RPE-specific genes are thought to not only disrupt intracellular metabolism, but also alter the extracellular interactions, and consequently, physiological responses of the complex. For example, mutations in the BEST2 gene cause X-linked retinoschisis and degeneration of PRs due to a primary chondro-lamellar defect in the neighboring RPE cells; yet, the pathophysiology of the interaction between RPE and PR cells preceding the formation of retinoschisis lesions remains not well-understood. In this study, we identified, in both photoreceptor and retinal pigment epithelial cells, the primary abnormality at the RPE-PR interface, and showed that the underlying structural defect in the RPE microvillar ensheathment destabilizes its interaction with PR outer segments, and results in a diffuse retinal-wide microdegeneration better well clinically-detectable disease. We also show that this earliest disease expression is strongly modulated by exposure to ambient light, thus aiding BEST2-disorders to a increasing number of retinopathies displaying hypersensitivity to light. We postulate that the abnormal response of the BEST2-mutant retina to light stimuli could be related to the markedly reduced light peak/dark trough ratio in EOG, a finding consistent in all, even presymptomatic, patients affected with bestronic phenotypes. Moreover, the abnormal modulation of the RPE-PR distance by light exposure would lead to a chronic retinal remodeling following the detachment, and these downstream consequences add new insight into the non-phototransductive genotype-phenotype correlation in bestrophinopathies. Most importantly, we used AA2-mediated BEST2 gene augmentation therapy to demonstrate the correction of both the gross subretinal lesions as well as the subclinical light-mediated microdegeneration with amelioration of the hypersensitivity to light post AA2-BEST2 therapy.

RPE Contributions to Photoreceptor Outer Segment Renewal

Finnemann, S.C., Esposito, N.J.
Fordham University, Biological Sciences, Bronx, United States

Diurnal photoreceptor outer segment renewal is a fundamental retinal process that involves communication between photoreceptor neurons and neighboring retinal pigment epithelial (RPE) cells. Deficiency in this process contributes to age-related changes of the human retina and causes select forms of retinitis pigmentosa.

At light onset, rods in wild-type mouse retina externalize the anionic inner membrane leaflet phospholipid phosphatidylserine (PS) specifically at their distal outer segment tip, which serves as “eat me” signal triggering tip clearance phagocytosis by RPE cells. MFG-E8 and ProteinS/Gas6 are secreted PS-binding proteins in the subretinal space that serve as specific and essential ligands for two known phagocytic receptors of the RPE, αvβ3 integrin and Mertk, respectively. Notably, photoreceptors in mutant mice lacking αvβ3 or integrin do not increase PS exposure at light onset although unlike RPE cells - they do not express αvβ3 receptors. These findings imply that photoreceptors do not expose PS cell-autonomously. Here, we explore the capacity of rod photoreceptors from wild-type and mutant mouse retina to respond to PS exposure to addition of purified proteins known to localize in the subretinal space in situ. We find that photoreceptor PS exposure in response to purified proteins differs by time of day and by mouse strain tested. Testing purified proteins with single amino acid substitutions further illuminates the specific molecular requirements for PS exposure by photoreceptor outer segments.
RP6 results in an AMD-like phenotype in mice and rats. It was recently reported that βA3/βC-crystallin may exhibit a circadian pattern of expression. We hypothesized that oscillations in βA3/βC-crystallin expression might regulate circadian entrainment in RPE cells. We experimentally validated that the expression of βA3/βC-crystallin in WT RPE oscillates during the day with one of the peaks coinciding with the peak of presumptive phagocytic activity. Pathway enrichment analysis of genes differentially expressed in the RPE from WT compared to Cryba1 KO mice revealed enrichment in genes involved in circadian rhythm (p = 0.0069). We also found that protein levels of GSK3β oscillate in WT RPE, coinciding with βA3/βC-crystallin oscillations. Importantly, in mice lacking Cryba1, the amplitude of GSK3β oscillation is decreased. Moreover, knockout of Cryba1 almost eliminated oscillations of β-Catenin (Ser 33/37), a downstream target of GSK3β, which in WT RPE closely followed the expression pattern of GSK3β. GSK3β is a known regulator of circadian rhythm, and we are the first to demonstrate that βA3/βC-crystallin is an upstream regulator of GSK3β expression, suggesting its additional role in the entrainment of circadian rhythmicity. This work proposes a novel functional link between the retinal circadian rhythmicity and regulation of lysosomal activity that needs to be maintained to prevent onset and progression of AMD.

Calciﬁcation of Bruch’s Membrane in ABC6 Knockout Mice and Other Models for Pseudoxanthoma Elasticum (PXE) and AMD

Bergen, A.A., ten Asbroek, A., Koster, C.

AMC, Clinical Genetics and Ophthalmology, Amsterdam, Netherlands

Pseudoxanthoma elasticum (PXE) is a heritable disorder characterized by ectopic calcification of connective tissue in Bruch’s membrane of the eye, skin and blood vessel walls. Previously, using homozygosity mapping avant le tete in a large PXE family, followed by candidate gene sequencing, we found that mutations in the ABC6 transporter gene are the cause of PXE. Subsequently, we made monoclonal antibodies and conﬁrmed that the ABC6 protein is expressed in liver and kidney only. Next, we made a truncating ABC6 knockdown, which essentially recapitulates all the pathological features of the disease in the animal model. We found in the ABC6-/- mice that dietary intake of Mg++ can reduce the progression of calcification. Most importantly, we found that hepatic ABCG1-mediated ATP release is the main source of systemically circulating inorganic phosphate (Pi). Consequently, patients with PXE and ABCG1 -/- knock out mice have reduced plasma Pi levels, which essentially explains the ectopic calcification in the peripheral tissues, including the eye. In the PXE mouse eye, both the elastic and collagen ﬁbers of Bruch’s membrane are calciﬁed, leading to a brittle membrane that is susceptible for tears (angioid streaks). Clinical trials to treat PXE patients using supplementation of a Pi source (bisphosphonates) are currently ongoing. The recent ﬁnding that renal drusen, a hallmark for age-related macular degeneration (AMD), may consist of a lipid core, surrounded by a crust of calcium-based hydroxyapatite that subse- quently interacts with proteins, has renewed interest in PXE research as a model for interaction between systemic minerals and the eye. We will discuss similarities and differences between PXE, AMD and aging eyes, and introduce new mouse model data relevant for calciﬁcation of Bruch’s membrane.

The Role of EFEMP1 Gene in Malattia Leventinese, AMD, and beyond

Marmorestein, L.

Mayo Clinic, Ophthalmology, Rochester, United States

A mutation (R345W) in EFEMP1 gene (Fibulin-3) causes Malattia Leventinese (ML), a hereditary dominant macular degenerative disease. With the exception of an earlier age of onset, ML/MLD patients exhibit symptoms and histopathology compatible with the diagnosis of age-related macular degeneration (AMD). Both ML/MLD and AMD show presence of sub-RPEBL space (basal lamina deposit, BLamD) containing basal laminar epithelial (RPE) deposits including basal laminar deposit (BLamD), basal linear deposit (BLinD), and drusen. BLamD is the ﬁrst deposit to develop, and lipoprotein derived debris accumulates within BLamD, and subsequently builds up in Bruch’s membrane to form BLinD and soft drusen. Fibulin-3 is an extracellular enzyme inhibitor expressed throughout the body. The R345W mutation does not impair Fibulin-3’s function. In the retina, Fibulin-3 is predominantly secreted by RPE cells apically to the interphotoreceptor matrix (IPM) with a small amount present basally in Bruch’s membrane. In contrast, mutant ﬁbulin-3 containing the R345W mutation accumulates in Bruch’s membrane. Although no mutation is identiﬁed in AMD, Rﬁbulin-3 also accumulates in Bruch’s membrane containing basal deposits in AMD patients. Deletion of ﬁbulin-3 prevents BLamD from forming even under stress conditions known to provoke it. Thus, ﬁbulin-3 accumulation at the site of basal deposits appears to be essential for deposit development. RPE cells carrying the R345W mutation overproduce cholesterol-rich micelles and that mutant ﬁbulin-3 is released basally in these micelles. The turnover of ﬁbulin-3 in the IPM is regulated by low density lipoprotein receptor-related protein 2 (LRP2)-mediated endocytosis and lysosomal degradation in RPE cells, but mutant ﬁbulin-3 in the IPM is transcytosed to the basa side of RPE cells. This indicates that the R345W mutation causes ﬁbulin-3 accumulation in Bruch’s membrane by altering the secretion pathway of newly synthesized protein and the turnover of ﬁbulin-3 in the IPM. Fibulin-3 accumulation leads to increased levels of proteoglycans in Bruch’s membrane. Proteoglycans are known to affect Bruch’s membrane’s permeability and retain lipoproteins. Mice carrying the R345W mutation develop BLamD with entrapped lipid-deposited debris. These data suggest that ﬁbulin-3 accumulation promotes BLamD formation and progression from BLamD to lipoderm-rich deposits associated with ML/MLD and other macular degenerations like AMD.

Late-onset Retinal Degeneration: Molecular Mechanisms and Phenotypic Consequences of C1QTNF5 Mutations

Dinculescu, A.

University of Florida, Gainesville, FL, United States

Purpose: The mutation S163R in the globular domain of C1QTNF5 causes an autosomal dominant disorder known as late-onset retinal degeneration (L-ORD), characterized by the presence of thick extracellular deposits between the RPE and choroid. A recent study has identiﬁed novel disease-causing L-ORD mutations in C1QTNF5, G126C and P188T. Here, we used the AAV vector technology to examine the expression pattern of C1QTNF5 mutant proteins following speciﬁc RPE targeting. Approach: We generated scAAV vectors (AAV2 quad YF capsid) expressing S163R, G126C and P188T mutants under the control of an RPE-speciﬁc BEST1 promoter. All mutants were HA-tagged at the C-terminal end for speciﬁc detection, and were analyzed in vitro following transient transfections in HEK cells, and in vivo following subretinal delivery in C57BL/6 mice. Eyes were examined by non-invasive imaging methods, including fundus imaging and spectral-domain optical coherence tomography (SD-OCT). In order to evaluate the effects of each mutant on retinal function, full-field ERG was performed in wild-type and HA (S163R) double-mutated and, photopic cone-mediated conditions. Mutant protein expression was detected by immunohistochemistry and Western Blotting using an anti-HA antibody. Histological examination of AAV-injected eyes by light microscopy was also performed following staining with haematoxylin and eosin of paraffin sections. Results: We have previously shown that AAV-expressed wild-type C1QTNF5 is secreted apically from the RPE towards the outer limiting membrane, a behavior consistent with that of the endogenous protein. Consistent with our previous study, mutant S163R-C1QTNF5 exhibits a reversely polarized distribution in vivo, being routed towards the basa rather than apical RPE. In contrast, none of the newly identiﬁed L-ORD mutations examined were routed towards the basa RPE. They were either retained in the RPE, or secreted apically. All mutants led to a signiﬁcant decrease in retinal ERG function, and accumulation of autofluorescent lipofuscin material. Conclusions: Each L-ORD mutant displays a unique expression pattern, with distinct pathological consequences in vivo when expressed in murine RPE cells. In contrast to S163R-C1QTNF5, none of the mutants examined led to basal deposits. These distinct behaviors suggest
that each of the three L-ORD mutant proteins triggers RPE dysfunction and photoreceptor cell death through different mechanisms.

Considerations in Studying Transport across Bruch’s Membrane

Johnson, M.
Northwestern University, Biomedical Engineering, Evanston, United States

Vitamins, signaling molecules, and other factors needed for photoreceptor function are carried to the RPE by lipoprotein particles passing through Bruch’s membrane, as do the RPE-produced lipopolysaccharides that are eliminated in the opposite direction. These transport processes are driven by molecular diffusion. A number of studies have examined these transport processes and found that macromolecules can be significantly hindered in their transport across Bruch’s membrane due to interactions with Bruch’s membrane extracellular matrix. Age-related lipopolysaccharide accumulation in Bruch’s membrane further hinders this transport process. Characterization of transport across Bruch’s membrane is frequently done in vitro using Ussing chambers. While these experiments are relatively easy to conduct, there are a number of issues that need to be recognized in design of these experiments and interpretation of the resulting data. These include:

(i) the importance of an osmotic leak test,
(ii) the unavoidable unstirred layers that significantly impact transport processes in Ussing chambers,
(iii) the role of osmotically-generated flows that slow diffusion, and
(iv) the need to normalize transport rates of molecules by their diffusion coefficient in free solution.

When these factors are properly considered, the molecular weight cut-off for transport through Bruch’s membrane is greater than, at least, 500 kD. Furthermore, the presence of unstirred layers means that Bruch’s membrane is not the only source of diffusion resistance in Ussing chambers, which can confound interpretation of experimental results. Steps that would enhance the interpretation of such experiments include:

(a) use of a low molecular weight compound such as uracil or taurine to characterize the unstirred layers in the system,
(b) use of flow and non-gentle stirring to mix upstream and downstream compartments of Ussing chambers to reduce the size of unstirred layers, and
(c) use of low protein concentrations to minimize osmotically-generated flows.

New Insights in the Nucleoredoxin-like 1 Metabolic and Redox Signaling

Léveillard, T.
Sorbonne Université, Genetics, Paris, France

The maintenance of central vision has been at the center of our attention for two decades. The analysis of the secondary degeneration of cones in murine models of retinitis pigmentosa revealed that rods secreted a truncated thioredoxin-like protein, called rod-de-riived cone viability factor (RdCVF) that is produced by alternative splicing of the nucleoredoxin-like 1 (NXL1) gene. This enzymatically inactive protein acts by binding to the basigin-1 (BSG1)-GLUT1 (SLC2A1) complex at the surface of the cones, stimulates glucose uptake and aerobic glycolysis to provide the necessary triglycerides for daily renewal of the cone outer segments. When rods degenerate through the action of cell-autonomous mechanisms triggered by a mutation in any of the 63 genes causing the disease known as the supply of glucose to the cones is reduced, the outer segments of the cones is sufficiently reduced, so that they shorten leading to the deficit of central vision. Glucose also provides, via the pentose phosphate pathway, the reducing power of the other spliced variant of the NXL1 gene in cones, the thioredoxin enzyme RdCVFL. The involvement of this metabolic and redox signaling in age-related macular degeneration was revealed through the role allele in the SLC16A4 gene encoding for a lactate transporter expressed on the basal side of the retinal pigmented epithelium, since lactate, a product of aerobic glycolysis, must be transported out of the retina. Alternative splicing of the NXL1 gene dates from 600 Million years as observed in Hydra vulgaris, where the main function of the gene was to protect the organism against oxidation via its thioredoxin product. 500 Million years ago, with the first animal with a dual retina emerged, RdCVF expressed specifically by the first rods binds to basigin-1 at the cone surface. The evolutionary constraint at the origin of this metabolic and redox signaling is linked to the positive autoregulatory feedback loop exerted by RdCVF on RdCVFL function. What drive the directionality of the metabolic flux from rods to cones? We now reveal that cones express specifically in a rod- and glucose-dependent manner the enzyme that regulates the production of the major allosteric activator of glycolysis, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2. This enzyme is able ectopically to protect cones. Increase glucose uptake by cones is relayed by this enzyme that accelerates aerobic glycolysis for the maintenance of cone outer segments.

Role of Aerobic Glycolysis in Photoreceptors

Punzo, C., Petit, L.
University of Massachusetts Medical Scholl, Ophthalmology, Worcester, United States

Aerobic glycolysis, which commonly favors cell anabolism, is the default metabolic state of photoreceptors. It is thought to be crucial in order for photoreceptors to sustain outer segment growth. However, its role in photoreceptor maintenance and function has only recently started to be understood thanks to the advance of tools that allow for glycolysis specific gene deletion in photoreceptors. Here we present data from experiments in which we disrupted one of the gatekeepers of aerobic glycolysis, namely hexokinase-2 (HK2). We found that loss of HK2 in rods does not affect rod survival or integrity but rather rod function. Rod compensate for the loss of HK2 by increasing oxidative phosphorylation and the number of rod mitochondria. While this results in a 33% drop in retinal lactate production, this drop did not affect retinal pigmented epithelium (RPE) health. This suggests the RPE is either able to adapt to this change or that lactate taken up by the RPE is less essential for RPE metabolism than thought. Interestingly, loss of HK2 in cones leads to a compensatory mechanism whereby cones start to take up lactate form rods. Consequently cones are not affected by loss of HK2. In contrast, in the absence of rod-produced lactate HK2 becomes important to maintain cone function. For instance, concomitant loss of HK2 in rods and cones affect cone function. Additionally, when glucose becomes limiting to cones and rod-produced lactate is absent, such as in retinitis pigmenta HK2 becomes essential for cone survival. In summary, the data suggest that aerobic glycolysis optimizes photoreceptor function but is not crucial for photoreceptor maintenance when nutrients are plentiful as other cellular pathways suffice to generate the building blocks needed to maintain outer segment growth. However, under nutrient-restricted conditions aerobic glycolysis becomes a necessity. It optimizes outer segment growth and maintains normal photoreceptor function by diverting most of the glucose into the anaerobic pathway.
associated with the disease. We have shown that the rods secrete a protein, the Rod-derived Cone Viability Factor (RDVF), which protects the cones via its binding complex formed of the basigin-1 (BSG1), its cell-surface receptor and the glucose transporter GLUT1 (SLC2A1). In this study, we demonstrate that there is a key element involved in the direction of the metabolic flux out of cone photoreceptors - the 6-phosphofructo-2-kinate/fructose-2,6-biphosphatase 2. This enzyme 6-phosphofructo-2-kinate/fructose-2,6-biphosphatase 2, is a bifunctional enzyme that regulates the fructose 2,6 bisphosphate in the glucose pathway. This enzyme has kinase and phosphatase domain, and is expressed mainly in cones, in a rod-dependent manner. The presence of this enzyme in cones was confirmed by in situ hybridization and immunohistochemistry. Moreover, a visible staining pattern restricted to the inner segments of cones was observed. Although the gene encoding this enzyme is known in the literature to be highly expressed in the heart, our studies demonstrate that it is highly expressed in the retina as well. In addition, we show that the loss of this enzyme in the cones of the rd1 mouse precipitates their death. In the cones of wild-type retina, the 6-phosphofructo-2-kinate/fructose-2,6-bisphosphatase 2 enzyme is particularly phosphorylated at the kinase activating serine residue, suggesting that the enzyme acts essentially as a kinase, and not as a phosphatase. The fructose 2,6 bisphosphate produced by the kinase activity of 6-phosphofructo-2-kinate/fructose-2,6-bisphosphatase 2 is the allosteric activator of phosphofructokinase, which catalyses the major limiting reaction of glycolysis. We show that ectopic expression of 6-phosphofructo-2-kinate/fructose-2,6-bisphosphatase 2 induces cone survival and that the expression of its messenger RNA is increased by glucose, the effector of RDVF. Furthermore, the activity of its promoter is stimulated by glucose. Altogether, 6-phosphofructo-2-kinate/fructose-2,6-bisphosphatase 2 is involved in stimulating aerobic glycolysis in cones. This activation of the BSG1/GLUT1 by RDVF, secreted by rods. This defines the directionality of the metabolic flux between rod and cone photoreceptors.

The human choroid is a complex tissue composed of a rich tracelulare matrix (ECM) in which different non-vascular cells such as fibroblasts and melanocytes are embedded. Fibroblasts maintain the composition and structural organisation of the choroid by secreting the ECM components. The functions of choroidal melanocytes, apart from providing pigmentary protection, are poorly understood. Using different combinations of 3D tissue models, we aimed at deciphering the interrelationship between the choroidal fibroblasts and the ECM they produce, the choroidal melanocytes and the retinal pigment epithelium (RPE). The self-assembly approach of tissue engineering was used in order to form sheets of ECM with cultured fibroblasts. Similar to native human choroids, these choroidal stromal subtypes were composed of collagens (types I, IV, VI, XII, XIV and XVIII) and proteoglycans such as perlecan, decorin and lumican. The proteomics analysis revealed an upregulation of 11 collagen genes following the 3D culture of fibroblasts. Differences in their secretome cytokine profile were also observed, such as an enhanced expression of IL-6 in the conditioned medium. When melanocytes were seeded on these stromal substrates and grown in low serum conditions, their survival was enhanced contrary to the same cells cultured in 2D. We also assessed the oxidative stress cell level of melanocytes and RPE cells. Separately, melanocytes and RPE cells showed high oxidative stress levels that diminished to basal levels when both cell types were cultured in 3D. The results suggest that melanocytes influence antioxidant defenses of RPE cells, and vice versa. To conclude, we demonstrated that choroidal fibroblasts (and their ECM) influence the choroidal melanocyte cell survival, and that in turn these latter confer a protective role to the RPE. 

DEFECTIVE CHOROIDAL BLOOD FLOW BAROREGULATION AND RETINAL DYSFUNCTION AND PATHOLOGY FOLLOWING SYMPTOMATIC DENERVATION OF CHOROID

Reiner, A.1,2, Li, C.1, Fitzgerald, M.1,2,3

1University of Tennessee Health Science Center, Anatomy & Neurobiology, Memphis, United States
2University of Tennessee Health Science Center, Ophthalmology, Memphis, United States
3Christian Brothers University, Biology, Memphis, United States

We sought to determine if symptomatic denervation of choroidal impairs choroidal blood flow (CBF) regulation and harms retina. Rats received bilateral superior cervical ganglionectomy (SGc), which we showed by immunolabeling for vesicular monoamine transporter-2 (VMAT2) versus vasoactive intestinal polypeptide (VIP) depleted choroid of sympathetic but not parasympathetic inner-vation. Control rats were subjected to sham surgery in which the SGc was exposed but not removed. The flash-evoked scotopic electroretinogram (ERG) and visual acuity were measured 2-3 months after SGc or sham surgery, and vasoconstrictive ChBF baroregulation during high systemic arterial blood pressure (ABP) induced by L-1-nitroarginine methyl ester (LNAME) was assessed by Laser Doppler Flowmetry (LDF). Eyes were harvested for histological evaluation following completion of functional testing. We found that ChBF increased in parallel with ABP in SCg rats over an ABP range of 90-140% of baseline ABP, while in sham rats ChBF remained stable and uncorrelated with ABP over this range. ERG a-wave amplitudes and b-wave latencies and amplitudes, and visual acuity were significantly reduced after SGc compared to sham surgery. In SCg retina, Müller cell GFAP immunolabeling was upregulated 2.5 fold, and Iba1+ microglia were increased 3-fold. Dopaminergic VMAT2 amacrine cells in inner plexiform layer were reduced in SCg rats, and photoreceptors were slightly depleted. Functional deficits and pathology were correlated with impairments in sympathetic regulation of ChBF in response to ABP rise. These studies indicate that sympathetic denervation of choroid impairs ChBF baroregulation during elevated ABP, leading to choroidal overperfusion. This defect in ChBF regulation is associated with impaired retinal function and retinal pathology. As sympathetic ChBF baroregulatory defects have been observed in young individuals with complement factor H polymorphisms associated with risk for age-related macular degeneration (AMD), our results suggest these defects may harm retina, perhaps contributing to AMD pathogenesis. Suppressed by NIH-EY-05298 (AR), P30EY003039 (Vision Core), The Methodist Hospitals Endowed Professorship in Neuroscience (AR), NSF 1551947 (SV) and IRG 17-104327. To the Department of Ophthalmology of the University of Texas Health Science Center (MECF), and an unrestricted grant from Research to Prevent Blindness (MECF).

MODELING MAST CELL INVOLVEMENT IN GEOGRAPHIC ATROPHY

Lutty, G.1,2, Balodesching, R.1, Ogura, S.1, Kambhampati, S.1, Ge, C.1, McLeod, D.S.1, Edwards, M.1, Edwards, M.1, Bhutto, I.1,2

1Johns Hopkins University School of Medicine, Ophthalmology, Baltimore, United States
2University of Pittsburgh School of Medicine, Ophthalmology, Pittsburgh, United States
3University of Tennessee Health Science Center, Anatomy & Neurobiology, Memphis, United States
4University of Tennessee Neuroscience Institute (MECF), and an unrestricted grant from Research to Prevent Blindness (MECF).

Macrophages (Mps) and mast cells (MCs) are resident inflammatory cells in choroid. However, little is known about their function in relation to aging and ocular diseases. Studies from our own group have shown that UCMS are a key factor to the immune privilege. When co-cultured directly with activated T-cells, the UCMS inhibit CD3/CD28 induced T-cell proliferation. The aim of this study has been to look further into the interactions between UCMS and infiltrating monocytes and to investigate whether these two cell types have an influence on each other on a transcriptional- and protein level. Analysing the transcriptional expression levels of the co-cultured cell cultures, fol-
RPE9 - Pathological immune processes in the RPE-choroid: role of immune cells

Identification of Myeloid Cell Populations at Sites of Laser-induced Choroidal Neovascularization by Single-cell Profiling

Schleicht, A.1, Wieghofer, P.1,2, Zhang, P.1, Boneva, S.1, Gruber, M.1, Laich, Y.1, Böck, M.1, Thien, A.1, Sankowski, R.1, Schlunck, G.1, Agostini, H.1, Prinz, M.2, Lange, C.1

1Eye Center, Medical Center, University of Freiburg, Freiburg, Germany, 2Institute of Neuroophthalmology, Medical Faculty, University of Freiburg, Freiburg, Germany, 3Institute of Anatomy, Leipzig University, Leipzig, Germany

Purpose: Myeloid cells (MC), such as resident microglia (MG) and infiltrating blood-derived monocytes (MO), are key players in the formation of choroidal neovascularization (CNV). However, the specific discrimination between MG and MO is challenging and the precise function of MG during CNV development remains unclear. In this study, we used MG-specific reporter mice to perform single-cell RNA-Sequencing (scRNA-Seq) analysis of MG cells with and without laser-induced CNV to further elucidate their function in CNV development.

Methods: Adult Cx3cr1creERT2; Rosa26-fam11 mice were used for RNA-Seq analysis. Six laser spots were applied to each eye by an Ar-gon laser (332nm) at equal distance from the optic disc. Laser setting was 150 mJ, 100 ms with a spot size of 100 μm to induce CNV formation, while untreated littersmates served as controls. Retinal MG cells were sorted by FACS (gating strategy: CD45+CD11b+Ly6C+Ly6G-) on the third day after laser injury to perform scRNA-Seq follow-up. Gene Ontology (GO) Cluster Analysis. Protein expression of SPP1, one of the most outstanding factors, was investigated by ELISA (n ≥ 7 mice per group) and immunohistochemistry. As a therapeutic approach, antibodies against SPP1 were injected intravitreally one day after laser injury and CNV lesion size was quantified.

Results: scRNA-Seq analysis demonstrates that MG strongly change their expression profile following laser-induced CNV formation and that different MG cell populations are present at sites of laser-induced CNV. In the CNV-associated MG cluster GO terms involved in migration, immune response and DNA synthesis were enriched and genes associated with proliferation, hypoxia-sensing and angiogenesis were significantly upregulated. SPP1, also known as Osteopontin, was highly upregulated in CNV-associated MG. Immunohistochemistry and ELISA protein analysis reveals that SPP1 accumulates in and around the neovascular membranes. Intravitreal injection of anti-SPP1 antibodies lead to an increase in the CNV area when compared to control treated eyes.

Conclusion: Our study demonstrates that retinal MG cells strongly change their expression profile following laser-induced CNV formation. CNV-associated MG cells secrete a plethora of molecules among which SPP1 may have anti-angiogenic and thus neuroprotective properties.

Complement Dysfuction and Innate Immune Activation in the RPE Choroid with Age

Wolf, A., Rashid, K., Langmann, T.

University of Cologne, Laboratory for Experimental Immunology of the Eye, Department of Ophthalmology, Cologne, Germany

Age-related macular degeneration (AMD) is a leading cause of vision loss in the elderly. Associated with chronic retinal microglia in the retina. Our previous work showed that SPP1 is a marker for reactive retinal microglia and that the selective SPP1 ligand BDB173 exerts strong anti-inflammatory and neuroprotective effects on microglia in light-induced retinal degeneration. Here, we hypothesized that BDB173 dampens mononuclear phagocyte reactivity and limits choroidal neovascularization (CNV) in the murine model of laser-induced retinal injury. In our studies, retinal laser-induced CNV was induced by DMSO-treated C57BL/6 mice and C57Bl/6j mice which received 10 mg/kg BDB173 by intraperitoneal injection and in mice harboring the conditional deletion of TSP1 in microglia (Cx3cr1CreERT2; TSP1fl/fl) upon administration of tamoxifen. Analysis tools were fundus fluorescence angiography (FFA), lectin staining and optical coherence tomography (OCT) 3, 7 and 14 days after laser coagulation. In addition, microglia morphology in laser-induced lesions was analyzed by iba1-staining of retinal and RPE/choroid flat mounts. To investigate gene expression and cytokine levels of pro-inflammatory and pro-angiogenic markers at different time points after laser damage we performed qRT-PCR and ELISA assays. Immunohistological analysis of iba1-stained...
retinal flat mounts showed a reduced number of activated pha-
gocytes in the laser lesions in XBD173-treated mice compared to
controls. XBD173-treated animals also displayed decreased vascu-
lar leakage and less CNV compared to controls. XBD173 potently
reduced gene transcription and cytokine levels of pro-inflamma-
tory and pro-angiogenic markers after laser coagulation compared
to controls. Moreover, specific deletion of TSPO in microglia using
Cx3cr1CreERT2:TSPOfl/fl mice effectively attenuated early microglia and
macrophage activation in lesion areas and significantly reduced
vessel leakage and CNV size. In conclusion, XBD173 treatment as
accompanied with the histopathology of retinal degenerative diseases
Abnormal accumulation of phagocytes in the subretinal space is ac-
-panied with the histopathology of retinal degenerative diseases.

Protective Specialization of Subretinal Microglia to the Retinal Pigment Epithelium in Retinal Degeneration
Yu, C.; O’Koren, E.; Saban, D.
Duke University, Durham, United States

Abnormal accumulation of phagocytes in the subretinal space is ac-
-panied with the histopathology of retinal degenerative diseases, such as age-related macular degeneration and retinitis pigmentosa.
A large portion of these patients usually have abnormalities of the retinal pigment epithelium (RPE). Current thinking holds that subre-
tinal phagocytes contribute to this pathology, although very little is
known about their function and ontology. Here, by applying microg-
lia lineage-tracing in two separate retinal degeneration models - acute
light damage model and Rhcd10-KO knockin model, we identified that
subretinal phagocytes are endogenous microglia originating from the
plexiform layers, as opposed to recruited monocyte-derived ma-
crophages. Moreover, using single cell RNA-seq and in situ validation, we identified the unique microglial subtype in the subretinal space associated with
degeneration. Interestingly, single cell analysis suggested a protec-
tive phenotype for these subretinal microglia, a change that was es-
tablished through a step-wise transcriptomic reprogramming upon
degeneration. Congruently, conditional microglial depletion resulted in
excessive accumulation of photoreceptor debris as well as defects in
cytoskeletal elements of the RPE. Thus, our findings indicate that the
subretinal space is a microglia-restricted phagocyte niche, and,
in contrast to conventional thinking, these microglia confer protecti-
on to the RPE in degeneration settings. Together, these data provide
important new insights into microglial dynamics and functionality in
retinal degenerative diseases.

Choroidal Blood Flow Autoregulation in Health and Disease
Garhofer, G.
Medical University of Vienna, Department of Clinical Pharmacology,
Wien, Austria

Autoregulation is the ability of a vascular bed to maintain blood
flow despite changes in perfusion pressure. For a long time, the
choroid has been assumed to have no autoregulation and reacts
strictly passive to changes in ocular perfusion pressure. However,
newer results show that choroidal blood flow regulates in a com-
plex way during both changes in mean arterial pressure and intracr-
nular pressure. Further, there is evidence from animal as well as
human studies that choroidal blood flow autoregulation in the eye is
not only dependent on the level of ocular perfusion pressure, but
also depends on the absolute values of intracranial pressure and
systemic blood pressure. The current talk seeks to summarize our
current understanding of the physiology of choroidal blood flow
autoregulation. Further, recent findings of animal models and re-
sults of human studies on choroidal autoregulation in healthy and
diseased eyes will be presented.

Mechanisms Underlying the Myogenic Constriction of Retinal Arterioles
McGahon, M.1, Kur, J.1, Fernandez, J.1, O’Hare, M.1, Esqui-
yva, G.2, Needham, M.2, Schoffeld, N.1, McGruen, G.1, Cur-
tis, T.1
Queen’s University of Belfast, Centre for Biomedical Sciences (Education), Belfast, United Kingdom, 1Queen’s University of Bel-
fast, Centre for Experimental Medicine, Belfast, United Kingdom,
2Naresuan University, Department of Pharmaceutical Chemistry
and Pharmacognosy, Phitsanulok, Thailand, 1Queen’s University of
Belfast, School of Medicine, Dentistry and Biomedical Sciences, Bel-
fast, United Kingdom

ALTERATIONS IN RETINAL PERFUSION LEAD TO NUMEROUS SIGHT THREATENING DISORDERS INCLUDING DIABETIC RETINOPATHY, GLAUCOMA AND RETINAL BRACH VEIN OCCLUSIONS. UNDERSTANDING THE MOLECULAR ME-
CHANISMS INVOLVED IN THE CONTROL OF BLOOD FLOW THROUGH THE RETINA AND HOW THESE ARE ALTERED DURING OCULAR DISEASE COULD LEAD TO THE IDENTIFICATION OF NEW TARGETS FOR THE TREATMENT OF THESE CONDITIONS. RETINAL ARTERIOLES ARE THE MAIN RESISTANCE VESSELS OF THE RETINA AND, CONSEQUENTLY, PLAY A KEY ROLE IN REGULATING RETINAL HEMODYNAMICS THROUGH CHANGES IN LUMINAL DIAMETER. IN RECENT YEARS, WE HAVE
made significant progress in understanding the generation of myo-
genic constriction in retinal arterioles. We have shown that Ca2+
sparks play an excitatory role in pressurized arterioles, promoting
myogenic tone. This contrasts with the generally accepted model in
which sparks promote relaxation of arterial vessels. We have also
found that Ltype and Ttype Ca2+ channel activity promotes myo-
genic constriction while BK and K+ channels oppose the genera-
tion of myogenic tone. Our studies have also indicated that Ca2+
channels contribute to agonist- but not pressure-induced tone develop-
ment in retinal arterioles. To identify the primary mechanosensor
involved in myogenic signalling in retinal arterioles, we studied the
potential involvement of transient receptor potential (TRP) chan-
nels. These studies revealed that retinal vascular smooth muscle
cells express a range of mechanosensitive TRP channels, but that
only TRPV2 appears to contribute to myogenic signalling in this va-
scular bed. Our findings provide new insights into the molecular
mechanisms controlling myogenic constriction in the retina and
provide a basis for future studies aimed at better understanding
the mechanisms responsible for the disruption of retinal perfusion
in ocular disease.

Imaging the Choroid with Optical Coherence Tomography Techniques
Ferrara, D., Waheed, N., Duker, J.
Tufts University School of Medicine, Ophthalmology, Boston, United States

The body of knowledge of in vivo investigation of the choroid has been
markedly enhanced by recent technological advances in optical
coherece tomography (OCT). New insights elucidating the morphological features of the choriocapillaris and choroidal vascula-
ture, in both physiological and pathological conditions, indicate
that the choroid plays a pivotal role in many posterior segment di-
seases. In this lecture, a review of the histological characteristics of
the choroid, which must be considered for the proper interpretati-
on of in vivo imaging, is followed by a comprehensive discussion of
fundamental principles of the current state-of-the-art in OCT, includ-
ing cross-sectional OCT, en face OCT, and OCT angiography with
newer approaches in understanding the molecular mechanisms
in ocular disease.

Autocontrol of Choroidal Blood Flow: What’s the Anatomy?
Schoeni, F.1,2, Trost, A.1, Bogner, B.1, Rungie, C.1, Bruckner, D.1, Strohmaier, C.1, Reitsamer, H.1,2, Kaser-Eichberger, A.1
1Paracelsus Medical University, University Clinic of Ophthalmology and Optometry, Research Program for Experimental Ophthalmolo-
y and Glaucoma Research, Salzburg, Austria, 2Head of Research Program for Experimental Ophthalmology and Glaucoma Research, Salz-
burg, Austria

The choroid, the middle layer of the eye, is one of the tissues with
the highest blood flow rates in the human body, and essential for
retinal oxygen and nutrition supply. It is densely innervated by all
parts of the autonomic nervous system, i.e., sympathetic (from the
superior cervical ganglion) and two parasympathetic pathways
(from the ciliary and pterygopalatine ganglion), and receives also
primary afferent (sensory) nerve fibers of trigeminal origin. Ad-
ditionally, neurons within the choroid, the intrinsic choroidal
neurons (ICN), contribute to this innervation. Although known for
some 150 years, the function of ICN is still unknown, as is the
interplay with the various external autonomic sources of choroidal
innervation. However, a choroidal local control based on retinal
needs is discussed. We here present an overview of choroidal
innervation and the specific choroidal features in human and animal
models with emphasis on the chicken eye, and we discuss these
features in the context of choroidal blood flow and choroidal ac-
commodation. While a unique marker of intrinsic innervation is
not available so far, we further demonstrate approaches in experi-
mental ophthalmology that could offer solutions in answering
the interplay of the various extrinsic and intrinsic sources. For
that, we use a combination of immunohistochemistry, surgical
lesion techniques, OCT, laser-Doppler flowmetry and tonometry.
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benstein Foundation

Investigating the Molecular Mechanisms of Macular Degeneration: The hiPSC Approach
Singh, B., Dalvi, S., MacDonald, L., Soto, C., Galloway, C.
University of Rochester, Department of Ophthalmology and Biome-
deral Genetics, Rochester, United States

Age-related macular degeneration (AMD) and related macular dys-

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Investigating Bestrophinopathies Using Induced Pluripotent Stem Cells

Carr, A.-J.1, Nonniste, B.1, Nymark, S.1, Smith, J.1, Da Cruz, C.1, Coffey, P.1

1UCL Institute of Ophthalmology, London, United Kingdom; 2Tampere University of Technology, Dept. of Electronics and Communication, Tampere, Finland; 3Moorfields Eye Hospital NHS Foundation Trust, London, United Kingdom

Bestrophinopathies are a group of clinically distinct inherited diseases that primarily affect the retina. These diseases, including Best disease (BD), autosomal recessive bestrophinopathy (ARB), and autosomal dominant vitreoretinoidopathy (ADVIRC), are caused by mutations in Bestrophin1 (BEST1), a protein found exclusively in the retinal pigment epithelial (RPE) cells of the eye. To further understand the effects of BEST1 mutations in RPE cells, we used induced pluripotent stem cells (iPSCs) to develop cellular disease models. We recruited patients with Best disease, ARB, and ADVIRC who donated a punch skin biopsy. Fibroblast cells were cultured from the biopsy and reprogrammed into iPSCs using episomal vectors. Patient iPSCs were cultured on matrigel in Essential 8 stem cell medium until colonies overgrew, reaching confluency. At this point, the cells were cultured in EHS medium without BFGF for 6-8 weeks, permitting spontaneous differentiation. RPE cells were identified in the cultures by pigmentation, colonies were manually isolated, dissociated and cultured as a monolayer. Differentiation of RPE cells into pigmented cobblestone monolayers was performed using a combination of mouse and human RPE culture protocols. Patient-derived RPE monolayers displayed pigmented cobblestone morphology and expressed a panel of RPE cell markers at the gene and protein level. BEST1 protein was detected in control BD and ADVIRC patient cells by immunocytochemistry and western blot. In control, non-diseased iPSC-RPE BEST1, is expressed at the basolateral membrane. BEST1 localized to the apical and basal membrane in ADVIRC-derived iPSC-RPE, whilst BEST1 protein in BD-derived cells did not localize correctly to the membrane. We were unable to detect BEST1 protein in cells created from the ARB patient (premature stop mutation) at the transcript or protein level. Understanding how different mutations affect BEST1 function and influence RPE cell behaviour will be essential in developing new therapies for these diseases.

RPE-Choroid

ORAL PRESENTATIONS

RPE-Choroid

ORAL PRESENTATIONS
Towards Personalized Prediction of AMD

Delcourt, C., Eye-Risk Consortium

University of Bordeaux, Inserm, Bordeaux Population Health Research Center, UMR1219, Bordeaux, France

In the last decade, major moves have been made towards predictive, preventive, personalized and participatory (P4) medicine. In particular, prediction models, based on genetic and non-genetic risk factors, are increasingly used by clinicians, in order to identify high-risk patients, decide therapies, recommendations and follow-up frequency, as well as select patients for clinical trials. For instance, the Framingham Risk Score is widely used in cardiovascular disease. Within the Eye-Risk project, we are currently developing comprehensive prediction models for advanced AMD, integrating all genetic, environmental and biomarkers identified within the project. The development of such prediction models is made possible by the large-scale epidemiological data collected in EYE-RISK, combined with comprehensive information on phenotype, genetics and environmental risk factors. Moreover, an AMD genetic test is currently being developed and will be included in the prediction models. In parallel, we are developing a user-friendly website, which will help clinicians and the general public to use this prediction model in routine. The website will help clinicians and patients to assess AMD risk factors (smoking, diet, cardiovascular risk factors) using standardized questionnaires and describe retinal abnormalities that are predictive of development of advanced AMD. Genetic testing will be offered through the website, using saliva samples to be sent to a specific laboratory. Results of the genetic testing will be communicated through the website and included in the prediction model. The website will also help people identifying modifiable risk factors (in particular smoking, diet and cardiovascular risk factors), on which they can act to prevent AMD. A monitoring of AMD risk will be possible, through repeated measurement of retinal abnormalities and modifiable AMD risk factors. Overall, the Eye-Risk projects aims at making P4 medicine for AMD widely available to the community.

RCB1 - Dynamic cellular processes in the photoreceptor inner / outer segment

Mitochondrial Ca\(^2+\) and Photoreceptor Function

Brockerhoff, S., Hutto, R., Bisbach, C., Bauer, B., Hurley, J.

University of Washington, Biochemistry, Seattle, United States

Introduction: We are investigating how mitochondrial Ca\(^2+\) levels influence photoreceptor function. In many cells, cellular Ca\(^2+\) fluxes are coordinated with mitochondrial Ca\(^2+\) dynamics. Ca\(^2+\)-dependent changes in the activity of enzymes involved in mitochondrial energy metabolism lead to metabolic changes, which can alter the cellular energy balance, but also modulate protein function at both the translational and the transcriptional level. The identification of the channel that transports Ca\(^2+\) into mitochondria, the mitochondrial Ca\(^2+\) uniporter (MCU), has made it possible to alter mitochondrion Ca\(^2+\) using genetic strategies in animal models.

Approach: To study the unique roles of mitochondrial Ca\(^2+\) on photoreceptor physiology we are using rod-dominated mouse models lacking MCU and cone-rich zebrafish models either over-expressing or lacking MCU. We are using standard methods to examine retinal and mitochondrial morphology such as IHC, light and electron microscopy. We examine Ca\(^2+\) homeostasis through live or in situ imaging of retinas from transgenic strains that express genetically encoded Ca\(^2+\) indicators within photoreceptors. For metabolic flux analyses we follow the fate of \(^{13}C\)-labeled fuels by extracting metabolites from isolated retinas following incubation.

Results: Cone-specific overexpression (OE) of MCU by approximately 200X dramatically increases basal [Ca\(^{2+}\)\(_{\text{cyt}}\)]. This results in pronounced differences in both the spontaneous changes and the clearance kinetics of cytosolic Ca\(^{2+}\) compared to normal. MCU OE also dramatically changes cone mitochondrial morphology starting at 4 days of age, but cones are largely preserved and do not degenerate for many months. In contrast, deletion of MCU in either rods or cones does not result in major morphological changes in retina. Although we detect robust expression of MCU in wild type mouse rod photoreceptors, the lack of MCU does not change glucose flux through glycolysis or the citric acid cycle under the conditions analyzed so far.

Conclusions: Our research demonstrates a key role for mitochondria in regulating photoreceptor Ca\(^{2+}\) homeostasis and highlights unique functions for MCU in retina.

Photoreceptor Dysfunction Associated with Rhodopsin Mislocalization

Imanishi, Y.

Case Western Reserve University, Cleveland, United States

Rhodopsin is a prototypical ciliary G-protein coupled receptor, and primarily localized to the outer segment disk membranes. In individual rod photoreceptors, approximately 10 million molecules of rhodopsin are synthesized and transported to the outer segment every day. Because of the massive synthesis, the concentration of rhodopsin is extremely high (~ 4 mM) within the disks, allowing efficient capture of photons. In inherited photoreceptor degenerative disorders such as retinitis pigmentosa, a massive amount of rhodopsin is mislocalized to the plasma membrane instead of being targeted to the outer segment disk membranes. The mislocalized rhodopsin is known to cause rod photoreceptor degeneration and dysfunction, but the mechanisms leading to the degeneration/dysfunction are still unclear. Light activation of mislocalized rhodopsin is known to exacerbate the degeneration; nevertheless, degeneration can be initiated without the light activation. Among retinitis pigmentosa patients, the most severe forms of rod degeneration are observed for those with class I rhodopsin mutations, which disrupt the VxPx outer segment-targeting signal located at the carboxyl terminus of rhodopsin. Because of the disruption, class I rhodopsin mutants are mistrafficked to rod photoreceptor plasma membranes. Using live imaging techniques, we studied the renewal of class I mutant (Q344ter) rhodopsin and found that the plasma membrane-mislocalized rhodopsin is actively eliminated while newly synthesized rhodopsin is continuously delivered to the plasma membrane. The elimination of class I mutant rhodopsin occurs through two mechanisms: (1) internalization and subsequent lysosome-mediated degrada- tion within rod photoreceptors. (2) Secretion of rhodopsin-laden vesicles to the extracellular space. We also determined that secreted rhodopsin-laden vesicles are actively engulfed and phagocytosed by RPE cells. Therefore, secreted rhodopsin molecules are cleared from the extracellular space. These elimination mechanisms also disrupt the plasma membrane homeostasis and likely cause collateral damage to the rod photoreceptor neurons. Taken together, our studies reveal the previously unexpected dynamics of protein transport and renewal in diseased photoreceptor cells and provide insights into the mechanisms leading to photoreceptor degeneration or dysfunction in inherited blinding disorders.
New insights into the Mechanism of Photoreceptor Disc Morphogenesis

Archavsky, V.1,2, Spencer, W., Salinas, R., Pearing, J., Ding, J., Lo, W.-K., Besharse, J., Burns, M.3
1Duke University, Durham, United States, 2Morehouse School of Medicine, Atlanta, United States, 3Medical College of Wisconsin, Milwaukee, United States, 4UC-Davis, Davis, United States

The structural organization of the photoreceptor outer segment is highly conserved across vertebrate animals. Unlike all other cilia structures, outer segments are filled with hundreds of flattened membrane vesicles, called photoreceptor discs. Discs provide vast membrane surfaces for efficient light capture, without which our ability to see would be restricted to bright daylight. Yet, it has remained a mystery how such elaborate structure could have evolved from a primary cilium. A recently discovered property shared with photoreceptor discs, the ability to see would be restricted to bright daylight. Yet, it has remained a mystery how such elaborate structure could have evolved from a primary cilium. A recently discovered property shared with photoreceptor discs, the primary cilium, is that they are ciliated, phosphorylated, and bound to rhodopsin. We generate a PRCD knockout mouse, which shows slow photoreceptor cell death, yet produces normal rod photoreceptors. In neurons, peripheral membrane proteins are enriched in specialized cell compartments, including synapses and sensory organelles, where they play vital roles in transducing and transmitting information about the environment. Despite its importance for cell health and its roles in disease, the mechanisms underlying protein enrichment in subcellular compartments are not understood. To approach this question, we examined the roles of lipidation and electrostatic surface charge on the subcellular compartmentalization and transport of peripheral membrane proteins in photoreceptors. Amino acid sequences encoding acylation and prenylation motifs were attached to fluorescent proteins by peptidelinkers containing systematically varied charge patches and expressed in Xenopus laevis rod photoreceptors. Importantly, these linkers did not consign recognition sequences for known prenyl and acyl binding proteins, PDE6d and Unc119. Probe distribution was assessed via live cell confocal microscopy and protein dynamics were measured by live cell confocal microscopy and protein dynamics were measured by FRAP. We show that peripheral membrane proteins modified by acylation are enriched within the rod outer segment, a sensory cilium, regardless of linker charge. In contrast, prenylation combined with positive surface charge resulted in outer segment depletion and apical membrane enrichment. Any lipidation with positive linker charge resulted in enrichment of probes in the presynaptic spherule. Despite strong enrichment, the peripheral membrane proteins readily exchange amongst the membrane compartments, with the exception that transport through the connecting cilium is significantly impeded. Thus, the first intrinsic peripheral membrane protein compartmentalization code for a sensory neuron was identified.

The Intrinsic Peripheral Membrane Protein Compartmentalization Code of Photoreceptor Neurons

Calvert, P., Mara, N.
SUNY Upstate Medical University, Center for Vision Research, Syracuse, United States

In neurons, peripheral membrane proteins are enriched in specialized cell compartments, including synapses and sensory organelles, where they play vital roles in transducing and transmitting information about the environment. Despite its importance for cell health and its roles in disease, the mechanisms underlying protein enrichment in subcellular compartments are not understood. To approach this question, we examined the roles of lipidation and electrostatic surface charge on the subcellular compartmentalization and transport of peripheral membrane proteins in photoreceptors. Amino acid sequences encoding acylation and prenylation motifs were attached to fluorescent proteins by peptidelinkers containing systematically varied charge patches and expressed in Xenopus laevis rod photoreceptors. Importantly, these linkers did not consign recognition sequences for known prenyl and acyl binding proteins, PDE6d and Unc119. Probe distribution was assessed via live cell confocal microscopy and protein dynamics were measured by FRAP. We show that peripheral membrane proteins modified by acylation are enriched within the rod outer segment, a sensory cilium, regardless of linker charge. In contrast, prenylation combined with positive surface charge resulted in outer segment depletion and apical membrane enrichment. Any lipidation with positive linker charge resulted in enrichment of probes in the presynaptic spherule. Despite strong enrichment, the peripheral membrane proteins readily exchange amongst the membrane compartments, with the exception that transport through the connecting cilium is significantly impeded. Thus, the first intrinsic peripheral membrane protein compartmentalization code for a sensory neuron was identified.

Disc Morphogenesis

New Insights into the Mechanism of Photoreceptor Retinal Cell Biology

J.1 Lo, W.-K.2 Besharse, J.
University of California, Los Angeles, Jules Stein Eye Institute, Los Angeles, United States

The signaling of many G-protein coupled receptors (GPCRs) is dependent on their localisation in cilia. Rhodopsin (RHO) is concentrated within the most elaborate of all cilia, the photoreceptor outer segment. However, the mechanisms that mediate the routing and subsequent retention of ciliary GPCRs, including RHO, are not well understood. Here, we have found, using FRAP imaging of live mouse photoreceptor cells expressing RhoEGFP, as well as immunoelectron localization of RHO, that RHO follows a plasmalemmal route prior to degeneration onset. In addition, we have found that ciliary GPCRs have fundamentally different properties with regard to ciliary trafficking: whereas Somatostatin Receptor 3 (SSTR3) is well retained within cilia, we observed relatively rapid exchange between ciliary and plasmalemmal pools of RHO in IERT-RPE1 cells. Replacement of the entire cytoplasmic C terminus of RHO with that of SSTR3 reduced this exchange, suggesting an involvement of the RHO C terminus with plasmalemmal trafficking.

 Opsin’s Route to the Cilium

Chadha, A., Volland, S., Williams, D.

Western University, Pathology and Laboratory Medicine, London, Canada

In neurons, peripheral membrane proteins are enriched in specialized cell compartments, including synapses and sensory organelles, where they play vital roles in transducing and transmitting information about the environment. Despite its importance for cell health and its roles in disease, the mechanisms underlying protein enrichment in subcellular compartments are not understood. To approach this question, we examined the roles of lipidation and electrostatic surface charge on the subcellular compartmentalization and transport of peripheral membrane proteins in photoreceptors. Amino acid sequences encoding acylation and prenylation motifs were attached to fluorescent proteins by peptidelinkers containing systematically varied charge patches and expressed in Xenopus laevis rod photoreceptors. Importantly, these linkers did not consign recognition sequences for known prenyl and acyl binding proteins, PDE6d and Unc119. Probe distribution was assessed via live cell confocal microscopy and protein dynamics were measured by FRAP. We show that peripheral membrane proteins modified by acylation are enriched within the rod outer segment, a sensory cilium, regardless of linker charge. In contrast, prenylation combined with positive surface charge resulted in outer segment depletion and apical membrane enrichment. Any lipidation with positive linker charge resulted in enrichment of probes in the presynaptic spherule. Despite strong enrichment, the peripheral membrane proteins readily exchange amongst the membrane compartments, with the exception that transport through the connecting cilium is significantly impeded. Thus, the first intrinsic peripheral membrane protein compartmentalization code for a sensory neuron was identified.

The Intrinsic Peripheral Membrane Protein Compartmentalization Code of Photoreceptor Neurons

Calvert, P., Mara, N.
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MicroRNAs in diabetic retinopathy: modulators of oxidative stress and inflammation beyond gene expression

Bartoli, M., Gutsaeva, D.
Medical College of Georgia - Augusta University, Department of Ophthalmology, Augusta, United States

Diabetic retinopathy is characterized by neurovascular injury resulting from the interaction of oxidative stress and chronic sub-clinical inflammatory processes. Epigenetic mechanisms involving altered expression and activity of microRNAs (miRs) have emerged as potential pathogenic hubs contributing to oxidative and pro-inflammatory processes in the diabetic retina. We have investigated the role of a number of miRs of which expression is altered in the diabetic condition. In particular, we have analyzed the expression of miRs on retinal barrier dysfunction through suppression of endogenous antioxidants and induction of inflammatory processes in retinal endothelial and epithelial cells. In addition, we have explored a novel signaling function of miRs, particularly miR-21, exerted through direct interaction with RNA-binding proteins. MiRs binding to toll-like receptors leads to their activation and consequent induction of pro-inflammatory intracellular signaling pathways. Our results demonstrate a significant impact of miR-dependent epigenetic and signaling functions in mediating diabetes-induced dysfunction of retinal barrier cells.

Investigating Donor-host Photoreceptor Interactions

Wallace, V.1,2
1University Health Network, Krembil Research Institute, Toronto, Canada, 2University of Toronto, Department of Ophthalmology and Vision Sciences, Toronto, Canada

The prospect of replacing cells of the central nervous system by cell transplantation remains a focal point of vision repair science. Rods and cone photoreceptors can be prospectively isolated and transplanted into the retinas of wildtype and retinal degeneration mouse models. We and others recently discovered that donor and host photoreceptor cell-cell interactions play a role in determining survival and function of transplanted photoreceptors. This is the first demonstration that cell-cell interactions play a role in determining survival and function of transplanted photoreceptors. This is the first demonstration that cell-cell interactions play a role in determining survival and function of transplanted photoreceptors. This is the first demonstration that cell-cell interactions play a role in determining survival and function of transplanted photoreceptors.
vision recovery involves the transfer of photoreceptor material between donor and host cells. Identifying the mechanism(s) involved in donor/host material exchange will help us to understand the mechanistic underpinnings of cell-based vision rescue, address safety concerns raised by donor/host intercellular communication, and will reveal exciting insight into this uncharacterized communication in the context of cell transplantation.

Retinal Repair through Photoreceptor Transplantation


Age-related macular degeneration (AMD) and inherited retinal degenerations represent the leading causes of blindness in industrialized countries. Despite different initiating causes, they share a common final pathophysiology, the loss of photoreceptors. Replacement by transplantation may offer a potential treatment strategy for both patient populations. The last decade has seen remarkable progress in the ability to generate different cell types, including photoreceptors, from a variety of murine and human pluripotent stem cells. The main challenge now for developing an effective therapy is the requirement to establish efficient synaptic connectivity. However, transplanted photoreceptors only need to make a few short synaptic connections to bipolar cells in the remaining circuitry. Furthermore, the macula occupies a small area and relatively few functional photoreceptor cells may be required for useful vision, so even low efficiency photoreceptor transplantation may result in clinical benefit. In this talk I will outline the progress that has been made in restoring vision in mice through photoreceptor transplantation and the challenges ahead to deliver an effective treatment for patients with retinal degeneration.

Growth of Mammalian Retina by mTORC1-induced RPC Proliferation and Retinal Neurogenesis

Choi, J.-H., Jo, H.S., Kim, J.W., Korea Advanced Institute of Science and Technology (KAIST), Department of Biological Sciences, Daejeon, Korea, Republic of

Development of vertebrate retina is a process that adds new-born neurons in temporally-ordered fashion. To accomplish the retinal histogenesis, photoreceptor progenitors (RPCs) not only undergo a sequential transition of neurogenic competence, but they also proliferate until the post-natal days. We investigated whether the alteration of RPC proliferation property affects retinal histogenesis by activating the mammalian target of rapamycin (mTOR) signaling pathway, which is known to promote cell proliferation and tissue growth. We found that the activation of the mTOR complex 1 (mTORC1) from the deletion of tuberous sclerosis complex 1 (Tsc1) enhances the RPC proliferation without interfering neurogenic competence. Importantly, the mTORC1-dependent retinal growth and development media-te immunoproteasome, which promote cell cycle progression by inducing rapid protein turnover. Together with our previous finding of the accelerated retinal histogenesis by the activation of PI3K-Akt pathway, these results reveal that the PI3K-Akt-mTORC1 signaling pathway coordinately regulates RPC proliferation and neuronal differentiation to support the retinal growth during development.

Impacts of Neurogenic Factors on Retinal Ganglion Cell Development in the Avian Retina and in Human ES Cell-derived Retinal Organoids

Zhang, X., Barnes, S., Yang, X.-J., University of California Los Angeles, Ophthalmology, Los Angeles, United States

The vertebrate retinogenesis is regulated by cell-intrinsic determinants and cell-to-cell signaling events. The normal production of retinal ganglion cells (RGCs) has been shown to require the function of ATOH7 protein, one of the basic-helix-loop-helix transcription factors expressed by retinal progenitor cells. Since retinal progenitors do not appear to uniformly express neurogenic bHLH factors at any given developmental stage, we have investigated whether elevating their expression among uncommitted progenitors can impact neurogenesis and influence cell fate choices. Towards this goal, we have used the embryonic chicken retina and ES cell-derived human retinal organoids as models. In the embryonic chicken retina, retinal-mediated expression of ATOH7 is sufficient to induce precocious RGC formation and expansion of the neurogenic territory. ATOH7 overexpression among neurogenic progenitors significantly enhances RGC production and also leads to a minor increase of cone photoreceptor genesis. We provide evidence that elevating ATOH7 levels accelerates cell cycle progression and promotes cell cycle exit. In the 3D human retinal organoids, inducible lentiviral mediated expression of either ATOH7 or Neurog2/Ngn2 promotes cell cycle withdrawal and results in increased production of functional postsynaptic neurons. Quantitative analyses further reveal that induction of ATOH7 or Neurog2/Ngn2 yields different neuronal marker profiles, thus suggesting distinct roles for the two bHLH factors during early retinogenesis. Together, our results demonstrate that these two proneural factors promote neuronal production among uncommitted early progenitors. Moreover, elevated ATOH7 expression levels strongly bias towards the RGC fate specification, thus suggesting a useful strategy to enhance RGC production from pluripotent stem cell-derived retinal epithelium.

RCB3 - Epigenetic, miRNA and transcriptional modulators of retinal disease

Using gene editing to obtain systems-level insights into AMD

Iyengar, S., Case Western Reserve University, Cleveland, United States

Age-related macular degeneration (AMD) is multifactorial, blinding eye disorder influenced by both genetic and non-genetic risk factors. In prior work, we identified >34 loci for AMD via a genome-wide association study (GWAS), showing that key pathways for complement, lipid and inflammation are potentially dysregulated. We are continuing this line of work in the currently funded Million Veteran Program (>650,000 participants), a multi-ethnic cohort with European-American (17838 cases, 142,522 controls), African Americans (1247 cases, 26,720 controls), and Hispanic American (544 cases, 7,425 controls) participants. We will add to the body of literature on AMD genetic risk files across ethnic groups, and expand the initial list of pathways. Despite successes in identifying locations of AMD risk variants, turning these variants into therapeutic paradigms has been challenging because the actual gene target is frequently not definitively identified, and therefore mechanistic insight is difficult to derive. An overwhelming number of AMD susceptibility variants (approximately three-quarters) reside in intronic or regulatory regions of the genome, where their pathogenic potential and clinical relevance is not immediately obvious. To help guide the interpretation of such variants for retinal diseases, we developed enhancer and promoter epigenetic maps for the human fetal retinal pigment epithelium (HRPEI), a cell type that is directly relevant to AMD, but is not well represented in ENCODE or GTEx. We have examined the data surrounding the AMD loci and observe that regulatory variation (e.g. enhancers and promoters) predominate at these susceptibility loci. Data from these studies and gene editing experiments of the ARMS2 locus will be presented.

Her9/HeS4 is a Notch-independent Regulator of Vertebrate Photoreceptor Differentiation

Morris, A., Wilson, S., Coomer, C., University of Kentucky, Lexington, United States

Her9 is a bHLH-D transcriptional repressor and is the zebrafish homolog of human HEAS4. Previously, we found that her9 expression is upregulated in a background of chronic rod photoreceptor specific degeneration and regeneration in the adult zebrafish retina. In this study, we investigated the function of her9 in retinal development, identifying signaling pathways upstream of her9 expression, and characterized the phenotypes of her9 homozygous mutants. We found that her9 expression in the developing retina was induced by retinoid acid (RA), but not by Notch-Delta signaling. Expression of her9 co-localized with retinal blood vessels, and overexpression of her9 resulted in ectopic retinal vasculature. Using CRISPR/Cas9 mutagenesis, we recovered two her9 mutant alleles that result in a frame-shift and premature termination codon upstream of the bHLH domain. Homozygous her9 mutants display craniofacial defects, enlarged livers, digestive system defects, circling swimming behavior, and a poor visual background adaptation (VBA) response. Her9 homozygous mutants also failed to develop a swim bladder and did not survive past 12 dpf. In the retina we observed reduced numbers of rod and cone photoreceptors, a thin or absent retinal ciliary marginal zone, and disorganized Müller glia in her9 mutants. Taken together, our results demonstrate that Her9/HeS4 is regulated by RA signaling and is required for proper retinal development.

As He4 is absent from the mouse genome, our zebrafish models provide an excellent resource to further understand the function of this transcription factor in retinal development and photoreceptor differentiation.
Retinal Cell Biology

RCB4 - Diabetic retinopathy - Where do we stand?

Molecular Mechanisms of Diabetic Retinopathy - Arginase as a Therapeutic Target

Caldwell, R.1, Caldwell, R.W.2

1Medical College of Georgia at Augusta University, Vascular Biology Center, Augusta, GA, United States, 2Medical College of Georgia at Augusta University, Pharmacology, Augusta, United States

Neurovascular injury is an early feature of diabetic retinopathy (DR) but conventional therapies—anti-VEGF injections or laser photocoagulation—target macula edema and neovascularization, which occur much later. Moreover, neither treatment promotes repair. Thus, there is a great need for therapies to limit the early damage. Our studies have demonstrated the significant involvement of the urea/arginase axis in early neurovascular injury during retinopathy. Arginase has 2 isoforms. The cytosolic isoform A1 is strongly expressed in the liver, where it is the central player in the urea cycle. The mitochondrial isoform A2 is expressed in extra-hepatic tissues, especially kidney. Both are expressed in retina and brain, both are strongly linked to CNS diseases. Arginase metabolizes L-arginine to form polyamines, proline, and glutamate. Polyamines and proline are beneficial for repair after injury but can promote fibrosis. Glutamate and the catabolic products of polyamine oxidation can induce oxidative stress and DNA damage. Excessive arginase activity reduces the L-arginine supply for nitric oxide synthesis (NOS) and decreases its production of NO. Our studies in models of DR and ischemic retinopathy have shown diverse effects of A1 and A2. We have found that increased expression/activity of A1 is involved in hyperglycemia-induced endothelial cell dysfunction and premature senescence by a mechanism involving A1-induced decreases in the NO production by endothelial NOS. In contrast, A2 is involved in ischemia-induced inflammatory injury by mechanisms involving decreased expression of A1 in macrophages and increased expression of inducible NOS (iNOS). Transcription of iNOS depends critically on the availability of extracellular L-arginine. Thus, increases in A1 can reduce iNOS protein and limit its production of NO. Our data show that ischemia-induced decreases in A1 expression/activity promote increases in iNOS, induce the formation of pro-inflammatory macrophage/microglia, and trigger cell death via activation of the tumor necrosis factor receptor interacting protein 3 kinase/dynamin-related protein 1 pathway. Deletion of A1 globally or in myeloid-derived cells exacerbates this injury, whereas intravitreal injection of recombinant A1 is protective. Thus, manipulation of the arginase pathway could provide a new therapeutic strategy for controlling injury in DR and other forms of ischemic retinopathy depending on the timing and specific disease context.

Neuronal Dysfunction in Diabetic Retinopathy

Pardue, M.T.1,2, Allen, R.L.3, Motz, C.1, Chesler, K.3, Aung, M.3, Thule, P.1, Ivonne, P.M.4

1Atlanta VA Medical Center, Decatur, United States, 2Georgia Institute of Technology, Atlanta, United States, 3Emory University, Atlanta, United States

Diabetic retinopathy is a major cause of vision loss in working age adults. Current clinical diagnosis is based on structural changes in the vasculature which cause leakage and neovascularization. In the last ten years, several studies have shown that deficits in retinal neurons occur prior to these vascular lesions. Our goal is to detect functional deficits caused by diabetic retinopathy at the earliest possible stage in order to begin treatment with neuroprotective agents that may slow or halt the progression of vision loss. To this end, we have carefully evaluated the temporal progression of functional changes that occur in the retina of STZ-induced and Goto-Kakizaki diabetic rats that model Type I and Type II diabetes, respectively. Our data show that visual function measured with optomotor response is decreased as early as 2-4 weeks after induction of hyperglycemia. In addition, retinal dysfunction occurs by 4 weeks post-hyperglycemia as indicated by a selective delay in electroretinogram oscillatory potentials in response to dim flash stimuli. This delay in response to dim flashes indicates that diabetes affects rod pathways first. Furthermore, we have shown that neuro-vascular coupling, as measured with functional hyperemia, is also abnormal by 2 weeks post-hyperglycemia. These results all occur prior to the structural changes in retinal vasculature. Using these signs of retinopathy, we have shown that neuroprotective agents, like exercise, synthetic bile acids (taurosodesoxycholic acid), and L-DOPA, slow the loss of visual and retinal function.

RCGen Study: Novel Genetic Variants in Extreme and Advanced Phenotypes of Diabetic Retinopathy

Dass, A.1, Pacheco, S.1, Monnichkaraj, F.1, Schork, N.1, Duggan, D.1, McGuire, P.1, RCGen Study Group

1University of New Mexico, Surgery/Ophthalmology, Albuquerque, New Mexico, United States, 2Translational and Genomic Research Institute, Phoenix, United States

Purpose: Landmark clinical studies have shown that genetic risk factors may play an important role in the development and progression of diabetic retinopathy (DR). Using a well-defined, phenotypic strategy, we aimed to identify rare genetic variants in DR progression, or protection by using whole exome sequencing (WES). Methods: Two cohorts of patients from the Diabetic Retinopathy Genetics (RCGen) study. Cohort 1: “extreme” phenotype (no DR in spite of ≥25 years of diabetes) (n=6). Cohort 2: proliferative diabetic retinopathy (PDR) (n=4) were chosen. Whole exome sequencing (WES) was done on DNA isolated from white blood cells using SureSelect All Human XT v5 exome kits, on IllumiNovaSeq platform, followed by an in-house down-stream analysis pipeline. Results: We identified enrichment of four heterozygous missense variants in CO3H, COLBA1, ZNF395 and PLEKHS5 genes in Cohort 2 (PDR). Further we found a protective rare variant of a gene, NXK-2.3 in Cohort 1 (extreme phenotype). Conclusion: We identified coding sequence variants in a novel set of genes involved in the angiogenesis/inflammatory pathway that contributes to DR progression, or protection.

Drac1-Nox2 Signaling Axis in the Pathogenesis of Diabetic Retinopathy: Complexity of Simplicity

Kowurlu, A.1, Kowurlu, R.2

1Wayne State University, Detroit, United States, 2Wayne State University School of Medicine, Detroit, United States

Increased intracellular generation of reactive oxygen species (ROS) has been implicated in the pathology of diabetic retinopathy. Accumulating evidence implicates NADPH oxidases (Nox) as one of the major sources of cellular ROS. Of this class of enzymes, the phagocyte-like Nox (Nox) has come under intense scrutiny as one of the “culprits” for the induction of cellular damage. Functional regulation of Nox is fairly complex due to its membranous (p22phox and p67phox) and cytosolic (p40phox, p47phox, p67phox and Rac1) cores; the majority of these core members require specific post-translational modifications (phosphorylation and prenylation) for their translocation to the membrane and the assembly of Nox holoenzyme. To complicate this further, the participation of Rac1 in the activation of Nox2 requires intricate interplay between its regulatory factors including GDP-dissociation inhibitors and guanine nucleotide exchange factors (GEFs). Therefore, optimal efficacy of Nox2 depends upon precise regulation of complex signaling steps and the interplay of a variety of regulatory factors. Recent work from our laboratories is focused on understanding putative regulatory roles of Rac1-Nox2 signaling axis in mitochondrial dysfunction in diabetic retinopathy, and has documented that Rac1-Nox2 activation precedes mitochondrial damage. We have identified several Rac1 positive interacting proteins and Rac1-dependent pathways that may be essential for Rac1 activities. The GEF for the activation of Rac1 since N5C2376F, a specific inhibitor of Tiam1-Rac1 signaling pathway, markedly attenuates Rac1 activation, ROS generation, p38 MAPK activation, mTorDNA damage and cell apoptosis. Based on these and other complementary findings we propose that the Rac1-Nox2 signaling axis is activated in the initial stages of diabetes to increase intracellular ROS leading to mitochondrial damage, accelerated capillary cell apoptosis and the development of retinopathy. Strategies for targeting this complex, and yet precisely regulated, signaling module could have the potential to halt the progression of diabetic retinopathy in the early stages of the disease.

A Diabetic Milieu Induces Premature Senescence in Retinal Endothelial Cells

Bertelli, P.M., Peixoto, E., O’Neill, C., Guduric-Fuchs, J., Stitt, A.W., Medina, R.J.

Queen’s University Belfast, The Wellcome-Wolfson Institute for Experimental Medicine, Belfast, United Kingdom

Purpose: Diabetic Retinopathy (DR) is a highly frequent microvascular complication in diabetic patients. Diabetes is associated to endothelial dysfunction, which is characterised by increased vascular permeability, deficit in blood perfusion, vessel loss and increased inflammation. The aim of this study is to evaluate the impact of the diabetic milieu in the endothelial cell phenotype and to senescence and gain new insights into the pathogenesis of DR. Methods: Human Retinal Microvascular Endothelial Cells (HRMECs) were cultured in normal and high-glucose (NG: Sm2D-glucose; HG: 30mM D-glucose). Senescence Associated (SA)-β-Galactosidase staining was used for Population Doubling Level (PDL) assessment. Cellular functionality was tested using a Matrigel-based 3D tube formation assay. Cellular senescence was studied using Transwell-based dye permeability assay and trans-endothelial electrical resistance measurements using the xCellence. Oxygen-induced retinopathy (OIR) and Streptozotocin (STZ) mouse models were used to assess senescence in vivo. Retinas were dissected for mRNA and immunohistochemistry. Gene expression profile was assessed via RT-qPCR. Results: HG HRMECs exhibit significantly lower PDLs when compared to NG-HRMECs (p<0.001), from 4 weeks until cells reached their Hayflick limit. The number of SA-β-Galactosidase positive cells was significantly higher in late-passage HG-HRMECs when compared to age matched late-passage NG-HRMECs (p<0.001). 4-week HG treated cells showed less tubulogenic potential than NG-HRMECs (p<0.05). HG-HRMECs exhibited significant upregulation of SASP components, such as IL6 and IL8. Furthermore, senescent HG-HRMECs showed higher permeability when compared to early-passage cells. There were significantly higher SA-β-Galactosidase staining in OIR retinas (p<0.001). Both OIR and STZ showed increased gene expression of senescent markers, such as p53, p16 and SASP components by RT-qPCR (p<0.05). Conclusion: Our results suggest that diabetic conditions induce cellular senescence in retinal endothelial cells in vitro and in vivo. Senescent HRMECs are characterised by increased permeability and decreased tubulogenic potential. Furthermore, we provide evidence to demonstrate that there is accumulation of senescent cells in the ischaemic and diabetic mouse retina.
Cone-rich Rodents as Useful Animal Models of Human Visual Physiology and Pathology

Hicks, D.
CNRS UPR3222 (IN), Strasbourg, France

Despite their enormous utility in mechanistic analyses of human pathophysiology, “conventional” rodent species like mice and rats present certain drawbacks, especially in relation to vision research. Being of nocturnal lifestyle, such species have reversed sleep-wake cycles compared to humans, and a different circadian organization. This is further reflected in ocular structure and function (size of eyes and lens, presence of tapetum, rod-dominated light response, unique rod chromatin organization, paucity of cones). Finally, some transgenic mouse models of retinal degeneration do not fully reflect the human situation. For these reasons, we speculated that diurnal rodents might represent certain advantages, and we began exploring the potential interest of some species for biomedical research. We concentrated on two species, Arvicanthis anseroii and Psammomys obesus. Both have cone-rich retinas (>30-40% cones vs 1-3% in rats and mice), approximately similar to the human macula. Single flash electroretinograms (ERGs) are dominated by cone responses, even at quite low intensities. Flicker ERGs show robust patterns at 30 Hz, similar to humans, whereas at such frequencies mouse flicker ERGs are essentially flat. We investigated vulnerability to light- and drug-induced photoreceptor degeneration and found Arvicanthis to be extremely resistant to such paradigms. Under conditions of very high stress, Arvicanthis retinas exhibited dose-, time- and region-dependent loss of photoreceptors. We hypothesize that this species possesses intrinsic neuroprotective mechanisms to prevent such damage and are currently investigating possible pathways. We also used this species to study the effects of the light-dark cycle on the retinal circadian clock and its biological output. Compared to mice and rats, Arvicanthis exhibits stronger amplitude rhythms in both circadian clock genes and output genes and cell physiological behaviors (photoreceptor phagocytosis). Whereas constant darkness did not greatly affect the majority of such measures, a single night of constant moderate light had dramatic effects on virtually all measures. Psammomys is known as a useful model of type 2 diabetes, and we showed this species also recapitulates many of the features of diabetic retinopathy in humans. Hence these and other diurnal animals represent valuable adjuncts to biomedical research.

Assessing the Roles of Irbp and Irbp-like in the Zebrafish Eye

Collery, R.
Medical College of Wisconsin, Milwaukee, United States

Mutations in human interphotoreceptor retinoid-binding protein (IRBP) are associated with high myopia and retinal degeneration, and mice in which IRBP has been inactivated also show excessive eye growth and loss of photoreceptors. Since being first described in 1977, much of the role of IRBP in the eye has been discovered, including retinoid transport, fatty acid binding, and protection of the photoreceptors from damage. However, there are still some mysteries that surround IRBP. How does its absence lead to retinits pigmentosa? How does its absence lead to myopia? What proteins does IRBP interact with? Does IRBP have roles outside of the interphotoreceptor matrix? We propose to use zebrafish to help answer these questions. Zebrafish have duplicated their ancestral irbp gene, and now have two copies: irbp and irbp-like. These two genes have diverged following duplication and may have undergone subfunctionalization - the separation of single roles to two genes that previously were both performed by one gene alone. Since IRBP carries appears linked to multiple processes in species with a single gene, we will investigate both IRBP and IRBP-like for their functions. We have used CRISPRs to inactivate irbp and irbp-like, both singly and together. We have developed promoter tools that will allow us to identify the cells expressing irbp-like in the inner retina. Finally, we have identified RPE-based receptors for IRBP and IRBP-like.

High Resolution Imaging and Correlative Histology in Cone-dominant Mammals

Sajdak, B.S.1,2, Salmon, A.E.1, Cava, J.1, Allen, K.P.1, Freling, S.1,2, Fitzpatrick, D.1, Merriman, D.K.1, Carroll, J.1,2
1Medical College of Wisconsin, Cell Biology, Neurobiology, and Anatomy, Milwaukee, United States, 2Medical College of Wisconsin, Ophthalmology & Visual Sciences, Milwaukee, United States

The canine species has dichromatic color vision with short-wavelength (S) and long/medium-wavelength (L/M) cone sensitivities, and a distinctive ability to perceive chromatic variations with peak spectral sensitivities of 450-455 nm and 555 nm. In the macaque, differentiation of rod- and cone-mediated responses by electroretinogram (ERG) is dogs is less clear, and standards have been developed based on ISCEV standards for human observers, methods to differentiate S- and L/M-cone responses have not been described. We developed flicker protocols designed to elicit biphasic responses from each of the 3 photoreceptor subclasses. ERG responses were measured with sine-wave modulation of photoreceptor excitation at different temporal frequencies (between 4 and 58 Hz) and mean luminances (between 3.25 and 130 cd/m²) on 3 different nor-
Retinal Regeneration Therapy Using iPSC Derived Retina

Mandai, M.

RIKEN Center for Biosystems Dynamics Research, Laboratory for Retinal Regeneration, Kobe, Japan

In view of clinical application of iPSC-derived retina (iPSC-retina) transplantation for patients with retinal degeneration, we tested the integration and function of iPSC-retina after transplantation using animal models of end stage retinal degeneration. In the end-stage retinal degeneration mouse model (rd1), iPSC-retina transplanted at embryonic stage developed photoreceptor layers after transplantation with outer segment formation and also formed synapses with host bipolar cells. The isolated host retinas with miPSC-RPE-transplanted also showed light responses as evaluated by multiple electrode array system (MEA). Human ES/iPSC-retina also developed photoreceptor layers after transplantation with >10% cone photoreceptors in immune-deficient rats and mice with retinal degeneration, and showed light responses similar to miPSC-retina by MEA. We also developed focal photoreceptor ablation models in monkeys using cobalt chloride or laser photoacoagulation and confirmed the maturation and integration of iPSC-RPE retina after transplantation. Substantial amount of photoreceptors were observed to survive for over 2 years in a monkey. Since remaining inner cells in graft iPSC-retina were considered to interfere with host-graft integration, we developed genetically engineered iPSC-retinas in which bipolar cells failed to mature and degenerate by knocking out the genes involved in bipolar cell maturation. In ISGs, transplantation of bipolar cells resulted in reduction and photoreceptor ratio increased. We observed more host-graft synapse formation with better light responsiveness in ISG transplants.

Deconstructing Retinal Organoids: To Assess the Heterogeneity, Maturity and Transplantation Potential of hESC-derived Photoreceptors

Collin, L.1, Zerti, D.1, Queen, R.1, Santo-Ferreira, T.2,3, Cox, head, H.1, Hussain, R.1, Steel, D.1, Mellough, C.1,4, Ader, M.2, Sernago, E.1, Armstrong, L.1, Lako, M.2

1Newcastle University, Newcastle upon Tyne, United Kingdom, 2Center for Regenerative Therapies, Dresden, Germany, 3Roche Pharmaceuticals, Basel, Switzerland, 4Lions Eye Institute Ltd., Perth, Australia

Irreversible loss of photoreceptors is a hallmark of outer retinal diseases, which cause a significant proportion of permanent blinding conditions. The ability to differentiate human embryonic stem cells (hESCs) into retinal organoids, which recapitulate many characteristics of human retina development, represents a source of human tissue for transplantation, disease modelling and regenerative medicine studies. Recent improvements in organoid culture and technologies such as genome engineering and single cell RNA-sequencing provide an opportunity to gain greater insight into the biology and therapeutic potential of lab-generated retina. To this end, hESCs were engineered to generate a core-end homeobox (Crx)-green fluorescence protein (GFP) reporter line. Upon differentiation to retinal organoids GFP-positive cells were detected and isolated. Single cell transcriptional analysis of GFP-positive cells revealed a largely homogenous population with a dominant cluster, 72%, expressing genes commonly found in photoreceptor precursors. A secondary cluster exhibited significant differential expression of cholesterol and lipid biosynthesis, mitochondrial biogenesis and cell cycle regulation genes. However, this cluster did not overlap with photoreceptor, other retinal cell types or pinealocyte expression profiles, indicating a subpopulation of matured, yet undifferentiated retinal progenitors. By single cell RNA-sequencing, we demonstrated that GGPS cells were derived from Crx-positive progenitors by in vitro transplantations into the sub-retinal space of an end-stage mouse model of retinal degeneration, Pde6brd1-C3h (rd1). Three weeks post-transplantation GFP-positive donor cells were observed in contact with host second order neurons and expressed marker genes of photoreceptors and ribbon synapse formation, indicating a potential donor-host interaction. The ability to generate a reporter line to identify photoreceptors as they emerge in organoids, coupled with their purification and in-depth analysis by single cell transcriptomics, allows insight into the heterogeneity and identity of these cells during in vitro differentiation. In turn, this facilitates the transplantation of a largely homogenous population of photoreceptor precursors into models of outer retinal disease to assess their engraftment potential. This study outlines a potential strategy to investigate, identify, quality control and manufacture a source of transplantable photoreceptors for future cell replacement therapies.

Modeling Retinal Ganglion Cell Development and Disease with Human Pluripotent Stem Cells

Langer, K.1,2,3, Fligor, C.1, Vij, R.1, Oliemacher, S.1, Sriradha, A.1,4, Moyer, J.1,2,3

1Indiana University, Biology, Indianapolis, United States, 2Indiana University, Department of Medical and Molecular Genetics, Indianapolis, United States, 3Indiana University, Stark Neurosciences Research Institute, Indianapolis, United States

Human pluripotent stem cells (hPSCs) can serve as effective in vitro models of both neural development as well as neurodegeneration, as they can give rise to all cell types of the body and be expanded indefinitely. Additionally, when derived from specific patient populations, they can serve as powerful tools for disease modeling as well as pharmaceutical screening. Retinal ganglion cells (RGCs) serve as the essential connection between the eye and the brain, with this connection disrupted in blinding disorders such as glaucoma, causing severe degeneration and eventual death of RGCs. Previously, we have demonstrated the ability to derive RGCs from iPSCs, with resulting cells expressing a variety of RGC-associated...
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with RGCs organizing within a discrete inner layer displaying pro-

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Retinal Vascular Wall Thickness in Hypotensive Subjects, the Naïve Vascular State: Data and Theoretical Model

Gask, T.

Indiana University Optometry, Bloomington, United States

A project was done measuring retinal arterial vascular wall thicknesses using the Indiana adaptive optics scanning laser ophthalmoscope. Wall thicknesses were measured from the smallest retinal arterioles to the largest vessels near the optic disk. This project’s original purpose was to determine the arterial wall remodeling done in patients with well controlled primary hypertension. This data will be briefly presented. In addition to the usual control group of subjects with normotension, a distinct second control group of subjects who had never had a systolic blood pressure over 100 mmHg in their lives, i.e. a lifetime hypertensive group, was measured. The results for this group will be presented as they showed remarkably regular low-like data. We interpret this as representing the naïve state of the ocular arterial vascular system. Possible interpretations of the basis for this data will be discussed based on Laplace’s Law and laws governing collapsible tube theory.

Statistical Assessment of Blood Vessel Activity as a Surrogate of in vivo Retinal Vascular Function

Nunez do Rio, J.M.1,2, Bergelles, C.1,2, Houston, S.1,2, Greenwood, F.1, Dubis, A.M.1,2

1UCL Institute of Ophthalmology, London, United Kingdom, 2Biomedical Research Centre, Moorfields Eye Hospital and UCL Institute of Ophthalmology, London, United Kingdom. *Welcome/EPSRC Centre for Interventional and Surgical Sciences, UCL, London, United Kingdom, Moorfields Eye Hospital NHS Foundation Trust, London, United Kingdom

Introduction: Understanding retinal vascular function is crucial to elucidate the pathology of vascular eye diseases and can serve as a surrogate of non-retinal vascular function. Adaptive optics (AO)-aided imaging techniques have recently allowed the visualization of inner retinal microvasculature and individual moving cells. While these techniques enable the detection of the vasculature, the extraction of consistent blood cell motion information remains a challenge, which hinders the measurement of accurate cell velocity. We propose a surrogate metric for blood cell velocity based on a statistical assessment of blood cell activity within vessels. Methods: Raw videos from a custom built split detection AO scanning laser ophthalmoscope (AOSLO) were pre-processed to de-warp and feature stabilized. The capillary network was extracted from a vessel contrast image computed from the arithmetic standard deviation. Blood cells in the vasculature network were segmented automatically exploiting its model of appearance in split detection videos: one side of the cone being substantially darker than the other one. A SIFT-based descriptor is computed for each pixel candidate, which is labeled as blood cell if the closest descriptor in a reference database belongs to a positive sample. The blood vessel content metric was then determined by the ratio of blood cell area to total vessel area. Results: The proposed blood flow metric was analyzed for different vessels and video sequences in both synthetic videos and split detection AOSLO videos. Synthetic videos simulate blood flow patterns on split detection frames and cell probability and speed are manually selected. Experiments on different video sequences show vessels with higher cell activity - cell probability- present higher values of the proposed metric. The same analysis was performed on 3 AOSLO video sequences in which vessel activity was manually graded by an expert. High activity vessels showed values of <0.5 whereas low activity vessels present values of <0.3 (example illustrated by Table 1). Conclusions: We introduce a surrogate metric and an automatic algorithm to analyze blood vessel activity in split detection AOSLO videos. Experiments provide evidence of the correlation between the level of activity within the vessel and the distribution of the metric. Acknowledgments: This project was supported by grants from the NIHR BRC at Moorfields Eye Hospital and the Rosetrees Trust.

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(Table 1. AOSLO data: vessel activity vs proposed metric)
Retinal membrane guanylyl cyclase 1 (RetGC1) plays important role in rod and cone physiology. Two types of regulatory proteins control enzymatic activity of RetGC1: Ca²⁺-sensitive guanylyl cyclase activating proteins (GCAPs) and retinal degeneration-3 (RD3) protein, which inhibits RetGC1 activity and controls RetGC1 accumulation in outer segment. Mutations in human RetGC1 (GUCY2D) cause two main types of the congenital blindness. Mutations that disable the cyclase activity cause recessive blindness at birth, Leber congenital amaurosis type 1 (LCA1), mostly a non-degenerate loss-of-function hereditary retinal disease. Different from the LCA1, another type of severe congenital blindness linked to the RetGC1 gene, dominant-cone rod dystrophy type 6 (CORD6), is a progressive early-onset loss of functional photoreceptors. The most frequent mutations in GUCY2D found in the CORD6 patients are substitutions of the R838 to phenylalanine or histidine, R838B does not inactivate the cyclase, but rather alter its regulation by calcium sensor proteins (GCAPs), in vitro and in vivo. In addition, R838B substitution makes RetGC1 less sensitive to inhibition by retinal degeneration-3 protein (RD3). RD3 deficiency causes a recessive blindness accompanied by severe photoreceptor degeneration in rd3/rd3 mouse strain and in human patients of Leber congenital amaurosis 12. The lack of RD3 diminishes the content of RetGC1 in the photoreceptor outer segments of the rd3/rd3 mouse. However, the severity of photoreceptor degeneration in mouse model cannot be explained solely by the reduction of the cyclase and is consistent with RD3 being required for blocking of RetGC1 activation by GCAP. Mutations in the calcium binding domain (e.g., R846P and R846N) caused a shift in Ca²⁺-sensitive regulation by guanylate cyclase-activating proteins (GCAPs). Mutations in the cyclase catalytic domain led to a loss of enzyme function in the mutant p.R869X, but not in p.R902L. Surprisingly, the p.R902L mutation increased the guanylate cyclase activity more than 20-fold showing a high GCAP independent activity. Thus, the GC-C catalytic core can be affected in complete opposite ways.

Pre-clinical Development of an AAV Based Gene Therapy for the Treatment of Retinal Dystrophy Due to Recessive Mutations in GUCY2D

Boyce, S.L.¹, Peterson, J.P.¹, Lukason, M.¹, Fajardo, D.¹, Zhang, H.², O’Blriand, C.², Baek, R.², Plummer, C.¹, Sherak, C.¹, Jacobson, S.G.¹, McVie-Wylie, A.¹, Boyce, S.E.²

¹University of Florida, Gainesville, United States, ²Sanofi-Genezyme, Boston, United States, ³University of Pennsylvania, Ophthalmology, Philadelphia, United States

Here we present experiments leading up to an AAV based gene therapy for the treatment of retinal disease due to recessive mutations in GUCY2D, the leading cause of the most severe form of early-onset retinal dystrophy, Leber congenital amaurosis (LCA1). GUCY2D encodes retinal guanylate cyclase-1 (retGC1), a protein expressed exclusively in photoreceptor outer segments that plays a role in the recovery phase of phototransduction. Despite a high degree of visual disturbance stemming predominantly from a loss of cone photoreceptor function, LCA1 patients retain normal photoreceptor laminar architecture, except for foveal outer segment abnormalities and, in some patients, foveal cone loss. Adeno-Associated Virus (AAV) has emerged as the vector of choice for targeting therapeutic genes to the retina. Taken together LCA1 is an outstanding candidate for AAV mediated gene replacement therapy. Here we present IND-enabling studies conducted to evaluate 1. photoreceptor transduction mediated by an AAV5 vector containing photoreceptor specific promoter driving GFP in non-human primate (NHP); 2. the minimal effective dose using clinically representative AAV-GUCY2D vector in retGC1 knock-out mice, 3. safety studies in NHP and the establishment of a no observable adverse effect level (NOAEL). Taken together, these results from these studies establish a reasonable safety window for first in human clinical trials to treat LCA1.

Retinal Guanylyl Cyclase RetGC1 and its Regulatory Proteins in Congenital Eye Diseases

Peshenko, I.
Pennsylvania College of Optometry, Salus University, Elkins Park, United States

Retinal membrane guanylyl cyclase 1 (RetGC1) plays important role in rod and cone physiology. Two types of regulatory proteins control enzymatic activity of RetGC1: Ca²⁺-sensitive guanylyl cyclase activating proteins (GCAPs) and retinal degeneration-3 (RD3) protein, which inhibits RetGC1 activity and controls RetGC1 accumulation in outer segment. Mutations in human RetGC1 (GUCY2D) cause two main types of the congenital blindness. Mutations that disable the cyclase activity cause recessive blindness at birth, Leber congenital amaurosis type 1 (LCA1), mostly a non-degenerate loss-of-function hereditary retinal disease. Different from the LCA1, another type of severe congenital blindness linked to the RetGC1 gene, dominant-cone rod dystrophy type 6 (CORD6), is a progressive early-onset loss of functional photoreceptors. The most frequent mutations in GUCY2D found in the CORD6 patients are substitutions of the R838 to phenylalanine or histidine, R838B does not inactivate the cyclase, but rather alter its regulation by calcium sensor proteins (GCAPs), in vitro and in vivo. In addition, R838B substitution makes RetGC1 less sensitive to inhibition by retinal degeneration-3 protein (RD3). RD3 deficiency causes a recessive blindness accompanied by severe photoreceptor degeneration in rd3/rd3 mouse strain and in human patients of Leber congenital amaurosis 12. The lack of RD3 diminishes the content of RetGC1 in the photoreceptor outer segments of the rd3/rd3 mouse. However, the severity of photoreceptor degeneration in mouse model cannot be explained solely by the reduction of the cyclase and is consistent with RD3 being required for blocking of RetGC1 activation by GCAP. Mutations in the calcium binding domain (e.g., R846P and R846N) caused a shift in Ca²⁺-sensitive regulation by guanylate cyclase-activating proteins (GCAPs). Mutations in the cyclase catalytic domain led to a loss of enzyme function in the mutant p.R869X, but not in p.R902L. Surprisingly, the p.R902L mutation increased the guanylate cyclase activity more than 20-fold showing a high GCAP independent activity. Thus, the GC-C catalytic core can be affected in complete opposite ways.
Role of structural dynamics in retinal binding and release to rhodopsin

Oregon Health and Science University, Biochemistry and Molecular Biology, Portland, United States

Structural studies have greatly advanced our understanding of how the visual GPCR rhodopsin interacts with its light-absorbing chromophore 11-cis retinal (11CRR) and its agonist, all-trans retinal (ATR). However, it is still not clear how these ligands enter and leave the receptor, what keeps them there, or why these processes are affected by the binding of signaling partners transducin and arrestin to activated receptor. In the process of studying these phenomena, we recently found evidence that rhodopsin behaves like a traditional ligand binding GPCR, with conformational selection governing the preference for binding of 11CRR vs. ATR (Schafer and Farrens, JBC, 2015). Unexpectedly, we also found evidence that under some conditions, after ATR is released from photo-activated rhodopsin (so called MII), the ATR can re-bind to the receptor in equilibrium (Schafer et al., PNAS, 2016).

In my talk, I will discuss the background and ramifications of these findings, and how we are using novel rhodopsin-based fluorogen biosensors to study the dynamics and energetics of these processes. I will also discuss our findings that transducin, arrestin and rhodopsin kinase all interact with the same site on rhodopsin through a hydrophobic contact-driven mechanism (Jones Brunette et al., Biochemistry, 2016), and how these interactions might play a role in ATR equilibrium binding.

References

Molecular Mechanism of Arrestin-1 Binding to Rhodopsin

Gurevich, V.
Vanderbilt University, Pharmacology, Nashville, United States

Arrestin 1 is the key player in the two-step quenching of light-activated rhodopsin with sub-second kinetics. Arrestin 1 demonstrates 10-20-fold higher binding to active phosphorylated rhodopsin than to other functional forms. This selectivity is achieved via a “coincidence detector” type of mechanism: arrestin 1 has sensors responding to the active state of rhodopsin and rhodopsin-attached phosphates, and only simultaneous engagement of both sensors induces the conformational changes in arrestin 1 necessary for the arrestin-receptor interaction. Mutagenesis, NMR and EPR studies, as well as X-ray crystallography revealed the identity of both activation and phosphate sensors in arrestins and the nature of the binding-associated conformational changes. The interaction mechanism appears to be conserved in other arrestin family members that bind non-visual GPCRs. This information guides targeted construction of arrestins with special functional characteristics and identifies the elements that have distinct conformations in free and receptor-bound arrestins as likely docking sites for non-receptor signaling partners. DEER distance measurements between selected points in arrestin-1 and rhodopsin revealed multiple distances for each pair, indicating that the complex is dynamic and likely has different “flavors”. It is reasonable to hypothesize that different shapes of the arrestin-receptor complex direct signaling to distinct pathways.

Funding: NIH grants RO1 EY015150, R35 GM122491, and Vanderbilt University Cornelius Vanderbilt Chair.

Efficiency of Rod Transduction Activation by a Single Opsin Molecule

Kefalov, V.1, Sato, S.1, Jastrzebska, B.2, Engel, A.2, Palczewski, K.3
1Washington University School of Medicine, Ophthalmology and Visual Sciences, Saint Louis, United States, 2Case Western Reserve University, Pharmacology, Cleveland, United States

Bleaching adaptation in rod photoreceptors is mediated by apo-opsin, which activates photo-transduction with an estimated effective activity 10-fold lower than that of photoactivated rhodopsin (Meta II). However, it is unclear whether opsins has low constitutive activity or it exists in equilibrium between a predominant inactive state and an intermittent active state. To address this question, we sought to record responses produced by individual opsin molecules in mouse rods. We used mice lacking the dominant calcium feed-back on cGMP synthesis, mediated by Guanylyl Cyclase Activating Proteins 1/2 (GCAPs). This boosts the rod single-photon responses, making them readily detectable by suction electrode recordings. To introduce a small fraction of opsin, we exposed dark-adapted GCAPs-/- rods isolated retinas to brief bleaching light that photoactivated ~1% of rhodopsin. This bleach produced a dramatic increase in the frequency of discrete photoreceptor-like events in GCAPs-/- rods. The bleach-induced activity also persisted in rods containing rhodopsin locked in its ground state by 11-cis 7-ring-retinal, ruling out transactivation of rhodopsin by opsin. Although individual photoresponse-like events were not readily observable in wild type rods, a similar bleach-induced increase in their activity was detected by power spectral analysis. Together, our results suggest that bleaching adaptation in rods is mediated by opsin that exists in equilibrium between a predominant inactive and an intermittent Meta II-like state.

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Vanilloid TRP Channels Regulate Neuronal Signaling in the Mammalian Retina

Krajci, D., Redmon, S., Yarishkin, O., Lak, M.
University of Utah School of Medicine, Ophthalmology & Visual Sciences, Salt Lake City, United States

Activity of CNS neurons is strongly influenced by their glial and endothelial neighbors and by physical parameters (pressure, strain, temperature) from the ambient milieu. Impaired sensing and transmission of ambient information is known to compromise cellular function and survival in the brain and the retina, especially in debilitating diseases such as glaucoma, diabetic retinopathy and traumatic brain/injury. To obtain insight into the molecular pathways that underlie the transduction of tensile & compressive stress, swelling and neuroinflammation we investigated the role of vanilloid transient receptor potential (TRPV) channels in retinal ganglion cells (RGCs). Muller glia, microglia, astrocytes and microvascular endothelial cells. Transcript analysis from immunoseparated populations revealed cell-type specific expression of Trpv transcripts that was strongly influenced by non-canonical functions of mechanical strain. Protein expression of TRPV1 and TRPV4 isoforms in retinal cells was confirmed with fluorescent reporters, antibody labeling, and by recording membrane currents and calcium signals in GCaMP5/G6-expressing transgenic retinas and isoform-specific knockout animals. We found that TRPV1 is confined to specific subsets of RGCs but is expressed in most Muller cells and microglia. The cognate TRPV4 channels is localized to these cell classes and is co-expressed in a cell-type specific expression of G protein signaling family, RGS7 and RGS11 that modulate the channel regulation by Ga and Gbg subunits of the heterotrimeric G protein Go. Affinity purification studies identified that these Gbg exist in macromolecular complex with an orphan receptor GPR179. Deletion of GPR179 in mice abolished targeting of RGS proteins and disrupted TRPM1. The complex is further scaffolded through interactions with the principle neurotransmitter receptor mGlur6. The positioning and function of the TRPM1 channel and mGlur6 requires contribution of several related cell-adhesion like proteins identified in proteomics screens: NYK, LRIT3, LRIT1, and ELFN1. Recent findings pertaining to dissection of this molecular organization will be presented. Overall, these results indicate that multiple elements of the synaptosomal signaling defects observed in glaucoma and diabetic retinopathy by stimulating the downstream activation of purinergic receptors, proteases and caspases whereas pharmacological and genetic targeting was neuroprotective. Overall, our results suggest that vanilloid TRP channels play key roles in RGC signaling and neuro-gliovascular interactions at the inner blood-retina barrier. Supported by the NIH, Willard L. Eccles Foundation, Glaouema Research Foundation.

In vitro Modulation of Retinal TRPV4 - Implications for Neuronal Survival and Neuroinflammation

Taylor, L., Abdshill, H., Nääv Ottosson, J., Ghosh, F.
Lund University, Dept of Ophthalmology, Lund, Sweden

Transient Receptor Potential Vanilloid 4 (TRPV4) polymodal ion channel activity has been implicated in retinal ganglion cell degeneration during biomechanical insult, such as hypoxia-tis stress. We have utilized a previously described in vitro adult full-thickness explant model of retinal detachment, in which the degree of biomechanical disturbance (mechanical tissue support) can be controlled (no support; partial support; no-thickness support), and examined TRPV4 expression, ganglion cell survival, glial activation and neuroinflammatory signaling. We have also explored the effect of modulating TRPV4 signaling at various time points in no support vs partial support explants. In adult rabbit explants, maximizing tissue support resulted in an upregulation of TRPV4 expression in ganglion cells, as well as an increased ganglion cell survival in comparison to partial support counterparts, despite showing no significant differences with regards to glial activation, neuroinflammatory mediators or cytokine release. In partial support adult pig explants cultured for 1, 2 and 5 days, treatment with the specific TRPV4 antagonist RN-1734 resulted in a significantly increased ganglion cell and photoreceptor survival, and attenuated the glotic response compared to untreated explants or explants treated with selective TRPV4 agonist GSK1016790A. Against treatment resulted in a significantly decreased survival of ganglion cells already after 24h. Free-floating explants (no tissue support) cultured for 24h and treated with RN-1734 similarly displayed an increased ganglion cell survival compared to untreated counterparts. However, no differences were observed when comparing partial and no support explants cultured for 24h with RN-1734, with regard to ganglion cell survival or cytokine release. Our results suggest that TRPV4 signaling is an important contributor to the ganglion cell degeneration in both the rabbit and pig retinal detachment model, and that modulating this response may be of key importance to preserve neuronal cell health and glial homeostasis in biomechanically-induced retinal pathology. Thus, TRPV4 may be an interesting pharmaceutical target to explore for treatment of retinal degenerative disease.

RCB11 - Sterol biosynthesis and elimination in retinal structure and function

Pikuleva, I.
Case Western Reserve University, Ophthalmology and Visual Sciences, Cleveland, United States

Cholesterol is a major lipid in the retina, whose levels are maintained by balancing the pathways of cholesterol input and output. Retinal cholesterol input includes local biosynthesis and tissue uptake of cholesterol-containing lipoprotein particles from the systemic circulation. Retinal cholesterol output represents local metabolism and elimination by retinal, as well as lipoprotein-mediated reverse cholesterol transport. Previously, this laboratory established that local cholesterol biosynthesis is the major source of cholesterol for the retina, accounting (at least in mice) for 72% of total retinal cholesterol input. We also found that the regulation of retinal cholesterol input at transcriptional levels is weak and likely encompasses post-translational mechanisms. The role of cholesterol output by metabolism to oxysterols was established as well. CYP27A1 and CYP46A1 were found to be important for normal structure and function of retinal vasculature and in addition, for the control of transcriptional regulation of retinal cholesterol output by liver X receptors. Cholesterol transport within the retina and exchange between different cell types is still poorly understood. Hence we conducted retinal characterization of mice lacking Apoe, a major apolipoprotein on retinal particles carrying cholesterol inside the retina. We documented that this genotype had almost a 3-fold increase in retinal cholesterol, yet an apparent lack of retinal cholesterol depositions. Retinal proteomics of wild type mice revealed that the most abundant cholesterol-related proteins belonged to the pathways of cholesterol biosynthesis (DHCR7), uptake (SCARB2, LRP1, LRMP1, and MEOX2), post-translational regulation (PCMK9), enzymatic cholesterol elimination (CYP27A1), lipoprotein- (APOA1, APOA4, APOE, APOJ, PON1, and PON2) and non-lipoprotein-mediated sterol transport (OSBP1 L2; OSBP1 L2, L2, and L80). Comparative studies of sterol, protein and mRNA levels in the Apoe-/- wild type retina identified the compensatory mechanisms that were caused by a lack of Apoe and underlie, at least in part, only a minor retinal phenotype of Apoe-/- mice. Finally, retinal proteomics pointed to non-canonical functions of Apoe. Collectively, the data obtained provide a more comprehensive understanding of retinal cholestetrol transport and homeostasis. Supported in part by EY018383 and EY011373.

Statins, RPE and Drusen

Chen, X., Vavvas, D.
Massachusetts Eye and Ear, Department of Ophthalmology, Boston, United States

In contrast to the less prevalent neovascular AMD, currently there is no effective therapy for the more prevalent non-neovascular age-related macular degeneration (AMD). Shared risk factors between cardiovascular disease and AMD as well as analogies in histopathology of the lipid rich atheromas and subRPE drusen deposits have prompted investigators to examine if therapies effective in atherosclerosis may affect AMD progression. Epidemiological studies have given us mixed results. This is likely to the heterogeneity of AMD and large variation in statins dose and lipophilicity. Cardiovascuclar Phase III studies have shown that dose of statins matter. Intensive high dose statin reduce atheroma burden, whereas moderate rate amount slows down, but do not reverse atheromatous disease burden. For this reason, we wanted to investigate the effect of high dose lipophilic study on a specific subset of AMD patients with large lipid rich confluent drusenoid pigment epithelial detachments. We performed an open label, prospective phase I/II multicenter pilot trial...
study with 26 high-risk AMD patients (mean age 68.1 years) recei-
ving 80mg atorvastatin for a minimum of 1 year. 23 patients com-
pleted at least 12 months of treatment. Average follow up was 16 
months (range 12-24). Eight (31%) had nearly complete disap-
pearance of drusen, 2 (8%) had partial disappearance of drusen. There 
was vision gain vs. non-responders of 1 line (p < 0.06). No patients 
progressed to late AMD (vs. 3-4 expected, alpha error 2.1%). The 
average time interval for response (resolution of the drusenoid de-
posits without atrophy) was 11.7 months (range 3-22). Women had 
a higher odds ratio (7.71) of being responders, but this failed to re-
ach statistical significance (95% confidence limits 0.746-79.7746, 
p = 0.0886). We had genetic data from only 6 patients (3 respon-
ders, 3 non-responders) that showed CHN SNP rs10486863 GG to 
be associated with responders. Since there were no difference in 
the cholesterol levels between responders vs non-responders ad-
ditional non-cholesterol lowering properties of atorvastatin may 
be important. In vitro high dose atorvastatin decreases inflammatory 
response of ARPE-19 cells and may increase phagocytosis ability. 
These findings suggest that there is potential for high dose statins 
to be effective in at least a subpopulation of AMD patients with 
large lipid rich subRPE deposits. Further studies of statins and AMD 
are warranted that takes into account dose and lipophilicity.

Cholesterol-24S-hydroxylase in the Retina: From Expression to Pathophysiological Implication in Glaucoma

Léger-Charnay, E.1, Gambert-Nicot, S.1,2, Masson, E.Y.1, Acar, N.1, Bron, A.M.1,2, Breitlon, L.1
1Centre des Sciences du Goût et de l’Alimentation, Eye, Nutrition and Cell Signalling Research Group, Dijon, France, 2University Hospi-
tal, Department of Clinical Chemistry, Dijon, France, 3University, Department of Ophthalmology, Dijon, France

Cholesterol-24S-hydroxylase, also called CYP46A1, is a choleste-
rol-metabolizing enzyme that converts cholesterol into 24-hydro-
xysterol. 24S-hydroxycholesterol is a more polar lipid than 
cholesterol, and is therefore considered as a mechanism for the 
removal of cholesterol from neurons.

The role of CYP46A1 in the maintenance of cholesterol homeosta-
sis has been thoroughly studied in the brain, and in the context of 
neurodegenerative diseases.

Nevertheless, far less is known on the importance of CYP46A1 in 
the retina. Glaucoma is a neurodegenerative disease that is charac-
terized by the loss of retinal ganglion cells. One peculiarity of retinal 
ganglion cells is the specific expression of CYP46A1. Clinical as well 
as experimental animal experiments have been carried out to better 
decipher the importance of CYP46A1 in the retina. The purpose of 
the present paper is to highlight data on the involvement of CYP46A1 
in the functioning of the retina and its pathophysiologic implicati-
s, with a special emphasis on glaucoma.

A Computational Model of Retinal Cholesterol Dynamics: Insights into the Pathophysiology of Dry AMD

Zekavat, S.1,2, Lu, J.1, Maugais, C.1, Maier, N.1
1Yale School of Medicine, New Haven, United States, 2Broad Institu-
tee of MIT & Harvard, Medical and Population Genetics, Cambridge, United States, 3Massachusetts General Hospital, Cardiovascu-
lar Research Center, Boston, United States, 4Genentech, South San 
Francisco, United States, 5Roche Innovation Center Basel, Basel, Switzerland

AMD is the leading cause of blindness in the elderly and begins 
with the currently untreated “dry” form, characterized by chole-
sterol deposits in the outer retina. Glaucoma is a neurodegenerative 
disease that is characterized by the loss of retinal ganglion cells. One 
peculiarity of retinal ganglion cells is the specific expression of 
CYP46A1. Clinical as well as experimental animal experiments 
have been carried out to better decipher the importance of 
CYP46A1 in the retina. The purpose of the present paper is to highlight 
data on the involvement of CYP46A1 in the functioning of the retina 
and its pathophysiologic implications, with a special emphasis on 
glaucoma.

Vitamin-D3 Acts as a Potent Vascular Inducer in Hyperoxic Insult: Primary Human Retinal Pigment Epithelium

Ponnalagu, M.1, Subramani, M.1, Vinekar, A.2, Anandula, V.A.1, Valiyar, A.K.1, Shetty, R.1, Das, D.1
1Narayana Nethralaya Foundation, GROW Lab, Bangalore, India, 2Narayana Nethralaya Eye Institute, Department of Pediatric Reti-
na, Bangalore, India, 3Narayana Nethralaya Hospital, Microbiology, & Molecular Diagnostics, Bangalore, India, 4Narayana Nethralaya 
Hospital, Cornea & Refractive Service, Bangalore, India

VIT-D3 is known to be a potent inhibitor of angiogenesis in cancer 
studies and in mouse oxygen induced ischemic retinopathy 
model (hyperoxic state). In the early disease condition (stage-1, hy-
peroxia) of retinopathy of prematurity (ROP), regressed vasculari-
zation is observed. VIT-D3 is multifunctional, and its role in hypero-
xia, in modulating the ocular neovascularization is not known. This 
in vitro study was carried out to evaluate the regulation of VIT-D3 on 
specific vascular factors and tubulogenesis in a hyperoxic condition.

Methods: Human cadaver primary RPE (PRPE) cells were used 
for the study. PRPE cells were cultured in 40%O2 for 5 days in the 
presence or absence of VIT-D3 [1x, 25-OHvitamin-D3 (D3)]. Tubulogenesis 
assay was carried out with primary HUVEC cells to functionally 
understand the outcome of VIT-D3 treatment. The qRT-PCR, sandwich-ELISA and immunofluorescence staining was carried 
out to analyze the gene and proteins of interest.

Results: VEGF and its receptor VEGF-R2 proteins were signifi-
cantly upregulated (P<0.05) in the presence of VIT-D3, which 
was disfigured in hyperoxic conditions. The tubulogenesis assay 
in hyperoxia conditioned PRPE supernatants showed a decrease in 
the mean tube length (P<0.001). Remarkably, VIT-D3 conditioned 
PRPE supernatants increased the mean tube length (P<0.001) 
when compared to hyperoxia. Further, in addition to VEGF, notch 
signalling which is vital for angiogenesis is deregulated in hyperox-
ia and revamped with VIT-D3 treatment (NOTCH receptor-1, DLL-
4, JAG-2, hes-1, and hes-5;P< 0.05). VIT-D3 also induced the tight 
junction protein ZO-1 in PRPE and hyperpolarized the transmain-
brane potential, which was depolarized in hyperoxic conditions.

Conclusion: This data suggest that nutritle VIT-D3 may act as a 
potent inducer of neovascularization under hyperoxia conditions.

Further, it provides a clue to investigate the potential of VIT-D3 as 
a therapeutic agent for the early stage of ROP (stage-1, hyperoxic 
state), which in turn may play a role in preventing the progression 
of the disease.

Kir4.1 Channel Function in the Post ischemic Retina of Various Transgenic Mouse Lines with Altered Müller Cell Gliosis

Pannicke, T.1, Frommerherz, I.1, Wunderlich, K.A.2, Rei-
chenbach, A.1, Perez, M.T.2, Pekny, M.1, Grosche, A.1
1University of Leipzig, Paul Flechsig Institute, Leipzig, Germa-
y, 2Lund University, Department of Clinical Sciences, Division of 
Ophthalmology, Lund, Sweden, 3University of Tübingen, Molecular 
Neurosciences, Center for Integrative Neurosciences, Tübingen, Ger-
many, 4University of Gothenburg, Center for Brain Repair and Reha-
bilitation, Department of Clinical Neuroscience and Rehabilitation, 
Gothenburg, Sweden, 5Ludwig-Maximilians-Universität Munich, 
Department of Physiological Genomics, Munich, Germany

Müller gli, the dominant glia cells of the retina, intimately interact 
with neurons in the healthy and disease retina. Still rather little is 
known how alteriations in this neuron-glia crosstalk is affected by 
the typical glicastic activation Müller gli cells show in response to 
every type of tissue damage. We investigated these changes in a 
model of transient ischemia evoked by elevation of the intracereal 
pressure in wild type (WT) mice and various genetic mouse mo-
dels including mice deficient in the glial-specific nucleotide recep-
tor P2Y1 (P2Y1R-KO) mice in which the ectosytic glicostatorm 
release is disrupted. Moreover, we investigate the Müller glial re-
sponse upon transient ischemia in GFAP/vimentin double knock-
out mice. We found significant, but partially janus-faced effects 
of the genetic modification in Müller cells (and thus, altered Müller 
glia function) onto the survival rate of neighbouring neurons.

Of note, the effect of an altered Müller gliosis [whether it was posi-
itive or negative] strongly depended on the respective neuro-
nal cell type and their need for glial support. Especially neurons 
of the inner retina benefited from a deficiency of P2Y1, while 
le vel of vimentin and GFAP had the inverse effect. In contrast, 
we found enhanced photoreceptor degeneration in P2Y1R-KD 
mice. Importantly, Kir4.1 channel function was better maintai-
ned in glicotic Müller cells of P2Y1-deficient mice and it was large-
ly diminished in glia of GFAP/vimentin double knockout animals.

We conclude that a better preserved Kir4.1 channel function is key 
for the retinal ion- and volume homeostasis stabilized by Müller 
glia and that neurons of the inner retina are those that primarily 
rely on this glicastic support. In the end, our results also emphasize 
the importance and need for future studies to improve our understan-
ding of mechanisms involved in Müller cell gliosis. Moreover, our 
studies demonstrate that modulation of single aspects of Müller 
glia cell gliosis could be considered as therapeutic option focusing 
on maintaining gliconic functions to putatively improve treatment of 
various retinal diseases.
Diabetes and Circadian Regulation of Kir4.1 Channels

Bhatwadelkar, A.

Indiana University, Indianapolis, United States

The Müller cell, a major glia of the retina regulate K⁺ balance via inwardly rectifying Kir4.1 channels. In diabetic retinopathy (DR), the Müller cells are dysfunctional and swollen due to downregulation of the Kir4.1 channels. Circadian rhythms regulate biochemical and physiological functions of the body. The suprachiasmatic nucleus (SCN), a master pacemaker of circadian rhythms is located centrally while at the cellular level, the clock genes orchestrate biological rhythms. This presentation will review the role of diabetes and circadian rhythms in regulating Kir4.1 channels in retinal Müller cells. We will discuss the role of clock regulatory mechanisms and their involvement in insulin resistance and diabetes. We previously demonstrated that Kir4.1 channels in the retina exhibit a diurnal rhythm. The Kir4.1 channels maintain the oscillatory pattern in vitro in Müller cells and are under regulation of clock genes Bmal and Per2. However, with diabetes, there is a loss of normal rhythm and dampening in the levels of Kir4.1 channels. Diabetes-induced increases in pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) is detrimental to Kir4.1 channels. We recently reported that advanced glycation end product modification of basement membrane downregulates the activity of Kir4.1 channels. In this study, we will further discuss emerging role of circadian rhythm in regulating Kir4.1 channels in the retina. The insulin receptor substrate 1 (IRS-1) is an important downstream regulator of insulin signal. The deletion of IRS isoforms leads to Müller cell gliosis and apoptosis. We will discuss a critical role of IRS-1 in regulating Kir4.1 channels in the Müller cells. Overall, this session will provide novel insights into the regulation of Kir4.1 channels in diabetes.

Involvement of TRPV1 and TRPV4 Channels in Pathological Retinal Angiogenesis


*Queen’s University Belfast / Wellcome-Wolfson Institute for Experimental Medicine, Belfast, United Kingdom, 1Queen’s University Belfast / Wellcome-Wolfson Institute for Experimental Medicine, Belfast, United Kingdom, 2Queen’s University Belfast / Centre for Biomedical Sciences Education, Belfast, United Kingdom

Visual loss in retinal ischemic diseases, such as retinopathy of prematurity and diabetic retinopathy, is closely associated with the development of pathological retinal angiogenesis. Current anti-VEGF or laser photocoagulation therapies to treat retinal neovascularization are either not effective in all patients or inherently destructive to the retina. Therefore, new therapeutic targets are urgently required. In the present study, we have investigated the contribution of TRPV1 and TRPV4 channels to retinal angiogenesis in vitro and ischemia-driven retinal neovascularisation in vivo. TRPV1 and TRPV4 mRNA and protein expression were identified in primary bovine retinal endothelial cells (RMECs) using RT-PCR and western blot analysis. Strong immunolabelling for these channels was also detected in RMECs and their functional expression confirmed by whole-cell patch-clamp recording. Pharmacological inhibition of TRPV1 and TRPV4 channels suppressed in vitro retinal angiogenesis through a mechanism specifically involving the modulation of tubulogenesis. TRPV1 and TRPV4 channel blockade had no effect on VEGF-induced angiogenesis in vitro. Inhibition of either channel reduced retinal neovascularisation and promoted reparative angiogenesis in the oxygen-induced mouse model of retinopathy. Using proximity ligation assays and patch-clamp recording, we observed that TRPV1 and TRPV4 form functional heteromeric channel complexes in RMECs in vitro. We conclude that retinal endothelial TRPV1 and TRPV4 channels may provide new targets for therapeutic intervention in ischemia-induced vascular proliferative diseases of the retina.

Voltag-gated Ion Channels in RPE Co-regulate the Uptake of Photoreceptor Outer Segments

Nymark, S., Johansson, J.K., Korkka, I., Skottman, H., Halilainen, T.O.

1Tampere University of Technology, Faculty of Biomedical Sciences and Engineering, Tampere, Finland, 2University of Tampere, Faculty of Medicine and Life Sciences, Tampere, Finland

Renewal of retinal photoreceptors occurs via outer segment disk shedding and subsequent phagocytosis by retinal pigment epithelium (RPE). This process is diurnally synchronized and precisely regulated by various signaling pathways. In RPE, changes in intracellular calcium concentration have been shown to regulate phagocytosis; however, the overall molecular mechanisms including the participating ion channels are not fully characterized. We investigated the co-regulation of phagocytosis by voltage-gated calcium channels (CaV) whose importance has been previously demonstrated in the literature, and membrane channels (NaV), that were previously identified both in human embryonic stem cell (hESC) -derived and mouse RPE. For this, we performed patch clamp recordings in hESC-derived RPE to characterize the CaV, and NaV currents, and used immunolabeling to investigate the channel subtype distribution and localization in hESC-derived and mouse RPE. The phagocytosis was assayed in vitro by incubating purified porcine photoreceptor outer segments (PODS) on hESC-RPE and ex vivo by preparing mouse eyes with the retina intact at various time points of the circadian cycle. Immunogold electron microscopy (EM) was used to further determine the localization of NaV channels during phagocytosis. Our patch clamp recordings showed the presence of CaV currents in hESC-derived and mouse RPE characteristic to L-type CaV channels. In addition, we identified NaV currents in hESC-deri- ved RPE that were primarily carried by subtypes Na1.4, Na1.6 and Na1.2. These channels were further observed both in hESC-deri- ved and mouse RPE by immunolabeling and proteomics. During phagocytosis, pharmacological inhibition of L-type CaV channels by nifedipine decreased the amount of bound or internalized PODS particles by 62%, while their activation by (+)-BayK8644 led to 30% decrease. Interestingly, inhibition of T-type CaV channels by ML218 resulted in 32% increase in the number of bound or internalized PODS particles. Inhibiting NaV channels with TTX and subtype specific blockers resulted in 41% reduction of the ingested PODS particles, while not affecting their binding. Taken together, our data indicates that both Na1 and Na3 channels in RPE co-regulate the phagocytosis of photoreceptor outer segments. Further studies are needed to elucidate their concerted functioning in RPE.
Rod Bipolar cell Networks in Early Retinal Remodeling

Pfeiffer, R. 1, Anderson, J. 1, Emrich, D. 1, Dahal, J. 1, Sigulinskii, C. 1, Morrison, H. 1, Yang, J.-H. 1, Watt, C. 1, Rapp, K. J., Garcia, J. 1, Kondo, M. 2, Terasaki, H. 2, Marc, R. 1, Jones, B. 1

1Moran Eye Center at the University of Utah, Ophthalmology, Salt Lake City, Utah, United States
2Mie University, Ophthalmology, Mie, Japan

ORAL PRESENTATIONS
Retinal Cell Biology

Retinal remodeling is a form of negative plasticity that occurs as a consequence of retinal degenerative diseases. Part of retinal remodeling involves anomalous sprouting of processes, termed neuritis. The synaptic structures and partners of the neuritis are not yet defined, leading to uncertainty about the consistency of network motifs between healthy and degenerate retina. Our goal is to map out the identities and network relationships of bipolar cell networks using a connectomics strategy. Retinal connectomes or ultrastructural maps of neuronal connectivity have substantially contributed to our understanding of retinal network topology, providing unique insight against which pathological network topologies can be evaluated. We have generated the first pathoconnectome (RPC1), or connectome of pathological tissue, of early retinal remodeling at 2nm/pixel, and are currently investigating the impact of remodeling on network architecture. The tissue for RPC1 was obtained from a 10mo transgenic P347L rabbit model of autosomal dominant retinitis pigmentosa. Tissue for RPC1 was fixed in mixed aldehydes, osmicated, dehydrated, embedded in resin, and semithin sections were placed on grids, stained, and imaged using a JOEL JEM-1400 TEM using SerialEM software. Every 30th section was det for computational molecular phenotyping (CMP), and probed for small molecules: glutamate, glutamine, glycine, GABA, taunine, glutathione, or TEM compatible proteins GFAP and GS. The pathoconnectome volume is explored and annotated using the scissors software suite. RPC1 was selected as an example of early retinal remodeling, demonstrating Muller cell hypertrophy, metabolic dysregulation, and degeneration of rod outer segments, indicating phas 1 remodeling and neuronal sprouting. We have observed the presence of both cone pedicles and rod spherules within the OPL to be synapticly active with neurites from some rod bipolar cells forming functional synapses with both rod spherules and cone pedicles. These rod bipolar cells also exhibit structural alterations that are currently being evaluated using pathoconnectome motifs and comparing them to networks established from our previous connectome, RCL, generated from a healthy rabbit. These fieds allow us to evaluate and analyze the impact of retinal remodeling on retinal networks which may have important implications for therapeutic interventions being developed which rely on inner retina network integrity.

Plasticity of Retinal Bipolar Cells

Kerschensteiner, D.
Washington University School of Medicine, Saint Louis, United States

Bipolar cells are second-order neurons of the visual system. They relay photoreceptor signals from the outer retina to amacrine and ganglion cells in the inner retina. I will discuss how plasticity of bipolar cell dendrites and axons guides the development of reliably functioning retinal circuits. A large variety of genetic causes result in photoreceptor death in the retina (i.e., retinal degenerations). Photoreceptor death removes input from bipolar cells and causes visual impairment in more than 1,2000 people worldwide. Retinal degenerations often progress slowly, leaving a window of opportunity, in which rewiring of bipolar cells with remaining cones could improve vision. We developed a mouse model in which photore ceptors can be removed with precise control. Whereas developing regeneration such as circuit formation, axon guidance and recovery of visual functions. These models provide a foundation to address three key bottlenecks in regeneration and should have wide implications for the treatment of retinal degenerative diseases, including LCA.

Müller Glia Proliferation and Retinal Regeneration Is Triggered by Acute Damage but Not Progressive Photoreceptor Degeneration in Zebrafish cep290-/- Mutants

Perkins, B., Fogerty, J., Cancio, L., Stupay, R.
Coe Eye Institute / Cleveland Clinic, Cleveland, United States

Leber’s Congenital Amaurosis (LCA) is the most common blinding disorder in children. LCA has been linked to mutations in at least 20 genes, and mutations in the gene CEP290 are among the most common causes of LCA. Unlike mammals, zebrafish possess the innate ability to regenerate retinal neurons following light damage, mechanical injury, or laser ablation. This response is characterized by Müller cell de-differentiation and proliferation to produce retinal progenitors that regenerate retinal neurons. While zebrafish regenerate lost neurons following acute injury, little is known about photoreceptor regeneration in genetic models of retinal degeneration. We used the zebrafish cep290-/- mutant to investigate Müller cell regeneration in a model of inherited retinal dystrophy. Zebrafish cep290-/- mutants undergo a slow, progressive cone degeneration with rhodopsin mislocalization, indicating rod dysfunction. We observed a significant increase in the number of cells staining positive for proliferating cell nuclear antigen (PCNA) in the outer nuclear layer (ONL) of cep290-/- mutants. This was consistent with proliferation of rod progenitor cells. In contrast, no increase in the number of cells expressing PCNA was seen in the inner nuclear layer, suggesting that Müller glia fail to proliferate in cep290-/- mutants. Quantitative RT-PCR revealed that the expression of astacin and lin28 were unchanged. Accumulation of 4CA, a marker of microglia and macrophages, was observed in the subretinal space of cep290-/- mutants, indicating a neuroimmune response. To determine if cep290-/- mutant retinas the capacity for regeneration, we used an OCT-guided laser to ablate photoreceptors. Following laser injury, Müller cell proliferation was observed in cep290-/- mutant retinas at 3 days post-injury. The results suggest that robust regeneration occurs following acute injury to zebrafish retinas but that the progressive degeneration observed in the cep290-/- mutants may be insufficient to trigger Müller cell proliferation. Ongoing work will determine if upstream signals of retinal damage are upregulated in cep290-/- mutant retinas and whether cone photoreceptors regenerated following laser injury resemble wild-type photoreceptors. By identifying the mechanisms that can activate retinal regeneration in zebrafish it will ultimately be possible to translate this knowledge into treatments for humans with retinal degenerative diseases, including LCA.
Müller glial cell lines originating from cadaveric human retina, have restored some retinal function in rodent models of glaucoma and photoreceptor degenerations. The discovery of methods to induce 3D retinal organoid formation using embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC) has provided a platform to study the development and maturation of human Müller glia in vitro. The study of these cells within retinal organoids would not only enhance our understanding of the role of these cells during retinal development but also in disease processes. We have therefore investigated Müller glia development in 3D retinal organoids derived from human ESC and iPSC. Human iPSC and ESC lines were differentiated into 3D retinal organoids using a slight modification of published methods. After initiation of differentiation, retinal organogonans were grown until day 280 and examined at various time points for markers of Müller glia, including CRALBP, vimentin, nestin, glutamine synthetase, and Rx. The results showed that protein expression of the Müller glia marker CRALBP can be detected by immunohistochemistry in retinal organoids from day 20 onwards, but vimentin and CD29 can be observed much earlier in the organogonan development. We hypothesise that Müller glia start to differentiate earlier in retinal development than previously thought, with the expression of vimentin, and more mature Müller glia express CRALBP from approximately day 70 during organogonan development. These results aim to enhance our understanding of Müller cell development in the human retina, that will enable value for future investigations into the regenerative capabilities of these cells.

The Role of Proliferation in Müller Cell Remodeling and Subretinal Glial Scar Formation Induced by Retinal Detachment

Lewis, G.1, Warrington, R.1, Luna, G.1, Fisher, S.1, Coffey, P.1,2, Radeke, M.1

CNRS, Universite Paris-Sud, CURTO - Retina France, Orsay, France

Retinal Müller glial cells are able to re-acquire a stem/progenitor phenotype and regenerate new neurons following injury. Although highly effective in some species, this phenomenon is extremely limited in mammals. Understanding the molecular mechanisms underlying Müller cell recruitment in species with different regenerative capacities is an active area of investigation. We generated Xenopus retinal degenerative models to investigate the hitherto unexplored issue of Müller cell regenerative potential in amphibians. We found that a subset of Müller cells re-enter the cell cycle following retinal damage/regeneration. These activated Müller cells produce new photoreceptors. This constitutes the first evidence so far for Müller cell-driven regeneration in this species. To identify new players acting in promoting Müller cell proliferation, we focused our work on the phosphorylation of SMAD3 in the Müller cells in both species and the phenotypical changes were followed by OCT. The results were correlated with morphological changes detected by histological analyses. In the OCT, a subtle and well-delimited hyper-reflective signal was detected at Day 3 corresponding to cavity formation in the ONL due to the photoreceptor loss seen in H&E staining. After Day 7 both OCT and histological analyses confirmed re-established normal retinal morphology in the zebrafish. Meanwhile, in the murine retina, the hyper-reflective signal was still visible, that corresponds to massive loss of nuclei within the ONL until the last time point investigated. To identify differences in the modulation of TGFβ isoforms as well as the downstream mediator SMAD3, we quantified changes on gene expression and investigated the expression of pro-inflammatory cytokines (IL-1β, IL-6, TNFα). We found that TGFβ1 and TGFβ3 were significantly reduced at day 3, while the number and size of vimentin labeled Müller cell scars in the subretinal space were significantly reduced at Day 7 in comparison to control detachments. These data suggest that proliferation initiated within a few days after RD is a critical step for the subsequent formation of subretinal glial scars. Therapies designed to inhibit proliferation during the early phase of detachment may, therefore, be a viable approach to preventing the formation of subretinal fibroses in patients.

Diabetic retinopathy (DR), the leading cause of blind- ness among working aged adults, is a microvascular complication of diabetes acting in Müller cell response to injury, we are focussing our interest on YAP/TAZ regulation in the pathogenesis of Diabetic Retinopathy (DR). The identification of Nr3c1 as a promising candidate gene, specifically expressed in Müller glia of diabetic mice revealed an upregulation of genes associated with inflammation, oxidative stress defense and inter- mediate filaments. While genes involved in blood vessel integrity, energy supply for neurons and homeostatic functions were down-regulated. Opossum analysis showed that the glucocorticoid recep- tor Nr3c1 binding site cluster was downregulated in Müller glia of diabetic mice and we confirmed the reduced expression level of Nr3c1 target genes by mass spectrometric proteome data. In line with these finding, the Nr3c1 transcript was significantly downre- gulated in Müller cells from diabetic mice and we detected a nuc- lear expression of Nr3c1 specifically in Müller cells. These results verify this TF as an interesting candidate for further investigations.

Conclusion: The identification of Nr3c1 as a promising candidate demonstrates the feasibility of our approach to shed light on novel mechanisms of Müller cell-specific genes involved in DR pathology. In a next step, we aim to test whether modulation of Nr3c1 expression in Müller cells of diabetic mice holds therapeutic potential by interfering with DR pathomechanisms, and, consequently, supports neuronal survival in the diseased tissue.

The Hippo/YAP Pathway and Müller Glial Cell Reactivation in the Retina

Hamon, A., All, D., Bitard, J., Roger, J., Derron, M.

University of Bern, Experimental Ophthalmology, Bern, Switzerland

Mammals are not endowed with the ability to regenerate complex structures as the retina lower vertebrates. Moreover, the molec- ular and cellular conditions enabling possible regeneration of mammalian tissues are not known. Therefore, we focus on Müller cells as they are able to re-enter the cell cycle, dedifferentiate into retinal progenitor cells and restore visual function in zebrafish. For this, we compared the SMAD2 of Müller cells during retinal degeneration/regeneration in zebrafish and mouse focusing in particular on the TGFβ superfamily ligands TGFβ-1 and TGFβ-3. A 532 nm diode laser was used to induce a focal damage to the retina in both species and the phenotypical changes were fol- lowed by OCT. The results were correlated with morphological changes detected by histological analyses. In the OCT, a subtle and well-delimited hyper-reflective signal was detected at Day 3 corresponding to cavity formation in the ONL due to the photoreceptor loss seen in H&E staining. After Day 7 both OCT and histological analyses confirmed re-established normal retinal mor- phology in the zebrafish. Meanwhile, in the murine retina, the hyper-reflective signal was still visible, that corresponds to massive loss of nuclei within the ONL until the last time point investigated. To identify differences in the modulation of TGFβ isoforms as well as the downstream mediator SMAD3, we quantified changes on gene expression (pPCR, iSH) and protein (IHC) levels showing that TGFβ1 and TGFβ3 isoforms were modulated differently in the laser damage area comparing mouse and zebrafish. Indeed, TGFβ1 gene expres- sion was halved starting from Day 3 and remained at a low level until Day 14 in the zebrafish. In the mouse, TGFβ1 was already upregulated at Day 1 and then it slightly decreased until Day 14. Regarding TGFβ3 gene expression, a strong upregulation starting from Day 1 was found in the zebrafish while in the mouse no chan- ges in expression of TGFβ3 was detectable over time. Furthermore, the phosphorylation of SMAD3 in the Müller cells in both animal models was analyzed. In the zebrafish, P-SMAD3 signal was visible in the Müller cells within the damage area only at Day 3. Howev- er, P-SMAD3 signal was already detectable at Day 1 in the mouse. Modulation of the identified endogenous repair mechanism could suggest new strategies for stimulating retinal regeneration in mammals and, in particular, in humans.
were complemented by pro-inflammatory mediators (e.g. IL-6 and tumor necrosis factor alpha) to the retina. Microglia co-cultured with Tregs exhibited a reduction in activated markers such as vascular endothelial growth factor. Further, these treatment approaches resulted in the de-activation of retinal microglia. Diabetic retinopathy (DR) remains a leading cause of blindness in the United States. Research into pathological mechanisms of DR has increased focus on retinal damage caused by diabetes. However, diabetes likely impairs the retina's reparative mechanisms, thus contributing to the accumulation of unrepair damage in the retina. In order to test this hypothesis, we utilized the mouse intraocular pressure model used ischemia-reperfusion (IR) model of disease to determine the effects of diabetes on resolution of inflammation and restoration of the blood-retinal barrier (BRB) following retinal injury. We showed that the natural progression of the retinal IR injury response includes self-resolving inflammation coinciding with restoration of the BRB. IR injury causes rapid alterations and loss of endothelial tight junction (TJ) proteins that make up the iBRB. Barrier restoration did not coincide with re-synthesis of TJ proteins, but rather, with reorganization of TJ complexes at endothelial cell borders. Restoration of the iBRB normally occurs 2-3 weeks after IR injury, but was defective in diabetic mice. Whereas IR produced 45% less (p<0.01) vascular leak in diabetes than controls at 2 days following injury, diabetic mice exhibited a 2.4-fold higher (p<0.05) leak at 4 weeks after IR. This defect corresponded with amplification of innate immune responses. Microglia responded to IR by transiently proliferating and migrating from plexiform layers toward the ganglion cell layer. Infiltrating leukocytes exhibited a temporal progression, with granulocytes and Ly6C^hi inflammatory monocytes predominating at 1 day and Ly6C^med repactive monocytes peaking at day 7. There were no significant differences in accumulation of granulocytes or Ly6C^med monocytes between control and diabetic mice. Surprisingly, at 2 weeks following IR diabetic mice exhibited a persistent 2.3-fold increase in inflammatory cells (p<0.01) compared to controls, and at 2 weeks following IR. Thus, diabetes may not only damage the retina but also inhibit IRB repair by altering the inflammatory response to damage and impeding inflammatory resolution. This model may provide an opportunity to examine mechanisms governing maintenance and restoration of the BRB in the adult and a means to test novel therapeutic approaches to treat diabetic macular edema.

The Role of the SIRT1/LXR Signaling Axis in Retinal Endothelial Cell Inflammation and Metabolism
Hammer, S.
Michigan State University, East Lansing, United States
Purpose: Liver X receptors (LXRs) are hypothesized to serve as a link between lipid metabolism and inflammation by promoting cholesterol efflux as well as exhibiting anti-inflammatory properties. Studies have shown that SIRT1 promotes insulin secretion; reduces glucose tolerance and plays a critical role in regulating inflammation. SIRT1 has also been shown to interact with LXR to promote LXR activation. The purpose of this study was to investigate the effect inflammation plays in SIRT1-LXR activation and subsequent metabolic changes in retinal endothelial cells.
Methods: Bovine retinal endothelial cells (BRECs) were isolated and validated according to a previously published protocol. BRECs were treated with diabetic relevant stimuli TNFa (10ng/ml); LXR activator, DMHCA (1uM); or SIRT1 activator, SRT1720 (1uM). In order to model calorie restriction in vitro BRECs were starved (0% FBS) for 24hrs.SIRT1, IL1beta, ABCA1 and ABCG1 were analyzed by qRT-PCR. SIRT1 activity was measured via histochemical assay (HdacA). LXR acylation was measured via western blot analysis. Cellular cholesterol concentrations were measured using the Amplex Red Cell Membrane Assay Kit. Results: Treatment with pro-inflammatory cytokine, TNFa (10ng/ml) for 24hrs significantly increased cholesterol levels (p<0.033), IL1beta expression (p=0.334), n=8, IL6 (p=0.001, n=3) expression and resulted in decreased levels of HDAC activity (p=0.023, n=3) in BRECs. Activation of LXR (DMHCA, p=0.0178, n=8) or SIRT1 (SRT1720, p=0.0084, n=6) prevented TNFa-induced inflammation. Serum starvation resulted in a significant increase in HDAC activity (p=0.0005, n=6), SIRT1 (p=0.0065, n=3), ABCA1 (p=0.0377, n=3) and ABCG1 (p=0.017, n=3) expression levels. Last, SIRT1 activation decreased a decrease in LXR acylated levels in BRECs. Conclusion: The work presented here suggests that serum starvation promotes activation of the SIRT1-LXR pathway. Targeting the SIRT1-LXR pathway has dual benefits of preventing low-grade retinal inflammation as well as promoting metabolic reprogramming.

Cytokine-induced ECM Alterations in DR Pathogenesis
Giblin, M.1, Penn, J.1,2
1Vanderbilt University, Cell and Developmental Biology, Nashville, United States, 2Vanderbilt Medical Center, Ophthalmology and Visual Sciences, Nashville, United States
Basement membrane (BM) thickening is one of the earliest structural abnormalities observed in diabetic retinopathy (DR). Recent studies suggest that this is a product of increased extracellular matrix (ECM) deposition and that it contributes to pathogenic retinal cell behaviors. Yet, to date, studies regarding BM alterations and DR remain inconclusive. The purpose of this study was two-fold; first, to investigate how diabetes-relevant stimuli affect expression of ECM and second to examine how ECM deposited under diabetes-relevant conditions affects human retinal microvascular endothelial cells (HREMC) expression of adhesion proteins that are known contributors to pathogenic leukostasis. HREMC and human retinal pericytes (HRP) were treated with diabetes-relevant stimuli; high glucose (25mM D-glucose) and inflammatory cytokines (TNFa and IL-1beta, 10ng/ml). Conditions were systemically optimized and expression of primary BM components collagens IV (COL4), fibronectin (FN), agrin (AGRN) and perlecan (HSPG2) was measured by qRT-PCR. High glucose produced no significant changes in HREMC or HRP. HREMC, TNFa caused a 1.7- and 2.3-fold increase in COL4 and AGRN, respectively, and a 5-fold decrease in FN and HSPG2 (all p<0.01). IL-1beta induced a 1.8- and 5-fold increase in COL4 (p<0.01) and AGRN (p<0.02), respectively. In HRP, TNFa and IL-1beta caused a 2.7-fold (p<0.01) and 1.8-fold (p<0.01) induction of COL4, respectively. To study how ECM alterations affect HREMC behavior, HREMC or HRP were treated with IL-1beta or TNFa, respectively, for 48hrs before cultures were decellularized. Naive HREMC were then plated on decellularized ECM and collected 16hrs later for qRT-PCR. ECM derived from IL-1beta-conditioned HREMC caused 4.2 (p<0.01, 2.2 (p<0.01) and 1.6 (p<0.01) fold-increments in SELE, ICAM and VCAM, respectively, in naive HREMC. ECM deposited from TNFa-conditioned HREMC caused 349, 6.5- and 3.3-fold increments in SELE, ICAM and VCAM, respectively, in naive HREMC. Overall, cytokines caused more potent alterations in ECM expression than conditions designed to simulate hyperglycemia. Interestingly, contrasting expression changes in BM-exposed HREMC suggest that ratios of BM constituents may be profoundly altered in diabetic retinal BM. Finally, decellularization experiments suggest that diabetes-relevant alterations in ECM composition alone are sufficient to alter adhesion molecule expression by HREMC, indicating that BM thickening may drive other pathogenic behaviors in DR.

TNXIP is Required for HFD-induced Retinal Leukostasis, Endothelial Inflammation and Microvascular Lesions through Autocrine Activation of NLRP3 Inflammasome
Mohamed, L.1, El-Remessy, A.2
1College of Pharmacy, California Northstate University, Pharmaceutical and Biomedical Sciences, Elk Grove, United States, 2Charlie Norwood VA Medical Center, Augusta Biomedical Research Corporation, Augusta, United States
Our group has shown that high fat diet (HFD) uniquely triggers expression of retinal thioredoxin interacting protein (TNXIP) and...
that TXNIP is required for activation of NOD-like receptor protease (NLRP3)-inflammasome. Retinas from wild type mice fed with 60% HFD for 8-18 weeks (WT-HFD) showed increased number of adherent leukocytes, barrier dysfunction and degenerated acellular capillary formation compared to WT mice on normal diet (WT-ND), but not in TXNIP knockout (TKO) mice. Here, we dissect the specific contribution of endothelial-to-endothelial link to HFD-induced leukostasis and NLRP3-inflammasome activation. After 8-weeks of HFD, peripheral blood mononuclear cells (PBMCs) isolated from WT-HFD showed increased adhesion to mouse endothelial cells (EC) by 2-fold, compared with PBMCs from WT-ND control group. Whereas, PBMCs isolated from TKO-ND or TKO-HFD showed no significant changes compared with WT-ND group. In parallel, isolated PBMCs from obese nondiabetic human subjects also showed increased adhesion to cultured human retinal ECs by 1.6-fold. Next, TXNIP overexpression (TXNIP+) in human retinal ECs triggered activation of the NLRP3-inflammasome evidenced by the upregulation of NLRP3 and cleaved-IL-1β expression by 2.5- and 2.4-fold, respectively, and a trend of increased cleaved caspase-1 expression compared with cells transduced with empty vector (EV)-controls. These effects coincided with significant increases in TNF-α, ICAM-1 and PECAM-1 expression by 1.85, 1.5 and 1.65-fold, respectively in the TXNIP+ group compared with EV-controls. Treatment with IL-1β receptor antagonist suppresses the effect of TXNIP overexpression on inflammasome activation and EC inflammation. Finally, after 18-weeks of HFD, retinal vascular branching density was decreased by 0.7-fold in WT-HFD compared with WT-ND group. In contrast, TKO mice had comparable vessel branching density to WT-ND controls. Together, our findings highlight the essential role of TXNIP expression on both leukocytes and ECs in activation of NLRP3-inflammasome, leukostasis and inflammation which involves an IL-1β receptor-autocrine fashion. This work establishes the early pre-diabetic impact of HFD-induced obesity on retinal inflammation and microvascular morphological changes independently from frank diabetes. Targeting TXNIP can provide attractive therapeutic modalities against HFD-induced NLRP3-inflammasome activation in both leukocytes and ECs.

**Inflammation and Pericyte Health in Diabetic Retinopathy**

Beleady-Adams, T.1, Sheibani, N., Baucum, A., Dharmara- jan, S., Wang, S.2

1Indiana University-Purdue University Indianapolis, Biological Sciences, Indianapolis, United States, 2University of Wisconsin School of Medicine and Public Health, Ophthalmology and Visual Sciences, Madison, United States

**Purpose:** Diabetic retinopathy is the impairment or loss of vision due to complications in the retinal neurovasculature as a result of diabetes. These complications arise due to the loss of the integrity of the blood retinal barrier. One of the earliest signs of diabetic retinopathy is the loss of pericytes associated with retinal vascularization. Inflammation is proposed to be important in the initiation and progression of diabetic retinopathy; however, the role of inflammation in pericyte loss is not well understood. The goal of these studies was to elucidate the connection between pericyte loss and inflammation, and investigate a role for interferon gamma (IFNγ) in retinal pericyte loss.

**Methods:** Retinal whole-mounts from Ins2Akita+/+ (Akita), Lep db/db (DB) and wild type (WT) control mice aged 3, 6, 12, 18 and 24 weeks were subjected to immunohistochemistry, cellular and vasculature quantitation, enzyme-linked immunosassays, and RT-qPCR. Isolated retinal pericytes were subjected to acute or chronic treatments with IFNγ and platelet-derived growth factor B (PDGFβ) signaling and pericyte health was investigated by western blot and immunocytochemistry.

**Results:** Numbers of pre-retinal pericytes from 3-24 weeks are shown in Table I. No change was detected in the number of retinal microglia; however, small increases in microglial cells in comparison to none-diabetic mice were present at several stages. RT-qPCR analysis revealed inflammatory and glissi markers to be upregulated as early as 3 weeks, with peak inflammation occurring at 12 weeks (Akita) or 18 weeks (DB). DB mice showed a greater upregulation of inflammatory markers than the Akita mice. Protein and mRNA levels of IFNγ were increased in both model systems. Chronic treatment of retinal pericytes with IFNγ led to a decrease in the protein levels of PDGFβR, and were accompanied by decreases in the activation of protein kinase C delta (PKCδ) and AKT. Finally, treatment of retinal pericytes led to an increase in the activated form of protein kinase C delta (PKCδ).

**Conclusion:** We conclude the following: 1) inflammation is present at the same time as pre-retinal pericyte loss occurring in two diabetes models, 2) the peak of mRNA levels was 12 weeks for the Akita mouse, but was shifted to 18 weeks for the DB mouse, 3) increased levels of IFNγ were present in both model systems, 4) treatment of pericytes with IFNγ reduced PDGFβR signaling and increased levels of cleaved activated PKCδ.

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**Detection of Early Microvascular Retinal Changes in Type I Diabetic Mice with OCT-angiography**

Ambati, B.1, Uehara, H.2, Lesuna, T.3

1University of Utah, Salt Lake City, United States, 2University of Utah, Ophthalmology, Salt Lake City, United States

**Purpose:** Detection of early diabetic retinopathy can be very helpful in initiating appropriate treatment. In this study, we sought to determine whether Optical Coherence Tomography Angiography (OCTA) can detect early vascular abnormalities in type 1 diabetic mice. **Methods:** 6-8 month old male type 1 diabetic Ins2 Akita/+ and age matched C57BL/6J mice were used. OCTA was performed by Heidelberg SPECTRALIS OCT Angiography Module with 30-degree lens + mouse adapter lens. After anesthesiaing mice with ketamine/xylazine, 1% tropicamide was applied on eyes to dilate the pupil. Then, Canter-Nissel contact lens (BOZ1 1.70, DIAM 3.20, PWR 0.00) was mounted to avoid corneal drying and obtain clear image. We acquired the OCTA image from the peripheral nasal position. We analyzed vascular volume density from the retinal surface (inner limiting membrane) to 120um depth with 4um steps in order to obtain vascular volume density vs depth (H-N4 each group).

**Results:** Vascular volume density of both mouse strains showed three different peaks. By comparing with the OCT image, the first peak (surface), second peak (intermediate) and third peak (deep) were located in nerve fiber layer/ganglion cell layer, inner plexus layer/inner nuclear layer and outer plexus layer/nuclear layer, respectively. We calculated vascular volume density of these peaks separately. In the first peak, the vascular volume density was 14.3 ± 3.5 % (±S.D, diabetic Ins2 Akita/+) and 14.3 ± 5.9 % (control) (no significant difference). In the second peak, the vascular volume density showed 13.9 ± 5.4 % (diabetic Ins2 Akita+/+) and 17.0 ± 3.8 % (control) (no significant difference). In the third peak, the vascular volume density showed 17.2 ± 2.8 % (diabetic Ins2 Akita+) and 19.0 ± 2.0 % (control) (p< 0.01 by student t-test). Also, total vascular density was 10.7 ± 3.2 % (diabetic Ins2 Akita+/+) and 17.2 ± 2.0 % (control) (p< 0.05).

**Conclusions:** OCTA successfully detected the retinal vascular alteration between type I diabetic mice and control mice. The diabetic mice showed the reduced vascular volume density especially in the deep vessels. Importantly, we detect the difference without retinal blood leakage/hemorrhage/neovascularization. Our analysis (vascular volume density vs retinal depth) could be useful to detect early diabetic retinopathy phenotypes.
Leucine-rich Alpha-2-glycoprotein-1 Disrupts Vessel Maturation in Developmental Retinal Angiogenesis

Hoeh, A.E., O'Connor, M.N., Kallenberg, D., Moss, S.E., Greenwood, J.
Institute of Ophthalmology, London, United Kingdom

Previous work from our laboratory has shown that leucine-rich alpha-2-glycoprotein-1 (LRG1) is not involved in developmental angiogenesis but is induced in disease causing disruption of the normal angiogenic process. Accordingly, LRG1 contributes to dysfunctional blood vessel growth in mouse models of ocular neovascularisation such as oxygen-induced retinopathy and laser-induced choroidal neovascularization. Here, we tested the hypothesis that introducing LRG1 during development of angiogenesis will corrupt the process and drive dysfunctional vessel formation. We examined the effect of exogenously introduced LRG1 during vascularization of the retina, which occurs in the first 3 postnatal weeks in mice. Human recombinant LRG1 was injected intravitreally in C57BL/6 pups on postnatal day 5. Retinal flatmounts were immunostained for endothelial markers, collagen IV and pericyte markers. LRG1 injected eyes showed a slightly larger vascularized area compared to control eyes. The total vessel length and number of vessel junctions were reduced, indicating a lower vessel density in the LRG1 treated retinas. High magnification confocal images of the retinal capillaries were taken of areas close to the disc and at the leading edge of vessel growth to examine vascular maturation which developed before and after the injection of LRG1. A feature of vessel maturation is the tight association with pericytes. Overlap analysis of endothelial (CD31) and pericyte (NG2) immunostaining showed significantly reduced pericyte coverage of the capillaries in both areas in LRG1 treated eyes compared to controls. Likewise, the overlap of the endothelium with collagen IV, a basement membrane marker, was reduced in the LRG1 treated retinas. These results indicate that the introduction of LRG1 during retinal blood vessel development leads to structurally abnormal vessels with reduced pericyte coverage and support the hypothesis that LRG1 is a vascular disrupting factor. This also highlights the therapeutic potential of LRG1 inhibition in the treatment of ocular diseases which are characterized by the growth of structurally and functionally abnormal blood vessels. This work was supported by the Deutsche Forschungsgemeinschaft (DFG), the Wellcome Trust (206413) and by the National Institute for Health Research University College London Hospitals Biomedi Cal Research Centre.

Hyperoxia in ROP in Retinal Vascular Endothelial Cells and in Vivo Models

McDonald, D.M.
Queen’s University Belfast, Belfast, United Kingdom

Retinopathy of Prematurity (ROP) is a serious complication of premature birth and is a major cause of visual loss in young children. In this disease, hyperoxia, used prophylactically to aid survival, causes vascular damage (phase 1) and induces detrimental sight-threatening retinal neovascularization (phase 2) when oxygen is removed. Current treatments that focus on the second phase of the disease are not always effective and have serious and irreversible side-effects. The ideal therapy for ROP would be to target the initial vaso-degenerative stage, in order to preserve endothelial integrity and prevent disease progression. Here we will present our research showing that hyperoxia stunts normal endothelial cell (EC) proliferation which is consistent with the arrest of normal vascular growth observed in vivo. This growth arrest is caused in part by an increase in hyperoxia-induced oxidative and nitrosative stress. Conversely, our research also shows that reducing oxidative stress specifically in endothelial cells (EC) is protective and reduces vascular regression in the oxygen induced retinopathy model of ROP. More recently, we have identified that the expression and activation of the cell growth and differentiation mediator Notch 1 is induced in EC following hyperoxia implying a major role for Notch 1 in modulating EC responsiveness in hyperoxia and ROP. Notch 1 is an important negative regulator of VEGF function and acts to limit blood vessel growth to enhance vascular stability and quiescence. Here our recent findings suggesting a role for Notch 1 in hyperoxic EC will also be discussed.

RCB18 - Role of mitochondrial damage in diabetic retinopathy

Burns, S., Sapoznik, K., Othman, H., Sawides, L., Luo, T., Gast, T., Elsner, A.
Indiana University, Bloomington, United States

Early diabetic retinopathy (DR) is clinically characterized by retinal lesions such as microangiopathy, microvascular abnormalities and increased retinal thickness, typically detected using color fundus photography and OCT. In this study we used an AOSLO, to provide high resolution imaging. Both confocal and multiply scattered light images were collected. Confocal images show local structure and index of refraction changes across the retinal tissue. Multiply scattered red light images reveal details of vessel walls, erythrocytes and leukocytes, as well as neural and glial cells and areas of retinal edema. We imaged 56 diabetic funds and 40 controls using both on super peripheral capillary layer both around the foveal and in a contiguous 3 degree tall temporal strip out to 7 degrees temporal. We also imaged patient specific features, such as areas of ischemia, vascular remodeling, or other regions of interest. The entire AOSLO protocol requires about 30 minutes of patient time. Changes to both the photoreceptors and microvasculature were present. For photoreceptors, the confocal imaging provides high contrast images. Approximately 25% of diabetic (14/56) has localized regions of cones with very low reflectivity. These persisted up to 3 years (the maximum follow up to date) and seem to be associated with outer retinal thickening on OCT and may presage outer retinal ischemic changes. For vascular imaging multiply scattered light images provide the best visualization. The wall to lumen ratio (WLR) in the diabetes was increased relative to normal subjects (p<0.001). There is widespread clinically undetected changes in the vasculature. These include capillary looping, capillary dropout and ghost vessels and thickening of arteriolar walls, especially for small vessels. The branching patterns of the vasculature was also altered in diabetics, and regions of microcystic changes were sometimes related to areas with changes such as capillary looping. The vascular wall and other retinal changes were qualitatively similar to changes seen in diabetics with more severe retinopathy, but not as widespread and both smaller and more localized in nature. The degree of WLR thickening varies across a retina, and was more pronounced in regions with signs of clinically recognized DR, as compared to regions without clinical signs of DR. The local variation in the WLR, with more severely affected regions having a larger WLR may be a metric for local ischemia.

Deciphering Mitochondrial Homeostasis on an Epigenetic Platform

Kowluru, R.
Wayne State University, Detroit, United States

Mitochondrial damage plays a key role in the development of diabetic retinopathy by accelerating apoptosis of capillary cells, which precedes the development of histopathology characteristic of diabetic retinopathy. Mitochondria copy numbers are also decreased, their DNA is damaged and the transcription of mitochondrial DNA-encoded genes is reduced, which compromises the electron transport chain system. Expression of a gene is also regulated by epigenetic modifications, the enzyme-mediated modifications that do not alter the DNA sequence. In diabetic environment, the epigenetic machinery is disturbed, and epigenetic modifications including DNA methylation and histone methylation/ acetylation regulate the expression of many genes implicated in the development of diabetic retinopathy. This presentation will highlight the role of epigenetic modifications in maintaining mitochondrial homeostasis including transcription of mtDNA and stability.
Conclusions: reduced autophagic flux from autophagosomes to lysosomes. But rather to the impairment of mitophagy resulting from mitochondrial biogenesis (since its machinery remained depressed), I.G. retina (after 2- and 8-month disease duration) and in retinal Müller cells. MQC are modified during the progression of diabetes in the retina. Methods: Mitochondrial contents were evaluated in human and mouse retina (Ins2Akita mice) at different stages of diabetes. The dysregulation of MQC was further investigated in the diabetic murine retina (after 8- and 10-month disease duration) and in retinal Müller cells (primary and MIO-M1 cell line) maintained under high glucose (HG = 30 mM) or l-glucose (LG = 30 mM, osmotic control) for 5 days.

Results: Mitochondrial contents were significantly diminished at the early stages of diabetes, as shown in diabetic patients with no clinical signs of retinopathy and Ins2Akita mice after 2 months of hyperglycaemia. At this stage, Ins2Akita retinae exhibited impairment of the mitochondrial biogenesis machinery (decreased levels of nuclear PGC-1α and mitochondrial proteins, as revealed by increased mitosomelye density in Ins2Akita mitophagy reporter mice (mtO2c-lacz)). Cultured primary and MIO-M1 Müller cells maintained under HG or LG conditions displayed increased mitophagy. suggesting that this effect is linked to hyperglycaemia-induced osmotic changes. In contrast, mitochondrial biogenesis was impaired in HG, and enhanced in LG-treated cells, indicating that they could be incorporated in potential complex I markers. The OphNdi vector shows increased efficacy compared to Ndi1 for a comparable quantity of vector, in principle enabling a wide array of neurological disorders. The retina is acutely sensitive to mitochondrial dysfunction due to its high metabolic demand. Several mutations have been identified in subunits of complex I of the electron transport chain that result in retinopathies. Here we investigate the S.cerevisiae gene NDI1, a retinoblastoma insensitive NADH-oxidoreductase, which has shown potential as a therapeutic for complex I-related disorders. We have examined the efficacy of an NDI1-based therapy to provide a potential complex I surrogate and explore if this therapeutic approach can be optimised for greater efficacy.

The NDI1 transgene was codon optimised (OphNDI; GenArt). HEK 293 cells transfected with OphNDI or NDI1 showed a significant increase in levels of RNA and protein after optimisation. The NADH oxidation activity of HEK 293 cells transfected with OphNDI was significantly increased when compared to both endogenous complex I activity (p < .001) and wild type NDI1 activity (p < .001). The basal Oxygen Consumption Rate of cells transfected with OphNDI and NDI1 showed a modest increase when compared to eGFP transfected cells. However, there was a significant increase of the OCR of OphNDI cells post rotenone treatment (complex I inhibitor) compared to NDI1 (p < .001) and eGFP (p < .001). HEK 293 cells transduced with AAV2-2 OphNDI showed a significant increase in the OCR compared to AAV2-2 eGFP (p < .001) and AAV2-2 NDI1 (p < .001). Analysis of HA-tagged OphNDI and mitochondrial dsRed showed very strong correlation indicating that the mitochondrial localisation of OphNDI had not been altered by the optimisation process. OphNDI performs significantly better at protecting against Reactive Oxygen Species (ROS) and altered by the optimisation process. OphNDI performs significantly better at protecting against Reactive Oxygen Species (ROS) and mitochondrial dysfunction. Here we studied the consequences for BRB function in a human diabetic retina (Macular Degeneration type 2) where Müller cells die in a specific region in the retina. Although we found increased retinal vasculature permeability in many locations of the affected region, there were also regions with a seemingly tight retinal vasculature despite a complete absence of Müller cells. This suggests that Müller cells may not be critical for maintaining the BRB in human retina.

Potential Role of VEGF Signaling in Müller Glia: Implication to Neuroprotection in Long-term Anti-VEGF Therapies for Diabetic Retinopathy, Age-related Macular Degeneration, and Other Hypoxic Retinal Vascular Diseases

Le X.1, Fu, S.1,2, Zhu, M.1

1University of Oklahoma Health Sciences Center, Department of Medicine and Harold Hamm Diabetes Center, Oklahoma City, United States; 2The Second Affiliated Hospital of Nanchang University, Ophthalmology, Nanchang, China

To determine the function of vascular endothelial growth factor (VEGF) signaling in Müller glia (MG) in diabetic retinopathy (DR) and hypoxic retinal diseases, such as age-related macular degeneration (AMD) and its potential role in neuroprotection during anti-VEGF therapies, we generated MG-specific VEGF receptor-2 (VEGFR2) knockout mice and characterized this animal model under diabetic/hypoxic conditions. MG-specific VEGFR2 KO mice show significant reduction of retinal MG and retinal neurons under normal conditions, suggesting that VEGF signaling through VEGFR2 is not required for MG viability normally. In STZ-induced diabetic model, the conditional VEGFR2 demonstrated a gradual reduction in Müller glial density, which reaches to a significant level 10 months after the onset of diabetes. This observation is accompanied by an age-dependent decrease of both scotopic and photopic ERG amplitudes, accelerated loss of rod and cone photoreceptors, ganglion cell layers, and inner nuclear layer neurons, and a significant reduction of retinal ganglion cell-line-derived neurotrophic factor (BDNF) and brain-derived neurotrophic factor (BDNF). Similar results were also observed in hypoxic conditional VEGFR2 mice. Mechanistic analysis using primary Müller cells derived from conditional VEGFR2 KO mice and a rat Müller cell line mRC1 suggests that 1) disrupting VEGFR2-mediated Akt survival signal caused the loss of MG and 2) trophic factor VEGF, BDNF, and GDNF played a synergistic role in MG survival under diabetic and hypoxic conditions. Our results suggest that VEGFR2-mediated Akt survival signal is essential to the viability of MG, which in turn, provides neuroprotection through BDNF and GDNF that also supports the survival of MG. As the diabetic/hypoxic MG-specific VEGFR2 KO mice have very thin retina (severe neurodegenerative changes in all retinal layers), which bears striking resemblance to that in a substantial portion (36%) of wet AMD patients treated with anti-VEGF drugs for 5-year
Acute Laser-induced Chorioretinal Damage Elicits Distinct Cytokine/Chemokine Spatiotemporal Profiles in Rats

Uhlès, S., Grüner, S., Mary, I.-L., Revelant, F., Lazendic, M., Zirwes, E., Brechelsen, M., Bieler, T., Ullmer, C., Jayagopal, A., Hoffmann-La Roche, Ltd., Pharma Research and Early Development (pRED), Neuroscience, Ophthalmology and Rare Diseases, Roche Innovation Center Basel, Basel, Switzerland

Retinal degenerative diseases are leading causes of vision loss, and chronic neuroinflammation is a common feature. Microglia are the resident immune cells of the retina that surveil neurovascular networks, sense damage, and respond by activating degenerative or regenerative processes. Depending on the insult they release pro- or anti-inflammatory cytokines, activate neuronal cell death, and phagocytose damaged tissue to promote wound healing and/or wound healing. Learning how to control these responses could have vast therapeutic potential for diverse retinopathies. Therefore, we characterized the molecular profiles of migrating microglia/macrophages after inducing laser-induced chorioretinal damage using laser capture microdissection (LCM).

Laser-induced chorioretinal lesions were induced in eyes of 10 week old mice using C57/BL6 mice. Thirty mice were treated with 700 µW laser to induce lesions and immunostained for Iba1 and IB4, and processed for LCM or qPCR. Laser damage elicits an immediate and transient increase in injury-induced immune cell activation markers at the lesion, restored microglia homoeostasis 14d post-injury. Understanding the temporal microglia response to chorioretinal damage and identifying key genes controlling them, could inform future therapies for diverse retinal diseases.

The Role of Retinal Microglia during Formation of Retinal Neovascularisation in the OIR Mouse Model

Böck, M.1, Hagemeyer, N.2, Viehwerth, P.2,1, Schlecht, A.1, Zhang, R.1, Boneva, S.1, Lai, Y.1, Thiene, A.1, Stahl, A.1, Schluck, G.1, Agostini, H.1, Prinz, M.3, Lange, C.3

1 Eye Center, Medical Center, University of Freiburg, Freiburg, Germany, Institute of Neuroanatomy, Medical Faculty, University of Freiburg, Freiburg, Germany, Institute of Anatomy, Leipzig University, Leipzig, Germany

Purpose: Myeloid cells such as resident retinal microglia (MG) or infiltrating blood-derived macrophages (Mφ) accumulate at sites of retinal neovascularisation (RNV). The relative distribution of MG and Mφ as well as their distinct function during the development of RNV, however, remain unclear. The aim of this study is to determine the differential distribution of MG and Mφ at sites of RNV and to assess the transcriptional profile of retinal MG using specific transgene mice in the oxygen-induced retinopathy (OIR) mouse model.

Methods: Conditional OcX3cr1YFP::Rosa26-TomYFP reporter mice were injected with Tamoxifen at postnatal day 1 (P1), thereby inducing the expression of the fluorescent protein Tomato specifically in microglial cells. Pups and their dam were kept in 75% oxygen from P7 to P12 and returned to normoxic conditions specifically in myeloid cells. Pups and their dam were kept in 75% oxygen from P7 to P12 and returned to normoxic conditions at 28h to 5d after trauma. After this time, microglia underwent a specific labelling of retinal MG from P12 onwards. Due to steady cell turnover, the tomato signal is lost in peripheral blood monocytes within 12 days after injection. This allows specific differentiation of MG and Mφ at sites of RNV. Immunofluorescence and flow cytometry analysis indicate that MG are the predominant microglial cell population (Mean ± SD = 93.8 ± 4.0%) while Mφ appear rarely at sites of RNV (Mean ± SD = 6.2 ± 4.0%).

Conclusion: Our data suggest that resident MG constitute the predominant immune cell population at sites of RNV in ischemic retinal disease of mice while blood-derived Mφ play a minor role. The transcriptional analysis suggests that MG proliferate during ischemic retinal disease and may modulate RNV by expressing cytokines such as the anti-angiogenic interferon beta.
ORAL PRESENTATIONS

Epidemiology of Eye Disease & Global Eye Health

EED2 - Global perspectives on AMD and diabetic retinopathy

**Global Perspectives on AMD and Diabetic Retinopathy**

Lengelly, L.1, Eye Risk Consortium, E.-R.2, QDITF Consortium, Q.1, Peto, T.1, Eye-Risk Consortium

1Queen’s University Belfast / Wellcome-Wolfson Institute for Experimental Medicine, Belfast, United Kingdom, 2Hot2020 grant, Rotterdam and Tuebingen, Netherlands, 3London School of Hygiene and Tropical Medicine, London, United Kingdom, 4Moorfields Eye Hospital, Ophthalmic Image Analysis unit, London, United Kingdom

Diabetes mellitus (DM) is one of the major non-communicable chronic diseases and according to the IAPB Visual Atlas 2017, the fastest growing cause of preventable/treatable vision loss. World Health Organization data show that the total number of people with diabetes will double to 366 million by 2030, this increase will be particularly dramatic in the developing world. Diabetic retinopathy (DR) is no longer the leading cause of visual loss in people in the working age-group in Iceland, England and Wales, where systematic screening and treatment programmes have been in place for several years. Using such a model as an example, we will discuss the impact of such programmes will have around the World. The number of sight threatening DR cases (including severe non-proliferative DR, proliferative DR (PDR) and diabetic macular edema (DME)) will rise substantially and will have a major impact on health spending as well. With DR being an independent risk factor for other DM related complications, ophthalmology is at the forefront of being able to have a major impact worldwide on reducing not only blindness rates but helping to reduce other DM related complications as well. Recent studies show, such as the International Diabetes Federation’s Barometer Project, that The hallmark of Age Related Macular Degeneration (AMO) is drusen, and yellowish deposit at usually just exterior to the retinal pigment epithelium. In most developed countries AMD is the leading cause of blindness in the older age-group, with longer life-expectancy overall, studies are now confirming that AMD is becoming an issue in the developing world as well. Both DM/DR and AMD can be addressed using lifestyle factors and these might be extremely powerful on population level if used correctly. We will be exploring such factors, smoking, diet, genetic background and environmental interactions to help with reducing the burden of DR/AMD in the communities as treatment for these diseases is expensive and time-consuming so prevention must be the key.

Dietary Patterns and Risk of Age Related Macular Degeneration

Woodside, J.

Queens University Belfast, Centre for Public Health, Belfast, United Kingdom

It has been known for some time that dietary factors can modulate AMD risk. Epidemiological studies have demonstrated that diets high in antioxidant nutrients (vitamins C and E, carotenoids such as lutein and zeaxanthin and fruit and vegetables rich in these nutrients) or zinc are associated with a decreased occurrence of AMD. Studies have also shown that a high dietary intake of trans fats is a risk factor for late AMD, while a higher intake of fish or olive oil is protective against AMD. However the evidence from clinical trials is less consistent. Most studies to date have focused on individual food groups or nutrients, yet in ‘real life’ settings individuals eat a combination of foods within a mixed diet rather than isolated foods and nutrients and it is known that diet is a multifactorial lifestyle behaviour, with particular foods frequently consumed together, depending on the cultural, geographical and economic context of the individual. Therefore nutrition researchers are increasingly attempting to analyse relationships between dietary patterns or overall diet and disease, rather than specific foods or nutrients thus permitting the synergistic effects of food intake to be examined in relation to risk of disease. Dietary patterns rather than individual components are also increasingly being studied in relation to AMD, and will be examined.

Racial Differences in Age-related Macular Degeneration and in the Use of Anti-VEGF Agents to Treat Age-related Macular Degeneration among U.S. Medicare Beneficiaries

Frie, L., Mahr, M.

Mayo Clinic, Ophthalmology, Rochester, United States

Our purpose was to determine racial differences in age-related macular degeneration (AMD) and in the use of anti-vascular endothelial growth factor (anti-VEGF) in the treatment of AMD among U.S. Medicare beneficiaries. We performed a cross-sectional Medicare database study by utilizing the 2014 Medicare 5% Limited Dataset Standard Analytic Files which represents a 5% sample of approximately 28,238,660 fee-for-service Medicare beneficiaries ≥ 65 years of age. We identified all beneficiaries who received a diagnosis of AMD and who received intravitreal anti-VEGF injections, stratified by race, gender, and age. Logistic regression analysis was used to determine racial differences in the likelihood of an AMD diagnosis and anti-VEGF treatment for AMD, adjusted for age and gender. Among 28,238,660 Medicare beneficiaries in 2014, 2,210,000 (7.8%) were diagnosed with AMD. Approximately 360,640 (1.3%) beneficiaries received one or more anti-VEGF intravitreal injections in the treatment of AMD. After adjustment for age and gender, an AMD diagnosis was 74% less likely in African Americans (OR 0.26, 95% Confidence Interval [CI] : 0.25, 0.27), 44% less likely in Latinos (OR 0.56, CI: 0.53, 0.60), and 39% less likely in Asian Americans (OR 0.81, CI: 0.77, 0.85) compared to whites. Anti-VEGF injections for AMD were 86% less likely in African Americans (OR 0.14, CI: 0.12, 0.16), 61% less likely in Latinos (OR 0.39, CI: 0.33, 0.45), and 48% less likely in Asian Americans (OR 0.52, CI: 0.46, 0.60) when compared to whites. The odds of an AMD diagnosis and the use of anti-VEGF agents to treat AMD increased with age (P< 0.001). In conclusion we found racial differences in the prevalence of an AMD diagnosis and in receiving anti-VEGF injections for AMD among fee-for-service Medicare beneficiaries ≥ 65 years of age. African Americans, Latinos, and Asian Americans were 19%–74% less likely to have an AMD diagnosis and 48%–86% less likely to receive anti-VEGF intravitreal injections for AMD relative to whites.

EED4 - Myopia: Seeing the blackboard: approaches to the problem of childhood myopia in low and middle-income countries

Morgan, I.

Australian National University, Canberra, Australia

Myopia is aetiological heterogeneous, and includes many rare genetic forms, which affect in total only a few percent of the population, and school myopia, which appears in association with education. It is this form which has increased dramatically in East and Southeast Asia over the last 60 years. The prevalence of high, and potentially pathological myopia has also increased, raising the specter of increased levels of visual impairment and blindness, and thus making prevention of myopia onset and progression towards high myopia essential. Based on epidemiological evidence that children who spend more time outdoors are less likely to become myopic, clinical trials have shown that increasing time outdoors in schools leads to reduced incident myopia. This approach is now implemented nationally in Taiwan, with initial positive results. However, this approach faces considerable barriers, given the wide-spread emphasis on high educational achievements, particularly in East Asia, and work is proceeding on bright classrooms and bright study lamps as possible alternatives more compatible with study. This approach depends on the ability of higher light outdoors to induce greater release of retinal dopamine, which slows axial elongation. This mechanism has strong support from animal studies. Whether time outdoors also slows myopia progression is currently controversial, but a recent trial, combined with strong seasonal effects on progression, suggest that this may be the case. It is likely that students who become myopic despite preventive measures will also need approaches more specifically designed to target progression. A wide range of these are available clinically, ranging from atropine eye-drops to myopia prevention spectacles and contact lenses. Low dose atropine eye-drops (0.01-0.05%) are as effective, if not more effective than the higher doses of atropine traditionally used. In this area it is surprising that dopaminergic drugs have not been used, given the role of dopamine appears to play. Other possibilities are being explored. Orthokeratology, which involves reshaping of the cornea is now widely used. There are however concerns about the long-term safety of these rather invasive treatments, and attempts are currently underway to develop myopia prevention spectacles which would be the least invasive option. In a broader context, reforms to highly competitive school systems which impose high study burdens from an early age will need to be considered.

Effect of Providing Near Glasses on Productivity Among Presbyopic Rural Indian Tea Workers: The PROSPER (PRODucTivity Study of Presbyopia Elimination in Rural-dwellers) Randomized Controlled Trial

Congdon, N.1, Reddy, P.2, Mackenzie, G.1, Gogate, P.2, Wen, Q.1

1Queen’s University Belfast, CPH, Lisburn, United Kingdom, 2Avaind Eye Hospital, Madurai, India, 3Riemann Limited, London, United Kingdom, 4Community Eye Care Foundation, Pune, India, 5Queen’s University Belfast, Belfast, United Kingdom

Background: Presbyopia, age-related decline in near vision, is the commonest cause of vision impairment globally, but no trials have assessed workplace effects. We studied impact of glasses on productivity of presbyopic tea-workers. Methods: Tea-pickers aged 240 years in Assam, India, were examined near visual acuity (NVA)≥ 6/12 in both eyes, correctable to 6/65-7/75 with near glasses; unaided distance vision 26/7; and no eye disease were randomised (1:1, stratified by age, sex, productivity) to receive free glasses optimising NVA at working distance (cost including delivery: US$10.20/person), either immediately or after 4 weeks. Randomised Controlled Trial (RCT) investigator-masked, intention to treat) was difference between groups in change in mean daily weight of tea picked (productivity), between 4-week baseline and 11-week evaluation period (cost including delivery: US$10.20/person), either immediately (Intervention) or at closeout (Control). Main study outcome (investigator-masked, intention to treat) was difference between groups in mean daily weight of tea picked (productivity), between 4-week baseline and 11-week evaluation period. Glasses compliance was assessed at 7 un-announced visits. Results: Among 2,699 permanent workers, 1,297 (48·1%) met vision criteria and were randomised to intervention (n=737, 50·1%, mean age 47·2 [range 40-59] ye-
The study was funded by Lions Clubs International Foundation. We also thank Mr. Zethembe Mseleku, Mr. Steven Maviswa and Miss Vimba Chibango for their input in the study.

**Barriers to Glaucoma Care in Low Resource Areas**

Olawoye, O., African Glaucoma Consortium

Compared to Caucasians, the prevalence of primary open-angle glaucoma and glaucoma blindness has been shown to be four to eight times higher in persons of African descent, whether in Africa,3,4 North America or the Caribbean.5 Compared to other populations, glaucoma in Africans is associated with a higher IOP, occurs 10-15 years earlier,6,7 and has a more rapid progression and severe presentation,8 with up to 50% of patients already blind in one eye at diagnosis.9 For this reason, glaucoma is a leading cause of blindness in sub-Saharan Africa, (SSA) and the proportion of blindness attributable to glaucoma is steadily rising as the population ages and causes such as cataract are addressed. During this presentation the major barriers to care in low-resource settings of Sub-Saharan Africa will be highlighted.

**Managing Glaucoma in Low Resource Areas: Role of Selective Laser Trabeculoplasty**

Sponrol, W.

WESMDPA, San Antonio, United States

Ninety percent of the world’s 285 million visually impaired individuals live in developing countries. Preventable causes account for 80% of these. Just under 80 million currently have glaucoma, 11 million of whom have been permanently blinded. The highest prevalence is found among individuals of African heritage, and Hispanic individuals are also at very high risk. The glaucoma problem among Africans is exacerbated by several key factors: earlier and more rapidly progressive disease, a much higher prevalence, and the lack of available healthcare resources, including unaffordable glaucoma medications that are inadequately distributed. Meta-analyses of multiple studies carried out among various populations around the world indicate that selective laser trabeculoplasty (SLT) is comparable in its IOP reducing efficacy with both standard medical therapy andargon laser trabeculoplasty. Groundbreaking work in the Caribbean nation of St. Lucia by Reali indicated that the response to SLT among its predominantly black population produced IOP reductions of substantially greater magnitude than were typically observed elsewhere. These initial findings were reaffirmed in a subsequent major study that included a novel staged design for eliminating any bias produced by regression to the mean. A study comparing ethnic subpopulations of comparable socioeconomic receiving identical care in Durban, South Africa demonstrated that the black population of that city demonstrated significantly greater IOP reduction than members of its sizable Indian population. The IOP reductions among black subjects in St. Lucia and Durban were often of a magnitude comparable to those expected with successful filtering surgery. The ability to carry out a minimally invasive non-invasive procedure that might preclude the need for costly medication in a high proportion of African glaucoma sufferers is of monumental importance. The skills required and the risks incurred in performing SLT are minuscule in comparison to incisional antimitabolite filtration or tube shunt procedures. There is a clear need to embrace this technology and make it as widely available as possible throughout Africa, the Caribbean and urban centers throughout the world.

**The African Glaucoma Consortium - A Model for Regional Problem-solving**

Reali, T.

West Virginia University, Ophthalmology, Morgantown, United States

Glaucoma is a leading cause of irreversible blindness in Africa. Both the prevalence of glaucoma and the incidence of glaucoma-related vision loss are higher in Africa than in other global regions. High rates of vision loss are attributable in large part to obstacles to care. These include lack of awareness of glaucoma, lack of screening, scarcity of trained medical professionals to diagnose and treat the disease, limited access to therapies, therapeutic non-adherence, and costs associated with care. A comprehensive strategy to reduce glaucoma blindness in Africa will require addressing all of these issues and others. The African Glaucoma Consortium is a strategic alliance of stakeholders from in and out of Africa devoted to identifying and solving the problems that perpetuate high glaucoma blindness rates. The Consortium comprises ophthalmologists, scientists, representatives of government and non-government organizations, and partners in pharmaceutical and medical device manufacturing companies. These united stakeholders are committed to developing an integrated network of glaucoma centers of excellence throughout Africa to provide high quality clinical care, conduct impactful community-based research to identify optimal regional disease management, and to train existing and future providers to upskill and expand capacity. In this presentation, we will describe the current activities of the African Glaucoma Consortium and outline future activities focused on preserving sight in Africans with glaucoma.
ORAL PRESENTATIONS

Epidemiology of Eye Disease & Global Eye Health

EED6 - Big data & image analysis

Reading Centres and Telemedicine in Ophthalmology

Balaskas, K.
Moorfields Eye Hospital NHS Foundation Trust, London, United Kingdom

The role of Ophthalmic Reading Centres is evolving in light of progress in technology that enables tele-ophtalmology services. Harnessing the potential of web-based and cloud-based review platforms the review process for imaging-based clinics in Ophthalmology can be centralised in Ophthalmic Reading Centres creating economies of scale and increasing capacity. The role of the Reading Centre thus becomes multi-faceted in that it needs to be involved in:

- Training and accreditation of allied-health professionals involved in delivering tele-ophtalmology services, both in terms of image acquisition and the image review and management decision process.
- Establishing, testing and validating tele-ophtalmology platforms to facilitate the virtual review process, including cloud-based technology.
- Lead research that aims at validating tele-ophtalmology models of service delivery.
- Contribute to the development, testing and validation of Artificial Intelligence management decision support systems in the context of tele-ophtalmology services.

Medical Image Processing and Analysis - An Overview

Aslam, T.
Manchester Royal Eye Hospital, Manchester, United Kingdom

Developments in imaging technologies have allowed visualisation of ever greater levels of detail of retinal anatomy and pathology such as through increasing resolution of optical coherence tomography (OCT) scans. Newer aspects of imaging are also increasing in importance such as enhanced depth imaging and wide-field imaging and newer modalities of imaging have emerged such as OCT angiography, constantly adding to the range and utility of multimodal imaging in patient care.

As imaging technologies rapidly develop, so too do computing power and capability, and there is incredible potential in combining all these technologies to advance medical practice. Indeed, image processing, analysis and computer vision techniques are increasing in prominence in all fields of medical science but especially in modern ophthalmology, with its dependence on visually oriented signs and imaging. This presentation will discuss the principles behind image analysis, exploring and explaining basic principles and potential of processing, analysis and machine vision, leading to discussions of current artificial intelligence techniques and beyond.

Artificial Intelligence in Ophthalmology - The Moorfields-DeepMind Collaboration

Keane, P.
Moorfields Eye Hospital NHS Foundation Trust, London, United Kingdom

Ophthalmology is among the most technology-driven of the all the medical specialties, with treatments utilizing high-spec medical lasers and advanced microsurgical techniques, and diagnostics involving ultra-high resolution imaging. Ophthalmology is also at the forefront of many trailblazing research areas in healthcare, such as stem cell and gene therapies. Moorfields Eye Hospital in London is the oldest eye hospital in the world. Every year, >700,000 patients attend Moorfields - more than double the number of the largest eye hospitals in North America. Together with the adjacent UCL Institute of Ophthalmology, Moorfields is among the largest centres for vision science research in the world. In July 2016, Moorfields announced a formal collaboration and data sharing agreement with DeepMind Health. This collaboration involves the sharing of >1,000,000 anonymised retinal scans with DeepMind to allow for the automated diagnosis of diseases such as age-related macular degeneration (AMD) and diabetic retinopathy (DR).

In my presentation, I will describe the motivation - and urgent need - to apply deep learning to ophthalmology, the processes required to establish a research collaboration between the NHS and a company like DeepMind, the goals of our research, and finally, why I believe that ophthalmology could be first branch of medicine to be fundamentally reinvented through the application of deep learning.

Implementation of Virtual Clinics for Medical Retina Patients in a Tertiary Eye Care Referral Centre

Kortuem, K.1,2, Balaskas, K.2, Rajendram, R.2, Hamilton, R.2, Keane, P.A.2, Sim, D.2
1University Eye Hospital Munich, Munich, Germany, *Moorfields Eye Hospital, London, United Kingdom

Background: The increasing incidence of medical retinal diseases has created capacity issues across the United Kingdom. In this study, we describe the implementation and outcomes of virtual medical retina clinics (VMRC) at Moorfields Eye Hospital, South Division, London. It represents a promising solution to ensure that patients are seen and treated in a timely fashion.

Methods: First attendances in the VMRC (September 2016 to May 2017) were included. It was open to non-urgent external referrals and to existing patients in a face-to-face clinic (F2FC). All patients received visual acuity testing, dilated fundus photography and OCT scans. Grading was performed by consultants, fellows and allied health care professionals. Outcomes of these virtual consultations and reasons for F2FC referrals were assessed.

Results: A total number of 1729 patients were included (1543 were internal and 186 external referrals). The majority were diagnosed with diabetic retinopathy (75.1% of internal and 46.8% of external referrals). Of the internal referrals, 14.6% were discharged, 54.5% continued in VMRC, and 30.9% were brought to a F2FC. Of the external referrals, 45.5% were discharged, 37.1% continued in VMRC and 17.4% were brought to a F2FC. The main reason for F2FC referrals was image quality (34.7%).

Conclusion: VMRC can be implemented successfully utilising existing resources within a hospital eye service. It may also serve as a first-line rapid-access clinic for low-risk referrals. This would enable medical retinal services to cope with increasing demand and efficiently allocate resources to those who require treatment.
Conjunctival melanoma, a rare ocular tumor, is associated with a high recurrence rate and a significant mortality with lymphatic metastatic spread. Recent evidence suggest that conjunctival and cutaneous melanoma partially share similar molecular genomic alterations including mutation burden, mutation spectrum and driver mutations, as well as copy number alterations. We will review the efforts undertaken in understanding the genomic landscape of conjunctival melanoma and its impact on molecular pathways.

Emerging Insights on Ocular Adnexal Lymphoma

Sullivan, T.1,2

1University of Queensland, Ophthalmology, Brisbane, Australia, 2Royal Brisbane and Women’s Hospital, Ophthalmology, Brisbane, Australia

Ocular melanoma includes intraocular (uveal) melanoma and conjunctival melanoma, both rare malignancies. Although they affect the same organ, these two conditions differ significantly clinically and biologically, and hence, there are some differences in their treatment, particularly with respect to metastatic disease. Uveal melanoma (UM) is the most common ocular melanoma, with an incidence of 6-7/100,000/year. Although treatment of the 1980s, resulting in a 5-year survival rate of only 25% for metastatic disease.

Conjunctival melanoma, on the other hand, is a rare malignancy, with an incidence of 0.2-3.8 million individuals/year in the Caucasian population, with only rare cases being reported in the non-Caucasian races. Whilst they appear histologically similar to UM, conjunctival melanomas do not have GNAQ/GNA11 mutations, as well as copy number alterations. We will review recent findings in conjunctival melanoma, including the development of targeted therapies.

Conjunctival melanoma is treated using a combination of surgery, cryotherapy and/or radiation therapy. Metastasis occurs via the lymphatics to lymph nodes. In the case of BRAF mutant conjunctival melanoma, disseminated conjunctival melanoma can be treated with BRAF inhibitors. New therapies are being trialed.

Emulating the Role of Oncogenic Cysteinyl Leukotriene Receptor 2 in Uveal Melanoma Using CRISPR/Cas9 Genome Editing

Slater, K.1, Garcia-Fernandez, J.1, Kennedy, B.1

1University College Dublin, Conway Institute of Biomolecular & Biomedical Science, Dublin, Ireland, 2Genomics Medicine Ireland Limited, Dublin, Ireland

Introduction: Uveal melanoma is a form of eye cancer that arises from melanocytes within the uveal tract. Approximately 50% of uveal melanoma patients develop metastases. The prognosis for metastatic patients is poor: 8% survive beyond 2 years. Identification of oncogenic mutations that drive uveal melanoma pathogenesis has furthered the understanding of the genetic basis of the disease. However, the lack of successful therapies or a proven standard-of-care treatment has evolved with low dose (2 x 2 Gy) and improved radiotherapy delivery, immunotherapy and targeted therapies, as well as addressing predisposing factors playing a role in addition to traditional chemotherapy and radiotherapy regimens.


Ocular Pharmacology, Therapeutics & Drug Delivery

Updated on the Genomics of Uveal Melanoma

Klicic E, ROMS

Erasmus MC, Rotterdam, Netherlands

Activating hotspot mutations in GNAQ/GNA11 occur in 83-95% of all uveal melanoma (UM) and have been identified as early events in tumorigenesis without any prognostic association. Hemizygous mutations in BAP1 were found in most of the non-Caucasian 3, UM, resulting in inactivation of BAP1 protein and loss of expression. Overall an aberrant BAP1 is associated with metastatic disease within five years after diagnosis. SF3B1 mutations are correlated with late metastasis with a median of approximately 8.2 years. EIF1AX mutations are associated with favourable prognostic features and prolonged survival.

In general, BAP1 negative UM are characterized by monosome 3 (95% of cases) and gain of the entire long (q) arm of chromosome 8 (80%). Gain of chromosome 8q is often accompanied by loss of chromosome 8p (85%) and 8q (73%), and loss of chromosomes 6q (52%) and 11q (45%), respectively. EIF1AX mutated UM show gain of chromosome 6q in 65% of the cases. The highest percentages of aneuploidy are found in BAP1 negative UM (11.8%) followed by SF3B1 mutated UM (9.1%). EIF1AX mutated UM harboured the least percentage of aneuploidy (1.7%). In SF3B1 mutated UM most chromosomal structural variants are found.

SF3B1 mutation was associated with lower expression of oncogenic driver genes, such as BAP1, and a poorer overall survival.
**ORAL PRESENTATIONS**

**Ocular Pharmacology, Therapeutics & Drug Delivery**

**Analyzing the Function of BAP1 in Uveal Melanoma**

Turunen, J.

Helsinki University Hospital, Ophthalmology, Helsinki, Finland

Mutations in the BRCA1 associated protein 1 (BAP1) gene cause the BAP1 tumour predisposition syndrome (BAP1-TDPS) with an increased risk of cancers, especially uveal melanoma (UM), mesothelioma, cutaneous melanoma and renal cell carcinoma. UM is cancer most commonly reported in patients with BAP1-TDPS (31%). The BAP1 is localized on chromosome 3p21.1, and loss of this chromosome (monosomy 3) in UM is associated with risk of metastasis. Loss of nuclear localization of BAP1 in tumour tissue also is associated with adverse outcome. BAP1 is a nuclear-localized deubiquitinating hydroxylase. While BAP1 has been categorized as a tumour suppressor, it is not known if a single function of BAP1, or multiple mechanisms, are responsible for this action. BAP1 has multiple proposed functions, including involvement in DNA damage response, transcriptional activation, chromatin remodelling, and cell cycle regulation. It seems that both nuclear localization and deubiquitinating activity are necessary for BAP1 to act as a tumour suppressor. In addition, BAP1 also appears to have functions in the cytoplasm. Currently, it is not exactly known how a loss of BAP1 leads to tumorigenesis of UM, but active research is ongoing to clarify this.

**The Immune Microenvironment in Ocular Melanoma**

Jager, M.1, Cao, J.

Leiden University Medical Center, Ophthalmology, Leiden, Netherlands

In general, inflammation is considered a bad prognostic sign in uveal melanoma. This is probably due to the presence of many pro-angiogenic macrophages in the tissues. High-risk uveal melanoma contain an inflammatory phenotype, which is associated with bad survival, and is determined by the tumor’s mutations and chromosome constitution. However, the expression of immunologically relevant molecules may not only affect the natural behavior of the tumor, but also therapy. This is because the field of oncology has changed in recent years, thanks to new insights into the way the immune system is regulated. Immune checkpoint inhibitors are now applied in many malignancies. Immune checkpoint inhibitors are monoclonal antibodies that target multiple mechanisms, are responsible for this action. BAP1 has multiple proposed functions, including involvement in DNA damage response, transcriptional activation, chromatin remodelling, and cell cycle regulation. It seems that both nuclear localization and deubiquitinating activity are necessary for BAP1 to act as a tumour suppressor. In addition, BAP1 also appears to have functions in the cytoplasm. Currently, it is not exactly known how a loss of BAP1 leads to tumorigenesis of UM, but active research is ongoing to clarify this.

**Toll-like Receptors, Melanocytes and Ocular Melanoma**

Madjian, M.C.1,2, Conway, R.M.2, McCluskey, P.J.2, Cleon- ca, A.V.1,3

1UNSW, Optometry and Vision Science, Sydney, Australia, 2University of Sydney, Save Sight Institute, Sydney, Australia, 3Australian National University, John Curtin School of Medical Research, Canberra, Australia

Toll-like receptors (TLRs) are a family of highly conserved pattern recognition receptors that sense microbial components/antigens (pathogen-associated molecular patterns (PAMPs)), activating innate immune responses and inducing pro-inflammatory cytokines and chemokines. TLRs can also be activated by endogenous proteins (danger associated molecular patterns (DAMPs)) that may be released associated with tissue damage, for example, extracellular matrix (ECM) proteins. TLRs are widely distributed in human eye tissues, on immune and non-immune cells, including iris pigment epithelium, choroidal and retinal vascular endothelium, retinal pigmented epithelium and most recently, choroidal melanocytes. The expression patterns and possible functions of TLRs in primary uveal melanoma remains to be fully explored. Primary human choroidal melanocytes (HCMs) and human uveal melanoma cell lines (92.1, Meli02, OCM1) were studied for expression of TLR1-10 and MYD88 genes (RT-PCR). We also examined for expression of TLR proteins (if antibodies available) using fluorescence immunocytochemistry and confocal microscopy. HCMs and melanoma cells were stimulated with synthetic TLR agonists (Pam3CSK4 (TLR2)/2) or LPS (TLR4), Flagellin (TLR5), FL-S1 (TLR6), Imiquimod (TLR7), sRNA40 (TLR8), and cell morphology, viability and production of pro-inflammatory cytokines (IL-8 and MCP-1/CCL2 ELISA) were assessed. We also immunolabelled paraffin sections of control and primary melanoma human eyes for TLR1 to 6. TLR gene expression patterns varied between HCMs and uveal melanoma cell lines, although all showed MYD88 gene expression. TLR agonists induced IL-8 and MCP-1 production when HCMs were stimulated with the agonists Pam3CSK4 (TLR2)/2 or LPS (TLR4), Flagellin (TLR5), and FL-S1 (TLR6). However for melanoma cell lines, MCP-1 was predominantly produced following Pam3CSK4 (TLR2)/2, Poly I:C (TLR3) and Flagellin (TLR5) stimulation. Strong immunolabelling of primary melanomas was found for TLR1, 2, 4 and 6, and more so in tumours with mixed/epithelioid morphology. The expression of TLR family members in uveal melanoma and a germline mutation in MBD4, which led to many genetic aberrations and therefore probably many new peptides. We investigated whether these molecules are being expressed on conjunctival melanoma. What activates these receptors, including endogenous proteins, remains to be discovered.

**Arginase I Promotes Retinal Neurovascular Protection from Ischemia through Suppression of Macrophage Inflammatory Responses**

Fouda, A.Y., Xu, Z.1, Shosha, E., Lemptali, T., Toque, H.A.2, Rodriguez, P.C.3, Smith, S.B.1, Narayanay, S.P.1, Caldwell, R.B.1, Caldwell, R.W.1

1Medical College of Georgia, Augusta University, Vascular Biology Center, Augusta, United States, 2Medical College of Georgia, Augusta University, Department of Pharmacology, Augusta, United States, 3Moffet Cancer Center, Tampa, United States, 3Medical College of Georgia, Augusta University, Cellular Biology & Anatomy, Augusta, United States

Arginase 1 promotes retinal neurovascular protection from ischemia/reperfusion (IR) injury. Arginase 1, competitively inhibits nitric oxide synthase (NOS) for L-arginine (L-arg) and nitric oxide (NO) formation by NOS leading to vascular dysfunction when endothelial NO is involved, but prevents inflammation when inducible NOS is involved. Studies were performed using wild type (WT) mice, global A1−/− knock out (KO), endothelial specific A1 KO, and myeloid specific A1 KO mice subjected to retinal IR injury. Global as well as myeloid specific A1 KO mice showed worsened IR-induced neuronal and retinal thinning. Deletion of A1 in endothelial cells had no effect, while treatment with pegylated (peg) A1 improved neuronal survival in WT mice. In addition, A1−/− KO mice showed worsened vascular injury manifested by increased acellular capillaries. Western blot analysis revealed reduced inflammatory markers with A1 deletion. In vivo experiments showed that macrophages lacking A1 exhibit increased inflammatory response and decreased the LPS-induced microvascular reprogramming. Moreover, intravitreal injection of A1 macrophages or systemic macrophage depletion with clodronate liposomes increased neuronal loss after IR injury. These results demonstrate that A1 reduces IR-induced retinal neurovascular degeneration via down-regulating macrophage inflammatory responses. Increasing A1 is a novel strategy for limiting neurovascular injury and promoting macrophage-mediated repair.

**OPT3 - New candidate drugs and targets for angiogenesis**

Semaphorins as Angiogenic Modulators in the Retina

Stahl, A.

Eye Center, University Medical Center Freiburg, Freiburg, Germany

Semaphorins are known modulators of axonal sprouting and angiogenesis. In the retina, we identified a distinct expression pattern of Semaphorin 3F in the outer layers. Interestingly, the photoreceptor layers are physiologically avascular. Using two different functional in vitro models, we found potent anti-angiogenic effects of Sema 3F on both retinal and choroidal vessels. This was confirmed in two different in vivo mouse models (Vitr knockout and Laser-induced CVD) in which we found protective effects of Sema 3F against the formation of pathologic neovascularization in the outer retina. In addition, human RPE isolates from patients with exudative AMD showed reduced Semaphorin 3F expression in 10 out of 15 patients, confirming a potential role of Sema 3F in maintaining outer retinal avascularity. Looking beyond the retina, we also studied Semaphorin 3F expression in the cornea. Similar to the outer retina, the cornea is physiologically avascular thus posing a similarly intriguing target for Sema 3F modulation. Harnessing Sema 3F for therapeutic purposes may protect the outer retina against subreti nal neovascularization and limit pathologic corneal vascularization.

**Solute Epoxydase Hydrolyase as a Target for Anti-angiogenic Therapies**

Corson, T.

Indiana University School of Medicine, Indianapolis, United States

The blockade of pathological angiogenesis is key to treating neo vascular eye diseases, which include proliferative diabetic retinopathy, wet age-related macular degeneration, retinopathy of prematurity, and others. Anti-vascular endothelial growth factor (VEGF) agents have been successful in this context, but side effects and non-responders limit their universal adoption. Thus, there is a strong need to find new targets for antiangiogenic therapy development. We have pursued chemical proteomics approaches to find protein targets of novel antiangiogenic compounds. One po
test this hypothesis we undertook PK studies in rabbit eyes. To measure injection compared to anti-VEGF agents currently in clinical use. To investigate the effect of opticin, we hypothesised that opticin binds to vitreous collagen fibrils, we hypothesised that opticin was a specific anti-angiogenic lead compound that we developed in cell-based assays. Of 70 compounds tested, >10 compounds showed robust anti-angiogenic activity. Efficacy was determined in a mouse choroidal sprouting assay. cysteinyl leukotriene receptor antagonist with anti-angiogenic activity as a novel regulator of ocular angiogenesis. Here, we characterise the features which confer anti-angiogenic activity. All experiments were carried out under institutional ethical exemptions. Potent, selective SRPK1 inhibitors reduced angiogenic VEGF levels, with permeabilities ex vivo up to 4.25 x 10^-6 cm/s (compared with 0.35 x 10^-6 cm/s for pazopanib). Multiple regression analysis gene expression data using gene signatures which correlated with antiangiogenic activity and in vivo retinal PK. SRPK1 inhibitors were equally distributed across the retina and detected in the vitreous at 4 h at multiple orders of magnitude higher concentrations than pazopanib. Lead compounds had prolonged exposure compatible with twice daily eye drop administration and potentially inhibited laser-CNV following eye drop administration in mice (EC50= 0.5 µM, n=6-8, P< 0.05). Ex vivo permeability screening enabled modelling and design of novel compounds with improved permeability and optimisation for in vivo retinal delivery. Increased potency and ocular permeability of the novel SRPK1 inhibitors show potential to reach therapeutic levels in the retina following eye drop administration and improve treatment for patients with wAMD and DME.

**Opticin and Its Potential as an Anti-angiogenic Therapeutic for Pre-retinal Neovascularization**

Bishop, P.N.1, Klaska, I.P.2, White, A.1, Griffiths, J.1, Cooper, G.J.S.1,2,3,4,5, Aarons, L.1, Unwin, R.1, Bainbridge, J.W.B.1

1University of Manchester, Faculty of Biology, Medicine and Health, Division of Evolution & Genomic Sciences, Manchester, United Kingdom, 2LICL Institute of Ophthalmology, Department of Genetics, London, United Kingdom, 3University of Manchester, Faculty of Biology, Medicine and Health, Division of Cardiovascular Sciences, Manchester, United Kingdom, 4University of Manchester, Faculty of Biology, Medicine and Health, Division of Pharmacy & Pharmacology, Manchester, United Kingdom

Opticin is a naturally occurring, anti-angiogenic glycoprotein that we discovered in the vitreous humour. Here we investigated the potential of intravitreal injection of opticin as a treatment for conditions characterized by pre-retinal neovascularization, such as proliferative diabetic retinopathy and retinopathy of prematurity. Recombinant human opticin was expressed in 293-EBNA cells and purified. We used a standard murine oxygen-induced retinopathy model to compare the effects of intravitreal injection of opticin and Eylea on pre-retinal neovascularization, using PBS injection as a control; both opticin and Eylea demonstrated efficacy and there was no significant difference in their effects. Next we compared opticin and Eylea using a modified oxygen-induced retinopathy model that was designed to specifically measure the effect of treatment upon neovascular regression; with this modified model both opticin and Eylea accelerated vascular regression, but the effect of Eylea 1 day after administration was superior to that of opticin. Because opticin binds to vitreous collagen fibrils, we hypothesised that it would have a relatively long half-life following intravitreal injection compared to anti-VEGF agents currently in clinical use. To test this hypothesis we undertook PK studies in rabbit eyes. To measure the opticin concentration we developed a selected reaction monitoring mass spectrometry-based assay that could quantify and differentiate between rabbit and human opticin. The PK studies demonstrated that following intravitreal injection, the vitreous collagen-associated human opticin had a half-life of 8 days, whereas the unbound human opticin had a half-life of 4.6 days in the vitreous. Safety studies in mice demonstrated that opticin injection into the vitreous did not affect retinal vascular development. Retinal perfusion function measured using electroneutrophage in conclusion, these studies suggest that opticin would be a safe and effective treatment for conditions characterised by pre-retinal neovascularization. The collagen-associated opticin has a longer half-life than the variant human opticin, and genetic approaches is antiangiogenic in multiple models. Opticin and epoxy lipid metabolism more broadly, is a rich area for development of novel therapeutic strategies for neovascular eye diseases.
Successful Subretinal Transplantation of Clinically Compatible Polarized RPE Cell Sheets Derived from Human Pluripotent Stem Cells in Rodents and Primates

Goureau, O.

Institut de la Vision, Sorbonne Université, INSERM, CNRS, Paris, France

In developed countries, retinal degenerative diseases affecting Retinal Pigmented Epithelium (RPE), including Age-related Macular Dystrophy and inherited retinal diseases such as Retinitis Pigmentosa (RP), are the predominant causes of human blindness worldwide. Despite the scientific advances achieved in the last 30 years, there is no cure for such diseases. Replacement of defective retinal pigment epithelium (RPE) by new RPE cells derived from human pluripotent stem cells provides a novel rational approach for treating these forms of blindness. First attempts in clinical trials demonstrated safety for the delivery of such cells as a suspension. Transplanting a more physiologically functional epithelium of RPE cells is the next challenge to effectively cure patients. We developed, under clinically compatible conditions, a tissue-engineered product (TEP) consisting of RPE cells derived from human embryonic stem cells disposed on a biocompatible substrate (human amniotic membrane), allowing the formation of a 3-D functional sheet, suitable for transplantation. Through a new surgical approach to engrat the TEP into the subretinal space of Royal college of Surgeons (RCS) dystrophic rats, we demonstrated that TEP transplantation improved photoreceptor rescue and the visual function compared to RPE injected as a simple cell suspension (Ben M Berek et al., Sci. Trans. Med. 2017).

A specific device was developed to test the safety of the surgery and local tolerance in non-human primates (NHP), by transplanting in one eye the TEP in the macular region. Retinal integrity and functionality assessed at different time points (week 1 to 6 post-surgery) through eye fundus, Optical-coherence tomography and electroretinography (ERG). TEP transplantation did not cause any long-lasting inflammation and ERG responses were not modified by the surgery. Moreover, morphologic and histologic studies indicated that RPE cells were integrated into the host retina and were able to interact with photoreceptors. Our results lay the foundations for clinical studies early 2019

Autologous Cell Therapy for Macular Degeneration: From Proof of Concept to a Clinical Trial

Amaral, J.1, Charles, S.2, Khristov, V.3, Rising, A.2, Bunea, I.1, Marinitski, A.1, Li, Y.1, Sharma, R.1, Jha, B.S.1, Dejene, R.1, Campos, M.1, Miller, S.1, Bharti, K.1

1National Eye Institute, Ocular Stem Cell & Translational Research Unit, Bethesda, United States, 2Charles Retina Institute, Memphis, United States, 3National Eye Institute, Section on Epithelial and Retinal Physiology and Disease, Bethesda, United States

In order to find the optimal maturation level for hPSC-RPE sheet transplantation and criteria to evaluate that prior transplantation, we developed, under clinically compatible conditions, a tissue-engineered product (TEP) consisting of RPE cells derived from human embryonic stem cells disposed on a biocompatible substrate (human amniotic membrane), allowing the formation of a 3-D functional sheet, suitable for transplantation. Through a new surgical approach to engrat the TEP into the subretinal space of Royal college of Surgeons (RCS) dystrophic rats, we demonstrated that TEP transplantation improved photoreceptor rescue and the visual function compared to RPE injected as a simple cell suspension (Ben M Berek et al., Sci. Trans. Med. 2017). A specific device was developed to test the safety of the surgery and local tolerance in non-human primates (NHP), by transplanting in one eye the TEP in the macular region. Retinal integrity and functionality assessed at different time points (week 1 to 6 post-surgery) through eye fundus, Optical-coherence tomography and electroretinography (ERG). TEP transplantation did not cause any long-lasting inflammation and ERG responses were not modified by the surgery. Moreover, morphologic and histologic studies indicated that RPE cells were integrated into the host retina and were able to interact with photoreceptors. Our results lay the foundations for clinical studies early 2019.

Overexpression of β-Secretase 1 (BACE1) by AAV-mediated Gene Delivery Provides Protection in a Mouse Model of AMD

Qi, X.1,2, Mitter, S.1, Qiugile, J.1,2, Godoy, J.1,2, Da Silva, J.1,2, Grant, M.3, Boulton, M.1

1University of Alabama at Birmingham, Ophthalmology and Visual Sciences, Birmingham, United States, 2Indiana University, Ophthalmology and Visual Sciences, United States, 3University College London, London, United Kingdom

β-site amyloid precursor protein cleaving enzyme (β-secretase or BACE1) is strongly implicated in amyloid deposition associated with Alzheimer’s disease (AD). We have previously reported that BACE1 is expressed in the normal retina and that knockdown of BACE1 in mice results in retinal abnormalities and increased levels of oxidative stress. In this study, we delivered an AAV1-vector containing a BACE1 gene with a gfp tag driven via a small CBA promoter by a 0.5 µL subretinal injection two weeks after initiation of an SOD2 knockdown model of AMD in C57BL/6J mice or age matched control C57BL/6J mice. Two different AAV1 concentrations were used: “low” = 1 x 10^11 and “high” = 2 x 10^11 viral particles/mL. Animals were assessed at one and three months after BACE overexpression by fundus angiography, ERG, OCT, Histopathology and immunohistochemistry. In normal mice, fundus angiography, electroretinography and OCT suggested no adverse effects due to increased expression of BACE1 at the low dose for both 1 and 3 months. However, we observed some pathological changes in the retinal vasculature and RPE layer as early as 1 month following infection with high dose BACE1. RPE
Development of Intravitreal Pharmacokinetic Toolbox

del Amo Páez, F. M.1,2

University of Manchester, Manchester, United Kingdom, 1University of Eastern Finland, Kuopio, Finland

There are eye diseases of various kinds. Some of them are vision threatening. However, effective ways of drug administration remain a challenge, especially in the case of treating diseases of the posterior segment of the eye such as age-related macular degeneration, diabetic retinopathy or endophthalmitis. The drug has to be injected into the vitreous to exert the effect in the posterior ocular target sites (retina, choroid). It is essential to understand the key physiological factors affecting the intravitreal pharmacokinetics (PK) of the different therapeutic entities: small molecular weight drugs, biologicals and drug delivery systems. I have developed a toolbox that can be used for PK model and typical values of the primary PK parameters (clearance, CL and volume of distribution, Vd) of intravitreal drugs. The CL and Vd are utilized for reliable estimations of concentration profiles of the intravitreal drug in solution or in delivery systems. Additionally, I present new insights on the generally asked questions such as: Does vitrectomy play a role in dosing regimen decisions? May inflammation affect drastically the intravitreal half-life of drugs? Can the dosage of the drug be calculated on the basis of animal PK data? The present analysis and tools can benefit the development of new intravitreal drugs to treat retinal illnesses.

Semi-mechanistic Models of the Ocular Pharmacokinetics and Pharmacodynamics of Macromolecules Administered by Intravitreal Injection for the Treatment of Retinal Diseases: Theoretical Insights into the Durability of Anti-VEGF Effects

Mazer, N.A.1, Hutton-Smith, L.A.2, Gaffney, E.A.2, Byrne, H.M.2, Malini, P.K.2, Caruso, A.3

1Roche Innovation Center Basel, Pharmaceutical Sciences, Basel, Switzerland, 2University of Oxford, Wolfson Centre for Mathematical Biology, Oxford, United Kingdom

Retinal diseases, such as neovascular age-related macular degeneration, diabetic retinopathy, and diabetic macular edema, are currently treated with anti-VEGF macromolecules administered by intravitreal (IVT) injection on a monthly, bimonthly, or as-needed basis. Extending the duration of VEGF suppression and therapeutic response following each injection, i.e., the so-called “durability” of anti-VEGF effects, and thereby decreasing the frequency of IVT injections, is an active area of pharmaceutical research and development. To support these efforts, we have developed a series of semi-mechanistic models of ocular pharmacokinetics (PK) and pharmacodynamics (PD) that have been used to analyze and simulate data obtained in clinical and pre-clinical studies of macromolecules administered by IVT. In Professor Arts’s Unit’s session on “Emerging Toolbox of Ocular Pharmacokinetics and Pharmacodynamics” we will present two talks on these models. The first talk will focus on PK aspects and the second talk on PD (anti-VEGF) aspects. In these semi-ocular PK/PD models, we provided new theoretical insights into the pharmacological and biological factors that influence the durability of anti-VEGF therapy. Among these factors, particular focus will be given to the IVT dose, VEGF-bindiding affinity and the macromolecular permeabilities of the inner limiting membrane and retinal pigmented epithelium.

Studying the Barrier Role of the Vitreoretinal Interface upon Intravitreal Injection of Nanomedicines

Devoldere, J.1, Peynsaert, K.1, Braeckmans, K.2, De Smedt, S.1, Renault, K.2

1Ghent University, Lab General Biochemistry and Physical Phama-

cy, Ghent, Belgium, 2Ghent University, Biophotonics Imaging Group, 

Ghent, Belgium, 3Ghent University, Ghent Research Group on Nano-

medicines, Ghent, Belgium

Retinopathies are characterized by the degeneration of retinal cells, situated at the back of the eye. Several viral and non-viral nanoparticles are being optimized to deliver therapeutics such as proteins or nucleic acids to the back of the eye, to halt or slow down retinal cell death. To reach the retinal cell types, intravitreal or subretinal injections are performed. Subretinal injections deliver therapeutics between the photoreceptors and RPE cells. They are efficient, but rather invasive and difficult to perform. Intravitreal (IVT) injection delivers the therapeutics into the vitreous, are minimally invasive and more easy to perform. After IVT injection, however, therapeutics need to diffuse to the back of the eye before reaching the retinal cells which are separated from the vitreous by the Inner Limiting Membrane (ILM). It is more and more recognized that IVT injected therapeutics and nanoparticles might suffer from a limited mobility and penetration through the vitreoretinal interface (e.g. vitreous and ILM), hampering their efficiency. We recently developed two ex vivo bovine eye models that are suitable to study the barrier role of the vitreoretinal interface. The IVT Mobility Model allows to measure the mobility of fluorescent nanoparticles in intact vitreous on a single particle level by tracking their movement by fluorescent Single Particle Tracking (fSPIT). In the ILM Model, we use bovine ex vivo retinal explants with the vitreous still attached. In regular ex vivo explants, the vitreous is removed from the eye cup, distorting the barrier function of the ILM. Studying the diffusion of semi-invasive dyes in these models, we have been able to show that ILM is indeed a barrier for molecules that are not able to cross the ILM after IVT injection and to determine the low in vivo expression obtained after IVT injection of mRNA nanoparticles. Overall, both models can help to investigate delivery bottlenecks for nanoparticles after IVT injection.

Optimisation of Topical Retinal Drug Delivery of Small Molecule Inhibitors of Pro-angiogenic VEGF-A Splicing

Batson, L.1, Toop, H.1, Liddell, S.1, Stewart, E.1, Habgood, A.1, Murphy, A.1, Daubney, J.1, Gutierrez-Cabaliero, C.1, McKechnie, K.1, Morris, J.1, Bates, D.1

1Exonate Ltd, Nottingham, United Kingdom, 2University of New South Wales, Sydney, Australia

Development of non-invasive therapies for wet age-related macular degeneration (wAMD) and diabetic macular oedema (DMO) has been unsuccessful, possibly due to insufficient pharmacokinetics/pharmacodynamics (PK/PD) profiles to deliver efficacious doses to the retina. Delivery of potent small molecules to the retina as eye drops would be a treatment paradigm shift but remains an unmet need due to incomplete understanding of drug properties required. We hypothesised that trans-scleral permeability modelling could identify physicochemical properties required for eye drop delivery enabling optimisation of inhibitors of the pro-angiogenic VEGF-A splicing kinase SRPK1. We found that metoprolol, a potent SRPK1 inhibitor, displayed significantly reduced in vivo activity in wAMD and DMO models compared to propranolol and pindolol. Further studies are required to determine the PK/PD profile of these inhibitors in a range of ophthalmological disease models and in human eyes.

Optical properties required for eye drop delivery enabling optimisation of inhibitors of the pro-angiogenic VEGF-A splicing kinase SRPK1. We found that metoprolol, a potent SRPK1 inhibitor, displayed significantly reduced in vivo activity in wAMD and DMO models compared to propranolol and pindolol. Further studies are required to determine the PK/PD profile of these inhibitors in a range of ophthalmological disease models and in human eyes.

flattened to reveal extensive numbers of GIP™ cells that colo-
culated with BACE1 expression at both 1 and 3 months after gene delivery. In the SOD2 knockdown model we observed a reduced ERG response together with significant retinal thinning, disorgan-
ized tight junctions and loss of the RPE blood-retinal barrier at 3 
months. This was associated with a dramatic increase in the oxida-
tive damage marker, 4HNE. By contrast, overexpression of BACE1 
at either the low or high doses showed a significantly improved 
function. In SOD2 model, however, demonstrated minimal retinal thinning, an intact RPE monolayer with orga-
nized tight junctions and an intact blood retinal barrier at both 1 
and 3 months in SOD2 knockdown mice. Moreover, 4HNE levels 
were greatly reduced indicating reduced oxidative stress. Our data 
suggest that BACE1 plays a key protective role in maintaining reti-
na/RPE homeostasis and that BACE1 may offer a new avenue for 
therapeutic intervention in AMD.

Melanin Binding Kinetics in the Eye

Urri, A.1,2

1University of Helsinki, Helsinki, Finland, 2University of Eastern Fin-

land, Kuopio, Finland

Melanin binding of drugs has been known for decades, but the ground rules determining its impact on pharmacokinetics and phar-
macodynamics have been missing. We studied melanin binding of various drugs and showed that the binding is rather non-specific, but varies greatly with the chemical structure. Melanin binding can lead to distribution coefficients of 1000-10,000 (cell/medium) the-
reforming large depot of bound drug in the cells. This may lead to prolonged activity if the drug is high binder and has medium or low permeability in the cellular membranes (melanosomal mem-
brane, plasma membrane). These tools help in designing ocular drugs that may benefit from the melanin binding in terms of tissue targeting and long duration of action.

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**Ocular Pharmacology, Therapeutics & Drug Delivery**

**Mitochondrial Targeting for Eye Disease**

Prusky, G.1, Alam, N.1, Soong, Y1, Lui, S.1, Sztot, H.1

1Burke Neurological Institute, White Plains, United States, 2Weil Cornell Medicine, Physiology and Biophysics, New York, United States

Diabetes and age are associated with progressive visual decline for which there is no satisfactory preventative or corrective treatment. Mitochondria in these conditions are unable to produce sufficient adenosine triphosphate (ATP) to meet metabolic demand, which leads to numerous complications including cellular oxidative stress. Retinal pigment epithelium (RPE) cells are particularly vulnerable to these effects because of their exceptionally high metabolism. We thus hypothesized that RPE would be especially affected by diabetes and age and contribute to visual decline, and that reme- diation of mitochondrial function would improve visual function. Optokinetic measures of spatial visual behavior, measures of retinal symptomatology, and measures of retinal structure and function, were made longitudinally in mouse models of diabetes, and in aging mice. Daily systemic or eye drop treatments were made with SS-31 (a.k.a. Elamipretide), a water-soluble mitochondrial-targeting peptide with a high affinity for cardioliopin, which promotes efficient electron transfer, improves ATP production, and attenuates the production of mitochondrial reactive oxygen species. Progressive visual decline emerged in vehicle-treated diabetic models before typical diabetic symptoms were apparent, but with time, more pronounced visual dysfunction was accom- panied by impaired glucose clearance, and elevated blood glucose and bodyweight. Treatment initiated later in the course of disease reversed visual decline and partly restored visual function. Similarly, progressive visual decline emerged with age, moderate visual dysfunction could be fully restored with SS-31 treatment, and severe visual dysfunction could be partly restored. Visual dysfunction was accompanied by evidence of RPE cellular and mitochondrial pathology, and outer retinal dysfunction, which were normalized by SS-31 treatment. The data provide evidence that diabetes- and age-related visual decline is a treatable complication related to mitochondrial dysfunction on that affects the outer retina. They also indicate that optokinetic measures of spatial visual function may provide early evidence of diabetic- and age-related visual decline, and afford an advanced opportunity for therapeutic intervention.

**Non-invasive Delivery Utilising Cell Penetrating Peptides to Deliver Therapeutics for the Treatment of Ocular Disease**

de Cogan, F.1, Slope, L.1, Lynch, A.1, Xu, H.2, Peacock, A.F.1, Chen, M.2

1University of Birmingham, Institute of Microbiology and Infection, Birmingham, United Kingdom, 2Queen’s University Belfast, Belfast, Centre for Clinical and Experimental Medicine, Belfast, United King- dom

Age-related macular degeneration is a leading cause of blindness in the developed world. While anti-VEGF therapies such as ran- izubumab and bevacizumab have been shown to halt the progress- sion of neovascular age-related macular degeneration the meth- odology of delivery (intravitreal injection) has significant side effects. This work presents a novel platform technology which can deliver a range of molecules from an eye drop to the posterior segment of the eye. The platform technology is formed of cell penetrating peptides (CPP) which can be modified and optimised for each therapeutic required. The CPP facilitate the passage of large mole- cules through the ocular tissue and into the posterior segment of the eye, thus removing the need for intravitreal injection. Therapeutics and CPP were mixed in phosphate buffered saline and vortexed for 30 seconds. The mixture was then applied topically to ex-vivo and in vivo eyes. Animals were grouped into:

1) CPP + therapeutic
2) CPP alone
3) therapeutic alone
4) negative control (phosphate buffered saline)

A single eye drop was applied to the front of the eye and the ani- mal left for set time periods. After each time period the animals were sacrificed and the ocular tissues dissected, freeze-thawed and homogenised in phosphate buffered saline. The le- vels of therapeutic were determined using fluorescence or ELI- SA. CPPs could successfully deliver therapeutics into the eye with CPP + therapeutic giving significantly higher levels than control for all therapeutics tested. CPP delivered 1.1 ± 0.3 µg/mL (beva- cizumab) or 15 ± 10 µg/mL (bovine serum albumin) or 0.5 ± 0.1 µg/mL (ovalbumin) to the eye. All of these are significantly hig- her than the therapeutic applied without CPP, CPP alone and phosphate buffered saline controls p < 0.05. The levels of therapeu- tics in the eye in both rodents and rabbits demonstrated a clearance profile over 24 hours in rodents and 7 days in rabbits. The CPP platform technology can be used to deliver a range of therapeutics in both in vivo and ex vivo eyes. The endurance of the therapeutic in the eye was dependent on the size of the eye, informing a clinical dosing regime in humans.

**OPT6 - Drug delivery technologies for retinopathies**

Targeting Semaphorin 3A for Retinal Vascular Disease

Sapiela, M.1, Beaulieu, N.1, Binet, F.1, Beauchemin, K.1, La- plante, P.1, Clement, J.1

University of Montreal, Montreal, Canada, 1SemThera Inc, Montreal, Canada

Neurovascular cross-talk and its influence on retinal vascular pa- thology has been receiving increasing interest in recent years as neuron-derived factors may provide important pharmacological targets for retinal vasculopathies. We previously demonstrated in both humans and rodents that under pathological conditions, retinal ganglion cell-derived Semaphorin 3A (SEMA3A) is a potent inductor of retinal inflammation, retinal edema, vascular deviation and retinal cell senescence. SEMA3A was first described as a gui- dance cue for nascent neurons, but it is now well accepted that it also influences vascular endothelial cells and both the innate and adaptive immune response. We will describe the benefits of target- ing SEMA3A with novel biological inhibitors in retinal vasculopathy cases such as that of diabetes and AMD.

**OPT7 - Ocular drug delivery**

Simultaneous Co-delivery of Therapeutics with Biodegradable Microparticles. Potential Treatment of Retinal Diseases


Pharmaceutical Innovation in Ophthalmology Research Group UCM 920415, Complutense University, Pharmaceutics and Food Technology, Faculty of Pharmacy, Madrid, Spain

Multifactorial neurodegenerative disorders of the back of the eye are leading causes of irreversible blindness worldwide. Depending on the disease, the neuronal damage is extended to different parts of the retina. Neuroprotective therapies are aimed to preserve the neuronal structure and/or function in response to injury. As neurodegeneration occurs through different pathways, effective treatments may require a multifactorial approach. Furthermore, the chronicity of the diseases demands maintained concentrati- ons of the active substances in the target site during long term. This can be done by using frequent intracocular injections. However, invasive administrations are related with adverse effects and the risk increases with the number of injections. Intracocular drug delivery systems are emerging tools for the treatment of chronic diseases as they are able to deliver drugs for a long period of time. Among them, biodegradable microparticulate carrier systems are of great interest as they can provide simultaneous co-delivery of...
the loaded drugs in a controlled fashion. The main advantages are that micelles can be injected through conventional needles and are useful for personalized therapies as different amounts of particles can be injected according to patient’s needs. Furthermore, this novel multi-therapy strategy allows the co-incorporation of several active substances into a single microcarrier diminishing the amount of administered biomaterial inside the eye.


Penetration Enhancers in Ocular Drug Delivery: in vitro and in vivo Studies

Khutoryansky, V.
University of Reading, School of Pharmacy, Reading, United Kingdom

Cornea is a multilayered membrane that acts as one of the main barriers in drug delivery to the eye. Poor permeability of the cornea could potentially be overcome by the use of penetration enhancers in topical liquid formulations. We have evaluated the ability of different classes of compounds (cyclohexestins [1], chelating agents [2] and crown ethers [3]) to enhance permeability of rifabutin through bovine cornea in vitro. The presence of these penetration enhancers in liquid formulations results in morphological changes in the corneal structure. The mechanisms of their permeability enhancing effects are related to the extraction of either cholesterol or calcium ions from the corneal surface. We have also conducted some in vivo experiments on permeability enhancement in rats and found that the permeability enhancers efficient in vitro do not always exhibit the same enhancement potential in dynamic in vivo conditions [3]. In a study exploring the combination of penetration enhancers with mucoadhesive polymers we established that polymers inhibit penetration enhancement effects but prolong retention time of the formulations on the cornea [4]. Some other strategies in formulating liquid dosage forms with permeability enhancement effects will be discussed in this talk.

References:

The Development and Commercialisation of Sustained Release Ocular Drug Delivery Systems

O’Rourke, M.
Re-Vano Therapeutics, Belfast, United Kingdom

The global ophthalmic pharmaceutical industry is estimated to reach $29 billion by 2022 due in part to a growing elderly population, an increase in obesity rates with a corresponding increase of diabetic eye disease [1]. The number of people visually impaired in the world is approx. 295 million, with at least 39 million blind and 225 million having low vision. 65% of people visually impaired and 82% of all blind are 50 years and older. The costs of treating serious blinding eye diseases like wet age related macular degeneration alone are $343 billion including $355 billion in direct health care costs. There is an urgent need to develop new therapies and technologies including sustained release drug delivery systems, providing controlled release for approx 4-12 months for the treatment of chronic and blinding eye diseases including retinal disease and glaucoma. However anterior diseases including dry eye are also providing significant opportunities for drug delivery systems replacing or reducing topical delivery. These will increase patient’s and doctor’s convenience by reducing the dosing frequency including the ability to reduce frequent intra-ocular injections for chronic conditions of the retina. However drug delivery using current systems is usually ineffective due to the corneal barrier. The permeability enhancers most commonly used are benzalkonium chloride and carbomer, which typically suspend in the tear film and are subject to washout. Due to the increased use of penetration enhancers many formulations may provide increased ocular bioavailability.

Lipid-DNA Nanoparticles - A Versatile Vehicle for Anterior Segment Drug Delivery

Schnichels, S., de Vries, J.W.I., Strudel, L., Bartz-Schmidt, K.U., Herrmann, A., Spitzer, M., Hurst, J.

1University Eye Hospital Tübingen, Tübingen, Germany, 2Owl-Leibniz-Institute for Interactive Materials, Aachen, Germany, 3Universitäts-Augenklinik Hamburg Eppendorf, Hamburg, Germany

The efficiency of drug delivery is impaired by the short survival time of the drug on the eye surface. As a consequence, topical administration of ocular therapeutics requires high drug doses and frequent administration, still rarely providing high bioavailability. One opportunity in the treatment of ophthalmic diseases lies in nanotechnology. Nucleic acids represent very appealing building blocks for the construction of nanoparticles (NPs) with great application potential in the field of drug delivery. Introducing hydrophilic chains at the nucleoside thymine enabled the synthesis of amphiphilic DNA strands (12mers with 4 lipid modified thymines at the 5’ end), which self-assemble into micelles. In a single step they can be equipped with different drugs by hybridization with an aptamer not requiring any chemical modification of the active pharmacuetical ingredient (API). Four drugs-two antibiotics and two glaucoma drugs were bound via aptameric interactions to the carrier system: Neomycin, Kanamycin, Travoprost and Brimonidine. The effects of the fluorescently labeled DNA-NPs were compared in vivo and in vitro to formulations with the pristine drugs. After different incubation times, the adhesion to the corneas was assessed via fluorescence microscopy and fluorophotometry. Our DNA-NPs adhered to the corneal surface for extended periods of time. The efficiency of Travoprost delivery was measured with liquid chromatography-mass spectrometry (LC-MS) and compared to the commercially available formulation. Quantification revealed that our NPs enable delivery of at least double the amount of the drug at every time-point investigated and most importantly four times more Travoprost at the four hour time-point compared to the efficacy and functionality of the API. Mouse corneas were infected with Pseudomonas. Antibiotics delivered with our NP showed a much faster and more effective bactricidal effect. With our NP the same efficacy can be reached with a 10% reduced concentration. Finally, the NPs were confirmed to be applicable even for human tissue. Our data successfully prove the applicability of a DNA-based drug delivery system for the treatment of a major eye disease, i.e. glaucoma or corneal infections. These results will fuel further research into easy-to-prepare and modular drug delivery platforms tackling also other indications of the anterior of the eye.

Novel Glaucoma Therapy Lowers IOP by 40% after a Single Dose

Jablonski, M., Doaa, M., Moustafa Ibrahim, M.
The University of Tennessee Health Science Center, Ophthalmology, Memphis, United States

Purpose: Elevated IOP is one of the most significant risk factors of visual field loss in glaucoma, therefore IOP reducing therapy is the first-line therapeutic option. Unfortunately, current IOP-lowering therapies have multiple deficiencies including rapid drainage, short corneal contact time and minimal corneal penetration, all of which lead to reduced efficacy and poor patient compliance. Our study was designed to develop a novel topical formulation as a once-daily IOP-lowering drop.
In our recent systems genetics study, we identified Cas-
caza2d as a novel modulator of IOP and confirmed that modula-
tion of the activity of the gene product, CACNA2D1, lowers IOP in a dose-dependent manner (Nat Comm 2017; 8:1755). To expand on this finding and move toward a once daily IOP-lowering drop, we engineered a W/O/W microemulsion containing a BCS class I drug (highly soluble and highly permeable), and characterized the formulation using multiple in vivo and in vitro evaluations.

Methods: Our microemulsion is multilayered and was engineered using highly biocompatible components with in situ gelling properties that improve bioadhesion, control corneal penetration and provide continuous release of drug for up to 24h. Because our microemulsion contains a miniscule particle size (20nm), it is transparent and does not blur vision. Our formulation is safe, as demonstrated with MTT and slit-lamp biomicroscopic exams. It also markedly enhances the efficacy of the drug. Using Dutch belted rabbits, we effectively demonstrate that a single drop of our microemulsion induces ~40% reduction in IOP that returns to baseline at 3h after application (AUC=169.3mmHg.hr). In the absence of our microemulsion, the same drug produced only a 27% IOP reduction that returned to baseline at 10h (AUC=38mmHg.hr).

Conclusions: We have engineered a novel topical formulation that supports once daily dosing of an IOP-lowering therapy. It is safe and biocompatible. Moreover, it greatly improves IOP-lowe-
ing efficacy both in vivo and in vitro. Our data suggest that our formulation has increased mucoadhesive properties, controlled corneal permeability, miniscule particle size, and sustained release behavior. If replicated in prospective clinical trials, our novel formulation could revolutionize glaucoma therapy.

The P2X7 receptor for ATP links elevated IOP with cytokine signaling in optic nerve head astrocytes

Mitchell, C.

University of Pennsylvania, Philadelphia, United States

Glaucomatous pathogenesis has traditionally been associated with elevation of IOP, but recent findings also suggest a role for increa-
sed inflammatory signaling. We hypothesized that mechanosensi-
tive release of ATP from optic nerve head astrocytes and autostimu-
lation of the P2X7 receptor for ATP linked mechanical forces with inflam-
matory signals in glaucoma. A sustained elevation of extra-
cellular ATP was found in the retina and optic nerve head in multi-
ple models of chronic ocular hypertension. Elevated IOP increased expression of cytokine IL-6 in rat retina at the mRNA and protein level. This rise in both message and protein was prevented by intra-
ocular injection of P2X7 receptor antagonist Brilliant Blue G. Injection of the P2X7 receptor agonist BzATP increased IL-6 expression. Elevated IOP also increased IL-6 expression in wild type mice but not P2X7 KO mice. Together this suggests the P2X7 receptor is ne-
cessary and sufficient for the rise in IL-6 in response to IOP elevati-
on. Given that the mechanical strains associated with elevated IOP are focused on the optic nerve head, we asked whether optic nerve head astrocytes contribute to the IL-6 response. Moderate stretch of isolated optic nerve head astrocytes led to ATP release that was blocked by carboxenoxolone and probenecid, consistent with release of ATP via pannexin hemichannels. This released ATP activated P2X7 receptors on the astrocytes to raise intracellular Ca2+ and in-
crease expression of IL-6 mRNA. This IL-6 priming was blocked by P2X7 receptor antagonist A830177 and Brilliant Blue G, and was absent in astrocytes from P2X7 KO mice. Stretched astrocytes also released cytokine IL-6; this release was mimicked by agonist BzATP. In summary, ATP release through pannexin channel and autostimu-
lation of the P2X7 receptor contributes to the rise in cytokine IL-6 that accompanies IOP elevation. This likely reflects a widespread link and identifies an important target for reducing inflammation in glaucoma.

Ocular Pharmacology, Therapeutics & Drug Delivery
CTGF Expression is Induced by Mechanical Forces in Astrocytes of the Glial Lamina under Normal and Glaucomatous Conditions


University of Regensburg, Institute of Human Anatomy and Embryology, Regensburg, Germany; *Otto-von-Guericke-Universität, Medizin, Leibniz-Institut für molekulare Pharmakologie, Berlin, Germany. 

Purpose: To evaluate the remodeling of the lamina cribrosa is associated with reactive astrocytes and increased CTGF synthesis. The TGF-β2 mediated ECM synthesis is dependent on its downregulation.

Methods: CTGF expression was analyzed in heterogeneous CTGF mice. Tangential sections of the glial lamina of 1- and 2-month-old β1β-CGF1 mice and latticere controls were stained against GFP and CTGF while phalloidin-labelling and staining against fibronectin was only performed on glial laminae of 2-month-old mice. CTGF and GFAP mRNA expression was analyzed by real-time RT-PCR in the ON and ONH of β1β-CGF mice at both ages. Astrocytes were treated with TGF-β2 and CTGF or seeded on PDMS substrata of different stiffness. Cells were affected by staining assay real-time RT-PCR, Western blotting and immunocytochemistry.

Results: ON astrocytes are the main source of CTGF expression. As expected, the TGF-β2 mediated ECM synthesis is dependent on its downregulation. This paucity of information on genetic variants associated with disease traits in persons of African ethnicity limits their opportunity to benefit from personalized medicine.

Conclusion: The primary Open-Label African American Glaucoma Genetics (POAGG) study has enrolled 3000 glaucoma patients and 4765 controls. The goal is to identify genotypic and phenotypic markers of POAG to define disease traits and risk characteristics which may ultimately result in better therapeutic options. Thus far, mitochondrial DNA haplogroups associated with more severe disease and phenotypic characteristics related to risk have been identified. Custom content from whole genome sequencing of members of this cohort has also been added to the illumina multi-ethnic array, thus providing a resource for large scale genotyping that is necessary to develop personalized medicine.

OPT9 - Personalized medicine and other innovations

Personalized Medicine: Equal Access for All?

Miller-Elis, E., Addis, Y., Sanker, P., Cui, Q., O’Brien, J.

Scheie Eye Institute/University of Pennsylvania School of Medicine, Ophthalmology, Philadelphia, United States.

The goal of personalized medicine is to offer more precise therapy tailored to the genotype of an individual patient. Primary open angle glaucoma (POAG) is a heterogeneous disease, yet all current therapy is geared towards lowering intraocular pressure (IOP). Some patients respond well to this treatment while others progress despite aggressive IOP reduction. Thus, a better understanding of pathophysiology and genetic risk is needed to develop more targeted therapy. Persons of African ancestry are disproportionately affected by POAG and tend to have more aggressive disease. Numerous studies have identified genes associated with glaucoma, but this highest risk group has been underrepresented. To benefit from personalized medicine, adequate genetic material and biomarkers are needed. According to a 2016 Nature publication [1] samples of African ancestry make up less than 4% of the GWAS catalog while those of European ancestry make up approximately 86%. This paucity of information on genetic variants associated with disease traits in persons of African ethnicity limits their opportunity to benefit from personalized medicine.

Objective: To address this disparity, the primary Open-Label African American Glaucoma Genetics (POAGG) study has enrolled 3000 glaucoma patients and 4765 controls. The goal is to identify genotypic and phenotypic markers of POAG to define disease traits and risk characteristics which may ultimately result in better therapeutic options. Thus far, mitochondrial DNA haplogroups associated with more severe disease and phenotypic characteristics related to risk have been identified. Custom content from whole genome sequencing of members of this cohort has also been added to the illumina multi-ethnic array, thus providing a resource for large scale genotyping that is necessary to develop personalized medicine.

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The promise of individual or personalized medicine, described in genomics-based knowledge that enables physicians to make rational choices about medical therapy based on the individual health records for specific action. Based on genome-wide technology and large data sets, personalized medicine offers the following:

1. A comprehensive medical history and physical examination
2. Specialized genetic testing to identify genetic factors that affect each of us as biologic individuals
3. How to interpret genetic results; and

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3. How to interpret genetic results; and

Our understanding of the mechanisms of ophthalmic diseases and the genetic drivers underlying them, there are barriers that prevent the implementation of pharmacogenomics into clinical practice. Some of these barriers include ethical and practical questions such as:

1. Who to genotype?
2. When to genotype?
3. How to interpret genetic results?
4. Whether to integrate the results of genetic information into health records for specific action.

For major ophthalmic diseases such as macular degeneration or primary open angle glaucoma, there are no accurate biomarkers to predict which groups of patients will or won’t respond to respectively, anti-VEGF or intraocular pressure (IOP) lowering medications. It is well-known that topical or intravitreal administration of steroids in animal models for both primary rod photoreceptor loss during hereditary retinal degeneration (RD). The HDAC inhibitors trichostatin A (TSA) and suberanilohydrazide (SAHA) were tested for their ability to prevent photoreceptor loss in the context of RD. Initially, this was done on retinal explant cultures derived from animal models for both primary rod photoreceptor degeneration, i.e., a situation representing the disease Retinitis Pigmentosa (rd2, rd10 mice) and for primary cone degeneration, as it occurs in the disease Achromatopsia (cfd2 mice). Both TSA and SAHA had a marked protective effect in rd2 and rd10 mouse retinal explant cultures and significantly increased rod photoreceptor survival. Similarly, TSA treatment preserved cone photoreceptors in cfd2 mouse retina in vitro. In cfd2 retina TSA also had a marked positive effect on the nucleokinetic migration of cone photoreceptors. Surprisingly, in cfd2 mice in vivo, a single intravitreal injection of TSA achieved a significant protection of cone photoreceptors, the effects of which were still detectable up to fifteen days post-injection. Taken together, these studies highlight the possibility to use pharmacological HDAC inhibition for photoreceptor protection, and may forward the future development of therapies for RD.

Retinitis Pigmentosa (RP) is a class of hereditary retinal dystrophy associated with photoreceptor loss and blindness. Among several actors mediating cell death, we observed an abnormal cell cycle re-entry in photoreceptors undergoing degeneration in rd1 mice and identified the Polyclamp repressive complex I (PRC1) core component BMI1 as a critical molecular factor orchestrating the cell death mechanism. The Bmi2 deletion leads to a marked photoreceptor protection in the rd1 retina, but independently of the conventional Ink4a/Arf pathways (Zencak et al., 2013), suggesting that BMI1 acts on other targets. We thus studied through the degenerative process the potential role of components of the PRC2 interact- ing with BMI1, such as Ezh2, which methylates Histone 3 to repress gene expression. We observed by immunohistochemistry, a hypermethylated of histone 3 (H3K27me3) in photoreceptors of
A Selective Histone Deacetylase 6 Inhibitor, Tubastatin A, Rescues Visual Capacity in Rafferia, a Novel Zebrasfish Retinal Mutant Model

Sundaramurthi, H.*,1,2,3, English, M.*,1, Starostik, M.*,1, Carter, S.*, Swaroop, A.*,1, Reynolds, A.*,1, Kennedy, B.1

1University College Dublin, Systems Biology Ireland, Dublin, Ireland. 2University College Dublin, UCD School of Medicine, Dublin, Ireland. 3University College Dublin, UCD School of Biomolecular & Biomedical Science, UCD Conway Institute, Belfield, Ireland, *National Eye Institute, National Institutes of Health, Bethesda, United States.

Inherited retinal dystrophies (IRDs) affect 1 in 3000 people worldwide, and it is characterized by progressive vision loss that ultimately results in blindness. Due to the genetic and clinical heterogeneity of these diseases, effective treatment options are not widely available. Histone deacetylase inhibitors (HDACi) have gained extensive molecular mechanisms of disease and the H3K27me3 mark. The most genes enriched by H3K27me3 (fold enrichment > 3) are involved in cellular processes regulating the defense mechanism, cell cycle, cellular trafficking and cell death. These results are consistent with our previous findings on photoreceptor death mechanism characterization and suggest that photo-receptor degeneration occurs with a loss of cell identity in parallel to activation of different cell death pathways. Links with miRNAs regulation and HDAC will be discussed.

A New Pluripotent Epigenetic Repressor of Diverse Genes, OBP801, Remarkably Prevents Chorioretinal Fibrosis and Choroidal Neovascularization


1Kyoto Prefectural University of Medicine, Kyoto, Japan. 2Ono Pharm Co., Ltd., Tokyo, Japan

Purpose: To investigate the effects of a new pluripotent epi-genetic repressor, OBP801, on RPE fibrosis and laser-induced choroidal neovascularization (CNV) to prevent the pathologi-cal progression of age-related macular degeneration (AMD).

Methods: The integral analysis of gene expression, involved in fibrosis and CNV aggravation, were performed using cultured human RPE cells and in vivo chorioretinal tissues in murine models of laser-induced CNV. Transforming growth factor beta 2 (TGF-β2, 30ng/ml) and tumor necrosis factor alpha (TNF-α, 10ng/ml) were used in inducing fibrotic change of RPE cells. The expression of α-smooth muscle actin (α-SMA), and diverse fibrosis related genes were ex-a mine by immunostaining, western blotting, and polymerase chain reaction (PCR). CNV was induced with a 532-nm laser. Laser spots (200 mw, 50 µm, 100 ms) were created in each eye using a slit lamp delivery system. After burns, 1 ml 5 mM OBP801 (OBP, 118 µg/kg) was injected intravitreally. Fluorescein angiograms (FAs) and histologic examinations were performed. CNV formation was confirmed with the fluorescent staining at retinal flat mounts. To evaluate the tissue fibrosis, frozen sections and choroidal flat mount were stained with anti-α-SMA and type I collagen antibody.

Results:
1. The expression of α-SMA was enhanced by TGF-β2 and TNF-α. The elevated gene expressions were abolished by OBP (1mM) or control histone deacetylase (HDAC) inhibitor, SAHA at 1000 time higher concentration of 1 mM.
2. The activity of HDAC was unaffected, but that of HAT was reduced by TGF-β2, TNF-α or TGF-β2+TNF-α. OBP reduced the former (p<0.01) but not the latter.
3. The integral analysis revealed that OBP suppressed the ex-pression of a wide variety of genes involved in fibrosis (αSMA, SERPINH1, ITGB5, MMP2, 9, 14), CNV (PDGFA, VEGFA) and tissue scar formation ( collage).
Recently, we discovered heterozygous loss-of-function mutations in the ternat intra-ocular cell gene, TEK. This is now established as the second leading cause of primary congenital glaucoma (PCG) after CYP1B1-related PCG, accounting for approximately 5% of cases.1 The TEK protein, also known as TIE2, is a receptor that regulates vascular homeostasis via phosphorylation signaling, and is an essential component in sustaining vascular stability. TEK is highly expressed in the endothelium of Schlemm’s canal (SC) a lymphatic/vascular/hybrid vessel.2 In mice, homozygous disruption of the TEK gene or both Angpt1 and Angpt2 in ganding-encoding genes, result in high intra-ocular pressure (IOP), leading to blindness in children. The condition is clinically and genetically heterogeneous, with both primary and secondary causes. Anterior segment dysgeneis are associated with developmental defects of the anterior ocular chamber and can cause secondary glaucoma by impairing drainage of the aqueous humor, thereby increasing IOP. Biacellular pathogenic variants in the CRAMD8 gene have previously been associated with anterior segment dysgenesis, myopia, and ectopia lentis but not with childhood glaucoma. Purpose: Childhood glaucoma is a significant cause of irreversible blindness in children. The condition is clinically and genetically heterogeneous, with both primary and secondary causes. Anterior segment dysgenesigs are associated with developmental defects of the ocular anterior chamber and can cause secondary glaucoma by impairing drainage of the aqueous humor, thereby increasing IOP. Biacellular pathogenic variants in the CRAMD8 gene have previously been associated with anterior segment dysgenesis, myopia, and ectopia lentis but not with childhood glaucoma. Methods and results: We performed whole genome sequencing on an individual with a suspected diagnosis of primary congenital glaucoma and his unaffected parents, and identified biacellular predicted deleterious variants in CRAMD8. We then performed whole exome sequencing on 84 additional probands with a suspected diagnosis of primary congenital glaucoma recruited in the Australian and New Zealand Registry of Advanced Glaucoma, from which we identified biacellular variants in an additional two families. In total, we have identified five novel predicted deleterious variants in CRAMD8 in four individuals from three families. CRAMD8 variants explained 3.6% (6/164) of cases in our cohort. Common ocular features included corectopia, iris stromal hypoplasia, iris transillumination defects, iridodermal, ectopia lentis, and myopia. Cataracts were present in all individuals above 20 years of age. All four individuals required surgery to control intraocular pressure and glaucoma. We detected CRAMD8 expression to be highest in tissue of neuroectodermal origin (including the ciliary body and corneal epithelium of adult anterior segment), suggesting a role for CRAMD8 in drainage structures of the eye. Conclusion: We extend the phenotypic spectrum associated with CRAMD8 variants to anterior segment dysgenesis with congenital glaucoma, adding CRAMD8 to the list of childhood glaucoma-related genes.

Strain Dependent Differences Modulating Ocular Phenotypes in Lmx1b Mutant Mice

Tolman, N.1, Macalino, D.1, Kearney, A.1, Macnicoll, K.1, Kuhlait, K.1, Nair, S.1, Cross, S.1, John, S.1

The Jackson Laboratory, Bar Harbor, United States, UCSF School of Medicine, University of California San Francisco, San Francisco, United States, University of Edinburgh, Edinburgh, United Kingdom

Mutations in Lmx1b disrupt anterior segment development and are associated with elevated IOP and glaucoma. However, there is a wide range of phenotypic variability between individuals with LMX1B mutations. Mice with a dominant negative value to aspartic acid in Lmx1b (Lmx1bΔ6) mutation display similar phenotypes to humans with LMX1B mutations. Characterizing genetic modifiers of LMX1B mutations can provide novel mechanistic information. To identify the genetic modifiers in Lmx1bΔ6 mutant mice, we performed a mapping cross between B6 and 129 backgrounds. We backcrossed the Lmx1bΔ6 allele (also known as Lmx1bD6) onto the C57BL/6j (B6), 129S6/SvEvTac (129), C3H/HeJ (C3H), and D2/J Gpnnm wt (D2) backgrounds for at least 6 generations. Lmx1bΔ6 and wild-type (WT) control littermates were examined for ocular phenotypes by slit-lamp, cannulated IOP measurements, histology, and immunofluorescence on whole-mounted eyes. To find genetic modifiers in Lmx1bΔ6 mutant mice, we performed a mapping cross between B6 and 129 backgrounds. We found differences in the presentation of ocular phenotypes between inbred strains with the Lmx1bΔ6 allele. B6.Lmx1bΔ6 mice had severe anterior segment dysgenesis, which included malformed corneal, irido-corneal strands, corneal haze and corneal scleralization. We also found elevated IOP at early ages and significant incidence of glaucomatous nerve damage in B6.Lmx1bΔ6 mice. 129.Lmx1bΔ6 mice displayed mild anterior clinical features compared with the other strains and modestly elevated IOP. However, 129.Lmx1bΔ6 mice did not show any detectable glaucomatous nerve damage. 129.Lmx1bΔ6 caused moderate phenotypes on C3H and D2 backgrounds compared to the 129 (resistant) and B6 (susceptible) backgrounds. A mapping cross between B6 and 129 mice with the Lmx1bΔ6 allele identified a locus on chromosome 18 that confers differential susceptibility to Lmx1bΔ6-induced abnormal phenotypes. Our results suggest genetic background profoundly influences susceptibility to glaucoma-related clinical features in Lmx1bΔ6 mice, with an important modifier locus on chromosome 18. Using genetic approaches to map modifier loci in Lmx1b mutant mice is a valuable approach to discover genes and pathways that modulate susceptibility to glaucoma.
OGM2 - Beyond the genome: Functional genomics in genetic eye disease

The Non-coding Morbid Genome of Inherited Retinal Diseases

De Baere, E.1
Ghent University and Ghent University Hospital, Ghent, Belgium

Integrated genome and transcriptome sequencing reveal an increasing number of non-coding mutations in Mendelian disorders including inherited retinal diseases (IRD). Of these the majority are deep-intronic splicing mutations, often leading to pseudo-exon inclusion and to the non-coding morbid genome of both Mendelian and complex diseases. Here, we report on the non-coding genome sequences of 36 families with isolated or syndromic IRD. We identified a total of 3,562 non-coding variants using a novel pipeline that integrates three RNA-seq datasets and 454 sequencing. It comprises a deep sequencing approach for the identification of splicing variants enriched in patients compared to controls. Our data revealed a high burden of deep-intronic and deep-exonic variants in IRD. We identified 206 non-coding variants not only for a selected group of families, but at a population-wide level. As all deep-intronic variants could be corrected using AONs using patient-derived photoreceptor progenitor cells. For two previously discovered neighboring deep-intronic variants (c.4539+2001G>A and c.4539+2028C>T) splice defects could only be shown and corrected by strengthening cryptic splice sites (c.769-784C>T, c.859-506G>C, c.4539+1100A>G, c.4539+1106G>C/T) and one (c.1387+4353C>G) by disrupting a predicted exonic splice silencer. Another variant (c.4253+43G>A) resulted in partial exon 28 deletion. These splicing defects could be corrected using antisense oligonucleotides (AONs) in HEK293T cells and fibroblasts. For two previously discovered neighboring deep-intronic variants (c.4539+2001G>A and c.4539+2028C>T) splice defects could only be shown and corrected by AONs using patient-derived photoreceptor progenitor cells. Splice assays for several other published deep-intronic variants are underway. To analyze the ABCA4 gene in 412 additionally genetically unsolved STGD1 cases, we designed 260 single molecule molecular inversion Probes (smMIPs) to sequence the 50 coding exons and flanking splice sites. A total of 12 selected deep-intronic regions containing 13 deep-intronic variants. We found 70 deep-intronic variants in 66 probands, the most frequent of which were c.4253+43G>A (n=29) and c.5196+1137G>A (n=13). In conclusion, we solved 24/45 (53%) of mono-allelic STGD1 cases previously sequenced for coding variants. By smMIPs-based ultra-quick sequencing we could genetically validate ~40% of previously genotyped STGD1 probands, half of whom carry deep-intronic variants. As all deep-intronic variants could be corrected using AONs, there is great potential to treat STGD1 patients carrying non-coding variants. The smMIPs platform is currently being expanded to cover the entire ABCA4 gene and will be used to test >1000 unsolved worldwide collected STGD1 probands.

Identification of Pseudo-exons due to Deep-intronic ABCA4 Variants in Stargardt Disease Using Midigens and Patient-derived Photoreceptor Progenitor Cells

Cremers, E.1, Sangermano, R.2, Garanto, A.2, Khan, M.2, Bauwens, M.1, Albert, S.1, Khan, M.I.1, Cornelis, S.1, Elmenik, D.2, Manders, E.1, Runhart, E.1, Arno, G.1, Fakim, A.1, Webster, A.1, Dhaenens, C.-M.1, Weber, B.2, de Baere, E.1, van den Born, L.I.1, Hoyng, C.1, Collin, R.1

1Radboud University Medical Center, Department of Human Genetics, Nijmegen, Netherlands, 2Radboud University Medical Center, Amsterdam, Netherlands, 3Ghent University and Ghent University Hospital, Ghent, Belgium

In 45 STGD1 cases with one ABCA4 variant we performed Haloplex- or whole genome sequencing of the 128 kb ABCA4 gene, which resulted in 101 independent novel intronic variants. At least two of five algorithms of Alamut Visual software predicted strengthening of cryptic splice sites for seven variants; three variants in two alleles showed a high frequency in our probands, and one variant resided in a previously identified pseudosexon (PE) that is present in a very-low expressed mRNA splice variant. The effect of these 11 variants on mRNA was tested in vitro by transfecting mutant midigens in HEK293T cells. Six variants found in 24/45 probands resulted in splice defects. Four variants led to PEs by strengthening cryptic splice sites (p.769-784C>T, c.859-506G>C, c.4539+1100A>G, c.4539+1106G>C/T) and one (c.1387+4353C>G) by disrupting a predicted exonic splice silencer. Another variant (c.4253+43G>A) resulted in partial exon 28 deletion. These splice defects could be corrected using antisense oligonucleotides (AONs) in HEK293T cells and fibroblasts. For two previously discovered neighboring deep-intronic variants (c.4539+2001G>A and c.4539+2028C>T) splice defects could only be shown and corrected by AONs using patient-derived photoreceptor progenitor cells. Splice assays for several other published deep-intronic variants are underway. To analyze the ABCA4 gene in 412 additionally genetically unsolved STGD1 cases, we designed 260 single molecule molecular inversion Probes (smMIPs) to sequence the 50 coding exons and flanking splice sites. A total of 12 selected deep-intronic regions containing 13 deep-intronic variants. We found 70 deep-intronic variants in 66 probands, the most frequent of which were c.4253+43G>A (n=29) and c.5196+1137G>A (n=13). In conclusion, we solved 24/45 (53%) of mono-allelic STGD1 cases previously sequenced for coding variants. By smMIPs-based ultra-quick sequencing we could genetically validate ~40% of previously genotyped STGD1 probands, half of whom carry deep-intronic variants. As all deep-intronic variants could be corrected using AONs, there is great potential to treat STGD1 patients carrying non-coding variants. The smMIPs platform is currently being expanded to cover the entire ABCA4 gene and will be used to test >1000 unsolved worldwide collected STGD1 probands.

Comprehensive Characterization of cis-regulatory Elements in Human Retina

Cherry, T.1, Yang, M.1, Tao, P.1, Harmin, D.2, Timms, A.1, Greenberg, M.1

1University of Washington, SCR/Center for Developmental Biology and Regenerative Medicine, Seattle, United States, 2Harvard Medical School, National Eye Institute, National Institutes of Health, Bethesda, United States, 3Seattle Children’s Research Institute, Center for Developmental Biology and Regenerative Medicine, Seattle, United States

Cis-regulatory elements orchestrate the dynamic and diverse transcriptional programs that assemble the human visual system during development and maintain its function throughout lifetime. Genetic variation within these cis-regulatory elements plays a central role in phenotypic variation in complex traits including the risk of developing disease. Using an integrative epigenomic approach we identified and characterized cis-regulatory elements in the adult human retina, macula, and RPE/chorioid. This comprehensive resource of tissue-specific regulatory elements, transcription factor binding, and gene expression programs in three regions of the human retina reveals novel features of regulatory elements that shape tissue-specific gene expression programs and defines regulatory variants with the potential to contribute to mendelian and complex disorders of human vision.

Dissection of Cis-regulatory Elements of PITX2

Semina, E.

Medical College of Wisconsin, Pediatrics, Milwaukee, United States

Axenfeld-Rieger syndrome (ARS) comprises a group of conditions characterized by abnormal development of the anterior segment of the eye and, in many cases, other systemic features. Mutations in two factors, PITX2 and FOK1, explain the majority of ARS cases. Both genes encode DNA-binding transcription factors, with PITX2 belonging to the bicoid-like homeodomain family. Complete loss-of-function alleles in PITX2 lead to typical ARS, while mutant proteins that retain partial wild-type activities associate with milder ocular phenotypes, suggesting that human development is highly sensitive to PITX2 dosage. Our studies demonstrated a high level of sequence and functional conservation between human and zebrafish PITX2/pitx2. Discovery and functional analyses of conserved noncoding sequences upstream of human and zebrafish PITX2/pitx2 predicted their possible role in human disease and subsequently identified a novel mechanism of ARS through deletions of these distant regulatory sequences in human patients. To further explore these findings, we used CRISPR/Cas9 technologies to generate a series of zebrafish lines carrying varying genomic deletions upstream of pitx2 and corresponding to human regions affecting transcription of these regulatory elements.
fected in ARS and/or encompassing the potential enhancers acting in eye development. As anticipated, the select regulatory deletions did not cause a complete loss of pitx2 expression, consistent with the presence of numerous additional elements likely contributing to the transcriptional regulation of pitx2. The CES-1/5-11 and CES-7/10 regions contain several regulatory elements that were previously characterized as ocular enhancers, CES, CE7 and CE10. And another deletion removed region CE4/9 that directed reporter expression in the pericellular mesenchyme, brain and craniosoma regions in transgenic fish. Tentative studies of the developing anterior segment structures in the deletion mutants identified a reduction of the anterior chamber depth and numerous defects in the development of the cornea and iris via electron microscopy, and its role in development of the anterior segment structures and other parts of the eye. Understanding the mechanisms by which PITX2 expression is controlled through individual and combinatorial contributions of regulatory elements will provide insight into its pathway and possibly explain some of the phenotypic variability.

JNK Dependent MAPK Signaling Has Multiple Roles in Axon Injury Induced Retinal Ganglion Cell Death

Lioby, B. 1, Yang, H. 1, Syc-Mazurek, S. 1, Harder, J. 1, John, S. 1, Stowell, G. 2

1University of Rochester Medical School, Flaum Eye Institute, Rochester, United States, 2The Jackson Laboratory, Bar Harbor, United States, 3Howard Hughes Medical Institute, Bar Harbor, United States

MAPK signaling, particularly JNK dependent MAPK signaling, is thought to be a critical mediator of neurodegeneration after axonal injury, a key, early event in glaucoma. We have shown that JNK2 and JNK3 are important mediators of RGC somal degeneration after mechanical axonal injury (optic nerve crush, ONC) and appear to act through their canonical target, the transcription factor JUN. We tested the importance of Jun and Jnk2/3 in the loss of RGC somas and axons in ocular hypertension. DBA/2J (D2) mice. Jun deficiency provided significant, but incomplete protection to RGC somas and no protection to RGC axons. Interestingly, unlike after ONC, Jnk2/3 deficiency did not protect RGC somas nor prevent JUN activation in D2 mice, suggesting JNK1 may be activated by ocular hypertensive injury and independently contribute to pro-death signaling. Also, Jnk2 deficiency alone increased the rate at which ocular hypertensive injury led to RGC axonal and somal degeneration, implicating JNK2 in a novel pro-survival function in glaucoma, possibly through JNK2 activity in a cell other than RGCs. Since Jun deficiency did not completely protect RGC somas or RGCs in glaucoma (in both cases reducing ~70% of the somas) another mechanism(s) must contribute to RGC death. A likely candidate for JUN independent RGC death was the ER stress-induced cell death pathway mediated by Ddit3. Combined deficiency of both Jun and Ddit3 significantly increased somal degeneration after ONC compared to deficiency of either molecule alone. In fact, deficiency of both Jun and Ddit3 provided near complete protection of RGC somas for extended periods of time. Both Jun and Ddit3 are transcription factors, thus, to understand the transcriptional networks these molecules regulate, RNA-seq was performed on RNA isolated from Jun, Ddit3, Jun/Ddit3 deficient retinas 3 days after ONC. Preliminary analysis of this experiment shows that of the 1497 differentially expressed genes in response to ONC, single Ddit3 and Jun deficiency prevented ~700 and 900 of those changes respectively. Furthermore, Ddit3/Jun deficiency prevented nearly all changes, highlighting the profound effect these molecules have on transcriptional changes after axonal injury. These experiments show that JNK signaling has multiple roles in ocular hypertensive RGC death and that the JNK signaling and ER stress signaling pathways are major regulators of somal degeneration, at least after mechanical axonal injury.

Exploring the Pathological Contribution of HDAC3 in the Death of Retinal Ganglion Cells


1University of Wisconsin-Madison, Ophthalmology and Visual Sciences, Madison, United States, 2McPherson Eye Research Institute, Madison, United States, 3Duke University Eye Center, Durham, United States, 4University of Wisconsin-Madison, Madison, United States, 5University of Wisconsin-Whitewater, Whitewater, United States

After optic nerve damage, retinal ganglion cells (RGCs) undergo rapid epigenetic deacetylation of histones resulting in silencing of active gene expression and the widespread formation of heterochromatin. Associated with this event is the translocation of histone deacetylase 3 (HDAC3) from the cytoplasm to the nucleus. Because HDAC3 plays a role in a variety of neurodegenerative conditions, we investigated further the pathological contribution of this enzyme in RGC death. Conditional knock-out of HDAC3, or treatment with HDAC3-selective inhibitors, significantly reduced histone deacetylation and heterochromatin formation, and attenuated the rate of RGC loss in acute and chronic models of optic nerve damage. Forced expression of HDAC3 in mouse RGCs increased the sensitivity of these cells to acute optic nerve damage, and induced moderate, but slowly progressive spontaneous RGC loss. We examined further the molecular pathways activated by HDAC3 in mouse 661W tissue culture cells, which can be neurally differentiated in vitro. Forced expression of HDAC3 was readily tolerated in undifferentiated cells, but generated rapid cell loss within 24 hours of the onset of differentiation. This was associated with activation of a GFP-BAX fusion protein and activation of caspase 3. In 661W cells with an edited Bax gene, or cells with Bax transcription knocked-down with siRNA, significantly reduced the level of induced HDAC3, indicating that this enzyme activated the intrinsic apoptotic pathway. While others have indicated that HDAC activity results in promotion of p33-dependent pro-apoptotic gene expression, we find no initial evidence of p33-dependent transcription by forced HDAC3 expression in these cells. Preliminary experiments, however, indicate that the pathological effect of forced HDAC3 expression in neuron-like cells may, in part, be a function of exit from the cell cycle. Funding: R01 EY012223, P30 EY016665, Research to Prevent Blindness, Inc., T32 GM081601.
Retina Circuit Disassembly and Connectivity in Glaucoma

Ota, Y., Xu, A., Mai, K., Tran, A., Della Santina, L.
University of California, San Francisco, Ophthalmology, San Francisco, United States

Precise connections between synaptic partners are shaped during development to ensure proper neural circuit function, but how these connections are disassembled and rearranged after injury is less well understood. In glaucoma, the ganglion cell is injured and eventually dies but very little is known about how the presynaptic circuitry and retinal connectivity are perturbed. Furthermore, our ability to diagnose and treat glaucoma at an optimal stage before irreversible ganglion cell death occurs requires a comprehensive understanding of inner retina circuit disassembly and its potential for plasticity in adult retina. Previous studies from us and others have identified the alpha OFF-transient RGC as selectively vulnerable to glaucomatous injury. Furthermore, we have found that presynaptic axons in the OFF sublamina of the inner plexiform layer (IPL) appear to be more susceptible to injury compared to those in the ON sublamina. Here we sought to determine the specificity and timing of circuit disassembly in the adult diseased retina. We examined the effects of intracranial pressure elevation on the order of pre-vs. postsynaptic excitatory synaptic component loss. We found that at the bipolar cell-ganglion cell synapse, axons are disassembled before PSD95, a shared phenomenon across the RGC types examined. However, when bipolar cell connectivity to specific ganglion cell types was investigated, we discovered that different RGC types employed distinct strategies of disconnection. Finally, the alpha ON-sustained RGC lost inputs from its major excitatory partner, the type 6 bipolar cell, and only the contralateral retina. These findings expand knowledge of how adult neural circuits can adapt to injury and permit the design of novel psychophysical testing paradigms and identify potential targets for RGC regeneration or neuroprotection.

Efficient Simultaneous Mapping of Many Retinal Ganglion Cell Center-Surround Receptive Fields

Troy, J.R.1, Zhao, Y.1, Chen, H.2, Liu, X.3
1Northwestern University, Biomedical Engineering, Evanston, United States, 2University of Virginia, Biolog and Psychology, Charlottesville, United States, 3Universidad de Murcia, IMIB, Ophthalmology, El Palmar, Murcia, Spain

Purpose: We investigate short- and long-term effects of intraorbital optic nerve (ION) axotomy on RGCs, a type of injury involved in glaucoma.

Methods: To compare the time-course and magnitude of retinal nerve fiber layer (RNFL) thinning with that of retinal ganglion cell (RGC) loss after ION transection in adult rats, at various survival intervals (ranging 3 days-4 months) retinas were imaged with SD-OCT to measure the RNFL and retinal thickness, then whole-mounted and immunoreacted for Brn3a and neurofilaments to identify RGCs and their intra-retinal axons. To examine in albino mice RGC survival and activation of caspase 3 after ION axotomy alone or with BDNF treatment we performed crush or transection and retinas were analyzed from 1 to 10d after. Additional groups were treated right after injury intravitreally with a single injection of BDNF (2.5 µg) or vehicle. Brn3a (RGCs) and cleaved-caspase 3 (c-casp3) were immunodetected and their numbers quantified. To analyze the long-term effect ION injury in adult rats, wholemounts were examined from 15d to 15m using modern techniques to identify, count and map the distribution of RGCs (identified with Fluorogold or Brn3a and melanopsin antibodies) and cells in the ganglion cell layer (GCL) (DAPI-staining).

Results: Brn3a+RGCs decreased to approximately 80, 52, 17, 9, 5 and 3% at 7, 12, 21 days, 1, 2 and 4 months, respectively, while RNFL thinning was first significant at 12d (10%-diminution) and increased up to 4m (7%-diminution) and RGC axon immunodetection decreased from 12 days onwards. Adult mice RGC loss followed the same temporal pattern after ION crush or transection and within the first 7 days was 65% loss with a peak at 4 days. Brn3a down-regulation coincides with c-casp3 expression, and BDNF results in delayed RGC loss by one day. Adult rat ION resulted within two weeks in a massive loss of Brn3a+RGCs and m+RGCs throughout the retina. At 15 months, this loss was even greater for the Brn3a+RGCs but not for the m+RGCs and represented less than 1% or 35% of the original population, respectively, indicating a significantly greater survival for m+RGCs.

Conclusions: After ION, RGC death is more severe and precedes thinning of the RNFL. Brn3a is upregulated at the beginning of RGC death which is caspase dependent apoptosis. Axotomy does not result in loss of other non-RGC neurons in the GCL, nor in the contralateral retina, and significantly more m+RGCs survive than Brn3a+RGCs.

OGM4 - AMD genetics and cell biology

Hallam, D., Collin, J., Steel, D., Kavanagh, D., Lako, M.
Newcastle University, Newcastle, United Kingdom

Age related macular degeneration (AMD) is the most common cause of blindness, accounting for 8.7% of all blindness globally. Vision loss is caused ultimately by apoptosis of the retinal pigment epithelium (RPE) and overlying photoreceptors. Treatments are evolving for the wet form of the disease, however these do not exist for the dry form. Complement factor H (CH) polymorphism in exon 9 (Y402H) has shown a strong association with susceptibility to AMD resulting in complement activation, recruitment of phagocytes, retinal pigment epithelium (RPE) damage and visual decline. We have derived and characterised induced pluripotent stem cell (iPSC) lines from two patients with AMD and low risk genotype and two patients with advanced AMD and high risk genotype and generated RPE cells that show local secretion of several proteins involved in the complement pathway including factor H (FH), factor I (FI) and factor H like 1 (FH-L1). The iPSC RPE cells derived from high risk patients mimic several key features of AMD including increased inflammation and cellular stress, accumulation of lipid droplets, impaired autophagy and deposition of ‘drusen’ like deposits. The low and high risk RPE cells respond differently to intertumoral expression to UV light which leads to an improvement in cellular and functional phenotype only in the high risk AMD RPE cells. Taken together our data indicate that the patient specific iPSC model provides a robust platform for understanding the role of complement activation in AMD, evaluating new therapies based on complement modulation and drug testing.

The Impact of Systemic Inflammation on the Retina: Implications for Age-related Macular Degeneration

Ibbett, P.1, Lotery, A.2, Teeling, J.3
1University of Southampton, Biological Sciences, Southampton, United Kingdom, 2University of Southampton, Vision Group, Southampton, United Kingdom

Age-related macular degeneration (AMD) is a neurodegenerative disease of the retina and the leading cause of blindness in the UK. Genetic studies and mouse models demonstrate a clear role for local immune activation in AMD pathology, and increasing evidence suggest that systemic inflammation can further contribute to the progression of AMD by exacerbating ongoing inflammation in the retina, leading to earlier development of blindness. To study the possible underlying mechanisms we induced local retinal inflammation in mice, resulting in deposition of complement in the subretinal space, along with the activation of microglia. These inflammatory changes were accompanied by transient deficits in electrophysiological recordings. Systemic inflammation, induced by a bacterial infection, results in retinal inflammation. Increased expression of MHCI, MHCIIC, ICAM-1 and VCAM-1 on retinal blood vessels, indicates that retinal blood vessels are activated. Furthermore, increased expression of FoxC1, CD11b and CD11c on microglia was observed, as well as recruitment of T cells to the retina. These changes in inflammatory cells were accompanied by increased protein expression of a range of proinflammatory cytokines (IL-1β, IL-6, INFγ, mIL-12, TNFα, IL-10), which were upregulated for up to 4 weeks after the infection. Systemic inflammation in mice with ongoing local retinal inflammation show higher numbers of activated myeloid cells in the subretinal space and choroid. Thus, systemic infection may recruit myeloid cells to the subretinal space, where there is chronic local inflammation and degeneration, which could accelerate diseases progression. We are currently investigating if systemic infections influence disease progression in other experimental models of AMD and in humans.

ORAL PRESENTATIONS
Ophthalmic Genetics/Genomics
Molecular Genetics of Age Related Macular Degeneration
Lottery A., AMD Genetics
University of Southampton, Southampton, United Kingdom

This talk will give an overview of our understanding of the molecular genetics of age related macular degeneration and how these insights are being translated into potential new therapies. Genotype-phenotype correlations will be discussed as well as insights gained from studying early onset forms of macular degeneration.

Complement Gene Expression and its Modulation in Human Retinal Pigment Epithelium
McHarg, S.1, Bayatti, N.1, Perveen, R.1,2, Brace, N.1, Booth, L.1, Unwin, R.1, Black, G.1, Day, A.1, Clark, S.1, Bishop, P.1,2
1University of Manchester, School of Biological Sciences, Manchester, UK, 2Manchester Royal Eye Hospital, CMFT, Manchester, UK

Introduction: Retinal pigment epithelial (RPE) cells are involved in the pathological processes underpinning age-related macular degeneration (AMD). Complement has been clearly implicated in the pathogenesis of AMD with genetic variants in complement genes modifying AMD risk. Here, we examine the transcription and regulation of complement gene transcription in both human primary RPE and RPE cell lines, whether it is altered by genetic variants in complement genes, and identify regulatory pathways of complement gene transcription.

Methods: RPE cells isolated from adult human donor eye tissue were cultured and their RNA extracted. Quantitative polymerase chain reaction (qPCR) was used to determine the expression of a range of complement genes including complement factor H (CFH) and complement component C3 (C3). Illumina HiSeq RNA expression analysis was performed on RNA from 12 primary RPE cultures.

Results: qPCR analysis of primary human RPE cell cultures demonstrated significantly elevated CFH expression in donors who are genetically high-risk for developing AMD (p = 0.0084) as compared to those who are low-risk. Further qPCR analysis demonstrated that the expression levels of CFH and C3 correlated significantly (R2 = 0.521, p = 0.0001). This observation was investigated further by RNA-seq transcriptome analysis of primary RPE cells which had high CFH and C3 expression, versus those with low expression. Furthermore, there is co-regulation of the majority of alternative and classical complement genes expressed (terming complement pathway genes and the lectin pathway are not expressed). Several canonical pathways relating to inflammation and immunology were upregulated in donors with elevated CFH/ C3 expression including IFNγ, TNFα and IL-1β pathways. Analysis of CFH and C3 putative promoter sites identified the transcription factor binding sites (CEBPβ, IRF1 & 2, and STAT3A), all of which were significantly upregulated in the high expressing CFH/C3 RPE cells.

Discussion: The expression of the majority of complement genes detected in primary RPE cultures is co-ordinated and there are interactions between complement gene expression and the expression of other inflammatory pathways implicated in AMD in these cells.

Genome-wide DNA Methylation Study in Dry Age-Related Macular Degeneration: Novel Candidate Loci Identified and Validated
Porter, L.F.1,2, Saptarsi, N.1, Fang, X.1, Ratli, S.1, Bishop, P.1,2, den Hollander, A.1,2, Venkata Chavali, M.1,2, Clark, S.1,2, Lilloo, L.1, Luminita, P.1
1University of Liverpool, Eye and Vision Science, Liverpool, United Kingdom, 2Radboudumc, Ophthalmology, Nijmegen, Netherlands

Purpose: Epigenetic mechanisms of gene regulation, including DNA methylation, are of considerable interest in age-related macular degeneration (AMD). Our goal is to identify differently methylated loci (DML) in the retinal pigment epithelium of patients with early/intermediate dry AMD. Materials and methods: Epigenome-wide case/control study was performed using the Illumina Human Methylation450 BeadChip Array on a total of 44 samples of RPE/choroid extracted DNA from donor eyes with early and intermediate dry AMD and control eyes. Data analysis was performed in R. Modelling with an interaction term was employed to detect differentially methylated probes with fitted model parameters evaluated by t-test and corrected for multiple testing. We applied combinations of FDR and delta beta to our data-set with cut-off criteria FDR less than 0.2 and delta beta greater than 0.1 (10% methylation difference). Bisulphite pyrosequencing was applied for technical replication and biological validation on a total of 55 samples. qPCR on human donor RPE was performed to explore gene expression changes associated with loci of differential methylation. Results: We identified 8 differentially methylated CpG loci, and 4 differentially methylated regions in Dry AMD RPE/choroid samples with 3 CpG-probes reaching genome-wide significance (P<5x10⁻⁶). Validation of DNA methylation changes using pyrosequencing confirmed a reduction in methylation around cg18934822 in an intronic region of the SKI proto-oncogene in dry AMD, and an increase in the methylation of cg22086264 GT2/H4 in dry-AMD amongst other changes. qPCR confirms expression changes in these genes. Conclusions: We have performed the largest study on DNA methylation changes occurring in RPE in the prevalent early and intermediate forms of dry AMD. Our sample size is significantly higher than those used in other epigenomic studies of AMD with a focus exclusively on the less studied forms of AMD and the RPE. Epigenetic dysfunction of RPE has recently been suggested to drive the disease. We identify and validate novel candidate loci for dry AMD functional studies into tissue-specific disease mechanisms.

Whole Genome Analysis of Inherited Retinal Disease Patients Reveals Mutations Intractable to Other Detection Strategies
Arno, G.1,2, Carls, K.J.1,2, Nilböck, M.1, Waseem, N.1, Cheetham, M.E.1, Michaelides, M.1,2, Moore, A.T.1,2, Raymond, F.L.1,3, Webster, A.R.1,2
1UCL Institute of Ophthalmology, London, United Kingdom, 2 Moorfields Eye Hospital NHS Foundation Trust, London, United Kingdom, 3University of Cambridge, NHS Blood and Transplant Centre, Department of Haematology, Cambridge, United Kingdom, 4MC-UK, University Hospitals NHS Foundation Trust, Cambridge Biomedical Campus, NIHR BioResource - Rare Diseases, Cambridge, United Kingdom, 5UCSF School of Medicine, University of California San Francisco, Ophthalmology Department, San Francisco, United States, 6Cambridge Institute for Medical Research, University of Cambridge, Department of Medical Genetics, Cambridge, United Kingdom

Purpose: To identify and analyse pathogenic variants in whole genome sequencing (WGS) data from a cohort of patients with inherited retinal disease (IRD). Methods: Patients were recruited for WGS studies from the inherited eye disease clinics at Moorfields Eye Hospital, London, UK. 657 singletons and 250 families (trios or duos) underwent WGS using the Illumina TruSeq PCR-free kit on an Illumina HiSeq 2500 generating a minimum coverage of 15x for >95% of the genome. The Illumina pipeline including CANVAS and MANTA algorithms were used for single nucleotide (SNV) and structural variant (SV) calling. Candidate pathogenic non-coding variants were investigated in patients unsolved by prior IRD gene exonic variant analysis and focusing on those patients in whom there was evidence for a single gene (eg. suggestive clinical phenotype, carrier of single mutation in a recessive gene). Candidate intransive variants underwent in silico cryptic splicing analysis and likely pathogenic alleles were selected for functional testing.

OGMS - 100.000 genome project - Ocular results

The 100,000 Genome Project - An Overview
Boardman-Pretty, F., Genomics England, O.B.O.

Genomics England, London, United Kingdom

To bring the predicted benefits of genomics to National Health Service (NHS) patients, the UK Prime Minister launched the 100,000 Genomes Project in late 2012. The project will sequence around 70,000 patients. Participants are NHS patients with a rare disease, plus their families, and patients with cancer. A network of 13 NHS Genomic Medicine Centres have now collected over 76,000 samples and 69,000 genomes have been sequenced. Genome analyses have been returned to recruiting centres for 8,000 families.

To deliver the programme, a novel framework has been established to enable large-scale recruitment, clinical data collection and the development of a semi-automated interpretation pipeline. In the cancer programme, substantial work has been required to redesign and build new patient and sample processing practices. In the rare disease programme, there has been a particular focus on the ‘mainstreaming’ of recruitment, much of which now occurs outside clinics previously engaged in genomics. A major component of our work has been development of specifi-cally-developed data formats to allow NHS users, researchers and partner companies who provide components of the system to interact with the data via application programming interfaces (APIs). The approach benefits from the centralisation of sequencing and bioinformatic infrastructure while enabling recruiting clinical and laboratory control over validation and reporting of results and forming a platform for research and innovation. The Genomics England Clinical Interpretation Partnership (GE-CIP) was launched in 2015 with the goal of driving up the value of the data from the 100,000 Genomes Project for the NHS. GE-CIP now comprises over 2,900 researchers from over 350 institutions worldwide and research is underway, with over 1,300 researchers with access to the data within a secure Research Environment.

This talk will describe the approaches taken and lessons learnt in developing the programme and its infrastructure, share early insights from analysis of the genomes, discuss research using the Project data, and describe the future plans to support genome sequencing in routine diagnostic care in the NHS.
Ocular maldevelopment causes over a third of visual impairment and blindness worldwide. It is the most common cause of childhood sight impairment registration in the UK accounting for near 20% of cases. The first signs of the developing eye are seen at day 22 when the optic sulci are seen as furrows at the top of the neural tube. By week 7 the eye has formed its main structures, and the globe is intact. Aberrations in both the environment and genes acting in these early weeks are what lead to ocular maldevelopment. This ranges from structural globe anomalies (microphthalmia, anophthalmia and coloboma [MAC]), primary congenital glaucoma, anterior segment dysgenesis, congenital cataracts to foveal hypoplasia and retinal dysplasia. In a prospective UK incidence study 2% of structural globe anomalies were attributed to environmental factors such as maternal infections, vitamin A deficiency and teratogenic exposure. The ocular gene panel uses exome sequencing to look at all the coding regions and splice sites of all known eye disease genes of which there are approximately 450. For some subsets of ocular maldevelopment such as bilateral congenital cataract, the detection rate is reaching 80%, but in contrast patients with syndromic and non-syndromic MAC have a less than 7% diagnostic rate, despite 90 disease-causing genes being identified. Hence, we must begin to employ whole genome sequencing to improve our diagnostic rates of ocular maldevelopment, improve genetic counselling, clinical risk stratification and help us to develop treatments. There are now small molecule drug trials underway for conditions such as aniridia, which requires molecular characterisation of the causative mutation. And with the advent of nonsense suppression, antiseNSE oligonucleotides for splice site mutations, finding the genetic diagnosis is key.

Results: 272/657 families remained unsolved after exonic SNV and SV analysis in a panel of 224 known and candidate IRD genes and togeth-er harbored 55,000 rare variants (MAF < 0.0001) across the entire gene panel. Of these, 6,883 variants were predicted to alter splicing. Likely pathogenic intronic variants identified included a CRB1 variant (c.1879T>1203C>6) found in trans with a previously re-portedly deleted coding mutation, in a family of three affected siblings with clinically suspected CRB1-retinopathy. A second simi-larily affected proband was found to harbor the same deep intronic mutation in trans with a different reported mutation. Spline prediction analysis indicates this variant may activate an intronic mutation in trans with a different reported mutation.

Using NGS to Find ‘Difficult’ Alleles

Zeilis, C.
Institut de la Vision - Fondation Voir & Entendre, Paris, France

Conclusions: WGS enables interrogation of variants on a scale ne-ver before possible. We report potential pathogenic mutations in IRD genes intractable to other mutation detection strategies. In si-lico and in vitro functional validation (where possible) confirms the pathogenicity of these candidate mutations.

Utility of WGS in ocular maldevelopment management
Moosajee, M.
UCL Institute of Ophthalmology, Great Ormond Street Hospital for Children, and Moorfields Eye Hospital, London, United Kingdom

Over 80 novel IRD mutations have been discovered to date, this includes several novel large structural variants including inversions and deletions spanning several kilobases. Our findings have also proved useful in a clinical setting where candidate vari-ants have helped to resolve some previously ambiguous disease phenotypes. Our study has also led to the identification of several actionable variants in our cohort where there is active recruitment for natural history studies or clinical trials of new therapeutics.

Conclusions: The aim of the study is to characterise the genetic architecture of IRD in Ireland and in principle potentially enable clinical trials to be more accessible for some patients where appro-priate. Thus far, during the study, genetic analysis of IRD patients has helped to resolve ambiguous phenotypes and to identify cau-sative mutations in nearly 70% of cases. The continuous expansion of our cohort has enabled us to better interrogate the sequencing data and interpret the potential pathogenicity of novel variants when detected. In addition to this, the growing body of data from NGS studies of IRD globally should facilitate better correlations between genotype and phenotype and further refine methods for diagnoses and prognoses.

ORAL PRESENTATIONS
Ophthalmic Genetics/Genomics

Hypermorphic alleles associated with non-syndromic retinal disease - an advantage of the unbiased nature of next-generation sequencing
Webster, A.
Moorfields Eye Hospital, UCL Institute of Ophthalmology, London, United Kingdom

Whole exome and genome sequencing allows the unbiased appraisal of all genes in a person’s genome, where previously ana-lysis was carefully targeted to those genes already associated with the person's phenotype. Here we present patients with non-syn-dromic retinal disease in which likely-disease causing alleles in ‘unexpected’ genes were detected. Through cohorts of probands entering two related projects in the UK, the NHR-Bioresource and Genomics England, both involving whole-genome sequencing, we demonstrated the detailed retinal phenotypes of patients harboring specific alleles of genes normally causing severe neurologi-cal failure in affected persons, including CNL1 (MFSDB), CNL3 and HGSNAT. Moreover, mild phenotypes were associated with specific alleles of genes previously associated with early-onset blindness, in which, alleles confer partial function and include families segregating GUCY2D, TULP1 and CERKL. The detailed appraisal of such pati-ents is more than unravel the molecular and cellular pathology, than of those with more severe visual failure. The unbiased nature of modern sequencing is continuously refining the spectrum of phenotypes conferred by reported genes, beyond the experience of highly specialised clinicians.
Ophthalmic Genetics/Genomics

ORAL PRESENTATIONS

OGM6 - Mendelian glaucoma genetics

Molecular Basis for Glucocorticoid-induced Ocular Hypertension Responsiveness

Clark, A.F.

University of North Texas Health Science Center, North Texas Eye Research Institute, Fort Worth, United States

Glucocorticoids (GCs) are unsurpassed in their therapeutic anti-inflammatory and immunosuppressive activities and are commonly used to treat a wide variety of ocular conditions. Unfortunately, there are serious ocular side effects associated with prolonged GC therapy, including the development of GC-induced ocular hypertension (GC-OHT) and iatrogenic open-angle glaucoma. GC-OHT is due to increased resistance of aqueous outflow through the trabecular meshwork (TM). However, not everyone develops GC-OHT during GC therapy. Approximately 40% of the general population are “steroid responders” (i.e., develop GC-OHT), while the vast majority (>90%) of GC patients are steroid responders. The molecular basis for this steroid responsiveness was unknown until recently. There are two major alternatively spliced isoforms of the glucocorticoid receptor. GRα is the physiological and pharmacological ligand binding receptor responsible for the majority of GC biological activities. GRβ lacks the ligand binding domain and acts as a dominant negative regulator of GC activity. Elevated expression of GRβ occurs in a number of GC resistant diseases. We compared GRβ expression in normal and glaucoma human TM cells and found that GTM cells have significantly lower levels of GRβ, compared GRβ expression in normal and glaucoma human TM cells.

Primary open angle glaucoma (POAG) is a leading cause of blindness. Current treatments of POAG are aimed at reducing intraocular pressure (IOP), the most important risk factor for the development and progression of the disorder. However, current glaucoma treatments do not address the underlying disease mechanisms.

Mutations in the myocilin gene (MYOC) are the most common known genetic cause of glaucoma. Three developments provide us with an opportunity to investigate novel treatments of glaucoma directed at the underlying disease mechanisms:

1. The development of a POAG mouse model (tg-MYOCY437H) that expresses mutant myocilin encoded by the MYOC gene;
2. The use of this mouse model to confirm that mutant myocilin accumulates in the endoplasmic reticulum (ER) and induces ER stress in the trabecular meshwork (TM) of the eye leading to glaucoma;
3. The development of genome editing nucleases as a valuable tool for gene therapy applications.

The fact that myocilin-associated POAG results from dominant gain of function mutations and the fact that ER stress induced apoptosis in the TM leads to POAG provided an opportunity to explore novel glaucoma treatments by in vivo inactivation of MYOC and/or myocilin apoptosis pathway genes using the Tg-MYOCY437H mouse model.

We have used this and other mouse models to explore the use of a novel genome editing method (CRISPR) for glaucoma treatment. Our experiences with genome editing in both mouse models and a human anterior segment organ culture system will be presented.

Genome Editing in the Treatment of Glaucoma

Sheffield, V.1, Jain, A.1, Clark, A.2, Fingert, J.1, Zode, G.2

1University of Iowa Institute for Vision Research, Pediatrics and Ophthalmology, Iowa City, United States, 2University of North Texas Health Science Center, North Texas Eye Research Institute, Fort Worth, United States

Two genes, optineurin (OPTN) and TANK binding kinase 1 (TBK1), are known to cause open angle glaucoma that occurs with low intraocular pressure (normal tension glaucoma). The Glu508lys mutation in OPTN and TBK1 gene duplications or triplications are each associated with “1% of cases of NTG. TBK1 encodes a kinase that phosphorylates and activates OPTN, an autophagy receptor protein. Consequently, we have hypothesized that mutations in TBK1 or OPTN may cause glaucoma by dysregulating autophagy in key ocular tissues. Here we present studies of the mechanisms by which TBK1 gene duplications cause glaucoma with transgenic mice and patient derived cells.

TBK1 Gene and Normal Tension Glaucoma Pathophysiology

Fingert, J.

Carver College of Medicine, University of Iowa, Ophthalmology, Iowa City, United States

We recently identified two protein isoforms of the TGFβ superfamily activator inhibitor-1 (PAI-1) and fibronectin (FN), leading to decreased outflow facility (C) and increased IOP. Bone morphogenetic proteins (BMPs), via BMP receptors, antagonize these effects of TGFβ2 on cultured TM cells via expression of inhibitor of DNA-binding (ID) proteins ID1 and ID3. Over-expression of hTGFβ2 in mouse eyes increases TM expression of FN and PAI-1, decreases C, and elevates IOP. We investigated the effects on IOP and C of co-expression of hTGFβ2 and either ID1 or ID3 in mouse eyes.

Methods: Retired breeder Balb/cJ mice (P, 40-48 weeks, 20-38g) were used. Bilateral baseline IOPs were measured (TonoLab). Animals were anesthetized and the left eyes were given an intravitreal injection of Ads.CMV.hTGFβ2 (10^11 cfu) or Ads.CMV.ID1 or Ads.CMV.ID3 or Ads.Null (control) as a single 2ul bolus (5x10^12 cfu, n=3/group). 2 days later, a 2nd injection was given, thus co-expressing two different proteins in some eyes, and only one protein, or no protein, in others. 3 days following the 2nd injection, IOP was assessed at 3x per week for 3 weeks. C was then measured.

Results: Over-expression of hTGFβ2, C226/228S and either ID1 or ID3 in mouse eyes.

- Co-expression of hTGFβ2 and ID1 increased IOP (14.0+/-0.8 mmHg with ID1 vs 13.0+/-2.0 mmHg (uninjected control), N/S). A similar result was seen with ID3 (19.0+/-1.0 mmHg with ID3) vs 18.0+/-2.0 mmHg (uninjected control), N/S).

Conclusion: ID1 and ID3 suppressed the TGFβ2-mediated elevation in IOP and decrease in C. These proteins may show promise as candidates for development of additional IOP-lowering therapies in POAG.

Ophthalmic Genetics/Genomics

ORAL PRESENTATIONS

ID1 & ID3 Proteins Block TGFβ2-Induced Ocular Hypertension and Decreased Aqueous Humor Outflow Facility in Mice

Miller, J.C., Mody, A.A., Wordinger, R.J., Clark, A.F.

University of North Texas Health Science Center, Pharmacology & Neuroscience (North Texas Eye Research Institute), Fort Worth, United States

Purpose: Elevated intraocular pressure (IOP) is a major risk factor for primary open-angle glaucoma (POAG). POAG is associated with elevated levels of TGFβ2, in the trabecular meshwork (TM), which increases deposition of the extracellular matrix proteins plasminogen activator inhibitor-1 (PAI-1) and fibronectin (FN), leading to decreased outflow facility (C) and increased IOP. Bone morphogenetic proteins (BMPs), via BMP receptors, antagonize these effects of TGFβ2 on cultured TM cells via expression of inhibitor of DNA-binding (ID) proteins ID1 and ID3.

Two genes, optineurin (OPTN) and TANK binding kinase 1 (TBK1), are known genetic cause of glaucoma. Three developments provide us with an opportunity to explore novel glaucoma treatments by in vivo inactivation of MYOC and/or myocilin apoptosis pathway genes using the Tg-MYOCY437H mouse model.

We have used this and other mouse models to explore the use of a novel genome editing method (CRISPR) for glaucoma treatment. Our experiences with genome editing in both mouse models and a human anterior segment organ culture system will be presented.

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1University of Iowa Institute for Vision Research, Pediatrics and Ophthalmology, Iowa City, United States, 2University of North Texas Health Science Center, North Texas Eye Research Institute, Fort Worth, United States

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Carver College of Medicine, University of Iowa, Ophthalmology, Iowa City, United States

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Conclusion: ID1 and ID3 suppressed the TGFβ2-mediated elevation in IOP and decrease in C. These proteins may show promise as candidates for development of additional IOP-lowering therapies in POAG.
The Role of Cell Stress Responses in Retinal Degeneration

Cheetham, M.

UCI Institute of Ophthalmology, London, United Kingdom

The main cellular responses to proteotoxic stress are the heat shock response (HSR) and unfolded protein response (UPR). The adaptive programmes respond to stress by reducing translation and increasing the transcription of protective factors, but prolonged stress can also lead to the initiation of cell death related pathways. The induction of the HSR and UPR is observed in models of several forms of inherited retinal dystrophies, yet the role of the HSR and UPR in the pathogenesis of retinal degeneration is currently unclear. Here I will discuss the HSR and UPR in the context of the photoreceptor stress response and the evidence for their role in photoreceptor cell death.

Integrated Stress Response, Protein Synthesis and New Therapeutic Strategies for Treatments of Retinal Degenerative Disorders

Gorbatyuk, M., Starr, C., Pitale, P.

University of Alabama at Birmingham, Birmingham, United States

Varied animal models of inherited retinal degeneration (IRD) express aberrant mutant proteins in photoreceptors that are characterized by aggregation of misfolded proteins and chronic activation of the integrated stress response (ISR). Cellular features of the ISR include downregulation of CAP-dependent general protein synthesis, through activation (phosphorylation, p) of eukaryotic initiation factor 2α (eIF2α), and upregulation of certain transcription factors that promote CAP-independent or IRES-mediated translation. As an example of the latter, translation of activating transcription factor 4 (ATF4) is found to be elevated during ISR activation in degenerating retinas. In general, translational control is carried out by two independent nodes. In addition to p-eIF2α-mediated control, the second node of translational regulation is presented by the p-6-KT-p-40SR-p-eIF4F Binding Protein 1 (p-eIF4F-BP1) axis. Therefore, we aimed to investigate whether degenerating retinas manifest chronic translational attenuation; whether translational halt is a cellular defense mechanism during the course of retinal degeneration or a consequence of chronic ISR activation contributing to retinal pathogenesis; and whether reprogramming the translational rate could retard the onset of retinal degeneration in mice. We assessed degenerating retinas of rd16, rd10, and T17M rhodopsin mice at postnatal days 15 and 20 and found that the rates of translation are significantly reduced in the range of 40-70%. Also, in addition to elevation in p-eIF2α levels, these retinas demonstrated a reduction in activation of the p-ATF4-p-mTOR-p-eIF4F axis, suggesting impairment of the second node of translational control.

Mind the gap: Cellular proteotoxic stress pathways in retinal degeneration

Cheetham, M., Gorbatyuk, M., Starr, C., Pitale, P.

University of Alabama at Birmingham, Birmingham, United States

We assessed the role of the ISR in degenerating retinas in the context of the photoreceptor stress response and the evidence for their role in photoreceptor cell death.

Stem Cell Modeling of ATFB6 Cone Photoreceptor Diseases

Lin, J.

University of California San Diego, La Jolla, United States

Activating Transcription Factor 6 (ATFB6) is an important regulator of endoplasmic reticulum (ER) function and homeostasis. Loss of function ATFB6 mutations are responsible for several types of early-onset cone photoreceptor disease, including achromatopsia (ACHM) and cone-rod dystrophy (CORD). To investigate the mechanism by which ATFB6 dysfunction leads to retinal disease, we collected fibroblasts from ACHM and CORD kindreds bearing ATFB6 mutations. We found that ATFB6 mutations compromised transcriptional activity by impaired intracellular trafficking and direct damage to DNA binding. We found that homozygous ATFB6 mutant fibroblasts were significantly more vulnerable to cell death in response to chemical agents that trigger ER stress and protein misfolding. We reprogrammed patient fibroblasts into induced pluripotent stem cells (iPSCs). We successfully differentiated ATFB6 iPSCs into cone photoreceptor sheets or retinal organoids. These stem cell models provide a platform to investigate the role of ATFB6 in cone photoreceptor diseases.

Mitochondrial Delivery of NR2f to the Nucleus Suppresses Proteotoxicity

O’Mealey, G.1, Pfaffer, K.1, Berry, W.2, Janknecht, R., Chan, J.3, Pfaffer, S.4

Oklahoma Medical Research Foundation, Oklahoma City, United States, 1University of Oklahoma Health Sciences Center, Dept of Surgery, Oklahoma City, United States, 2University of Oklahoma Health Sciences Center, Dept of Cell Biology, Oklahoma City, United States

Leber’s congenital amaurosis (LCA) is the most common inherited blinding disease in children. Mutations in 24 genes including guanylate cyclase 1 (GCLC), rhodopsin isomerase (RPE65, LCA2), and lecithin-retinol acyltransferase (LRAT, LCA14) have been associated with LCA. RPE65 and LRAT are critical in recycling 11-cis-retinal in retinal pigment epithelium while GC1 is important in synthesizing cyclic guanosine monophosphate in photoreceptors. Three mouse models GC1-/-, Rpe65-/-, and Lrat-/- (Rpe65-/- and Lrat-/- have similar phenotypes for LCA1, LCA2, and LCA14, respectively; widely used for mechanistic and therapeutic studies. Common pathologic features regarding cones are, 1) mislocalization of both short-wavelength opsin (S-opsin) and medium-wavelength opsin (M-opsin); and 2) S-opsin enriched ventral/conal cones degenerate much more rapidly than M-opsin enriched dorsal cones. The purpose of this study is to determine the mechanism of rapid “S-cone” degeneration in LCA1, 2, and 14. We found that mislocalized M-opsin was degraded whereas mislocalized S-opsin accumulated in both GC1-/- and Lrat-/- cones before the onset of massive ventral/conal cone degeneration. In transduced cells, human opsin and mouse S-opsin, but not mouse RPE65 or human red/green opsins, aggregated to form cytoplasmic inclusions and caused ER stress. Genetic deletion of S-opsin reduced ER stress and completely prevented the rapid ventral/conal degeneration in Lrat-/-S-opsin mice. Genetic deletion of S-opsin also completely prevented rapid ventral/conal degeneration in GC1-/-S-opsin mice. We have identified a phenylalanine-rich region in the S-opsin family (SW1) but absent from the medium/long-wavelength opsin family, which is responsible for S-opsin aggregation. Based on the mechanism we discovered, we designed two strategies to protect cones in LCA.

1. Systemic delivery of an ER chaperone, tauroursodeoxycholic acid, effectively reduced ER stress and preserved ventral/conal cones in Lrat-/- mice. 2. Intravitreal injection of nanoparticule-encapsulated small molecules that inhibit S-opsin aggregation protected ventral/conal cones from degeneration in Lrat-/- mice. Collectively, our data suggest that S-opsin aggregation-induced apoptotic ER stress plays a unified role in the rapid degeneration of “S-cones” in all three LCA models. The concept here is applicable to a wide range of cone dystrophies due to cone opsin mislocalization.

A Unified Mechanism for Multiple Forms of Cone Photoreceptor Degeneration

Fu, Y.1, Zhang, T., Enchemchukwu, N.2

1Boylor College of Medicine, Ophthalmology, Houston, United States, 2University of Utah, Salt Lake City, United States

Nears_InfraRed Imaging of Lymphatic Function and Cerebrospinal Fluid Outflow

Proulx, S., Mia, O., Ries, M., Ditmar, M.

ETH Zurich, Zurich, Switzerland

Standardized quantitative methods for measuring lymphatic function are lacking. We have developed a number of assays using near-infrared fluorescence imaging to evaluate many different aspects of the lymphatic system. New inert tracers that can be detected with high contrast by near-infrared fluorescence imaging systems have enabled quantitative assessments of lymphatic clearance, collecting lymphatic vessel contractility and lymphatic transport to the systemic blood circulation. We have applied this technology to disease models of lymphedema, cancer and chronic inflammation and clinical translation is underway. The textbook understanding of cerebrospinal fluid (CSF) outflow is that it drains through arachnoid punctures from the subarachnoid space to the dural venous sinuses. However, many reports have described a role for lymphatic vessels in this process. We utilized lymphatic-reporter mice and high-resolution stereomicroscopy to characterize the anatomical routes and dynamics of outflow of CSF.
After infusion into a lateral ventricle or cisterna magna, near-infra-
red tracers rapidly reached lymph nodes using routes along cranial
nerves, such as olfactory and optic nerves, through foramina in the
skull. Surprisingly, we found that lymphatic vessels are the major
outflow pathway for bulk flow tracers in mice, implying that the
lymphatic system and not the venous system is responsible for CSF
efflux. Less CSF lymphatic outflow was found in aged compared to
young mice, suggesting that the lymphatic system may represent a
target for age-associated neurological conditions. Data will also be
presented characterizing the relationship between lymphatic out-
flow and potential influx of CSF into the brain along paravascular
spaces.

Determining Mechanisms of Aqueous Humor Drainage Using Mice

John, S.1, Jeffrey, M.2, Tolman, N.1, Baldwin, T.1, DeVries, M.1, Kuzhiat, K.1

1Jackson Laboratory, Bar Harbor, United States, 2Tufts University, Boston, United States

This talk will summarize our recent findings relevant to Schlemm’s
canal and developmental glaucomas. The trabecular meshwork
(TM) and Schlemm’s canal (SC) drain aqueous humor (AHQ) from
the eye. The resistance to AHQ drainage (outflow) across the in-
ner wall of SC is an important determinant of IOP. Abnormally in-
creased resistance underlies high IOP in glaucoma. The molecular
mechanisms of AHQ drainage are only partially understood. To
improve knowledge of ocular drainage structure development and
mechanisms of outflow, we are developing new procedures and
tools for studying SC and TM. This presentation will summarize
recent findings, focusing on SC. Importantly, SC endothelial cells
(SECs) have a novel, specialized phenotype with properties that are
a blend of blood and lymphatic endothelia. We demonstrated that
SC is a polarized vessel with lymphatic expression being primarily
present in the inner wall endothelium. Thus, SC is a unique ves-
sel with endothelial cells that are highly specialized for its complex
functions. SECs express Prox1, a master regulator of lymphatic fate,
and other key vascular signaling/lymphatic molecules (e.g. TEK,
KDR). The lymphatic features are polarized being largely restricted
to the functionally specialized inner wall. SC development is KDR
(VEGFR2) dependent but differs to previously known processes of
vascular development. Mice with mutations affecting either TEK
or its ligand ANGPT2 develop an abnormal SC and TM. Following
the mouse studies, mutations in these genes were found to cause
developmental glaucomas in human patients. Beyond the eye, these
findings are of high interest in the vascular/lymphatic biology
communities.

IND3 - Dementia in the Eye

Neuropathological Hallmarks of Alzheimer’s Disease in Post Mortem AD Retinas
den Haan, J.1, Morrema, T.H.J.1, ten Brink, J.B.1, Verbraak, F.D.1, de Boer, J.F.1, Schelten, P.1, Rozemuller, A.J.2, Berg,
A.A.B.1, Bouwman, F.H.1, Hoozemans, J.J.1

1VU University Medical Center, Alzheimer Center, Neuroscience, Amster-
dam, Netherlands, 2VU University Medical Center, Department of Pathology,
Amsterdam, Netherlands, 3Academic Medical Center, University of Amsterdam, Department of Ophthalmology,
Department of Ophthalmology, Amsterdam, Netherlands, 4VU Medical Center, Ophthalmology, Amsterdam, Netherlands,
5VU University Medical Center, Department of Physics, Bio Laser Lab Amsterdam, Amsterdam, Netherlands

Purpose: In vivo labeling of retinal amyloid-beta (Abeta) might be a
promising non-invasive biomarker in AD. However, literature
on the presence of amyloid-beta in AD retinas is conflicting. One
group showed presence of amyloid plaques in AD retinas, while
three other groups were unable to replicate these findings([1]-[6]).
We therefore set out to assess the presence of amyloid-beta
(AB) and amyloid precursor protein (APP) in post mortem re-
tinas in a well characterized cohort of AD cases and controls.

Methods: We included 6 AD patients and 6 controls who donated
brains and eyes to the Netherlands Brain Bank between 2010 and
2015. Neuropathological diagnosis of AD was made following NIA-
AA criteria based on formalin-fixed paraffin-embedded tissue blocks
including the frontal cortex, temporal pole cortex, parietal cortex,
occipital pole cortex, and the hippocampus([1]). Patients had Braak
stages IV>V and amyloid scores C while controls had Braak stages II
and amyloid stages ranging from O to C (Table 1). Snap frozen eyes
were thawed in 4% PFA and dissected in four quadrants through the
horizontal and vertical meridian. Nasal-inferior and superior-temp-
oral quadrants were cut in 5 and 10 µm sections (Figure 1). Sections
were DAB-stained using the following primary antibodies: APP[AP-
P166], 6E10(Aβ1-16), 12F4(Aβ1-42), 4G8(Aβ17-24), and IC-16(Aβ1-16).

Results: AD retinas showed intracellular staining of ganglion cells
with 6E10 and 12F4 antibodies, slightly more prominent compa-
red to control retinas. This intracellular localization was similar to
the localization of APP. Stainings for IC-16 and 4G8 on the other
hand, were negative. In addition, incidentally small extracellular
inclusions (10-20µm) were seen in RNF1 and GCL of superior parts
of AD retinas exclusively with 6E10 and 12F4 antibodies (Figure 3).

Conclusions: Our results imply that AD pathological hallmarks ap-
ppear differently in the retina compared to the cerebral cortex.
Intracellular staining in the retina is most likely associated with APP
accumulation. To further explore this we will expand our cohort
and assess tau H1C in AD retinas.
The past few years have seen an enormous development in the field of ultrasonic measurement techniques to determine concentrations of neurodegeneration-related biomarkers in body fluids and tissues. The presentation will give an update on these methods, review how they can be used in CSF and blood to diagnose and monitor neurodegenerative diseases and discuss what roles they could play in the study of neurodegenerative changes in the eye. As an example, data on vitreous fluid concentrations of the axonal injury marker neurofilament light (NfL) in relation to macular holes, epiretinal membranes, vitreous macular traction, and vitreous floaters will be presented. The lower limit of quantification of the NfL assay was 1.2 pg/ml and repeatability and intermediate precision were 3.7-8.8% for two internal control samples representing low and medium NfL concentrations. NfL concentrations were measurable in all vitreous fluid samples (range 13.9 to 1273 pg/ml). Vitreous fluid NfL concentrations were similar in men and women and did not correlate with age. There was no difference in vitreous fluid NfL concentrations between phakic and pseudo-phakic patients and there was no relation to time after cataract surgery. Patients with epiretinal membranes had significantly higher vitreous fluid NfL concentrations than patients with vitreous floaters (p=0.03) or macular holes (p=0.02), whereas the epiretinal membrane and vitreous macular traction groups were similar. The latter result should be interpreted with caution because of the low sample number in the vitreous macular traction group (n=4). Study designs to assess the potential association of vitreous fluid NfL amyloid beta and tau concentrations with Alzheimer’s pathology in the brain will be discussed. The potential of using these markers in blood and CSF to improve the etiological classification of patients with cognitive impairment who undergo eye examination to study the association of degenerative changes in the brain and eye will also be discussed. 

Investigating Tau Pathology through the Eye: A Preclinical and Clinical Study of Frontotemporal Dementia

Harrison, L.E.1, Whitaker, R.2, Bertelli, P.M.1,2, O’Callaghan, J.M.1,2, Cisinsky, L.1,2, Boccetta, M.1, Ma, D.1, Fisher, A.1, Ahmed, Z.1, Murray, T.K.1, O’Neill, M.J.1, Rohrer, J.D.1,2, Lythgoe, M.F.1, Lengyel, I.1,2

1 University College London, London, United Kingdom, 2 University College London, UCL Institute of Ophthalmology, London, United Kingdom, 3 Queens University Belfast, Centre for Experimental Medicine, Belfast, United Kingdom

Visual impairments, such as difficulties in reading and finding objects, perceiving depth and structure from motion, and impaired stereopsis, have been reported in tauopathy patients. Previously however, these defects have been attributed to cortical tau pathologies, rather than changes in the neural retina and optic nerve themselves, for example impairment of the recently described ‘glymphatic’ clearance system, leading to accumulation of neurototoxic tau in regions associated with visual processing. Tau mediated pathogenic mechanisms have been previously shown to be involved in retinal degeneration in glaucoma patients. However, there is lack of evidence regarding the effect of tau on retinal neurodegeneration in tauopathies such as frontotemporal dementia (FTD). Moreover, recent evidence in support of the existence of glymphatic clearance mechanisms in the eye and optic nerve suggest that the neural retina and optic nerve themselves may be prone to neurotrophic accumulation of tau in degenerative tauopathies. In the current study we sought to investigate tau pathology in the visual system of an animal model of FTD, the rTg(tauP301L)4510 mouse, through localisation and quantification of tau pathology in the neural retina and vitreous humor. To achieve this, we firstly showed that the expression of a human tau isoform with a P301L mutation is sufficient to result in neurodegeneration in the eye and optic nerve. More specifically, we demonstrated that: 1) tauopathy is evident in the peripheral retina of FTD patients; 2) human tau pathology in the eye is not accompanied by any neuronal loss in the retina or optic nerve; 3) tauopathy is evident in the eye of a transgenic mouse model of FTD, the rTg(tauP301L)4510 mouse model of FTD. Significant tau deposition is also observed in the neural retina of this transgenic line, concurrent with degeneration in tau loaded layers. These data suggest that it is likely that ophthalmic tau pathology also exists in the eyes of FTD patients, highlighting the role of clearance systems, such as glymphatic function, in the aetiology of tauopathic disease processes.
Eye Morphogenesis in the Blind Mexican cavefish

Deyos, L.1, Hinaux, H.1, Recher, G.1, Klee, F.1, Edouard, J.-J.2, Blin, M.1, Sohn, F.1, Déaux, S.1

ChRs, Paris-Saclay Institute of Neuroscience, Gif sur Yvette, France; CNRS/INRA, Amagón Platform, Gif sur Yvette, France

The fish Astyanax mexicanus comes in two forms: a normal ri-
ver-dwelling form, and a blind cave-dwelling morph. Although the adult cavefish is completely colorless, eyes first develop in embryos. The embryonic cavefish eyes suffer several molecular and morpho-
genesis defects: the lens undergoes apoptosis around 24 hours post-fertilization, and the retina shows a coloboma phenotype with an apparent lack of optic fissure closure. Through the analysis of regionalization and cell specification markers gene expression pat-
terns and through 3D-time live imaging of the morphogenesis of the lens and the optic vesicle in the two morphs, we have examined the comparative development of the cavefish and surface fish eyes. We found that the early eye field and the early lens placode are reduced in size in cavefish, and that cell movements responsible for the invagination of the optic vesicle to form the optic cup are impaired. Red molecular potential and cellular mechanisms will be discussed. Work supported by ANR, Equipe FRM, and AVIESAN/INSERM grants.

Mechanisms Underlying Lens Transdifferentiation in Neural Retina Cells and Pituitary Precursors

Kondoh, H.

Kyoto Sangyo University, Faculty of Life Sciences, Kyoto, Japan

Multiple developmental pathways lead to lens differentiation in addition to the form which are commonly re-
ferred to as lens transdifferentiation. During normal lens develop-
ment, the first step, beginning with the lens placode formation and leading to lens vesicle development, is triggered by the co-
operation of two transcription factors (TFs), SIX2 and PAX6. The second step of mature lens differentiation requires the action of several TFs, including PROX1 and PITX3. Lens regeneration from the dorsal iris in newts fully recapitulates the two-step process of normal lens development, wherein the first step is triggered by FGFR2 signaling and the second step by canonical WNT signaling. However, the following two cases illustrate different situations. Chicken embryonic neural retina cells, expressing high levels of SIX2 and PAX6, developed into lens cells at a low frequency under the spreading culture condition, where cell-cell interaction are reduced compared with that in vivo. Inhibition of NOTCH signaling by addition of DAPT to the neural retina culture dramatically ac-
tivated lens development, causing most cells to differentiate into lenses. Following DAPT addition, Prox1 expression was quickly activated, which was followed by Pitx3 activation. This indicates that the first step of lens expression is already primed in retinal cells owing to the endogenous expression of SIX2 and PAX6, how-
ever, full lens development is suppressed by NOTCH signaling.

An analogous condition appears applicable to pituitary precursors, which express SIX2, PAX6, and PITX3, in addition to pituitary-spe-
cific TFs LHX3 and NKX2.2. In mutant embryos of Japanese medaka and zebrafish defective in hedgehog signaling, the pituitary pre-
cursors developed into lens tissues, presumably due to the lack of LHX3 and NKX2.2 expression. Thus, lens developmental potential in the pituitary precursors is suppressed by hedgehog signaling. Thus, the endogenous expression of SIX2 and PAX6 can prime tis-
sues to develop into lenses, but this potential is repressed in tissu-
es other than the ocular lens via tissue-dependent mechanisms. Repression of noncanonical developmental potentials in a tissue may be applicable to a wide range of cell types other than the lens.

Specification of the Retina Pigment Epithelium and Its Implication in Vertebrate Optic Cup Morphogenesis

Bovolenta, P.1,2

CSIC-UCM, Madrid, Spain; CNB-BM, ICIC, Madrid, Spain

The optic primordium is initially organized as a vesicle evaginated from the anterior neural plate. During eye morphogenesis the optical neuroepithelium folds over itself and gives rise to a cup-shaped structure called the optic cup. Concomitant to this process, eye progenitors become specified and differentiate into three cell subpopulations: the neural retina, the optic stalk and the reti-

cal pigment epithelium (RPE). Upon specification, RPE cells un-
dergo considerable shape and molecular transformations, which are thought to confer distinctive biomechanical properties on the RPE as a whole. How these transformations occur and whether they are required for optic cup folding is still poorly understood. To address these questions, we have identified a genomic regula-
tory element that allows us to manipulate gene expression specifici-
fication starts in a very discrete dorsal/proximal region of the optic vesicle, which initially presents a pseudostratified organization. RPE cells then transit through a cuboidal shape to finally acquire a squa-
mous configuration. These changes are sufficient to cover the back of the optic cup with little contribution of cell proliferation. Tissue specific interference with the acto-myosin cytoskeleton indicates that RPE flattening depends on its own acto-myosin activity and not on specific interference with the acto-myosin cytoskeleton.

Red molecular potential and cellular mechanisms will be discussed. Work supported by ANR, Equipe FRM, and AVIESAN/INSERM grants.

Single-cell RNA-Seq Analysis of Mouse Retinal Development Identifies NFI Factors as Essential Regulators of Late-born Cell Specification and Mitotic Exit

Blackshaw, S.

Johns Hopkins University School of Medicine, Neuroscience, Balti-
more, United States

The mammalian retina is an excellent model for understanding the molecular mechanisms that regulate temporal patterning and neurogenesis, but a comprehensive picture of gene regulatory networks that control these processes is lacking. To generate this, we conducted an extensive RNA-Seq analysis of the developing mouse retina at single cell resolution. We have profiled gene expression at 10 different time points ranging from embryonic day 11 to postnatal day 14 using single-cell RNA-Seq analysis of mouse retina, profiling over 120,000 individual cells. We are able to recover developmental trajectories of individual retinal cell fates, part-
tners of co-regulated genes involved in each of these developmen-
tal processes, and to identify the genes and gene networks that directly influence competence, neurogenesis and cell fate specification during development. We have used these data to identify a comprehensive set of candidate genes that regulate progenitor competence and retinal neurogenesis, and identify the NFI family of transcription factors as necessary and sufficient for generation of late-born retinal cell cells as well as mitotic exit by retinal progenitors. This work represents an important resource for further studies of retinal development and additionally provides a template for investigation of temporal patterning in all areas of the developing CNS.

Analysis of Retinal Development at Single-cell Resolution Identifies NFI Factors as Essential for Mitotic Exit and Specification of Late-born Cells

Blackshaw, S.

Johns Hopkins University School of Medicine, Neuroscience, Balti-
more, United States

Precise temporal control of gene expression in neuronal progeni-
tors is necessary for correct regulation of neurogenesis and cell fate specification. However, the tremendous cellular heterogeneity of the developing CNS has posed a major obstacle to identifying the gene regulatory networks that control these processes. To ad-
dress this, we used single-cell RNA-sequencing to profile ten de-
velopmental stages encompassing the full course of retinal neuro-
genesis. This allowed us to comprehensively characterize changes in gene expression that occur during initiation of neurogenesis, changes in developmental competence, and specification and differentiation of each of the major retinal cell types. These data identify a rapid and major transition in gene expression between early and late-stage retinal progenitors, as well as a distinct class of neurogenic progenitors. Using these findings as a starting point, we have identified the NFI family of transcription factors (NFIx, NFIy, and NFIz) with enriched expression within late RPCs and as regula-
tors of both the specification of late-born bipolar interneurons and Muller glia and the onset of proliferative quiescence.

Temporal Requirement of Mab21l2 during Eye Development in Chick Reveals Stage Dependent Functions for Retinogenesis

Sahari, S., Gunhaga, L.

Umea Centre for Molecular Medicine, Umeå, Sweden

Purpose: Different missense mutations in the single exon gene Mab21l2 have been identified in unrelated families with var-ious bilateral eye malformations, including microphthalmia, anophthalmia and coloboma, but the molecular function of Mab21l2 during eye development still remains largely unknown.

Methods: We have established an in vivo Mab21l2-deficient eye development model in chick, by using a Mab21l2 RNA interfer-
ence construct that we electroporated in vivo in prospective reti-
 nal cells. In addition, we designed a Mab21l2 gain-of-function electroporation vector. Mab21l2-modulated retinoblasts were analy-
sed on consecutive sections in terms of morphology, and mole-
cular markers for apoptosis, cell proliferation and retinogenesis.

Results: Our Mab21l2-deficient chick model mimics human ocu-
lar phenotypes. When Mab21l2 is down-regulated prior to op-
tic vesicle formation, the embryos develop anophthalmia, and Mab21l2 inhibition by optic cup stages results in a microph-
thalmic colobomatous phenotype. Our results show that inhi-
bition of Mab21l2 affects cell proliferation, cell cycle exit, and the expression of Atoh7/Atoh5, NeuroD4/Atoh4, Islet, Pax6, AP-2α, and Prox1. In addition, Mab21l2 over-expression hampers cell cycle exit and differentiation of retinal progenitor cells (RPCs).

Conclusions: Our results highlight the importance of a regula-
ted temporal expression of Mab21l2 during eye development; i) at early stages Mab21l2 is required to main-
tain RPC proliferation and expansion of cell number, ii) before retinogenesis a decrease in Mab21l2 expression in pro-
fiterating RPCs is required for cell cycle exit and differentiation, iii) during retinogenesis Mab21l2 is chronologically up-regulated in RPCs, followed by differentiated horizontal and amacrine cells, and cone photoreceptor cells.
Loss of αB Crystallin-derived Chaperone Peptides in AMD

Mammalian Target of Rapamycin as a Potential Target of Neovascular Age-related Macular Degeneration: A Evidence in Laser-induced CNV Mouse Model

PREVENTIVE MECHANISM AND THERAPEUTIC POTENTIAL OF αB CRISTALLIN-DERIVED CHAPERONE PEPTIDES IN AMD

IND6 - Proteinopathy in AMD: protein misfolding, clearance and putative therapy targets

Cystatin C in the Regulation of Proteostasis in RPE Cells

Session: Cross-Discipline

Cystatin C in the Regulation of Proteostasis in RPE Cells

Paraoanj, L.

University of Liverpool, Institute of Ageing and Chronic Disease, Eye and Vision Science, Liverpool, United Kingdom

Loss of proteostasis in the RPE, both intra- and extracellularly, is key to understanding the mechanisms responsible for the cascade of events leading to AMD, associated with different levels/forms of proteins, excessive protein misfolding, misfolding, aggregation, and degradation leading to loss/gain-of-function phenotypes. RPE invests a remarkable metabolic effort in synthesizing and maintaining appropriate levels of a broad range of proteolytic enzymes and their inhibitors; these are among the most abundantly expressed proteins by RPE, with the potent cytoskeletal protein inhibitor cystatin C in the top 2%. We have used cystatin C as a model for studying various aspects of proteostasis that are essential for the normal RPE functions, that change with aging and become impaired in degenerative processes, such as compromised intracellular trafficking, organelle interactions, protein misprocessing and imbalance of proteolytic activities. Cystatin C inhibits cathepsins B, H, L, S, in the posterior eye it is expressed almost exclusively by the RPE and secreted basolaterally, supporting a function related to Bruich’s membrane/choroid. Variant B cystatin C (CAMP2/1) is associated with increased risk of developing exudative AMD and presents leader sequence-related protein misprocessing and imbalance of proteolytic activities. The retinal-pigmented epithelium (RPE) is a single layer of cells interposed between the neurosensory retina and choroid. The RPE has a variety of critical functions and are among the most active phagocytes in the body and as a result, they have a high metabolic activity. Because they are post-mitotic cells with high metabolic activity, RPE cells rely on a high rate of autophagy to maintain their health. Thus, to ensure functional integrity of both the photoreceptors and the RPE, autophagy and phagocytosis, which are highly dependent upon lysosomes, must maintain intact in the RPE. Our recent work focuses on understanding the mechanisms that regulate both autophagy and phagocytosis in RPE cells, which is based on our discovery that αB/A1-crystallin (encoded by the gene Cryba2), an abundant lens protein, is also ubiquitously expressed in the RPE. We found that αB/A1-crystallin is localized to the lysosomal lumen where it binds with the V$_{$_1}$ subunit of lysosomal V-ATPase and modulates mechanistic target of rapamycin, complex 1 (mTORC1) signaling, which is a critical mediator of nutrition and metabolism. Lysosomal calcium plays an essential role in regulating mTORC1 signaling. Recently, it has been suggested that mTORC1 is a new calmodulin-dependent kinase. Interestingly, our proteomics study has identified an abnormal phosphorylation profile of proteins involved in calcium signaling in the RPE (Bruich’s membrane-choroid complex) of Cryba2 knockout mice compared to age-matched control mice. Specifically, we found reduced phosphorylation of calcium/calmodulin-dependent protein kinase type 1 (CaMK1D), calcium regulated heat shock protein 1 (HSPH1), nucleobindin-1 (NUCB1), doublecortin like kinase 1 (DCLK1) and spectrin beta (SPTB2). Further, our data suggest that RPE cells lacking Cryba2 have abnormal calcium homeostasis. Our studies delineate a unifying mechanism as to how lysosomal function, and hence autophagy and phagocytosis, is regulated in RPE cells during health and disease.
The Circadian Clock Modulates Photoreceptor Functioning and Viability during Aging

Tozini, G.
Morehouse School of Medicine, Atlanta, United States

Existence of daily rhythms in behavior and physiology is one of the major hallmarks of life on Earth. This characteristic has developed throughout evolution from bacteria or unicellular algae to vertebrates, in accordance with periodic variation of environmental factors such as the 24 h day/night cycle. Not surprisingly, circadian rhythms (circadian = about 24 h) are controlled by conserved, cell-autonomous and self-sustaining mechanisms. The day/night cycle is the major environmental factor able to entrain clocks, also known as a Zeitgeber (time-giver). In mammals, eyes constitute the only light input pathway to the circadian system. The retina was actually the first tissue outside of the suprachiasmatic nuclei or the hypothalamic tissue described to display circadian clock properties, based on its capacity to synthesize and release melatonin in vitro with a 24 h rhythm. This observation triggered thorough analysis of retinal physiology over the 24 h cycle and many molecular and cellular processes are presently established to be under clock control. These extend from the expression of photopigments to visual processing and they also include processes linked to retinal survival such as rhythms in retinal pigment epithelium phagocytosis and the vulnerability to phototoxicity. In this presentation we describe how dysfunction of the circadian clock or by its outputs affects photoreceptor functioning and viability. In particular we will focus on how removal of the clock gene Bmal1 and of melatonin signaling affects photoreceptor viability and functioning during aging.

Melatonin is a key player in the circadian physiology of the retina

Laurent-Gyde, V.1, Giansesi, C.1, Tozini, G.2, Hicks, D.1
1Institut des Neurosciences Cellulaires et Intégratives-CNRS UPR3212, Neurobiology of Rhythms, Strasbourg, France, 2Morehouse School of Medicine, Department of Pharmacology and Toxicology and Neuroscience Institute, Atlanta, United States

Melatonin is described as a "zeitgeber" hormone mainly produced in the pineal gland but is also present at significant levels in the neural retina. The indolamine exerts local effects on neuronal electric activity or light sensitivity. We aimed to characterize melatonin synthesis, circadian regulation and potential roles as a zeitgeber in the retina. For this study, we used Angiogenesis or formation of new vessels is a metabolically demanding process. An important function of the circadian clock is to regulate the metabolic machinery in anticipation of metabolic demands. Systemic loss of this synchrony between energy sources or growth factors that regulate vascular growth and function can be an additional risk factor for increased susceptibility towards vascular diseases. The molecular links between loss of circadian function and its influence on the growth and maintenance of the ocular vascular networks are relatively unexplored. Here, we present evidence that Bmal1 is required both in the mouse retinal neurons and the endothelial cells for vascular growth and patterning. Loss of Bmal1 from the retinal neuron results in increased retinal vascular density. However, the endothelial clock exerts an exactly opposite influence on the retinal vasculature, where by loss of endothelial Bmal1 results in reduced branching and less proliferation. To identify the molecular targets, we performed a ChIP-Seq analysis and show that Bmal1 in the neurons regulate the expression of the anti-angiogenic molecule sFlt1 (Vegf receptor 1) and EphinB2. Loss of Flt1 from the neurons results in a similar phenotype as that of Bmal1. Finally, to assess the influence of clock genes in vascular homeostasis and maintenance we have used the laser-induced choroidal neovascularization assay to determine if the loss of clock genes affect the vascular barrier function and integrity of the vasculature. Our data suggest that these functions are compromised by the loss of Bmal1. In conclusion, the circadian clock genes have differential effects on retinal vascular growth, remodeling, and function. We speculate that the circadian disruption could shift the balance of pro versus anti-angiogenic factors.

Multiple Effects of Retinal BMA11 Disruption: Structure and Function during Development and Aging

Iwane, P.M.
Emory University, Ophthalmology, Atlanta, United States

The retina and RPE contain networks of circadian clocks that regulate gene expression, post-translational modifications, rod-cone coupling, photoreceptor outer segment disc shedding, photic responses, and visually-guided behavior. Previous studies have shown that there are circadian rhythms in contrast sensitivity and photopic ERG B-wave amplitudes, but not scotopic A- or B-wave amplitudes. I will review recent studies from our lab and others investigating the mechanisms involved. We have conditionally disrupted the clock gene, Bmal1 (Arntl), from retina (rBmal1 KO). These mice show an 85-95% loss of Bmal1 mRNA compared to controls. In adult mice, this reduces, but does not abolish, the amplitude of the rhythm of contrast sensitivity measured with the optometer response, suggesting that retinal clocks as well as clocks outside the retina contribute to this visually guided reflex behavior. The circadian rhythm of photopic ERG B-wave amplitude of rBmal1 KO mice is nearly abolished and, strikingly, the mice fail to light adapt day or night. Although the scotopic ERG A- and B-wave amplitudes do not show circadian rhythms, the B-wave amplitudes are reduced in the rBmal1 KO mice compared to controls. SD-OCT was used to follow possible changes in retinal structure during postnatal development and aging. As early as 3 months of age, the outer plexiform layer (OPL), inner nuclear layer (INL), and inner plexiform layer (IPL) were thinner in the rBmal1 KO mice compared to controls. No thinning of the outer nuclear layer (ONL) or ganglion cell nerve fiber layer (GCL) was observed at this age. The thinning of the OPL, INL and IPL progressed with advancing age, and by 12 months of age thinning was significant. Statistically significant reductions in ONL thickness were observed in the rBmal1 KO mice. The GCL thickness of rBmal1 KO mice was significantly thinner than control at 18 and 24 months of age. Consistent with the thinned OPL and the reduced scotopic B-wave amplitudes, abnormal dendrites of rod bipolar cells were observed in 3 month-old and older rBmal1 KO mice. In addition, disruption of rBmal1 was found to accelerate the age-related cone photoreceptor death compared to age-matched controls. The remaining cones in 26 month-old rBmal1 KO mice had greatly reduced outer segment length compared to controls. These studies indicate that retinal Bmal1 plays important roles in retinal structure and function during development and aging.

An Opposing Role for Neuronal and Endothelial Bmal1 in Retinal Vascular Growth, Remodeling, and Homeostasis

Rao, S.1, Jidigam, V., Fuller, R., Singh, R., Sawant, O., Lang, R.1
1Cleveland Clinic, Ophthalmic Research, Cleveland, United States, 2Cincinnati Children’s Hospital, Pediatric Ophthalmology/Visual Systems Group, Cincinnati, United States

Bmal1 in Retinal Vascular Growth, Remodeling, and Homeostasis

We have demonstrated a ChIP-Seq analysis and show that Bmal1 in the neural retina, rich in cone photoreceptors (33% against 2% in mouse). Cones are essential for visual acuity and color vision and are thought to be involved in the synthesis of melatonin. We report that Arylalkylamine N-acetyltransferase (AA-NAT) expression and activity span over the 24 h periods, albeit a tendency to increase during the night. Hydroxyindole-O-methyltransferase (HIDMT) activity and melatonin secretion are both constitutive and with a low level. AA-NAT and HIDMT are localized in cone photoreceptors as well as ganglion cells layers. Melatonin receptors MT1 are distributed in photoreceptors, inner nuclear layer and ganglion cells layers. These data prove that melatonin is strongly driven by retinal clock control and can be considered as a major output involved in retinal physiology. We therefore worked with a specific melatonin-prophetic mouse model C3H/HeJ (without rd1 mutation). We demonstrated that loss of melatonin signaling leads to an increase of cone degeneration during aging and that the clock-controlled AKT-FOXO pathway plays a role in this event. Moreover, photoreceptor survival is strongly dependent on its daily disk shedding and concomitant phagocytosis by the retina pigmented epithelium (RPE). Hence, we investigated the daily rhythm of phagocytic activity by the retinal pigment epithelium in the MT1 and MT2 knock-out mice. Our data indicate that in C3H/HeJ/Mt1 and Mt2 knockout mice, the peak of phagocytosis is advanced by 3 h with respect to wild-type mice. As such, phagocytosis occurred in dark rather than after the onset of light, although the mean phagocytic activity over the 24 h period did not change among the three genotypes. Nevertheless, this advanced profile of daily phagocytic rhythms may produce a significant decrease in the ONL Bwave amplitude in rBmal1 KO mice at 1 month and older. In addition, disruption of rBmal1 was found to accelerate the age-related cone photoreceptor death compared to age-matched controls. The remaining cones in 26 month-old rBmal1 KO mice had greatly reduced outer segment length compared to controls. These studies indicate that retinal Bmal1 plays important roles in retinal structure and function during development and aging.

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1Cleveland Clinic, Ophthalmic Research, Cleveland, United States, 2Cincinnati Children’s Hospital, Pediatric Ophthalmology/Visual Systems Group, Cincinnati, United States

Angiogenesis or formation of new vessels is a metabolically demanding process. An important function of the circadian clock is to regulate the metabolic machinery in anticipation of metabolic demands. Systemic loss of this synchrony between energy sources or growth factors that regulate vascular growth and function can be an additional risk factor for increased susceptibility towards vascular diseases. The molecular links between loss of circadian function and its influence on the growth and maintenance of the ocular vascular networks are relatively unexplored. Here, we present evidence that Bmal1 is required both in the mouse retinal neurons and the endothelial cells for vascular growth and patterning. Loss of Bmal1 from the retinal neuron results in increased retinal vascular density. However, the endothelial clock exerts an exactly opposite influence on the retinal vasculature, where by loss of endothelial Bmal1 results in reduced branching and less proliferation. To identify the molecular targets, we performed a ChIP-Seq analysis and show that Bmal1 in the neurons regulate the expression of the anti-angiogenic molecule sFlt1 (Vegf receptor 1) and EphinB2. Loss of Flt1 from the neurons results in a similar phenotype as that of Bmal1. Finally, to assess the influence of clock genes in vascular homeostasis and maintenance we have used the laser-induced choroidal neovascularization assay to determine if the loss of clock genes affect the vascular barrier function and integrity of the vasculature. Our data suggest that these functions are compromised by the loss of Bmal1. In conclusion, the circadian clock genes have differential effects on retinal vascular growth, remodeling, and function. We speculate that the circadian disruption could shift the balance of pro versus anti-angiogenic factors.
The Ohio State University, Columbus, United States

Roles for Notch Signaling during Early Eye Morphogenesis

La Torre, A.1, Lum, N.1, Brown, N.1

1University of California, Davis, United States, 2University of California, Cell Biology & Human Anatomy, Davis, United States

Notch pathway gene expression and mouse germ line mutant pheno
notypes suggest this signaling pathway functions at the earliest stages of vertebrate eye development. Because previous mouse mutant analyses were hampered by systemic growth defects and embryonic death during gastrulation, a conditional mutant strategy has been needed to study this pathway during ocular morphogenesis. Using a BAC transgenic Rax-Cre mouse line, we analyzed the consequences of removing canonical Notch signaling, as well as constitutive over-expression of the Notch receptor intracellular domain (N1-ICD). Although the loss of Notch signaling results in primarily neurogenic phenotypes, we found that too much Notch signaling, prior to E9.5, was catastrophic for optic vesicle outgrowth and patterning, while also impacting ventral hypothalamic development. These defects arise downstream of the onset of early patterning genes such as foxa2 and Pdx1. We conclude that Notch signaling levels must be tightly controlled during the earliest phases of ocular growth. Our models for Notch cross-regulation of other signaling pathways (e.g., Hedgehog signaling) will also be discussed.

Extrinsic Regulation of Retinal Pigment Epithelium and Optic Cup Morphogenesis

Cechmanek, P.1, Hehr, C.1, McFarlane, S.1

1University of Calgary, Calgary, Canada, 2University of Calgary, Cell Biology and Anatomy, Calgary, Canada

The retinal pigment epithelium (RPE) sits at the back of the neural retina, and serves a critical role in photoreceptor survival and function. During embryonic development, RPE progenitors first emerge in the doral neural plate, and subsequently the RPE stretches ventrally to surround the entire neural retina. The cellular behaviours and molecular mechanisms that drive this process are largely unknown. We have used the zebrafish as a model to study the events of embryonic RPE embryogenesis, taking advantage of the speed of eye development, the transparency of the embryos for ready imaging of fluorescently labeled RPE and retinal progenitors in the live embryo, and the availability of loss-of-function approaches to study the transient (anti-sense morphologolinorolucodites (MO)) or permanent loss (CRISPR transgene) of molecules we hypothesize to be important. We first characterized the normal expansion of the RPE domain to cover the eye, which happens rapidly in zebrafish over a 10 hour period (14-24 hours post-fertilization (hpf)). We identified two phases of expansion; Phase 1 (10-14 hpf) involves a previously uncharacterized expansion of the RPE domain within the inner leaflet of the eye vesicle in an anterodorsal direction, through the de novo specification of RPE progenitors. During this phase RPE progenitors turn on RPE specific differentiation genes; Phase 2 (17-24 hpf) involves a previously reported stretching of the RPE as the eye vesicle invaginates around the developing lens. Our data suggest that Phase 2 depends on retinoid signaling between the transmembrane Semaphorin (SemA) ligand expressed by neural retina progenitor, and the corresponding Plexa1b receptor expressed by RPE progenitors. Knockdown of SemA or Plexa1b by a MO approach causes an optic cup morphogenesis defect, which impacts both the RPE cells and the ventral neural retina cells of the inner eye vesicle leaflet; the ventral retina cells move around the distal rim of the optic cup, and the RPE cells do not elongate and come to congregate next to the misplaced neural retinal cells. A related defect is seen in two semaCRISPR mutant alleles, SemA and SemB.

The Rho GTPase Cdc42 is Required for Filopodia Formation during Closure of the Optic Fissure

Fuhrmann, S.

Vanderbilt Medical Center, Ophthalmology and Visual Sciences, Nashville, United States

During normal eye development, morphogenesis of the optic cup is a critical step; in particular, formation of the transient optic fissure requires coordinated invagination of the ventral optic cup and the optic stalk. The fissure margins subsequently fuse leaving only a small opening for ganglion neuron axons exiting the neural retina and vasculature. Defects in closure of the optic fissure result in coloboma accounting for more than 10% of childhood blindness. Even though the importance of optic fissure closure for eye development and functioning is well appreciated, yet the cellular and molecular mechanisms underlying the closure process are still obscure. Classical electron microscopic studies revealed that thin cytoplasmic extensions from the fissure margins across the gap during fissure closure. We hypothesized that these extensions could represent filopodia-like structures. As the Rho GTPase Cdc42 can induce filopodia formation, we predicted that Cdc42 is critical for proper contact between the fissure margins and the optic cup through regulation of filopodia assembly. We employed a tamoxifen-inducible mouse line, Hes1CreERT2, for temporally controlled and tissue-specific Cdc42 inactivation. Normal optic fissure starts closing around embryonic day 11 (E11) and fusion is completed by E12.5. To avoid potential effects on other functions of Cdc42 such as apical polarity, we disrupted Cdc42 as late as possible by activating Hes1CreERT2 for 18-24 hours before analysis at E11.5. Our immunohistochemistry data with a variety of markers shows that tissue patterning and apical polarity is unchanged. However, closure of the optic fissure is consistently disrupted in Cdc42 mutant eyes. To analyze the formation of filopodia extensions and cell-cell interactions during optic fissure closure, we used high resolution confocal microscopy with Airyscan. In control eyes, we can visualize cytoplasmic bridges and filopodia-like structures extending between the optic fissure margins. Cdc42 mutant optic cups show defects in filopodia formation; in particular the number of filopodia-like structures is severely reduced. Given the role of Cdc42 in filopodia and cell adhesion, our data suggest that it supports optic fissure closure by filopodia formation in cells lining the optic fissure margin.

Analysis of Transcriptomic Change during Periocular Neural Crest Cell Differentiation into Corneal Cells

Bi, L., Ma, J., Dwigle, P.

Rice University, BioSciences Department, Houston, United States

The pericorneal neural crest cells (pNC) are a subset of multipotent cranial neural crest population that contribute to several ocular tissues including the corneal endothelium and stromal keratocytes, the most distal portion of the corneal stroma. The migration and differentiation of these cells requires the function of several transcription factors like Lhx2 and Pax6. We conclude that mutations affecting these factors result in abnormal corneal development, and the molecular mechanisms underlying their generation was investigated using mouse models. We found that pNC differentially expressed between E10.5 and E14.5 cornea, and 3617 genes was expressed genes in chick, while 865 genes were differentially expressed between E10.5 pNC and E14.5 cornea, and 3617 genes were differentially expressed between E10.5 pNC and E16.5 cornea. Initial analysis using the gene regulatory network of cranial neural crest as a guide, showed that several transcription factors expressed by migratory neural crest cells were down regulated in the pNC. KEGG pathway analysis of both chick and mouse pNC during differentiation into corneal cells revealed significant expression of growth factor families (TGFβ/BMP, Wnt, Shh, and Notch), and extracellular matrix and cell adhesion molecules. We validate the expression and localization of novel genes during corneal development, which may play crucial roles during pNC differentiation into corneal cells.
Nonhuman Primate Models of Inherited Retinal Degenerative Diseases

Neuringer, M.1, Renner, L.1, Stoddard, J.1, McGill, T.1

1Oregon Health & Science University, Neuroscience & Ophthalmology, Beaverton, United States, 2Oregon Health & Science University, Oregon National Primate Research Center, Beaverton, United States

Nonhuman primates are the only animals to possess a retina with all the features of the human macula, and thus they can provide valuable models for studying retinal disease. We have identified several naturally-occurring retinal disease models in macaque monkeys. First, rhesus macaques show a high prevalence of age-related maculopathy which shares common phenotypic features and genetic and nutritional risk factors with human age-related macular degeneration (AMD). Drusen in these animals express key markers of drusen in human AMD, including complement components and hyaluronan. Appearance of drusen and their progression is dramatically accelerated by a life-long diet lacking carotenoids and omega-3 fatty acids, and some cases show progression to RPE atrophy. In Japanese macaques, we identified a dominant-ly inherited pattern of early-onset peripapillary drusen, and showed acceleration of progression by a high-fat Western diet. A similar syndrome has also been documented in cynomolgus macaques. In addition, we recently identified and characterized two other naturally-occurring monogenic retinal disease syndromes. The first, in Japanese macaques, is a form of Batten disease, a fatal neuro-degenerative lysosomal storage disease, due to a mutation in the CLN7 gene. Autofluorescence storage material is visible in multiple retinal layers by fundus autofluorescence imaging by 2 months of age and increases rapidly after 3 years. By 5 years, retinal structure and function is profoundly impaired but with nearly complete sparing of the fovea. Genetic screening has identified 28 carriers and 3 living homozygotes, targeted breeding is underway, and we conclude that immune privilege of the eye can become compromised following RPE cell transplantation, immunomodulatory approaches will be needed to enhance survival of RPE cell transplantation, and the non-human primate model system is well-suited for the development and evaluation of these approaches and therapies.

Support: Research to Prevent Blindness, BrightFocus Foundation, Foundation Fighting Blindness, NIH grants R01EY21214, R30EY015572 and P51OD011092.

Translational Cell-based Therapy Research in the Non-human Primate

McGill, T.1, Wilson, D.1, Renner, L.1, Stoddard, J.1, Lauer, A.1, Neuringer, M.1

Subretinal transplantation of cell suspension into rodent models with photoreceptor degeneration has repeatedly been demonstrated to rescue behaviorally measured vision, maintain electrophysiological responses from the retina and the brain, and slow the degeneration of rod and cone photoreceptors for extended periods. While these studies are critical to the development of cell-based therapies, translation from rodent models directly into human clinical trials skips an important intermediary preclinical step that is needed to address critical issues for intraocular cell transplantation. The non-human primate provides an optimal model for evaluation of surgical variables related to the administration of cells into a large eye with a macula and fovea, and evaluation of the host immune system response to cell transplantation. Recently, we developed a novel method for the delivery and encapsulation of RPE cells into the subretinal space using a two-step trans-retinal and trans-scleral approach. This approach achieved 100% successful delivery of cells into the subretinal space, with evidence of cell leakage in only 1 of 36 eyes due to a non-sealed retinotomy. Using this approach, we have evaluated the immunological consequences of cell transplantation using allogeneic ES- and iPS-derived RPE cells with and without immunosuppression as well as autologous IPS-derived RPE cells. Independent of immunosuppression condition, within weeks of implantation all allogeneic RPE cells suffered graft failure characterized by a strong microglial, T- and B-cell response. Autologous IPS-derived RPE did not survive past 4 weeks survival, but minimal inflammatory T- or B-cells were observed. Thus, we conclude that immune privilege of the eye can be compromised following RPE cell transplantation, immunomodulatory approaches will be needed to enhance survival of RPE cell transplantation, and the non-human primate model system is well-suited for the development and evaluation of these approaches and therapies.

Support: Research to Prevent Blindness, BrightFocus Foundation, Foundation Fighting Blindness, NIH grants R01EY21214, P30EY015572 and P51OD011092.
Mesenchymal Stem Cell-derived Exosomes Promote Retinal Ganglion Cell Survival after Optic Nerve Crush and in Rodent Models of Glaucoma

Tomarev, S.1, Ahmed, Z.2, Mead, B.1,2

1National Eye Institute, National Institutes of Health, Section of Retinal Ganglion Cell Biology, Laboratory of Retinal and Molecular Biology, Bethesda, United States, 2University of Birmingham, Neurotrauma Research Group Neurobiology Section, Clinical and Experimental Medicine, Birmingham, United Kingdom.

The loss of retinal ganglion cells (RGCs) and their axons is one of the leading causes of blindness and includes traumtic (optic neuropathy) and degenerative (glaucoma) eye diseases. The present study aimed to investigate neuroprotective effects of human bone marrow mesenchymal stem cell (BMSC)-derived exosomes on RGCs in three different rodent glaucoma models and in a rat optic nerve crush (ONC) model. Exosomes were isolated from huma BMSC and fibroblasts. Both BMSC and fibroblasts secrete similar numbers of exosome (approximately 10^9/24h/100,000 cells). Exosomes were injected weekly (a laser photoagulation and a microbead rat models of glaucoma and a rat ONC model of optic neuropathy) or monthly (a microbead rat model and DBA/2J mice) into the vitreous at a concentration 3x10^6 (rat) or 1x10^7 (mouse). Following intravitreal transplantation, exosomes successfully integrated into the inner retinal layers, including RGC layer. After ONC (21d), BMSC-derived exosomes, but not fibroblast-derived exosomes, promoted a statistically significant increase in survival of RGCs and regeneration of their axons while partially preventing RGC axonal loss and RGC dysfunction. All three rodent glaucoma models exhibited elevated IOP, RGC dysfunction and loss as well as axonal degeneration. In both rat glaucoma models, BMSC but not fibroblast exosomes promoted significant preservation of RGC function, as measured by positive scotopic threshold response amplitudes, and significant neuroprotection of RGCs. BMSC but not fibroblast exosomes prevented axonal degeneration as evaluated by retinal nerve fiber layer thickness and parapheylidyelamine axonal scaling. Monthly BMSC exosome injections but not fibroblast exosome injections provided long-term neuroprotective effects (one year) in the DBA/2J model. Knockdown of the AGO2 gene encoding a factor that negatively regulates miRNA expression resulted in decreased survival of RGCs in the DBA/2J model. Knockdown of the AGO2 gene encoding a factor that negatively regulates miRNA expression resulted in decreased survival of RGCs in the DBA/2J model. Knockdown of the AGO2 gene encoding a factor that negatively regulates miRNA expression resulted in decreased survival of RGCs in the DBA/2J model. Knockdown of the AGO2 gene encoding a factor that negatively regulates miRNA expression resulted in decreased survival of RGCs in the DBA/2J model. Knockdown of the AGO2 gene encoding a factor that negatively regulates miRNA expression resulted in decreased survival of RGCs in the DBA/2J model. Knockdown of the AGO2 gene encoding a factor that negatively regulates miRNA expression resulted in decreased survival of RGCs in the DBA/2J model. Knockdown of the AGO2 gene encoding a factor that negatively regulates miRNA expression resulted in decreased survival of RGCs in the DBA/2J model. Knockdown of the AGO2 gene encoding a factor that negatively regulates miRNA expression resulted in decreased survival of RGCs in the DBA/2J model.

Cross Talk between Non-pigmented Ciliary Epithelium and Trabecular Meshwork Cells Is Taking Place by Exosomes

Belt-Yannai, E., Lerner, N., Tabak, S., Schreiber-Avisar, S.

Ben-Gurion University, Clinical Biochemistry and Pharmacology, Beer-Sheva, Israel.

Delicate balance control of the intracellular pressure is essential for proper functioning of the visual system. The trabecular meshwork was found to respond to changes in the intraqueous pressure as details of the mechanisms standing behind are not fully understood. Signals originated from the ciliary epithelium were suggested to act as a potential source for trabecular meshwork pressure responses. Extracellular vesicles known as exosomes are 50-150nm sized double layer vesicles produced within multivesicular bodies and are secreted following fusion with the plasma membrane. Exosomes contain elements of their membrane origin, such as lipids and receptors, miRNAs, lipids and proteins. Exosomes are a candidate for signal transfer between aqueous humor producing site and the aqueous humor drainage site. Primary non-Pigmented Ciliary epithelium (NPC) and NPCE cell line derived EVs were extracted and analyzed by size and concentration by Tunable Re...
Gial involvement in Retinal De- and Re- generation in Zebrafish and Mouse

Enzmann, V.1, Conedera, F.1,2, Mercader Huber, N.1, Tschopp, M.1

1 Bern University Hospital, Ophthalmology, Bern, Switzerland, 2 Université de Bern, Biomedical Research, Bern, Switzerland, 3 University of Bern, Graduate School for Cellular and Biomedical Sciences, Bern, Switzerland, 4 University of Bern, Anatomy, Bern, Switzerland, 5 Kantonsspital Aarau, Ophthalmology, Aarau, Switzerland

Retinal degenerations are heterogeneous eye diseases causing permanent vision loss. Therapeutic modalities that can reverse these processes are still not available. A new strategy for restoring sight is based on the regenerative capabilities of zebrafish. There, Müller cells (MCs) respond to retinal injury by re-entering the cell cycle, dedifferentiating into retinal progenitor cells and replacing lost cell types. None of these steps occurs spontaneously in the mammalian retina. To further characterise the role of MCs during degeneration/regeneration, laser-induced retinal damage was induced in a species with high regeneration capacity (zebrafish) and another with low regeneration capacity (mouse). Phenotypical changes were monitored and correlated with morphological alterations detected by histological analyses overtime. Laser damage was identified as a hyper-reflective band in the ONL in both animal models by OCT. This subtle and well-delineated signal detected at day 3 correlated to cavitory formation in the ONL due to photoreceptor loss found in the histological sections. After day 7, both OCT and histological analyses confirmed that the outer retina re-established its normal morphology in the zebrafish but not in the mouse where the hyper-reflective signal was still visible. MC behavior was characterized on gene (qPCR, ISH) and protein (IHC) levels. Thereby, activated GFAP-positive MCs were found within the injury site starting from day 3 in the zebrafish. At day 7 PCNA was co-localised in the INL and ONL indicating MC proliferation. In the mouse, both GFAP and PCNA signals were upregulated at day 3, while PCNA expression was downregulated at day 14, GFAP was still expressed indicating glial scar formation. Furthermore, MC-microglia bidirectional interaction help shape the overall injury response in the retina. PLX3397 was used to ablate microglia/macrophages in the zebrafish. Unlike in the untreated zebrafish, no GFAP expression was detected during retinal degeneration/regeneration in the PLX3397 treated animals. PCNA expression was anyhow identified in the laser injury site at days 3 and 7. Differentially regulated MC activation/proliferation and development of a gial scar after laser-induced retinal injury result in differences in regeneration capacity of zebrafish compared to mouse. Thereby, microglia - MC interaction plays a pivotal role during successful regeneration.

Activation marks. 5-mC redistribution was further investigated by whole genome bisulfitesequencing of the chick RPE methylome before and after reprogramming. Sequencing revealed a global reduction of 5-methylcytosine as well as differential methylation at retina-associated genes. Altogether, these results suggest that RPE reprogramming is accompanied by functional modifications to the epigenetic landscape.

Comprehensive Transcriptomic and Epigenomic Analyses of Retinal Müller Glia during Different Damage Paradigms in Zebrafish, Chick and Mouse

Hyde, D.1,2, Boyd, P.1,3, Lehne, M.1,2, Hoang, T.1, Wang, J.1, Ash, J.1, Fischer, A.1, Qian, J.1, Blackshaw, S.1

1 University of Notre Dame, Biology, Notre Dame, United States, 2 University of Notre Dame/Center for Stem Cells and Regenerative Medicine, Notre Dame, United States, 3 Johns Hopkins University, School of Medicine, Baltimore, United States, 4 Johns Hopkins University/Wilmer Eye Institute, Baltimore, United States, 5 University of Florida School of Medicine, Ophthalmology, Gainesville, United States, 6 Ohio State University College of Medicine, Neuroscience, Columbus, United States

Retinal damage induces zebrafish Müller glia to reprogram and re-enter the cell cycle to produce neuronal progenitor cells that continue to proliferate and differentiate to regenerate the lost cells. In contrast, the damaged chick retina undergoes only a limited regenerative response and the damaged mouse retina fails to regenerate, with the Müller glia undergoing gliosis. While several groups examined gene expression changes in damaged/regenerating zebrafish retinas, comparisons between different damage models and cross-species analyses have not been performed. We tackled these knowledge gaps by comparing light-damaged zebrafish retinas, which lose rod and cone photoreceptors, NMDA-damaged zebrafish retinas, in which amacrine and ganglion cells die, and zebrafish retinas co-injected with TNFa and a gamma secretase inhibitor, which stimulates Müller glia to reprogramming and proliferation, including several transcription factors and chromatin remodeling proteins. We conditionally knocked down the expression of several candidate proteins to test their role in Müller glia programming and proliferation. These tests involved quantitative real-time expression of mRNAs to assess gene expression, immunohistochemistry to assess Muller glia proliferation, and Western blots to assess protein expression. We will discuss the roles of these transcription factors and chromatin remodeling candidates in zebrafish Müller glia reprogramming and proliferation.
Mechanistic Insights into Ciliary Signalling and Function Using Gene Discovery and Functional Genomics

Johnson, C.
University of Leeds, Section of Ophthalmology & Neurosciences, Leeds, United Kingdom

Background: Primary or sensory cilia are microtubule-based organelles that extend from the apical surface of most mammalian cells. Cilia participate in diverse roles in cell signalling, chemosensory, mechanosensation, and photoreception, and defects in primary ciliogenesis underlie the ciliopathies, a growing group of genetic disorders. Affected individuals invariably present with cystic kidney disease and cognitive defects. The molecular mechanisms by which inositol polyphosphates regulate ciliary function remain an active area of research.

Hypothesis: Can gene discovery, pathogenic variant modelling and functional genomics approaches be used to provide new insights into disease pathogenesis?

Methods: Whole exome sequencing, high content imaging and RNA sequencing were performed to identify novel ciliopathy genes. Cultures of patient fibroblasts were used to model pathogenic variants.

Results: We describe whole genome sRNA-based reverse genetics screens for defects in ciliogenesis and/or maintenance, length, morphology and number. Validated hits from the screens have identified components of the ubiquitin-proteasome system, G protein-coupled receptors, ion channels, regulators of the actin cytoskeleton and cell polarity, and pre-mRNA processing factors (PRPFs). Exome sequencing data also identified recessive mutations in the screen candidate gene C21orf32/LRRK7 as a cause of Jeune syndrome, a ciliopathy characterised by skeletal dysplasia and retinal degeneration. Further work has highlighted a retinal tissue-specific role for PRPF11 in the modulation of splicing fidelity for pre-mRNAs encoding cilary proteins.

Conclusions: Ciliopathies remain a group of conditions that are both medically challenging and important to diverse fields of basic scientific research. They remain a top priority for targeted therapeutic interventions such as anti-cilinucleotide or gene therapies. Our approaches using reverse genetics screens and gene discovery provide novel unbiased insights into essential ciliary processes and identify roles for unanticipated pathways in human genetic disease.

Phosphatidylinositol 3-phosphate 5-kinase Rescues Cone Photoreceptor Degeneration due to the Absence of Phosphatidylinositol 3-phosphate

Rajala, R.
University of Oklahoma Health Sciences Center, Ophthalmology and Physiology, Oklahoma City, United States

Phosphatidylinositol 3-phosphate (PI(3)P) is only generated by class III phosphoinositide-3-kinase VPS34. We bred mice expressing a cre-recombinase in cones to mice with a floxed VPS34 allele to generate offspring with conditional deletion of VPS34 in cones. Loss of VPS34 resulted in an age-dependent cone degeneration. We found that cones lacking VPS34 have significantly lower cone function compared to cones with competent VPS34. Loss of VPS34 in cones has no effect on rod structure and function. To probe the mechanism of cone degeneration, we examined PI(3)P-binding partner FYVE-domain containing phosphatidylinositol 3-phosphate 5-kinase (PIKfyve). Proteins containing the FYVE domain bind to PI(3)P. PIKfyve binds to PI(3)P at the membrane and phosphophytates phosphatidylinositol (PI(3)P) to PI(3)P. We hypothesized that cone degeneration in Vps34-/- mice could be due to the lack of PI(3)P generation as a result of the absence of PIKfyve tethering to the PI(3)P in the membrane. Subretinal injection of BODIPY FL-PI(3)P or lipid nanoparticles-complexed myristoylated PIKfyve rescued VPS34-mediated cone degeneration. PI(3)P serves multiple roles in the Pds due to its low abundance. Our findings suggest that VPS34 regulates the generation of PI(3)P in cones. Our data also show that PI(3)P is essential for cone photoreceptor structure and function.

Essential Roles for Phosphoinositides in Retinal Neurons and Retinal Pigment Epithelium

He, F., Agosto, M., Wensel, T.
Baylor College of Medicine, Biochemistry and Molecular Biology, Houston, United States

Despite their well-recognised roles in signalling, vesicle trafficking and cellular homeostasis in all tissues and cell types, the roles of phosphoinositides (PI) in the retina have received relatively little attention. Only recently have tools with sufficient sensitivity and spatial resolution become available to explore retinal phosphoinositides rigorously. We have used phosphoinositide binding domains of high affinity and specificity to measure fmol levels in isolated mouse rods, and to image membranes containing specific PI in rod, ON-bipolar cell and retinal pigment epithelium (RPE) cells. We have also used cell-type-specific knockouts of the enzyme responsible for production of phosphatidylinositol 3-phosphate (PI(3)P), Vps34, to determine its functional importance in these same retinal cell types. The results show that phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2 or PIP2) is distributed throughout the plasma membranes of cells, but excluded from rod outer segments; phosphatidylinositol 4-phosphate (PI(4)P) is largely confined to a sub-compartment of the Golgi apparatus, with lower levels also found in plasma membrane; PI(3)P is found in a distinct population of unidentified membrane organelles, as well as in the membranes of RPE phagosomes containing shed tips of rod outer segments and in autophagosomes. Levels of PI(4)P, PI(4,5)P2, and PI(3)P in isolated rods are relatively low following light adaptation, but are greatly increased upon prolonged exposure to light. In contrast, levels of PI(3,4,5)P3 are so low as to be undetectable in light or dark. Cell type-specific knockouts of Vps34 revealed an essential role for PI(3)P in hometostasis in all three cell types. Knockout caused defects in endosome processing, phagosome processing, and autophagy, leading to cell death. Mechanistic studies reveal that autophagy and phagocytosis in RPE are distinct pathways, with LC3 recruitment requiring PI(3)P in phagosomes, but not in autophagosomes.

Ocular Imaging of Brain Disease

Schmitter, T.
Singapore Eye Research Institute, Singapore, Singapore

The human brain is inaccessible for high-resolution imaging. As such there is an intensive search for biomarkers of neurodegenerative eye disease that can be easily measured. The retina is an attractive approach in this respect, because it shares functional and structural similarities with the brain. Novel ocular imaging modalities have indeed provided some insight into brain disease. The present talk discusses the state of the art.

Translational Ophthalmology: Current Challenges at the Interface between Research and Clinic

Zeitz, O., Strauss, O., Jousen, A.M.
Charte University Medicine Berlin, Ophthalmology, Berlin, Germany

The treatment of neurovascular diseases of the macula was revolutionized by the introduction of intravitreal therapy with VEGF inhibitors. This has attracted a lot of interest in translational research for eye disease. Despite significant investments in research and development over the last two decades, the process is limited and gradual since a few years. After several spectacular failures, major breakthroughs for wide-spread and sight-threatening eye diseases are not on the near-to mid-term horizon. In this presentation, we will review recent development failures in the field of macular diseases and other eye diseases. The majority of these projects came with high hopes, but ultimately failed. Attrition was reached in several cases only after large scale investments for phase 3 programs. The reasons for these late stage failures will be discussed. In brief, the translational research process in macular diseases lacks structure and predictive value of preclinical models and early clinical markers of efficacy is not established. In addition, disease mechanisms are understood incompletely and superficially and in conclusion, more research on research in eye diseases is required in order to re-structure the translational research cascade.
**System Medicine in Ophthalmology**

**Joussen, A.**

Charité University Medicine Berlin, Berlin, Germany

Diabetic retinopathy (DR) is the most common complication of diabetes and remains the leading cause of blindness among working-age individuals in developed countries. Given that the global prevalence of diabetes is expected to increase from 347 million in 2011 to 552 million in 2030 the impact of DR is set to intensify. Currently, there is no robust physiopathological basis for the treatment approach given to each patient, with available clinical, genetic and molecular data unable to reliably stratify the majority of patients. We present a novel integrated approach to understanding the functional impairment, morphological alteration and underlying relevant biological pathways in DME, enabling identification and stratification of specific endophenotypes within this disease. To identify potential biomarkers to differentiate endophenotypes and to predict disease progression, state-of-the-art functional and imaging technologies, alongside Patient Related Outcome measures (PROs) and a range of molecular analyses are implemented in existing patient cohorts and experimental models. The results are validated in prospective studies. Given that the diagnostic accuracy, predictive value and cost-effectiveness of stratification measures, as well as the treatment response, likely depend on the stage of the disease at which they are studied, both early and advanced stages of disease will be targeted. While the research in Diabetic eye disease is still ongoing, we present first results of a System medicine approach in Patients with choroidal melanoma, dissecting molecular pathways and integrating clinical data.
Conclusion: Eye disease, and vitamin A therapy (p-value < 0.05). There was no significant association between DED and smartphone addiction and determine the risk factors of DED in participants associated with smartphone addiction.

Methods: This cross-sectional study was conducted in May 2017. Participants were selected from the Faculty of Medicine at King Abdulaziz University in Jeddah, Saudi Arabia. Stratified random sampling technique was used to recruit participants according to their academic year with equal allocation of males and females. All participants owning smartphones were included in the study. DED was assessed using the Ocular Surface Disease Index (OSDI) questionnaire and the Smartphone Addiction Scale short version (SAS-SV) was used to assess smartphone addiction among the participants.

Results: A total of 443 completed questionnaires were received, providing a response rate of 94%. There was an almost equal distribution of our sample by gender, with 225 males (50.8%). Almost half of the participants were found to have DED (49.4%). There was no significant association between DED and smartphone addiction (odds ratio (OR)=0.69, 95% confidence interval (CI): 0.44-1.1, p-value = 0.102). However, significant associations were observed between the DED and contact lens use, eye drops use, eye disease, and vitamin A therapy (p-value < 0.05).

Conclusion: The usage of contact lenses, eye drops, and vitamin A therapy were found to be associated with DED. Furthermore, there was a statistically significant association between DED and pre-existing eye disease. Despite the prevalence of DED, there was no statistically significant association between DED and smartphone addiction in our study.
Gene Expression Analysis of Conjunctival Epithelium of Stevens-Johnson Syndrome Patients in the Chronic Stage

Ueda, M.1, Nishigaki, H.1, Sotozono, C.1, Ohsako, S.1, Yokoi, N.1, Kinoshita, S.1

1Kyoto Prefectural University of Medicine, Department of Frontier Medical Science and Technology for Ophthalmology, Kyoto, Japan
2Kyoto Prefectural University of Medicine, Department of Ophthalmology, Kyoto, Japan

Purpose: To investigate the pathology of ocular surface complications of SJS/TEN with SOC in the chronic stage, we performed comprehensive gene expression analysis of conjunctival epithelium of SJS/TEN with SOC in the chronic stage in this study.

Methods: We performed gene expression analysis of conjunctival epithelium from each 3 individuals of SJS/TEN patients and conjunctivochalasis patients as controls using oligonucleotide microarrays, GeneChip.

Results: The 65 transcripts were up-regulated more than 10-fold and showed significant differences (ANOVA p-value < 0.05) in conjunctival epithelium from each 3 individuals of SJS/TEN patients and conjunctivochalasis patients as controls using oligonucleotide microarrays, GeneChip.

Discussion: The expression of many transcripts were increased in conjunctival epithelium of SJS/TEN patients as controls using oligonucleotide microarrays, GeneChip.

Mega-dose Dietary Riboflavin and Direct Sunlight UV Exposure in the Treatment of Keratoconus and Post-refractive Surgery Ectasia

Jarstad, J.1,2,3,4, McNabney, L.1,2, Taranassi, M.1, Schaeffer, A.1, Jarstad, K.1, Fraunfelder, R.1

1University of Missouri, Department of Ophthalmology, Columbia, United States
2Evergreen Eye Center, Refractive Surgery, Federal Way, United States
3University of Tennessee, Ophthalmology, Memphis, United States
4Tulane University, Ophthalmology, New Orleans, United States

Corneal collagen cross-linking has emerged as a highly successful treatment for keratoconus and post-refractive surgery kerectasia. The use of topical concentrated riboflavin solution, along with a calibrated ultraviolet light source has been shown to successfully cross-link and stiffen the corneal collagen stroma and arrest and in most cases, reduce the effect of corneal steepening in several degenerative corneal conditions including keratoconus and post-refractive surgery ectasia.

Corneal cross-linking with the Avedro commercial UV light source and concentrated topical riboflavin solution produced in a standard dose is widely accepted and was FDA approved in 2018 as a treatment for keratoconus and post-refractive surgery ectasia.

The financial treatment cost, along with the high cost of the Avedro machine, has limited ophthalmologist’s and patient’s access to this new effective treatment.

We report cases at three separate institutions where patients, either on their own or under the suggestion of their ophthalmologist ingested high doses of dietary riboflavin and were exposed to direct sunlight in an attempt to flatten corneae by flattening by both topographic and keratometric measurements. No adverse effects were observed or reported in patients taking up to 1500 milligrams of dietary riboflavin per day and spending 15 minutes daily outdoors without sunglasses while walking vigorously facing the sun.

We conclude that high-dose dietary riboflavin and natural UV exposure from sunlight may have a place in the treatment of keratoconus and post-refractive surgery kerectasia as an economical alternative or adjunctive form of treatment.

It may be especially useful in pediatric and pregnant patients who are more likely to regress after medical cross-linking therapy and may not be candidates for commercial cross-linking.

Optimal Cryopreservation Conditions for Limbal Stem Cells

Ghareeb, A.1, Osei-Bempong, C.1, Figueiredo, F.1, Armitage, W.1

1University of Newcastle, Institute of Genetic Medicine, Newcastle Upon Tyne, United Kingdom
2University of Bristol, Division of Ophthalmology, Newcastle Upon Tyne, United Kingdom
3Keio University School of Medicine, Ophthalmology, Tokyo, Japan
4Kitasato University Kitasato Institute Hospital, Ophthalmology, Minato-ku, Japan
5Tokyo Dental College Ichikawa General Hospital, Ophthalmology, Ichikawa, Japan

Cryopreservation has the potential to make autologous limbal stem cells readily available for ex-vivo expansion and transplant.

An investigation of optimal cryopreservation conditions is essential to improve low-temperature banking of limbal stem cells.

Our aims were twofold: to identify optimal cryopreservation conditions from a group of candidate cryoprotectants employed at different concentrations and to look for the presence of Side Population (SP) cells in cryopreserved limbal cultures.

Suspension cultures obtained from donated human corneoscleral rims and grown on 3T3 feeder cells were added to carrier medium cooled to 4°C. Dimethyl Sulfoxide (DMSO), Propylene Glycol (PG) or Ethylene Glycol (EG) was added in equal volume at a concentration of 10%, 20% or 30% to achieve concentrations of 5%, 10%, and 15%.

After 10 minutes, cryoprotectants were diluted in 2 steps with equal volume of medium before centrifugation and resuspension. Cells were incubated with Trypan blue and visualised with a haemocytometer to measure membrane integrity before and after cooling. AlamarBlue reduction as measured by fluorescent emission was used as an assay of metabolic activity.

Colonies forming efficiency was measured using the number of membrane intact cells. Suspension cultures were cooled with 5% PG at its optimal concentration and stored frozen for 1 week at -100°C. Cells with the SP phenotype were identified by FACS.

We found that increasing cryoprotectant concentration greater than 5% reduces membrane integrity, metabolic activity and colony-forming efficiency (p<0.001, p=0.002, P<0.0001), while changing cryoprotectant was not a significant determinant of colony-forming efficiency.

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A Nonlinear Viscoelastic Model of Corneal and Whole-eye Motion under Air-puff Loading by a Dynamic Scheimpflug Analyzer

Nguyen, B.A.1, Reilly, M.A.1,2, Roberts, C.J.1,2
1Ohio State University, Biomedical Engineering, Columbus, United States, 2Ohio State University, Ophthalmology & Visual Science, Columbus, United States

The CorVis ST is a dynamic Scheimpflug analyzer which employs an air-puff to deform the cornea and clinically evaluate its biomechanical response, outputting several parameters which describe motion of the cornea and whole eye. There is a lack of consensus on the relative contributions of ocular and periocular tissues to describing this complex motion. A one-dimensional nonlinear viscoelastic model was developed to quantitatively capture essential features of corneal and whole-eye motion (WEM) observed in vivo and ex vivo during air-puff loading. The initial model was a system of linear elastic springs and inertial masses representing the cornea, eye and periocular tissues, but did not reproduce the observed response. This model was iteratively refined by adding viscous dashpot elements and a nonlinear stiffening spring until the numerical simulation sufficiently described both corneal motion and WEM. The values of spring and dashpot constants were estimated using an iterative approach to minimize the error between the simulation result and the in vivo data. Ex vivo studies were conducted on human donor eyes (5 pairs) where the sclera of one eye was stiffened with 4% glutaraldehyde for 30 minutes and the paired eye served as control. The eyes were placed in a rigid holder to restrict WEM, and the model was run to fit the ex vivo data. The peri-ocular tissues were well represented by a Kelvin-Voigt viscoelastic element, and the cornea was best described as a 3-parameter viscoelastic solid. The scleral contribution to limiting corneal deformation by constraining corneal lateral expansion was modeled as a non-linear stiffening spring, and was confirmed by the ex vivo data. The parameter describing scleral stiffness significantly increased (p < 0.05) by an average of 33%, while the parameters describing the cornea did not change significantly post-scleral stiffening (mean change < 2%). We found that the corneal viscoelasticity must be considered under air-puff loading to match CorVis ST data. The impact of the sclera on limiting corneal motion is also described. Without the non-linear stiffening spring, the peak of corneal motion would coincide with the peak of the pressure profile, which is not observed in vivo. When WEM is restricted ex vivo, the peaks of corneal motion and pressure coincide. The model was able to successfully recreate these important in vivo and ex vivo events.

Asymmetrical Endothelial Cell Migration from in vitro Quarter-DMEK Grafts

Miron, A.1, Spinozzi, D.1, Brunsmma, M.1, Lie, J.1,2, Birbal, R.3, Baydoun, L.1, Oeliferich, S.7, Melles, G.2
1NIiOS (Netherlands Institute for Innovative Ocular Surgery), R&D, Rotterdam, Netherlands, 2NIiOS (Netherlands Institute for Innovative Ocular Surgery), Amelins EyeBank, Rotterdam, Netherlands, 3NIiOS (Netherlands Institute for Innovative Ocular Surgery), Cornea Clinic, Rotterdam, Netherlands

Purpose: To investigate in vitro central and peripheral corneal endothelial cell migration from Quarter-Descemet Membrane Endothelial Keratoplasty (Quarter-DMEK) grafts.

Methods: Quarter-DMEK grafts were obtained from 10 corneas ineligible for transplantation but with intact and viable endothelial cells. Ten Quarter-DMEK grafts were “sandwiched” between 2 glass slides and cultured over 1 week in a humidified atmosphere at 37°C and 5% CO2. Cell migration was evaluated by light microscopy at standardized time intervals. In addition, immunohistochemistry analyses was performed to assess the detailed structural organization of endothelial cells in the corneal center and far periphery.

Results: Endothelial cell migration occurred from the radial cut graft edges, but not from the far peripheral area. Cell migration followed three different migration patterns:

1. Individual in-cell migration,
2. Uncoordinated cell migration of cell clusters, and
3. Collective migration in which endothelial cells moved as a sheet. Immunostaining showed presence of endothelial cells up to the far periphery but with different expression patterns of phenotypic markers ZO-1, N-Cad, and Vimentin compared to central endothelial cells.

Conclusion: In vitro endothelial cell migration from Quarter-DMEK grafts occurs along the radial cut edges with a decrease in migration activity towards the central corneal periphery. No migration occurred along the outer peripheral corneal edge possibly due to a different anatomical matrix in the far periphery. Hence, endothelial cells from the far periphery may not contribute to corneal clearance of the adjacent bare area after Quarter-DMEK surgery, but these cells may constitute a valuable cellular reserve on the graft.

Effect of Extraction Method and Location on in vitro Stromal Cell Behavior

Volatier, T.
University of Newcastle, Gateshead, United Kingdom

Background: The cornea is the clear window at the front of the eye and its clarity is vital for the transmission of light to the retina at the back of the eye, enabling visual perception. The cornea functions as a physical barrier, protecting the inner contents of the eye, while also providing a significant portion of the refraction needed for vision. The stroma is the collagen-rich stroma, which contributes the majority of the cornea’s volume. Within the stroma are the cells that maintain the collagen matrix; these cells are keratocytes or stromal stromal cells. When investigating stromal cell behaviour, cells will typically be extracted from the limbal region, where the cornea meets the conjunctiva. Cells harvested from this region express stem cell markers and survive longer in culture. Conversely, keratocytes isolated from the central region of the stroma may be less proliferative in culture. In addition to the challenge of extraction location, the method of extraction may also affect cell behaviour.

Aims: Investigating the effect of extraction location (central button and limbal ring) and extraction method (enzymatic and migratory) on the behaviour of corneal stromal cells in vitro.

Methods: Stromal cells were isolated from either central or limbal stroma by either collagenase digestion or explant out growth. Following extraction cells were expanded in serum containing media before re-planting at a cell density in serum free media. Cell number was followed over 8 days by microscopic examination and the Alamar blue assay.

Results: Stromal cells extracted from the limbal ring via enzyme digestion survived in greater numbers for longer periods of time than cells extracted via explant migration or cells extracted from the central button. Cells extracted via explant out growth survived re-planting in serum-free media poorly (20-50% cells adhered out of all cells detached from original flask). The digestion of explants after explant migration revealed a separate population of cells in the central button that survives significantly better than classically enzyme digested cells.

Conclusion: Different extraction methods and locations lead to different populations of cells that can be isolated from the corneal stroma.

A Mesenchymal Stem Cells Patch for Corneal Wound Healing

Al-Jaibaji, O., Szwiklo, S., Connon, C.
Newcastle University, Newcastle Upon Tyne, United Kingdom

Background: Corneal damage and opacity have been estimated to cause blindness in 8 million people worldwide each year. While corneal transplantation is the most utilised surgical intervention for treating corneal damage, it still has significant limitations such as corneal availability and compatibility. Thus, novel therapeutic strategies to treat corneal damage would greatly improve healthcare.

Aims: This work aims to form a stem cell-based patch, that is a bioactive, bio-compatible and cost-effective material capable of stimulating the wound repair process. This therapeutic biomateri- al promotes corneal regeneration by stimulating the production of exogenous therapeutic factors from alllogenic mesenchymal stem cells (MSCs).

Method: This study is focused on in vitro evaluation of the hypo- thermo-stored encapsulated MSCs at 15°C and their ability to heal corneal wounds using scratch assay. Alginate hydrogels were used for their safety, cost-effectiveness, and biocompatibility to encap- sulated MSCs to form a transplantable patch that could support their viability and maintain their production of paracrine factors. Hypothermic storage was employed to represent a future method of storage and distribution of cell therapy between manufacturing site and end-user.

Results: MSCs hypothermic storage for 72 hours at 15°C did not affect their viability. Non-stored encapsulated MSCS enhanced corneal wound recovery compared to hydrogel (p = 0.0031) and to medium controls (p = 0.0056). Following 15°C hypothermic storage, encapsulated MSCs showed significant improvement in in vitro corneal scratch-wound healing when compared to hydrogel (P < 0.0001) and to medium controls (P < 0.0001). Interestingly, 15°C storage showed a significant difference of (P = 0.0245) when compared to non-stored encapsulated MSCs, suggesting that hypothermic storage of encapsulated MSCs improved cells ability to produce growth factors. All the while encapsulation maintains MSCs phenotypic markers following storage and co-culture with human corneal cells.

Conclusions: This work presents a novel strategy that could sig- nificantly impact the corneal treatment by promoting healing via growth factors and, thus, establish a new paradigm for regener- ative corneal therapy. We are closer to the formation of stored stem cell patch. A bioactive, bio-compatible and cost-effective material capable of stimulating the wound repair process.
Safety of Wearing Contact Lenses for Patients Using Selective Serotonin Reuptake Inhibitors (SSRIS)

Liu, Y.-P.1, Liu, Y.-J.1, Liu, Y.-S.1
1Taipei City Hospital Songde Branch, Taipei City, Taiwan, Province of China, 2Taipei City Hospital, Taipei City, Taiwan, Province of China

Objective: Due to the frequent use of contact lenses by modern humans, and the widespread usage of SSRIs, which is considered to be one of the antidepressants used by most people, leads to increased adverse effects such as elevated intraocular pressure, uveal effusions and angle-closure glaucoma, we reviewed English lectures to assess the effect of wearing contact lenses on eye side effects associated with SSRIs.

Methods: To review the English lectures of Google Scholar and PubMed, which were related to the evidence of wearing contact lenses, changing intraocular pressure, and the role of SSRIs in controlling intraocular pressure in patients with SSRIs treatment.

Results: Acute angle-closure glaucoma and elevated intraocular pressure are the most important manifestations of SSRIs associated ocular adverse events. Case reports by Andrew and others showed that any patient using paroxetine may cause angle-closure glaucoma. In addition, some literatures has reported that people wearing contact lenses have a risk of having increasing intraocular pressure, especially in the case of prolonged wearing of contact lenses, therefore contact lenses must be carefully selected.

Conclusion: Elevated intraocular pressure is an evident cause of glaucoma. Thus, wearing contact lenses should be considered as a risk. In addition, SSRIs may have a side effect on elevating intraocular pressure. Therefore, it is worth noting that patients using SSRIs may have the same side effects when wearing contact lenses. In conclusion, patients who are advised to take SSRIs and have been in the cases of intraocular pressure elevation after taking SSRIs should be ensured the safety use of contact lenses.

Acute Ophthalmoplegia in Herpes Zoster Ophthalmicus: Clinical Features and Radiographic Findings

Zhou, W.
Tan Tock Seng Hospital, Ophthalmology, Singapore, Singapore

Objective: To describe the clinical features and radiographic findings of a series of patients with herpes zoster ophthalmicus (HZO) and associated acute ophthalmoplegia.

Methods: Medical records of 4 cases and existing literature on HZO with acute ophthalmoplegia were reviewed.

Results: Three males and one female with a median age of 6 years (range 59-68) presented with HZO. 3 of them were diabetic, and another one of them had systemic lupus erythematosus (SLE). The onset of acute ophthalmoplegia from initial presentation of HZO was 16 days (range 7-35 days). Clinical findings of ophthalmoplegia included oculomotor nerve palsy (75%), abducens nerve palsy (75%) and a mixture of both (50%). MRI features demonstrated enhancement of the cavernous sinus (100%), orbital apex (75%), superior orbital fissure (50%) or myositis (25%). Interestingly, one of the patients was found to have enhancement along the route of the trigeminal nerve extending from the brainstem. 3 patients had lumbar puncture, which showed pleocytosis with 1 of them having positive polymerase chain reaction (PCR) of varicella zoster virus (VZV). Treatment with intravenous acyclovir was administered in all patients and in 2 cases systemic corticosteroids were also given. Ophthalmoplegia improved in all patients over weeks to months.

Conclusions: Ophthalmoplegia is not an unusual complication of HZO, with third and sixth nerve palsy being the commonest. Systemic diseases such as diabetes or immunosuppressive status predispose the patients with HZO to central nervous system involvement. Cavernous sinus enhancement is a common radiographic feature of HZO related ophthalmoplegia. Improvement of symptoms and signs with systemic antiviral therapy can be expected. The role of systemic steroids in treatment of orbital disease is yet to be determined.

Three Techniques to Adjust IOP Immediately Following Femtosecond Laser-Assisted (FLACS) & Micro Incision Cataract Surgery (MICS) to Avoid IOP Spikes (the #1 Post-Operative Complication in Cataract Surgery)

An, J.A.1, Jarstad, J.S.2, Buckner, B.R.2
1University of Missouri School of Medicine, Ophthalmology - Glaucoma Service, Columbia, United States, 2University of Missouri School of Medicine, Ophthalmology, Columbia, United States

Objective: To determine the accuracy of current techniques to estimate intraocular pressure immediately following FLACS and MICS on the operating table.

Methods: In 176 consecutive uncomplicated FLACS & MICS patients, IOP was estimated with palpation by an experienced cataract/glaucoma surgeon (20,000+ cases) and experienced senior eye residents-in-training. Intraocular pressure (IOP) was then verified with a Barraquer (15-21mmHg) sterile surgical tonometer (Ocular Inc. $340.00 USD) and Tono-Pen AWA (Reichert $4000 USD).

IOP was checked before & adjusted immediately after cataract surgery & IOP repeated day one post op. A control group did not have IOP adjusted if IOP < 30mmHg by Tono-Pen at immediate case completion.

Results: Immediate post-operative IOP thought to be safe by palpation ranged from 9 to 67mmHg when verified by tonometry at case completion in the operating theatre. There was less than 5mmHg average IOP difference from measurements immediately post-op to day one post-op in clinic. As many as 33% of post-op cataract patients required IOP adjustment in clinic at their one day post-op visit if IOP was not adjusted immediately post-op.

An unexpected finding was that eyes that had immediate post-op IOP adjustment in theatre were up to 4x less likely to develop cystoid macular oedema. There was an unexpected financial benefit to adjusting IOP immediately at case completion in a savings of $44,000 per ophthalmologist, per year was calculated by an industrial engineer in lost opportunity cost savings by not having to treat elevated IOP in clinic.

Conclusion: Immediate IOP adjustment in the operating theatre at case completion has both medical and financial implications in treating and avoiding post operative pressure spike, the number one complication in femtosecond laser-assisted (FLACS) and micro incision cataract surgery (MICS).

Targeting of CD44 by Hyaluronic Coated-nanoparticles in Outflow Tissues - A New Therapeutic Approach for Glaucoma

Fromel, F.1, Guter, M.A.2, Dillingen, A.E.1, Perkumas, K.M.3, Stamer, W.D.1, Breunig, M.3, Fuchshofer, R.1
1Institute of Human Anatomy and Embryology, University of Regensburg, Regensburg, Germany, 2University of Regensburg, Department of Pharmaceutical Technology, Regensburg, Germany, 3Duke University, Department of Ophthalmology, Durham, United States

Objective: Current glaucoma medications rely on decreasing IOP by administering topical eye drops which have a long list of drawbacks such as poor compliance and inadequate application. Consequently, there is a strong need for new therapeutic concepts to prevent vision loss. In the present study, we have identified CD44 as a potential target receptor for nanoparticle (NP) delivery in trabecular meshwork (TM) and Schlemm canal (SC) cells. We developed hyaluronic (HA) coated-NP (HA-NPs) with the intention to target the CD44 receptor and to deliver siRNAs to outflow pathway cells.

Methods: CD44 expression was assessed in TM cells after treatment with TGF-B2 (1ng/ml) and CTGF (50ng/ml), in eyes of the ββ1-CGTH glaucoma mouse model, in SC cells as well as outflow tissues derived from glaucomatous and healthy donor eyes by RT-qPCR, Western Blot and/or immunohistochemistry (IHC). LBL-coated NPs were assembled in a modular approach. The particles have four layers: siRNA layer is sandwiched between two polycatonic layers, followed by a final layer based on HA. Porcine and human perfusion samples were used to study outflow tissues derived from glaucomatous and healthy donor eyes by RT-qPCR, Western Blot and/or immunohistochemistry (IHC). HA-NPs were analyzed in TM cells by silencing CTGF with a specific siRNA. Statistical analyses were performed with Student’s t-test.

Results: CD44 expression was significantly increased after TGF-B2 (2.21±0.4) and CTGF (3.81±0.7) treatment in TM cells and in outflow tissues of CTGF-overexpression mice in comparison to controls. Elevated CD44 expression was detected in SC cells and in outflow tissues of eyes from glaucomatous donors compared to healthy eyes. The uptake of HA-NPs was 3-fold more efficient (p<0.05) in TM cells compared to PEI-NPs. In porcine and human perfusion model, HA-NPs were found in a higher concentration within the outflow tissue than PEI-NPs. IHC analyses showed a clustering of PEI-NP in the corneoscleral TM, whereas HA-NPs were found throughout the entire outflow tissues. siRNA delivery and silencing of CTGF were significantly efficient with HA-NPs (0.5±0.1; p<0.05). PEI-NPs had no effect (0.9±0.2).
Early Deficits in Visual and Retinal Function in the Rat Microbead Model of Glaucoma

Hannon, B.G.1, Fu, J.2,3, Kim, R.K.2, Feola, A.I.2,3, Ethier, C.R.2,3, Pardue, M.T.1,2
1Georgia Institute of Technology, Georgia Woodruff School of Mechanical Engineering, Atlanta, United States, 2Georgia Insitute of Technology, Wallace H. Coulter Department of Biomedical Engineering, Atlanta, United States, 3Atlanta VA Medical Center, Center for Visual and Neurocognitive Rehabilitation, Atlanta, United States

The microbead model of ocular hypertension (OHT) is widely used in glaucoma research1, with multiple studies characterizing elevated intraocular pressure (IOP) and axon loss over time. However, early deficits in visual function have not been thoroughly evaluated in this model. We aim to assess visual and retinal function acutely after induction of OHT using the microbead model. 19 male, retired breeder (6-12 months old) Brown Norway rats were anesthetized and received a unilateral intracameral injection of magnetic microbeads (25μl, 15ml/g, 1.44μm diameter). IOP was measured by rebound tonometry (Tonolab) every 2 days for the first week following microbead injection, and subsequently twice/week. Visual function was evaluated using spatial frequency and contrast sensitivity thresholds from optomotor response (OMR) every 2 days for the first week following injection and weekly thereafter. In a preliminary subset of animals, retinal ganglion cell function was evaluated using scotopic threshold response (STR) from full-field flash electrotoretinography (ERG, 4.906cd/m2) 8 days post-injection. IOP in microbead-injected eyes was elevated compared to naïve controls and remained depressed up to 28 days post-injection. Preliminary ERG findings indicate a significant decline in positive STR amplitude at 8 days post-injection.

Our data suggest that significant deficits in visual function, measured via OMR, are present 1 day after microbead injection. Similar significant decline in IOP/OMR function has been reported in a mouse microbead model as early as 3 days post-injection2; to our knowledge, no studies have shown similar trends in rat models. Our ERG data suggest that deficits in retinal function take longer to present themselves than OMR deficits, but still occur on a short time-scale (at some point within 8 days). At present we cannot say whether these results are specific to the microbead model, but future studies should consider the presence of early functional deficits in rodent models of OHT/glaucoma.

Neuroprotection of Retinal Ganglion Cells with Metallothionein-2 in FLOREC Retinal Explants Culture

Pietrucha-Dutczak, M.1, Ajeleti, M.1, Machowicz, J.1, Wojtyniak, A.1, Wittek, P.1, Kocot, E.1, Wawrzonkowski, P.1, Lewin-Kowalki, J.1, Smedowski, A.1,2
1Medical University of Szlesia, Department of Physiology, Katowice, Poland, 2Medical University of Szlesia, Department of Ophthalmology, Katowice, Poland

Aim: To evaluate retinal ganglion cells (RGC) survival under metallothionein-2 (MT2) treatment in FluoroGold-labeled organotypic rat retinal explants culture (FLOREC).

Materials and methods: Eight Wistar rats received FluoroGold (FG) injection into superior colliculi to retrogradely label RGC. After 5 days, rats were sacrificed by anesthetics overdose, eye balls were removed, retinas isolated and placed in tissue culture inserts. Explants (n=32) were cultured in neuronal-specific medium with or without addition of 1 μg/ml of MT2 and/or 1 μg/ml of gentamicin (LDLR2 receptor blocker). The culture medium was exchanged every second day and collected for LDH assay. After 7 days, 16 explants were fixed with 4% PFA and stained with DAPI and FG. After 5 days, rats were sacrificed by anesthetics overdose, eye balls were removed, retinas isolated and placed in tissue culture inserts. Explants (n=32) were cultured in neuronal-specific medium with or without addition of 1 μg/ml of MT2 and/or 1 μg/ml of gentamicin (LDLR2 receptor blocker). The culture medium was exchanged every second day and collected for LDH assay. After 7 days, 16 explants were fixed with 4% PFA and stained with anti-b-tubulin antibody and TUNEL for apoptotic cells. The RGC density was evaluated for FG labeling using ImageJ. Other 16 explants were utilized for Western Blotting (WB) to evaluate HuR protein content.

Results: Density of FG-positive cells was significantly higher in treated explants compared to control. In treated explants the LDH activity decline was significantly slower, suggesting more cells surviving after 7 days of culture that the LDH activity in untreated explants was decreased to 22% at day 3 and to 53% in non-treated explants; p< 0.001). In WB, the MT2 treatment delayed HuR protein content increase, probably by reducing oxidative stress, which is an enhancer of HuR expression. This would not have been present if the megalin receptor (LDLR2) was blocked with gentamicin.

Conclusion: Treatment with MT2 exerts neuroprotective effect expressed in prolonged survival of RGC in rat retinal explants. The possible mechanism includes suppression of oxidative stress in retinal cells.

Functional Analysis of MANF in Retinal Ganglion Cells by Oxidative Stress

Ko, J.1, Okumichi, H.1, Kimchi, Y.1
1Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Japan, 2Hiroshima University Graduate School of Biomedical, Hiroshima, Japan

Background and aims: The neuroprotection of retinal ganglion cells is important for development of new drugs for glaucoma. We have now examined the effects of mesenchephalic astrocyte derived neurotrophic factor (MANF) on the retinal ganglion cells survival by oxidative stress.

Methods: Primary retinal ganglion cells from 4-5day rats after birth were cultured. And, the cells were treated with 500 mM hydrogen peroxide (H2O2), at the same time, stimulated by MANF. After cultivation for indicated time, using RT-PCR and immunoblot analysis, the expression levels of the several survival markers of retinal ganglion cells were studied. Also, the extension of neurites of retinal ganglion cells after culture was examined by Immunofluorescence analysis.

Results: Immunoblot and Immunofluorescence analysis were indicated the down regulated expression levels of the several survival markers of RGC. Field of view of neurites were decreased in RGC with 500 mM H2O2. But, in additional MANF, we found blocked down-regulated expression of neural markers and extension of neurites by oxidative stress in RGC. So, the stimulation with MANF was blocked the damage from oxidative stress in RGC.

Conclusions: These results suggest that MANF may play an important role in the survival of RGC with oxidative stress. In brief, it is possible that MANF is important key factor in neuroprotection of RGC.

Novel Low Molecular Weight Compound OBP-801 Ameliorates Detrimental Scarring Formation Accompanied in Glaucoma Filtration Surgery

Yamamoto, Y.1,2, Mukai, A.1, Ueno, M.1, Hamuro, J.1, Ura, Y.1, Kinoshita, S.1, Solduzo, C.1
1Kyoto Prefectural University of Medicine, Kyoto, Japan, 2Baptist Eye Institute, Kyoto, Japan, 3Oncolyces BioPharma Inc., Tokyo, Japan

Purpose: To evaluate the practical usefulness of OBP-801, a pluripotent epigenetic repressor of diverse genes activated and involved during scar formation in glaucoma filtration surgery (GFS).

Methods: GFS was performed by introducing a siliconula cannula through a scleral tunnel under a conjunctival flap, resulting in aqueous-filtering blebs. Rats that underwent GFS were divided into four treatment groups.

Group I rabbits received 200μl subconjunctival injection of balanced salt solution 30 minutes before the GFS surgery, and Group II rabbits received 100μl subconjunctival injection of 0.02% mitomycin C (MMC) solution 30 minutes before the GFS surgery.

Group III rabbits received 200μl subconjunctival injection of 10 nM OBP-801 solution (315 pg/kg) 30 minutes before the GFS surgery, and Group IV rabbits received 80 μl eye drops of 100 μM OBP-801 solution (2517 pg/μl) 30 minutes before the GFS surgery and two times per day on days 1, 2, 4, 5, 6, 7 after the surgery. The contralateral eyes of rabbits were used as the nonsurgical control. Clini- cal score of IOP, bleb vascularity, and slit-lamp examination were performed. Blebs were harvested at days 2, 5, 10 and three or more replicates of three blebs per time point were investigated for the expression of genes using microarrays. On postoperative day 30, the bleb tissues were collected to evaluate the tissue fibrosis by staining with hematoxylin and eosin, a-smooth muscle actin (αSMA), and collagen-1. Alpha-SMA and collagen-1 expres- sion in conjunctiva were analyzed by Western blot.

Results: OBP-801 treatment after GFS showed no signs of edema, corneal opacity, endophthalmitis, or cataract formation. Morpho- metric analysis of OBP-801 treated eyes showed lower bleb height compared to MMC treated eyes. Importantly, OBP-801 treated eyes maintained the low IOP stably until the end of the tested period, day 30, whereas the repression of increased IOP was not confirmed in MMC, or balanced salt solution group. OBP-801 treated eyes showed normal vascular appearance, in contrast to the avascular appearance in MMC treated eyes. In accordance with the clinical efficacy, OBP-801 treatment reduced the expression levels of αSMA, and collagen-1 in microarray analysis, immuno- staining, and Western blot analysis, compared to MMC, or balanced salt solution treated eyes. Conclusions: OBP-801 can be a potent useful adjunct at the very low dose to improve the GFS outcome.

Asessing Shear Stress in Schlemm's Canal Using Shear Stress-Responsive Reporter Adenoviruses

McDonnell, F.1, Perkumas, K.M.1, Ashpole, N.E.1, Kalnits- ky, J.1, Stamer, W.D.1,2
1Oule University Eye Center, Ophthalmology, Durham, United States, 2Oule University, Biomedical Engineering, Durham, United States

Introduction: Elevation of intraocular pressure (IOP) leads to narrowing of the Schlemm’s canal (SC) lumen, causing increased shear stress. SC endothelial cells produce nitric oxide (NO) in a shear-dependent manner through activation of endothelial nitric oxide synthase (eNOS); creating a feedback loop that increases outflow facility. Using two engineered adenoviruses constructs which contain the eNOS promoter driving either Secreted Alkaline Phosphatase (eNOS-SEAP) or GFP (eNOS-GFP), we tested the effects of shear stress on confluent endothelial cell monolayers in vitro and ex vivo.

Methods: HUVECs or SC cells were transduced with eNOS-SEAP or eNOS-GFP adenovirus (5.5x1010IU/kg) for 8 hrs. Cells were then exposed to flow resulting in shear stress of 0.1 or 10 dynes/cm2. In parallel, human anterior segments were perfused at 2.5 μl/min for 24-48 hrs prior to retroperfusion with both the eNOS-SEAP and eNOS-GFP adenoviruses (2x1016IU/ml). Perfusion was restarted at 2.5 μl/min in one anterior segment and a new flow rate in the contralateral segment that doubled IOP; giving low and high shear stress in the SC lumen, respectively. SEAP secretion was measured using a SEAP reporter assay kit. GFP expression was measured using Western blotting, confocal microscopy and flow cytometry. Nitrile concentration was measured using a Griess Reagent assay.

Results: In response to 10 dynes/cm2 shear stress, HUVECs pro- duced 4.9-fold more SEAP and 6-fold more GFP than cells exposed to 0.1 dynes/cm2. Similarly, SC cells exposed to 10 dynes/cm2 shear stress increased SEAP secretion by 1.4 fold. Fluorescence intensity of GFP by confocal microscopy increased 1.7-fold and by flow cytometry increased 3-fold when compared to controls. Human anterior segments perfused at a higher flow rate, and therefore increased IOP showed a 2.5 fold increase in SEAP secre- tion in effluent and qualitatively higher levels of GFP expression in SC lumen compared to those of lower flow rates. Elevated IOPs corresponded to increased nitrile concentrations in perfusion effluent.

Discussion: The eNOS-SEAP and eNOS-GFP adenoviruses are effective tools to study SC responses to shear stress and better understand conventional outflow dynamics; showing the capacity to quantify and localize shear stress of endothelial cells in culture, and SC cells in situ in an organ culture model.
Systemic Vascular Risk Factors for Multiple Retinal Nerve Fiber Layer Defects

Jung, K.I., Park, C.K.
The Catholic University of Korea, Department of Ophthalmology, Seoul, Korea, Republic of

Multiple retinal nerve fiber layer (RNFL) defects develop uncommonly, even though glaucomatous RNFL loss is typically observed as one RNFL defect in each quadrant. We investigated the risk factors associated with multiple RNFL defects to increase our understanding of the nature and pathogenesis of various RNFL defect patterns. Data from subjects with multiple RNFL defects (28 patients) and glaucoma patients without multiple RNFL defects (194 patients) were analyzed. The term “multiple RNFL defects” refers to three or more isolated defects separated by a comparably normal area. Patients with multiple RNFL defects showed a higher prevalence of hypertension, end-stage renal disease, and cerebrovascular disease than those without multiple RNFL defects, both before and after propensity score matching for age and mean deviation (P<0.05). The number of patients with paravenous visual field points depressed < 5% on pattern deviation plots was higher in subjects with multiple RNFL defects than in those without multiple RNFL defects (P=0.048). In conclusion, the presence of multiple RNFL defects had clinical relevance for systemic vascular risk factors and a higher risk of paravenous scotoma. Clinicians should be aware of the possibility of concomitant systemic vascular disease when evaluating patients with multiple RNFL defects.

Analyzing the Role of Cav-1 in Mediating Acute IOP Induced Inner Retinal Deficits

Abassi, M., Gupta, V., Chitranshi, N., Graham, S., Vision Neuroscience
1Moirae University, Clinical Medicine, Sydney, Australia; 2Save Sight Institute, The University of Sydney, Sydney, Australia

**Purpose:** Glaucoma is characterized by progressive loss of retinal ganglion cells (RGCs) and the enhanced intraocular pressure (IOP). More recently, genome-wide association studies have linked variation in Cavolin1/2 (Cav1/2) gene loci as a risk factor in glaucoma. However, the biochemical role of Cav-1 and the potential mechanisms underlying this genetic association remain to be explored. We recently demonstrated that Cav-1 knockout mice (KO) exhibit reduced inner retinal function compared to the wildtypes (WT) littermates. Here we investigated the effects of genetic ablation of Cav-1 on the inner retinal function in mice by experimentally inducing acute elevation of IOP.

**Methods:** A total number of n=16, 6-8-week-old WT and Cav-1 KO mice were used in this study. To induce the acute IOP ocular hypertension paradigm (70 mmHg), mice eyes were connected to a 33-gauge needle linked to an external saline reservoir for 1 hr and animals were subsequently euthanized 2 weeks later. Retinal function was evaluated by electroretinogram (ERG) and positive scotopic threshold response (pSTR) recordings. TUNEL assay was performed to assess any apoptotic changes in the retina. Brn3a immunofluorescence staining was used as a marker to assess and quantify GCL changes.

**Results:** Exposure to acutely elevated IOP induced a significantly greater loss in the average pSTR amplitude in WT (50±10%; p<0.001) compared to that of Cav-1 deficient mice (35±15%; p<0.05) (n=8 each). Similarly, ERG a- and b-wave amplitudes were significantly reduced in WT eyes (47±8%) compared to the Cav-1−/− mice (25±10%) retinas (n=8; p<0.05). High IOP induction resulted in apoptotic activation in the retina, however the WT eyes demonstrated significantly more TUNEL immunoreactivity compared to the Cav-1−/− littermates. Immunostaining of retinal sections further revealed prominent loss of Brn3a positive ganglion cell population in the WT group (50±13%) compared to that of Cav-1−/− animals (34±9%).

**Conclusion:** Our findings suggest that Cav-1 adaptor protein ablated retinas show partial resistance to functional loss induced by acutely elevated IOP. This could potentially be caused by adaption of the retinas to pre-existing molecular stress in Cav-1 null mice. Alternately, it could be attributed to impairment of a pathological process involving Cav-1 that is otherwise activated in the retina under glaucomatous conditions.

Endosomal Function and Glaucoma

Mckay, B.S.1, Sillik, S.A.1, Figueroa, A.G.1, Dismuke, W.M.2, Locke, C.J.1, Congrove, N.R.1, Stamer, W.D.2
1University of Arizona, Ophthalmology and Vision Science, Tucson, United States; 2Duke University, Ophthalmology, Durham, United States

Multiple genes either linked to or causative for open angle glaucoma (POAG) function in the endosomal pathway. These include myocilin, optineurin, and cavolin-1, each of which are widely expressed yet genetic changes cause a very limited phenotype. Exosomes, small extracellular vesicles that function in inter-tissue communication, are produced in the endosomal pathway, specifically the multivesicular body (MVB). We showed that the myocilin is recruited to GPR143 during receptor mediated endocytosis, and then is released on the surface of exosomes. In previous work we demonstrated that ligand activation of GPR143 halted exosome release from retinal pigment epithelial (RPE) cells. Together, this suggests a relationship between exosomes, GPR143 signalling, and myocilin that may impact glaucoma. Here we tested whether GPR143 controls exosome release from the ciliary epithelium (CE). Using ciliary body tissue isolated from porcine eyes we collected exosomes from conditioned control medium, or medium containing 1.0μM L-DOPA; the ligand of GPR143. After 30 minutes of treatment, exosomes were isolated from conditioned media by differential ultracentrifugation, then analyzed for protein content and characterized by nanoparticle tracking analysis. Aqueous humor (AH) was analyzed in parallel to determine whether the exosomes in AH matched those released by the CE. Results illustrate that CE releases two populations of different sized exosomes, 105 and 130nm in diameter. Western blot analysis confirmed that myocilin was released with the exosomes from CE. AH exosomes included both the 105 and 130nm populations, but also included an additional 70nm diameter population. In contrast, RPE released 4 distinct populations of exosomes 45, 60, 75, 90nm in diameter. Activation of GPR143 by L-DOPA halted exosome release from the CE. However, GPR143 pharmacology was complex in CE, as dopamine, a GPR143 antagonist exhibited variable effects on exosome release resulting in either increased (n=9) or decreased (n=9) exosome release. In conclusion, we found that control of exosome release by GPR143 is tissue specific, but the control may be complex when extended beyond L-DOPA and GPR143. Our results suggest that exosomes offer a viable controlled communication system in the anterior segment likely impacted by proteins linked to glaucoma.
The Activation of Endocannabinoid Signaling and Microglial Activity after Optic Nerve Injury

Luker, M. M. 1 Szczesniak, A.-M. M. Kelly, M. E. M.

1Dalhousie University, Pharmacology, Halifax, Canada. 2Dalhousie University, Halifax, Canada

Glucoma is an age-related, multifactorial blinding eye disease that results in optic nerve damage and retinal ganglion cell (RGC) loss. It is accompanied by a neuroinflammatory response, involving early and exacerbated activation of retinal glial cells and increased production of inflammatory mediators. The activation of the endocannabinoid system (ECS) through cannabinoid (CB) receptors has been shown to induce anti-inflammatory responses and promote the survival of neurons. However, little is known about the molecular events that link endocannabinoid (eCB) signaling to RGC death. We hypothesized that a better characterization of ECS involvement in RGC loss and retinal pathology could develop potential therapeutic targets of glaucoma. Here, we used an optic nerve transection (ONT) as an experimental model of RGC injury to determine if there are changes in ECS signaling in the axotomized retina.

The optic nerve of mice aged 2-4 months old were transected. Retinas were harvested 2, 4, and 7 days after ONT. Total RNA was isolated and reverse transcribed into complementary DNA from isolated and reverse transcribed into complementary DNA from the axotomized retina. Following optic nerve crush (ONC), the retinal ganglion cells (RGCs) of wild type mice undergo apoptosis and the vast majority are eliminated from the injured eye 3 weeks after ONC. Converse- ly, the RGC somas of Bax-/- mice survive indefinitely following ONC. Thus, Bax is an intriguing therapeutic target for the prevention of RGC loss in glaucoma. It is unknown if lowering the quantity of BAX in RGCs protects them from apoptosis following optic nerve injury. Therefore, we used Bax-/- mice to test further the hypothesis that a lowered quantity of BAX in RGCs would prevent them from undergoing apoptosis after ONC. We found that Bax-/- mice did not lose a significant percentage of their RGCs until 12 weeks after ONC and that even at 24 weeks post-ONC, only 12% of cells in the RGC layer had been lost. Next, we sought to assess the status of apoptotic machinery in these cells in the months after ONC. We used immunofluorescence to visualize cells that expressed the RGC marker, Brn3a, and a contributor to RGC apoptosis, phosphorylated c-Jun (p-cJun). Within 7 days, p-cJun accumulated in the majority of Brn3a positive cells. This accumulation persisted for 7 weeks but by 12 weeks there was a decrease in the percentage of cells expressing p-cJun that was not accompanied by a decrease in the percentage of cells expressing Brn3a, suggesting that these cells are deactivating their apoptotic program. Next, we tested the hypothesis that, 12 weeks after optic nerve crush, RGCs damaged by ONC would be protected from apoptosis. We did this by using an AV2-Pgk-GFP-BAX virus to introduce exogenous GFP-BAX into the RGCs of Bax-/- mice at 8-12 weeks after ONC. We found that 12 weeks after ONC, there was no difference in the percentage of RGCs with punctate (active) BAX in the damaged eye as the contralateral eye. Finally, we used qPCR to assess the transcriptional program of damaged RGCs in the months following ONC. We found that the transcript abundance of several BH3-only genes, but principally Noxa and Hrk, as well as markers of glial activation and Jun-directed transcription, were upregulated in damaged retinas immediately following ONC, but that this activation abated 12 weeks. Taken together, these results suggest that lowering BAX dosage in RGCs prevents them from undergoing apoptosis, without interfering with the cell’s ability to activate upstream apoptotic pathways, and that RGCs will eventually deactivate their apoptotic program in spite of their severed axons.

The Apoptotic Machinery in the Retinal Ganglion Cells of Bax Heterozygous Animals Is Deactivated 7 Months After Optic Nerve Crush

Donahue, R., Grosser, J., Nickells, R.

University of Wisconsin School of Medicine and Public Health, Ophthalmology and Visual Sciences, Madison, United States

Following optic nerve crush (ONC), the retinal ganglion cells (RGCs) of wild type mice undergo apoptosis and the vast majority are eliminated from the injured eye 3 weeks after ONC. Conversely, the RGC somas of Bax-/- mice survive indefinitely following ONC. Thus, Bax is an intriguing therapeutic target for the prevention of RGC loss in glaucoma. It is unknown if lowering the quantity of BAX in RGCs protects them from apoptosis following optic nerve injury. Therefore, we used Bax-/- mice to test further the hypothesis that a lowered quantity of BAX in RGCs would prevent them from undergoing apoptosis after ONC. We found that Bax-/- mice did not lose a significant percentage of their RGCs until 12 weeks after ONC and that even at 24 weeks post-ONC, only 12% of cells in the RGC layer had been lost. Next, we sought to assess the status of apoptotic machinery in these cells in the months after ONC. We used immunofluorescence to visualize cells that expressed the RGC marker, Brn3a, and a contributor to RGC apoptosis, phosphorylated c-Jun (p-cJun). Within 7 days, p-cJun accumulated in the majority of Brn3a positive cells. This accumulation persisted for 7 weeks but by 12 weeks there was a decrease in the percentage of cells expressing p-cJun that was not accompanied by a decrease in the percentage of cells expressing Brn3a, suggesting that these cells are deactivating their apoptotic program. Next, we tested the hypothesis that, 12 weeks after optic nerve crush, RGCs damaged by ONC would be protected from apoptosis. We did this by using an AV2-Pgk-GFP-BAX virus to introduce exogenous GFP-BAX into the RGCs of Bax-/- mice at 8-12 weeks after ONC. We found that 12 weeks after ONC, there was no difference in the percentage of RGCs with punctate (active) BAX in the damaged eye as the contralateral eye. Finally, we used qPCR to assess the transcriptional program of damaged RGCs in the months following ONC. We found that the transcript abundance of several BH3-only genes, but principally Noxa and Hrk, as well as markers of glial activation and Jun-directed transcription, were upregulated in damaged retinas immediately following ONC, but that this activation abated 12 weeks. Taken together, these results suggest that lowering BAX dosage in RGCs prevents them from undergoing apoptosis, without interfering with the cell’s ability to activate upstream apoptotic pathways, and that RGCs will eventually deactivate their apoptotic program in spite of their severed axons.

Hand-Held Spectral Domain Optical Coherence Tomography Measurements of Anterior Chamber Angle in Children with Congenital Glaucoma

Edawari, B., Shah, S., Proudflock, F., Gottlob, I.

University of Leicester, Department of Neuroscience, Psychology and Behaviour, Ulverscroft Eye Unit, Leicester, United Kingdom

Purpose: To compare anterior chamber angle measurements of children with congenital glaucoma and age-matched controls using hand held spectral domain optical coherence tomography (HH-OCT).

Methods: We compared 33 children treated and operated for congenital glaucoma (mean age 4.3 ± 3.6 years; range from 2 months to 9.7 years) to 170 healthy children (mean age 3.8 ± 2.9 years; range from 2 days to 10.2 years). All participants’ eyes were imaged using HH-OCT (Leica Microsystems Ltd) without sedation. Customized ImageJ macro was used to measure nasal and temporal Schwalbe’s line opening distance (SLAOD) and trabecular iris surface area (SLTISA), after manual identification of Schwalbe’s line (SL). Mixed models adjusted for age, gender, eye variation and season were used to compare between anterior chamber angle measurements of congenital glaucoma and controls.

Results: Children with congenital glaucoma had significantly wider nasal SLAOD and SLTISA throughout childhood compared to controls (both p values < 0.05) (Table 1). The temporal SLAOD and SLTISA became significantly wider in congenital glaucoma compared to controls by one year of age (both p values < 0.001.)

Nasal angle widening developed earlier than the temporal angle in both groups. The nasal angle was significantly wider in congenital glaucoma compared to controls by the age of three months (p > 0.05). In contrast, the temporal angle was not significantly different in both groups at age of 3 months (p > 0.05). During the first year, the nasal SLAOD and SLTISA, both increased by 19% (from 717 to 854 µm) and 358% (from 0.07 to 0.31 mm2) in congenital glaucoma and by 109% (from 315 to 656 µm) and 111% (from 0.12 to 0.26 mm2) in controls, respectively. Widening of angle could be used prognostically for congenital glaucoma.

Conclusion: Anterior chamber angle became wider in congenital glaucoma compared to age-matched healthy children regardless of whether treatment was with medication or surgical intervention. HH-OCT is a promising non-invasive tool to understand the developmental changes in congenital glaucoma and to evaluate angle widening could be used prognostically for congenital glaucoma management.

<table>
<thead>
<tr>
<th>Time point</th>
<th>SlAOD (µm)</th>
<th>SLTISA (mm2)</th>
<th>SlAOD (µm)</th>
<th>SLTISA (mm2)</th>
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</thead>
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<tr>
<td>3 months</td>
<td>-1.4 (328.6, 325.9) 0.993</td>
<td>-0.06 (0.19, 0.08) 0.410</td>
<td>351.2</td>
<td>546.2</td>
</tr>
<tr>
<td>1 year</td>
<td>145.0 (148.9, 201.1) 0.000</td>
<td>0.04 (0.02, 0.07) 0.000</td>
<td>196.8</td>
<td>152.9</td>
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<tr>
<td>3 years</td>
<td>169.9 (104.1, 235.8) 0.000</td>
<td>0.06 (0.03, 0.10) 0.000</td>
<td>172.2</td>
<td>225.5</td>
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<tr>
<td>5 years</td>
<td>178.8 (110.2, 247.5) 0.000</td>
<td>0.07 (0.04, 0.13) 0.000</td>
<td>166.8</td>
<td>219.5</td>
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<tr>
<td>10 years</td>
<td>242.5 (160.6, 324.3) 0.000</td>
<td>0.11 (0.07, 0.14) 0.000</td>
<td>163.0</td>
<td>107.6</td>
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</tbody>
</table>
Cataracts Are a Non-cancerous Effect of Ionizing Radiation - A Lifetime Study in Mice

Dalke, C.1, Kunze, S.1, Pawliczek, D.1, Neff, F.2, Rößler, U.1, Greiter, M.3,4,5,6,7, Schlattl, H.3,4,5,6,7, Hernandez, F.1, Reddy, A.1, David, L.3,4,5,6,7, Paillard, S.1,2, Lovicu, F.J.1,2, Lens Research Laboratory (02NUK045A), Université de Rennes 1 - Faculté de Médecine - Développement de Rennes, Rennes, France, 1Oregon Health & Science University, Department of Biological Sciences, Portland, United States, 2Stony Brook University, Stony Brook, United States.

Ionizing radiation is one of several risk factors for cataract formation, but the effects of low dose ionizing radiation are not well understood and the mechanisms of radiation-induced cataracts are still unclear. In a lifetime study with mice we analyzed radiation effects on the eye and other organs.

Deficiency of the conserved RNA-binding protein (RBP) Celf1 causes lens defects and cataract in vertebrates. Celf1 regulates the proteome by distinct post-transcriptional control (PTC) mechanisms such as pre-mRNA splicing, mRNA decay, and translational control. These Celf1-mediated PTC outcomes depend on its interaction with other regulatory proteins. However, the specific proteome impact of Celf1 that determines its function in lens has not been investigated. Furthermore, an integrated analysis of the proteome and transcriptome regulated by Celf1 in mouse lenses has not been reported. Here we report novel Celf1-interacting proteins and several high confidence Celf1 targets that are differentially expressed in Celf1 deficient mouse lenses.

Immunoprecipitation (IP) was performed with Celf1-specific antibody (test) or IgG (control) on protein lysates prepared from mouse embryonic day (E) 16.5 and early post-natal day (P) 5 wild-type mouse lenses, and the mouse lens epithelium-derived cell line 21EM15. High-throughput tandem mass spectrometry (MS/MS) was then performed using Q-Exactive LC-MS/MS system. Tandem mass tag (TMT) labelled quantitative proteomics was performed with 5 TMTplex® and 5 control samples. Each sample was triplicated digested and labelled with TMT tags. Normalized samples were run on the Orbitrap Fusion. The data was analyzed with Proteome Discoverer v.1.4 and EdgeR.

Using MS/MS and prioritized protein interaction, we identified 79 high-confidence candidate proteins in the Celf1-pulldown from 21EM15 lens cell line and E16.5 and P5 lens lysates. Functional annotation clustering analysis identified several RBPs (Elavl1, Elavl2, Elavl3, Hnrnp, Hnrnpd, splicing factors (Prpf35, Snpn40), translation factors (Eif5a, Eif4a2, Eif3e and ribonucleases (Pann3, Rnasel). Further, Elavl1 was co- immunoprecipitated with Celf1 in 21EM15. Relative quantitative proteomics identified 60 differentially expressed proteins in Celf1+/– lenses. Integrated analysis of this with RNA-Seq and microarray identified several differential expressed candidate proteins and transcripts such as heat shock protein (Hspd1), transcription factors (Cdx2, Zfp945). Kinochore interacting protein (Zwint) and Fmnl (Bn–A) agreeing in all three data sets.

Together, these data define the Celf1 interactome in the lens and identify high priority Celf1 targets providing new directions for investigating RBP-mediated PTC in lens development and cataractogenesis.
Investigating Retinal Biomarkers for Usher Syndrome in Preparation for Gene Therapy

Gill, J.S.1, Mitsios, A.2, Houston, S.1,2, Theofylaktoptoulos, V.2, Dubis, A.M.1,3, Moosajee, M.1,2

1UCL Institute of Ophthalmology, London, United Kingdom, 2NIHR Biomedical Research Centre at Moorfields Eye Hospital NHS Trust and UCL Institute of Ophthalmology, London, United Kingdom

Purpose: Usher syndrome (USH) represents a large sub-group of inherited retinal diseases that lead to 18% of all retina pigmentosa cases. Recent advances in non-viral gene therapy have raised possibilities of treatment for retinal diseases such as USH. However, detailed longitudinal imaging data is required in preparation for clinical trials to establish rates of progression and inform trial design for slow progressing, degenerations which are binocularly/

Longitudinal Phenotyping of Non-syndromic Retinitis Pigmentosa Caused by Mutations in USH2A

Ang, Y.L.1, Mitsios, A.1,2, Dubis, A.1, Moosajee, M.1,2,3

1UCL Institute of Ophthalmology, London, United Kingdom, 2NIHR Biomedical Research Centre at Moorfields Eye Hospital and UCL Institute of Ophthalmology, London, United Kingdom, 3Great Ormond Street Hospital for Children NHS Foundation Trust, London, United Kingdom

Introduction: USH2A mutations are the commonest cause of Usher syndrome and account for 23-25% of non-syndromic autosomal recessive retinitis pigmentosa (RP). There is a lack of non-syndromic USH2A-RP natural history data in the literature. In this study we evaluated best corrected visual acuity (BCVA), structural SD-OCT and fundus autofluorescence (FAF) in a group of molecularly confirmed non-syndromic USH2A-RP patients to assess progression and potentially determine suitable outcome measures to guide future therapy trials.

Methods: Patients were identified from the Moorfields Eye Hospital Inherited Eye Disease Database. Patients who have molecularly proven USH2A variants, at least 3 visits withgradable SD-OCT and FAF data, and no history of congenital hearing loss were included. Ellipsoid zone (EZ) length, outer nuclear layer (ONL) and photoreceptor layer thickness at the fovea were measured from OCT. Ring appearance, hyperautofluorescence (hyperAF) ring area were correlated to timepoint 2 (0.7 to 0.65), but this was not statistically significant (p=0.11). Baseline EZ length and ring area, respectively, were highly correlated between eyes (r2=0.76; 0.66). Baseline EZ length was also correlated to EZ length at timepoint 3 and rate of EZ length decline (r=0.96; 0.51 respectively). Rate of EZ length decline for the whole cohort was 142.4±41µm/yr (149.0±53µm/yr for age ≤40y; 138.4±51µm/yr for age ≥40y; range -20.23 to 973.91). There was a correlation between the rate of decline of both the EZ length and ring area, respectively, from baseline to timepoint 1 (r=0.72, 0.66). Rate of ring area decline for the whole group was also correlated to EZ length at timepoint 3 and rate of EZ length decline (r=0.76; 0.51 respectively). Rate of EZ length decline for the whole cohort was 142.4±41µm/yr (149.0±53µm/yr for age ≤40y; 138.4±51µm/yr for age ≥40y; range -20.23 to 973.91). Baseline EZ length and area of ring were correlated (r=0.6).

Conclusions: EZ length and hyperAF ring area could be used as outcome measures in trials. Rate of decline of EZ length and ring area were higher in younger patients <40y compared to those ≥40y. Interestingly, the baseline EZ length was highly variable across all ages.

Imaging Dementia through the Eye

Csincsik, L.1,2, Mac Gillivray, T.1, Pellegrini, E.1, Flynn, E.1, Papanastasios, G.1, Shakespeare, T.1, Crutch, S.1, Ritchie, C.1, Peto, T.1,2, Lengyl, E.1,2

1Queen’s University Belfast, Belfast, United Kingdom, 2University College London, London, United Kingdom, 3University of Edinburgh, Edinburgh, United Kingdom, 4The George Washington University, Washington DC, United States, 5Moorfields Eye Hospital NHS Foundation Trust, London, United Kingdom

Purpose: Pathological changes in the eye have been reported in a range of neurodegenerative diseases. Thinning of the retinal nerve fibre layer (RNFL) increased accumulation of extracellular drusen deposits and vascular changes might mirror changes in the brain. Here we report on dementia studies examining retinal images to gain insights into retinal changes in patients and healthy controls (HC) using Optos Ultra-wide field (UWF) and Optos Optical Coherence Tomography (OCT) imaging.

Methods: UWF images were graded for the prevalence of Age-Related Macular Degeneration (AMD)-like pathologies and analysed for Retinal Vascular Parameters (RVPs). OCT scans were analysed for macular and peripapillary (pp) thickness as well as pp vascular calibre (VC) changes.

Study 1: Only UWF images were acquired of 48 AD patients and 59 HC at baseline (BL) and after a 2-year follow-up (FU). Patients with AD were slightly older (79.2±18.4) than HC (70.7±10.4) (p<0.0002).

Study 2: UWF images and OCT scans were acquired of 33 Posterior Cortical Atrophy (PCA) patients, 28 AD patients and 71 HC. There was no significant difference in age between PCA, AD and HC (66.7±8.6 vs. 65.8±6.3 vs. 66.1±7.5; p=0.1).

Results:

Study 2: Grading for AMD-like pathologies at BL, revealed a significantly higher prevalence of a hard drusen phenotype in the periphery of AD (21/55, 25.4%) compared to HC (2/48, 4.2%) (x2=9.9, df=4, p=0.04). Increased drusen number was also observed at the 2-year FU in AD compared to HC. RVP analysis found a decreased arteriole FD (p=0.05) and increased venular WGs (p=0.01) in AD compared to HC. At FU a trend remained in both measures between the two groups (p=0.2). From BL to FU no progression was detected in any of the RVPs.

Study 2: UWF imaging detected higher prevalence of RPD in AD (30%) than in PCA (10%) or HC (17%) (x2=6.879, df=2, p=0.032).

Analysis of OCT scans showed a significant reduction in pp outer plexiform − inner nuclear layer (OPL-ILN) thickness in PCA compared to HC (64.15µm±7.45µm vs 68.7±9.45µm, p=0.049) and a significant increase in ppV in both PCA (457.05µm±89.85µm) and AD (478.4µm±83.4µm) in HC (411.5µm±82.75µm) (p<0.05).

Conclusion: These findings suggest that some retinal imaging parameters could become a valuable tool in detecting and monitoring the progression of dementia. However, there are challenges in imaging AD and PCA patients and care needs to be taken to acknowledge limitations during image analysis.
Intravitreal Anti-vascular Endothelial Growth Factor Therapies for Diabetic Macular Edema with Practical Protocol

Oshitari, T.1,2
1Chiba University Graduate School of Medicine, Ophthalmology and Visual Science, Chiba, Japan, 2International University of Health and Welfare, Ophthalmology, Narita, Japan

Purpose: To determine the efficacy of intravitreal aflibercept and ramipril in an experimental protocol on diabetic macular edema (DME).

Methods: The medical records of 46 eyes of 40 patients who had received IVR and 49 eyes of 36 patients who had received IVA were reviewed. All of the patients had DME. The best-corrected visual acuity (BCVA), and the retinal macular thickness (CMT) were measured at the baseline and at 1, 3, and 6 months after the IVA or IVR. To determine the efficacy of IVA after one year of treatments, the medical records of 52 eyes of 43 patients with DME who had received IVA treatments were reviewed. The BCVA and the CMT were measured at the baseline and at 1, 3, 6, and 12 months after the IVA.

Results: After 6 months, the mean numbers of IVA injections was 2.6±1.1 and IVA was 2.7±1.4. After 12 months, the mean number of injections of IVA was 3.8±2.4. The mean BCVA was significantly better than the baseline only at 1 and 3 months after IVA and at 1, 3, and 6 months after IVR (P<0.05). After 12 months, the BCVA and CMT were significantly improved after the IVA at all follow-up times (P<0.05). The BCVA was better in eyes with a serous retinal detachment (SRD) than without a SRD (P=0.01). The effects of IVA persisted longer than that of IVR for at least 6 months. There was a significant correlation between the photoreceptor outer segment (PROS) length and BCVA at the baseline and at 12 months after IVA (P<0.05).

Conclusion: A fewer number of anti-VEGF injections significantly improved the BCVA and the CMT in eyes with DME. The effectiveness of both IVA and IVR was not dependent on the presence of SRD. The PROS length may be a predictive marker for visual outcomes after one-year treatment with IVA for DME.

Evaluation of Intravitreal Anti-vascular Endothelial Growth Factor Injection on Renal Function in Patients with Diabetic Macular Oedema

Gallagher, B.1, Douglas, T.1, Little, J.-A.1, Silvestri, G.1, McKay, G.2
1Queens University Belfast, Belfast, Belfast, United Kingdom, 2Ulster University, Belfast, United Kingdom, 3Belfast Health and Social Care Trust, Belfast, United Kingdom

Purpose: Hypertension, proteinuria and renal thrombotic microangiopathy have previously been reported following intravenous administration of anti-vascular endothelial growth factor (anti-VEGF) in oncology. Intravitreal administration with reduced dosage has improved the safety profile in ophthalmology. Nevertheless, given the reported systemic effects and several reports of acute kidney injury following anti-VEGF injection, further consideration of renal safety is necessary. We aimed to assess the long-term effect of multiple intravitreal anti-VEGF injections on the rate of decline of estimated glomerular filtration rate (eGFR).

Methods: A retrospective audit of 92 patients receiving treatment for diabetic macular oedema (DMO) was undertaken. Serum creatinine measurements (to estimate eGFR), number and type of intravitreal anti-VEGF injections and potential confounding variables were collected from electronic healthcare records. A co-efficient of eGFR change over time was calculated from measurements taken before, during and after the injection period. Linear regression modelling was used to assess variation in the number of anti-VEGF injections and change in eGFR in unadjusted and adjusted analyses.

Results: A total of 92 patients with DMO (57.6% male, 78.3% type 2 diabetes mellitus (T2DM)) were included in the audit. The mean duration of diabetes was 16.1 years and median HbA1c was 66.9 mmol/L. There was a high prevalence of co-morbidities with 82.6%, 76.1% and 33.7% of patients having hypertension, hyperlipidaemia and chronic kidney disease, respectively. On average, 26.9 intravitreal anti-VEGF injections were given per patient over a mean duration of 31 months. Renal function declined from a mean baseline eGFR of 75.4 ± 21.0 mL/min/1.73m² to a follow up mean eGFR of 66.6 ± 22.7 mL/min/1.73m². However, no association between increasing number of intravitreal anti-VEGF injections and eGFR slope was detected (beta = 0.035; p=0.206, confidence intervals [CI]: -0.2, 0.89), which remained non-significant following adjustment for hypertension, cerebrovascular disease and T2DM (beta = 0.036; p=0.198, CI: -0.019, 0.092).

Conclusion: This audit suggests regular long-term intravitreal VEGF inhibition does not significantly alter the rate of eGFR beyond that of natural decline. Further evaluation of the long-term renal safety of intravitreal anti-VEGF injections particularly in a larger sample size of high-risk groups is warranted.

Comparing Intravitreal (IVT) Pharmacokinetics of Selected Antiangiogenic Agents in Rabbits Using ESI-LC-MS/MS

Halder, N., Das, U.K., Velpandian, T.
AIIMS, Ocular Pharmacology & Pharmacay, New Delhi, India

Background: For Ocular neovascularization, small molecules targeting other than VEGF have been explored in this study. So far no intravitreal pharmacokinetic study has been reported for selected antiangiogenic agents such as curcumin, emodin, thalidomide and valdecoxib. Therefore, the present study was designed to develop IVT formulation of these agents and to evaluate IVT pharmacokinetics in rabbits using cassette-dosing approach.

Objectives:
1) To develop and evaluate the IVT formulations of curcumin, emodin, thalidomide and valdecoxib.
2) To develop and validate ESI-LC-MS/MS and HPLC methods for simultaneous quantification of these agents and bevacizumab (standard) respectively in biological fluids.
3) To perform the IVT pharmacokinetics of developed formulations and bevacizumab in rabbits.

Methods: Under sterile conditions, IVT formulations were prepared, gamma sterilized and evaluated. ESI-LC-MS/MS and HPLC analytical methods were developed and validated as per USFDA guidelines. For IVT pharmacokinetics, New Zealand albino rabbits of either sex weighing 1.3-2.9 kg were used after obtaining ethics permission from Institutional Ethics Committee (911/IAEC/16).

Results: Serum IVT formulations of all 5 drugs were reconstituted with 1 mL of normal saline in sterile conditions. Bevacizumab was taken as a standard. An aliquot of 0.1 mL at a dose of 10 μg each drug and 1.25 mg of bevacizumab were intravitreally injected asceptically into the left eye of rabbits (n=4 for each interval) through pars plana 3-4 mm posterior from the limbus at 12 o’clock position. Rabbits were euthanized at 1, 4 and 8 hr using carbon dioxide. Ocular fluids, tissues, and plasma were collected, stored at -80°C and subjected for analysis using LC-MS/MS.

Conclusion: Serum IVT formulations for bevacizumab in rabbits, emodin, thalidomide and valdecoxib have been successfully developed and evaluated. IVT cassette dosing pharmacokinetics showed a rapid clearance of curcumin from vitreous cavity followed by thalidomide > valdecoxib > emodin. At 8 hrs, the concentration of all the drugs was found to be less than their IC50 value.

Acknowledgement: This audit was supported by the University of Delhi for financial assistance.

Tetramethylpyrazine Attenuates Intracellular Inflammation in Experimental Autoimmune Uveitis Through Modulating Stat3 and Stat4 Pathways

Lei, B.1, Lin, R.2
1Henan Eye Institute/Henan Eye Hospital, Henan Clinical Research Center for Ocular Diseases, Zhengzhou, China, 2First Affiliated Hospital of Chongqing Medical University, Chongqing, China

Purpose: To investigate the protective effect of tetramethylpyrazine (TMP) against retinal inflammation in experimental autoimmune uveitis (EAU) mice and explore its underlying mechanism.

Methods: Mice were subcutaneously injected by IRBP peptide with CFA to induce EAU. TMP (50 mg/kg/d) or vehicle was administered intraperitoneally starting from day 2 prior to day 14 after IRBP peptide immunization as the preventive group, and from day 8 to day 14 as the effector phase, respectively. The clinical and histological scores were used to evaluate the severity of ocular inflammation in EAU mouse eyes. The pro-inflammatory cytokines IL-6, TNF-α, IL-1β, MCP-1, anti-inflammatory cytokine IL-10, Th1 cell signature cytokine IFN-γ and Th17 cell signature cytokine IL-17 were detected by real-time PCR. Retinal function was assessed by ERG. The protein levels of p-STAT3 and p-STAT4 were detected by Western blotting.

Results: In prevention and effector phases, the clinical and histological scores were remarkable reduced in EAU mice treated with TMP systemically. Intraperitoneally administration of TMP significantly decreased the retinal mRNA expression of IL-6, TNF-α, IL-1β, MCP-1, IFN-γ and IL-17, but significantly enhanced the expression of IL-10 in EAU. Compared with the vehicle treated EAU groups, the ERG b- and a-wave amplitudes were alleviated in the SMP treated EAU eyes. Meanwhile, TMP significantly preserved the expression of p-STAT3 and p-STAT4 at the protein levels.

Conclusion: We found systemic administration of TMP significantly reduced ocular inflammation and retinal function impairment in both prevention and effector phases of EAU. Our results suggested the anti-inflammatory effect of TMP is associated with inhibition of the immune response pathways, including STAT3 and STAT4 signaling.
Efficacy of Nonsteroidal Anti-inflammatory Drugs (NSAIDs) in Patients with Retinopathy: A Systematic Review and Meta-analysis

Enabi, S.1,2, Hailouli, O.4, Abdelkarem Faraj, H.1, Makram, O.M.1,4, Ahmad Qureshi, Z.2,3, Said Elkolloky, S.1, Abdelmongy, M.8, Enabi, S.7, Fouad, I.H.7, Huy, N.T.10,11

1Tripoli University, Faculty of Medicine, Tripoli, Libya, 2Minia University, Faculty of Medicine, Minia, Egypt, 3Al-Azhar University, Faculty of Medicine, Cairo, Egypt, 4The University of Jordan, Faculty of Medicine, Amman, Egypt, 5October University 6University of Medicine, Giza, Egypt, 7University of Health Sciences, Faculty of Medicine, Lahore, Pakistan, 8Menoufia University, Faculty of Medicine, Menoufia, Egypt, 9Tripoli University, Faculty of Science & Technology, Faculty of Medicine, Sixth of October City, Egypt, 10Sulaiman AlRajhi Colleges of Medicine, Faculty of Medicine, Qassim, Saudi Arabia, 11Nagasaki University, Department of Clinical Product Development, Institute of Tropical Medicine (NEKKEN), Leading Graduate School Program, and Graduate School of Biomedical Sciences, Nagasaki, Japan, 12Tun Duc Thang University, Evidence Based Medicine Research Group & Faculty of Applied Sciences, Ho Chi Minh City, Viet Nam

Introduction: Nonsteroidal anti-inflammatory drugs (NSAIDs) are used routinely by ophthalmologists to treat allergic conjunctivitis, postoperative inflammation and to reduce cystoid macular edema. Recent evidence that NSAIDs play a role in reducing the prostaglandin inflammatory effects in the pathogenesis of diabetic retinopathy and age-related macular degeneration has been established. Also, the US Food and Drug Administration (FDA) has approved topical NSAIDs for retinal diseases. However, clinical evidence demonstrating a consistent therapeutic benefit of NSAIDs for retinal is lacking or have a small sample size studies. Our study aimed to recapitulate and summarize the findings from the literature to draw out quite definite conclusion about NSAIDs efficiency in the treatment of retinal diseases.

Methods: A systematic search of 9 databases was performed to include RCTs that address the NSAID use and efficacy in patients with retinal diseases. The methodological quality of each RCT was assessed using the Cochrane Collaboration’s tool. The study protocol has been registered in PROSPERO with registration number CRD42016032829.

Results: Our results showed an obvious advantage of using non-steroidal anti-inflammatory drugs (NSAIDs) in patients with retinopathy after cataract surgery. For perifoveal thickness, there was a significant (P < 0.001) difference between NSAIDs group and the matched control group (MD [95% CI] = -34.54 [-46.68, -22.2]). In the same context, total macular volume (TMV) showed a significant difference (P < 0.001) between NSAIDs patients and their matched controls (MD [95%CI] = -0.56 [0.83, -0.29]). Although this difference extended to include all other outcomes but the significance faded away. There was no significance difference between NSAIDs and control groups for central foveal thickness (CFT), best corrected visual acuity (BCVA) and intraocular pressure (IOP) with [MD (95% CI) = 3.2 [-7.75, 1.34], P = 0.167], [MD (95% CI) = -0.01 [-0.25, 0.22], P = 0.923] and [MD (95% CI) = -0.44 (-1.23, 0.35), P = 0.275], respectively.

Conclusions: Using NSAIDs in patients with retinopathy after cataract surgery seems to have an advantage on different levels compared to controls. However, the significance of these advantages still controversy and further and larger studies are needed for a stronger evidence.

Obtaining a Stable and Reliable Diluted Pupil Using a Gentamicin Combination in Patients between the Ages of 30 - 50

Zolowski, R.1, Roberts, D.1, Lake, R.1, Ashianti-Zarandi, J.3, Sammak, N.1,4, McArdle, G.2

1Illinois College of Optometry, Chicago, United States, 2Lentiscus Research Group, Naperville, United States

Appropriate mydriasis is an integral part of a successful cataract extraction (CE). Pupil size is probably the most important variable in cataract surgery, as well as ophthalmological and optometric clinical practices. Maintenance of mydriasis contributes to procedural safety and efficiency.

Methods: To make these tools safe and quicker are being assessed to reduce the clinical costs of repeated administration of drops. An ASCRS Cataract Clinical Committee white paper investigated various methods in the management of the pupil dilatation in intra-operative flow. Pupil size is probably the most important variable in cataract surgery, as well as ophthalmological and optometric clinical practices. Maintenance of mydriasis contributes to procedural safety and efficiency.

Results: Methods to make these tools safe and quicker are being assessed to reduce the clinical costs of repeated administration of drops. An ASCRS Cataract Clinical Committee white paper investigated various methods in the management of the pupil dilatation in intra-operative flow. Pupil size is probably the most important variable in cataract surgery, as well as ophthalmological and optometric clinical practices. Maintenance of mydriasis contributes to procedural safety and efficiency.

Conclusions: Among groups including control, STZ-induced diabetic and metformin treated diabetic mice, no abnormality was evident in fundus examination in any mice. FA recording analysis revealed a significant prolonged mean filling time of both central retinal artery and microvascular beds in STZ-induced diabetic mice when compared with age-matched control mice (both p < 0.01). Metformin treatment for 10 weeks after diabetes induction completely reversed the prolonged microvascular bed filling time (p < 0.001). The central arterial filling time appeared minimally affected by metformin. Although there was a slightly prolonged venous filling time in diabetic and metformin treated groups, the difference did not reach statistical significance when compared to control mice. ERG analysis revealed substantial reduction of scotopic and photopic ERGs (p < 0.001) in the STZ-induced diabetic mice. Metformin treatment had significant effect in preserving the b-wave amplitude (p < 0.01), but had less effect on the a-wave amplitude (p = 0.54). However, no obvious apoposis was observed in the retina of diabetic mice with or without metformin treatment.

Conclusions: Early metformin treatment significantly improves retinal capillary perfusion and protects the photoreceptor function in diabetic mice. These data further supplement our previous findings of metformin’s multiple beneficial effects on retinal functional preservation in diabetic retinopathy.

Metformin Improves Retinal Capillary Perfusion and Reserves Retinal Function in Diabetic Mice

Qiao, X.1, Li, Y., Zhou, T., Edwards, P., Gao, H.

Henry Ford Health System, Detroit, United States

Purpose: Retinal microvascular pathologies and neuronal dysfunction are well-known components of diabetic retinopathy (DR). We reported that long-term metformin treatment was associated with significantly reduced severity of DR in patients with type 2 diabetes, probably by its anti-angiogenesis and anti-inflammation effects. This study examined the effects of metformin on retinal blood flow and retinal function in diabetic mice.

Methods: Diabetes was induced in C57BL/6 mice by streptozotocin (STZ) injection 5 in consecutive days. Metformin hydrochloride solution was administered by gavage at 200 mg/kg/day after STZ injection for 10 weeks. Retinal blood flow was examined by fluo- rescein angiography (FA) video recordings after tail vein injection of 100 µl of 1% sodium fluorescein. Scotopic electroretinography (ERG) was used to assess photoreceptor and bipolar cell functions. TUNEL stain was applied to retinal sections for detection of neuron apoptosis.

Results: Among groups including control, STZ-induced diabetic and metformin treated diabetic mice, no abnormality was evident in fundus examination in any mice. FA recording analysis revealed a significant prolonged mean filling time of both central retinal artery and microvascular beds in STZ-induced diabetic mice when compared with age-matched control mice (both p < 0.01). Metformin treatment for 10 weeks after diabetes induction completely reversed the prolonged microvascular bed filling time (p < 0.001). The central arterial filling time appeared minimally affected by metformin. Although there was a slightly prolonged venous filling time in diabetic and metformin treated groups, the difference did not reach statistical significance when compared to control mice. ERG analysis revealed substantial reduction of scotopic and photopic ERGs (p < 0.001) in the STZ-induced diabetic mice. Metformin treatment had significant effect in preserving the b-wave amplitude (p < 0.01), but had less effect on the a-wave amplitude (p = 0.54). However, no obvious apoposis was observed in the retina of diabetic mice with or without metformin treatment.

Conclusions: Early metformin treatment significantly improves retinal capillary perfusion and protects the photoreceptor function in diabetic mice. These data further supplement our previous findings of metformin’s multiple beneficial effects on retinal functional preservation in diabetic retinopathy.

Reassessment of Treatment Resistant Exudative AMD Diagnosis with Indocyanine Green Angiography Prior Switching Anti-VEGF Treatment to Afiblbercept

Demircan, A.

Beyoglu Eye Research and Education Hospital, Istanbul, Turkey

Purpose: To re-evaluate treatment resistant exudative age-related macular degeneration (AMD) cases with indocyanine green (ICG) angiography prior switching intravitreal anti-vascular endothelial growth factor (VEGF) injection treatment to afiblbercept and investigate the effect of the drug on visual and anatomic outcomes over different AMD subgroups.

Materials and methods: Fifty-one eyes of 44 patients with exu- dative AMD diagnosis resistant to ranibizumab and bevacumab injections were included to this retrospective study. Before switch- ing to afiblbercept injection, subjects underwent ICG angiography for further investigation. The cases are classified according to the AMD subtype which was found out by angiography. The patients switched to intravitreal afiblbercept injections with initial three loading doses. The primary outcomes were changes in best corrected visual acuity (BCVA) and central macular thickness in third and sixth months following switch procedure.

Results: Out of 51 eyes, 38 (74%) had polypoidal choroidal vascu- lopathy and 10 (20%) had occult choroidal neovascular membrane (CNV) based on ICGA findings. There was a mean increase in BCVA for eyes with occult CNV in third month (from baseline 0.61±0.42 to 0.48±0.35, p=0.003) compared to the maintenance of vision for eyes with PCV (from baseline 0.75±0.46 to 0.79±0.46) and these changes remained stable until sixth month. Mean CMT was re- duced from 390±103 µm to 326±72 µm in third month (p=0.001) and preserved until sixth month. Similar decreases were observed for occult CNV and PCV subgroups (p=0.009 and p=0.001 respec- tively).

Conclusion: Switching therapy to afiblbercept might have different visual and anatomical outcomes for treatment resistant AMD cases according to underlying lesions. Diagnostic reassessment of these cases with ICGA seems to be beneficial in predicting these results.
Real-life Evidence on Treatment Outcomes of Poloidal Choroidal Vasculopathy in Turkey
Alkin, Z.1, Demir, G.1
1 Beyoglu Eye Research and Education Hospital, Istanbul, Turkey, 2 Beyoglu Eye Training and Research Hospital, Istanbul, Turkey
Purpose: To evaluate outcome of anti-vascular endothelial growth factor (VEGF) therapy alone and combination treatment with anti-VEGF injection and photodynamic therapy (PDT) for the treatment of poloidal choroidal vasculopathy (PCV) in the real-life setting.
Methods: This was a cross-sectional, retrospective study. Cauca- sian patients who were diagnosed with PCV and had more than 1 year of follow-up were included. Diagnosis of PCV who made through the clinical examination, optical coherence tomography, fluorescein angiography and indocyanine green angiography imag- ing. Combination treatment included intravitreal ranibizumab (0.5 mg/0.1 ml) or intravitreal aflibercept (2 mg/0.1 ml) or intravitreal bevacizumab (1.25 mg/0.1 ml) plus standard PDT. Best-corrected visual acuity (BCVA) measurement, fundus examination and OCT were performed at each visit. Data were collected at day 0, 3 months, 6, 9, 12, 24, 36 and 48 months.
Results: The study included 99 eyes of 103 patients, 35 female and 64 male. The mean age was 69.6±9.5 years. At baseline, the mean BCVA (logMAR) was 0.77±0.50. Patients received 12.4±6.1 injections on average 38.6±13.4 months. Sixty-four eyes had 95 PDT treatment sessions and anti-VEGF injections with a mean number of 12.2 while remaining 39 eyes were treated only with anti-VEGF injections with a mean number of 12.6. Overall, the mean BCVA was 0.69±0.44 at 3 months, 0.67±0.43 at 6 months, 0.70±0.42 at 12 months, 0.75±0.44 at 24 months, 0.82±0.45 at 36 months, and 0.81±0.45 at 48 months. Central macular thickness was 371±105 μm at baseline, 321±81 μm at 3 months, 320±84 μm at 6 months, 335±84 μm at 12 months, 315±86 μm at 24 months, 336±102 μm at 36 months, and 334±99 μm at 48 months. Ana- tomically, OCT data showed a decline during follow-up.
Conclusion: Anti-VEGF therapy and combination treatment with anti-VEGF injection and PDT resulted in an initial improvement in visual acuity; however, this was not maintained over time. In clinical practice, fewer injections are administered than in clinical trials in PCV patients in Turkey.

The Evaluation of Topical SPL, a Novel Dendrimer Antiviral, against Adenovirus in NZW Rabbit Ocular Models
Romanowski, E.1, Yates, K., Shankis, R., Kowalski, R.
University of Pittsburgh, The Charles T. Campbell Ophthalmic Mi- crobiology Laboratory, UPMC Eye Center, Pittsburgh, United States
Purpose: There is no FDA or EMA approved antiviral therapy for adenovirus (Ad) ocular infections. Dendrimers are novel na- nonscale macromolecules that have the ability to be designed to myeloid cells that can be used to deliver specific agents. A dendrimer (Polyethylene Glycol)-paracetamol (Ptx3) significantly increased CD163 expression (P<0.01) over unstimulated controls. These results underscored that Ptx3 levels are significantly reduced in human and animal models of DR.

Comparison of an Intravitreal Dexahemathone Implant and Anti-VEGF Drugs in Treatment of Macular Edema: A Meta-analysis of Randomized Controlled Trials
Ming, S.1, Bo, L.2
1 Henan Eye Institute, Henan Eye Hospital, Clinical Research Center for Eye Diseases, Henan Provincial People's Hospital, Zhengzhou, China, 2 Henan Eye Institute, Henan Eye Hospital, Henan Provincial People's Hospital, People's Hospital of Zhengzhou University, Zhengzhou, China
Objective: To compare the efficacy and safety of an intravitreal dexahemathone implant (DEX implant) and anti-VEGF agents in treating macular edema (ME).
Methods: We searched the databases of Medline, Cochrane, and the website of www.clinicaltrials.gov. Two researchers inde- pendently identified the eligible studies, extracted data and evalu- ated methodological quality of the included studies with same cri- teria. Random or fixed effect model was utilized to synthesize the data by judging the existence of heterogeneity. Only head-to-head randomized clinic trials were included. We chose mean change in best-corrected visual acuity (BCVA) and mean change in central retinal thickness (CRT) as efficacy outcomes. DEX implant and anti-VEGF agents were designed as intervention and control group respectively. Weighted mean difference (WMD) were calculated to combine outcomes. The higher of WMD of BCVA change and lower of CRT change, the higher efficacy of DEX implant compar- ing with anti-VEGF drugs. Sub-analysis was based on the dosage of DEX implant. Safety was evaluated by adverse events (AEs).
Results: Seven eligible RCTs were included for inclusion with 1068 eyes. Overall, the WMD of BCVA and CRT between the two arms was -3.42 letters (95% CI: -6.01 ~ -0.84, P = 0.009) and 3.05mm (95% CI: -5.84 ~ 6.54, P = 0.92). In the subgroup of “DEX implant at 2-6 month interval”, the comparative efficacy of BCVA and CRT was -1.57 letters (95% CI: -3.97 ~ 0.83, P = 0.20) and -0.23 ± 0.04 mm (95% CI: -1.8 ~ 1.37, P = 0.003) respectively. In the subgroup of “DEX implant at <6 month interval”, the comparative efficacy of BCVA and CRT was -1.57 letters (95% CI: -3.97 ~ 0.83, P = 0.20) and -0.23 ± 0.04 mm (95% CI: -1.8 ~ 1.37, P = 0.003) respectively. The DEX implant had higher risks of elevated IOP and cataracts (RR = 7.21, 95% CI: 2.30 ~ 22.56, P = 0.0007) and cataract (RR = 2.77, 95% CI: 1.54 ~ 5.00, P = 0.0007).
Conclusion: Comparison to anti-VEGF agents, DEX implant exhib- ited equivalent functional efficacy and better anatomical efficacy for treatment of ME, only when the implant is administrated at less than 6-month interval. However, the efficacy should be bal- anced against a higher incidence of ocular AEs, especially elevated IOP and cataracts.

Penetrx 3 Enhances Myeloid Cell Phagocytic Function
Queens University Belfast, Belfast, United Kingdom
Diabetic retinopathy (DR) remains a leading cause of visual impair- ment and blindness. The pathogenesis of vascular complications in DR is driven by endothelial damage. In addition, there is emerg- ing evidence underscoring the importance of inflammation and myeloid cells in DR. Penetrx 3 (Ptx3) is a pattern recognition re- ceptor released in response to pro-inflammatory stimuli. Bio- logical actions for Ptx3 include regulation of inflammation and angio- genesis. The current study was designed to investigate the impact of diabetes-like conditions on Ptx3 expression and its role in my- eloid cell biology. First, myeloid angiogenic cells (MACs) and hu- man microglia cells (HMCs) were exposed to 25 mM D-glucose to model diabetes or to 1% O2 to mimic tissue hypoxia. Expression of Ptx3 mRNA in MACs but not in HMCs was significantly (P < 0.01) downregulated after 7 days exposure to high glucose. This was in agreement with a reduced protein expression of PTK3 in MACs. Furthermore, there was increased mRNA expression of IL-8 in both MACs and HMCs cells (P < 0.05 & P < 0.001). In response to hypoxia, both MACs and HMCs cells displayed significantly (P < 0.05 to P < 0.001) decreased PTK3 expression at 2, 4, and 6 hours. These results underscored that PTK3 levels are significantly re- duced in myeloid cells under diabetic-like conditions. Therefore, we tested whether supplementation of exogenous recombinant PTK3 has any effects on human myeloid cells. We investigated phagocytic using pH sensitive fluorescent bioparticles. Both MACs and HMCs cells were incubated with recombinant PTK3 (100 ng/ ml) for 24 hours before exposure to phorphio™ Red Bioparticles. MACs and HMCs cells pre-treated with recombinant PTK3 demon- strated significantly enhanced phagocytosis when compared to vehicle controls (P < 0.001). These results confirmed that Ptk3 promotes phagocytosis in myeloid cells. Furthermore, treatment of MACs with Ptk3 significantly increased CD16 expression (P < 0.05), which suggest that Ptk3 promotes an anti-inflammatory phenotype. These findings support our hypothesis that PTK3 plays a role in modulating myeloid cell function. This may be of thera- peutic benefit for DR, but further research is warranted. This research project is funded by Novo Nordisk.
Ocular Pharmacology, Therapeutics, and Drug Delivery

Targeting SRPK1 with Novel Potent and Selective Inhibitors Blocks Choroidal Neovascularization through Modulating VEGF-A Alternative Splicing

Stewart, E.1, Batson, J.1, Blackley, Z.1, Gutierrez-Caballero, C.1, Murphy, A.1, Daubney, J.1, Liddell, S.1, Habgood, A.1, Toop, H.1, McXechnie, K.1, Morris, J.1, Bates, D.1
1ExxonE Ltd, Nottingham, United Kingdom, 2University of New South Wales, Chemistry, Sydney, Australia

Current standard treatments for neovascular age-related macular degeneration involve the intravitreal injection of anti-VEGF agents and block all VEGF-A isoforms, including the alternatively spliced anti-angiogenic VEGF-A<sub>165a</sub> isoforms. Splicing of VEGF-A is controlled by the splicing kinase SRPK1 through phosphorylation and nuclear translocation of the splicing factor SRSF1. Inhibition of SRPK1 restores the endogenous anti-angiogenic system and produces VEGF-A<sub>165a</sub>. Our aim is to develop SRPK1 inhibitors which can alter the balance of VEGF-A splicing from the angiogenic to the anti-angiogenic isoform, with physicochemical properties enabling eye drop delivery to the retina.

Novel compounds, synthesised based on the structure of SRPK1, were tested for SRPK1 potency and selectivity in vitro in kinase assays. Compounds with SRPK1 IC<sub>50</sub> < 100 nM were tested for cell efficacy using immunoprecipitation, immunofluorescence and NanoBRET assays. The effect on VEGF-A splicing was tested using ELISA and VEGF-A<sub>165a/b</sub> reporter assay. In vivo efficacy was evaluated by laser-CNV

In vitro kinase assays identified compounds that are selective for SRPK1 and potentially inhibit SRPK1 enzyme activity. A novel NanoBRET assay was established to directly quantify the interaction of SRPK1 with the substrate SRSF1 in cells. Compounds dose-dependently inhibited SRSF1 phosphorylation and release from the SRPK1-SRSF1 complex, leading to a reduction in nuclear localization of SRSF1. VEGF-A isoform specific reporter assays and ELISA showed that compounds dose-dependently and specifically reduced angiogenic VEGF-A<sub>165a</sub> levels. Novel compounds potently inhibited laser-CNV following eye drop administration in mice. These compounds potentially offer more specific, efficacious and safer therapeutics for patients with neovascular age-related macular degeneration

Ocular Pharmacokinetics of Cyclosporine after Ocular Administration of OTX-101, a Novel Nanomicellar Formulation of Cyclosporine in New Zealand White Rabbits

Weiss, S.L.1,2, Arentz, R.1, Kramer, W.G.1, Velagataleti, P.1, Gilger, B.C.1,4
1Ocular Technologies Sarl, Delray Beach, FL, United States, 2Novo-Ion, Inc., Randolph, NJ, United States, 3Sun Pharmaceutical Industries, Hoofddorp, Netherlands, 4Kramer Consulting LLC, North Potomac, MD, United States, 5North Carolina State University, Raleigh, NC, United States, 6Powered Research, Raleigh, NC, United States

OTX-101 is a novel, clear, aqueous, nanomicellar solution of cyclosporine (CsA) under development for the treatment of keratoconjunctivitis sicca (KCS). A pharmacokinetic (PK) study was conducted in New Zealand white rabbits to determine the ocular tissue distribution of OTX-101 (0.01%, 0.05%, 0.1% CsA) compared with a currently marketed ophthalmic emulsion of CsA 0.05% ( comparator) (35 μL/application). In the single-dose phase, rabbits received a single bilateral topical ocular instillation of OTX-101 (0.05% CsA) or comparator. In the repeat-dose phase, OTX-101 (0.03%, 0.05%, 0.1% CsA) or comparator was instilled bilaterally 4 times/day for 7 days. PK parameters [maximum concentration (C<sub>max</sub>), mean exposure (AUC) and area under the concentration time curve from 0 to last measurable concentration] were analyzed in whole blood, tears, and ocular fluids/tissues (e.g. conjunctiva, cornea, eyelid). Tolerance assessments included examination of clinical signs (e.g., ill health, behavioral changes) and cage-side monitoring.

Ocular tissue samples and whole blood from 112 rabbits were analyzed; 2 rabbits per time point, both eyes included. Following a single dose, CsA concentrations were higher in most ocular tissues for OTX-101 0.05% vs comparator; C<sub>max</sub> (ng/g) was 862 vs 239 for cornea, 807 vs 307 for cornea, and 102 vs 29.7 for sclera. Following the single dose, systemic exposure was minimal (< 7 ng/mL) in both treatment groups. After repeat doses, the ratio of the CsA AUC<sub>0-7</sub> for OTX-101 0.05% vs comparator was 2.00 in the cornea, 1.15 in the superior bulbar conjunctiva, and 1.85 in the sclera. CsA concentrations increased in a dose-related manner after administration of OTX-101, with a 1.43-fold increase in the superior bulbar conjunctiva and a 3.29-fold increase in the lacrimal gland for the 0.1% vs 0.05% dose. No mortality nor treatment-related clinical signs were noted in either study phase.

Since outcomes for self-administered topical ocular drugs are often suboptimal due to poor drug penetration or patient adherence, the goal is to achieve the highest practical target tissue concentrations, without acute or chronic toxicity. Single and repeat topical administration of OTX-101 0.05% resulted in proportionally higher tissue concentrations of CsA vs comparator in many ocular tissues. OTX-101 demonstrated dose-related increases in CsA concentration and was well tolerated.

Sponsored by Ocular Technologies Sarl, now a subsidiary of Sun Pharmaceutical Industries, Inc.
Mitochondrial Morphology Change and the Expression of Mitochondrial Fission/Fusion Genes in RPE Cells under Oxidative Stress

Liu, X.1, He, Y.1,2
1Xian Medical University, Xi’an, China, 2Baqiao District Textile City, Xi’an, China

Purpose: To assess the expression of mitochondrial fission genes (Fis1, Dnm1, MTP18) and fusion genes (Mfn1, Mfn2) in different concentrations of hydrogen peroxide (H2O2) under the oxidative damage in ARPE-19 cells.

Methods: The obtained ARPE-19 cells were divided into the normal control group and the oxidative damage groups treated by different concentrations of H2O2. The oxidative damage groups were treated by H2O2 at the concentrations of 75, 150 and 200 µmol/L respectively for 24 h. RT-PCR assay was applied for cell viability. The cell morphology change was observed by phase contrast microscope. The mitochondrial morphology change was recorded by transmission electron microscope. The mRNAs levels of mitochondrial fission/fusion genes (Fis1, Dnm1p, MTP18) and fusion genes (Mfn1, Mfn2) were measured by RT-PCR and Real-time PCR.

Results: With the increased concentration of H2O2, ARPE-19 cells were shrinking aggressively and cell death increased. The mitochondrial structures were destroyed with membranes and cristae. RT-PCR results showed the decreased expression of Fis1 gene, compared with the normal control group in different H2O2 treatment (P < 0.05). There were no significant differences in different concentration of H2O2 groups. The expression of fission genes MTP18, Dnm1 and fusion genes Mfn1, Mfn2 showed no significant change. Real-time PCR results demonstrated that the expression of Fis1 gene decreased with different concentrations of H2O2 treatment, whereas the expression of Mfn2 gene increased in the treatment of 200 µM H2O2. There were no significant differences of other genes expression.

Conclusion: The abnormal expression of mitochondrial fission gene Fis1 and fusion gene Mfn2 caused mitochondrial dysfunction in ARPE-19 cells, indicating the imbalance of mitochondrial dynamics, which might participate for these cell death in oxidative stress environment.

The Role of the LIM Homeodomain 2 (Lhx2) in Differentiation of the Mammalian Retinal-Pigmented Epithelium (RPE)

David, A.1, Cohen, M.1,1, Iedelson, M.2, Reubinoff, B.2, Elkon, R.2,3, Ruth Ashery-Padan
1Tel Aviv University, Department of Human Molecular Genetics and Biochemistry, Sackler Faculty of Medicine, Tel Aviv, Israel, 2The Hadassah Hebrew University Medical Center, The Goldyne Savad Institute of Gene Therapy & Department of Gynecology, Jerusalem, Israel

Normal vision depends on the retinal pigmented epithelium (RPE), a metabolic cell layer vital for development and function of the adjacent retinal photoreceptors. However, the transcriptional regulatory program in the course of RPE differentiation and maintenance is still unclear. Lhx2 is a LIM homeodomain (LIM-HD) family member required for the early morphogenesis and patterning of the vertebrate eye. Our current objective is to uncover the roles of Lhx2 complex in the differentiation of the mammalian RPE. We have established Lhx2 conditional mutant (cKO) in the mouse RPE. Initial morphological and immunofluorescence analyses of Lhx2 cKO RPE mutants show altered morphology and reduced expression of early RPE key transcription factors.

To study the role of Lhx2 in human RPE and to further identify direct targets of Lhx2 we utilize RPE generated from human embryonic stem cells (hES-RPE) for chromatin immunoprecipitation followed by sequencing (ChIP-Seq) and for functional studies using lentiviral knockdown approach. Considering the importance of RPE for retinal physiology and the recent advance in using hES-RPE for cell-replacement therapy this study will contribute to uncover gene regulatory networks (GRNs) downstream of Lhx2, involved in RPE fate and function.
Novel Frameshift Mutation in MAB21L2 in Two Patients with Bilateral Colobomata without Skeletal Malformation

Wendlandt, M., Neuhn, L., Neuhn, T., Holinski-Feder, E.
MGZ - Medizinisch Genetisches Zentrum, Munich, Germany

The phenotypic spectrum of pathogenic variants in the MAB21L2 gene include microphthalmia/anophthalmia, coloboma and skeletal dysplasia syndrome. So far, only very few disease-associated variants have been described which elucidate the function of this gene. We identified the heterozygous MAB21L2 frameshift mutation c.58del (Cys20Valfs*37), which leads to a premature stop codon, in two unrelated patients with a distinct phenotype involving. In addition, we report another patient with bilateral isolated bilateral colobomata without skeletal or intellectual involvement. In addition, we report another patient with bilateral isolated bilateral colobomata without skeletal or intellectual involvement.

Epigenetic Regulation of the LOXL1 Gene in Pseudoxofoliation Glaucoma

Evers, S.1, Greene, A.1, McDonnell, F.1, Irinten, M.1, Der- van, E.1, O’ Brien, C.1, Wallace, D.1
1University College Dublin, Dublin, Ireland, 2Duke University, Durham, United States, 3Mater Misericordiae University Hospital, Dublin, Ireland

Glaucoma is an optic neuropathy and a leading cause of irreversible blindness worldwide. Although there are many factors that may contribute to disease predisposition, severity and progression, pseudoxofoliation (PXF) syndrome represents a significant and identifiable cause for developing open-angle glaucoma. Single nucleotide polymorphisms (SNPs) within the lysyl oxidase like (LOXL1) gene have been identified as a major risk factor for PXF syndrome. Additionally, a recent study has proposed LOXL1 deficiency in the lamina cribrosa region as a candidate risk factor for PXF. DNA methylation is a significant means of epigenetically regulating gene expression in physiological/pathophysiological states, including in ocular diseases. Epigenetic silencing of LOXL1 by DNA methylation has been demonstrated in several human diseases and neoplastic malignancies. In particular, epigenetic silencing of LOXL1 through promoter hypermethylation has been demonstrated in an autosomal recessive cutis laxa case and found to be reversible by an inhibitor of DNA methyl transferase activity. This may represent a novel approach to exploit for therapeutic intervention in the future. Whether LOXL1 is epigenetically regulated in pseudoxofoliation glaucoma (PXF) has yet to be elucidated. Herein, it was investigated if DNA methylation may play a role in the regulation of LOXL1 in PXF using human tendon fibroblasts isolated from PXF patients and control patients. Initially, quantitative PCR and immunoblotting demonstrated decreased LOXL1 expression in PXF patients versus the cataract controls at both mRNA and protein levels. Additionally, DNA methylation analysis showed an increase in global methylation in PXF patients when compared to the cataract controls. Bisulfite sequencing confirmed the DNA methylation status of the LOXL1 promoter in PXF and cataract controls. Additionally, inhibition of DNA methyltransferase activity using the inhibitor 5-azacytidine increased LOXL1 expression in PXF patients. Collectively, these results provide a greater understanding into the role of DNA methylation in the regulation of the LOXL1 gene in PXF.

Conditional Lack of Bcl-2 in Pericytes and Astrocytes Distinctly Impacts Retinal Vascularization

Sorensen, C., Zaitoun, I., Sheibani, N.
UWSMPh, Madison, United States

Bcl-2 is the founding member of a family of proteins that positively or negatively modulate apoptosis. Bcl-2 expression plays a vital role during angiogenesis as evidenced by decreased retinal vascularization and endothelial cell and pericyte density as well as an inability to undergo pathologic neovascularization in mice globally lacking Bcl-2. Here we assessed the contribution Bcl-2 expression in pericytes or astrocytes has on postnatal retinal vascular development and pathologic neovascularization by generating mice carrying a conditional Bcl-2 allele (Bcl-2-cre) and Pdgrfα-cre (Bcl-2-cre) or Gfap-cre (Bcl-2-cre mice). Bcl-2-cre and Bcl-2- mice had decreased retinal vascular density and fewer retinal arteries and veins which may be attributed at least in part to increased levels of apoptosis. We also observed delayed spreading of the superficial retinal vascular layer in Bcl-2 mice similar to that previously observed in global Bcl-2-/- mice. Formation of the deep vascular plexus in retinas from Bcl-2- mice was also decreased. Laser-induced choroidal neovascularization was reduced in Bcl-2-/- mice compared to littermate controls or Bcl-2AC mice. Furthermore, pathologic retinal neovascularization in oxygen-induced ischemic retinopathy was not affected by lack of Bcl-2 expression in either pericytes or astrocytes, as observed in mice globally lacking Bcl-2. Together these studies illustrate that Bcl-2 expression in retinal pericytes or astrocytes play distinct roles in retinal vascularization and homeostasis, which only partially mimics the Bcl-2-/- phenotype.

A Novel Missense Mutation, G2284E, in the Zinc Finger Protein (ZNF469) Gene Contribute to the Pathogenesis of Korean Keratoconus Patients

Joo, C.-K.1, Mok, J.1
1Seoul St. Mary’s Hospital, College of Medicine, The Catholic University of Korea, Department of Ophthalmalogy & Visual Science, Seoul, Korea, Republic of, 2Catholic Institutes for Visual Science, The Catholic University of Korea, Seoul, Korea, Republic of

Purpose: To investigate whether the Zinc Finger Protein gene (ZNF469) is a candidate gene in the pathogenesis of keratoconus in Korean, we performed sequencing screening of ZNF469 gene in Keratoconus patients. Methods: One hundred fifty patients with sporadic Keratoconus, which visited the Eye Center of Seoul St. Mary’s Hospital and 100 control individuals were enrolled into this study. To screen genetic variations in ZNF469, we investigated using polymerase chain reaction and direct sequencing. The Sorting intolerant Form Tolerant (SIFT) program was used to predict the effect of amino acid substitution on the ZNF469 protein. Results: In this study, we detected 16 non-synonymous variations and 8 synonymous variations. Among them, 5 non-synonymous variations, G1279S, E1593X, E2149K, G2284E, and P2475S, and three synonymous variations, G1131G, S1614S, A1621A were observed only keratoconus patients. Particularly, two novel mutations in ZNF469, G2284E and P2475S were detected in 20.8% and 1.7% of keratoconus patients and were predicted as damaging mutations by computational methods using SIFT. Fourteen SNPs were observed both populations, among them, the C allele of the G235R was associated with increased occurrence of keratoconus, while the C allele of the T1859T and the A allele of the E630Q was correlated with a decreased occurrence of keratoconus. Conclusions: This is the first report of genetic variation screening of ZNF469 in Korean Keratoconus patients and our results suggested that non-synonymous variations in ZNF469, particularly G2284E, has a pathogenic role in Korean patients with Keratoconus.
Decreased Uncoupling Protein 2 Expression in Aging Retinal Pigment Epithelial Cells

He, Y., Wang, X.
The Second Affiliated Hospital of Xi’an Medical University, Xi’an, China

Purpose: To analyze the expression of UCP2 in RPE cells at the different human age, further explore the possible new target of RPE cells protection.

Methods: ARPE19 cells and the primary RPE cells at the different age (9-20yo, 50-55yo, 60-70yo, >70yo) were cultured and harvested. The expression of UCP2 in these cells was detected by RT-PCR, Western Blot and confocal microscopy.

Results: Cells from the donors more than 60 yo are larger and more fibroblastic in appearance compared to ARPE19 cells and in the younger primary cultured human RPE cells at the age of 9-20yo and 50-55yo, whereas lower expression of UCP2 was measured in the older primary cultured RPE cells at the age more than 60yo.

Conclusions: Expression of UCP2 gene was decreased in aged RPE cells, promoting the lower ability of anti-oxidation in these cells. It is indicated that UCP2 gene might be a new target of RPE cells, promoting the lower ability of anti-oxidation in these cells.

Regulation of mRNA Decay by Zfp36l1 and Zfp36l2 in Retinal Development and Maintenance

Mu, X.1, Wu, F.1, Kaczynski, T.2, Liu, T.2, Turner, M.1
1University of Buffalo, Ophthalmology, Buffalo, United States
2University of Buffalo, Biochemistry, Buffalo, United States. The Babraham Institute, Laboratory of Lymphocyte Signalling and Development, Cambridge, United Kingdom

Gene regulation, which takes place at multi-levels, plays important roles in retinal development and function. So far, studies of gene regulation in the retina have largely focused on the transcription level, but not on post-transcriptional levels. Far less is known about how post-transcriptional regulation impacts retinal development and maintenance, despite the fact that post-transcriptional mechanisms are critically involved in various other biological processes.

We tried to address this issue by studying two members of the TTP (tristetraprolin) mRNA binding protein family, Zfp36l1 and Zfp36l2 (collectively called Zfp36l1/2). This family of CCCH zinc finger proteins are highly conserved through evolution and are involved in diverse biological processes. They carry out their functions by binding to the AU-rich elements (AREs) in the 3’ UTR of target mRNAs to promote their decay. By in situ hybridization and immunofluorescence staining, we found that Zfp36l1/2 were expressed in retinal progenitor cells during development and Müller glial cells and photoreceptors in the mature retina. To further study their functions, we have created retna-specific knockouts in mice. A large number of mice from the two genes. Our analysis of the mutant retinas showed that, whereas the single knockout retinas appeared largely normal, the double knockout retina did not develop normally and degenerated rapidly after development completed. RNA-seq analysis indicated the Zfp36l1/2 interact with multiple signaling pathways to impact the balance between proliferation and differentiation. Our results suggest that regulation of mRNA decay plays essential roles in the retina, and that Zfp36l1/2 are two critical regulators of mRNA decay functioning redundantly in both retinal development and maintenance.

Protective Effects of Single or Combinations of Neuroprotective and Regenerative Agents against Degeneration of RGCs in Optic Nerve Crush Rat Model

Bikbov, G.1, Kitamura, Y.1, Baba, T.1, Yamamoto, S.1, Oshita, T.2,3
1Chiba University, Chiba, Japan, 2International University of Health and Welfare, Nanto, Japan

Purpose: To determine the effectiveness of single or combination of topical neuroprotective factors in protecting retinal ganglion cells (RGCs) in the optic nerve crush rat model.

Methods: The left optic nerves were crushed to induce RGC death in 36 adult Sprague-Dawley rats (Japan SLC Co., Hamamatsu, Japan). The right eyes were used as control. Rats were divided into 7 groups with 6 animals in each group for the different therapeutic agents including 100 mM TUDCA, 100 mM citicoline, 10 ng/ml NT-4, combined TUDCA/citicoline (Doublet), and combined TUDCA/citicoline/NT-4 (Triplet). After 2 weeks, the number of surviving RGCs was determined by Brn3a mouse antibody (Santa Cruz Biotechnology) immunostaining of whole-mount retinas. Crosssections of the optic nerve were immunostained for anti-200 kD neurofilament heavy antibody (Abcam, Japan) to study regeneration. To examine the adverse effects, crosssections of the sclera and epiretinal neovascular membranes were immunostained for VEGF antibody (Santa Cruz Biotechnology).

Results: The density (cell number/mm²) of RGCs was 4.64±7.31 at 2 weeks after the optic nerve crush without any treatment which was significantly lower than in the normal control at 1857.3±34.7 (P<0.001). The density of RGCs in the TUDCA treated eyes was 678.4±144.6/mm², in the citicoline treated eyes was 701.1±356.7, in the NT-4 treated eyes was 70.4±29.8, in the Doublet treated eyes was 795±563.6, and in the Triplet treated eyes was 701.1±356.7. All densities were significantly higher than that of RGCs without treatment (P<0.001). The density of RGCs in the NT-4 treated eyes was lower than in other treated groups, and the densities in the other groups were not significantly different among them. A decrease of neurofilament signal was observed in all groups except the Triplet group. Neovascularizations of the cornea, iris, and retina were observed in 62% of the Doublet group eyes. Intense VEGF immunopositive signaling was observed in the sclera and epiretinal neovascular membranes.

Conclusions: Topical instillations of different neuroprotective factors had neuroprotective and regenerative effects on the RGC in an optic nerve crush rat model with the Triplet group to be the most effective. Adverse effects such as neovascularizations were observed in the Doublet group.

Correlation between Uncorrected Visual Acuity and Macular Distortion in Idiopathic Epiretinal Membrane Patients

Lee, J.1, Bae, S.2
1Military Manpower Administration of South Korea, Seoul, Korea, Republic of, 2Military Manpower Administration of South Korea, Daegu, Korea, Republic of

Purpose: To evaluate the association between degree of retinal abnormalities and uncorrected visual acuity (UCVA) in idiopathic epiretinal membrane (ERM) patients with a small amount of refractive error.

Methods: We retrospectively reviewed 49 eyes (37 patients) of idiopathic ERM patients. We investigated the association between visual acuity and macular abnormalities (macular thickness (MCT), outer retinal integrity score, and inner retinal irregularity index) that was assessed by optical coherence tomography using multiple linear regression analysis. We defined visual acuity difference (VAD) as the difference between UCVA and best-corrected visual acuity (BCVA). We divided patients into two groups according to VAD size and compared clinical characteristics between the two groups. We also investigated factors associated with VAD using multiple linear regression analysis.

Results: BCVA showed significant association with CMT and outer retinal integrity score, while UCVA showed significant association with CMT and outer retinal integrity index. Patients with a large VAD showed a similar level of BCVA compared to the small VAD group (logarithm of the minimum amount of resolution (logMAR), large VAD group 0.11 ± 0.11 vs. small VAD group 0.13 ± 0.12, p = 0.585). However, UCVA was worse (logMAR, large VAD group 0.4 ± 0.14 vs. small VAD group 0.18 ± 0.14, p < 0.001) and inner retinal irregularity was higher (large VAD group 1.06 ± 0.04 vs. small VAD group 1.04 ± 0.03, p < 0.001) in patients with a large VAD. On multiple linear regression analysis, the absolute value of spherical equivalent (standardized coefficient β 0.521, p < 0.001) and inner retinal irregularity index (standardized coefficient β 0.448, p < 0.001) were significantly associated with VAD.

Conclusions: UCVA was associated with inner retinal irregularity in idiopathic ERM patients with a mild degree of refractive error. Inner retinal irregularity was also associated with degree of VAD, suggesting that the effect of refractive error correction is greater in patients with more distorted retina.
IL-1b in ROP and Choroidal Degeneration

Chetnab, S.1 Zhou, E.1 Nadeau-Vallee, M.1 Beaudy-Richard, A.1 Quiniou, C.1 Rivera, J.-C.1 Prairie, E.1 Dabou, R.1

1Université de Montréal, Ophthalmology, Montreal, Canada, 2McGill University, Ophthalmology, Montreal, Canada, 3Université de Montréal, Pharmacology, Montreal, Canada, 4Université de Montréal, Neurosciences, Montreal, Canada, 5McGill University, Pharmacology, Montreal, Canada

Retinopathy of prematurity (ROP), the most common cause of blindness in premature infants, has long been associated with inner retinal alterations. However, recent studies reveal outer retinal dysfunctions in patients formerly afflicted with ROP. We have recently demonstrated that choroidal involution occurs early in retinopathy. Here, we investigated the mechanisms underlying the choroidal involution and its long-term impact on retinal function. An oxygen-induced retinopathy (OIR) model was used. In vitro and ex vivo assays were applied to evaluate cytotoxic effects of interleukin-1β (IL-1β) on choroidal endothelium. Electroretinogram (ERG) was used to evaluate visual function. We found that pro-inflammatory IL-1β was markedly increased in RPE/choroid and positively correlated with choroidal degeneration in the early stages of retinopathy. IL-1β was found to be cytotoxic to choroid in vitro, ex vivo and in vivo. Long-term effects on choroidal involution included a hypoxic outer neuroretina, associated with a progressive loss of RPE and photoreceptors, and visual deterioration. Early inhibition of IL-1β receptor preserved choroid, decreased subretinal hypoxia and prevented RPE/photoreceptor death, resulting in life-long improved visual function in IL-1 receptor antagonist (IL-1ra)-treated OIR animals. Using an antenatal-triggered ROP induced by IL-1β, we again found that prenatal antagonism of IL-1R preserved choroid and ensuing photoreceptor degeneration. Together, these findings suggest a critical role for IL-1β-induced choroidal degeneration in outer retinal dysfunction. Early life therapy using IL-1ra preserves choroid and prevents protracted outer neuroretinal anomalies in OIR, suggesting IL-1β as a potential therapeutic target in avoiding protracted photoreceptor anomalies in OIR, suggesting IL-1β as a potential therapeutic target in avoiding protracted photoreceptor anomalies in OIR, suggesting IL-1β as a potential therapeutic target in avoiding protracted photoreceptor anomalies in OIR, suggesting IL-1β as a potential therapeutic target in avoiding protracted photoreceptor anomalies in OIR.
Increased Levels of Lysyl Oxidase in the Vitreous Humor of Diabetic Patients with Advanced Diabetic Retinopathy

Subramanian, M.1, Stein, T.2,3, Ness, S.1, Siegel, N.1, Roy, S.1

1Boston University School of Medicine, Department of Ophthalmology, Boston, United States, 2Boston University School of Medicine, Department of Pathology and Laboratory Medicine, Boston, United States, 3VA Boston Healthcare System, Boston, United States, 4Boston University School of Medicine, Departments of Medicine and Ophthalmology, Boston, United States

Purpose/Objective: Lysyl oxidase (LOX) has been implicated in the abnormal functionality of the thickened vascular basement membrane in diabetic microangiopathy, breakdown of blood-retinal barrier and retinal vascular cell loss in high glucose conditions associated with diabetic retinopathy. The purpose of this study is to determine if patients with advanced diabetic retinopathy expressed altered levels of LOX in the vitreous humor.

Methods: A total of 59 vitreous specimens were obtained from 31 subjects with advanced proliferative diabetic retinopathy (PDR), and 28 non-diabetic subjects; the subjects were age-matched and gender-matched (57±12 years vs 53±16 years, respectively; and 19 males and 12 females vs 17 males and 11 females, respectively). The specimens were obtained during vitrectomy which were clinically indicated for various vitreoretinal conditions, including complications of diabetic retinopathy (DR). Total LOX protein levels were measured in the vitreous specimens using ELISA.

Results: ELISA measurements showed a significant increase in LOX levels in the vitreous specimens of diabetic patients with advanced PDR compared to those of non-diabetic individuals (p<0.01). Additionally, vitreous LOX was detectable in a significantly higher number of diabetic patients with advanced PDR compared to those of non-diabetic subjects by a threefold increase; 58% vs 18%, respectively. There were no differences in the vitreous LOX levels between male and female subjects in both the diabetic and non-diabetic groups. The vitreous specimen of one non-diabetic subject showed an unexpectedly high level of LOX, however, this particular individual had a diagnosis of acute endophthalmitis, which was the clinical indication for vitrectomy, and the significant ocular inflammation associated with the infection may have contributed to the elevated LOX levels.

Conclusions: The findings indicate that increased LOX is closely associated with the development and progression of diabetic retinopathy and may be a potential therapeutic target for treatment of advanced PDR.

COMP-Ang1 Stabilizes Hyperglycemic Disruption of Blood-retinal Barrier Phenotype in Human Microvascular Endothelial Cells

Rochfort, K.1,2, Barabas, P.1, Carroll, L.S.3, Curtis, T.M.1, Ambati, B.K.1, Barron, N.3, Cummins, P.M.1

1Dublin City University, School of Biotechnology and National Institute for Cellular Biology, Dublin, Ireland, 2Queen’s University Belfast, Belfast, School of Medicine, Dentistry and Biomedical Sciences, Belfast, United Kingdom, 3University of Utah, John A. Moran Eye Centre, Salt Lake City, United States, 4University College Dublin, NIBRT, Dublin, Ireland

Introduction: Diabetic retinopathy (DR) is the leading global cause of blindness in working individuals. Characteristic features of DR include elevated microvascular endothelial leakage, inflammation, inner retinal ischemia and neuroglial dysfunction. In view of limitations associated with existing therapies (e.g. anti-VEGF strategies), improved DR treatments are clearly warranted. COMP-Ang1 (i.e. the short Coiled-coil domain of cartilage Glycosaminoglycan Protein combined with Gngioprotein-1) has been reported to exhibit vaso normalization and neuroprotective properties. The objective of this study was to investigate the ability of COMP-Ang1 to reverse blood-retinal barrier (BRB) destabilization resulting from hyperglycemic challenge in vitro.

Methods: Cultured human retinal microvascular endothelial cells (HRMvECs) were exposed to glucose at normoglycemic (5.0 mM), pre-diabetic (15 mM), and diabetic (30 mM) concentrations for 1, 6, 12, 24 and 48 hours in the absence and presence of 100 ng/ml recombinant COMP-Ang1. Mannitol controls were included in all experiments. Post-treatment, cells were harvested for analysis of tight junction gene expression by qPCR. Cells were also monitored for paracellular permeability and production of reactive oxygen species (ROS) by transendothelial marker exchange and rhoderythrin flow cytometry, respectively.

Results: Glucose treatment of HRMvECs decreased mRNA expression of tight junction proteins (VE-cadherin, occludin, claudin-5, and zonula occludens-1) in a dose- and time-dependent manner. In parallel studies, glucose treatment increased HMRMvEC permeability to FITC-Dextran 40 kDa and induced a surge in ROS production. In all studies, co-treatment of cells with COMP-Ang1 significantly reduced the injurious impact of glucose on BRB properties and ROS induction.

Summary: COMP-Ang1 can partially block the pro-oxidant actions of glucose on HMRMvECs and also help to normalize glucose-mediated injury to HMRMvEC barrier properties. These studies highlight the potential value of COMP-Ang1 as a DR therapy and provide a useful model for quantitatively assessing the efficacy of COMP-Ang1 treatment regimens and delivery strategies.

BA3/A1-crystallin is a Potential Regulator of Receptor Tyrosine Kinases (RTKs) Endocytosis and Maintain the Polarity of RPE Cells

Shang, P.1, Hose, S.L.1, Bhutto, I.A.1, Zigler Jr, J.S.1, Sinha, D.1,2

1University of Pittsburgh, Department of Ophthalmology, Pittsburgh, United States, 2Johns Hopkins University School of Medicine, Wilmer Eye Institute, Baltimore, United States

Purpose: We have previously shown that BA3/A1-crystallin plays a pivotal role in the lysosomal-mediated clearance process in RPE cells. Our recent studies suggest multifaceted roles of this protein in intracellular vesicle trafficking and organelle function. This study was undertaken to determine how BA3/A1-crystallin modulates receptor tyrosine kinases (RTKs) endocytosis and the related signaling pathways in RPE polarization.

Methods: A phospho-proteomics experiment was performed on RPE-choroid complex preparations from wildtype (WT) and Cryba1 (encoding BA3/A1-crystallin) KD mice. QPCR and western blotting techniques were used to detect target gene and/or protein expression. Immunostaining of RPE flatmounts was used to investigate cell phenotypes and protein expression. Mouse primary RPE cells were cultured in polarized or non-polarized status.

Results: Protein-protein interaction array showed high probability of interaction between BA3/A1-crystallin and PTPN11 (Protein tyrosine phosphatase), BA3/A1-crystallin and EGFR, indicating that BA3/A1-crystallin may be involved in the EGFR/RTK signaling pathway. Reduced tyrosine phosphate activity was detected in Cryba1 KO RPE cells. The phosphoproteomics dataset suggested that the phosphorylation levels of proteins related to vesicle trafficking were altered in KO RPE cells, such as FNBP1, B1IN, DYN3, which are key regulators in clathrin-coated vesicle budding and fission. Rb65 expression was reduced in KO RPE cells, suggesting abnormally reduced endocytic activity in KO RPE cells. Mouse primary RPE cell culture experiments suggested that BA3-crystallin is only expressed in polarized RPE cells, indicating the spatial restriction of RTKs activity through a polarized endocytic cycle. In addition, cytokinetic organization is abnormal in Cryba1 KO mice: misshapen RPE cells and altered β-tubulin solubility were observed. Cryba1 KO RPE cells showed epithelial-to-mesenchymal transition (EMT) phenotype: reduced expression of E-Cadherin, increased Snai1, Snai2 and Vimentin expression, as well as the β-catenin translocation from plasma membrane to cytoplasm were observed in Cryba1 KO RPE cells.

Conclusions: BA3/A1-crystallin may modulate RTKs endocytosis and dephosphorylation by regulating PTPN11 activity and thereby modulate RTKs signaling pathway in RPE cells. An aberrant RTKs signaling may result in vesicle mis-trafficking and the failure of Cryba1 KD RPE cells to establish normal polarity, triggering the onset of EMT.
A Novel Murine Model for Age-related Macular Degeneration Induced by Combined Chronic Exposure to Light and Hydroquinone

**Jing, Z.**
Eye Hospital, China Academy of Chinese Medical Sciences, Beijing, China

**Objective:** To develop a murine model for age-related macular degeneration by using combined chronic exposure to light and hydroquinone, and to characterize the pathophysiology, ultrastructural and retinic function, thus to provide a suitable model for AMD pathogenesis and treatment research.

**Results:** ERG test showed that retinal function of the mice in the model group was lower than that of normal group. Light microscopy showed that RPE layer had atrophy changes in the model group, and the number of photoreceptor cells was 164.67±34.37, while it was 243.33±152.21 in the normal group. Transmission electron microscopy showed that photoreceptor outer segment of the mice in the model group mice turned loose and deformed, partially fragmented, the microvilli of RPE cells got shorter, the structure of Bruch’s membrane became thickened irregularly. And endothelial cells from choroidal capillary basement membrane penetrated into the Bruch’s membrane. TUNEL test showed that there were many positive cells in RPE layer and photoreceptor layer in the model group. Apoptosis was not found in the normal group. It was shown by immunofluorescence that marked VEGF positive staining was found in the RPE layer in the model group, while no specific staining was found in the normal group. Immunofluorescence of CD31 showed that there was scattered positive staining in the outer plexiform layer and ganglion cell layer in the normal group, while it could also be found in the RPE layer in the model group, which indicated the development of neurovascularization.

**Conclusion:** The mice treated with combined chronic exposure to light damage mimic the development and characteristic of human AMD very well, which may provide a reliable animal model for the further study of AMD pathogenesis and management.

Effect of a Traditional Chinese Medicine, BSYJ on Photoreceptor Apoptosis in RCS Rat with Inherited Retinal Degeneration

**Lina, L.**
Eye Hospital, China Academy of Chinese Medical Sciences, Beijing, China

**Objective:** To observe the effect of a Traditional Chinese Medicine, BSYJ on photoreceptor apoptosis in RCS rat (BACE1) and investigate the mechanism.

**Methods:** 3-week-old RCS rats were randomly divided into two groups, gavaged with either BSYJ solution (BSYJ group, n=12) or distilled water (DW group, n=12). 12 age-matched healthy SD rats were fed regularly as normal control. At day 7, day 28 after treatment pathophysiological technique and TdT-mediated dUTP nick end labeling (TUNEL) method were used to detect the changes of photoreceptor cell numbers and apoptosis rate. Quantitative real time polymerase chain reaction (q-PCR) was used to determine the effect of BSYJ on the expression of oligar neurotrophic factor (CNTF), brain derived neurotrophic factor (BDNF) and basic fibroblast growth factor (BFGF).

**Results:** At day 7 and day 28 after treatment, pathological observation showed that photoreceptor cell numbers in BSYJ group were 140±9 and 80±9, the numbers in DW group were 113±8 and 44±6. The photoreceptor numbers in BSYJ group were significantly more than that of DW group (both P<0.05). It was shown by TUNEL detection that the apoptosis rates of photoreceptor cell in BSYJ group were 31.67±5.39% and 29.68±4.31% at day 7 and day 28 after treatment, the apoptosis rates in DW group were 50.34±5.21% and 44.02±7.17%. The apoptosis rates in BSYJ group were significantly lower than that of DW group (both P<0.05). It was shown by q-PCR detection that expression of CNTF in BSYJ group were 31.67±5.39% and 29.68±4.31% at day 7 and day 28 after treatment (both P>0.05). And the difference of BFGF expression in BSYJ group were 31.67±5.39% and 29.68±4.31% at day 7 and day 28 after treatment (both P>0.05).

**Conclusion:** BSYJ could protect photoreceptor apoptosis in RCS rats significantly, and the mechanism might be related to the promotion of retinal neurotrophic factor expression. Acknowledgement: National Natural Foundation of China (No: 81473736, 81674033, 81102618); TCL “Beit and Road Initiative” Cooperative project funded by China Academy of Chinese Medical Sciences (GH2017-04-02).

BACE1 Plays a Critical Role in Retinal Pigment Epithelial Cell Homeostasis

**Mitter, S.**, Qi, X., Boehm, S., Godoy, J., Silver, S., Quigley, J., Barodia, S., Goldberg, M., Grant, M., Boulton, M.

**University of Alabama at Birmingham, Ophthalmology and Visual Sciences, Birmingham, United States, University of Alabama at Birmingham, Birmingham, United States, Indiana University, Ophthalmology, Indianapolis, United States, University of Alabama at Birmingham, Neurology, Birmingham, United States**

**Background:** BACE1 is a key enzyme facilitating the generation of neurotoxic β-amyloid peptide implicated in Alzheimer’s disease. We have previously reported significant retinal abnormalities including retinal thinning, reduced retinal vasculature and increased lipofuscin in BACE1+/- mice. In this study, we investigated the role BACE1 in maintaining Retinal Pigment Epithelium (RPE) cell homeostasis in vitro under oxidative stress.

**Methods:** BACE1 activity in cultured RPE cells was inhibited by either transfection of RPE with anti-BACE1 siRNA or pharmacological inhibition using BSI IV. Cells were then subjected to oxidative stress with rotenone. Mitochondrial membrane potential (Δψm) was determined using TMRM cationic dye and flow cytometry, mitochondrial morphology was analyzed using Mito-RFP reporter, confocal microscopy and Mito-Morphology macro within ImageJ software. Mitophagy was measured using our custom reporter similar to Mito-QC and by Pink1 and Parkin western analysis. Autofluorescent granule accumulation in cells was quantified and BACE1 colocalization with lysosomes and mitochondria was detected by immunohistochemistry; lysosomal membranes were isolated by selective disruption of lysosomes using methionine methyl ester followed by sucrose density gradient ultracentrifugation.

**Results:** BACE1 knock down in RPE cells resulted in reduced mitoΨm, and disorganized network when cells were exposed to oxidative stress (rotenone, 5 or 10μM for 4hrs) compared to scrambled siRNA transfected cells. Mitophagic proteins were elevated in BACE1 attenuated RPE under oxidative stress with increased number of autophagosomes positive for mitochondrial cargo while the mitophagic markers PARK1 and Parkin protein levels were increased. Confocal microscopy revealed BACE1 co-localized with both mitochondria and lysosomal compartments. Inhibition of BACE1 activity resulted in increased lipofuscin accumulation and a dramatic reduction of lysosomal protein, LAMP2. Purified lysosomal membranes were positive for mature di- merized (110KDa) BACE1 underlining the possibility that BACE1 is an active enzyme and not mere cargo for lysosomal degradation.

**Conclusions:** Our results suggest that BACE1 may play a critical role in the maintenance of RPE cell homeostasis.
We recently reported remarkable rescue of cone photoreceptor function, though the mechanisms of neuroprotection are unknown. Sig1R is a novel target for treatment of neurodegenerative diseases. These observations led us to explore the role of Sig1R activation in protecting mitochondrial and lysosomal dynamics in the retina. Here, we investigated the contribution of C1q to the progression of retinal degenerations. The results showed that administration of C1q knockout mice had increased levels of nuclear DNA damage. These mice have a short lifespan and develop an aging phenotype caused by an accumulation of DNA damage and genotoxic stress. These mice have a short lifespan and develop age-related changes, which may also contribute to disease in humans. Aging remains a major risk factor for many common blinding disorders including age-related macular degeneration, diabetic retinopathy and glaucoma. Age-related eye diseases are associated with a chronic inflammatory profile, with dysregulation of innate immunity and upregulation of inflammatory cytokines in the ocular compartment as key features of disease. Little is known about the specific processes and molecular pathways that underlie age-related changes, which may also contribute to disease in the retina and RPE-choroid. In order to identify such processes in a feasible time frame, we chose to examine the ocular phenotype of a mouse model of monogenetic progeroid syndrome, the Xpg-/- mouse. This model lacks a key exonuclease required for transcription-coupled DNA repair, and shows a fast aging phenotype caused by an accumulation of DNA damage in postmitotic tissues. These mice have a short lifespan and develop degenerative liver and brain pathology due to accumulation of genotoxic stress. These mice have a short lifespan and develop a type of a mouse model of monogenetic progeroid syndrome, the Xpg-/- mouse. 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Age-related eye diseases are associated with a chronic inflammatory profile, with dysregulation of innate immunity and upregulation of inflammatory cytokines in the ocular compartment as key features of disease. Little is known about the specific processes and molecular pathways that underlie age-related changes, which may also contribute to disease in the retina and RPE-choroid. In order to identify such processes in a feasible time frame, we chose to examine the ocular phenotype of a mouse model of monogenetic progeroid syndrome, the Xpg-/- mouse. This model lacks a key exonuclease required for transcription-coupled DNA repair, and shows a fast aging phenotype caused by an accumulation of DNA damage in postmitotic tissues. These mice have a short lifespan and develop degenerative liver and brain pathology due to accumulation of genotoxic stress. These mice have a short lifespan and develop age-related changes, which may also contribute to disease in humans. 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These mice have a short lifespan and develop degenerative liver and brain pathology due to accumulation of genotoxic stress. These mice have a short lifespan and develop age-related changes, which may also contribute to disease in humans. Aging remains a major risk factor for many common blinding disorders including age-related macular degeneration, diabetic retinopathy and glaucoma. Age-related eye diseases are associated with a chronic inflammatory profile, with dysregulation of innate immunity and upregulation of inflammatory cytokines in the ocular compartment as key features of disease. Little is known about the specific processes and molecular pathways that underlie age-related changes, which may also contribute to disease in the retina and RPE-choroid. In order to identify such processes in a feasible time frame, we chose to examine the ocular phenotype of a mouse model
Investigating the Transcriptome and Epigenome of Reprogrammed Zebrafish Retinal Müller Glia during Retinal Damage

Boyd, P.1, Lahne, M.1, Hoang, T.2, Wang, J.1, Ash, J.1, Fisch- er, A.1, Qian, J.1, Blackshaw, S.1, Hyde, D.R.1

1University of Notre Dame, Department of Biological Sciences, Notre Dame, United States, 2Johns Hopkins University, Department of Neuroscience, Baltimore, United States, 3Johns Hopkins University/Wilmer Eye Institute, Wilmer Eye Institute, Baltimore, United States, 4University of Florida School of Medicine, Department of Ophthalmology, Gainesville, United States, 5Ohio State University College of Medicine, Department of Neuroscience, Columbus, United States

In response to retinal damage, zebrafish Müller glia reprogram and reenter the cell cycle to produce neuronal progenitor cells, which continue to proliferate and differentiate into the lost retinal cell types. While several groups examined gene expression changes in damaged and regenerating zebrafish retinas, comparison between different damage models has not been performed. We set out to fill this knowledge gap by comparing light-damaged retinas, which lose rod and cone photoreceptors, NMDA-damaged retinas, in which amacrine cells and a subset of ganglion cells die, and retinas conjoined with TNFα and the gamma secretase inhibitor RO4929097, which stimulates a robust regeneration response on freshly dissected flat mounts collected from Rho-EGFP mice expressing a single-step fusion of phagosomes with lysosomes, as is often portrayed. Indeed, phagosomes appear to interact with early endosomes or genetic mutation of the gene, results in multiple diseases including cancer. Our previous studies have shown that miR-204 is relatively enriched in human retinal pigment epithelium (RPE) and plays an important role in maintaining the epithelial phenotype and its physiological functions in vitro. Genetic ablation of miR-204 in mice led to deposits in the sub-retinal space and hyper-auto-fluorescent spots that persisted with age. These changes were observed in parallel with decreased response amplitudes in the a- and b-waves of the electroretinogram (ERG) and a decrease in the fast oscillation (FO) of the DC-ERG. The current study shows that development of hyper-auto-fluorescence in miR-204 −/− mice coincides with increased Iba-1 + microglia in the subretinal space, buildup of undigested rhodopsin both in RPE and microglia, and increase in autophagy expression. Therefore, miR-204 plays a key role in maintaining the structure and function of the RPE/retina by regulating local autophagic protective responses which prevent intracellular accumulation of undigested photoreceptor outer segment (POS) that can generate oxidative stress and lead to disease onset.

Disruption of MicroRNA 204 Triggers Autophagy-related Retinal Degeneration

Zhang, C.1, Miyagishima, K.1, Dong, L.1, Sharma, R.2, Rising, A.1, Dejene, R.1, Wang, Y.1, Maminishkis, A.1, Miller, S.1

1National Institute of Health, SERPO/OGVBR/NEI, Bethesda, United States, 2National Institute of Health, Genetic Engineering Facility/NEI, Bethesda, United States, 3National Institute of Health, UDCSC/OGVBR/NEI, Bethesda, United States

miR-204 plays a fundamentally important role in pulmonary, renal, mammary, and ocular functions and aberrant expression or genetic mutation of the gene, results in multiple diseases including cancer. Our previous studies have shown that miR-204 is relatively enriched in human retinal pigment epithelium (RPE) and plays an important role in maintaining the epithelial phenotype and its physiological functions in vitro. Genetic ablation of miR-204 in mice led to deposits in the sub-retinal space and hyper-auto-fluorescent spots that persisted with age. These changes were observed in parallel with decreased response amplitudes in the a- and b-waves of the electroretinogram (ERG) and a decrease in the fast oscillation (FO) of the DC-ERG. The current study shows that development of hyper-auto-fluorescence in miR-204 −/− mice coincides with increased Iba-1 + microglia in the subretinal space, buildup of undigested rhodopsin both in RPE and microglia, and increase in autophagy expression. Therefore, miR-204 plays a key role in maintaining the structure and function of the RPE/retina by regulating local autophagic protective responses which prevent intracellular accumulation of undigested photoreceptor outer segment (POS) that can generate oxidative stress and lead to disease onset.
Retinoic Acid and Insulin Play a Critical Role in the Regulation of the Blood-Retinal Barrier in Diabetes

Pollock, L.1, Xie, J.2, Bell, B.3, Anand-Apte, B.1,2
1Cole Eye Institute / Cleveland Clinic, Ophthalmic Research, Cleveland, United States, 2Cole Eye Institute / Cleveland Clinic, Cleveland, United States, 3Cleveland Clinic Lerner College of Medicine, Ophthalmology, Cleveland, United States

Macular edema is one of the major causes of loss of visual acuity in diabetic retinopathy. The accumulation of extracellular fluid in the retina occurs due to disruption of one or both of the blood retinal barriers (BRB) (inner and outer). Despite the apparent success of treatment with laser photocoagulation and anti-VEGF drugs, only a small proportion of patients recover good vision. Thus it is critical to identify new molecular mechanisms and therapies for diabetic macular edema.

We have previously reported that diabetic mice on high doses of insulin show a marked increase in BRB breakdown similar to the increased incidence of macular edema seen in type 2 diabetic patients on insulin. To test the hypothesis that insulin can directly induce breakdown of the BRB we evaluated the distribution of tight junctions in endothelial cells as well as RPE cells in culture following treatment with insulin and observed a dramatic disruption of tight junctions that was mediated by EGF. Retinoic acid (RA) has been previously shown to regulate the EGF signaling pathway. We evaluated the role of RA on BRB development and maintenance using a novel zebrafish transgenic line Tg(l-fabp:DBP-EGFP:flk1:mcherry) that expresses vitamin D binding protein (a member of the albumin gene family) tagged to green fluorescent protein. Treatment of zebrafish with antagonists of retinal dehydrogenase as well as inverse agonists of the RA receptor, resulted in breakdown of the BRB that could be rescued by all-trans RA. In addition, we demonstrated that Cyp26a1, which catalyzes RA degradation also demonstrated that Cyp26a1, which catalyzes RA degradation also resulted in BRB breakdown. To determine whether these pathways play a role in diabetic retinopathy and potentially macular edema, we established an in vivo model of diabetes-induced BRB breakdown in Tg(l-fabp:DBP-EGFP:flk1:mcherry) fish. Similar to our data in mice and humans, we observe that insulin can induce leakage of plasma proteins into the retina of zebrafish. All trans RA could prevent this BRB breakdown suggesting that RA could potentially play a critical role in the maintenance of BRB in diabetic retinopathy. 

Acknowledgements: This work was supported by NIH grants EY016490, EY015638, EY026181, T32EY024236, P3OEY025585, a Research to Prevent Blindness Challenge Grant; Foundation Fighting Blindness Center Grant.
Epidemiology of Eye Disease & Global Eye Health

Ocular Axial Length and its Associations in Russia: The Ural Eye and Medical Study

Bikkov, M.1, Kazakbaeva, G.1, Zainullin, R.1, Gilmarshin, T.1, Bikkova, G.1, Jonas, J.2
1Ufa Research Institute, Ufa, Russian Federation, 2Medical Faculty Mannheim of the Ruprecht-Karls-University of Heidelberg, Department of Ophthalmology, Mannheim, Germany

Purpose: To assess the normal distribution of axial length and its associations in a typically multi-ethnic population in Russia. Since high myopia can lead to myopic maculopathy and optic neuropathy, myopia has been estimated to become the most common cause for irreversible blindness worldwide. It is therefore important to know the prevalence of refractive errors in the general population.

Methods: The population-based Ural Eye and Medical Study was carried out in a rural and an urban area in the region of Ufa / Bashkortostan 1400 km East of Moscow. Out of 7328 eligible individuals aged 40+ years, 5,899 (80.5%) individuals participated and underwent an detailed ophthalmologic and general examination.

Axial length was measured sonographically.

Results: Mean axial length was 23.33 ± 1.10 mm (median: 23.23 mm; range: 19.78-32.87mm) for the right eyes and 23.29 ± 1.09 mm; range: 19.78-32.20mm for left eyes. The 95% confidence interval (CI) ranged from 21.40 mm to 25.91 mm. Prevalence rates of any myopia (defined by axial length ≥ 24.00 mm), minor myopia (axial length: 24.0 to < 24.50 mm), moderate myopia (axial length: 24.5 to < 25.00 mm), high myopia (axial length: 25.0 to < 25.50 mm), and high myopia (axial length: ≥ 25.50 mm) were 78/5709 or 1.4% (95% CI: 1.1, 1.7), respectively. In multivariate regression analysis, longer axial length was associated with higher level of education (P = 0.001; standardized regression coefficient β: 0.07), higher age (P = 0.001; β:0.06), higher weight (P = 0.001; β:0.02), higher height (P = 0.001; β:0.01), thinner lens thickness (P = 0.001; β: -0.07), and lower corneal refractive power (P = 0.001; β: -0.38; B: -0.23; 95% CI: -0.24, -0.21).

Conclusions: The prevalence of axial myopia, in particular of high axial myopia, is markedly lower in the Russian population than in East Asian populations. As in other ethnic groups, longer axial length was correlated with higher level of education, urban region of habitation, taller body height and higher intraocular pressure. It was not related with the prevalence of age-related macular degeneration, diabetic retinopathy, region of habitation and ethnicity.

Nucleic Acid Stimulation Increases Barrier Function in Immortalized Corneal and Conjunctival Epithelium via Toll-like receptor 3

BAN, Y.1,2, Azita, Y.1, Sotozo, C.1, Kinoshita, S.3
1Kyoto Chubu Medical Center, Ophthalmology, Nantan, Japan, 2Kyoto Prefectural University of Medicine, Ophthalmology, Kyoto, Japan, 3Kyoto Prefectural University of Medicine, Frontier Medical Science and Technology for Ophthalmology, Kyoto, Japan

Purpose: We previously reported that polyinosinic-polycytidylic acid [poly(I:C)], a synthetic analog of viral double-stranded RNA, strengthens both the tight junction barrier and the glycocalyx barrier of corneal and conjunctival epithelia. Poly(I:C) is a ligand of toll-like receptor 3 (TLR3), a member of innate immune receptors family. In this study, we investigated whether or not the increase of the barrier function by poly(I:C) in immortalized corneal and conjunctival epithelium is via TLR3.

Methods: Immortalized human corneal-limbal epithelial (HCLE) cells and human conjunctival epithelial (HCjE) cells were cultured on 12-mm Transwell filters (n=12) at a density of 4x105 cells/cm2. The cultured cells were then stimulated with 25μg/ml of polyinosinic-polyribocytidylic acid [Poly(I:C)], an analog of viral double-stranded RNA that is produced during viral replication. To block TLR-3, TLR3/dsRNA Complex Inhibitor (EMD Millipore Corp., Billerica, MA) was added to the medium until the final concentration became 40μM for the HCLE cells and 25μM for the HCjE cells. TLR3/ dsRNA Complex Inhibitor contained cytosolic dimethyl sulfoxide (DMSO), so the same concentration of DMSO was added to the medium as a control. After 24-hours exposure to Poly(I:C), transepithelial electrical resistance (TER) was measured using Endohm electrodes (World Precision Instruments, Sarasota, FL).

Results: Poly(I:C) challenge increased the TER after 24-hours exposure to Poly(I:C) (p< 0.01). Inhibition of TLR3 blocked TER increase of the barrier function by poly(I:C) in immortalized corneal and conjunctival epithelium is via TLR3.

Conclusions: The findings of this study show that Poly(I:C) challenge increases the barrier function of corneal and conjunctival epithelia via TLR3. Our findings revealed that Poly(I:C) challenge increases the barrier function by poly(I:C) in immortalized corneal and conjunctival epithelium is via TLR3.

Evaluation of the Suitability of Biocompatible Carriers as Artificial Transplants Using Cultured Porcine Corneal Endothelial Cells

Spinozzi, D.1, Miron, A.1, Bruinsma, M.1, Dapena, I.1,2, Rat, F.1,2, Dierlacher, E.1,2, Melles, G.1,2
1NIVOS, Rotterdam, Netherlands, 2Melles Cornea Clinic, Rotterdam, Netherlands, 3Linköping University, Department of Biomedical Engineering, Linköping, Sweden, 4Amintrans EyeBank, Rotterdam, Netherlands

Purpose: In vitro assessment of bioengineered carriers and human anterior lens capsules (HAlC) carrying cultivated porcine corneal endothelial cells (pCEC) as tissue-engineered grafts for Descemet membrane endothelial keratoplasty (DMEK). Materials: pCEC were isolated and cultured up to P2 before being seeded onto LinkCell™ biogenicinated matrices of 20μm (LK20) or 100μm (LK100) thickness, and on HAlC. During expansion on the carriers, pCEC viability and morphology were assessed by light microscopy. Expression of ZO-1 and Na+/K+ -ATPase was measured by immunohistochemistry. pCEC viability was measured by glucose levels in the culture medium at different time points. Biomechanical properties of the pCEC-carryer constructs were evaluated by simulating DMEK surgery in vitro using an artificial anterior chamber (AC) and a human donor cornea denuded of DM and de-swelling after in vitro surgery was checked by optical coherence tomography (OCT).

Results: pCEC seeded on LK20, LK100, and HAlC, formed a uniform monolayer of hexagonal, tightly packed cells that expressed Na+/K+ -ATPase and ZO-1. Glucose level in the culture medium decreased by an average of 53±13% and 62±10% for pCEC seeded on LK20 and HAlC, respectively. During in vitro surgery the pCEC-LK20 and pCEC-LK100 constructs were handled like Descemet stripping endothelial keratoplasty (DSEK) grafts, i.e. folded like a ‘taco’ for insertion due to the challenges related to the rolling and sticking of the graft to the injector. The pCEC-HAlC construct behaved similar to the DMEK reference model during implantation and unfolding in the artificial AC, showing good adhesion to the bare stroma. Furthermore, two days after the in vitro surgery, corneal thickness decreased by an average of 12% indicating deturgescence capacity of the pCEC-HAlC constructs.

Conclusions: HAlC was the most suitable carrier for cultivated pCEC in vitro DMEK surgery with good intraoperative graft handling and inducing postoperative corneal deturgescence, while the LK20 carrier showed good biocompatibility, but required an adapted surgical protocol, that is a DSEK protocol. Both carriers might be good candidates for potential future clinical applications.

MONDAY, 10 SEPTEMBER 2018

Cornea and Ocular Surface

TUESDAY, 11 SEPTEMBER 2018
**Integrative Analysis of Cytokines and miRNAs in the Aqueous Humor of Bullous Keratopathy Patients to Develop Prognostic Biomarkers**

*Ueno, M.\(^1\), Yoshii, K.\(^1\), Fujita, T.\(^1\), Uehara, A.\(^1\), Asada, K.\(^1\), Sotozono, C.\(^1\), Kinoshita, S.\(^2\), Hamuro, J.\(^1\)*

\(^1\)Kyoto Prefectural University of Medicine, Department of Ophthalmology, Kyoto, Japan, \(^2\)Kyoto Prefectural University of Medicine, Department of Mathematics and Statistics in Medical Sciences, Kyoto, Japan.

Purpose: We recently developed a novel form of regenerative medicine for bullous keratopathy (BK) involving a cell-injection therapy using cultured human corneal endothelial cells (hCECs). The regenerative ability of the injected cells is influenced by both the quality of the hCECs and the anterior chamber (AH) environment. The purpose of this study was to examine cytokine profiles in AH to develop prognostic biomarkers for BK.

Methods: AH specimens were collected just prior to surgery in BK patients undergoing Descemet’s stripping automated endothelial keratoplasty. The patients were classified into the following 3 BK groups:

1. pseudophakic BK and argon laser iridotomy-induced BK
2. penetrating keratoplasty. The patients were classified into the following 3 BK groups:
3. >30% decrease in CEC density, and
4. 0% decrease of CEC density.

Topical TRPM8 Antagonist AMTB Reduces the Activity of Cold Thermosensitive Trigeminal Neurons Innervating the Ocular Surface

*Diaz-Tahoces, A., Velasco, E., Alexandre-Carrera, F., Luna, C., Acosta, M.C., Belmonte, C., Gallar, J.*

Instituto de Neurociencias, Universidad Miguel Hernández-CSIC, San Juan de Alicante, Spain.

Cold thermosensitive trigeminal ganglion (TG) neurons are characterized by the presence of TRPM8, an ion channel involved in sensory transduction of cold stimuli. Previous experiments performed in TRPM8 knockout mice showed that both the background activity and the frequency and firing pattern evoked in high background cold thermoreceptor neurons (HBCNs) by cooling are dependent on TRPM8 channel.

In the present work, we explored the effects of TRPM8 antagonist N-(3-aminopropyl)-2-[(3-methylphenyl)-methyl]oxlyl]-N-[2-thienylmethyl]benzamide hydrochloride salt (AMTB) on the background and stimulus-evoked impulse activity of cold thermosensitive TG neurons innervating the ocular surface anesthetized adult wild type rats of both sexes.

Impulse activity of TG neurons was recorded extracellularly with tungsten electrodes (1-2MΩ) and stored for off-line analysis. A microprobe thermometer was placed on the corneal surface for simultaneous temperature recording. Background activity (BA) at 34°C and cooling-evoked activity (CEA) were evaluated. Cooling stimuli of high intensity (inducing -5°C to -20°C corneal temperature change) and low intensity (-0.1°C to -5°C change) were applied. BA and CEA before and 15 min after topical treatment with 1% AMTB were compared.

Thirteen HBCNs with RF located in the cornea or the bulbar conjunctiva were recorded. BA of HBCNs was 5.16±0.71 imp/s and BA after AMTB treatment was 3.04±0.75 imp/s (p=0.016). HBCNs exhibited an increase in firing rate proportional to the cooling stimulus intensity, being the mean CEA evoked by high-intensity stimuli significantly higher than CEA evoked by low-intensity cooling (8.75±0.93 vs. 4.89±0.62 imp/s, p< 0.001). After AMTB treatment, HBCNs responded to cold only in a 7.4% of the 121 cooling stimuli applied (p< 0.00001) and, when present, responses to high- and low-intensity cooling were not significantly different (5.67±2.47 vs. 4.58±1.39 imp/s).

Results suggest that corneal high background cold thermosensitive neurons encode temperature changes occurring on the ocular surface. This response to cooling stimulation is mediated mainly by TRPM8 channels, being abolished after topical treatment with TRPM8 antagonist AMTB.


Influence of Material Stiffness on a Corneal Epithelial Cell Line

*Masterton, S.\(^1\), Ahearne, M.\(^2\)*

\(^1\)Trinity College Dublin, Trinity Centre for Biocomputing, Dublin, Ireland, \(^2\)Trinity College Dublin, Department of Mechanical and Manufacturing Engineering, Dublin, Ireland.

Introduction: Corneal blindness is one of the most common causes of blindness worldwide, shortage of donor tissue and increased graft failure makes treatment options limited (1). The corneal epithelium, the outer layer of the cornea, can be damaged by injury or disease causing pain and loss of vision. Tissue engineering techniques to grow corneal epithelium could aid in the short-age of available tissue. To do this, the factors that control corneal epithelial behaviour must be understood. Mechanical stress on cells can affect their activity (2), however, little is known about its effect on the corneal epithelium. This study examines how material stiffness regulates cell behaviour using a human telomer-a eam immortalised corneal epithelial cell line (hTCEpi) to determine optimal conditions for generating new tissue.

Materials and methods: Polydimethylsiloxane (PDMS), was used to study the effect of material stiffness on corneal epithelial cells. Blends of PDMS were prepared and tensile tested. An elastic modulus from 12kPa to 2.24MPa was achieved. PDMS was cast into cell culture dishes and seeded at a density of 5,000 cells/cm² for 7 days. Cell morphology, viability, protein expression of a prolifera-tion marker phosphorylated extracellular regulated kinase (pERK), mature corneal epithelial marker cytokeratin 3 (CK3) and mRNA expression of CK3 was carried out.

Results: Preliminary data show that stiffer substrates promote the ‘cobblestone’ morphology observed in these cells. Stiffer substrates had a significant increase in cell viability and an in-crease in pERK protein expression. CK3 was not expressed in any groups. Therefore, RT-PCR was performed to see if differences in mRNA expression for CK3 was observed, stiffer substrates ex-pressed increased CK3 expression. Further replications of these experiments will be performed to confirm these findings.

Discussion: Our data shows that stiffer substrates increase cellular viability and proliferation as well as increased mRNA expression of the mature corneal epithelial marker CK3. In addition to this, stiffer substrates produce the typical ‘cobblestone’ cell morphology that is observed in corneal epithelial cells. This information may...
be used in the design of biomaterials of particular stiffnesses for corneal tissue regeneration.

References:

Purpose: Human corneal endothelial progenitor cells (HCEPs), which has been selectively isolated and differentiated into human corneal endothelial cells (HCECs), are crucial for repairing corneal endothelial damage. In this study, we evaluated the roles of a ROCK inhibitor, Y-27632, on the isolation and expansion of HCEPs, and assessed the in vitro effects of different concentrations of Y-27632 on HCECs cultured by isolating HCEPs.

Methods: HCEPs were isolated and expanded in a medium with and without 10 µM Y-27632, and then differentiated into HCECs in a medium with FBS. The characteristics of HCEP and HCEC were confirmed by immunofluorescence staining. The proliferation, viability, morphology, and wound healing ability of HCECs were assessed in nerve regeneration.

Results: Y-27632 enabled the isolation and expansion of HCEPs from the corneal endothelium. HCECs cultured from the isolated HCEPs showed an optimal increase in proliferation and survival in the presence of 10 µM Y-27632. As the concentration of Y-27632 increased, HCECs became elongated, and actin filaments were redistributed to the periphery of cells. Y-27632 also caused a concentration-dependent enhancement in the wound healing ability of HCECs.

Conclusion: Y-27632 enabled the isolation and expansion of HCEPs. It also enhanced the proliferation, viability, and migration of HCECs cultured by isolating HCEPs.

Laminin N Terminus α31 Distribution and Influence on Matrix Organisation Indicates a Role Controlling the Maturation of Corneal Epithelium after Wounding

Troughton, L., Iorio, V., Barrera, V., Hamill, K.
University of Liverpool, Eye and Vision Science, Liverpool, United Kingdom

Laminin N terminus α31 (LαNt α31) is a relatively understudied laminin and netrin-related protein. Previously it has been shown to influence epidermal keratinocyte migration however, the mechanism has not been determined. Here we investigated the distribution and function of this new protein during corneal epithelial wound repair. Using immunohistochemistry processing of ex vivo porcine alkali corneal burn wound models we identified dramatic shifts in LαNt α31 distribution during wound closure and maturation. In intact, undamaged pig anterior segments LαNt α31 was highly enriched in the conjunctival and limbal epithelium with relatively low expression in cornea, however, within the cornea it was concentrated at the basal aspect of the epithelial sheet. In wound healing conditions the distribution changed, basal LαNt α31 expression was observed in the newly formed epithelium until three days after the wound closed i.e. when the nascent epithelium matured. To study mechanism, we developed an adenoviral-driven overexpression system for use in primary and immortalised cultured corneal keratinocytes. LαNt α31 overexpressing cells displayed reduced wound closure rates and increased cell spread area compared with controls, suggesting a change in cell-matrix adhesion. Interestingly, indirect immunofluorescence microscopy and live imaging of fluorescently tagged laminins revealed LαNt α31-induced changes to laminin organisation during times of new matrix deposition. Consistent with these data, immunoprecipitation and live imaging of GFP tagged-LαNt α31 indicated interaction between LαNt α31 and laminin 332. Finally, we investigated the consequences of these matrix changes upon adhesive complex formation and noted aberrant distribution of focal adhesion proteins as well as early maturation of hemidesmosomes in LαNt α31 overexpressing cells. Together these data suggest that LαNt α31 controls the switch from a migratory to stable-adhered state for corneal epithelial cells during wound repair, influencing the maturation of the epithelium. These findings could have implications for conditions of poor or ineffective wound repair such as recurrent corneal erosions.

Deep Anterior Lamellar Keratoplasty with Xenonograft. First Clinical Results

Baldis, M.
Ophthalmica Eye Institute, Anterior Segment, Thessaloniki, Greece

To describe our initial experience with heterologous bio-engineered corne (xenograft). Acornea is a bio-engineering cornea, derived from porcine cornea. It is extracellular matrix (ECM) of porcine stroma prepared through inactivation of virus and decellularisation. The main component of ECM is collagen. Acornea possesses the collagen fiber structure of natural cornea, preserving bowman’s membrane and the anterior partial cornea stroma. It is sterilized with Gamma ray. The first case a 75 year old female suffered from severe microbial keratitis, postoperative after complicated cataract operation. She presented with central corneal melt and perforation. The second case is an 81 year old rheumatoid arthritis female with paraen- tral keratolysis and descemetomecule. In both cases post-operative lamellar keratoplasty performed despite the perforation. In both cases complete epithelial regeneration and matrix synthesis achieved in less than 5 days. Mechanical structural stability, bio-compatability, transparency were similar to human graft. These are the first successful cases performed with heterologous bio-engineering cornea in the western world (more than 80 cases performed in China).

Epithelial Repair and Re-innervation after Corneal Alkali Burn

Martinez-Garcia, M.C., Lorenzo-Martín, E., Herrera-Pérez, C., Gallego-Munoz, P.
University of Valladolid, Biología Celular, Valladolid, Spain

Tears contribute to maintaining a healthy ocular surface by providing nutrients and growth factors. At the same time, corneal nerve fibres release neuromediators that provide trophic support to the ocular surface. When corneal nerves are damaged, as in alkali burn, corneal healing and tear secretion are impaired. Nerve regeneration during healing of alkali burn allows us to observe the relationship between clinical signs, histology and the re-innervation process. Left eyes of New Zealand adult rabbits were burned for 60 seconds with an 8-mm filter paper soaked in 0.5N NaOH. Clinical signs such as oedema (pachymetry), tear secretion (Schirmer test), ulcer and epithelial defects (fluorescein and lissamine green solution) were evaluated every day during the first week and after 15, 30 and 90 days. Animals were sacrificed and corneas excised at 15, 30 and 90 days. Hematoxylin-Eosin (H&E) stained sections were used for histologic assessment of epithelial and stroma regeneration. Immunohistochemistry with TUBB (Tubulin beta-3) was performed in ‘in toto’ corneas and in sections displaying nerve regeneration. Pachymetry was significantly increased during the 90 days. Tear secretion was similar to control at day 15 and 30 and decreased at day 90. A high percentage (50-70%) of fluorescein testing positive eyes was found during the first seven days, although at day 15 and 30 they had decreased to 40%. This test showed a highly variable evolution with closed and re-opened wounds. At 90 days, negative fluorescein tests were found. However, at this time, positive lissamine green were localized in the wound area. Histological evaluation showed, from day 15 to day 90, a statisti-cal significant decrease in epithelial thickness compared to the control. This epithelium was irregularly damaged. At day 30, stroma displayed irregularities in collagen lamellae disposition, these irregularities being lower at 90 days. TUBB labelled thick branches in the deep stroma, running irregularly around the collagen lamel-las. At day 90, there were also thick branches in the deep stroma and the subbasal plexus appeared although there were no nerve endings between the epithelial cells. Sensory denervation produces unstable epithelial closure, possibly due to abnormal development of the epithelial basement membrane. Rabbit alkali burn could be a good model to study re-innervation of cornea and, as a consequence, might provide a good model for dry eye.

Corneal Cold Thermoreceptors Activity Decreases with Age

Gallar, J., Mizerska, K., Luna, C., Quirce, S., Acosta, M.
1 Universidad Miguel Hernandez-CSIC, San Juan de Alicante, Spain, 2Well Medicine, Ophthalmology, New York, United States

TRPM8-dependent activity of cold thermoreceptors is involved in the maintenance of basal tearing. The purpose of this work was to analyze the activity of corneal cold thermoreceptors in young and adult guinea pigs (1-18 months old). Blinking rate and tear film (tearing rate and TRU) changes with age were also explored. The spontaneous (at 34ºC) and stimulus-evoked activity (cooling the bath solution from 34ºC to 20ºC) of corneal cold nerve termi-nals were recorded from ex-vivo eyes of guinea pigs of different ages.
Aqueous Humour Outflow Involves Metabolic Activity

Reina-Torres, E., Sherwood, J.M., Overby, D.R.

Imperial College London, Dept. of Bioengineering, London, United Kingdom

The bulk of aqueous humour (AH) drains through the trabecular meshwork (TM), which generates a resistance that determines intraocular pressure (IOP). Elevated TM resistance is responsible for IOP elevation in glaucoma. Classic studies argued that AH outflow is unaffected by temperature reduction or metabolic inhibitors, leading to the conventional view that AH outflow does not require metabolic activity. In this study, we re-examined the effects of temperature and metabolic inhibitors on AH outflow facility in mice.

Outflow facility (C, 1/resistance) was measured in enucleated eyes from C57BL/6 male mice (9-14 weeks old) using Perfusion. We considered 4 experimental sets for this project: 1) we measured C over a range of pressure steps (-18 mmHg) in paired eyes with one eye at 35°C and the contralateral eye at 22°C (n=10 pairs), 2) we measured C at 8 mmHg in individual eyes while temperature was changed from 35 to 22°C (n=9 eyes), 3) we repeated (2) except temperature was changed from 22 to 35°C (n=9 eyes), and 4) we measured C in an additional 7 pairs of eyes perfused at 35°C with a cocktail of metabolic inhibitors (11mM 2-Deoxy-D-glucose + 4mM sodium azide + 100mM 3P0 in PBS) versus isometric vehicle. Paired t tests were used to determine statistical significance.

In the first experimental set, C was 283% [125, 551] (geometric mean [95% CI]) higher in eyes at 35°C versus 22°C (p<0.0003). This difference was larger than could be attributed to changes in AH viscosity. In the second set, reducing temperature from 35°C to 22°C decreased C by 25% [10, 38], (p=0.021), which could be attributed to viscosity. In the third set, increasing temperature from 22°C to 35°C increased C by 29% [146, 541] (p=0.003). Perfusion with metabolic inhibitors reduced C by 25% [10, 37] (p=0.009). Inhibiting metabolic activity in the TM reduces outflow facility. Differences between increasing and decreasing temperature in sets 2 and 3 could be explained if the TM shuts down shortly after temperature change.

Regulation of Connective Tissue Growth Factor by miRNA-18a in the Human Trabecular Meshwork Cell Response to TGFβ: Therapeutic Opportunities

Knox, J., Lister, K.,1,2 Hamill, K.,1 Willoughby, C.,1,2

1University of Liverpool, Department of Eye and Vision Sciences, Liverpool, United Kingdom, 2Ulster University, School of Biomedical Sciences, Coleraine, United Kingdom

Connective tissue growth factor (CTGF) is a transforming growth factor β (TGFβ) inducible gene that is strongly associated with fibrosis and with aberrant extracellular matrix (ECM) deposition in the trabecular meshwork, resulting in elevated intraocular pressure and primary open angle glaucoma. miRNAs play an important role in the regulation of CTGF expression, which is tissue and disease specific. Previous studies have identified key miRNAs involved in the regulation of CTGF in other tissues and disease conditions, including fibrosis: miRNA-18a, -19a, -19b, -19a, -19b, -26a, -26b, -133b and -205. In this study, primary human TM (HTM) cells (n=5) were treated with 5 nM TGFβ for 24 hours and the expression of these specific miRNAs measured by qPCR. CTGF transcript abundance and protein expression were also measured; CTGF was increased in HTM cells after TGFβ treatment. While the response of the majority of the selected miRNAs to TGFβ treatment was variable among the donors, miRNA-18a showed a consistent upregulation with a 1.5-fold increase in protein expression (P=0.01). To further investigate the role of miR-18a in regulating CTGF, HTM cells (n=3) were treated with 5 nM TGFβ and co-transfected with 100 nM of miRNA mimic, 100 nM miRNA inhibitor or 100 nM of a scramble control for 24 hours. The miRNA and protein expression of CTGF was measured following these transfections and compared to HTM treated with TGFβ without co-transfection. Cells co-transfected with a miR-18a mimic showed a 0.6-fold reduction in the transcript abundance of CTGF (95% CI of diff. 0.0908 to 0.7570, P=0.03), and a 0.4-fold reduction in the protein abundance (95% CI of diff. 0.2004 to 0.9782, P=0.01). Together, these data show that miR-18a plays an important role in regulating the expression of CTGF induced by TGFβ in the HTM. Increasing the level of miR-18a in the HTM may be a valuable therapeutic strategy for treating the TGFβ-induced changes in the trabecular meshwork in glaucoma.

Downregulation of Tight Junctions in Schlemm’s Canal Endothelia Decreases Intraocular Pressure and Increases Outflow Facility in a Model of Steroid-induced Ocular Hypertension

Kelly, R.A.1, Cassidy, P.S.1, Reina-Torres, E.1, O’Callaghan, J.T.1, Sherwood, J.M.3, Humphries, M.M.1, Lawson, M.1, Campbell, M.1, Stam, D.W.1, Overby, D.R.1, Humphries, P.1

1Trinity College Dublin, Dublin, Ireland, 2Imperial College London, London, United Kingdom, 3RxGen, Connecticut, United States, 4University of North Carolina, Charlotte, United States, 5Imperial College London, London, Ireland

Mice were implanted subcutaneously with osmotic mini-pumps delivering dexmethasone, while control mice were implanted with pumps containing cyclosporin as a vehicle control. IOP was measured by rebound tonometry. Mice were then injected intracamerally with 20-1 and tricellulin targeting siRNA in one eye, while contralateral control eyes received non-targeting siRNA injections. At 48 hours post injection, IOP was measured again. 72 hours after siRNA injection, eyes were enucleated and perfused using the perfusion system. DEX treated animals underwent a significant IOP elevation of 4.98 mmHg at week 4 over baseline levels, while vehicle treated animals had no significant change in IOP, 0.86 mmHg. 48 hours post siRNA treatment showed DEX treated mice had a significant IOP reduction in IOP in TGFβ siRNA eyes, 11.2%, compared to baseline. In the vehicle treated normotensive mice, T-siRNA eyes also had a significant mean reduction in IOP of 6.38%. NT-siRNA injected eyes did not show significant reduction in IOP in either DEX or vehicle treated mice. Additionally, analysis showed while both NT-siRNA and T-siRNA injected eyes showed no significant differences in IOP before injection, at 48 hours post injection, eyes injected with T-siRNA had significantly lower IOP than those injected with NT siRNA, showing a mean difference of 1.93 mmHg, while vehicle treated mice had a mean difference of 0.65 mmHg. In DEX treated mice, eyes injected with T-siRNA had a significant increase in outflow facility of 63% over contralateral NT-siRNA injected controls. Similarly, in normotensive vehicle treated mice T-siRNA induced an increase in outflow facility of 38% as compared to NT-siRNA. In the DEX induced murine model of ocular hypertension, we have demonstrated here that siRNA mediated downregulation of ZO-1 and tricellulin significantly reduces IOP in both hypertensive mice and normotensive controls, with hypertensive mice showing a greater relative reduction in IOP. We additionally demonstrated that this therapeutic approach significantly increases outflow facility in this hypertensive model, and in wildtype controls, with a greater relative change seen in hypertensive animals.

Scleral stiffening has been suggested as a potential therapy for glaucoma and myopia. Genipin, a widely used collagen-crosslinker, has been shown to stiffen the sclera in vivo and ex vivo. However, the long-term effects of in vivo ocular administration of genipin have not been investigated. Here, we aim to determine whether retrobulbar injection of genipin causes an immune or inflammatory response in the retina.

Male retired breeder (age: 10 months) Brown Norway rats were subdivided into 2 groups. We delivered single 150 µl retrobulbar injections such that Group 1 (genipin/HBSS) rats received 15µM genipin in Hank’s Balanced Salt Solution (HBSS) unilaterally and HBSS contralaterally and Group 2 (HBSS/control) rats received HBSS unilaterally and no injection (naïve control) in the contralateral eye. Four weeks post injection, rats were sacrificed, eyes were enucleated, and the optic cup was prepared for cryosectioning. 10µm thick sections (n = 10/eye) from the peripapillary region were mounted on sections from a rat with microbead-induced experimental glaucoma were used as positive controls (+ ctrl). Fluorescence was quantified using mean gray values from a region of interest the inner and outer limiting membranes and with a width of 100µm (ImageJ).

In this preliminary study, we report data from 1 genipin/HBSS rat and 1 HBSS/naïve rat. There was minimal effect on Iba1 signal

Research at the Ocular Genetics Unit at the University of Dublin, Trinity College was supported by the European Research Council (ERC-2012-AAd 322656-Oculus), and Enterprise Ireland. We also acknowledge an equipment grant from Science Foundation Ireland (12/ERC/B2359) in support of this project.

An Assessment of Inflammatory Response after Retrobulbar Injection of Genipin

Kim, R.K.1, Hannon, B.G.2, Read, A.T.1, Gao, K.1, Pardue, M.T.1,2, Ethier, C.R.1

1Georgia Institute of Technology, Wallace H. Coulter Department of Biomedical Engineering, Atlanta, United States, 2Georgia Institute of Technology, George W. Woodruff School of Mechanical Engineering, Atlanta, United States, 3Atlanta VA Medical Center, Center for Visual and Neurocognitive Rehabilitation, Atlanta, United States

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from HBSS or genipin, with genipin Iba-1 signal always < 0.4% of + ctrl (Table). Genipin had a modest effect on GFAP signal (13.4% of + ctrl), but this was less than GFAP in the contralateral HBSS eye (13.4% of + ctrl). More variability was seen in GFAP signal, e.g. genipin/HBSS rat vs. HBSS control. Differences in signal between HBSS-treated eyes can be attributed to biological variability. Genipin treatment did not lead to increased GFAP or Iba1 expression compared to the contralateral HBSS eye, and GFAP and Iba1 levels were < < than in a + ctrl eye. Thus, retrolubar injection of genipin does not appear to cause an immune or inflammatory response in the retina. Additional eyes must be assessed to confirm this finding.

2. Levy et al. IOVS. 2015.
Support: NIH EY025286 (CRE)

<table>
<thead>
<tr>
<th>Percent Fluorescence Signal Normalized to Positive Control</th>
<th>Naive/HBSS Group</th>
<th>HBSS/Genipin Group</th>
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<tbody>
<tr>
<td>Naive Eye</td>
<td>HBSS Eye</td>
<td>HBSS Eye</td>
</tr>
<tr>
<td>Genipin Eye</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFAP</td>
<td>0.040 ± 0.079%</td>
<td>2.96 ± 5.88%</td>
</tr>
<tr>
<td>Iba-1</td>
<td>0.036 ± 0.044%</td>
<td>0.217 ± 0.416%</td>
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[Values are mean ± SD computed from n=8 sections/eye. Each section was immunolabeled for both antigens.]

AP-2β Expression Is Required in the Peripapillary Mesenchyme for Normal Development of the Trabecular Meshwork

West-Mays, J.1 Akula, M.1, Ball, A.1, Williams, T.1
1McMaster University, Hamilton, Canada, University of Colorado School of Medicine, Department of Ophthalmology, Boulder, Denver, United States

Previously, our lab has shown that conditional deletion of Activating Protein 2 beta transcription factor (AP-2β) in the peripapillary mesenchyme (POM) of the eye (AP-2β NCC KO mice) resulted in anterior segment defects, including absence of a corneal endothelium, iridocorneal adhesions and altered morphology of the outflow pathway structures. These defects resulted in abnormal high intraocular pressure (IOP). To better understand the requirement of AP-2β in development of the outflow structures we examined the embryonic patterns, differentiation and ultrastructure of the developing trabecular meshwork (TM) in the AP-2β NCC KD mice. Wnt1Cre+/-;Tcfap2b+/-; mice were bred with Tcfap2blox/lox mice to generate Wnt1Cre+/-;Tcfap2b-/-;lox mice (AP-2β NCC KD) and were examined by transmission electron microscopy (TEM) and immunohistochemistry for expression of myocilin and α-SMA. Ultrastructural analysis of the TM region at postnatal day 14 (P14) revealed an increase in spacing between trabecular beams of control mice that was not seen in NCC KO mice. Further analysis revealed that the NCC KD mice had a reduced number of TM cells as compared with controls. At P14, myocilin was expressed in the TM region of control mice, but was greatly reduced in NCC KD mice and by 2 to 3 months of age a significant reduction in the number of myocilin-positive cells in the TM region of NCC KO mice was observed when compared with controls. A similar, significant reduction in α-SMA expression was also observed in the TM region of the NCC KD mice when compared to control mice. Together these data show that deletion of AP-2β in the POM resulted in altered numbers and differentiation of TM cells. Since our previous studies have shown that the AP-2β NCC KD mutant mice have aberrantly high IOP, these findings indicate that AP-2β is required in the developing TM in order to ensure IOP homeostasis.


Uddin, M.1, Koina, M.2, Hu, P.1, Behar-Cohen, F.1, Channling, T.1
1The University of Sydney, Discipline of Anatomy & Histology, Rose Institute, Sydney, Australia, Canberra Hospital, ACT Pathology, Garran, Australia, 2Centre de Recherche des Cordeliers, INSERM, UMRS 872 Team 17, Paris, France

Lymphatics play a role in fluid homeostasis and immune surveillance. With our earlier demonstration of lymphatics in human choroid, this study aims to provide evidence of lymphatics including their precise location & to detail changes in lymphatics & immune response in glaucoma. Retrolaminar optic nerve meninges from 18 adult eyes (glaucoma & age matched controls) were examined. Histological, ultrastructural characterization & multi-marker IHC was carried out using D2-40 (podoplanin), UVE-1, VEGFR3 (lymphatics); UEA lectin, CD34 (blood vessel); Iba-1 (macrophage); MHC II (antigen presenting cell, APC); GFAP, vimentin (glia), & AQPA (water channel protein) markers. Indian ink & FITC-dextran were injected into supra-choroidal space of adult Lewis rats to examine lymphatic drainage. Ultrastructural features of lymphatics including anchoring filaments, luminal flocculent protein but absence of erythrocytes, basal lamina, Weibel-Palade bodies, & fenestrance were shown. Lymphatics were evident in arachnoid mater adjacent to sub-choroidal space (SAS) in close association with meningeothelial cells (MEC). MEC expressed D2-40 constitutively. IHC showed D2-40+/UVEA collapsed & thin-walled lymphatics in retrolaminar optic nerve meninges. D2-40+/UVEA lymphatic-like structures were also found in dura close to D240/UVEA blood vessels. Tracer studies in rat showed presence of Indian ink filled lymphatic capillary in arachnoid mater showing lymphatic outflow from choroid plexus to sub-choroidal space. In glaucoma, arachnoid & dural lymphatics had larger lumens indicating lymphatic filling, thicker wall & greater density (78%); increased density (56%) & intensity of D2-40+ expression (69%) of MEC; & increased density of Iba-1+ macrophages (37%), MHC II+ APCs (47%) & Iba-1+/MHC II+ macrophages (75%), p < 0.05 compared to control eyes. This study has shown in retrolaminar optic nerve, lymphatics are located in both dura & arachnoid mater, where arachnoid lymphatics are spatially related to MEC. This intimate association leads us to suggest that MEC and arachnoid lymphatics form the structural basis for functional interaction between lymphatics and CSF in healthy CNS. The findings also showed increased APCs, lymphatic filling & density of MEC in glaucoma. With MEC functions of CSF drainage, pro-inflammatory cytokine expression & waste transport (via AQPA) our findings suggest that lymphatic/MEC interactions are integral to glaucoma pathogenesis.

Glaucoma Risk Gene Tmco1 Binds to RNA in the Nucleus and Affects Trabecular Meshwork Cell Viability

Sharma, S.1, Martin, S.1, Wood, J.1, Childow, G.1, Casson, R.1, Craig, J.1
1Flinders University, Ophthalmology, Adelaide, Australia, 2The University of Adelaide, Ophthalmic Research Laboratories, Adelaide, Australia

Glaucoma is the leading cause of irreversible blindness in the world. Progressive loss of retinal ganglion cells and corresponding visual field loss are the hallmarks of the disease. Elevated intraocular pressure (IOP) due to compromised trabecular meshwork function is a major risk factor for developing glaucoma. Common single nucleotide polymorphisms (SNPs) in the TMCO1 gene located on human chromosome 1q24 are strongly associated with the risk of blindness and less severe open-angle glaucoma. SNPs in this gene are also associated with IOP, and significantly increase the risk of glaucoma linked with elevated IOP. Thus the aim of the present study is to determine the role of TMCO1 in the trabecular meshwork in glaucoma. Our previous work showed that TMCO1 gene is ubiquitously expressed in ocular tissues including the trabecular meshwork, and the encoded protein primarily localises to the nucleus of both ex vivo and in vivo. The present study shows that in NTM-S normal trabecular meshwork cells, in the nucleus, TMCO1 protein binds to RNA, and siRNA-mediated knockdown of the encoding gene reduces cell viability. Further, levels of the protein in GTM-3 glaucomatous trabecular meshwork cells are lower than in NTM-S cells. However the levels of TMCO1 mRNA in peripheral blood from glaucoma patients and controls are unaltered. The present data suggests that TMCO1 may contribute to reduced cellularity of the trabecular meshwork reported in patients with glaucoma thus explaining its association with IOP, and stronger association with glaucoma linked with elevated IOP. TMCO1 is evolutionarily highly conserved and recent research indicates that it is a multifunctional protein. It reportedly regulates calcium storage in the endoplasmic reticulum, and cell proliferation in cancer cells and acts as a tumour suppressor. Our work suggests its role in regulating cellularity in normal cells. Further research is required to understand the mechanism of regulation of trabecular meshwork cellularity by TMCO1 in glaucoma.

Funding support: This work was funded by the National Health and Medical Research Council, Australia (grant #1031347), and the Ophthalmic Research Institute of Australia.
Comparison of Pattern Electroretinograms of Glaucoma Patients with Initial Parafocal Scotoma versus Initial Peripheral Nasal Step

Department of Ophthalmology and Visual Science, Seoul St. Mary’s Hospital, College of Medicine, The Catholic University of Korea, Seoul, Korea, Republic of

**Purpose:** To determine whether pattern electroretinogram (PERG) amplitude is associated with the location of initial visual field (VF) defect in primary open-angle glaucoma

**Methods:** Data from Twenty-nine glaucoma patients with an initial parafocal scotoma (IPS) within the central 10° of fixation, 23 glaucoma patients with an initial peripheral nasal step (IPNS), and 27 normal control subjects were analyzed in this study. Electroretinograms (ERGs) were obtained using a commercial ERG stimulator (Neuro-ERG). The N95 amplitudes of the IPS and the IPNS groups were compared. The thickness of the ganglion cell-inner nuclei-plexiform layer (GCIP) was measured using spectral-domain optical coherence tomography.

**Results:** The reproducibility of ERG measurements at the N95 amplitude was excellent (ICC=0.827). A lower N95 amplitude was observed in both IPS and IPNS compared to the normal control group (P<.001). The N95 amplitude of the IPNS group was significantly lower than that of the IPS group (P=0.034). Average GCIP thickness correlated positively with N95 amplitude (r=−0.368, P=0.002), but did not correlate significantly with global mean sensitivity (r=0.228, P=0.03) or mean deviation on 24-2 standard automated perimetry (r=0.173, P=0.176).

**Conclusions:** The N95 amplitude was lower in glaucoma patients with paracentral scotoma than in those with peripheral nasal step. The location of initial VF defects seems to have affected the PERG amplitude observed in glaucoma patients. Therefore, it is necessary to take into account the location of VF defects in evaluating PERGs of glaucoma patients.

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Preliminary Study of the Effect of Prostaglandin Analogs on Corneal Biomechanics and IOP Measurement Error with Goldmann Applanation Tonometry

Roberts, C.J.1, Mahmoud, A.M.1, Jain, S.G.1
1The Ohio State University, Ophthalmology & Visual Science; and Biomedical Engineering, Columbus, United States, 1The Ohio State University, Ophthalmology & Visual Science, Columbus, United States

Prostaglandin Analogs (PGs) are a common medical treatment for glaucoma with a primary mechanism of action to lower intraocular pressure (IOP) by increasing uveoscleral outflow through upregulation of matrix metalloproteinases and remodeling of extracellular matrix. This may also affect the cornea, and changes in corneal biomechanics have been reported. It is hypothesized that a portion of the IOP-lowering effect of PGs is due to measurement error in Goldmann Applanation Tonometry (GAT) due to a softened cornea. Prostaglandin naïve subjects were prospectively recruited with 21 subjects and 41 eyes enrolled. IOP was measured with GAT, PASCAL Dynamic Contour Tonometer (DCT), and corneal compensated IOPc with the Ocular Response Analyzer (ORA). Biomechanical Response was assessed using corneal hysteresis (CH) from the ORA, and four measures of stiffness from the Corvis ST: stiffness parameter at first aperture (SP-A1) and at highest concavity (SP-CH), deformation amplitude (DA-Ratio), and Inverse Radius (InvRad) which is analogous to concave curvature. All measures were obtained at baseline (visit 1) prior to initiation of PG therapy, and at 1 month (visit 2) and 4 months (visit 3) after baseline. The parameter GATminusDCT was calculated between visits to indicate GAT measurement error. Statistical analysis was performed with SAS, and included t-tests between visits and linear regressions of ΔSP-A1 and ΔInvRad, indicating that greater GAT measurement error was associated with greater corneal softening. Treatment with Prostaglandin Analogs generates a reduction in corneal stiffness that leads to underestimation of IOP using Goldmann and Support Applanation tonometry. It is recommended to use a different tonometric technology when using PGs in order to accurately monitor reduction in IOP.

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Visual Evoked Potentials Detect Functional Correlates of Demyelination, Remyelination and Axon Loss in Feline Models of Common Optic Nerve Pathologies

McLellan, G.J.1,2,3, Heidari, M.1, Snyder, K.C.1,2,3, Teixeira, L.B.2, C.1, Okawa, K.1, Chan, K.1,3, Lindemann, J.1,3, Hennes-Bean, E.A.1, Kiland, J.A.1,3, Dejanovich, S.3, Radcliff, A.3, Verhoeve, J.N.1,3, Duncan, I.D.1,3
1University of Wisconsin-Madison, Ophthalmology and Visual Sciences, Madison, United States, 2University of Wisconsin-Madison, Ophthalmology and Visual Sciences, Madison, United States, 3University of Wisconsin-Madison, Medical Sciences, Madison, United States, 4University of Wisconsin-Madison, Pathobiological Sciences, Madison, United States

Visual evoked potentials (VEPs) provide an assessment of optic nerve (ON) function in patients with ON disease, including multiple sclerosis (MS) and glaucoma. Prolonged VEP latency and reduced VEP amplitude reflect slowed conduction and loss of axons, respectively. Myelin repair in MS and preservation of axons in the ON in both MS and glaucoma are major treatment goals in patients with these common diseases. While VEP abnormalities are considered to reflect demyelination and axon loss in the ON, direct evidence that remyelination leads to improvement of VEP parameters, or that VEP correlates with ON axon numbers is lacking. We addressed this lack of function-histopathology correlations in two feline optic neuropathy models: 1) feline iridectomy diet-induced demyelination (FIDID), in which there is extensive demyelination of the entire CNS, with neurologic recovery in association with global remyelination on discontinuation of the iridectomized diet, and 2) feline congenital glaucoma (FCG) due to LTBP2 mutation, in which IOP elevation is associated with variable loss of ON axons. Flash VEPs were recorded prior to the onset of clinical signs of neurological disease in 10 FIDID cats and at variable time points thereafter, and in 13 cats with FCG. Electroretinography and optical coherence tomography were also used to evaluate retinal function and structure. Following fixation ONs were processed for light and electron microscopy. In FIDID, there was a clear association between demyelination and prolonged VEP latency in all cats during acute disease, and partial normalization of latency in nerves which showed extensive remyelination. Cats with FCG exhibited variable axon loss in PPD- and Richardson-stained sections, with strong correlation between axon count and VEP P2 amplitude (Pearson r=−0.62; P=0.02) and root mean square of early VEP wavelets (r=−0.74; P<0.004). These data validate and support VEPs as a surrogate marker for axon loss, demyelination and remyelination of ON in glaucoma and MS models.

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The Role of Schlemm’s Canal Endothelial Cell Connections in Giant Vascular and Pore Formation: A 3D Electron Microscopy Study

Gong, H., Lal, J., Yu, S., Getchevski, D., Huang, D.
Boston University School of Medicine, Boston, United States

**Purpose:** We investigated whether connections between Schlemm’s canal (SC) inner wall (IW) endothelial cells and juxtamural connective tissue (JCT) cells/extracellular matrix (ECM) plays a role in giant vascular (GV) and pore formation by comparing IW endothelial cells from two groups: perfusion- and immersion-fixed eyes.

**Methods:** Normal human eyes (N=5) were either immersion- or perfusion-fixed. The IW of SC and JCT were imaged using serial block-face scanning electron microscopy. A total of 24 IW cells were 3D-reconstructed from ~5,600 serial images. In each cell, cell-cell and cell-matrix connections, GVs and intracellular pores (i-pores) were 3D-reconstructed and analyzed. The amount of overlap between adjacent IW cells was measured to calculate mean Overlay Length (OL) in each cell; border pores (B-pores) were 3D-reconstructed and analyzed. All data were compared between the two groups.

**Results:** The connectivity between the IW cells and the JCT cells/ECM was found surprisingly substantial in both immersion- and perfusion-fixed eyes. Total number of connections per cell significantly decreased (30±6 vs. 82±6; P=0.01) and GV volume significantly increased (495.36±134.21 µm3 vs. 43.06±13.55 µm3; P<0.01) in perfusion-fixed eyes compared to immersion-fixed eyes. A negative correlation was observed between the number of cell-cell connections and the summed GV volume per cell in all eyes. A total of 6-i pores and 2-B pores were found in cells of perfusion-fixed eyes, whereas no GVs were observed in immersion-fixed eyes. The OL significantly decreased in cells from the perfusion-fixed eyes (0.73±0.13 µm vs. 2.83±0.25 µm; P<0.01) compared to immersion-fixed eyes. All B-pores were found only in regions where overlap was minimal (OL<0.1 µm).

**Conclusions:** We developed an effective method for the quantification and analyses of cellular connections, GVs, and pores. Our data suggest that cellular connections may play a role in GV and pore formation. Altering cellular connectivity may therefore be used as a strategy to develop new therapeutics to decrease outflow resistance and lower IOP.

This study was supported by NIH/NEI EY022634, EY019696, BrightFocus Foundation 20160999, and The Massachusetts Lions Eye Research Fund.
**Glaucoma**

**TGFβ2 Regulates the Expression of ECM and Associated Proteins by Modulating miRNA Expression in Human ONH Cells**

Lopez, N.1, Clark, A.2, Tovar-Vidales, T.1

1University of North Texas Health Science Center, Pharmacology and Neuroscience, Fort Worth, United States, 2University of North Texas Health Science Center, Fort Worth, United States

**Purpose:** Glaucoma is a neurodegenerative disease of the optic nerve that affects more than 60 million people worldwide. Elevated intraocular pressure is a major risk factor for glaucoma and leads to pathological fibrotic changes at the optic nerve head (ONH). Pro-fibrotic cytokine TGFβ2 is elevated in the ONH of glaucoma eyes and has been shown to induce the synthesis of extracellular matrix (ECM) including fibronectin (FN) and collagens. TGFβ2 has been shown to modulate the expression of microRNAs (miRNAs) in fibrotic diseases including glaucoma. The purpose of this study was to determine whether TGFβ2 modifies the expression of miRNAs in ONH cells and if transfection of candidate miRNA mimics and inhibitors alters TGFβ2 induced expression of ECM proteins.

**Methods:** Primary human glial fibrillary acidic protein (GFAP) positive ONH astrocytes (ONHA) and GFAP negative lamina cribrosa (LC) cells were grown to 100% confluency. LC cells and ONA were treated with SnR/mi TGFβ2 or with control to determine differentially expressed miRNAs. Human ONA and LC cells were transfected with candidate miRNA mimics (10nM) or non-targeting siRNA to determine the expression of FN and collagen type IV. ONH tissue sections were analysed for FN and collagen IV expression in glaucomatous and aged matched normal donors.

**Results:** miRNA PCR arrays showed that TGFβ2 treatment downregulated the expression several miRNAs in ONHA and LC cells. Transfection with mir-200b-3p and mir-211-5p mimics and inhibitors show that FN and collagen type IV (COL IV) are targets and regulators show that FN and collagen type IV (COL IV) are targets and regulate TGFβ2 induced expression of FN and COL IV in ONHA. This correlates to an increased expression of FN and COL IV in the glaucomatous lamina cribrosa compared to aged matched normal donors.

**Conclusion:** TGFβ2 is capable of modulating the expression of several miRNAs in cultured human ONHA. Transfection of candidate miRNAs downregulated ECM proteins including FN and COL IV in ONHA, while inhibitors reversed this effect. Thus, TGFβ2 modulation of miRNAs could lead to an increase in FN and COL IV expression and remodeling of the ONH in glaucoma.

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**An Unstable Relationship: Dimerization of Human γS-crystallin Leads to Non-cooperative Unfolding and the Formation of an Aggregation-prone Intermediate Associated with Cataract**

Thorn, D., Grosas, A., Mabbitt, P., Ray, N., Jackson, C., Carver, J.

The Australian National University, Research School of Chemistry, Canberra, Australia

The loss of lens transparency underlying age-related cataract is generally attributed to cumulative post-translational modifications to the lifelong crystallin proteins. These modifications lead to a loss of protein stability and a subsequent propensity to unfold, aggregate and precipitate. While the γ-crystallins are conventionally considered to be monomeric proteins, human γS-crystallin (γSc) readily forms dimers via oxidation of a cysteine conserved in mammalian γS proteins at position 24, thereby covalently linking the N-terminal domains. In the present work, we verify these findings using cysteine knockouts, and characterize the γSc dimer in terms of its stability and aggregation propensity under conditions of thermal stress. Thermal unfolding experiments performed using a variety of biophysical techniques, including tryptophan fluorescence and circular dichroism spectroscopy, indicate that the γS dimer unfolds non-cooperatively; it populates a partially folded intermediate during unfolding, whereas the monomer unfolds via an apparent two-state transition. Using a pair of γSc mutants devoid of tryptophan residues in either their N- or C-terminal domains, we show that dimerization of γSc dimer leads to a reduction in the stability of its N-terminal domain, thereby decoupling the unfolding of the two domains. Moreover, light scattering experiments show that the γSc dimer precipitates at significantly lower temperatures than its monomeric counterpart. This suggests the folding intermediate formed by the dimer is more prone to aggregation than the native, unmodified state of the protein. Based on these findings, we propose a novel pathway through which age-related modifications of lens proteins contribute to the development of cataract.

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**Effect of Intracameral Dexamethasone Injection at Conclusion of Cataract Surgery on Macular Thicknes**

Hussien, A.1, Bessa, A.1, Ibrahim, T.1

1Alexandria University, Ophthalmology, Alexandria, Egypt, 2Southend University Hospital, Southend-on-Sea, United Kingdom

**Context:** To measure the central macular thickness (CMT) in diabetic patients after instilling intracameral dexamethasone at the end of cataract surgery and compare this measure with the control group.

**Design:** Prospective case-control study.

**Participants:** 100 eyes of 100 diabetic patients undergoing cataract extraction.

**Methods:** 50 eyes received intracameral dexamethasone 0.4mg/0.1ml at the end of surgery and 50 eyes received sham treatment as a control group. The CMT was measured before, one month, and three months after the surgery.

**Results:** The mean CMT in was 261.32 ± 9.45µm in the dexamethasone injected group while in the control group was 275.76± 21.36µm (p < 0.05) at the end of the first month postoperatively. At the end of the third postoperative month the mean CMT was 262.34 ± 10.77µm and 264.82 ± 9.73µm in the dexamethasone injected group and the control group, respectively, (p > 0.05). The mean Intraocular Pressure (IOP) in the dexamethasone injected eyes was 14.98 ± 2.82 and 15.1 ± 2.82mmHg before and at the end of the first month following the surgery, respectively, (p > 0.05). The mean CMT in was 261.32 ± 9.45µm in the dexamethasone injected eye. The mean CMT in was 261.32 ± 9.45µm in the dexamethasone injected group while in the control group was 275.76± 21.36µm (p < 0.05) at the end of the first month postoperatively. At the end of the third postoperative month the mean CMT was 262.34 ± 10.77µm and 264.82 ± 9.73µm in the dexamethasone injected group and the control group, respectively, (p > 0.05). The mean Intraocular Pressure (IOP) in the dexamethasone injected eyes was 14.98 ± 2.82 and 15.1 ± 2.82mmHg before and at the end of the first month following the surgery, respectively, (p > 0.05).

**Conclusion:** The CMT of eyes which received intracameral dexamethasone is significantly lower than the control group at the end of the first postoperative month, suggesting a possible role of intracameral dexamethasone in suppressing the early inflammatory response that can be linked to post cataract surgery macular edema in diabetics. A non-significant increase in the IOP has been observed in the dexamethasone injected group this makes intracameral dexamethasone injection a possible safe practice at the end of cataract extraction in diabetics.
An Approach to Understanding Lens Epithelium Homeostasis - The Effect of Low Dose Ionizing Radiation on Proliferation, Cell Density and Cellular Organisation

Uwineza, A.1,2, Kalligeraki, A.1, Barnard, S.1,2, Obara, B.3, Jarrin, M.1, Ainsbury, E.1, Quinlan, R.1, LDensRad Consortium

1 Durham University, School of Biological and Biomedical Sciences, Durham, United Kingdom, 2 Public Health England, Centre for Radiation, Chemical and Environmental Hazards, Oxford, United Kingdom, 3 Durham University, Department of Computer Science, Durham, United Kingdom

Due to reanalysis of old datasets together with new epidemiological studies, the International Commission on Radiological Protection (ICRP) revised its recommendation of cataract development threshold of deterministic effects from 2 Gy for acute exposure and 5 Gy for protracted doses to a nominal threshold of 0.5 Gy independent of the dose rate. Simultaneously, the ICRP recognised that the details of the underlying biological mechanism explaining how ionising radiation (IR) causes cataract are not completely understood. The lens comprises of a single layer of lens epithelial cells (LECs) covering the anterior half of the lens. These cells migrate from the central zone on the anterior to the germinative zone where their proliferation peaks and they start differentiating in the transition zone. These differentiating LECs encroach onto the germinative zone where their proliferation peaks and they start differentiating in the transition zone. These differentiating LECs have been developed to dispense them during surgery, and IOLs have been used as platforms for their assembly. While research on LECs has shown promising results, there are still some issues that need to be addressed. One of them is the achievement of a sustained release over extended periods of time or only in pathological conditions. The present project aims at solving this problem by creating a hydrogel-based drug delivery system formulated to release a therapeutic agent known to reduce PCO only when this syndrome starts to develop. This delivery system uses an IOL as a substrate, thus making it minimally invasive for the patient. The therapeutic agent that has been used in this study is an anti-VEGF molecule that has shown to reduce PCO in recently published studies. The efficiency of this system has been evaluated through in vitro studies of release in a 2D cell culture system, and its effects on the cellular responses have been assessed.

Comparison of Contact Lens Hygiene Compliance and Self-management Behaviours between Medical and Non-medical Students in Saudi Arabia

Alqahtani, S., Fayez, F., Alqahtani, H., Alrowibah, H., Al-dulhumah, A., Nisan, S.

Princess Nourah Bint Abdullahman University, Medicine College, Riyadh, Saudi Arabia

Objectives: To compare contact lenses hygiene compliance and the self-management behavior. With a focus on wearing habits, cleaning and maintaining contact lenses by different methods. As well as, habits of sharing and self-prescription between medical and non-medical students in Saudi Arabia.

Method: Five hundred young contact lenses wearers with an average age of 18-22 years were conveniently selected from the student population at Princess Nourah Bint Abdullahman University, Riyadh. After taking verbal consent from the participants, their level of contact lenses hygiene compliance and self-management was assessed through a questionnaire. All data were analyzed using SPSS software.

Results: The mean ±SD age of the participants was 20.87±1.696 years. Out of 500 students 37.8% of them were medical students and 62.2% were non-medical students. 56.4% of students were wearing contact lenses for the cosmetic reasons while 43.6% of students were using them for the correction of their myopic refractive error. Most of the students were using daily wear soft contact lenses (96.6%) by self-prescription (51.4%) and majority of them were buying them from general retail store (83.6%) instead of some proper optician. The self-management behavior was statistically significant among non-medical students (p=0.026).

Conclusions: This study concludes that self-management with the contact lenses use is very common among non-medical students in Saudi Arabia. Although they are good at hygiene compliance, their knowledge about the risks and the complications of contact lenses use as well as their knowledge about lens care accessories were significantly low.

Cataracts are the primary cause of blindness worldwide. Currently, the most effective treatment available is surgery with implantation of an intraocular lens (IOL). Even though this procedure has proven effective in restoring vision, cataract shows a very high recurrence rate. This is due to the wound-healing response triggered by the lens epithelial cells (LECs) that remain in the portion of the natural lens that is left after surgery. As a result, these cells undergo a transdifferentiation process, they encroach onto the posterior side of the implanted IOL and deposit aberrant extracellular matrix components. Subsequently, a secondary cataract forms, constituting a syndrome that is also known as posterior capsule opacification, or PCO. Due to the difficulties encountered when trying to treat PCO, prevention of this disease is preferable. This can be achieved by formulating IOLs with biomaterials that present properties that can modulate cell adhesion and by including elements of design that control cell migration. Another approach is the administration of chemical agents that block the signalling pathways that lead to the development of PCO. Molecules that cause cell death or that prevent cell adhesion, migration or proliferation have also been tested. Drug delivery systems have been developed to dispense them during surgery, and IOLs have been used as platforms for their assembly. While research on this area has shown promising results, there are still some issues that need to be addressed. One of them is the achievement of a sustained release over extended periods of time or only in pathological conditions. The present project aims at solving this problem by creating a hydrogel-based drug delivery system formulated to release a therapeutic agent known to reduce PCO only when this syndrome starts to develop. This delivery system uses an IOL as a substrate, thus making it minimally invasive for the patient. The therapeutic agent that has been used in this study is an anti-VEGF molecule that has shown to reduce PCO in recently published studies. The efficiency of this system has been evaluated through in vitro studies of release in a 2D cell culture system, and its effects on the cellular responses have been assessed.

Development of Intraocular Delivery System for Controlled Release of Therapeutic Agents Used in the Treatment of PCO

Hidalgo-Alvarez, V., Wormstone, M., Saeed, A.

University of East Anglia, Norwich, United Kingdom

Cataracts are the primary cause of blindness worldwide. Currently, the most effective treatment available is surgery with implantation of an intraocular lens (IOL). Even though this procedure has proven effective in restoring vision, cataract shows a very high recurrence rate. This is due to the wound-healing response triggered by the lens epithelial cells (LECs) that remain in the portion of the natural lens that is left after surgery. As a result, these cells undergo a transdifferentiation process, they encroach onto the posterior side of the implanted IOL and deposit aberrant extracellular matrix components. Subsequently, a secondary cataract forms, constituting a syndrome that is also known as posterior capsule opacification, or PCO. Due to the difficulties encountered when trying to treat PCO, prevention of this disease is preferable. This can be achieved by formulating IOLs with biomaterials that present properties that can modulate cell adhesion and by including elements of design that control cell migration. Another approach is the administration of chemical agents that block the signalling pathways that lead to the development of PCO. Molecules that cause cell death or that prevent cell adhesion, migration or proliferation have also been tested. Drug delivery systems have been developed to dispense them during surgery, and IOLs have been used as platforms for their assembly. While research on this area has shown promising results, there are still some issues that need to be addressed. One of them is the achievement of a sustained release over extended periods of time or only in pathological conditions. The present project aims at solving this problem by creating a hydrogel-based drug delivery system formulated to release a therapeutic agent known to reduce PCO only when this syndrome starts to develop. This delivery system uses an IOL as a substrate, thus making it minimally invasive for the patient. The therapeutic agent that has been used in this study is an anti-VEGF molecule that has shown to reduce PCO in recently published studies. The efficiency of this system has been evaluated through in vitro studies of release in a 2D cell culture system, and its effects on the cellular responses have been assessed.

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Most of the students were using daily wear soft contact lenses (96.6%) by self-prescription (51.4%) and majority of them were buying them from general retail store (83.6%) instead of some proper optician. The self-management behavior was statistically significant among non-medical students (p=0.026).

There was no significant difference between the two groups regarding the compliance of the contact lens hygiene but, the knowledge and the awareness about the risks and complications was statistically higher in the medical students (p=0.028).

Most of the students in our study had rated themselves as average wearers.

Conclusion: This study concludes that self-management with the contact lenses use is very common among non-medical students in Saudi Arabia. Although they are good at hygiene compliance, their knowledge about the risks and the complications of contact lenses use as well as their knowledge about lens care accessories were significantly low.
Quantifying the Ellipsoid Zone Loss in Childhood-Onset Stargardt Disease

Georgiou, M.1, Tanna, P.1,2, Kallizotos, A.1,3, Michaelides, M.1,2

1UC Institute of Ophthalmology, London, United Kingdom, 2 Moorfields Eye Hospital NHS Foundation Trust, London, United Kingdom

Forty-five molecularly confirmed subjects were imaged under a standard protocol bilaterally, over the foveal centre. The area of EZ loss was calculated with en-face analysis, twice by a single observer. The borders of the EZ were marked on the NIR-R fundus image for each consecutive B-scan and the points were used to calculate the area of EZ loss using the Region Finder Tool (Heidelberg Eye Explorer software). In addition to the area, the width of the lesion was measured on a horizontal transfoveal scan line twice by a single observer, using the caliper tool. Any set of measurements with a percentage difference greater than 5% were rejected and not further analysed.

The mean (range, SD) age was 12.5 (8.0-17.9, ±3.3) years. Seven subjects had lesions beyond the area imaged with SD-OCT at baseline, so 14 eyes (14/90; 15.6%) were excluded from further analysis; their age was not statistically different from the rest of the cohort (mean, range, SD): 10.56; 8.3-12.9, ±1.7 years.

For 23 eyes (23/90; 25.6%) from 15 patients, the percentage difference between the 2 area measurements at baseline was greater than 5% and hence were excluded from further analysis; there was no difference in age or lesion size between these subjects and the remaining cohort. Fifty-three eyes (53/90; 58.9%) were available for cross-sectional analysis. The mean values for EZ area and EZ width were 6.44 (±2.93; 4.13) mm² and 2967 (±771; ±1657; ±1456) μm, respectively.

From these 53 eyes, follow-up data were available for 42 eyes. From the latter the lesion extended beyond the OCT width in 2 eyes, and in another five the percentage difference between measurements was greater than 5%, and so were excluded from longitudinal analysis. Thirty-six eyes from 20 subjects had a mean follow up of 28.3 (10.5-62, ±18) months and a mean rate of EZ area loss of 0.52 mm²/year (0-1.56, ±0.84). The mean rate of EZ width follow up of 28.7 (3-52, ±13.8) months and a mean rate of EZ area 105.74 ±115.8 μm/year. The mean annual percentage change was greater for EZ area loss (8.07%) compared to EZ width (3.36%).

En face SD-OCT analysis can be used to evaluate the rate of progression in STGD. The size of the lesion and the coalescence of flecks can be a challenge in accurately measuring EZ area. The rate of progression was highly variable among subjects. Our rate of progression for a childhood-onset STGD cohort is greater than a recently reported study using similar methodology for an older cohort.

Reversible Cone Photoreceptor Dysfunction in Mice Lacking the Antioxidant Enzyme Methionine Sulfide Reductase A.

Mazzoni, F., Dun, Y., Vargas, J., Finne mann, S.

Fordham University, Department of Biological Sciences, Center for Cancer, Genetic Diseases and Gene Regulation, Bronx, New York, United States

RPE cells express high levels of the enzyme Methionine Sulfide Reductase A (MsrA) which counteracts oxidative protein damage by catalyzing reduction of methionine sulfoxide to methionine. Our earlier studies showed that MsrA in cultured RPE cells plays a role in mitochondrial function in energy metabolism and in protection from oxidative stress. Oxidative stress is associated with aging and many retinal diseases including age-related macular degeneration (AMD). Here, we study retinal phenotype and function of mice lacking MsrA (MsrA+/−) fed either standard diet or diet supplemented with grapes, which contain a complex mix of natural antioxidants (grape diet).

Testing retinal light responses in electroretinograms (ERGs) of six-week and six-month-old MsrA−/− mice, we found severely reduced photopic-a-waves but normal scotopic-a-waves indicative of dysfunction specifically of cone photoreceptors at both ages. MsrA−/− mice raised in constant darkness or feeding grape diet until 6 weeks of age showed normal cone photoreceptor responses. Remarkably, feeding grape diet for one month to adult MsrA−/− mice that had been raised under normal lighting conditions and fed standard diet was sufficient to restore cone function to levels found in age-matched wild-type mice. Morphological analysis of retina-RPE tissue confirmed and extended these functional data showing subtle cone abnormalities that were rescued in MsrA+/− mice fed with grape diet. Furthermore, RPE/choroid but not neural retina from MsrA−/− mice fed grape diet showed a moderate increase in mitochondrial-cytochrome C and a robust decrease in the ER stress marker C/EBP homologous protein (CHOP) at the protein level. In contrast, we detected little change in oxidative stress response and autophagy markers. Our results show that MsrA−/− mice provide a small animal model of reversible cone-specific photoreceptor dysfunction. This model may have utility in analysis of cone-specific disease mechanisms and rescue studies that could be relevant to AMD.

Citrulline Protects Human Retinal Pigment Epithelium Cells against Oxidative Stress

Jacquemot, N.1, Hassel, C.1, Blavignac, C.2, Loi, C.1, Moinard, C.1, Oedema: Impact on Clinical Outcomes

Moorfields Eye Hospital NHS Foundation Trust, London, United Kingdom

Objective: To evaluate the pragmatism and generalisability of randomised clinical trials (RCTs) on ranibizumab for the treatment of diabetic macular oedema (DMO) and to determine whether clinical outcomes of patients receiving this treatment differ based on whether or not they fulfil the eligibility criteria set by these RCTs.

Methods: Application of a trial assessment tool (PRECIS) and prospective evaluation of a retrospective cohort of consecutive patients.

Subjects: Patients with DMO undergoing treatment with ranibizu

Results: Of 1588 eyes identified through an electronic database, was evaluated by two graders.

Conclusions: CIT is able to protect human RPE cells from oxidative stress. This suggests potential protective effect of CIT against retinal diseases associated with oxidative stress including AMD.
Conclusions: RGCs evaluating ranibizumab for DME were found to be more pragmatic than exploratory using the PRECIS-2 tool. No statistically significant differences in outcomes were observed between "eligible" and "ineligible" patients; ineligible patients still benefit from ranibizumab therapy.

Therapeutic Effect of Mesenchymal Stromal Cells in the Cornea in a GVHD Murine Model

Velasco, A.1, Sanchez-Guijo, F.2, Hernández-Gaíllo, E.1, Alijón, J.2, Martínez-Carrasco, R.*

1University of Salamanca. IBASL, INCyL, Biología Celular, Salamanca, Spain, 2Hospital Clínico de Salamanca. IBASL, Hematology, Salamanca, Spain, 3University of Salamanca, Hospital Clínico de Salamanca, IBASL, Ophthalmology, Salamanca, Spain, 4Universidade de Salamanca, Hospital Clínico de Salamanca, IBASL, Biología Celular, Salamanca, Spain

Hematopoietic stem cell transplantation is used with increasing frequency for hematological disorders. However, as the mortality of these patients is reduced, their quality of life is negatively impacted by the development of GVHD. Among the consequences of GVHD, ocular involvement is one of the main causes of this loss of quality of life. Hence, developing new strategies for the treatment of this condition is required. Alloreactive T lymphocytes are the main effectors of GVHD. In GVHD, T lymphocytes invade the cornea, causing severe damage. In this context, the treatment with MSCs is promising, given their capacity to modulate immune responses.

To assess this, GVHD was induced in mice after hematopoietic stem cell transplantation (HSCT) between MHC-mismatched donors and recipients. After 10 days post-HSCT, intravital fluorescence studies were performed to determine the infiltration of CD3+ cells in the cornea and the appearance of epithelial alterations. Quantitative polymerase chain reaction was used to evaluate changes in cytokines, Pax6 expression, and SPRR1B expression. To evaluate the effect of total body irradiation on the cornea, causing severe damage. In this context, the treatment with MSCs is promising, given their capacity to modulate immune responses.

Intravenous Treatment of Choroidal Neovascularization by Photo-targeted Nanoparticles

Yanfei Wang1, Chi-Hsiu Liu2, Tianjiao Ji1, Manisha Mehta1, Weiping Wang1, Elizabeth Marino1, Jiu Chen2, and Daniel S. Kohane,*

1Laboratory for Biomaterials and Drug Delivery, Department of Anesthesiology, Division of Critical Care Medicine, Boston Children’s Hospital, Harvard Medical School, 300 Longwood Avenue, Boston, Massachusetts 02115, United States 2Department of Ophthalmology, Boston Children’s Hospital, Harvard Medical School, Boston, MA 02215

Choroidal neovascularization (CNV) is the major cause of vision loss in wet age-related macular degeneration (AMD). Current therapeutic options include photodynamic therapy and anti-vascular endothelial growth factor (VEGF) antibodies. In the current study, we compared the upstream promoter sequences of the human Nefh gene to the Nefh gene of murine origins, to identify novel promoters that may be more suitable for gene therapy applications. We have previously reported preferential expression in murine RGCs from a 2.2 kb murine Nefh promoter when delivered intravitreally (Hanlon et al., 2017). In the current study we compared the upstream promoter sequences of the human Nefh gene to the Nefh gene of murine origins, to identify novel promoters that may be more suitable for gene therapy applications. We have previously reported preferential expression in murine RGCs from a 2.2 kb murine Nefh promoter when delivered intravitreally (Hanlon et al., 2017). In the current study we compared the upstream promoter sequences of the human Nefh gene to the Nefh gene of murine origins, to identify novel promoters that may be more suitable for gene therapy applications. We have previously reported preferential expression in murine RGCs from a 2.2 kb murine Nefh promoter when delivered intravitreally (Hanlon et al., 2017). In the current study we compared the upstream promoter sequences of the human Nefh gene to the Nefh gene of murine origins, to identify novel promoters that may be more suitable for gene therapy applications. We have previously reported preferential expression in murine RGCs from a 2.2 kb murine Nefh promoter when delivered intravitreally (Hanlon et al., 2017). In the current study we compared the upstream promoter sequences of the human Nefh gene to the Nefh gene of murine origins, to identify novel promoters that may be more suitable for gene therapy applications. We have previously reported preferential expression in murine RGCs from a 2.2 kb murine Nefh promoter when delivered intravitreally (Hanlon et al., 2017).
Delayed Treatment with AAV2.COMP-Ang1, a Potent, Bioengineered Angiopoietin-1 Replacement Therapy, Prevents Proliferative Vascular Changes Associated with Progression of Diabetic Retinopathy

Carroll, L., Uehara, H., Choi, S., Ambati, B.

University of Utah School of Medicine, John A. Moran Eye Institute, Salt Lake City, United States

Progression of diabetic retinopathy (DR) is often well underway prior to its diagnosis, with peripapillary capillary dropout, altered blood flow, and neuroglial dysfunction long preceding clinical detection. Fewer than half of all diabetic macular edema (DME) patients receiving anti-VEGF therapies such as ranibizumab and VEGF Trap show clinically meaningful improvements in visual improvement, yet anti-VEGF therapies are usually preferable to patients receiving anti-VEGF therapies such as ranibizumab and VEGF Trap. The effect on Müller cells should be further analyzed.

Introduction: The pivotal trials for the fluocinolone acetonide (FAc) implant were the Fluocinolone Acetonide for Diabetic Macular Edema (FAME) trials. However, these trials pre-dated the use of more recently licensed anti-VEGF and steroid therapies in diabetic macular oedema (DMO) which are now commonly used before a switch to FAc. We aim to assess outcomes in DMO patients treated with the FAc implant at the Manchester Royal Eye Hospital since 2014.

Methods: This was a retrospective consecutive case series of 21 eyes from 17 patients (1 phakic, 20 pseudophakic eyes) with chronic DMO. The mean duration of DMO at baseline was 44 months (range 13–72). 19 of 21 eyes had previously been treated with up to 4 different types of anti-VEGF and steroid intravitreal injections (IVI). Previously treated eyes received a mean of 13.2 IVI; prior injection numbers ranged from 1–36. The mean follow-up period was 27 months, range 6–36. Central foveal thickness (CFT) was monitored along with visual acuity (VA) and intraocular pressure (IOP). An additional analysis was conducted looking only at those patients with full 3-year follow-up.

Results: At the last observation point, CFT decreased by 151.5±13.81 µm (mean±SD) from a baseline of 410.3±138.1 µm, and 76.2% of eyes had a CFT of <300 µm. VA also improved by 3.8±1.18 letters from a baseline of 53.4±19.8 letters, with 84% of eyes maintaining or gaining VA from baseline.

8 of 21 eyes required new or altered IOP lowering therapies with 2 requiring subsequent surgery and 2 requiring laser treatment. 4 eyes had an increase to ≥30mmHg during follow up. A total of 5 eyes received a mean of 12.2 additional anti-VEGF injections in combination with FAc. 2 eyes required post-implant laser.

Discussion: Our real-world experience demonstrates that treatment with the FAc implant led to drying of the macula and VA stabilization/gain in most patients, despite significant prior treatment. Overall, the 3-year completer group (EYV) showed greater mean VA gains despite both groups achieving mean CMT of around 250µm. This might be related to a shorter duration of DMO (12.8 months) and fewer prior therapies overall in the 3Yr group.

Conclusion: Our real-world experience demonstrates that treatment with the FAc implant led to drying of the macula and VA stabilization/gain in most patients, despite significant prior treatment. Overall, the 3-year completer group (EYV) showed greater mean VA gains despite both groups achieving mean CMT of around 250µm. This might be related to a shorter duration of DMO (12.8 months) and fewer prior therapies overall in the 3Yr group.


Efficacy of Allergic Immunotherapy on Vernal Keratoconjunctivitis

Lu, K.-L., Vance, G., Gardener, J.
Royal Victoria Infirmary, Newcastle upon Tyne, United Kingdom

Introduction: Vernal keratoconjunctivitis (VKC) is a rare allergic eye disease characterised by epiphora, photophobia and cobble-stone giant papillae on the tarsal conjunctiva. Traditionally, management of VKC is symptomatic treatment with topical mast cell stabilisers and corticosteroids. In contrast, immunotherapy tackles the pathophysiology of the disease and has been shown to improve long-term outcomes of other allergic conditions. However, no studies to date have investigated the role of immunotherapy in managing VKC specifically. In this study, we aim to confirm the efficacy of allergen immunotherapy in treating VKC.

Methods: Patients with a confirmed diagnosis of VKC by senior ophthalmologists who were treated with allergen immunotherapy in the 2013-16 period are identified using electronic database held by specialist paediatric allergy MDT. This is analysed by patient demographics, patient-reported symptoms score and medications score.

Results: 9 patients are identified for the study. This includes 8 boys and 1 girl, with a mean age of 10 (7-13). 7 were treated with monotherapy: SCIT (n=3), DRAUVAC (n=2) and GRAZAX (n=2), while 2 were treated with dual therapy by DRAUVAC and GRAZAX. Among them, 1 patient experienced no change in the combined symptoms-medications score while the rest have a reduction in score ranged 10-100%. The mean reduction in combined symptoms-medications score of all 9 patients at one-year post-treatment was 46% (p=0.006).

Discussion: By having both nasal-ocular symptom score and medication score as our primary endpoints, we have demonstrated the use of immunotherapy is effective in treating VKC patients. Unfortunately, the challenge of immunotherapy remains that it is allergen-specific therefore a particular allergen has to be identified prior to treatment. Moreover, immunotherapy has been reported to carry a higher risk of adverse events compared to conventional anti-allergic medications. Further studies need to stratify its risks and safeness in our paediatric cohort.

Homozygous Mutations in a Novel Deubiquitylase Gene Are Associated with Leber Congenital Amaurosis

State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-Sen University, Guangzhou, China

Leber congenital amaurosis (LCA) is the earliest and most severe form of inherited retinal dystrophies. LCA is characterized by severe vision loss in the first year of life accompanied by severely reduced or extinguished electroretinography. In approximately 60% of probands, genetic defects can be detected in the 25 known LCA-causing genes, including RPE65 for which gene therapy is available in the clinic. Identification of additional genes responsible for the remaining portion of LCA probands would be valuable for their prevention and treatment. Through whole-exome sequencing of 3280 probands with various forms of hereditary eye diseases, rare biallelic mutations in a novel deubiquitylase gene X were identified in two singleton families with LCA. Sanger sequencing confirmed that the two novel mutations were homozygous in the two probands but heterozygous in their parents in both families. These two mutations, c.1636A>T (p.Lys546*), and c.935G>A (p.Arg312Gln), were predicted to be damaging and were absent in the remaining 3278 probands. Immunohistochemistry analysis of human retina sections with anti-X antibodies revealed enriched expression in the inner segments of rod and cone photoreceptors, especially in cones, suggesting an important role of X in the maintenance of photoreceptor cells. Knockdown of X in zebrafish resulted in small eye size and decreased expression of green cone opsin. These effects could be rescued by coinjection with wild-type X mRNA. Identification of X mutations as a cause of LCA may further enhance our understanding of the molecular network involved in the pathogenesis of LCA.

The Role of Genomic Analyses in Pathogen Identification in Keratitis

Glenn, M., Kaye, S., Neal, T., Fairley, D., Keating, G., Simpson, D.

1 Centre for Experimental Medicine, Queens University, Belfast, United Kingdom, 2 Royal Liverpool and Broadgreen University Hospitals NHS Trust, Liverpool, United Kingdom, 3 Belfast Health and Social Care Trust, Belfast, United Kingdom, 4 HiberGene Diagnostics, Dublin, Ireland

Background: Microbial Keratitis is a serious infection of the cornea and a major cause of corneal opacity and vision loss worldwide. The majority of keratitis infections in developed regions are caused by bacterial pathogens, with other microorganisms including fungi and acanthamoeba also having the potential to cause infection. Genomic analysis of bacterial isolates has the potential to provide a wealth of clinically valuable information. This includes the ability to identify the specific strain of the infecting bacteria along with the detection of antibiotic resistance determinants and virulence factors. Metagenomic analysis of a sample from infected corneas offers the potential to identify causative agents which remain undetected using traditional culture-based diagnosis. Current techniques for pathogen identification are laborious, time-consuming and associated with poor successful culture rates.

Methods/results: Samples from keratitis patients were collected using corneal impression membranes (CIMs) and subsequently cultured on agar media to isolate causative agents. To date, 100 CIMs have been obtained, 46 of which were culture positive for a variety of species including Pseudomonas aeruginosa, Staphylococcus aureus, Coagulase negative staphylococcus, Staphylococcus epidermidis and Streptococcus pneumoniae. DNA has been extracted from pure cultures of these isolates and a miniaturised Nextera XT library preparation has been optimised and sequencing (Illumina) is ongoing. The feasibility of metagenomics analysis directly from CIMs has been assessed under control conditions using RT-PCR to quantify available DNA.

Discussion/conclusion: The clinical role of next-generation sequencing is currently limited due to associated costs and required computational resources, however in future this method could replace current techniques for pathogen identification. Whole genome sequencing can be used to characterise the complete genomes of common corneal infectious agents and identify key sequences which will provide insight into their pathogenicity and antimicrobial resistance. Metagenomics provides a research tool to better understand the aetiology of corneal infections and shows future potential to be the centre of culture-independent pathogen identification.
Prevalence of Age-related Macular Degeneration Associated Genetic Risk Factors and 4-year Progression Data in the Irish Population

Connolly, E.1, Rhatigan, M.1, O’Halloran, A.M.1, Muldrew, K.A.2, Chakravarty, U.1, Cahill, M.1, Kenny, R.A.1, Doyle, S.L.1

1Trinity College Dublin, Clinical Medicine, Dublin, Ireland. 2Trinity College Dublin, Medical Gerontology, The Irish Longitudinal Study on Ageing (TILDA), Dublin, Ireland. *Queens University Belfast, Centre for Public Health, Belfast, United Kingdom. "Royal Victoria Eye and Ear Hospital, Dublin, Ireland

As the leading cause of blindness in the over 50’s in developed countries, the global burden of Age-related Macular Degeneration (AMD) is expected to rise with the growing aging population. Due to the multifactorial nature of AMD the exact etiology of the disease remains unclear, however, several lifestyle and genetic factors have been identified as significantly associated with disease incidence and progression. Many of these genetic factors have been found in genes of the alternative complement pathway and a single nucleotide polymorphism (SNP) in the regulatory protein Complement Factor H (CFH) has emerged as a strong genetic risk variant associated with AMD. To investigate the prevalence of these risk variants in the Irish population we collaborated with The Irish Longitudinal study on Aging (TILDA) to sequence 6 SNPs in CFH, ARMS2, C3 and CFB of the complement pathway along with TIRAP and SKIV2L. Using multinomial regression analysis adjusting for lifestyle and biological factors, we calculated the Odds Ratio (OR) for each genotype as an indicator for Early or Late AMD and we found that CFH and ARMS2 were significantly associated with the incidence of AMD in the Irish population. Homozygosity for both these SNPs were found in the mutant eyes. In addition, we also demonstrated that NICD-induced RPE transformation was mediated by Hippo/Yap1 signaling pathway. Knockout of NICD of the complement pathway along with TIRAP and SKIV2L. Using multinomial regression analysis adjusting for lifestyle and biological factors, we calculated the Odds Ratio (OR) for each genotype as an indicator for Early or Late AMD and we found that CFH and ARMS2 were significantly associated with the incidence of AMD in the Irish population. Homozygosity for both these SNPs were found in the mutant eyes. In addition, we also demonstrated that NICD-induced RPE transformation was mediated by Hippo/Yap1 signaling pathway. Knockout of NICD-overexpressed RPE cells reduced EMT. Our results suggest that Notch signaling pathway plays important role in control of RPE size, diploidy, and EMT, involving YAP1 activity.

YAP1 Mediates Notch Induction of Epithelial-mesenchymal Transition (EMT) in RPE

Lu, Q. Li, Q.

University of Louisville, Ophthalmology and Visual Sciences, Louisville, United States

Epithelial-mesenchymal transition (EMT) of retinal epithelium (RPE) cells is a major pathologic change in development of proliferative vitreoretinopathy (PVR). Constitutive activation of RBP-Jκ-dependent Notch signaling during murine embryonic development leads to hyperproliferation and tumor formation in adult RPE. However, the molecular mechanism underlying these phenotypic changes has not been well studied. We generated a mouse model with constitutively activation of Notch signaling in adult RPE by Best1-Cre mediated over expression of the Notch intracellular domain (NICD). NICD overexpression in adult RPE induced a large number of cells that carried double nuclei, and characterized with dramatically increased cell size. Hypertrophy and EMT of RPE cells were induced. The affected RPE cells invaded into choroid with loss of RPE differentiation marker - RPE65. β-catenin nuclear translocation and RPE proliferation were observed. The cells that had undergone EMT were labeled with GFP induced by Best1-Cre to identify their RPE origin. Detachment of neuronal retina occurred in the mutant eyes. In addition, we also demonstrated that NICD-induced RPE transformation was mediated by Hippo/Yap1 signaling pathway. Knockout of Yap1 in NICD-overexpressed RPE cells reduced EMT. Our results suggest that Notch signaling pathway plays important role in control of RPE size, diploidy, and EMT, involving YAP1 activity.

Leucine-rich Repeats of PXDN Is Essential for Lens Development

Kuang, L. Yan, X.

Shenzhen Eye Hospital, Jinan University, Shenzhen, China

Purpose: Mutations in Peroxidasin (PXDN) cause severe inherited eye disorders in humans, such as congenital cataract, corneal opacity and developmental glaucoma. As there are multiple domains in PXDN, including Leucine-rich repeats (LRR), immunoglobulin (Ig) domain, peroxidase domain and VW domain. The function of each domain remains unknown. To investigate the role of LRR domain during the eye development and disease, we generated a novel PXDN knockout mouse with two exons of LRR domains are deleted and performed preliminary characterization.

Methods: CRISPR/Cas9 system was used to make large DNA fragment deletions, including LRR3 and LRR4 domain deletion, about 130-177 aa in size. The eyes of the LRR-deficient mice were analyzed by histology and immunohistochemistry.

Results: We successfully generated a new PXDN knockout mouse line with LRR deficiency, which resulted in the genetic deletion of two exons (LRR3 and LRR4) of the LRR domain. This deletion in the two exons causes severe anterior segment dysgenesis and microphthalmia in postnatal periods, which resembles the manifestations in patients with PXDN mutations. During embryonic periods, the lens capsule is disrupted and broken, and lens matrix is intruded into the anterior chamber.

Conclusions: LRR domain of PXDN is essential for lens development, especially for lens capsule development. Deficiency of LRR domain of the PXDN leads to lens capsule broken (congenital cataract), and later anterior segment dysgenesis and microphthalmia. Our model also gives a pathological mechanism of anterior segment dysgenesis and microphthalmia due to abnormal lens development.
Regulatory Placental Growth Factor (PLGF) Bioactivity in the Outer Retina as a New Therapeutic Approach to Age-related Macular Degeneration

Cunningham, F.1, Van Bergen, T.2, Feyen, J.H.M.1, Canning, P.1, Lengyel, T.1, Stitt, A.W.1

1Queen’s University Belfast, The Wellcome-Wolfson Institute for Experimental Medicine, Belfast, United Kingdom; 2ThromboGenics NV, Leuven, Belgium

PLGF and the differential activation of its receptor system have been implicated in retinal vascular diseases. However, recent reports suggest that PLGF could also be involved in key aspects of age-related retinal pathology, including retinal pigment epithelium (RPE) dysfunction and outer retinal atrophy. Using in vitro and in vivo models of oxidant-induced RPE damage, this study aims to examine the PLGF receptor axis in RPE. The ARPE-19 cell line was used as a model of RPE in vitro. PCR and western blot were used to analyse expression of PLGF receptors at days 7, 14, 21, and 28 post-plating. Transient electrophilic resistance was used to evaluate barrier function of ARPE-19 exposed to PLGF, and immunostaining was used to visualise tight junction integrity. RPE dysfunction was modelled using ARPE-19 exposed to sodium iodate (NaIO3). The cytotoxic effects of NaIO3, induced oxidative stress on ARPE-19 were evaluated by MTT and LDH Cytoxicity assays.

NaIO3 reduced the viability of ARPE-19, as measured by MTT as a consequence. This reflects cellular mitochondrial activity and suggests that NaIO3 affected mitochondrial respiration. Future experiments investigating ARPE-19 mitochondrial function under control conditions and post-NaIO3 exposure are planned, alongside additional readouts of RPE function, including phagocytosis. ARPE-19 express PLGF receptors, suggesting this cell line can respond to this cytokine. Now, PLGF signalling pathways in RPE will be elucidated by analysis of signalling molecule phosphorylation by western blot. The effects of PLGF treatment on NaIO3-exposed ARPE-19 will also be evaluated. Development and characterisation of an in vivo NaIO3-induced choroidal atrophy animal model will also be performed alongside in vitro studies. Future in vivo experiments will involve investigating the effects of PLGF blockade in this animal model.

CRB2 Is Involved in the Apical Polarization of RPE Cells by Participating in Tight Junction Maintenance and Cell Cycle Arrest

Segurado, A.1, Escudero Paniagua, A.1,2, Valle, V.1, Fernández-Delón, J.1, Velasco, A.1, Lillo, C.1

1University of Salamanca, Institute of Neurosciences of Castilla y León, Salamanca, Spain; 2UCSA Stein Eye Institute Westwood, Department of Ophthalmology, Los Angeles, United States

Apical polarities are essential for the precise performance of epithelial cell functions. It is determined by the expression of three polarity protein complexes named Scribble, Par and Crumbs. While Scribble complex promotes the differentiation of the basolateral domains, both the Par and the Crumbs complexes promote the apical side identity. The Crumbs complex is typically composed by PALS1, PATJ and the CRB proteins. We have described the expression of one of the CRB proteins in retinal pigment epithelial (RPE) cells, the CRB2 protein. To better understand the highly regulated process of polarization, we have studied the role of the polarity protein CRB2 in human RPE cells during differentiation in vitro and in mature murine RPE cells in vivo.

To do this, we have analyzed the temporality of the sequential expression and localization of the polarity proteins during human fetal RPE cells differentiation. We have knocked down CRB2 in cultured RPE cells and analyzed their proliferation rate, expression and localization of proteins related with the establishment of junctional complexes and those involved in polarization compared to control. We have also measured the transepithelial electrical resistance (TER), a direct measure of the strength of the cell-cell junctions, during ordinary differentiation and after a severe disruption of junctional complexes with an acute depletion of Ca2+. Calcium switch assay. Finally, we have analyzed the role of CRB2 in adult mouse RPE cells in vivo by knocking down CRB2 with sub-retinal injections of lentiviral particles containing a shRNA directed against the mouse CRB2 mRNA.

The results showed that CRB2 is the last of the whole pool of polarity proteins to be positioned at the cell membrane. Subsequently, the absence of the CRB2 protein from these cells results in a delay in the formation of cell-cell junctions and in an increase in cell proliferation in human RPE cells. In addition, our studies in vivo show that knocking down CRB2 in RPE cells affects the distri- bution of different apical polarity proteins and perturbed the retinal homeostasis manifested by the invasion of activated microphtalmia cells into the subretinal space. Together our results demonstrate that CRB2 is a key protein for the development and maintenance of a polarized epithelium.

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Glycogen Synthase Kinase 3 Restricts the Genesis of Displaced Ganglion Cells during Retinal Development

Rojo, J.1, Braginskaja, E.I., Vigouroux, R.1, Chedotal, A., Swaroop, A., Perron, M.1

1CNRS / CERTO, Neuro-PS1, Orsay, France, 2Institut de la Vision, Paris, France, 3National Eye Institute, Bethesda, United States

Glycogen Synthase Kinase 3 alpha (GSK3a) and beta (GSK3b) are at the crossroad of multiple signaling pathways and act as molecular switches mediating their output to regulate numerous cellular processes. Several studies have contributed to delineate GSK3 function in central nervous system development. Here, we have investigated the function of GSK3 signalling during retinal development.

We generated mice with partial or complete loss of Gsk3a and Gsk3b using e-Cre mouse line, allowing deletion of Gsk3 alleles as early as E10.5 in retinal progenitors. The phenotype was evaluated by immunohistochemistry and electoretinogram analyses. Retinal ganglion cell (RGC) bodies and axonal projections to the brain were tracked using retrograde and anterograde labelling, respectively.

Complete loss of Gsk3 in retinal progenitors led to severe morphological defects with progressive death of the pool of proliferative retinal progenitors and lack of neuronal differentiation resulting in microphthalmia. In contrast, expression of even one Gsk3a or Gsk3b allele led to differentiation of a functional retina.

Further phenotypic analysis, however, revealed the presence of a large number of Brn3a-positive displaced ganglion cells (dRGCs) located in the inner nuclear layer (INL), while only a few dRGCs were present in the control retina. Using retrograde labelling, we demonstrated that dRGC axons reached the optic nerve validating their RGC identity. EdU injection at E12.5 demonstrated that dRGCs were born at the same time as orthotopic RGCs (oRGCs) in both Gsk3a- and Gsk3b-deficient mice. Gsk3a and Gsk3b would thus be involved in the survival of dRGCs.

In conclusion, this study identifies GSK3 as the first regulator of the genesis and survival of a rare subtype of RGC of as yet unknown function.
Is the Retina a Reliable Mirror of Alzheimer’s Disease Brain? Screening of Molecular and Cellular Parameters

Rogéndes Neves, A.C.1,2, Carecho, R.I.1, Baptistia, F.I.1,2, Moreira, F.I.1,2, Ambrósio, A.F.1,2

1Coimbra Institute for Clinical and Biomedical Research (ICBRI), Faculty of Medicine, University of Coimbra, Coimbra, Portugal
2CNC IBIIL Consortium, University of Coimbra, Coimbra, Portugal

Alzheimer’s disease (AD) diagnosis is difficult and relies on invasive methods. Therefore, it is crucial to identify early biomarkers. Some AD patients have visual complaints, even before the first symptoms of dementia. Being the retina an extension of the brain, we aimed checking whether the retina also undergoes changes similar to those occurring in the AD brain. Retina (RET), hippocampus (HIP) and cortex (CTX) homogenates from male triple transgenic (3xTg-AD; AD model) and age-matched wild-type (WT; C57BL6/129S) mice at 4, 8 and 12 months (M) old were used to assess proteins associated with AD pathology, cell degeneration and neuroinflammation. Glial cell distribution and reactivity as well as retinal ganglion cell (RGC) loss were assessed by immunohistochemistry. Cell death was evaluated by TUNEL assay.

Increased A-beta levels were detected in the HIP and CTX of 3xTg-AD mice at all timepoints. No differences were detected in pre- and postsynaptic proteins (syntaxin, synaptophysin and PSD95) in all regions analyzed, with the exception of an increase of PSD95 in the HIP and syntaxin in CTX of 3xTg-AD at 4M. Also, no changes were detected in the choline acetyltransferase. Moreover, we did not detect RCG loss in the retina of 3xTg-AD, which is in line with the absence of cell death in the retina and brain of 3xTg-AD mice. Regarding glial reactivity, there were fluctuations in GAP43 levels in all regions. No changes were detected in vimentin levels ( Müller cells marker) or in its distribution. However, it was detected a significant increase in the number of microglial cells (Iba-1 cells) in the RET of 3xTg-AD at 12M. Interestingly, transcript levels of antioxidant genes in RPE were significantly upregulated in the DJ-1 KO mice when compared to controls (p< 0.05). There was also significantly less SVP28, Nrf2, and NQO1 in the retinas of diabetic rats compared to controls (p< 0.05). Diabetes-induced reductions in the spatial frequency threshold and contrast sensitivity correlated with apoptosis, as well as the morphological changes in both the inner and outer retina and the content of SVP38 (0.0424< p< 0.0001). OCT imaging suggests that, in the streptozotocin-diabetic rat model, neurodegeneration due to apoptosis and loss of synaptic protein leads to thinning of the inner retina but is also accompanied by swelling of the outer retina, presumably caused by edema due to vascular permeability. These abnormalities in retinal morphology, including the outer retina swelling, are strongly associated with vision loss, as is the increase in cell death and reduction in the abundance of the synaptic protein, SVP38.

Diabetes-induced Visual Dysfunction in Rats is Due to a Combination of Inner Retina Thinning and Outer Retina Swelling

Barber, A.J.1, Wang, W.-W.1, Nasrallah, Z.1,2, Baccouche, B.1, Kim, S.D.1

1Penn State College of Medicine, Hershey, United States, 2Zucker School of Medicine at Hofstra/Northwell, New York, United States
3Université de la Manouba, Tunis, Tunisia

Diabetic retinopathy (DR) is one of the leading causes of vision loss in adults. Features of DR include deterioration of visual function, apoptosis and neurodegeneration, and blood-retinal barrier permeability leading to edema. But the relationship between visual dysfunction and neural and vascular degeneration is not well established. The aim of this study was to correlate visual function with the presence of retinal layers and some diabetes-induced molecular markers including apoptosis and expression of synaptic and anti-oxidant response proteins.

Male Long-Evans rats were made diabetic by injection with streptozotocin, inducing hyperglycemia > 250 mg/dl. Visual function was determined by behavioral test of the optomotor reflex. The thickness of retinal cell layers was measured in vivo by spectral domain optical coherence tomography (OCT). After sacrifice, apoptosis was measured by cell death ELISA. The protein content of synaptophysin (SVP38), nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and NAD(P)H dehydrogenase quinone 1 (NQO1) were determined by western blot.

Diabetic rats had significantly reduced optomotor function compared to controls 10 weeks after induction of hyperglycemia (p< 0.0001). OCT revealed that after 16 weeks of diabetes the ganglion cell layer was significantly thinner (p< 0.05) and the outer nuclear layer was significantly thicker compared to controls (p< 0.05). There was also significantly less SVP28, Nrf2, and NQO1 in the retinas of diabetic rats compared to controls (p< 0.05). Diabetes-induced reductions in the spatial frequency threshold and contrast sensitivity correlated with apoptosis, as well as the morphological changes in both the inner and outer retina and the content of SVP38 (0.0424< p< 0.0003), but not the anti-oxidant response proteins.

The Sodium Iodate (NaIO3) Model of Degeneration: Deciphering the Oxidative Stress Regulatory Mechanisms in the Retina and RPE

Upadhayay, M.1; Bonilha, V.1,2

1 Cleveland Clinic Foundation, Ophthalmic Research, Cleveland, United States, 2Cleveland Clinic Lerner College of Medicine, Ophthalmic Research, Cleveland, United States

Sodium iodate (NaIO3) is an oxidizing agent known to induce preferential degeneration and atrophy of RPE cells with secondary effects on photoreceptors and the choroidiscapsular. We used this model to gain mechanistic insights into oxidative stress-related RPE degeneration in a concentration-dependent manner. The Parkinson’s disease (PD)-associated gene DJ-1 encodes a protein that responds to and protects neurons from oxidative stress by increasing cell survival and antioxidant potential. In adult rodent retinas, DJ-1 is highly expressed in the RPE and photoreceptor cells (inner segments and cell body) while DJ-1 also displays lower expression in the outer plexiform layer. Loss of DJ-1 results in mild structural and physiological changes in the retinas of the aged DJ-1 knockout (DJ-1 KO) mice in association with increased oxidative stress. Mice (C57Bl/6J and DJ-1 KO) received a single tail vein injection of 10, 15 and 20mg/kg body weight of NaIO3 parallel groups of mice were injected with PBS. Histological analysis of the retinas of mice were performed in toludine blue section and flatmounted retina and RPE/choroid seven days post-injection. Total RNA was extracted, and cDNA was generated for RT-PCR study (CENTRO-01-0145-FEDER-000008: BrainHealth 2020). DJ-1 expression in the retina and RPE was evaluated by immunohistochemistry and cell death was evaluated by TUNEL assay.
renders the retina and RPE susceptible to increased degeneration after exposure to NaIO. Our data also suggest that retina and RPE are differentially affected by the lack of expression of Di-1 and by exposure to NaIO.

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CNG Channel Inhibition as a Treatment Option for Rod Degeneration in Retinitis Pigmentosa

Das, S.1,2, Rieger, N.1, Paquet-Durand, F.1

1University of Tuebingen, Institute for Ophthalmic Research, Tuebingen, Germany, 2Graduate School of Cellular & Molecular Neuroscience, Tuebingen, Germany

Primary rod photoreceptor degeneration in the hereditary disease retinitis pigmentosa is believed to be caused by high Ca2+ influx. Cyclic nucleotide gated channels (CNGC), present in the outer segment of photoreceptors, mediate the influx of Ca2+ during the phototransduction cascade. Previously, genetic knockout of CNGC showed partial photoreceptor rescue and improved retinal function in the rd1 retinal degeneration mouse model (Paquet-Durand et al., Hum. Mol. Genet. 2004;13, 2098–2106). In this work, we explored CNGC as a possible target for a pharmacological intervention in retinitis pigmentosa using retinal explant cultures derived from rd2 and wild-type (wt) mice. The experiments were done under completely controlled conditions, serum free conditions on organotypic retinal explants cultured from post-natal day (P) 5 to 11. In our initial studies, we used L-cis-diltiazem, a well-known CNGC blocker and obtained a dose-response curve for its effect on rd2 cultures. Here, at 100 µM L-cis-diltiazem exhibited its effect on well-known CNGC blocker and obtained a dose-response curve for its effect on rd2 cultures. At 100 µM L-cis-diltiazem exhibited a minor protective effect. This was contrasted by the effects of the D-cis-diltiazem enantiomer, which blocks voltage gated calcium channels (VGCC), and which had a detrimental effect on rd2 explant cultures at the same concentration. To study possible toxicity of both diltiazem enantiomers, their effect was tested also on wt retinal explants. Interestingly, in the wt situation, both L- and D-cis-diltiazem appeared to have detrimental effects.

To further develop inhibition of CNGC pharmacologically, we tested different cyclic GMP analogues, predicted to be highly efficacious CNGC inhibitors, and obtained corresponding dose–response curves to find the most effective analogue and concentration. Our preliminary data suggest that blocking CNGC can significantly reduce photoreceptor degeneration in rd1 retina. On the other hand, we found a significant negative effect on cell survival when using a VGCC blocker.

In summary, our work confirms a role for CNGC in the photoreceptor degenerative process and highlights CNGC as a target for therapeutic intervention. In the future, antisense oligonucleotides designed to specifically downregulate channel subunits, may be investigated as an alternative and potentially more specific approach to targeting CNGC.

Multimomics Study of Primary Human Foetal Retinal Pigment Epithelial Cells: Effects of Zinc Supplementation

Emti, F.1, Dammeyer, S.1, Klose, F.1, Cincisik, L.1, Simpson, D.1, Deffing, M.1, Lengyel, L.I.1, Eye-Risk Consortium

1Queen’s University, Belfast, United Kingdom, 2Institute for Ophthalmic Research, Universitätsklinikum Tuebingen, Tubingen, Germany

Purpose: Retin pigment epithelial (RPE) cells are common model system to study sub-RPE deposit formation, the hallmark of age-related macular degeneration (AMD). In AMD, cellular zinc levels decline. Zinc supplementation can attenuate the progression to late AMD, potentially at least in part, via inhibiting complement cascade. However, the molecular pathways involved in the effect of zinc are not fully explored, and as such, we set up a multimomics study and examined the effect of changing apical or basal zinc in our primary RPE cell model.

Methods: We cultured primary human foetal RPE cells from 3 individuals on transwell inserts (Corning), coated with Geltrex. After one week, the apical or basal compartments were replaced with medium containing 125 µM added zinc or left untreated and cultured for a further 4 weeks. Thereafter, apical and basal secretome and the RPE cells were harvested for genotyping, RNAseq, lipidomics and proteomics analysis or fixed for fluorescence and electron microscopy (EM).

Results: A total of 5006 genes, 484 proteins and 43 lipids were up- or down-regulated in the apical or basal compartments. RPE cell morphology and functional activity showed no obvious differences between the two conditions. In addition, a number of genes were up- or down-regulated in both the apical and basal compartments.

Conclusion: Our findings indicate that zinc supplementation may modulate the expression of genes involved in photoreceptor degeneration and AMD.

Gap Junctions Formed by Connexin 43 in Astrocytes Contribute to Neuronal Damage after Ischemia-reperfusion Injury

Toychiev, A., Srinivas, M.

Sun College of Optometry, Biological and Vision Science, New York, United States

Background: Retinal ischemia is a common cause of blindness in a number of ocular neurodegenerative diseases. Neuronal loss in response to retinal ischemia is triggered by factors released by several cell types, including glial cells. Astrocytes provide structural and functional support for ganglion cells in normal conditions, but they become reactive in response to injury. Reactive astrocytes can exert neurotoxic or neuroprotective actions. Astrocytes abundantly express connexin 43 (Cx43), a transmembrane protein that forms gap junction (GJ) channels and hemichannels. We have recently shown that deletion of Cx43 in astrocytes curtailed retinal hypoxia and pathologic neovascularization and improved neuronal function in a model of oxygen-induced retinopathy (Slavé et al., 2018). In this study, we examine the role of Cx43 in astrocyte reactivity and neuronal loss seen in retinal ischemia-reperfusion injury.

Methods: Wild-type (WT) and astrocyte-specific Cx43 knockout (KO) C57BL/6J mice were subjected to retinal ischemia by transient elevation of intraocular pressure (75-80 mmHg) for 60 min followed by reperfusion. Expression of Cx43 and reactivity of astrocytes was assessed with immunofluorescent staining of retinal injury and functional support for ganglion cells in normal conditions, astrocytes were assessed with immunofluorescent staining of retinal injury and functional support for ganglion cells in normal conditions.

Results: Ischaemia-reperfusion caused hypertrophy of retinal astrocytes and an increase in Cx43 expression that mainly co-localized with glial-specific markers in the ganglion cell layer. Ischemia-reperfusion resulted in a decrease in the RGC numbers compared to contralateral eyes. Deletion of Cx43 in astrocytes did not affect neuronal function under normoxic conditions, but caused a significant (p < 0.05) increase in the survival of RGC cells (33 % cell loss in KO mice vs. n = 4 vs. 77 % cell loss in WT, n=6) in ischemia-reperfusion. Cx43 KO in ischemia-reperfusion also appeared to be associated with a decrease in astrocyte hypertrophy; however additional studies quantifying the extent of remodeling are required.

Conclusion: Results suggest that astrocytic Cx43 contributes to neurodegeneration in retinal ischemia-reperfusion injury. The mechanism by which Cx43 exerts its deleterious effect on neuronal survival is currently being investigated.

Impact of α-adducin Deletion on the Retinal Structure and Function

Campos, E.1,2, Oliveira, B.1,2,3, Martins, J.1,2,4, Sousa, M.M.5, Ambrosio, A.F.1,2

1University of Coimbra, Institute for Clinical and Biomedical Research (iCBR), Coimbra, Portugal, 2University of Coimbra, CIBM Centre, Coimbra, Portugal, 3University of Coimbra, Faculty of Technology, Coimbra, Portugal, 4University of Coimbra, Coimbra Institute for Biomedical Imaging and Translational Research (CIBIT), Coimbra, Portugal, 5University of Porto, Institute for Research and Innovation in Health (i3S), Porto, Portugal

Adducins are membrane-skeletal proteins composed by three subunits (α, β, γ), differentially expressed in a developmental and tissue-specific manner, and are localized at restrictin-actin junctions. In the tetrameric adducin formed by the α-β heterodimer, the α monomer is essential. These proteins promote the association of spectrin with actin and cap the fast growing end of actin filaments. It is also known that adducins are important for the maintenance of the structure and function of neuronal axons and synapse formation. Furthermore, they have been associated with neurodegenerative conditions including amyotrophic lateral sclerosis and cerebral palsy. Recently, it was shown that the absence of α-adducin causes progressive axon enlargement, namely, in the optic nerve, followed by axon degeneration and loss. These findings challenged us to investigate the impact of the deletion of α-adducin on the retinal structure and function, using an adducin knockout mouse.

The deletion of α-adducin induced retinal structural malformations that were observed in about 26% of its extension. By immunohistochemistry using antibodies specific for retinal cell types, it was detected an apparent worsening of the cones organization, a significant increase in the length and abnormal morphology of bipolar and horizontal cells, as well as a significantly higher density of ganglion and amacrine cells. A clear pro-ophthalmologic scenario was confirmed by the abnormal microglial density, distribution and reactivity, particularly in the ganglion cell and inner plexiform layers. Nevertheless, no cell death by apoptosis was detected.
Retinal Cell Biology

TUESDAY, 11 SEPTEMBER 2018

Retinal Ganglion Cells Are Protected through Hypothermia Treatment in a Porcine Retina Organ Culture Model

Hurst, J., Kuehn, S., Herms, F., Malia, A., Bartz-Schmidt, K.U., Dick, B., Joachim, S., Schnichels, S.

University Eye Hospital Tübingen, Tübingen, Germany, Ophthalmology, Ruhr-University Bochum, Bochum, Germany

Purpose: Cobalt is important for the neuronal integrity but high quantities induce cytotoxic mechanisms. Treatment with Cobalt-chloride (CoCl2) led to strong degeneration of porcine retina through hypoxic mechanisms. Neurons of the inner retina layers are particularly affected. In the present project this model is used to study possible protective effects of hypothermia treatment.

Methods: Porcine retina explants were cultivated for four and eight days at 37°C. Four groups were compared: control, control+30°C, CoCl2, CoCl2+30°C. Hypothermia (30°C) was applied in parallel to hypoxic damage using 300µM CoCl2 for 48 h. Retinal explants were subject to immunohistochemistry, qRT-PCR, and Western blot analysis. The number of retinal ganglion cells (RGC), as well as microglia, bipolar, and amacrine cells was evaluated and compared between groups.

Results: At four days of cultivation, in the control group 30.9±1.1 Iba1+ cells/mm were counted, exposure with CoCl2 led to 14.9±1.0 Iba1+ cells/mm, p<0.001. At four days, 80.7±7.1 Co1+ cells/mm; eight days: 13.3±0.9 Co1+ cells/mm compared to control (four days: 154.1±1.1 Co1+ cells/mm; eight days: 40.1±4.2 Co1+ cells/mm, p<0.05). Histopathology in the CoCl2+30°C group could stop this effect and rescued the number of Iba1+ cells/mm (four days: 37.1±4.7 Iba1+ cells/mm, p<0.01; eight days: 19.5±0.8 Iba1+ cells/mm, p=0.03).

Conclusions: The cultivation of porcine retinae with CoCl2 lead to a hypoxia-like degeneration processes. The hypothermia treatment induced cell protection and saved especially retinal ganglion cells from apoptosis. Hence, this system may serve as a first analysis model for therapy screening for retinal diseases. This alternative model is suitable for drug and treatment screening and will therefore reduce the number of animal studies.

Novel Function of β-catenin in Regulation of RPE Adhesion Junctions

Li, Q., Scott, P., Kaplan, H., Dean, D., Lu, Q.

University of Louisville, DOVS, Louisville, United States

Polarization of epithelial cells into apical and basal compartments is key to their function. The retinal pigment epithelium (RPE) is a specialized epithelial monolayer connected by tight, adherens, and desmosome junctions laterally among adjacent cells and anchored to basal Bruch’s membrane by hemidesmosome and desmosome-like junctions that generally link the basal lamina via desmosome junctions laterally among adjacent cells and anchored to basal Bruch’s membrane by hemidesmosome and desmosome-like junctions, creating a tight blood-retinal barrier (BRB) that separates the inner space of the eye from the blood stream. Tight junctions form laterally between epithelial cells within their apical domain. Adherens junctions, comprised of β-, P-cadherins, and β-, α-catenins in RPE, mediate cell-cell interactions by providing anchors for actin cytoskeletal filaments that basal domain. Desmosome junctions are spot-like adhesions anchoring keratin intermediate filaments to cadherin-catenin complex via desmoplakin adaptor proteins. The basal surface of RPE is anchored to Bruch’s membrane via hemidesmosome junctions and desmosome-like junction that generally link the basal lamina via transmembrane integrin on the basal membrane of epithelial cells. It is unclear if β-catenin and its associated cadherin-catenin complex have any role in maintaining basal polarity and formation of basal infoldings in the adult RPE. Although adherens junctions are generally restricted to cell-cell junctions in the lateral region of epithelial monolayers, we found that RPE-specific P-, N-catenins were also present along with β-catenin and α-catenin along the basal membrane of the RPE layer. We used condition knockout (ΔKO) of β-catenin via Best1-promoter-Cre to examine β-catenin functions in adult RPE. Two different mutants were examined – one only disrupted the nuclear transcription factor function of β-catenin and the other one is a null mutation causing complete lack of β-catenin protein. While no effect was observed for the mutant with disrupted only nuclear function of β-catenin, β-catenin in null mutation led to disorganizing RPE morphological changes with expansion in cell body, focal adhesion disruption and detachment from Bruch’s membrane. P-cadherin was significantly reduced and α-catenin was completely lost from the basal membrane of the affected RPE cells. These β-catenin CKO mice mimicked many symptoms found in the patients with mutations in P-cadherin or α-catenin lead to Hypothryrosis with Juvenile Macular Dystrophy, Ectodermal Dysplasia, Ectrodactyly and Macular Dystrophy, and butterfly-shaped pigment dystrophy, respectively.

Insulin Activates the PI3K/Akt/GSK3 Pathway in Culture Retin Pigment Epithelium

Salceda, R., Morales-Galeana, M., Sánchez-Chávez, G.

Universidad Nacional Autónoma de México, Instituto de Fisiología Celular, Neurociencias, Mexico, D.F., Mexico

Retinal pigment epithelium (RPE) is part of the blood retinal barrier, and maintains the structural and functional integrity of the retina. Insulin is a hormone with pleiotropic effects and has been recognized to participate in retina development. Insulin receptors in RPE have been demonstrated although their functions are unknown. Our aim was to investigate whether insulin activates its signaling cascade in RPE. Then, by the Western blot technique, we studied the effect of insulin on the phosphorylation of protein kinases known to participate in the insulin signaling pathway, in RPE rat primary cultures. We demonstrated that insulin induced a time and dose dependent increase in the phosphorylation level of Akt (protein kinase B) and glycogen synthase kinase 3 (GSK3) as well as a decrease in the phosphorylation of glycogen synthase. Insulin-induced phosphorylation changes of these protein kinases were dependent of phosphoinositide 3-kinase activity (PI3K).

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Implication of Various Oxidative Stress Pathways in the Phenotype of Prpf31-Mutant Mice

Hamieh, A., Hadjout, N., Millet-Puel, G., Leveillard, T., Nandrot, E.

Sorbonne Université, INSERM, CNRS, Institut de la Vision, Paris, France

Mutations in the ubiquitous PrpF mRNA Processing Factors 3, 8, and 31 (PRPF3, PRPF8, Band 31) constitute the second most prominent cause of autosomal dominant retinitis pigmentosa (aDRP). Human ARPE-19 cells down-regulated for PRPF31 and Prpf31 mutant primary RPE cells display a 40% decrease of RPE phagocytosis, suggesting similar pathological processes occur in both species. With age, mouse Prpf31 mutant RPE cells exhibit structural abnormalities including the formation of cytoplasmic vacuoles. We thus investigated RPE-related stress pathways and lipid accumulation in 3- to 18-month-old Prpf31-mutant mice to dissect the full series of molecular events. Gene and associated protein expression levels for the mitochondrial respiratory chain, detoxifying enzymes and the endoplasmic reticulum (ER) unfolded protein response (UPR) pathways were analyzed. Functional evaluation of mitochondrial activity was assessed using the Seahorse technology on fresh tissues and RPE lipid accumulation was quantified on histological sections. In RPE/chorioid samples, complexes IV and V of the mitochondrial respiratory chain seemed affected in Prpf31-mutants: Complex IV expression increased from 6 months of age, while decreased ATP7e and protein expressions were detected at 6-12 and 6-18 months of age, respectively. Lower ATP6 expression was confirmed on tissue sections. A tendency for overall diminished expression of the mitochondrial SD2D superoxide dismutase was observed at all ages in contrast to other cytoplasmic detoxifying enzymes such as SOCl and catalase. Indeed, this deregulation in mitochondrial proteins expression was reflected by higher oxygen consumption rates in Prpf31-mutant mice from 6 months of age. Activation of UPR pathways was evidenced by the clavage of ATF6 and increased CHOP expression as early as 3 months of age. Some mitochondrial, ER and detoxification pathway perturbations were also observed in retinal extracts, showing that oxidative processes are not limited to the RPE. Prpf31-mutant RPE gradually accumulate more lipid droplets than wildtype RPE in number and in size since the age of 3 months. Currently, nature and amount of accumulated lipids are being assessed by thin layer chromatography analysis. Taken together our results suggest that oxidative imbalance and associated stress take place very early at different levels in Prpf31-mutant RPE cells, reinforcing the idea that the RPE is at the center of the phenotype development in PRPF-related RP cases.
Results: Data are shown in Table 1. A trend of increasing organoid lamination was observed during the course of development. The number of organoids with irregular boundaries peaked around Day 48 and normalized to having more continuous surface contour and area. Free floating, non-adherent cells were detectable; however, no definitive trend was seen. Multiphoton imaging provided definitive structural information that was not detectable with brightfield microscopy. High lamination rates, high nuclei presence, low free-floating cell concentration, and regular boundaries are associated with organoids that are perceived to be of high quality by brightfield microscopy.

Conclusions: Evaluation of intrinsic fluorophores provides detailed structural information which can improve the selection of transplantable retinal organoids for optimal outcomes.

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Pre-transplant Analysis of Retinal Organoids Using Dual-wavelength Excitation of Metabolic Markers

Sellers, M.1,2,3, Kalakuntla, T.1, McLelland, B.T.1, Kravieva, T.1, Collin, J.1, Mellough, C.1, Lako, M.1, Tromberg, B.1, Browne, A.4

1University of California, Irvine, Physical Medicine & Rehabilitation, Irvine, United States. 2University of California, Irvine, Stem Cell Research Center, Irvine, United States. 3University of California, Irvine, Institute for Regenerative Medicine, University of California, Irvine, Department of Microbiology, Molecular Genetics and Biochemistry, Irvine, United States. 4Center for Regenerative Medicine, Beckman Laser Institute and Medical Clinic, Irvine, Irvine, United States.

Purpose: To determine the structural arrangement, surface contour of retinal organoids, and presence for observed changes in organoid lamination based on nuclear morphology.

Eight retinal organoids were monitored by serial weekly imaging excitation at 800 nm was employed to simultaneously excite and image intrinsic auto-fluorescence of metabolic markers, such as NADH and ROS. We have employed 2-photon cellular level to reveal structural arrangement of cell layers while indicating metabolic activity (Browne et al. 2017, IOVS). 2-photon intrinsic auto-fluorescence of metabolic markers, such as NADH and ROS, allowed for observed changes in organoid lamination based on nuclear morphology. The number of organoids with irregular boundaries peaked around Day 48 and normalized to having more continuous surface contour and area. Free floating, non-adherent cells were detectable; however, no definitive trend was seen. Multiphoton imaging provided definitive structural information that was not detectable with brightfield microscopy. High lamination rates, high nuclei presence, low free-floating cell concentration, and regular boundaries are associated with organoids that are perceived to be of high quality by brightfield microscopy.

Retinal Cell Biology

Retinal Cell Biology

A Lipidic Phloroglucinol Derivative Against Carbohydry and Oxidative Stress Involved in Macular Dystrophy

Brabet, P.1, Cubizolle, A.1, Guillou, L.1, Jacquemont, N.3, Vercauteren, J.1, Durand, T.1, Cia, D.1, Crauste, C.1

1Institut des Neurosciences de Montpellier, UMR INSERM 1051, Montpellier, France. 2Université de Médicine et de Pharmacie, UMR INSERM 1107, Clermont-Ferrand, France. 3Institut des Biomolécules Max Mousseron (IBMM), UMR5247-CNRS-UM ENSC, Montpellier, France.

Carbonyl and oxidative stress (COS) mechanisms play a substantial role in Stargard’s disease and in age-related macular degeneration (AMD). In the photoreceptor, light-induced all-trans retinal (aTRA) reactivity generates carbonyl and oxidative stress by condensation with phosphatidylethanolamine (PE) and formation of autofluorescent bisretinoid such as A2PE. We have formerly reported the efficacy of new pharmacological molecules from alkylated phloroglucinol (PG) derivatives conjugated with DHA to reduce aTRA-induced damages in outer retinal cells (Crauste C, Eur JOC 2014; Cia D, J Cell Mol. Med. 2016). These encouraging in vitro results drive us to assess therapeutic potential of A Lipidic Phloroglucinol Derivative Against Carbonyl and Oxidative Stress Involved in Macular Dystrophy.

Abca4-deficient mouse model of Stargardt’s disease and to clarify mechanisms of cell protection against aTRA toxicity. Abca4-deficient 129/SV x C57BL/6J-eYFP mice were treated by intravenous injection with IP-DHA complexed to BSA. Mice were exposed white fluorescent light for 2 hours and then kept in the dark for 5 days before analysis of retinal morphology and visual function. The number of photoreceptors was determined using histological H&E staining and SD-OCT, their light responses were quantified by full-field ERG. Retinal content in retina was determined by HPLC. Cultured RPE cells were treated with protective doses of ip-DHA and/or aTRA, and free aTRA concentration, COS production, activities and expression of antioxidant and detoxifying enzymes were measured. The vein injection is the most effective and quick to reach retina, which does not require anesthesia affecting the visual cycle. The light exposure caused 50-60% photoreceptor loss in BSA-injected mice compared to dark-adapted control. IP-DHA treatment enabled to recover 75-80% of intact photoreceptors and ERG a-wave and b-wave amplitudes were fully recovered. Both 11-cis and 13-cis were reduced by IP-DHA in retina. IP-DHA decreased free aTRA in RPE cell and prevented aTRA-induced ROS production. Moreover, IP-DHA activated antioxidant catalase activity and induced Nrf2 translocation to the nucleus. Consequently, the expression of phase II enzymes such as glutathione-related enzymes and NADPH oxidase was increased.

We demonstrated that IP-DHA protects against retinal degeneration in a mouse model of acute stress induced by light. This effect can be partly due to the reduction of retinaldehydes by IP-DHA. EYS is a major causative gene of autosomal recessive retinitis pigmentosa (RP) but is lost in rodents, the most prevalent animal model. On the other hand, the ortholog, eys, exists in zebrafish and homologous mouse model of Stargardt’s disease and to clarify mechanisms of cell protection against aTRA toxicity. We have formerly reported the efficacy of new pharmacological molecules from alkylated phloroglucinol (PG) derivatives conjugated with DHA to reduce aTRA-induced damages in outer retinal cells (Crauste C, Eur JOC 2014; Cia D, J Cell Mol. Med. 2016). These encouraging in vitro results drive us to assess therapeutic potential of a Lipidic Phloroglucinol Derivative Against Carbonyl and Oxidative Stress Involved in Macular Dystrophy.
may shed light on EYS function. Here, we generated zebrafish eys+/-; LRP5+/− heterozygous mutant line and performed microarray analysis together with eys+/- and LRP5−/− mutants using the adult eye to test the possibility and also possible interaction of eys and LRP5 genes. In eys+/-; LRP5+/− heterozygous mutants, we found remarkable decrease in expression levels of LRP5 gene compared to the wildtype adult siblings (fold change, 1/17.6). The mouse ortholog, Rbp2, is known to cause photoreceptor outer segment abnormality under vitamin A deficient diet condition. Therefore, the result suggests that the digenic heterozygous mutations, eys+/-; LRP5−/−, can cause photoreceptor degeneration in zebrafish, which would in turn support the recent human case report. Furthermore, Rbp2 expression decreased by 2.19 times in the eys+/- eye, which was quite smaller than eys+/-; LRP5+/− heterozygous mutations. The result may suggest that pathogeneses causing RP are different between EYS+/-; LRP5−/− and EYS−/− mutations. Besides, no change was observed in the expression level in either of zebrafish orthologs of Prominin-1 (Prom1) gene, prom2a or prom1b in eys+/- compared to the wildtype siblings. Genetic interaction between Eys and Prom1 was shown in Drosophi la2. However, the result suggests that there is no genetic interaction between eys and prom2a or prom1b genes in zebrafish although the possibility of their interactions at protein levels is currently not omitted.

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Learning the Hard Way: Role of Vascular Stiffening in Early Diabetic Retinopathy and AMD

Ghosh, K.1, Yang, X.2, Cabrera, A.1, Santiago-Tierno, I.1, Das, A.1, Kern, T.3

1University of California, Riverside, Riverside, United States, 2National Eye Institute, Bethesda, United States, 3University of New Mexico, Albuquerque, United States, 4Case Western Reserve University, Cleveland, United States

Vascular inflammation is strongly implicated in the loss of retinal and choroidal vessels associated with the early stages of diabetic retinopathy (DR) and age-related macular degeneration (AMD).

Since vascular atrophy is considered to be a risk factor for end-stage neovascularization associated with these conditions, there is a need to understand the mechanisms underlying vascular inflammation in early DR and AMD. Work in our lab has identified vascular ‘stiffening’ as a new paradigm of vascular inflammation associated with these conditions. Specifically, our studies have revealed that retinal and choroidal vessels in vivo and cultured vascular endothelial cells (ECs) in vitro undergo significant stiffening in diabetes and aging that, in turn, exacerbates the inflammatory effects of high glucose, leukocyte-secreted factors, and complement activation, major risk factors for DR and AMD. In diabetes, vascular stiffening, caused by upregulation of the collagen-crosslinking enzyme lysyl oxidase (LOX), exerts these inflammatory effects by modulating the intracellular mechanotransduction pathway involving Rho/ROCK and mechanosensitive ion channel TRPV4 that, in turn, control ICAM-1 expression and clustering necessary for monocyte-EC adhesion. Separately, our AMD studies revealed that the retinal pigment epithelium (RPE), essential for photoreceptor development and function, requires a functional primary cilium for complete maturation, and RPE maturation defects in ciliopathies precede photoreceptor development. Our results shed light onto the mechanisms by which signaling pathways, regulated by primary cilia disassembly, facilitate tissue maturation and provide novel insights into ciliopathy-induced retinal degeneration.

Ciliary Regulation of Wnt Signaling during RPE Maturation Is Essential for Visual Function

Patnaik, S., Kretschmer, V., May-Simera, H.

Johannes Gutenberg University, Mainz, Germany

Primary cilia are conserved organelles that mediate extracellular and intracellular communications. They are crucial for organogenesis and homeostasis in numerous tissues. Cilia defects cause ciliopathy disorders with retinal degeneration as a prominent phenotype. Although the primary cause of retinal degeneration in ciliopathy patients is due to defective cilia function in the retinal photoreceptors, contributions from other ciliated cell types in the eye has till now been largely ignored. We recently showed that the retinal pigment epithelium (RPE), essential for photoreceptor development and function, requires a functional primary cilium for complete maturation, and RPE maturation defects in ciliopathies precede photoreceptor development. The RPE is a ciliated monolayer in the eye that borders the retina and is vital for visual function. Here we show that primary cilia are transiently expressed during RPE development and that as the RPE matures, primary cilium retraction, which is accompanied by altered expression levels of cilia disassembly components. We see that ciliary associated Bardet-Biedl Syndrome (BBS) proteins protect against this HDAC6 mediated ciliary disassembly recruitment of Inversin. This consequently affects ciliary regulation of Wnt signaling. Moreover, BBS proteins also stabilize cilia disassembly components by controlling proteasomal degradation. Our results shed light onto the mechanisms by which signaling pathways, regulated by primary cilia disassembly, facilitate tissue maturation and provide novel insights into ciliopathy-induced retinal degeneration.

Retinal mTORC Expression and Protein Synthesis

Gardner, T.1, Losiewicz, M.2, Elghazi-Cras, L.2, Fangar, D.3, Rajala, R.3, Fort, P.1, Abcouwer, S.2

1Kellogg Eye Center, University of Michigan Medical School, Ophthalmology & Visual Sciences, Molecular & Integrative Physiology, Ann Arbor, United States, 2Kellogg Eye Center, University of Michigan Medical School, Ann Arbor, United States, 3University of Michigan Medical School, Biological Chemistry, Ann Arbor, United States, 4Dean McGee Eye Institute, University of Oklahoma Medical School, Ophthalmology & Physiology, Oklahoma City, United States

We have shown that rodent retina has a high basal rate of protein synthesis that decreases during diabetes (Diabetes 63:3077, 2014), and that inhibition of glycolysis reduces total protein synthesis (Am J Physiol Endo Metab 309:E546, 2015). We are now testing the hypothesis that mTOR complex 1 (mTORC1) and/or mTORC2, the central effector of the PI3-K signaling pathway, regulates retinal protein synthesis and/or metabolism. Total mTOR protein expression was quantified by immunoblotting and is equivalent in normal rat retina and liver, but greater than in the brain. The contents of Raptor and Rictor, co-effectors of mTORC1, were also significantly greater in retina and brain than in liver. Expression of the critical mTORC1 activating partner protein, mSin1, was highest in the retina, whereas the inhibitory partner, DEPTOR, was highest in liver. TSC2 was found in both mTORC1 and mTORC2 complexes by co-immunoprecipitation. Additionally, immunolocalization studies revealed that across rat, mouse and human tissues, the mTORC1 substrate, p70S6K1, that regulates protein synthesis in many tissues, was expressed predominantly in ganglion cells, the inner plexiform layer and inner nuclear layer. The mTORC2 substrates, PRKalpha and Akt, showed a similar pattern. Pharmacologic mTOR inhibition reduced retinal protein synthesis. The SUNSET method of protein synthesis showed predominant puromycin labeling in ganglion cells and photoreceptor outer segments. Ganglion cells are also the primary site of 18S and 28S ribosomal mRNA expression. Furthermore, inhibition of glycolysis with 2-deoxyglucose significantly reduced protein expression in rat retinas. Collectively, these data reveal a high level of metabolic activity in inner retinal neurons and suggest potential roles for mTOR complex signaling in this process. Understanding the regulation of retinal homeostatic mechanisms may be key to maintaining vision in patients with diabetes and other disorders.
Conclusion: The four leading causes of unilateral blindness were: cataract 43% (21/49), corneal pathology 18% (9/49), glaucoma 12% (6/49), and phtisis 10% (5/49). Out of the 101 patients that were either bilaterally blind or had a reversible condition, 12 or 25% were irreversibly blind with an additional 6 patients (12%) having a reversible condition for which the means to correct were unavailable. Of the 49 unilaterally blind patients, 12 or 25% were reversibly blind with an additional 6 patients (12%) having a reversible condition for which the means to correct were unavailable. Of the 52 bilaterally blind patients, 13 or 25% were reversibly blind with an additional 6 patients (12%) having a reversible condition for which the means to correct were unavailable.

Results: 138 new consultations (44 females, 94 males) were seen in a four week period. 38% (52/138) of patients presented with unilateral blindness and 36% (49/138) presented with unilateral blindness. Of the 52 bilaterally blind patients, 13 or 25% were reversibly blind with an additional 6 patients (12%) having a reversible condition for which the means to correct were unavailable.

Purpose: Evaluate the incidence of blindness, both reversible and irreversible in an isolated, rural, clinic-based population in OUESville, Extreme North, Congo.

Methods: Visual acuity, slit-lamp examination, applanation tonometry, and dilated examinations with the direct ophthalmoscope were performed. Based on the diagnoses, an assessment of treatability was established to determine reversibility of blindness. Blindness was defined as a visual acuity of count fingers or less.

Result: Of count fingers or less.

Consultations in Ouesso, Extreme North, Congo: Incidence of Reversible and Irreversible Blindness -2017

Mampouya Diandomba, A.
Maien Ngouabi University of Brazzaville, Ophthalmology, Brazzaville, Congo

Purpose: Evaluate the incidence of blindness, both reversible and irreversible in an isolated, rural, clinic-based population in OUESville, the Extreme North, Congo and present data on the variety of pathology present.

Methods: Visual acuity, slit-lamp examination, applanation tonometry, and dilated examinations with the direct ophthalmoscope were performed. Based on the diagnoses, an assessment of treatability was established to determine reversibility of blindness. Blindness was defined as a visual acuity of count fingers or less.

Result: Of count fingers or less.

High Serum Iron Levels Can Promote Retinal Degeneration

Baumann, B.1, Song, Y.1, Shu, W. 1,2, Lakhal-Littleton, S.3, Dunia, J.1
1University of Pennsylvania, Ophthalmology, Philadelphia, United States, 2Shanghai General Hospital, Shanghai Jiao Tong University, School of Medicine, Ophthalmology, Shanghai, China, 3University of Oxford, Oxford, United Kingdom

Purpose: The elevated iron levels detected in AMD retinas may promote oxidative damage (PMID: 12912886). Recently, we described early onset AMD in a patient who had received IV iron treatment for iron deficiency anemia (PMID: 27565570). We also found that iron can promote cell death caused by retinal bioregulators (PMID: 28686088). The purpose of the current study is to determine whether elevated serum iron can penetrate the blood retinal barrier and promote retinal degeneration.

Methods: We increased serum iron levels in mice through IV iron injection, IP iron injection, and by generating a liver-specific hepcidin knockout mouse, which has unfettered iron absorption from the gut throughout its lifespan.

Results: Both IV and IP iron injection resulted in increased RPE iron levels, and, if sustained, RPE degeneration. Liver specific hepcidin knockout led to age-dependent increase in retinal and RPE iron levels. The RPE became hypertrophic and autofluorescent by age 12 months. Electron microscopy of the RPE revealed abundant lipofuscin-like vesicles. Photoreceptor cell death and diminished ERG amplitudes were observed.

Conclusions: Elevated serum iron levels lead to increased RPE iron levels, and, if sustained, RPE degeneration. Liver specific hepcidin knockout led to age-dependent increase in retinal and RPE iron levels. The RPE became hypertrophic and autofluorescent by age 12 months. Electron microscopy of the RPE revealed abundant lipofuscin-like vesicles. Photoreceptor cell death and diminished ERG amplitudes were observed.

The Vasoreparative Potential of Endothelial Colony-forming Cells in the Ischemic Retina Is Enhanced by a Non-hematopoietic Erythropoietin Mimetic

Caning, P.1, O’Leary, O.1, Reid, E.1, Brines, M.1, Cerami, A.1, Brazil, D.1, Medina, R.1, Stitt, A.1
1Queen’s University Belfast / Wellcome-Wolfson Institute for Experimental Medicine, Belfast, United Kingdom, 2Arim Pharma-ceuticals Inc, Torrytown, United States

Purpose: Retinal ischaemia remains a common sight threatening end-point in blinding diseases such as diabetic retinopathy and retinopathy of prematurity. Endothelial colony forming cells (ECFCs) represent an advantageous therapeutic subpopulation of endothelial progenitor cells for promoting reparative angiogenesis in the ischaemic retina. The present study has investigated the potential of enhancing this cell therapy approach by the dampening of the pro-inflammatory milieu typical of ischaemic retina.

Methods: The effects of ARA290 on pro-survival signalling and function were assessed in ECFC cultures in vitro. Efficacy of ECFC transplantation therapy to promote retinal vascular repair in the presence and absence of ARA290 was studied in the oxygen induced retinopathy (OIR) model of retinal ischaemia.

Results: ARA290 activated pro-survival signalling and enhanced cell viability in response to H2O2-mediated oxidative stress in ECFCs in vitro. Preconditioning of ECFCs with EPO or ARA290 prior to delivery to the ischaemic retina did not enhance vasoreparative function. ARA290 delivered systemically to OIR mice reduced pro-inflammatory expression of IL-1β and TNF-α in the mouse retina. Following intravitreal transplantation, ECFCs incorporated into the damaged retinal vasculature and significantly reduced avascular area. The vasoreparative function of ECFCs was enhanced in the presence of ARA290 but not EPO.

Discussion: Regulation of the pro-inflammatory milieu of the ischemic retina can be enhanced by ARA290 and may be a useful adjunct to ECFC-based cell therapy for ischemic retinopathies.
An Usher Syndrome Type II A Knockin Model Exhibits Late-onset Retinitis Pigmentosa
Naash, M., Mwoyosi, M., Al-Ubaidi, M.
University of Houston, Biomedical Engineering, Houston, United States

Purpose: Usher syndrome (USH) causes combined blindness/deafness; disease mechanisms of this sensory loss remain elusive. To study the role of USH2A in visual impairment we generated and characterized a knockin (KI) mouse model expressing the human c.2299delG frameshift mutation.

Methods: The c.2299delG mutation causes a frameshift and premature stop codon. Message and protein levels were evaluated by RT-PCR, western blots and immunoblotting. Transmission electron microscopy (TEM) and immunohistochemistry (IHC) were used to assess the onset of structural defects while electroretinography (ERG) and optokinetic were used to evaluate the onset of visual loss at different ages of KI and wild-type (WT) mice.

Results: The KI mutation generates a stable truncated message and protein. Retinal evaluations show a delay in the recovery of dark-adapted scotopic ERG and delay in the light-dependent and protein. Retinal evaluations show a delay in the recovery of scotopic ERG and in arrestin translocation. This light dependent rescue is dependent on mice being born in the higher lighting conditions seen in KI animals is only evident at P30, not in older animals, and is dependent on mice being born in the higher lighting conditions (1200 lux).

Conclusion: This mouse is the first USH2A model with a human mutation and the only USH model that shows late-onset retinal degeneration. Data are consistent with USH patients having a late onset visual impairment. Interestingly, this is the first USH model to show a light dependent rescue of retinal dysfunction at early ages. Full characterization of this model will lead to a better understanding of the human disease and will be a valuable resource in developing targeted therapies for treatment.

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An Usher Syndrome Type II A Knockin Model Exhibits Late-onset Retinitis Pigmentosa

Retinal Cell Biology

Flavin Homeostasis in the Mouse Retina during Aging and Degeneration
Al-Ubaidi, M., Sinha, T., Makia, M., Naash, M.
University of Houston, Houston, United States

Involvement of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) in cellular homeostasis has been well established for tissues other than the retina. Here, we present an optimized method to effectively extract and quantify FAD and FMN from a single neural retina and its corresponding retinal pigment epithelium epithelium (RPE). Optimizations led to detection efficiency of 0.1 pmol for FAD and FMN while 0.01 pmol for riboflavin. Interestingly, levels of FAD and FMN in the RPE were found to be 1.7 and 12.5 folds higher than their levels in the retina, respectively. Both FAD and FMN levels in the RPE and retina gradually decline with age and preceded the age-dependent drop in the functional competence of the retina as measured by electroretinography.

Further, quantifications of retinal levels of FAD and FMN in different mouse models of retinal degeneration revealed differential metabolic requirements of these two factors in relation to the rate and degree of photoreceptor degeneration. We also found 2-fold reductions in retinal levels of FAD and FMN in two mouse models of diabetic retinopathy. Altogether, our results suggest that retinal levels of FAD and FMN can be used as potential markers to determine state of health of the retina in general and more specifically the photoreceptors.

Funding: This project was supported by NEI (R01 EY026499 and EY10609).

Flavin Homeostasis in the Mouse Retina during Aging and Degeneration

Retinal Cell Biology

NDR Kinases Regulate Retinal Development, Homeostasis and Gene Expression
Léger, H., Luca, F.
University of Pennsylvania School of Veterinary Medicine, Biomedical Sciences, Philadelphia, United States

Ndr2/Slk38 encodes a protein kinase associated with the Hippo tumor suppressor pathway. Recent studies suggest that Ndr2 protein kinase is important for retina maintenance, although its precise retinal functions are unknown. Notably, an Ndr2 loss-of-function allele causes early retina degeneration (erd) in dogs, characterized by opsin mislocalization and concurrent increases in photoreceptor apoptosis and proliferation. Separate studies indicate that Ndr2 and its paralog Ndr1/Slk38 regulate cell proliferation, morphogenesis and gene expression in other tissues. To elucidate the retinal functions of Ndr2 and Ndr1, we generated Ndr1 and Ndr2 single knockout (KO) mice and characterized their retinal phenotypes by immunofluorescence microscopy, immunoblotting and gene expression analysis. Although retinal lamination appeared normal in these mice, Ndr deletion caused a subset of Pax6-positive amacrine cells to proliferate in differentiated retinas, while concurrently decreasing the number of GABAergic and Pax6-positive amacrine cells. Retinal transcriptome analyses revealed that Ndr2 deletion increased expression of neuronal stress genes and decreased expression of synaptic organization genes.

Consistent with the latter, Ndr deletion dramatically reduced levels of Aak1, an Ndr substrate that regulates vesicle trafficking. Our findings indicate that Ndr kinases are important regulators of amacrine and photoreceptor cells and suggest that Ndr kinases inhibit the proliferation of a subset of terminally differentiated cells and modulate interneuron synapse function via Aak1. Further analyses of retinal NDR mechanisms may influence the development of therapeutic interventions for retinal degenerations and methods to stimulate photoreceptor regeneration.

Funding: This project was supported by NEI (R01 EY026499 and EY10609).

NDR Kinases Regulate Retinal Development, Homeostasis and Gene Expression

Retinal Cell Biology

Functional Roles of the Fragile X Syndrome-related Gene in the Retina
Chaya, T.1, Sugita, Y.1, Ishikane, H.1, Furukawa, T.1
1Institute for Protein Research, Osaka University, Suita, Japan
2Graduate School of the Humanities, Senshu University, Kawasaki, Japan

Fragile X syndrome (FXS) is known as one of the most prevalent forms of inherited mental retardation. FXS is triggered by the expansion of CGG trinucleotide repeats in the Fragile X mental retardation 2 (FMR2) gene, which results in transcriptional silencing and reduction of its coding protein, Fragile X mental retardation protein (FMRP). FMRP is the mRNA-binding protein that is highly expressed in neurons and regulates axonal and dendritic transport as well as translation of hundreds of target mRNAs. Therefore, dysregulation of its target mRNAs by the reduced expression of FMRP is generally recognized as the molecular pathophysiology for FXS. Although it is known that some individuals with FXS display visual sensory impairments in addition to mental retardation, the mechanism underlying sensory abnormality remains poorly understood.

We observed that Fmr1 and Cytoplasmic FMR1-interacting protein 2 (Cyfip2) are similarly expressed in the inner retina. To address in vivo Cyfip2 function in the retina, we generated and analyzed the mouse line where Cyfip2 is conditionally deleted in the retina (Cyfip2 CKO mouse). Although no significant difference in the retinal layer structure was detected between control and Cyfip2 CKO mice, the expression level of some neuronal function-associated genes decreased in the Cyfip2 CKO retina. In addition, optokinetic response analysis showed that Cyfip2 CKO mice exhibit high visual sensitivity, which resembles the visual abnormality observed in FXS patients. These results suggest that Cyfip2 is essential for gene expression regulation and normal visual function. Our results may shed light on the mechanism for abnormal visual perception observed in FXS.

Functional Roles of the Fragile X Syndrome-related Gene in the Retina

Retinal Cell Biology
Insights into Rab28-associated Retinal Degeneration from C. elegans and Zebrafish
Carter, S., 1 Jenson, V., 1 Sanders, A., 1 Kennedy, J., 1 Gomez, D., 1 Leroux, M., 1 Blacque, O., 1 Kennedy, B. 1

1University College Dublin, School of Biomolecular and Biomedical Science, Dublin, Ireland, 2Simon Fraser University, Department of Molecular Biology and Biochemistry, Burnaby, Canada, 3University College Dublin, Systems Biology Ireland, Dublin, Ireland

Cilia are microtubule-based organelles important for normal development, tissue homeostasis and sensory transduction. Various small GTPases facilitate cillum formation and function, serving as important regulators of cilary protein trafficking, including intraflagellar transport (IFT). A number of small G-proteins are also associated with human cilipathy phenotypes such as retinal degeneration. To identify new cilary transport-associated proteins, we screened available C. elegans RNA-Seq library datasets for genes with expression profiles matching those of known ciliary genes. From this analysis we found that cone-rod dystrophy-associated Rab28 (Rab28) is expressed specifically in ciliated sensory neurons, where it accumulates at the periciliary membrane (PCM) and underpins bidirectional IFT. This localisation and transport behaviour is dependent on GTP binding and the BBSome, an IFT cargo-adaptor complex which recruits GTP-bound Rab28 to the PCM. Functional analyses using a null allele revealed that C. elegans Rab28 is dispensable for cillum structure, function and IFT. However, ciliary extracellular vesicles (ectosomes), known to be important for phorotocceptor outer segment formation, accumulate in the neurons of Rab28 null worms. Additionally, Rab28 is a genetic modifier of the sensory phenotypes displayed by mutants of the Bardet-Biedl syndrome genes bbs-8 and bbs-11, indicating that, like BBS proteins, it may function in pathways important for ciliary cargo transport. To investigate the retinal functions of Rab28, we generated zebrafish rab28 knockout lines by CRISPR. Rab28 null fish display normal development and tissue homeostasis, and there are concerns about the long-term effects of VEGF inhibition. In a previous study we have shown that multiple intravitreal injections of anti-VEGF induced retinal neurodegeneration in inls2(Akita) diabetic mice. In this study we analysed the retinal vasculature in inls2(Akita) diabetic mice following multiple intravitreal injections of anti-VEGF.

Methods: Inls2(Akita) diabetic mice were injected intravitreally with 1 µl of anti-VEGF antibody (0.2 µg/µl) every 2-3 weeks for a total of 5 injections. Mice injected with an IgG control (0.2 µg/µl) and un-injected mice were used as control. Non-diabetic siblings of Inls2(Akita) mice received the same treatment. Four weeks after the last injection, eyes were enucleated and the expression of VEGF variants and tight junction genes were analysed by qPCR. Furthermore, the effects of sustained VEGF inhibition on the retinal and choroidal vasculature were analysed in retinal and choroidal flat mounts.

Results: Gene expression analysis revealed significantly increased levels of VEGF-A in Inls2(Akita) diabetic mice after 6 months of diabetes duration when compared to age-matched non-diabetic siblings. Tight junction gene expression was unaffected by repeated intravitreal anti-VEGF treatment. The number of acellular vessels in the retina of diabetic mice although the difference was not statistically significant. There were no significant differences in the vessel area to the retinal and choroidal vasculature in Inls2(Akita) diabetic and non-diabetic mice.

Conclusions: Our results suggest that five consecutive intravitreal anti-VEGF injections do not cause any structural changes, as assessed by vessel area, to the retinal and choroidal vasculature in Inls2(Akita) diabetic and non-diabetic mice.
Compromised Heterogeneity, Autophagy and Reduced Metabolic Efficiency in Retinal Pigment Epithelium (RPE) Contribute to Age-related Retinal Degeneration During “Physiological Ageing”

Shahhossein-Dastjerdi, S.1, Koina, M.2, Fatseas, G.1, Chan- Ling, T.3
1University of Sydney, Sydney, Australia, 2Canberra Hospital, Can- berra, Australia

To undertake a quantitative, ultrastructurally characterization of human RPE during fetal & early childhood development & “physi- ological ageing”. 10 fetal specimens (8-36 weeks’ gestation (W)); - human RPE during fetal & early childhood development & “physi- ological ageing” though limited in severity & extent, lead us to suggest...
Cav1.4 in Photoreceptor Synapses: More than a Pore

University of Iowa, Biochemistry, Iowa City, United States, University of Iowa, Molecular Physiology & Biophysics, Iowa City, United States, University of Iowa, Biostatistics, Iowa City, United States

Purposes: Cav1.4 is a photoreceptor specific, voltage-gated calcium channel clustered at the presynaptic active zone or ‘ribbon’ of photoreceptors. Ca²⁺ influx via Cav1.4 is essential for communication across the first visual synapse; additionally Cav1.4 has been found to be required for synaptic development. Loss of Cav1.4 or altering the activity of the channel can result in either a stationary loss of synaptic development, and limited vision in Cav1.4 knock out mouse rods and assay for vision.

Methods: We used in vivo electrophysiology to transiently express Cav1.4 in a subset of Cav1.4 knock out mouse rods and assayed for rescue of synaptogenesis using morphological markers, electrophysiological recordings of bipolar neurons, and a behavioral assay for vision.

Results: This approach allowed for rescue of Cav1.4 expression, synaptic development, and limited vision in Cav1.4 knock out mice. Furthermore, we used an inducible version of Cav1.4 to demonstrate that even in a mature mouse retina synaptic development could be induced. We next focused on testing if Cav1.4 could trigger synaptogenesis in both immature and mature mouse retinas. The second was to probe the mechanism of Cav1.4 mediated synaptogenesis.

Conclusions: We propose that Cav1.4 serves as a scaffold to organize development of the presynaptic ribbon complex. This function is distinct from role played by Cav1.4 in signaling for neurotransmitter release. The observation that adding Cav1.4 back to either immature and mature photoreceptors triggers synaptogenesis, indicates that these synapses retain a sufficient degree of plasticity to support future restorative therapies.

Hybrid Phagosome Degradation Pathways Are Required for Lipid Homeostasis

Boesze-Battaglia, K.1, Bell, B.1, Philp, N.2  
1University of Pennsylvania, Philadelphia, United States, 2Thomas Jefferson University, Philadelphia, United States

The mammalian retina relies on heterophagy and selective autophagy to maintain visual function and retinal-RPE homeostasis. Three genes encode the highly homologues, (LC3) proteins, LC3α (LC3A), LC3β (LC3B) and LC3γ (LC3C). Whether the LC3 isoforms are functionally redundant or play a specialized role in maintaining RPE homeostasis is the focus of our studies. Within the retinal pigment epithelium (RPE) of the eye, the daily phagocytosis of lipid-rich photoreceptor outer segments provides a constant degradative burden to these terminally differentiated cells. The purpose of this study is to determine the role of the LC3B isomert in retinal lipid homeostasis. We examined the structure and function of the LC3B -/- mouse retina as a function of age using in vivo ocular imaging and electrophotography coupled with ex vivo, lipid metabolism as well as IHC of lipid aggregates. Deletion of the LC3B isoform resulted in defects within the RPE including increased phagosome accumulation, decreased lipid metabolism and subsequent increase in sub-retinal lipid deposits. Age-dependent degeneration of the RPE occurred, with elevated levels of oxidized cholesterol, subretinal migration of activated microglial cells, deposition of 4-HNE lipid peroxidation products, RPE hypertrophy, and loss of retinal function. These observations are consistent with a critical role for LC3-dependent processes in the maintenance of normal lipid homeostasis in the aging eye and suggest that LC3 isoform specific disruption in autophagic processes contribute age-related disease.

Ablation of the Rod Specific Protein Retbindin Impairs the Metabolic Homeostasis of the Retina Leading to Progressive Degeneration of Both Rods and Cones

1University of Houston, Biomedical Engineering, Houston, United States, 2Department of Ophthalmology, Angleton-Danville, United States, 3University of Houston, Biomedical Engineering, Houston, United States

We have previously reported a rod and cone degeneration model following the knockout of the retina specific flavin binding protein retbindin (Rtbdn-/-). We showed a reduction in retinal flavin and ATP levels concomitant with reduction in ERG response at P120 and the onset of degeneration. Flavins being directly involved in multiple metabolic pathways, goal of the study was to identify the underlying mechanism of this retinal degeneration using metabolomics analyses on Rtbdn-/- P45 retinas.

We found that individual glycolytic products are elevated in the Rtbdn-/- (N=14) while intermediates of the TCA cycle were largely unchanged. This was confirmed by using C13-Glucose (N=10) where by almost all the glycolytic products were enriched several fold with the glucose isotope. This suggested that the glycolytic flux is elevated in the Rtbdn-/- . Since FAD and FMN are essential for the TCA cycle and ETC, using C13-Glucose we evaluated whether the TCA cycle flux would be compromised. We consistently observe lower intensity of C13-Glucose labeled TCA cycle intermediates in the Rtbdn-/- retinas. This is in agreement with the untargeted metabolomics analyses, where we observe significantly elevated aconitate (a critical TCA cycle intermediate) levels in the Rtbdn-/- retinas, indicating reduction of downstream enzyme activity. The C13-Glucose data further showed that labeled isotope was incorporated in glutamine at threonate the rate of wild type. This indicates that glutamine and potentially other amino acid metabolism was also compromised. A separate untargeted lipophilic metabolomic analyses (N=9) revealed that in the absence of adequate flavins, almost all the intermediates of fatty acid synthesis, acylcarnitine fatty acid metabolism, ceramides, sphingomyelins and ketone bodies were significantly elevated in the Rtbdn-/- animals while specifically the species of PE-DHA were significantly reduced.

In summary, elimination of retbindin led to reduced flavins and significant metabolic dysregulation with possible inhibition of certain enzymes involved in mitochondrial metabolism like isocitrate dehydrogenase. Also, the accumulations of fatty acid and lipid metabolism intermediates are in agreement with clinical reports of patients suffering from flavoprotein mutations specific to other organs and thus shed a new light on retinal pathology. Our findings will also yield a novel window to studies addressing the role of flavins and flavin binding proteins in retinal cell biology.
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