Progress reports on research supported by ORIA Institute grants

Genetics of Corneal Thickness in Glaucoma

Dr Kathryn Burdon

ORIA/Glaucoma Australia Inc – Two year project 2007/2008

This two-year project has completed the proposed aims. Data analysis and preparation of publications will continue for the next few months. Three conference presentations have resulted from this work. Four manuscripts are in preparation or under review and a review of the area has recently been accepted for publication. The large amount of data collected has provided the basis for an NHMRC project grant application which began in 2009.

Aim 1. Investigation of inter-strain variability of CCT in mice and rats and subsequent genetic mapping of CCT in divergent strains of mice

We have measured central corneal thickness (CCT) in fourteen strains of laboratory mice (Table 1). This clearly shows that CCT varies between strains. A high level of statistical significance was achieved with several comparisons, providing additional evidence of the genetic component of this trait, suggesting that genes can be identified for this glaucoma risk factor (manuscript in preparation). We observed that the six strains with the thickest CCT were all albino and the pigmented animals had thinner CCT on average.

Table 1: Mean CCT for 14 strains of mice and correlation with coat colour

Strain	Coat Colour	n	Mean CCT(µm)
DBA/1J	Grey	6	70.6
DBA2	Grey	5	71.1
C3H/HeJ	Brown	5	73
C57B1/KALWRIJ	Black	5	73.6
AJ	White	28	74.4
CBA/C7H	Brown	6	79.3
C57BL/6J	Black	12	82.6
C3H	Brown	7	84.8
SJL/J	White	5	86.8
FUB/NJ	White	6	87.4
BALB/C	White	25	87.6
Nod LT	White	6	87.9
129/SV	White	4	88.6
AKR	White	6	95.5

To investigate this further, we measured CCT on three strains that had spontaneous mutations in genes related to *pigment*, *tyrosinase*, *p gene* and *slc45* (Table 2). These experiments showed a significant difference in the CCT of mice with a null mutation of the *tyrosinase* gene and *slc45* genes, but not for the *p* gene (manuscript in preparation). The point mutation in the *tyrosinase* gene results in complete albinism in the homozygous mutants. To further investigate the genetics of CCT, we measured the trait in a strain with a known mutation in the *col1a2* gene, encoding collagen 1A, a major structural component of the cornea. These experiments clearly showed a significant difference in CCT between wildtype and homozygous mutant mice (Table 2). Further, electron microscopy of the corneas shows significantly thinner collagen fibrils in the mutant mice, indicating that this mutation affects the structure of the cornea (manuscript submitted). This finding has lead directly to collaboration with Dr Bang Bui at University of Melbourne who

is now testing the potential for glaucomatous damage in the *col1a2* mice with thick and thin corneas to investigate further the link between CCT and glaucoma.

Table 2: CCT of mouse strains with known mutations in pigment or corneal candidate genes

Gene	Genotype	Coat Colour	n	Mean CCT (µm)	P-value
Tyrosinase	wildtype	Black	12	82.6	0.007
	mutant	Albino	22	71.8	
P-Gene	wildtype	Brown	7	84.8	0.312
	mutant	Yellow	10	88.7	
SLC45	wildtype	Black	7	76.4	0.042
	mutant	Grey	11	68.8	
Col1a2	wildtype	Black	19	87.7	0.006
	mutant	Black	10	74.5	

Due to the innate measurement error involved in measuring such small thicknesses in mice, we have determined that it is difficult to assign a specific animal a CCT measurement. Thus, we have not pursued the planned breeding program and subsequent linkage analysis, which relies on accurate phenotyping of individual mice. Instead, we have conducted expression arrays in our thickest and thinnest strains of mice to identify genes that are differentially expressed in thick and thin corneas. Following the success of the project in mice, we have also measured several strains of rat and have conducted the same expression analysis on rat tissue. Rat is proving to be a useful model due to the larger cornea and has allowed us to expand the research into investigating the proteomics of CCT as well as genetics. The large amounts of array data are currently undergoing analysis and will be assesssed in conjunction with our human genome wide association scan recently funded by the NHMRC.

Aim 2. Candidate gene analysis in human samples

In funding this project, the review panel reduced the budget stating that it was unnecessary to genotype 1000 individuals. We disagree with this assessment as there is no real way to know the genetic architecture of this trait, and thus the power calculations are completely arbitrary. We have modified our approach in order to type up to 1000 people within the new budget. In total 143 SNPs were selected from 17 candidate genes including structural corneal proteins, developmental transcription factors, genes associated with disease where extreme CCT has been noted and pigment related genes (based on the mouse work outlined above). All SNPs were genotyped in 400 samples from the Blue Mountains Eye Study (BMES), representing the top and bottom quintiles of the CCT distribution. SNPs that were found to be associated in this comparison of thinnest vs. thickest were then genotyped in the remaining 600 BMES samples with CCT measurements.

Table 3: Most significantly associated SNPs with CCT as a quantitative trait in 1000 'normal' BMES samples

Gene	SNP	P-value	CCT difference by genotype (µm)
COL1A1	rs2696297	0.005	21
	rs1046329	0.006	16
COL1A2	rs1034620	0.010	6
PAX6	rs3026398	0.019	5
MC1R	rs2270459	0.0008	10
	rs3212346	0.006	7
Fibrillin	rs17352842	0.022	5

Several genes were found to be consistently associated with CCT (Table 3). Of note are the *COL1A1*

and *COL1A2* genes, consistent with the findings from the mouse study described above. This study shows for the first time, that common variants in both genes are associated with normal central corneal thickness variation of up to 21µm on average (manuscript submitted). Other genes showing significant associations include *PAX6* (transcription factor, aniridia gene), *MC1R* (pigment related), and *Fibrillin* (Marfan syndrome gene) (manuscripts in preparation). This study is the first to identify genetic variants contributing to normal corneal thickness variation. These positively associated genes are now being assessed in our glaucoma cohorts.

Publications and presentations

1. David P Dimasi, Kathryn P Burdon, Jamie E Craig. The genetics of corneal thickness. *British Journal of Ophthalmology* Accepted 11 June 2009.
2. 2008 Human Genetics Society of Australasia – 32nd Annual Scientific Meeting, Adelaide, August 2–6. Dimasi DP, Burdon KP, Hewitt AW, Mitchell P, Mackey DA, Craig JE. Identification of genes determining the normal population distribution of central corneal thickness (oral presentation).
3. 2008 European Glaucoma Society Conference, Berlin, June 1–6. David P Dimasi, Jern Chen, Kathryn P Burdon, Alex W Hewitt, Ravi Savarirayan, David A Mackey, Paul Mitchell, Jamie E Craig. The role of *COL1A1* and *COL1A2* genes in central corneal thickness variation in normal individuals and Osteogenesis Imperfecta (poster).
4. 2009 7th Australian Human Gene Mapping Conference, Katoomba, April 14–17. Platform Presentation. Dimasi DA, Chen JY, Davey R, Savariryan R, Mackey DA, Mitchell P, Burdon KP, Craig JE. Novel quantitative *trait loci* for central corneal thickness identified by candidate gene analysis of osteogenesis imperfecta genes (oral presentation).

Genetic Study of Diabetic Retinopathy

Dr Kathryn Burdon and Dr Sue Abhary

ORIA/RANZCO Eye Foundation Grant

In total, over the last two years (including one year of funding), we have recruited over 1000 patients with various stages of diabetic retinopathy (DR) and either Type 1 (T1DM) or Type 2 Diabetes Mellitus (T2DM) (Table 1). Patients with proliferative (PDR), severe non-proliferative (NPDR) or clinically significant macular edema (CSME) are also classified as having 'Blinding DR' and are analysed together as a group, as well as separately.

Table 1: Number of patients recruited by type of diabetes and stage of retinopathy. Some patients belong to more than one group.

Group		T1DM	T2DM
1	No DR	103	304
2	Minimal DR	44	55
3	Mild DR	34	72
4	Moderate DR	12	59
5	PDR	73	138
6	Severe NPDR	5	40
7	CSME	37	151
8	Blinding DR (groups 5+6+7)	115	329
	Total Patients	**308**	**751**

Extensive data on relevant clinical covariates has been collected. These data show that the expected correlations exist in our cohort, primarily that nephropathy, degree of glycemic control (HbA1c) and duration of diabetes are all significantly correlated with DR, indicating the generalisability of our findings to other populations of DM and DR patients. These correlations alter slightly when the cohort is stratified by type of diabetes as would be expected (not shown). Of interest are the differing correlations between PDR and CSME, where sex is a risk factor for PDR but not CSME and smoking trends as a risk factor for CSME but not PDR. This supports the different etiologies of these components of the DR phenotype and justifies analysing these phenotypes as separate entities.

The first 554 subject samples to be collected have been used in candidate gene studies of *VEGFA*, *EPO* and *CA1* (Table 2). *VEGFA* has long been postulated as a causative factor in DR given its role in angiogenesis (in relation to PDR) and vascular permeability (in relation to CSME). We have typed 15 tag SNPs and found several to be associated in both types of diabetes. Relatively large odds ratios were detected for several SNPs indicating a major effect for this gene This has been accepted for publication in IOVS[1]. Interestingly, different SNPs were seen to be associated in T1DM than T2DM. The reason for this is unknown and it will be interesting to see if similar results are obtained at other genes or in the larger cohort now recruited.

Table 2: Candidate gene studies in first 554 participants

Gene	No. SNPs typed	SNP or Haplotype	Allele or Genotype	P-value T1DM	P-value T2DM	OR (95%CI)
VEGFA	15 tags	rs699946	AA	**0.007**	0.64	4.1 (1.5-11.4)
		rs833068	GG	**0.017**	0.280	3.1 (1.3-7.2)
		rs3025021	C	0.940	**0.002**	3.8 (1.5-10.0)
		rs10434	G	0.660	**0.002**	2.6 (1.3-5.3)
EPO	3	Haplotype*	GCC	Not sig	**0.008**	
CA1	10 tags	No associations observed				

*Haplotype of SNPs rs1617640, rs507392 and rs551238

EPO, encoding erythropoietin, was also recently found to be associated with DR. Although the mechanism of action with regards to DR is unknown, this protein is expressed by the retina, particularly under ischemic conditions. Our dataset finds significant association with the same SNP haplotype has previously reported. This manuscript is undergoing revisions at *Archives of Ophthalmmology*.

Carbonic Anhydrase (*CA1*) was chosen as a candidate following a report that the protein is found at greater levels in the vitreous of PDR patients and caused macular edema in a rodent model. Ten tag SNPs were typed, however no associations were identified, suggesting that the presence of high levels of CA in the vitreous may be a result of PDR rather than a cause, or genetic loci other than the gene itself are responsible for regulating CA levels in the retina. This work has been accepted for publication in *Molecular Vision*[2].

In addition to the genetic studies we have undertaken preliminary investigations of serum markers in blinding DR. Asymmetrical dimethylarginine (ADMA), symmetric dimethylarginine (SDMA) and L-arginine are serum analytes that play a role in nitric oxide (NO) bioavailability. The NO pathway is likely to be involved in DR via its effects on the microvasculature. ADMA, SDMA and L-arg are reportedly elevated in the plasma and aqueous of DR patients. Our own data strongly support an association of these markers with DR (manuscript under review at *Diabetes Care*). We have assayed ADMA, SDMA and L-arg by mass spectroscopy in patients with blinding DR and found significantly higher levels in these subject when compared to control subjects with DM but no DR ($p<0.001$), particularly in T1DM. This is the largest DR cohort to have been investigated for these analytes and confirms the postulated association between ADMA or SDMA and DR seen in much smaller cohorts.

Although this study aimed to conduct preliminary candidate gene studies, the long term aim is to conduct a genome-wide association scan. The funding provided for this project has allowed significant progress to be made in the recruitment of a suitable cohort for genome-wide studies and has allowed the

candidate gene studies, demonstrating the feasibility of the approach. This data has been used as the basis for an application to the NHMRC in the current funding round. The size of our data set is now such that we have been able to attract International collaborators to set up reciprocal replication agreements, whereby each group will attempt to replicate the findings of the other.

Publications and presentations

1. Abhary S, Burdon KP, Gupta A, Lake S, Petrovsky N, Craig JE. Common sequence variation in the *VEGFA* gene predicts risk of diabetic retinopathy. *Invest Ophthal Vis Sci.* Accepted 6 June 2009.
2. Abhary S, Burdon KP, Gupta A, Petrovsky N, Craig JE. Diabetic retinopathy is not associated with carbonic anhydrase gene polymorphisms. *Molecular Vision.* Accepted 29 April 2009.
3. Abhary S, Hewitt AW, Burdon KP, Craig JE. A systematic meta-analysis of genetic association studies for diabetic retinopathy. *Diabetes.* Accepted 27 May 2009.
4. 2008 Royal Australian and New Zealand College of Ophthalmologists annual meeting. The genetic study of diabetic retinopathy. Abhary S, Burdon KP, Petrovsky N, Craig JE (oral presentation).
5. 2008 Human Genetics Society of Australasia – 32nd Annual Scientific Meeting, Adelaide, August 2–6. The Genetic Study of Diabetic Retinopathy. Abhary S, Burdon KP, Petrovsky N, Craig JE (oral presentation).

Age-related mitochondrial dysfunction and the vulnerability of retinal ganglion cells to oxidative stress in glaucoma

Prof Jonathan G Crowston

ORIA/RANZCO Eye Foundation Grant – Two year project 2007/2008

Hypothesis

1. Mitochondrial dysfunction associated with increasing age increasing age sensitizes retinal ganglion cells to injury.
2. Treatments that delay aging protect retinal ganglion cells from injury

Study aims

The specific aims of this study are to investigate the effect of aging on the functional response of retinal ganglion cells to acute IOP elevation in mice; and investigate whether calorie restriction alters age-related vulnerability to injury.

Delaying age-related vulnerability of the optic nerve to glaucoma damage

Glaucoma is a disease of the optic nerve that is estimated to affect over 60 million people worldwide, of these 10% are expected to go blind from this disease. The two most common risk factors for glaucoma are increased intraocular pressure and advanced age. We propose that aging of the optic nerve increases its vulnerability to glaucoma stress, and that the aged nerve is less able to repair itself from damage.

We are aging rodents and investigating the response of the retinal ganglion cells in the optic nerve to a glaucoma challenge by increasing the pressure in the eye (IOP) then measuring cellular function with the electroretinogram (ERG). By examining the ERG response to IOP (Figure 1 – grey area) we can determine the sensitivity of cells to stress. By releasing the pressure we can then see the recovery of cells after stress (Figure 1 – white area) back to 100% function. This data uniquely combines specific analysis of retinal ganglion cell function *in vivo* over real time in response to IOP.

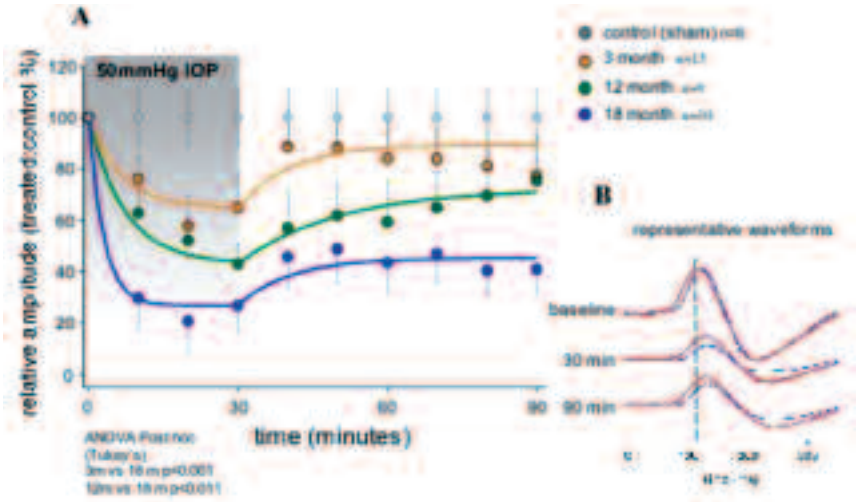


Figure 1: Old eyes show greater dysfunction and slower recovery to IOP stress

Electroretinogram ganglion cell response (positive scotopic threshold response pSTR) demonstrates greater amplitude loss during intraocular pressure challenge (50 mmHg for 30 minutes) and slower recovery in old animals compared to young animals.

As seen in Figure 1, younger mice (3 months) are less sensitive to IOP challenge than older mice (18 months). Younger mice also recover from the IOP challenge better than the older mice. This suggests that aged retinal ganglion cells are more vulnerable to damage and are less able to recover from stress.

Calorie restriction reverses IOP induced oxidative stress

Calorie restriction or intermittent fasting, has been shown to reduce oxidative stress, increase lifespan and delay age related diseases such as cancer and neurodegeneration in animal models. In the latter case calorie restriction has shown to improve resistance of cultured neurons to damages. Calorie restriction decreases oxygen consumption by mitochondria, limiting the formation of damaging reagents called free radicals. We are investigating this as a mechanism to reverse or delay the aging process and protect the optic nerve against IOP challenge. Calorie restriction took place on 12 month mice, for 6 months, and these mice were compared to 18 month *ad-libitum* mice. Figure 2 shows that 18 month calorie restriction mice respond to IOP stress in a similar way to 3 month mice, unlike the 18 month *ad-libitum* mice. This indicates that calorie restriction protects the optic nerve against IOP challenge and may delay or reverse the aging process.

Mitochondrial dysfunction contributing to vision loss

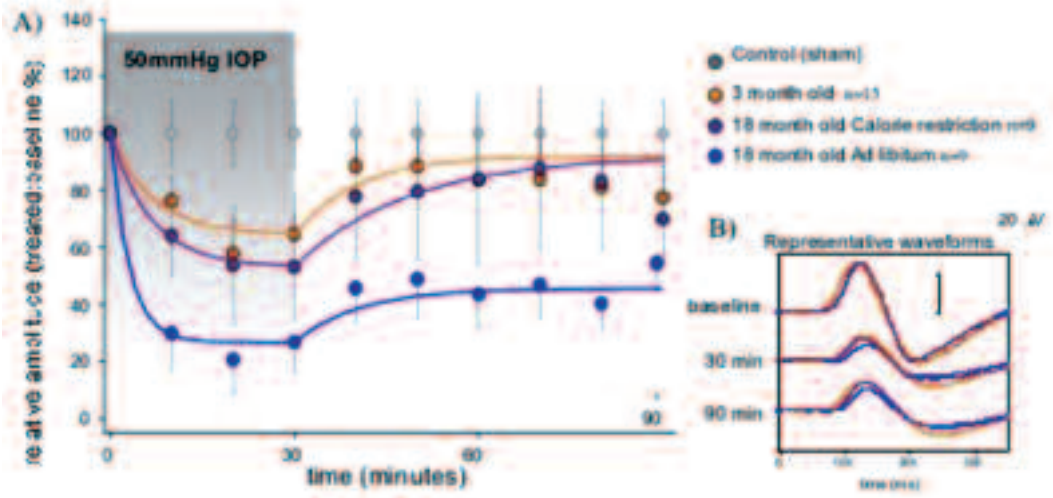
Mitochondrial oxidative phosphorylation (OXPHOS) dysfunction is believed to play an important role in loss of the optic nerve in aging, as well as in inherited optic nerve diseases. We hypothesise that defects in mitochondria may predispose the cells of the optic nerve (retinal ganglion cells) to injury and death. We also hypothesise that modulating the bioenergetic activity of mitochondria may provide resistance against IOP injury.

Defects in mitochondrial energy production contribute to aging of the optic nerve

Transgenic mice with a defect in the mitochondrial DNA repair mechanisms experience accelerated aging. We show below that the older these mice are, the more defective their retinal ganglion cells are (Figure 3). We show that these mice with defective mitochondrial function have impaired inner retinal function with increased age. Thus aging and mitochondrial dysfunction both contribute to optic neuropathy.

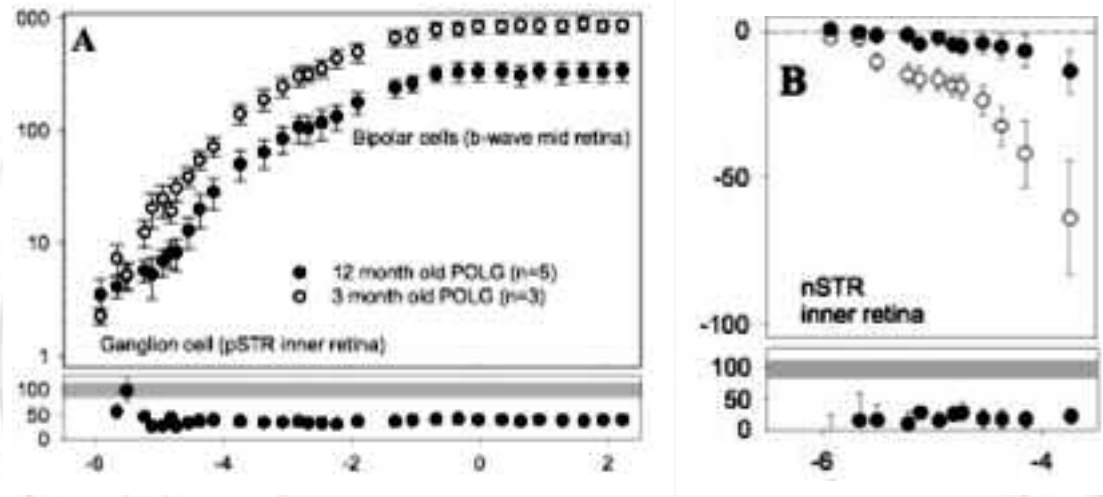
Summary

These data indicate that aging predisposes the optic nerve to IOP-related injury and that the effect of aging can be reversed by diet manipulation. Preliminary data point to mitochondrial dysfunction underlying this effect. Future work will evaluate the effect of pharmacological agents that increase mitochondrial function on theis injury paradigm.



A) 18 month old mice which had 6 months of calorie restriction showed significantly less amplitude loss during intraocular pressure challenge (50 mmHg for 30 minutes) and slower recovery compared to age-matched mice that were fed *ad libitum*. Eighteen month old calorie restricted mice showed similar IOP related function response as young 3 month old mice. **B)** Representative STR waveforms.

Figure 2: Calorie restriction reversed aged-related increased dysfunction and slower recovery to IOP challenge



A) Maximum positive component of electroretinogram waveform from dim (ganglion cell pSTR range) to bright (b-wave bipolar cell range) intensities. Twelve month old POLG mice showed >50% reduction in response amplitude for both ganglion cell and bipolar cell response. This degree of reduction is much more severe than could be accounted for by aging alone. **B)** Inner retina nSTR showed almost complete loss of response in older POLG mice.

Figure 3: Mitochondrial DNA POLG defect causes severe age-related acquired optic neuropathy

Publications

1. Leung CK, Lindsey JD, Crowston JG, Chen LJ, Chiang SW, Weinreb RN (2008) Longitudinal profile of retinal ganglion cell degeneration after optic nerve crush with blue-light confocal scanning laser ophthalmoscopy. *Invest Ophthalmol Vis Sci* 49: 4898–902.

2. Van Bergen NJ, Wood JP, Chidlow G, Trounce IA, Weinreb RN, Ju W-K, Crowston JG (2009) Re-characterisation of the RGC-5 retinal ganglion cell line. *Invest Ophthalmol Vis Sci* in-press, April 2009.

3. Kong G, Trounce IA, Van Bergen N, Crowston JG (2009) Mitochondrial dysfunction in glaucoma. *Journal of Glaucoma* 18: 93–100.

4. Yu X Kong, Crowston JG, Vingrys AJ, Trounce IA, Bui BV. Functional Changes in the Retina During and Following Acute Intraocular Pressure Elevation in Mice. *Invest Ophthalmol Vis Sci* in-press June 2009.

Oxidative phosphorylation defects and the optic nerve response to acute IOP injury in the xenomitochondrial mouse

Prof J Crowston and Dr Ian Trounce

ORIA/Glaucoma Australia Inc Grant

Background

- The pathophysiology of how aging increases risk of glaucoma is not well understood.
- Energy (ATP) production by mitochondria through oxidative phosphorylation (OXPHOS) is compromised in normal aging and age-related neurodegenerative diseases.
- Xenomitochondrial mouse is a novel animal model with nuclear-mitochondria DNA mismatch-polymorphisms useful for examining the role of mild OXPHOS impairment in mediating age-related vulnerability to stress.

Hypothesis

Impairment of mitochondrial oxidative phosphorylation sensitises retinal ganglion cells to intraocular pressure-induced oxidative stress.

Study aims

To evaluate the retinal ganglion cell response to acute IOP elevation in the xenomitochondrial mouse; and to characterise the nature and extent of OXPHOS impairment in xenomitochondrial tissue and cybrids.

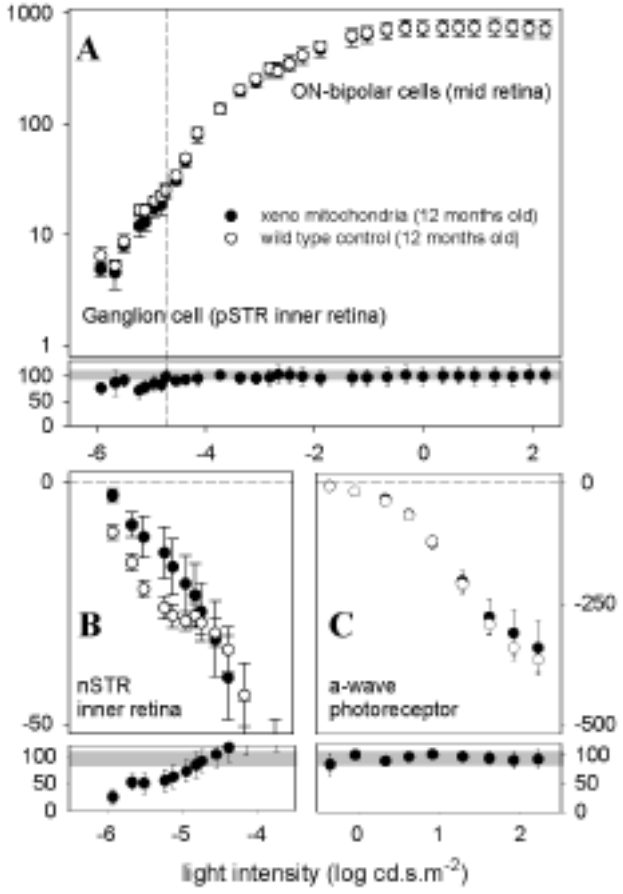
Experimental methods 1

To evaluate the response of the retinal ganglion cells to acute IOP elevation in the xenomitochondrial mouse. These experiments will determine whether mild impairment in OXPHOS in the xenomito-chondrial mouse alters retinal ganglion cell vulnerability to oxidative damage induced by acute IOP elevation.

Experiment and results 1:
Mild impairment in OXPHOS has a selective effect on inner retinal (retinal ganglion cell) function

- Electroretinograms (ERG) were performed on anaesthetised (ketamine:zylaxine) 12-month-old xenomitochondrial mice and age-matched wildtype C57/BL6 controls. (Figure 1) Retinal function was analysed in detail by separating the overall ERG into ganglion cell (pSTR), inner retinal function (nSTR), bipolar cell (b-wave) and photoreceptor (a-wave) function.
- Ganglion cell function in xenomitochondrial mice was found to be ~20% less compared to age matched controls. Inner retinal function (nSTR), which has ganglion cell and amacrine cell contributions, showed greater impairment 57±5% compared to controls.
- Bipolar cell and photoreceptor cell functions were unaffected in 12-month-old xenomitochondria mice compared to age-matched controls.

This suggests that in 12-month-old xenomitochondrial mice, mitochondrial impairment has a greater effect on inner retinal function compared to outer retinal function. Initial experiments were performed on only four mice. In light of the small differences observed, compared to control wild type mice, we have elected to age the remaining xenomitochondrial mice to 18 months to maximise any differences. It is hypothesised that mitochondrial function will decline further in a non-linear manner with further aging.



Electroretinogram (ERG) performed on 12-month-old xenomitochondrial mice (dark symbols: n=4) and age-matched controls (white symbols: n=11) and the response amplitudes were measured and plotted against flash intensity.

(**A**) 12 month old xenomitochondrial mice showed ~20% reduction ganglion cell response amplitude compared to age matched controls. Bipolar cell response is within normal range of age-match controls.

(**B**) Inner retina nSTR showed 57±5% loss in xenomitochondrial mice compared to controls.

(**C**) Outer retina photoreceptor response showed no impairment in response amplitude in xenomitochondrial mice.

Figure 1: Xenomitochondrial mice causes mild selective inner retinal impairment compared to controls

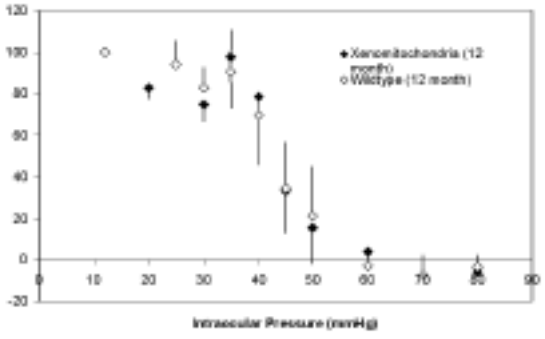
Retinal neuronal function during and following IOP challenge in xenomitochondrial mice

Xenomitochondrial mice 12-month-old and age-matched wildtype controls were subjected to acute intraocular pressure elevation challenge by cannulation of anterior chamber with fine glass needle. Functional changes in the retinal neurons were monitored using ERG.

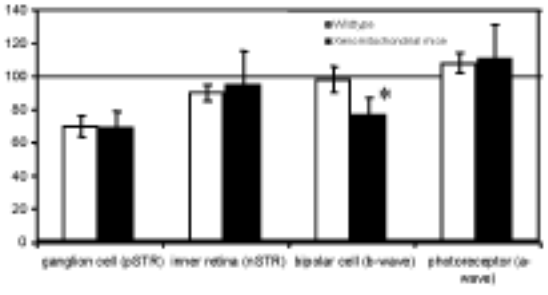
Intraocular pressure was elevated in steps of 5–10 mmHg from baseline up to maximum of 80 mmHg, where ischemia may play a role. Changes in ganglion cell (pSTR) function of the two cohorts relative to their own baseline with IOP is shown in Figure 2. Xenomitochondrial mice and wildtype mice showed similar response to IOP induced functional loss. ERG function measurements were repeated in both xenomitochondrial and wildtype mice one week following the acute IOP elevation experiment. Both cohorts have persistent impairment of ganglion cell function (pSTR) of ~30%. Xenomitochondrial mice showed less bipolar cell recovery compared to wildtype mice (98% vs 77%, $p = 0.04$).

This suggests that while xenomitochondrial mice experience similar impairment compared to wildtype mice when exposed to raised IOP, there are subtle differences in the rate of recovery of the cell components following such challenge.

A



B



A) Effect of increasing level of IOP on ganglion cell function (pSTR) as measured by ERG. Xenomitochondrial mice (dark symbols) showed similar response compared to wildtype (white symbols) mice.

B) Recovery at 1 week following acute IOP elevation of various retinal neuronal cell components is shown. Both xenomitochondrial and wildtype mice showed significant persistent ganglion cell impairment (~30% loss). Xenomitochondrial mice showed significantly less (p=0.04) bipolar cell recovery compared to wildtype mice.

Figure 2: Retinal function response to IOP in Xenomitochondrial mice

Experimental methods 2

To characterise the specific OXPHOS defects in xenomitochondrial mouse cell cybrids.
Although mild metabolic defects have been demonstrated in the xenomitochondrial mouse, a more detailed analysis of OXPHOS enzyme complexes is required to better characterise this model and determine the key players that may modulate cellular resistance to oxidative pressure-induced oxidative damage.

Experiment and results 2:

Xenomitochondrial mouse cell cybrids show reduced OXPHOS enzyme respiratory capacity

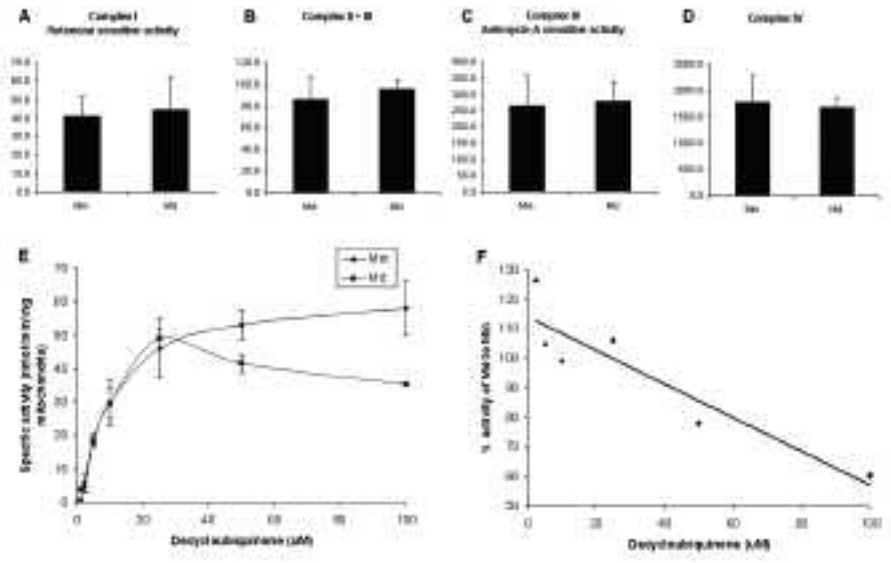
Fibroblast cybrids were used to for enzymology studies. Mus musculus fibroblast cells were used as controls. Cultured fibroblasts were expanded, mitochondria isolated and OXPHOS enzyme rates measured.

Using the standard enzyme kinetic assays there was no difference in the respiratory capacity of complex I (Fig 3A), complexes II+III (Fig 3B), complex III (Fig 3C) or complex IV (Fig 3D) between xenomitochondrial cybrid fibroblasts (Md) and control Mus musculus fibroblasts (Md).

However decreased respiratory capacity of Complex I of the xenomitochondrial cybrid fibroblasts compared to control cells was found by titration of Complex I substrate (decylubiquinone) concentration (Fig 3E). This proportional activity of xenomitochondrial to control cells decreased linearly with increasing substrate (Fig 3E).

Summary

Xenomitochondrial mice provide a unique genetic model of mild mitochondrial dysfunction which results from introducing mismatch polymorphisms between mitochondrial DNA and nuclear DNA. Reduction in ganglion cell and inner retinal neuronal function was detected in xenomitochondrial mice compared to controls at 12 months of age. Subtle impairment in functional recovery particularly of bipolar cells from IOP challenge was found in xenomitochondrial mice. *In vitro* experiments suggest that in an unstressed environment there is no difference between xenomitochondrial and wildtype enzyme activity. Impairment in mitochondrial respiration capacity can be found when mitochondrial enzyme complexes were stressed by altering conditions of their environment.



A)–D) No significant difference was found in OXPHOS enzyme activity for complexes I, II+III, III and IV between xenomitochondrial fibroblasts (Md) and control Mus musculus fibroblasts (Md).
E) Xenomitochondrial fibroblast showed decreased respiratory capacity compared to control as shown by decrease in enzyme activity with increasing substrate concentration for Complex I.
F) Proportional activity of xenomitochondrial to control cells decreased linearly as substrate increased.

Figure 3: OXPHOS enzyme analysis of xenomitochondrial fibroblast cells.

Our results suggest that further detailed studies examining the changes in mitochondrial activity and biogenesis following IOP in xenomitochondrial mice will contribute to understanding of the role mitochondria play in age-related increase in neuronal vulnerability to stress.

Publications

Kong GY, Van Bergen NJ, Trounce IA, Crowston JG. Mitochondrial dysfunction and glaucoma. *J. Glaucoma* 2009 Feb; 18(2):93–100.

Van Bergen NJ, Trounce IA, Wood JP, Chidlow G, Casson RJ, Crowston JG. Re-Characterisation of the RGC-5 Retinal Ganglion Cell Line, *IOVS 2009* May 14 [Epub ahead of print].

Kong Y, Bui BV, Trounce IA, Vingrys AJ, Crowston JG. Functional Changes in the Retina During and Following Acute Intraocular Pressure Elevation in Mice, *IOVS* (in-press accepted June 2009).

Mechanisms of lithium's promotion of the lens epithelial phenotype and inhibition of TGF beta-induced cataract

Prof JW McAvoy and Dr FJ Lovicu

ORIA/GJ Williams Grant

The TGFß family of growth factors induce cataracts that involve disturbed epithelial growth. Our major focus has been to identify ways of promoting the normal behaviour of lens epithelial cells and blocking the cataractous effects of TGFß. Recently we showed that the simple salt, lithium chloride, promotes normal

epithelial cell behaviour and blocks the effects of TGFß. This finding has major clinical significance and the aim of this project was to determine how lithium brings about these beneficial effects.

Does lithium inhibit activation of cytoplasmic kinases?

Because LiCl so effectively promotes the quiescent polarized lens phenotype, it is important to understand the molecular basis of this phenomenon. In addition to well-known effects on promoting Wnt/ß-catenin signalling, there is now a growing appreciation for lithium's other effects on key cell signalling pathways. As our previous studies showed that growth factor-induced lens epithelial cell proliferation depends on ERK signalling and as more recent results showed LiCl inhibited cell proliferation, we assessed the effects of LiCl on ERK1/2 activation. Our results have shown that LiCl significantly inhibits ERK1/2 phosphorylation in lens epithelial explants cultured at low cell density. LiCl also inhibits ERK1/2 activation by TGFß (and FGF). In the absence of LiCl, phospho-ERK1/2 is clearly activated in most of the proliferating, depolarised cells in low density explants; whereas in the presence of LiCl little phospho-ERK is evident. Further investigations into the mechanisms of lithium's action, showed that LiCl promotes the maintenance of the receptor tyrosine inhibitor, Sprouty2 (Spry2), in lens epithelial explants. This is consistent with our findings that members of the Spry family, as well as the Sef family, of receptor tyrosine kinase inhibitors are expressed in the lens epithelium. Given this finding we have proposed that Sef/Spry expression/activity in the lens epithelium is critical for regulating ERK1/2 signalling, and subsequently regulating cell proliferation and differentiation. In other words we propose that Sef/Spry are important factors for maintaining the lens epithelial phenotype. This has opened up a new area of research for us and is the basis of current grant applications to study the role of endogenous receptor tyrosine kinase inhibitors (Sef, Spry and the newly identified Spreds) in maintaining the epithelial phenotype.

Does TGFß promote Wnt/ß-catenin/TCF signalling?

Studies have concentrated on identifying the various signalling pathways that are modulated by TGFß during destabilisation of the lens epithelium and cataract formation. This focus was considered necessary because studies in other systems showed that TGFß-induced EMT is associated with ß-catenin/TCF signalling and this is characterised by nuclear translocation of unphosphorylated (stabilised) ß-catenin. Similarly our lens studies showed that TGFß-induced EMT/fibrosis is linked with nuclear translocation of stabilised ß-catenin. This raises the question of the ß-catenin/TCF signalling pathway being a key mediator of TGFß-induced EMT.

Support for involvement of Wnt signalling in TGFß-induced EMT/fibrosis comes from our most recent studies that have shown substantial upregulation of Wnts accompanies the development of cataract (Wnts are the main ligands for activation of ß-catenin/TCF signalling). For example, when whole rat lenses are cultured with TGFß opaque subcapsular plaques develop after five days. These have been shown in earlier studies to have the same morphological and molecular features of anterior subcapsular cataract in humans. As shown by RT-PCR and in situ hybridisation, several Wnts are strongly expressed in these TGFß-induced subcapsular plaques (Figure). In fact, out of the six Wnts assessed only one, Wnt 7a, is not upregulated.

Thus, given the ability of TGFß to upregulate Wnts and Frizzleds, this indicates that Wnt/ß-catenin/TCF signalling pathways may be involved in TGFß-induced EMT and other events in cataract development. Whilst nuclear localisation of ß-catenin and upregulation of signalling components are good indicators of Wnt/ß-catenin/TCF signalling, the acid test comes from studies on ß-catenin/TCF reporter mice. We now have several lines of these imported from USA and plan to use these to further investigate the role of Wnt/ß-catenin/TCF in TGFß-induced cataract. We are also aware that upregulation of Wnts and Frizzleds may be an indicator of activation of a ß-catenin independent Wnt pathway, namely, the planar cell polarity pathway. This possibility will also be tested in future studies.

Significance

The main findings from this project have given us major insights into important novel mechanisms and molecules that regulate the behaviour of lens cells. Overall, these studies have provided further insights into mechanisms of growth factor signalling in regulation of key processes in pathological lens development. In

addition, in relation to cataract prevention it has led to the identification of new targets for key therapeutic agents that will slow or prevent cataracts that involve EMT and aberrant lens cell growth. Finally, our aim of elucidating the mechanisms and molecules underlying the formation and maintenance of lens cells brings us closer to achieving our long-term goal of regenerating normal lens structure and function following cataract surgery.

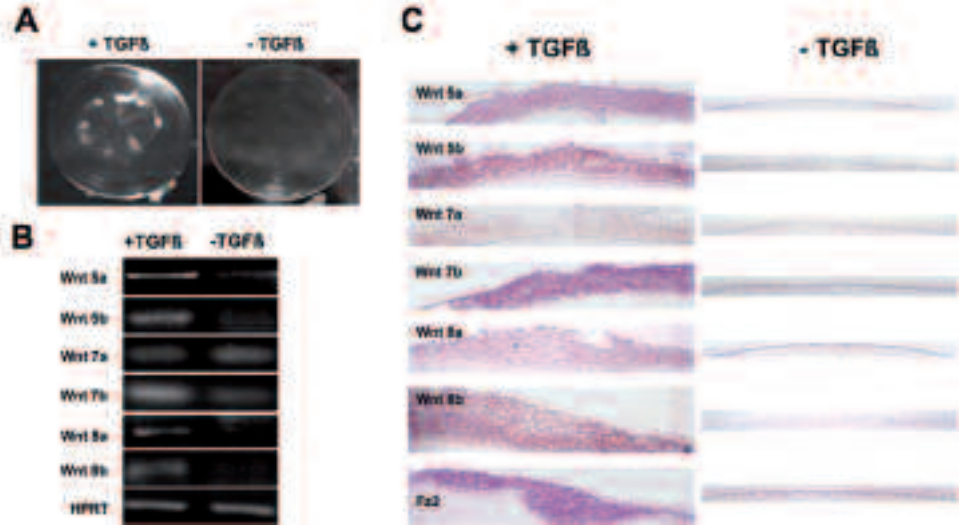


Figure. **A**) After 5 days culture with TGFß2 (500pg/ml) lenses develop subcapsular opacities, whereas in the absence of TGFß, lenses remain transparent. **B**) RT-PCR shows that capsule preparations (contain the epithelium and associated plaques) from lenses cultured with TGFß2 express Wnt 5a, 5b, 7b, 8a and 8b, much more strongly than lenses cultured without TGFß2. HPRT was used as a loading control. Of all the Wnts studied, Wnt 7a was the only isoform that was not upregulated by TGFß2. **C**) In situ hybridisation also showed that all the Wnts studied (with the exception of Wnt 7a) were all more strongly expressed in the subcapsular plaques of lenses cultured with TGFß2 than in lenses cultured without TGFß2. Fz2 expression was also shown to be upregulated in the presence of TGFß.

Association of macular pigment density with severity and progression of age-related macular degeneration

Dr L Robman

ORIA/Renensson Bequest Grant

Upon receiving granted money, the entire sum of the grant has been spent in 2008 to upgrade the confocal laser ophthalmoscope HRA to Macular Pigment Densitometer in Heidelberg Engineering, Heidelberg, as was indicated in the proposal.

The instrument was upgraded and supplied with the software for digital image analysis of macular pigment density (lutein and zeaxanthin) and density of lipofuscin in the macular area.

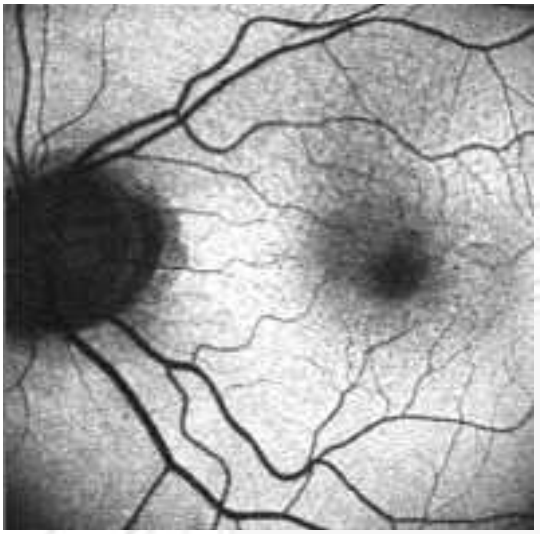
Since installation, the 2-wavelengths autofluorescent imaging, with subsequent analysis of macular pigment density, has been conducted on 160 participants from four categories:

1. The longitudinal study on visual function tests as predictors of AMD progression (Aged Macular/Functional Macula, AM/FM)
2. The Age-Related Macular Degeneration Statin Study (ARMSS) – randomised placebo-controlled trial of the effect of statin on the course of high risk age-related macular degeneration.

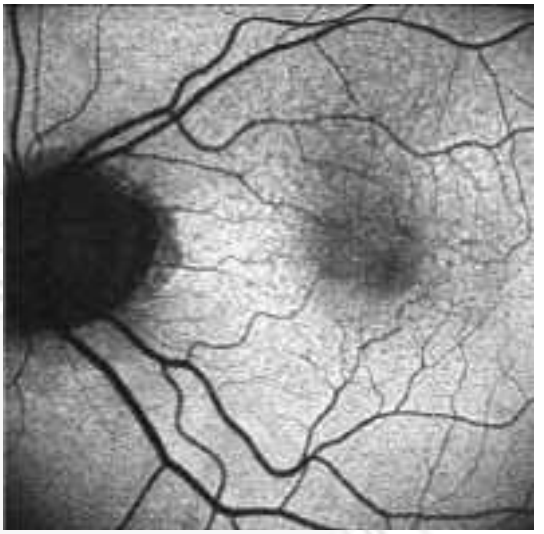
3. The Macular Telangiectasia (MACTEL) project – a multi-centred, international observational study that clinically examines the natural history of Idiopathic Juxtafoveal Macular Telangiectasia. Centre for Eye Research Australia is one of the centres for this study.
4. On the subjects with no AMD, to define the range and distribution of normal macular pigment measurements in Australians of different ages and background.

Data collection continues. Two research assistants were taught by the grantee to conduct the auto-fluorescent imaging and the Macular Pigment Density analysis on a regular basis.

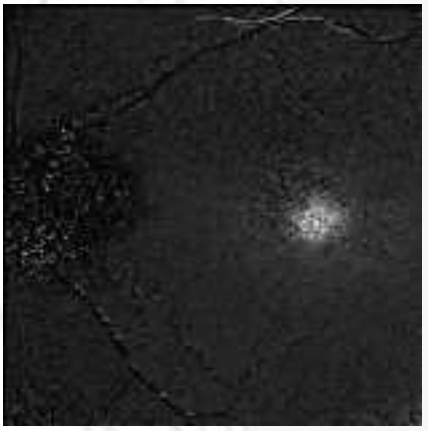
The advantage of this methodology is its capacity to isolate the density of the yellow colour belonging to the macular pigment, which is characterised by the specific wavelength spectrum, from all the other colour influences (Figures 1 and 2).



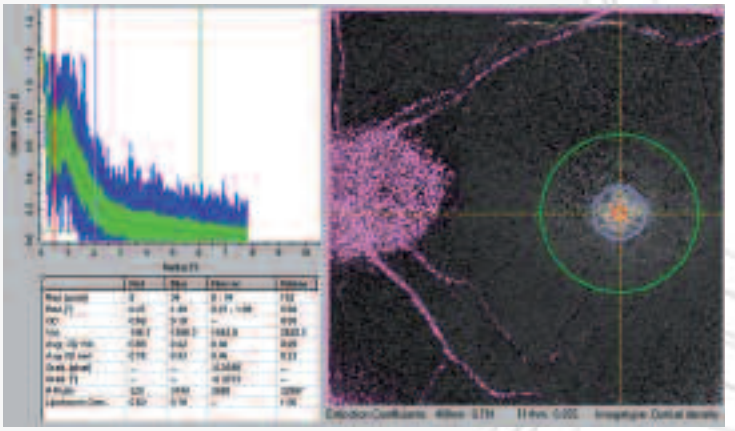
A. Autofluorescent image taken with the 488 nm wavelength light



B. Autofluorescent image taken with the 514 nm wavelength light

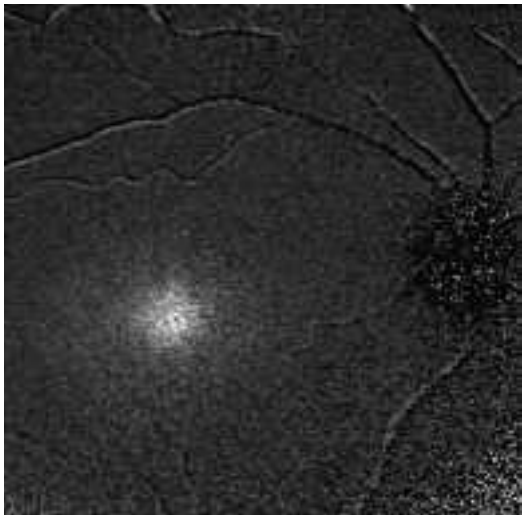


C. Subtraction of the 488-image from 514-image shows the picture of the yellow pigment of wave-length between 488 and 514 nm

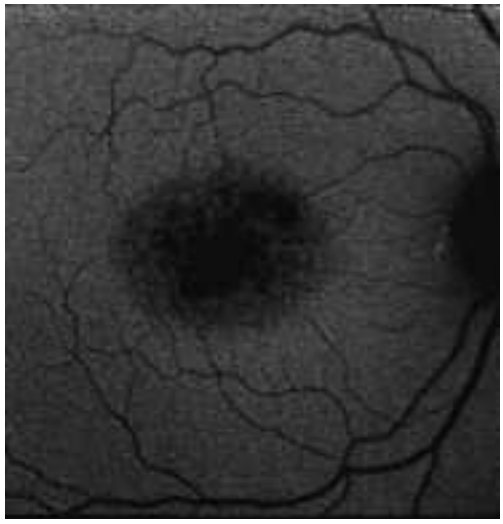


D. Radial, centred on fovea, density of macular pigment can be analysed, with analysis showing the histogram of the density distribution, and average radial density at various selected distances from the centre.

Figure 1. Macular pigment density analysis with 2-wavelengths methodology.



A. Normal eye. High density of macular pigment in the central area around the fovea.



B. Macula affected by Idiopathic Juxtafoveal Macular Telangiectasia. Lack of macular pigment.

Figure 2. The contrasts in distribution of macular pigment.

Genetics of Retinal Vascular Calibre: The Australian Twin Eye Study

Prof D Mackey, Dr Cong Sun and Dr J Ruddle

ORIA/Esme Anderson Grant

Aims

1. Measure retinal vascular calibre and other retinal vascular features (tortuosity, branching angles) from 1,000 twin pairs.
2. Determine the heritability of retinal vascular calibre by comparing the concordance of retinal vascular calibre between MZ and DZ twins.

Significance: systemic vascular disease (stroke, heart disease, cardiovascular mortality) as a whole is a major public health concern in Australia, and many are listed as Australia's National Health Priority Areas. Retinal vascular calibre changes (e.g. retinal arteriolar narrowing and venular dilation) are related to risk of systemic vascular disease, and thus examination of the eye provide a unique means for early identification of high-risk and asymptomatic people. Specific findings are detailed below. We are also able to correlate the early birth data with retinal vascular calibre to test the underlying mechanisms of fetal origins of adult disease in the twins. These findings provide new insights into the aetiology of complex systemic vascular disease, and may also indicate potential prevention and better medical treatment at an early stage of these systemic conditions in the future.

1. Findings from the Genetic Analyses:

Research into the genetic effects and specific genes associated with retinal vascular calibre, a risk marker of cardiovascular diseases, may provide new insights into the genetic contribution of early microvascular disease. A combined 374 monozygotic and 536 dizygotic twin pairs and 322 siblings from the Twins Eye Study in Tasmania (TEST) and the Brisbane Adolescent Twin Study (BATS) underwent complete ophthalmic examinations including retinal photography, and bilateral retinal vascular calibre was measured. Structural equation modeling was used to estimate the heritability. Genome-wide linkage analysis was conducted on 836 individuals from 381 BATS families with adjustments for age, sex, and other covariates.

The heritability for retinal arteriolar calibre was 59.4% (95% confidence interval [CI] 53.2% to 64.7%) and 56.5% (50.1% to 61.9%) in TEST and BATS, respectively, and for venular calibre was 61.7% (55.6% to 67.0%) and 64.2% (58.7% to 68.8%), after adjusting for age, sex, and body mass index. Two multipoint peaks detected on chromosomes 3p12.3 and 8p23.1 for retinal arteriolar calibre had suggestive linkage, with the highest multipoint peak logarithm of odds (LOD) score of 2.24 on chromosome 8p23.1 (genome-wide p-value = 7.0 10-4). Two suggestive LOD scores for venular calibre were identified on chromosomes 2p14 and 9q21.13. The largest multipoint LOD score was 2.69 on chromosome 2p14 (genome-wide p-value = 2.0 10-4). In this large twin population, genetic factors appear to play a significant role in the variation of retinal vascular calibre. Several putative loci were identified for retinal vascular calibre.

2. Findings from the study on early birth data with retinal vascular calibre

Recent studies reported an association between smaller birth size and narrower retinal vascular calibre, but it remains unclear whether this association is due to confounding by shared environment or genetic factors. At mean age of 9.3 years, 266 twins (49 monozygotic and 84 dizygotic pairs) in the Twins Eye Study in Tasmania underwent an ophthalmic examination including retinal photography. Retinal vascular calibre was measured using a validated protocol. A subset of these twins was in two other studies, which prospectively collected data on birth parameters and antenatal maternal factors. We conducted the main analysis using linear mixed models, accounting for birth set clustering. Both the within-pair ($ß_W$ -9.73, 95% confidence interval [CI] -14.68, -4.77 µm per 5 cm decrease in birth length) and between-pair associations ($ß_B$ -7.15, 95%CI -11.54,-3.01) with retinal arteriolar calibre were significant and of similar magnitude (difference in effect, p=0.61), after adjusting for age, gender, maternal smoking, mean arterial blood pressure and other confounders. These associations remained within dizygotic and monozygotic pairs. Analyses of head circumference and retinal arteriolar calibre were similar to those of birth length ($ß_W$ -2.41, 95%CI -5.09,0.28; $ß_B$ -2.60, 95%CI -5.00,-0.19). For birthweight, only a between-pair association was evident (-7.28, 95%CI -13.07,-1.48). This study demonstrates a consistent association between smaller birth size and narrower retinal arterioles in twins. The independent effect of shorter birth length on retinal arteriolar calibre supports a role for twin-specific supply line factors affecting fetal growth on vascular structure. This report adds to the growing literature that suggests fetal programming *in utero* may be a long term determinant of human vasculature development, with implications for later cardiovascular disease risk.

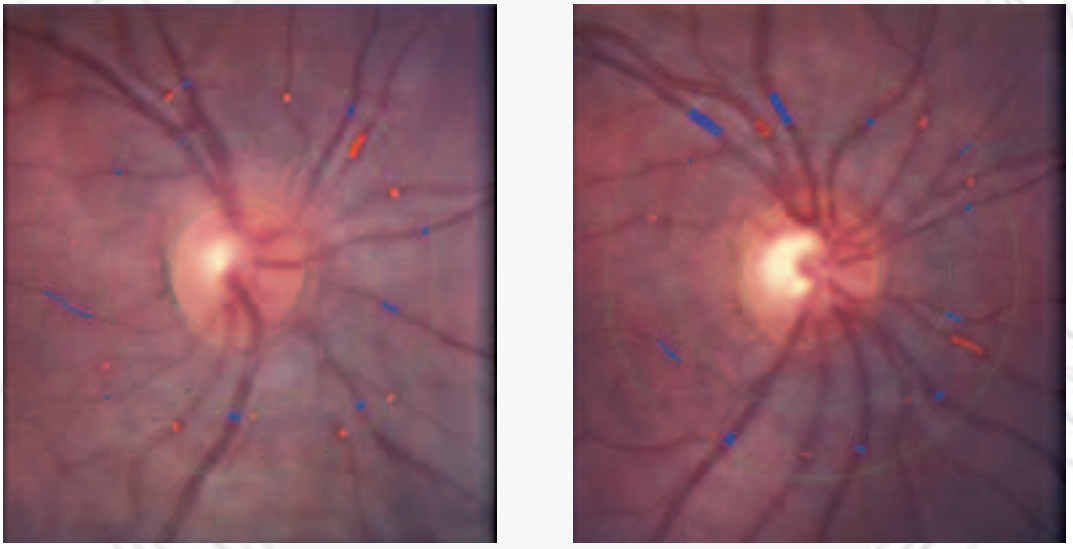


Figure 1. Retinal Vessel Measures in identical twins

Achievements to date

1. Vessel calibre measurements of 4,233 images from both eyes of more than 1000 twin pairs have been completed.
2. Data cleaning, analysis and manuscript preparation have also been completed.

Publications

Sun C, Ponsonby AL, Wong TY, Brown SA, Kearns LS, Cochrane J, MacKinnon JR, Ruddle JB, Hewitt AW, Liew G, Terrence Dwyer, Scurrah K, Mackey DA. Effect of Birth Parameters on Retinal Vascular Caliber: The Twins Eye Study in Tasmania. *Hypertension*. 2009; 53(3):487-93.

Sun C, Wang JJ, Mackey DA, Wong TY. Retinal vascular caliber: systemic, environmental, and genetic associations. *Surv Ophthalmol.* 2009; 54(1):74-95.

The role of osteopontin in the retina

Dr G Chidlow and A/Prof R Casson

ORIA/Glaucoma Australia Inc Grant

Aims

The cytokine osteopontin has been implicated in the response of various tissues to injury, but little is known about the function of osteopontin in the retina. We have new evidence that osteopontin is rapidly and transiently synthesised by retinal microglia after ischemic and excitotoxic injuries. The research questions being investigated in the current project are as follows:

1. *What factors lead to production of osteopontin by retinal microglia following ischemia/excitotoxicity?*

We propose to ascertain whether the pro-inflammatory mediators TNF-α and IL-1β are responsible for synthesis and release of osteopontin by microglia.

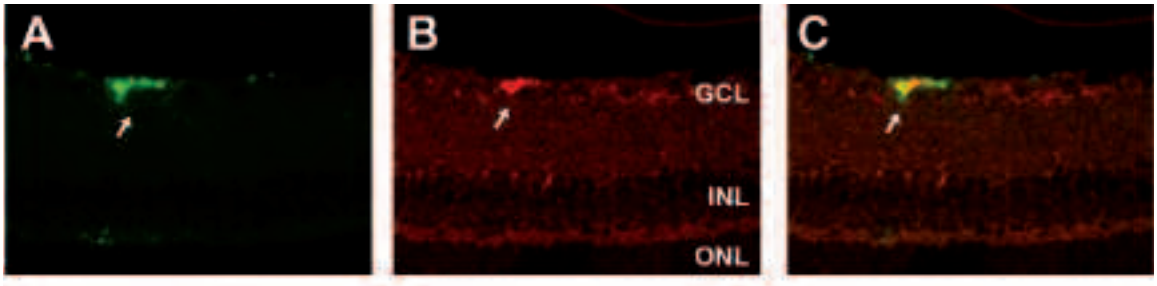
2. *What is the effect of an increased level of OPN on neuronal survival?*

An accumulating body of evidence indicates that, in certain tissues, osteopontin exerts an anti-inflammatory, pro-survival action on neurons in pathological situations similar to those employed in our study. We hypothesise that osteopontin is a survival factor for injured retinal neurons.

1. What factors lead to the production of osteopontin?

Our previous work has shown that ischemic and excitoxic injuries to the rat retina lead to the synthesis of the cytokine osteopontin by microglia. In the current project to date, we have demonstrated that administration of the pro-inflammatory toxin lipopolysaccharide results in the activation and proliferation of microglia in the rat retina *in vivo* and also in tissue cultures. Furthermore, we have shown that, in these retinas, there is an increase in synthesis of various cytokines and inflammatory mediators, including IL-1β, TNF-α, IL-6, COX-2 and iNOS, as well as osteopontin. Using double-labelling immunohistochemistry, we have identified activated microglia as the source of IL-1β, TNF-α, IL-6 and osteopontin, and we have shown co-localisation of osteopontin with all three of these mediators in overlapping subsets of microglia. Shown below is an example of co-localisation of osteopontin (red) and IL-1β (green) in an activated microglia within the nerve fibre layer following lipopolysaccharide injection.

The combined results show that osteopontin is produced within the same time period and by some of the same cells as various classical pro-inflammatory mediators, but the data do not provide information on the cellular mechanism that elicits osteopontin synthesis, in particular whether IL-1β or TNF-α are directly responsible for induction of osteopontin synthesis. Experiments are currently underway to address this particular question. Rats have been given an intraocular injection of IL-1β or TNF-α and the retinas are being analysed at various time points to determine whether microglia have synthesised osteopontin. The results from these experiments will be available shortly.



2. What is the effect of an increased level of osteopontin on neuronal survival?

In order to establish whether osteopontin secreted following an ischemic-like injury is a survival factor for retinal neurons, it has been necessary both to characterise suitable tissue culture systems to test the hypothesis and to optimise methodologies for measuring neuronal death. During the past year, we have evaluated the efficacy of Fluoro-Jade C, a novel fluorescent dye that labels dying neurons in the brain, for detection of neuronal degeneration in the retina and optic nerve. The accumulated results, which have been published in Experimental Eye Research (see below), indicate that Fluoro-Jade C is a useful tool in the retina and optic nerve for visualisation of neuronal degeneration in excitotoxic and ischemic paradigms of injury. It is simple to perform, cost- and time-effective, highly reproducible, labels degenerating dendrites as well as cell bodies and can be used in multiple label studies.

With regard to tissue cultures, during 2008, we established primary cultures of mixed neonatal rat retinal cells and adult rat retinal ganglion cells, and, furthermore, acquired an immortalised ganglion cell line (RGC-5) from collaborators in Melbourne. We have been conducting detailed experiments documenting cellular responses to anoxic/excitotoxic challenges with regards the primary cell cultures and have recently finished characterising a means of terminally differentiating the RGC-5 cell line into a neuronal-like phenotype. This latter work has resulted in a presentation at the Association for Research into Vision and Ophthalmology meeting in the USA in May 2009 and two publications (see below). We are currently delineating the optimal means to induce hypoxic/anoxic death in differentiated RGC-5 cells. When this information is available we will be conducting the osteopontin-protection studies on all three cell culture models.

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Investigating the endothelial cell morphological changes in retinal veins at sites of predilection to occlusion

A/Prof IL McAllister, Dr WH Morgan, Dr LRS Vijayasekaran, Dr PK Yu and Dr C Balaratnasingham

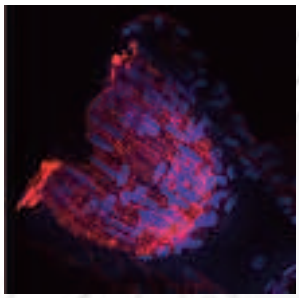
ORIA/RANZCO Eye Foundation Grant

We proposed to study the morphology of endothelium in human retinal veins in regions prone to occlusion. Specifically, we are interested in the pre-laminar, laminar and post-laminar regions of the optic nerve, and the artery-vein crossing point within the retina.

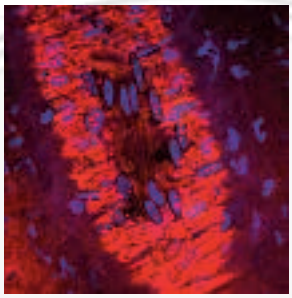
Human cadaver donor eyes were successfully cannnulated, and the endothelial and vascular smooth muscle cell f-actin labelled for study. After overnight fixation, the optic nerve was embedded in saturated sucrose solution for thick vibratome sectioning and subsequent imaging. Due to the auto-fluorescent properties of the myelin and sclera, various treatments (BABB, Methyl Salicylate, Methanol, Ethanol, DMSA, H_2O_2, Dent's Fix etc.) were trialled on the optic nerve sections to change its optical diffraction properties for improving visibility. Unfortunately, these treatments proved unsuccessful as the chemical would also remove the Alexa Fluor 546 phalloidin attached f-actin label. After many trials of different methods, it was found that thick vibratome sections around 70 to 100μm without any treatment were suitable for confocal imaging the central retinal vessels wall.

In donor eyes without known eye pathologies, we were able to study the central retinal artery and central retinal vein in the three regions specified. Whilst the pre-laminar and post-laminar regions had reasonably broad length of vessel wall lying flat for imaging; due to the physical constriction presented by the collagen plates at the laminar region, both the central retinal artery and the central retinal vein were visibly narrowed and the vessel path tortuous in this part of the nerve.

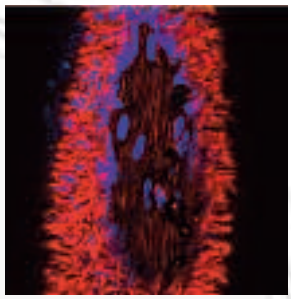
Seven eyes were imaged for CRA. Peripheral border staining, as well as intracellular stress fibres were found within the CRA endothelium. From the peripheral border staining, the shape of the CRA endothelium was found to be most elongated in the post laminar region and least elongated in the pre-laminar region. Intracellular stress fibres were found in endothelia from all three regions studied. Dual staining for nucleic acid using Hoechst allowed the endothelial nuclei to be visible (blue) in relation to the peripheral cell border. There was no difference in the shape or position of the endothelial nuclei in the three regions studied.



Prelaminar CRA



Laminar CRA



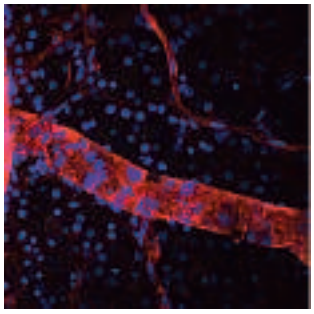
Post-laminar CRA

It was more difficult to obtain data for CRV as venous endothelium generally stained less intensely than arteriole endothelia. The curvy nature, more pliable/collapsible vessel wall and physical constriction by the laminar cribrosa made it more difficult to obtain venous data. However, data collection is still in progress and a paper on the subject of CRA and CRV is in preparation.

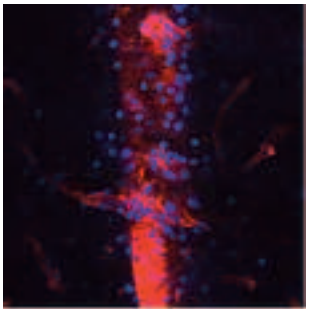
The retina of perfusion stained eyes were flat mounted for confocal imaging. Arterio-venous crossings (AVC) were identified using epifluorescence and low magnification lens and the microfilament distribution pattern recorded by confocal imaging at high magnification (x40 or x60 lenses). At these AVCs, the venules would dive underneath in a curving fashion to circumvent the arterioles lying above it.

In eyes from donors with no direct link in their cause of death and cardiovascular diseases, the venous endothelium were either free from microfilament staining in their cytoplasms or had dotty spread of microfilament staining. Comparison of the upstream and downstream venous segment found little difference in the microfilament staining characteristics within the venous endothelial cytoplasm. However, venous endothelium of donor eyes with cardiovascular related morbidities had prominent stress fibres within their cytoplasm and some regional differences were observed. In general, there appear to be a greater presence of stress fibres in the immediate regions around the crossing point compare with area further upstream or downstream. Peripheral border staining of the endothelium enabled quantitative measurement of these venous endothelium. In general, the venous endothelium at the crossing point are smaller and rounder in shape. The venous endothelia downstream from the crossing point tend to be longer and slimmer than those upstream from the crossing point.

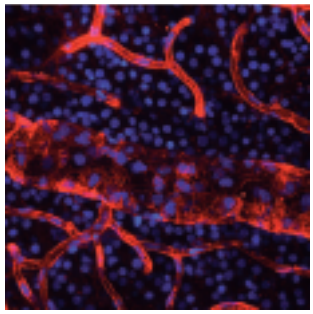
We have received two eyes from glaucoma donors recently and are in the process of collecting data for them.



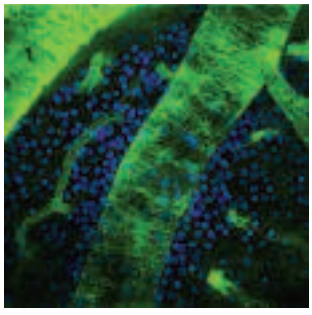
Pre AV crossing

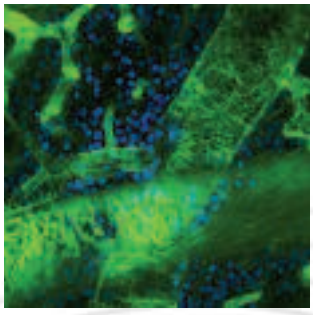


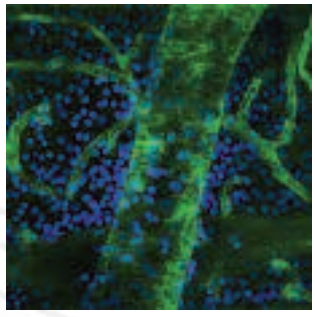
AV crossing



Post AV crossing







Using state-of-the-art protein and DNA methods to discover the causes of the pseudoexfoliation syndrome, a cause of glaucoma blindness

A/Prof JE Craig and Dr A Hewitt

ORIA/Glaucoma Australia Inc Grant

Overview

Pseudoexfoliation (PEX) syndrome is an age-related disorder characterized by accumulation of fibrillar extracellular deposits in the anterior ocular structures. Its prevalence varies in different populations but increases with age. Individuals with PEX are at higher risk of developing glaucoma. Associated cataract is the most common cause of surgical intervention required by PEX patients. These patients have higher rate of complications from cataract surgery. Retinal vein occlusion associated with PEX can lead to profound visual loss. Besides ocular pathology PEX also increases the risk of heart disease and stroke. Both genetic and environmental factors are thought to play a role in this disease. Based on some molecular analyses, overexpression and reduced turnover of the extracellular matrix has been suggested to be involved in the disease pathology. However the disease pathogenesis is poorly understood.

This project aimed to determine the genetic and molecular factors contributing to PEX syndrome. We chose to employ a combination of genetics and proteomics approaches to investigate this disease. Both approaches have generated successful outcomes. Our findings supported by reports from other groups have advanced the understanding of PEX syndrome.

Genetic analysis in PEX syndrome

In 2007, a genome wide-association study reported strong association of the lysyl oxidase-like 1 (*LOXL1*) gene, involved in elastin cross-linking in the extracellular matrix, with PEX syndrome in the Nordic population. Two coding variants rs1048661 (Arg141Leu) and rs3825942 (Gly153Asp) in exon 1 of *LOXL1* were strongly associated with the risk of developing PEX. The highest risk haplotype (G_G) was found in

81.4% cases and 49.8% controls. We rapidly replicated this finding in the BMES (Blue Mountain Eye Study Cohort) PEX cases and found similar association of these two *LOXL1* variants in the Australian population. These findings were published in *Human Molecular Genetics*[1]. Since then several studies have reported association of the same two *LOXL1* variants with PEX in various populations. In the Japanese population the T_G haplotype is the highest risk haplotype. Thus *LOXL1* is a significant contributor to PEX. Interestingly, the highest risk *LOXL1* haplotype is also the most frequent haplotype in the populations studied to date. In addition, despite similar allele frequencies at the *LOXL1* gene in various populations, the disease prevalence varies considerably among populations. Thus additional factors seem to contribute to the disease risk.

In an attempt to identify additional genetic factors contributing to PEX, we undertook a candidate gene approach. With the funding support from the ORIA, we analysed the clusterin (*CLU*) gene. *CLU* is a secreted molecular chaperone involved in preventing aggregation of unfolded proteins. It is a component of the PEX deposits, is differentially expressed in the anterior segment of PEX eyes compared to normal eyes and present at lower levels in the aqueous humor of PEX eyes compared to normal eyes. Hence *CLU* is a potential candidate for association with the disease. We analysed the genetic variants across *CLU* in BMES PEX cases and controls and detected only nominal association of one nucleotide variant rs3087554 (p=0.044) with the disease. A single haplotype of the genetic variants in the *CLU* gene also showed minor disease association. This analysis suggested that *CLU* is not a major contributor to the disease risk. The findings of this work were published in *Molecular Vision*[2].

Proteomics analysis in PEX syndrome

In addition to genetic analysis, we performed proteomics analysis to identify the additional constituents of pathological PEX deposits. We receive lens capsules from PEX and non-PEX cases undergoing cataract surgery for use in this study. With funding support from the ORIA, we established a novel methodology of surgically removing PEX material from the anterior lens capsule of affected eyes before capsulorhexis and chemically cleaving the 'isolated' PEX material for protein identification by mass spectrometry. Mass spectrometry was performed at the state-of-the-art Flinders Proteomics Facility. Our novel methodology led to the identification of LOXL1 and apolipoprotein E as novel components of PEX material. Presence of these proteins in the pathological deposits was confirmed by immunohistochemistry. These findings have been recently published in *Experimental Eye Research*[3]. Presence of LOXL1 protein in PEX deposits is supported by an independent study conducted in Germany. Our work also suggests the presence of additional as yet unidentified protein constituents in PEX material.

The recent research into the PEX syndrome has generated much excitement among the scientific community indicated by presentations at recent international conferences. We were invited to present our work on PEX at the International Congress of Eye Research in Beijing in 2008. We have also presented this work at other international conferences. Our preliminary work funded by the ORIA and Glaucoma Australia Inc formed the basis for receiving funding from the NH&MRC for further investigation into the PEX syndrome.

Publications

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2. Burdon KP, Sharma S, Hewitt AW, McMellon AE, Wang JJ, Mackey DA, Mitchell P, Craig JE (2008) Genetic analysis of the clusterin gene in pseudoexfoliation syndrome. Mol Vis. 14:1727-36.
3. Sharma S, Chataway T, Burdon KP, Jonavicius L, Klebe S, Hewitt AW, Mills RW, Craig JE. Identification of LOXL1 protein and Apolipoprotein E as components of surgically isolated pseudoexfoliation material by direct mass spectrometry. Exp Eye Res doi:10.1016/j.exer.2009.05.001.

Growing corneal stem cells on silk

Prof L Hirst, Dr Z Barnard and Dr Zainuddin

ORIA/RANZCO Eye Foundation Grant

Specific aims of the project and progress

1. To establish a reproducible method of manufacturing silkworm silk fibroin membranes, which can be sterilised, but maintain the necessary structural, mechanical and surface properties, and display long-term biodegradability.

The method for producing the silk fibroin membranes detailed within our original research plan has been successfully established for the project. Production of silk fibroin membranes is now carried out routinely with further modifications to the membranes such as thickness and porosity also being investigated.

Sterilisation of the membranes is achieved by soaking in 70% ethanol for 1 hour and then washing overnight in phosphate buffered saline. Autoclaving of the membranes is also successful but the ethanol treatment is the standard protocol we use. Sterilised membranes have been stored for 12 months without significant changes to structure.

2. To investigate in detail the in vitro proliferation of progenitor limbal epithelial cells on fibroin membranes as compared to their growth under animal product-free conditions, and on amniotic membrane or tissue culture plastic as substrates.

Experimental work examining the attachment and growth of both a human corneal epithelial cell line as well as primary donor-derived cell cultures to silk fibroin membranes, amniotic membrane and tissue culture plastic has been completed. The results demonstrate that attachment of the human corneal cell line and primary donor-derived cell cultures to all three substrates are not statistically different, however we have observed some variation in the morphology of the cells on the different substrates. The presence of serum in the culture media also had a significant effect in enhancing attachment regardless of the substrate being seeded.

3. To examine by a variety of molecular biology techniques the phenotypes of the cell cultures in order to assess any differences in the expression of established molecular markers of these cells.

The design of the procedure to complete this aim has been developed and the initial experiments have been performed. Several more runs of the protocol need to take place to gather sufficient data to address our hypothesis effectively. A thorough selection of antibodies raised against relevant molecular markers has been attained and the immunohistological procedure has begun.

Once completed both the results of the molecular biology analysis and the attachment assays will be submitted for a follow up publication to our first paper, which was presented initially as a talk: T.V. Chirila, Z. Barnard, Zainuddin, D.G. Harkin, I.R. Schwab and L.W. Hirst. *Bombyx mori Silk Fibroin Membranes as Potential Substrata for Epithelial Constructs Used in the Management of Ocular Surface Disorders*. CSIRO Conference "Fibrous Proteins: transforming structural knowledge into new materials", Mount Eliza, Melbourne, Australia, 31 March – 3 April 2008.

Publication

Chirila TV, Barnard Z, Zainuddin, Harkin DG, Schwab IR and Hirst LW 2008. Bombyx mori Silk Fibroin Membranes as Potential Substrata for Epithelial Constructs Used in the Management of Ocular Surface Disorders. Tissue Eng Part A. 2008 Jul;14(7):1203-11. PMID: 18380593

Ocular developmental disorders: molecular genetics and gene function

Dr Robyn Jamieson, Dr John Grigg, Prof Patrick Tam and Prof Frank Billson

ORIA GRANT

The Eye & Developmental Genetics Research group aims to understand the genetic causes of diseases which lead to blindness in humans. This provides the basis for the development of improved treatments and prevention of vision loss. We are taking a number of approaches to elucidate the underlying genetic causes of vision problems. These include the study of patients and families with ocular disease and associated chromosomal anomalies, families which are suitable for linkage analysis, and animal models of ocular disease processes.

In eye development and for the maintenance of normal eye function, it is critical that cells are able to turn on the correct genes at the right times and in the right place. Understanding of how these processes work is for the benefit of the development of treatments for conditions such as retinitis pigmentosa and other retinal dystrophies. One of the signalling pathways important for correct spatial and temporal gene expression in the eye is the Wnt signalling pathway. In this project, we are examining mice with aberrations in the Wnt signalling pathway and have found that there are abnormalities affecting several parts of the eye, including the retina and the anterior segment. Slit lamp and fundal examination of the mice, as well as histological analyses, show that the Wnt signalling pathway is important in normal development of the eye, as well as in maintenance of normal eye health particularly of the retina. Our examination of several markers of specific cell types in the early developing anterior segment and the retina are indicating the way various components of the eye are affected by aberrations in this pathway. We have identified coding sequence variations in candidate disease genes in this pathway in patients affected with ocular abnormalities and we are currently testing these for presence in other family members and control subjects. Understanding of the function of this signalling pathway in the eye will contribute to future development and precision in therapeutic approaches in anterior segment problems and retinal dystrophies of the eye.

The precise mechanisms regulating the complex genetic interactions leading to proper eye formation are not fully known. SOX2 is a critical factor in eye development, but there are still questions about how its functions are regulated during the formation of the various eye components. By examining a multigenerational family with eye anomalies, we have identified a novel SOX2 mutation that affects the partner-factor interaction domain of SOX2. It is critical that appropriate partner-factor proteins are able to bind to this part of SOX2 for normal eye development to proceed. This is the first time that such a mechanism concerning SOX2 and its function has been recognised in human patients. This is also the first time that a SOX2 mutation has been identified in a multigenerational eye anomaly family and this work was recently published in the European Journal of Human Genetics[1]. This work was also recently presented at the annual meeting of the Association for Research in Vision and Ophthalmology[2]. Support from the ORIA is gratefully acknowledged in publications and presentations of this work.

Publications and presentations

[1]Mihelec M, Abraham P, Gibson K, Krowka R, Susman R, Storen R, Chen Y, Donald J, Tam PPL, Grigg JR, Flaherty M, Gole G, Jamieson RV. Novel SOX2 partner-factor domain mutation in a four generation family. May 27, 2009 [Epub ahead of print].

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Jamieson RV. Hereditary optic neuropathies. Neuro Ophthalmology 2009 Conference, Sydney Eye Hospital, January 2009.

Jamieson RV. Signalling in early lens and eye development. Australian Ophthalmic and Vision Science Meeting, Canberra, December 2008.

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1. Mihelec et al, European Journal of Human Genetics, 2009 May 27.
2. Jamieson et al, Association for Research in Vision and Ophthalmology, A359, May 2009

Factors involved in the development of ocular surface tumour

Dr Nick Di Girolamo, Dr Michele Madigan and Dr Max Conway

ORIA GRANT

Ocular surface squamous neoplasia (OSSN) is a dysplastic or carcinomatous lesion arising from epithelial cells in the conjunctiva, limbus or cornea and is considered an uncommon disease with a prevalence of 0.2–3.5 cases per 100,000. Lesions are slow growing, usually confined to the ocular surface and rarely metastasize. Increased incidence of OSSN has been reported in the last decade and despite improvements in treatment, recurrence rates range from 5-53%. The pathogenesis of OSSN has yet to be definitively attributed to a specific etiological factor. Several risk factors have been reported and include chronic ultraviolet (UV) exposure, Human Papilloma Virus, Human Immunodeficiency Virus and smoking. The role of limbal epithelial stem cells in the development of this disease has been proposed but has remained controversial. It has been speculated that OSSN arise from dysfunctional limbal stem cells having been altered by mutagenic agents, such as UV radiation. Despite multiple theories, evidence for UV radiation as the prime etiological trigger is likely, as epidemiological studies show a linear relationship between incidence rates and proximity to the equator. Interestingly, this disease shares some striking similarities to skin neoplasia whose origins are more tightly defined. Independent of cellular and DNA injury, UV radiation can induce phosphorylation events in cytokine and growth factor receptors which in turn activate down-stream transcription factors.

Only a limited number of molecular studies have been undertaken to delineate the pathogenesis of OSSN. An area lacking attention is the potential contribution of matrix metalloproteinases (MMPs) and their natural inhibitors, the tissue inhibitors of MMPs (TIMPs). These molecules have been heavily implicated in tissue invasion and metastasis that characterizes many human neoplasias. In this study we hypothesised that there is a differential pattern of MMP and TIMP expression in OSSN that may be a consequence of UV radiation exposure at the ocular surface. We identified a pattern of MMP and TIMP expression in OSSN tissue that implied these molecules may play a critical role in the development of this disease. Moreover, we demonstrate that cells derived from dysplastic ocular tissue are more sensitive to UVB radiation than normal conjunctival cells, with regards to MMP production, thereby providing preliminary clues as to pathogenesis of OSSN and potential preventative measures that can be recommended for at-risk subjects.

Although the precise contribution of MMPs and TIMPs in OSSN remains to be clearly defined, to our knowledge, this study is the largest of its kind on the localisation of relevant effector molecules. In the absence of an animal model, our *in vitro* system is the first to model one component of OSSN (the epithelium) using UV radiation as the stimulatory agent to determine its role on the expression of proteolytic enzymes relevant to neoplasia. Furthermore, the genetic switch, transforming dysplastic lesions into neoplastic carcinomas has yet to be determined. Investigations into differentially expressed genes using gene microarray analysis in dysplasia versus malignant carcinoma have been planed and we predict they may yield clues as to likely therapeutic targets.

Publication

Ng J, Coroneo MT, Wakefield D, Di Girolamo N. Ultraviolet radiation and the role of matrix metalloproteinases in the pathogenesis of ocular surface squamous neoplasia. *Invest Ophthalmol Vis Sci*. 2008; 49:5259-5306.

Improving outcomes for corneal transplants 2008–2009

Prof KA Williams, Dr HM Brereton and Prof DJ Coster

ORIA/RANZCO Grant

Overview and Aims

Corneal transplant surgery can restore vision to many people who are visually impaired. However, a significant proportion of such transplants fail because the recipient recognises the transplant as being foreign and rejection ensues. Our goal is to reduce the impact of rejection by transferring genes to the donor cornea *ex vivo*, prior to surgery. We have been able to prolong corneal graft survival significantly in an outbred experimental animal model by such a gene therapy approach with adenoviral vectors, but we have been unable to produce long-term expression of the therapeutic gene with these vectors and the grafts ultimately fail. We are now exploring a lentiviral vector as a means of long-term gene transfer to the cornea.

Our specific aims are:

(i) to construct and characterise lentiviral vectors carrying transgenes controlled by a constitutive promoter, or by a glucocorticosteroid-inducible element;

(ii) to construct and test multicistronic lentiviral vectors containing the novel 2A sequence, to will allow expression of multiple transgenes in the cornea;

(iii) to test these vectors for their ability to prolong rat and ovine corneal allograft survival.

Background

Our gene therapy vectors, designed by Professor Donald Anson, are recombinant HIV-1-based lentiviruses (LV) that are pseudotyped with vesicular stomatitis virus glycoprotein G, and that are non-replicative and self-inactivating. The basic vector contains the strong viral promoter, SV40. In previous work funded by the ORIA, we assessed transgene expression in corneal endothelium following transduction with lentiviral vectors containing a number of other promoters. Vectors containing the phosphoglycerate kinase promoter, the elongation factor-1 alpha promoter, or the myeloproliferative sarcoma virus promoter were not significantly more effective than the basic vector in driving transgene expression. Two lentiviral vectors containing the SV40 promoter and either the ovine or the rat interleukin 10 (IL10) transgene were then constructed. Lentivirus-mediated expression in transduced ovine and human corneal endothelium was assessed by fluorescence microscopy, real-time quantitative reverse transcription PCR (qRT-PCR) and ELISA, following alterations of transduction period duration (2–24 hours) and vector dose, as well as in the presence or absence of polybrene. A 24 hour transduction of ovine corneal endothelium with the lentiviral vector encoding IL10 resulted in expression levels which were increasing after 15 days of organ culture but logarithmically lower than those achieved by adenovirus. Shortening the lentiviral transduction period to 2 hours led to a reduction in expression, but the addition of polybrene (40 _g/ml) to the transduction mixture restored expression to levels comparable to those attained after a 24 hour transduction period, although at the cost of associated toxicity for corneal epithelium. Ovine corneas were transduced *ex vivo* with a LV-SV40-interleukin 10 (IL10) construct and transplanted orthotopically to the eyes of recipient sheep. Corneal allograft survival was prolonged by a median of 7 days in the LV-SV40-IL10-treated recipients, compared with the control group (p=0.026). Thus although the lentiviral vector showed some promise for corneal gene therapy, we considered further vector modification to increase transgene expression, and the use of

multigenic vectors was warranted, to attempt to further prolong corneal allograft survival. Considerable work addressing the first two aims, above, has already been performed, whereas work on the third is continuing.

Progress to date on the current grant

Aim 1. We have continued to explore different promoter constructs. We first compared gene expression controlled by the SV40 and the cytomegalovirus (CMV) promoters. The CMV promoter produced faster, stronger transgene expression in ovine cornea than did the SV40 promoter, and is probably the construct of choice. We have also assessed the efficacy of a glucocorticosteroid-inducible promoter in controlling transgene expression following lentivirus-mediated gene transfer to ovine and human corneas. A glucocorticosteroid response element (GRE5) was cloned into a lentiviral vector (LV-GRE-IL10) encoding the model transgene interleukin 10. Transgene expression by LV-GRE-IL10-transduced A549 cells, ovine corneas, and human corneas cultured with or without dexamethasone, was quantified by an IL10-specific enzyme-linked immunosorbent assay. IL10 levels were 30-40-fold higher in supernatants from LV-GRE-IL10-transduced A549 cells cultured with dexamethasone than in controls. Dexamethasone withdrawal resulted in restoration of baseline IL10 levels. Supernatants from LV-GRE-IL10-transduced ovine and human corneas cultured in dexamethasone contained 9–10 times more IL10 than supernatants from transduced corneas cultured without dexamethasone. We conclude that the GRE5 promoter in a lentiviral vector can drive rapid, sustained and inducible transgene expression in both ovine and human corneas in the presence of dexamethasone. A steroid-inducible promoter may be useful for controlling transgene expression in gene-modified donor corneal allografts, given that topical glucocorticosteroids are administered to virtually every corneal transplant recipient. This work has now been accepted for publication in *Br J Ophthalmol*.

Aim 2. Given the complexity and redundancy of the immune response to a foreign graft, it has always been apparent to us that the use of a cocktail of gene therapy vectors, each carrying cDNA for a different therapeutic transgene, or the use of a multicistronic vector carrying multiple transgenes, might be necessary to achieve the maximum therapeutic effect. Lentiviral vectors typically have a reasonably-sized expression cassette and in theory, can carry more than one transgene. However, construction of multicistronic vectors that work well in mammalian cells has proven difficult. A possible solution to this problem has recently been reported. Incorporation of a so-called 2A sequence from a Picornavirus such as foot-and-mouth disease virus (FMDV) into the expression cassette of a viral vector, between multiple transgene sequences, results in production of a polyprotein expressed from a single open-reading frame. The polyprotein then self-cleaves at a post-translational level into individual proteins at the 2A motifs. Using this system, efficient production of multiple, functional transgenic proteins in a single cell has been reported. Using a plasmid containing the FMDV 2A sequence from colleagues in Melbourne, we have now constructed several vectors containing two transgenes, specifically a model secreted protein and a cytoplasmic reporter gene (enhanced yellow fluorescent protein, eYFP), separated by the 2A sequence. To monitor gene expression, the human cell line HEK-293A and rat corneas were transduced with the lentiviral vectors. Transgene expression at the mRNA and protein levels was quantified by qRT-PCR and by flow cytometry, respectively. Each transgene was expressed in the appropriate cellular compartment, but the second transgene in the string was expressed to a lower level than was the first, irrespective of the order of genes in the string. Other constructs are currently being prepared. The chief investigators acknowledge the essential contributions of Dr Douglas Parker, Ms Sarah Brice and Ms Alison Clarke to this work.

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