HERITABLE INFLUENCES IN EXPERIMENTAL RETINOPATHY OF PREMATURITY

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SUMMARY OF THESIS

Retinopathy of prematurity (ROP) is a potentially blinding eye condition of premature infants exposed to oxygen therapy. Different inbred rat strains exhibit differential susceptibility to oxygen-induced retinopathy (OIR), a robust animal model of ROP which mimics the pathophysiology seen in human disease. Susceptibility to OIR has previously been shown to segregate with ocular pigmentation, and is inherited in an autosomal dominant fashion in pigmented rat strains. The mode in which susceptibility to OIR is inherited in albino rat strains has not previously been determined. Using genetic cross, and backcross analysis, it was determined that susceptibility to OIR is inherited in the same autosomal dominant manner in albino rats, making it likely that genetic modifiers other than ocular pigmentation contribute to disease susceptibility. These modifiers could potentially be involved in the oxygen sensing pathway, which is central to the development of OIR and ROP.

Differences in retinal gene expression are likely to underlie the differential susceptibility to OIR that is exhibited by the two inbred strains of albino rats used in this study. MicroRNAs (miRNAs) may also contribute to the OIR phenotype by regulating these changes in gene expression. To test this hypothesis, RNAs from the retinae of two different rat strains exposed to oxygen therapy for 3, 5 or 6 days were analysed for gene and miRNA

expression using Affymetrix and Exiqon microarrays, respectively. A bioinformatics approach using the freely available online database, Database for Annotation, Visualisation and Integrated Discovery (DAVID), was used to assist in functional grouping of differentially regulated genes, many of which were found to be associated with response to hypoxia.

Candidate genes were identified from the gene expression microarray data based on their regulation by oxygen and on a search of the literature. The gene candidates EGL nine homolog 3 (EGLN3) and EGL nine homolog 1 (EGLN1) belong to a class of oxygen-dependent prolyl hydroxylases which are responsible for regulating levels of hypoxia-inducible factor-α (HIF-α) in normoxia. HIF-α is a master transcription factor which upregulates the expression of target genes in response to hypoxia. An additional candidate, insulin-like growth factor binding protein 3 (IGFBP3) was also chosen based on evidence from the literature showing that early expression of IGFBP3 was protective against the disease in a mouse model of OIR. Expression of candidate genes was confirmed using relative quantification real-time RT-PCR analysis.

miRNAs are non-coding RNAs which regulate gene expression at a post-transcriptional level and have been associated with a wide variety of physiological and pathological conditions including retinal development and OIR.

Initial analysis of microarray data from all 3 time points showed a total of 15 miRNAs to be differentially expressed after correction for multiple comparisons. miRNAs were identified as candidates if they targeted oxygen-related genes, were regulated by both oxygen and strain or had significant adjusted p values ($p<0.05$). miRNAs which are differentially regulated by exposure to oxygen therapy in a strain-dependent manner may contribute to differences in the disease phenotype that is exhibited by these two albino rat strains. Three miRNAs of interest, miR-30e, miR-338-3p and miR-210, were chosen for confirmation by real-time RT-PCR analysis. These miRNAs are predicted to target elements of the HIF-α oxygen sensing pathway.

Identifying the molecular basis of susceptibility to OIR may help to identify infants at risk of developing ROP and identify new therapeutic targets for treatment.

PUBLICATIONS ARISING FROM THIS THESIS

Tea, M., Fogarty, R., Brereton, H.M., Michael, M.Z., Van der Hoek, M.B., Tsykin, A., Coster, D.J., and Williams, K.A., Gene expression microarray analysis of early oxygen-induced retinopathy in the rat. J Ocul Biol Dis Infor, 2009. **2**(4): p. 190-201.

PRESENTATIONS ARISING FROM THIS THESIS

Tea MN, van Wijngaarden P, Michael M, Brereton HM, Coster DJ, Williams KA. Altered mRNA and miRNA expression and genetic susceptibility to experimental Retinopathy of Prematurity (Poster). Lorne Genome 2011, Lorne, February 13-15.

Tea MN, van Wijngaarden P, Michael M, Brereton HM, Coster DJ, Williams KA. Altered miRNA and gene expression and genetic susceptibility to experimental Retinopathy of Prematurity (Poster). RNAi and miRNA Europe 2010, Dublin, September 14-15.

Tea MN, van Wijngaarden P, Michael M, Brereton HM, Coster DJ, Williams KA. Altered gene expression and genetic susceptibility to experimental Retinopathy of Prematurity (Poster). Lorne Genome 2010, Lorne, February 14-16.

Tea MN, van Wijngaarden P, Michael M, Brereton HM, Coster DJ, Williams KA. Genetic susceptibility to experimental Retinopathy of Prematurity: The role of microRNAs (Presentation). **53rd Annual Conference of Australian Society for Biochemistry and Molecular Biology** (ComBio) 2009, Christchurch, December 6-10.

Tea MN, van Wijngaarden P, Michael M, Brereton HM, Coster DJ, Williams KA. Genetic susceptibility to experimental Retinopathy of Prematurity: The role of microRNAs (Presentation). ASMR SA Division: Annual Scientific Meeting 2009, Adelaide, June 2.

DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Signed: _____ Date: _____

Melinda Tea

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ABBREVIATIONS

≤	less than or equal to
≥	more than or equal to
~	approximately
°C	degrees Celsius
µg	microgram (10^{-6} g)
µl	microlitre (10^{-6} l)
µM	micromolar (10^{-6} M)
µm	micrometer (10^{-6} m)
AAKT	*apoptosis-associated tyrosine kinase*
AE	*amplification efficiency*
Aga	*Anopheles gambiae*
AGO1-4	argonaute 1/2/3/4 protein
ANG1	angiopoietin 1
ANG2	angiopoietin 2
ANOVA	analysis of variance
ARBP	acidic ribosomal phosphoprotein
ARVO	Association for Research in Vision and Ophthalmology
BEAT-ROP	Bevacizumab Eliminates the Angiogenic Threat of Retinopathy of Prematurity
bFGF	basic fibroblast growth factor
BN	Brown Norway rat strain
BNIP3	BCL2/adenovirus E1B 19 kDa-interacting protein 3
bp	base pairs
CA	California
CBP	creb-binding protein
cDNA	complementary DNA
CI	confidence interval
cm	centimetre
CO_2	carbon dioxide
COX 2	cyclooxygenase 2
Ct	cycle threshold
DA	Dark Agouti rat strain
DAVID	Database for Annotation, Visualisation and Integrated Discovery
ddH_2O	double distilled water

DE	Delaware
DEPC	diethylpyrocarbonate
DMOG	dimethyloxalylglycine
DNA	deoxyribonucleic acid
dNTP	dinucleotide triphosphate
DOPA	dihydroxyphenylalanine
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EC	endothelial cell
EDTA	ethylene-diamine-tetraacetic-acid
EFNA3	Ephrin-A3
EGLN1	EGL nine homolog 1/PHD2
EGLN2	EGL nine homolog 2/PHD1
EGLN3	EGL nine homolog 3/PHD3
EPO	erythropoietin
EtOH	ethanol
$F_{(x,y)}$	F statistic (degrees of freedom, error)
F344	Fischer 344 rat strain
FDR	false discovery rate
FEVR	familial exudative vitreoretinopathy
FIH-1	factor-inhibiting HIF-1
FRA	F344 room air-exposed rat
FO_2	F344 cyclic hyperoxia-exposed rat
g	gram
g	gravity
GEO	gene expression omnibus
GO	gene ontology
GS-IB4	*Griffonia simplicifolia* type I isolectin B4-Alexa 488™ conjugate
h	hour
HK2	hexokinase 2
HIF-α	hypoxia inducible factor-α
HIF-1	hypoxia inducible factor-1
HIF-1/2α	hypoxia inducible factor-1/2α
HPRT	hypoxanthine guanine phophoribosyl transferase
HRE	hypoxia response element
Hsa	*Homo sapien*
HUVEC	human umbilical vein endothelial cell

HW	Hooded Wistar rat strain
ICROP	International Classification of Retinopathy of Prematurity
IGF-1	insulin-like growth factor-1
IGFBP2/3	insulin-like growth factor binding protein 2/3
IL	Illinois
Kb	kilobases
l	litre
LE	Long Evans rat strain
LNA	locked nucleic acid
log	logarithm
M	molar
m	metre
MA	Massachusetts
mg	milligram (10^{-3} g)
min	minutes
miRNA	microRNA
ml	millilitre (10^{-3} l)
mm	millimetre(10^{-3} m)
mM	millimolar (10^{-3} M)
Mmu	Mus musculus
MNE	mean normalised expression
Mo	Missouri
mRNA	messenger ribonucleic acid
MW	molecular weight
n	number/sample size
NaCl	sodium chloride
ND	not determined
NE	normalised gene expression
ng	nanogram (10^{-9} g)
NJ	New Jersey
nm	nanometre (10^{-9} m)
NSW	New South Wales
O_2	oxygen, cyclic hyperoxia exposure
OIR	oxygen-induced retinopathy
OR	Oregon
PBS	phosphate buffered saline
p300	creb-binding protein homolog

PCA	principal components analysis
PCR	polymerase chain reaction
PDGF-β	platelet derived growth factor-β
PEDF	pigment epithelium derived factor
PHD	Prolyl hydroxylase
PHD1	PHD1/EGLN2
PHD2	PHD2/EGLN1
PHD3	PHD3/EGLN3
pre-miR	primary precursor microRNA transcript
pri-miR	precursor microRNA transcript
R^2	coefficient of determination of a linear *regression*
RA	room air
Ref	reference
RIN	RNA integrity number
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNA Pol II	RNA polymerase 2
Rno	*Rattus norvegicus*
ROP	retinopathy of prematurity
RPE	retinal pigment epithelium
rpm	revolutions per minute
RT	room temperature
RT-	reverse transcriptase-free; negative control cDNA
RT-PCR	reverse transcription-polymerase chain reaction
sec	second
SA	South Australia
SCD	sickle cell disease
SCF	stem cell factor
SD	Sprague Dawley rat strain. Note that in the context of statistical analysis, standard deviation is also abbreviated to SD.
SDRA	Sprague Dawley room air-exposed rat
SDO_2	Sprague Dawley cyclic hyperoxia-exposed rat
SLC16A3	Solute carrier family 16, member 3
snRNA	small nuclear RNA
snoRNA	small nucleolar RNA
SV40	Simian Vacuolating *Virus* 40
TBE	tris borate EDTA

TGF-β	transforming growth factor-β
Tie2	tyrosine kinase with Ig and epidermal growth factor homology domain receptor 2: receptor for angiopoietin-1 & -2
T_m	melting temperature
TRBP	TAR RNA-binding protein
TX	Texas
TYR	tyrosinase
U	units
Ub	Ubiquitin
UK	United Kingdom
UNG	Uracil-DNA glycosylase enzyme
USA	United States of America
UV	ultraviolet light
V	volt
v	version; volume
v/v	unit volume per unit volume
VEGF	vascular endothelial growth factor (VEGF A unless otherwise specified)
VEGFR-2	vascular endothelial growth factor receptor-2
VHL	von Hippel-Lindau protein
VIC	Victoria
w/v	unit weight per unit volume
WA	West Australia
WF	Wistar-Furth rat strain
WI	Wisconsin
x	times / multiplication factor