CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

2.1.a Water

Double glass-distilled water (ddH_2O) was used to prepare buffers and solutions unless otherwise stated. RNase-free water (autoclaved Milli-Q® water, Millipore, Billerica, MA, USA) was used for total RNA extractions (section 2.4.d). Water containing no nucleic acids or nucleases (Ultra Pure water, Fisher Biotech, West Perth, WA, Australia) was used for reverse transcription-polymerase chain reaction (RT-PCR).

2.1.b General chemicals

General chemicals were obtained from AJAX chemicals (Auburn, NSW, Australia), BDH Chemicals (Kilsyth, VIC, Australia) or Sigma Chemical Company (St Louis, MO, USA). Chemicals were of analytical reagent grade unless otherwise specified. Recipes for buffers and solutions are shown in section 2.2.

2.1.c Enzymes

Total RNA extracts were treated with DNase prior to cDNA synthesis using recombinant DNase I (Turbo DNA-free®, Ambion, Austin, TX, USA). RNA reverse transcription for mRNA expression work was performed using Superscript III® Reverse Transcriptase (SuperScript III®

First-Strand Synthesis System, Invitrogen, Carlsbad, CA, USA). Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was used for quantitative real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) in these experiments. RNA reverse transcription for microRNA (miRNA) expression work was performed using Multiscribe Reverse Transcriptase (TaqMan® MicroRNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA, USA). TaqMan® Fast Universal PCR Master Mix (Applied Biosystems) was used for quantitative real-time RT-PCR in the miRNA validation experiments. All enzymes were stored at -20°C.

2.1.d PCR primers

Primers used in real-time RT-PCR experiments to validate mRNA results were made by GeneWorks Pty Ltd (Thebarton, SA, Australia) at sequencing-grade purity. Guidelines for primer design are shown in section 2.4.l. Primer sequences are listed in Table 2.1.

Primers used in real-time RT-PCR miRNA validation experiments were commercially available from Applied Biosystems (Foster City, CA, USA). Sequences of the mature miRNAs for which the assays were designed are shown in Table 2.2.

Table 2.1 Quantitative real-time RT-PCR mRNA primer sequences. Primers were designed for use with SYBR® Green (Applied Biosystems, Foster City, CA, USA). Nucleotide position refers to the region of the mRNA sequence flanked by the primers and the exons to which the primers hybridise are also shown. All primers amplify a single product, the identity of which was subsequently confirmed by sequencing.

Gene (Accession No.)	Primer	Sequence (5'→ 3')	Nucleotide Position	Amplicon Size (bp)
Acidic Ribosomal Phosphoprotein (NM_022402.1)	ARBP(for) ARBP(rev)	AAAGGGTCCTGGCTTTGTCT GCAAATGCAGATGGATCG	766-856 exons 5/6	91
Hypoxanthine Guanine Phosphoribosyltransferase (NM_012583.2)	HPRT(for) HPRT(rev)	TTGTTGGATATGCCCTTGACT CCGCTGTCTTTTAGGCTTTG	629-733 exons 9/10	105
Insulin-like Growth Factor Binding Protein 2 (NM_103122.2)	IGFBP2 (for) IGFBP2 (rev)	GCGGGTACCTGTGAAAAGAG CCACATGGTTCTCCACCAG	346-445 exons 1/2	100
Insulin-like Growth Factor Binding Protein 3 (NM_012558.1)	IGFBP3 (for) IGFBP3 (rev)	CGAGTCTAAGCGGGAGACAG CTGGGACTCAGCACATTGAG	901-993 exons 2/3	93
EGL nine homolog 3 (NM 019371.1)	EGLN3 (for) EGLN3 (rev)	TTGGGACGCCAAGTTACATG GGGCTCCACGTCTGCTACAA	1003–1078 exons 2/3	76

Table 2.1 (continued) Quantitative real-time RT-PCR mRNA primer sequences.

Gene (Accession No.)	Primer	Sequence (5'→ 3')	Nucleotide Position	Amplicon Size (bp)
EGL nine homolog 1 (NM_178334.3)	EGLN1 (for) EGLN1 (rev)	GGCGTAACCCTCATGAAGTACAG TGCATCGAAATACCAAACGGTTATT	800-870 exons 3/4	70
BCL2/adenovirus E1B 19 kDa-interacting protein 3 (NM 053420.2)	BNIP3 (for) BNIP3 (rev)	GCGCACAGCTACTCTCAGCA GTCAGACGCCTTCCAATGTAGA	478-627 exons 4/5	150
Solute carrier family 16, member 3 (NM 030834.1)	SLC16A3 (for) SLC16A3 (rev)	ATGTGTGTGAACCGCTTTGG (for) GACCCCTGTGGTGAGGTAGATC	327-449 exons 2/3	123
Hexokinase 2 (NM 012735.1)	HK2 (for) HK2 (rev)	CAACATTCTCATCGATTTCACGAA GATGGCACGAACCTGTAGCA	2463-2607 exons 16/17	145

Table 2.2 Sequences of the mature miRNAs and small RNAs investigated by quantitative real-time RT-PCR. Specific primers were commercially available from Applied Biosystems (Foster City, CA, USA). All primers are human, rat and mouse reactive unless otherwise specified. Applied Biosystems assay ID numbers are provided.

miRNA	Assay ID	Mature miRNA sequence
miR-210	000512	CUGUGCGUGUGACAGCGGCUGA
miR-30e	002223	UGUAAACAUCCUUGACUGGAAG
miR-338	000548	UCCAGCAUCAGUGAUUUUGUUGA
miR-16	000391	UAGCAGCACGUAAAUAUUGGCG
miR-379	001138	UGGUAGACUAUGGAACGUAGG
miR-191	002299	CAACGGAAUCCCAAAAGCAGCUG
let-7d	002283	AGAGGUAGUAGGUUGCAUAGUU
4.5S RNA(H) Assay 1	001716	GCCGGTTGTGGTGGCGCACACCGGTAGGATTTGCTGAAGGAGGCAGAGGCAGGAG GATCACGAGTTCGAGGCCAGCCTGGGCTACACATTT
4.5S RNA(H) Assay 2	001717	AAAAATGTGTAGCCCAGGCTGGCCTCGAACTCGTGATCCTCCTGTCTCTGCCTCCTTCAG CAAATCCTACCGGCGTGCGCCACCACAACCGGC

Table 2.2 (Continued) Sequences of the mature miRNAs and small RNAs investigated by quantitative real-time RT-PCR.

miRNA	Assay ID	Mature miRNA sequence
snoRNA202 (mouse specific)	001232	GCTGTACTGACTTGATGAAAGTACTTTTGAACCCTTTTCCATCTGATG
snoRNA (rat specific)	001718	AGTTGAGGTCACACGCTGGTCGATGAACTCCTAAGTGTAGGTAGTGTGCTAAACGAG CGGCAAG
U6 snRNA	001973	GTGCTCGCTTCGGCAGCACATATACTAAAATTGGAACGATACAGAGAAGATTAGCAT GGCCCCTGCGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTT
RNU6B (human specific)	001093	CGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTTT
Y1 (rat specific)	001727	TGAGTTATCTCAATTGATTGTTCACAGTCAGTTACAGATTGAACTCCTGTTCTACACTTTCC CCCCTTCTCACT
U87 (rat specific)	001712	ACAATGATGACTTATGTTTTTGCCGTTTACCCAGCTGAGGGTTTCTTTGAAGAGAGAATCTT AAGACTGAGC

2.1.e Experimental animals

2.1.e.1 Ethical treatment of animals

All animal experimentation was approved by the institutional Animal Welfare Committee. Experiments met the standards described in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research [171], and those set out by the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes [172].

2.1.e.2 Rats

Inbred Fischer 344 (F344) and Sprague Dawley (SD) rats were obtained from the institutional animal facility. Annual allozyme electrophoresis for 15 genetic markers confirmed rat strains were inbred, shown by an absence of genetic variability within each strain and by no evidence of genetic contamination (Genetic Monitoring Service, Evolutionary Biology Unit, South Australian Museum). Rats were allowed unlimited access to rat chow ("New Joint Stock" Ridley Agriproducts, Murray Bridge, SA, Australia) and water and were exposed to a 12 h light–dark cycle. Temperature was maintained at 24°C in a humidified atmosphere. Animals were euthanased with an inhaled overdose of isoflurane anaesthetic.

2.1.f Miscellaneous materials

Table 2.3 Miscellaneous materials. A description of the miscellaneous items is shown below, along with the source of each item

Item	Description	Source
Agarose	Analytical grade	Promega, WI, USA.
DNA ladder 20 bp	20 bp – 1 Kb fragments in 20 bp increments	GeneWorks, Thebarton, SA, Australia
DNA ladder 2 log	100 bp- 10 Kb fragments	New England Biolabs, Beverly, MA, USA
GelRed™	1/10 000 v/v dilution	Biotum Inc., CA, USA
Isolectin GS-IB4	*Griffonia simplicifolia* type I isolectin B4-Alexa 488™ conjugate (excitation 495 nm; emission 519 nm)	Molecular Probes, Eugene, OR, USA
Sodium chloride for irrigation	0.9% w/v isotonic, non-pyrogenic, sterile	Baxter, Old Toongabbie, NSW, Australia
RNA extraction column	RNeasy® micro-kit	Qiagen, Hilden, Germany
DEPC	Diethylpyrocarbonate	Sigma, St. Louis, MO, USA
0.22 μm filter	0.22 μm Minisart filter	Sartorius Stedhim Biotech GmbH, Goettingen, Germany
Isoflurane (I.S.O) Inhalation Anaesthetic	Isoflurane 1ml/ml	Veterinary Companies of Australia Pty Ltd, Kings Park NSW

2.2 BUFFERS AND SOLUTIONS

2.2.a DEPC-NaCl

1 ml Diethylpyrocarbonate (DEPC)

Up to 1l 0.9% NaCl

Leave solution at room temperature overnight and autoclave prior to use to inactivate DEPC.

2.2.b Chrome alum-subbed microscope slides

Rinse glass slides thoroughly with ddH_2O and air dry in slide racks.

Prepare 0.05% $Cr(SO_4)_2.12H_2O$ in ddH_2O.

Place slide racks into chrome alum solution for 5 mins.

Air dry then wipe clean with cotton gauze prior to use.

2.2.c Phosphate Buffered Saline (PBS 10 x)

28.55 g $Na_2HPO_4.2H_2O$

(or 22.85 g Na_2HPO_4)

6.25 g $NaH_2PO_4.2H_2O$

70 g NaCl

Up to 1 l ddH_2O

Autoclave then dilute to desired concentration using ddH_2O.

Adjust pH to 7.3 prior to use.

2.2.d Sodium Hydroxide stock 10M

40 g NaOH pellets

Up to 100 ml ddH_2O

Add pellets slowly to water in fume hood (reaction is exothermic).

Dilute stock in ddH_2O to achieve desired molarity.

2.2.e Phosphate buffers 0.2M (pH 7.4)

Solution A

3.12 g $NaH_2PO_4.2H_2O$

Up to 100 ml ddH2O

Solution B

14.19 g NaH_2PO_4

Up to 500 ml ddH_2O

To make to 0.1 M phosphate buffer, add 95 ml solution A to 405 ml solution B. Adjust pH to 7.4. Filter sterilise using 0.22 μm filter.

2.2.f Paraformaldehyde (4%) in 0.1M phosphate buffer

4 g paraformaldehyde

Up to 60ml 0.1M phosphate buffer (section 2.2.e).

Heat to 65°C with mixing until dissolved (approximately 30 min). To this add 10 M NaOH drop-wise to clear the solution then cool. Make up to 100 ml with 0.1 M phosphate buffer. Filter (0.2 µm) to remove debris. Store at 4°C.

2.2.g Electrophoresis gel DNA/RNA loading buffer

1 ml glycerol

1 ml DEPC-H_2O

3 mg bromophenol blue

3 mg xylene cyanol

Mix 1 µl of loading buffer with 5 µl of DNA or RNA and load into agarose gel.

2.2.h EDTA Buffer (0.5 x)

113 g Ethylene-diamine-tetraacetic-acid (EDTA)

Dissolve in 400 ml ddH_2O and pH to 8.0 with HCl.

Make up to 500 ml with ddH_2O and autoclave.

2.2.i Tris Borate EDTA buffer (TBE 10 x)

108 g Tris base

55 g Boric acid

40 ml 0.5 M EDTA pH 8

Dissolve in 1 l ddH_2O and autoclave. Dilute with ddH_2O prior to use. RNase-free water is used in place of ddH_2O for RNA electrophoresis.

2.3 ANIMAL AND TISSUE TECHNIQUES

2.3.a Oxygen-induced retinopathy

2.3.a.1 Oxygen chamber

A custom-built oxygen chamber designed to deliver oxygen to neonatal rat litters under controlled conditions was constructed by Dr Anne-Louise Smith (Biomedical Engineering Department, Flinders Medical Centre, SA, Australia) (Figure 2.1).

The small Perspex chamber was constructed in the bottom of a wooden cabinet and lined with rubber seals to make the chamber relatively air-tight. A release valve was built into the chamber to prevent the build-up of excess pressure. Gas was vented via passive release into the atmosphere by a small opening at the top of the chamber through which an oxygen monitor was fed to the bottom of the chamber. Oxygen and air were delivered from two separate flow meters and blended prior to entry into the chamber via tubing. The blended oxygen and air mixture was pumped in at the top of the chamber and the mixture directed to the bottom of the chamber using additional tubing.

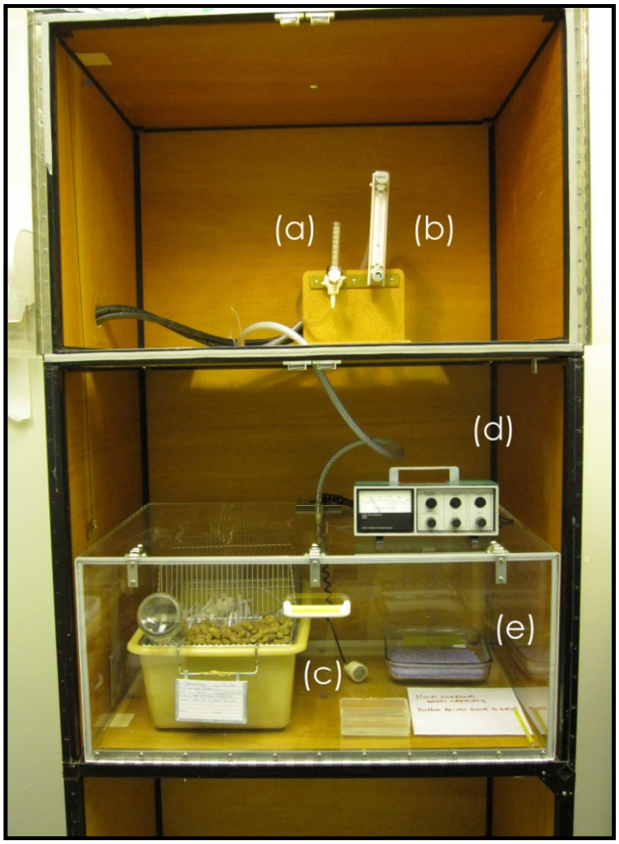

Figure 2.1 Oxygen chamber. Oxygen (a) and air (b), controlled by two separate flow meters were delivered to the oxygen chamber. A Perspex chamber was constructed within a wooden cabinet and made relatively air-tight using rubber seals. A single litter of rats was able to be housed within the chamber at a time. Oxygen levels in the chamber were monitored continuously using a fuel cell oxygen sensor (c) and an oxygen monitor (d) attached to a data recorder. Medical grade CO_2 absorbent was used to absorb expired CO_2 from within the chamber (e).

The oxygen flow meter was set to deliver oxygen at a rate of 2-2.5 l/min and a low flow air meter was set to deliver air at a rate of 1.2 l/min. These flow rates were found to maintain stable oxygen levels when rat litters were placed in the chamber. Medical grade CO_2 absorbent containing no alkali hydroxide (LoFloSorb, Intersurgical Ltd., Wokingham, Berkshire, UK) was used to absorb expired CO_2 from within the chamber.

Oxygen levels were continuously monitored with a fuel-cell oxygen monitor (Hudson Oxygen Monitor, model 5550, Temecula, CA, USA) and were recorded with a data logger (Tinytag, Gemini Dataloggers Ltd, Chichester, West Sussex, UK) for subsequent analysis (Tinytag Explorer, v2.4, Gemini Dataloggers Ltd) for the duration of each experiment. The oxygen monitor was regularly calibrated with room air and 100% oxygen prior to starting experiments and at the cessation of experiments to ensure the accuracy of monitoring and to calculate any drift in the monitor. Oxygen was maintained at 80 ± 5% and a maximum drift of ± 7% was considered acceptable.

A small container of water was placed at the bottom of the chamber to provide humidity. The temperature within the chamber and enclosing room was kept constant at 24°C and a 12 h light-dark cycle was maintained.

2.3.a.2 Exposure of neonatal rats to cyclic hyperoxia

The oxygen exposure protocol used in this study is a modification of those used in other studies of OIR in the rat [95, 173-176]. Within 12 h of birth, female rats and their litters were placed in a humidified chamber (Figure 2.1) and exposed to alternating 24 h cycles of hyperoxia (80% oxygen in air) and normoxia (21% oxygen in air) for a maximum of 14 days (Figure 2.2). This protocol was chosen because it reliably induces retinopathy in susceptible strains, without evidence of systemic toxicity [96]. Survival rates were in excess of 96% for hyperoxia-exposed neonatal rats, and there was no obvious

maternal oxygen toxicity. Mean litter size for F344 and SD rats were 11 and 9 pups, respectively. Age and strain-matched room air (normoxia)-exposed rats were used as controls. Pups from a minimum of two litters for each of the two rat strains being studied were analysed at each time point of interest. Multiple litters were used to distinguish between intra-strain and inter-strain variation. When an entire litter was not required for an experiment, individual rats were selected at random.

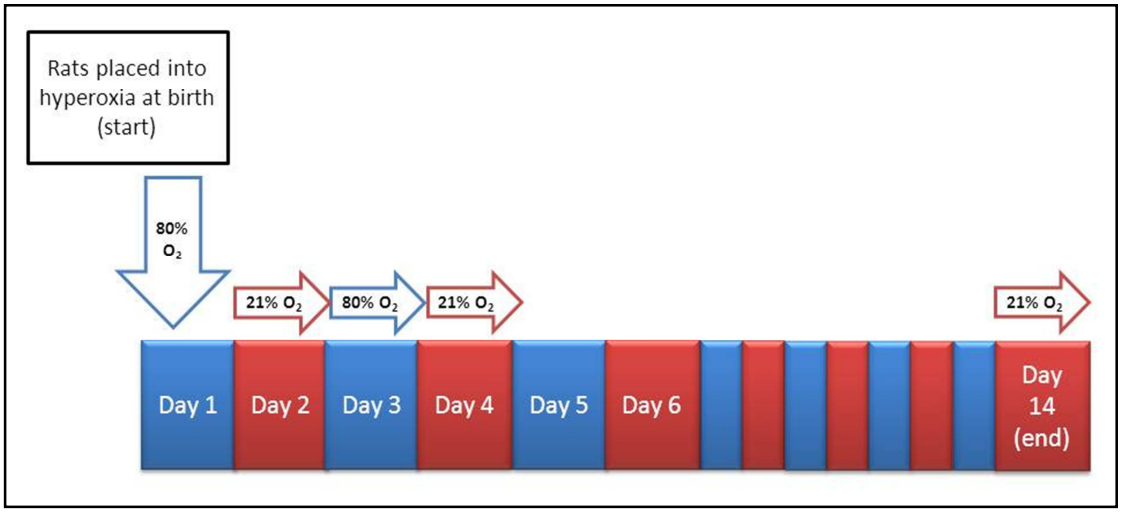

Figure 2.2 Cyclic hyperoxia exposure protocol. Within 12 hours of birth, newborn rats were placed in the oxygen chamber and exposed to 80% oxygen for 24 hours (blue boxes). The end of this time period was considered day 1. Over the next 24 hours, rats were exposed to room air (21% oxygen; red boxes), which was considered relatively hypoxic for the avascular retinae. The end of this period was considered day 2. Rats were then continuously exposed to this hyperoxia/hypoxia cycle for 14 days, at the end of which rats were euthanased for retinal dissection.

2.3.b Retinal dissection and histochemistry

Rats were killed with an inhaled overdose of isoflurane anaesthesia at designated time points, depending on experimental requirements. For each rat, the right eye was taken for retinal dissection and subsequent total RNA extraction as described in section 2.4.d, whilst the left eye was taken for retinal dissections and histochemistry labelling as described below.

Eyes were gently enucleated using fine curved forceps and curved-tip scissors. Eyes were fixed in 2% w/v paraformaldehyde in PBS (pH 7.4) for 90 min on ice. Retinal flat-mounts were performed using a modified method of Stone [177] and Chan-Ling [178]. Retinae were dissected in the lid of a petri-dish with 1-2 ml of ice cold PBS under an operating microscope (Wild Heerbrugg M690, Heerbrugg, Switzerland). Fat and connective tissue were dissected away from the eye to expose the sclera. A short section of the optic nerve was left attached to the globe to allow for manipulation of the eye without excessive pressure being placed on the globe. Fine-toothed forceps were used to hold the globe firmly in place by the optic nerve. An incision with a scalpel (No. 11 blade) was made at the corneoscleral limbus and fine-toothed forceps were used to turn the globe whilst microdissection scissors were used to continue to incision until the cornea was completely excised. The cornea, iris and crystalline lens were discarded.

More ice cold PBS was placed in the eye cup and microdissection scissors were used to cut the hyaloid vessels and vitreous gel from their retinal attachments. Particular attention was paid to the regions adjacent to the ora serrata and around the optic nerve head, where the attachments were most tight [178]. Care was taken at all times to remain above the surface of the retina.

Using a scalpel, four equally-spaced incisions extending from one third the way in from the optic nerve head, out to the peripheral retina, were made to flatten the tissue. Incisions were placed so as to avoid major vessels. The flattened eyecup was turned retina side-down, the optic nerve stump was grasped with forceps and the tissue was carefully transferred, face-down, to a chrom-alum subbed microscope slide. The remainder of the dissection was performed on the microscope slide.

A drop of chilled PBS was added to the tissue and two angled 26G lacrimal cannulae (BD Visitec, Becton, Dickson and Company, Waltham, MA, USA) attached to 1 ml syringes were used as blunt probes and inserted into the sub-retinal space via the cut edges of the eyecup. The choroid and sclera were carefully separated from each quadrant of the retina and excised with microdissection scissors. The optic nerve stump and the surrounding rim of choroid and sclera were removed and the retina was carefully turned over with two blunt probes. The ora serrata was cut away from the peripheral

retina. Fine forceps and microdissection scissors were used to remove the remaining vitreous and hyaloid vessels. A drop of ice cold PBS was added to prevent dehydration of the retina. The microscope slide was transferred to a humid box for isolectin histochemistry.

Within 6 h of enucleation, the dissected retinae were stained for 18 h with fluorochrome-conjugated *Griffonia simplicifolia* type I isolectin B4 (GS-IB4; Alexa Fluor 488 conjugate; Molecular Probes, Eugene, OR, USA) to highlight the retinal vasculature. Isolectin binds to alpha-galactosylated glycoprotein residues on vascular endothelial cells and macrophages [179].

Using a modified method of Cunningham [180], retinae were permeabilised with ice cold 70% v/v ethanol for 30 min at 4ºC, followed by PBS-1% v/v Triton X-100 (Ajax Chemicals, Sydney, NSW, Australia) for 20 min. Each retina was washed twice with PBS and incubated with 50 µl of 8 µg/ml isolectin in PBS overnight, in the dark at 4°C. Each retina was then washed a further seven times with PBS, covered with PBS: glycerol (2:1 v/v, pH 7.4) and cover slipped.

2.3.c Image analysis of isolectin-labelled retinae

Imaging of labelled retinae was performed within 12 h of staining. Images were captured using a fluorescence microscope (Olympus Optical Co. Ltd., Tokyo, Japan) attached to a CCD-digital camera (Roper Scientific, Trenton,

NJ, USA) and image acquisition software (CoolSNAP, Roper Scientific). Serial, overlapping high-resolution images of the entire retina were taken using a 4 x objective lens. Between 20 and 40 individual serial images were merged to create a montage (Adobe Photoshop CS3, Adobe systems Inc., San Jose, CA, USA).

The montages of all isolectin-labelled flat-mounts were assessed macroscopically prior to analysis of retinal avascular area and retinal blood vessel tortuosity for artefacts such as the introduction retinal tears which may have occurred during the flat mounting process. These artefacts may have affected the percentage of total avascular area in the retina and not be a true representation of susceptibility to OIR in these rats. In these instances, these flat-mounts were considered technical failures as total avascular area and retinal blood vessel tortuosity scores were not able to be determined, therefore were omitted from further analysis.

2.3.c.1 Avascular area analysis of isolectin-labelled retinae

The montages were cropped to show only the flat-mounted retina. The entire flat-mount was then manually outlined using the magnetic lasso and the measurement tools in Adobe Photoshop CS3 (Adobe systems Inc.). Avascular areas in the central and peripheral retina were separately outlined and measured as a percentage of the total retinal flat-mount by a masked observer. The central avascular area was defined as the capillary-free region

surrounding the optic nerve head and the peripheral avascular areas were defined as capillary-free regions showing no continuation with the optic nerve head. Repeat analyses by the same observer were concordant (mean difference 0.9 ± 1.2%). Analysis by a second masked observer was not performed.

2.3.c.2 Retinal vessel tortuosity analysis of isolectin-labelled retinae

A semi-quantitative grading system was used to analyse retinal vessel tortuosity [181]. Retinal montages of cyclic hyperoxia-exposed rats were compared to reference images of room air-exposed control rats and graded for vascular tortuosity (Figure 2.3). Each quadrant of the retina was divided into three approximately equal segments and each clock hour assessed in a masked fashion for the degree of vessel tortuosity present in the primary blood vessels compared to the reference images. A score of one was given per clock hour for normal first-order vessels, up to a score of four, when highly tortuous vessels were present. A minimum score of 12 and maximum score of 48 was possible for each retinal flat-mount.

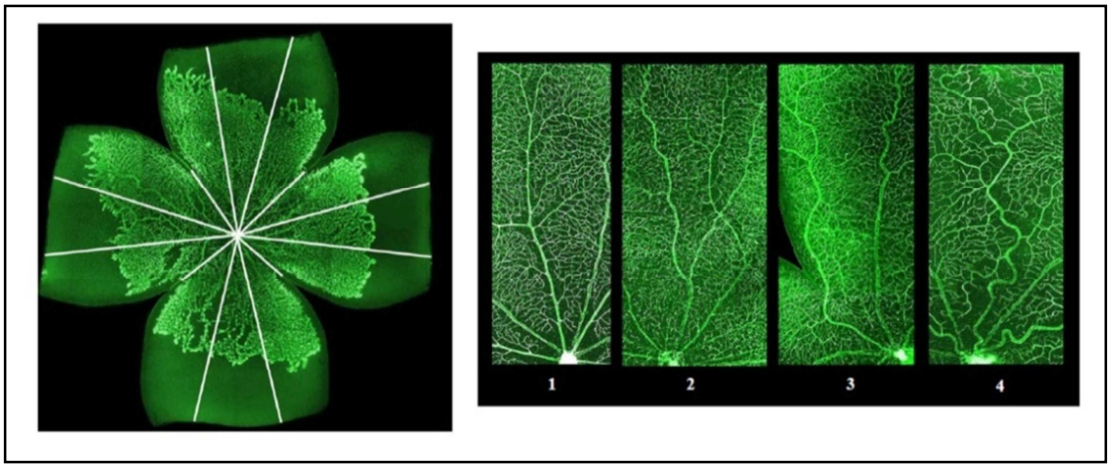

Figure 2.3 Clock hour method for scoring retinal vessel tortuosity (modified from van Wijngaarden [181]). Each retinal quadrant was divided into three segments (left) and each clock hour was scored for the degree of vessel tortuosity observed in the first-order vessels compared to the reference images (right). Key: 1= normal; 2 = mild tortuosity; 3 = moderate tortuosity; 4 = marked tortuosity. A minimum score of 12 was possible when no tortuosity was present in all 12 clock hours, and a maximum score of 48 was possible when highly tortuous blood vessels were observed in all 12 clock hours.

2.4 MOLECULAR TECHNIQUES

2.4.a RNA collection time line

Total RNA was collected from both retinae of rats as described in section 2.4.d. These rats differed from those that were used for the analyses of retinal avascular area and tortuosity scoring in section 2.3.b. The retinae were collected from rats at different time points during the 14 day room air or cyclic hyperoxia exposure period, depending on experimental requirements for the use of total RNA. Four experimental conditions were tested: F344 room air-exposed (RA), F344 cyclic hyperoxia-exposed (O_2), SD RA and SD O_2.

2.4.b Collection of rat retinae for microarray analysis

Total RNA was collected from the retinae of rats exposed to room air or cyclic hyperoxia at postnatal days 3, 5, and 6 for concurrent microarray analysis of gene and microRNA expression. Generally a minimum litter size of 9 pups was required during the course of RNA collection. Pups were removed from each litter on different days depending on the experimental requirements, until no pups remained. As a positive control for the cyclic hyperoxia exposure protocol, both retinae from a representative day 14 rat were flat-mounted and assessed for evidence of oxygen-induced retinopathy. Each representative day 14 cyclic hyperoxia-exposed rat showed incomplete retinal neovascularisation, consistent with exposure to cyclic hyperoxia.

An initial microarray screen was performed on RNA collected from rats at day 3. A second microarray screen required new litters to be bred so that the retinae of these rats could be collected on consecutive days (days 5 and 6) from within the same litter for RNA extraction. These litters were separate to those used at day 3.

Cyclic hyperoxia-exposed rats collected at days 3 and 5 were removed from the oxygen chamber after a 24 h period of hyperoxia. Rats collected at day 6 were removed from the oxygen chamber after a 24 h period of relative hypoxia (Figure 2.4). These three time points early in the cyclic hyperoxia exposure period were chosen to investigate changes that may be causative of

the disease, rather than changes that are a consequence of the initial changes. Examining changes in gene and microRNA expression that occur between days 5 and 6 enabled identification of changes which were specific either to hyperoxia at day 5 or exposure to relative hypoxia at day 6.

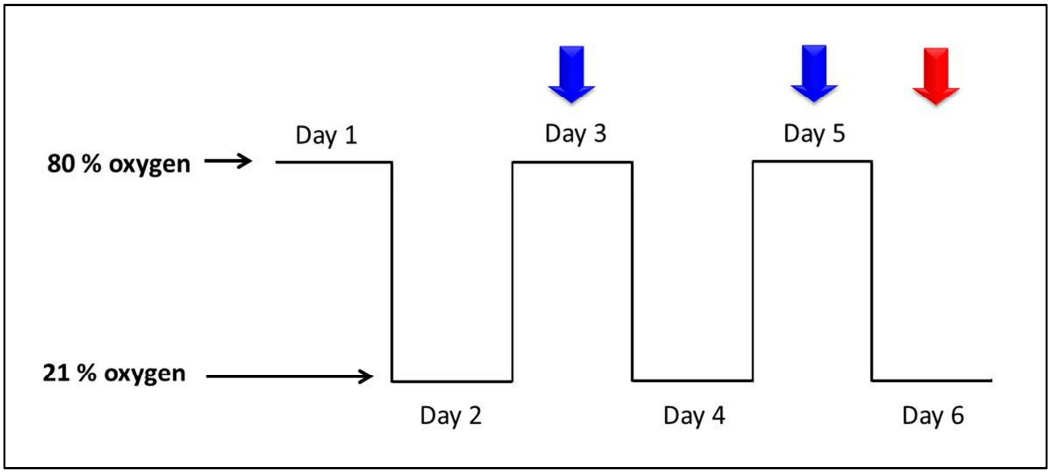

Figure 2.4 Time points at which retinae were collected for total RNA extraction from cyclic hyperoxia-exposed rat litters. Retinal RNA was collected at 3 different time points during the 14 day cyclic hyperoxia exposure period as indicated by the arrows. Blue arrows: retinae collected at the end of a 24 h period of hyperoxia; red arrow: retinae collected at the end of a 24 h period of relative hypoxia. Differential gene and miRNA expression could then be attributed to either exposure to cyclic hyperoxia at days 3 and 5, or to relative hypoxia at day 6.

For each experimental condition, total RNA from the retinae of 3 randomly selected rats were taken from a minimum of 2 different litters and pooled to make a test sample for microarray analysis (Figure 2.5). Each rat was represented equally in the pool in terms of RNA yield with approximately 4 µg of RNA included for each rat. RNA from the remaining rats were used to make a second and third pool as required. Total RNA was kept for each of

the individual rats for downstream quantitative real-time PCR analysis of gene and microRNA expression levels. Samples were outsourced to the Adelaide Microarray Centre (Adelaide, Australia) for microarray analysis as described in sections 2.4.g and 2.4.s.

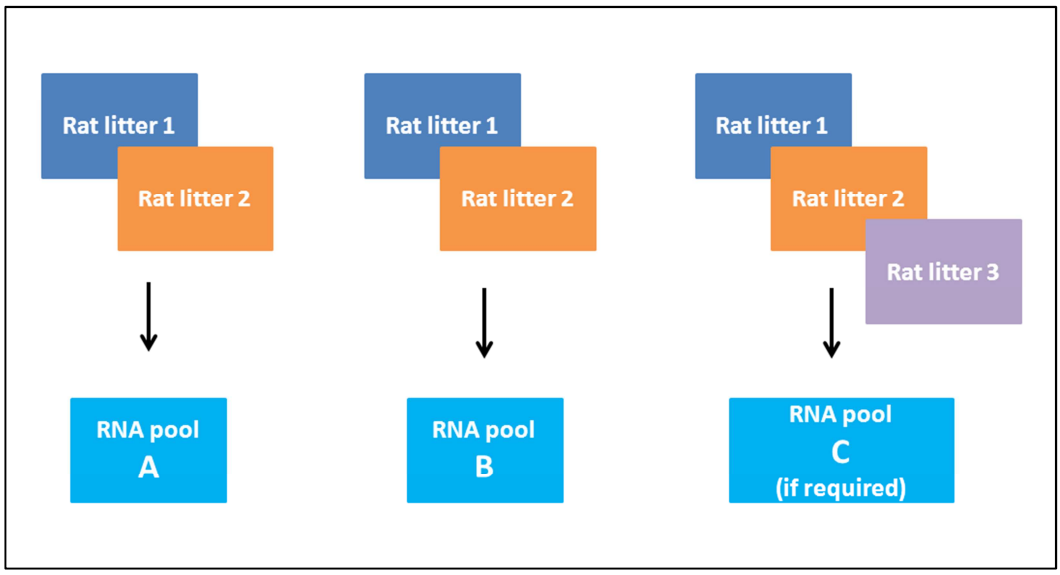

Figure 2.5 Schematic representation of the retinal RNA pools submitted for microarray analysis. Retinal RNA was collected at days 3, 5, or 6 from a minimum of 2 litters for each experimental condition. Three randomly selected rats were chosen from rat litter 1 and rat litter 2 to form RNA pool A. Another 3 randomly selected rats were chosen from the same 2 litters to form RNA pool B. If a third RNA pool was required, then any remaining rats from the first 2 litters were used and supplemented with randomly selected rats from rat litter 3 to create RNA pool C.

2.4.c Collection of rat retinae for real-time RT-PCR analysis

Relative quantification real-time RT-PCR was used to confirm microarray results on either the same RNA pools tested in the microarray experiments and/or on RNA from individual rats collected from different litters. At day 3, initial confirmation experiments were performed on the same pooled RNA

samples used in the microarray experiments. Subsequent gene and microRNA expression analysis was performed on RNA from individual rats that had been previously pooled for the microarray analysis, as well as on RNA from individual rats collected at later date. At days 5 and 6, microarray findings were confirmed using RNA from individual animals which differed to those used in the microarray pools due to limitations in RNA yield from these rat retinae.

To confirm microarray findings in individual animals, rats were collected from a minimum of two litters for each experimental condition being tested. Both retinae from each rat were collected for total RNA extraction at postnatal days 3, 5 and 6 for both room air and cyclic hyperoxia-exposed rats to enable time-course analysis of gene and microRNA expression. RNA from individual rats was not pooled at these time points. A minimum of 4 rats per experimental condition were used to confirm microarray findings, with the number of rats tested per group dependent on the quality of the total RNA being tested.

2.4.d Total RNA extraction from rat retinae

Neonatal rats were euthanased with an inhaled overdose of isoflurane anaesthetic. The eyes were immediately enucleated and placed into chilled DEPC-treated saline. The neural retina, including the RPE layer was dissected from the whole eye using instruments treated with RNase Zap

(Ambion, Austin, TX, USA) and rinsed with ddH_2O followed by DEPC-NaCl. Care was taken to manipulate the retinal tissue as little as possible to maximise retainment of the RPE. The dissection was performed in a petri dish over ice and tissue handling was kept to a minimum to prevent RNA degradation. Both retinae from an individual rat were placed in a single 1.5 ml tube, snap frozen in liquid nitrogen and then stored at -80°C until required. Gloves were worn at all times to prevent the introduction of RNases.

Total RNA was isolated from the pooled retinae of individual rats using TRIzol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's method. RNase-free reagents, tubes and pipette tips were used at all times. Briefly, 500 µl of TRIzol® was added to the tube containing the frozen retinae. The retinae were not allowed to thaw prior to addition of the TRIzol® reagent to prevent the degradation of RNA. The tissue was homogenised using a plastic pestle (Edwards Instrument Co., Narellan, NSW, Australia) attached to a Dremel MultiPro™ drill. The homogenised tissue was allowed to dissociate further in TRIzol® at room temperature for a minimum of 30 min before 100 µl of Chloroform (BDH Chemicals, Kilsyth, VIC, Australia) was added and the tube shaken vigorously for 15 sec. The sample was left at room temperature for a further 2-3 min then centrifuged for 15 min at 4°C at ~ 13 000 *g*.

After centrifugation the mixture separated into 3 phases. The upper aqueous phase was transferred into a fresh 1.5 ml tube. The remaining phases were stored at -80°C for protein extraction from the interphase. RNA was precipitated by adding 250 µl of isopropanol (Sigma St Louis, MO, USA) and left to stand for 15 min at room temperature. The sample was centrifuged for 10 min at 4°C at ~ 13 000 *g* and the supernatant removed. The pellet was washed with 750 µl of 75% ethanol prepared with RNase-free MilliQ water and centrifuged for 5 min at 4°C at ~ 5 000 *g*. The supernatant was removed and the pellet air dried for 10 min at room temperature. The pellet was dissolved in 30 µl of RNase-free MilliQ water and kept on ice.

2.4.e Quantification and determination of RNA integrity

Total RNA was quantified using the Nanodrop 8000 (Thermo Scientific, Wilmington, DE, USA). The spectrometer was blanked using 1.5 µl of RNase-free MilliQ water, and then 1.5 µl of RNA solution was quantified. RNA yield from neonatal and adult rats was typically between 16 - 45 µg (both retinae). The absorbance ratio of the RNA at 260 and 280 nm was used as a guide in determining RNA purity. Samples with a ratio of 1.8 or more were considered to be acceptable. The ratios typically ranged from 1.83 – 2.22.

RNA integrity and residual DNA contamination was also assessed using agarose gel electrophoresis. Gloves were worn at all times to prevent the introduction of RNases. An electrophoresis tank, comb and gel tray were

washed with detergent and rinsed well with ddH_2O to minimise DNA and RNase contamination. The comb and gel tray were further rinsed with 70% ethanol prepared with RNase-free water and allowed to dry overnight. A 1 % agarose gel was made using 0.5 x RNase free TBE in a clean flask dedicated to RNA work. GelRed™ (Biotum Inc., CA, USA) was used at a 1/10 000 v/v dilution to visualise RNA under low intensity UV light. Approximately 1.5 µg of total RNA was mixed with 3 µl of loading dye and loaded on the gel. The gel was run at 100 volts for 75 mins. Total RNA with a 28S:18S ratio of approximately 2:1 showing little or no smearing and minimal DNA contamination was considered acceptable for further use.

2.4.f Preparation of test RNA samples for mRNA microarray analysis

RNA of greater purity was required for mRNA microarrays analysis; therefore total RNA was purified using a Qiagen RNeasy Micro column purification kit (Qiagen, Hilden, Germany) as detailed below, prior to submission to the Adelaide Microarray Centre.

Briefly, RNA samples were made up to a volume of 100 µl and 350 µl of Buffer RLT containing 1% v/v 2-mercaptoethanol (Sigma) was added and mixed well. To this 250 µl of 96-100% RNase-free ethanol was added and the sample was mixed by pipetting before being transferred to an RNeasy MinElute spin column and centrifuged for 15 sec at ≥ 8000 *g*. The flow

through was discarded and 700 µl of Buffer RW1 was added to the spin column then centrifuged for 15 sec at ≥ 8000 *g* to wash the spin column membrane. The flow-through and collection tube were discarded.

The spin column was placed in a fresh 2 ml collection tube and 500 µl of Buffer RPE was added, and then centrifuged for 15 sec at ≥ 8000 *g*. The flow-through was discarded and 500 µl of 80 % ethanol prepared with RNase-free water added to the spin column then centrifuged for 2 min at ≥ 8000 *g* to wash the spin column membrane. The flow through and collection tube were discarded. The spin column was placed into a fresh collection tube and centrifuged at full speed with the lid open for 5 min to dry the spin column membrane and prevent any residual ethanol from contaminating downstream reactions. The flow through and collection tubes were discarded.

The spin column was then placed in a fresh 1.5 ml tube and 14 µl of RNase-free water was added directly to the centre of the membrane. The spin column was allowed to stand for 1 min prior to centrifugation at full speed for 1 min to elute the RNA. The eluted RNA was kept on ice. RNA concentration was determined as described previously, and the A260:280 ratio was used to assess RNA purity.

Samples containing 3-5 μg of RNA were submitted to the Adelaide Microarray Centre. Prior to performing the arrays, samples were further analysed by the facility to determine RNA integrity using the Agilent Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA, USA). The Bioanalyser software generates an RNA integrity number (RIN) for each sample. The RIN is an indication of RNA degradation and is scored on a scale of 1-10, 1 indicating degraded RNA and 10 indicating fully intact RNA. Samples with a RIN of 7 or above were deemed suitable for further use in mRNA microarrays. The RIN typically ranged from 9.3 – 10.0. Two to 3 samples were provided for each of the 4 experimental conditions.

2.4.g Affymetrix mRNA microarrays

Affymetrix GeneChip Gene 1.0 ST Rat Arrays (Affymetrix Inc., Santa Clara, CA, USA) covering 27,342 genes using 722,254 distinct 25-mer oligonucleotide probes were used for gene expression analysis. The array is based on the rat genome assembled in November 2004 (UCSC rn4; Baylor HGSC build 3.4) with comprehensive coverage of RefSeq NM transcripts and GenBank putative full length transcripts from April and January of 2007, respectively.

Each of the 27,342 genes is represented on the array by approximately 26 probes spread across the full length of the gene, targeting multiple exons in a transcript. This provides a more complete and more accurate picture of gene

expression than 3′-based expression array designs where truncation or alternate splicing of the 3′ end of the gene may result in inaccurate assessment of gene expression.

Arrays were performed at the Adelaide Microarray Centre following the manufacturer's protocol. Briefly, cDNA was synthesised and purified prior to labelling with a fluorescent dye, then hybridised to the microarray slide before the slide was washed and dried prior to scanning. These methods are freely available and can be accessed online at http://www.microarray.adelaide.edu.au/protocols/.

Single colour arrays allowed for comparisons to be made between all experimental conditions as shown in Table 2.4. The number of RNA pools submitted for microarray analysis per time point is shown in Table 2.5. Additional samples were required for day 3 F344 cyclic hyperoxia-exposed and SD room air-exposed rats based on the results of principal components analysis as described in section 4.2.a.1.

Comparison	Differential gene expression association
FRA vs. SDRA	Strain-related
FO_2 vs. FRA	Oxygen-related differences in F344 rats resistant to OIR
SDO_2 vs. SDRA	Oxygen-related differences in SD rats susceptible to OIR
FO_2 vs. SDO_2	Differences associated with strain AND exposure to cyclic hyperoxia
Interaction (Strain*Treatment)	Differences associated with interaction between strain (F344 and SD combined) AND treatment (RA and cyclic hyperoxia exposure combined)

Table 2.4 Comparisons of each experimental condition for Affymetrix microarrays The association between the comparison being performed and the resulting gene expression change is shown in the table above. FRA = F344 room air-exposed; SDRA = SD room air-exposed; FO_2 = F344 cyclic hyperoxia-exposed; SDO_2 = cyclic hyperoxia-exposed.

Experimental condition	Day 3	Day 5	Day 6
FRA	2	2	2
FO_2	3	2	2
SDRA	3	2	2
SDO_2	2	2	2

Table 2.5 Number of RNA pools submitted for Affymetrix microarray analysis at each time point. Each RNA pool consisted of 3 randomly selected rats taken from a minimum of 2 litters. FRA = F344 room air-exposed; SDRA = SD room air-exposed; FO_2 = F344 cyclic hyperoxia-exposed; SDO_2 = cyclic hyperoxia-exposed.

2.4.h DNase treatment of RNA extracts

Contaminating genomic DNA was removed from total RNA using DNaseI (Turbo DNA-free, Ambion) according to the manufacturer's instructions for rigorous treatment of RNA. Briefly, 3 µl of DNaseI buffer and 2 µl of

recombinant DNaseI were added to approximately 6 µg of total RNA made to a final volume of 30 µl using Ultra Pure water (Fisher Biotech, WA, Australia). The mixture was incubated at 37°C for 30 min, before another 1 µl of recombinant DNaseI was added, followed by a second 30 min incubation at 37°C. From here, 6 µl of DNaseI inactivation slurry was added and mixed by intermittent vortexing at room temperature over a period of 5 min. The inactivated mixture was then centrifuged at 10,000 *g* for 1.5 min, and the supernatant containing the RNA was transferred to a clean 1.5 ml tube and stored on ice. Where possible, total RNA extractions and cDNA synthesis were performed on the same day to prevent RNA degradation upon freezing and thawing of the samples. Where this was not possible, DNase-treated RNA was stored at -80°C until required.

2.4.i Preparation of the standard mRNA sample

To allow for standardisation, a standard cDNA pool was made from retinal RNA pooled from several F344, SD and pigmented Dark Agouti (DA) rats from various developmental stages as shown in Table 2.6. DA rats were included in the standard mRNA sample to allow for the analysis of gene and microRNA expression in the pigmented rat strain; however, this did not eventuate.

Total RNA was extracted from both retinae of a single rat per time point unless otherwise indicated. A proportion of the total RNA from each

individual rat was kept aside for the preparation of standard microRNA sample as described in section 2.4.t. The remainder of the total RNA was DNase-treated as described in section 2.4.h prior to being pooled. Each sample was represented equally within the pool in terms of RNA yield with 400 ng of RNA included for each rat.

Strain	Experimental exposure	Postnatal day
F344	Room air	2, 3, 5, 9, 14
SD	Room air	2 (2 rats), 8, 9, 14
DA	Room air	3, 5, 6, 9, 14
F344	Cyclic hyperoxia	2, 3, 5, 6, 8, 9, 14
SD	Cyclic hyperoxia	2, 3, 5, 6, 9
DA	Cyclic hyperoxia	2, 3, 14

Table 2.6 Rat retinae used in the pooled cDNA standard sample. RNA was extracted from both retinae of each rat and DNase-treated prior to pooling for reverse transcription to cDNA to make the cDNA standard sample. A single rat was used per time point unless otherwise indicated.

2.4.j cDNA synthesis of the standard mRNA sample

Two cDNA synthesis reactions were performed in bulk, using a scaled up version (10 x reaction volume per 1.5 ml tube) of the reaction described in section 2.4.k for individual test samples. A total of approximately 20 µg of RNA was converted into cDNA. A reverse transcriptase-free control pool was prepared in parallel with the standard pool to act as negative control for the real-time RT-PCR but scaled down to prepare 120 µl of a standard pool

reverse transcriptase negative control. The standard cDNA pool was frozen in aliquots at −80°C and diluted to a working concentration of 1/5 as required.

2.4.k cDNA synthesis of test samples

For each cDNA sample, 1 µg of Turbo DNase-treated RNA was reverse-transcribed in 20 µl reactions using a first-strand cDNA synthesis kit (SuperScript III® First-Strand Synthesis System, Invitrogen) according to the manufacturer's instructions. Briefly, 1 µl of random hexamers (50 ng/µl) and 1 µl of dNTP mix (10 mM) were added to 1 µg of DNAse-treated RNA and the volume made up to 10 µl with DEPC-treated water in a 1.5 ml tube. The mixture was vortexed and briefly pulse centrifuged to bring the contents of the tube to the bottom, then incubated at 65°C for 5 min prior to cooling on ice for a minimum of 2 min. Ten µl of cDNA synthesis mix was added (2 µl of 10X RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M dithiothreitol {DTT}, 1 µl of RNaseOUT and 1 µl of SuperScript III® reverse transcriptase) before mixing and gentle centrifugation. The reactions were incubated at 25°C for 10 min, following by a 50 min incubation at 50°C. The reaction was terminated with a 5 min incubation at 85°C. The contents of the tube were briefly pulse spun and 1 µl of RNase H was added prior to a final 20 min incubation at 37°C. A reverse transcriptase-free control sample was prepared in parallel with each cDNA sample, with DEPC-treated water substituted for the reverse transcription enzyme in these reactions. These samples acted as

negative controls for the real-time RT-PCR. Neat cDNA and 1/5 dilutions of each sample were prepared with Ultra Pure water (Fisher Biotech) and stored at -20°C until required.

2.4.l PCR primer design for gene expression

Primers for candidate genes were designed using Primer3 software [182] according to stringent criteria for optimal design as shown in (Table 2.7) [183]. Primers were tested *in silico* for specificity against sequences for *Rattus norvegicus* using NCBI BLAST software to identify intron-exon boundaries (www.ncbi.nlm.nih.gov/BLAST). Primers were designed to span introns for easy discrimination between products derived from genome amplification and those derived from cDNA amplification on the basis of size. Amplicons were designed to be between 65-100 bases in length.

Primer Properties	
Length	15-20 bases
Sequence	G/C content 20-70% ≤ 2 G/C in the last 5 bases of the 3' end Forward and reverse primers bind to separate exons
Melting temperature	Minimum: 58°C Maximum 60°C ≤ 2°C difference between forward and reverse primer pair

Table 2.7 Optimal primer design criteria. Parameters for optimal primer design as recommended by Bustin [183].

Primer pairs were aligned with the rat genome using NCBI BLAST to identify potential genomic DNA amplification and were considered acceptable for testing if the genomic amplicon was more than twice the length of the target cDNA amplicon. Primers were synthesized by Geneworks Ltd (Thebarton, South Australia, Australia) and tested in real-time RT-PCR with SYBR® Green chemistry.

Primer PCR amplification efficiencies were determined for each set of primers using seven 1/5 serial dilutions of the standard cDNA sample to generate a standard curve. The mean Ct value for each dilution was plotted against the log cDNA concentration and the gradient of the regression line of the standard curve was used to calculate the PCR amplification efficiency. Amplification efficiencies were used in the quantification of gene expression [184].

Real-time RT-PCR products of all genes of interest were purified and sequenced for comparison with the predicted amplicon to confirm sequence identity. The melt curve for each real-time PCR product was also compared to that of the corresponding sequenced product of the gene of interest to confirm its identity.

2.4.m Agarose gel electrophoresis

PCR products were analysed on 1% agarose gels stained with GelRed™ (Biotum Inc.) Each gel was prepared by adding 0.8 g of agarose (Promega, WI, USA) to 80 ml of 0.5X TBE buffer and heating in a microwave oven until completely dissolved. Eight µl of GelRed™ (1/10 000 v/v dilution) was added to the agarose gel solution and mixed gently before being poured into a gel tray with a well comb in place. The gel was allowed to set for approximately 20 min before the gel comb was removed. The gel was then transferred to an electrophoresis tank containing 0.5 x TBE. One µl of loading dye was added per 5 µl of PCR product and mixed prior to loading into each well. Two molecular weight markers were used for product size estimation: 2 log DNA ladder (New England Biolabs, Beverly, MA, USA) and a 20 bp DNA ladder (Geneworks). PCR products underwent electrophoresis for approximately 1 h at 100 volts before being visualised under low intensity UV light.

2.4.n Agarose gel PCR product purification

PCR products were run on 1% agarose gels and visualised under low intensity UV light. DNA was extracted from the gels using clean scalpel blades, and purified using the Qiaquick column purification system (Qiagen). Purified DNA was eluted in 10 mM Tris–Cl pH 8.5 then quantified using the Nanodrop 8000 (Thermo Scientific) as described in section 2.4.e prior to sequencing.

2.4.o PCR product sequencing

Purified PCR products were sequenced by Mr Oliver van Wageningen (DNA sequencing facility, Flinders University, SA, Australia). PCR products were labelled using the BigDye® Terminator v3.1 Cycle Sequencing Kit and resolved using the ABI 3100 Genetic Analyser (Applied Biosystems).

2.4.p General precautions for PCR

Polymerase chain reactions were prepared in a dedicated room free of contamination from PCR-products. Gloves and a clean gown were worn at all times and consumables free of nucleic acids and nucleases were used (Edwards Instrument Company).

2.4.q Quantitative real-time RT-PCR for gene expression

Quantitative real-time RT-PCR was performed with reference to the MIQE guidelines [185]. Each 20 µl reaction mixture contained 10 µl of Power SYBR® Green PCR Master Mix (Applied Biosystems), 2 µl each forward and reverse primers (0.5 µM final concentration), and 6 µl cDNA sample diluted 1/5 with Ultra Pure water (Fisher Biotech). Reactions were run on a StepOnePlus Real-Time PCR System (Applied Biosystems). Cycling conditions were: initial denaturation (95°C, 10 min) followed by 40 cycles of denaturation (95°C, 15 sec), annealing, and extension (60°C, 1 min). A melt

curve was performed at the end of each run 60-99°C at 0.5°C intervals to confirm a single product was amplified and its identity.

Candidate genes were tested in triplicate wells for each sample. A working dilution of cDNA of 1/5 for all genes of interest, including the reference genes was used based on the findings from the standard cDNA sample dilution series for the standard curve as described in 2.4.l. Each real-time RT-PCR run was designed to test a maximum of 2 genes of interest alongside 2 reference genes in as many samples as possible. The standard cDNA pool was included in triplicate in each PCR run. A single RT-free negative control for each sample and 2 water controls (no-template control) were also included in each experiment. A negative control sample was considered to be negative for product amplification if the cycle threshold (Ct) was at least 5 cycles greater than that of the corresponding test sample. The Ct was defined as the cycle at which the fluorescence was raised above the background fluorescence; which coincided with the approximate starting point of the exponential phase.

Data from the PCR reactions were analysed using StepOne Plus software provided with the system. The same manual baseline was used for all genes between real-time PCR runs to omit any background fluorescence present in the wells of the StepOne Plus instrument. A gene-specific manual threshold value based on the approximate start point of the exponential phase of the

reaction was also used to ensure consistent analysis between real-time PCR runs.

2.4.r Relative quantification of retinal gene expression

An expression value for each gene of interest in each sample triplicate was calculated relative to the standard cDNA pool using a delta Ct method with adjustment for amplification efficiency [184, 186]. The relative expression value was normalised to the reference genes acidic ribosomal phosphoprotein (ARBP) and hypoxanthine guanine phosphoribosyl transferase (HPRT) using the geometric mean of the two reference genes in the same sample to calculate a normalisation factor [184, 187]. This normalisation factor was applied to each sample triplicate and the mean expression value was determined by averaging the normalised relative expression values for the 3 replicates of that sample. The reference genes ARBP and HPRT were chosen as they are stably expressed during cyclic hyperoxia and early development in a number of rat strains [188].

2.4.s Exiqon microRNA microarrays

For the day 3 total RNA samples, Exiqon microRNA version 8.1 all species array library (Exiqon A/S Vedbaek, Denmark) were printed onto slides at the Adelaide Microarray Centre, and total RNA on slides were labelled, hybridised and scanned as described by Neal et al., [189]. Statistical analysis was performed as described in section 2.5.c. Each slide consisted of

approximately 1500 microRNA probes from 50 different species including rat, human and mouse. The capture probes were complementary to mature miRNAs registered in miRBase Release 8.1 database [190] and covered 92.3% of the miRNAs registered in miRBase Release 9.0. The array also contained an additional 146 capture probes which target proprietary miRNAs that have been discovered by Exiqon but have yet to be included in miRBase. Several control capture probes were included on each array including positive and negative controls, as well as control probes to ensure optimal labelling and hybridisation of samples.

Exiqon microRNA version 11 all species array library (Exiqon A/S) became available for the day 5 and 6 total RNA samples and were printed onto slides at the Adelaide Microarray Centre [189]. Each slide consisted of approximately 1769 microRNA probes from 50 different species including rat, human, mouse and their related viruses. The capture probes were complementary to mature miRNAs registered in miRBase Release 11.0. The array also contained an additional 43 control capture probes for quality control purposes.

Exiqon arrays utilise Locked Nucleic Acid (LNA™) technology to lock the oligonucleotides on the array in the ideal conformation required to increase the affinity and specificity of the probe for its microRNA target via more efficient hybridisation. This is particularly important due to the short length

of mature miRNAs and the fact that closely related miRNAs may differ from each other by as little as a single nucleotide.

Two-coloured microRNA microarrays were performed. Eight µl of each sample at a concentration of ~1000 ng/µl was provided to the microarray facility. Half the sample was labelled with the fluorescent dye Cy3 and the other half with Cy5. Each sample was applied to 2 separate arrays. The dye swap was performed to prevent the introduction of dye bias of the samples into the data, as well as acting as a technical replicate. Each sample was applied to the array in a competitive hybridisation reaction.

Eight 2-coloured arrays were performed in total as outlined in Table 2.8. The use of 2-coloured arrays only allowed comparisons to be made between the two samples tested on each array. The same array design was used at each time point. Each sample was tested in duplicate and 2 pooled samples were provided for each experimental condition. These pools were the same pools used in the Affymetrix arrays as described in section 2.4.b. Two samples for each experimental condition were sufficient for the Exiqon microarrays.

A number of different comparisons were made to provide insight into the biological relevance of the microarray data as shown in Table 2.9.

Array	Cy3	Cy5
1	SDO_2 a	FO_2 a
2	FO_2 b	SDO_2 b
3	FO_2 a	FRA a
4	FRA b	FO_2 b
5	SDRA a	SDO_2 a
6	SDO_2 b	SDRA b
7	FRA a	SDRA a
8	SDRA b	FRA b

Table 2.8 Description of the comparisons made on the 8 arrays performed at each time point. Each array was performed in duplicate to include a dye swap which prevented the introduction of dye bias of the samples into the data, as well as acting as a technical replicate. a,b two different pools of RNA to act as biological replicates. FRA = F344 room air-exposed; SDRA = SD room air-exposed; FO_2 = F344 cyclic hyperoxia-exposed; SDO_2 = cyclic hyperoxia-exposed.

Comparison	Differential gene expression association
FRA vs. SDRA	Strain-related
FO_2 vs. FRA	Oxygen-related differences in F344 rats resistant to OIR
SDO_2 vs. SDRA	Oxygen-related differences in SD rats susceptible to OIR
FO_2 vs. SDO_2	Differences associated with strain AND exposure to cyclic hyperoxia
Interaction (Strain*Treatment)	Differences associated with interaction between strain (F344 and SD combined) AND treatment (RA and cyclic hyperoxia exposure combined)

Table 2.9 Comparisons of each experimental condition for the Exiqon microarrays. The association between the comparison being performed and the resulting gene expression change is shown in the table above. FRA = F344 room air-exposed; SDRA = SD room air-exposed; FO_2 = F344 cyclic hyperoxia-exposed; SDO_2 = cyclic hyperoxia-exposed.

2.4.t Preparation of the standard microRNA sample

As described in section 2.4.i, a proportion of the total RNA from each individual rat was kept aside for the preparation of a pooled standard miRNA sample. Unlike cDNA prepared from mRNA, cDNA prepared from miRNAs do not remain stable upon storage; therefore a new cDNA synthesis reaction was prepared from each individual sample, as well as the standard miRNA pool for each real-time RT-PCR run performed [191]. A reverse transcriptase-free control pool was also freshly prepared in parallel, with standard pool in each run, to act as negative control for the real-time RT-PCR.

2.4.u Quantitative real-time RT-PCR for microRNA expression

Taqman® miRNA assays (Applied Biosystems) were used to confirm the results of the Exiqon miRNA microarrays. The assay consisted of a 2-step protocol which accurately detects and quantifies mature miRNA species. The first step involved the reverse transcription of each miRNA species of interest from total RNA to produce cDNA using miRNA specific primers. The second step involved the amplification of target cDNA using sequence-specific primers for each miRNA of interest. A miRNA which was indifferent to strain, remained stable with cyclic hyperoxia exposure and showed no known functional relevance to the biological system being investigated acted as a housekeeping gene and was reverse transcribed and amplified for each RNA sample (refer to section 2.4.w).

Conversion of total RNA to single-stranded cDNA was performed in accordance with the manufacturer's instructions for the assays with slight modifications as detailed below. Briefly, total RNA samples were diluted to a concentration of 4, 20 or 40 ng/µl depending on the optimised template concentration determined for each miRNA of interest as described in section 2.4.v, and placed on ice. A total of 5-40 ng of RNA was used in each reverse transcription reaction. RNA used in these reactions was not heat denatured prior to use, to prevent the reduction in yield of cDNA from some miRNA species. All steps were performed on ice.

The reverse transcription reaction was modified to 7.5 µl rather than 15 µl reactions and the volumes adjusted accordingly. A bulk reverse transcription master mix was prepared containing 100 mM dNTPs (with dTTP, 0.075 µl/reaction), Multiscribe™ Reverse Transcriptase 50 U/µl (0.5 µl/reaction), 10 x Reverse Transcriptase Buffer (0.75 µl/reaction), RNase Inhibitor 20 U/µl (0.095 µl/reaction) and nuclease-free water (2.08 µl/reactions). The reverse transcription master mix (3.5 µl/reaction) was then aliquoted into a 96 well reaction plate with 0.1 ml well volume (Applied Biosystems) and miRNA specific RT primer (1.5 µl/reaction) added. The plate was gently vortexed and briefly pulse spun to bring the solution to the bottom of the well. The diluted total RNA (2.5 µl/reaction) was added to the tube to bring the final volume to 7.5 µl.

The reactions were incubated for 30 min at 16°C, followed by a second 30 min incubation at 42°C. The reaction was then terminated at 85°C for 5 min before being held at 4°C until required for real-time qPCR which was performed on the same day. The reverse transcription and real-time PCR were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems).

A PCR master mix was prepared for each miRNA of interest and the reference small RNA gene. Each reaction contained 20 x Taqman® miRNA assay mix (0.5 µl/reaction), Taqman® 2 x Fast Universal PCR Master Mix No Amperase UNG (5 µl/reaction) and 3.8 µl of nuclease-free water (3.8 µl/reaction). From this bulk mixture, 9.3 µl was aliquoted into a 96 well reaction plate (Applied Biosystems) and 1 µl of the reverse transcription product was added to each tube, giving a final reaction volume of 10.3 µl. Each reaction was performed in triplicate. Nuclease-free water was used in place of reverse transcription template as a negative control for non-specific amplification.

The reactions were placed in the thermal cycler and the AmpliTaq® DNA polymerase activated at 95°C for 20 sec. This was followed by 40 cycles of denaturing at 95°C for 1 sec then annealing and extension at 60°C for 20 sec. Data from the reactions were analysed using StepOne Plus software provided with the system. A miRNA-specific manual threshold value based

on the approximate start point of the exponential phase of the reaction was used to ensure consistent analysis between real-time PCR runs.

2.4.v Determination of primer amplification efficiencies and optimal template concentration for quantitative real-time RT-PCR

Primer PCR amplification efficiencies were determined for each set of primers using six 2-fold serial dilutions of the standard miRNA sample, and were used for the quantification of miRNA expression [192]. To determine the optimal total RNA template concentration required for quantitative real-time RT-PCR for each miRNA of interest, the standard miRNA sample was diluted to 4, 10, 20 and 40 ng/µl. This was equivalent to a final template concentration of 10, 25, 50 and 100 ng of total RNA respectively. Each dilution of the standard miRNA sample was tested in parallel for each miRNA of interest and a manual threshold value specific for each miRNA was applied to maintain consistency between real-time PCR runs. The optimal template concentration for each miRNA of interest was determined based on the template concentration at which PCR product amplification was most efficient.

2.4.w Selection and validation of a small RNA reference gene

A total of twelve commercially available small RNA endogenous controls and stably expressed miRNAs which were manually identified in the Exiqon

microarray data to be stable with each comparison were tested prior to real-time quantification of miRNA expression (Table 2.10).

Taqman assay type	Assay name	Species reactivity
Endogenous control	4.5S RNA(H) "Variant 1"	Rno-
	4.5S RNA(H) "Variant 2"	Rno-
	snoRNA202	Hsa-, mmu-
	snoRNA	Rno-
	U6 snRNA	Hsa-, mmu-, rno-
	RNU6B	Hsa-
	Y1	Rno-
	U87	Rno-
Stably expressed miRNA	hsa-miR-16	Hsa-, mmu-, rno-
	mmu-miR-379	Hsa-, mmu-, rno-
	hsa-miR191	Hsa-, mmu-, rno-
	hsa-let-7d	Hsa-, mmu-, rno-

Table 2.10 Endogenous small RNA controls and stably expressed miRNAs tested for suitability as reference genes for normalisation of miRNA expression. A total of twelve small RNAs were tested Assay IDs can be found in Table 2. 2. Hsa = *Homo sapien*; mmu = *Mus musculus*; rno = *Rattus norvegicus*.

Optimal template concentrations and primer PCR amplification efficiencies for each small RNA endogenous control were determined using the standard miRNA sample. Based on these results, 4 of the small RNA reference genes were further tested for stable expression between rat strains and treatment, using representative total RNA samples as shown in Table 2.11 and on the standard miRNA sample. Raw Ct threshold values were plotted against strain and/or treatment then visually assessed for stable expression across all

samples. The most stable small RNA was chosen for normalisation of miRNA expression as described in the next section.

Strain	Experimental exposure	Postnatal day
F344	Room air	5, 6, 9, 14
SD	Room air	5, 6, 9, 14
F344	Cyclic hyperoxia	5, 6, 9, 14
SD	Cyclic hyperoxia	5, 6, 9, 14

Table 2.11 Expression of each small RNA reference gene was determined using representative total RNA samples. Raw Ct values were plotted against strain and/or treatment to determine if these small RNA reference genes were stably expressed.

2.4.x Relative quantification and normalisation of retinal microRNA expression

Current convention for quantification of miRNA expression is to use a single small RNA reference gene for data normalisation [193]. MiRNA expression in each sample was determined and normalised to the reference miRNA, miR-16, using the readily available software application Q-gene (http://www.gene-quantification.de/download.html).

The standard miRNA sample described in section 2.4.t was run in parallel with the test samples in each run to ensure there was little inter-run variation. Mean normalised miRNA expression was calculated using two equations as shown in Figures 2.6 and 2.7, which take into account the PCR

amplification efficiencies of both the target miRNA and the reference miRNA [192, 194]

$$NE = \frac{(E_{target})^{CT_{target}}}{(E_{ref})^{CT_{ref}}}$$

Figure 2.6 Equation 1 used by Q-gene for the calculation of normalised gene expression [194]. NE, normalised gene expression; E_{target}, PCR amplification efficiency of the target gene; E_{ref}, PCR amplification efficiency of the reference gene; Ct_{target}, threshold cycle of the PCR amplification of the target gene; Ct_{ref}, threshold cycle of the PCR amplification of the reference gene. The Ct was defined as the cycle at which the fluorescence was raised above the background fluorescence; which is approximately at the start of the exponential phase.

$$MNE = \frac{\frac{(E_{target})^{Ct_{target,\ well\ 1}}}{(E_{ref})^{Ct_{ref,well\ 1}}} + \frac{(E_{target})^{Ct_{target,\ well\ 2}}}{(E_{ref})^{Ct_{ref,well\ 2}}} + \frac{(E_{target})^{Ct_{target,\ well\ 3}}}{(E_{ref})^{Ct_{ref,well\ 3}}}}{3}$$

Figure 2.7 Equation 2 used by Q-gene for the calculation of mean normalised gene expression [194]. Mean normalised expression (MNE) is calculated using the arithmetic mean of three normalised expression values. Red: well 1 of triplicate wells; blue: well 2 of triplicate wells; orange: well 3 of triplicate wells.

2.4.y microRNA databases and microRNA target prediction programs

Since the first miRNA was discovered in 1993, the field of miRNA research has advanced rapidly as more and more new miRNAs are identified. Initially miRNAs were collated in a central miRNA registry which was designed to enable researchers to register novel, experimentally supported miRNA sequences prior to their publication, and to provide a searchable database of published miRNA sequences [190]. The miRNA registry has since been superseded by the miRBase registry [195], which builds upon the old registry by providing additional information on miRNA sequence and annotation, as well as miRNA target prediction using the miRanda, PicTar and TargetScanS algorithms [196, 197]. Since August 2007, 18,226 hairpin precursor miRNAs entries which express 21,634 mature miRNA products in 168 species have been registered in miRBase release 18.0. MiRBase is provided by the Sanger Institute and is freely available online at http://mirbase.org/.

The mRNA transcripts which were predicted to be targets of the miRNAs of interest were determined using two freely available online target prediction programs: miRGen (http://www.diana.pcbi.upenn.edu/cgi-bin/miRGen/v3/Targets.cgi) and microCosm Targets (formerly miRBase Targets) http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/.

The primary aim of miRGen is to study the relationship between the location of miRNAs in the genome and their function, and to provide information on target prediction [197]. Target prediction is accomplished by integration of miRGen with TarBase, a database of experimentally supported miRNA targets (for review see [198]), and by the union or intersection of results from 4 commonly used target prediction programs in a variety of different combinations [197]. miRGen is freely available online at http://www.diana.pcbi.upenn.edu/miRGen/v3/miRGen.html. MicroCosm Targets uses different algorithms to predict targets for miRNAs across many species based on miRNA sequences in the miRBase Sequence database [190, 195, 196, 199] and most genomic sequence from EnsEMBL.

2.5 STATISTICAL ANALYSIS

All statistical analyses were carried out using the software package PASW Statistics version 18.0 (SPSS Inc., Chicago, IL, USA) unless otherwise specified.

2.5.a Retinal avascular area and vascular morphology

Percentage avascular area and retinal vessel tortuosity scores were analysed using Chi-Square and Kruskal-Wallis tests with the significance (alpha) level set at 0.05. Two-tailed Mann-Whitney U-tests were used for comparisons between subsets of data with Bonferroni adjustments for multiple

comparisons applied post-hoc. Adjusted significance levels were set at 0.017. Summary data were expressed as means with 95% confidence intervals (95% CI).

2.5.b Affymetrix gene expression microarrays

Affymetrix raw gene array data were processed at the Adelaide Microarray Centre using the Partek Genomics Suite software (Partek Inc., St. Louis, MO, USA). Briefly, .cel files were imported using RMA background correction, Partek's own GC content correction, and mean probe summarization. Differential gene expression was assessed by ANOVA with the p value adjusted using step-up multiple test correction [200] to control the false discovery rate (FDR) which was set at 0.05. Adjusted p values <0.05 were considered to be significant. A total of 27,342 p values were analysed at day 3 and 29,214 p values analysed each at day 5 and at day 6.

2.5.c Exiqon microRNA microarrays

Exiqon raw miRNA array data were processed at the Adelaide Microarray Centre using the software package LIMMA R (WEHI, Melbourne, VIC, Australia) [189]. Differentially expressed miRNAs were identified using linear models and empirical Bayesian moderation of standard errors of the estimated log-fold changes within LIMMA R. This method provides more stable inference and improved power for experiments with small numbers [201]. Differential miRNA expression was assessed by ANOVA, with the p

value adjusted using step-up multiple test correction to control the false discovery rate (FDR) [200]. The FDR was set to a significance level of 0.05 and represented the number of false positives that may be present within the dataset. Adjusted p values <0.05 were considered to be significant. A total of 1,536 miRNAs from 50 different species including rat, human and mouse were analysed at each time point.

2.5.d Real-time PCR data

Prior to statistical analysis of gene and miRNA expression, samples considered to be technical failures and outliers were removed from the data set. Tests of homogeneity of variance (Levene's tests of equality of error p >0.05) were applied to the remaining gene and miRNA expression data. Where appropriate, data were either $\log_{10}$ or square root transformed to enable statistical analysis using two-away analysis of variance with the significance (alpha) set at 0.05. Post-hoc testing was not applied as both categorical independent variables (strain and treatment) did not meet the minimum requirements of 3 levels for this to be carried out.