CHAPTER 5: RESULTS
ALTERED MIRNA EXPRESSION IN OXYGEN-INDUCED RETINOPATHY

5.1 ABSTRACT

Differential susceptibility to OIR exhibited by two albino inbred rat strains is likely to be a result of changes in retinal gene expression, which may be post-transcriptionally regulated by small non-coding RNAs called microRNAs (miRNAs). Published studies of miRNA expression in OIR have been limited to the late proliferative stages of the disease which occur in response to relative hypoxia. Changes which occur in response to hyperoxia and relative hypoxia in the early stages of OIR have not previously been studied. Exiqon microarrays were used to analyse the differential expression of over 1500 miRNAs in response to cyclic hyperoxia in F344 (resistant to OIR) and SD (susceptible to OIR) rats. Three different miRNAs, miR-30e, miR-338 and miR-210 were identified to be differentially expressed in a strain-dependent manner in response to relative hypoxia at day 6.

miR-30e is predicted to target the asparaginyl hydroxylase FIH, which regulates the transcriptional activity of HIF-α in normoxic conditions. miR-338 is predicted to target HIF-α directly to regulate the hypoxic response, and transcription of miR-210 is known to be upregulated by hypoxia. miR-210 is a master regulator of the hypoxic response and is known to regulate VEGF-induced endothelial cell survival, migration and tube formation. Downregulation of miR-210 in resistant F344 rats may be protective against the poorly regulated angiogenesis observed in susceptible SD rats. Strain-

dependent differential expression of these miRNAs, in particular miR-210, may contribute to the differential susceptibility to OIR exhibited by F344 and SD rats, as these miRNAs play a role in the angiogenic response to relative hypoxia.

5.1a Introduction

MicroRNAs (miRNAs) are short non-coding sequences of RNA approximately 21 nucleotides in length that post-transcriptionally regulate gene expression [101]. Binding of miRNAs to their target mRNA results in either mRNA degradation or translational repression [102-105]. miRNAs may act together in a complex regulatory network to target hundreds of genes within a pathway, or several miRNAs may act together to regulate a single mRNA target [106].

miRNAs expressed in the eye show distinct tissue specificity and spatio-temporal expression during embryonic and postnatal development, suggesting miRNAs play an important role in regulating ocular development, differentiation and function [134, 135, 137]. miR-31 and miR-184 are constitutively expressed in the cornea and lens, two avascular tissues in the eye, suggesting they may play a role in suppressing ocular neovascularisation [136]. Expression of miR-31, miR-150 and miR-184 have been shown to be significantly decreased in the retina in response to relative hypoxia in a mouse model of OIR [136]. Ocular injection of the precursors of

these 3 miRNAs, pre-miR-31, pre-miR-150 and pre-miR-184, suppressed hypoxia-induced retinal neovascularisation. miR-31 may regulate retinal neovascularisation through post-transcriptional modification of HIF-1α, which alongside platelet-derived growth factor-beta (PDGF-β), was confirmed to be a target of miR-31. miR-150 was confirmed to target VEGF and PDGF-β, whereas Frizzled family receptor 4 (FZD4) remained an unconfirmed target of miR-184 [136].

Several miRNAs have been identified that are expressed by the retina and endothelial cells, and are also known to be upregulated by hypoxia, including miR-23a, miR-107, miR-181a, and miR-210 [133, 134, 145-147]. The upregulation of miR-210 in response to hypoxia appears to play a role in the angiogenic response to hypoxia by promoting endothelial cell survival, migration and tube formation [147]. This complex interplay of miRNA regulation of retinal angiogenesis via the HIF-α oxygen sensing pathway may contribute to the pathophysiology of OIR/ROP.

To date, studies of the differential expression of miRNAs in OIR have been limited to the late, proliferative stages of the disease which occurs in response to relative hypoxia or changes which occur at the end of a sustained period of hyperoxia [136, 139]. The changes in miRNA expression which occur in response to hyperoxia and relative hypoxia in the early stages of OIR, or in different inbred strains, have not previously been studied.

5.1.b Specific aims

The specific aims of this chapter were to investigate if miRNAs play a role in regulating the changes in retinal gene expression that may underlie the differential susceptibility to OIR exhibited by F344 and SD rats, and to determine at what time point any changes were occurring.

Exiqon microarrays were used to investigate changes in the expression of hundreds of retinal miRNAs simultaneously using a high-throughput approach. This enabled the identification of differentially expressed miRNAs for further study. Exiqon microarrays were used to identify miRNAs which were differentially expressed in a strain-dependent manner, and/or were regulated by oxygen, and/or were predicted to target genes involved in the HIF-α oxygen sensing pathway to regulate gene expression levels. Relative quantification real-time RT-PCR provided a sensitive method for the comparison of candidate miRNA levels and was used to confirm the microarray findings [227].

5.2 EXIQON MICROARRAY RESULTS

Exiqon microarrays were performed using pooled RNA samples from F344 room air-exposed (FRA), F344 cyclic hyperoxia-exposed (FO_2), SD room air-exposed (SDRA) and SD cyclic hyperoxia-exposed (SDO_2) rats at postnatal days 3, 5 and 6. Each pool consisted of RNA from 3 rats, from a minimum of

2 litters at each time point. Changes in miRNA expression were examined at days 3, 5 and 6 of the cyclic hyperoxia exposure period and microarray results were confirmed by real-time RT-PCR using commercially available Taqman miRNA assays on RNA from individual animals. An electronic version of the datasets from days 3, 5 and 6 may be made available upon request.

5.2.a Analysis of microarray data

Preliminary Exiqon microarray data analyses were performed by Mark van der Hoek (Adelaide Microarray Centre, Adelaide, Australia) using the software package LIMMA R, as described by Neal et al., [189, 201]. Briefly, images were scanned and median spot pixel intensity values were extracted with the aid of the Spot v3 plugin (CSIRO, Clayton South, VIC, Australia) within the statistical software package R. Background intensities were subtracted and global loess normalisation was applied. Log2 transformation of mean intensities was performed and Cy5/Cy3 rations obtained [189]. Global loess normalisation was used to adjust for the effects due to variation in microarray technology, as opposed to effects resulting from biological differences between RNA samples [259]. As a result, genes within the array were not used for data normalisation.

Differentially expressed miRNAs were identified using linear models and empirical Bayesian moderation of standard errors of the estimated log-fold

changes within LIMMA R, which 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. Five comparisons were made to determine whether changes in miRNA expression were due to strain and/or oxygen exposure or the interaction between strain and oxygen (Table 5.1).

Comparison	**Differential miRNA 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 cyclic hyperoxia exposure
Interaction strain*treatment	MiRNAs differentially expressed in response to the interaction between strain (F344 and SD together) and treatment (RA and cyclic hyperoxia exposure together)

Table 5.1 Exiqon microarray comparisons performed at days 3, 5 and 6. Five different comparisons were made to identify differences in gene expression associated with strain, oxygen exposure or both. Differences due to strain and oxygen, and differences associated with the interaction between the two variables were of greatest interest. FRA = F344 room air-exposed; SDRA = SD room air-exposed; FO_2 = F344 cyclic hyperoxia-exposed; SDO_2 = SD cyclic hyperoxia-exposed.

Correction for multiple comparisons yielded very few miRNAs with significant adjusted p values at days 3, 5 and 6 (Table 5.2). The details of the differentially expressed miRNAs, including fold changes, are found in Table 5.3.

Comparison	**Number of miRNAs with significant adjusted p values**		
	DAY 3	**DAY 5**	**DAY 6**
FRA vs. SDRA	4	5	0
FO2 vs. FRA	2	2	0
SDO2 vs. SDRA	0	0	0
FO2 vs. SDO2	0	0	2
Interaction strain*treatment	0	0	0

Table 5.2 Summary of significant adjusted p values at each time point. The number of miRNAs with significant p values after using step-up correction for multiple comparisons for each time point and each comparison is shown. Significance level: 0.05; False Discovery Rate level 0.05; total number of p-values at each time point = 1, 536. FRA = F344 room air-exposed; SDRA = SD room air-exposed; FO_2 = F344 cyclic hyperoxia-exposed; SDO_2 = SD cyclic hyperoxia-exposed.

5.2.b Identification of candidate microRNAs

A bioinformatics approach was next used to investigate the miRNA expression data generated by the microarray screens to identify not only miRNAs that had significant adjusted p values ($p<0.05$), but also those that were regulated by both strain and oxygen, or oxygen alone, or were predicted to target oxygen-related genes.

Table 5.3 miRNAs with significant adjusted p values at each time point. miRNAs were human, mouse and rat specific unless otherwise specified. Fold changes are shown in brackets. A single positive control miRNA returned a significant adjusted p value after correction for multiple comparisons at day 5 in cyclic hyperoxia-exposed F344 rats compared to room air-exposed control F344 rats. Aga= *Anopheles gambiae*; SV40 = *Simian Vacuolating Virus* 40; Rno = *Rattus norvegicus*. FRA = F344 room air-exposed; SDRA = SD room air-exposed; FO_2 = F344 cyclic hyperoxia-exposed; SDO_2 = SD cyclic hyperoxia-exposed.

Comparison	**MiRNAs with significant adjusted p values (Fold changes)**		
	DAY 3	**DAY 5**	**DAY 6**
FRA vs. SDRA	miR-34a (↑ 1.7 fold FRA) miR-100 (↑ 1.7 fold FRA) miR-99a (↑ 1.6 fold FRA) miR-19a (↑ 1.3 fold FRA)	miR-338-3p (↓ 2.0 fold FRA) sv40-miR-S1-5p (↓ 2.0 fold FRA) miR-135b (↓ 2.4 fold FRA) miR-30e (↓ 2.1 fold FRA) miR-96 (↓ 2.7 fold FRA)	-
FO2 vs. FRA	miR-210 (↓ 1.6 fold FO2) aga-miR-210 (↓ 1.6 fold FO2)	sv40-miR-S1-5p (↑ 1.4 fold FO2) Positive control miR ID# 42735 (↑ 1.4 fold FO2)	-
SDO2 vs. SDRA	-	-	-
FO2 vs. SDO2	-	-	rno-miR-190b (↓ 1.8 fold FO2) miR-338-3p (↓ 2.0 fold FO2)
Interaction strain*treatment	-	-	-

Two freely-available online target prediction programs were used to identify miRNAs which were predicted to target genes within the HIF-α oxygen sensing pathway, as described in section 2.4.w. MiRGen combines a number of widely used target prediction programs to identify miRNA targets including those which have been experimentally validated and can be found at (http://www.diana.pcbi.upenn.edu/cgi-bin/miRGen/v3/Targets.cgi). MicroCosm Targets (formerly known as miRBase Targets) found at http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/, was also used. MicroCosm Targets computationally predicts targets for miRNAs across many species, based on miRNA sequences in the miRBase Sequence database and most genomic sequence from EnsEMBL.

Using these miRNA target prediction programs, miRNAs of interest were submitted to each database and the predicted mRNA target output lists were analysed manually to identify target genes which were involved in the HIF-α oxygen sensing pathway. miRNAs which were predicted to target elements of the HIF-α oxygen sensing pathway in a strain-dependent manner were of greatest interest, because this post-transcriptional mechanism may contribute to differential susceptibility to OIR in F344 and SD rats.

5.2.b.1 Identification of microRNA candidates at day 3

Four miRNAs were found to be upregulated to a statistically significant extent in F344 room-air exposed rats compared to SD room air-exposed rats at day 3 (Figure 5.1). These miRNAs: miR-34a, miR-100, miR-99a and miR-19a were all found to be differentially expressed in response to strain and were all upregulated in F344 rats compared to SD rats.

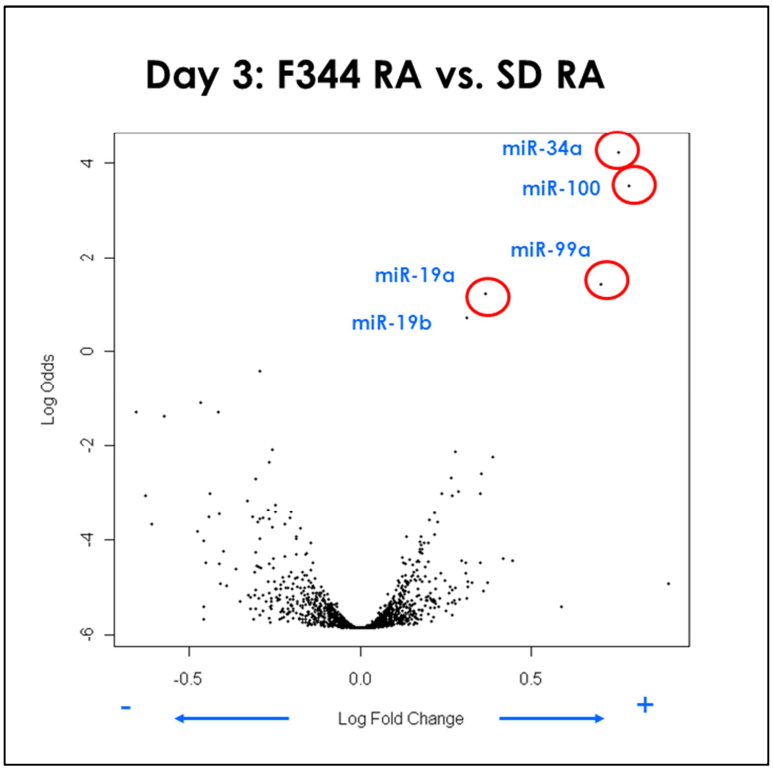

Figure 5.1 Volcano plot showing miRNAs which are differentially expressed in response to strain at day 3. The log odds (y axis) that a miRNA is differentially expressed is plotted against the $\log_2$ fold change in expression of that particular miRNA (x axis). All miRNAs were human, mouse and rat specific unless otherwise specified. miRNAs that had statistically significant p values after correction for multiple comparison are circled in red ($p<0.05$) and all were upregulated. "-" refers to miRNAs which are downregulated and "+" refers to miRNAs which are upregulated. FRA = F344 room air-exposed; SDRA = SD room air-exposed.

Analysis of F344 cyclic hyperoxia-exposed rats, compared to room air-exposed F344 rats, identified miR-210 to be downregulated in response to hyperoxia at day 3 to a statistically significant extent (Figure 5.2).

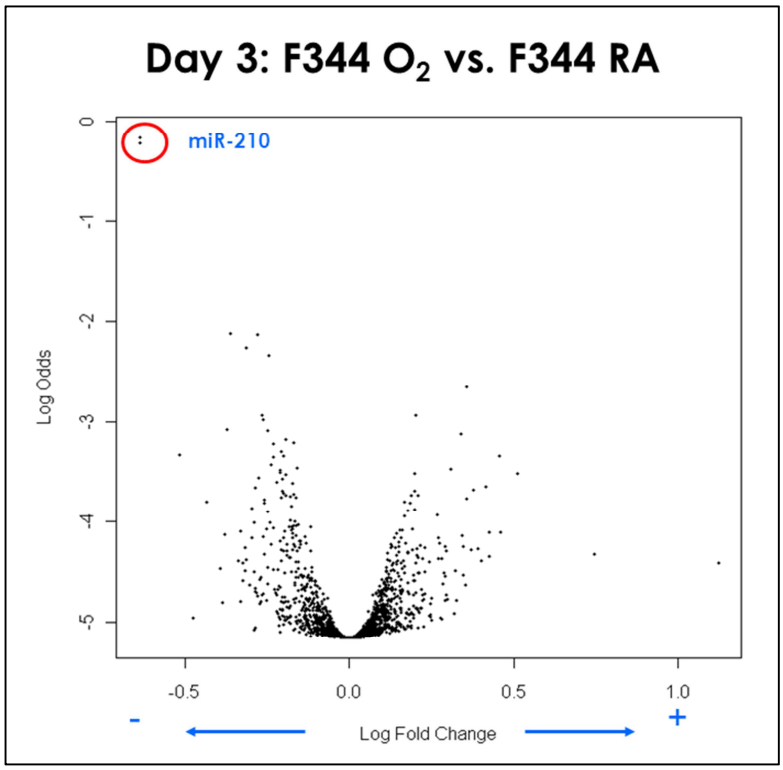

Figure 5.2 Volcano plot showing miRNAs which are differentially expressed in response to cyclic hyperoxia at day 3 in F344 rats. The log odds (y axis) that a miRNA is differentially expressed is plotted against the log_2 fold change in expression of that particular miRNA (x axis). All miRNAs were human, mouse and rat specific unless otherwise specified. miRNAs that had statistically significant p values after correction for multiple comparison are circled in red; $p<0.05$. miR-210 had two significant adjusted p values, one derived from the human, mouse and rat specific miRNA, and the other reactive to *Anopheles gambiae* (African malaria mosquito). "-" refers to miRNAs which are downregulated and "+" refers to miRNAs which are upregulated. FO_2 = F344 cyclic hyperoxia-exposed; FRA = F344 room air-exposed.

The interaction effect between strain (F344 and SD rats taken together) and treatment (room air and cyclic hyperoxia exposure taken together) revealed no miRNAs to be differentially expressed to a statistically significant level (Figure 5.3).

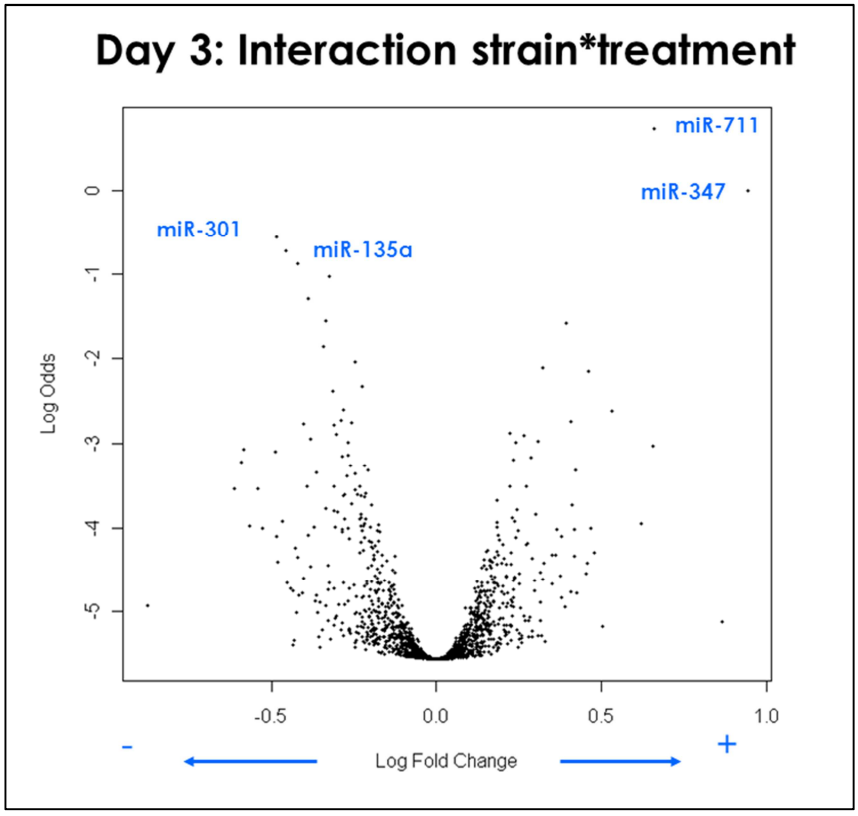

Figure 5.3 Volcano plot showing miRNAs which are differentially expressed in response to the interaction between strain and treatment at day 3 in response to hyperoxia. The log odds (y axis) that a miRNA is differentially expressed is plotted against the log_2 fold change in expression of that particular miRNA (x axis). All miRNAs were human, mouse and rat specific unless otherwise specified. No miRNAs were found to be statistically significant after correction for multiple comparisons, $p<0.05$. "-" refers to miRNAs which are downregulated and "+" refers to miRNAs which are upregulated.

5.2.b.2 Identification of microRNA candidates at day 5

Five miRNAs were found to be statistically significant after correction for multiple comparisons in the F344 room air-exposed vs. SD room air-exposed comparison at day 5 (Figure 5.4). miR-338-3p, sv40-miR-S1-5p, miR-30e, miR-135b and miR-96 were all found to be differentially expressed in response to strain and were all downregulated in F344 rats compared to SD rats.

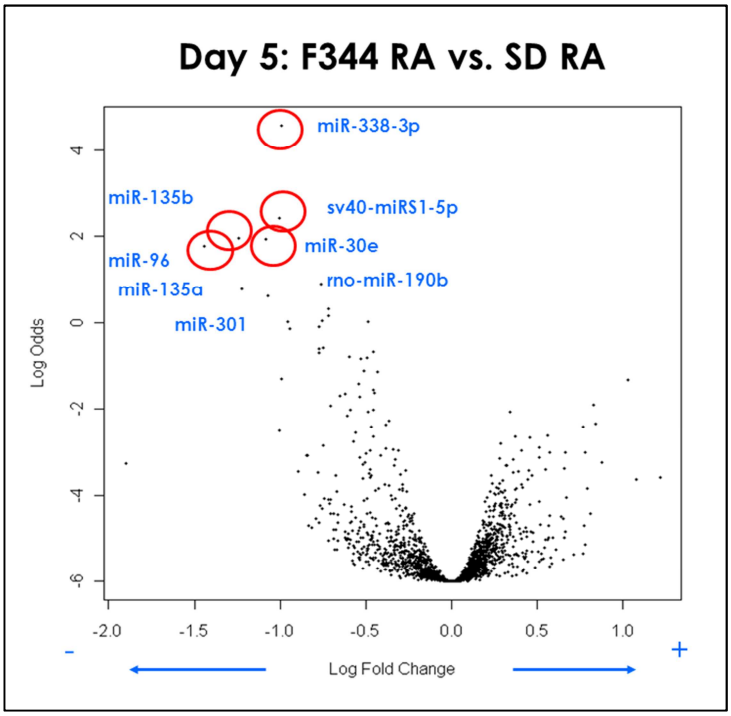

Figure 5.4 Volcano plot showing miRNAs which are differentially expressed in response to strain at day 5. The log odds (y axis) that a miRNA is differentially expressed is plotted against the log_2 fold change in expression of that particular miRNA (x axis). All miRNAs were human, mouse and rat specific unless otherwise specified. miRNAs that had statistically significant p values after correction for multiple comparison are circled in red ($p<0.05$) and in this case were all found to be downregulated. "-" refers to miRNAs which are downregulated and "+" refers to miRNAs which are upregulated. FRA = F344 room air-exposed; SDRA = SD room air-exposed.

Two miRNAs were found to be upregulated in response to hyperoxia at day 5 to a statistically significant extent in F344 cyclic hyperoxia-exposed rats compared to room air-exposed rats (Figure 5.5). One of these miRNAs, sv40-miR-S1-5p was a Simian Virus 40-specific miRNA, and is likely be an artefact of the microarray screening procedure. As a result, sv40-miR-S1-5p was not investigated any further. The other miRNA was a positive control miRNA. Since the significance (alpha) level for the false discovery rate was set at 0.05, it is possible that false positive results may occur, which reinforces the need to confirm results of microarrays using real-time RT-PCR.

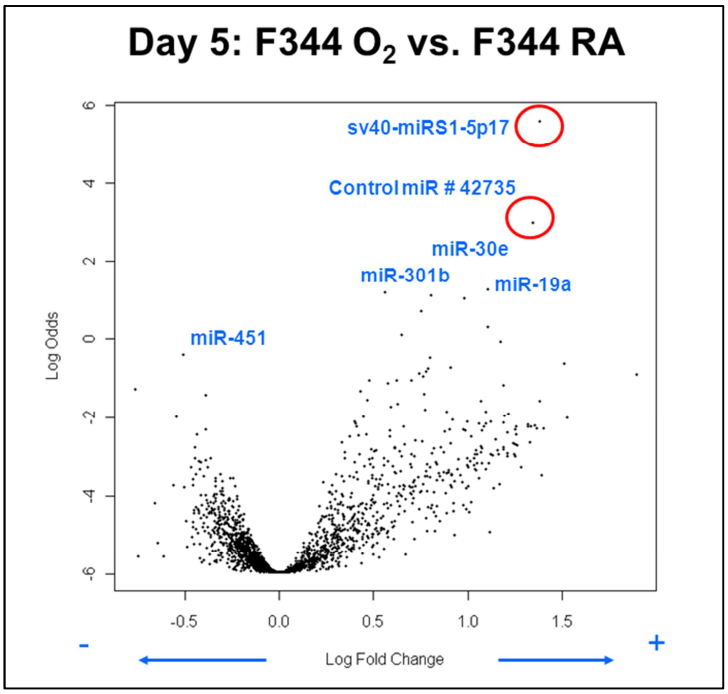

Figure 5.5 Volcano plot showing miRNAs which are differentially expressed in response to cyclic hyperoxia at day 5 in F344 rats. The log odds (y axis) that a miRNA is differentially expressed is plotted against the log_2 fold change in expression of that particular miRNA (x axis). All miRNAs were human, mouse and rat specific unless otherwise specified. miRNAs that had statistically significant p values after correction for multiple comparison are circled in red; $p<0.05$. "-" refers to miRNAs which are downregulated and "+" refers to miRNAs which are upregulated. FO_2 = F344 cyclic hyperoxia-exposed; FRA = F344 room air-exposed.

Analysis of the interaction effect between strain and treatment at day 5 in response to hyperoxia found no miRNAs were differentially expressed to a statistically significant level (Figure 5.6).

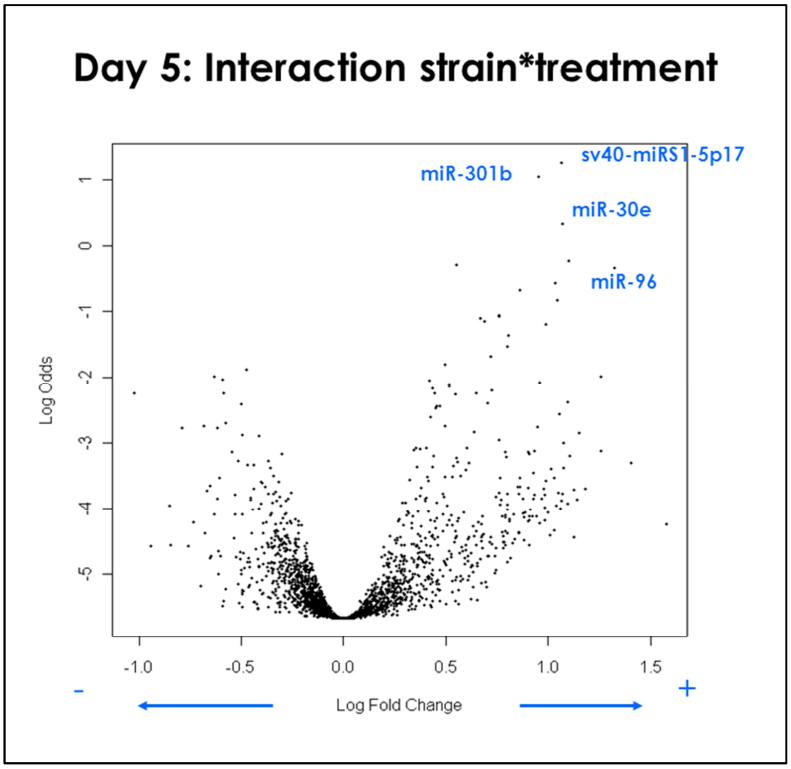

Figure 5.6 Volcano plot showing miRNAs which are differentially expressed in response to the interaction between strain and treatment at day 5 in response to hyperoxia. The log odds (y axis) that a miRNA is differentially expressed is plotted against the log_2 fold change in expression of that particular miRNA (x axis). All miRNAs were human, mouse and rat specific unless otherwise specified. No miRNAs were found to be statistically significant after correction for multiple comparisons, $p<0.05$. "-" refers to miRNAs which are downregulated and "+" refers to miRNAs which are upregulated.

5.2.b.3 Identification of microRNA candidates at day 6

Two miRNAs were found to be statistically significant after correction for multiple comparisons in the F344 cyclic hyperoxia-exposed vs. SD cyclic hyperoxia-exposed rats at day 6 in response to relative hypoxia (Figure 5.7). Rat-specific miR-190b and human, mouse and rat specific miR-338-3p were both downregulated in a strain-dependent manner in response to relative hypoxia in F344 rats compared to SD cyclic hyperoxia-exposed rats.

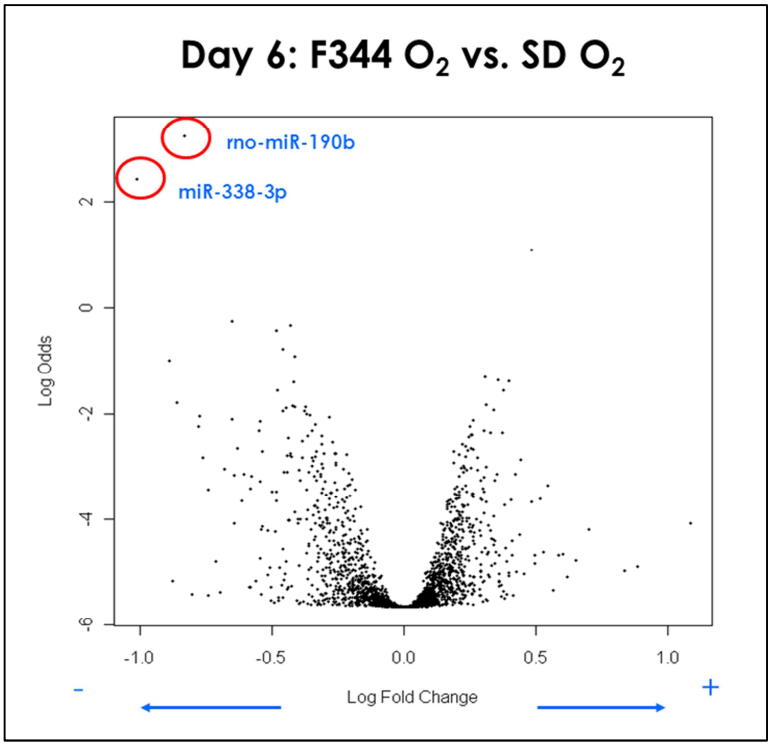

Figure 5.7 Volcano plot showing miRNAs which are differentially expressed in response to cyclic hyperoxia exposure at day 6 in F344 and SD rats. The log odds (y axis) that a miRNA is differentially expressed is plotted against the log_2 fold change in expression of that particular miRNA (x axis). All miRNAs were human, mouse and rat specific unless otherwise specified. miRNAs that had statistically significant p values after correction for multiple comparison are circled in red; $p<0.05$. miR-190b was found to be rat specific with no human equivalent. "-" refers to miRNAs which are downregulated and "+" refers to miRNAs which are upregulated. FO_2 = F344 cyclic hyperoxia-exposed; SDO_2 = SD cyclic hyperoxia-exposed. Rno = *Rattus norvegicus*.

Analysis of the interaction effect between strain and treatment at day 6 in response to relative hypoxia found no miRNAs to be differentially expressed to a statistically significant level (Figure 5.8).

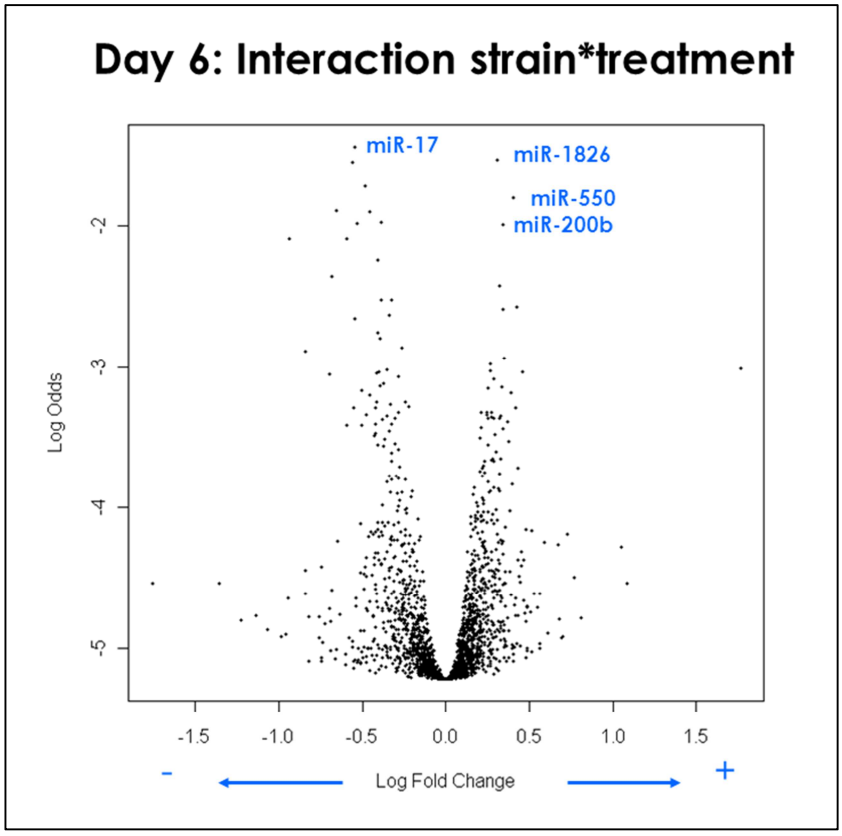

Figure 5.8 Volcano plot showing miRNAs which are differentially expressed in response to the interaction between strain and treatment at day 6 in response to relative hypoxia. The log odds (y axis) that a miRNA is differentially expressed is plotted against the log_2 fold change in expression of that particular miRNA (x axis). All miRNAs were human, mouse and rat specific unless otherwise specified. No miRNAs were found to be statistically significant after correction for multiple comparisons, $p<0.05$. "-" refers to miRNAs which are downregulated and "+" refers to miRNAs which are upregulated.

5.2.b.4 Summary of microRNA candidates chosen for confirmation by quantitative real-time RT-PCR

A total of 15 miRNAs were found to be differentially expressed to a statistically significant level after corrections for multiple comparisons at days 3, 5 and 6. Nine of these were strain-related differences in miRNA expression, 4 were differentially expressed in response to exposure to hyperoxia in F344 rats and 2 were differentially expressed in response to relative hypoxia in a strain-dependent manner at day 6. The false positive miRNA (control miRNA 42735) was excluded from all further analyses from this point on.

As described in section 5.2.b, the fourteen miRNAs of interest were submitted to the online mRNA target prediction programs miRGen and MicroCosm Targets. Manual analyses of the mRNA target output lists showed that these miRNAs were predicted to target various elements of the HIF-α pathway as shown in Figure 5.9. Each program uses different algorithms for the prediction of mRNA targets; therefore different results may be obtained depending on the program used and some concordance was observed between the two programs, as shown in Figure 5.9.

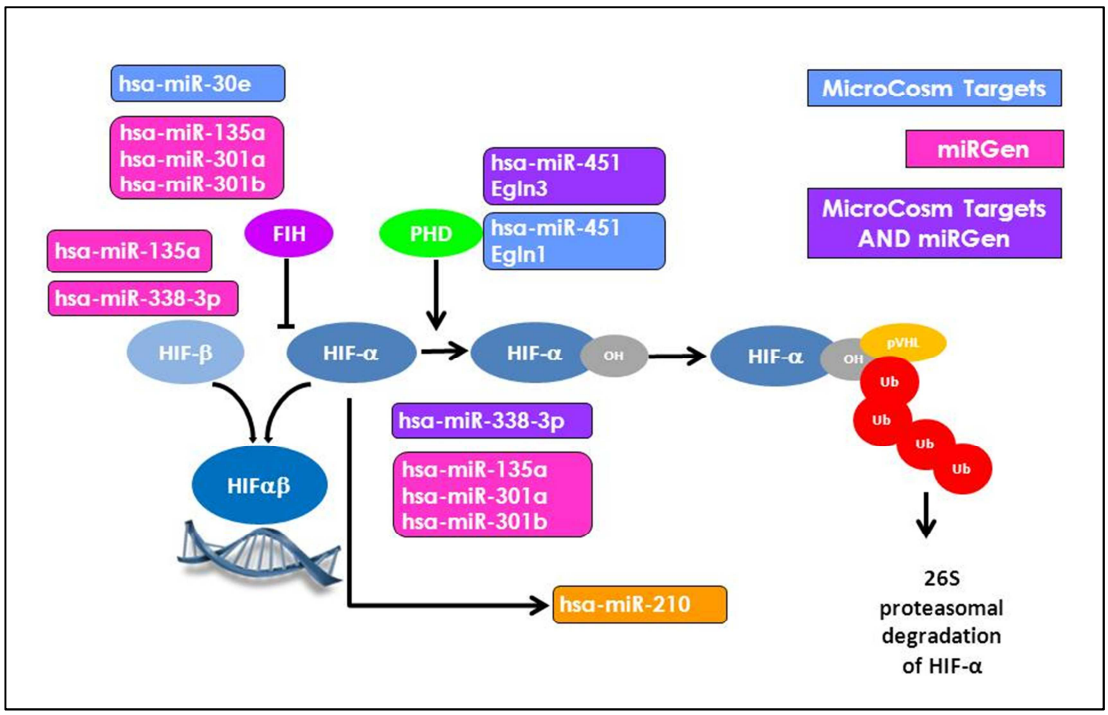

Figure 5.9 Simplified schematic representation of the HIF-α oxygen sensing pathway showing miRNAs of interest which are predicted to target elements of the pathway. Two different target prediction programs were used; MicroCosm Targets are shown in blue boxes and miRGen shown in pink boxes. Purple boxes represent miRNAs whose targets show concordance between the two programs. Orange box represents miRNA whose target has previously been validated experimentally. Ub = Ubiquitin; PHD = Prolyl hydroxylase domain protein; FIH = Factor inhibiting HIF (Asparaginyl hydroxylase domain protein); pVHL = von Hippel Lindau protein.

The asparaginyl hydroxylase FIH was a predicted target of miR-30e, expression of which was found to be statistically significantly different at day 5 in the F344 room air vs. SD room air comparison. Other miRNAs predicted to target FIH include miR-135a, miR-301a and miR-301b, which appeared in the upper end of the volcano plot of the day 5 F344 room air vs. SD room air comparison shown in Figure 5.4, but did not reach statistical significance after correction for multiple comparisons. These 3 miRNAs were also

predicted to target HIF-α directly and miR-135b was also predicted to target HIF-β.

Another miRNA of interest, miR-338-3p was found to be statistically significant at day 5 in the F344 room air vs. SD room air comparison (Figure 5.4), but of greater interest was the fact that it showed a statistically significant difference in expression at day 6 in the F344 cyclic hyperoxia-exposed vs. SD cyclic hyperoxia-exposed (Figure 5.7). miR-338-3p was predicted to target both HIF-α and HIF-β directly.

At day 3, miR-210 reached statistical significance in the F344 cyclic hyperoxia–exposed vs. F344 room air-exposed comparison (Figure 5.2). miR-210 is considered to be a master post-transcriptional regulator of the hypoxic response and has been shown to be upregulated in response to relative hypoxia.

Considering the differential expression of the prolyl hydroxylases EGLN3 and EGLN1 in response to relative hypoxia in F344 and SD rats at day 6, miRNAs predicted to target EGLN3 and EGLN1 were also investigated. miR-451 was predicted to target both these genes; however it did not reach statistical significance after correction for multiple comparisons in any of the comparisons tested, at any of the time points. Preliminary quantitative real-time RT-PCR experiments for miR-451 were unsuccessful, as the miRNA was

in such low abundance that expression levels could not be reliably determined. As a result, miR-451 was not investigated any further.

As a result of these preliminary analyses, miR-30e, miR-338-3p and miR-210 were chosen for further investigation using time-course analyses of miRNA expression at days 3, 5 and 6 in individual rats (Figure 5.10).

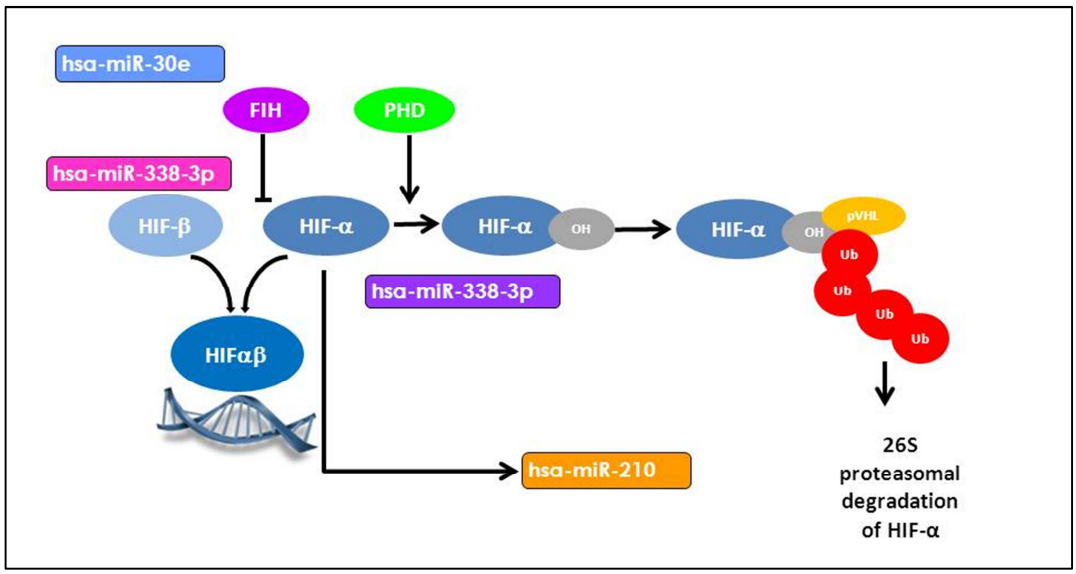

Figure 5.10 MiRNAs which are predicted to target elements of the HIF-α oxygen sensing pathway that were chosen for confirmation of microarray results. miR-30e was chosen as it is predicted to target FIH, miR-338-3p is thought to directly target HIF-α and HIF-β for post-transcriptional regulation and miR-210 is known to be upregulated by hypoxia. These 3 miRNAs all had adjusted p values <0.05. MicroCosm Targets are shown in blue boxes and miRGen shown in pink boxes. Purple boxes represent miRNAs whose targets shows concordance between the two programs. Orange box represents miRNA whose target has previously been validated experimentally. Ub = Ubiquitin; PHD = Prolyl hydroxylase domain protein; FIH = Factor inhibiting HIF (Asparaginyl hydroxylase domain protein); pVHL = von Hippel Lindau protein.

5.3. SELECTION AND VALIDATION OF SMALL RNA REFERENCE GENES FOR NORMALISATION OF MICRORNA EXPRESSION

Twelve commercially available small RNA endogenous controls and stably expressed miRNAs which were manually identified in the Exiqon microarray data to be stable with strain and treatment were tested prior to real-time RT-PCR quantification of miRNA expression. Primer amplification efficiencies for each small RNA endogenous control were determined using the standard miRNA sample (described in section 2.4.t) and are shown in Table 5.4. The stability of these small RNA reference genes with strain and treatment was also tested using representative samples from days 5, 6, 9 and 14 (Table 5.4).

Mean raw Ct threshold values for miR-16, U6 snRNA, 4.5S RNA (H) "Variant 1" and U87 were plotted against strain (Figure 5.11) or treatment (Figure 5.12) and visually assessed for stable expression across all samples. These reference genes were chosen as optimal template concentrations, primer amplification efficiencies and stable expression with strain and treatment were observed in preliminary experiments. U6 snRNA was found to be the least stable with strain or treatment. 4.5S RNA (H) “Variant 1” was relatively stable in F344 rats, but not in SD rats, and was also found to be relatively stable with exposure to room air but not with exposure to cyclic hyperoxia. U87 and miR-16 were found to be relatively stable with strain and treatment, however the PCR amplification efficiency for U87 was only 35.0% compared to 79.0% for miR-16. In addition to this, U87 is a rat–specific small RNA

endogenous control, whereas miR-16 is human, mouse and rat specific, making miR-16 a more relevant normaliser of miRNA expression for future use with human samples. Therefore miR-16 was chosen for normalisation of miRNA expression using the software application Q-gene as described in section 2.4.x.

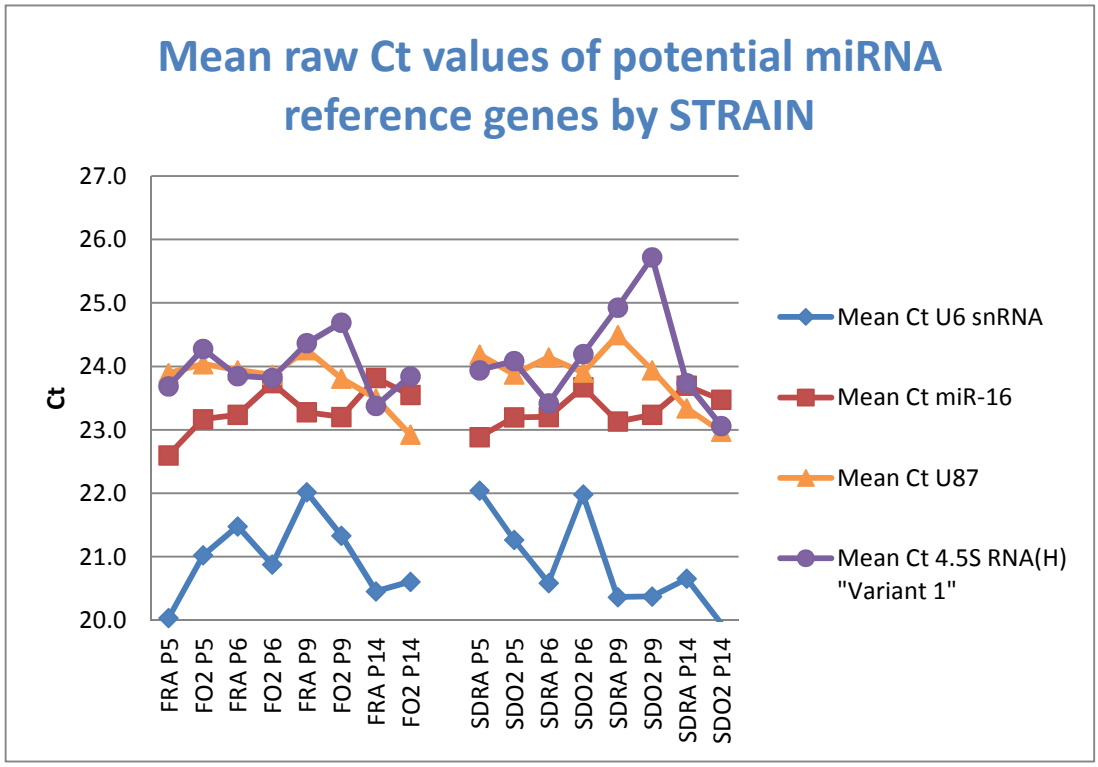

Figure 5.11 Endogenous small RNA controls and stably expressed miRNAs tested for suitability as reference genes for normalisation of miRNA expression tested by strain. Stability of U6 snRNA, miR-16, U87 and 4.5S RNA (H) "Variant 1" were tested on representative retinal RNA samples from days 5, 6, 9 and 14. U6 snRNA and 4.5S RNA (H) "Variant 1" were found to be least stable with strain, whereas miR-16 and U87 were found to be more stable. FRA = F344 room air-exposed; SDRA = SD room air-exposed; FO_2 = F344 cyclic hyperoxia-exposed; SDO_2 = SD cyclic hyperoxia-exposed. P5 = postnatal day 5; P6 = postnatal day 6; P9 = postnatal day 9; P14= postnatal day 14.

Table 5.4 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. PCR primer amplification efficiencies were determined using the miRNA standard pool, as described in section 2.4.t. Hsa = *Homo sapien;* mmu = *Mus musculus*; rno = *Rattus norvegicus*. ND = not determined due to low miRNA abundance

Assay type	Assay name	Species specificity	Stability with strain and cyclic hyperoxia exposure	MiRNA primer amplification efficiency
Endogenous control	4.5S RNA(H) "Variant 1"	Rno-	Stable in both	31.8%
	4.5S RNA(H) "Variant 2"	Rno-	ND – low miRNA abundance	ND – low miRNA abundance
	snoRNA202	Hsa-, mmu-	ND – low miRNA abundance	ND – low miRNA abundance
	snoRNA	Rno-	Not stable in SD cyclic hyperoxia-exposed vs. SD room air-exposed rats	ND – low miRNA abundance
	U6 snRNA	Hsa-, mmu-, rno-	Stable in both	57.5%
	RNU6B	Hsa-	ND – low miRNA abundance	ND – low miRNA abundance
	Y1	Rno-	Not stable with cyclic hyperoxia exposure	ND – low miRNA abundance
	U87	Rno-	Stable in both	35.0%
Stably expressed miRNAs	Hsa-miR-16	Hsa-, mmu-, rno-	Stable in both	79.0%
	Mmu-miR-379	Hsa-, mmu-, rno-	Not stable with strain or oxygen	ND – low miRNA abundance
	Hsa-miR191	Hsa-, mmu-, rno-	Not stable with strain or oxygen	ND – low miRNA abundance
	Hsa-let-7d	Hsa-, mmu-, rno-	Not stable in SD cyclic hyperoxia-exposed vs. SD room air-exposed rats	ND – low miRNA abundance

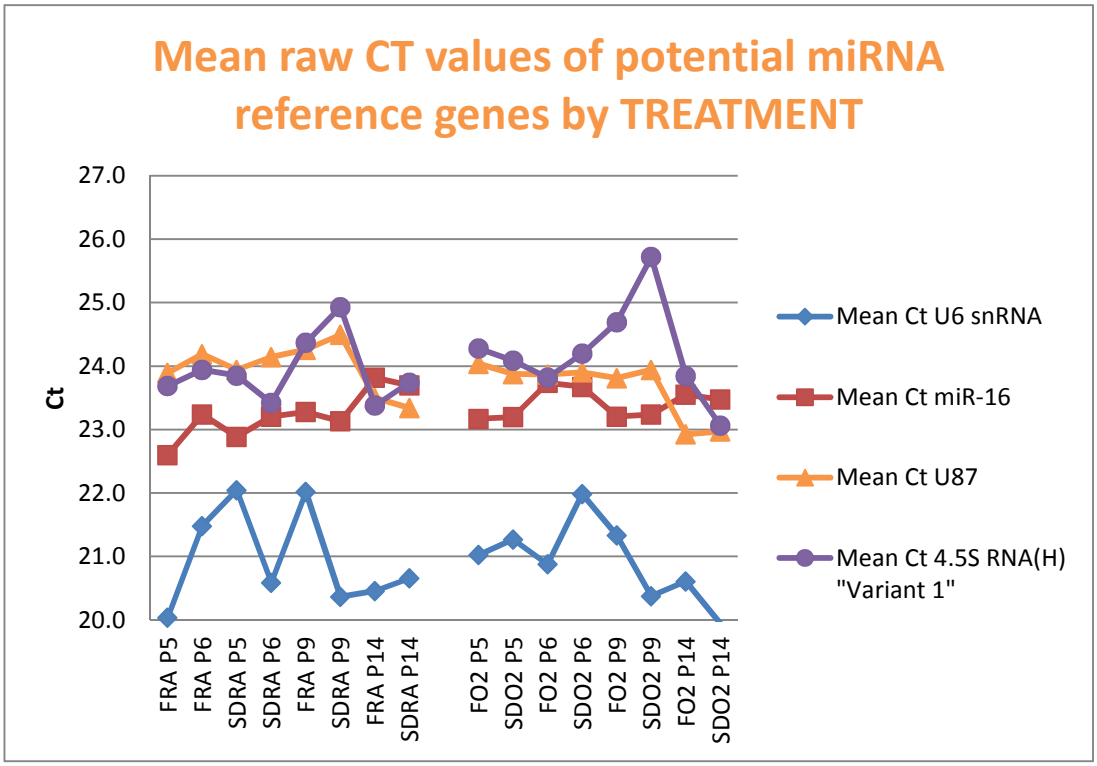

Figure 5.12 Endogenous small RNA controls and stably expressed miRNAs tested for suitability as reference genes for normalisation of miRNA expression tested by treatment. Stability of U6 snRNA, miR-16, U87 and 4.5S RNA (H) "Variant 1" were tested on representative retinal RNA samples from days 5, 6, 9 and 14. U6 snRNA and 4.5S RNA (H) "Variant 1" were found to be least stable with treatment, whereas miR-16 and U87 were found to be more stable. FRA = F344 room air-exposed; SDRA = SD room air-exposed; FO_2 = F344 cyclic hyperoxia-exposed; SDO_2 = SD cyclic hyperoxia-exposed. P5 = postnatal day 5; P6 = postnatal day 6; P9 = postnatal day 9; P14= postnatal day 14.

5.4. INVESTIGATION OF MICRORNA EXPRESSION IN INDIVIDUAL RATS

Time-course analysis of miR-30e, miR-338 and miR-210 expression was performed at days 3, 5 and 6, in individual rats. Between 3 and 11 rats were analysed for each treatment group, depending on the quality of the total RNA used for cDNA synthesis. miR-30e was chosen as it was predicted to target FIH, miR-338 was chosen as it was predicted to target HIF-α and HIF-β, and miR-210 was chosen as it is known to be upregulated by HIF-α in response to relative hypoxia. miRNA expression levels were determined relative to a standard pooled miRNA sample and were normalised against a single miRNA reference gene, miR-16, as described in section 2.4.x. Graphical representation of these results, along with the statistical analyses on these data can be found in Appendix 1.

Data were transformed where necessary prior to statistical analysis using two-way analysis of variance (ANOVA) to ensure data were normally distributed. Two-way ANOVA was used to compare the effects of strain (F344 and SD together), treatment (room air and cyclic hyperoxia together) and the interaction between strain and treatment (strain*treatment) on miRNA expression. The significance (alpha) level was set at 0.05.

Note that miR-338-3p was available in an Applied Biosystems Taqman® miRNA assay in a human and mouse specific assay. miR-338-3p is one of two

possible mature miRNA cleavage products from the miRNA stem-loop precursor miR-338 (miRBase accession number MI0000814). A human and rat specific assay for miR-338 was available from Applied Biosystems and differed from the miR-338-3p assay by a single nucleotide as shown in Table 5.5, therefore the human and rat specific miR-338 assay was chosen for use with the rat retinal samples.

Assay ID	Assay name	Species reactivity	Mature miRNA sequence
002252	miR-338-3p	Human mouse	UCCAGCAUCAGUGAUUUUGUUG
000548	miR-338	Human, rat	UCCAGCAUCAGUGAUUUUGUUG**A**

Table 5.5 Applied Biosystems Taqman® miRNA assays for miR-338-3p and miR-338. miR-338-3p is a mature miRNA derived from the miR-338 stem-loop precursor and the two assays differ from each other by a single nucleotide. As the miR-338 assay was rat specific and the miR-338-3p was not, the former was chosen for use with the rat retinal samples.

A collective summary of time-course analysis of miR-30e, miR-338 and miR-210 expression is discussed next and includes graphical representation of the results.

5.5 SUMMARY OF MICRORNA EXPRESSION IN INDIVIDUAL RATS FOLLOWING EXPOSURE TO CYCLIC HYPEROXIA

5.5.a. miR-30e expression in individual rats at days 3, 5, and 6

Overall, miR-30e was downregulated in response to hyperoxia in F344 rats at days 3 and 5, whereas SD rats showed an upregulation of miR-30e (Figure 5.13). The interactions between strain and treatment at these time points were statistically significant, as was the interaction at day 6 in response to relative hypoxia (Table 5.6). At day 6, miR-30e was relatively unchanged in response to relative hypoxia in F344 rats but was upregulated in SD rats.

Variable	**Day 3**	**Day 5**	**Day 6**
Strain	F(1,37)=2.311, p=0.138	F(1,34)=8.600, p<0.01	F(1,28)=0.836, p=0.370
Treatment	F(1,37)=0.229, p=0.636	F(1,34)=0.0001, p=0.992	F(1,28)=3.309, p=0.082
Interaction Strain*Treatment	F(1,37)=15.125, p<0.001	F(1,34)=8.634, p<0.01	F(1,28)=8.162, p<0.01

Table 5.6 Summary of the results from two-way ANOVA analysis for miR-30e expression at days 3, 5 and 6 in response to strain, treatment and strain*treatment The significance (alpha) level was set at p<0.05 at days 3 and 5 and p <0.01 at day 6. A statistically significant strain*treatment interaction was observed in response to hyperoxia at days 3 and 5 and in response to relative hypoxia at day 6.

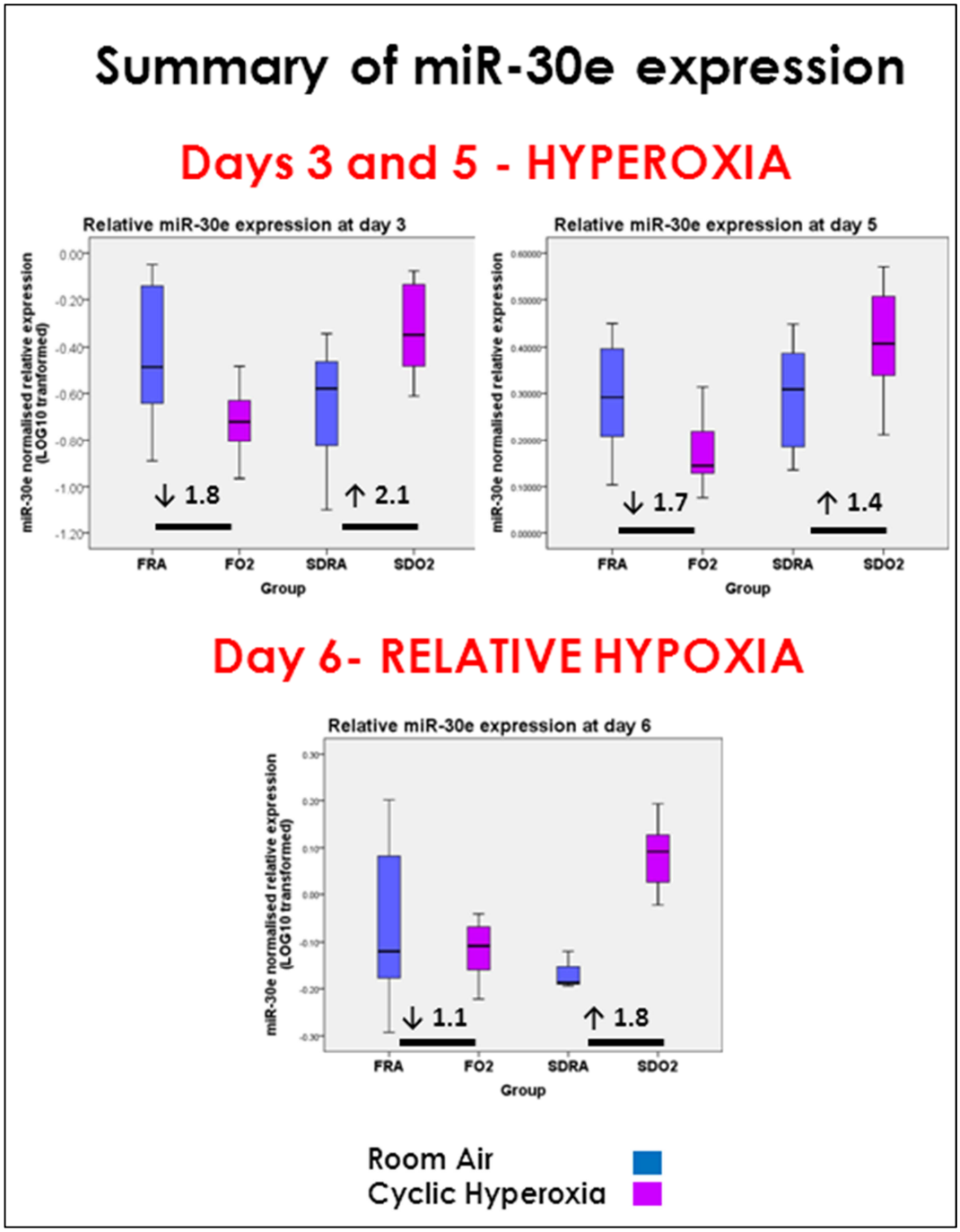

Figure 5.13 Summary of miR-30e expression at days 3, 5 and 6 in response to cyclic hyperoxia. Quantification was performed on individual rats. Statistical analysis was performed using two-way ANOVA. Expression levels were normalised to the small RNA reference gene miR-16. Numbers and arrows indicate direction of fold changes in cyclic hyperoxia. The significance (alpha) level was set at $p<0.05$ at days 3 and 5, and $p<0.01$ at day 6. Statistically significant interactions between strain and treatment were observed at days 3 and 5 in response to hyperoxia.

5.5.b miR-338 expression in individual rats at days 3, 5, and 6

Expression of miR-338 was slightly downregulated in response to hyperoxia at days 3 and 5 in F344 rats (Figure 5.14). At day 3, SD rats showed an upregulation of miR-338 expression in response to hyperoxia, whereas at day 5, miR-338 was downregulated. The interactions between strain and treatment at days 3 and 5 were not statistically significant (Table 5.7). At day 6, miR-338 was downregulated in response to relative hypoxia in F344 rats but was greatly upregulated in SD rats. The interaction between strain and treatment at this time point was statistically significant.

Variable	**Day 3**	**Day 5**	**Day 6**
Strain	$F(1,33)=4.824$, $p<0.05$	$F(1,28)=0.280$, $p=0.601$	$F(1,26)=6.337$, $p<0.05$
Treatment	$F(1,33)=1.244$, $p=0.274$	$F(1,28)=6.071$, $p<0.05$	$F(1,26)=5.444$, $p<0.05$
Interaction Strain*Treatment	$F(1,33)=0.047$, $p=0.829$	$F(1,28)=1.326$, $p=0.259$	$F_{(1,26)}=15.034$, $p<0.001$

Table 5.7 Summary of the results from two-way ANOVA analysis for miR-338 expression at days 3, 5 and 6 in response to strain, treatment and strain*treatment. Significance (alpha) level was set at 0.05. A statistically significant strain*treatment interaction was observed in response to relative hypoxia at day 6.

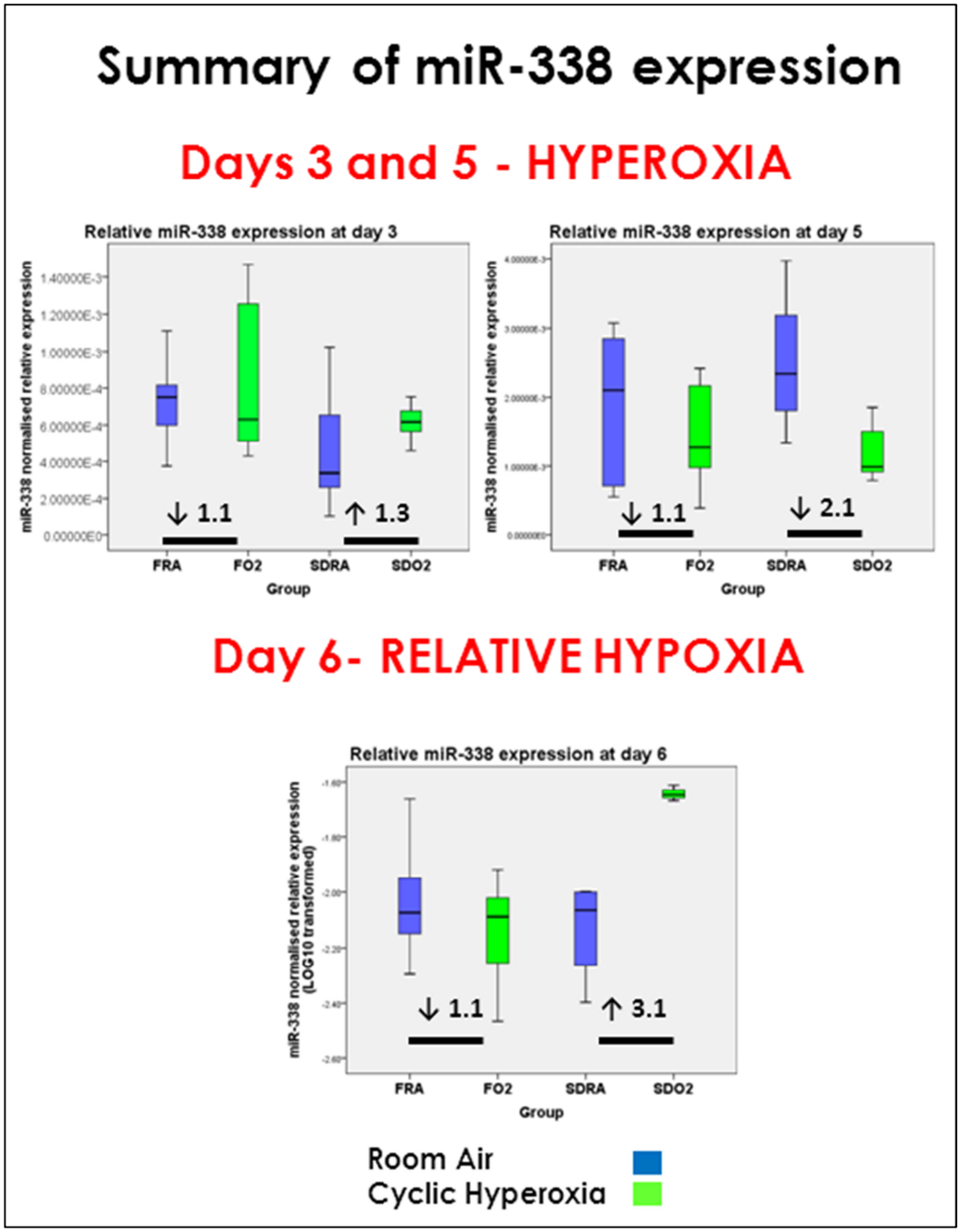

Figure 5.14 Summary of miR-338 expression at days 3, 5 and 6 in response to cyclic hyperoxia. Quantification was performed on individual rats. Statistical analysis was performed using two-way ANOVA. Expression levels were normalised to the small RNA reference gene miR-16. Numbers and arrows indicate direction of fold changes in cyclic hyperoxia. A statistically significant strain*treatment interaction was observed at day 6 response to relative hypoxia.

5.5.c miR-210 expression in individual rats at days 3, 5, and 6

Expression of miR-210 was downregulated in response to hyperoxia at days 3 and 5 in both F344 and SD rats (Figure 5.15). At day 6, miR-210 was downregulated in response to relative hypoxia in F344 rats but was upregulated in SD rats. The interaction between strain and treatment was statistically significant at day 3 in response to hyperoxia and at day 6 in response to relative hypoxia (Table 5.8).

Variable	Day 3	Day 5	Day 6
Strain	F(1,37)=4.735, p<0.05	F(1,34)=13.569, p<0.001	$F_{(1,28)}$=9.359, p<0.01
Treatment	F(1,37)=62.990, p<0.001	F(1,34)=63.116, p<0.001	$F_{(1,28)}$=6.533, p<0.05
Interaction Strain*Treatment	F(1,37)=4.975, p<0.05	F(1,34)=5.416, p=0.0269	$F_{(1,28)}$=29.477, p<0.001

Table 5.8 Summary of the results from two-way ANOVA analysis for miR-210 expression at days 3, 5 and 6 in response to strain, treatment and strain*treatment. Significance (alpha) level was set at 0.05. The significance (alpha) level was set at p<0.05 at days 3 and 6 and p <0.01 at day 5. A statistically significant strain*treatment interaction was observed in response to hyperoxia at days 3 and 5 and in response to relative hypoxia at day 6.

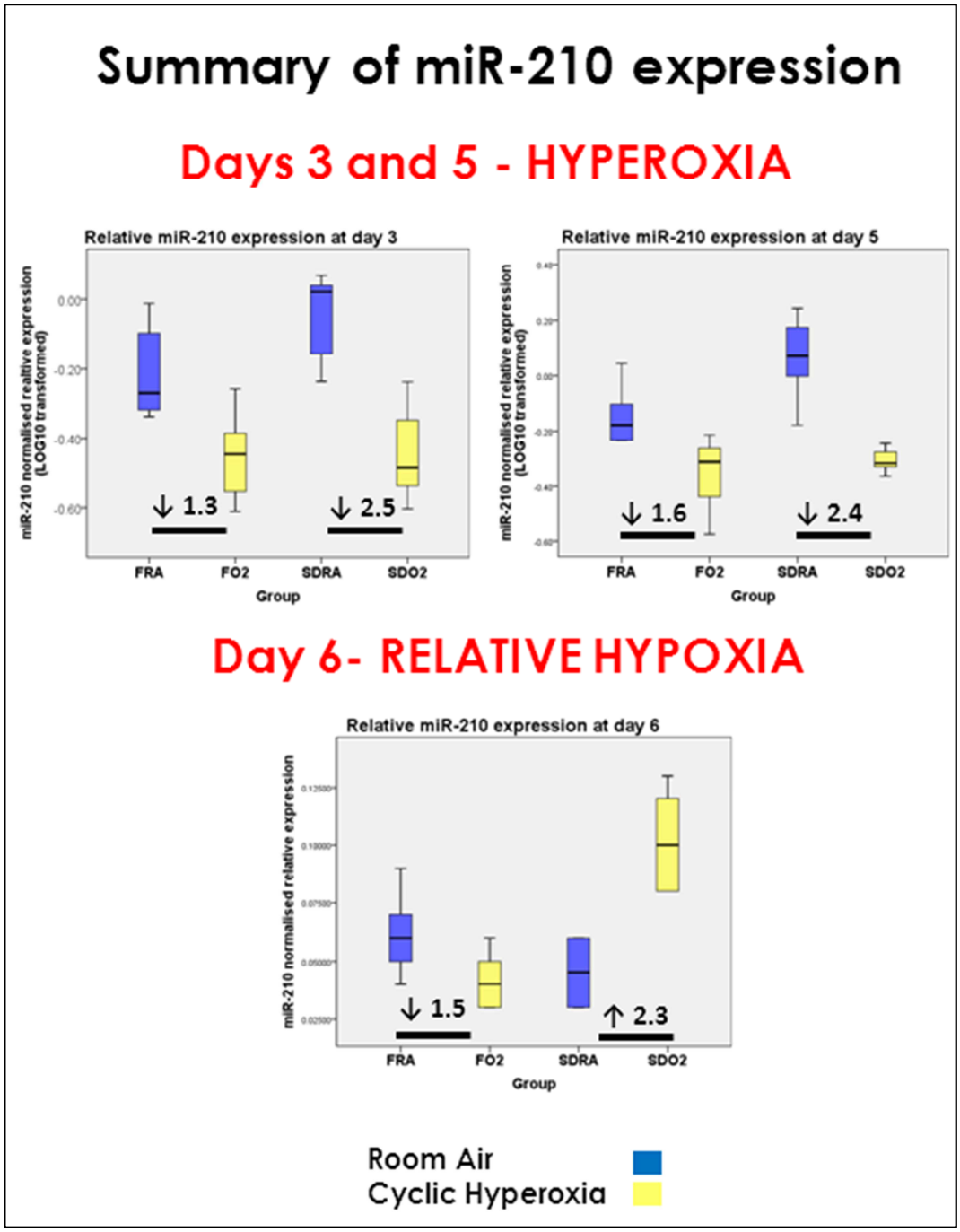

Figure 5.15 Summary of miR-210 expression at days 3, 5 and 6 in response to cyclic hyperoxia. Quantification was performed on individual rats. Statistical analysis was performed using two-way ANOVA. Expression levels were normalised to the small RNA reference gene miR-16. Numbers and arrows indicate direction of fold changes in cyclic hyperoxia. The significance (alpha) level was set at $p<0.05$ at days 3 and 6, and $p<0.01$ at day 5. Statistically significant interactions between strain and treatment were observed at day 3 in response to hyperoxia and at day 6 in response to relative hypoxia.

5.6 DISCUSSION

5.6.a Summary of findings

Exiqon microarrays were used to investigate changes in retinal miRNA expression in two strains of rats which differ in their response to oxygen therapy. We considered that any changes in miRNA expression and the corresponding changes in gene expression might contribute to the differential susceptibilities to OIR exhibited by albino inbred F344 and SD rats.

Three miRNAs of interest were identified to be differentially expressed to a significant extent after correction for multiple comparisons and were predicted to target elements of the HIF-α oxygen sensing pathway. miR-30e was predicted to target the asparginyl hydroxylase FIH, a regulator of HIF-α whereas miR-338-3p was predicted to target the two HIF subunits HIF-α and HIF-β. miR-210 was also chosen for investigation, as it is considered to be a master post-transcriptional regulator of the hypoxic response and has previously been shown to be upregulated in response to hypoxia [145, 147]. Taqman® real-time RT-PCR was used to investigate the relative expression of these miRNAs at days 3, 5 and 6 in F344 and SD rats.

miR-30e was downregulated in response to hyperoxia at days 3 and 5 in F344 rats but was upregulated in SD rats at the same time points. The interaction between strain and treatment with cyclic hyperoxia was significant at these

time points. In response to relative hypoxia at day 6, miR-30e was also differentially expressed between strains in response to relative hypoxia, with F344 rats showing relatively unchanged expression of miR-30e, whereas SD rats showed an upregulation of expression. The interaction between strain and treatment was also statistically significant at day 6 in response to relative hypoxia.

miR-338 showed a statistically significant strain*treatment interaction in response to relative hypoxia at day 6 with a downregulation of miR-338 expression observed in F344 rats and a greater upregulation observed in SD rats. The interaction between strain and treatment in response to hyperoxia was not statistically significant at days 3 or 5.

miR-210 was found to be downregulated in both strains in response to hyperoxia at days 3 and 5. The interaction between strain and treatment was found to be statistically significant at day 3; however it was not statistically significant at day 5. At day 6, strain-dependent differential expression of miR-210 was evident with a downregulation of the miRNA seen in F344 rats and an upregulation seen in SD rats in response to relative hypoxia. The interaction between strain and treatment at day 6 was also statistically significant.

These strain-dependent changes in retinal miRNA expression and their ability to modify gene expression at a post-transcriptional level may contribute to the differential susceptibility to OIR exhibited by resistant F344 and susceptible SD rats, given that these miRNAs are predicted to target genes within the HIF-α oxygen sensing pathway. Whether these miRNAs act independently or in concert to regulate gene expression currently remains unknown.

5.6.b. Microarray analysis of differential microRNA expression in the early stages of OIR

Changes in miRNA expression which occur early in OIR have not previously been investigated. Changes in retinal miRNA expression in cyclic hyperoxia-exposed resistant F344 and susceptible SD rats were evident as early as day 3, so it may be that miRNAs are acting to regulate gene expression at this early stage of disease. In addition, a number of miRNAs were found to be differentially expressed in a strain-dependent manner in response to relative hypoxia at day 6, the same time point at which statistically significant changes in retinal gene expression were found to have occurred.

Investigations at the late time points in OIR by Shen and colleagues showed that miR-31, miR-150 and miR-184 were important regulators of retinal neovascularisation, as discussed briefly in section 1.6.h [136]. Ocular injection of the precursor miRNAs pre-miR-31, pre-miR-150 and pre-miR-184 was able

to reduce retinal neovascularisation in response to relative hypoxia in the proliferative (late) stages of a mouse model of OIR. Injection of pre-miR-31 alone was able to reduce VEGF-induced retinal neovascularisation and was thought to occur indirectly via the suppression of HIF-1α activity.

Analysis of the Exiqon microarray results presented here showed that miR-31, miR-150 and miR-184 were not differentially regulated to a statistically significant extent after correction for multiple comparisons in any of the comparisons investigated at any of the 3 early time points. As priority was given to miRNAs which were differentially expressed at early time points in OIR, these miRNA were not investigated any further, although it is possible that they may also contribute to disease pathogenesis. Taken together these studies support the role of miRNAs in the regulation of HIF-α expression and the angiogenic response to relative hypoxia in the context of OIR. Ocular injection of miRNAs or their antagonists may be a potential therapeutic option for the treatment of ROP in the future.

5.6.c Biological interpretation of strain-related differences in microRNA expression in response to cyclic hyperoxia and susceptibility to OIR

Strain-dependent changes in miRNA expression at day 6 in response to relative hypoxia may have downstream effects on retinal mRNA expression. Overall, miR-30e, miR-338 and miR-210 were found to be downregulated in

F344 rats (resistant to OIR) in response to relative hypoxia at day 6, potentially resulting in *upregulation* of gene expression, whereas the overall upregulation of these miRNAs in SD rats (susceptible to OIR) may result in *downregulation* of gene expression (Table 5.9).

MiRNA of interest	**Fold change direction in F344 rats at day 6**	**Fold change direction in SD rats at day 6**
miR-30e predicted to target FIH	↓	↑
miR-338 predicted to target HIF-α and HIF-β	↓	↑
miR-210 upregulated by hypoxia via HIF-α	↓	↑

Table 5.9 Summary miRNA changes in cyclic hyperoxia-exposed F344 and SD rats at day 6 in response to relative hypoxia. F344 rats showed an overall downregulation expression of these miRNAs in response to relative hypoxia whereas SD rats showed an overall upregulation in expression of these same miRNAs.

Considering these miRNAs are predicted to act on regulators of HIF-α expression such as FIH and HIF-α and HIF-β directly which in turn upregulate the expression of other miRNAs such as miR-210, the effect of these changes in susceptibility to OIR is not straightforward. Whilst these fold changes in the Exiqon microarrays (Table 5.3) and quantitative real-time RT-PCR results are modest, they still reach statistical significance after correction for multiple comparisons and may also exert a biological effect. Fold changes as small as 1.5 fold have been shown to be significant

biologically and have been associated with cancer and endometriosis [260-262].

5.6.c.1 Strain-dependent differential expression of miR-30e and the HIF-α oxygen sensing pathway

An upregulation of miR-30e was observed in response to relative hypoxia in susceptible SD rats. As miR-30e is predicted to target FIH, this may result in decreased expression of FIH in hypoxic conditions, allowing HIF-α to interact with the co-activator complex CBP/p300 which is required for transcriptional activation of HIF-α [38-43]. The activated complex can then bind to the relative hypoxia response element (HRE) of HIF-α target genes activating the hypoxic response.

In contrast, miR-30e was downregulated in resistant F344 rats, which may result in the opposite effect where miR-30e is unable to downregulate FIH expression in response to relative hypoxia. This may potentially allow the asparaginyl hydroxylase to remain active in hypoxic conditions and inhibit HIF-α from binding to CBP/p300, thereby repressing the response to relative hypoxia in resistant F344 rats. Whether or not FIH is subject to the same type of feedback loop that is observed with EGLN3 and EGLN1 remains uncertain, however it has been suggested that FIH may be able to act in hypoxic conditions as well as normoxic conditions, so it is possible that FIH

may be post-transcriptionally regulated by miRNAs [40]. FIH is thought to be involved in the "fine-tuning" of the hypoxic response [53].

Single nucleotide polymorphisms in the pre-miR-30e transcript have previously been associated with differential susceptibility to major depression disorders and schizophrenia [263, 264]. Interestingly, functional annotation of differentially expressed genes at days 5 and 6 (sections 4.2.b.2.a and 4.2.b.2.b) showed these genes were related to synaptic transmission and behaviour. It may be that the differential expression of genes related to the neurological system may be in part regulated by miR-30e; however without validating the predicted targets of miR-30e, this remains to be seen. To date, this is the first study to identify miR-30e as a potential modifier of susceptibility to OIR. Further investigation is required to confirm if miR-30e does in fact target FIH for post-transcriptional regulation.

5.6.c.2 Strain-dependent differential expression of miR-338 and the HIF-α oxygen sensing pathway

miR-338 was found to be upregulated in response to relative hypoxia in susceptible SD rats at day 6. An upregulation of miR-338, which is predicted to target HIF-α directly, may lead to the repression of HIF-α activation by limiting HIF-α expression. In comparison, the downregulation of miR-338 expression observed in resistant F344 rats may enable HIF-α to become

activated in response to relative hypoxia and upregulate the expression of genes involved in the hypoxic response.

Like miR-30e, miR-338 has previously been linked to the neurological system and altered miR-338 expression has been associated with the differentiation of neuroblasts [265]. miR-338 is also co-transcribed from within the intron of the apoptosis-associated tyrosine kinase (AATK) gene. This gene acts to repress the expression of a family of mRNAs which negatively regulate neuronal differentiation, under the control of miR-338 [266]. These data suggest that miR-30e and miR-338 may play a role in neuronal development and differentiation. Considering that a single miRNA has the potential to target hundreds of genes, miR-30e and miR-338 may also be involved in regulating gene expression changes in OIR. This is the first time miR-338 has been associated with differential susceptibility to OIR and experimental validation of HIF-α as a target of miR-338 is required.

5.6.c.3 Strain-dependent differential expression of miR-210 and the HIF-α oxygen sensing pathway

Upregulation of miR-210 in response to hypoxia has been well established in cancer cells lines and has previously been used as a prognostic indicator in human breast cancer [145, 162]. miR-210 contains a HRE to which HIF-1α is able to bind to upregulate transcription [145, 147, 267], however, there is

conflicting evidence as to whether or not HIF-2α is also able to induce expression of miR-210 [145, 268].

miR-210 is thought to play a role in endothelial cell survival, migration and tube formation in response to hypoxia, as inhibition of miR-210 expression in hypoxic conditions has been shown to prevent the formation of capillary-like structures and VEGF-induced endothelial cell migration [147]. miR-210 was found to be upregulated in susceptible SD rats in response to relative hypoxia. An upregulation of miR-210 in SD rats may therefore result in increased angiogenesis by promoting endothelial cell survival, migration and tube formation. miR-210 has also been experimentally validated to target Ephrin-A3 (EFNA3) which has been associated with vascular remodelling [269]. In contrast, resistant F344 rats showed a downregulation of miR-210 expression in response to relative hypoxia. A downregulation of miR-210 may result in decreased VEGF-mediated endothelial cell migration and tube formation in resistant F344 rats.

5.6.c.4 Summary of strain-dependent differential microRNA expression in susceptibility to OIR

Overall, expression of miR-30e, miR-338 and miR-210 was found to be downregulated in resistant F344 rats and upregulated in susceptible SD rats (Table 5.9). These miRNAs are predicted to target FIH, HIF-α and to be upregulated by HIF-α, respectively. Whether or not these miRNAs do in fact

target the mRNAs for regulation, and whether or not they act together, or independently to contribute to differential susceptibility to OIR, is currently unknown.

Given the role of miR-210 in regulating VEGF-mediated endothelial cell survival, migration and tube formation, its upregulation in response to relative hypoxia at day 6 in susceptible SD rats may promote the angiogenic response that is absent in resistant F344 rats. However, analysis of retinal avascular area and blood vessel tortuosity as a measure of susceptibility to OIR suggests that normal angiogenesis occurs to a greater extent in resistant F344 rats, which have small retinal avascular areas at day 14. In comparison, susceptible SD rats exhibit larger retinal avascular area and increased blood vessel tortuosity at the same time point. It may be that the retinal angiogenesis occurring at day 6 in SD rats is poorly regulated and aberrant, potentially as a result of the dysregulation of the HIF-α negative feedback loop controlled by EGLN3 and EGLN1 in these rats. The downregulation of miR-210 in response to relative hypoxia at day 6 in resistant F344 rats may also be protective against OIR, preventing aberrant retinal neovascularisation. Further investigations are required to determine whether either of these hypotheses hold true.

5.6.d CONCLUSION

Strain-dependent changes in retinal miRNA expression were identified and found to occur at day 6 in response to relative hypoxia in the early stages of OIR. miR-30e, miR-338 and miR-210 have not previously been implicated in the pathogenesis of OIR but given their predicted targets within the HIF-α oxygen sensing pathway, they may play a central role in the post-transcriptional regulation of retinal gene expression in this context. Experimental validation of the targets of miR-30e and miR-338 are required to elucidate their role in susceptibility to OIR. miR-210 is likely to play a role in OIR as it is upregulated by HIF-α in response to hypoxia and has been experimentally validated to target genes involved in angiogenesis. Regulation of gene expression using miRNAs is an attractive target for therapy to control a variety of physiological and pathological processes including angiogenesis, inflammation and cancer, and could potentially be used as an intervention in ROP [148, 270, 271].