CHAPTER 6: DISCUSSION

6.1 OVERVIEW

I have identified the mode in which susceptibility to OIR is inherited in two inbred albino rat strains which exhibit differential susceptibility to OIR. I have also identified strain-dependent changes in retinal gene and miRNA expression that might underlie this differential susceptibility. This final discussion synthesizes the major findings of this study and provides a biological interpretation of how changes in retinal gene and miRNA expression might influence differential susceptibility to OIR. The major findings of this study provide a solid foundation for further avenues of investigation into differential susceptibility to OIR and may be relevant to human ROP.

6.2 FINDINGS FROM THESE STUDIES

6.2.a Inheritance of susceptibility to OIR in albino F344 and SD rats

This is the first study to determine the mode in which susceptibility to OIR is inherited in an albino inbred rat strain. In SD rats, susceptibility to OIR was shown to be inherited loosely following an autosomal dominant mode of inheritance. However, the heritable trait was not fully penetrant, suggesting that genetic modifiers may play a role in disease susceptibility and result in differences in the disease phenotype. Strain-related changes in gene and

miRNA expression may underlie the differential susceptibility to OIR that is exhibited by the F344 and SD strains examined here.

6.2.b Changes in retinal gene expression and susceptibility to OIR

6.2.b.1 Prolyl hydroxylase expression and susceptibility to OIR

This study is the first to associate the HIF-α oxygen sensing pathway and its regulators in *differential* susceptibility to OIR. Strain-dependent changes in expression of prolyl hydroxylases (PHDs) which regulate the HIF-α oxygen sensing pathway were identified to occur in early OIR.

Significant changes in the expression of two prolyl hydroxylases, EGLN3/PHD3 and EGLN1/PHD2, were found to occur at day 6 in response to relative hypoxia in resistant F344 and susceptible SD rats. Overall, cyclic hyperoxia-exposed F344 rats showed significantly *increased* expression of EGLN3 and EGLN1 at day 6 in response to relative hypoxia compared to room air-exposed rats, whereas EGLN3 and EGLN1 expression was *decreased* in SD rats.

EGLN3 and EGLN1 are known to regulate expression of HIF-α in conditions of sustained hypoxia via a negative feedback loop as described in section 4.4.c.2 [54, 247]. I hypothesize that the increase in EGLN3 and EGLN1 expression in resistant F344 rats in response to relative hypoxia may enable the prolyl hydroxylases to remain active under these conditions and

negatively regulate HIF-α abundance (Figure 6.1a). On the other hand, a decrease in EGLN3 and EGLN1 expression, as observed in SD rats, may result in dysregulation of the HIF-α negative feedback loop (Figure 6.1b).

The inability of the prolyl hydroxylases to regulate HIF-α expression in conditions of sustained hypoxia may allow HIF-α to accumulate and become activated. Activated HIF-α may then bind to the HRE of hypoxia response genes such as VEGF and upregulate their expression to promote the formation of the vessel abnormalities characteristic of OIR, such as increased retinal vessel tortuosity [34, 35, 45, 244].

Altered expression of EGLN3 and EGLN1 and their regulatory effects on the HIF-α oxygen sensing pathway have not previously been implicated in the pathogenesis of OIR and may provide a new therapeutic target for early intervention in ROP.

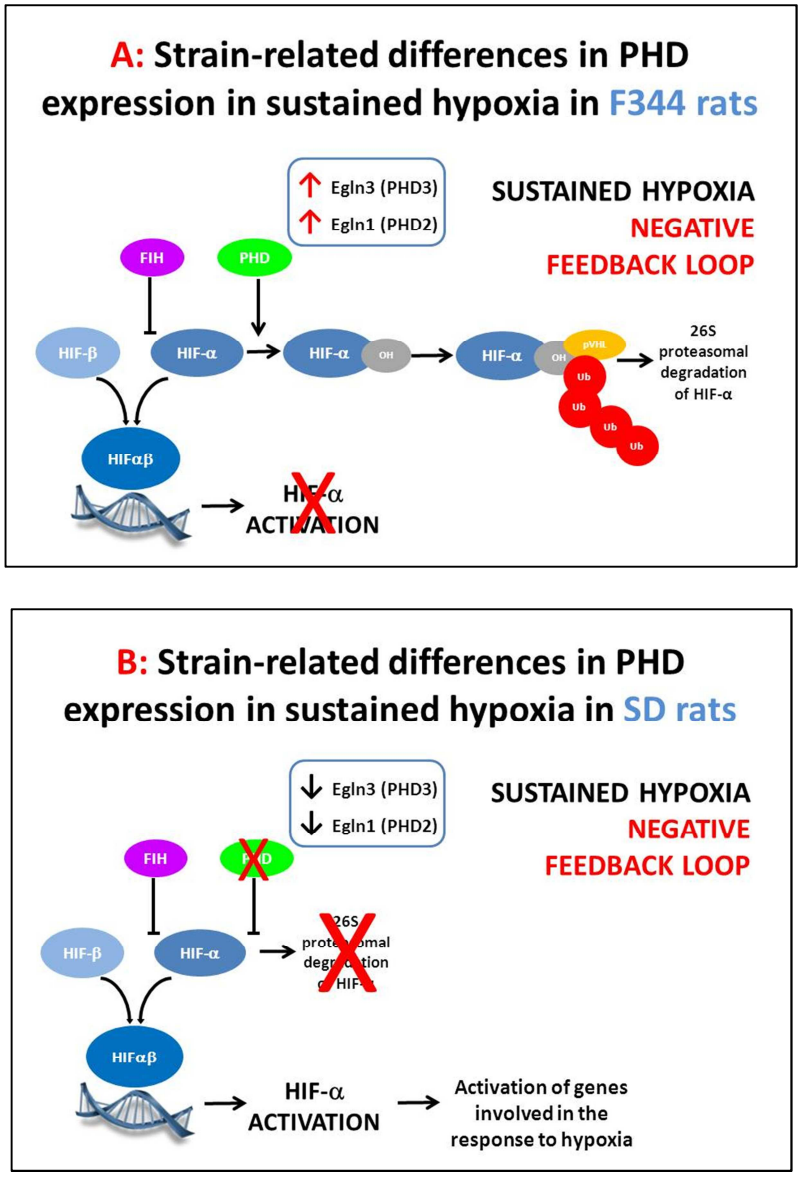

Figure 6.1 (A) Upregulation of EGLN3 and EGLN1 in response to sustained hypoxia in F344 rats results in HIF-α degradation. Under conditions of sustained hypoxia, HIF-α is subject to a negative feedback loop by the oxygen-dependent prolyl hydroxylases. EGLN3 and EGLN1 are able to remain active in F344 rats and hydroxylate HIF-α. This enables the von Hippel Lindau protein (pVHL) to bind to the hydroxylated HIF-α and target it for polyubiquitination and proteasomal degradation. **(B) Downregulation of EGLN3 and EGLN1 in response to sustained hypoxia in SD rats results in HIF-α activation.** The reduced levels of EGLN3 and EGLN1 in SD rats may prevent HIF-α from being negatively regulated in response to sustained hypoxia. This may allow HIF-α to become activated and upregulate genes involved in the response to hypoxia which may have detrimental effects. Ub = Ubiquitin; PHD = Prolyl hydroxylase domain protein; FIH = Factor inhibiting HIF (Asparaginyl hydroxylase domain protein); pVHL = von Hippel Lindau protein.

6.2.b.2 Insulin-like growth factor binding protein 3 expression and susceptibility to OIR

Strain-dependent changes in IGFBP3 expression were also identified in early OIR. At day 6, a statistically significant interaction between strain and treatment was observed. IGFBP3 was shown to be stably expressed in resistant F344 rats in response to relative hypoxia, whereas it was decreased in susceptible SD rats. This stable expression of IGFBP3 in F344 may provide some protection against hyperoxia-induced vessel loss, resulting in a resistant phenotype. This is consistent with a previous finding that early expression of IGFBP3 is protective against OIR [236]. Low expression of IGFBP3 has been associated with proliferative stages of OIR, and persistent vaso-obliteration, consistent with the large avascular areas observed in the susceptible phenotype exhibited by SD rats [236]. The evidence presented here suggests strain-dependent changes in IGFBP3 expression may contribute to differential susceptibility to OIR.

6.2.c Changes in retinal microRNA expression and susceptibility to OIR

miRNAs are able to regulate gene expression post-transcriptionally and may play a role in regulating changes in retinal gene expression associated with susceptibility to OIR. I identified several miRNAs that were differentially expressed in response to relative hypoxia at day 6 in a strain-dependent

manner. These miRNAs are predicted to act on regulators of HIF-α expression such as FIH (miR-30e), HIF-α and HIF-β directly (miR-338) and in the case of miR-210, are known to be upregulated by HIF-α [145, 147]. As some of these miRNAs are predicted to target regulatory elements of the HIF-α oxygen sensing pathway, as well as genes within the pathway, the effect of these changes on susceptibility to OIR is not straightforward. The potential effect of these changes on gene expression at day 6 in response to relative hypoxia is described below for each individual miRNA.

6.2.c.1 Biological interpretation of strain-dependent changes in miR-30e expression and the HIF-α oxygen sensing pathway

Increased miR-30e expression was observed in response to relative hypoxia at day 6 in susceptible SD rats. As miR-30e is predicted to target FIH, this may result in decreased expression of FIH in hypoxic conditions, allowing the recruitment of the transcriptional co-activator complex CBP/p300 [43]. This in turn allows HIF-α to bind to HIF-β and subsequently bind to the hypoxia response element of HIF-α target genes, activating the hypoxic response (Figure 6.2a). In contrast, miR-30e expression was decreased in resistant F344 rats, which may result in the opposite effect, in which FIH expression is not downregulated in response to relative hypoxia in these rats (Figure 6.2b).

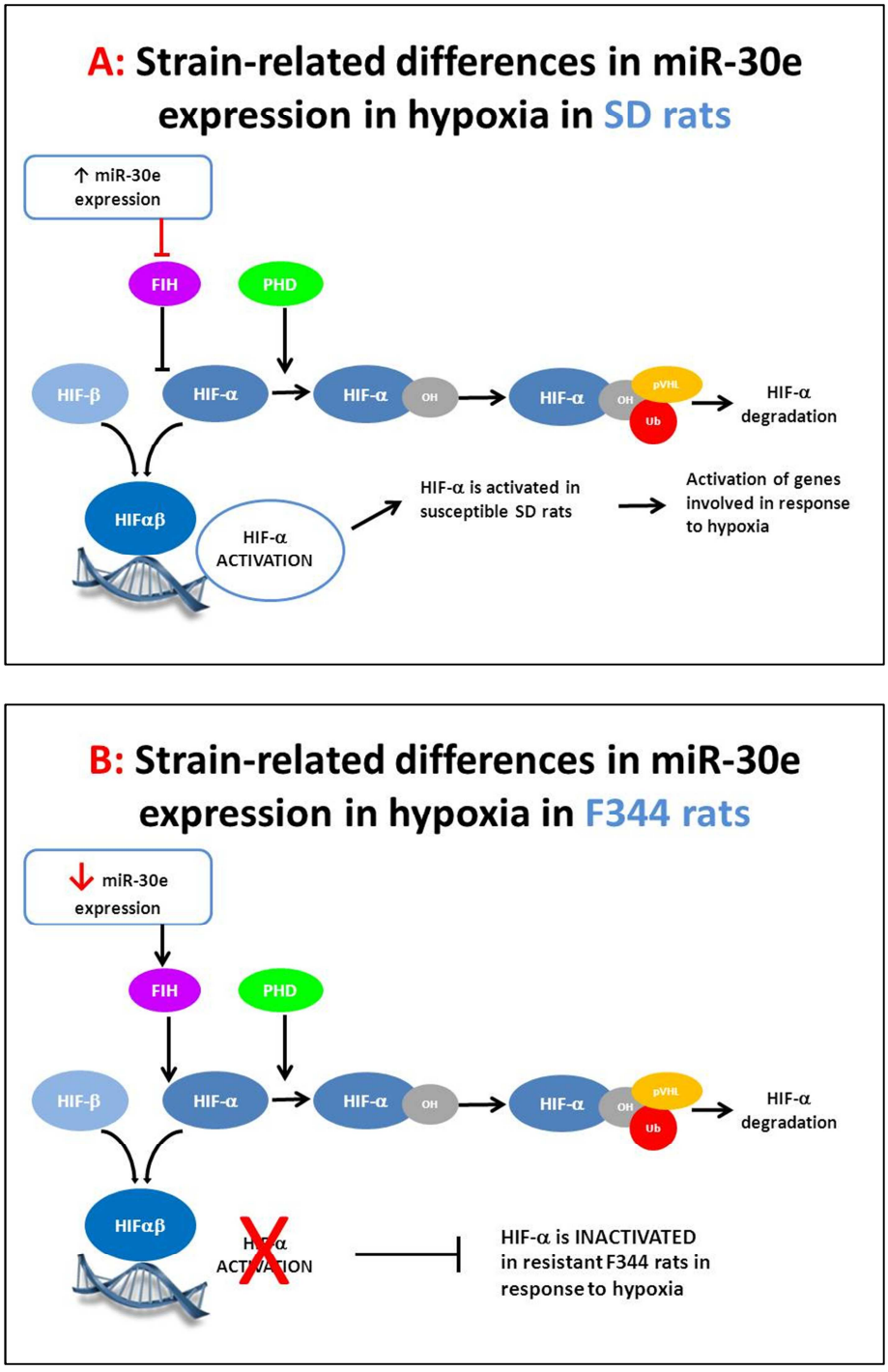

Figure 6.2 (A) Effect of miR-30e upregulation in SD rats at day 6 in response to relative hypoxia. Increased miR-30e expression may lead to the repression of FIH, allowing HIF-α to bind to HIF-β and upregulate the expression of genes involved in the hypoxic response in SD rats. **(B) Effect of miR-30e downregulation in F344 rats at day 6 in response to relative hypoxia.** Decreased miR-30e expression may lead to the upregulation of FIH in hypoxic conditions, preventing HIF-α from binding to HIF-β, resulting in HIF-α degradation in F344 rats. Ub = Ubiquitin; PHD = Prolyl hydroxylase domain protein; FIH = Factor inhibiting HIF (Asparaginyl hydroxylase domain protein); pVHL = von Hippel Lindau protein.

The decrease in miR-30e expression in resistant F344 rats may plausibly allow FIH to remain active in hypoxic conditions, and inhibit the transcriptional activity of HIF, 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].

Post-transcriptional regulation of FIH by miR-30e is yet to be determined.

6.2.c.2 Biological interpretation of strain-dependent changes in miR-338 expression and the HIF-α oxygen sensing pathway

miR-338 expression was increased in response to relative hypoxia in SD rats at day 6. An increase in miR-338, which is predicted to target both HIF-α and HIF-β, may prevent HIF-α activation by limiting the amount of both HIF subunits available for activation in response to relative hypoxia (Figure 6.3a). This would result in the absence of the hypoxic response in susceptible SD rats.

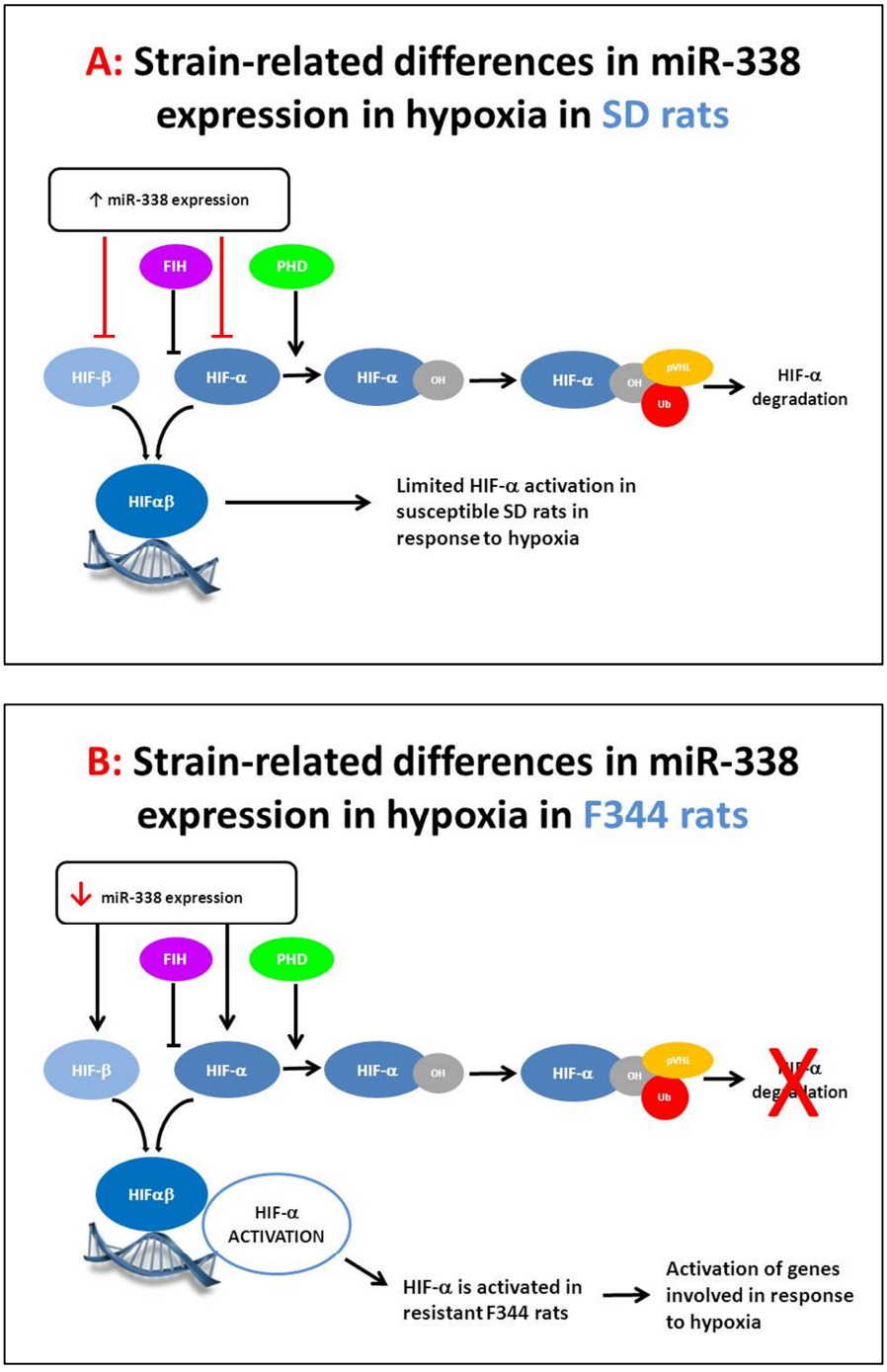

Figure 6.3 (A) Effect of miR-338 upregulation in SD rats at day 6 in response to relative hypoxia. Increased miR-338 expression may lead to repression of HIF-α activation and a reduction of the hypoxic response in SD rats as HIF-α is degraded. **(B) Effect of miR-338 downregulation in F344 rats at day 6 in response to relative hypoxia.** Decreased miR-338 expression may lead to HIF-α activation and the induction of the hypoxic response by upregulating gene expression in F344 rats. Ub = Ubiquitin; PHD = Prolyl hydroxylase domain protein; FIH = Factor inhibiting HIF (Asparaginyl hydroxylase domain protein); pVHL = von Hippel Lindau protein.

In comparison, the decrease in miR-338 expression observed in resistant F344 rats at day 6 may increase the amount of the HIF-α and HIF-β subunits available for activation in response to relative hypoxia. This could enable HIF-α to form a heterodimer with HIF-β and bind to the hypoxia response element of genes associated with the response to hypoxia and upregulate their expression (Figure 6.3b). This sequence of events may account for the increased retinal neovascularisation that is observed in resistant F344 rats compared to susceptible SD rats, in response to cyclic hyperoxia.

HIF-α and HIF-β are yet to be experimentally validated as targets for miR-338, therefore further functional characterisation studies are required.

6.2.c.3 Biological interpretation of strain-dependent changes in miR-210 expression and the HIF-α oxygen sensing pathway

Upregulation of miR-210 in response to relative 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 hypoxia response element to which HIF-1α is able to bind to upregulate transcription of the miRNA [145, 147, 267]; however, there is conflicting evidence as to whether 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 [147]. Inhibition of miR-210 expression in

hypoxic conditions *in vitro*, 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 (Figure 6.4a). 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 which has been associated with vascular remodelling [269].

In contrast, F344 rats showed a downregulation of miR-210 expression in response to relative hypoxia (Figure 6.4b). This may result in decreased VEGF-mediated endothelial cell migration and tube formation in resistant F344 rats. Further studies examining phenotypic changes which might occur in response to intraocular injection of miR-210 may yield further information regarding the role miR-210 plays in susceptibility to OIR.

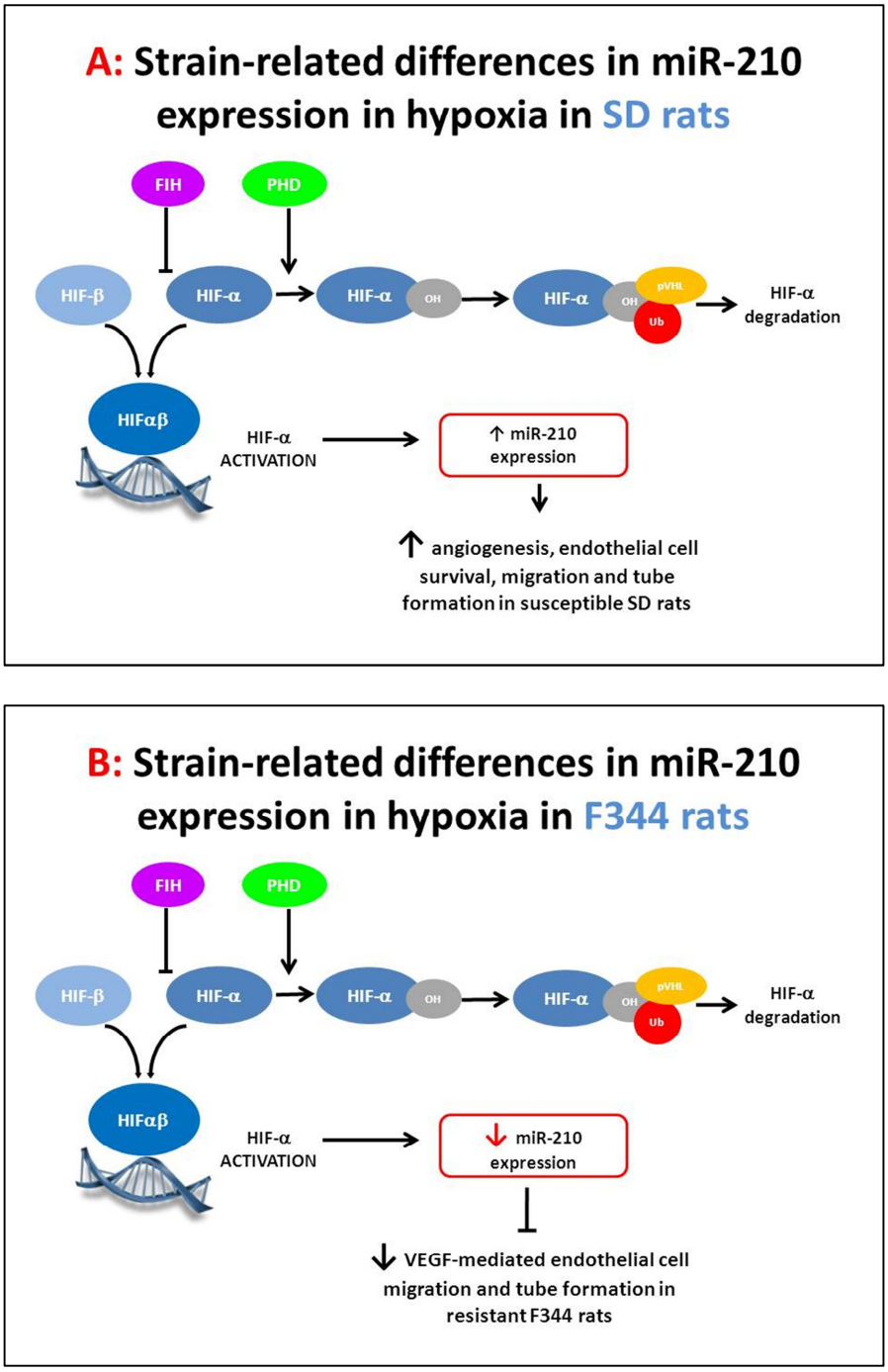

Figure 6.4 (A) Effect of miR-210 upregulation in SD rats at day 6 in response to relative hypoxia. Increased miR-210 expression may lead to an increase in the angiogenic response and promote VEGF-mediated endothelial cell survival, migration and tube formation in susceptible SD rats. **(B) Effect of miR-210 downregulation in F344 rats at day 6 in response to relative hypoxia.** Downregulation of miR-210 expression may lead to a decrease in VEGF-mediated endothelial cell migration and tube formation in resistant F344 rats. Ub = Ubiquitin; PHD = Prolyl hydroxylase domain protein; FIH = Factor inhibiting HIF (Asparaginyl hydroxylase domain protein); pVHL = von Hippel Lindau protein.

6.2.d Synthesis: strain-dependent changes in gene and microRNA expression in susceptibility to OIR

To recapitulate, strain-dependent differences in EGLN3, EGLN1 and IGFBP3 were observed at day 6 in response to relative hypoxia in two inbred albino rat strains which differ in their susceptibility to OIR (Table 6.1). In resistant F344 rats, expression of the PHDs EGLN3 and EGLN1 was upregulated in response to relative hypoxia, while IGFBP3 expression remained stable. In this same strain, expression of miR-30e, miR-338 and miR-210 was decreased. In contrast, susceptible SD rats showed decreased expression of EGLN3, EGLN1 and IGFBP3 in response to relative hypoxia at day 6. Expression of miRNAs miR-30e, miR-338 and miR-210 were all increased.

Gene or miRNA of interest	**Regulation in F344 rats at day 6**	**Regulation in SD rats at day 6**
EGLN3 and EGLN1	↑	↓
IGFBP3	Stable expression	↓
miR-30e Predicted to target FIH	↓	↑
miR-338 Predicted to target HIF-α and HIF-β	↓	↑
miR-210 Upregulated by HIF-α in hypoxia	↓	↑

Table 6.1 Summary of gene and miRNA expression changes in cyclic hyperoxia-exposed F344 and SD rats at day 6 in response to relative hypoxia. Overall, resistant F344 showed stable or increased expression of the genes of interest, whereas the miRNAs of interest showed decreased expression. In comparison, overall expression of the genes of interest in susceptible SD rats decreased, whereas in this strain an increase in miRNA expression was observed.

Taken together, it is reasonable to hypothesise that these changes in gene and miRNA expression contribute to the two different susceptibility phenotypes observed in these two rat strains (Figure 6.5a and 6.5b). In resistant F344 rats, the prolyl hydroxylase negative feedback loop may remain active, preventing the accumulation of HIF-α in conditions of sustained hypoxia. Other elements of the HIF-α oxygen sensing pathway such as FIH, HIF-α and HIF-β may also be subject to modification at a post-transcriptional level. Alongside this, stable expression of IGFBP3 may have a protective effect in the resistant F344 rats. The changes in gene and miRNA expression might result in the overall reduction in the response to hypoxia and be expressed as a resistant phenotype.

In contrast, decreases in prolyl hydroxylase expression in susceptible SD rats may result in dysregulation of the PHD negative feedback loop in sustained hypoxia, resulting in poorly regulated, aberrant retinal neovascularisation. Low expression of IGFBP3 in SD rats may also underlie susceptibility to OIR, with low expression previously associated with persistent vaso-obliteration [236]. These changes, in conjunction with post-transcriptional modification of gene expression by the miRNAs of interest could result in an overall increase in the hypoxic response which is expressed as a susceptible phenotype.

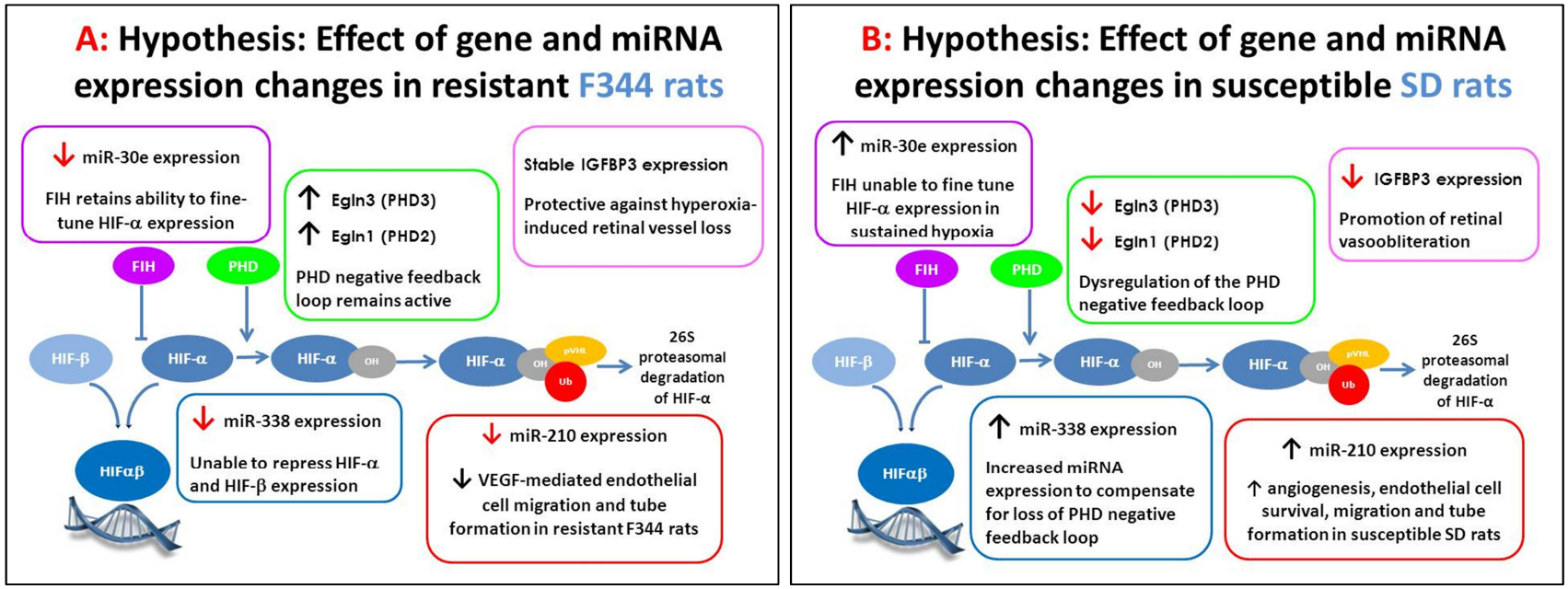

Figure 6.5 (A) Hypothesis of the biological effects of gene and miRNA expression changes in susceptibility to OIR in F344 rats. The potential biological effects of gene and miRNA expression changes are shown. The prolyl hydroxylase negative feedback loop may be able to remain active in resistant F344 rats, preventing the accumulation of HIF-α in conditions of sustained hypoxia. These changes, as well as the potential post-transcriptional modifications of FIH, HIF-α and HIF-β expression by these miRNAs of interest could result in the overall reduction in the response to hypoxia, and be expressed as a resistant phenotype. **(B) Hypothesis of the biological effects of gene and miRNA expression changes in susceptibility to OIR in SD rats.** The potential biological effects of gene and miRNA expression changes are shown. The prolyl hydroxylase negative feedback loop may be unable to remain active in susceptible SD rats in response to sustained hypoxia resulting in HIF-α accumulation. The inability of the other miRNAs of interest to modify HIF-α expression may allow HIF-α to become active and could result in an overall increase in hypoxic response, and be expressed as a susceptible phenotype.

6.2.e A note on the bioinformatics approach

A microarray approach was undertaken to investigate changes in global gene and miRNA expression in the context of susceptibility to OIR. Microarrays offer an advantage over a candidate gene approach as they can identify changes in the expression of hundreds to thousands of genes within the same samples simultaneously, making them a useful screening tool [225, 226]. In addition, microarrays make no assumptions about the relevance of each gene to the condition being investigated. The disadvantage of this approach is that it is not always possible to validate the results of the microarrays for each gene or miRNA that is differentially expressed to a statistically significant level. Therefore, certain filters must be applied to the results, be it an arbitrary fold change cut off or functional annotation, in order to obtain a manageable number of candidate genes or miRNAs for further investigation. In the process of choosing these candidates, it is quite possible that genes or miRNAs which are associated with susceptibility to OIR may be erroneously discarded. However, the use of this approach enabled a pathway not previously associated with differential susceptibility to OIR to be identified.

The biological effects of changes in miRNA expression in susceptibility to OIR are yet to be determined. The targets of miR-30e and miR-338 have not previously been validated in the context of OIR, and therefore the putative biological effects on expression of FIH, and HIF-α and HIF-β, respectively, have yet to be confirmed. MiR-210 is known to be upregulated by hypoxia

and to exert an angiogenic effect in human umbilical vein endothelial cells, however the effect miR-210 has on retinal endothelial cells in the context of susceptibility to OIR is currently unknown [145, 147, 267]. The potential regulation of the HIF-α oxygen sensing pathway by these miRNAs adds another layer of complexity to differential susceptibility to OIR. Further studies are required to determine the exact role, if any, that each of these changes may play in susceptibility to OIR.

Considering that miRNAs have the ability to modify gene expression post-transcriptionally, this may result in changes in protein expression. Expression levels of FIH, HIF-α and HIF-β protein were not investigated. Confirmation of the miRNA targets followed by investigation of protein expression levels in both strains in response to cyclic hyperoxia would strengthen the hypothesis that miRNAs are involved in the regulation of the HIF-α oxygen sensing pathway and might contribute to differential susceptibility to OIR. Identifying changes in the expression of proteins associated with susceptibility to OIR might identify biomarkers that could eventually be used as indicators of disease progression and severity [272].

6.3 AN OVERVIEW OF RELATED STUDIES

6.3.a Role of genetic modifiers in inheritance of susceptibility to OIR

Susceptibility to OIR in SD rats was found to loosely follow an autosomal dominant mode of inheritance; however the fact that susceptibility trait did not appear to be fully penetrant suggests that genetic modifiers may be involved. One such genetic modifier in OIR/ROP that has previously been studied is ocular pigmentation [79-81, 96, 100, 203, 204, 209, 210]. As discussed in Chapter 3, formal backcross analysis by van Wijngaarden and colleagues using an albino (F344, resistant to OIR) and pigmented (DA, susceptible to OIR) rat strain showed that all F1 (F344 x DA) generation rats had ocular pigmentation and coat colouring similar to the DA rats, and all rats were found to be susceptible to OIR. The same observation was made in regards to the pups of the F1 x DA backcross where all rats were susceptible to OIR. The F1 x F344 backcross produced rats with a range of ocular pigmentation and coat colouring, and here susceptibility to OIR was found to segregate with ocular pigmentation. From these studies it was concluded that susceptibility to OIR in pigmented DA rats was inherited in an autosomal dominant mode [203]. Ocular pigmentation may be one of many genetic modifiers involved in susceptibility to OIR. The fact that the same mode of inheritance underlies susceptibility to OIR in albino and pigmented rat strain indicates that ocular pigmentation is not the sole modifier in susceptibility to OIR and that other genes are involved.

It has been proposed that differences in susceptibility to OIR may be associated with the ability or inability of different strains to overcome hyperoxia-mediated vaso-obliteration, as suggested by Dorfman and colleagues [204]. Hyperoxia is known to induce retinal vascular endothelial cell apoptosis resulting in vaso-obliteration of retinal vessels, and inhibits responses critical for normal retinal vascular development such as cellular proliferation and differentiation [273-275]. Hyperoxic and hypoxic stresses can affect the expression of genes including the pro-angiogenic growth factor VEGF and the anti-angiogenic growth factor PEDF, both of which are known to be important in retinal angiogenesis [28, 98, 100]. Indeed, susceptibility to OIR in Brown Norway (BN) rats has been associated with increased expression of VEGF accompanied by decreased expression of the anti-angiogenic factor PEDF [98, 100].

HIF-α is known to upregulate the expression of VEGF in response to hypoxia, and *in vitro* experiments show that HIF-α is also involved in the regulation of PEDF expression in response to hypoxia in human retinal cells [8, 32, 34, 222]. Given that oxygen is central to the pathogenesis of ROP and OIR, defects in the oxygen sensing pathway may be responsible for differential susceptibility to OIR. The changes in gene and miRNA expression presented in this thesis support the argument that genetic modifiers other than ocular pigmentation play a role in susceptibility to OIR.

6.3.b Role of prolyl hydroxylases in susceptibility to OIR

Whilst this is the first study to associate prolyl hydroxylase regulation of the HIF-α oxygen sensing pathway with *differential* susceptibility to OIR in two different rat strains, there have been three other studies that have linked prolyl hydroxylases with the development of susceptibility to OIR. All three studies used the murine model of OIR as described by Sears et al. [276]. These studies did not take into account strain differences in susceptibility to OIR, despite reports that the rate at which normal retinal vascular development occurs differs between strains of mice [277, 278].

The first is a study by Sears and colleagues that showed inhibition of prolyl hydroxylases during hyperoxia was able to prevent OIR [276]. This study used a different model of OIR, in which neonatal mice at postnatal day 7 were subjected to 75% oxygen for 5 days. After this period of sustained hyperoxia, mice were allowed to recover for 5 days in room air, or normoxic conditions. To determine the effects of prolyl hydroxylase inhibition on OIR, a prolyl hydroxylase (PHD) inhibitor, dimethyloxalylglycine (DMOG) was administered via injection into the peritoneal cavity of hyperoxia-exposed mice at postnatal day 6 (24 h prior to hyperoxia exposure) and at postnatal day 8 (24 h after exposure to hyperoxia). Injection of DMOG at these two time points prevented hyperoxia-induced retinal vessel dropout, and resulted in a decrease in the vascular tortuosity and neovascular tufts that are characteristic of OIR. Injection of DMOG during the hypoxic phase at

postnatal day 11 (24 h prior to hypoxic exposure) and at postnatal day 13 (24 h after exposure to relative hypoxia) did not have a significant effect on reducing vascular drop out, tortuosity or neovascular tufts [276].

DMOG inhibits the expression of all 3 PHDs, and considering the activity of each PHD is dependent on context, and that the different isoforms have varying affinities for HIF-1α and HIF-2α [54], it may be that the inhibition of a particular PHD, not necessarily all 3 PHDs, is enough to negate the detrimental effects of hyperoxic exposure. It is also possible that in the instance that one or more of the prolyl hydroxylases is inhibited, the remaining hydroxylases may compensate for the loss of function. The role of the PHDs in the HIF-α negative feedback loop was not discussed in the study by Sears et al [276].

In the context of the work presented in this thesis, strain-dependent differential expression of PHDs EGLN3 and EGLN1 was statistically significant at day 6 in response to relative hypoxia. EGLN3 and EGLN1 expression was generally upregulated in resistant F344 rats, whereas expression was downregulated in susceptible SD rats in response to relative hypoxia. In response to hyperoxia, the interaction between strain and treatment *was* significant for EGLN3/PHD3 (Table 4.37) but was not statistically significant for EGLN1/PHD2 (Table 4.38).

At day 3, EGLN3 expression was found to be unchanged in resistant F344 rats in response to hyperoxia compared to room air-exposed rats but was downregulated 3.2 fold in SD rats cyclic hyperoxia-exposed rats compared to room air-exposed rats (Figure 4.21). The effect of downregulation of EGLN3 in response to hyperoxia in susceptible SD rats is not currently known. At the same time point, EGLN1/PHD2 expression was largely unchanged in response to hyperoxia in both strains compared to room air-exposed rats (Figure 4.15). More experiments are required to determine whether or not this stable expression of EGLN3/PHD3 (and to a lesser extent EGLN1/PHD2) in response to hyperoxia in F344 rats is enough to reduce the vascular drop out, tortuosity and neovascular tufts observed in these rats.

Since the finding that inhibition of PHDs during hyperoxia prevented OIR was first published, other studies have shown an association between expression of two specific prolyl hydroxylases, EGLN1/PHD2 and EGLN2/PHD1, in susceptibility to OIR [279, 280].

Duan et al. showed that significant stabilisation of HIF-1α, and to a lesser extent, HIF-2α, was evident in the retinae of EGLN1/PHD2 deficient mice at the cessation of the hyperoxia exposure period [279]. The stabilisation of the transcription factor during the hyperoxia exposure period was considered to be protective against hyperoxia-induced vaso-obliteration [279]. Upon return of the mice to the relative hypoxia of room air, minimal retinal

neovascularisation was observed in EGLN1/PHD2 deficient mice, during what is traditionally the vaso-proliferative phase of OIR. Interestingly, a combined EGLN2/EGLN3 deficiency was not shown to be protective against hyperoxia-induced vessel loss. The authors suggested that inhibition of EGLN1/PHD2 during the vaso-obliteration stage of the disease may be protective against OIR. The role of the EGLN1/PHD2 in the HIF-α negative feedback loop in response to sustained hypoxia was not discussed.

My own data showed that EGLN1/PHD2 expression was relatively unchanged in response to hyperoxia at days 3 and 5 in resistant F344 rats and susceptible SD rats, compared to room air-exposed controls. It was at day 6, in response to relative hypoxia, that EGLN1/PHD2 showed regulation by oxygen, and was significantly downregulated in susceptible SD rats, whereas it was upregulated in resistant F344 rats. Whether or not the stable expression of EGLN1/PHD2 in response to hyperoxia at days 3 and 5 is protective against hyperoxia-induced vaso-obliteration in either strain is unknown.

A study by Huang and colleagues found that absence of a different prolyl hydroxylase, EGNL2/PHD1, was also able to prevent hyperoxia-induced vascular obliteration in mice with OIR [280]. Mice deficient in PHD1 had reduced vessel dropout, vascular leakage and apoptosis in their retinae. The protective effects of the absence of EGNL2/PHD1 were proposed to occur

via the accumulation of HIF-1α due to the lack of degradation by EGNL2/PHD1 [280]. HIF-1α was found to be stabilised in PHD1 deficient mice whereas HIF-2α expression was not detected, possibly due to low abundance [280]. In contrast, *in vitro* studies have found that EGNL2/PHD1 has a greater affinity for HIF-2α than HIF-1α [54]. Huang et al. suggest that EGNL2/PHD1 may have affinities for both HIF-α isoforms and that this difference may be explained by the inability to detect small changes in HIF-2α expression [280]. EGLN2/PHD1 has not been shown to be upregulated in hypoxia and is not thought to be involved in the HIF-α negative feedback loop [54].

This mechanism of HIF-1α stabilisation due to lack of EGLN2/PHD1 expression is similar to that proposed by Duan et al., in that lack of EGLN1/PHD is protective against hyperoxia-induced vaso-obliteration [279]. Huang and colleagues suggested that a deficiency in EGLN2/PHD1 results in HIF-1α accumulation, as it does not undergo PHD-induced proteasomal degradation [280]. The accumulation of HIF-1α induces expression of VEGF in the hyperoxic conditions, leading to a reduction in vessel loss. When animals are returned to the relative hypoxia of room air, there is no drive towards angiogenesis, as hyperoxia-induced vessel loss has been limited, preventing OIR.

Expression of EGLN2/PHD1 in response to both hyperoxia and relative hypoxia was not investigated in my study as it did not show regulation by hyperoxia at day 3. The fact that similar conclusions were able to be drawn from these studies suggests that EGNL2/PHD1 and EGLN1/PHD2 prevent OIR via regulation of HIF-α expression, particularly HIF-1α. In light of these findings further studies are required to determine if strain-dependent changes in EGLN1/PHD2 and EGLN2/PHD1 expression play a role in differential susceptibility to OIR. Further investigations regarding post-transcriptional modification of all 3 PHDs by miRNAs are also warranted.

6.3.c Role of IGFBP3 in susceptibility to OIR

In the retina, IGFBP3 appears to promote vascular survival and regrowth. However this may occur independently of IGF-1, as rodent models of OIR using IGFBP3 knockout mice suggest that IGFBP3 acts independently of IGF-1 to promote vessel regrowth [236, 257]. IGF-1 levels remain the same in wild type and IGFBP3 knockout mice [236]. The exact interplay between IGF-1 and IGFBP3 in the retina remains uncertain.

Clinical studies in premature infants have found that increased levels of *serum* IGFBP3 are protective against retinal vessel loss and therefore protective against proliferative ROP [236]. Low serum IGF-1 levels have also been associated with development of ROP in premature infants [250]. Here, low IGF-1 levels were also associated with reduced VEGF-mediated

endothelial cell survival, resulting in retinal vessel loss followed by aberrant neovascularisation. Together, these data suggest that low IGF-1 and IGFBP3 levels are detrimental to normal retinal neovascularisation and restoring levels may provide some therapeutic benefit. Lofqvist and colleagues suggest that the IGF-1/IGFBP3 ratio is similar between premature infants who go on to develop proliferative ROP and those who do not, lending some weight to this hypothesis [236].

Monitoring of serum IGF-1 and IGFBP3 levels has been proposed as a means to identify infants who are at high risk of developing severe ROP [236, 250, 258]. In a trial involving 50 premature infants, postnatal weight and serum IGF-1 and IGFBP3 levels were routinely measured and were successfully used to identify infants who went on to develop proliferative ROP and those that did not. Monitoring these factors may be a valuable screening tool for the future. A clinical trial to determine if restoring serum IGF-1 levels is able to prevent ROP and associated complications of premature birth is currently underway in Sweden (ClinicalTrials.gov Identifier: NTC01096784).

Serum IGF-1 and IGFBP3 levels were not examined in the course of this thesis and further studies are required to elucidate the role these proteins might play in differential susceptibility to OIR. Whether or not stable expression of IGFBP3 in resistant F344 rats is enough to be protective against hyperoxia-induced vessel loss, or whether downregulation of IGFBP3 in

susceptible SD rats results in aberrant retinal neovascularisation, is yet to be determined.

6.3.d Role of microRNAs in susceptibility to OIR

Few studies have examined the role of miRNAs in susceptibility to OIR, however, post-transcriptional modification of gene expression by miRNAs has been shown by others to regulate ocular neovascularisation and contribute to susceptibility to OIR [136]. As discussed in Chapter 5, expression of miRNAs which target genes associated with the response to hypoxia, such as HIF-1α (miR-31) and VEGF (miR-150), was found to be downregulated in response to relative hypoxia in OIR [136]. Injection of precursor miRNAs, pre-miR-31, pre-miR-150 and pre-miR-184 at the end of the hyperoxic exposure period was able to suppress hypoxia-induced retinal neovascularisation.

A study by Bai and colleagues identified a different miRNA, miR-126, to be downregulated in response to hyperoxia and associated with susceptibility to OIR [139]. In this model of OIR, postnatal day 7 mice were exposed to hyperoxia for 5 days, given an intraocular injection of miR-126 at the end of the hyperoxic exposure period, then allowed to recover in room air for 5 days. At the end the hypoxic exposure, inhibition of pathological retinal neovascularisation was observed [139]. MiR-126 was shown to regulate retinal neovascularisation by post-transcriptional modification of VEGF, IGF-

2 and HIF-1α expression. These studies of the differential expression of miRNAs in OIR have been limited to the late, proliferative stages of the disease which occur in response to relative hypoxia [136], or to changes which occur at the end of a sustained period of hyperoxia [139]. Changes which occur in the early stages of OIR in response to hyperoxia (or relative hypoxia in the cyclic hyperoxia model) as in my work, have not previously been studied, nor has the effect of strain on differential susceptibility to OIR. Changes occurring at these time points may also contribute to differential susceptibility to OIR.

6.3.e Temporal gene and microRNA expression in differential susceptibility to OIR

The time point at which genes and miRNAs are differentially expressed in response to hyperoxia and relative hypoxia in differential susceptibility to OIR is important. The majority of studies that examine changes in gene expression in OIR are limited to time points late within the induction period when the OIR is approaching or has reached its maximum [96, 98, 100, 281]. Changes which occur at this point in time are likely to be a consequence of earlier events which contribute to differential susceptibility to OIR.

Previous studies from our laboratory examined the expression of various genes related to angiogenesis at different time points along the cyclic hyperoxia exposure protocol in 3 strains of rats which showed differential

susceptibility to OIR [30]. Retinal expression of VEGF, VEGF receptor 2 (VEGFR2), angoipoietin-2 (ANG2), cyclooxygenase-2 (COX2), Tie2, erythropoietin (EPO), IGF-1 and PEDF were examined at postnatal days 2, 3, 8, 9, 14 and 18 of cyclic hyperoxia in F344 rats (resistant to OIR), as well as in SD and DA rats (susceptible to OIR).

In this study, the differential expression of these genes at postnatal day 9 in response to hyperoxia was found to be important. Expression of VEGF, VEGFR2 and ANG2 was found to be significantly greater in resistant F344 rats compared with susceptible DA rats until postnatal day 9. By postnatal day 14 and 18, the reverse was observed, and susceptible SD and DA rats showed a significant increase in the expression of VEGF, EPO, VEGFR2, ANG2, IGF-1, COX2 and PEDF compared to resistant F344 rats [30]. Up to postnatal day 9, the drive towards angiogenesis, as reflected by the VEGF/PEDF ratio, was greater in the resistant F344 rats compared to susceptible DA rats. At the cessation of cyclic hyperoxia at day 14, the VEGF/PEDF ratio became higher in the susceptible DA rats compared to the resistant F344 rats. van Wijngaarden and colleagues proposed that susceptibility to OIR was associated with changes in gene expression which occurred early in OIR in response to cyclic hyperoxia exposure, rather than changes which occurred later in response to sustained relative hypoxia [30].

Other than the work presented in Chapter 4 from our laboratory [282], one other study has examined gene expression changes that occur in early and late OIR [283]. A comprehensive microarray study was undertaken by Recchia and colleagues to investigate genes and pathways which were involved in retinal neovascularisation in two rodent models of OIR at early and late time points within the *hypoxic* exposure period [283]. Changes in gene expression in response to cyclic hyperoxia exposure were determined relative to expression in room air-exposed rats.

SD rats were used in this model and exposed to cyclic hyperoxia for 14 days after birth followed by the relative hypoxia of room air up to postnatal day 20. Retinal RNA was collected for analysis at postnatal day 15 (early OIR in response to hypoxia) and at postnatal day 20 (late OIR in response to hypoxia). Similarly, mice were exposed to 5 days of sustained hyperoxia from postnatal day 5-12 after birth, and retinal RNA was collected at postnatal day 13 (early OIR in response to hypoxia) and at postnatal day 18 (late OIR in response to hypoxia). Statistically significant changes in the expression of EGLN3, BNIP3 and IGFBP3 were also identified by this study [283]. However, the context in which these changes were found was different to ours, in that whilst this study by Recchia and colleagues purported to investigate changes in gene expression which occur early and late in OIR, it did not take into account changes which occur in response to *hyperoxia* nor did it take into account strain-dependent differences in gene expression. It

does, however, support our findings that changes in the expression EGLN3, BNIP3 and IGFBP3 are likely to be involved in susceptibility to OIR.

The microarray screen performed by Recchia et al. found EGLN3 expression was upregulated by 2.5 fold in response to relative hypoxia in SD rats in early OIR (postnatal day 15) in response to relative hypoxia, and BNIP3 to be upregulated 2.3 fold in mice (postnatal day 13). At the later time points, SD rats showed further upregulation of EGLN3 expression in response to relative hypoxia at postnatal day 20 with a 3.0 fold change. At postnatal day 20, mice also showed an increased in EGLN3 expression with a 5.3 fold upregulation observed, and in addition to this, IGFBP3 was also found to be upregulated, showing a 5.8 fold increase at postnatal day 18 [283].

Statistically significant changes in the expression of these genes were also observed in our laboratory upon confirmation of the microarray screen. Changes in gene expression found in our study occurred early in the hyperoxia/relative hypoxia exposure period, in contrast to that of Recchia et al.

Strain-dependent differences in miRNA expression in susceptibility to OIR have not previously been reported. Changes in miRNA expression have been examined in the context of changes which occur in response to hyperoxia or relative hypoxia in OIR [136, 139]. Depending on the time point examined

and whether animals are being removed from hyperoxia or relative hypoxia, the miRNAs which are differentially expressed to a statistically significant extent can vary greatly and may explain why different miRNAs are identified from different studies to be important in susceptibility to OIR [136, 139]. As my results have shown, the expression of miRNAs varies not only between exposure to hyperoxia and relative hypoxia as one might expect, but also at different time points in the hyperoxic period.

Changes in miRNA expression occurring in response to sustained hyperoxia are important, as intraocular injection of certain miRNAs can act to repress hypoxia-induced retinal neovascularisation characteristic of OIR [136, 139]. However, changes that occur in response to exposure to relative hypoxia, as proposed early in this chapter, may also play a role in susceptibility to OIR. The model of OIR used by Shen and colleagues [136] and Bai et al. [139], consists of period of sustained hyperoxia, followed by a period of sustained relative hypoxia to induce the OIR phenotype. In contrast, the cyclic hyperoxia exposure period used in my studies cycles between periods of hyperoxia and relative hypoxia to induce the OIR phenotype and more closely mimics the supplemental oxygen therapy used in the neonatal intensive care unit [96]. Identifying changes in miRNA expression which occur in response to both hyperoxia and relative hypoxia is likely to be of importance.

In addition to these oxygen-dependent changes in miRNA expression, it may be that multiple miRNAs are involved in regulating the changes in gene expression that contribute to differential susceptibility to OIR. These miRNAs may be acting in concert, or independently to target various arms of the HIF-α oxygen sensing pathway to influence susceptibility to OIR.

The timing at which changes in gene and miRNA expression occur has implications for the time point at which therapeutic interventions are most beneficial. VEGF therapy has been shown in animal models of ROP to be beneficial during phase I of the disease when normal retinal vascularisation is attenuated in response to high oxygen and there is no stimulus for angiogenesis [9, 71, 284, 285]. Introduction of VEGF therapy during phase II of the disease, where the angiogenic factors are already liberated in the hypoxic retina, tends to have a detrimental effect on disease progression [72, 284, 285]. As a result of these studies, anti-VEGF therapy was proposed to be most beneficial when introduced during phase II of the disease.

Off-label use of anti-VEGF therapy in the treatment of ROP has been reviewed in detail by Micieli et al., who concluded that there were still many uncertainties surrounding the correct dose, timing and frequency of treatments required to be beneficial, and that possible side effects and long term outcomes had yet to be determined [286]. Results from the BEAT-ROP clinical trial involving intravitreal injection of the anti-VEGF antibody

bevacizumab to treat ROP have done little to resolve these outstanding issues [287]. The benefits, safety and efficacy of anti-VEGF for the treatment of ROP is yet to be determined and these issues have been reviewed elsewhere [288-291].

6.4 CONCLUDING REMARKS

Oxygen-induced retinopathy as a robust animal model of ROP has provided a wealth of evidence that has been used to investigate the mechanisms by which OIR is inherited, and how changes in gene and miRNA expression may underlie disease pathogenesis. In this thesis, I have identified several genes and miRNAs which could in the future be used as biomarkers for disease pathogenesis, or as potential therapeutic targets for intervention in ROP. However, further studies are required to elucidate the time point at which any therapy which aims to restore or diminish the expression of relevant genes and their protein products is most beneficial, so as not to exacerbate progression of the disease. OIR as a model would be of great value in testing these biological agents, and the findings of these studies may have great implications for human ROP and could improve visual outcomes in premature infants.