CHAPTER 1: INTRODUCTION

1.1. OVERVIEW

This thesis is centred on the role of heritable influences in susceptibility to retinopathy of prematurity (ROP). This introductory chapter will give a brief account of retinal vascular development and the pathogenesis of neovascular disorders of the retina, in particular, ROP. There will be a focus on the role of angiogenic factors in physiological and pathological angiogenesis and how microRNAs may act to regulate genes important in the development of ROP. A majority of the literature cited in this chapter precedes the start of my PhD candidature in February 2008. The aims of the work described in this thesis will be outlined at the end of the chapter.

1.2 RETINAL ANATOMY

The eye is a complex organ whose function is to focus light on the retina [1]. The retina lines the posterior segment of the eye and is terminated anteriorly by the transition zone between the ora serrata and the ciliary body (Figure 1.1) [1].

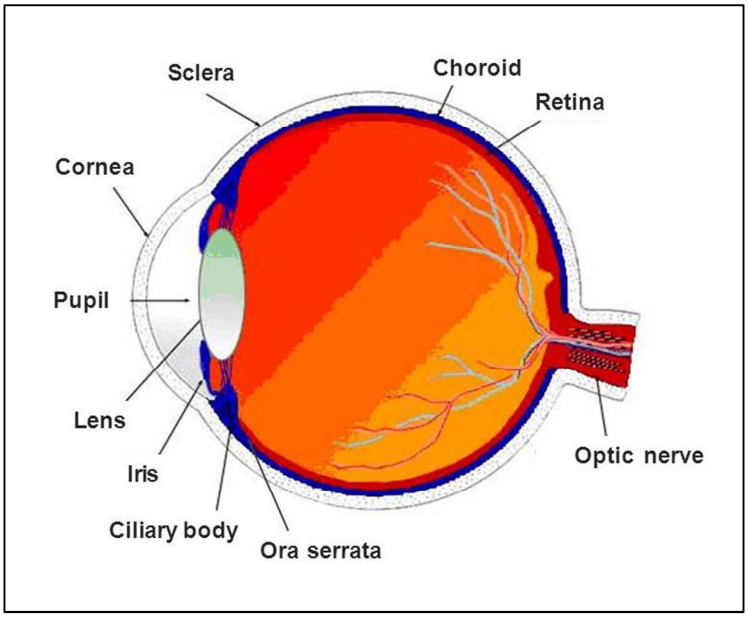

Figure 1.1 A schematic diagram showing the retina lining the posterior segment of the eye. Adapted from Kolb et al. [2]

The retina consists of the neural (or sensory) retina and the retinal pigment epithelium (RPE), each having a different function. The neural retina is made up of three general layers. From posterior to anterior, these are the photoreceptor cell layer, the bipolar neurons and the ganglion cell layer. Light is passed between each layer of the neural retina and converted into electrical impulses which are then assembled into vision by the central nervous system. The neural retina plays a role in maintaining high visual acuity, colour perception, and preventing retinal blood vessels from penetrating the vitreous. The RPE acts to transport nutrients and metabolites from the choroid into the retina for optimal photoreceptor function. The melanin-rich cells of the RPE also aid in the absorption of scattered light, to increase visual acuity [1, 3].

1.3 BLOOD SUPPLY TO THE RETINA

The retina consists of three separate vascular beds: the hyaloid, retinal and choroidal circulations. The hyaloid circulation is active during early embryonic development, when both the hyaloid and choroidal circulations support the metabolic activities of the retina. The hyaloid vascular system then regresses as the retinal vascular system begins to develop [4-6].

Vascularisation of the retina occurs during late gestation and supplies the inner half of the retina. The outer half of the retina is composed of avascular neural tissue which has a high metabolic demand and is therefore supplied by the choroidal circulation due to its high flow rate [5, 7]. Whilst the retinal and choroidal circulations act independently of each other, together, they provide a continuous supply of oxygen and nutrients to support the retina [8].

1.4 RETINAL VASCULAR DEVELOPMENT

Retinal vascular development in humans is almost complete at full term. However, many animals such as rats and mice have a rudimentary retinal vasculature and a majority of the development occurs in the postnatal period [9].

Retinal vascular development involves two phases: vasculogenesis followed by angiogenesis [9, 10]. Vasculogenesis is the *de novo* formation of blood vessels from endothelial precursor cells. It involves the migration, proliferation and differentiation of these angioblasts into endothelial cells (EC) to an area not previously vascularised to form a primitive blood vessel network [11]. Angiogenesis refers to the formation of new blood vessels from the pre-existing vasculature, vessels that are subsequently pruned and remodelled with the aid of mural cells such as smooth muscle cells and pericytes to form a mature endothelial network [11].

Retinal vascular development in humans commences in the middle of the second trimester and takes place over a period of approximately 20 weeks [12]. Vascularisation of the retina occurs in two phases. In the first phase, a basic vascular network is laid down in the central retina by vasculogenesis [13, 14]. In the second phase, angiogenesis drives vascularisation of the inner retina causing it to become more densely vascularised, resulting in regional tissue hypoxia in the outer retina. This regional hypoxia triggers another wave of angiogenesis and the blood vessels continue to develop until they reach the peripheral retina and vascularisation is complete [10, 14].

1.4.a Growth factors involved in retinal vascular development

A number of growth factors play integral roles in the development of the retinal vasculature. Vascular endothelial growth factor (VEGF) is a potent

angiogenic factor that is critical for both physiological and pathological angiogenesis. The role of VEGF in retinal development has been well characterised and will be discussed shortly [5].

Other growth factors involved in retinal vascular development include platelet derived growth factor-β (PDGF-β), transforming growth factor-β (TGF-β) and the angiopoietins. PDGF-β is expressed by endothelial cells undergoing angiogenic sprouting and is required for the proliferation and recruitment of pericytes, which have been shown to be integral in promoting vessel stability, to newly formed blood vessels [15-17]. TGF-β is a cytokine involved in cellular proliferation and differentiation and is expressed by endothelial cells, smooth muscle cells and pericytes. TGF-β is thought to recruit these mural cells during embryonic development to aid in vessel stabilisation [17].

Angiopoietin1 (ANG1) and angiopoietin2 (ANG2) are the ligands of the Tie2 receptor tyrosine kinase and play differing roles in angiogenesis [18]. Ang1 activates its receptor to stabilise blood vessel growth and appears to play a role in maintaining the quiescence and stability of mature vessels [17, 19]. In contrast, induction of ANG2 by endothelial cells acts in an antagonistic fashion to destabilise blood vessel formation, which is required for both physiological and pathological angiogenesis [19].

Other growth factors have also been implicated in both physiological and aberrant angiogenesis of the retina, including insulin-like growth factor-1 (IGF-1) and pigment epithelium derived growth factor (PEDF).

IGF-1 is a growth factor important in foetal growth and development [20]. It is thought to be involved in the pathogenesis of a number of diseases affecting the retina including diabetic retinopathy and retinopathy of prematurity [21, 22]. IGF-1 is expressed by pericytes, smooth muscle cells and endothelial cells and in association with VEGF has been shown to promote angiogenesis [23, 24].

PEDF is an angiogenic growth factor with neurotrophic and angiogenic effects that is able to reduce the migration and VEGF-mediated proliferation of endothelial cells [25-27]. In animal models, PEDF has also been shown to reduce retinal neovascularisation by altering the VEGF/PEDF ratio, considered to be a surrogate marker of the angiogenic balance within the retina [25, 28, 29]. An increase in VEGF coupled with a decrease in PEDF causes the VEGF/PEDF ratio to increase, favouring angiogenesis. An increase in the VEGF/PEDF ratio has been observed amongst strains of rats shown to be susceptible to oxygen-induced retinopathy (OIR), an animal model of ROP [30].

1.4.a.1 Vascular endothelial growth factor

Retinal vascular development is mediated by the hypoxia-induced spatial and temporal expression of VEGF by the astrocytes and Müller cells of the neural retina, in response to an increase in the metabolic demand during development [14, 31]. An increase in VEGF expression is often observed preceding the vascular front until the blood vessels reach the peripheral retina and vascularisation is complete [12].

VEGF also plays a key role in the growth of aberrant blood vessels in the retina in response to hypoxia [8, 32]. Normally, a delicate balance exists between pro- and anti-angiogenic factors to maintain vessel quiescence [33]. However, when there is an exposure to a stimulant such as hypoxia, an "angiogenic switch" may be triggered in favour of pro-angiogenic factors, resulting in angiogenesis [33]. The expression of VEGF is upregulated by the transcription factor hypoxia-inducible factor-α (HIF-α) [34, 35].

1.4.b Hypoxia-inducible factor-1α

HIF-1 is a heterodimer consisting of HIF-1α subunit and the constitutively expressed HIF-1β subunit [36]. HIF-1 activity is controlled by regulating levels of the rate-limiting HIF-1α subunit and preventing its association with the HIF-1β subunit [37]. Control of HIF-1α levels is also regulated by preventing its interaction with its essential transcriptional co-activator complex CBP/p300 [38-43]. HIF-2α or HIF-like factor (HLF) is closely related

to HIF-1α and shares 48% sequence homology with the transcription factor. It has also been shown to heterodimerise with the HIF-1β subunit, bind hypoxia response elements in target genes and to be selectively expressed in endothelial cells [44, 45]. The mechanisms by which HIF-α levels are regulated in normoxia and hypoxia will be discussed later in this chapter.

1.4.b.1 HIF-1α and HIF-2α in the retina

HIF-1α has been implicated in retinal vascular development and a number of oxygen-dependent diseases including von Hippel-Lindau disease, diabetic retinopathy and retinopathy of prematurity [8, 46]. Increased HIF-1α levels have been observed in rats with oxygen-induced retinopathy (OIR) [47]. Induction of OIR in neonatal rats caused an upregulation of HIF-1α which also correlated with increased expression of VEGF mRNA [47].

HIF-2α is also crucial for normal retinal vascular development, as shown by studies in HIF-2α knockdown mice with OIR, where lack of HIF-2α resulted in degeneration of the neural retina due to poor retinal vascularisation [48]. Another study showed that mice with OIR and which had only one functional HIF-2α gene had a reduced capacity to respond to changes in oxygen levels, resulting in decreased retinal neovascularisation [49]. The authors attributed this finding in part to an absence of the pro-angiogenesis factors required to promote an angiogenic response.

1.4.b.2 HIF-α degradation in normoxia

During normoxia there is constant, rapid turnover of HIF-1α [50]. The tumour suppressor protein von Hippel-Lindau (VHL) is a component of the E3 protein-ubiquitin ligase system [51]. Binding of VHL to HIF-α is mediated by the hydroxylation of specific conserved proline residues within the central oxygen-dependent degradation domain of HIF-α, targeting it for ubiquitinylation and proteasomal degradation [52, 53].

Prolyl hydroxylases (PHDs) are a class of 2-oxoglutarate-dependent enzymes which are dependent on oxygen for their function [53]. There are 3 prolyl hydroxylases: PHD1, PHD2 and PHD3, all of which have been shown to have different functions in regulating HIF-1α and HIF-2α levels [54]. Prolyl hydroxylation is the main mechanism by which HIF-α levels are regulated [35]. Another class of 2-oxoglutarate-dependent enzyme is the asparaginyl hydroxylase, factor-inhibiting HIF-1 (FIH-1), which regulates HIF-α transcriptional activity by hydroxylation of an asparagine residue in the c-terminal activation domain of HIF-α, to prevent the recruitment of the transcriptional co-activator complex CBP/p300 [38-43].

1.4.b.3 Transcriptional activity of HIF in hypoxia

Hypoxic induction of HIF-1α and HIF-2α requires the inhibition of the oxygen-dependent prolyl and asparaginyl hydroxylases to prevent the

proteasomal degradation of HIF and to allow assembly of the CBP/p300 transcriptional co-activator complex, respectively (Figure 1.2) [38-43].

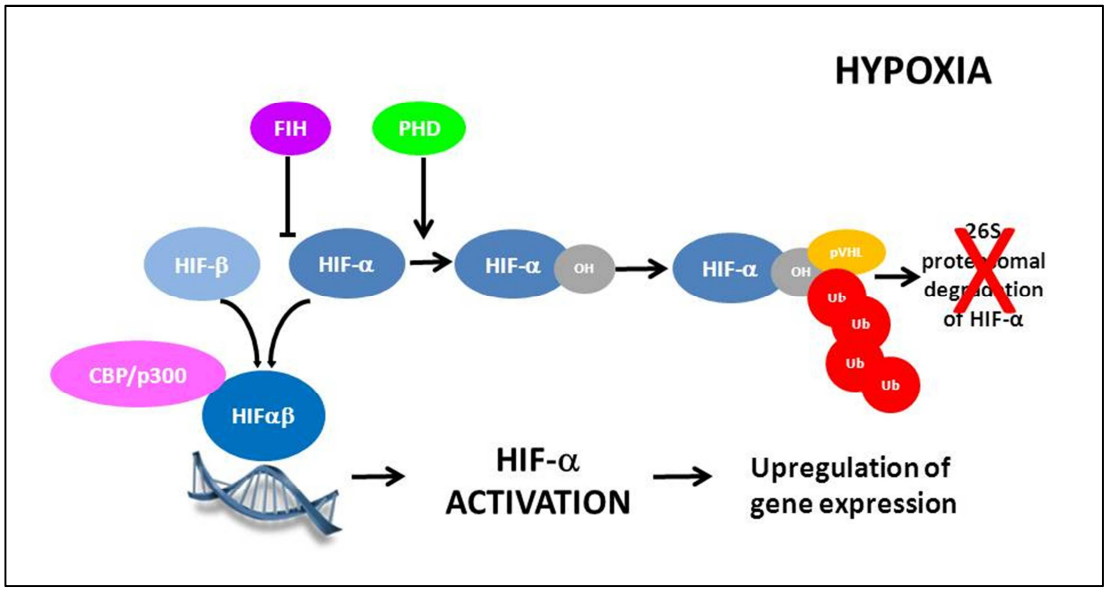

Figure 1.2 Simplified schematic representation of the activation of HIF-α in hypoxic conditions. In hypoxic conditions, the oxygen-dependent hydroxylases FIH and PHD have limited activity, allowing HIF-α to accumulate. HIF-α can then to bind to the constitutively expressed HIF-β subunit and promote the recruitment of the co-activators CBP/p300 required for HIF-α activation. HIF-α can then bind to the hypoxia response element in the promoter of target genes to induce gene expression. Ub = Ubiquitin; PHD = Prolyl hydroxylase domain protein; FIH = Factor inhibiting HIF (Asparaginyl hydroxylase domain protein); CBP/p300 = transcriptional co-activator proteins; pVHL = von Hippel Lindau protein.

Hypoxic conditions deprive these enzymes of oxygen, therefore limiting their function, allowing the HIF-α subunits to accumulate and subsequently translocate to the nucleus where they can bind to HIF-1β and form the activated HIF complex [37]. The activated transcription factor can then bind the hypoxia response element (HRE) in the promoter of specific genes

including VEGF [35, 37]. The ability of activated HIF to bind to the HRE of genes involved in the angiogenic response to hypoxia is critical, as deletion of the HRE in the promoter of the VEGF gene has been shown to result in reduced neovascularisation in mouse models of choroidal and retinal neovascularisation [55].

1.5 RETINOPATHY OF PREMATURITY

Retinopathy of prematurity (ROP) is a potentially blinding condition which affects infants born prematurely who undergo supplemental inspired oxygen therapy. ROP was first described in the 1940s by Terry as retrolental fibroplasia in extremely premature infants [56]. A decade later, ROP was attributed to the use of uncontrolled supplemental oxygen therapy which was used as a means of overcoming the breathing difficulties experienced by premature infants [57, 58]. Studies shortly thereafter confirmed that high levels of oxygen resulted in vaso-obliteration of retinal blood vessels [59, 60]. Limiting the use of supplemental oxygen therapy reduced the incidence of ROP; however, it was associated with increased morbidity [61, 62]. Despite advances in neonatology, ROP still remains a leading cause of morbidity in preterm infants [63-69].

Premature infants are born with an incompletely developed retina which is characterised by an avascular peripheral zone [70]. As retinal vascular

development is incomplete at this stage, the retina is particularly sensitive to changes in oxygen levels, and the aberrant retinal neovascularisation triggered by the use of supplemental oxygen therapy may result in progression to ROP [10, 14, 70].

Retinopathy of prematurity is a multifactorial disease that is biphasic in its progression. The first phase of ROP is characterised by a delay in retinal vascular development as the VEGF-driven retinal vascular development that normally occurs *in utero* ceases upon exposure to the relative hyperoxia of the postnatal environment [9, 71]. The second phase of ROP is initiated by hypoxia and results in the liberation of various growth factors which promote the proliferation of aberrant blood vessel at the border of the vascularised and avascularised retina [72]. These vessels can form a fibrovascular scar that can ultimately result in retinal detachment and blindness [70].

Technological advances over the last two decades have resulted in an increased survival of extremely premature infants with the potential to develop ROP. In response, an International Classification of Retinopathy of Prematurity (ICROP) was developed to characterise the stages during the development of ROP [73]. Since its introduction, it has been revised twice, to more accurately define the characteristics of the disease, and to incorporate

recent observations, concepts and clinical tools which better classify the stages of ROP and aid in disease management [74].

Treating ROP represents a challenge for ophthalmologists as a proportion of infants with threshold disease will develop more aggressive ROP, while others who show the same clinical manifestations may experience spontaneous regression of the disease [75]. The mechanism by which this occurs is currently unknown. Treatment of the disease with retinal ablation is associated with its own co-morbidities. As a result, all infants who reach a particular stage of the disease are subjected to retinal ablation to destroy avascular areas of the retina, reduce the oxygen demand of the tissue, and to eliminate the growth of aberrant blood vessels [76, 77]. Identifying risk factors and heritable traits which contribute to disease susceptibility would enable infants not at risk of developing severe disease to be spared unnecessary intervention.

1.5.a Clinical and genetic risk factors of ROP

Low gestational age, low birth weight and the use of supplemental oxygen therapy are all well documented clinical risk factors of ROP [78]. There is now increasing evidence emerging from clinical studies suggesting a heritable component to the disease, supporting the role of genetic factors in susceptibility of infants to ROP.

Ethnicity plays an uncertain role in susceptibility to ROP. Some studies of infants with ROP have shown that ocular pigmentation is associated with decreased risk of severe ROP, whereas other studies have shown the opposite [79-81]. The role of ocular pigmentation in susceptibility to ROP remains unclear, and differences in cultural and economic approaches in neonatal intensive care world-wide have made it increasingly difficult to identify causative changes that result in differential susceptibility to ROP [69].

Various polymorphisms and mutations in genes involved in the retinopathies including ROP have been identified. Polymorphisms in the VEGF gene have been associated with increased risk and severity of disease; however there is also evidence to the contrary [82-84]. Random forest analysis of selected published genetic variants of genes associated with ROP including VEGF, IGF-1 receptor and angiopoietin2 has suggested that there is negligible contribution of these polymorphisms to risk and severity of ROP [85]. Other studies by Cooke and colleagues found that the VEGF – 634 G allele was associated with increased risk for disease progression, however the reverse was found by Vannay et al., where the VEGF – 634 G>C mutation was found to be more prevalent in infants with more severe disease than those with less severe disease [82, 83]. In contrast, a study by Shastry et al., found no association between the VEGF – 634 G>C mutation and progression to more severe disease [84]. However, it was noted by the

authors that a large scale study of the mutation in the VEGF gene in ethnically diverse preterm infants was required before this method could be used to identify at high-risk infants in the clinic in the future. The use of gene polymorphisms as predictors of disease progression at birth remains an attractive concept despite the conflicting evidence.

Mutations in genes associated with a group of phenotypically similar retinopathies including ROP, Norrie disease and familial exudative vitreoretinopathy (FEVR) have also been identified. In particular, mutations in the Norrie disease gene have been associated with infants with ROP, however the association is unclear, with some reports suggesting that mutations in the gene may be responsible for some cases of advanced ROP and while others showed no increased risk of progression to severe disease [86-92]. As shown by these studies, the role of polymorphisms and mutations in susceptibility to ROP remains uncertain.

Whilst none of the polymorphisms and mutations which have been identified has been found to be causative of ROP, they still may provide some insight into the genetic basis of ROP. The identification of genetic influences could contribute to the understanding of the condition and provide a means for stratifying infants at risk of developing severe disease. Animal models of ROP provide a robust model for studying these influences.

1.5.b Oxygen-induced retinopathy as an animal model of ROP

Oxygen-induced retinopathy (OIR) is an animal model which mimics the pathophysiology of ROP to some degree. A neonatal rat is born with an immature retina, comparable to that of a 24-26 week old human embryo, and as most of the retinal vascularisation in rats and mice occurs *ex utero* this allows vascular development of the retina to be readily studied [9, 93]. Other animals have also been used in models of OIR, as reviewed by Madan and Penn [94].

Several different rodent models of OIR are commonly used. Regardless of the method used, the aim of the exposure of neonatal rats and mice to varying levels of oxygen is to recreate the biphasic progression and phenotype of the disease seen in humans [23]. Exposure to hyperoxia effectively mimics the first phases of the disease in which there is a delay in retinal vascular development and in the development of the neural retina. The second phase is mimicked by exposure of the neonates to the relative hypoxia of room air, which promotes the growth of aberrant blood vessels [95]. Other models of OIR utilise combinations of exposure to cyclic hyperoxia and relative hypoxia, followed by a prolonged period of room air exposure to more accurately reflect the supplemental inspired oxygen therapy to which premature infants are exposed in the neonatal intensive care unit after birth [96].

There are, however, limitations to using OIR as a model of ROP since the retinal vasculature of small rodents is often quite rudimentary, and does not reflect the complexity of the human retinal vasculature [9]. Therefore any observations made from studies of OIR must be treated with caution when translating them into clinical practice for infants affected by the disease. Nonetheless, OIR represents a robust model for studying the pathophysiology and genetics of ROP and findings from studies in animal models of retinal disease are relevant to human disease, as demonstrated by Francis et al., who studied age-related macular degeneration in rhesus monkeys and found that the same polymorphisms in genes associated with age-related macular degeneration in humans were also responsible for increased susceptibility to the disease in monkeys [97].

1.5.b.1 Strain-related differences in susceptibility to OIR in the rat

Studies in different outbred and inbred strains of rat have shown varying susceptibility to OIR. Some studies have shown that susceptibility to OIR segregates with ocular pigmentation [96, 98]. However, studies of infants with ROP have shown the reverse, where reduced ocular pigmentation is associated with greater disease severity, suggesting that other factors may be involved in disease progression [79]. Differential susceptibility to OIR in different inbred rat strains has also been associated with differential expression of pro- and anti-angiogenic factors [28, 30, 98-100]. Strain-dependent differences in differential susceptibility to OIR are a major focus

of the work presented in this thesis and will be discussed at length in the following chapters.

1.6 MICRORNAS

MicroRNAs (miRNAs) are short non-coding sequences of RNA approximately 21 nucleotides in length that regulate gene expression at a post-transcriptional level [101]. Binding of microRNAs to their target mRNA results in either mRNA degradation or translational repression, depending on the complementarity between the miRNA and the mRNA target [102-105]. A single microRNA has the potential to target hundreds of genes, and the pathways regulated by microRNAs may be involved in the pathogenesis of various diseases including ROP [106].

1.6.a Discovery of microRNAs

MicroRNAs were first discovered in the nematode *Caenorhabditis elegans* in 1993 when Lee and colleagues identified two small RNA transcripts of approximately 22 and 61 nucleotides in length that were complementary to the 3' untranslated region of lin-14 mRNA and did not code for protein [101]. These transcripts, lin-4, were found to be essential for controlling the normal timing of developmental events in *C. elegans* by negatively regulating levels of LIN-14 protein. A second miRNA, let-7 was not discovered until several years later and was shown to target a number of different genes [107]. Let-7

was also found to be important in regulating developmental timing in *C. elegans* and was later shown to be conserved among different species including arthropods and vertebrates [108]. A majority of cloned miRNAs have been shown to be conserved in closely related mammals [109].

1.6.b Biogenesis of microRNAs

The biogenesis of miRNAs involves miRNA gene transcription and maturation via a number of cleavage steps before the mature miRNA can be incorporated into an effector complex to act on its target mRNA [110]. Most miRNA genes are located in the non-coding regions of the genome, however a number of miRNA genes have also been found within the introns of protein coding and non-coding RNAs [109, 111, 112]. Clustered miRNAs are transcribed to form a single polycistronic transcript which is subsequently cleaved into individual miRNAs during maturation [113].

MicroRNA gene transcription is mediated by RNA polymerase II (RNA Pol II), through which genes are transcribed into a primary precursor transcript (pri-miRNA) of up to several hundred kilobases in length that contains local hairpin structures (Figure 1.3) [114-116]. The pri-miRNAs are then cleaved into shorter precursor miRNAs (pre-miRNA) of ~ 70 nucleotides in length in a stem loop structure [113, 116, 117]. This initial step in miRNA maturation occurs in the nucleus under the direction of the RNase III enzyme Drosha [117].

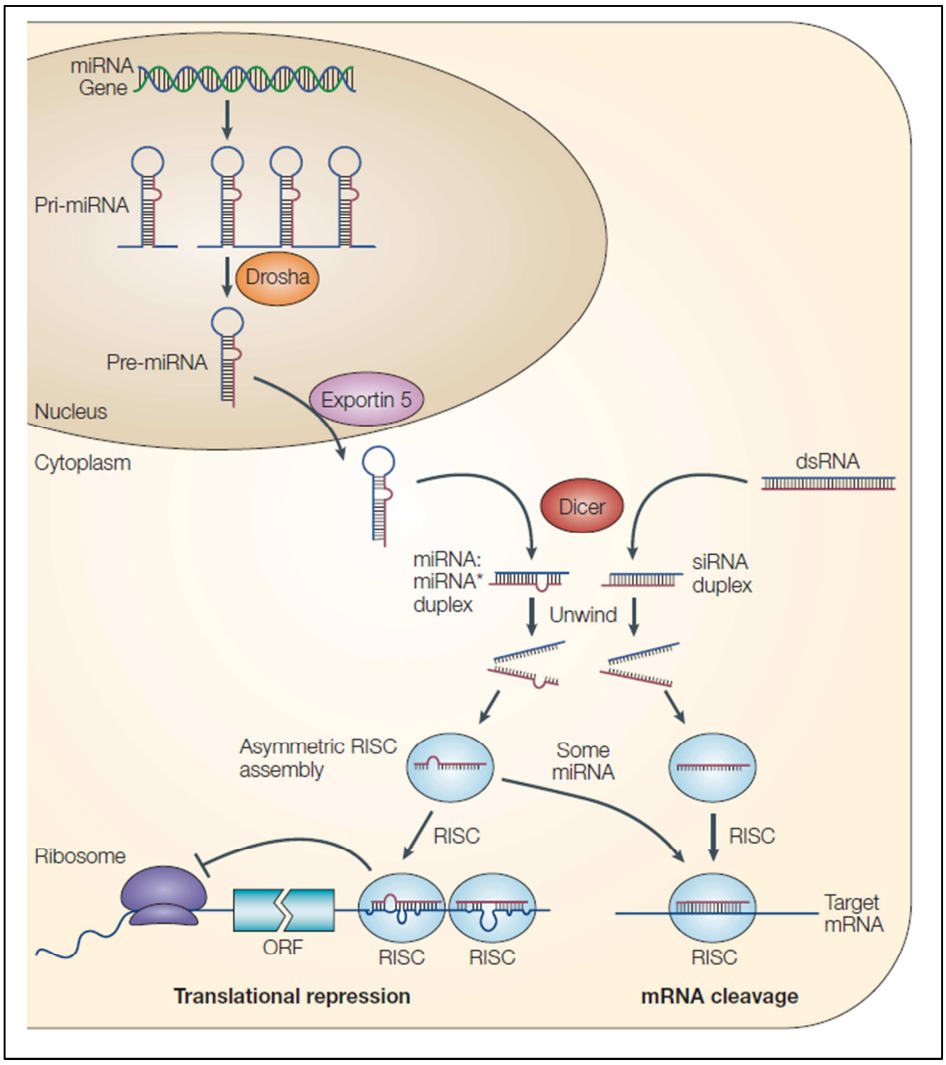

Figure 1.3 Schematic representation of the biogenesis of miRNAs and mechanisms of post-transcriptional regulation of gene expression as described by He et al., [118]. miRNAs are transcribed from miRNA genes by RNA Pol II to form a primary miRNA transcript. This transcript is cleaved by the RNase III enzyme Drosha into a precursor miRNA that is then exported out of the nucleus by Exportin5. The precursor miRNA is further cleaved by the RNase III enzyme Dicer to form a mature miRNA duplex. The double stranded miRNA then undergoes strand selection whereby one strand is degraded, while the other strand is incorporated into the RNA-induced silencing complex or RISC, of which the Argonaute proteins are core components. Once bound to the RISC, the miRNA can then go on to bind to the 3'UTR sequence of target mRNAs. Depending on the degree of complementarity to the mRNA target and the miRNA within the RISC, the target is either cleaved when there is a perfect match, or translational repression occurs when there is an imperfect match. In both cases, transcript levels are reduced.

Precursor miRNAs are then transported out of the nucleus into the cytoplasm for further processing [119, 120]. Once in the cytoplasm, the pre-miRNA is cleaved by the RNase III enzyme Dicer to form a ~22 nucleotide double stranded miRNA duplex [121, 122]. The miRNA duplex then undergoes strand selection, whereby one strand is incorporated into the RNA-induced silencing complex (RISC) and the other strand is degraded [116].

1.6.c Formation of the RISC and mechanism of microRNA action

The RISC acts as the effector complex for the degradation of complementary single stranded RNA targets [123]. The mature miRNA strand binds to the RISC in association with Dicer, TAR RNA-binding protein (TRBP) and one of the Argonaute proteins AGO1-4, to recognise the miRNA target through anti-sense base-pairing to sequences in the 3' untranslated region of the mRNA target [101, 102, 124, 125]. Whilst the 4 members of the Argonaute family are known to be involved in miRNA repression, only AGO2 is known to play a role in guiding the mRNA target cleavage [102].

Post-transcriptional regulation of gene expression occurs either by the cleavage of the mRNA target or by translational repression, depending on the complementarity of the miRNA seed sequence and the mRNA target. Cleavage of the target occurs when there is perfect complementarity between the miRNA and the mRNA target [102, 103]. An incomplete match may

result in translational repression of the protein and/or destabilisation of the mRNA via deadenylation and decapping of the mRNA target, resulting in degradation [104, 105, 126-128]. In mammals, miRNAs have been shown to predominantly mediate their regulatory effects through destabilisation of their mRNA targets [126].

1.6.d MicroRNA expression in the eye

miRNAs were first discovered in the eye while Hipfner and colleagues were studying the role of the gene bantam in regulating *Drosophila* growth [129]. They found that over-expression of the bantam gene product resulted in an overgrowth of wing and eye tissue. Bantam was later shown to encode a miRNA that promotes cellular proliferation and prevents apoptosis during eye development in *Drosophila* [130]. Since then, a number of different research groups have identified miRNAs in the eyes of various animals including zebrafish, newt and rat [131-133], with a focus on those microRNAs found in mouse and human eyes. Ocular expression of selected miRNAs is shown in Table 1.1. miRNAs expressed in the eye display tissue specificity and spatio-temporal expression during embryonic and postnatal development, suggesting that miRNAs may play a role in the regulation of ocular development, differentiation and function [134, 135]. Few miRNAs found in the eye have yet to be fully characterised and a large number of targets have not been identified or experimentally verified.

Table 1.1 Selected miRNAs expressed in the eye. A list of miRNAs expressed in the eye of various species is provided.

miRNA	Tissue	Species	Expression
miR-31	Retina, cornea	Mouse	Expressed in the cornea [135] Downregulated in OIR [136]
miR-96	Retina	Mouse	Expressed as a cluster with miR-181 and miR-183 in mice, an equivalent cluster is found in humans [137] Mutation in miR-96 gene associated with progressive hearing loss in humans, although the effect on vision is unknown [138]
miR-106a	Retina	Mouse	Upregulated in OIR [136]
miR-124	Retina	Human, rat	Expressed in the retina [133]
miR-124a	Eye, retina	Newt, mouse	Expressed during embryonic development in the mouse [134] Expressed in the newt eye [131]
miR-126	Retina	Mouse	Downregulated in OIR [139]
miR-146	Retina	Mouse	Upregulated in OIR [136]
miR-150	Retina	Mouse	Downregulated in OIR [136]
miR-181	Retina	Mouse	Upregulated in OIR [136]
miR-181 a, b	Eye, retina, lens	Mouse, newt	Expressed in the newt eye [131] Expressed in the mouse retina [135] Expressed during embryonic development in the mouse [134]

Table 1.1 (Continued) Selected miRNAs expressed in the eye.

miRNA	Tissue	Species	Expression
miR-182	Retina, neural retina	Mouse	Expressed in the retina [135, 137] Expressed during embryonic development [134]
miR-183	Retina	Mouse	Expressed in the retina [135, 137]
miR-184	Cornea, lens and retina	Mouse	Expressed in the retina, cornea and lens [135, 137] Downregulated in OIR [136]
miR-199a	Retina	Mouse	Upregulated in OIR [136]
miR-204	Cornea	Mouse	Expressed in the cornea[135] Expressed during embryonic development [134]
miR-210	Retina	Mouse	Expressed in the mouse retina and during embryonic development [137]
miR-214	Retina	Mouse	Upregulated in OIR [136]
miR-424	Retina	Mouse	Upregulated in OIR [136]
miR-451	Retina	Mouse	Upregulated in OIR [136]

1.6.e MicroRNAs and hypoxia

Hypoxia plays a major role in the pathogenesis of many diseases including tumour growth and in several retinopathies such as retinopathy of prematurity. The miRNA cluster miR-17-92 has been associated with a number of cancers and contains the following 7 miRNAs: miR-17-5p, miR-17-3p miR-18a, miR- 19a, miR-20a, miR-19b and miR-92-1 [140].The miR-17-92 cluster has been shown to target HIF-1α which in turn is known to target over 70 genes including VEGF [39, 141-143]. Two members of the miR-17-92 cluster, miR-17-5p and miR-20a have also been predicted to target VEGF [144].

A microRNA signature of hypoxia has been identified using cancer cell lines [145]. Several miRNAs have been shown to be increased in response to hypoxia including miR-23a, miR-107, miR-181a, and miR-210. Interestingly, miR-23a, miR-107 and miR-181a are also expressed in the retina and by endothelial cells [133, 134, 146].

HIF-1α has been shown to induce miR-210 expression in response to hypoxia [145, 147]. Blockade of miR-210 expression under hypoxic conditions prevents the formation of capillary-like structures and VEGF-induced endothelial cell migration. Inhibition of miR-210 expression in normoxia and hypoxia appears to inhibit cell growth and promote apoptosis, suggesting

miR-210 plays a role in endothelial cell survival, migration and tube formation [147].

1.6.f MicroRNA and angiogenesis

MicroRNAs have been shown to play a role in vascular disease, inflammation and angiogenesis, including tumour angiogenesis and metastasis, through miRNA-regulated epithelial mesenchymal transition [148-150]. The importance of miRNA expression and function in angiogenesis has been supported by recent studies.

The knock-down of Dicer expression *in vitro* has been shown to reduce endothelial cell proliferation, migration and cord formation in response to the pro-angiogenic factors, basic fibroblast growth factor (bFGF) and VEGF [146, 151]. Another study confirmed that endothelial cell proliferation and cord formation were reduced by knockdown of Dicer, however VEGF-induced endothelial cell migration remained unaffected [146]. *In vivo* studies of Dicer knock-down have shown reduced postnatal angiogenesis in response to VEGF in a number of models including tumour angiogenesis, hindlimb ischaemia and wound healing [152]. Many miRNAs have been found to be expressed by endothelial cell including miR-16, miR-21, miR-23a, miR-29, miR-100, miR-107, miR-126, miR-181a, miR-221 and miR-222 [146, 148]. miR-29a has been also associated with complications of diabetes,

however whether or not it plays a role in ROP or diabetic retinopathy, another proliferative disease of the retina, remains to be seen [153, 154].

1.6.f.1 Pro-angiogenic miRNAs

The miR-17-92 cluster is considered to be pro-angiogenic due to its ability to augment tumour angiogenesis when activated by the proto-oncogene myc [155]. Myc-induced miR-17-92 expression downregulates the expression of the anti-angiogenic protein thrombospondin-1, independent of VEGF levels, resulting in better vascularised tumours. Conditioned medium from myc over-expressing cells has also been shown to promote angiogenesis in a model of corneal neovascularisation [156].

1.6.f.2 Anti-angiogenic miRNAs

Studies of miR-221 and miR-222 in human umbilical vein endothelial cells (HUVECs) have shown that over-expression of these miRNAs is able to reduce tube formation and wound healing in response to the growth factor, stem cell factor (SCF) [157]. SCF has been shown to promote endothelial cell survival, migration and capillary tube formation through its receptor c-Kit [158]. miR-221 and miR-222 are thought to exert their effects by down-regulating c-Kit expression [157]. Other anti-angiogenic miRNAs have been identified in HUVECs, including miR-23a whose target includes the receptor for the pro-angiogenic bFGF [157]. miR-23a has also been shown to be expressed in rat and human retina [133].

1.6.g Role of microRNAs in a mouse model of oxygen-induced retinopathy

Shen and colleagues have confirmed the importance of miRNA in the regulation of angiogenesis in response to retinal hypoxia [136]. Using a mouse model of OIR, they identified several miRNAs to be differentially expressed in response to oxygen. Seven miRNAs were shown to be up-regulated and three down-regulated. The down-regulated miRNAs; miR-31, miR-150 and miR-184, were of particular interest as their predicted targets are known to be involved in angiogenesis. miR-31 and miR-184 have also been previously shown to be highly expressed in the lens, cornea and retina [135, 137]. Intraocular injection of precursor miRNAs pre-miR-31, pre-miR-150 and pre-miR-184 were all able to reduce retinal neovascularisation in the OIR model. Intraocular injection of pre-miR-31 and pre-miR-150, but not pre-miR-184, was able to reduce choroidal neovascularisation in a separate model of angiogenesis [136].

1.6.h MicroRNAs as therapeutic drugs

Targeting miRNA expression in order to regulate angiogenesis may be of therapeutic value in treating diseases such as tumour growth and the proliferative retinopathies and furthermore, there is great interest in using miRNA signatures as biomarkers for the diagnosis, prognosis and treatment of human diseases. The use of miRNAs as diagnostic or prognostic markers

for cancers such as breast, prostate and pancreas, as well as melanoma, is currently being investigated [159-162]. The use of mouse models to determine the exact role miRNAs play in cancer and how they may be used to treat the disease is reviewed in detail by Kasinki et al. [163]. The use of miRNAs as a therapeutic tool is not limited only to cancer, but can be applied to a wide variety of pathological processes, as reviewed in Gambari et al. [164].

There are several issues that warrant attention before miRNA-mediated therapy becomes a viable treatment modality. The mechanisms by which miRNAs regulate gene expression have not been fully elucidated and therefore possible off-target effects or negative outcomes have not been determined [165]. Off-target effects as a result of slightly different mRNA target sequences can also influence the silencing function of the small RNA [166].

Another issue, relating to the use of miRNAs as diagnostic or prognostic markers of disease, is the use of small RNA reference genes for the normalisation of miRNA expression levels. A study by Gee et al. found that some small RNAs that are typically used for the normalisation of miRNA expression levels are located within the introns of genes that are dysregulated in cancer [167]. As a result, these small RNAs were considered unsuitable for use as reference genes, as they did not accurately reflect the

associations between the expression of the miRNAs of interest and tumour pathology or disease outcome [167].

Despite these caveats, miRNAs are likely to be useful therapeutic agents in the future, with efforts being made to resolve these issues. Off-target effects have been limited by the use of locked nucleic acid-based technology [168]. For example, miRNAs antagonists were successfully used to inhibit miRNAs which belonged to the same family and shared the same seed sequence, resulting in the upregulation of the direct targets of these miRNAs [168]. Negligible off-target effects were reported by the authors. The careful selection of small RNA reference genes following confirmation of their stability in the relevant cancerous tissue, rather than normal tissue, could limit the bias introduced by the use of incorrect reference genes and help to overcome this particular issue [167].

Studies in primates have shown that the small RNA SPC3649, which is complementary to the 5′ end of miR-122, was able to reduce the hepatitis C viral load of infected animals [169, 170]. A phase I clinical trial was successfully completed, which determined that SPC3649 could be used safely in humans and that the drug was well tolerated in healthy individuals (ClinicalTrials.gov Identifier: NCT00979927). A phase II clinical trial is currently underway to test the drug (now marketed as Miravirsen), in

patients with chronic hepatitis C who have not undergone any other treatment (ClinicalTrials.gov Identifier: NCT01200420).

1.7 ROP: WHAT WE KNOW AND WHERE TO NOW

ROP is an iatrogenic, complex disease. Differences in the approaches to providing neonatal intensive care world-wide have made it difficult to identify the causative factors that result in differential susceptibility to ROP [69]. The increase in the number of premature infants surviving has meant that it is becoming increasingly important to identify infants at risk of developing severe disease from those infants in whom disease will regress. A genetic test would enable the resource-intensive ophthalmic screening of infants to be managed effectively, and to be provided to those infants who need it most. It would also spare infants in which disease will regress, from receiving unnecessary treatment, which in itself, is associated with morbidity. Determining the molecular and genetic basis of ROP will aid in stratification of infants based on disease risk, and may identify new therapeutic targets for prevention and treatment of disease.

1.8. HYPOTHESIS AND AIMS

Heritable factors are likely to underlie and contribute to differential susceptibility to retinopathy of prematurity and a rat model of ROP will be used to test this hypothesis. The specific aims of the work presented in this thesis are:

1. to determine the mode in which susceptibility to OIR is inherited in two strains of albino inbred rats who differ in their response to oxygen therapy;
2. to investigate if changes in retinal gene expression may contribute to differential susceptibility to OIR and to determine at what time point any changes are occurring; and
3. to determine if microRNAs play a role in regulating these changes in gene expression.

By investigating these three aspects of susceptibility to OIR, it is anticipated that greater insight will be gained, to enable better diagnosis and treatment of ROP.