HYPOXIA – REGULATED, GLIAL CELL-SPECIFIC GENE THERAPY TO TREAT RETINAL NEOVASCULARIZATION

by

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This dissertation was prepared under the direction of the candidate's dissertation advisor, Dr. Janet Blanks, Complex System and Brain Sciences, and has been approved by the members of his supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

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Diabetic retinopathy is an ischemic retinal neovascular disease causing vision loss among adults. The studies presented involve the design and testing of a gene therapy vector to inhibit retinal neovascularization, similar to that found in diabetic retinopathy. Gene therapy has proven to be an effective method to introduce therapeutic proteins to treat retinal diseases. Targeting a specific cell type and expression of therapeutic proteins according to the tissue microenvironment should have an advantage over traditional gene therapy by avoiding unwanted transgene expression. Hypoxia plays a significant role in the pathophysiology of many retinal ischemic diseases. Retinal Müller cells provide structural and functional support to retinal neurons, as well as playing a significant role in retinal neovascularization. Targeting Müller cells may be an effective strategy to prevent retinal neovascularization under pathological conditions.

We designed a vector using a hypoxia-responsive domain and a GFAP promoter to drive cell-specific transgene expression in Müller cells under conditions of hypoxic stress in the pathologic retina. Both our *in vitro* and *in vivo* studies confirmed the hypoxia inducibiltity and cell specificity of our regulated promoter. To test its therapeutic efficiency, endostatin was included as a therapeutic transgene in our construct. The vector was tested in the oxygen induced retinopathy (OIR) rodent model involving postnatal day 7 (P7) mouse pups exposed to 75% oxygen for 5 days, returned to room air for another 5 days and sacrificed at P17. This model exhibits central retinal vasobliteration and massive abnormal peripheral neovascularization by P17. P7 mice received an intravitreal injection of 1 ul of the therapeutic vector. At P17, the animals were sacrificed and the extent of neovascularization analyzed and transgene expression quantified.

The hypoxia regulated, glial specific vector successfully reduced abnormal neovascularization in the periphery by 93% and reduced the central vasobliterated area by 90%. A substantial amount of exogenous endostatin was produced in the retinas of P17 OIR mice. A significant increase in human endostatin protein and reduced vascular endothelial growth factor (VEGF) were identified by Western blot and ELISA, respectively. These findings suggest hypoxia-regulated, glial cell-specific scAAV mediated gene expression may be useful to prevent blindness found in devastating retinal diseases involving neovascularization.

DEDICATION

This thesis is dedicated to my loving mother and father. Without their blessings and sacrifices, I would have never achieved this highest degree.

HYPOXIA – REGULATED, GLIAL CELL-SPECIFIC GENE THERAPY TO TREAT RETINAL NEOVASCULARIZATION

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LIST OF ABBREVIATIONS

AAV:	Adeno Associated Virus
AMD:	Age Related Macular Degeneration
bFGF:	Basic Fibroblast Growth Factor
CNV:	Choroidal neovascularization
DR:	Diabetic Retinopathy
ELISA:	Enzyme Linked Immunosorbent Assay
Endo:	Endostatin
GFAP:	Glial Fibrillary Acidic Protein
GFP:	Green Florescent Protein
HEK:	Human embryonic kidney cell line
HRE:	Hypoxia Regulated Element
MMP:	Matrix metalloproteinase
NV:	Neovascularization
OIR:	Oxygen Induced Retinopathy
PBS:	Phosphate Buffered Saline
PEDF:	Pigment Epithelial Derived Factor
PFA:	Paraformaldehyde
REG:	Hypoxia Regulated GFAP
RPE:	Retinal Pigment Epithelium
scAAV :	Self complementary AAV
VEGF:	Vascular Endothelial Growth Factor

OVERVIEW OF THE DISSERTATION

This dissertation focuses on the possibility of using hypoxia-regulated, cell-specific gene therapy to treat neovascularization that occurs at the inner border of the retina and the vitreous. This type of neovascularization mimics the pathology found in the devastating disease, diabetic retinopathy. Several successful clinical trials already exist that use gene therapy to treat other forms of retinal diseases. The eye has proven to be an excellent model for gene therapy since it is easy to access all ocular tissues as well as, the eye is considered an immune privileged site.

Research presented in the thesis addresses key questions related to restricting expression of a foreign gene in a cell-specific manner. Since diabetic retinopathy is a proliferative neovascular disease that is affected by oxygen availability in retinal tissues, we have also used an hypoxia responsive element so that the vector can be switched on only during episodes of tissue hypoxia.

The introductory chapter (Chapter-1) presents the etiology of diabetic retinopathy as well as available treatments. Evidence is presented that this disease is amenable to treatments that target angiogenesis. We present the reasoning behind selection and design of the promoters necessary to “sense” hypoxia and then activate the transgene in a cell specific manner. We chose to target Müller cells, the major glial cell type in retina. Therefore, we decided to use another promoter, the gene for Glial Fibrillary Acidic

Protein (GFAP), the signature protein found in glial cells throughout the central nervous system, including the retina.

The materials and methods used for the study are presented in Chapter-2. The experimental procedures, animal models and criteria for data analysis are outlined. The results are presented in chapter-3, starting with *in-vitro* testing of our plasmid and vector that verify cell specificity and hypoxia regulation. The *in vitro* study was extended to an *in vivo* model using the standard laboratory model of oxygen induced retinopathy (OIR). To test whether our vector can stop or retard the progression of neovascularization seen in this model, we chose to deliver the transgene for the anti-angiogenic protein, endostatin. This decision was made on the basis of extensive literature supporting the beneficial effects of endostatin to reduce tumor angiogenesis and ocular neovascularization.

The thesis concludes with a discussion of our findings (chapter-4) that focus on key therapeutic aspects of the vector, a comparison between regulated and non-regulated vectors, and the uniqueness of our approach. Finally, we consider the limitations of our proposed therapy, as well as the far-reaching implications for future clinical intervention using our strategy to treat diabetic retinopathy.

CHAPTER-1: INTRODUCTION

Diabetic retinopathy

Diabetic retinopathy (DR) and diabetic macular edema are common vision complications of diabetes mellitus and frequent causes of vision loss among adults in the US. (Kempen et al., 2004, Roy et al., 2004, Bhavsar, 2006, Bhavsar and Tornambe, 2006). DR involves occlusion and leakage of retinal vessels, leading to macular edema in the nonproliferative phase and formation of highly permeable new vessels (neovascularization, NV) on the retinal surface in the proliferative phase. These new vessels are associated with fibrous tissue that attaches to the vitreous gel on one side and the retina on the other. Contraction of fibro-vascular tissues during eye movement leads to distortion and retinal detachment and ultimately causes loss of vision. This research project focuses on developing a treatment strategy to prevent the neovascularization in DR. Before discussing our strategy it is necessary to understand the retinal vascular supply and retinal angiogenesis.

Vascular supply in retina

Retinal dysfunction in diabetes is associated with changes in the retinal neurovascular unit (which refers to the physical and biochemical relationship among

neurons, glia and vasculature). The inner retina has capillary beds in the ganglion cells layer and inner nuclear layer (Figure 1.1). These capillaries deliver oxygen and nutrients to Müller cells, astrocytes, amacrine cells and ganglion neurons. The outer retina which consists of photoreceptor cells and Müller cell processes receives nutrients and oxygen diffusing from choroidal vessels through the pigmented epithelial cell layer.

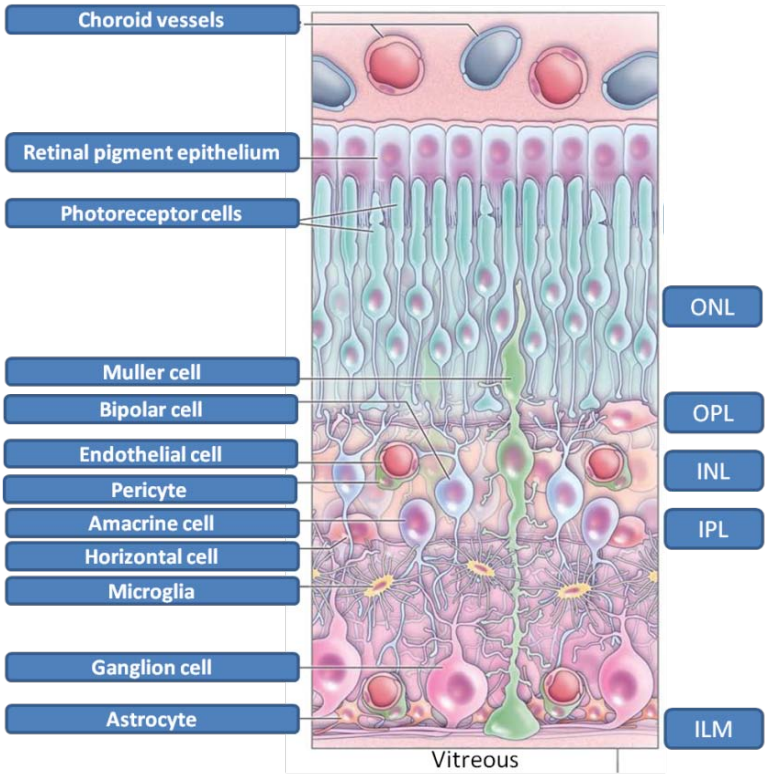

Figure 1. 1: Morphology of mammalian retina (modified from Antonetti, D. 2012). (ONL), Outer nuclear layer; (OPL), Outer plexiform layer; (INL), Inner nuclear layer; (IPL), Inner plexiform layer; (ILM), Inner limiting membrane.

Retinal angiogenesis

Angiogenesis is a complex multi-step process in which vascular endothelial cells sprout from existing vessels. This process starts with dissolution of the basement membrane surrounding vessels which releases endothelial cells. Pericytes are responsible for migration, proliferation and formation of matured capillaries. Angiogenesis involves the interactions between endothelial cells, pericytes, growth factors and components of the extra-cellular matrix. The net outcome of angiogenesis is determined by the balance of angiogenic or angiostatic molecules (Hanahan et al., 1996). Although there are several growth factors associated with retinal angiogenesis, vascular endothelial growth factor (VEGF) acts as a strong endothelial cell mitogen or growth factor to promote both physiological and pathological angiogenesis.

Role of VEGF in ocular angiogenesis

Several ocular cell types, including Müller cells, RPE cells, pericytes, vascular endothelial cells, and ganglion cells express VEGF (Pierce et al., 1995, Dorey et al., 1996, Aiello, 1997, Mousa et al., 1999). Hypoxia also up-regulates VEGF expression both *in vitro* (Shweiki et al., 1992) and *in vivo* (Adamis et al., 1994). VEGF messenger RNA expression is found in Müller cells, scattered astrocytes, and amacrine cells as well as RPE, during hypoxia induced neovascularization in rats (Dorey et al., 1996). VEGF acts through the VEGF receptors (VEGFR) which are high affinity tyrosine receptor kinases expressed on vascular endothelial cells. Upon binding to the receptor, VEGF mediates phosphorylation of several proteins. Ultimately, VEGF induces proliferation,

migration, permeability and prolongs survival of endothelial cells. The levels of VEGF are significantly increased in experimental models of retinal ischemia (Miller et al., 1994, Pierce et al., 1995, Dorey et al., 1996), in patients with proliferative diabetic retinopathy (Adamis et al., 1994, Aiello et al., 1997, Boulton et al., 1997), in choroidal neovascularization (Kvanta et al., 1996), retinopathy of prematurity (Stone et al., 1995), and in retinal vein occlusion (Aiello et al., 1997). The critical role of VEGF in neovascularization makes it an attractive target for angiostatic strategies both in the eye and elsewhere.

Etiology of diabetic retinopathy

Although the exact mechanism by which diabetes causes diabetic retinopathy is not clear, hyperglycemia seems to be the most influential factor. Chronic hyperglycemia causes loss of pericytes followed by constriction and regression of retinal capillaries. It injures the endothelial cells and impairs the association between pericytes and endothelial cells. Pericytes are critical for functioning retinal capillaries since they stabilize and prevent proliferation of endothelial cells (Orlidge and D'Amore, 1987, Antonelli-Orlidge et al., 1989). Due to regression of retinal capillaries, vascular supply to the surrounding tissue is reduced. Some areas of retina develop ischemia due to reduced levels of glucose and oxygen. In addition to low oxygen, oxidative stress and inflammation impair the primary function of pericytes in stabilizing retinal capillaries. During diabetic retinopathy the balance between pro-and anti-angiogenic factors are disturbed which promotes pathological angiogenesis (Simo et al., 2006).

Angiogenic factors, including VEGF, initiate vasodilation in existing vessels causing increased vascular permeability and degradation of the surrounding matrix resulting in the of release of endothelial cells. Endothelial cells migrate and proliferate to form numerous capillary tubes which penetrate the inner limiting membrane and grow into the vitreous gel (Witmer et al., 2003). Pericytes play a critical role in stabilization of migrating endothelial cells and the formation of new capillaries. In the case of diabetic retinopathy, loss of pericytes due to physiological conditions leads to formation of unstable vessels (Li et al., 1997, Enge et al., 2002, Hammes et al., 2002). When newly formed vessels proliferate from the retinal surface toward or into the vitreous, it is termed "proliferative retinopathy." The newly formed abnormal vessels are leaky and often bleed into the vitreous causing reduced vision. As these vessels are associated with fibrous tissue that attaches to vitreous gel on one side and the retina on the other, contraction of these fibro-vascular tissues lead to distortion and retinal detachment, and, ultimately cause loss of vision.

Current treatment for neovascularization in diabetic retinopathy

The standard strategy to treat neovascularization in diabetic retinopathy involves photocoagulation and pharmacological blockade of VEGF. Laser photocoagulation is currently the standard of care (Nadal et al., 2012). Laser photocoagulation uses scattered laser burns in peripheral retina and improves perfusion and increases oxygenation to a reduced area of viable retina (Bhavsar, 2006, Bhavsar and Tornambe, 2006). Although this treatment reduces the risk of severe vision loss, photocoagulation affects peripheral vision as well as night and color vision (Brucker et al., 2009). Surgical removal of the

vitreous gel, termed vitrectomy, is performed when bleeding develops in the vitreous (vitreous hemorrhage). Retinal detachment, and/or severe scar tissue formation also occurs in DR. Vitrectomy isn't beneficial if patients are diagnosed with the late phase of diabetic retinopathy. Even with treatment, vision may continue to decline along with other complications. Despite reduction in retinal neovascularization, both photocoagulation and vitrectomy increase the risk of future vision defects.

FDA approved anti-VEGF pharmacologic agents are commercially available to stop choroid neovascularization in age related macular degeneration (AMD) an ocular disease causing blindness among aged populations. These agents are in clinical trials to treat diabetic macular edema and proliferative diabetic retinopathy. They include aptamers like Pegaptanib (Macugen), (Adamis et al., 2006, Giuliari et al., 2009, Gonzalez et al., 2009, Rinaldi et al., 2012), humanized monoclonal antibodies like Bevacizumab (Avastin), (Minnella et al., 2008, di Lauro et al., 2010) , and Ranibizumab (Lucentis) (Messias et al., 2012, Nguyen et al., 2012, Rauen et al., 2012) . All of these drugs act to downregulate VEGF activity. Because such anti-angiogenic agents have a short half-life (Stewart et al., 2012), patients must have multiple injections to be effective. Although the above strategies have been of some benefit in reducing VEGF levels (Chung et al., 2011), the possibility of potential vision impairment (Bhavsar et al., 2009, Bandello et al., 2012, Mitchell et al., 2012) and enhanced inflammation due to multiple injections can not be avoided (Rangasamy et al., 2012). Hence current strategies are not adequate for the treatment of diabetic retinopathy. Treatments involving local and sustained delivery of anti-angiogenic molecules could be a potential strategy to treat retinal neovascularization.

Novel strategy for the treatment of retinal angiogenesis

Gene transfer mediated by nanoparticles (Kachi et al., 2006, Zhang et al., 2010) transposons (Fjord-Larsen et al., 2011), or viruses (Kachi et al., 2009, Calame et al., 2011, Bennett et al., 2012) offer an attractive strategy for the local delivery of small therapeutic proteins to treat a range of complex diseases. The retina is especially attractive for a gene therapy approaches because it is surgically accessible, offers an isolated compartment due to the presence of the blood/retina barrier and is an immunologically privileged site. The requirements for successful gene therapy include efficient and sustained gene transfer and choice of a gene product that is capable of eliciting therapeutic efficacy (Alexander et al., 1999). The expression of a therapeutic protein by a vector that is regulated by alterations in the tissue microenvironment and in a cell specific manner is likely to have added advantages over ubiquitous unregulated expression in all cells in the retina. In the case of diabetic retinopathy, chronic inhibition or complete inhibition of VEGF by sustained expression of anti-VEGF molecules may affect the normal physiologic role of VEGF. Regulated expression of anti-VEGF molecules during the disease process may be beneficial and avoid complications due to complete inhibition of VEGF.

The primary goal of this study is to test the hypothesis that a hypoxia-responsive domain can be employed for activating cell specific expression in Müller cells (the major glial cell type in retina) under conditions of hypoxic stress in the diseased retina. A promoter containing a hypoxia responsive domain together with a cell specific promoter could potentially be designed to retain cell specificity while also being hypoxia inducible.

In responding to hypoxia, this promoter would have the potential for activation during the early phase of hypoxia as well as the later ischemic phase found in the progression of such diseases as AMD and diabetic retinopathy. A variety of gene products may be appropriate for expression in a vector including pro-survival kinases, antioxidant enzymes, and secreted factors either for preventing angiogenesis or for eliciting neuroprotection. Our hypoxia responsive element (HRE) together with a retinal glial cell-specific promoter could be incorporated to investigate new therapies to treat a range of eye diseases such as inherited photoreceptor degeneration, AMD, diabetic retinopathy and glaucoma. Since our promoter requires hypoxia for its activation, the importance of tissue hypoxia is described below.

Hypoxia and HIF

Hypoxia is a key regulator in VEGF-induced ocular neovascularization. The sensing of cellular hypoxia depends on the action of a key oxygen-dependent sensing system involving the transcription factor hypoxia inducible factor-1 (HIF-1), a heterodimer formed between the constitutive and ubiquitously expressed monomers HIF-1α and HIF-1β (Jiang et al., 1996). In normoxia, transcription is prevented because HIF-1α is modified by hydroxylation of a proline residue and then processed for ubiquitin mediated proteasomal degradation (Semenza, 1999, Semenza et al., 1999). Under hypoxic conditions, however, HIF-1α dimerizes with its partner HIF-1β and translocates to the nucleus for activation of gene transcription (Figure 1.2). Transcriptional activation by HIF-1 occurs through binding of the factor to HREs in regulatory domains of target

genes including VEGF and other angiogenic factors (Semenza, 1999, Semenza et al., 1999, Wenger, 2002).

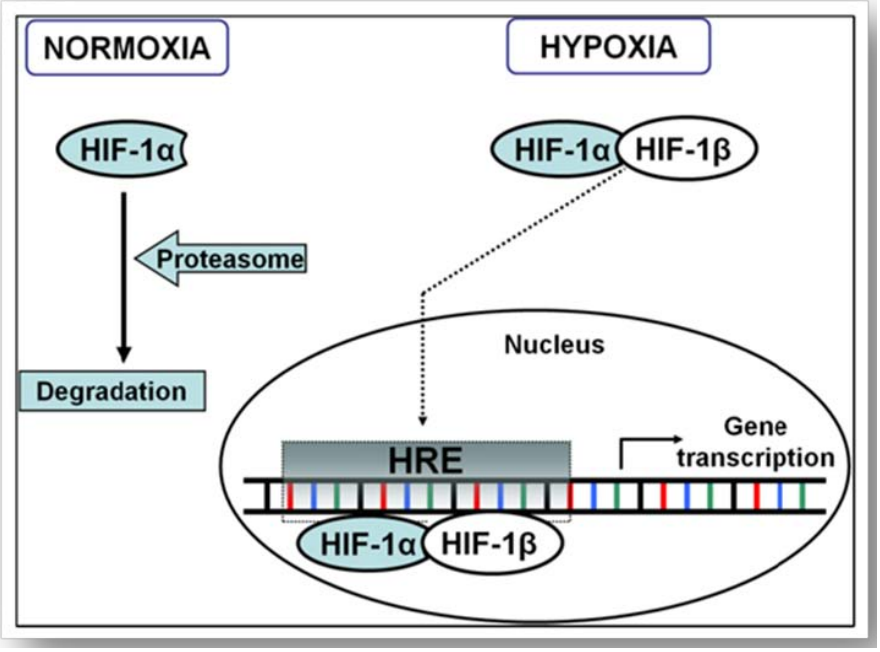

Figure 1. 2: HIF-1 signaling. Hypoxia induces the expression of hypoxia inducible factors (HIF-1) which are composed of two subunits HIF-1α and HIF-1β. During normoxia, HIF-1α is degraded by proteasomal ubiquitination. In the absence of oxygen, HIF-1α is stabilized by binding with HIF-1β to form HIF-1. The HIF-1 translocates into the nucleus and binds to the sequence of hypoxia responsive element (HRE) of several genes to induce gene transcription.

Use of hypoxia-regulated therapy

The potential value of cell specific and regulated gene therapy for the eye has been proposed for models of AMD and retinal ischemia (Bainbridge et al., 2003,

Dougherty et al., 2008). Alterations in retinal oxygen availability can form a basis for disease-appropriate patterns of transgene expression either at early stages of oxygen deprivation due to tissue stress or damage or at later stages of disease associated with tissue ischemia and cell necrosis. Oxygen is critical for maintaining retinal function and a reduction in oxygen levels serves as a trigger for pathological effects underlying AMD and diabetic retinopathy (Shimizu et al., 1981).

Hypoxia induced changes in the retina could also serve as a trigger for activation of gene therapy vectors designed for regulating transgene expression in response to depleted oxygen levels (Bainbridge et al., 2003, Dougherty et al., 2008). Such tight regulation of the expression from gene therapy vectors is likely to be particularly important in retinal tissue where there are several cell types with differing abilities to tolerate stress from hypoxia or elevated reactive oxygen species.

Therapeutic products synthesized by hypoxia-regulated vectors have included growth factors such as basic Fibroblast Growth Factor (bFGF) (Hu et al., 2009) and VEGF (Jiang et al., 2007), antioxidant components (Pachori et al., 2004), anti-angiogenic factors (Li et al., 2006) and pro-apoptotic components such as Bax (Ruan et al., 2001). The promoters of such hypoxia-regulated therapeutic vectors are designed to include a regulatory domain which incorporates multiple HREs which are known to bind the transcription factor HIF-1. We and others have reported that multimers of the HRE drive enhanced levels of gene expression relative to a single HRE (Prentice et al., 1997, Dougherty et al., 2008). For further control over basal levels of expression and of inducibility, we previously incorporated a neuronal silencing element into the promoters

in order to prevent “leaky” gene expression under normoxic conditions (Dougherty et al., 2008). HRE containing promoters which also contain silencer elements have been found to elicit inducibility in hypoxia of greater than 40 fold (Dougherty et al., 2008). As our strategy involves the design of a cell-specific vector, identification of potential retinal cell types are described below.

Choice of retinal cells to target gene therapy in DR

The question is which retinal cell type is the most appropriate target to deliver the therapy. Most of the cell types in retina are neurons (photoreceptor, ganglion, amacrine, bipolar and horizontal cells). The retinal pigment epithelial (RPE) cells are located over 300 microns from the border of the inner limiting membrane (ILM) with the vitreous, the site of neovascularization in diabetic retinopathy. We chose to target the major glial cell, the Müller cell in the retina. Müller cells extend from the outer retina to the vitreal surface where Müller end feet make up the ILM. (Figure-1.1)

Müller cells in the mammalian retina serve a variety of roles vital to the health and function of surrounding retinal neurons (Bringmann et al., 2006). Spanning the entire thickness of retina, Müller cells provide both physiological and structural support throughout the retina (Greenberg et al., 2007). Müller cells play a major role in the establishment of the blood retinal barrier by modulating production of VEGF (Tout et al., 1993). It has been reported that the signals from Müller cells induce the formation of the endothelial cell tight junctions both in the outer limiting membrane (OLM) and the ILM, the latter which is impaired during hypoxia (Tretiach et al., 2005).

In response to a pathological alteration, Müller cells become activated or reactive. Müller cells are activated by hypoxia and glucose deprivation. Gliosis, an important component of the injury response of Müller cells, leads to changes in several cellular processes which includes activation of microglia, vasculature alterations and immigration of blood derived leukocytes into the retinal tissue. As microglial cells are activated, they interact with Müller cells to produce several trophic factors; some of which promote survival while others promote photoreceptor cell death (Bringmann and Reichenbach, 2001, Harada et al., 2002, Bringmann et al., 2006).

In diabetic retinopathy, Müller cells secrete VEGF and other angiogenic factors which promote pre-retinal neovascularization (Ye et al., 2012). Increased GFAP expression by activated Müller cells is the primary response to the metabolic perturbation leading to vascular abnormalities (Bringmann and Reichenbach, 2001). The retinas from diabetic retinopathy patients are characterized by immunoreactivity of GFAP and HIF-1 (both are expressed in response to hypoxia) (Bek, 1997b, a, Lim et al., 2010). HIF-1 produced by Müller cells plays a key role in regulating retinal angiogenesis in oxygen induced retinopathy and diabetic retinopathy (Mowat et al., 2010, Lin et al., 2011). Deletion of the VEGF gene from Müller cells in a conditional knock-out mouse shows the importance of glial cells for VEGF production in oxygen-induced retinopathy models of angiogenesis (Bai et al., 2009, Wang et al., 2010). The possibility of using tissue specific promoters to target Müller cells in retina are described below.

Choice of Müller cell specific promoter

Promoters of several Müller cell associated genes which include CD44, GFAP, vimentin, retinal binding protein 1 (RLBP1), platelet derived growth factor receptor-alpha (PDGFR-A), glutamate ammonia ligase (GLUL), carbonic anhydrase II (CAR2), S100 protein-beta polypeptide-neuronal (S100B) and solute carrier family-1 (SLC1A3) are active in retinal Müller cell cultures (Geller et al., 2008). Among all CD44, GFAP and vimentin promoters have been used to test Müller cell specific transgene expression *in vivo* (Geller et al., 2007, Greenberg et al., 2007). For our project, we considered using the GFAP promoter to regulate Müller cell specific gene expression. GFAP is a 54-kDa type III intermediate filament protein and is the major component of glial filaments in astrocytes. GFAP is also expressed in Müller cells as well as non-myelinated Schwann cells, and Bergmann glia. Up-regulation of GFAP in Müller cells is regarded as an early cellular marker of the injury response in retinal pathology (Bringmann and Reichenbach, 2001). In the normal mammalian retina, the GFAP level is minimal but during photoreceptor degeneration (Fan et al., 1996), retinal detachment (Erickson et al., 1987), ischemia (Kim et al., 1998), or trauma (Li et al., 2012), GFAP is upregulated. GFAP induction in Müller cells is mainly controlled at the transcription level when different *cis* and *trans* acting elements bind to their proximal promoter region. This project involves using the GFAP promoter to drive expression of a therapeutic gene by Müller cells during pathological alterations in the retina.

Importance of GFAP expression in retinal hypoxia

Prolonged ischemia is known to promote Müller cell activation by upregulating GFAP expression (Fitzgerald et al., 1990, Kim et al., 1998, Kaur et al., 2006). Interestingly, exposure of the established Müller cell line rMC-1 to hypoxia for 24 hours elicited a decrease in GFAP expression relative to normoxic conditions (Loewen et al., 2009). GFAP induction in diabetic retinas (Lieth et al., 1998, Rungger-Brandle et al., 2000) has been reported in Müller cells of diabetic rats, and in astrocytes, but not Müller cells, of diabetic mice (Feit-Leichman et al., 2005). Induction of GFAP in Müller cells in rats correlated with pro-apoptotic changes in neurons observed after 2 to 5 months of streptozotocin induced experimental diabetes (Feit-Leichman et al., 2005).

Anti-angiogenic factors to stop neovascularization

Several endogenous angiogenesis inhibitors exist and are termed angiostatic proteins. An imbalance between angiogenic stimulators and inhibitors causes pathological angiogenesis. Pigment epithelium-derived factor (PEDF), endostatin, vasohibin and angiostatin were identified as potent inhibitors of neovasularization. Other anti-angiogenic factors include tissue inhibitor of metalloproteinases-3 (TIMP-3), soluble Tie-2, tumstatin, angiopoietin-2, thrombospondin-1, platelet factor-4, N-terminal fragment of human prolactin (16KhPRL) and soluble Flt1. PEDF, one of the most potent angiostatic proteins, acts through promotion of endothelial cell apoptosis (Cao et al., 2001). Endostatin is an endogenous inhibitor of angiogenesis which is well documented to inhibit angiogenesis and tumor growth *in vivo* (Berges and Pientka, 1999, Nguyen,

2000, Brower, 2001, Shi et al., 2002, Liu et al., 2007, Su et al., 2008). Phase 1 (Lin et al., 2007) and phase II (Kulke et al., 2006, Han et al., 2011) clinical trials for endostatin were initiated to treat cancer and tumor angiogenesis (Li et al., 2008, Zhou et al., 2011). Unfortunately these clinical trials did not prove effective.

However, inhibition of laser induced choroidal neovascularization in the mouse eye was first demonstrated using secretable endostatin (Mori et al., 2001). Intraocular expression of viral mediated endostatin successfully reduced pre-retinal neovascularization in the oxygen induced retinopathy mouse model (Auricchio et al., 2002). Subretinal delivery of tamoxifen-inducible endostatin reduced VEGF-induced retinal vascular permeability in transgenic mice (Takahashi et al., 2003). Lack of collagen XVIII/endostatin is involved in eye abnormalities due to delayed regression of blood vessels in the vitreous along the surface of the retina (Fukai et al., 2002) and it is critical for normal blood vessel formation (Marneros et al., 2004). Since endostatin was found to be effective in reducing ocular neovascularization in several disease models (Mori et al., 2001, Auricchio et al., 2002, Lai et al., 2007) , we decided to deliver endostatin in our gene therapy project as an anti-angiogenic molecule to inhibit experimentally induced retinal neovascularization.

Mechanism of endostatin action

Endostatin is generated from collagen XVIII by proteolytic cleavage of the C-terminal end (Sasaki et al., 1998). Collagen XVIII is an integral proteoglycan in endothelial and epithelial basement membranes (Halfter et al., 1998, Saarela et al., 1998).

Endostatin basically works through modulating multiple pathways in angiogenesis. Endostatin stabilizes blood vessels by suppressing VEGF-induced leakage and neovascularization. Endostatin is shown to have strong anti-angiogenic activity which inhibits the proliferation and migration of endothelial cells (Dhanabal et al., 1999). Recent studies show that endostatin alters the adhesion and migration of endothelial cells by interacting with the integrins (αvβ1, αvβ3 and αvβ5) (Rehn et al., 2001). It is also reported that endostatin reduces cell proliferation, motility and survival of endothelial cells by interfering with the VEGF receptor II (Kim and Hajjar, 2002). In a mouse model of retinal neovascularization, exogenous endostatin downregulated the expression level of VEGF and integrin β3 mRNA (Zhang et al., 2006a).

Endostatin prevents the activation of MMP2 (a matrix metalloproteinase that promotes angiogenesis by degrading the basement membrane) by binding to the catalytic domain of proMMP-2, (Kim et al., 2000) thus preventing invasion of endothelial cells (Lee et al., 2002). In addition, endostatin induces tyrosine phosphorylation of focal adhesion kinase (FAK) which disturbs the cell-matrix interaction and subsequently inhibits endothelial cell migration (Dixelius et al., 2002). Cell cycle progression is also inhibited by endostatin at the G1/S transition phase by down-regulating cyclin D. Endostatin also down-regulates several proliferative angiogenic genes by reducing the mRNA levels of MAPK pathway enzymes (Shichiri and Hirata, 2001). It has also been reported that endostatin promotes endothelial cell apoptosis by activating caspase 9 and down-regulating antiapoptic genes (Dhanabal et al., 1999).

Possible viral delivery options

Gene therapy for the retina has been used successfully in humans and animal models for the treatment of retinal diseases (Hauswirth et al., 2008, Simonelli et al., 2010, Beltran et al., 2012, Jacobson et al., 2012). A variety of recombinant viral vectors have been used effectively to deliver therapeutic genes to different cell types in the retina. The choice of a suitable vector for ocular gene transfer depends on consideration of the virus's natural tropisms and time course of expression. Recombinant AAV (rAAV) is considered an ideal choice for transducing photoreceptor cells. AAV is non pathogenic, stable, safe and an efficient gene transfer vector leading to constant production of the transgene product in many clinical applications. One of the major advantages of AAV is that it transduces non-dividing cells thus making it an attractive candidate for efficient gene transfer to adult retinal neurons. On the other hand, lentiviral vectors may stably transduce RPE cells but are less efficient than rAAV (Bainbridge et al., 2001). Although adeno viral vectors transduce cells in the outer retina, the immune response limits the expression of the transgene (Reichel et al., 2001). Retroviruses target dividing cells and hence they are used in gene therapy approaches for proliferative intraocular disorders (Sakamoto et al., 1995).

AAV mediated expression of anti-angiogenic agents to treat ocular neovascularization have been used in several animal models (Auricchio et al., 2002, Bainbridge et al., 2002, Lai et al., 2007, Pechan et al., 2009, Mao et al., 2011). The success of clinical trials using AAVs as a gene therapy approach to target retinal genetic disease like LCA favors its use as a viral vector (Hauswirth et al., 2008, Maguire et al.,

2009, Simonelli et al., 2010, Bennett et al., 2012). Studies on preclinical safety evaluation of subretinal injection of AAV2.sFLT-1 in non –human primates targeting retinal acquired neovascular diseases like AMD (Lai et al., 2011) is further reason to consider AAVs to deliver therapeutic genes to cure other retinal neovascular diseases. In the current project, a self complementary AAV vector is used to drive hypoxia-regulated cell specific promoters to express either reporter genes or a therapeutic transgene.

Choice of an animal model of retinal neovascularization

Several experimental animal models of retinal neovascularization exist and are widely used to test new methods for inhibiting neovascularization. The oxygen induced retinopathy (OIR) model is routinely used for retinal neovascularization because it mimics the pathology of both retinopathy of prematurity and proliferative diabetic retinopathy. The mouse (Smith et al., 1994) and rat model (Dorey et al., 1996, Penn et al., 2001) are currently used by many investigators. In the OIR model, a litter (postnatal day 7, P7) together with the nursing mother is exposed to 75% oxygen for 5 days and then returned to room air for 5 days. During the period of elevated oxygen exposure, regression of central vessels occurs by P12 (Figure 1.3). When the pups are removed from hyperoxia the central area remains hypoxic due to vessel loss. Cells within the hypoxic central retina, particularly the Muller cells, secrete VEGF and other angiogenic factors that elicit growth of new vessels around this poorly vascularized zone. The newly formed vessels in the peripheral retina are leaky and unstable. Many of the newly formed aberrant vessels grow into the vitreous.

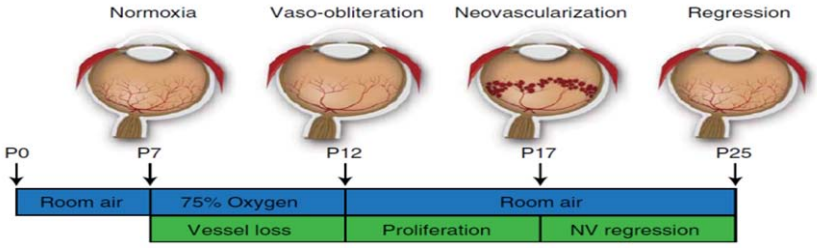

Figure 1. 3 : Schematic of oxygen induced retinopathy (OIR) in mouse. On postnatal day 7 (P7), mouse pups along with their mother are exposed to 75% oxygen for five days. During this period the developing vessels in the central retina degenerate (vaso-obliteration) by P12. When the pups are returned to room air (P12) they experience hypoxia leading to peripheral neovascularization. The neovascularization is maximum at P17 pups but regresses by P25 (Courtesy: Connor et al., 2009)

We tested the hypoxia-regulated, glial cell specific promoter driving either GFP or endostatin in the OIR model. It is anticipated that our approach with regulated expression of endostatin would have a significant impact on inhibition of neovascularization following intraocular delivery.

Significance of the project:

Ischemia-induced ocular neovascularization is a major cause of blindness. As both hypoxia and Müller cells play important roles in retinal angiogenesis, a hypoxia-regulated, aerobically-silenced vector targeting Müller cells would be applicable for

local, auto-regulated delivery of anti-angiogenic agents to hypoxic regions of retina. Such auto-initiated therapy would be useful for intervention in hypoxia induced neovascularization which is known to occur in a number of ocular diseases including retinopathy of prematurity, proliferative diabetic retinopathy, AMD and macular edema.

Research hypothesis

There is evidence that Muller cells experience hypoxia in retinal NV (Mowat et al., 2010, Lin et al., 2011) and Muller cell derived VEGF is a significant contributor to retinal NV (Bai et al., 2009, Ye et al., 2012) . Endostatin was also proven effective to treat ocular neovascularization in animal models (Mori et al., 2001, Auricchio et al., 2002). Our hypothesis is that neovascularization can be prevented by production of endostatin by Müller cells following intravitreal injection of an AAV vector using a hypoxia-regulated, Müller cell-specific promoter. The expression of endostatin should be restricted only to Müller cells and be produced only during ischemia in retinal tissue. The long range goal of this project is to evaluate regulated promoter constructs in retinal disease models to treat ocular neovascularization.

The following specific aims will test our hypothesis:

1. To develop and characterize a hypoxia-regulated promoter for Müller cell-specific gene expression.
2. To examine the hypoxia-regulated expression of a reporter gene both *in-vitro* and *in-vivo.*

3. To test the ability of the regulated vector to produce endostatin to inhibit hypoxia-induced neovascularization in the oxygen-induced retinopathy (OIR) mouse model of neovascularization.

The experimental design is described in Figure 1.4.

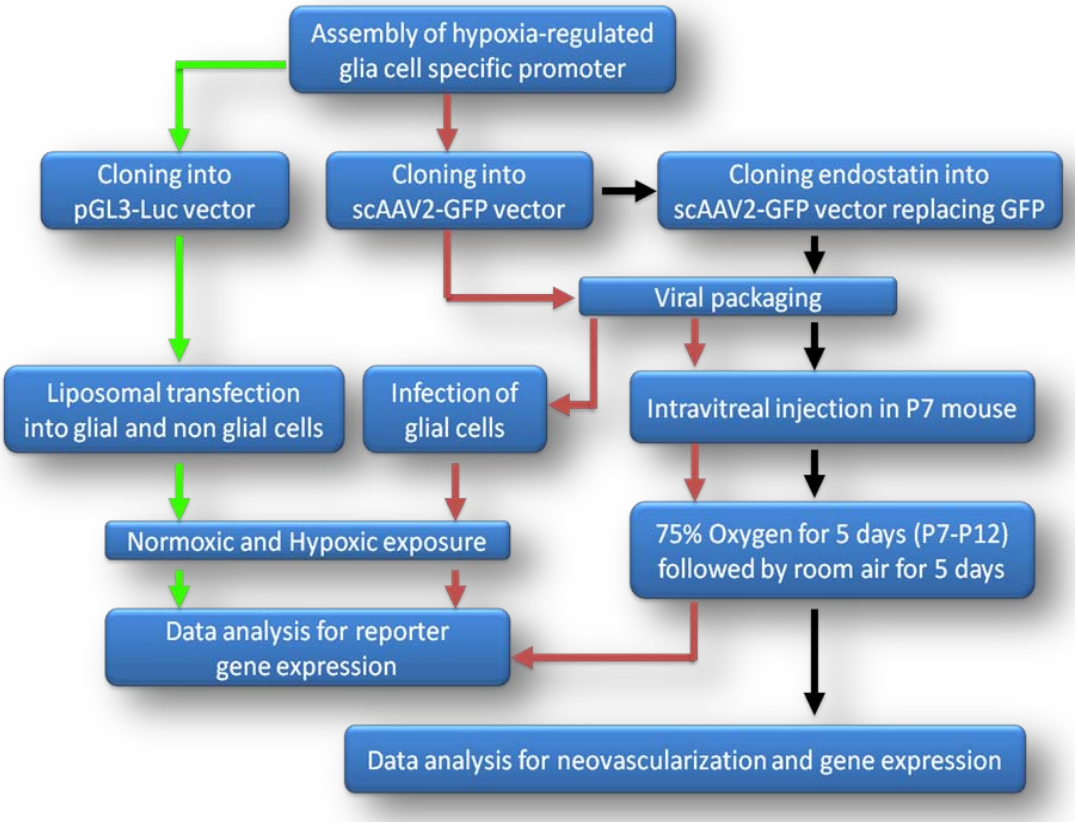

Figure 1. 4: Flowchart of experimental design. Green arrows indicate testing the regulated vector driving luciferase reporter gene in cell culture, red arrows: testing of vectors driving GFP reporter gene both *in vitro* and *in vivo* and black arrows indicate testing the vector driving endostatin in OIR model.

CHAPTER-2: MATERIALS & METHODS

Cell Cultures and Hypoxia treatment

Cells employed were the M10-M1 human Müller cell line (gift from Dr. Astrid Limb, University College, London), the ARPE-19 human retinal epithelial cell line (American Type Culture Collection, Manassas, VA), the human embryonic kidney cell line HEK 293 (Stratagene, La Jolla, CA), the glioma cell line C6 (gift from Dr. Ajay Verma, Uniform Health Services University, Bethesda, MD), and the mouse hippocampal neuronal cell line HT22 (gift from Dr. David R. Schubert, The Salk Institute, San Diego, CA). Cells were cultured using previously described methods and maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). Rat primary astrocytes were cultured from one day old pups using standard procedures.

All procedures involving mice were performed in accordance with the Association for Research in Vision and Ophthalmology's (ARVO) statement for the use of animals in ophthalmic research and approved by the Animal Care and Use Committee at Florida Atlantic University. Primary cultures of Müller cells were obtained from postnatal day 1 (P1) mice according to previously described methods with minor modifications. Briefly retinas from P5-P6 mice were dissected and dissociated with

activated papain (Worthington Biochemical Corp, Lakewood, NJ). After dissociation and centrifugation, cells were resuspended and plated on cell culture flasks for experimental treatment and analysis.

Cultures were exposed to hypoxic conditions at 1 % oxygen as previously described (Dougherty et al., 2008) using an anaerobic chamber. Oxygen levels were monitored continuously with an oxygen electrode (Engineered Systems & Designs, Newark, DE).

Choice of GFAP promoter for cell specificity

A previous study by Brenner and colleagues identified a segment from -1488 to -1434 in their ABC1D GFAP promoter that contains brain region specific elements and another segment from -1443 to -1399 required for silencing expression in neurons (Lee et al., 2008b). By combining specific enhancer domains from the full length (-2163) GFAP promoter, these investigators generated a highly active promoter containing enhancer domains A,B,C1 and D taken from -1757 to -1488 (AB), -1488 to -1256 (C1) and -132 to -56 (D) plus the proximal promoter region. For our hypoxia-regulated GFAP promoter construct, we employed a promoter sequence consisting of a hybrid of the 4 enhancer segments A, B, C1 and D. Brenner and colleagues also showed in transgenic mice that gfaABC1D generally drove beta galactosidase expression at a stronger level than was obtained in -2163 GFAP-lacZ mice. We preferred to use the shorter gfaABC1D (shorter version of GFAP promoter) for construction of our regulated plasmid vector.

Construction of hypoxia-inducible promoter (Reg-GFAP)

The GfaABC1D (681bp) domain (a gift from Dr. Michael Brenner) which contains subfragments of the GFAP promoter was previously reported to drive astrocyte-specific gene expression in brains of transgenic mice (Lee et al., 2008b) and the promoter subdomains of GfaABC1D were previously described by Lee et al., 2008. pGL3-HRSE-GFAP-Luc (Figure 2.1A) was constructed in the pGL3 (Promega) plasmid backbone by incorporation of the following promoter domains: 1) a silencing region (hypoxia responsive silencing element, HRSE; provided by Dr. Keith Webster, University of Miami Miller School of Medicine, (Dougherty et al., 2008), 2) six copies of the hypoxia response element (6 x HRE) of the phosphoglycerate kinase gene as previously described (Dougherty et al., 2008) and 3) GfaABC1D (please see above). This regulated plasmid, pGL3-HRSE-GFAP-Luc was given the name "Reg-GFAP" (Figure 2.1B).

Transfection and Dual Luciferase Assay

Cell lines and primary Müller cells were transfected with liposome reagents (Lipofectamine 2000 and Lipofectamine LTX, Invitrogen) with the exception of primary astrocytes which are known to require specially optimized transfection reagents (personal communication, A. Haces, Florida Atlantic University). For this reason, these cells were transfected with NovaFECTOR (VennNova, Pompano Beach, FL). To ensure comparable transfection efficiencies with different cell types, a strongly expressing promoter plasmid (CMV-Luciferase) was employed for comparison to "test" promoter plasmids in all plasmid transfection experiments. For an internal control in each experimental condition,

the TK renilla plasmid was co-transfected with the plasmid of interest. Data on promoter activities were obtained by the dual luciferase assay as previously described (Dougherty et al., 2008).

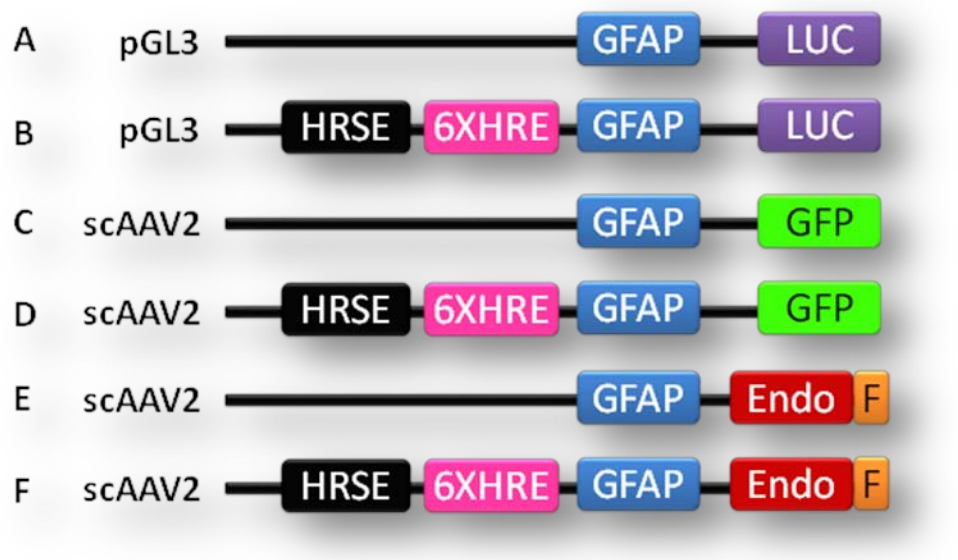

Figure 2. 1: Schematic diagram of vectors used in this study. (A) Conserved domains of GFAP promoter (686 bp) were incorporated into pGL3-based vector driving the Luciferase (Luc) reporter gene, (B) hypoxia-responsive and aerobically silenced elements (HRSE) and 6XHRE regulatory elements are incorporated into the 5'end of pGL3-GFAP-Luc vector, (C) GFAP promoter was ligated to self-complementary AAV plasmid vector by replacing the CMV promoter, (D) regulated element including GFAP promoter has been ligated to self-complementary AAV plasmid vector by replacement of CMV promoter, (E) Flag tagged Endostatin cDNA is cloned into plasmid C replacing GFP and (F) Flag tagged Endostatin cDNA is cloned into plasmid D replacing GFP.

Self Complementary AAV

For production of AAV and cellular transduction, standard methods were employed (Dougherty et al., 2008). ScAAV plasmid was a gift from Dr. D. McCarty (Ohio State University, Columbus, OH). Promoter cassettes were amplified by PCR and inserted into the scAAV plasmid (Figure 2.1 C and 2.1D). To make endostatin vectors, the endostatin open reading frame (Invivogen, San Diego, CA) without the stop codon was amplified by PCR and cloned into scAAV plasmid by replacing GFP. In order to detect exogeneous endostatin expression a FLAG epitope sequence with appropriate restriction sites was synthesized (IDT DNA Technology, Coralville, Iowa) and cloned into the 3' end endostatin (Figure 2.1 E and F). ScAAV serotype-2 viruses were produced at the Gene Therapy Vector Core , University of North Carolina (Chapel Hill, NC) and titers were determined by standard dot blot analysis. Primary Müller cells were transduced with scAAV six days before subjecting the cells to hypoxic conditions.

Intravitreal Injection and Oxygen Induced retinopathy (OIR) model

The OIR model was generated by standard methods (Smith et al., 1994). Briefly, P7 pups were anesthetized with ketamine/xylazine and subjected to intravitreal injection of scAAV. P7 pups with their mothers were then either maintained in normal room air, controls (21% oxygen) or exposed to 75% O2 for 5 days in a chamber, the experimental animals. Pups at P12, were moved to normoxic conditions in room air for a 5 more days and euthanized at P17.

Flat Mount

P17 pups were sacrificed using an over-dose of Ketamine / Xylazine. Eyes were enucleated, the cornea and lens removed and the eye cups fixed with 4% paraformaldehyde (PFA) for an hour. After several washes in PBS, the neuro-retina was isolated from the eye cup by removing the RPE cell layer (Connor et al., 2009). The retina was stained with 100µl of (10 µg/ /ml) Isolectin B4-594 (Alexa Fluor 594 – I21413, Molecular Probes) at room temperature overnight with gentle shaking. Four incisions were made to divide the retina into four equal-sized quadrants, which were carefully flattened on a slide, with the ganglion cells side facing down and cover-slipped with anti-fading mounting agent, Floromount G. Fluorescent images of the flat mounted retina were obtained using a Nikon inverted fluorescence microscope (Figure 2.2).

Immunohistochemistry

For analysis of GFP expression retinal sections were labeled with primary rabbit anti-GFP IgG (Cat # A11122, Invitrogen, Carlsbad, CA) and dylight-488 conjugated goat anti-rabbit secondary antibodies (Vector lab, Burlingame, CA). Images were obtained using a Nikon Eclipse confocal microscope.

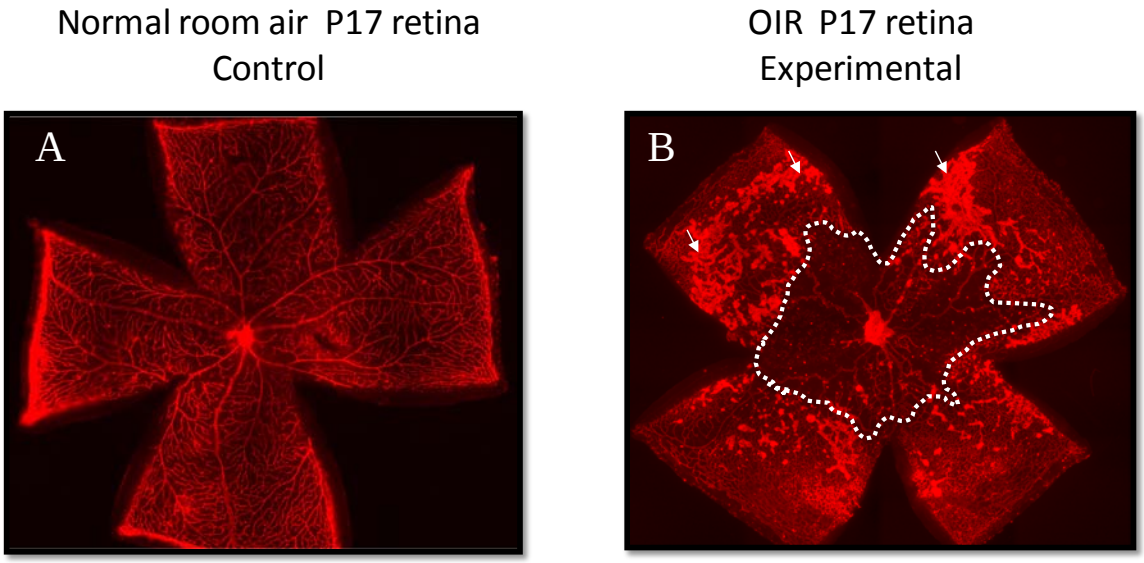

Figure 2. 2: Flatmounted retina labeled with IB4 lectin. The eyes were enucleated and fixed in 4% PFA for 2 hour after euthanizing the P17 mice. Cornea, iris, lens, sclera and RPE were removed from the eye cup. The retina was stained with Isolectin B4 overnight to label endothelial cells. (A) Flat mounted retina from the control mouse in normal room air. (B) Flat mounted retina from hyperoxia treated mouse which shows central vasoobliteration (dotted line) and peripheral neovascularization (arrows).

Analysis of vasoobliteration and neovascularization

Flat mounted retinas were used to quantify both retinal vasoobliteration and neovascularization using ADOBE photoshop 5.5 (Banin et al., 2006, Connor et al., 2009). The central avascular area and the total retinal area were documented separately. The

percentage of vasoobliteration was calculated by comparing the central avascular area to total retinal area (Figure 2.3).

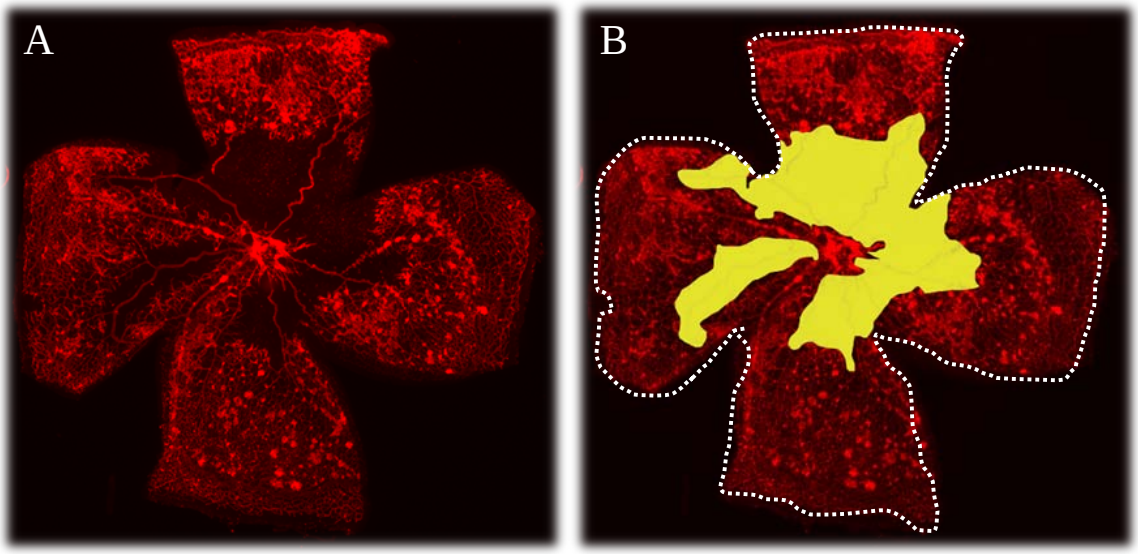

Figure 2. 3: Demonstration of quantification of central avascular area. (A) Flatmounted retina from hyperoxia exposed mice. (B) The same retina is digitalizaed to measure the central avascular area. Using Adobe photoshop the central avascular area (yellow zone) was measured and then the total area (white dotted line) of the retina was measured. Percentage (%) of central avascular area was calculated as (central avascular area/total retinal area) x 100.

To quantify the percentage of peripheral neovascularization, 2/3rd of each retinal quadrant was selected and the area of neovascular tufts compared with the total vascular area. For each retina, average data from 4 quadrants were used to calculate the percentage of peripheral neovascularization (Figure 2.4).

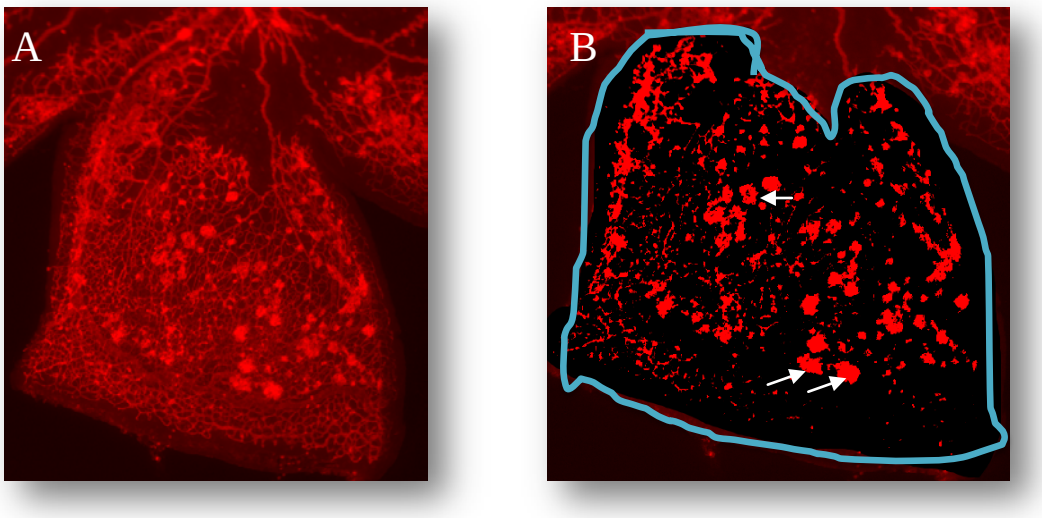

Figure 2. 4: Demonstration of quantification of peripheral neovascular area. (A) One quadrant of flatmounted retina from hyperoxia exposed mice. (B) The same retina digitalizaed to measure peripheral neovascular area. Using Adobe photoshop, the peripheral neovascular area (deep red area, arrows) was measured and then the total vascular area (blue line) of the retina was measured. Percentage (%) of peripheral neovascular area was calculated as (peripheral neovascular area ⁄ total retinal area) x 100.

Sample preparation for Western Blotting & ELISA

Sample preparations for both Western Blotting and ELISA were the same. Pups were sacrificed using an over-dose of Ketamine / Xylazine and eyes were collected in PBS. After removing the cornea, lens, sclera and RPE, the retina was collected in 100 ul of radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor. After

incubation for 30 minutes on ice, the retina was homogenized manually using a plastic homogenizer in an eppendorf tube. The tissue lysate was centrifuged at 14000 RPM in a 4 degree centrifuge for half an hour and the supernatant collected in a separate tube. The protein concentration in the retinal lysate was quantified using the standard BCA method.

Western Blotting

Standard western blotting procedures were followed to detect exogenous endostatin expression. Briefly around 20 μg of each sample were loaded in a tris glycine poly acrylamide gel (Novex) and proteins were transferred to nitrocellulose membranes. The membranes were blocked in 5% milk (in PBS) and incubated overnight with rabbit primary antibody. After several washes, the membranes were incubated with anti rabbit FLAG secondary antibody for one hour. HRP conjugated substrate was added to the membrane and the film was developed using an autoradiography machine.

ELISA

Endostatin concentration was measured using the RayBio® human endostatin ELISA Kit (Ray Biotech Inc. Norcross, GA, USA) . The concentration of endostatin expressed by either GFAP-Endo or REG-Endo vector was normalized to the PBS treated eyes. VEGF concentration was measured from the retinal lysates using the RayBio® Mouse VEGF ELISA Kit (Ray Biotech Inc. Norcross, GA, USA). Briefly 10ul of each sample was used to quantify VEGF and then normalized to μg of protein for each sample. Each experiment was performed in duplicate (n = 2), and each data point was performed in triplicate, according to the manufacturer's protocol.

Statistical Analysis

Data is expressed as mean +/- SEM. Significance was determined using analysis of variance. Differences between conditions were regarded as significant if $p<0.05$.

CHAPTER-3 : RESULTS

Cell specific expression of GFAP promoter

High levels of luciferase gene expression were observed in cultured mouse primary Müller cells and rat primary astrocytes following transfection of pGL3-GFAP-luc plasmid. The firefly luciferase activity of the GFAP promoter was measured as 28 ± 10 fold ($n=8$, $p<0.001$) and 30 ± 1.6 fold ($n=6$, $p< 0.05$) in the Müller cells and astrocytes, respectively (Figure 3.1A and 3.1B) compared to control TK renilla activity. In MIO-M1 cells, the GFAP promoter activity was measured as 0.75 ± 0.0.15 fold ($n = 9$, $p<0.001$) and minimal expression occurred in HEK cells (0.16 ± 0.01, $n = 8$, $p< 0.001$) (Figure. 3.1C). Although MIO-M1 cells exhibited relatively low levels of transfection, there was clear cell specificity in MIO-M1 cells relative to HEK cells. The GFAP promoter also shows basal or minimal activity in C6, HT22 and ARPE-19 cells (data not shown).

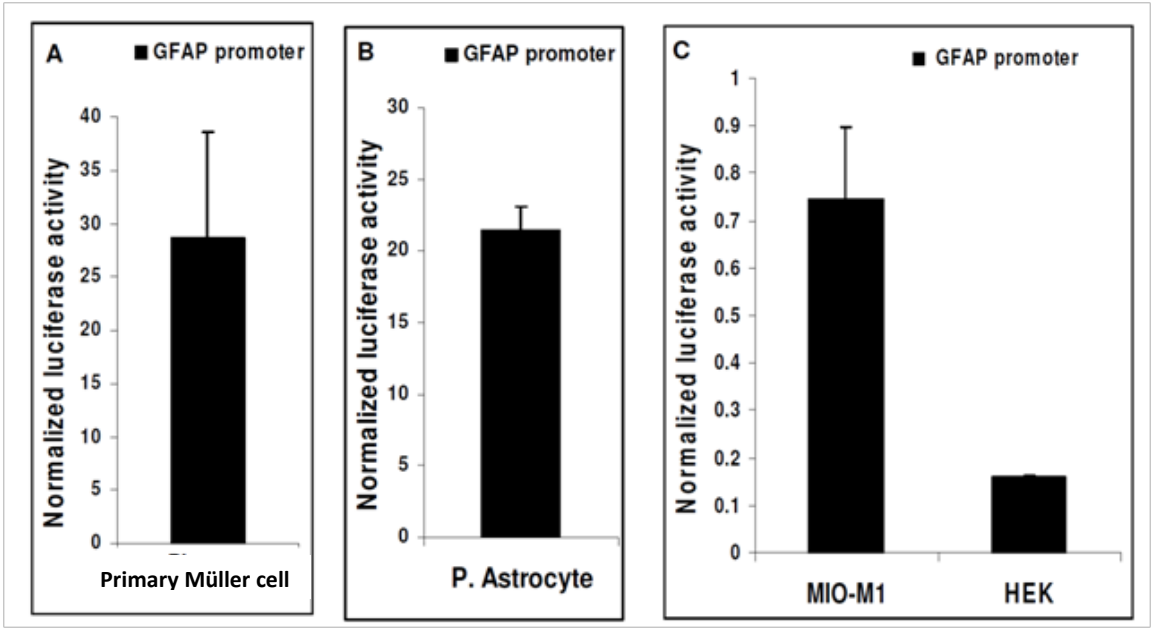

Figure 3. 1: Müller cell specificity of GFAP promoter. GFAP promoter driving the luciferase gene was transfected into MIO-M1, HEK cells, primary mouse Müller cell, and rat primary astrocytes. The promoter shows the maximal luciferase activity in (**A**) primary mouse Müller cells (28.6 ±10, $n = 8$, $P < 0.05$) and in (**B**) rat primary astrocytes (30 ±1.6, $n = 6$, $P<0.05$) compared with TK Renilla activity. (**C**) GFAP promoter restricts expression of reporter gene to MIO-M1 cells (0.75 ± 0.15, $n = 9$, $P < 0.001$) with basal expression in HEK cells (0.16 ± 0.01, $n = 9$, $P < 0.001$). (Note: The abscissa in **C** was reduced compared with **A** and **B** reflecting the low level of transfection in MIO-M1 cells).

In vitro activity by hypoxia regulated GFAP promoter

Aerobic silencing

The luciferase constructs bearing the GFAP promoter or the regulated GFAP promoter were transfected to mouse primary Müller cells and the human MIO-M1 cell line. The transfected cells were cultured in 21% oxygen. The suppressive action of the HRSE in normoxia was confirmed by dual luciferase assay data showing that when in normoxia, the regulated GFAP construct was silenced by 82% in mouse primary Müller cells (Figure 3.2B) and by 21% in MIO-M1 in comparison to the unregulated GFAP promoter (Figure 3.2A).

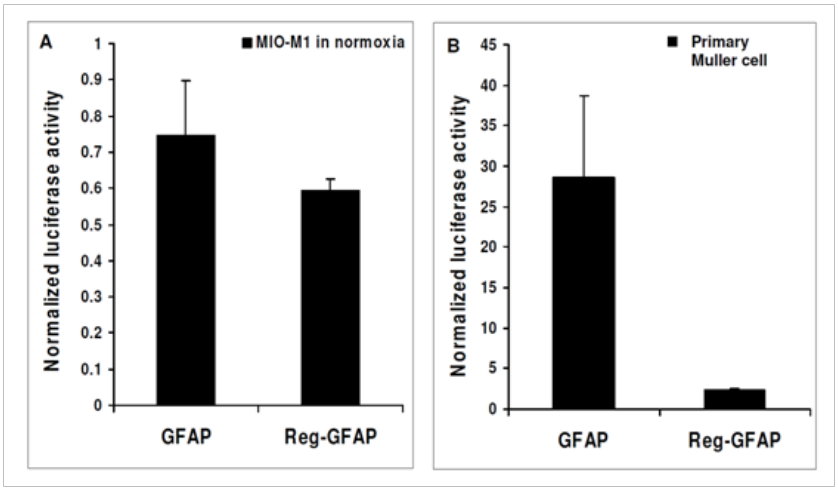

Figure 3. 2: Conditional silencing of regulated GFAP promoter. After 40-hour exposure to normoxia, the activity of the reg-GFAP promoter in comparison with the GFAP promoter was significantly reduced by 21% in (**A**) MIO-M1 and by 82% in (**B**) mouse primary Müller cells.

Hypoxic induction and cell specificity

HRE elements in the regulated promoter successfully responded to hypoxia resulting in dramatically elevated induction of luciferase activity. As shown by dual luciferase assay data in Figure. 3.3, the regulated GFAP promoter was induced in hypoxic mouse primary Müller cells approximately by 12-fold (29.5 ± 0.6 vs 2.4 ± 0.3, $n = 8$, $p>0.05$) and in hypoxic M10-M1 cells by ~16-fold (9.5 ± 0.9 vs 0.6 ± 0.03, $n = 9$, $p<0.001$). Hypoxic induction of the regulated promoter was observed in Müller cells but not in the following non glial cell lines: kidney HEK, glioma C6, hippocampal neuronal HT22, and retinal pigment epithelial ARPE-19 (Figure 3.3).

Silencing and hypoxia-induced expression of GFP

The GFAP and regulated promoters were successfully cloned into scAAV packaging plasmids and scAAV2/2 vectors were produced for both plasmids. Upon infection in reduced serum media, the vectors transduced mouse primary Müller cells. AAV2 transduced cells were exposed to hypoxia for another 6 days and GFP expression analyzed. Primary Müller cells infected with AAV2-GFAP-GFP express GFP in both normoxia (Figure 3.4A, C) and hypoxia (Figure. 3.4B, D), whereas AAV2-Reg-GFAP-GFP transduced cells expressed GFP only under hypoxic conditions (Figure 3.4F, H). (Note: GFP expression in normoxia (Figure 3.4E, G) was below detection limits under standard microscopic conditions.)

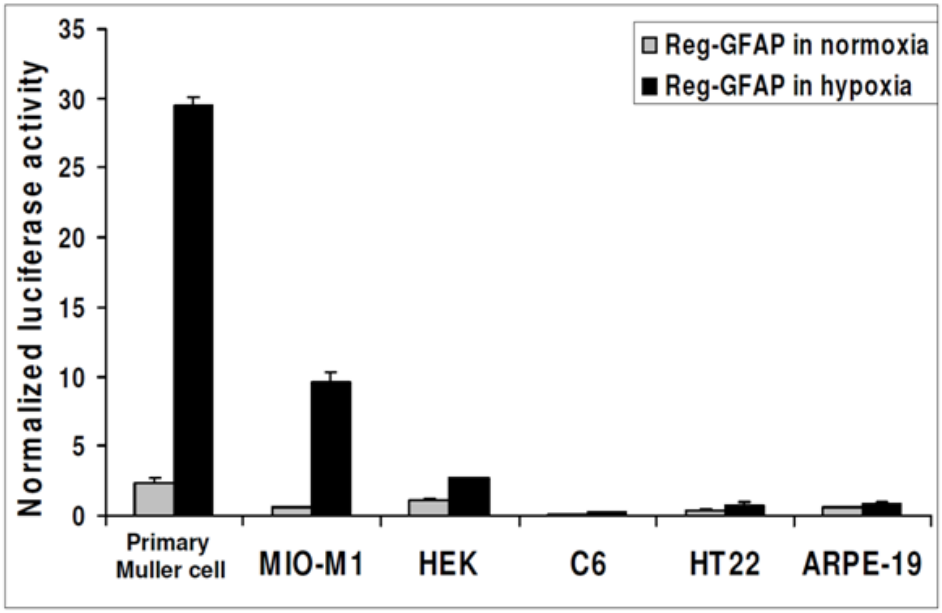

Figure 3. 3: Hypoxia induction and cell specificity of regulated promoter. There was approximately a 12-fold induction of the regulated GFAP promoter (29.5 ± 0.6, $n = 8$, $P < 0.05$) in mouse primary Müller cells after 40 hours of hypoxia. Similarly in MIO-M1 cells, the regulated GFAP vector shows approximately 16-fold induction (9.5 ± 0.9, $n = 9$, $P < 0.001$) greater than in normoxia. Basal or minimal expression of the promoter was evident in different cell lines such as HEK, C6, HT22, and ARPE-19.

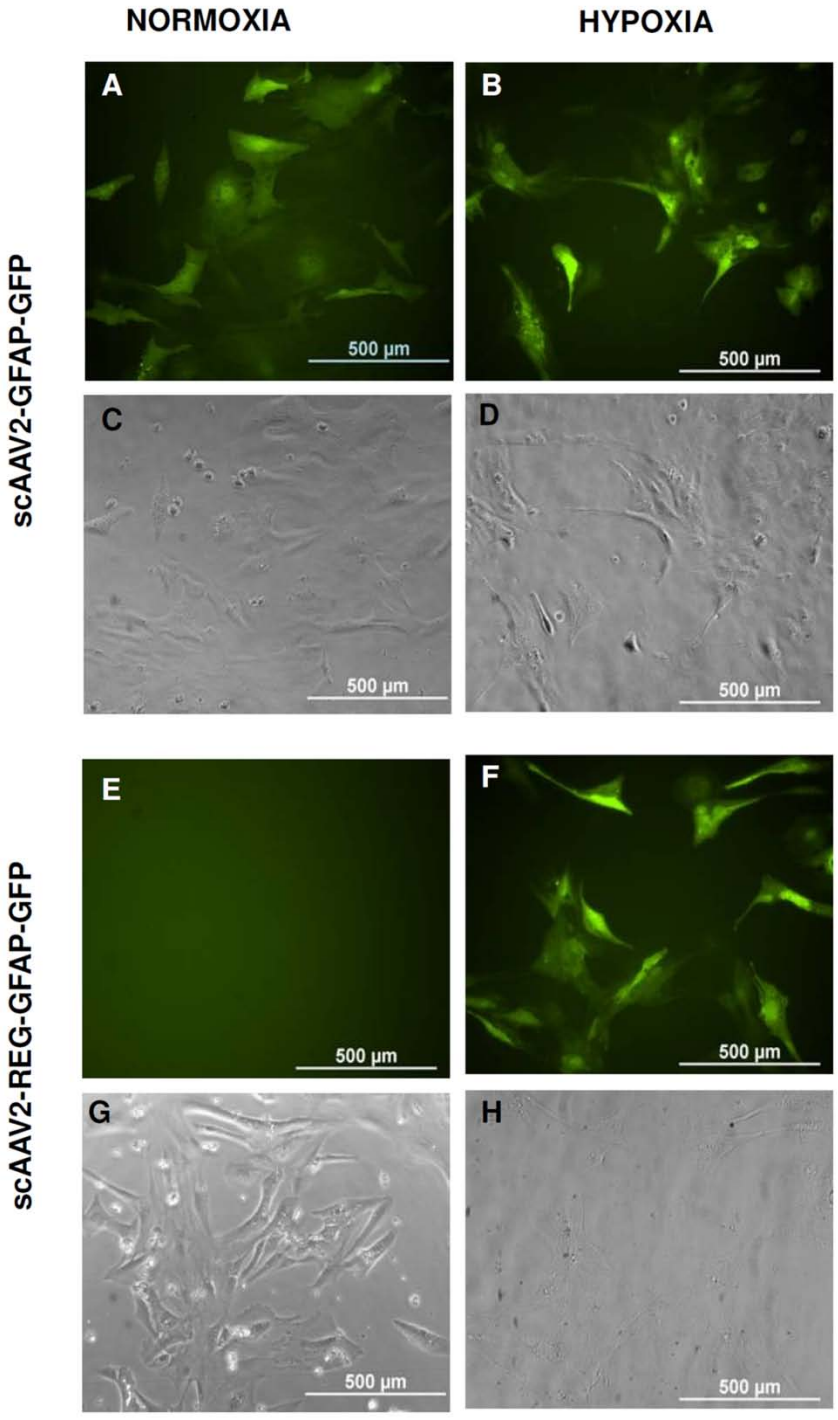

Figure 3. 4: GFP expression in transduced mouse primary Müller cells.

Images of scAAV2-GFAP-GFP and scAAV-reg-GFAP-GFP transduced primary

Müller cells under normoxia and hypoxia. GFP was expressed by the scAAV-GFAP-GFP vector in transduced mouse primary Müller cells under (A) normoxic and (B) hypoxic conditions. GFP expression was inactive in normoxia (E) and induced in hypoxic Müller cells transduced with scAAV2-reg-GFAP-GFP vector (F). (A, B, E, F) Fluorescent images; (C, D, G, H) phase images.

In-vivo silencing and hypoxia-induced expression of GFP

Flat mounted retinas from P17 mice demonstrate a normal vascular pattern while mice from the OIR model show an abnormal vascular pattern with a central avascular region (Figure 3.5A and 3.5B, respectively). ScAAV-GFAP-GFP injected eyes demonstrated GFP induction in Müller cells and astrocytes in P17 retinas of mice exposed to either room air [controls, (Figure 3.5C) or high oxygen (Figure 3.5D). In contrast, there was no GFP expression in the retinas of scAAV-reg-GFAP vector injected eyes of mice kept at room air (Figure 3.5E), thus confirming that the promoter is silenced in normoxia. Prominent induction of GFP expression was found almost exclusively in Müller cells in hypoxic regions of retinas of P17 mice in the OIR model, (Figure 3.5F). GFP was clearly localized in the Müller cell cytosol extending from the outer retina to the foot processes along the inner limiting membrane (ILM). The possibility that astrocytes along the ILM expressed GFP cannot be precluded**;** however, if so, the level is clearly reduced in comparison to the unregulated promoter. These results confirm hypoxia induction of the GFP and cell specificity of the regulated promoter *in vivo*.

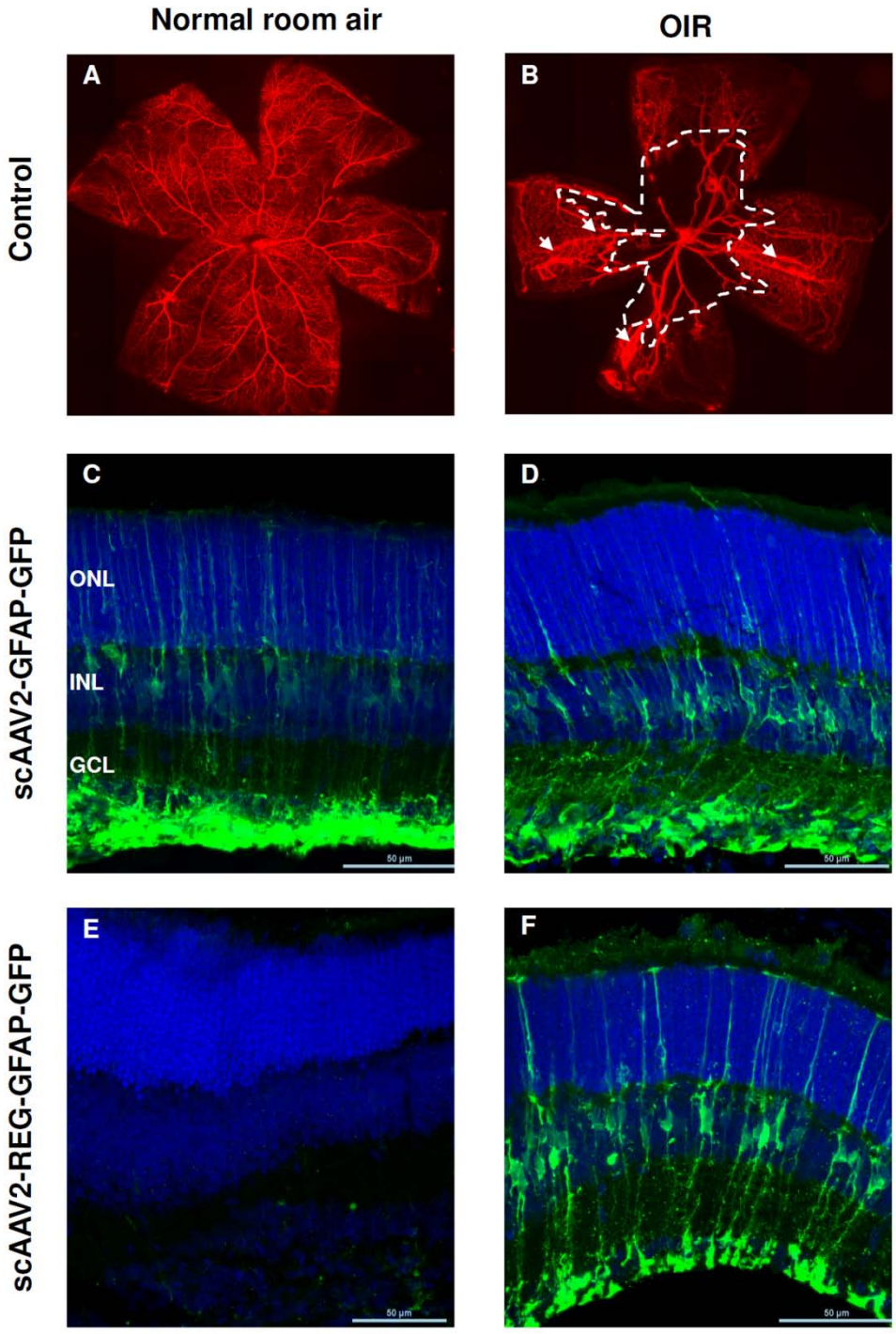

Figure 3. 5: Retinal flat mounts and Müller cell restricted GFP expression.

Retinal flat mounts prepared from P17 mice raised in room air (**A**) or exposed to

hyperoxia for 5 days (**B**) and perfused with Tomato lectin. Central vascular occlusion (*dashed line*) and increased vascular tufts (*arrows*) occurred in the OIR model. Injection of the unregulated scAAV-GFAP-GFP resulted in GFP expression in Müller cells and astrocytes in both room air controls (**C**) and oxygen exposed retinas (**D**). The regulated construct scAAV-reg-GFAP-GFP was silent in room air-exposed retinas (**E**) but in hypoxic retinas, GFP expression was evident in the soma as well as cell processes of Müller cells. There was less evidence of GFP expression in astrocytes of hypoxic retinas expressing the regulated construct (**F**) than in hypoxic or normoxic retinas expressing the unregulated construct (**C**, **D**). INL, inner nuclear layer; ONL, outer nuclear layer; GCL, ganglion cell layer.

Intravitreal delivery of scAAV2-Reg-Endo in OIR model

We tested the effect of scAAV mediated glial cell-targeted endostatin expression in the mouse OIR model. Exposure of neonatal mice to high oxygen (P7-P12) following a period in room air (P12-P17) results in central vasoobliteration on day 12 and peripheral retinal neovascularization by day 17. All the neonatal animals used for this study developed neovascularization. On day P7, 30 animals were injected with 1µl ($2x10^9$ viral particles) of either scAAV2-GFAP-Endo or scAAV2-REG-Endo in one eye and phosphate buffered saline or the empty AAV2 capsid vector in the other eye.

Flat mount techniques were successfully practiced in room air control animals before analyzing the experimental animal. The effect of both GFAP-Endo and REG-Endo

vector were clearly visible in flat mounted retinas (Figure 3.6 and 3.7). The peripheral neovascularization observed in both capsid and PBS treated eyes was almost identical. The neovascular area was clearly reduced in eyes expressing exogenous endostatin by either GFAP or REG vector compared to PBS or CAPSID treated eyes. To measure the exact effect in terms of reduction of the neovascular area further quantification was performed.

Reduction in neovascular area:

Demonstration of reduced neovascular area by scAAV2-GFAP-Endo and scAAV2-REG-Endo vector is shown in figures 3.6 and 3.7, respectively. There was no significant difference in the peripheral neovascular area found in PBS injected eyes (24.82 ± 3.2 %, n = 11) or Capsid injected eyes (22.82 ± 4.1%, n = 10). Retinal neovascular area (%) was significantly reduced by 90% in both scAAV2-GFAP-Endo (2.18 ± 0.5 %, n =9) and scAAV2-REG-Endo (1.61± 0.6 %, n = 9) injected eyes compared to either PBS or Capsid injected eyes in P17 OIR retina. (Figure 3.8).

Reduction of avascular area:

Demonstration of reduced avascular area by scAAV2-GFAP-Endo and scAAV2-REG-Endo vector is demonstrated in figures 3.6 and 3.7, respectively. Significant re-vascularization of the central avascular area was observed that corresponds to reduction in the central avascular zone both in scAAV2-GFAP-Endo (2.34 ± 1.4 %, n =9) and scAAV2-REG-Endo (2.31 ± 1.6 %, n = 9) injected eyes compared to either PBS (21.1 ± 1.9 %, n =11) or Capsid (21.87 ± 2.5 %, n = 10) injected eyes. There was no significant

difference in the central avascular area in eyes injected with either PBS or Capsid. (Figure 3.9)

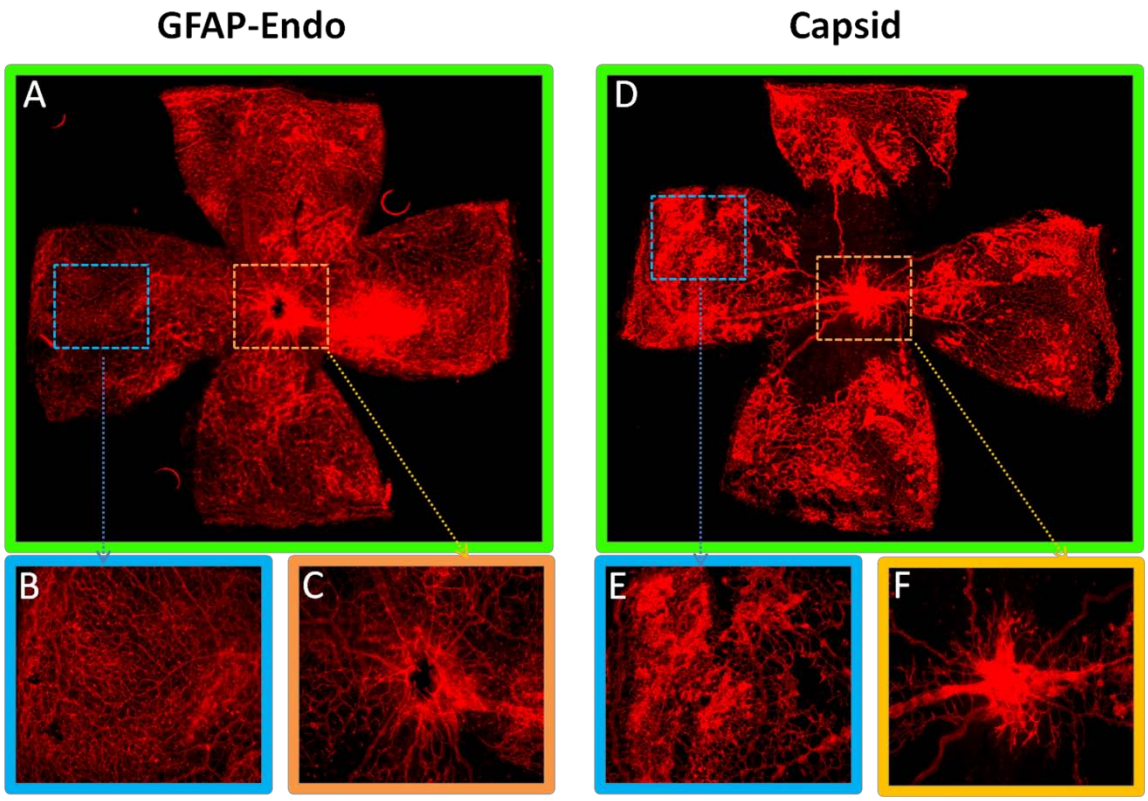

Figure 3. 6: Flat mounted retinas comparing GFAP-Endo with Capsid injected eye at P17. Retina from GFAP-Endo treated (A) eye shows reduction of central avascular area and peripheral neovascular area in comparison to contralateral capsid injected eye (D). B and E represent magnified view of peripheral neovascular area. C and F represent magnified view of central avascular area from each retina.

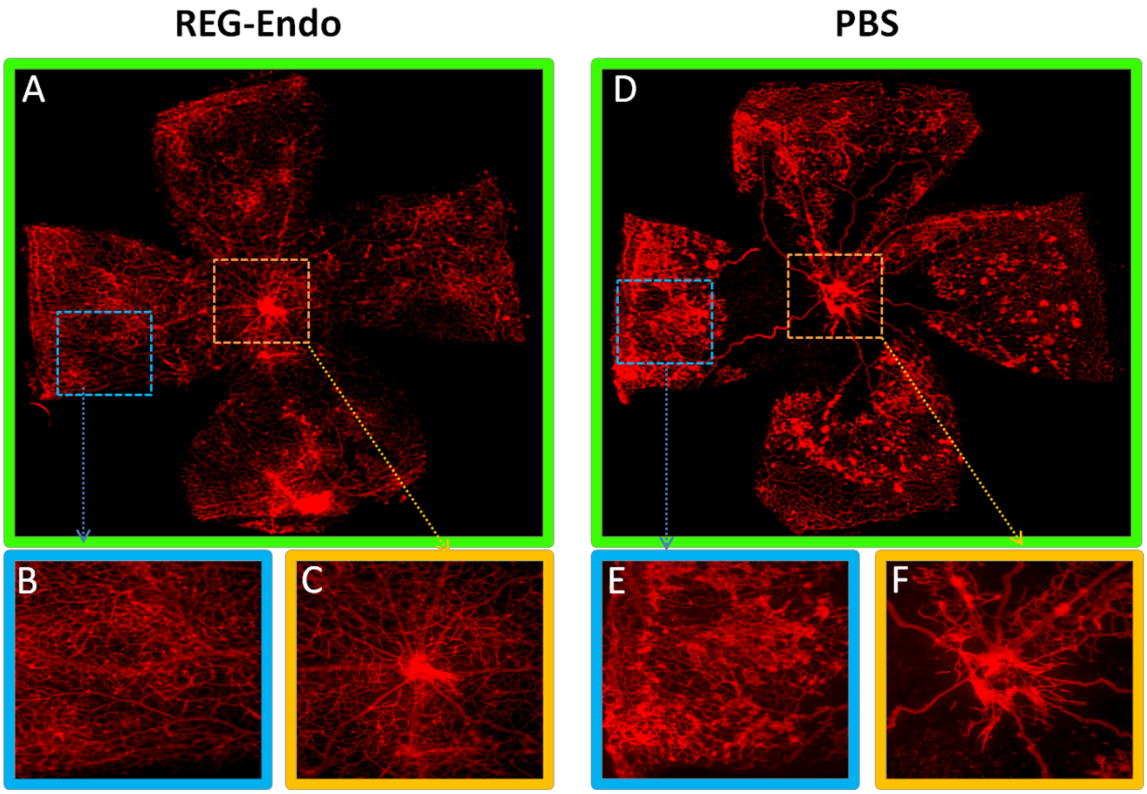

Figure 3. 7: Flat mounted retinas comparing REG-Endo with PBS injected eye at P17. Retina from REG-Endo treated (A) eye shows reduction of central avascular area and peripheral neovascular area in comparison to contralaeral PBS injected eye (D). B and E represent magnified view of peripheral neovascular area. C and F represent magnified view of central avascular area from each retina.

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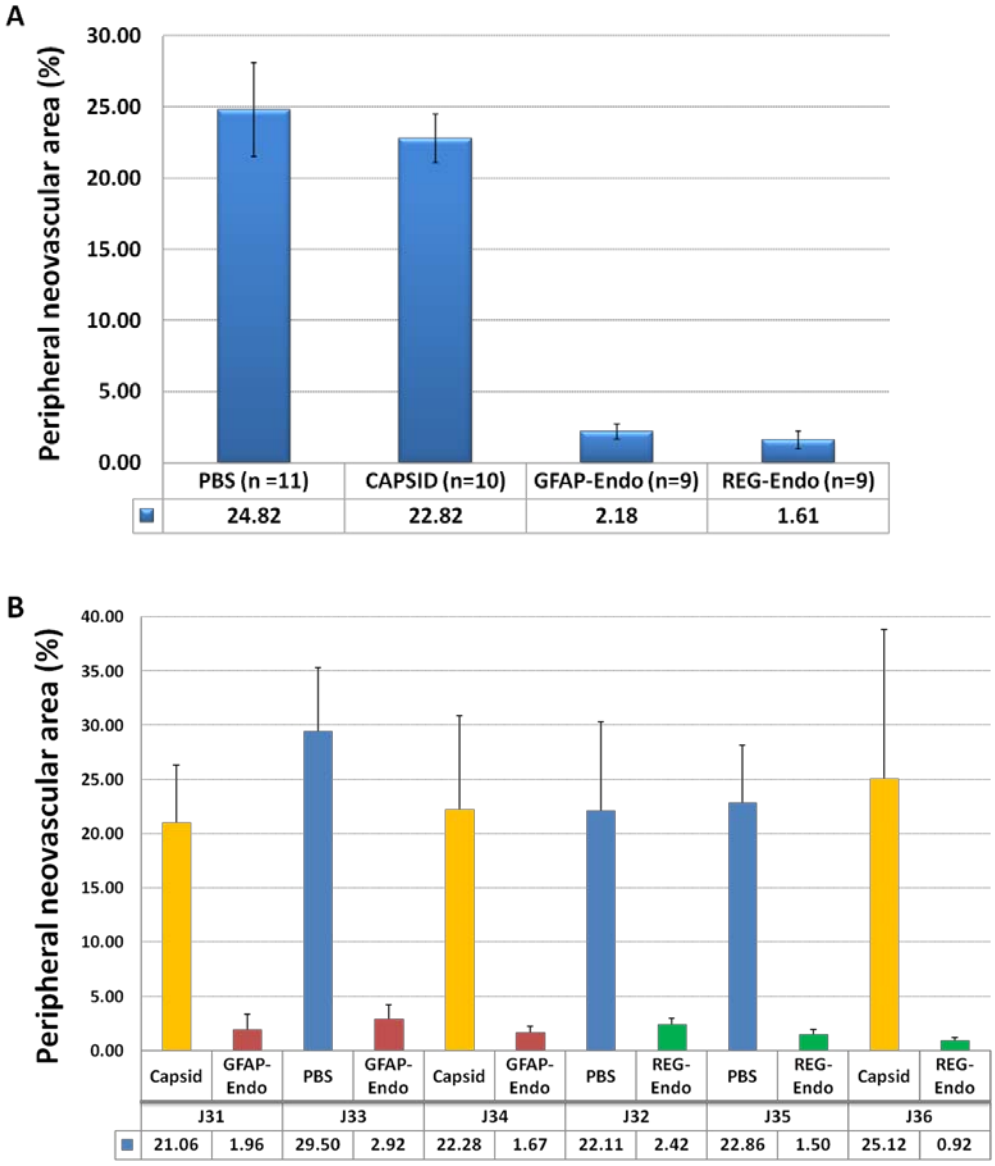

Figure 3. 8 : Reduction of peripheral neovascularization. A) Comparison of neovascular area in mice treated with endostatin vector in one eye and the contralateral eye treated with either PBS or Capsid. B) Comparison of average reduction in each mouse injected with endostatin vector in one eye and Capsid or PBS in contralateral eye. (Yellow bars: Capsid treated eyes; red bars: GFAP-Endo treated eyes; blue bars: PBS treated eyes; green bars: Reg-Endo treated eyes)

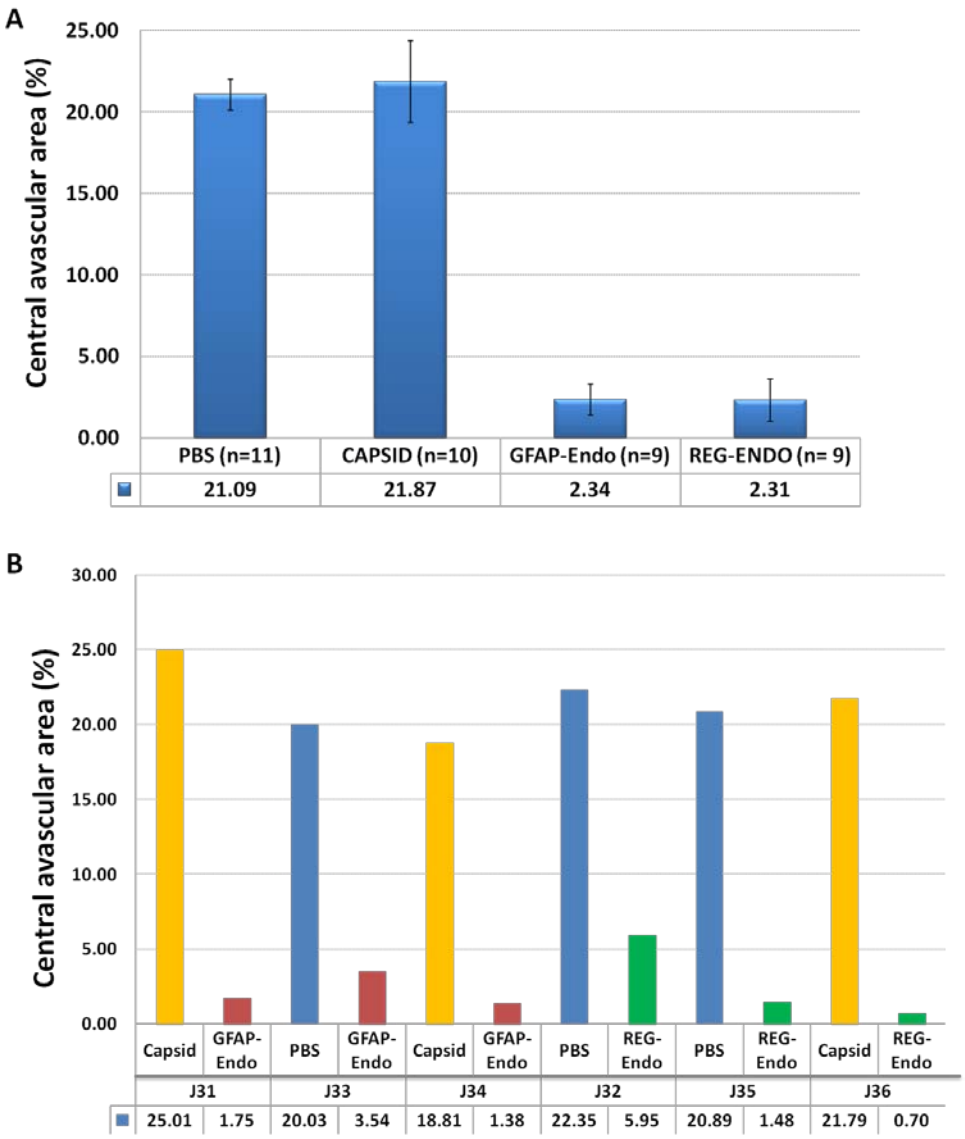

Figure 3. 9: Reduction of central avascular area. A) Comparison of central avascular area in each mouse treated with endostatin vector in one eye and contralateral eye is treated with either PBS or Capsid. B) Comparison of average reduction in each mouse injected with endostatin vector in one eye and Capsid or PBS in the contralateral eye. (Yellow bars: Capsid treated eyes; red bars: GFAP-Endo treated eyes; blue bars: PBS treated eyes; green bars: Reg-Endo treated eyes)

Detection of exogenous endostatin: Western Blotting & ELISA

Protein was successfully isolated from the retinas following an intravitreal injection of experimental vector, PBS or capsid. The amount of protein measured by BCA assay was approximately 1-3mg/ml per retina. To achieve sufficient amounts of protein for analysis, two retinas were pooled together for one data point. Western analysis of the retinal lysate demonstrated the 22 kDa endostatin band as detected by flag antibody (Figure 3.10). ELISA of human endostatin also confirmed exogenous expression of endostatin in eyes injected with either the scAAV2-GFAP-Endo or scAAV2-REG-Endo vector.

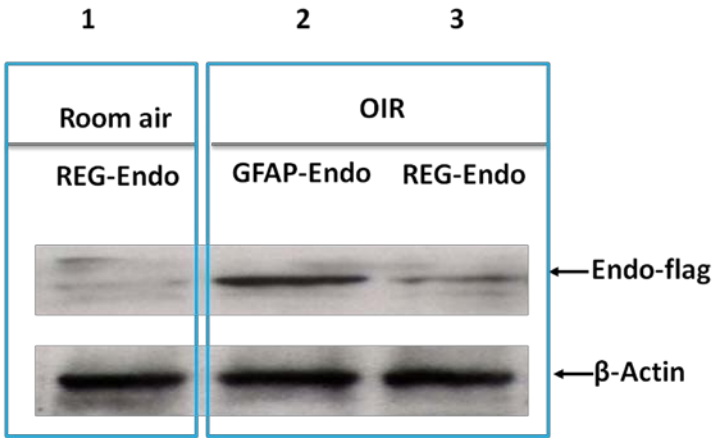

Figure 3. 10: Detection of flag tagged exogenous endostatin in mouse retina at P17: Lane 1 shows that there was no expression of endostatin in room air retina by the regulated vector as detected by flag antibody. Lane 2 and lane 3 indicate expression of endostatin in OIR retinas by both vectors as detected by flag antibody.

GFAP driven endostatin expression was detected both in retinal lysates from room air (2.36 ± 0.4 ng/mg) and OIR P17 (4.13 ± 0.3 ng/mg) mice. ELISA data confirm the increased expression of endostatin (2.74 ± 0.8 ng/mg) on day 17 by the regulated vector in OIR mice compared to room air (0.2 ± 0.3ng/mg) (figure 3.11). There was approximately 13- fold induction of exogenous endostatin in OIR retinas compared to room air retinas injected with regulated vector, a difference comparable with our *in vitro* data. No significant difference in was observed between endostatin levels w room air controls injected with regulated vector compared to PBS injected OIR retinas.

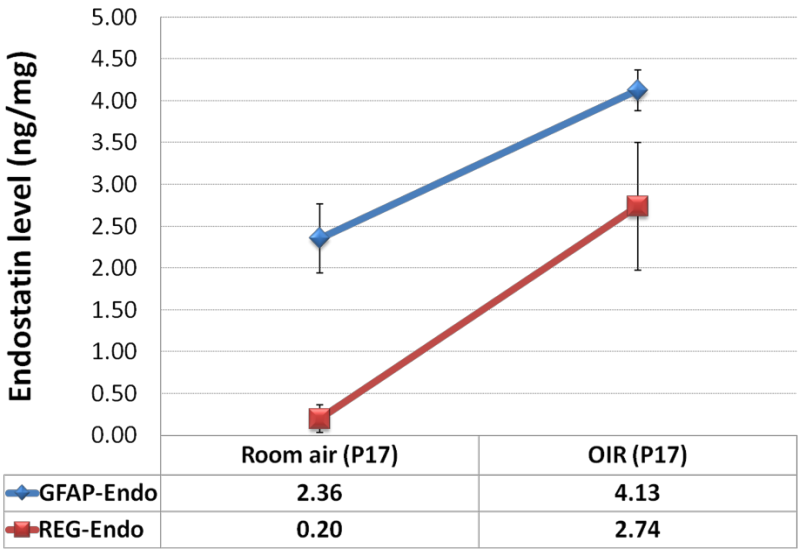

Figure 3. 11: Endostatin expression by vectors (ELISA). Significant reduction in endostatin levels was observed in retinas from eyes injected with the REG-Endo vector and left in room air compared to OIR mice receiving the GFAP-Endo vector. However, the endostatin level by the REG-Endo vector was dramatically increased (approximately by 13-fold) in the OIR retina compared to controls raised in room air.

Reduction of VEGF levels in OIR retina

VEGF levels were quantified from both normal room air and high oxygen treated animals. ELISA data shows that there is a significant increase in VEGF levels in mice exposed to high oxygen (365 ± 20 pg/mg) compared to room air animals (85 ± 23 pg/mg) injected with PBS in P17 mouse eyes (Figure 3.12). Intravitreal injection of either GFAP-Endo (144± 28 pg/mg) or REG- Endo (153 ± 38 pg/mg) significantly reduces VEGF levels in comparison to PBS treated eyes (365 ± 20 pg/mg) in the OIR model. No significant difference was observed between VEGF levels in eyes treated with either GFAP-Endo or REG- Endo vectors.

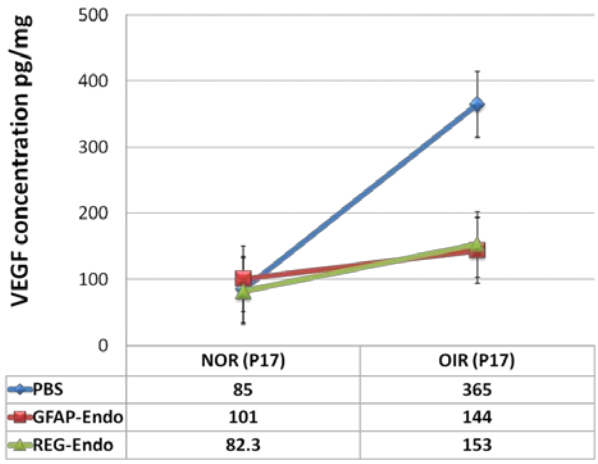

Figure 3. 12: Reduction in VEGF levels measured by ELISA. VEGF levels were significantly increased in retinas from PBS treated OIR mice at P17 compared to room air controls. There was also a significant reduction in VEGF levels in retinas treated with GFAP-Endo or REG-Endo compared to PBS controls from OIR mice at P17.

CHAPTER-4: DISCUSSION

The need for new therapy to treat diabetic retinopathy.

The progression of diabetes can include diabetic retinopathy which is the leading cause of blindness in working age populations in the US (Crosby-Nwaobi et al., 2012, Sivaprasad et al., 2012a, Sivaprasad et al., 2012b). Hyperglycemia is the primary cause of capillary degeneration and this leads to proliferative neovascularization on the retinal surface (Yu and Lyons, 2005, Devi et al., 2012). Both oxidative stress and inflammation contribute to the severity of diabetic retinopathy (Yu and Lyons, 2005). Photocoagulation and vitrectomy are standard treatments which are aimed at increasing oxygenation and removing vitreal haemorrhages respectively. However, neither of these treatments treat the pathophysiology. In diabetic retinopathy, there is impairment in the balance between pro- and anti-angiogenic factors. VEGF, a potent angiogenic factor, plays an important role in proliferative neovascularization. Intravitreal injection of anti-VEGF agents has been used to reduce VEGF levels in the diabetic retina. These agents may help to maintain the balance between VEGF and anti-angiogenic factors. For the best therapeutic results, patients need to have multiple injections. Unfortunately, these agents are not cost effective and also multiple intra ocular injections may increase the possibility of inflammation and future vision problems.

Currently, there is no effective treatment for diabetic retinopathy. A novel treatment strategy for diabetic retinopathy would be to deliver an angiostatic molecule to a specific cell type and to regulate transgene expression according to the tissue microenvironment. Which retinal cell type to target for gene therapy and how to regulate production of the therapeutic transgene product are important questions to be answered. The major retinal cell types include neurons (photoreceptors, ganglion, amacrine, bipolar and horizontal cells), glia (astrocytes and Müller cells) and RPE. RPE cells are located over 300 microns from the site of neovascularization in diabetic retinopathy which occurs at the border between the inner limiting membrane (ILM) and the vitreous. Müller cells extend from the outer (scleral) retina to the vitreal surface where Müller end feet make up the ILM. Our proposed hypoxia-regulated, cell-specific gene transfer will target Müller cells, the major glial cell type in retina.

Therapeutic strategy to prevent neovascularization in diabetic retinopathy

The goal of our study is to develop a gene therapy strategy aimed at preventing retinal neovascularization at its initial stages. An important concern in the design of an optimal gene therapy vector is that the foreign gene product should be expressed only in the location it is needed: in diseased tissue, but not in normal tissue, and at the appropriate time. Sustained expression of transgenes might have unwanted effects including the possibility of tissue toxicity in the long term (Toniatti et al., 2004). Furthermore, levels of expression of a therapeutic protein may need to be optimized, especially if the protein is intracellular (Pachori et al., 2004, Li et al., 2006). The ability of a particular promoter to control the bioavailability of a therapeutic gene product will

depend on the strength of the promoter when it is activated in a specific cell type. In designing the regulated and cell-specific vector, we chose to employ hypoxia responsive elements (HREs) (see page 11 for details) to take advantage of altered oxygen levels in the retina and a cell-specific promoter to target a specific cell type.

Consideration of oxygen levels in the retina

Incorporating cell specific promoters, as well as HRE promoters, is important for hypoxia-regulated gene therapy in the retina because it is known that oxygen concentrations and oxygen requirements vary remarkably across the retina. The soma of Müller cells extend from the deep retinal capillary bed (where oxygen tension in the rat is ~52 mm Hg or approximately 8% oxygen) through a "hypoxic" region in the inner retina of ~10 mm Hg (approximately 1.5% oxygen), to the inner limiting membrane where Müller cell end feet reside at about 73 mm Hg (approximately 11% oxygen) (Yu et al., 1994). In studies from Linsenmeier's group it was shown that O_2 concentrations at the level of photoreceptor inner segments were very low in dark adapted retina and slightly higher in light adapted retina (Wangsa-Wirawan and Linsenmeier, 2003). Hypoxia at this location is likely to be accompanied by hypoxia at the outermost Müller cell processes.

Importance of hypoxia responsive elements for retinal gene therapy

Local tissue hypoxia (Penn et al., 1994, Dorey et al., 1996) and inflammation (Joussen et al., 2002, Joussen et al., 2004, Zhang et al., 2006b, Kern, 2007) are associated with retinal pathologies that involve neovascularization (Chan-Ling et al., 1995, Stone et al., 1995, Zhang et al., 2003). In RCS rats, these stresses result in loss of photoreceptor

cells (Yu and Cringle, 2005). Hypoxia activates the HIF-1 transcription factor which then translocates into the nucleus and binds to the HRE regions found in the promoter region of several oxygen sensitive genes resulting in activation of transcription.

Hypoxic induction of exogenous gene products was previously shown in the retina using vectors that contain an HRE promoter, but expression was not cell specific (Bainbridge et al., 2003). The HRE promoters used in the original study lacked transcriptional repression in normoxia and thus both normoxic and hypoxic regions were treated. It is important to note that additional stimuli other than hypoxia have been shown to induce HIF-1 activity. Indeed, reactive oxygen species have been shown to activate HIF-1 via oxidation responsive domains on the HIF-1 protein (Wang and Semenza, 1993).

Inflammatory factors including interleukin1 beta (IL-1beta) and tumor necrosis factor (TNF-α and TNF-β) have been found to elicit responses through activation of HIF-1 (Jung et al., 2003, Zhou et al., 2003, Zhou et al., 2004, McMahon et al., 2006). The level of HIF-1 plays a critical role for activation of our regulated vector. It is important to choose a model where we know there will be enough hypoxia to turn on expression of the vector. An investigation by Del Niro et al (2010) showed that HIF-1 staining intensity was significantly elevated (by 31-fold) in the OIR model compared to mice developing in normoxia. Based on this information, we chose the OIR model to test the activation of our regulated promoter.

Hypoxia sensing by the HRE containing promoter in Müller cells

Cellular sensing of hypoxia depends on input from different parts of the Müller cell which are influenced by localized hypoxia. The biological outcome or actions of effector responses may not be restricted to specific parts of the cell but may be more general. Because of regional variations in levels of hypoxia in retinal tissue, cell-specific HRE vectors may demonstrate dynamic fluctuations in topographical expression patterns across the retina. An implication from our data is that the degree of activation of our promoter is likely to be influenced by the extent to which the Müller cell cytoplasm is exposed to hypoxic conditions. Furthermore the Müller cell may experience relative hypoxia where there is an imbalance between the amount of oxygen needed compared to the amount of oxygen being delivered to the tissue.

It is not certain whether the time course for the activated cellular phenotype is identical to that of induction of hypoxic responses. To confirm the cell specificity of our vector, the GFAP promoter was used along with hypoxia-regulated elements. In the event of exposure to hypoxia or oxidative stress, our HRE-driven GFAP promoter should provide finely tuned transcriptional activation that may show a pattern distinct from the GFAP induction associated with Müller cell activation. The question arises as to how our vector will be activated in Müller cells and astrocytes in the retina. As Müller cells express low levels of GFAP in normal retina, it is possible that our regulated promoter will be activated by brief transient hypoxic signals in the absence of endogenous GFAP induction (Sarthy et al., 1991). By contrast, astrocytes in the normal retina express GFAP

constitutively. Hence using our vector, foreign gene expression in retinal astrocytes would be silenced in normoxia but activated in hypoxia.

In vitro data confirm normoxia silencing and cell-specific hypoxic induction.

Our data confirmed Müller cell specificity of the ABC1D region of the GFAP promoter in the transfected human Müller cell line (MIO-M1) and in mouse primary Müller cells (Figure 3.1). The luciferase activity produced by the GFAP promoter used in this study was negligible in non-glial cell types (i.e., human embryonic kidney cell line). We also confirmed the glial specificity of the promoter in primary rat astrocytes. While Brenner et. al. 2008 reported that ABC1D was astrocyte-specific in transgenic mice, they also demonstrated that hybrid enhancers containing sub-regions of the GFAP promoter were more active than the full length (-2163) GFAP promoter due to removal of inhibitory domains (Lee et al., 2008a).

By employing our hypoxia-regulated, GFAP promoter, we were able to achieve low level expression under silenced, normoxic conditions in Müller cells and astrocytes (Figure 3.2) while ensuring high level transcriptional induction in hypoxia (Figure 3.3). Our promoter resulted in induction of 12-16 fold reporter activity in hypoxic cells compared to cells in normoxia. The regulated promoter was also inactive in other non-glial cell types (ARPE-19, C6, PC12, HEK etc.) in hypoxia. Our *in vitro* result is comparable (in terms of hypoxia regulation and cell specificity) to the hypoxia-regulated RPE specific plasmids in which Dougherty et al (2008) showed approximately a 40 fold induction of reporter gene expression in hypoxic ARPE-19 cells. The difference in the

degree of reporter gene expression in the two cell types may be linked to oxygen availability. Since RPE cells reside in high oxygen, it is possible that they experience more severe cellular hypoxia than Muller cells.

In vivo data confirm Müller cell-specificity and hypoxic induction

The OIR model was used to test HRE-driven GFP expression in mouse retina (Bainbridge et al., 2003). P17 Retinas from the OIR model are known to differ from normal retinas in terms of development of the retinal vasculature. High oxygen exposure (during P7-P12) resulted in regression of the normal retinal vasculature; upon return to room air, the regions of the retina now lacking sufficient vascular supply become hypoxic, which results in expression of pro-angiogenic factors and neovascularization. Our retinal flat mount data (Figure 3.5A & B) confirmed the presence of the central avascular zone, as well as peripheral neovascular tufts reported in the literature (Smith et al., 1994, Connor et al., 2009, DeNiro et al., 2010).

Since the hypoxic period starts after day 12 in the OIR model (Smith et al., 1994, Connor et al., 2009) , it was expected that the promoter would not be active until after P12. The vector should be activated by hypoxic induction. Induction of GFP expression was demonstrated in Müller cells of P17 oxygen-exposed retinas injected with the regulated vector on P7, while GFP expression was silenced in P17 retina from mice raised in room air (Figure 3.5 E). Although GFP expression was localized predominantly in Müller cells in retinas from P17 mice using the OIR model, the possibility of GFP expression in some retinal astrocytes near the border of the inner limiting membrane

cannot be excluded. Our results suggest that our GFAP promoter is more effective at rapid activation of Müller cells compared to previous results using other Müller cell specific vectors (Greenberg et al., 2007, Lamartina et al., 2007, Koerber et al., 2009, Aartsen et al., 2010).

Efficient and selective Müller cell specific transduction was achieved using a modified AAV variant ShH10, closely related to AAV serotype 6 (AAV6), capable of efficient, selective Müller cell transduction following intravitreal injection (Klimczak et al., 2009). A modified helper dependent adenovirus was used to analyze GFP expression in rat Müller cells (Lamartina et al., 2007). AAV2/6 mediated GFP expression by a 2.6 kb mouse GFAP promoter resulted in consistent but low level expression in Müller cells (Aartsen et al., 2010). A lentivirus vector with a CD44 promoter (1.8kb) or GFAP promoter (2.6kb) was used to target Müller cell specific GFP expression in rat retina. To our knowledge, no one has shown regulation of Müller cells in normal compared to diseased retinas. It is important to show inactivation of the promoter in normal conditions to avoid sustained gene expression. Our data suggest that the vector using a GFAP promoter can be regulated to turn on and off in response to retinal oxygen concentration (Figure 3.5F).

Endostatin delivered by our regulated vector reduces retinal neovascularization

Our hypoxia-regulated, glial-cell specific promoter produced endostatin in the OIR model and reduced neovascularization in retina. Administering recombinant endostatin protein stops neovascularization but this approach in the clinic is difficult due

to the high cost of production and short half life of the protein *in vivo* . To maintain therapeutic levels, several gene transfer methods have been tested. Sustained release of endostatin by an Adeno vector was first studied in the mouse choroidal neovascularization model (CNV) (Mori et al 2001). Later the same group used the VMD2- RPE specific promoter with flag tagged endostatin to inhibit CNV in the same model (Kachi et al., 2009). It is clear that modifying the 3' end of endostatin by adding the flag tag does not affect the activity of endostatin. The vector we designed was successful in reducing neovascularization, thus verifying the exogenous expression of endostatin using a flag antibody (Figure 3.10).

Quantification of the neovascular area in a retinal flat mount was the technique used in several studies to assess anti-NV treatment in the OIR model (Banin et al., 2006, Higuchi et al., 2008, Bartoli et al., 2009, Abdelsaid et al., 2010, Stahl et al., 2010, Zaniolo et al., 2011). In an OIR retina, the new vessels on the retinal surface (pre-retinal vessels) grow into the vitreous. Counting the pre-retinal endothelial cells on the retinal border from retinal sections is another technique to assess NV (Auricchio et al., 2002, Raisler et al., 2002). A comparison of quantification of the neovascular area using flat mounts versus counting endothelial cells from retinal sections provided similar results (Banin et al., 2006, Liang et al., 2012b). Therefore, we used flat mounts to quantify the central avascular zone and peripheral neovascular area (Connor et al., 2009). AAV mediated expression of endostatin in an OIR model reduced pre-retinal endothelial nuclei by 86% compared to control eyes (Auricchio et al., 2002). Endostatin delivery by an adeno vector also reduced endothelial cell nuclei by 75% in an OIR model (Le Gat et al., 2003).

Therefore, our results are comparable with other studies using endostatin as an anti-angiogenic factor since our vector was able to reduce the avascular area by 90% (Figure 3.9) and the neovascular area by 93% on P17 (Figure 3.8).

There is no doubt that exogenous endostatin expression reduced the central avascular area in the OIR retina (Figure 3.6 and Figure 3.7). It has been suggested that endostatin is involved in vascular remodeling by decreasing pathologic neovascularization and promoting physiological angiogenesis (Campochiaro and Hackett, 2003, Duh et al., 2004). Further research is required to understand how Müller cell-targeted endostatin production contributes to vascular remodeling in the OIR retina.

Pathological neovascularization depends upon the balance of angiogenic and angiostatic factors. For this reason, it is important to maintain the balance by providing exogenous angiogenic inhibitors. It is necessary to know the levels of angiogenic inhibitors required to shift the balance in favor of pathological angiogenesis. High levels of an inhibitory protein may not be needed to provide a substantial effect. Our regulated vector was able to produce approximately 2- 3ng of endostatin from 1mg of retina homogenate (Figure 3.11). The expression level of endostatin by a CMV promoter was approximately 10-14 ng compared to 1-2 ng from the VMD2-RPE specific promoter previously reported in the same model (Kachi et al., 2009). Safety and biodistribution studies using lentivirus-mediated endostatin (Retinostat, Oxford Biomedica) delivery in the rabbit neovascularization model produced approximately 600ng of endostatin (Binley et al., 2012).

Although the quantity of endostatin produced by our vector was sufficient to reduce NV, the low level of production may be attributed to the use of intravitreal injection and the number of transduced Müller cells. It could be predicted that most of the secretion of endostatin from the Müller cells into the vitreous occurs as NV develops at ILM in the OIR model. However it is not clear whether secretion from Müller cell end feet to the vitreous or, alternatively, secretion from the Müller cell body to the retinal layers, is optimal for stopping NV in response to tissue hypoxia. Further studies involving a comparison of the endostatin levels between the vitreous fluid and retina homogenates is necessary to determine the exact site of endostatin delivery. It is also important to remember that endostatin is a secreted protein with a shorter half life than other therapeutic proteins.

Reduction of VEGF levels in retina

In our model, VEGF levels were reduced in eyes receiving intravitreal delivery of endostatin compared to control eyes. VEGF is a significant contributor to retinal neovascularization. Although we did not find an absolute reduction of VEGF levels, the amount of reduction in VEGF due to exogenous endostatin expression was sufficient to produce a therapeutic effect. VEGF is also required for normal angiogenesis and complete inhibition may have deleterious effects as reported for other anti-VEGF agents. Our results are comparable to those for other agents used to reduce VEGF levels. Like others we found that there is a significant increase in VEGF levels in the retinas of the OIR mouse compared to mice kept in room air (Figure 3.12). In the OIR model we found approximately 60% reduction in VEGF levels in endostatin treated retinas. The

amount of VEGF produced in our OIR model was comparable with that of other studies (Huang et al., 2011).

Uniqueness or advantages of our regulated and cell specific vector

Gene therapy approaches in number of diseases are likely to benefit from targeted expression to specific cell types and also regulated expression that enables one to switch on transgene expression when it is needed and to switch off transgene expression when it is not required. Regulated therapy can be achieved by use of a drug responsive promoter (as in Tet-on) or by use of a promoter that responds to an endogenous stimulus, such as blood glucose levels, oxidative stress, inflammatory processes or hormone levels. Hypoxia is one endogenous stimulus that has proved to be an appropriate stimulus for several gene therapy strategies including anti-cancer approaches and treatment for ischemic disorders such as peripheral limb ischemia, ischemic heart disease and stroke.

In our strategy, Müller cell specificity of the promoter has important implications because the Müller cell traverses the width of the retina so it experiences a distinct temporal pattern of hypoxic exposure. The activation of our promoter is likely to differ in astrocytes and Müller cells. Astrocytes may experience different levels of hypoxia than the Müller cells and, therefore, the response in these two cell types could be different both in timing and in levels of activation. Moreover our promoter might be activated under oxidative conditions known to stimulate apoptosis in the diabetic retina (Kowluru et al., 1997, Kowluru, 2005, Kowluru et al., 2006).

It is essential to use sustained expression of a transgene to target a genetic disease in which large amounts of therapeutic protein may be needed to correct a gene defect. However others have suggested sustained delivery of a transgene may lead to tissue toxicity overtime (Toniatti et al., 2004). Regulated gene therapy has advantages over sustained gene expression in diseases where a small amount of transgene expression may be needed to produce significant therapeutic effects. There is a possibility that a patient diagnosed at the age of 40 who receives classic gene therapy treatment with a universal promoter like CMV, will have increased production of the therapeutic gene throughout their life, which may not be needed. If the transgenes are regulated in response to the pathologic state of surrounding tissue, it can be effective during the period of the disease and once the disease progression is stabilized, the gene expression will be turned off. If, in the future, the patient has a recurrence of the pathology then the expression will turn on.

One or two injections of our vector may be sufficient to produce the desired therapeutic effect throughout the disease period. This approach will reduce the cost of treatment, as well as reduce inflammation and vision complications compared to continuous injections of anti-angiogenic agents in patients.

Future directions

Vectors that successfully express endostatin lead to inhibition of retinal neovascularization and reduced VEGF levels. Despite its success, it is necessary to test other issues. It is not clear yet whether endostatin expression by a regulated vector would

restore retinal structure and function once the neovascularization is treated. Studies should be initiated to monitor the rescue of photoreceptor cells, measuring the width of different retinal layers and performing electroretinographic testing (the ERG) to assess proper functioning of the retina (Liang et al., 2012a, Liang et al., 2012b, Mowat et al., 2012).

The regulated vector could be tested in other preclinical models including the streptozotocin induced rat model of diabetic retinopathy (Penn et al., 2001, Ideno et al., 2007, Lamartina et al., 2007, Zhang et al., 2009) and the dog model of diabetic retinopathy (Frank, 1995, Lutty and McLeod, 2003, Lutty et al., 2011). Established models including laser induced choroidal neovascularization in the mouse (Lai et al., 2001, Mori et al., 2002) can be considered for monitoring gene expression by the regulated vector. Pigment epithelial derived factor (PEDF) which reduces neovascularization and inflammation (Park et al., 2011) and acts as a neuroprotective factor (Miyazaki et al., 2011) could be delivered to different neovascularization models using our regulated promoter. Studies of other angiogenic and growth factors in hypoxic Müller cells could also be initiated using our vector. Our vector should also be tested for activation by other stimuli such as oxidative stress or inflammation. Although our vector successfully inhibited neovascularization within a short period of time, the effectiveness in ensuring long term transgene expression still requires investigation.

Our regulated vector was activated in astrocytes in response to hypoxia. Long term gene therapy targeting astrocytes could be studied in models of stroke and glioma in which astrocytes are affected by tissue hypoxia to promote neuronal survival and reduce

tumor angiogenesis, respectively. Overall our strategy may provide the ground work for future gene therapy clinical trials.

Conclusion

There are requirements for exogenous gene transfer in a regulated manner to target a specific cell type to treat retinal diseases. The activation of a transgene in response to tissue microenvironment should have advantages compared to sustained expression. The major findings of our study are the development of tools which lead to dramatic reduction of peripheral neovascularization and central vaso-obliteration in the OIR mouse model. This was achieved by using a hypoxia-regulated, retinal glial cell specific promoter driving the expression of the human endostatin gene. Intravitreal injection of the regulated vector on P7 allowed more than 90% reduction of peripheral neovascularization. Our results demonstrated the effect of exogenous endostatin on VEGF expression levels in retina. These results suggest that the localized effect of endostatin in response to tissue ischemic conditions has an effect on retinal neovascularization by reducing pathological angiogenesis and promoting physiological angiogenesis.

REFERENCES

Aartsen WM, van Cleef KW, Pellissier LP, Hoek RM, Vos RM, Blits B, Ehlert EM, Balaggan KS, Ali RR, Verhaagen J, Wijnholds J (2010).GFAP-driven GFP expression in activated mouse Muller glial cells aligning retinal blood vessels following intravitreal injection of AAV2/6 vectors. PLoS One 5:e12387.

Abdelsaid MA, Pillai BA, Matragoon S, Prakash R, Al-Shabrawey M, El-Remessy AB (2010).Early intervention of tyrosine nitration prevents vaso-obliteration and neovascularization in ischemic retinopathy. J Pharmacol Exp Ther 332:125-134.

Adamis AP, Altaweel M, Bressler NM, Cunningham ET, Jr., Davis MD, Goldbaum M, Gonzales C, Guyer DR, Barrett K, Patel M (2006).Changes in retinal neovascularization after pegaptanib (Macugen) therapy in diabetic individuals. Ophthalmology 113:23-28.

Adamis AP, Miller JW, Bernal MT, D'Amico DJ, Folkman J, Yeo TK, Yeo KT (1994).Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. Am J Ophthalmol 118:445-450.

Aiello LP (1997).Vascular endothelial growth factor and the eye: biochemical mechanisms of action and implications for novel therapies. Ophthalmic Res 29:354-362.

Aiello LP, Bursell SE, Clermont A, Duh E, Ishii H, Takagi C, Mori F, Ciulla TA, Ways K, Jirousek M, Smith LE, King GL (1997).Vascular endothelial growth factor-induced retinal permeability is mediated by protein kinase C in vivo and

suppressed by an orally effective beta-isoform-selective inhibitor. Diabetes 46:1473-1480.

Alexander MY, Webster KA, McDonald PH, Prentice HM (1999).Gene transfer and models of gene therapy for the myocardium. Clin Exp Pharmacol Physiol 26:661-668.

Antonelli-Orlidge A, Saunders KB, Smith SR, D'Amore PA (1989).An activated form of transforming growth factor beta is produced by cocultures of endothelial cells and pericytes. Proc Natl Acad Sci U S A 86:4544-4548.

Auricchio A, Behling KC, Maguire AM, O'Connor EM, Bennett J, Wilson JM, Tolentino MJ (2002).Inhibition of retinal neovascularization by intraocular viral-mediated delivery of anti-angiogenic agents. Mol Ther 6:490-494.

Bai Y, Ma JX, Guo J, Wang J, Zhu M, Chen Y, Le YZ (2009).Muller cell-derived VEGF is a significant contributor to retinal neovascularization. J Pathol 219:446-454.

Bainbridge JW, Mistry A, Binley K, De Alwis M, Thrasher AJ, Naylor S, Ali RR (2003).Hypoxia-regulated transgene expression in experimental retinal and choroidal neovascularization. Gene Ther 10:1049-1054.

Bainbridge JW, Mistry A, De Alwis M, Paleolog E, Baker A, Thrasher AJ, Ali RR (2002).Inhibition of retinal neovascularisation by gene transfer of soluble VEGF receptor sFlt-1. Gene Ther 9:320-326.

Bainbridge JW, Stephens C, Parsley K, Demaison C, Halfyard A, Thrasher AJ, Ali RR (2001).In vivo gene transfer to the mouse eye using an HIV-based lentiviral vector; efficient long-term transduction of corneal endothelium and retinal pigment epithelium. Gene Ther 8:1665-1668.

Bandello F, Cunha-Vaz J, Chong NV, Lang GE, Massin P, Mitchell P, Porta M, Prunte C, Schlingemann R, Schmidt-Erfurth U (2012).New approaches for the treatment

of diabetic macular oedema: recommendations by an expert panel. Eye (Lond) 26:485-493.

Banin E, Dorrell MI, Aguilar E, Ritter MR, Aderman CM, Smith AC, Friedlander J, Friedlander M (2006).T2-TrpRS inhibits preretinal neovascularization and enhances physiological vascular regrowth in OIR as assessed by a new method of quantification. Invest Ophthalmol Vis Sci 47:2125-2134.

Bartoli M, Al-Shabrawey M, Labazi M, Behzadian MA, Istanboli M, El-Remessy AB, Caldwell RW, Marcus DM, Caldwell RB (2009).HMG-CoA reductase inhibitors (statin) prevents retinal neovascularization in a model of oxygen-induced retinopathy. Invest Ophthalmol Vis Sci 50:4934-4940.

Bek T (1997a).Glial cell involvement in vascular occlusion of diabetic retinopathy. Acta Ophthalmol Scand 75:239-243.

Bek T (1997b).Immunohistochemical characterization of retinal glial cell changes in areas of vascular occlusion secondary to diabetic retinopathy. Acta Ophthalmol Scand 75:388-392.

Beltran WA, Cideciyan AV, Lewin AS, Iwabe S, Khanna H, Sumaroka A, Chiodo VA, Fajardo DS, Roman AJ, Deng WT, Swider M, Aleman TS, Boye SL, Genini S, Swaroop A, Hauswirth WW, Jacobson SG, Aguirre GD (2012).Gene therapy rescues photoreceptor blindness in dogs and paves the way for treating human X-linked retinitis pigmentosa. Proc Natl Acad Sci U S A 109:2132-2137.

Bennett J, Ashtari M, Wellman J, Marshall KA, Cyckowski LL, Chung DC, McCague S, Pierce EA, Chen Y, Bennicelli JL, Zhu X, Ying GS, Sun J, Wright JF, Auricchio A, Simonelli F, Shindler KS, Mingozzi F, High KA, Maguire AM (2012).AAV2 gene therapy readministration in three adults with congenital blindness. Sci Transl Med 4:120ra115.

Berges RR, Pientka L (1999).Management of the BPH syndrome in Germany: who is treated and how? Eur Urol 36 Suppl 3:21-27.

Bhavsar AR (2006).Diabetic retinopathy: the latest in current management. Retina 26:S71-79.

Bhavsar AR, Googe JM, Jr., Stockdale CR, Bressler NM, Brucker AJ, Elman MJ, Glassman AR (2009).Risk of endophthalmitis after intravitreal drug injection when topical antibiotics are not required: the diabetic retinopathy clinical research network laser-ranibizumab-triamcinolone clinical trials. Arch Ophthalmol 127:1581-1583.

Bhavsar AR, Tornambe PE (2006).25 years of progress in the treatment of retinal diseases: where we have been, where we are now, and where we will be. Retina 26:S1-6.

Binley K, Widdowson PS, Kelleher M, de Belin J, Loader J, Ferrige G, Leroux-Carlucci MA, Esapa M, Chipchase D, Angell-Manning D, Ellis S, Mitrophanous K, Miskin JE, Bantseev V, Nork TM, Miller P, Naylor S (2012).Safety and Biodistribution of an EIAV-based Gene Therapy, RetinoStat(R), for Age-related Macular Degeneration. Hum Gene Ther.

Boulton M, Gregor Z, McLeod D, Charteris D, Jarvis-Evans J, Moriarty P, Khaliq A, Foreman D, Allamby D, Bardsley B (1997).Intravitreal growth factors in proliferative diabetic retinopathy: correlation with neovascular activity and glycaemic management. Br J Ophthalmol 81:228-233.

Bringmann A, Pannicke T, Grosche J, Francke M, Wiedemann P, Skatchkov SN, Osborne NN, Reichenbach A (2006).Muller cells in the healthy and diseased retina. Prog Retin Eye Res 25:397-424.

Bringmann A, Reichenbach A (2001).Role of Muller cells in retinal degenerations. Front Biosci 6:E72-92.

Brower V (2001).Endostatin "cell factories" shrink rodent brain tumors. Trends Mol Med 7:97-98.

Brucker AJ, Qin H, Antoszyk AN, Beck RW, Bressler NM, Browning DJ, Elman MJ, Glassman AR, Gross JG, Kollman C, Wells JA, 3rd (2009).Observational study of the development of diabetic macular edema following panretinal (scatter) photocoagulation given in 1 or 4 sittings. Arch Ophthalmol 127:132-140.

Calame M, Cachafeiro M, Philippe S, Schouwey K, Tekaya M, Wanner D, Sarkis C, Kostic C, Arsenijevic Y (2011).Retinal degeneration progression changes lentiviral vector cell targeting in the retina. PLoS One 6:e23782.

Campochiaro PA, Hackett SF (2003).Ocular neovascularization: a valuable model system. Oncogene 22:6537-6548.

Cao W, Tombran-Tink J, Elias R, Sezate S, Mrazek D, McGinnis JF (2001).In vivo protection of photoreceptors from light damage by pigment epithelium-derived factor. Invest Ophthalmol Vis Sci 42:1646-1652.

Chan-Ling T, Gock B, Stone J (1995).The effect of oxygen on vasoformative cell division. Evidence that 'physiological hypoxia' is the stimulus for normal retinal vasculogenesis. Invest Ophthalmol Vis Sci 36:1201-1214.

Chung EJ, Kang SJ, Koo JS, Choi YJ, Grossniklaus HE, Koh HJ (2011).Effect of intravitreal bevacizumab on vascular endothelial growth factor expression in patients with proliferative diabetic retinopathy. Yonsei Med J 52:151-157.

Connor KM, Krah NM, Dennison RJ, Aderman CM, Chen J, Guerin KI, Sapieha P, Stahl A, Willett KL, Smith LE (2009).Quantification of oxygen-induced retinopathy in

the mouse: a model of vessel loss, vessel regrowth and pathological angiogenesis. Nat Protoc 4:1565-1573.

Crosby-Nwaobi R, Heng LZ, Sivaprasad S (2012).Retinal Vascular Calibre, Geometry and Progression of Diabetic Retinopathy in Type 2 Diabetes Mellitus. Ophthalmologica.

DeNiro M, Al-Halafi A, Al-Mohanna FH, Alsmadi O, Al-Mohanna FA (2010).Pleiotropic effects of YC-1 selectively inhibit pathological retinal neovascularization and promote physiological revascularization in a mouse model of oxygen-induced retinopathy. Mol Pharmacol 77:348-367.

Devi TS, Lee I, Huttemann M, Kumar A, Nantwi KD, Singh LP (2012).TXNIP links innate host defense mechanisms to oxidative stress and inflammation in retinal Muller glia under chronic hyperglycemia: implications for diabetic retinopathy. Exp Diabetes Res 2012:438238.

Dhanabal M, Ramchandran R, Volk R, Stillman IE, Lombardo M, Iruela-Arispe ML, Simons M, Sukhatme VP (1999).Endostatin: yeast production, mutants, and antitumor effect in renal cell carcinoma. Cancer Res 59:189-197.

di Lauro R, De Ruggiero P, di Lauro MT, Romano MR (2010).Intravitreal bevacizumab for surgical treatment of severe proliferative diabetic retinopathy. Graefes Arch Clin Exp Ophthalmol 248:785-791.

Dixelius J, Cross M, Matsumoto T, Sasaki T, Timpl R, Claesson-Welsh L (2002).Endostatin regulates endothelial cell adhesion and cytoskeletal organization. Cancer Res 62:1944-1947.

Dorey CK, Aouididi S, Reynaud X, Dvorak HF, Brown LF (1996).Correlation of vascular permeability factor/vascular endothelial growth factor with extraretinal neovascularization in the rat. Arch Ophthalmol 114:1210-1217.

Dougherty CJ, Smith GW, Dorey CK, Prentice HM, Webster KA, Blanks JC (2008).Robust hypoxia-selective regulation of a retinal pigment epithelium-specific adeno-associated virus vector. Mol Vis 14:471-480.

Duh EJ, Yao YG, Dagli M, Goldberg MF (2004).Persistence of fetal vasculature in a patient with Knobloch syndrome: potential role for endostatin in fetal vascular remodeling of the eye. Ophthalmology 111:1885-1888.

Enge M, Bjarnegard M, Gerhardt H, Gustafsson E, Kalen M, Asker N, Hammes HP, Shani M, Fassler R, Betsholtz C (2002).Endothelium-specific platelet-derived growth factor-B ablation mimics diabetic retinopathy. EMBO J 21:4307-4316.

Erickson PA, Fisher SK, Guerin CJ, Anderson DH, Kaska DD (1987).Glial fibrillary acidic protein increases in Muller cells after retinal detachment. Exp Eye Res 44:37-48.

Fan W, Lin N, Sheedlo HJ, Turner JE (1996).Muller and RPE cell response to photoreceptor cell degeneration in aging Fischer rats. Exp Eye Res 63:9-18.

Feit-Leichman RA, Kinouchi R, Takeda M, Fan Z, Mohr S, Kern TS, Chen DF (2005).Vascular damage in a mouse model of diabetic retinopathy: relation to neuronal and glial changes. Invest Ophthalmol Vis Sci 46:4281-4287.

Fitzgerald ME, Vana BA, Reiner A (1990).Evidence for retinal pathology following interruption of neural regulation of choroidal blood flow: Muller cells express GFAP following lesions of the nucleus of Edinger-Westphal in pigeons. Curr Eye Res 9:583-598.

Fjord-Larsen L, Kusk P, Emerich DF, Thanos C, Torp M, Bintz B, Tornoe J, Johnsen AH, Wahlberg LU (2011).Increased encapsulated cell biodelivery of nerve growth factor in the brain by transposon-mediated gene transfer. Gene Ther.

Frank RN (1995).The galactosemic dog. A valid model for both early and late stages of diabetic retinopathy. Arch Ophthalmol 113:275-276.

Fukai N, Eklund L, Marneros AG, Oh SP, Keene DR, Tamarkin L, Niemela M, Ilves M, Li E, Pihlajaniemi T, Olsen BR (2002).Lack of collagen XVIII/endostatin results in eye abnormalities. EMBO J 21:1535-1544.

Geller SF, Ge PS, Visel M, Flannery JG (2008).In vitro analysis of promoter activity in Muller cells. Mol Vis 14:691-705.

Geller SF, Ge PS, Visel M, Greenberg KP, Flannery JG (2007).Functional promoter testing using a modified lentiviral transfer vector. Mol Vis 13:730-739.

Giuliari GP, Guel DA, Gonzalez VH (2009).Pegaptanib sodium for the treatment of proliferative diabetic retinopathy and diabetic macular edema. Curr Diabetes Rev 5:33-38.

Gonzalez VH, Giuliari GP, Banda RM, Guel DA (2009).Intravitreal injection of pegaptanib sodium for proliferative diabetic retinopathy. Br J Ophthalmol 93:1474-1478.

Greenberg KP, Geller SF, Schaffer DV, Flannery JG (2007).Targeted transgene expression in muller glia of normal and diseased retinas using lentiviral vectors. Invest Ophthalmol Vis Sci 48:1844-1852.

Halfter W, Dong S, Schurer B, Cole GJ (1998).Collagen XVIII is a basement membrane heparan sulfate proteoglycan. J Biol Chem 273:25404-25412.

Hammes HP, Lin J, Renner O, Shani M, Lundqvist A, Betsholtz C, Brownlee M, Deutsch U (2002).Pericytes and the pathogenesis of diabetic retinopathy. Diabetes 51:3107-3112.

Han B, Xiu Q, Wang H, Shen J, Gu A, Luo Y, Bai C, Guo S, Liu W, Zhuang Z, Zhang Y, Zhao Y, Jiang L, Zhou J, Jin X (2011).A multicenter, randomized, double-blind, placebo-controlled study to evaluate the efficacy of paclitaxel-carboplatin alone or with endostar for advanced non-small cell lung cancer. J Thorac Oncol 6:1104-1109.

Hanahan D, Christofori G, Naik P, Arbeit J (1996).Transgenic mouse models of tumour angiogenesis: the angiogenic switch, its molecular controls, and prospects for preclinical therapeutic models. Eur J Cancer 32A:2386-2393.

Harada T, Harada C, Kohsaka S, Wada E, Yoshida K, Ohno S, Mamada H, Tanaka K, Parada LF, Wada K (2002).Microglia-Muller glia cell interactions control neurotrophic factor production during light-induced retinal degeneration. J Neurosci 22:9228-9236.

Hauswirth WW, Aleman TS, Kaushal S, Cideciyan AV, Schwartz SB, Wang L, Conlon TJ, Boye SL, Flotte TR, Byrne BJ, Jacobson SG (2008).Treatment of leber congenital amaurosis due to RPE65 mutations by ocular subretinal injection of adeno-associated virus gene vector: short-term results of a phase I trial. Hum Gene Ther 19:979-990.

Higuchi A, Yamada H, Yamada E, Jo N, Matsumura M (2008).Hypericin inhibits pathological retinal neovascularization in a mouse model of oxygen-induced retinopathy. Mol Vis 14:249-254.

Hu HW, Li XK, Zheng RY, Xiao J, Zeng JQ, Hou ST (2009).bFGF expression mediated by a hypoxia-regulated adenoviral vector protects PC12 cell death induced by serum deprivation. Biochem Biophys Res Commun 390:115-120.

Huang H, Van de Veire S, Dalal M, Parlier R, Semba RD, Carmeliet P, Vinores SA (2011).Reduced retinal neovascularization, vascular permeability, and apoptosis in ischemic retinopathy in the absence of prolyl hydroxylase-1 due to the

prevention of hyperoxia-induced vascular obliteration. Invest Ophthalmol Vis Sci 52:7565-7573.

Ideno J, Mizukami H, Kakehashi A, Saito Y, Okada T, Urabe M, Kume A, Kuroki M, Kawakami M, Ishibashi S, Ozawa K (2007).Prevention of diabetic retinopathy by intraocular soluble flt-1 gene transfer in a spontaneously diabetic rat model. Int J Mol Med 19:75-79.

Jacobson SG, Cideciyan AV, Ratnakaram R, Heon E, Schwartz SB, Roman AJ, Peden MC, Aleman TS, Boye SL, Sumaroka A, Conlon TJ, Calcedo R, Pang JJ, Erger KE, Olivares MB, Mullins CL, Swider M, Kaushal S, Feuer WJ, Iannaccone A, Fishman GA, Stone EM, Byrne BJ, Hauswirth WW (2012).Gene therapy for leber congenital amaurosis caused by RPE65 mutations: safety and efficacy in 15 children and adults followed up to 3 years. Arch Ophthalmol 130:9-24.

Jiang B, Dong H, Zhang Z, Wang W, Zhang Y, Xu X (2007).Hypoxic response elements control expression of human vascular endothelial growth factor(165) genes transferred to ischemia myocardium in vivo and in vitro. J Gene Med 9:788-796.

Jiang BH, Rue E, Wang GL, Roe R, Semenza GL (1996).Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. J Biol Chem 271:17771-17778.

Joussen AM, Poulaki V, Le ML, Koizumi K, Esser C, Janicki H, Schraermeyer U, Kociok N, Fauser S, Kirchhof B, Kern TS, Adamis AP (2004).A central role for inflammation in the pathogenesis of diabetic retinopathy. FASEB J 18:1450-1452.

Joussen AM, Poulaki V, Tsujikawa A, Qin W, Qaum T, Xu Q, Moromizato Y, Bursell SE, Wiegand SJ, Rudge J, Ioffe E, Yancopoulos GD, Adamis AP (2002).Suppression of diabetic retinopathy with angiopoietin-1. Am J Pathol 160:1683-1693.

Jung YJ, Isaacs JS, Lee S, Trepel J, Neckers L (2003).IL-1beta-mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. FASEB J 17:2115-2117.

Kachi S, Binley K, Yokoi K, Umeda N, Akiyama H, Muramatu D, Iqball S, Kan O, Naylor S, Campochiaro PA (2009).Equine infectious anemia viral vector-mediated codelivery of endostatin and angiostatin driven by retinal pigmented epithelium-specific VMD2 promoter inhibits choroidal neovascularization. Hum Gene Ther 20:31-39.

Kachi S, Esumi N, Zack DJ, Campochiaro PA (2006).Sustained expression after nonviral ocular gene transfer using mammalian promoters. Gene Ther 13:798-804.

Kaur C, Sivakumar V, Foulds WS (2006).Early response of neurons and glial cells to hypoxia in the retina. Invest Ophthalmol Vis Sci 47:1126-1141.

Kempen JH, O'Colmain BJ, Leske MC, Haffner SM, Klein R, Moss SE, Taylor HR, Hamman RF (2004).The prevalence of diabetic retinopathy among adults in the United States. Arch Ophthalmol 122:552-563.

Kern TS (2007).Contributions of inflammatory processes to the development of the early stages of diabetic retinopathy. Exp Diabetes Res 2007:95103.

Kim IB, Kim KY, Joo CK, Lee MY, Oh SJ, Chung JW, Chun MH (1998).Reaction of Muller cells after increased intraocular pressure in the rat retina. Exp Brain Res 121:419-424.

Kim J, Hajjar KA (2002).Annexin II: a plasminogen-plasminogen activator co-receptor. Front Biosci 7:d341-348.

Kim YM, Jang JW, Lee OH, Yeon J, Choi EY, Kim KW, Lee ST, Kwon YG (2000).Endostatin inhibits endothelial and tumor cellular invasion by blocking the

activation and catalytic activity of matrix metalloproteinase. Cancer Res 60:5410-5413.

Klimczak RR, Koerber JT, Dalkara D, Flannery JG, Schaffer DV (2009).A novel adeno-associated viral variant for efficient and selective intravitreal transduction of rat Muller cells. PLoS One 4:e7467.

Koerber JT, Klimczak R, Jang JH, Dalkara D, Flannery JG, Schaffer DV (2009).Molecular evolution of adeno-associated virus for enhanced glial gene delivery. Mol Ther 17:2088-2095.

Kowluru RA (2005).Diabetic retinopathy: mitochondrial dysfunction and retinal capillary cell death. Antioxid Redox Signal 7:1581-1587.

Kowluru RA, Kern TS, Engerman RL (1997).Abnormalities of retinal metabolism in diabetes or experimental galactosemia. IV. Antioxidant defense system. Free Radic Biol Med 22:587-592.

Kowluru RA, Kowluru V, Xiong Y, Ho YS (2006).Overexpression of mitochondrial superoxide dismutase in mice protects the retina from diabetes-induced oxidative stress. Free Radic Biol Med 41:1191-1196.

Kulke MH, Bergsland EK, Ryan DP, Enzinger PC, Lynch TJ, Zhu AX, Meyerhardt JA, Heymach JV, Fogler WE, Sidor C, Michelini A, Kinsella K, Venook AP, Fuchs CS (2006).Phase II study of recombinant human endostatin in patients with advanced neuroendocrine tumors. J Clin Oncol 24:3555-3561.

Kvanta A, Algvere PV, Berglin L, Seregard S (1996).Subfoveal fibrovascular membranes in age-related macular degeneration express vascular endothelial growth factor. Invest Ophthalmol Vis Sci 37:1929-1934.

Lai CC, Wu WC, Chen SL, Xiao X, Tsai TC, Huan SJ, Chen TL, Tsai RJ, Tsao YP (2001).Suppression of choroidal neovascularization by adeno-associated virus vector expressing angiostatin. Invest Ophthalmol Vis Sci 42:2401-2407.

Lai CM, Estcourt MJ, Himbeck RP, Lee SY, Yew-San Yeo I, Luu C, Loh BK, Lee MW, Barathi A, Villano J, Ang CL, van der Most RG, Constable IJ, Dismuke D, Samulski RJ, Degli-Esposti MA, Rakoczy EP (2011).Preclinical safety evaluation of subretinal AAV2.sFlt-1 in non-human primates. Gene Ther.

Lai LJ, Xiao X, Wu JH (2007).Inhibition of corneal neovascularization with endostatin delivered by adeno-associated viral (AAV) vector in a mouse corneal injury model. J Biomed Sci 14:313-322.

Lamartina S, Cimino M, Roscilli G, Dammassa E, Lazzaro D, Rota R, Ciliberto G, Toniatti C (2007).Helper-dependent adenovirus for the gene therapy of proliferative retinopathies: stable gene transfer, regulated gene expression and therapeutic efficacy. J Gene Med 9:862-874.

Le Gat L, Gogat K, Bouquet C, Saint-Geniez M, Darland D, Van Den Berghe L, Marchant D, Provost A, Perricaudet M, Menasche M, Abitbol M (2003).In vivo adenovirus-mediated delivery of a uPA/uPAR antagonist reduces retinal neovascularization in a mouse model of retinopathy. Gene Ther 10:2098-2103.

Lee S, Kim K, Kim HA, Kim SW, Lee M (2008a).Augmentation of erythropoietin enhancer-mediated hypoxia-inducible gene expression by co-transfection of a plasmid encoding hypoxia-inducible factor 1alpha for ischemic tissue targeting gene therapy. J Drug Target 16:43-50.

Lee SJ, Jang JW, Kim YM, Lee HI, Jeon JY, Kwon YG, Lee ST (2002).Endostatin binds to the catalytic domain of matrix metalloproteinase-2. FEBS Lett 519:147-152.

Lee Y, Messing A, Su M, Brenner M (2008b).GFAP promoter elements required for region-specific and astrocyte-specific expression. Glia 56:481-493.

Li DR, Zhang F, Wang Y, Tan XH, Qiao DF, Wang HJ, Michiue T, Maeda H (2012).Quantitative analysis of GFAP- and S100 protein-immunopositive astrocytes to investigate the severity of traumatic brain injury. Leg Med (Tokyo) 14:84-92.

Li HL, Li S, Shao JY, Lin XB, Cao Y, Jiang WQ, Liu RY, Zhao P, Zhu XF, Zeng MS, Guan ZZ, Huang W (2008).Pharmacokinetic and pharmacodynamic study of intratumoral injection of an adenovirus encoding endostatin in patients with advanced tumors. Gene Ther 15:247-256.

Li W, Yanoff M, Liu X, Ye X (1997).Retinal capillary pericyte apoptosis in early human diabetic retinopathy. Chin Med J (Engl) 110:659-663.

Li YY, Qian GS, Huang GJ, Chen F, Qian P, Yu SC, Wang CZ, Li Q, Wang JC, Wu GM (2006).Enhancement of antiangiogenic effects of human canstatin with a hypoxia-regulated transgene vector in lung cancer model. Cancer J 12:136-146.

Liang X, Zhou H, Ding Y, Li J, Yang C, Luo Y, Li S, Sun G, Liao X, Min W (2012a).TMP prevents retinal neovascularization and imparts neuroprotection in an oxygen-induced retinopathy model. Invest Ophthalmol Vis Sci 53:2157-2169.

Liang XL, Li J, Chen F, Ding XY, Yang XX, Long LX (2012b).A comparing study of quantitative staining techniques for retinal neovascularization in a mouse model of oxygen-induced retinopathy. Int J Ophthalmol 5:1-6.

Lieth E, Barber AJ, Xu B, Dice C, Ratz MJ, Tanase D, Strother JM (1998).Glial reactivity and impaired glutamate metabolism in short-term experimental diabetic retinopathy. Penn State Retina Research Group. Diabetes 47:815-820.

Lim JI, Spee C, Hinton DR (2010).A comparison of hypoxia-inducible factor-alpha in surgically excised neovascular membranes of patients with diabetes compared with idiopathic epiretinal membranes in nondiabetic patients. Retina 30:1472-1478.

Lin M, Chen Y, Jin J, Hu Y, Zhou KK, Zhu M, Le YZ, Ge J, Johnson RS, Ma JX (2011).Ischaemia-induced retinal neovascularisation and diabetic retinopathy in mice with conditional knockout of hypoxia-inducible factor-1 in retinal Muller cells. Diabetologia 54:1554-1566.

Lin X, Huang H, Li S, Li H, Li Y, Cao Y, Zhang D, Xia Y, Guo Y, Huang W, Jiang W (2007).A phase I clinical trial of an adenovirus-mediated endostatin gene (E10A) in patients with solid tumors. Cancer Biol Ther 6:648-653.

Liu F, Tan G, Li J, Dong X, Krissansen GW, Sun X (2007).Gene transfer of endostatin enhances the efficacy of doxorubicin to suppress human hepatocellular carcinomas in mice. Cancer Sci 98:1381-1387.

Loewen N, Chen J, Dudley VJ, Sarthy VP, Mathura JR, Jr. (2009).Genomic response of hypoxic Muller cells involves the very low density lipoprotein receptor as part of an angiogenic network. Exp Eye Res 88:928-937.

Lutty GA, McLeod DS (2003).Retinal vascular development and oxygen-induced retinopathy: a role for adenosine. Prog Retin Eye Res 22:95-111.

Lutty GA, McLeod DS, Bhutto I, Wiegand SJ (2011).Effect of VEGF trap on normal retinal vascular development and oxygen-induced retinopathy in the dog. Invest Ophthalmol Vis Sci 52:4039-4047.

Maguire AM, High KA, Auricchio A, Wright JF, Pierce EA, Testa F, Mingozzi F, Bennicelli JL, Ying GS, Rossi S, Fulton A, Marshall KA, Banfi S, Chung DC, Morgan JI, Hauck B, Zelenaia O, Zhu X, Raffini L, Coppieters F, De Baere E,

Shindler KS, Volpe NJ, Surace EM, Acerra C, Lyubarsky A, Redmond TM, Stone E, Sun J, McDonnell JW, Leroy BP, Simonelli F, Bennett J (2009).Age-dependent effects of RPE65 gene therapy for Leber's congenital amaurosis: a phase 1 dose-escalation trial. Lancet 374:1597-1605.

Mao Y, Kiss S, Boyer JL, Hackett NR, Qiu J, Carbone A, Mezey JG, Kaminsky SM, D'Amico DJ, Crystal RG (2011).Persistent suppression of ocular neovascularization with intravitreal administration of AAVrh.10 coding for bevacizumab. Hum Gene Ther 22:1525-1535.

Marneros AG, Keene DR, Hansen U, Fukai N, Moulton K, Goletz PL, Moiseyev G, Pawlyk BS, Halfter W, Dong S, Shibata M, Li T, Crouch RK, Bruckner P, Olsen BR (2004).Collagen XVIII/endostatin is essential for vision and retinal pigment epithelial function. Embo J 23:89-99.

McMahon S, Charbonneau M, Grandmont S, Richard DE, Dubois CM (2006).Transforming growth factor beta1 induces hypoxia-inducible factor-1 stabilization through selective inhibition of PHD2 expression. J Biol Chem 281:24171-24181.

Messias A, Filho JA, Messias K, Almeida FP, Costa RA, Scott IU, Gekeler F, Jorge R (2012).Electroretinographic findings associated with panretinal photocoagulation (PRP) versus PRP plus intravitreal ranibizumab treatment for high-risk proliferative diabetic retinopathy. Doc Ophthalmol 124:225-236.

Miller JW, Adamis AP, Shima DT, D'Amore PA, Moulton RS, O'Reilly MS, Folkman J, Dvorak HF, Brown LF, Berse B, et al. (1994).Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with ocular angiogenesis in a primate model. Am J Pathol 145:574-584.

Minnella AM, Savastano CM, Ziccardi L, Scupola A, Falsini B, Balestrazzi E (2008).Intravitreal bevacizumab (Avastin) in proliferative diabetic retinopathy. Acta Ophthalmol 86:683-687.

Mitchell P, Annemans L, Gallagher M, Hasan R, Thomas S, Gairy K, Knudsen M, Onwordi H (2012).Cost-effectiveness of ranibizumab in treatment of diabetic macular oedema (DME) causing visual impairment: evidence from the RESTORE trial. Br J Ophthalmol 96:688-693.

Miyazaki M, Ikeda Y, Yonemitsu Y, Goto Y, Murakami Y, Yoshida N, Tabata T, Hasegawa M, Tobimatsu S, Sueishi K, Ishibashi T (2011).Pigment epithelium-derived factor gene therapy targeting retinal ganglion cell injuries: neuroprotection against loss of function in two animal models. Hum Gene Ther 22:559-565.

Mori K, Ando A, Gehlbach P, Nesbitt D, Takahashi K, Goldsteen D, Penn M, Chen CT, Melia M, Phipps S, Moffat D, Brazzell K, Liau G, Dixon KH, Campochiaro PA (2001).Inhibition of choroidal neovascularization by intravenous injection of adenoviral vectors expressing secretable endostatin. Am J Pathol 159:313-320.

Mori K, Gehlbach P, Yamamoto S, Duh E, Zack DJ, Li Q, Berns KI, Raisler BJ, Hauswirth WW, Campochiaro PA (2002).AAV-mediated gene transfer of pigment epithelium-derived factor inhibits choroidal neovascularization. Invest Ophthalmol Vis Sci 43:1994-2000.

Mousa SA, Lorelli W, Campochiaro PA (1999).Role of hypoxia and extracellular matrix-integrin binding in the modulation of angiogenic growth factors secretion by retinal pigmented epithelial cells. J Cell Biochem 74:135-143.

Mowat FM, Gonzalez F, Luhmann UF, Lange CA, Duran Y, Smith AJ, Maxwell PH, Ali RR, Bainbridge JW (2012).Endogenous erythropoietin protects neuroretinal function in ischemic retinopathy. Am J Pathol 180:1726-1739.

Mowat FM, Luhmann UF, Smith AJ, Lange C, Duran Y, Harten S, Shukla D, Maxwell PH, Ali RR, Bainbridge JW (2010).HIF-1alpha and HIF-2alpha are differentially activated in distinct cell populations in retinal ischaemia. PLoS One 5:e11103.

Nadal J, Carreras E, Canut MI (2012).Endodiathermy Plus Photocoagulation as Treatment of Sclerotomy Site Vascularization Secondary to Pars Plana Vitrectomy for Proliferative Diabetic Retinopathy. Retina.

Nguyen JT (2000).Adeno-associated virus and other potential vectors for angiostatin and endostatin gene therapy. Adv Exp Med Biol 465:457-466.

Nguyen QD, Brown DM, Marcus DM, Boyer DS, Patel S, Feiner L, Gibson A, Sy J, Rundle AC, Hopkins JJ, Rubio RG, Ehrlich JS (2012).Ranibizumab for diabetic macular edema: results from 2 phase III randomized trials: RISE and RIDE. Ophthalmology 119:789-801.

Orlidge A, D'Amore PA (1987).Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. J Cell Biol 105:1455-1462.

Pachori AS, Melo LG, Hart ML, Noiseux N, Zhang L, Morello F, Solomon SD, Stahl GL, Pratt RE, Dzau VJ (2004).Hypoxia-regulated therapeutic gene as a preemptive treatment strategy against ischemia/reperfusion tissue injury. Proc Natl Acad Sci U S A 101:12282-12287.

Park K, Jin J, Hu Y, Zhou K, Ma JX (2011).Overexpression of pigment epithelium-derived factor inhibits retinal inflammation and neovascularization. Am J Pathol 178:688-698.

Pechan P, Rubin H, Lukason M, Ardinger J, DuFresne E, Hauswirth WW, Wadsworth SC, Scaria A (2009).Novel anti-VEGF chimeric molecules delivered by AAV vectors for inhibition of retinal neovascularization. Gene Ther 16:10-16.

Penn JS, Henry MM, Tolman BL (1994).Exposure to alternating hypoxia and hyperoxia causes severe proliferative retinopathy in the newborn rat. Pediatr Res 36:724-731.

Penn JS, Rajaratnam VS, Collier RJ, Clark AF (2001).The effect of an angiostatic steroid on neovascularization in a rat model of retinopathy of prematurity. Invest Ophthalmol Vis Sci 42:283-290.

Pierce EA, Avery RL, Foley ED, Aiello LP, Smith LE (1995).Vascular endothelial growth factor/vascular permeability factor expression in a mouse model of retinal neovascularization. Proc Natl Acad Sci U S A 92:905-909.

Prentice H, Bishopric NH, Hicks MN, Discher DJ, Wu X, Wylie AA, Webster KA (1997).Regulated expression of a foreign gene targeted to the ischaemic myocardium. Cardiovasc Res 35:567-574.

Raisler BJ, Berns KI, Grant MB, Beliaev D, Hauswirth WW (2002).Adeno-associated virus type-2 expression of pigmented epithelium-derived factor or Kringles 1-3 of angiostatin reduce retinal neovascularization. Proc Natl Acad Sci U S A 99:8909-8914.

Rangasamy S, McGuire PG, Das A (2012).Diabetic retinopathy and inflammation: novel therapeutic targets. Middle East Afr J Ophthalmol 19:52-59.

Rauen PI, Ribeiro JA, Almeida FP, Scott IU, Messias A, Jorge R (2012).Intravitreal Injection of Ranibizumab during Cataract Surgery in Patients with Diabetic Macular Edema. Retina.

Rehn M, Veikkola T, Kukk-Valdre E, Nakamura H, Ilmonen M, Lombardo C, Pihlajaniemi T, Alitalo K, Vuori K (2001).Interaction of endostatin with integrins implicated in angiogenesis. Proc Natl Acad Sci U S A 98:1024-1029.

Reichel MB, Bainbridge J, Baker D, Thrasher AJ, Bhattacharya SS, Ali RR (2001).An immune response after intraocular administration of an adenoviral vector containing a beta galactosidase reporter gene slows retinal degeneration in the rd mouse. Br J Ophthalmol 85:341-344.

Rinaldi M, Chiosi F, Dell'omo R, Romano MR, Parmeggiani F, Semeraro F, Mastropasqua R, Costagliola C (2012).Intravitreal pegaptanib sodium (Macugen((R))) for treatment of diabetic macular ooedema: a morphologic and functional study. Br J Clin Pharmacol.

Roy MS, Klein R, O'Colmain BJ, Klein BE, Moss SE, Kempen JH (2004).The prevalence of diabetic retinopathy among adult type 1 diabetic persons in the United States. Arch Ophthalmol 122:546-551.

Ruan H, Su H, Hu L, Lamborn KR, Kan YW, Deen DF (2001).A hypoxia-regulated adeno-associated virus vector for cancer-specific gene therapy. Neoplasia 3:255-263.

Rungger-Brandle E, Dosso AA, Leuenberger PM (2000).Glial reactivity, an early feature of diabetic retinopathy. Invest Ophthalmol Vis Sci 41:1971-1980.

Saarela J, Rehn M, Oikarinen A, Autio-Harmainen H, Pihlajaniemi T (1998).The short and long forms of type XVIII collagen show clear tissue specificities in their expression and location in basement membrane zones in humans. Am J Pathol 153:611-626.

Sakamoto T, Kimura H, Scuric Z, Spee C, Gordon EM, Hinton DR, Anderson WF, Ryan SJ (1995).Inhibition of experimental proliferative vitreoretinopathy by retroviral vector-mediated transfer of suicide gene. Can proliferative vitreoretinopathy be a target of gene therapy? Ophthalmology 102:1417-1424.

Sarthy PV, Fu M, Huang J (1991).Developmental expression of the glial fibrillary acidic protein (GFAP) gene in the mouse retina. Cell Mol Neurobiol 11:623-637.

Sasaki T, Fukai N, Mann K, Gohring W, Olsen BR, Timpl R (1998).Structure, function and tissue forms of the C-terminal globular domain of collagen XVIII containing the angiogenesis inhibitor endostatin. Embo J 17:4249-4256.

Semenza GL (1999).Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 15:551-578.

Semenza GL, Agani F, Iyer N, Kotch L, Laughner E, Leung S, Yu A (1999).Regulation of cardiovascular development and physiology by hypoxia-inducible factor 1. Ann N Y Acad Sci 874:262-268.

Shi W, Teschendorf C, Muzyczka N, Siemann DW (2002).Adeno-associated virus-mediated gene transfer of endostatin inhibits angiogenesis and tumor growth in vivo. Cancer Gene Ther 9:513-521.

Shichiri M, Hirata Y (2001).Antiangiogenesis signals by endostatin. FASEB J 15:1044-1053.

Shimizu K, Kobayashi Y, Muraoka K (1981).Midperipheral fundus involvement in diabetic retinopathy. Ophthalmology 88:601-612.

Shweiki D, Itin A, Soffer D, Keshet E (1992).Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359:843-845.

Simo R, Carrasco E, Garcia-Ramirez M, Hernandez C (2006).Angiogenic and antiangiogenic factors in proliferative diabetic retinopathy. Curr Diabetes Rev 2:71-98.

Simonelli F, Maguire AM, Testa F, Pierce EA, Mingozzi F, Bennicelli JL, Rossi S, Marshall K, Banfi S, Surace EM, Sun J, Redmond TM, Zhu X, Shindler KS, Ying

GS, Ziviello C, Acerra C, Wright JF, McDonnell JW, High KA, Bennett J, Auricchio A (2010).Gene therapy for Leber's congenital amaurosis is safe and effective through 1.5 years after vector administration. Mol Ther 18:643-650.

Sivaprasad S, Gupta B, Crosby-Nwaobi R, Evans J (2012a).Prevalence of Diabetic Retinopathy in Various Ethnic Groups: A Worldwide Perspective. Surv Ophthalmol.

Sivaprasad S, Gupta B, Gulliford MC, Dodhia H, Mohamed M, Nagi D, Evans JR (2012b).Ethnic variations in the prevalence of diabetic retinopathy in people with diabetes attending screening in the United Kingdom (DRIVE UK). PLoS One 7:e32182.

Smith LE, Wesolowski E, McLellan A, Kostyk SK, D'Amato R, Sullivan R, D'Amore PA (1994).Oxygen-induced retinopathy in the mouse. Invest Ophthalmol Vis Sci 35:101-111.

Stahl A, Chen J, Sapieha P, Seaward MR, Krah NM, Dennison RJ, Favazza T, Bucher F, Lofqvist C, Ong H, Hellstrom A, Chemtob S, Akula JD, Smith LE (2010).Postnatal weight gain modifies severity and functional outcome of oxygen-induced proliferative retinopathy. Am J Pathol 177:2715-2723.

Stewart MW, Rosenfeld PJ, Penha FM, Wang F, Yehoshua Z, Bueno-Lopez E, Lopez PF (2012).Pharmacokinetic rationale for dosing every 2 weeks versus 4 weeks with intravitreal ranibizumab, bevacizumab, and aflibercept (vascular endothelial growth factor Trap-eye). Retina 32:434-457.

Stone J, Itin A, Alon T, Pe'er J, Gnessin H, Chan-Ling T, Keshet E (1995).Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia. J Neurosci 15:4738-4747.

Su C, Na M, Chen J, Wang X, Liu Y, Wang W, Zhang Q, Li L, Long J, Liu X, Wu M, Fan X, Qian Q (2008).Gene-viral cancer therapy using dual-regulated oncolytic adenovirus with antiangiogenesis gene for increased efficacy. Mol Cancer Res 6:568-575.

Takahashi K, Saishin Y, Silva RL, Oshima Y, Oshima S, Melia M, Paszkiet B, Zerby D, Kadan MJ, Liau G, Kaleko M, Connelly S, Luo T, Campochiaro PA (2003).Intraocular expression of endostatin reduces VEGF-induced retinal vascular permeability, neovascularization, and retinal detachment. FASEB J 17:896-898.

Toniatti C, Bujard H, Cortese R, Ciliberto G (2004).Gene therapy progress and prospects: transcription regulatory systems. Gene Ther 11:649-657.

Tout S, Chan-Ling T, Hollander H, Stone J (1993).The role of Muller cells in the formation of the blood-retinal barrier. Neuroscience 55:291-301.

Tretiach M, Madigan MC, Wen L, Gillies MC (2005).Effect of Muller cell co-culture on in vitro permeability of bovine retinal vascular endothelium in normoxic and hypoxic conditions. Neurosci Lett 378:160-165.

Wang GL, Semenza GL (1993).Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. Blood 82:3610-3615.

Wang J, Xu X, Elliott MH, Zhu M, Le YZ (2010).Muller cell-derived VEGF is essential for diabetes-induced retinal inflammation and vascular leakage. Diabetes 59:2297-2305.

Wangsa-Wirawan ND, Linsenmeier RA (2003).Retinal oxygen: fundamental and clinical aspects. Arch Ophthalmol 121:547-557.

Webster KA (2000).Therapeutic angiogenesis: a case for targeted, regulated gene delivery. Crit Rev Eukaryot Gene Expr 10:113-125.

Wenger RH (2002).Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression. FASEB J 16:1151-1162.

Witmer AN, Vrensen GF, Van Noorden CJ, Schlingemann RO (2003).Vascular endothelial growth factors and angiogenesis in eye disease. Prog Retin Eye Res 22:1-29.

Ye X, Ren H, Zhang M, Sun Z, Jiang AC, Xu G (2012).ERK1/2 Signaling Pathway in the Release of VEGF from Muller Cells in Diabetes. Invest Ophthalmol Vis Sci 53:3481-3489.

Yu DY, Cringle SJ (2005).Retinal degeneration and local oxygen metabolism. Exp Eye Res 80:745-751.

Yu DY, Cringle SJ, Alder VA, Su EN (1994).Intraretinal oxygen distribution in rats as a function of systemic blood pressure. Am J Physiol 267:H2498-2507.

Yu Y, Lyons TJ (2005).A lethal tetrad in diabetes: hyperglycemia, dyslipidemia, oxidative stress, and endothelial dysfunction. Am J Med Sci 330:227-232.

Zaniolo K, Sapieha P, Shao Z, Stahl A, Zhu T, Tremblay S, Picard E, Madaan A, Blais M, Lachapelle P, Mancini J, Hardy P, Smith LE, Ong H, Chemtob S (2011).Ghrelin modulates physiologic and pathologic retinal angiogenesis through GHSR-1a. Invest Ophthalmol Vis Sci 52:5376-5386.

Zhang B, Hu Y, Ma JX (2009).Anti-inflammatory and antioxidant effects of SERPINA3K in the retina. Invest Ophthalmol Vis Sci 50:3943-3952.

Zhang C, Wang YS, Wu H, Zhang ZX, Cai Y, Hou HY, Zhao W, Yang XM, Ma JX (2010).Inhibitory efficacy of hypoxia-inducible factor 1alpha short hairpin RNA plasmid DNA-loaded poly (D, L-lactide-co-glycolide) nanoparticles on choroidal neovascularization in a laser-induced rat model. Gene Ther 17:338-351.

Zhang M, Yang Y, Yan M, Zhang J (2006a).Downregulation of vascular endothelial growth factor and integrinbeta3 by endostatin in a mouse model of retinal neovascularization. Exp Eye Res 82:74-80.

Zhang SX, Wang JJ, Gao G, Shao C, Mott R, Ma JX (2006b).Pigment epithelium-derived factor (PEDF) is an endogenous antiinflammatory factor. FASEB J 20:323-325.

Zhang W, Ito Y, Berlin E, Roberts R, Berkowitz BA (2003).Role of hypoxia during normal retinal vessel development and in experimental retinopathy of prematurity. Invest Ophthalmol Vis Sci 44:3119-3123.

Zhou J, Fandrey J, Schumann J, Tiegs G, Brune B (2003).NO and TNF-alpha released from activated macrophages stabilize HIF-1alpha in resting tubular LLC-PK1 cells. Am J Physiol Cell Physiol 284:C439-446.

Zhou J, Schmid T, Brune B (2004).HIF-1alpha and p53 as targets of NO in affecting cell proliferation, death and adaptation. Curr Mol Med 4:741-751.

Zhou JF, Bai CM, Wang YZ, Li XY, Cheng YJ, Chen SC (2011).Endostar combined with chemotherapy for treatment of metastatic colorectal and gastric cancer: a pilot study. Chin Med J (Engl) 124:4299-4303.