MECHANISMS OF ACTION OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-3 IN PROMOTING REPAIR IN ISCHEMIC RETINAL VASCULAR MOUSE INJURY MODELS

By

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To my mother

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LIST OF ABBREVIATIONS

AKT	Protein kinase B
ALS	Acid-labile subuit
AMD	Age-related macular degeneration
BBB	Blood-brain barrier
BM	Bone marrow
BMD	Bone marrow derived
BMDC	Bone marrow derived cell
BRB	Blood-retinal barrier
BSA	Bovine serum albumin
Cdc6	Cell division cycle 6
cDNA	chromosomal deoxyribonucleic acid
cGMP	Guanosine 3',5'-cyclic monophosphate
CNV	Choroidal neovascularization
DAF-FM	4-amino-5-methylamino-2',7'-difluorofluorescein diacetate
DM	Diabetes mellitus
DMEM	Dulbecco's modified eagle medium
DMS	Dimethylsphingosine
DR	Diabetic retinopathy
EBM-2	Endothelial cell basal medium-2
EC	Endothelial cell
ECM	Extracellular matrix
EDG	Endothelial differentiation gene
EDTA	Ethylenediamine tetraacetic acid
Ena	*Drosophila melanogaster* protein enabled

eNOS	Endothelial nitric oxide synthase
ENDO	Endothelium derived nitric oxide
EOMs	Extraocular muscles
EPC	Endothelial progenitor cell
EPO	Erythropoietin
EVH	Ena/VASP homology
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
Flk	Fetal liver kinase
Flt-1	Vascular endothelial growth factor receptor-1
Flt-2	Vascular endothelial growth factor receptor-2
GCL	Ganglion cell layer
GFP	Green fluorescent protein
GM-SCF	Granulocyte/macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
GS isolectin	*Griffonia simplicifolia* isolectin
HIF-1	Hypoxia inducible factor-1
HMVEC-L	Human lung microvascular endothelial cells
HREC	Human retinal endothelial cells
HSC	Hematopoietic stem cell
HUVEC	Human umbilical vein endothelial cells
IACUC	Institutional Animal Care and Use Committee
IGF-1	Insulin-like growth factor-1
IGF-2	Insulin-like growth factor-2
IGFBP	Insulin-like growth factor binding protein

IGFBP-3	Insulin-like growth factor binding protein-3
ILM	Inner limiting membrane
INL	Inner nuclear layer
IOP	Intraocular pressure
KDR	Kinase insert domain-containing tyrosine kinase
LGN	Lateral geniculate nucleus
mRNA	messenger ribonucleic acid
NIH	National Institutes of Health
NO	Nitric oxide
NOD	Non-obese diabetic
NOS	Nitric oxide synthase
NPDR	Nonproliferative diabetic retinopathy
OIR	Oxygen-induced retinopathy
ONL	Outer nuclear layer
OPL	Outer plexiform layer
PBS	Phosphate buffered saline
PDR	Proliferative diabetic retinopathy
PECAM-1	Platelet/endothelial cell adhesion molecule-1
peNOS	phospo-endothelial nitric oxide synthase
PFA	Paraformaldehyde
PIGF	Placenta growth factor
PI3K	Phosphatidylinositol-3-kinase
RGC-5	Rat retinal ganglion cells
ROP	Retinopathy of prematurity
ROS	Retro-orbital sinus

RPE	Retinal pigment epithelium
RT-PCR	Real time polymerase chain reaction
S1P	Sphingosine-1-phosphate
S1PR	Sphingosine-1-phosphate receptor
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SDF-1	Stromal cell derived factor-1
SD	Standard deviation
Sphk	Sphingosine kinase
SR-B1	Scavenger receptor class B type 1
TBS	Tris buffered saline
TGF-β	Transforming growth factor
VASP	Vasodilator stimulated phosphoprotein
VCAM	vascular cell adhesion molecule
VEGFR-1	Vascular endothelial growth factor receptor-1
VEGFR-2	Vascular endothelial growth factor receptor-2
VEGF	Vascular endothelial growth factor
VPF	Vascular permeability factor
VSMC	Vascular smooth muscle cell
vWF	von Willebrand factor

Abstract of Dissertation Presented to the Graduate School
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Endothelial progenitor cells (EPCs) are bone-marrow derived cells, which give rise to mature endothelial cells. IGFBP-3 can promote EPC repair and maintainence of blood vessel integrity. This study was conducted to gain insight into IGFBP-3's cellular mechanism of action on EPC-mediated repair in damaged retinal vasculature.

To unveil how IGFBP-3 modulates EPC functional repair, studies were designed to focus on how IGFBP-3 promotes cell migration. For *in vivo* studies, 2 complementary vascular injury models were utilized: laser occlusion of retinal vessels in adult green fluroscent protein (GFP) chimeric mice and oxygen-induced retinopathy in mouse pups. Intravitreal injection of IGFBP-3 expressing plasmid into lasered adult gfp^+ chimeric mouse retinas stimulated homing of EPCs into retinal blood vessels. The gfp^+ EPCs also differentiated into various vascular cell types such as pericytes, astrocytes, and endothelial cells. In the OIR model, IGFBP-3 injection prevented cell death of resident vascular endothelial cells, while simulateneously increasing astrocytic ensheathment of retinal blood vessels. For EPCs to orchestrate these cytoprotective and homing effects, they must migrate into ischemic or damaged tissue. Experimental *in vitro* studies showed their migratory ability is mediated, in part, by endogenous nitric oxide (NO) generation. IGFBP-

3 treated EPCs significantly increased NO generation compared to untreated EPCs. IGFBP-3 can signal through the high-density lipoprotein receptor, Scavenger Receptor class B, type 1 (SR-B1), to increase NO production and activity in mature endothelial cells. In EPCs and mature endothelial cells SR-B1 blockade with a neutralizing antibody resulted in a decrease in NO production. Furthermore, when EPCs and mature endothelial cells underwent IGFBP-3 treatment, there was an increase in phospo-endothelial nitric oxide synthase (peNOS) protein expression. IGFBP-3 exposure led to the re-distribution of vasodilator stimulated phosphoprotein (VASP), a NO regulated protein, critical for cell migration of endothelial cells. Lastly, IGFBP-3 effects on vascular permeability were examined. Distinct differences in permeability were found depending on whether the IGFBP-3 was adminstered acutely or there was chronic exposure. IGFBP-3 acutely increased pemeability, while upon longer exposure IGFBP-3 reduced retinal vascular permeability, supportive of its vascular stabilizing ability.

In summay, identification of a new signaling receptor that IGFBP-3 can activate was uncovered. IGFBP-3 can activate the SR-B1 receptor to increase exogenous NO production in EPCs, which leads to increased cell migration of these cells. Due to the positive stimulatory effects of IGFBP-3 on cell migration, it can act as an *in vivo* homing, resulting in vascular repair and stabilization of injured mouse retinal vasculature. IGFBP-3 has the potential to be used as a therapeutic agent to treat ischemic vascular eye conditions.

CHAPTER 1
BACKGROUND AND SIGNIFICANCE

Sight is one of our most precious senses. When it is compromised or lost, it can significantly impair quality of life. As life expectancy continues to increase, blindness is an imminent threat for the aging population.[1-4] In fact, more than 2 million Americans age 50 and older have age-related macular degeneration (AMD) and more than 4.4 million Americans age 40 and older suffer from diabetic retinopathy, according to the National Eye Institute in 2008. Over the next 20 years the number of people with compromised vision and blindness will continue to climb, not only in the United States, but worldwide.[4,5] This will undoubtedly place a strain on not only the visually impaired, but their families, as well as society as a whole. Therefore, therapeutic strategies to combat blindness are necessary, such as stem cell based therapies.

Stem cell therapy in the eye has become an attractive therapeutic strategy to correct lost vision.[6-11] This is particularly true of ocular diseases with vascular complications, such as diabetic retinopathy.[12] Diabetic retinopathy results from uncontrolled hyperglycemia, which ultimately leads to neovascularization and aberrant vessel formation in the eye. The vessels that form in the eye are leaky, fragile, and unstable, which leads to obscured vision.[13] In order to overcome vision impairment, adult hematopoietic stem cells (HSCs) derived from the bone marrow, specifically endothelial progenitor cells (EPCs), can be potentially used to repair and re-endothelialize damaged retinal vessels in diabetic retinopathy patients.[14-17] Previously, EPCs have yielded improved blood perfusion in ischemic vascular injury animal models involving the hind limb[18-24] and heart.[25-31] Also, EPCs incorporate into damaged retinal blood vessels in ocular injury mouse models with promising results.[15-17,32-34] However, the extent of EPC repair and therapeutic benefit still requires improvement with regard to increased homing and

regenerative efficiency. When EPCs are provided the right cue and/or stimulus, such as a potent growth factor, their migratory efficiency and reparative capability can be greatly enhanced. But the question remains open as to what growth factor stimulus will best improve EPC mediated repair, particularly in the retinal vasculature.

Insulin-like Growth Factor Binding Protein-3 (IGFBP-3) has garnered much interest within the past few years as a potential therapeutic to treat ischemic vascular eye complications, especially in pre-mature infants with retinopathy of prematurity (ROP). This is based on studies using a mouse model of ROP, called oxygen induced retinopathy (OIR). By increasing exogenous levels of IGFBP-3 in the mouse pup eye reduced pathological neovascularization was observed.[35,36] Thus, IGFBP-3 has vascular protective effects. It is believed IGFBP-3's vascular protective nature may be due to its influence on EPC behavior. Exogenous IGFBP-3 administration has been shown to have a profound effect on EPC migration, tube formation, and differentiation, all of which allow the progenitor cells to home, stabilize and promote normal vessel development in the mouse OIR model.[36] IGFBP-3's cellular mechanism of action on EPC mediated vascular repair is not yet understood. Hence, this study was undertaken to illuminate how IGFBP-3 influences EPC driven repair in retinal vascular mouse injury models at both the cellular and molecular levels to better understand its therapeutic potential.

The Eye

The vertebrate eye is a well designed and highly efficient organ. All of the various cell types and structures contained in the eye work in harmony to create a clear image which is sent to the brain for visual processing. If any part of the eye is damaged, it can disrupt the intricate process of visual phototransduction. The eye, although an important organ, can be under appreciated for its intricacies. Nevertheless, the eye is truly spectacular in its anatomical shape, form, and function.

Eye Structure and Function

The eye is a complex organ composed of many structures. The ability to see is dependent on all the structures working together to create a clear image.[33] Figure 1-1 depicts the essential components of the eye. Light enters the eye through the cornea, which is considered the window of the eye due to its transparency and avascular nature.[37] After light rays pass through the cornea, they travel through the aqueous humor, which provides nourishment for the surrounding lens and cornea, as well as maintains intraocular pressure (IOP).[38] The aqueous humor is produced by the ciliary body. The ciliary body changes the shape of the lens for focusing. The iris is the pigmented part of the eye. It separates the anterior chamber from the posterior chamber and regulates the amount of light entering through the pupil.[39] The size of the pupil is regulated by the dilator and sphincter muscles of the iris and controls the amount of light that enters the eye. After light travels through the pupil, it passes through the lens. The lens is surrounded by ligaments (zonule fibers) that are attached to the anterior portion of the ciliary body. The lens changes shape, contracts or relaxes, by the ciliary muscles and attached ligaments.[40] Light then passes through a clear, jelly-like substance called the vitreous humor before it finally reaches the retina. The retina is a thin, multi-layered transparent, neuro-sensory tissue lining the back of the eye. It allows light rays to be converted into electrical impulses, which are transmitted to the optic nerve leading to the brain.[38] The optic nerve is a bundle of nerve fibers that carries visual information from the eye to the brain. It consists of approximately 1 million axons arising from the ganglion cells of the retina. The optic nerve runs from the optic disc through the optic foramen to the optic chiasma where it becomes the optic tract. The visual fibers synapse in the lateral geniculate nucleus (LGN). The cell bodies of this structure give rise to the neurons that comprise the visual pathway.

The eye is made up of three distinct layers: the external layer, the intermediate layer and

the internal layer. The external layer is formed by the sclera and cornea. The cornea, as described earlier, is quite refractive, thus provides the eye with exquisite focusing power.[38] The sclera is composed of a tough, fibrous tissue that serves to protect the eye. Extraocular muscles surround the eye and are attached to the sclera. There are a total of six extraocular muscles, four rectus muscles and two oblique muscles, which work together in accord to keep both eyes properly aligned.[41] The intermediate layer is divided into two parts: the anterior (iris and ciliary body) and the posterior part called the choroid. The choroid contains a layer of blood vessels and lies between the retina and sclera. The choroid supplies oxygen and nutrients to the outer layers of the retina. The choroid connects the ciliary body with the front part of the eye and is attached to the edges of the optic nerve.[42] The internal layer is the sensory part of the eye deemed the retina.

The Retina

The retina is one of the most important structures in the eye and is where a large amount of the eye's blood supply is located. Most eye diseases result in some degree of retinal damage.[43-45] The retinal blood vessels are frequently subjected to damage in diseases such as retinopathy of prematurity (ROP) and diabetic retinopathy (DR).[33,43,46] In these disease processes, neovascularization occurs, which is the formation of new blood vessels. The new blood vessels that form are abnormal in nature, thus unable to support normal blood flow and result in retinal ischemia.[46-48] The abnormal blood vessels that form in the retina, ultimately contribute to blindness due to the formation of edema, exudate build up, scar tissue accumulation, and/or retinal detachment.[13,33] It is these abnormal blood vessels that are a target for repair and stabilization. Fortunately, the retinal vasculature is easy to visualize *in vivo.* This facilitates studying pathological neovascularization and potential cell based therapies, such as with EPCs, to target this process from obliterating the retina.

Anatomy of the Retina

The retina is a multi-layered structure that is essential for visual processing. The human retina is approximately 0.2 mm thick and has an area of roughly 1100 mm.[38] The human retina contains well over 200 million neurons.[49] The retina captures light and converts it into electrical impulses by way of photoreceptor cells called rods and cones. There are close to 125 million rods in the human retina.[50] They are situated throughout the peripheral retina and function best in dim light. Hence, rods are responsible for peripheral and night vision. In contrast, the human retina contains 6 million cones, which function best in bright light and color perception.[51] The highest density of cones can be found in the macula. The center part of the macula, called the fovea, is densely packed with cones. The fovea permits greater light absorption by the dense array of photoreceptors, thus is the site of our most acute vision. The fovea contains no blood vessels, permitting increased visual acuity in the macular region. Nevertheless, the vascularization of the remainder of the macula is very dense and increases the likelihood of several vascular related diseases. The retina is loosely attached to the retinal pigment epithelium (RPE). These cells contain an abundant amount of pigment that is necessary for light absorption and transportation of oxygen, nutrients, and wastes between the photoreceptors and the choroid.[42] Bruch's membrane is tightly associated with the RPE, stabilizing the RPE layer by separating it from the blood vessels of the choroid. Oxygen diffuses across the Bruch's membrane and this membrane thickens with age.[42] Breaks in Bruch's membrane are the hallmark for choroidal neovascularization (CNV) in the retina. Beneath the Bruch's membrane is the choroid, which contains a network of blood vessels, nerves, and provides all of the nutritional needs of the RPE and the outer part of the sensory retina.

The human retina consists of ten layers (Figure 1-2). Among them, three layers of nerve cell bodies and two layers of synapses are responsible for converting a light signal into a neural

signal. The photoreceptor cell bodies form the outer nuclear layer (ONL).[52] While the inner nuclear layer (INL) contains the cell bodies of the bipolar, horizontal, and amacrine cells, the ganglion cell layer (GCL) contains the cell bodies of ganglion cells and displaced amacrine cells. The outer plexiform layer (OPL) is located between the outer nuclear layer (ONL) and inner nuclear layer (INL).[53] In the OPL, photoreceptors relay their information to the bipolar cells, as well as the horizontal cells. The bipolar cells then transfer information to the inner plexiform layer (IPL), which separates the INL and GCL. Bipolar cells are connected to the retinal ganglion cells in addition to amacrine cells in the IPL.[53] The ganglion cells are the output neurons of the retina that transmit information from the eye to the brain.

Retinal Blood Supply

The retina is a metabolically active tissue and requires among the highest blood flow rates of any tissue.[45,54] The blood supply to the retina originates from the ophthalamic artery.[37] There are two sources of blood supply to the mammalian retina: the central retinal artery and the choroidal blood vessels. The outer retina is supplied by the choriocapillaris, which is an extensive network of fenestrated capillaries. The choroid receives the greatest blood flow between 65-85%, which is critical for the maintenance of the outer retina, specifically the inner and outer segments of the photoreceptors.[42] The central retinal artery supplies the remaining 20-30% blood flow from the optic nerve head to nourish the inner retinal layers.

All blood vessels share a number of common features. For example, the insides of blood vessels are lined with endothelium, a thin layer of endothelial cells (ECs), which separates the blood from tissues.[55] Blood vessels are also covered with a specialized layer of connective tissue called the basement membrane followed by a layer of mural cells known as pericytes and vascular smooth muscle cells. Astrocytes, which are characteristically star-shaped glial cells, are also a component of blood vessels. They form a layer around blood vessels and provide

biochemical, as well as structural support to endothelial cells. Astrocytes help maintain the blood brain barrier (BBB) in cerebral blood vessels in the brain.[56,57]

Retinal blood vessels are remarkably similar to cerebral blood vessels in that they maintain the blood-retinal barrier (BRB), which is similar to the BBB.[58] The BRB consists of two distinct monolayers of cells: the retinal pigment epithelium (outer barrier) and the retinal capillary endothelial cells (inner barrier). Both monolayers form tight junctions, which are responsible for maintenance of the barrier. The inner BRB is covered with pericytes and glial cells. Glial Muller cells predominately support retinal endothelial cells and glial astrocytes are partly responsible for supporting endothelial functions at the inner BRB.[56] The inner BRB plays an important role in supplying nutrients to the neural retina and is responsible for the efflux of neurotransmitter metabolites from the retina to maintain neural functions. The outer BRB consists of specialized nonfenestrated capillaries and tight junctions within the RPE.[57] The outer BRB forms a transport barrier between the retinal capillaries and choroidal capillaries. Also, it prevents the passage of large molecules from the choriocapillaris into the retina.[48] Transmembrane proteins such as occludin, claudin, junctional adhesion molecule (JAM), as well as adherens junctions help maintain BRB integrity.[59]

The eye's blood supply is crucial for its proper function and any disruption in it or the BRB can have dire consequences on visual perception. The BRB maintains the ocular milieu by protecting the neural retina from circulating inflammatory cells and their cytotoxic products. This allows the retina to regulate its own extracellular chemical composition for proper neuronal function.[59] The retina essentially controls its own blood flow and BRB by a variety of cellular and chemical interactions. Therefore, any breakdown of the BRB, such as ischemic injury or inflammation, can lead to detrimental alterations in blood flow and increased vascular

permeability.[33,47] This in turn can lead to serious vascular eye diseases called retinopathies (Figure 1-3).

Retinopathies

Loss of vision is a difficult health problem to overcome. When vision is compromised it leads to disability, suffering, loss of productivity, and a lower quality of life. In the United States alone, more than 25 million people suffer from vision loss according to the 2008 National Health Interview Survey. Approximately 1.3 million people in the United States are legally blind. Many of these cases of blindness are attributed to retinopathies, which are ocular diseases in which deterioration of the retina is caused by abnormal neovascularization, resulting in vision impairment (Figure 1-3). Vascular retinopathies are the leading causes of visual disability and blindness worldwide.[46] Pathological growth of new blood vessels is the hallmark of retinopathies. Retinopathies affect all age groups. Retinopathy of prematurity (ROP) affects premature infants, while diabetic retinopathy (DR) strikes the working age population. Retinopathies are debilitating eye diseases, which are increasing in number and frequency.

Retinopathy of Prematurity

ROP is the leading cause of blindness in children in both developed and undeveloped countries.[60] Two major risks factors of ROP are the use of oxygen and a decreased gestational period.[61] ROP mainly affects premature infants weighing approximately 2.75 pounds or less that are born before 31 weeks of gestation.[62] In general, the more premature the baby and the lower the birth weight, the greater the risk for ROP.[63] Growth of the human fetal eye occurs within the last 12 weeks of full term delivery, 28-40 weeks gestation. Vessels reach the anterior edge of the retina and then regress at about 40 weeks of gestation. Therefore, infants born pre-maturely (before 31 weeks) have incompletely vascularized retinas with a peripheral avascular zone. [60,64] In premature infants, vascular growth that normally occurs *in utero* slows and is accompanied by

regression of developed retinal vessels.

ROP was first described by Terry in 1942. At this time, administration of high oxygen was considered the standard of care for premature infants to supplement their underdeveloped lungs and maintain adequate respiration.[65] Although the high oxygen assisted premature infants with their breathing, it was discovered upon removal from the high oxygen environment, the return to normal levels of atmospheric oxygen was often seen as a hypoxic environment in the eye.[66] As a result, many infants suffered pathological neovascularization within the retina. Despite adjustment of oxygen delivery and other medical advances, the total number of infants with ROP has not decreased over the years because of increased survival rates in very low birth weight infants.[64]

There are approximately 4 million babies born in the United States annually. According to the National Eye Institute, about 28,000 premature infants are born each year. Between 14,000 and 16,000 of these premature infants develop some degree of ROP. There are 5 stages of ROP, from a mild stage 1 to the most severe stage 5.[61] Most infants, close to 90%, have mild ROP and do not require extensive treatment. ROP in most cases regresses spontaneously.[67] Therefore, only a small number between 1,100 and 1,500 of the premature babies develop severe ROP and require significant medical care. [61] As a consequence, 400 to 600 infants each year in the United States become legally blind from ROP.

ROP progresses in two phases.[60,64,66] The first phase includes the hyperoxia extrauterine environment surrounding the baby and the supplemental oxygen administered to the baby. The growth inhibition of retinal vascular growth after birth and partial regression of existing retinal vessels is the first phase, followed by a second phase of ROP involving hypoxia-induced uncontrolled proliferative vessel growth. The pathological growth of vessels produces a fibrous

scar that extends from the retina to the vitreous and lens. Retraction of this scar tissue can separate the retina from the RPE, resulting in retinal detachment, bleeding, and ultimately blindness in neonates.[64]

The biphasic disease process of ROP is associated with unbalanced levels of growth factors.[64] Low levels of insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) are detected in phase I, whereas, excessively high levels of IGF-1 and VEGF are found in phase II.[68] IGF-1 plays a critical role in ROP in infants. Reducing IGF-1 levels inhibits vessel growth even in the presence of VEGF.[69] Low levels of IGF-1 reduces vascular density, which subsequently causes early vessel degeneration in phase I. The mean serum levels of IGF-1 in age-matched premature babies are directly correlated with the severity of ROP disease stages.[70] Likewise, infants with ROP have lower IGFBP-3 levels than those of healthy infants. In the second phase of ROP, which is driven by hypoxia, VEGF expression is increased in the retina. This results in pathological neovascularization because blood vessels grow toward the concentrated VEGF areas in the retina.[71] Inhibition of VEGF can prevent a certain degree of hypoxia-induced retinal neovascularization in the second phase of ROP. However, VEGF inhibition does not completely prevent neovascularization in the second phase of ROP, suggesting ROP is a multi-factorial process involving an interplay between a number of factors related to growth and development.[64]

There are several therapies, such as cryotherapy and laser photocoagulation, that can be used to reduce visual loss in ROP infants.[62] However, these therapies can reduce peripheral vision and include risks from anesthesia. Therefore, preventive and less invasive therapies for ROP are warranted. Also, understanding how IGF-1 and VEGF contribute to ROP and the use of these growth factors as potential therapeutic targets is crucial. The two phases of ROP are quite

distinct and require different therapeutic interventions. In phase 1, the hypoxia-induced vessel loss can be partially prevented by administering VEGF or PIGF-1.[60,72,73] While an injection of anti-VEGF aptamer, as well as anti-VEGF antibody can be used to treat neovascularization in phase II, pharmacological intervention related to the prevention of vessel loss may be a more effective therapeutic strategy.[64] This is true because the extent of the second destructive phase of ROP is determined by the amount of vessel loss in the first phase.

In 1994, Smith and colleagues developed a mouse model of ROP to study the molecular mechanisms involved in the disease.[71] This model was developed on the premise that retinal vessel development in mice is incomplete at birth.[74] Therefore, this model is intended to mimic the first and second phases of ROP in human pre-mature infants. Neonatal mice are exposed to 75% oxygen from postnatal day 7 until day postnatal 12. When neonatal mice are exposed to hyperoxia, vessel regression and the cessation of normal radial vessel growth occurs mirroring the first phase of ROP. The hyperoxia primarily targets capillaries adjacent to arteries in the center of the retina and does not affect larger, more mature veins and arteries.[75] Upon return to room air, the non-perfused portions of retina become hypoxic, resulting in retinal neovascularization. This neovascular phase in the OIR model is similar to the second phase of ROP in humans. The mouse ROP model has been used extensively to study neovascularization in the early developing vasculature and to unravel molecular changes in both phases of the disease. It has proven to be a reproducible and quantifiable model and is used in studies described in subsequent chapters.

Diabetic Retinopathy

Diabetes mellitus (DM) affects 100 million people worldwide.[5] A common complication of DM is diabetic retinopathy, which can have detrimental effects on vision. Nearly 4.1 million Americans are affected by diabetic retinopathy (DR).[76] Of these individuals, close to 900,000

are affected by retinopathy that is vision threatening. Vascular diseases are the principal cause for death or disability in people with diabetes. The metabolic dysfunctions that characterize diabetes include elevated blood glucose levels, increased levels of free fatty acids, and insulin resistance, all of which contribute to vascular complications.[77] According the National Eye Institute, the microvascular complications of DR is one of the most common ailments of diabetes and frequently results in visual loss. DR is known to affect diabetic patients who have had the disease for longer than 20 years and have poor glucose control.[78] Although the best way to prevent vision loss is to initiate treatment before symptoms develop, many diabetic patients do not experience visual complications until significant vascular damage has already occurred. DR is classified into two stages: nonproliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). PDR typically develops in patients with type 1 diabetes, while NPDR is more common in patients with type 2 diabetes (Figure 1-3).[76]

The progression of DR begins with increased reactive oxygen species (ROS) and changes in nitric oxide synthase (NOS) isoforms followed by apoptosis of pericytes and adhesion of leukocytes to the vessel wall that result in microvascular occlusion, basement membrane thickening, and increased vascular permeability.[13,77] As a result of these pathological changes, the blood vessels become leaky, allowing blood and vascular fluids to accumulate in the retinal tissue and form exudative deposits.[59,79] This then results in macular edema, which is commonly seen in patients with NPDR.[59] NPDR is classified according to three stages: mild, moderate, and severe. NPDR is associated with areas of capillary non-perfusion, which leads to hypoxia in the retina in the severe stages of the disease. To compensate for the decreased oxygen supply, angiogenic factors such as VEGF are released from the hypoxic retinal tissues and stimulate the growth of new blood vessels on the surface of the retina.[13,80] This late stage is called PDR. The

walls of the newly formed vessels are fragile and can easily break, allowing blood to leak out. This can cloud the vitreous and obscure vision. In the advanced stage of PDR, newly formed fibrous vascular tissue grows from the retinal surface into the vitreous cavity. This can cause retinal detachment leading to blindness.[59]

The current therapy for DR is laser photocoagulation.[76,81] However, this treatment often causes unwanted side effects such as neural tissue loss, peripheral vision loss, impairment of night vision and change in color perception. Additionally, in some patients, their retinopathy still continues to progress after laser treatment.[46] Therefore, new therapeutic treatments are greatly needed to treat diabetic retinal vascular disease. Pharmacological agents that directly inhibit angiogenesis have been developed to treat DR. Particular emphasis has been placed on inhibiting pro-angiogenic growth factors such as VEGF. The overexpression of VEGF plays a key role in the pathogenesis of diabetes and induction of retinal vascular dysfunction.[13] The development of agents that directly target VEGF and its receptors have been vigorously pursued in clinical research trials. The success of these agents has been limited in patients. However, the use of EPCs to repair damaged retinal vessels is a novel and exciting strategy to treat DR.

Neovascularization

Blood and lymphatic vessels are either developed by vasculogenesis or angiogenesis.[26,47,82] During *de novo* vasculogenesis, endothelial lineage committed angioblasts assemble to form new vessels during embryogenesis. During angiogenesis, sprouts form from pre-existing blood vessels and migrate into the surrounding tissue in the adult. This involves sprouting, pruning and intussusception of pre-existing vessels.[83] This process relies on proliferation, migration, and remodeling of fully differentiated endothelial cells. Most organs are vascularized by vascuologenesis, like the brain and kidney.[84] Neovascularization during adult life has long been attributed to angiogenesis only.[85] However, this dogma has recently been

challenged.[20,86-89] Studies have shown that EPCs also circulate postnatally in the peripheral blood and can be recruited and incorporated into sites of active neovascularization in ischemic hind limbs, ischemic myocardium, injured corneas and retina, cutaneous wounds, and even the tumor vasculature.[90] Therefore, retinal vascular development occurs by a combination of both vasculogenesis and angiogenesis, which is called neovascularization (Figure 1-4).[47] A variety of factors are known to contribute to neovascularization such as cytokines, chemotactic factors, and angiogenic factors. Essentially, EPCs can functionally revascularize ischemic tissues, participate in neovessel formation, and maintain overall vascular homeostasis (Figure1-5).[26,43,55,82,87,90-92]

Endothelial Progenitor Cells

The bone marrow (BM) is the major reservoir of adult stem cells.[93,94] The bone marrow microenvironment, commonly known as the bone marrow niche, remains relatively quiescent. It is comprised of stromal cells and extracellular matrix (ECM) components. A special subtype of BM-derived stem cells, known as EPCs, are able to differentiate into mature endothelial cells and incorporate into sites of neovascularization under physiological as well as pathological conditions, such as wound healing, organ regeneration, and tumor growth.[87,95-97] EPCs were first isolated by Asahara *et al.* in 1997.[89] Over the past ten years EPCs have been extensively studied.[17-20,27,34,43,91,98-114]

EPCs are a rare population in the peripheral blood and bone marrow. They represent between 0.01 and 0.001% of the total peripheral blood mononuclear cell fraction from a normal blood sample.[43,95] EPCs can be isolated from not only peripheral blood, but fetal liver or umbilical cord blood.[87,89] EPCs are characterized by specific antigens, such as $CD34^+$ in humans and c-kit^+/Sca-1^+ in mice, expressed on the surface of the cells.[27,93,95,115-118] Stem cells maintain immature, primitive markers so that they can differentiate or transdifferentiate into a wide spectrum of cell types. This process is known as stem cell plasticity.[94]

The identification of true EPCs has been highly debated in terms of their stem cell plasticity ability. Defining EPCs has been controversial to say the least because several studies have demonstrated overlapping antigens among other types of bone marrow-derived cells, such as monocytes.[116,119-121] Moreover, many have suggested size, cell cycle, cell adhesion, and other functional characteristics, rather than cell surface markers alone, may be more useful when isolating and characterizing EPCs.[112] Little effort has been expended to carefully characterize the definitive EPC. However, even with discrepancies in the EPC field regarding phenotypic characteristics that define EPCs, it is widely accepted that CD34, vascular endothelial growth factor receptor-2 (VEGFR-2), and CD133 (AC133) are the common antigens used in the sorting and isolation of human EPCs.[27,87,115,118] It has also been suggested that endothelial nitric oxide synthase (eNOS) is an additional marker used to define EPCs. EPCs have other characteristics of endothelial cells including acetylated low density lipoprotein uptake and endothelial specific lectin binding *in vitro*. Additionally, EPCs can produce nitric oxide (NO). With maturation, EPCs begin to lose expression of CD34 and CD133, and start to express CD31, also known as PECAM-1 (platelet /endothelial cell adhesion molecule), vascular endothelial cadherin, and von Willebrand factor.[122] The differentiation of EPCs occurs when circulating EPCs home to sites of injured vessels or integrate into mature endothelium, based on molecular stimuli that govern their rapid differentiation.[87]

EPC function and number can vary among individuals due to pathological, pharmacological, and physiological factors. In fact, the number of circulating EPCs and colony forming ability of EPCs are directly correlated with certain disease states.[43,86,104,113,123-130] Fewer $CD34^+$ EPCs are circulating in patients with diabetes[131-134] and peripheral artery disease.[135-137] Also, increased levels of oxidative stress and inflammatory cytokines have negative

consequences on EPC function and mobilization. On the other hand, increasing numbers of EPCs were found in patients with limb ischemia or vessel wall damage after coronary thrombosis, burn injury, or coronary bypass surgery to rescue damaged vessels.[113] Exercise and physical training have also been found to increase circulating EPCs.[126] Clearly EPC numbers vary among individuals. But it is clear reduced EPC numbers can have a negative impact on vascular homeostasis and consequently vascular repair.

EPC recruitment, as well as release from the bone marrow, is influenced by various exogenous factors (Figure 1-5). Pro-angiogenic growth factors such as granulocyte/macrophage colony-stimulating factor (GM-CSF) and erythropoietin (EPO) have been shown to modulate EPC functions that play a critical role in embryonic development, as well as in homeostasis in the adult.[14,82,87,138] However, three well studied stimuli, hypoxia, VEGF, and SDF-1, have a major impact on not only EPC mobilization, but their overall function and reparative capacity.[14,20,90] Each of these stimuli are discussed separately. However, hypoxia, VEGF, and SDF-1, frequently work together in accord through cross-talk signaling to influence EPC behavior and homing.

Hypoxia

Hypoxia occurs when there is an imbalance between oxygen supply and demand in cancerous or ischemic tissues. Consequently, hypoxia is a critical stimulus for expansion of the vascular bed.[139] In wounds, capillary injury generates a hypoxic environment, and altered oxygenation induces a reconstructive angiogenic response.[99] Hypoxia triggers vessel growth by signaling through hypoxia-inducible transcription factors (HIFs), to stimulate SDF-1, VEGF, EPO and other factors. It has been suggested HIF-1α may be ultimately responsible for initiating progenitor mobilization and targeting to sites of neovascularization.

Hypoxia serves as a critical cue for both physiological and pathological angiogenesis in the brain, heart, kidneys, lungs, and muscles. Hypoxia is also a potent stimulus for mobilization of bone marrow derived cells, such as EPCs.[14,20,32,140] When a gradient of hypoxia was created in skin wounds in mice, bone-marrow derived (BMD) cells followed the gradient, and the greatest number of cells homed to and integrated into vessels of ischemic tissue.[141] Kalka *et al*. transplanted human EPCs into athymic nude mice with hindlimb ischemia.[19] They found blood flow recovery and capillary density in the ischemic hindlimb was significantly improved in mice receiving human transplanted EPCs. Also, Annabi *et al.* report hypoxia promotes murine BMD stromal cell migration and tube formation.[140] They suggest hypoxia-driven angiogenesis may be a critical condition for remodeling by bone marrow-derived stem cells.

More or less, the proliferation, patterning, and assembly of recruited progenitors into functional blood vessels are influenced by tissue tension and hypoxia.[99] Hypoxia may be regarded as fundamental requirement for progenitor cell trafficking and function. Since the bone marrow environment itself is hypoxic, where EPCs originally reside prior to release into the circulation, ischemic tissue may represent a "conditional" stem cell niche, which may attract circulating EPCs.[99] This premise appears to hold true for the eye, in which EPCs are recruited to sites of ischemic ocular injury (Figure 1- 6).[16,17,34,92,102,138]

Takahashi *et al.* showed in 1999 that EPCs contribute to enhanced corneal neovascularization, which were mobilized from the bone marrow in response to ischemia and GM-CSF.[14] Two years later, Grant *et al.* showed that recruitment of endothelial progenitors to sites of retinal ischemic venous occlusion injury had a significant role in neovascularization in retina.[17] They concluded HSCs are major contributors to the functional vessel formation that occurs during neovascularization in the retina. Furthermore in 2006, Ritter *et al.* showed a

population of adult BM-derived myeloid progenitor cells migrated to avascular regions in the retina.[32] They found myeloid progenitors differentiate into microglia and promoted vascular repair in a model of ischemic oxygen induced retinopathy. Essentially, circulating EPCs and HSCs are mobilized endogenously in response to tissue ischemia. [25,28,30,31,33,36,43,104,140,142]

Vascular Endothelial Growth Factor (VEGF)

VEGF, also known as vascular permeability factor (VPF), plays an important role in both normal physiological angiogenesis and pathological angiogenesis associated with disease states such as diabetic retinopathy, rheumatoid arthritis, and solid tumor formation.[143,144] It modulates vascular growth and morphogenesis, vascular tone, as well as chemotaxis of endothelial cells.[145] VEGF is especially well known as a pro-angiogenic growth factor and is implicated in inducing microvascular hyperpermeability, which can both precede and accompany angiogenesis.[144] VEGF is a highly specific mitogen for endothelial cells and is a potent cell survival factor.[143] Signal transduction involves binding to tyrosine kinase receptors, resulting in endothelial cell proliferation, migration, and new blood vessel formation.[83]

The VEGF family consists of seven structurally related homodimeric glycoproteins: VEGF-A, placenta growth factor (PIGF), VEGF-B, VEGF-C, VEGF-D, orf virus-encoded VEGF-like proteins (VEGF-E), and a series of snake venoms (VEGF-F).[83,143] Although structurally similar, the VEGF homologs have distinct functions and roles and bind to specific subtypes of VEGF receptors (VEGFRs). VEGF exerts its effects by binding to one of its three receptors that belong to the superfamily of receptor tyrosine kinases.

VEGFR-1 is a 180 kDa glycoprotein expressed on many hematopoietic cells. It regulates blood vessel morphogenesis. [83] This receptor is also required for normal blood vessel development during embryogenesis. Homozygous deletion of VEGFR-1 is lethal in mice at embryonic day E8.5 due to severe malformation of the vasculature.[83] VEGFR-2, on the other

hand, regulates a diverse range of cellular functions such as angiogenesis, mitogenesis, cytoskeletal organization, cell migration, vascular permeability and mediates cell survival.[143] It is a 200 kDa glycoprotein expressed on hematopoietic, neural, and retinal cells. VEGFR-2 is considered the most important mediator in angiogenesis.[83] VEGFR-3 is synthesized as a 195 kDa precursor protein consisting of seven extracellular Ig-like domain, a transmembrane and an intracellular kinase domain. It regulates lymphangiogenesis and angiogenesis. Expression of this receptor starts at E8.5 of mouse development in all embryonic endothelial cells. After E8.5, VEGFR-3 expression is only seen on developing veins and lymphatics.[83] Later in development, the expression gradually becomes restricted to lymphatic vessels. VEGF-A binds to either VEGFR1 (Flt-1) or VEGFR2 (KDR/Flk-1). PIGF and VEGF-B binds only to VEGFR-1. VEGF-C and VEGF-D are specific ligands for VEGFR-2 and VEGFR-3, regulating both blood and lymphatic vessel development. Viral VEGF-E and some of the snake venom VEGF-F variants exclusively activate VEGFR-2.[83,143]

VEGF-A, commonly referred to as simply VEGF, has been extensively studied and is regarded at the most potent mediator of angiogenesis.[13] It is a 34-42 kDa, dimeric, disulfide-bound glycoprotein highly expressed in the eye, lung, kidney, heart, and adrenal gland. There are several splice variants of VEGF including VEGF-121, -145, -165, -189, and -206. VEGF and its receptors are naturally present in the retina. They are important for maintaining angiogenesis and homeostasis in the vascular bed and retinal tissue. VEGF, however, plays a role in not only normal angiogenesis, but also pathological neovascularization in the eye. Specifically, VEGF is involved in intraocular neovascular syndromes in diseases, such as diabetic retinopathy and retinopathy of prematurity. VEGF expression is induced when cells are subjected to hypoxia or hypoglycemia precipitating these disease states. In all these ocular disease states, VEGF not only

causes uncontrolled neovascular growth that damages the retina, but also promotes vascular leakage leading to blood barrier breakdown, which can lead to vision loss. The primary sources of VEGF in the eye include RPE and neural cells, such as ganglion cells and Muller cells. VEGF is both necessary and sufficient for the occurrence of pathological ocular neovascularization.

VEGF clearly regulates several key endothelial cell functions in both normal and pathological states. Yet even more noteworthy, VEGF also plays a role in HSC and EPC cell function. VEGF is a potent chemoattractant and plays a role in EPC recruitment. [146-148] It promotes monocyte chemotaxis.[149] Gill *et al*. demonstrated that in burn patients and those who undergo coronary artery bypass grafting, there is a rapid elevation of VEGF levels followed by immediate mobilization, within 6 to 12 hours of vascular trauma, of $VEGFR2^{+}$ AC133 cells into the peripheral circulation.[27] Recruitment of circulating EPCs to sites of active angiogenesis is mediated through VEGFR2. Galiano and coworkers show topical VEGF is able to improve wound healing by systemically mobilizing BM derived cells to wound injury where they accelerate repair.[150] Also, VEGF controls HSC and EPC survival. VEGF-deficient HSCs and bone marrow mononuclear cells show an inability to repopulate lethally irradiated hosts, despite co-administration of wild-type cells.[151] More interestingly, VEGF is induced by HIF-1α which then upregulates SDF-1, another potent EPC modulator and stimulus.[99] SDF-1 and VEGF can work in concert such that VEGF and SDF-1 induce each other to influence EPC behavior. SDF-1 can mobilize and sequester HSCs, while VEGF recruits HSCs and promotes their differentiation and proliferation. [152] VEGF clearly has a powerful influence on EPC and HSC recruitment, homing, as well as differentiation of these cells.

Stromal Derived Factor-1 (SDF-1)

Stromal cell derived factor-1 (SDF-1) belongs to a group of chemokine CXC subfamily,

originally isolated from murine bone marrow stromal cells. It is produced by multiple bone marrow stromal cell types, as well as epithelial cells. CXCR4, a 7-transmembrane spanning G protein coupled receptor and is the only known receptor for SDF-1.[153] Both hematopoietic and endothelial progenitor cells express CXCR4.[154] SDF-1 is a potent EPC cytokine and chemoattractant.[155-157] SDF-1 mediates homing of stem cells to the bone marrow by binding to its receptor CXCR4 on circulating cells. CXCR4 is also required for maintaining quiescence of primitive hematopoietic cells.[153]

The SDF-1/CXCR4 signaling pathway is critical during embryogenesis, vascular development, and cardiac development. Blockade of SDF-1 in ischemic tissue or CXCR4 on circulating cells inhibits progenitor cell recruitment to sites of injury.[158] Inhibition of the SDF-1/CXCR4 axis partially blocks the homing of progenitor stem cells to the ischemic myocardium.[159] Also, inhibition of CXCR4 by neutralizing anti-CXCR4 antibodies significantly reduces SDF-1 induced migration of EPCs *in vitro* and reduces *in vivo* homing of myeloid EPCs to the ischemic limb.[160] Overexpression of CXCR4 on stem and progenitor cells promotes their proliferation, migration, and *in vivo* engraftment of NOD/SCID mice.[103] SDF-1 gene expression is regulated by the transcriptional factor, HIF.[99] Progenitor cell mobilization is activated by hypoxia gradients through HIF-1 induction of SDF-1. HIF-1 induced secretion of SDF-1 in ischemic tissue has a direct correlation with reduced oxygen tension. It is proposed SDF-1 induces increased expression of metalloproteinase-9 (MMP-9) activity, which causes cleavage of membrane bound Kit ligand into soluble Kit ligand, known as stem cell factor (SCF). Hence, SDF-1 promotes stem cell mobilization into the circulation. SDF-1 likely acts to sequester HSCs at sites of injury since studies have shown it is required for adhesion of HSCs at sites of injury.[146,147]

SDF-1 has also been implicated in neovascularization. SDF-1 promotes revascularization of ischemic hind limbs through recruitment of $CXCR4^+$ hemangiocytes. [161] De Falco *et al.* report that SDF-1 expression following hind-limb ischemia was up-regulated in plasma and down-regulated in bone marrow, thus mobilizing c-kit^+ cells into the peripheral blood. Additionally, SDF-1 and CXCR4 contribute to the involvement of bone marrow derived cells and collaborates with VEGF in the development of several types of ocular neovascularization.[138] SDF-1 alone is not sufficient to recruit BMD cells to tissues. SDF-1 works in conjunction with others signals such as VEGF to promote BMD mobilization. SDF-1 plays an important role in regulating BM derived cell engraftment and function in vascular remodeling and neovascularization. However, SDF-1 is not likely to be the only cytokine that can profoundly impact stem cells. There are likely to be more factors identified that mobilize and influence stem cells in coming years. One such group of factors is the Insulin-like growth factor binding proteins (IGFBPs).

Insulin-like Growth Factor Binding Proteins (IGFBPs)

Insulin-like growth factor-1 (IGF-1) and II (IGF-II) modulate a diverse range of biological activities including growth, differentiation, survival, and regulation of cell metabolism.[162-164] In serum and extracellular fluid, circulating IGFs are sequestered into 150 kDa ternary complexes with IGF binding proteins (IGFBPs) and the liver derived glycoprotein (acid labile subunit, ALS).[163] This complex prolongs the half life of IGFs in the circulation and prevents them from crossing the capillary barrier. IGFBPs consist of six homologous secreted proteins, which specifically bind to IGF-1 with high affinity (Figure 1-7). There has been increasing research on IGFBPs in recent years regarding their role in angiogenesis. Of the six IGFBPs, IGFBP-3 or Insulin-like growth factor binding protein-3, has attracted considerable interest as a pro-angiogenic factor and EPC modulator.[35,36,165-167]

Insulin Like Growth Factor Binding Protein-3 (IGFBP-3)

IGFBP-3 was first described in 1989 as a transporter of 70-90% of all circulating IGF.[162] Today, IGFBP-3 is known as the most abundant binding protein in serum and milk. IGFBP-3 can either transport IGF-1 to its receptor, enhance IGF-1 actions, or sequester IGF-1 from its receptor.[164] It circulates in the serum, binding IGF-I or IGF-II in conjunction with ALS, to form a 150 kDa circulating complex at a serum concentration of 100 nM. Human IGFBP-3 is present in various glycosylated forms between 40 and 44 kDa and contains a total of 264 amino acids. It contains 3 functional domains: a nonconserved central domain and highly conserved cysteine-rich carboxyl and amino domains. Interestingly, IGFBP-3 contains a nuclear localization sequence (NLS), which is responsible for translocation into the nucleus via nuclear transport factor importin-β (Figure 1-8) where it can impact transcription.[163] IGFBP-3 is produced and released by Kupffer hepatic cells and endothelial cells.

The liver and kidney are the main sources of IGFBP-3.[163] The level of IGFBP-3 in the serum is regulated by not only its rate of synthesis, but also its post-translational modification and proteolysis. IGFBP-3 proteases include plasmin, matrix metalloproteases, kallikreins, prostate-specific antigen, and cathepsin. Normal individuals have minimal IGFBP-3 protease activity; however, protease activity is increased in individuals who are pregnant, diabetic or have acute catabolic illnesses.[163] Post-translational modifications of IGFBP-3 include phosphorylation, methylation, glycosylation, and ubiquitination.[163,168]

IGFBP-3 concentration in serum is also regulated by other factors such as IGF-1, HIF-1, VEGF, NO, and TGF-β. IGF-1 affects HIF-1, which increases VEGF and IGFBP-3 expression.[162,163] More importantly, IGFBP-3 is hypoxia-regulated. Hypoxia can induce IGFBP-3 mRNA through p53 dependent and independent mechanisms. Likewise, induction of IGFBP-3

mRNA was observed in response to hypoxia in wild-type embryonic stem cells. Furthermore, it is upregulated in ischemic tissues.[169,170] For example, IGFBP-3 mRNA is upregulated in ischemic brain, specifically in cerebral vascular endothelial cells.[171] The activation of IGFBP-3 is a likely mechanism by which endothelial cells respond to hypoxic insult and increase cell survival. IGFBP-3 is known to bind many factors involved in wound healing such as heparin, fibrinogen, humanin, plasminogen, plasmin, dermatan sulfate and fibronectin.[163] IGFBP-3 can also activate several receptor signaling pathways like TGF-β, the integrins, and proteoglycans (Figure 1-8). However, IGFBP-3 has no official cell surface receptor. There are two putative receptors: one that was cloned via a yeast two-hybrid system using the midregion of IGFBP-3 and another termed the low-density lipoprotein-related protein-1 (LRP-1)/α2M receptor.[164] However, these putative IGFBP-3 receptors have not been confirmed or validated. The exact cell surface receptor structure and signaling mechanism of IGFBP-3 remains unresolved.

IGFBP-3 has been recognized to have IGF-1 independent effects.[172,173] In fact, more research is being focused on IGFBP-3 IGF independent effects through the use of IGFBP-3 mutants that do not bind IGF-1 or IGF-1 analogs with reduced affinity for IGFBP-3. Also, available are IGFBP-3 fragments with total and partial loss of IGF affinity, as well as the existence of IGF-1 negative cell lines (breast cancer and chondrocytes).[164] IGFBP-3 depending on the cell type and environment, can be either pro- or anti-angiogenic.[163] IGFBP-3 has been widely studied in cancer regarding its anti-angiogenic effects. *In vitro*, IGFBP-3 can inhibit cell proliferation in human breast cancer cells devoid of IGF-1. Also, IGFBP-3, when co-treated with VEGF, inhibits VEGF induced vessel formation in human endothelial cellular vessel formation in matrigel. Furthermore, IGFBP-3 can interact with the retinoic acid receptor (RXR)-α, to reduce prostate tumor growth and prostate specific antigen *in vivo*. Likewise, *in vivo*, CD31

staining in microvessels and endothelial cells was reduced by half in tumors treated with IGFBP-3 versus control, indicating IGFBP-3 suppresses intra-tumoral angiogenesis.

Regarding IGFBP-3's pro-angiogenic effects, Granata *et al.* found IGFBP-3 can stimulate neovessel formation in human endothelial cells, but at a very high concentration, 1000 ng/ml, which is well above the physiologically relevant concentration.[174] Also, IGFBP-3 has been shown to have pro-angiogenic effects on EPCs with regard to enhancing their cell migration, proliferation, and tube formation *in vitro,* but at physiologically low concentrations.[36] Additionally, IGFBP-3 prevents oxygen-induced vessel loss and promotes vascular re-growth after ischemic insult *in vivo*, thus reduces pathological neovascularization.[35,36] IGFBP-3 is now considered more than just a binding protein, but an effector molecule with clear IGF independent effects on cell growth and proliferation.

Even though IGFBP-3 has been widely studied, it still remains an elusive growth factor with no known cell surface receptor. We propose it can signal through another receptor system, the scavenger type receptor (SR-B1) to activate downstream cell survival pathways, which promote its pro-angiogenic effects on EPCs and vascular reparative ability by increasing nitric oxide production (Figure 1-9).

Scavenger Receptor Class B type 1 (SR-B1)

In 1996, Krieger and co-workers identified the Scavenger Receptor class B type 1 (SR-B1).[175] SR-B1 is a cell surface multiligand receptor that can bind high density lipoproteins (HDL) and mediate exchange of lipids with cells. It is a member of the CD36 family of proteins and contains two transmembrane domains, short N and C-terminal cytoplasmic regions. It is 509 amino acids in length. The SR-B1 has a horseshoe-like membrane topology and is heavily N-glycosylated, like IGFBP-3.[176] It is also highly expressed in the liver and cells of the vascular wall, similar to that of IGFBP-3. Also, IGFBP-3 and HDL have been found to be

complexed together in the same lipid fraction when isolated. Therefore, it is reasonable to postulate SR-B1 may indeed uptake HDL complexed with IGFBP-3. Both IGFBP-3 and HDL are "sticky" molecules. Consequently, IGFBP-3 may activate SR-B1 and lead to subsequent downstream signaling that may be responsible for IGFBP-3's vascular protective nature. SR-B1 activation is well known to generate nitric oxide, which plays an important role in the cardiovascular system and plays an important role in endothelial cell migration. IGFBP-3 has been reported to increase nitric oxide production in EPCs, thus we hypothesize IGFBP-3 may signal through the SR-B1 pathway to increase NO production, leading to enhanced EPC cell migration.

SR-B1, along with HDL has sparked the interest of many researchers regarding their possible role on endothelial cell and EPC function. Recently, Van Eck *et al.* found SR-B1 in bone marrow-derived cells is either pro-atherogenic or anti-atherogenic, indicating a unique dual role in pathogenesis of atherosclerosis.[177] Furthermore, Mineo *et al.* report that HDL-SR-B1 promotes endothelial repair.[178] Likewise, Seetharam *et al.* demonstrated impaired reendothelialization was observed in SR-B1 knockout mice.[179] Even more interesting, HDL by itself has been found to play a role in progenitor mobilization for endothelium repair. Increased levels of HDL can improve EPC availability in patients with Type 2 Diabetes. Tso and co-workers report injection of HDL into mice increases $Sca1^+$ cells in damaged aortic endothelium.[180] Similarly, Sumi *et al.* found HDL directly stimulates EPC differentiation via PI3K/Akt pathway and enhances ischemia-induced angiogenesis.[181] Overall, SR-B1 signaling can influence EPC differentiation and migration.

EPC Homing and Cell Migration

Cell migration is an essential biological process involved in development, cell growth, wound healing, vascular remodeling, and angiogenesis. If cells fail to migrate, then biological

processes can be easily disputed in an organism, leading to alterations in homeostasis and development of pathological states. In order to ensure cells migrate, particularly EPCs, there are a number of stimuli that activate cell migration. EPCs require potent stimuli to attract, recruit, and direct them to areas in need of vascular repair or required vascular homeostasis. Some of these include nitric oxide (NO) and sphingosine-1-phosphate (S1P). Also, various cytoskeletal components are involved in cell migration, such as vasodilator associated stimulated protein (VASP). Each of these are discussed in detail in the context of their role in EPC homing.

Nitric Oxide (NO)

Vascular endothelial cells produce numerous vasoactive substances that play important roles in the regulation of vascular tone, inflammatory responses, and growth and migration of vascular smooth muscle cells (VSMCs).[182-185] Nitric oxide (NO) is one of the most important nonpeptide endothelium-derived vasoactive factors.[186] Endothelium-derived NO (ENDO) was initially identified as a main molecule representing the endothelium-derived relaxing factors. It was originally identified in 1980 by Furchgott and Zawadzki. NO is structurally one of the simplest biological molecules. NO is a small, diffusible, lipophilic free reactive radical that participates in a variety of signaling activities in nearly every organ system in the body.[186] NO has been shown to inhibit platelet aggregation, leukocyte-endothelium interaction, and VSMC proliferation and differentiation.[187] NO has multiple important regulatory roles in the maintenance of vascular homeostasis and angiogenesis. [188,189] [190,191]

NO is generated by NO synthases (NOS). NOS is a heme-containing enzyme that is linked to NADPH-derived electron transport. NOS catalyzes the oxidation of L-arginine to L-citrulline and NO, with tetrahydrobioterin and NADPH as essential cofactors.[192] Three NOS isoforms have been identified and named after the cell type or condition in which they were first discovered. They include: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible or

inflammatory (iNOS).[189] NO has a very short half life, roughly 5 seconds, therefore many studies on NO have relied on measuring NOS.

All three isoforms of NOS are found in different cell types in the eye.[193] nNOS is responsible for producing NO in photoreceptors and bipolar cells, whereas eNOS is present in vascular endothelial cells. iNOS is found in Muller cells and in the retinal pigment epithelium. It is involved in inflammatory processes and phagocytosis of the photoreceptor outer segment. iNOS is also thought to be responsible for the pathogenesis of diabetic retinopathy.[194,195] The retinal endothelium produces significant NO and as a result plays an important role in eye function and homeostasis. [187,195]

NO defects have been implicated in a wide range of diseases. It is well established that endothelial NO bioavailability is systemically reduced in patients with coronary artery disease and heart failure. In diabetic patients reduced NO bioavailability may result from altered NO metabolism.[123,131] In diabetic mice, vascular endothelial dysfunction is associated with uncoupling of eNOS within the endothelium that is caused by oxidation of its essential cofactor tetrahydrobiopterin, resulting in a specific loss of NO bioavailability.[13] The rise in cGMP accounts for many of the physiological effects of NO. The NO dependent cGMP response is rapidly and selectively reduced in diabetic rats, and the cGMP response to exogenous NO donor is progressively reduced. NO modulates various physiological processes and has a major role on endothelium health and maintenance.

NO also has an important function in the stem cell microenvironment as a molecular mediator in controlling the stem cell niche.[196] eNOS deficient mice have an impaired capacity to mobilize stem cells from the bone marrow as reported by Aicher and associates. Guthrie *et al.* found that NO/NOS pathway is a significant regulator of neovascularization. NO can modulate

hemangioblast and EPC activity by dictating the size and branching characteristics of blood vessels that are formed in response to ischemic retinal injury.[102] Moreover, Kaminski *et al.* show the presence of eNOS appears to be a crucial and specific factor for firm c-kit^{+} cell adhesion to the vascular endothelium.[197] Michurina *et al.* report NO is a regulator of HSC activity and Grant *et al.* show NO modulates HSC behavior and vascular phenotype in the retina. Essentially, NO is a multifactorial signaling molecule and plays a key role in EPC migration.

Sphingosine-1-Phosphate (S1P)

Sphingosine 1-Phosphate is a bioreactive sphingolipid metabolite that is an extracellular mediator.[198-201] It has been implicated in angiogenesis, wound healing, embryonic development, cytoskeletal organization, adherens junction assembly, vascular permeability, and morphogenesis. [198,202,203] Sphingosine Kinase (Sphk) catalyzes the formation of S1P from sphingosine, an abundant lipid in most cell membranes, while sphingosine lyase breaks down S1P in most cells. Basal levels of S1P in cells is low, but can rapidly increase when cells are exposed to mitogenic and/or angiongenic growth factors such as EGF, IGF-1, or VEGF.[200,204] These signals activate Sphk, the enzyme that phosphorylates sphingosine to produce S1P, which is ultimately responsible for increased levels of S1P. S1P levels inside cells are tightly regulated. The plasma concentration of S1P is approximately 200 nM and more than doubles in the serum to 500 nM.[198,199,202,205] Both red blood cells and endothelial cells are reported to be the major sources of S1P. However, activated platelets also store and produce large amounts of S1P.[198,205]

Sphk is an evolutionarily conserved lipid kinase, which consists of five conserved domains. [198,205] There are two isoforms of mammalian Sphk, Sphingosine Kinase type 1 (Sphk1) and Sphingosine Kinase type 2 (Sphk2) that have been characterized. Sphk1 is mainly expressed in the cytosol, whereas Sphk2 is localized to the nucleus.[206] Sphk1 and Sphk2 have contrasting biological roles. Sphk1 is considered anti-apoptotic, thus promoting cell growth and survival,

while Sphk2 enhances apoptosis. The biological roles of Sphk have been studied using *N,N*-dimethylsphingosine (DMS), a pharmacological inhibitor, which blocks both Sphk1 and 2. This inhibitor was used in *in vitro* studies, which are described in subsequent chapters.

S1P acts as a ligand for plasma membrane localized G protein coupled receptors (GPCRs) known as endothelial differentiation gene receptors (EDGR) or more commonly S1PRs.[207] S1P binds to the five members of this receptor family: S1PR1, S1PR2, S1PR3, S1PR4, or S1PR5 (previously referred to as EDG 1,5,3,6 and 8 respectively). The S1P receptors are expressed in wide range of organs and systems such as the cardiovascular, immune, reproductive, respiratory, and nervous systems.[200] They also have wide range of biological effects such as regulating cell migration, differentiation, proliferation, and survival. S1P receptors are particularly important in vascular maturation and angiogenesis.[208] S1PR1 null mice are embryonic lethal due to massive hemorrhaging that is caused by incomplete vascular maturation of arteries and capillaries.[208,209] Endothelial cells form a vascular network, but smooth muscle cells and pericytes fail to be recruited to stabilize the vessels in S1PR1 knockout mice. Also, *in vitro*, reduction of S1PR1 expression by antisense oligos inhibits endothelial cell proliferation, migration, and tube formation. S1PR1 plays an important in endothelial cells and consequently is the most highly expressed on endothelial cells.

S1P has been shown to have profound effects on cell migration, specifically on endothelial cells. S1P has an impact on the cytoskeletal actions such as aggregation, contraction, shape change, and adhesion. S1P activates the small GTPases Rac and Rho, thus acting as a chemoattractant for endothelial cells. S1P induces Rho-dependent integrin clustering in focal contact sites, which modulate cell adhesion, spreading, and migration.[210] S1P can also phosphorylate protein kinase Akt in endothelial cells, leading to cell migration. At low levels

S1P can even act as a chemoattractant for CD4 and CD8 T immune cells. S1P can also trigger invasion of primitive hematopoietic cells into stromal layers.[211]

Yet even more striking, S1P has also been shown to have a substantial role in the mobilization and homing of stem cell and progenitor populations. Whetton *et al.* was the first to show that murine HSCs that are $Lin^{-}Sca^{+}Kit^{+}$, express S1P receptors, specifically S1PR's1-4.[212] Additionally, it was shown S1P substantially enhanced the chemotactic migratory response of mouse HSCs to SDF-1.[213] With regard to human $CD34^{+}$ cells, Seitz *et al.* found that S1P can act as a direct chemoattractant for these progenitors *in vitro*.[214] Furthermore, they showed sustained S1P1R activation *in vivo*, via FTY720, a specific S1PR1 agonist, increased engraftment of human $CD34^{+}$ cells in NOD/SCID mice. Similarly, Walter *et al.* found that human EPCs treated with S1P or FTY720, increased flow in ischemic murine limbs via S1PR3.[213] Bonder *et al.* recently showed sphingosine kinase regulates the rate of EPC differentiation.[215] When Sphk1 levels are reduced, EPC mobilization of the bone marrow is increased, and the rate of endothelial cell differentiation is hastened. Together, these studies demonstrate an emerging picture for the importance of S1P in regulation HSCs and EPCs, specifically regarding their migratory potential. Thus, we propose IGFBP-3 may activate S1PR's in order to enhance EPC migration (Figure 1-9).

Vasodilator Stimulated Phosphoprotein (VASP)

Nitric oxide-dependent, vasodilator stimulated phosphoprotein (VASP) plays a pivotal role in cytoskeletal actin regulation. VASP belongs to a family of proline-rich proteins that include the *Drosophilia melangaster* protein Enabled (Ena), its mammalian ortholog Mena, and the Ena-VASP-like protein Evl.[216] All Ena/VASP family members share a highly conserved amino-terminal Ena/VASP homology 1 (EVH1) domain followed by a proline rich central region and a carboxy-terminal Ena/VASP homology 2 (ENVH2) domain.[217] The structure of ENV1

domain of Ena/VASP family has been identified by using X-ray crystallography and nuclear magnetic resonance spectroscopy. The EVH1 domain serves as an Ena/VASP protein-binding site for the focal adhesion proteins including vinculin, zyxin, and axon guidance proteins roundabout (Robo). EVH1 domain-protein interactions are necessary for localization of Ena/VASP family to focal adhesions, as well as to the periphery of protruding lamellipodia.[216,218] The central proline rich region has binding sites for several SH3 and EE domain containing proteins and profilin. The C-terminal EVH2 domain not only mediates tetramerization of Ena/VASP proteins, but also binds both monomeric (G) and polymerized (F) actin. The EVH2 domain functions appear to be important for both actin filament bundling and stabilization.[216,217,219]

VASP is a cytoskeletal actin filament protein, which is involved in platelet activation, cell adhesion, and migration.[220,221] VASP mutant mice exhibit defects in actin-dependent process of platelet aggregation.[222] The results from site-directed mutagenesis and overexpression studies, suggest the importance of Ena/VASP proteins in the developmental and physiological processes in various cell types. For example, VASP modulates T cell activation, phagocytosis, and epithelial morphogenesis. It also induces migration of neutrophils, fibroblasts, and neurons.[223,224]

In mammalian cells, Ena/VASP proteins localize within cells to areas of dynamic actin organization such as the leading edge of lamellipodia and at the tips of filopodia and other actin-dependent intracellular structures like cell-cell contacts, focal adhesions, and in periodic puncta along stress fibers. In endothelial cells, VASP functions in membrane ruffling, aggregation, as well as tethering of actin filaments during the formation of endothelial cell-substrate and cell-cell contacts. VASP plays a distinct role in endothelial cell migration and function. Oelze ***et al.***

reported the levels of vessel phospho-VASP leads closely follows changes in endothelial function and vascular oxidative stress.[225] Li Calzi *et al.* showed NO mediates cytoskeletal changes through site- and cell-type specific VASP phosphorylation in endothelial cells.[127] Hence, we postulate exogenous IGFBP-3 generated nitric oxide can promote VASP re-distribution in endothelial cells, which can ultimately enhance endothelial cell migration (Figure 1-9).

Significance

IGFBP-3 has been identified as an important binding and carrier protein for IGF. However, it physiological and systemic roles extend much further than a simple binding protein. In fact, considerable research has been conducted on IGFBP-3's IGF independent effects, as well as its pro- and anti- angiogenic effects. IGFBP-3 has direct IGF independent effects on cell migration, proliferation, differentiation, and apoptosis. However, IGFBP-3's mechanism of action still baffles many researchers, especially since no known cell surface receptor has been identified. IGFBP-3's pro-angiogenic role on EPC-mediated repair is especially confounding.

The goal of this study was to highlight IGFBP-3's role in enhancing cell migration of EPCs, and its vascular reparative effects. Here we demonstrate IGFBP-3 increases EPC migration through NO generation via activation of the SR-B1 receptor.

Specific Aims and Hypotheses

Three specific aims were set forth for the studies of IGFBP-3 in human EPCs and in two mouse retinal vascular injury models. The first aim was to examine the effects of IGFBP-3 on cell migration in both EPCs and endothelial cells. The second aim was to determine the mechanism of action of IGFBP-3 on promoting cell migration. The third aim was to determine whether IGFBP-3 is cytoprotective in two different types of mouse retinal vascular injury models.

The underlying hypotheses are: (1) IGFBP-3 can act as an EPC homing factor *in vivo* in an adult retinal ischemia- induced injury model by stimulating EPCs to migrate to sites of vascular injury. (2) IGFBP-3's mechanism of action on cell migration in both EPCs and resident vascular endothelial cells is generation of exogenous NO via SR-B1. (3) IGFBP-3 can aid in trafficking EPCs to unstable, damaged vessels for needed repair by influencing vascular permeability, which leads to vascular stabilization and cytoprotection of retinal blood vessels. Overall, it is hypothesized IGFBP-3 has vascular protective effects by influencing EPC and endothelial cell migration via NO generation by SR-B1, which leads to the homing and reparative effects of these cells in injured retinal vasculature.

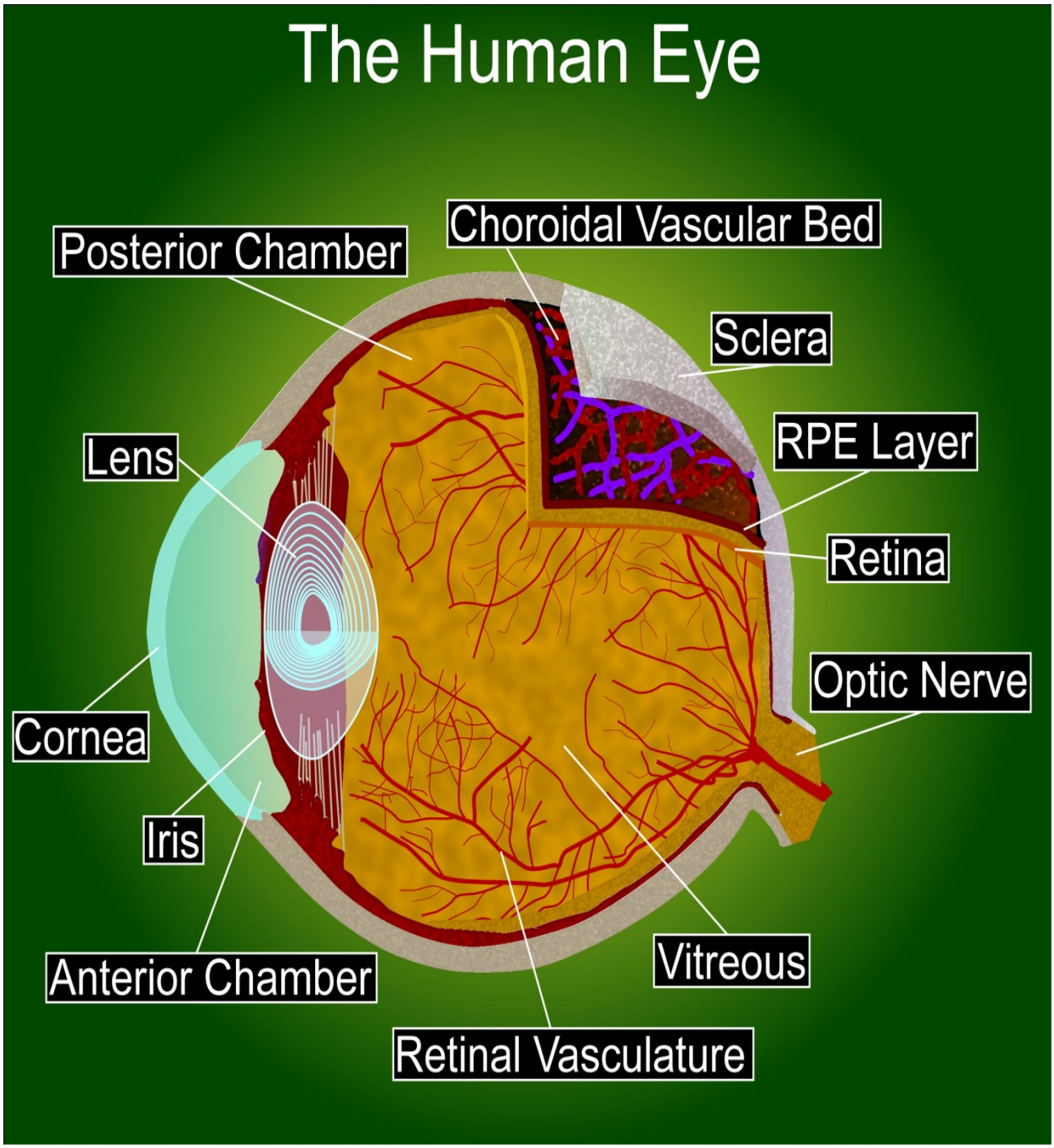

Figure 1-1. Anatomy of the human eye. The eye is made up of many structures, which are essential for clear visual perception.

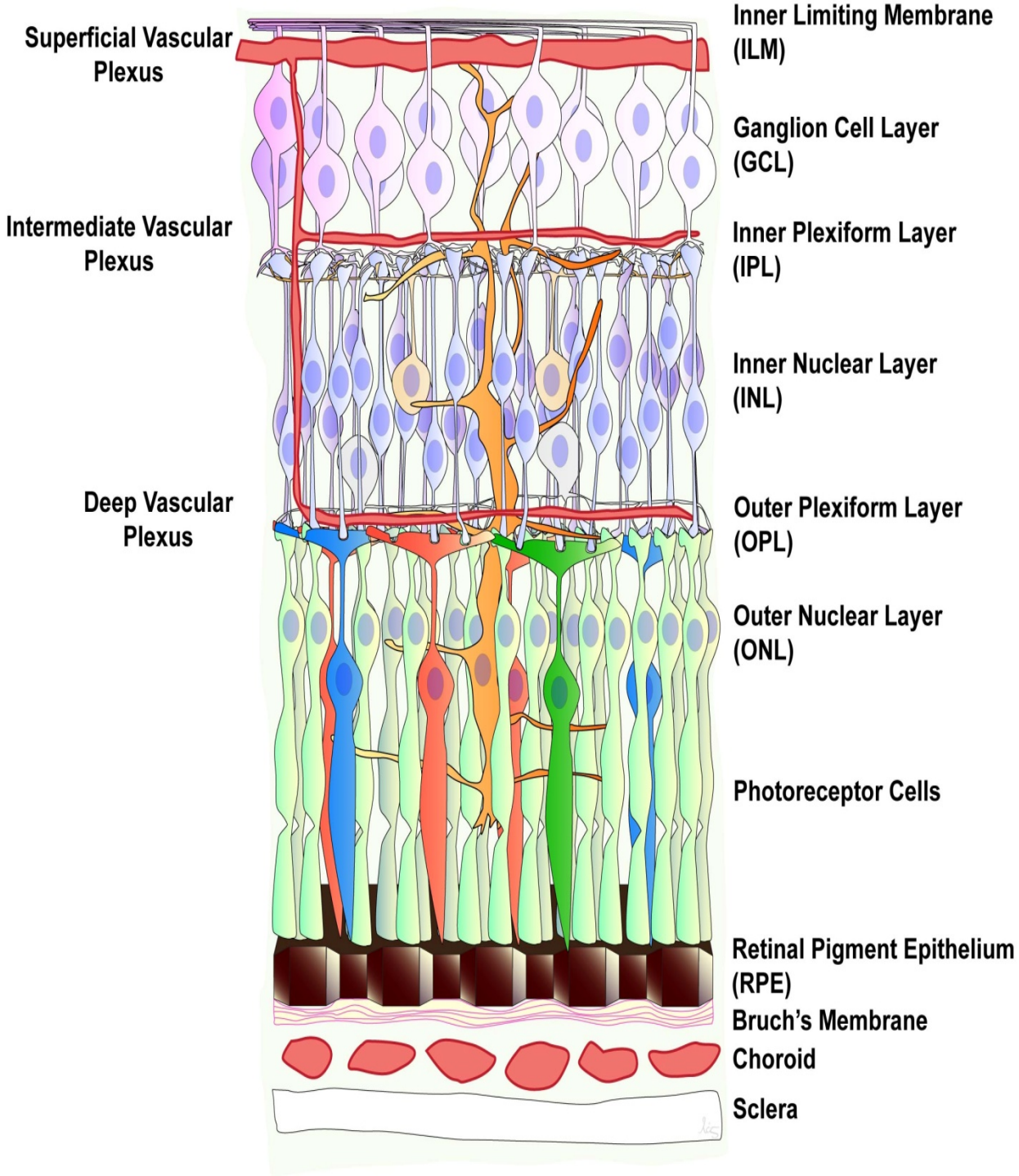

Figure 1-2. Human retinal layers. This diagram is a depiction of a retinal cross section of the human retina. From the most anterior layers of the retina, the retinal layers consist of the sclera, choroid, retinal pigment epithelium, rod and cone photoreceptors, outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, ganglion cell layer, and the inner liming membrane.

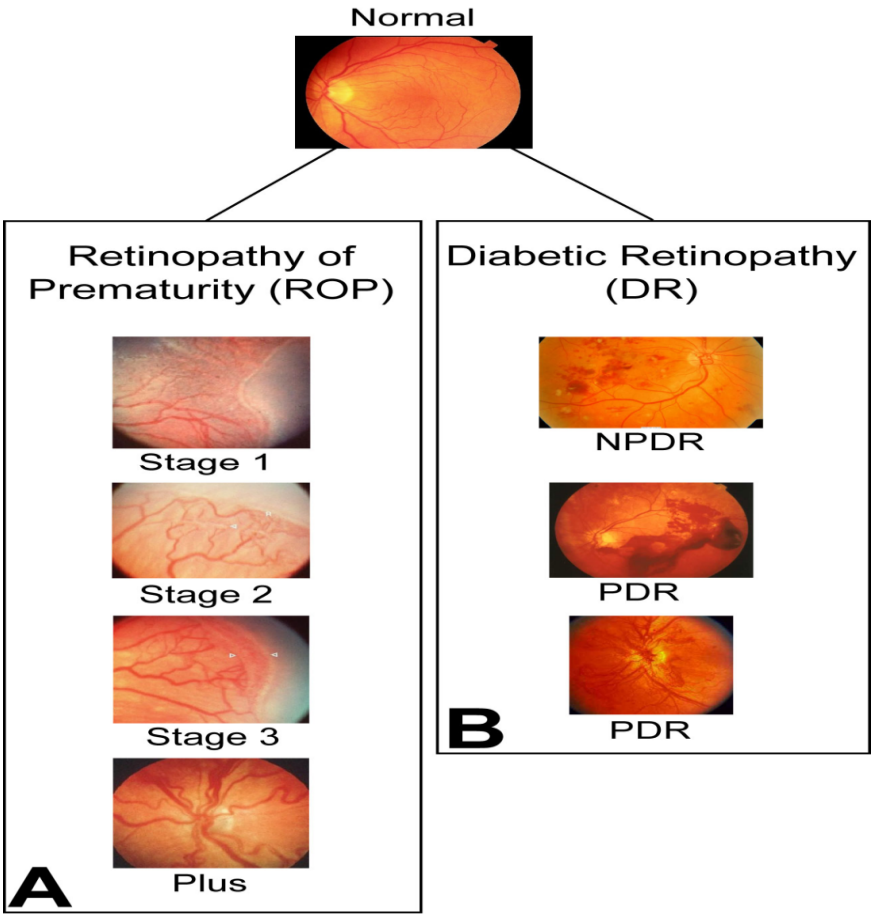

Figure 1-3. Retinopathies. A) Shows stages of ROP. Stage 1 involves a demarcation line where normal and abnormal vessels meet. Stage 2 is characterized by an intraretinal ridge that rises up from the retina as a result of abnormal vessel growth. Stage 3 contains a ridge which grows from the spread of abnormal vessels and extends into the vitreous. Stage 4 and 5 (plus) is characterized by retinal detachment with Stage 5 being total retinal detachment and blindness. B) Contains representative images of the two forms of Diabetic Retinopathy, Proliferative Diabetic Retinopathy (PDR) and Non-Proliferative Diabetic Retinopathy (NPDR). NPDR, also known as background retinopathy, involves the accumulation of blood and/or exudative deposits in the retina from tiny blood vessels that leak (microaneurysums). In advanced stages of NPDR fibrous vascular tissue accumulates in the retina leading to visual impairment. PDR is characterized by the formation of abnormal blood vessels (neovascularization) growing on the surface of the retina or optic disc. These blood vessels are typically fragile and easily ruptured.

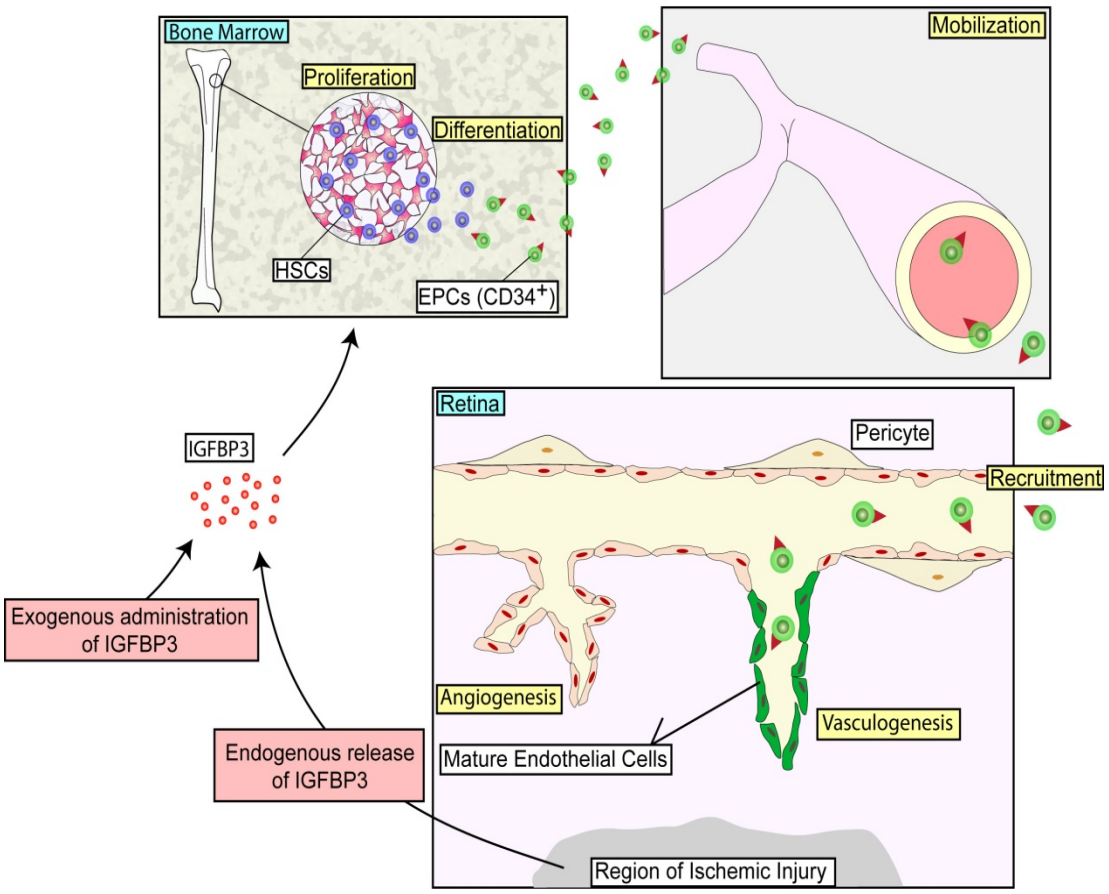

Figure 1-4. Blood vessel development. Blood vessels are either developed by vasculogenesis or angiogenesis. During vasculogenesis, endothelial progenitor cells ($CD34^+$ cells) can assemble to form new vessels during embryogenesis. During angiogenesis in the adult, sprouts form from pre-existing blood vessels and migrate into the surrounding tissue in the adult. This process relies on proliferation, migration, and remodeling of fully differentiated endothelial cells. Neovascularization can also occur with EPCs, which circulate postnatally in the peripheral blood. They can be recruited and incorporated into sites of active neovascularization in ischemic tissue by growth factors such as IGFBP-3, which are released by ischemic tissue in response to injury.

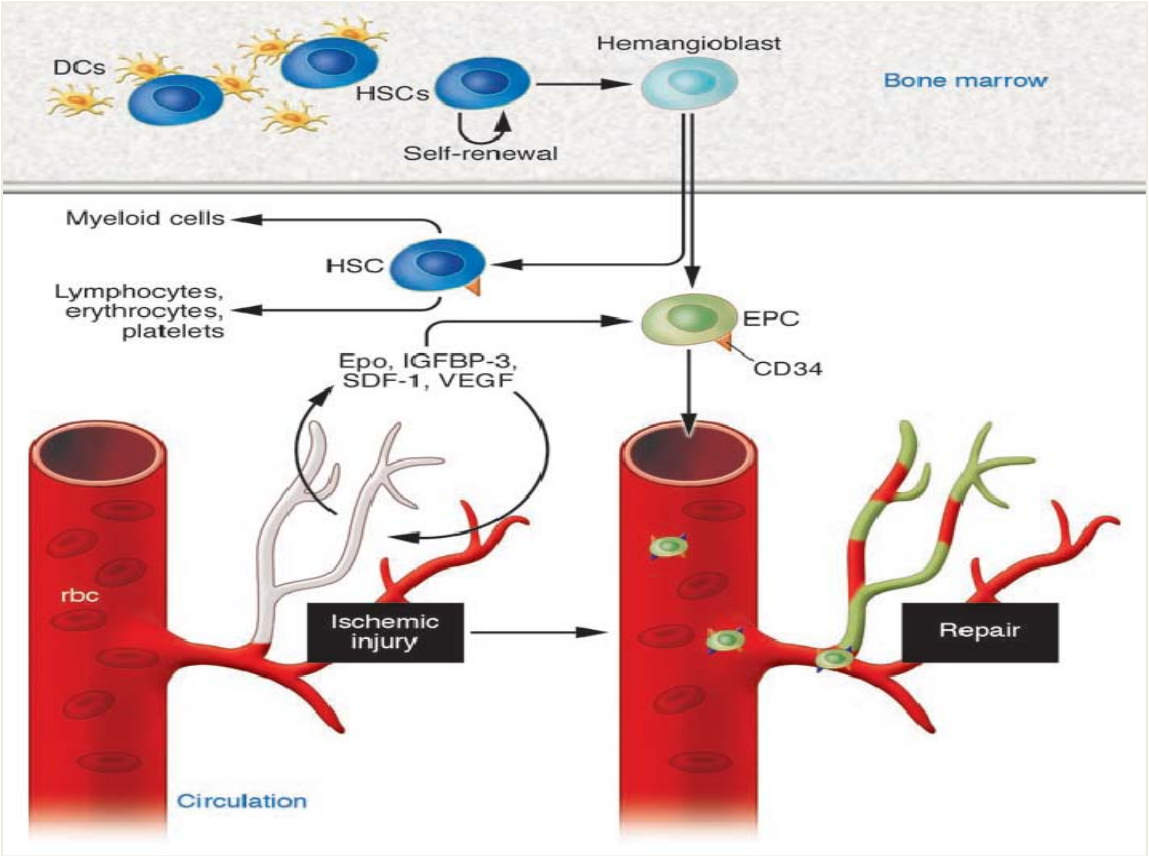

Figure 1-5. Hypoxia-regulated growth factors and bone-marrow derived cells. HSCs and EPCs are derived from a common precursor called the hemangioblast. These cells maintain primitive characteristics and can differentiate into a wide range of cells types as shown. $CD34^{+}$ cells are EPCs. These cells are mobilized by many factors such as SDF-1 and VEGF, which govern their migratory and homing ability into blood vessels undergoing ischemic insult. EPCs are recruited to areas of ischemia where they can participate in vascular repair and functional revascularization.

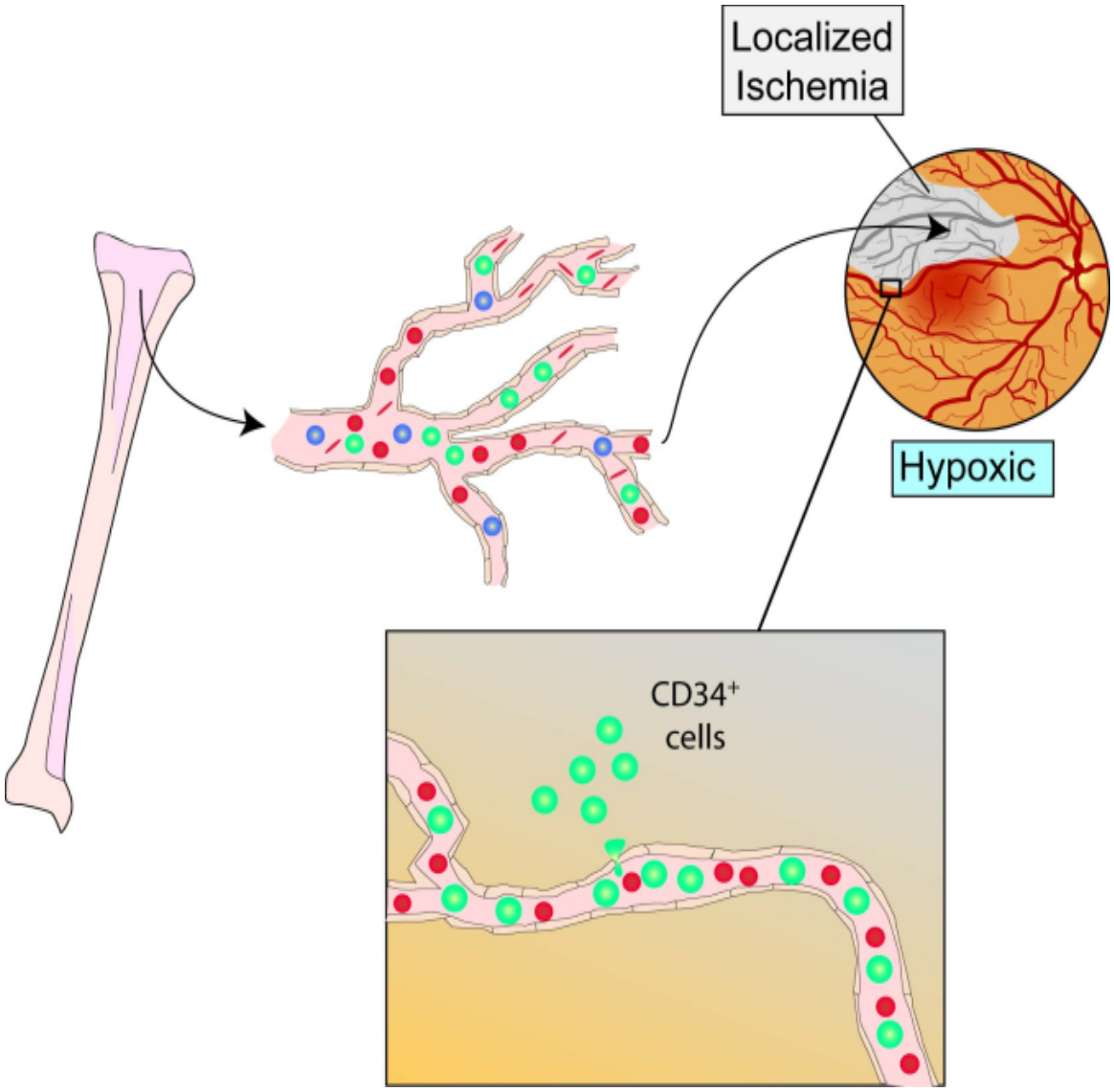

Figure 1-6. Recruitment of EPCs in ischemic tissue. Circulating EPCs, specifically $CD34^+$ cells, are recruited to areas of hypoxic vascular injury such as in the retina where they can home, integrate, stabilize, and promote proper vascular repair.

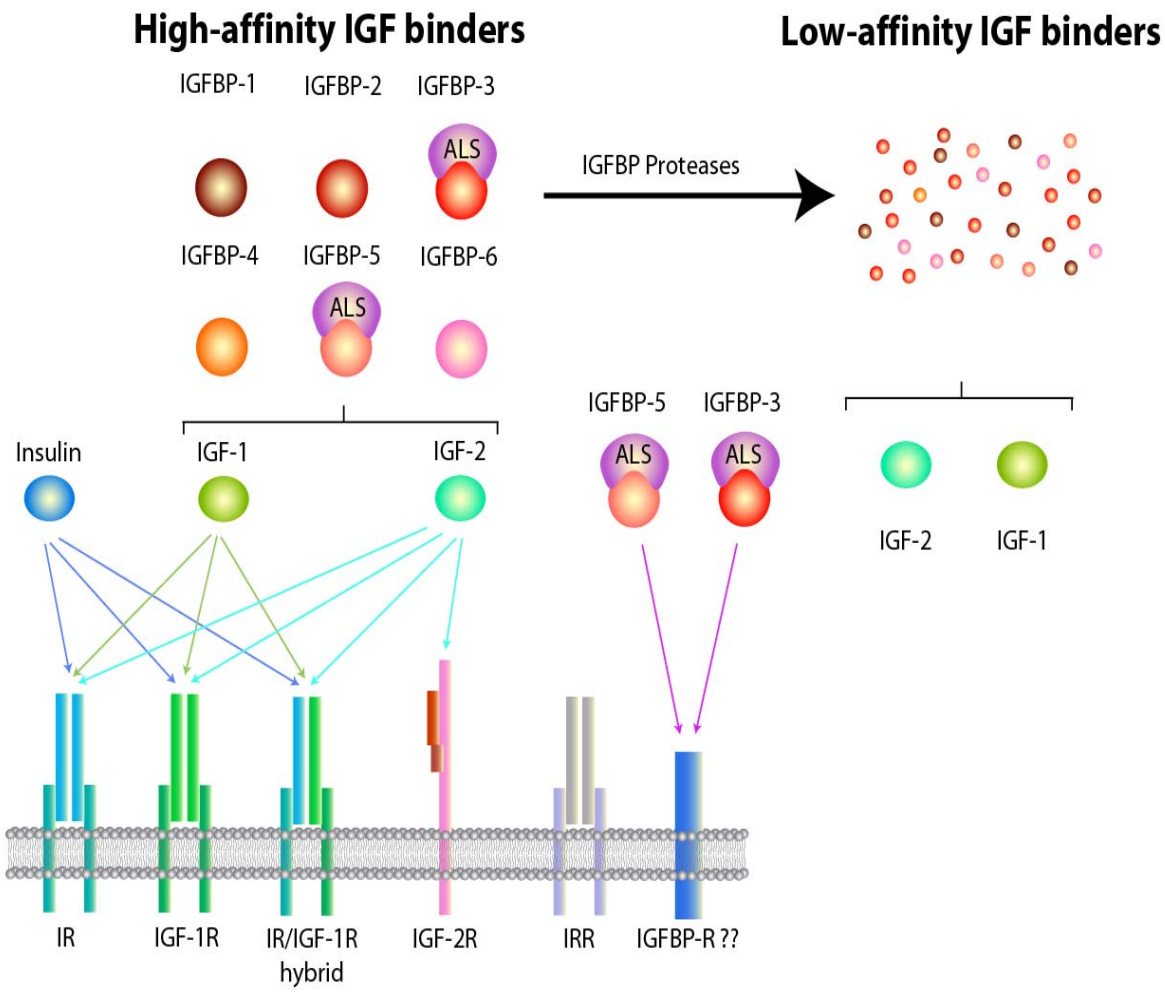

Figure 1-7. IGF system. Ligands (IGF-1, IGF-2 and insulin) IGFBP's (1-6), as well as receptors (IR, IGF-1R, hybrid IR/IGF-1R, IGF-2R, IRR, and IGFBP-R) are depicted. IGF-I interacts with IGF-1R, IR, hybrid IR/IGF-1R, and IGFBP's; IGF-2 interacts with IR, IGF-1R hybrid IR/IGF-1R, IGF-2R, and IGFBP's; insulin interacts with IR, IGF-1R, and hybrid IR/IGF-1R. Some IGFBP's are cleaved by IGFBP proteases releasing proteolysed fragments, which have low affinity for IGFs. IGFBP-3 and IGFBP-5 may act through their own receptor, which to date has not been identified.

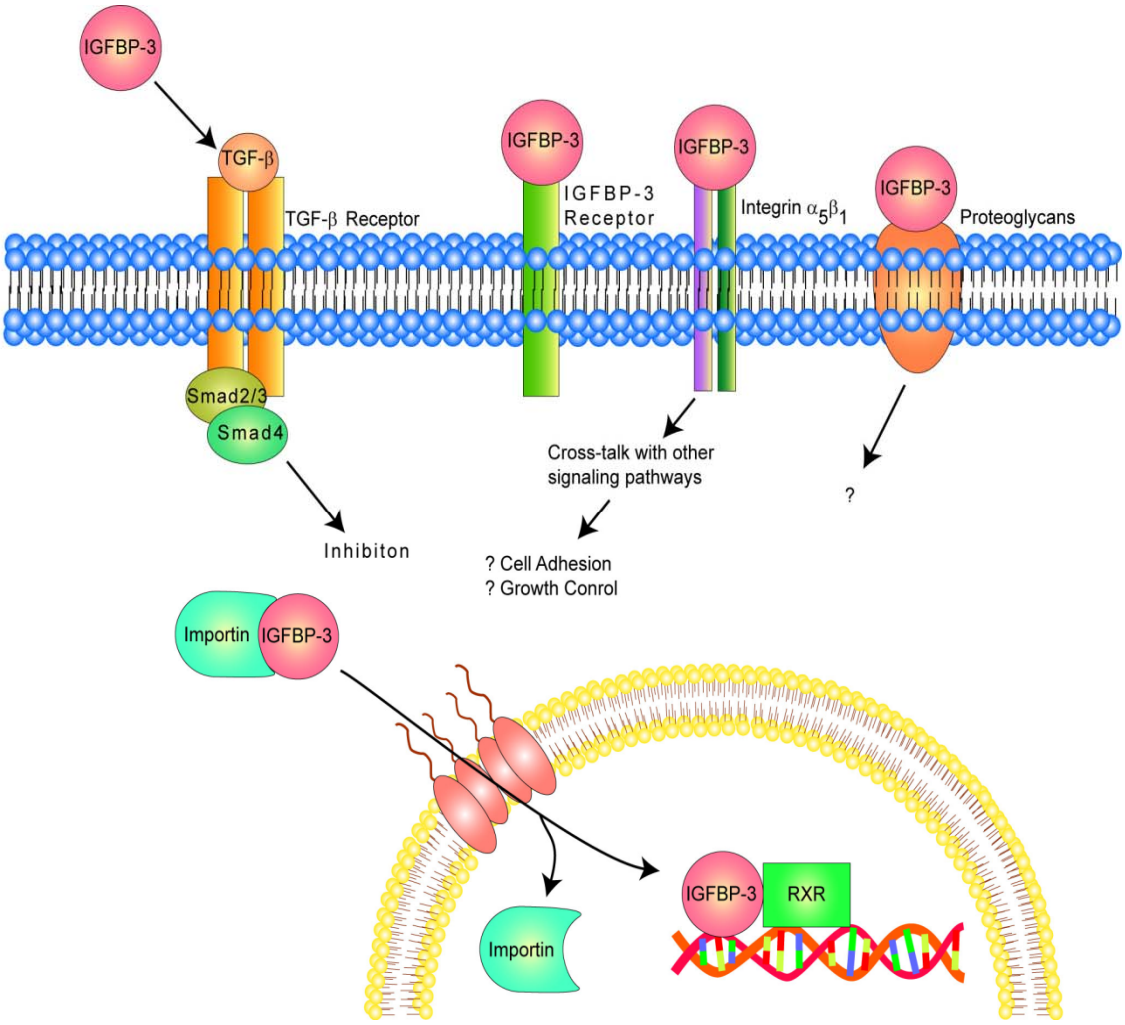

Figure 1-8. IGFBP-3 signaling and cross-talk. IGFBP-3 has no known receptor. However, it can signal through other receptors such as TGF-β receptor. IGFBP-3 can also interact with proteoglycans and integrins to control cell adhesion and/or growth. IGFBP-3 contains a NLS sequence which allows it to enter the nucleus via importin. In the nucleus IGFBP-3 can interact with the RXR receptor to influence transcription.

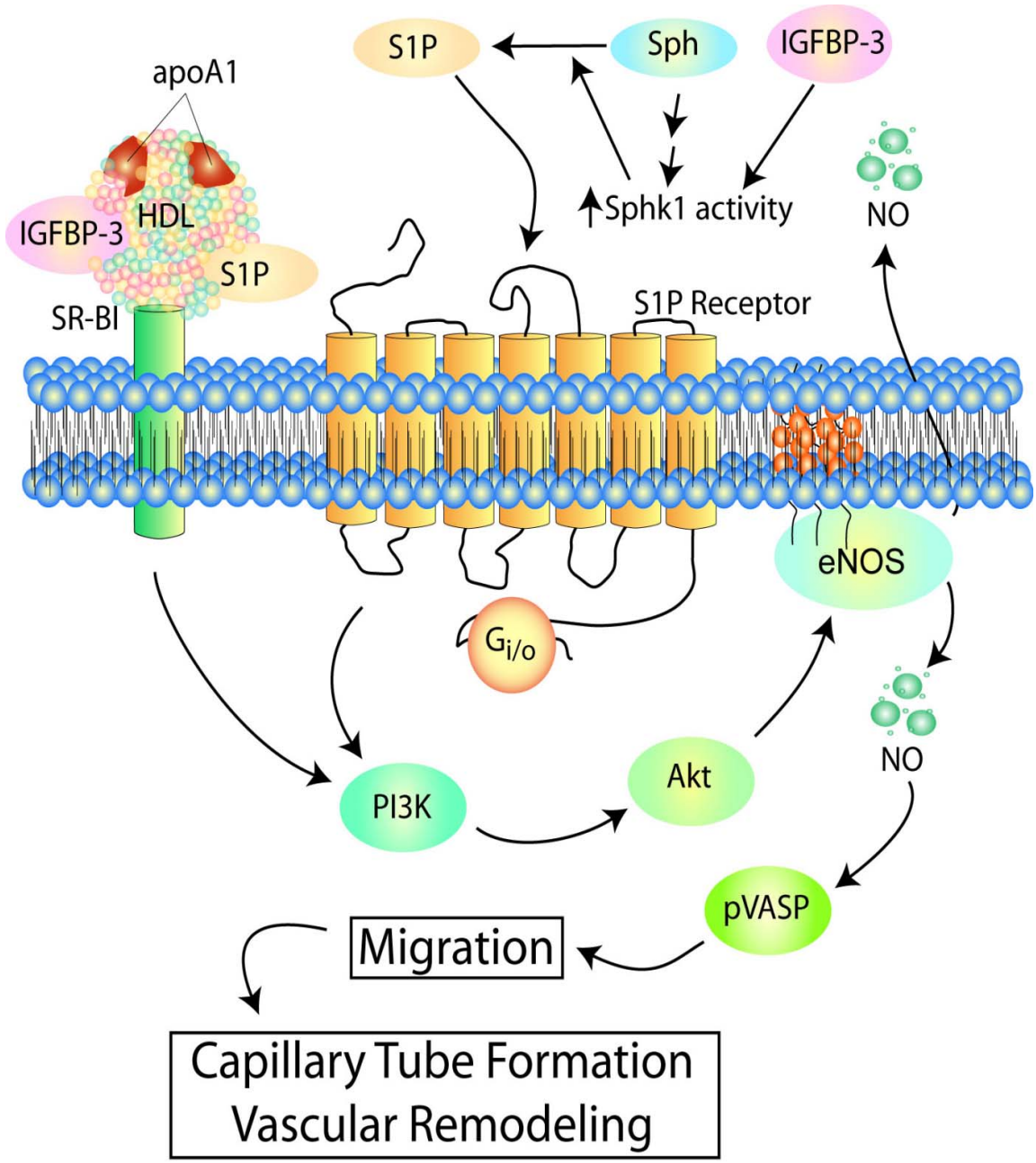

Figure 1-9. IGFBP-3 signaling to increase NO leading to cell migration. IGFBP-3 can activate the SR-B1 or S1PR to increase NO production and release via the PI3K/Akt cell survival pathway. IGFBP-3 also induces VASP re-distribution, which promotes cell migration. IGFBP-3 increases NO production in both EPCs and endothelial cells, which allows them to migrate to areas of needed repair or vascular instability. Likewise, NO production and release by these cells can also attract other cells to promote vascular remodeling in areas of vascular damage.

CHAPTER 2
METHODS AND MATERIALS

In Vivo Studies

All animal procedures conducted in this study were in agreement with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals (DHEW Publication No. NIH 80-23, Offices of Science and Health Reports, DRR/NIH, Bethesda, MD 20205). All protocols were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC). Timed pregnant C57BL/6 mice, adult female C57BL/6 mice, and C57BL/6-tg (UBC) transgenic gfp^+ mice were obtained from Jackson Laboratories (Bar Harbor, ME) and housed in a temperature controlled room with a 12 hour light/dark cycle in the University of Florida Health Science Center Animal Care Resource facilities. Approximately 50 mouse pups, 50 adult female C57BL/6 mice and 50 adult female chimeric gfp^+ mice were used in various studies. We performed a series of *in vivo* assays to assess IGFBP-3's effect on EPC migration, incorporation, and differentiation in the mouse retinal vasculature. We also evaluated IGFBP-3's anti-apoptotic effects in the mouse vasculature via TUNEL analysis and mass spectrophotometry. Lastly, we assessed IGFBP-3's effects on vascular permeability. Figures 2-1 through 2-3 summarize the *in vivo* studies conducted.

Oxygen Induced Retinopathy (OIR) Model

We used the OIR model developed by Smith *et al.*, which was described in chapter 1. It is also depicted in Figure 2-1. The OIR model induces the formation of pre-retinal neovascularization in mouse pups. On postnatal day 7 (P7), the pups and the nursing dam were placed into an oxygen (O_2) chamber and exposed to hyperoxic levels of O_2 (~75%) for 5 days. On P12 the mice were returned to room air. The return to normoxic conditions (21% O_2) after being held at high O_2 stimulates a hypoxic stimulus that initiates the formation of the pre-retinal

vasculature. The mice were sacrificed at either P12 or P17 and their retinas were harvested for various studies.

One cohort of mice (n=24) underwent the OIR model and were euthanized at P17 to examine TUNEL apoptosis analysis of IGFBP-3 injected and vector injected control mouse pups. We used the OIR model to assess whether IGFBP-3 has anti-apoptotic effects on specific vascular cell types such as endothelial cells, astrocytes, and pericytes. (Figure 2-2) The OIR model was ideal for this since the developing neonate vasculature is actively undergoing rapid cell turnover and remodeling, compared to the adult vasculature, which is relatively quiescent.

The mice were intravitreally injected with 0.5 μl in the right eye on P1 with either plasmid expressing mouse IGFBP-3 (2μg/ml) under control of a proliferating endothelial cell-specific promoter (Cdc6) (n=12) or the cloning vector as an injection control (n=12). By utilizing a proliferating endothelial cell-specific promoter IGFBP-3 expression was targeted to endothelial cells, thus to areas of neovascularization. For intravitreal injection into P1 mouse pups, ice-induced anaesthesia was performed by placing the neonate on a plastic shield over a layer of crushed ice for 1-2 minutes. The mice were euthanized at either P12 or P17 for simultaneous TUNEL analysis of retinal flatmounts (n=12) and for immunohistochemical staining of endothelial cells, pericytes, and astrocytes (n=12). The eyes were fixed with 4% paraformaldehyde for 1 hour at room temperature. The retinas were dissected from the posterior eye cup and incubated with antibodies for astrocytes (S-100, Sigma), endothelial cells (GS isolectin B4, Sigma) and pericytes (NG2, Chemicon) followed by staining with a commercial TUNEL detection kit (Roche, Switzerland) to detect cells undergoing DNA fragmentation. The eyes from the neonate mice injected with IGFBP-3 plasmid were compared with eyes from mice

injected with empty plasmid vector or the uninjected eye of the same animal (contralateral eye). Three different regions of the retinal flatmounts were studied: the central, mid-peripheral, and peripheral regions.

Statistical Analysis of Cell Death in OIR Model

In order to quantify cell death in the OIR model, representative fields of view from the central, mid-peripheral, and peripheral retinas were counted using a 20x objective as the field of view for analysis as modified by Hughes *et al*.[226] In each field of view $TUNEL^+$/GS $Lectin^+$ vascular endothelial cells, $TUNEL^+/NG2^+$ pericytes, and $TUNEL^+/S\text{-}100^+$ for astrocytes were counted. The data is collected as mean standard deviation (SD) N=6 per experimental group, and the statistical significance of differences among mean values was determined by one-way ANOVA. ANOVA statistical analysis was performed with SPSS 13.0 software (SPSS, Chicago, IL). A p-value of less than 0.05 was considered statistically significant.

Quantification of Astrocyte Ensheathment in OIR Model

We also assessed the frequency of S-100 ensheathment of retinal vessels in the OIR model. We studied the astrocytes in OIR model, since they serve as a template for developing vessels. Astrocytes also help to stabilize blood vessels and we propose IGFBP-3 may have an influence on these particular cells.

We determined the astrocyte ensheathment using a modified method previously described. Representative fields of view from the mid-peripheral retina were counted using a 20x objective as the field of view for analysis. Each confocal image was overlaid with a 10 x 10 equally spaced grid using Adobe Photoshop V 5.0. The grid was superimposed onto each image. The occurrence of S-100 labeling relative to lectin labeling at the 100 intersection points yielded the percentage of astrocyte ensheathment. The data was collected as mean standard deviation (SD) where n=6 per experimental group and the statistical significance of differences among

mean values was determined by t-Test. A p-value of less than 0.05 was considered statistically significant.

Electron Microscopy in the OIR Model

In order to assess apoptosis in the neural retina, we used electron microscopy to assess retinal cross sections for their morphology and cell structure. Electron microscopy's fine detail allowed us to clearly delineate whether neuronal cells in the retina were undergoing active apoptosis in IGFBP-3 treated versus untreated neonatal mouse retinas.

A second cohort of mice (n=12) was injected as previously described with a total of 6 mice injected with IGFBP-3 plasmid and 6 mice injected with the empty vector plasmid control. (Figure 2-2) The mice were euthanized at P17, whole eyes globes were harvested, and processed for electron microscopy analysis. The eyes were fixed with 4% paraformaldehyde/2.5% glutaraldehyde for 4 hours. The eyes were embedded in epoxy resin and stained for electron microscopy as previously described. [227]

Generation of Adult Chimeric gfp^+ Mice

Chimeric mice have been used extensively for *in vivo* studies to assess homing and the reparative capacity of EPCs, especially in ocular injury models. Green fluorescent protein (gfp^+) chimeric mice are easy to produce, have high engraftment efficiency, and are relatively stable over time. They serve as an excellent tool to study and track EPC recruitment, differentiation, and incorporation into the retinal vasculature.

Bone Marrow Isolation & Transplant

C57BL/6 Tg mice (Jackson Laboratories, Bar Harbor, ME) are homozygous for gfp expression under control of the ubiquitin promoter. These mice were bred and housed at the University of Florida. For the bone marrow isolation, male C57BL/6 mice were anesthetized by intraperiotoneal injection of a mixture of xylazine (30 mg/ml) and ketamine (14 mg/ml) at a dose

of 5 μl per 20 grams of body weight. The animals were sacrificed by cervical dislocation. The tibias and fibulas were removed and placed on ice. Both ends of all bones were removed and bones were flushed with ice-cold PBS using a 27 gauge needle. The bone marrow mixture was collected in a 15 ml conical tube, centrifuged at 19,000 g for 10 minutes at 4 degrees. The supernatant was discarded and the pellet was resuspended in 2 ml of PBS. Controls were prepared in 5 ml polypropylene tubes. The first control contained 1 ml of PBS with 30 μl of the resuspended bone marrow. The second control had 1 ml of PBS, 30 μl of bone marrow, and 1 μl of Sca-1-phycoerythrin antibody (Sca-1-PE, BD Pharmingen, San Diego, CA). The third control consisted of 1 ml of PBS, 30 μl of bone marrow, and 1 μl of c-kit-allophycocyanate antibody (c-kit-APC, BD Pharmingen). The sample contained the remaining bone marrow and 120 μl each of the Sca-1-PE antibody and the c-kit-APC antibody in a 15 ml conical tube. These were all incubated at 4 degrees for 15 minutes to allow the antibodies to attach to the antigens. PBS was added to the controls and sample. All tubes were centrifuged. The supernatant was discarded to remove unbound antibodies. The controls were resuspended in 500 μl of PBS, while the sample was resuspended in 10 ml of PBS. The total volume was then filtered using polystyrene tubes with cell strainer caps (Becton Dickinson, Franklin Lakes, NJ). The bone marrow cell population was then enriched for HSCs by fluorescence-activated cell sorting (FACS Calibur flow cytometer, BD Biosciences, San Jose, CA). Only cells that were c-kit^{+}/Sca-1^{+}/ gfp^{+} were selected and placed in PBS supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) on ice until needed. The cells were then centrifuged and resuspended in a volume adequate to yield 5,000 cells per 100 μl. Adult female C57BL/6 mice were anesthetized and lethally irradiated (950 rads) using a cesium source. The recipient mice (6-8 weeks age of age) were

injected in the retro-orbital sinus (ROS) with 100 μl of c-kit$^+$/sca-1$^+$/gfp$^+$ cells. The transplanted mice were followed and allowed to engraft for three months.

Chimeric Engraftment Confirmation

After three months, successful engraftment of the mice was confirmed by flow cytometry analysis of peripheral blood. Approximately 500 μl of blood was obtained from the transplanted mice by tail venipuncture and added to the same volume of PBS with 10 mM EDTA (ethylenediaminetetraacetic acid) to prevent coagulation. Two ml of Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ) was added to each sample and the samples were centrifuged at 125 g at room temperature for 30 minutes. After centrifugation, glass pipettes were used to collect the buffy coat (comprised of lymphocytes), which was then placed in a clean tube. A total of 2 ml of PBS was added and the samples were centrifuged at 55 g at 4˚ for 5 minutes to remove any residual Ficoll. After discarding the supernatant, 500 μl of PBS was added and the number of gfp$^+$ cells was determined by flow cytometry. Mouse samples in which the concentration of gfp$^+$ cells was higher than 80% were considered adequately reconstituted. Only these highly engrafted mice were used for experimentation.

Retinal Laser Vessel Occlusion Model

In order to assess whether IGFBP-3 enhances EPC homing and recruitment to retinal vessels, we had to induce ischemic injury to the adult retinal vessels. The mice were lasered by delivering ~50 spots at 150 milliwatts for 0.2 seconds with an Argon green laser in a transpupillary manner to the right eye to induce retinal vessel occlusion. Two groups of adult C57BL/6gfp$^+$ chimeric mice: laser only (n=15) and laser with IGFBP-3 injection (n=18) were subjected to laser injury in their right eye. A third group of chimeric mice received only IGFBP-3 injection into their right eye (n=16). The IGFBP-3 plasmid under the endothelial cell specific

promoter (Cdc6), was packaged into liposomes. Immediately following laser treatment to the right eye a total of 2 μl of IGFBP-3 plasmid (1μg/ml) packaged into liposomes was delivered intravitreally into the right eye of the designated groups with a 39 gauge Hamilton syringe (Reno, NV). All mice were sacrificed 3 weeks post laser treatment and their eyes were harvested for microscopy analysis. The mouse eyes were fixed for 1 hour at room temperature with 4% PFA. The neural retinas were dissected from the eye cup and washed with PBS prior to immunostaining.

Immunostaining & Microscopy Analysis

In order to visualize the retinal vasculature in the adult chimeric mice, retinas were stained with rhodamine-agglutinin (Vector Labs, Burlingame, CA), a blood vessel specific stain, which clearly labels both large and small vessels in the mouse retina. The retinas were permeabilized with 0.2% triton-X-100 and 0.2% BSA in 10mM HEPES buffer overnight at 4 degrees followed by incubation with rhodamine-agglutinin at 1:1000 overnight. The retinas were then stained with an anti-GFP antibody (Chemicon, Temecula, CA) at 1:500 to confirm gfp^+ cell incorporation into the retinal vasculature. Also, the adult retinas were stained with three additional markers to determine the fate of the gfp^+ cells. (Figure 2-3) These markers include: *Griffonia Simplicifolia* isolectin B4 (Sigma) for detection of endothelial cells, NG2 (Chemicon) for detection of pericytes and smooth muscle cells, and S100 (Sigma) for detection of astrocytes. After immunostaining, the mouse retinas were flatmounted by placing radial cuts in the tissue and mounting on a glass coverslip with VectaShield (Vector Labs). Retinas were examined using a Leica-argon krypton laser mounted on a Leica DMRBE epifluorescence photomicroscope (Leica, Wetzlar, Germany) using OpenLab Imaging software. Images were taken at 20x and 40x to examine fine detail of the retinal blood vessels.

Mass Spectrophotometry Analysis

Adult non-chimeric C57BL/6 mice were subjected to retinal vessel occlusion (n=6) and either injected (n=6) or uninjected (n=6) with IGFBP-3 plasmid as described earlier for mass spectrophotometry analysis. The mice were sacrificed three weeks later and their retinas were harvested to assess sphingosine-1-phosphate, ceramide, and sphingomyelin lipid ratios. Retinal homogenates were extracted with 200 µl/mg tissue of chloroform:methanol (2:1 v/v) by vortexing for 1 minute. After centrifugation at 3000g for 10 minutes, the lower organic phase was recovered and the aqueous upper phase was re-extracted with 200 µl/mg tissue of chloroform. The pooled organic phases were evaporated under nitrogen, resuspended in chloroform:methanol (2:1 v/v) and washed with HPLC-grade water. The solvent was again evaporated under nitrogen, and lipids were further dried overnight under vacuum. Lipid extracts were resuspended in 50 µl/mg tissue of isopropanol:methanol:chloroform (4:2:1v/v/v) and stored under nitrogen in glass vials in the dark at -80º C until further use.

Prior to mass spectrophotometry analysis, lipid extracts were diluted 1:20 in isopropanol/methanol/chloroform (4:2:1 v/v/v) containing 20 mM ammonium hydroxide. The final concentrations of internal standards added to the lipid extracts were 300 nM for GPCho and 12.5 nM for sphingolipids. All samples were centrifuged, loaded into Whatman Multichem 96 well plates (Fisher Scientific) and sealed with Teflon Ultra-Thin Sealing Tape (Analytical Sales and Services, Pompton Plains, NJ). Lipids were introduced to a Thermo model TSQ Quantum Ultra triple quadruple mass spectrometer (San Jose, CA) at a flow rate of ~250 nL/min via a chip-based nanoelectrospray ionization (nESI) source (Advion NanoMate, Ithaca, NY) operating in infusion mode using an ESI HD-A chip, a spray voltage of 1.4kV, a gas pressure of 0.3 psi and an air gap of 2µL. The ion transfer tube of the mass spectrometer was maintained at 150˚C. All MS/MS spectra were acquired automatically by methods created using Xcalibur software

(Thermo, San Jose, CA) with a scan rate of 500 m/z second^{1}, and Q2 collision gas pressure of 0.5 mtorr. GPCho and SM species were analyzed as $[M+H]^+$ ions by parent ion scanning of m/z 184 in positive ion mode, and as $[M+Cl-H]^-$ ions by neutral loss scanning of 50 Da in negative ion mode. Ceramide species were analyzed as $[M+H]^+$ions by parent ion scanning of m/z 264.4 in positive ion mode. GPIns species were analyzed as $[M-H]^-$ ions by parent ion scanning of m/z 241 in negative ion mode. Fatty acid constituents of GPCho species were confirmed in negative ion mode by product ion scanning of $[M+Cl-H]^-$ ions of interest. Collision energies for each MS/MS scan were optimized using commercially available synthetic lipid standards. MS/MS spectra were averaged over a period of 5 minutes, and a five point Gaussian smooth was applied to all spectra prior to data analysis.

Vascular Permeability

Non-chimeric C57Bl/6 mice were used for *in vivo* permeability experiments. In the first set of permeability experiments, there were a total of five experimental groups. These included IGFBP-3 alone (n=12), VEGF alone (n=12), VEGF followed by IGFBP-3 injected 6 hours later (n=12), VEGF followed by IGFBP-3 injected 24 hours later (n=12), and IGFBP-3 vehicle consisting of acetic acid (n=12). The mice were intravitreally injected with recombinant VEGF (100 ng/ml) and/or recombinant non-IGF binder mutant IGFBP-3 (100 ng/ml). Recombinant VEGF was administered to induce permeability followed by IGFBP-3 injection at 6 hours or 24 post VEGF injection. After 48 hours following the initial VEGF injection, the mice received a tail injection of FITC albumin. Two hours later the mice were sacrificed by cardiac perfusion with 4% PFA. The retinas were immediately harvested and processed for fluorescence analysis.

In the second set of permeability experiments, non-chimeric C57BL/6 mice underwent laser injury and IGFBP-3 intravitreal injection as previously described earlier in the chapter. There were a total of three experimental groups: laser only (n=12), IGFBP-3 injected only

(n=12), and laser and IGFBP-3 injected (n=12). After 4 days post laser treatment, the mice received a tail injection of FITC albumin. Two hours later the mice were sacrificed by cardiac perfusion with 4% PFA. The retinas were immediately harvested and processed for fluorescence analysis.

In Vitro Analysis

Various *in vitro* assays were conducted to assess IGFBP-3's effects on nitric oxide production, eNOS expression, and VASP re-distribution in endothelial cells. Figure 2-4 summarizes the *in vitro* experiments conducted. Four types of cells were used in the *in vitro* experiments. These include human $CD34^+$ cells (EPCs) from normal patients, human microvascular endothelial cells from the lung (HMVEC-L), human umbilical vein endothelial cells (HUVECs), and rat retinal ganglion cell line (RGC-5). Human recombinant IGFBP-3 protein (Upstate Cell Signaling) was used at a concentration of 100ng/ml in all *in vitro* experiments. This concentration is reflective of physiological levels found in human blood serum. We have previously shown this concentration has the desired maximum effect on EPCs.[36] Also, various inhibitors were used to block nitric oxide such as L-NAME, a blocking antibody to SR-B1, and inhibitors for Akt and PI3K. These inhibitors were used at concentrations previously reported in the literature for endothelial cells. Also, human HDL isolated from normal patient blood, was used at a concentration of 1mg/ml, within the physiological range found in human serum. Basically, the *in vitro* studies were conducted to seek a mechanism of action of IGFBP-3 in promoting vascular repair. Our *in vitro* studies were designed with emphasis on how IGFBP-3 influences cell migration, both EPC and mature endothelial cell migration.

EPC Isolation

Endothelial progenitor cells (EPCs) were isolated from peripheral blood from healthy patients. The blood was collected into cell preparation tubes (CPT; BD Biosciences, San Jose,

CA) and spun to obtain mononuclear cells. EPCs were separated from the mononuclear fraction using a $CD34^+$ isolation kit (StemCell Technologies, Vancouver, CA). Mononuclear cells (2 $x10^7$) were incubated with a $CD34^+$ selection cocktail for 15 minutes. A total of 50 μl of nanoparticles were added to the cells and incubated for an additional 10 minutes. The suspension volume was increased to 2.5 ml and the tube containing the cells was placed in a magnet for 5 minutes. The supernatant from the tube was poured off and the remaining $CD34^+$ cells in the tube were resuspended in culture media (StemSpan, Vancouver, BC, Canada). The isolated EPCs were used for western blot and nitric oxide studies.

Cell Culture of Endothelial Cells

Mature endothelial cells, HMVEC-L and HUVECs were maintained in culture with EBM-2 media enriched with aliquot growth factors (Lonza, Walkersville, MD). The cells were used at passage 2-4 with distinct cobblestone morphology, which is indicative of their endothelial phenotype. The RGC-5 cells were grown in DMEM supplemented with 20% FBS. All three cell types were grown at 37 degrees under 5% CO_2.

Nitric Oxide Measurement

In order to assess whether IGFBP-3 increases nitric oxide, a DAF-FM nitric oxide production assay and radioactive nitric oxide activity assay were performed. Also, eNOS expression and peNOS expression was evaluated via western blotting, as well as evaluation of expression levels of the S1PRs1-5 and SR-B1 in the mature endothelial cells and EPCs.

DAF-FM NO Production Assay

NO production was quantified in HUVECs, HMVEC-Ls, rat retinal ganglion cells (RGC-5) and human $CD34^+$ cells using NO sensitive cell permeant fluorescent dye DAF-FM. HUVECs, HMVEC-L, and RGC-5's were cultured on cover-slipped bottomed dishes (MatTek, Ashland, MA). $CD34^+$ cells in suspension were loaded with DAF-FM diacetate (Invitrogen) (10

μM for HUVECs, HMVEC-Ls, and RGC-5s; 20 μM for the $CD34^+$ cells) for 30 minutes in Dulbeccos's phosphate buffered saline with calcium and magnesium (Mediatech, Inc., Manasses, VA) supplemented with glucose (1 mg/ml) and L-arginine (1mM). DAF-FM loaded cells in dishes were placed on the stage of Axiovert inverted microscope with 20x fluar objective (Zeiss) for fluorescence imaging. In the case of $CD34^+$ cells, approximately 75 μl of cell suspension was placed in the coverslipped dish to perform imaging. Fluorescent images were obtained using a computer-controlled monochromator excitation light source (TILL Polychrome II, TILL-Photonics, Martinsried, Germany) and a cooled CCD camera with exposure control. Images were analyzed and fluorescence was measured in arbitrary units using Till vision.

To evaluate the effect of IGFBP-3 and/or HDL on NO production, cells were treated with these agents for 30 minutes. Some dishes were incubated with Scavenger Receptor (SR-B1, Novus Biologicals, Littleton, CO) blocking antibody for 30 minutes (1:100) before loading cells with DAF-FM. Changes in DAF fluorescence with different treatments were expressed as percent change. Results were evaluated for statistical significance by one-way ANOVA.

eNOS Activity Assay

Activation of eNOS by IGFBP-3 was evaluated by measuring L-citrulline synthesis in HUVECs using radioactive L-arginine as substrate. Briefly, the cell suspension was incubated with L-(^{14}C) arginine at 37 degrees with constant agitation in the present or absence of 500 μM L-NAME, a nitric oxide synthase inhibitor. Following incubation, cells were lysed by sonication for 10 seconds and the sample suspension was run through 1 ml columns of Dowex AG50WX-8. Radioactivity corresponding to (^{14}C) citrulline within the eluate was quantified by liquid scintillation counting. Enzyme activity was expressed as L-NAME inhibitable radioactivity/mg of cell protein. In order to evaluate the effects of different blockers on IGFBP-3 stimulated eNOS

activity, cell suspension was incubated with various blockers (SR-B1 antibody, PI3K inhibitor, or Akt inhibitor) for 30 minutes before the addition of IGFBP-3.

Western Blot Analysis

Since $CD34^+$ cells are nonadherent suspension cells, $CD34^+$ cells (10^6) were treated in eppendorf tubes with 100ng/ml of recombinant IGFBP-3 (UpState Biotechnology) 0, 10, 30 and 60 minutes. The cells were harvested at each time point by lysing them with a mixture of lysis buffer (Cell Signaling Technology, Danvers, MA) and proteinase inhibitor (Sigma). The homogenates were vortexed and then centrifuged at 1000 G for 1 minute to collect the supernatant. The protein concentration of the samples was assayed using a protein assay to assure equal protein loading (Pierce, Rockford, IL). Proteins were separated by 10-20% sodium dodecylsulfate-polyacrylamide gel electrophoresis, with 15 μg of protein loaded in each lane. Proteins were transferred to nitrocellulose membrane with 0.2 μm pore size (Bio-Rad Laboratories, Inc, Hercules, CA). Effective protein transfer was verified by Coomassie Blue protein staining. Membranes were blocked for 1 hour with Odyssey Buffer (LI-COR, Lincoln, Nebraska) and probed overnight in Odyssey blocking buffer at 4^oC with the rabbit polyclonal antibody for peNOS (Abcam used at 1:4000 dilution. A fluorescent conjugated donkey anti rabbit secondary antibody (Rockland Immunochemicals, Inc., Gilbertsville, PA) was applied for 1 hour at room temperature at a dilution of 1:5000. All samples were normalized to cofilin, rabbit polyclonal, (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1:1000 dilution. Immunoblots were visualized using an Odyssey LI-COR infrared imaging scanner. Image analysis software (LI-COR) was used to quantify the intensity of the specific bands.

Real Time Polymerase Chain Reaction (RT-PCR)

Total mRNA from human $CD34^+$ cells, RGC-5, HMVEC-L, and HUVECs or retina from adult mice was isolated using the total RNA Mini Kit (BioRad Laboratories, Inc, Hercules, CA).

The mRNA was transcribed using iScript cDNA synthesis kit (BioRad Laboratories, Inc, Hercules, CA) and real-time PCR was performed using ABI Mastermix (ABI Biosystems, Foster City, CA). Primers for the PCR were purchased from ABI BioSystems. All samples were normalized to β-actin or TATABP (mouse retina). Real time PCR was performed on an ABI 7500 Fast PCR machine for 60 cycles. All reactions were performed in triplicate.

Immunocytochemistry

In order to assess whether IGFBP-3 influences VASP expression in mature endothelial cells, immunocytochemistry was performed. Immunocytochemistry was used because if IGFBP-3 has an effect on VASP re-distribution and rearrangement, it can be clearly visualized using fluorescence microscopy versus assessing protein changes by western blotting.

Cell Preparation and Fixation

HMVEC-L (Lonza, Walkersville, MD) were cultured on fibronectin coated coverslips (BD Biosciences) with EBM-2 media (Lonza, Walkersville, MD). Cell passage 3 was used for experiments. Cells were either untreated (control), treated for 15 minutes with recombinant 100 ng/ml of IGFBP-3 (Upstate Biotechnology, Lake Placid, NY) or with IGFBP-3 preceded by 1 hour pretreatment with 100 mM L-NAME, a selective non-inhibitor of nitric oxide synthase. At the end of the treatment, the media was removed and cells were fixed at room temperature in 4% PFA in PBS, supplemented with calcium and magnesium ions, and adjusted to pH 7.4.

Immunostaining

After fixation, the cells were washed in PBS with 0.1% Triton-X 100 for 5 minutes at room temperature. After three additional PBS washes, cells were blocked with 10% normal goat serum with 1% BSA in PBS for 1 hour at room temperature to block nonspecific antigens.

Cells were incubated with 5 mg/ml mouse anti-VASP antibody (BD Biosciences) in blocking solution overnight at 4 degrees and then with FITC labeled goat anti-mouse IgG

(Abcam, Cambridge, MA) at 1:200 dilution in blocking solution for 1 hour at room temperature. Samples were mounted with Vectashield (Vector Labs) mounting medium. Samples were examined by fluorescence microscopy (Zeiss Axioplan 2), using a Zeiss Plan-neofluar 40x oil objective. Micrographs were captured using a QImaging 12 bit cooled CDC digital camera (QImaging, Surrey, BC, Canada) and processed using Openlab imaging software (Improvision, Waltham, MA) for Macintosh.

Quantification of Immunocytochemistry

The micrographs were analyzed as TIFF images. Analysis of the images was performed using Image J software from NIH. For each cell, three measurements were made by drawing a line from the outer edge of the nuclear membrane to the outer edge of the plasma membrane. A fluorescence profile was determined by plotting the profile of each line. This yielded a set of distance coordinates (x-values) and fluorescence values (Y-values) for each line. This was done for a set of five cells for each condition and resulted in 15 sets of X-Y values for each condition. The sets of distance values (Xs) were normalized by setting the distance from the nucleus to the outer plasma membrane of each set to one and then adjusting the remaining Y-values relative to this value so that all Y-values are within the range of zero and 100. Each set of 15 XY coordinates were graphed using Excel (Microsoft) and a graph with fifteen plots for each condition was obtained. The area under the curve was determined for each condition at the relative distance from the nucleus of 0.2-0.25, 0.5-0.55, and 0.95-1.0 using Scion image (based on NIH Image for MacIntosh by W. Rasband at NIH and modified for Windows by Scion Corporation). These areas were averaged and the standard error of the mean was determined.

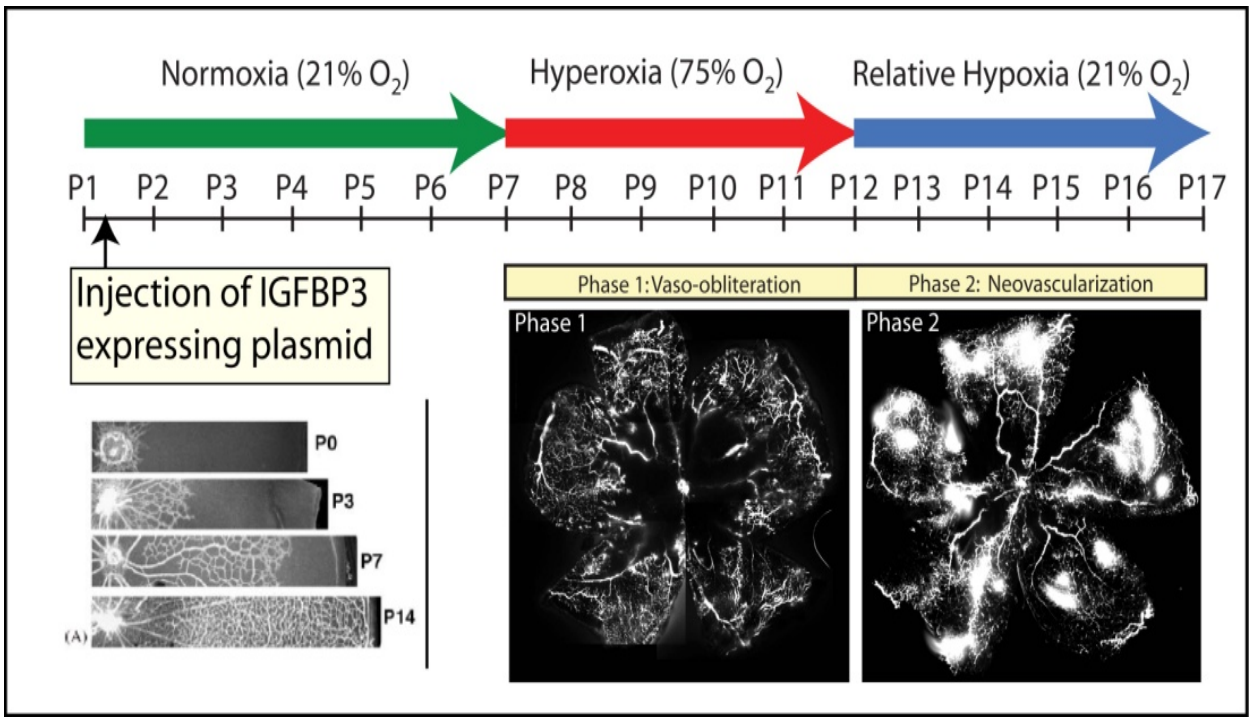

Figure 2-1. Oxygen-Induced Retinopathy (OIR) Mouse Model. Mouse pups are injected with IGFBP-3 expressing plasmid on P1 and maintained at normal room oxygen levels (21% O_2). On P7 the mice are transferred to ~75% oxygen until P12 when they are returned back to normal oxygen levels (21% O_2). The mouse pups are sacrificed at P17. Phase 1 (P7-P12) of the OIR model includes vaso-obliteration in which the retinal blood vessels regress followed by Phase 2 (P12-17) in which neovascularization or new retinal blood vessel formation occurs.

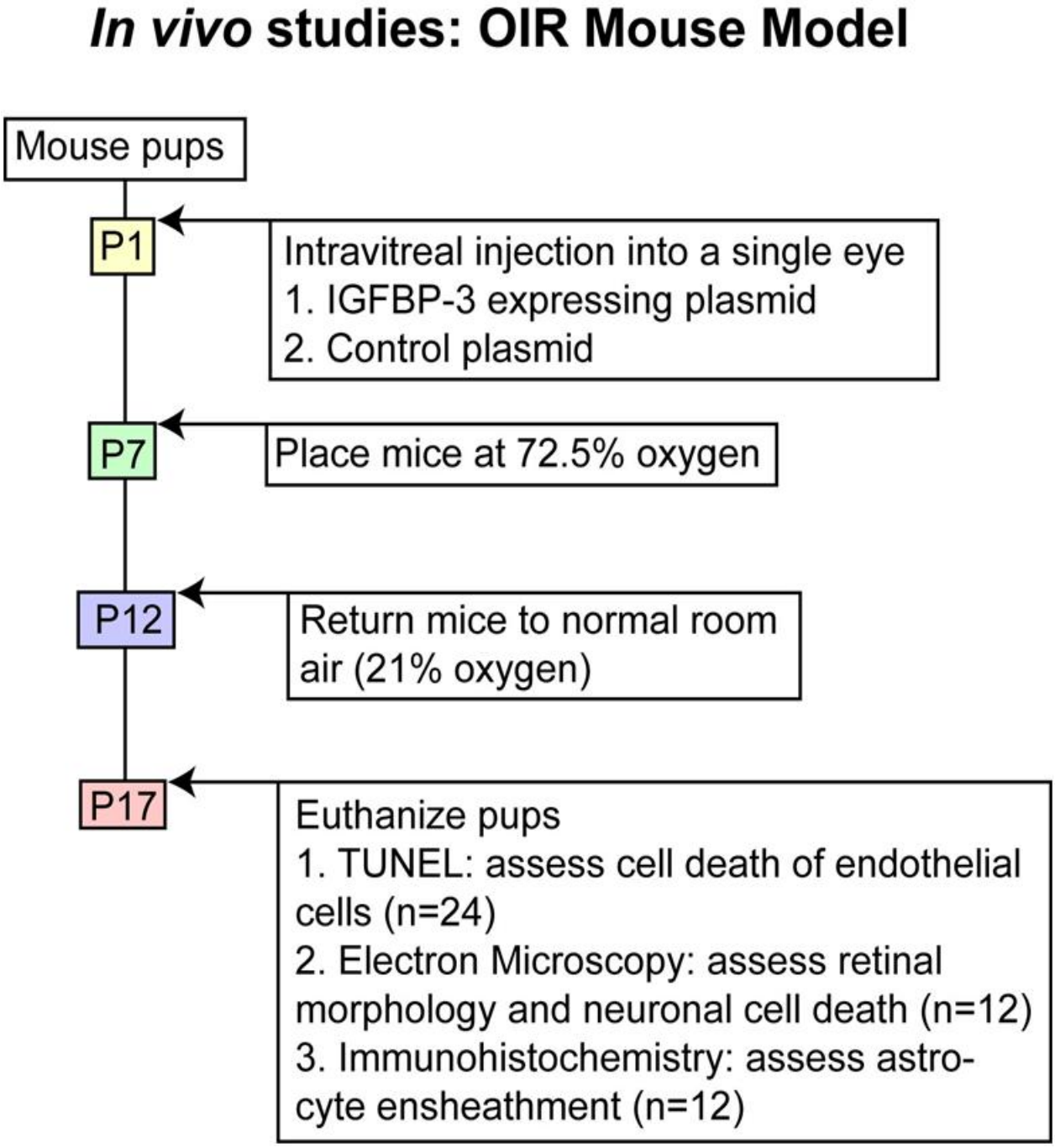

Figure 2-2. *In vivo* studies with IGFBP-3 plasmid in OIR Model.

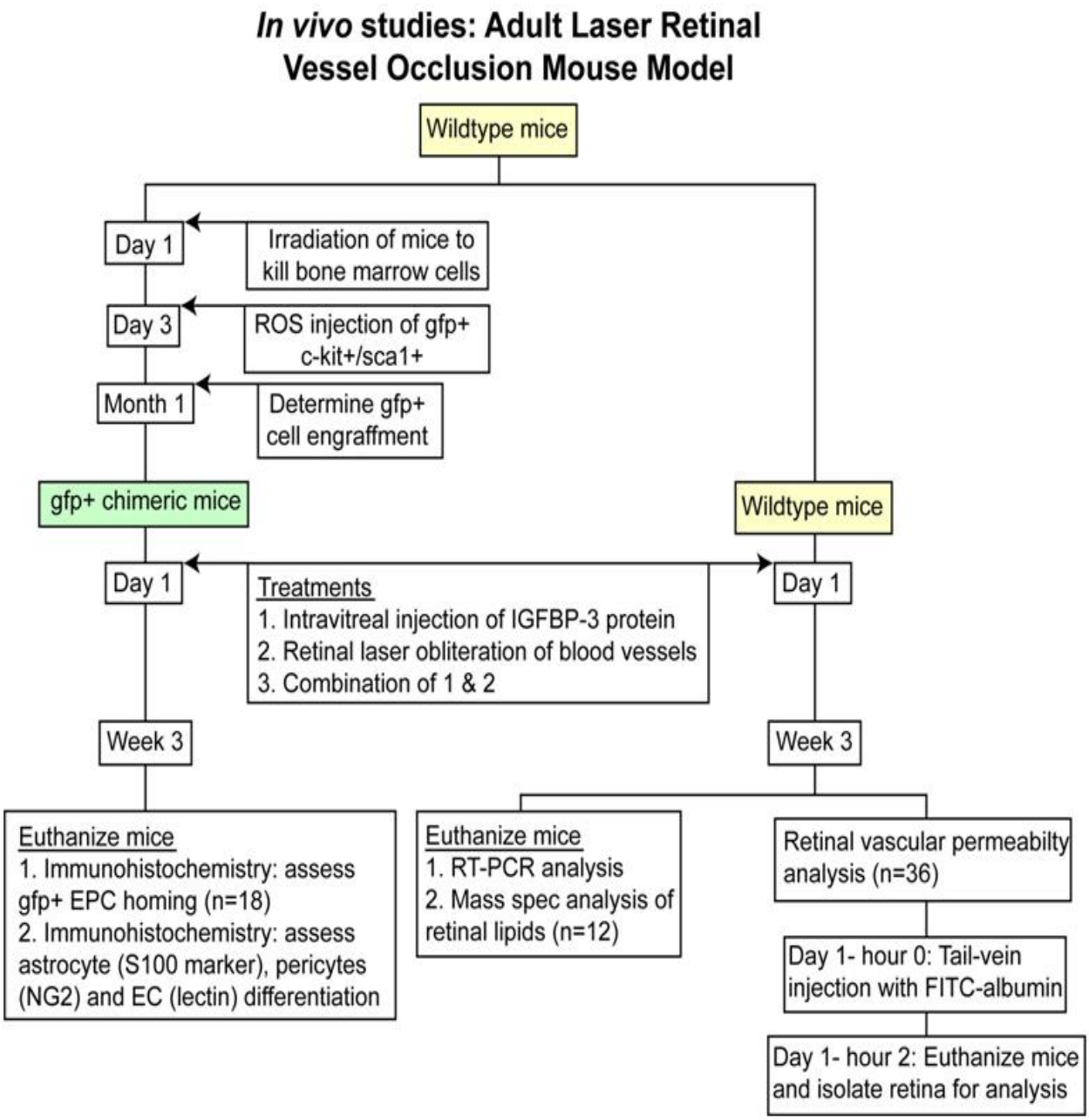

Figure 2-3. *In vivo* studies with IGFBP-3 plasmid in Laser Vessel Occlusion Model.

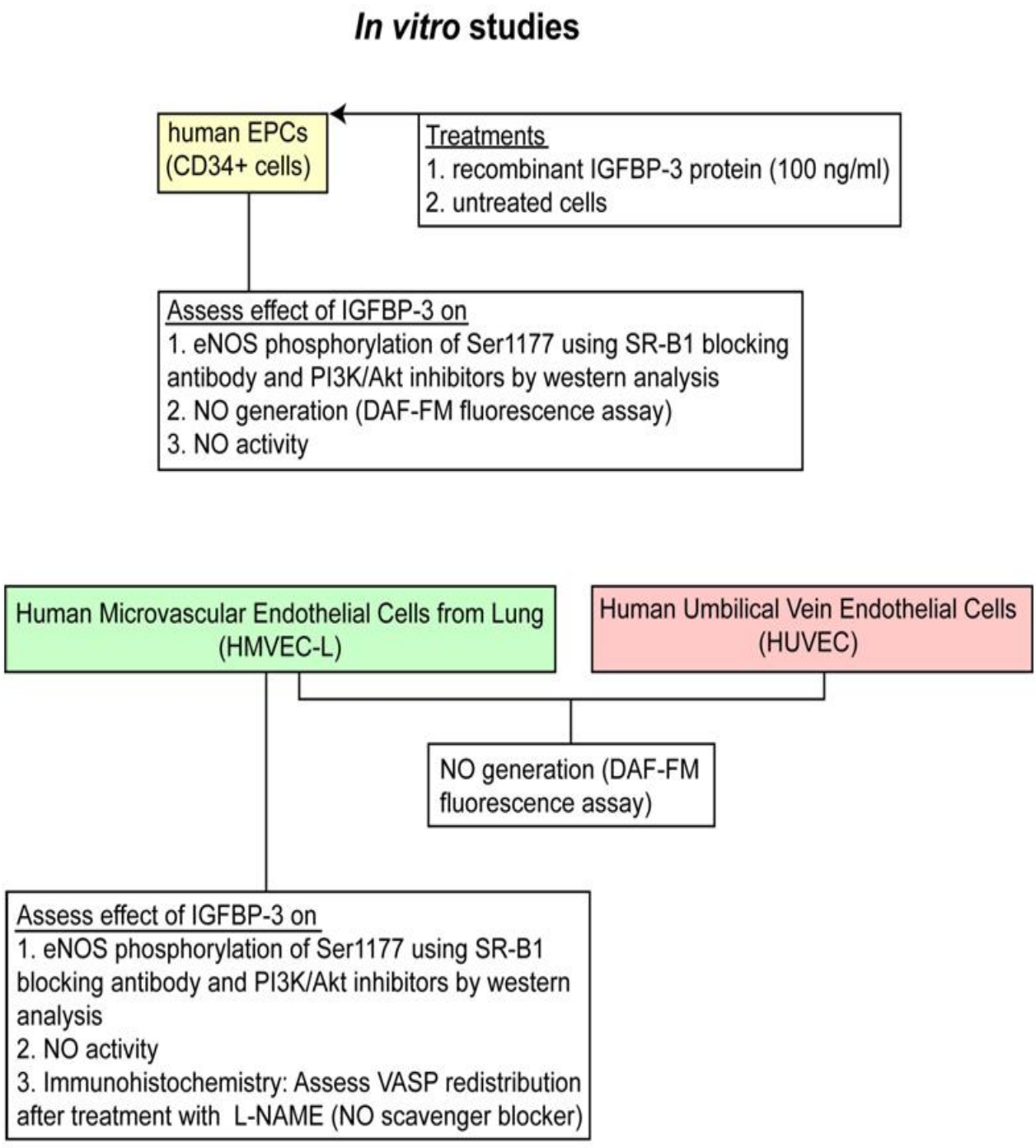

Figure 2-4. *In vitro* studies with IGFBP-3 recombinant protein.

CHAPTER 3
RESULTS

IGFBP-3 Prevents Endothelial Cell Death in the OIR Model

IGFBP-3 is known to have anti-apoptotic effects. We used the OIR model to assess cell death of endothelial cells in IGFBP-3 injected mouse retinas. IGFBP-3 plasmid was injected into the vitreous of mouse pups on P1 and then the mice were subjected to hyperoxia from P7-P12 (phase 1) and returned to room air P13-P17 (phase 2). The mouse pups were sacrificed at both P12 and P17 and their retinas harvested for TUNEL analysis. The OIR model is ideal to assess apoptosis because the early developing mouse vasculature undergoes constant remodeling through combined cell death and proliferation. Moreover, in the OIR model endothelial cells are the most sensitive to cell death. Therefore, if IGFBP-3 has an anti-apoptotic effect in ischemic mouse pup retinas, we would be able to readily detect this through TUNEL analysis of OIR retinal flatmounts. We found IGFBP-3 injected mouse pups subjected to OIR had significantly reduced cell death of endothelial cells in the retina compared to control plasmid injected OIR control mice in both phase 1 and phase 2 of the OIR model (Figure 3-1 D-F IGFBP-3 injected versus A-C control). This reduction in endothelial cell death was evident in the mid-peripehral ($p<0.05$) and peripheral regions ($p<0.05$) of the IGFBP-3 injected retinas. In contrast, IGFBP-3 injected OIR mice did not show a significant reduction in cell of pericytes or astrocytes in the retina (data not shown).

IGFBP-3 Increases Astrocytic Ensheathment of OIR Blood Vessels

Although IGFBP-3 injected OIR mice showed no decrease in the number of apoptotic astrocytic cells in the retina, we found that IGFBP-3 still had an influence on astrocyte morphology. IGFBP-3 injected OIR mice had much thicker and elaborate astrocytic

ensheathment of retinal blood vessels compared to uninjected control OIR mice as shown in Figure 3-2 A-H. Also, the IGFBP-3 injected eyes had far greater S-100^{+}/gfp^{+} immunoreactivity (red stain E-H) compared to uninjected mice (A-D). Quantification of the frequency of astrocytic ensheathment of retinal vasculature (Figure 3-2M) was significantly increased at both P12 and P17 stages of development in IGFBP-3 injected OIR mice compared to control injected OIR mice ($p<0.05$). Retinal astrocytes critically support the development of the retinal vasculature and can modulate angiogenesis during OIR. IGFBP-3 has a protective effect on retinal astrocytes in mice subjected to OIR by likely enhancing their stability, which lends support to the overall retinal vasculature.

IGFBP-3 Protects Retinal Neurons from Apoptosis in the OIR Model

In addition to evaluating cell death of endothelial cells, pericytes and astrocytes in OIR mouse retinas, we also assessed neuronal cell death via microscopy analysis. We found that IGFBP-3 injected OIR mice had reduced neuronal cell death in retinal cross sections compared to uninjected control OIR mice ($P<0.01$). Figure 3-3 A&B contains representative images and quantification. The black arrows denote cells undergoing apoptosis, which are stained darkly denoting their breakdown and condensation of genetic material. There are significantly fewer of these cells present in the IGFBP-3 injected OIR mice ($p<0.001$) compared to the control uninjected OIR mice (Figure 3-3G). The neuronal cells that appear to have the least cell death in the IGFBP-3 injected mice are ganglion cells located in the ganglion cell layer. Most cells undergoing cell death are located in the inner retinal layers, predominately in the inner nuclear and inner plexiform layers. Electron microscopy analysis further confirmed the presence of apoptotic cells in the retina of vector control injected mice (Figure 3-3 C&E) versus the IGFBP-3 injected mice (Figure 3-3 D&F), which showed few apoptotic cells in the retina.

IGFBP-3 Increases Incorporation of EPCs in Adult Retinal Blood Vessels

Besides assessing the cellular protective effects of IGFBP-3 in the OIR model, we also assessed the cytoprotective effects of IGFBP-3 in the adult vasculature of chimeric mice. The adult vasculature is relatively quiescent. Therefore, we questioned whether IGFBP-3 would still actively recruit EPCs into the retinal vasculature. We evaluated IGFBP-3's effect on EPCs in the adult vasculature both in the absence of laser injury, as well as in the presence of laser induced injury. Greater gfp^+ cell incorporation was observed in IGFBP-3 injected adult chimeric mice (Figure 3-4 D-F) and IGFBP-3 injected chimeric mice subjected to retinal vessel occlusion injury (Figure 3-4 G-I) compared to uninjected eyes at 3 weeks post laser injury (Figure 3-4 A-C). There is clear incorporation of gfp^+ HSCs/EPCs into the retinal vasculature of IGFBP-3 injected mice both lasered and unlasered. We also see some incorporation of gfp^+ HSCs/EPCs in the lasered only mice (Figure 3-4 J-L). However, most of the gfp^+ cells are outside the retinal vessels and do not appear to be directly incorporated into the retinal blood vessels.

IGFBP-3 Expressing Plasmid is Upregulated in the Adult Mouse Retina

In order to confirm the IGFBP-3 plasmid expressed IGFBP-3 following injection, we performed mRNA analysis of retinas injected with this plasmid. The IGFBP-3 plasmid begins to express IGFBP-3 at 48 hours and remains high at 72 hours post injection ($p<0.05$). The IGFBP-3 expressing plasmid remains significantly elevated until 1 week post injection in the mouse retina, after which time the expression levels begin to decline (Figure 3-5). Thus, the IGFBP-3 plasmid transiently upregulates IGFBP-3 expression in the retina.

IGFBP-3 Causes Differentiation of EPCs into Pericytes, Astrocytes, and Endothelial Cells

IGFBP-3 increased incorporation of gfp^+ HSCs/EPCs into retinal blood vessels. However, we questioned what the gfp^+ cells were in terms of their cell type. We stained wholemount retinas for mature vascular cells types such as endothelial cells, pericytes, and

astrocytes. In Figures 3-3.6 and 3-3.7 it is shown IGFBP-3 results in the differentiation of gfp^+ HSCs/EPCs into endothelial cells (Figure 3-3.6), pericytes (Figure 3-3.7), and astrocytes (Figure 3-3.7). There appears to be the greatest increase in gfp^+ cells differentiating into endothelial cells, pericytes, and astrocytes in the IGFBP-3 injected and lasered mice compared to the IGFBP-3 injected alone mice. However, this was not quantified.

IGFBP-3 Decreases the Ceramide/Sphingomyelin Ratio in Lasered Mice

In the OIR model, IGFBP-3 has anti-apoptotic effects on endothelial cells. We reasoned IGFBP-3 may also have anti-apoptotic effects in the adult vasculature. The ceramide/ sphingomyelin ratio is an indicator of a pro-inflammatory and pro-apoptotic state in a tissue. The graph in Figure 3-3.8 shows lasered mice have a significantly higher ceramide/sphingomyelin ratio in their retinas ($p<0.05$), at 3 weeks post laser treatment compared to untreated control. This is in contrast to laser and IGFBP-3 injected mice. This group has a ceramide/sphingomyelin ratio similar to that of untreated mice. Hence, IGFBP-3 can lower the pro-inflammatory and pro-apoptotic state in lasered retinas to that of control levels.

IGFBP-3 Increases NO Production in $CD34^+$ Cells and Endothelial Cells

IGFBP-3 stimulates incorporation of gfp^+ HSCs/EPCs in the retinal vasculature. These cells have to migrate in order to home to retinal blood vessels. IGFBP-3 may act as a potent migratory stimulus by recruiting EPCs into the retinal vasculature by stimulating NO generation. In Figure 3-3.9, IGFBP-3 at 100 ng/ml increases exogenous NO production in $CD34^+$ cells. The increase in DAF-FM fluorescence, which is an indicator of NO production, is statistically significant ($p<0.01$). Also, IGFBP-3 at a concentration of 100 ng/ml increases NO generation in endothelial cells as shown in Figure 3.3-11. There is a significant increase in NO generation by IGFBP-3 treatment in both cultures of HUVEC's, a well known macrovascular endothelial cell line, and HMVEC-L, a microvascular type of endothelial cell. IGFBP-3 clearly increases NO

generation in both human $CD34^+$ cells and human endothelial cells.

IGFBP-3 increases eNOS phosphorylation at Serine 1177 in $CD34^+$ Cells

NO production in $CD34^+$ EPCs must occur through NOS activation. We tested whether IGFBP-3 increases eNOS phosphorylation at Serine 1177. IGFBP-3 increased eNOS phosphorylation protein levels at Serine 1177 in EPCs. As depicted in Figure 3.3-10. IGFBP-3 increases Serine 1177 phosphorylation in a time-dependent manner. eNOS Serine 1177 phosphorylation significantly increases by 30 minutes ($p<0.05$) and significantly peaks at 60 minutes ($p<0.01$). Likewise, IGFBP-3 leads to increased eNOS phosphorylation at Serine 1177 in HUVECs at 60 minutes ($p<0.05$) as shown in Figure 3.3-13.

Blockade of SR-B1 Leads to Decreased NO Production in Endothelial Cells

IGFBP-3 clearly increases nitric oxide production. There are a number of ways nitric oxide can be generated exogenously in the cell. We postulated IGFBP-3 can signal through the HDL receptor called SR-B1 to increase intracellular levels of NO in endothelial cells. In Figure 3.3-11, we demonstrate blocking the SR-B1 with a blocking antibody leads to decreased NO production in mature endothelial cells treated with a physiologically relevant concentration of IGFBP-3 (100 ng/ml). We evaluated two types of endothelial cells, microvascular (HUVECs) and macrovascular (HMVEC-L) human endothelial cells. The decrease in NO generation when the SR-B1 was blocked was equivalent in both cell lines ($p<0.02$). The RGC-5 cell line is rat ganglion cell line that does not express SR-B1, hence serves as a negative control. There was no observed increase in NO upon addition of IGFBP-3 or HDL, nor was NO production altered upon blocking SR-B1 in this cell type. Interestingly, addition of exogenous HDL combined with IGFBP-3 led to increased NO production in both HMVEC-L ($p<0.001$) and HUVECs ($p<0.05$), but was most pronounced in the HMVEC-L. Addition of HDL alone produced NO levels similar to that of IGFBP-3 alone. Also, blocking the SR-B1 in HDL treated cells decreased NO

production significantly ($p<0.05$). However, blocking the SR-B1 in IGFBP-3 treated and HDL treated HMVEC-L did significantly lower NO production, but in the HUVECs the reverse is true ($p<0.01$). (Figure 3-11)

Blockade of SR-B1 Leads to Decreased NO Activity in Endothelial Cells

In addition to evaluating NO generation, NO activity was also assessed. In Figure 3.3-12C, NO activity was measured in HUVECs blocked for SR-B1 receptor and treated with IGFBP-3. There was a significant reduction in eNOS activity as measured by the conversion of L-arginine to L-citrulline in HUVECs treated with SR-B1 blocking antibody and IGFBP-3 ($p<0.05$).

Blockade of PI3K/Akt Reduces NO Generation and Activity

IGFBP-3 increases NO through activation of the SR-B1 receptor. To further understand the signaling cascade we examined the PI3K/Akt cell survival pathway, since IGFBP-3 promotes cell survival. Pharmacological inhibitors for PI3K (LY294002) or Akt (Triciribine) were used to block IGFBP-3 treated HUVECs. We found IGFBP-3 treated cells both inhibitors resulted in decreased NO production as shown in Figure 3.3-12B ($p<0.01$). Also, L-NAME treated HUVECs had decreased NO generation ($p<0.01$). We further confirmed these observations by evaluating eNOS activity in Figure 3.3-12C. IGFBP-3 stimulated eNOS activity was significantly reduced by pre-treatment with SR-B1 or LY294002 or triciribine ($p<0.05$). Additionally, in Figure 3.3-13, IGFBP-3 decreases eNOS phosporylation at Serine 1177 in HUVECs upon exposure to SR-B1 or LY294002 or triciribine either individually or in combination ($p<0.05$).

Blockade of Sphingosine Kinase Decreases NO Release in $CD34^+$ Cells

IGFBP-3 has previously been shown to increase the activity of sphingosine kinase (Sphk), the enzyme responsible for the generation of the potent pro-angiogenic factor

sphingosine-1-phosphate, S1P. Addition of the Sphk inhibitor, N,N-dimethylsphingosine as shown in Figure 3.3-9, resulted in a significant reduction in NO generation in response to IGFBP-3, as well as HDL, supporting that S1P generation can contribute to the effects of both HDL and IGFBP-3 on NO generation ($p<0.01$). $CD34^+$ cells and endothelial cells express S1PR, predominately S1PR1 and S1PR3, as well as Sphingosine Kinase 1 and 2 (data not shown).

IGFBP-3 Stimulates VASP Re-distribution in Endothelial Cells

Nitric oxide is a critical regulator of cell migration. VASP, vasodilator-stimulated phosphoprotein (VASP), plays a pivotal role in promoting actin filament elongation at the leading edge of the cell by forming an active molecular motor complex that propels motility. Migration of BMDCs into areas of ischemia is paramount to their ability to initiate and orchestrate repair. HUVECs treated with IGFBP-3 underwent significant VASP re-distribution ($p<0.05$) compared to untreated cells as depicted in Figure 3.3-14 A&C. IGFBP-3 results in lamellipodia formation at the periphery (Figure 3.3-14C). Pre-treatment with L-NAME, a NO scavenger, resulted in significant decreases in VASP re-distribution and lamellipodia formation as shown in Figure 3.3-14E ($p<0.05$). VASP biodistribution was quantified in Figure 3.3-14 H in endothelial cells. Due to $CD34^+$ cells being nonadherent, VASP re-distribution could not be studied in this cell type.

IGFBP-3 Decreases Vascular Permeability in Laser Injured Adult Mice

IGFBP-3 clearly has dramatic effects on the cell migration of endothelial cells and EPCs. IGFBP-3 facilitates their recruitment and incorporation into retinal blood vessels. We assessed IGFBP-3 on vascular permeability in the retinal vessel occlusion injury model. We found IGFBP-3 injected animals subjected to laser injury have decreased vascular permeability compared to laser alone treated mice (Figure 3-15). This effect was observed 4 days after laser injury when the IGFBP-3 expressing plasmid was at its optimal peak expression.

IGFBP-3 Increases Vascular Permeability in Unlasered Adult Mice

Besides evaluating vascular permeability in the laser injury model, we also decided to look at VEGF induced vascular permeability in normal unlasered mouse retina. VEGF, as mentioned previously, is one of the most, if not the most, potent vascular permeability factor. We injected VEGF intravitreally in normal mice and as expected there was an increase in vascular permeability *in vivo*. IGFBP-3 by itself, increased vascular permeability more so than VEGF. IGFBP-3 and VEGF injected together in combination increased vascular permeability at 12 hours, but fell at 24 hours to control levels. IGFBP-3 coupled with VEGF can increase vascular permeability transiently in normal mice (Figure 3-16). IGFBP-3 by itself significantly increases vascular permeability, suggesting IGFBP-3 has similar effects to that of IGFBP-3 in normal mouse retina. These results were further confirmed *in vitro*. IGFBP-3 by itself acutely increases vascular permeability, however, by 24 hours it declines to control levels (data not shown). IGFBP-3 and VEGF combination increases vascular permeability at 5 minutes, 1 hour, and 5 hours, but declined by 24 hours (data not shown). IGFBP-3 depending on the type of injury in the vasculature, laser injured versus VEGF injected, can impact vascular permeability in two distinct ways. Also, the exposure time to IGFBP-3 can influence vascular permeability.

IGFBP-3 Reduces Sphingomyelinase mRNA Expression

IGFBP-3 has an impact on vascular permeability in retinal blood vessels. We suspect IGFBP-3 influences sphingomyelinase, which in turn can affect vascular permeability. We found IGFBP-3 and laser mice have decreased acid and neutral sphingomyelinase levels in mouse retina at 4 days post laser treatment compared to laser only treated mice (Figure 3-17). Likewise, we confirmed these results in a sphingomyelinase activity assay. We found that laser treated and IGFBP-3 treated mice have lower sphingomyelinase activity compared to laser only treated mice (data not shown).

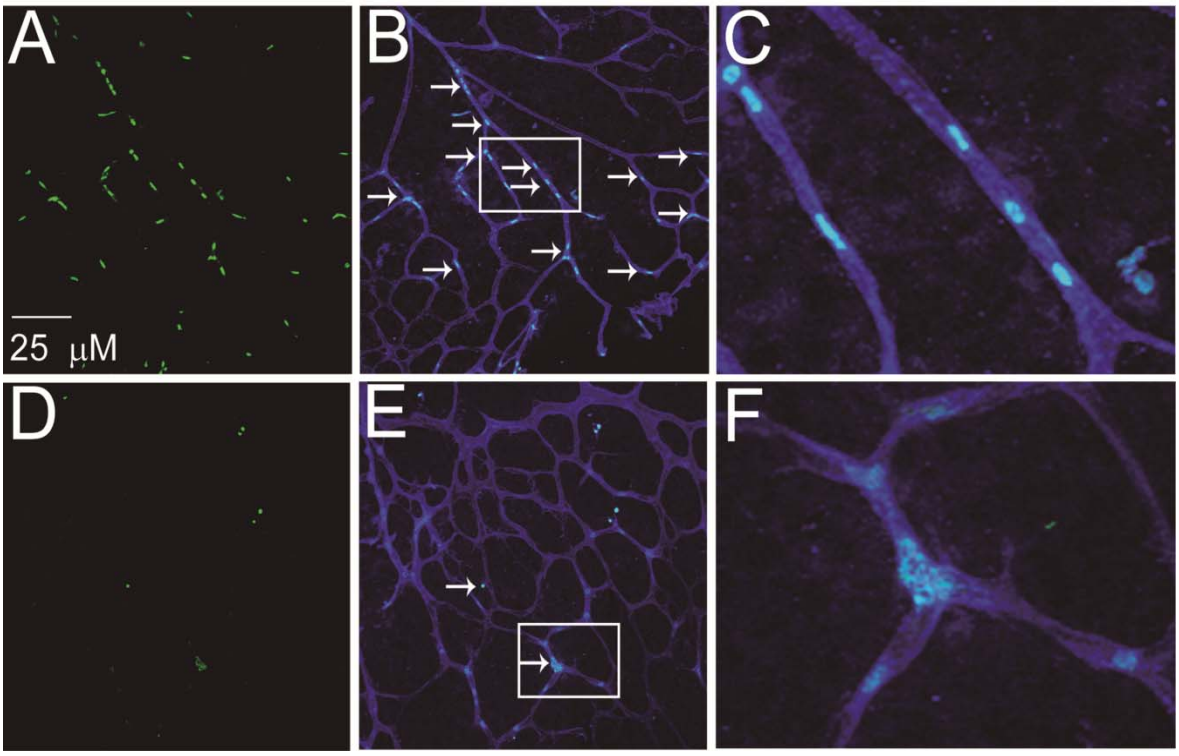

Figure 3-1. IGFBP-3 prevents endothelial cell death in the OIR model. Panels A-C) are uninjected control mice. A) shows an abundance of TUNEL+ green cells B) represents TUNEL$^+$ cells (white arrows), and GS lectin staining of endothelial cells (blue stain). C) depicts a higher magnification shown in white box in B of numerous colocalized TUNEL$^+$/GS lectin$^+$ staining endothelial cells. D-F) are IGFBP-3 injected mice. D) shows fewer TUNEL$^+$ green cells. E) shows a small number of TUNEL$^+$ cells (white arrows), and GS lectin staining of endothelial cells. F) represents a higher magnification of white box shown in E of colocalized TUNEL$^+$/GS lectin$^+$ staining of endothelial cells.

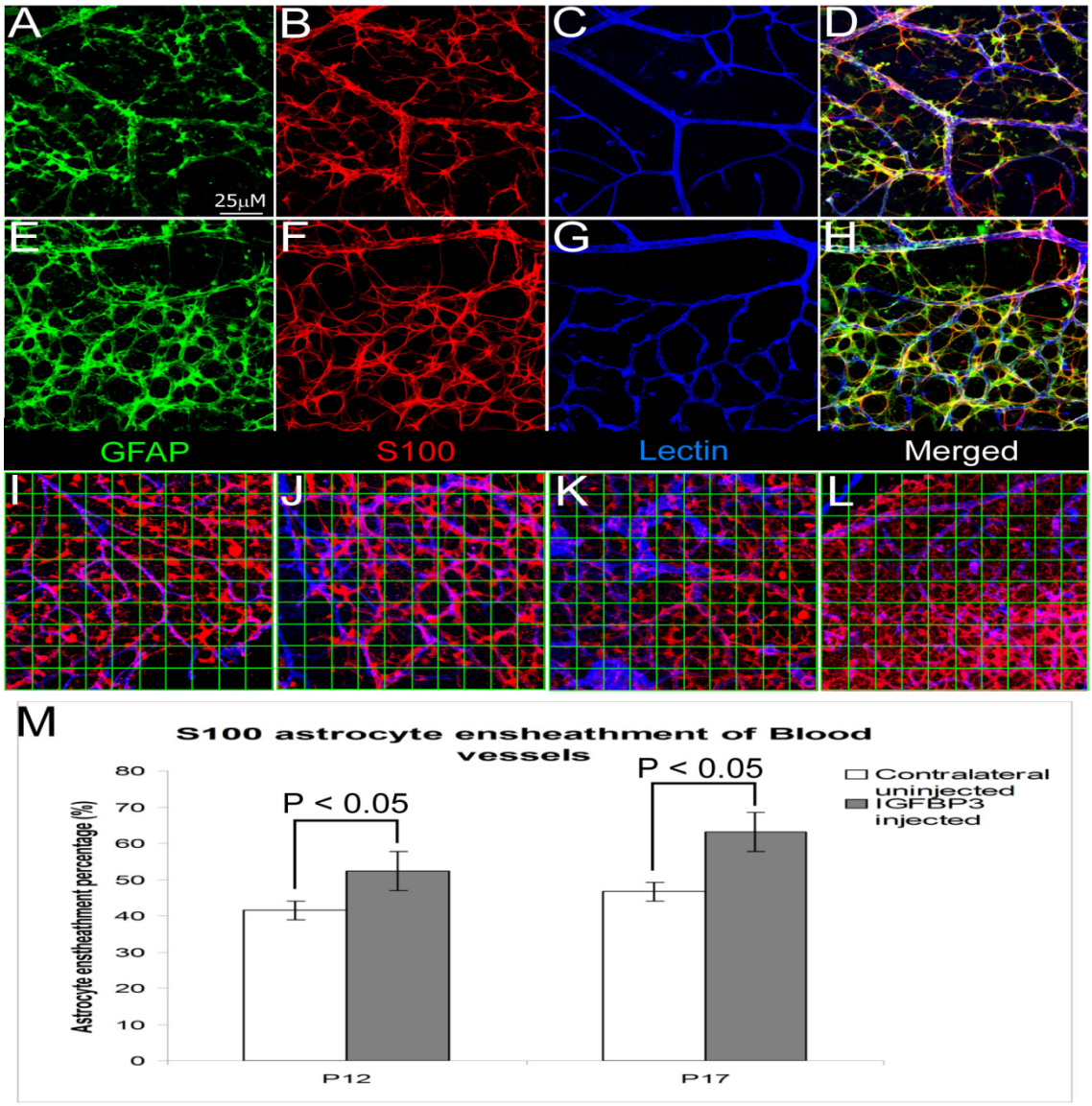

Figure 3-2. IGFBP-3 increases astrocytic ensheathment in OIR blood vessels. In comparison to control A-D), the S-100/GFAP ensheathment of underlying vasculature in the IGFBP-3 injected eyes E-H) was much more completed and astrocytes showed larger, thicker processes. I-L) show representative fields of view from the mid-peripheral retina using a 20x objective during normal development and during exposure to the OIR model. The ensheathment of GS lectin^{+} vascular endothelial cells (blue) by S-100^{+} astrocytes (red) and GFAP (green) were determined. S-100 ensheathment of underlying vasculature in the IGFBP-3 injected L) was much more complete than in control injected eyes K) at P17. M) Shows quantification of astrocytic ensheathment of retinal blood vessels. Astrocyte ensheathment was found to be more significant ($p<0.05$) in both P12 and P17 injected eyes in comparison to controls.

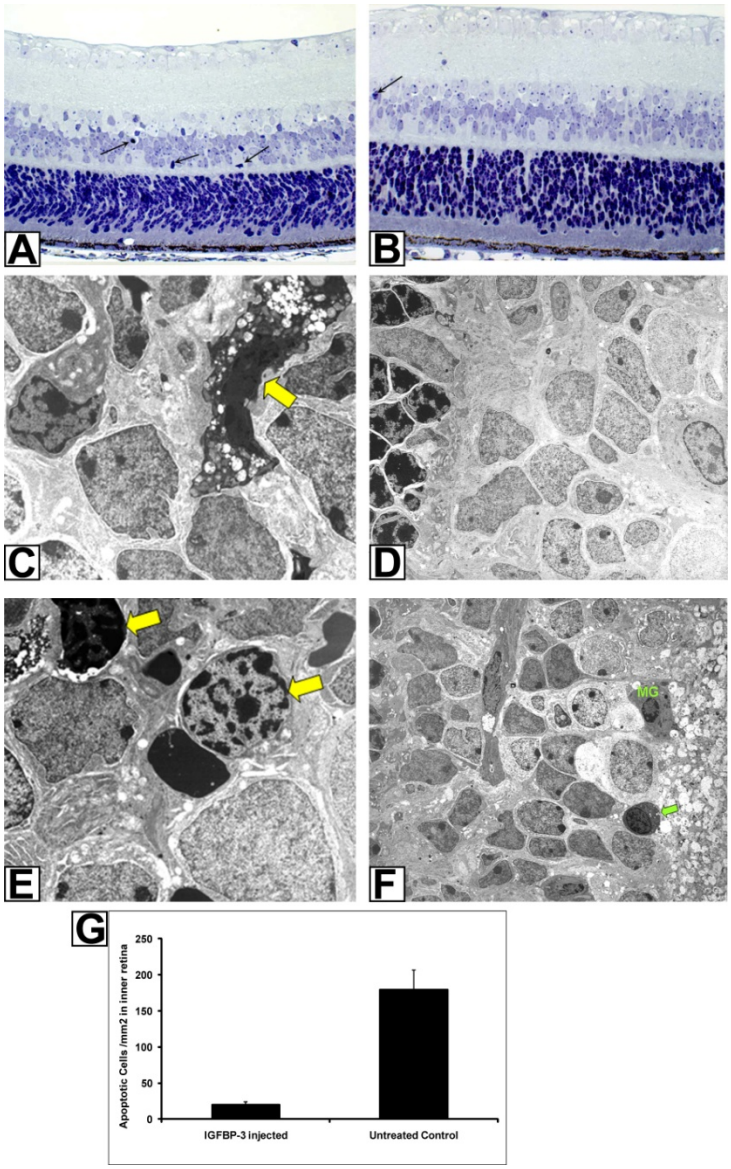

Figure 3-3. IGFBP-3 protects retinal neurons from apoptosis in the OIR model. Panels A-B) show representative light micrographs stained with Toluidine Blue to clearly visualize the retinal layers in OIR mice at P17. The vector control OIR mice A) have increased numbers of apoptotic cells in the inner retina (black arrows) compared to the IGFBP-3 plasmid injected mice B). Electron microscopy analysis revealed retinal cells undergoing active apoptosis as shown in C-F). C&E show obvious cells undergoing active apoptosis in the vector injected mice versus D&F which show little signs of retinal apoptosis in the IGFBP-3 injected OIR mice. Yellow arrows denote apoptotic cells. The graph in G) shows quantification of the number of apoptotic cells counted in the retinas of vector injected mice (untreated control) and the IGFBP-3 injected mice.

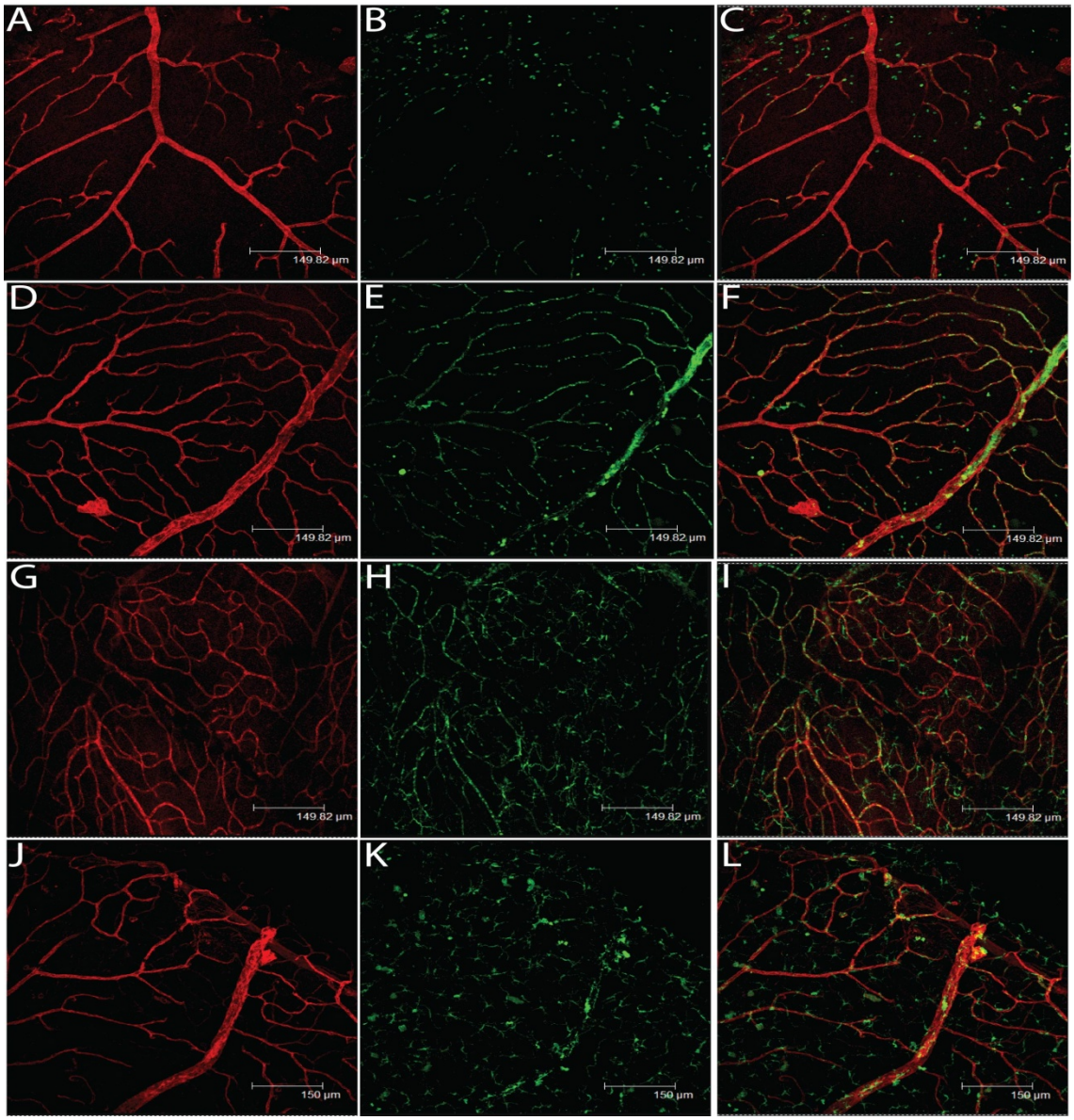

Figure 3-4. IGFBP-3 increases incorporation of EPCs in adult retinal blood vessels.Uninjected control mice A-C) show very little incorporation of gfp^+ EPCs/HSCs directly into the retinal blood vessels. IGFBP-3 injected mice D-F) show increased gfp^+ EPCs/HSCs incorporation and homing as well as mice lasered and injected with IGFBP-3 G-I). Laser only mice J-L) show the presence of gfp^+ EPCs/HSCs, however, these cells are present outside the blood vessels and do not incorporate directly into retinal blood vessels. The red stain represents rhodamine agglutinin (far left side panels) which is a blood vessel specific stain and the green stain is indicative of gfp^+ cells (middle panels). The merged images of the rhodamine stain and gfp^+ cells are shown in the far right panels.

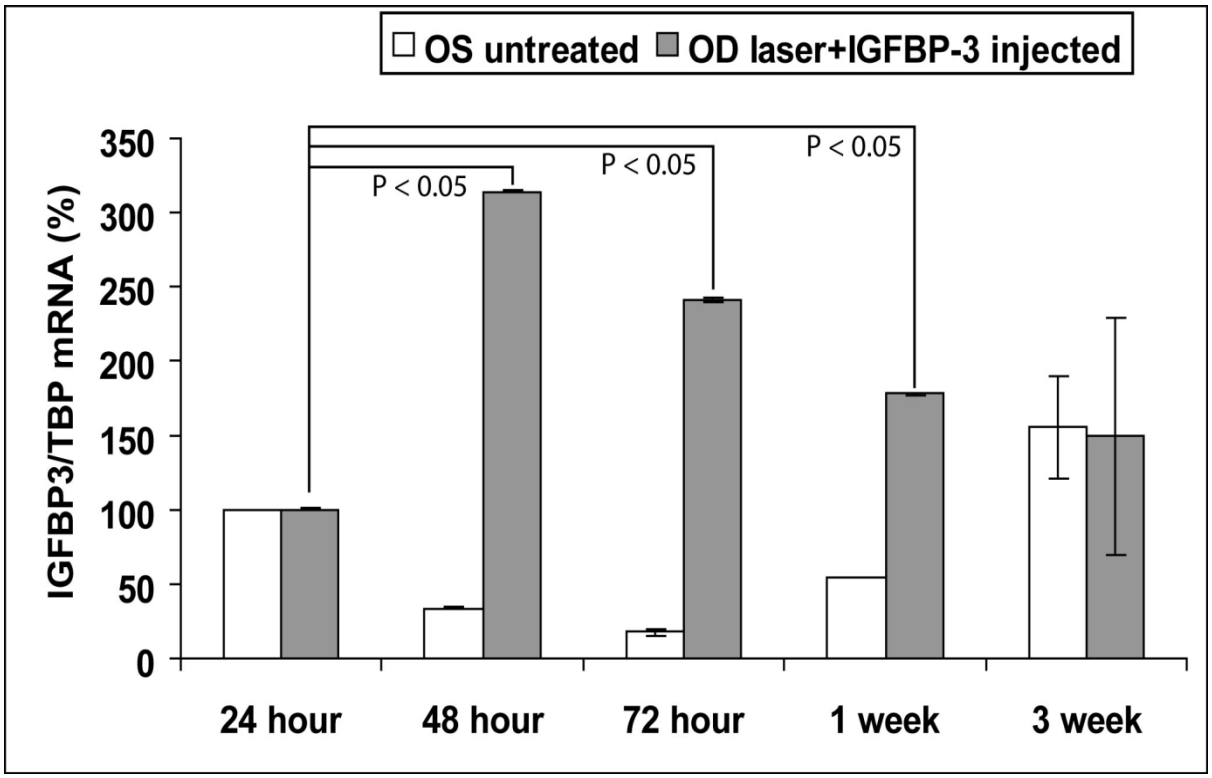

Figure 3-5. IGFBP-3 expressing plasmid is upregulated in the adult retina.RT-PCR analysis of mouse retina evaluating IGFBP-3 mRNA expression over a 3 week time course. IGFBP-3 mRNA remains highly expressed in the treated eye (OD) of mice subjected to laser injury followed by intravitreal injection of IGFBP-3 up to 1 week compared to the contralateral control eye (OS). By 3 weeks, IGFBP-3 expression levels in the treated eye (OD) returned to normal control levels. *$p<0.05$

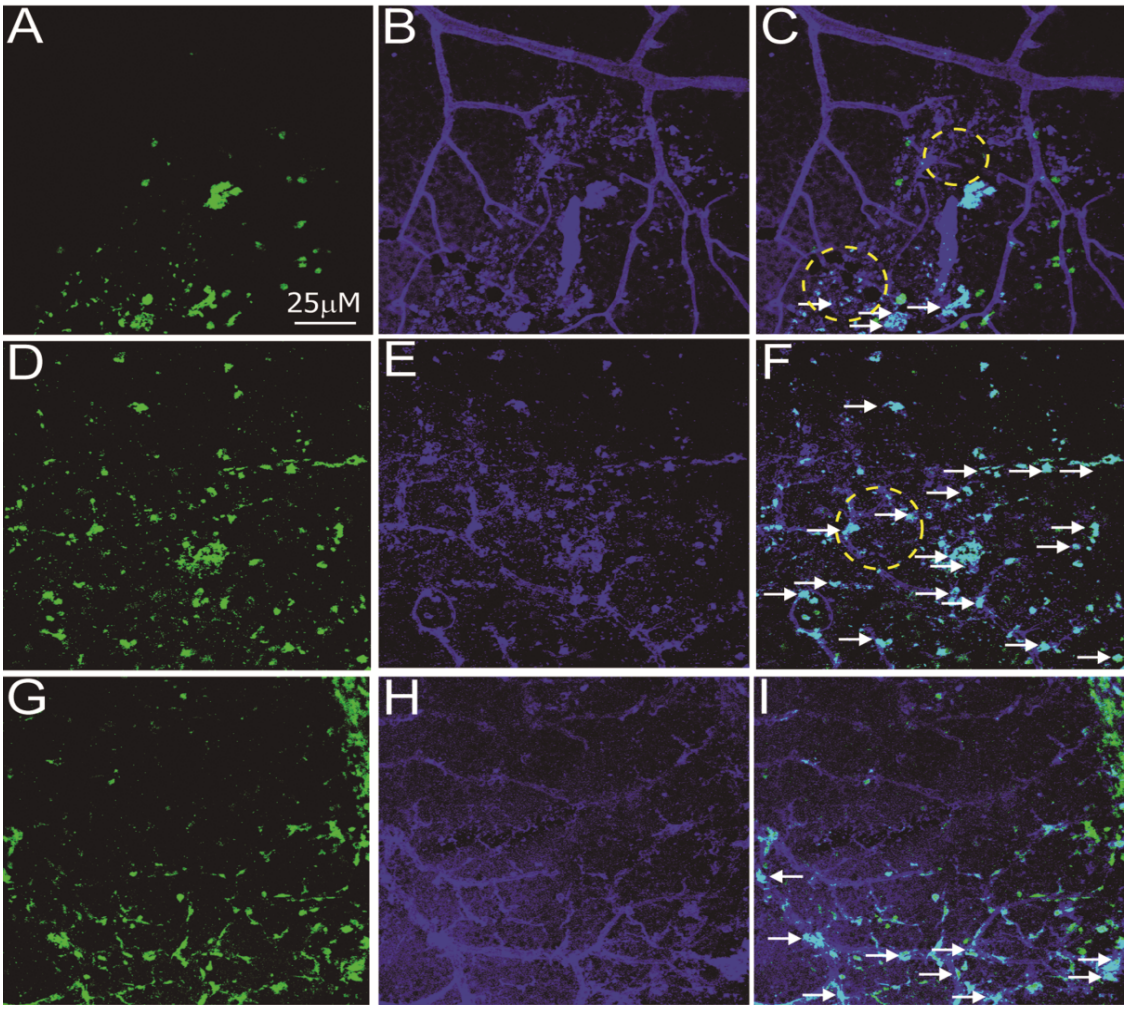

Figure 3-6. IGFBP-3 causes differentiation of EPCs into endothelial cells in the adult retina. The green stain is gfp+ cells and the blue represents GS isolectin B4 for detection of endothelial cells and activated microglia/macrophages. A-C) represents laser only retina with 2 laser burn sites indicated by pigment changes and tissue damage (dashed circles) and surrounding vascular remodeling. There are few gfp^+ cells colocalized with lectin indicating little differentiation of gfp^+ cells into endothelial cells. D-F) illustrates IGFBP-3 injected plus laser retina showing large incorporation and differentiation of gfp^+ cells, at and around the site of laser injury, participating in wound healing response. gfp^+ cells differentiate into GS Lectin labeled endothelial cells (white arrows). G-I) illustrates IGFBP-3 injected eyes showing significant gfp^+ BMDC incorporation and differentiation into GS Lectin labeled vessels (white arrows).

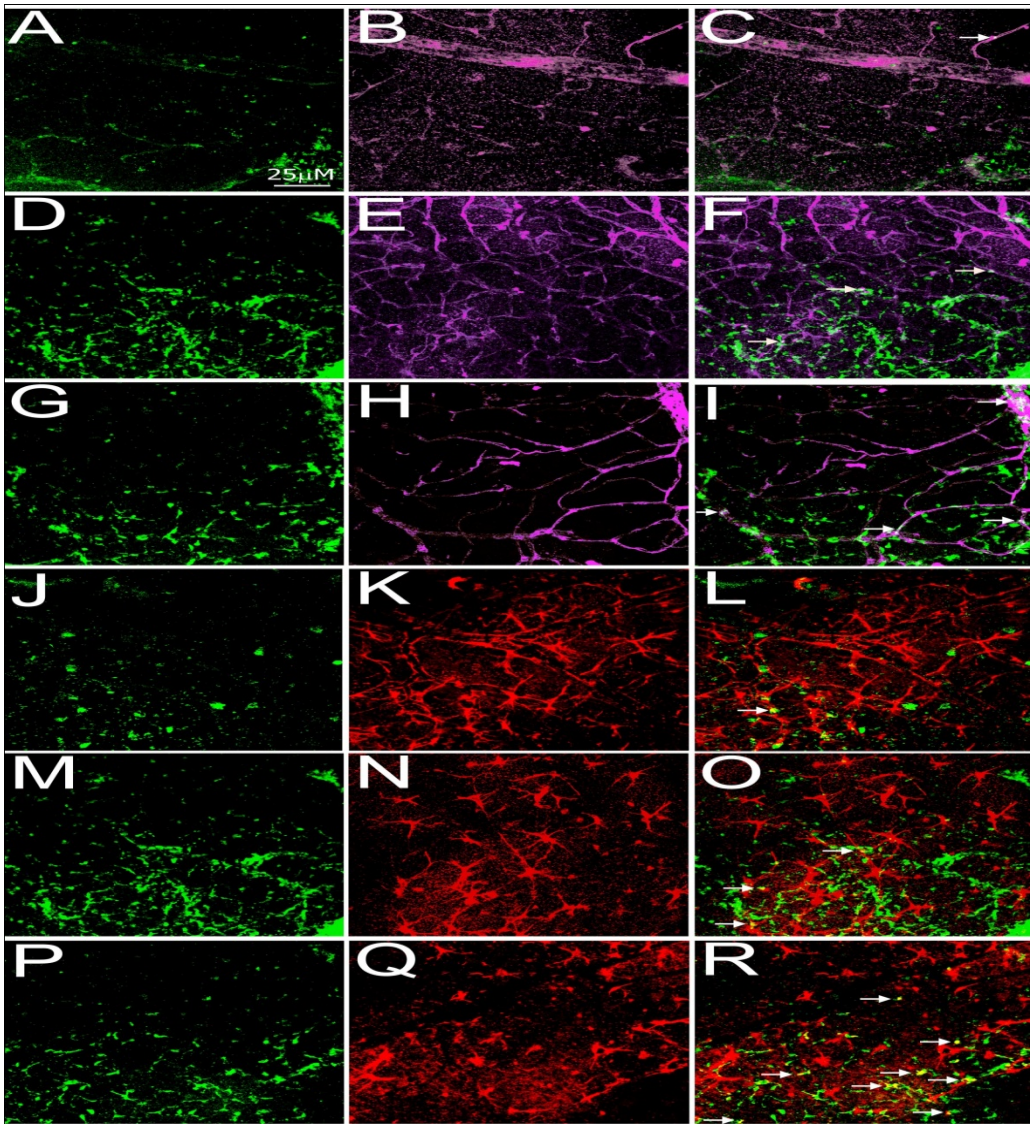

Figure 3-7. IGFBP-3 stimulates differentiation of EPCs into astrocytes and pericytes. A-I) The green stain is gfp^+ cells and the purple represents NG2 for detection of pericytes. A-C) Represents Laser only retina showing very little gfp^+ cells giving rise to $NG2^+$ pericytes. D-F) Illustrates IGFBP-3 injected laser retina showing increased gfp^+ cell differentiation into $NG2^+$ pericytes (white arrows). G-I) Illustrates IGFBP-3 injected eyes showing more gfp^+ cell differentiation into $NG2^+$ pericytes (white arrows). J-R) The green stain is gfp^+ cells and the red represents S100 for detection of astrocytes. J-L) Represents Laser only retina showing markedly increased S-100 immunoreactivity. gfp^+ HSC differentiated $S100^+$ astrocytes (white arrows) were evident though in very low numbers in the laser only eyes. M-O) Illustrates IGFBP-3 injected plus laser injured retina showing markedly increased S-100 immunoreactivity. gfp^+ HSC gave rise to $S100^+$ astrocytes (white arrows) which were evident in greater numbers in the laser plus IGFBP-3 injected eyes compared to laser only eyes. P-R) Illustrates IGFBP-3 injected eyes showing the most gfp^+ cell differentiation into $S100^+$ astrocytes (white arrows).

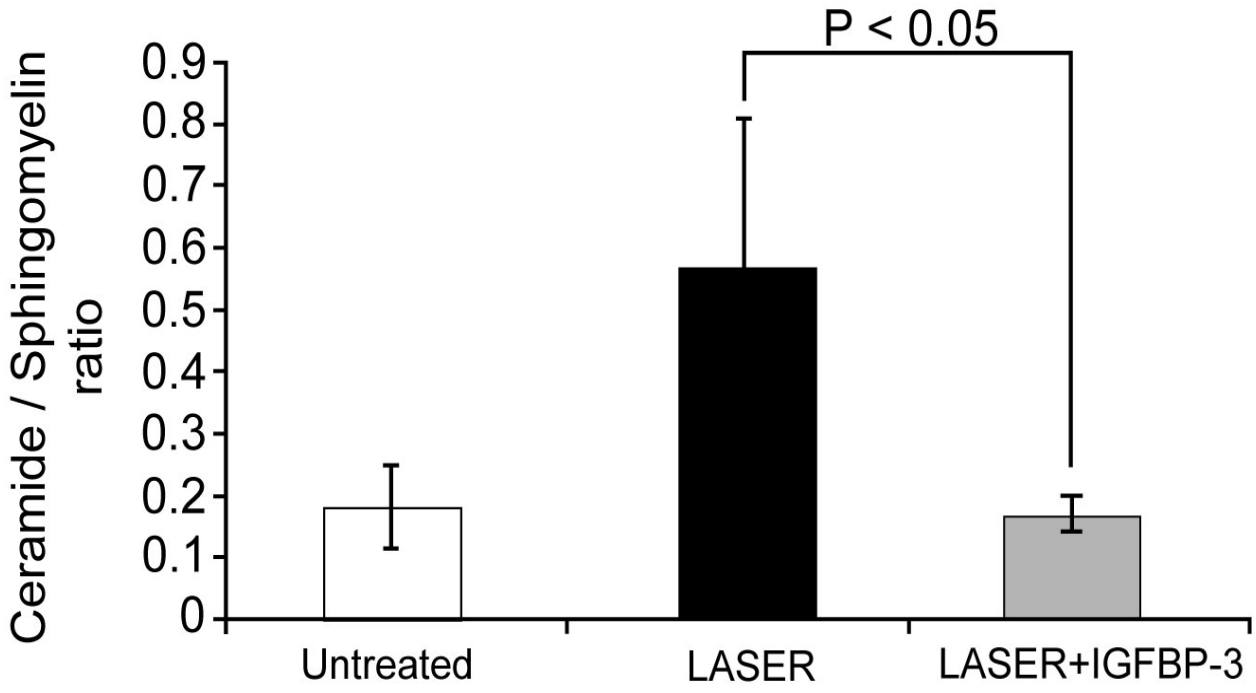

Figure 3-8. IGFBP-3 decreases the ceramide/sphingomyelin ratio in lasered retina. Retinal Lipids extracts from control, laser, or laser plus IGFBP-3 treated mice were analyzed for ceramide and sphingomyelin molecular species as their $[M+H]^+$ ions by precursor ion scanning of *m/z* 264.4 *m/z* 184, respectively, after alkaline hydrolysis of glycerophospholipids. The data was normalized to internal standards and ratios of total retinal ceramide/sphingomyelin were calculated. The results are the means ± SD of 3 independent experiments. *$p<0.05$ vs. control is statistically significant.

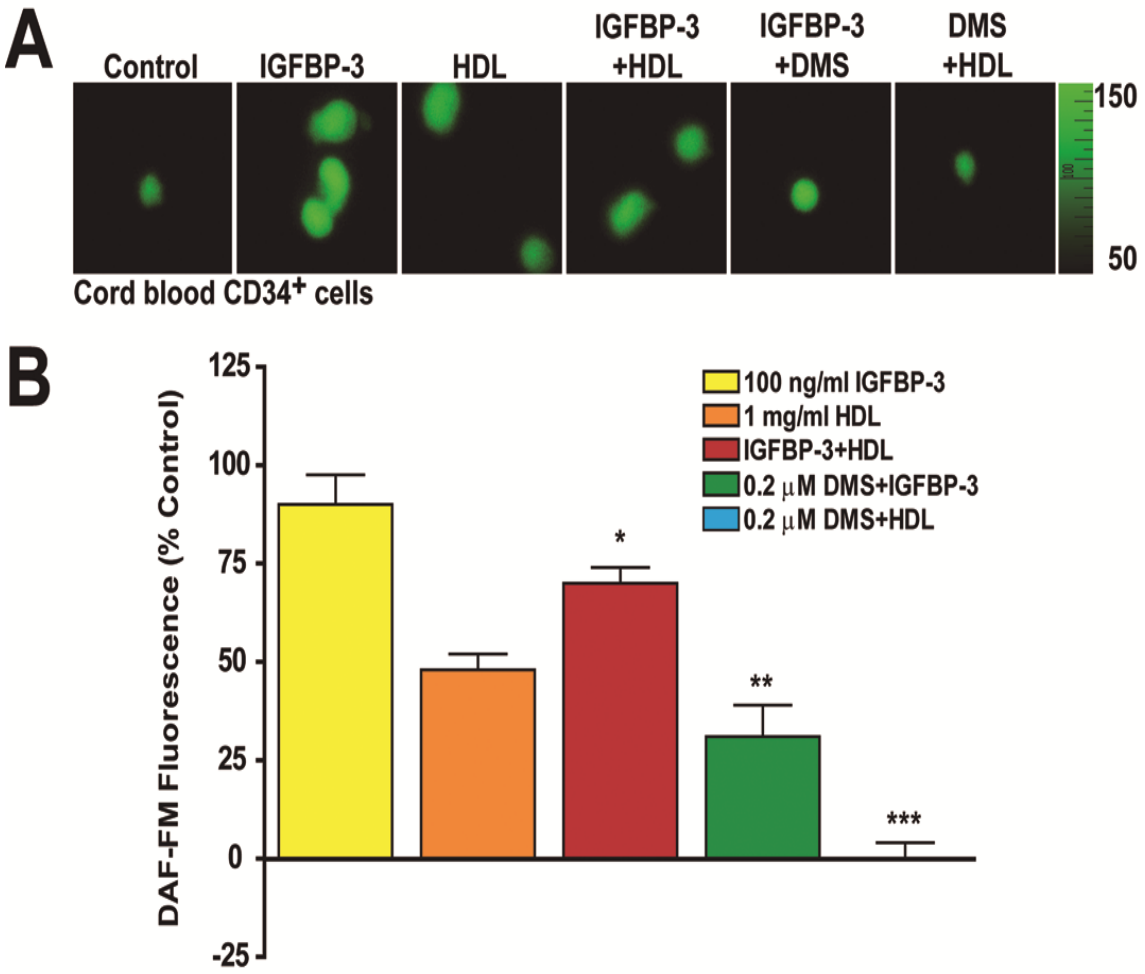

Figure 3-9. IGFBP-3 increases NO production in $CD34^+$ cells. Determination of NOrelease by DAF-FM fluorescence imaging in human CD34+ cells. A) Showsrepresentative images of cells treated with DAF-FM. Images obtained were cells that were either untreated (control) or treated as labeled. B) Graph displayschanges in fluorescence with different treatments expressed as percentageincrease over the control. *$p<0.01$ compared with IGFBP-3 or HDL alone;**$p<0.001$ compared with IGFBP-3; ***$p<0.0001$ compared with HDL.Representative results from 3 independent experiments are shown.

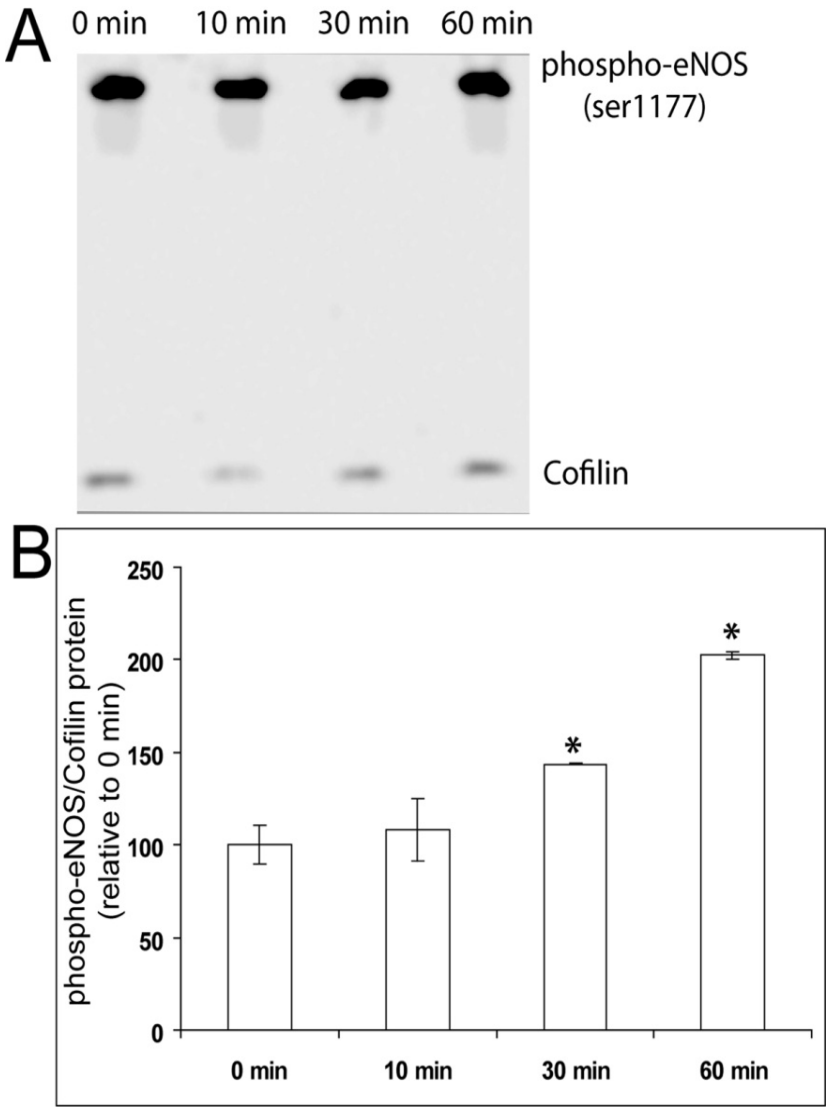

Figure 3-10. IGFBP-3 increases phosphorylation of eNOS at Serine 1177 in $CD34^+$ cells. A) Western Blot analysis depicting eNOS phosphorylation in human $CD34^+$ cells treated with 100 ng/ml of IGFBP-3 at 10,30, and 60 minutes. Protein expression levels were normalized to cofilin. B) Quantification of western blot in panel A. There is a time dependent increase in eNOS phosphorylation at Ser 1177 in human $CD34^+$ cells ($p<0.05$ and $p<0.01$) at 30 and 60 minutes, respectively.

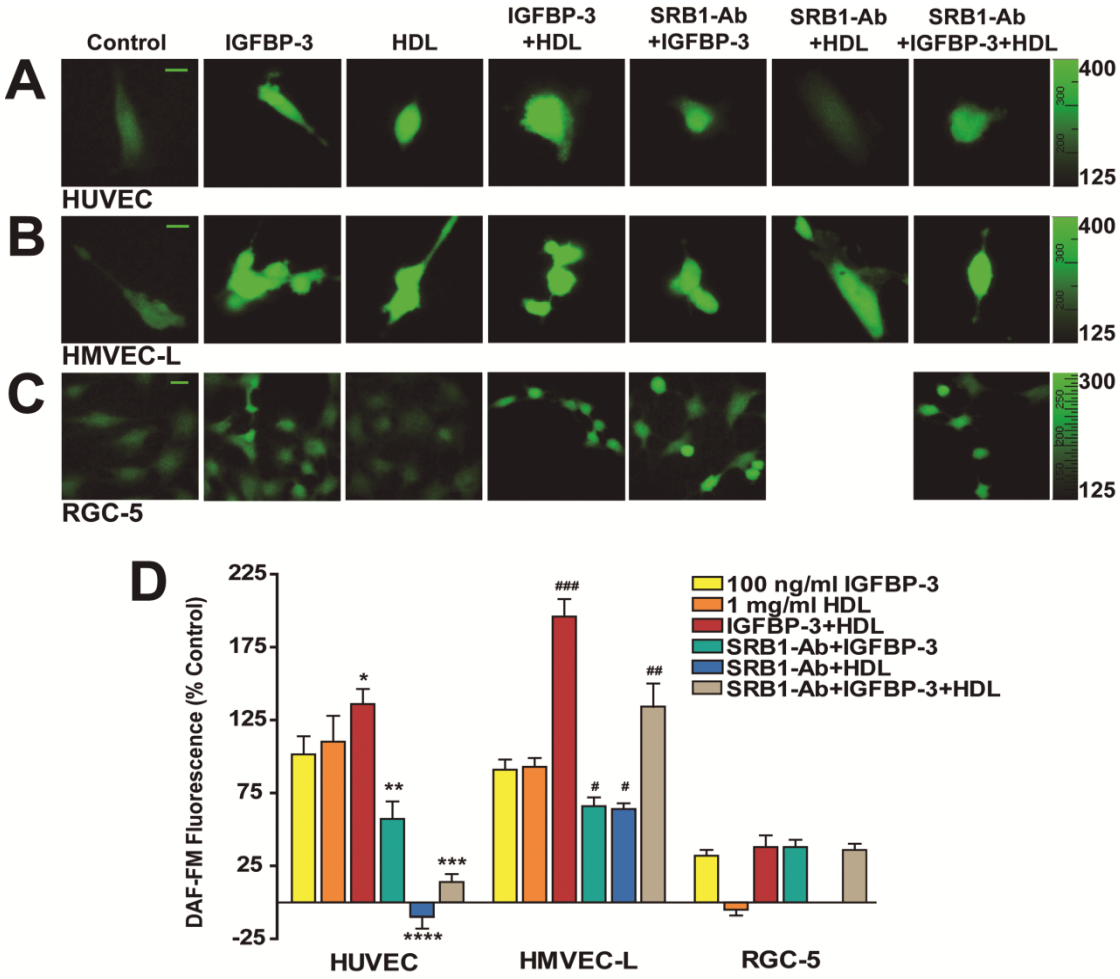

Figure 3-11. IGFBP-3 activates the SR-B1 receptor leading to NO generation in endothelial cells. Determination of NO release in response to IGFBP-3 and HDL by DAF-FM fluorescence imaging. NO release in response to IGFBP-3 and/or HDL in HUVECs, HMVEC-L, and RGC-5 cells. Treatment with SR-B1 blocking antibody significantly decreased NO release by either IGFBP-3 or HDL or the combination of IGFBP-3 and HDL. (A-C) show respective scales for fluorescence in 3 different cell types. Images obtained were cells that were either treated or untreated as labeled. (D) Changes in fluorescence with different treatments were expressed as percentage increase over the control. *$p<0.05$ and **$p<0.02$ compared with IGFBP-3; ***$p<0.01$ compared with HDL+IGFBP-3; ****$p<0.0001$ compared with HDL; #$p<0.05$ compared with IGFBP-3 or HDL alone; ##$p<0.01$ compared with HDL and IGFBP-3; ###$p<0.01$ compared with HDL or IGFBP-3. Representative results from 3 independent experiments are shown.

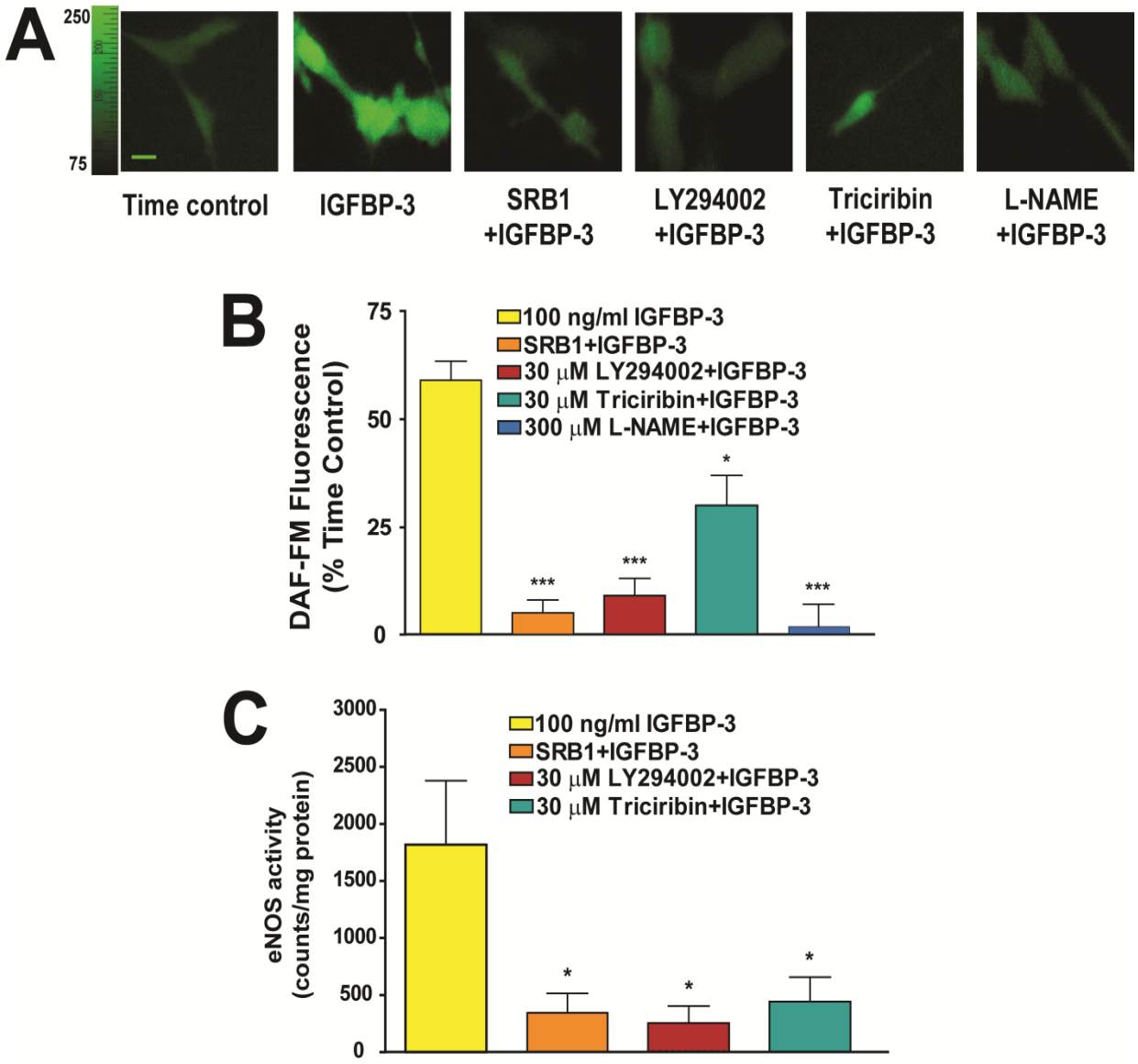

Figure 3-12. Blockade of PI3K/Akt reduces NO generation in endothelial cells. A) NO production in response to IGFBP-3 was measured by DAF-FM fluorescence in HUVECs and effects of different pharmacological blockers were evaluated (color scale for fluorescence). Images obtained were cells that were either untreated or treated as labeled. B) Changes in fluorescence with different treatments were expressed as percentage increase over the time control. NO release by IGFBP-3 was significantly decreased by pretreatment with SR-B1 blocking antibody (SR-B1-Ab), LY294002, triciribine, or L-NAME. *$p<0.01$ and ***$p<0.0001$ compared with IGFBP-3. C) eNOS activity expressed as L-NAME-inhibitable conversion of [14C]L-arginine to [^{14}C]L-citrulline was stimulated by 100 ng/mL IGFBP-3. Pre-treatment with SR-B1-Ab, 30 μmol/L LY294002 or 30 μmol/L triciribine significantly decreased IGFBP-3-induced eNOS activation ($p<0.05$). Representative results from 3 independent experiments are shown.

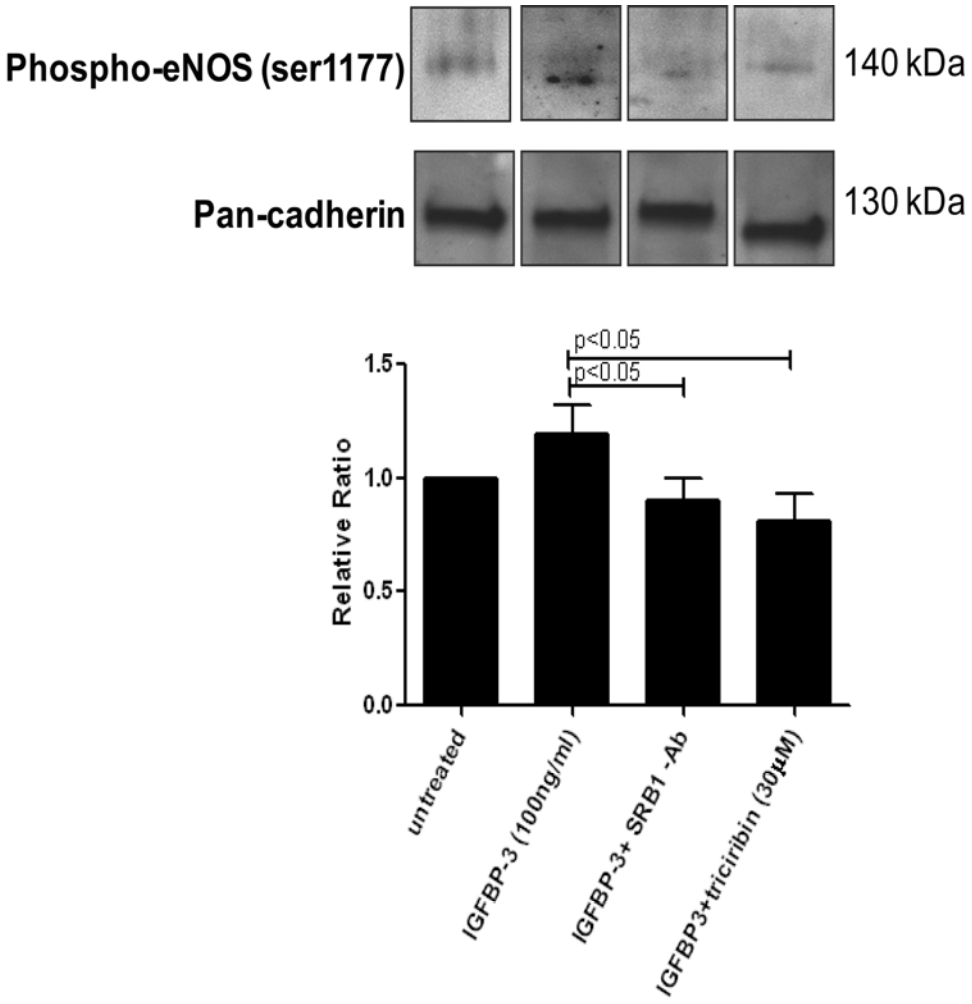

Figure 3-13. IGFBP-3 phosphorylates eNOS at Serine 1177 in HUVECs. HUVECs were treated with 100 ng/ml of IGFBP-3 for 60 minutes. Protein expression levels were normalized to pan-caderin. Phosphorylation was significantly increased in IGFBP-3 compared to untreated cells ($p<0.05$). In the presence of blockers SR-B1 or triciribine, the phosphorylation was significantly decreased. $p<0.05$

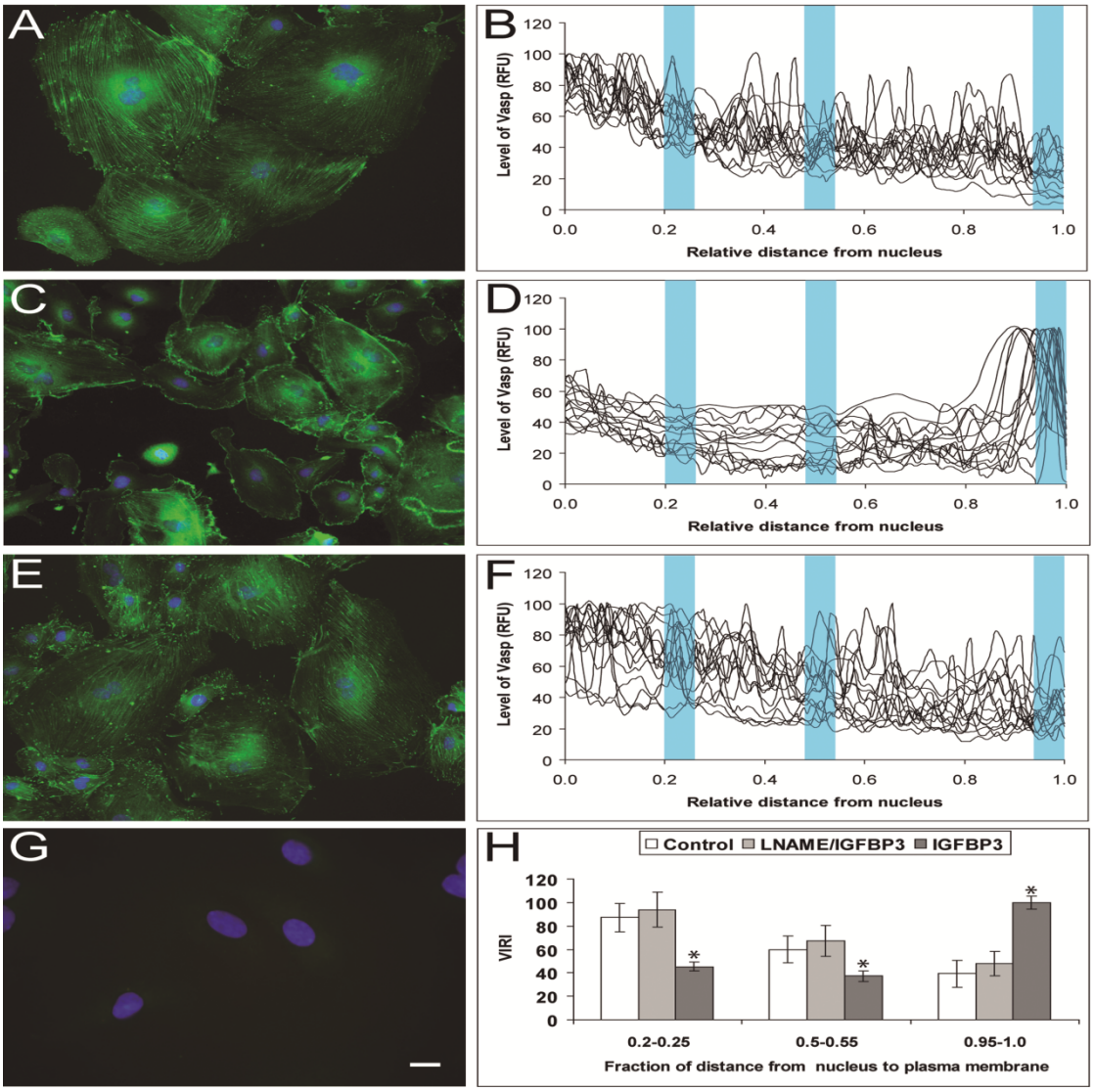

Figure 3-14. IGFBP-3 induces VASP re-distribution in HMVEC-L. HMVEC-L were treated with IGFBP-3 and VASP biodistribution was detected by immunofluorescence A,C,E,G) and quantified. A untreated control cell showing uniform VASP localization (green) along the actin filaments throughout the cytoplasm. C) IGFBP-3 induced VASP redistribution to llamellipodia at the leading edge of HUVECs. E) Pretreatment with an inhibitor of nitric oxide synthase (L-NAME) abolishes the effect of IGFBP-3 on VASP redistribution. G) Control in which VASP primary antibody was omitted. B,D,F) Quantification of VASP biodistribution in A,C, and E respectively. H) Area under the curve calculated from the three areas of interest in B,D, and F (region shaded blue) Green: VASP; Blue: DAPI (nuclei).

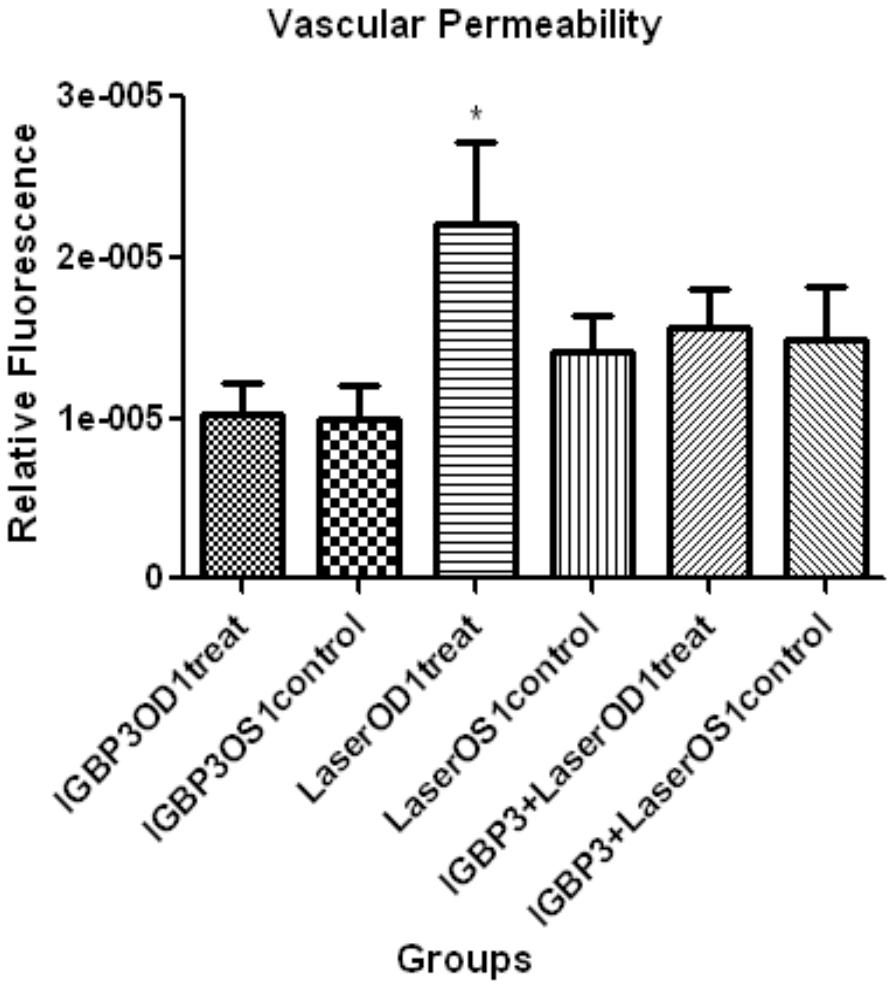

Figure 3-15. IGFBP-3 decreases vascular permeability in laser injured mice. Four days after laser treatment, lasered mice displayed a significant increase in vascular permeability. In mice lasered and injected with IGFBP-3 expressing plasmid, vascular permeability declined at four days post laser treatment compared to laser treated only mice. *$p<0.05$

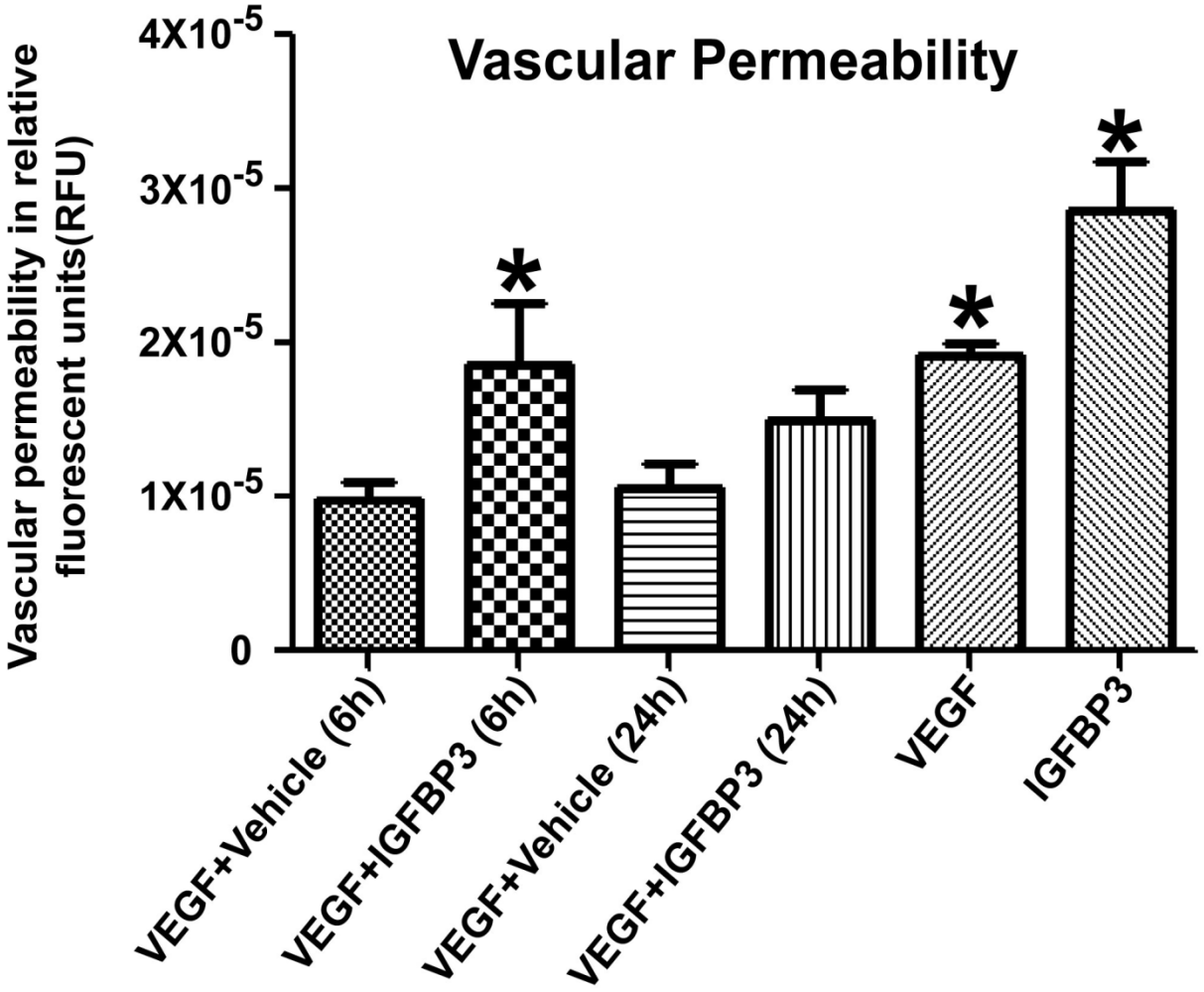

Figure 3-16. IGFBP-3 acutely increases vascular permeability in unlasered mice. IGFBP-3 and VEGF given in combination at 6 hours increases vascular permeability. At 24 hours, VEGF and IGFBP-3 combination leads to reduced vascular permeability. IGFBP-3 by itself increases vascular permeability greater than VEGF alone. *$p<0.05$

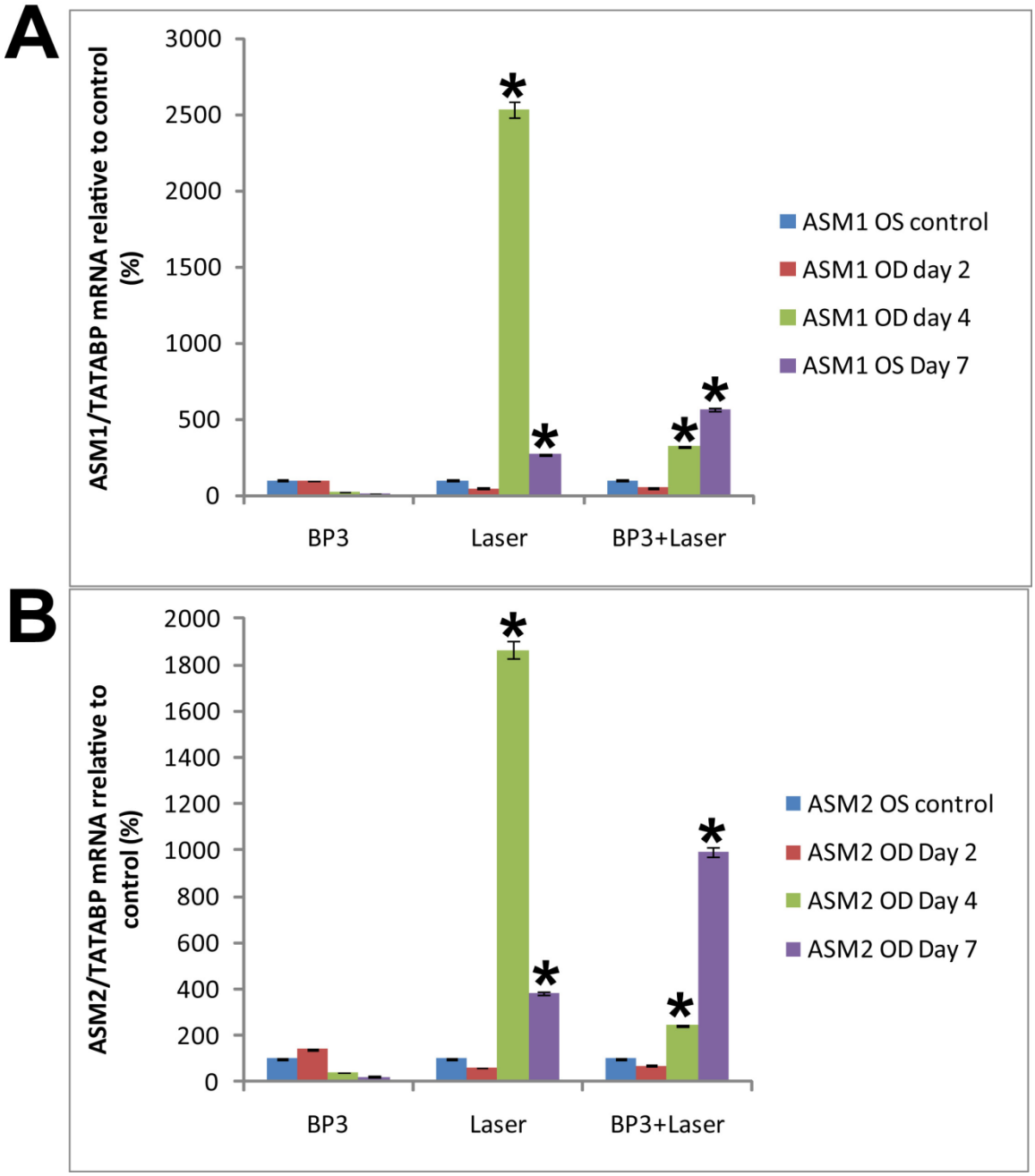

Figure 3-17. IGFBP-3 reduces sphingomyelinase mRNA expression levels. Four days after laser treatment, laser alone mice displayed a significant increase sphingomyelinase mRNA levels, both SMase 1 (acid form) as shown in A) and SMase 2 (neutral form) as shown in B). In laser treated mice injected with IGFBP-3 there is significant reduction in sphingomyelinase mRNA levels at 4 days post treatment. By 7 days the sphingomylinase mRNA levels reversed. OS (left eye) in the control untreated eye and the OD (right eye) is the treated. *$p<0.05$

CHAPTER 4
DISCUSSION

The main purpose of this study was to determine how IGFBP-3 influences EPC driven repair in mouse retinal vascular injury models at the cellular and molecular levels to better understand its therapeutic potential as a robust EPC migratory stimulus. Particular emphasis was placed on understanding how IGFBP-3 impacts both EPC and EC migration, which is key to vascular repair. Directing, homing, and trafficking cells to the proper areas in need of repair is crucial for vascular stabilization, remodeling, and revascularization. IGFBP-3 mediates NO generation, which allows for functional revascularization of ischemic injured retinal blood vessels by enhancing both EPC and endothelial cell migration (Figure 4-1).

IGFBP-3 Acts as an EPC/HSC Homing Factor and Provides Cytoprotection

In this study, the cellular and signaling mechanisms responsible for the vascular protective effects of IGFBP-3 were identified. The effects of IGFBP-3 on two distinct types of vasculature, a stable vascular bed (adult chimeric mice undergoing laser vessel occlusion) and an immature unstable vascular bed undergoing active endothelial cell proliferation and migration (hyperoxia-induced retina injury model) were examined.

In the adult model, IGFBP-3 enhanced repair by recruiting BMDCs to sites of laser occlusion within the ischemic retina. Even in the absence of injury, overexpression of IGFBP-3 by the resident retinal endothelium promoted extravasation of BMDCs from the circulation into perivascular regions and their incorporation into areas of vascular remodeling. The incorporation of EPCs/HSCs into the endothelium appears to be a result of direct integration and not due to cell fusion. Cell fusion can take place with adult stem cells, but usually occurs with embryonic stem cells. There is little evidence for significant fusion of BMC cells in the endothelium and in situations where BMD cells are recruited to repair acute damage in otherwise healthy tissue,

fusion rarely occurs.[112] As reported by Harris *et al.*, cell fusion was unlikely in their studies involving HSC donor-derived differentiation of RPE regenerating injured RPE.[92] Essentially, there are no clear reports of cell fusion of BMDCs in the retina and the observed results described here are unlikely due to cell fusion.

The reparative effects of IGFBP-3 are not limited to simply promoting BMDC homing, because IGFBP-3 reduced both endothelial and neuronal cell death, in the OIR model, as well as decreased inflammatory lipids in the adult retina. IGFBP-3 is known to have both pro- and anti-apoptotic effects. The fact that IGFBP-3 is anti-apoptotic gives credence to its vascular stabilizing and reparative nature, which are supported by the studies described, as well as those previously published by both Chang *et al.* and Lofquist *et al.* in 2007. During repair of the retina, IGFBP-3 increased astrocyte-endothelial cell interactions, likely leading to enhanced barrier properties of the neovasculature. Moreover, IGFBP-3 decreased vascular permeability *in vivo* in mice subjected to laser retinal vessel occlusion and is likely mediated through decreased pro-inflammatory sphingomyelinase levels in the retina. IGFBP-3 also stimulates the differentiation of EPCs/HSCs into not only endothelial cells, but also astrocytes and pericytes, which likely aids in decreasing vascular permeability and reducing retinal inflammation. Astrocytes specifically serve as a template for both developmental and injury associated angiogenesis. The fact that IGFBP-3 increases astrocytic ensheathment and stimulates differentiation of EPCs into astrocytes suggests that IGFBP-3 has a specific effect on astrocytes, which likely stabilizes the resident vasculature allowing for proper repair to occur.

Interestingly, however, in normal mice subjected to VEGF induced retinal vascular permeability, IGFBP-3 increases permeability suggesting IGFBP-3 can be pro-angiogenic or anti-angiogenic. Depending upon the injury state of the tissue, IGFBP-3 can act very differently.

Thus, IGFBP-3 may act to augment VEGF effects to increase vascular permeability in normal retina, while providing vascular stabilization in injured retina. *In vitro* data in bovine retinal endothelial cells also supports the *in vivo* findings that IGFBP-3 and VEGF administered in combination increase permeability. To date, this is the first in depth examination into IGFBP-3's effects on retinal vascular permeability.

IGFBP-3 Increases NO and Activates SR-B1 to Mediate its Protective Effects

NO is an essential signaling molecule that promotes revascularization and vascular remodeling. Endogenous NO generation by BMDCs is critical for their migration, which in turn is required for their reparative function. IGFBP-3 increased eNOS phosphorylation, leading to increased NO generation and subsequent VASP redistribution promoting cell migration. Previous work has shown IGFBP-3 promotes cell migration in EPCs. In this study IGFBP-3 treatment led to the redistribution of VASP to focal adhesions and pseudopodia. The rapid increase in VASP (within 15 minutes) suggests that VASP redistribution occurred, rather than a change in VASP protein expression.

Moreover, IGFBP-3 was shown to mediate IGFBP-3 induced NO generation and this occurred independent of HDL. Activation of eNOS by phosphorylation at Serine 1177 by IGFBP-3 is dependent on activation of SR-B1 and the downstream signaling pathway involving PI3K and Akt activity. NO released by IGFBP-3 in circulating EPCs and resident endothelial cells may modulate the function of BMDCs in an autocrine, as well as paracrine manner, thus contributing to vascular repair.

In agreement with Granata *et al.* in HUVECs, the studies conducted in $CD34^+$ cells support the notion that IGFBP-3 mediated NO generation is also dependent on activation of Sphk1, because NO generation is blocked by Sphk inhibition. S1P-mediated NO generation occurs by activation of endothelial differentiation genes, also known as S1P receptors. S1P,

much like IGFBP-3, increases NO generation, thus promoting migration of cells. Moreover, S1P, like IGFBP-3 has direct vascular protective effects. In the blood, S1P is associated with lipoproteins including low-density lipoprotein, very high density lipoprotein, and HDL, with the majority of S1P being bound to HDL. The studies performed support that in EPCs, both IGFBP-3 and HDL activate Sphk1, because inhibiting Sphk1 with N,N-dimethylsphingosine resulted in a loss of NO generation in response to either agents.

Although there was no observed increase in S1P *in vivo* in response to IGFBP-3, measurements were performed at 3 weeks following retinal injury. It is plausible any acute rise in S1P would have returned to baseline levels by this point. Despite the inability to detect S1P changes in the retina, changes in sphingolipids still persisted at 3 weeks. Most notably, the ceramide/sphingomyelin lipid ratio was completely normalized by IGFBP-3 to that of control levels. This is in contrast to the laser-treated eyes, which had a higher ceramide/sphingomyelin ratio, consistent with an inflammatory, pro-apoptotic state. This suggests IGFBP-3 mediates vascular protection through inhibition of inflammation, allowing IGFBP-3 to promote the recruitment of reparative BMDC population, EPCs, rather than a deleterious inflammatory population.

Basically, the data collected supports the hypothesis that IGFBP-3 mediates functional revascularization in the retinal by promoting the homing of beneficial EPCs, while reducing the number of detrimental inflammatory cells. Once EPCs are routed to areas of damage IGFBP-3 enhances their incorporation and differentiation into endothelial cells, which facilitates vessel formation and stabilization. The studies conducted suggest that these beneficial effects may be mediated by increased NO generation, which occurs via IGFBP-3 binding of SR-B1 and subsequent activation of the PI3K-Akt pathway. Furthermore, the studies support a second

mechanism by which IGFBP-3 stimulated NO generation by activation of Sphk-1 and generation of S1P. IGFBP-3 can also influence vascular permeability via sphingomyelinase, which is downstream of S1P. IGFBP-3 effects on vascular permeability can augment its vascular stabilization and reparative capacity. Hence, IGFBP-3 can modulate interactions between the scavenger receptor system and the S1P receptor system, which may serve to regulate both physiological and pathological angiogenesis, as well as vascular remodeling. Figure 4-1 summarizes the results showing the mechanism of action by which IGFBP-3 acts to increase NO generation in both EPCs and endothelial cells via SR-B1 and S1P to elicit its cytoprotective and reparative effects in injured mouse retinal vasculature.

IGFBP-3: New Perspectives and Remaining Questions

Although this study has identified a new receptor system that mediates the effects of IGFBP-3 with respect to NO generation, IGFBP-3 has been shown to bind several other receptors including transforming growth factor-β receptor, transferrin receptor, and low-density lipoprotein receptor-related protein (LRP). Also, IGFBP-3 has numerous binding partners such as plasminogen, plasmin, fibrin, humanin, and even caveolin.[164] Therefore, the diverse physiological effects of IGFBP-3 are likely mediated by multiple receptor systems. It is likely IGFBP-3 will be implicated in activation of other receptors due to its multi-faceted roles in being pro- and anti angiogenic as well as pro- and anti-apoptotic. S1PRs have already been linked to IGFBP-3's pro-angiogenic effects and additional new findings will likely surface in the future between S1P receptors and IGFBP-3. The role of IGFBP-3 and S1PR's in vascular permeability and their role in IGFBP-3 mediated nitric oxide generation especially require greater investigation. Through the use of IGFBP-3, S1PR, and eNOS knockout mice, these studies can be completed.

IGFBP-3 as a Therapeutic in the Future

IGFBP-3 can still interact with a wide range of binding partners and activate multiple receptors, which can mediate its IGF independent effects. IGFBP-3 will continue to stimulate the interest of researchers. The studies described support IGFBP-3 as a potential therapeutic to treat ischemic ocular disorders, but IGFBP-3 is likely to be suitable for modulation of various vascular and endothelial dysfunctions. Furthermore, IGFBP-3's effects on EPCs will be of further interest. The EPC biology field is beginning to undergo tremendous growth and exploration with regard to understanding the mechanisms by which EPCs home, engraft, and participate in repair. There are a plethora of systemic and local factors that influence EPC behavior. IGFBP-3 is just one of the many factors, which have a role in recruiting EPCs. It is possible other IGFBPs may also play a role in EPC recruitment and mobilization. A recent study by Bartling *et al.* suggests IGFBP-2 and IGFBP-4 can enhance the migration of human $CD34^-/CD133^+$ hematopoietic stem and progenitor cells.[228] They evaluated at IGFBP-2 and IGFBP-4 in lung epithelial cancer cells and showed $CD34^-/CD133^+$ cells can be mediated in part by IGF-independent action of IGFBP-2 and -4.

There is still much to uncover regarding IGFBPs and their effects on EPCs. Delivery, therapeutic dosing, and as well as how to best treat EPCs with IGFBP's are major clinical questions researchers must tackle before IGFBPs can be utilized in the clinical setting. Currently, there are ongoing clinical trials with IGFBP-3 administered to pre-mature babies to alleviate the effects of retinopathy of prematurity.[64] However, many unanswered questions remain such as the delivery method for IGFBP-3. In the case of the eye, local delivery may be appropriate, but for other vascular complications systemic delivery may be optimal. Also, whether a patient's EPCs should be pre-treated with IGFBP-3 in a way to prime them to increase their therapeutic reparative capacity is another key question that merits study or whether treating the injured tissue

with IGFBP-3 is the most effective approach. Basically, IGFBP-3 has the potential to be an effective vascular therapeutic. Cell based therapies involving IGFBP-3 are likely in the future, particularly for patients who suffer from ischemic vascular complications, such as retinopathies.

Conclusions

IGFBP-3 is clearly more than just a binding protein. It has significant vascular protective effects and has a profound impact on EPC migration and homing. Cell migration is vital for stem cell based therapies to be effective. Thus, IGFBP-3 has many positive attributes and qualities, which make it an ideal therapeutic for EPC mediated repair. IGFBP-3 will continue to captivate the interest of researchers. IGFBP-3 is still enigmatic in many ways and there is still much to learn about IGFBP-3. The results discussed here, as well as previous reports, provide a platform upon which to build greater knowledge about IGFBP-3's effects on EPC homing and recruitment. Overall, IGFBP-3 is a complex factor, but one with great promise and hope for stem cell based therapies for ischemic vascular diseases.

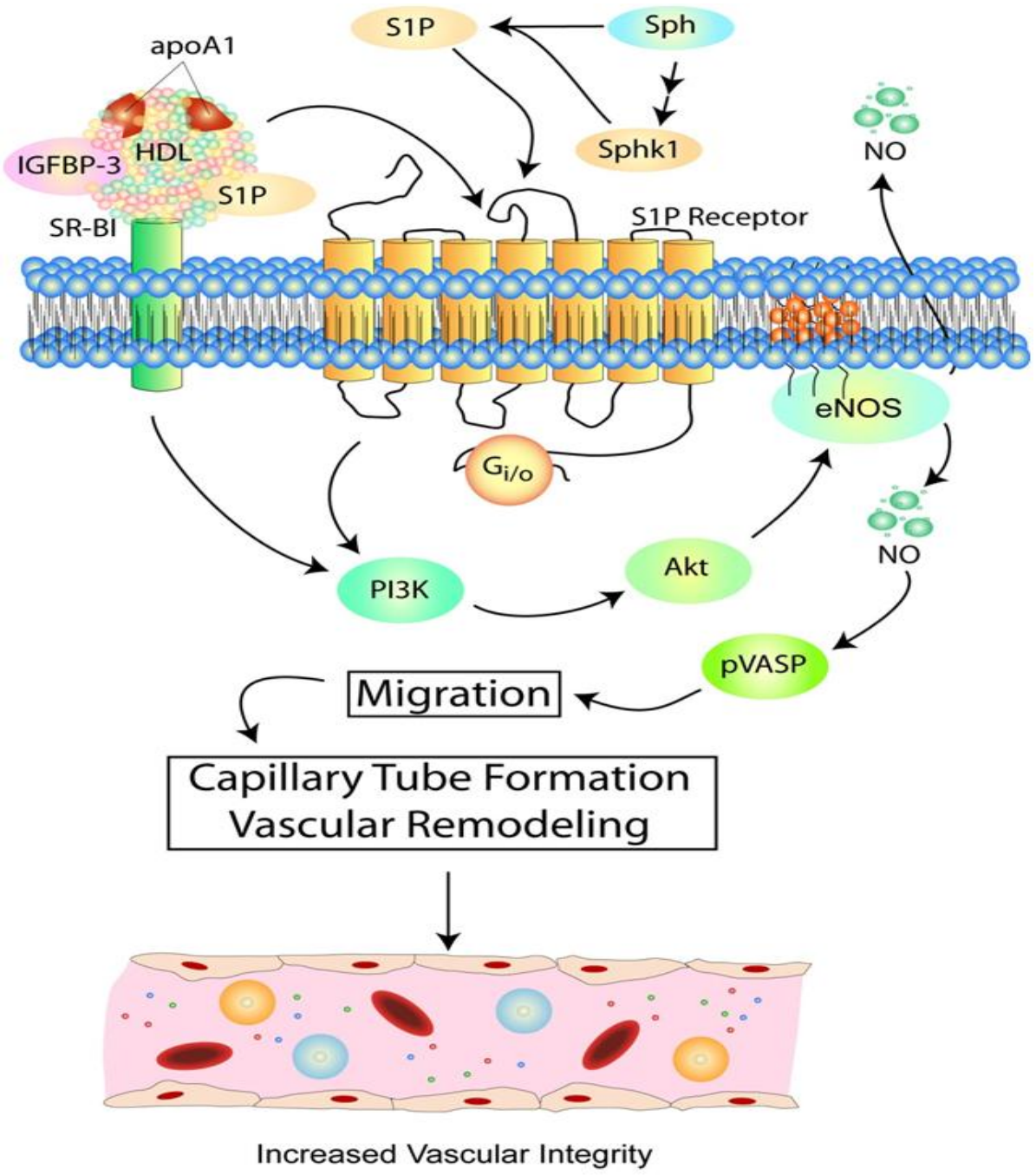

Figure 4-1. Mechanisms of action of IGFBP-3. IGFBP-3 can activate the SR-B1 or S1PR to increase NO production and release via the PI3K/Akt cell survival pathway. IGFBP-3 induces VASP re-distribution, which promotes cell migration. IGFBP-3 increases NO production in both EPCs and endothelial cells, which allows these cells to migrate to areas of needed repair or vascular instability. Likewise, NO production and release by these cells can also attract other cells to promote vascular remodeling in areas of vascular damage. IGFBP-3 can influence vascular permeability leading to vessel stabilization and increased blood vessel integrity. IGFBP-3 has vascular protective and reparative effects.

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BIOGRAPHICAL SKETCH

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