INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-3 REGULATES HEMATOPOIETIC STEM CELL AND ENDOTHELIAL PROGENITOR CELL FUNCTION DURING VASCULAR DEVELOPMENT

By

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To my beloved family

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LIST OF ABBREVIATIONS

ALS	Acid-labile subunit
AMD	Age-related macular degeneration
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BH4	Tetrahydrobiopterin
BM	bone marrow
BRB	Blood-retinal barrier
BSA	Bovine serum albumin
cAMP	Adenosine 3’ 5’-cyclic monophosphate
Cdc6	Cell division cycle 6
cGMP	Guanosine 3’, 5’-cyclic monophosphate
CNV	Choroidal neovascularization
CORMs	Carbon monoxide-releasing molecules
CPT	Cell preparation tube
DAF-FM diacetate	4-amino-5-methylamino-2’, 7’-difluorofluorescein diacetate
DAPI	4’, 6-diamino-2-phenylindole
DM	Diabetes mellitus
DMS	Dimethylsphingosine
DNA	Deoxyribonucleic acid
DR	Diabetic retinopathy
EBM-2	Endothelial cell basal medium-2
ECM	Extracellular matrix
EDG	Endothelial differentiation gene
EDTA	Ethylenediamine tetraacetic acid

Ena	*Drosophila melanogaster* protein enabled
eNOS	Endothelial nitric oxide synthase
EOMs	Extraocular muscles
EPC	Endothelial progenitor cell
EPO	Erythropoietin
ET	Endothelin enhancer
EVH	Ena/VASP homology
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Flk-1	Vascular endothelial growth factor receptor-2
Flt-1	Vascular endothelial growth factor receptor-1
Flt-4	Vascular endothelial growth factor receptor-3
GCL	Ganglion cell layer
GFP	Green fluorescent protein
GM-CSF	Granulocyte/macrophage colony-stimulating factors
GPCR	G protein-coupled receptor
GS isolectin	*Griffonia Simplicifolia* isolectic
HIF-1	Hypoxia inducible factor-1
HIV	Human immunodeficiency virus
HMVEC-L	Human lung derived microvascular endothelial cells
HO	Heme oxygenase
HPGM	Hematopoietic progenitor growth medium
HREC	Human retinal endothelial cells
HRE	Hypoxia response element

HSC	Hematopoietic stem cell
IACUC	Institutional animal care and use committee
IGF	Insulin-like growth factor
IGF-1R	Insulin-like growth factor-1 receptor
IGFBP	Insulin-like growth factor binding protein
IL	Interleukin
INL	Inner nuclear layer
iNOS	inducible/inflammatory nitric oxide synthase
IPL	Inner plexiform layer
KDR	Vascular endothelial growth factor receptor-2
MMP-9	Metalloproteinase-9
mRNA	messenger ribonucleic acid
NaCl	Sodium chloride
NIH	National institutes of health
nNOS	neuronal nitric oxide synthase
NO	Nitric oxide
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
PEI	Polyethylenimine

PFA	Paraformaldehyde
PKA	Protein kinase A
PlGF	Placenta growth factor
rAAV	recombinant adeno-associated virus
ROP	Retinopathy of prematurity
RPE	Retinal pigment epithelium
RT-PCR	Real time polymerase chain reaction
S1P	Sphingosine 1-phosphate
SCF	Stem cell factor
SDF-1	Stromal cell derived factor-1
SD	Standard deviation
sGC	soluble guanylyl cyclase
SK	Sphingosine kinase
TGF	Transforming growth factor
TPO	Thrombopoietin
VASP	Vasodilator stimulated phosphoprotein
VEGF R-1	Vascular endothelial growth factor receptor-1
VEGF R-2	Vascular endothelial growth factor receptor-2
VEGF	Vascular endothelial growth factor
VPF	Vascular permeability factor
ZO-1	Zoula-occludens-1

Abstract of Dissertation Presented to the Graduate School
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Retinal blood vessels develop by a combination of vasculogenesis and angiogenesis, called neovascularization. Endothelial progenitor cells (EPCs) which originated from lateral and posterior mesoderm contribute to neovascularizaiton in response to certain cues such as cytokines and hypoxic gradient. Aberrant vessel growth in retina results in vascular retinopathies, the leading cause of visual disability and blindness worldwide. The pathological outgrowth of new blood vessels involves the recruitment and proliferation of circulating EPCs. This study was initiated to better understand a complex network regulating neovascularization involving EPCs function. Many factors have been identified to promote trafficking, mobilization, and homing of EPCs. We demonstrated that insulin-like growth factor binding protein-3 (IGFBP-3) has a critical function in postnatal vasculogenesis both *in vitro* and *in vivo*. IGFBP-3 showed enhanced migration, tube formation and proliferation of EPCs *in vitro*. *In vivo*, IGFBP-3 inhibited pathological neovascularization by protecting developmental retinal vessels from oxygen induced regression. In EPCs, nitric oxide (NO) regulates migration through redistribution and phosphorylation of the motor protein vasodilator-stimulated phosphoprotein (VASP). IGFBP-3 has been shown to trigger EPC mobilization by generating NO and subsequently

activating VASP. The signaling mechanisms of IGFBP-3 on EPCs were also identified in this study. We have defined that down stream pathway of IGFBP-3 involve sphingosine kinase (SK)/sphingosine-1 phosphate (S1P) signal transduction. NO release from EPCs was reduced after treatment with a SK inhibitor, dimethylsphingosine (DMS) and could not be recovered by exposure to IGFBP-3. This result suggests that IGFBP-3 may act as an upstream mediator of SK/S1P signaling. We addressed the hypothesis that IGFBP-3 stimulates NO production and VASP phosphorylation through SK activation. Thus, IGFBP-3-induced angiogenesis of EPCs may be involved in a SK dependent signaling pathway. Whereas an inhibitory angiogenic role for IGFBP-3 has been widely reported, several other researchers have shown contradictory results in that IGFBP-3 also enhances angiogenic effects. Our results suggest that IGFBP-3 has an angiogenic/vasculogenic function in modulating the migratory ability of EPCs.

CHAPTER 1
BACKGROUND AND SIGNIFICANCE

Blood vessel development is mediated by angiogenesis as well as by vasculogenesis. In vasculogenesis stem cells are mobilized from the bone marrow and differentiate into circulating endothelial progenitor cells which are then integrated into the primary capillary plexus. This process is responsible for the development of the vascular system during embryogenesis. In contrast, angiogenesis is the formation of new blood vessels from sprouts on preexisting vessels, and occurs both during development and in postnatal phase. In adults, neovascularization involves the recruitment and proliferation of either endothelial cells from preexisting vessels or circulating EPCs originating from bone marrow. This process is involved in many physiological and pathological conditions such as tissue remodeling, regeneration, wound healing and tumorigenesis. Vessel development is regulated by a complex network of mediators and cellular interactions. Reduced oxygen tension, various cytokines, and angiogenic factors at least partially regulate neovascularization. This study was intended to better understand the mutifactorial processes of neovascularization.

The Eye

The eye exists in a relatively isolated compartment. The ocular tissue and vasculature are highly differentiated for conducting the complex process of visual transduction with little systemic exposure. The retina is an ideal model system to study molecular mechanisms of angiogenesis due to the unique vascular supply of the eye, the ability to visualize this vasculature *in vivo* and the ability to selectively express genes in the eye.

Anatomy

The eye is a complex organ composed of many parts. The ability to see is dependent on the actions of several structures. Figure 1-1 shows many of the essential components of the eye.

Initially, light enters the eye through a lubricating tear film that covers the cornea.[1] The cornea is the transparent outer covering of the eye and helps to focus incoming light. After light rays pass the cornea, they travel through a clear, watery fluid, called the aqueous humor.[2] The aqueous humor transports nourishment for the surrounding lens and cornea as well as maintaining a constant intraocular pressure.[2] The aqueous humor is produced by the ciliary body which also changes the shape of the lens for focusing.[3] The iris is the colored part of the eye. It separates the anterior chamber from the posterior chamber and regulates the amount of light entering through the pupil.[3] The size of the pupil is controlled by the dilator and sphincter muscles of the iris and regulates the amount of light that enters the eye.[3] After light travels through the pupil, it passes through the lens. The lens is suspended by ligaments (called zonule fibers) that are attached to the anterior portion of the ciliary body.[4] As a consequence of ciliary muscle actions, the contraction or relaxation of these ligaments changes the shape of the lens (a process called accommodation allowing the formation of a sharp image on the retina).[4] Light then passes a clear, jelly-like substance called the vitreous before it finally reaches the retina. The vitreous is a viscous, transparent liquid that fills the center of the eye.[1] It is composed mainly of water and comprises about 2/3 of the eye's volume, and helps to maintain eye shape.[1] The retina is a multi-layered sensory tissue lining the back of the eye that operates similar to the film in a camera. At the retina, the light rays are converted to electrical impulses which are transmitted via the optic nerve to the brain. The central portion of the human retina contains a yellow pigment called the macular pigment.[5] This pigment helps protect the sensitive receptors in the retina, particularly from the potentially harmful effects of blue light.[5] The density of the pigment has been shown to be linked to diet and can be reduced in a person who smokes.[5] The macula is the area of the retina that contains the highest concentration of photoreceptor cells.

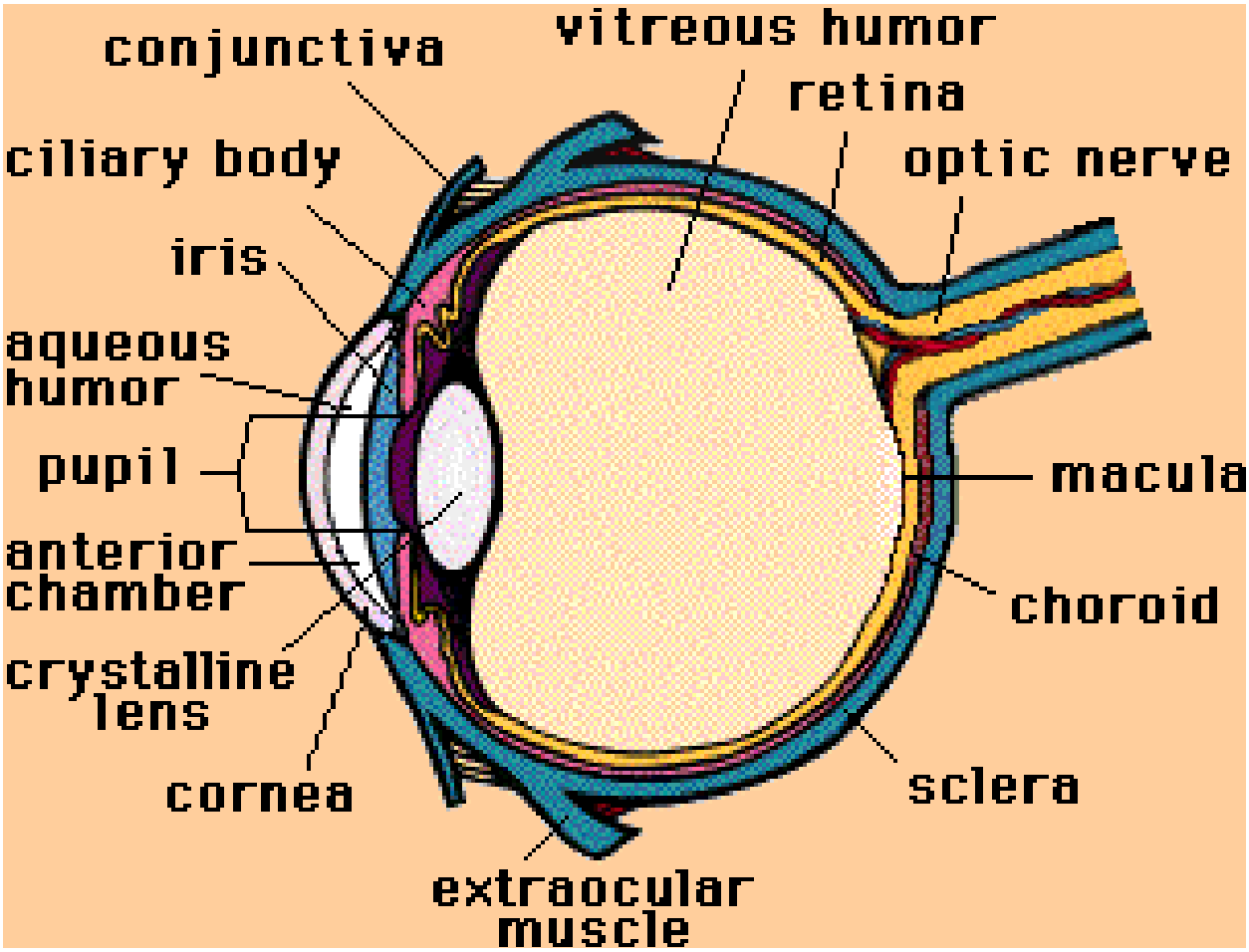


Figure 1-1. Anatomy of human eye.

At the very center of the macula is the fovea, the site of our sharpest vision.[6]

The optic nerve is a bundle of nerve fibers that carries visual information from the eye to the brain. The optic nerve runs from the optic disc through the optic foramen to the optic chiasma where it becomes the optic tract.[7] It is 5cm in length and surrounded by 3 layered membranes of the central nervous system: pia, arachnoid, and dura.[7]

The eye is comprised of three different layers and spatially divided into three chambers of fluid. The external layer is formed by the sclera and cornea.[2] The cornea is a refracting surface, providing 2/3 of the eye's focusing power.[2] The cornea is extremely sensitive and contains more nerve ending than anywhere else in the body.[2] The sclera is composed of tough, fibrous tissue that protects the inside of the eye.[2] Extraocular muscles are attached to the sclera and maintain the shape of the eye.[8] The six tiny muscles, known as the extraocular muscles (EOMs), surround the eye and control its movements.[8] The primary function of the four rectus muscles is to control the eye's movements from left to right and up and down.[9] The two oblique muscles allow the eye to rotate inward and outward.[9] All six muscles of both eyes work in unison so that the eyes are always aligned.[8] The intermediate layer is divided into two parts: anterior (iris and ciliary body) and posterior part, called the choroids.[10] The choroid contains a layer of blood vessels and lies between the retina and sclera.[10] The choroid supplies oxygen and nutrients to the outer layers of the retina.[11] The choroid connects the ciliary body with the front of the eye and is attached to edges of the optic nerve.[11] The internal layer is the sensory part of the eye called retina.

The eye consists of three chambers; the anterior chamber (between cornea and iris), posterior chamber (between iris, zonule fibers, and lens), and the vitreous chamber (between the

lens and the retina). The first two chambers are filled with aqueous humor and the vitreous chamber is filled with more viscous fluid, the vitreous humor.

The Retina

The retina is a multi-layered structure that is involved in signal transduction. It covers about 65 % of interior surface of the vitreous chamber.[2] The human retina is approximately 0.2 mm thick, and has an area of approximately 1100 mm.[2] Each retina is composed of about 200 million neurons.[12] The retina captures the light and converts it into electrical impulse using photoreceptors. There are two types of photoreceptors in the retina: rods and cones.[13] Approximately 125 million rods exist in human retina.[13] They are spread throughout the peripheral retina and function best in dim lighting; therefore, the rods are responsible for peripheral and night vision.[13] The retina contains approximately 6 million cones.[14] Cones function best in bright light and color perception.[14] The highest density of cones is in the macula.[15] The macular contains a very different retinal configuration towards the center called foveal region.[15] Cones are most densely packed within the fovea, the center portion of the macular. The fovea is maximally thinned and mainly consists of photoreceptors and their nuclei.[15] The purpose of foveal thinning is to permit greater light absorption by the dense array of photoreceptors.[6, 15] Another interesting aspect of the fovea is the absence of blood vessels over the photoreceptors. This absence of blood vessels contributes to increasing visual acuity in the macular region.[15] However, the vascularization in the rest of the macula is very dense and thus increases possibility of many vascular related diseases. The retina is loosely attached to the retinal pigment epithelium (RPE).[10] These cells contain a great amount of pigment that is necessary for light absorption and transportation of oxygen, nutrients, and cellular wastes between the photoreceptors and the choroids.[10] Bruch's membrane is tightly bound to the RPE, stabilizing the RPE layer by separating it from the blood vessels of the choriod.[10]

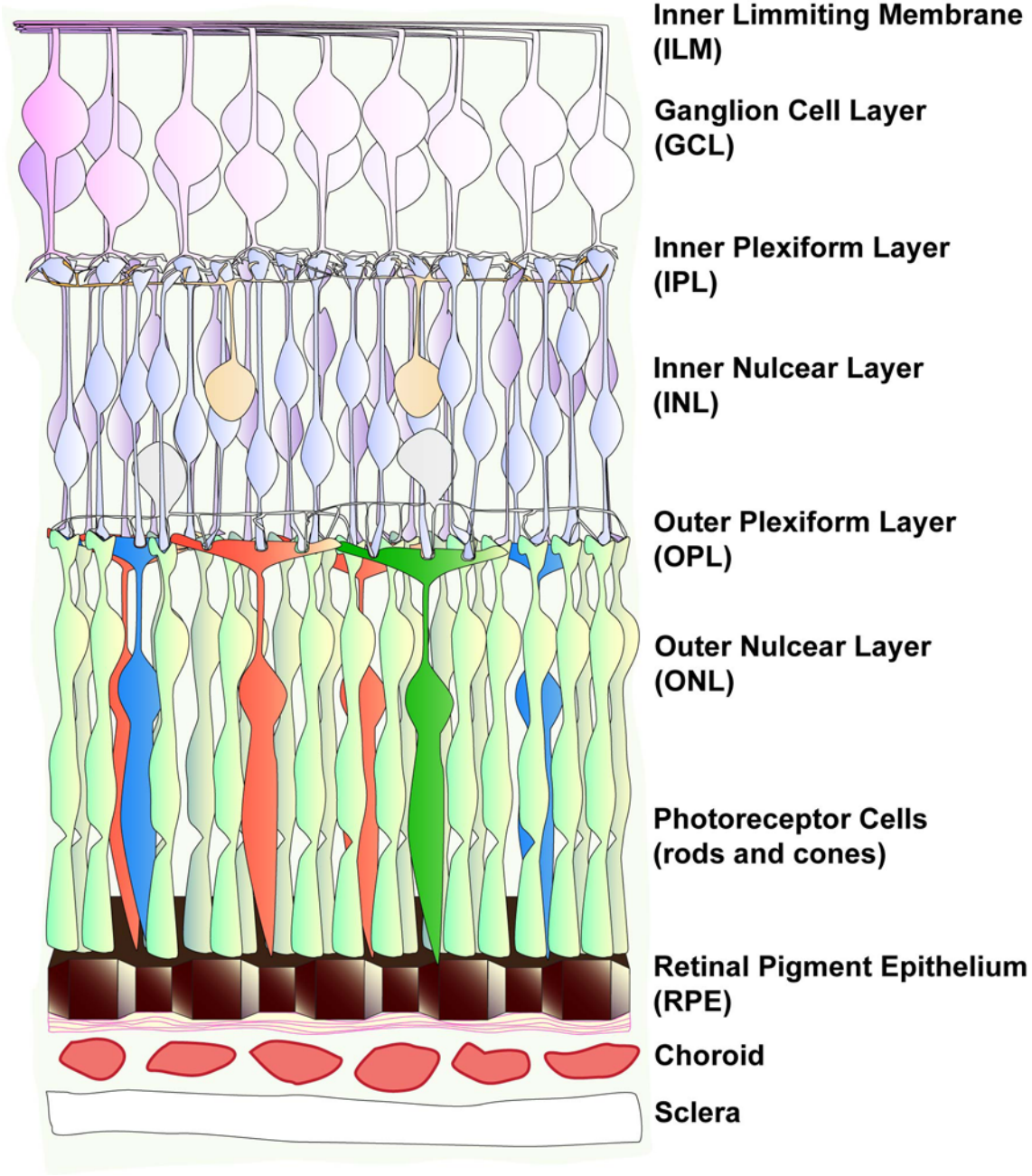


Figure 1-2. The ten layers of the retina. This drawing shows retinal and choroidal cross-section. From the most anterior layers of the retina, the ten layers of the retina consist of sclera, choroids, retinal pigment epithelium, rod and cone layer, outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, ganglion cell layer, and the inner limiting membrane.

Oxygen diffuses across the Bruch's membrane and this membrane grows thinker with age.[10] Breaks in Bruch's membrane are the hallmark for choroidal neovascularization (CNV) into the retina.[10] Beneath the Bruch's membrane the chroroid, containing its network of blood vessels, nerves, immune cells, and fibroblasts, supplies all of nutritional needs of the RPE and the outer part of the sensory retina.

Human retina consists of ten layers (Figure 1-2). Among them, three layers of nerve cell bodies and two layers of synapses are mainly responsible for converting a light signal into neural signal. The photoreceptor cell bodies form the outer nuclear layer (ONL).[16] While the inner nuclear layer (INL) contains cell bodies of the bipolar, horizontal and amacrine cells, the ganglion cell layer (GCL) contains cell bodies of ganglion cells and displaced amacrine cells.[16] The outer plexiform layer (OPL), the first area of neutrophil, is located in between the ONL and the INL.[17] In the OPL, the photoreceptors convey their information to the bipolar cells as well as the horizontal cells. Afterward, bipolar cells relay information to the inner plexiform layer (IPL), which separates the INL and the GCL.[17] Bipolar cells are connected to the retinal ganglion cells in addition to amacrine cells in the IPL.[17] The ganglion cells are the output neurons of the retina that transmit the signal from the eye to the brain.[16]

Retinal Blood Supply

The blood supply to the retina originates from the ophthalmic artery.[1] There are two sources of blood supply to the mammalian retina: the central retinal artery and the choroidal blood vessels.[1] The outer retina is supplied by the choriocapillaries. The choroidal arteries arise from long and short posterior ciliary arteries and branches of Zinn's circle around the optic disc.[10] Each of the posterior ciliary arteries is further classified into fan-shaped lobules of capillaries that supply localized regions of the choroids.[10,18] The arteries penetrate the sclera around the optic nerve and spread out to form vascular layers in the choroids.[10] The choroid

receives the greatest blood flow (65-85%), which is critical for the maintenance of the outer retina, particularly the photoreceptors.[10] The central retinal artery supplies the remaining 20-30% blood flow from the optic nerve head to nourish the inner retinal layers. The central retinal artery supplies the blood as it branches into smaller segments upon leaving the optic disc.[18] The vessels are further divided into either an artery or a vein[18]. The central retinal artery has 4 main branches in the human retina.[18] The arterial intraretinal branches then supply three layers of capillary networks: the radial peripapillary capillaries, an inner layer of capillaries, and an outer layer of capillaries.[18] The radial peripapillary capillaries are the most superficial layer of capillaries lying in the inner part of the nerve fiber layer, and run along the paths of the major superotemporal and inferotemporal vessels.[18] The inner capillaries are located in the ganglion cell layer both under and parallel to the radial peripapillary capillaries.[18] The outer capillary network runs from the inner plexiform layer to the outer plexiform layer though the inner nuclear layer.[18]

Retinal blood vessels that are similar to cerebral blood vessels maintain the blood-retinal barrier (BRB). The BRB consists of two distinct monolayers of cells: the retinal pigment epithelium (RPE: outer barrier) and the retinal capillary endothelial cells (inner barrier).[19] Both monolayers form tight junctions, which are operative in the maintenance of the barrier.

The concept of the BRB was first proposed by Schnaudigel in 1913 following the classical work of Ehrlich and Goldman who discovered the blood-brain barrier (BBB). Similar to the structure of BBB, the inner BRB is covered with pericytes and glial cells.[19] Glial Müller cells predominantly support retinal endothelial cells and glial astrocytes are partly responsible for supporting endothelial functions at the inner BRB.[20,21]

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.[20] The outer BRB consists of specialized nonfenestrated capillaries and tight junctions within the RPE.[21] The outer BRB forms a transport barrier between the retinal capillaries and the retinal tissue.[21] In addition, it prevents the passage of the large molecules from choriocapillaries into the retina.[21]

The eye is extremely sensitive to any disruption of its blood supply. The BRB maintains the ocular milieu and protects the neural retina from any pathological blood circulation. The breakdown of the BRB is a clinical hallmark of early diabetic retinopathy.

Retinopathies

Vision impairment, disability, and blindness are major public health problems. Significant suffering, disability, loss of productivity, and lower quality of life can affect millions of people. In the United States, more than 11 million people have some degree of visual impairment. Approximately 890,000 people in the US are legally blind. Retinopathies are ocular diseases in which deterioration of the retina is initiated by abnormal neovascularization, resulting in vision loss. Vascular retinopathies are the leading causes of visual disability and blindness worldwide. Pathological growth of new blood vessels in pre-retinal region is the hallmark of retinopathies. Retinopathies affect all age groups: retinopathy of prematurity (ROP) is a disease that occurs in premature babies. Diabetic retinopathy (DR) primarily affects the working age population, and age-related macular degeneration (ARMD) affects the aging population.

Retinopathy of Prematurity

ROP is the leading cause of blindness in children in both developing and developed countries. ROP mainly affects premature infants weighing about 1.25 kg (approximately 2.75lb) or less that are born before 31 weeks of gestation. At 16 weeks of gestation, blood vessels

gradually grow over the surface of the retina.[22] Active growth of the human fetal eye occurs within the last 12 weeks of full term delivery (28 to 40 weeks of gestation).[23] Vessels reach the anterior edge of the retina and then stop progressing at about 40 weeks of gestation.[22-25] A premature baby is placed into an oxygen chamber to assist the still developing lungs. Once in high oxygen the retinal vessel development is stopped. Upon removal from the high oxygen environment the return to normal levels of oxygen is seen as a hypoxic environment in the eye, and this stimulates the neovascularization within the retina.

There are approximately 3.9 million babies born in the U.S. annually. According to National Eye institute, about 28,000 premature infants are born. About 14,000 to 16,000 of these premature infants could potentially develop some degree of ROP. Although, approximately 90% of all the infants with ROP are in the milder stage and do not need treatment, the rest of 10 % (about 1,100 to 1,500) of the babies develop severe ROP and require medical treatment. As a consequence, about 400 to 600 infants each year in the U.S. become legally blind from ROP.

Many factors are likely to cause ROP. Once a premature baby is born, excessive oxygen supply is needed to help the development of the premature baby's lungs.[25] ROP was first described in 1942, but its cause was unknown at that time. Most of premature babies were treated with high oxygen whether they were having breathing problems or not.[22] After a while, it was found that although supplemental oxygen helped the premature babies who were having lung and breathing complications, the high oxygen destroyed blood vessels in the retina.[22] Despite adjustment of oxygen delivery and other medical advances, the total number of infants with ROP has not decreased because of the increased survival rates among the low birth weight infants.[22,26] ROP progresses in two phases.[24] The hyperoxia extrauterine environment surrounding the baby precedes the development of the first phase of ROP.[24] The growth

inhibition of neural retina and retinal vasculature in the first phase is followed by a second phase of ROP involving relative hypoxia-induced uncontrolled proliferative vessel growth.[24] The pathological growth of vessels produces a fibrous scar that extends from the retina to the vitreous gel and lens.[24] Retraction of this scar tissue can separate the retina from the retinal pigment epithelium (RPE), resulting in a retinal detachment, bleeding and blindness.[22,23]

This biphasic disease is associated with unbalanced levels of growth factors. 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.[27] IGF-1 plays a critical role in ROP infants. Reducing IGF-1 levels inhibits vessel growth even in the presence of VEGF.[28] Low levels of IGF-1 directly 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.[28,29] In the second phase of ROP, which is driven by hypoxia, VEGF expression is increased in the retina, resulting in pathological neovascularization.[30]

Because the retinal vessel development of mice is incomplete at birth, Smith *et al*. developed a mouse model of ROP to study the molecular mechanism in the disease.[30] This mouse model is intended to mimic the first and the second phases of ROP.

Traditional therapies such as cryotherapy and laser photocoagulation of other proliferative retinapathies can also be used to prevent blindness in ROP infants.[23] However, these methods can reduce peripheral vision and include risks from the anesthesia.[23] Therefore, preventive and less invasive therapies for ROP are desirable. Likewise, efforts to understand diseases that involve VEGF and IGF-1 are important to develop such medical treatments. The two phases of ROP require apposite approaches. In phase I, the hyperoxia induced vessel loss can be partially

prevented by administrating exogenous VEGF or PlGF-1.[31-33] While an injection of anti-VEGF aptamer as well as anti-VEGF antibody fragment can be used to treat neovascularization associated with phase II of ROP.[34] Pharmacological intervention related to the prevention of vessel loss may be more effective in the treatment of ROP since the extent of the second destructive phase of ROP is determined by the amount of vessel loss in the first phase.

Diabetic Retinopathy

Approximately 100 million people worldwide have been affected by diabetes mellitus (DM).[35] In the United States, 16 million individuals are diabetic, and about 40,000 patients per year are diagnosed with the ocular complications of DM in the U.S.[36] Among them, 5 to 10 percent are known to be insulin-dependent type 1 DM and 90 % to 95 % is known to be insulin-independent type 2 DM.[36] Vascular diseases are the principal causes for death or disability in people with diabetes.[35] The metabolic abnormalities that characterize diabetes such as elevated blood glucose levels, increased levels of free fatty acids, and insulin resistance cause vascular dysfunction.[35] According to the NIH, the microvascular complications of DR are the most common complication of diabetes and thus a leading cause of blindness. DR is known to affect approximately 75 % of diabetic patients within 15 years after onset of the disease.[37] Although the best way to prevent visual loss is to initiate treatment before symptoms develop, many diabetic patients are only diagnosed after visual complications have already begun. DR is divided into two stages: nonproliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR).[36] PDR typically develops in patients with type 1 diabetes, while NPDR is more common in patients with type 2 diabetes.[36]

The progression of DR begins with apoptosis of pericytes and adhesion of leukocytes to the vessel wall that lead to microvascular occlusion, basement membrane thickening, and increased vascular permeability.[38] At this stage, the blood vessels become leaky, allowing blood

and vascular fluids to accumulate in the retinal tissue and form exudate deposits.[38] These pathological processes then result in macular edema which is a common feature in patients with NPDR.[39] NPDR produces an increasing area of capillary non-perfusion which leads to hypoxia in the retina.[38,40] To compensate for the decreased oxygen supply, angiogenic factors are released from the hypoxic retinal tissues that stimulate the growth of new blood vessels on the surface of the retina.[37,41] This stage is called PDR. The walls of the new blood vessels are fragile and may break, allowing blood to leak out.[38] This can cloud the vitreous and compromise vision.[38] In an advanced stage of PDR, newly formed fibro vascular tissue grows from the retinal surface into the vitreous cavity.[36] This can cause retinal detachment which can result in blindness.[36,39]

To date, a common therapy for DR, including advanced PDR or diabetic macular edema, is laser photocoagulation. However, this method often causes common side effects such as neural tissue loss, peripheral vision loss, impairment of night vision, and change in color perception.[42] Moreover, in some patients, the retinopathy continues to progress after treatment.[42] Thus, there is a great need for the development of new therapies that treat diabetic retinal vascular disease. Recently, pharmacological agents that directly inhibit angiogenesis have been developed to treat DR.[34] VEGF plays a pivotal role in the retinal microvascular complications of diabetes.[37] The overexpression of VEGF plays a key role in diabetes inducing retinal vascular dysfunction.[37] The developments of agents that directly target VEGF and its receptors have been actively studied in clinical research.[43-45] The use of endothelial progenitor cells for drug delivery or molecular and genetic manipulation is a technique that takes a new approach in the treatment of DR.

Age-related Macular Degeneration

AMD is the most common cause of poor sight in people with age over 60.[46] Recently, it is reported that AMD affects approximately 11% of the U.S. population age 65 to 74.[46] In the western world, there are approximately 12 to 15 million cases of AMD.[46]

Two main types of AMD can be distinguished: the dry form (atrophic) and the wet form (exudative), based on the absence or the presence of choroidal neovascularization (CNV).[46] Dry AMD is more common than the wet form.[46] It develops very slowly and causes gradual loss of central vision. Dry AMD is characterized by the presence of drusen in the macular region.[47] The excreted materials, damaged photoreceptors and concentrated by-products of cellular metabolism affect the formation of drusen, which looks like yellow-gray nodules localized between the retinal pigment epithelium (RPE) and Bruch's membrane.[47] Increased drusen formation affects RPE function and eventually causes RPE alteration and depigmentation.[47] As a result of dry AMD, patients are likely to lose the central perception as well as color contrast sensitivity. Although wet AMD only consists of approximately 10% of all AMD cases, about 80% of severe vision loss is caused by the wet form as compared to 20% that are caused by the dry form. CNV determines the characteristics of the wet AMD. CNV is the process of the growth of immature blood vessels in the choroid, and the pathological new vessels penetrate the subretinal space. Over time, CNV causes hemorrhages, RPE detachment, scarring, and blindness.[11,47]

The main factor that causes AMD is not known. However, a number of risk factors have been identified that partially contribute to AMD such as age, gender (women are more likely to develop AMD), smoking, genetics and nutrition.[48] There are also molecular factors that are known to affect the development of AMD.[48] It is found that VEGF expression is increased in RPE cells of patients with AMD.[11,41] In experimental animal models, VEGF levels were found

to be significantly higher in the vitreous of wet AMD than healthy controls.[11,41,48] To date, the most promising results of a treatment for ARMD has been achieved with anti-angiogenic reagents that target VEGF.[42]

Neovascularization

Blood vessels are developed by vasculogenesis or angiogenesis. During vasculogenesis, endothelial cells differentiate from progenitor cells and angioblasts, which are already present throughout the tissue, and then link together to form vessels. During angiogenesis, sprouts form from preexisting blood vessels and invade into surrounding tissue. Most organs are vascularized by vasculogenesis, but brain and kidney are vascularizaed by angiogenesis. Retinal vascular development occurs by a combination of vasculogenesis and angiogenesis, called neovascularization. A variety of stimuli are known to contribute to neovascularization by recruiting stem or progenitor cells and inducing adhesion to activated ECs.

Cytokines, chemotactic factors, and angiogenic factor have been implicated as positive regulators of neovascularization. Some of these molecules are strongly induced by hypoxia in cultured cells, including tumor cell lines, cardiac myocytes, and vascular smooth muscle cells as well as in ischemic tissues.

Endothelial Progenitor Cells

Bone marrow (BM) is the major reservoir of stem cells in adults. The bone marrow microenvironment, in which bone marrow stem cells remain quiescent, is comprised of stromal cells and extracellular matrix (ECM) components. A special subtype of BM derived stem cells, termed endothelial progenitor cells (EPCs) that 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. EPCs can be isolated from peripheral blood, fetal liver, or umbilical cord blood.[49-51] EPCs are characterization by

specific antigens expressed on the surface of the cells. Stem cells maintain primitive characteristics so that they can differentiate or transdifferentiate into a wide range of cell types. This is called stem cell plasticity.[52] The identification of true EPCs has been challenged by the phenomenon of stem cell plasticity. Defining the validated EPCs has been debated because several studies have demonstrated overlapping antigens among subtypes of bone marrow derived cells including EPCs and mesenchymal stem cells.[52-58] Although it is not clear what markers define EPCs, it is widely accepted that CD34, vascular endothelial growth factor receptor-2 (VEGFR-2), and CD133 are the common antigens used in the enrichment of EPCs.[59-61] Schatterman, *et a*l. suggested that expression of endothelial nitric oxide synthase (eNOS) is a reliable marker for EPCs.[49,53] EPCs have other characteristics of endothelial cells including acetylated low density lipoprotein incorporation and endothelial specific lectin binding *in vitro*.[49,59,62] Furthermore, EPCs also show typical endothelial functional characteristics like formation of capillary tubes and production of nitric oxide (NO).[63,64] With maturation, EPCs begin to lose expression of CD34 and CD133 (i.e., early hematopoietic stem cell marker) or start to express CD31, also known as PECAM-1 (platelet/endothelial cell adhesion molecule), vascular endothelial cadherin, and von Willebrand factor.[49,57] The differentiation and maturation of EPCs occur when circulating EPCs move to the site of injured vessel or integrated into mature endothelium.[50]

Increasing evidences suggest that EPCs are preferentially recruited to sites of ischemia and tumor formation and incorporated into functional vasculature.[50,65,66] EPC recruitment as well as the release from BM is influenced by various factors. Proangiogenic growth factors such as VEGF, granulocyte/macrophage colony-stimulating factors (GM-CSF), SDF-1, and erythropoietin (EPO) have been shown to modulate EPC functions that play a critical role in

embryo development as well as in homeostasis in adult.[65,67] For instance, these factors are essential for EPCs differentiation and blood vessel development during embryogenesis and also contribute in increasing circulating numbers of EPCs in adults.[65,67] Transplantation of cultured EPCs successfully promotes therapeutic neovascularization in both ischemic hind limbs and acute myocardial infarction models.[50,65]

Both the number of circulating EPCs and colony forming ability of EPCs are correlated with some types of diseases. It was found that fewer $CD34^+$ EPCs are circulating in patients with diabetes, diabetic retinopathy, and peripheral artery disease.[68] 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 the damaged vessels.[65,68,69] Since new blood vessel growth from mature ECs has rarely been found in adults, and turnover of the quiescent endothelium is considerably low, the vascular repair may need the support of EPCs. The study of EPC biology will help to better understand postnatal vasculogenesis and also help to find novel therapies for the treatment of pathological neovascularization.

Hypoxia Inducible Factor-1

Hypoxia occurs when there is an imbalance between oxygen supply and demand in cancer or ischemic tissues. In wounds, capillary injury generates a hypoxic environment, and altered oxygenation induces the reconstructive angiogenic response.[70] Hypoxia serves as a critical cue for both physiological and pathological angiogenesis in the brain, heart, kidneys, lungs or muscles.[71] In stem cell research, hypoxia is considered a potent trigger for mobilization of bone marrow derived cells.

Hypoxia inducible factor-1 (HIF-1), a transcription factor, functions as a major regulator of O_2 homeostasis or an adaptor of O_2 deprivation. HIF-1 is a heterodimer composed of an oxygen related HIF-1α subunit and a constitutively expressed HIF-1β subunit.[72] In order to respond

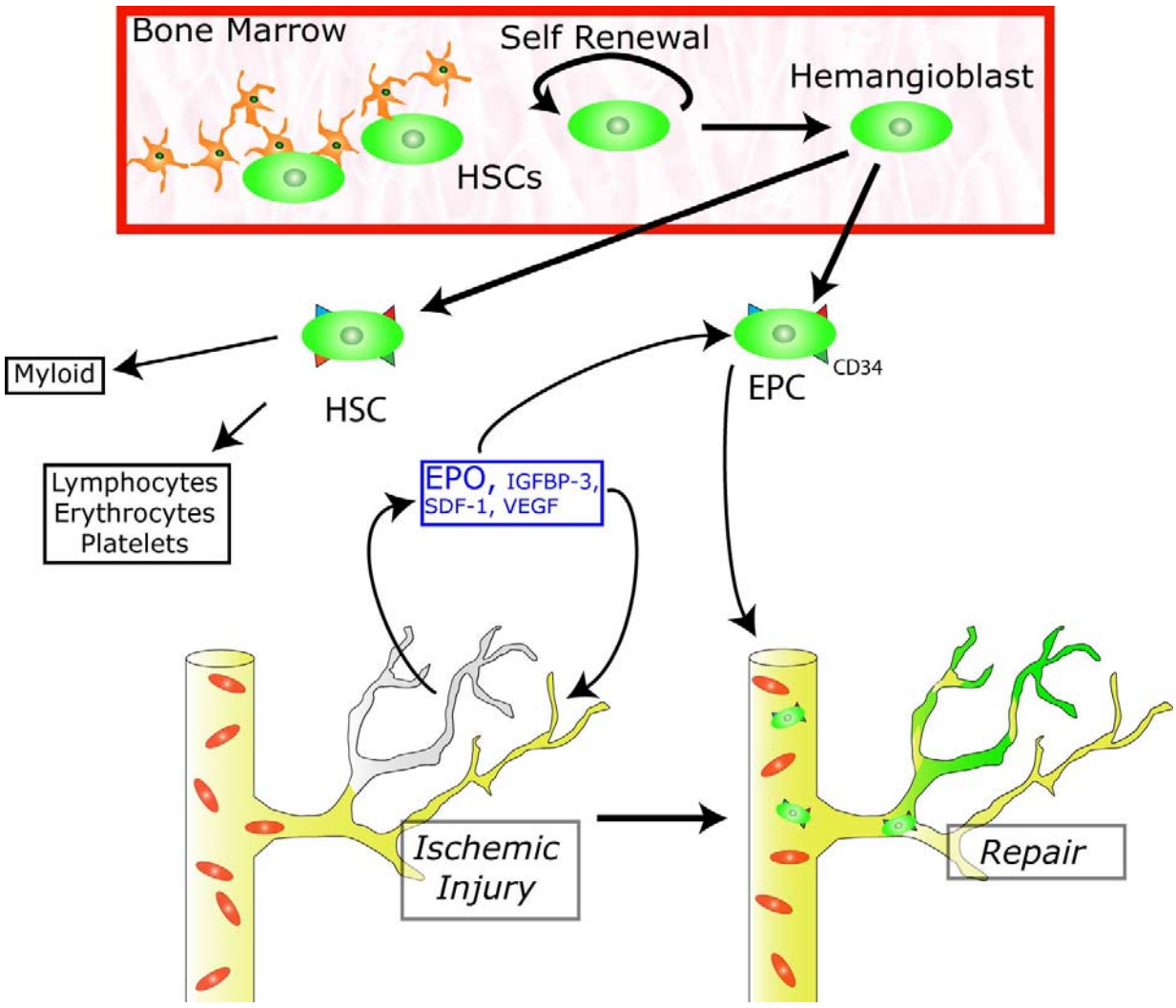


Figure 1-3. Hypoxia-regulated factors and BM-derived cells. HSC and EPC are originated from common precursor, hemangioblast. These cells maintain primitive characteristics so that they can differentiate themselves into a wide range of cell types. EPC has been primary material for this study and it is well characterized as CD34+cells. EPC mobilization is a complex process involving many mediators and cellular interactions. Reduced oxygen tension has been widely believed to trigger this process. Once tissue got an injury, the damaged tissue releases various cytokines and angiogenic factors. Respond to Hypoxia-regulated factors such as EPO, SDF-1, VEGF, and IGFBP-3, circulating EPCs migrate to ischemic area then re-endothelized damaged vessel

rapidly to hypoxia, HIF-1α is continuously synthesized and degraded under non-hypoxic conditions. Under hypoxic conditions, however, the degradation of HIF-1α is inhibited, so that the expression is increased exponentially as O_2 concentration declines, resulting in dimerization with HIF-1β.[73,74] Dimerized complex of HIF-1 binds to hypoxia response element (HRE) within a target gene, and recruits coactivator proteins, which lead to increased transcription of the target gene.[75]

More than 40 genes are known to be directly activated by HIF-1 at the transcriptional level.[76] Genetic studies revealed the presence of a functionally essential HIF-1 binding site in the target gene. The genes induced by HIF-1 regulate molecular mechanism for sensing and responding to changes in O_2 concentration. HIF-1 regulated genes contain a *cis*-acting transcriptional regulatory element, HRE, as a HIF-1 binding site. Recently, DNA microarray analysis showed that over 2% of all human genes are either directly or indirectly regulated by HIF-1 in endothelial cells.[76,77]

Physiological stimuli other than hypoxia can also induce HIF-1 activation and the transcription of hypoxia-inducible genes under non-hypoxia conditions. IGF-1 induces HIF-1α synthesis through phosphatidylinositol 3- kinase and MAP kinase pathways[78], and IGF-1 receptor tyrosine kinase induces HIF-1α protein synthesis, independent from oxygen concentration.[75] HIF-1 has been shown to activate transcription of the gene encoding VEGF.[79] HIF-1 induces VEGF secretion which subsequently induces upregulation of SDF-1 expression. In turn, SDF-1 has a reciprocal effect on inducing VEGF. In addition to growth factors, nitric oxide is also known to play a similar role enhancing HIF-1 activation under non-hypoxic conditions mediating prolyl hydorxylase activities.[80-82]

Vascular Endothelial Growth Factor

VEGF was discovered in 1983 and called vascular permeability factor (VPF) due to its blood vessel permeability increasing capacity.[83,84] In 1989, it was determined that VPF and the endothelial specific mitogen, VEGF was the same protein.[85-87] VEGF plays an important role both in normal physiological angiogenesis and in most of the pathological angiogenesis associated with diseases such as diabetic retinopathy, rheumatoid arthritis, and solid tumors.

The VEGF family consists of seven structurally related homodimeric glycoproteins: VEGF-A, placenta growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D, orf virus-encoded VEGF-like proteins (called as VEGF-E), and a series of snake venoms (collectively called as VEGF-F).[88-91] Despite structural similarity, the VEGF homologs play distinct roles and bind to specific subtypes of VEGF receptors. VEGF exerts its effects by binding to one of its three receptors that belong to the superfamily of receptor tyrosine kinsase. VEGF-A binds to either VEGF receptor 1 (VEGFR-1 / Flt-1) or VEGF receptor 2 (VEGFR-2 / KDR / Flk-1). However, PlGF and VEGF-B exclusively bind to VEGFR-1. VEGF-C and VEGF-D are specific ligands for VEGFR-2 and VEGFR-3 (Flt-4), regulating both blood and lymphatic vessel development. Viral VEGF-Es and some snake venom VEGF-F variants exclusively activate VEGFR-2.[84,88]

Within the VEGF family, most of the research has focused on VEGF-A (usually referred as VEGF). VEGF-A is regarded as the most potent mediator of angiogenesis. There are several splice variants of VEGF-A including VEGF-121, -145, -165, -189, and -206, while VEGF-165 is the predominant form.[67]

VEGF mobilization appears to be isoform specific. For instance, VEGF-165, rather than VEGF-189, induces a rapid mobilization of VEGFR-2^+ cells into circulation.[92] VEGF initiates embryonic vasculogenesis and triggers angiogenic sprouting by activating VEGF-R2 on vascular endothelial cells.[93,94]. VEGFR-2 is also essential for the development of HSCs during early

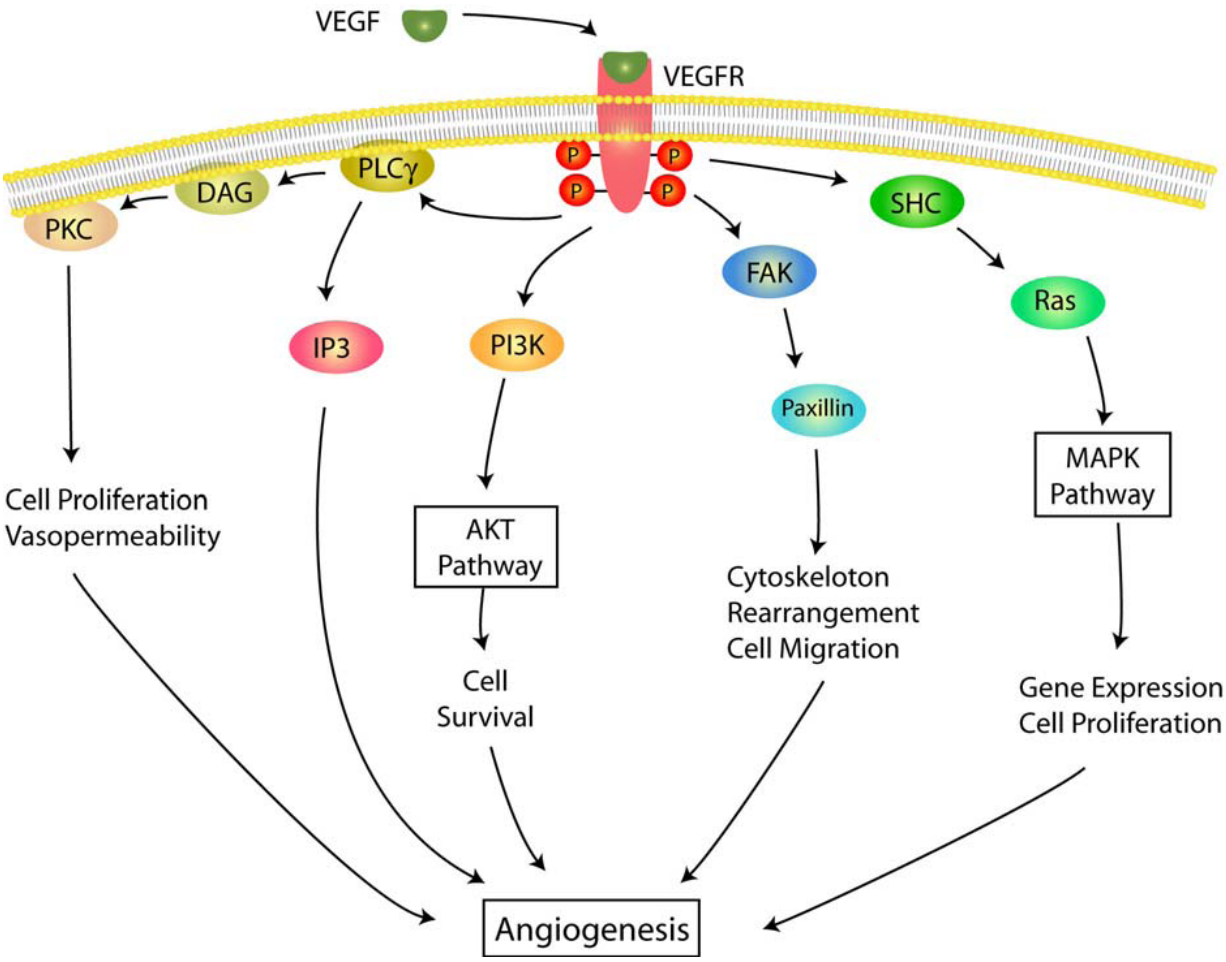


Figure 1-4. The VEGF (VEGF-R2) signaling pathway. Upon binding of VEGF, VEGF-R2 is activated by autophosphorylation, and initiates several signaling cascades all of which lead to angiogenesis.

embryonic development.[95] In addition, the activation of VEGFR-1 is sufficient to rescue HSC survival *in vitro* and hematopoietic repopulation *in vivo.*[96]

VEGF regulates several endothelial cell functions, including proliferation, differentiation, permeability, vascular tone, and the production of vasoactive molecules.[95] VEGF is also a chemoattractant and plays a role in EPC recruitment and induces *in vitro* differentiation of EPCs into mature endothelial cells.[97] Genetic modifications of VEGF have helped to understand its biology related to EPCs. Overexpression of VEGF in nonischemic mouse hearts can lead to the formation of endothelial cell-derived intramural vascular tumors.[98] In addition, VEGF gene transfer promotes EPC migration into ischemic regions. VEGF-deficient HSCs and bone marrow mononuclear cells show lack of ability to repopulate lethally irradiated hosts.[96]

Stromal Derived Factor-1

Stromal cell derived factor-1 (SDF-1) belongs to the group of chemokine CXC subfamily, originally isolated from murine bone marrow stromal cells.[99] It is produced by multiple bone marrow stromal cell types as well as epithelial cells in many organs.[100]

CXCR4, a 7-transmembrane spanning G protein-coupled receptor, is the only known receptor for SDF-1 and is also a coreceptor for human immunodeficiency virus (HIV) type 1.[101] SDF-1 is chemotactic for EPC. The chemokine SDF-1 or CXCL12 mediates homing of stem cells to bone marrow by binding to its receptor CXCR4 on circulating cells.[102]

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.[61] Overexpression of CXCR4 on stem and progenitor cells promotes its proliferation, migration, and *in vivo* engraftment of NOD/SCID mice.[103] SDF-1 gene expression is regulated by the transcription factor, HIF-1. Progenitor cell mobilization is triggered by hypoxia gradients through HIF-1

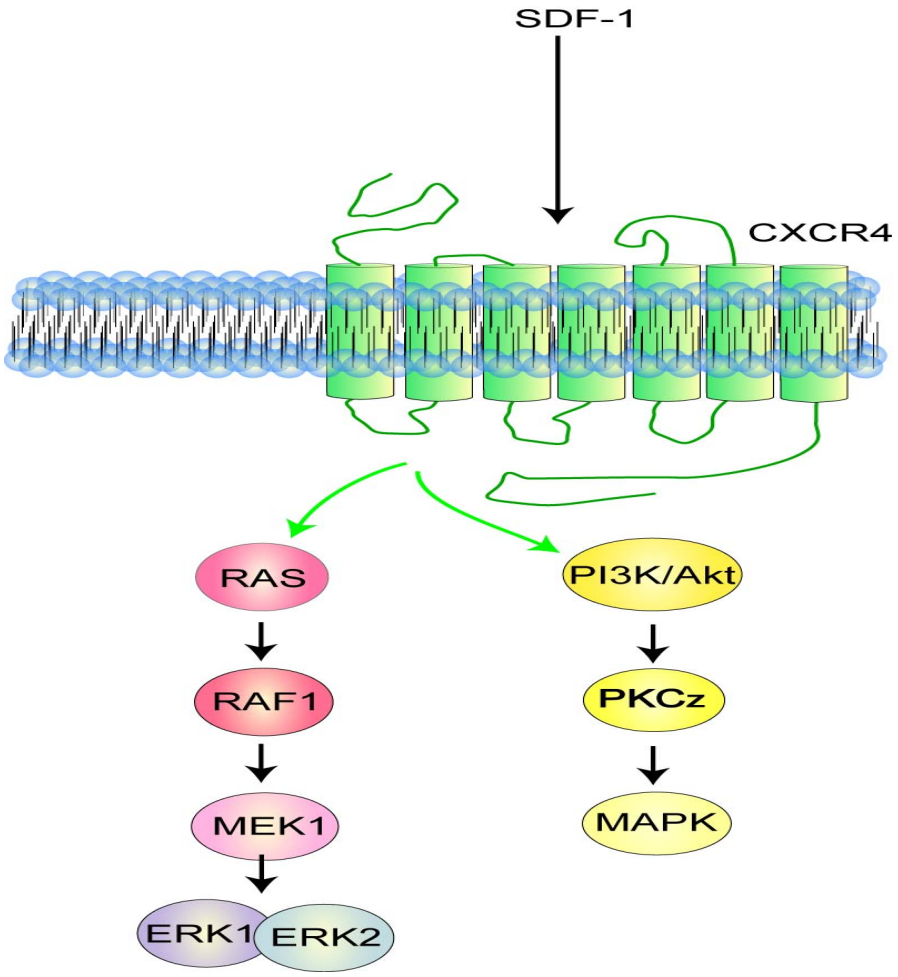


Figure 1-5. The interaction between SDF-1 and CXCR4. SDF-1 binding to its receptor, G-protein coupled CXCR4 leads to activation of ERK1/2 activation or MAPK activation. This interaction results in cell migration or proliferation.

induction of SDF-1. HIF-1 induced secretion of SDF-1 in ischemic tissue has a direct correlation with reduced oxygen tension.[70]

SDF-1 gene transfer induces EPC mobilization from bone marrow into peripheral blood and also improved perfusion to ischemic limbs. It is proposed that SDF-1 induces upregulation of metalloproteinase-9 (MMP-9) activity, which causes cleavage of membrane bound Kit-ligand into soluble Kit-ligand, stem cell factor (SCF). As a consequence, SCF promotes stem cell mobilization into the circulation. Recently, it was shown that SDF-1 is critical for the development of proliferative retinopathy.[104] Vitreous concentrations of SDF-1 and VEGF are increased in diabetic patients. Compared to VEGF, exogenous SDF-1 has a greater effect in causing retinal neovascularization in an animal model.[104] Intravitreal injection of blocking antibodies to SDF-1 disrupts retinal and choroidal neovascularization in mouse.[47,103] Blockage of SDF-1 is now being considered for potential treatment for ocular vascular diseases.

Insulin-like Growth Factor Binding Protein-3

Insulin-like growth factor-I (IGF-I) and II (IGF-II) modulate a diverse range of biological activities including growth, differentiation, survival, and regulation of cell metabolism.[105] In serum and the extracellular fluid, the majority of circulating IGFs are sequestered into 150 kDa ternary complexes with IGF binding protein (IGFBP) and the liver-derived glycoprotein (acid-labile subunit: ALS).[106] This complex prolongs the half-life of IGFs in the circulation and prevents them from crossing the capillary barrier.[107,108] IGFBPs consist of six homologous secreted proteins, which specifically bind to IGF-I with high affinity.

IGFBP-3, the most abundant binding protein in serum, is present in various glycosylated forms between 40 and 44 kDa. A number of investigators have reported that IGFBP-3 has IGF-1 independent cellular actions.[109,110] For instance, independent of IGF-I, IGFBP-3 regulates cell activities such as growth, proliferation, and apoptosis in both carcinoma cell lines and normal

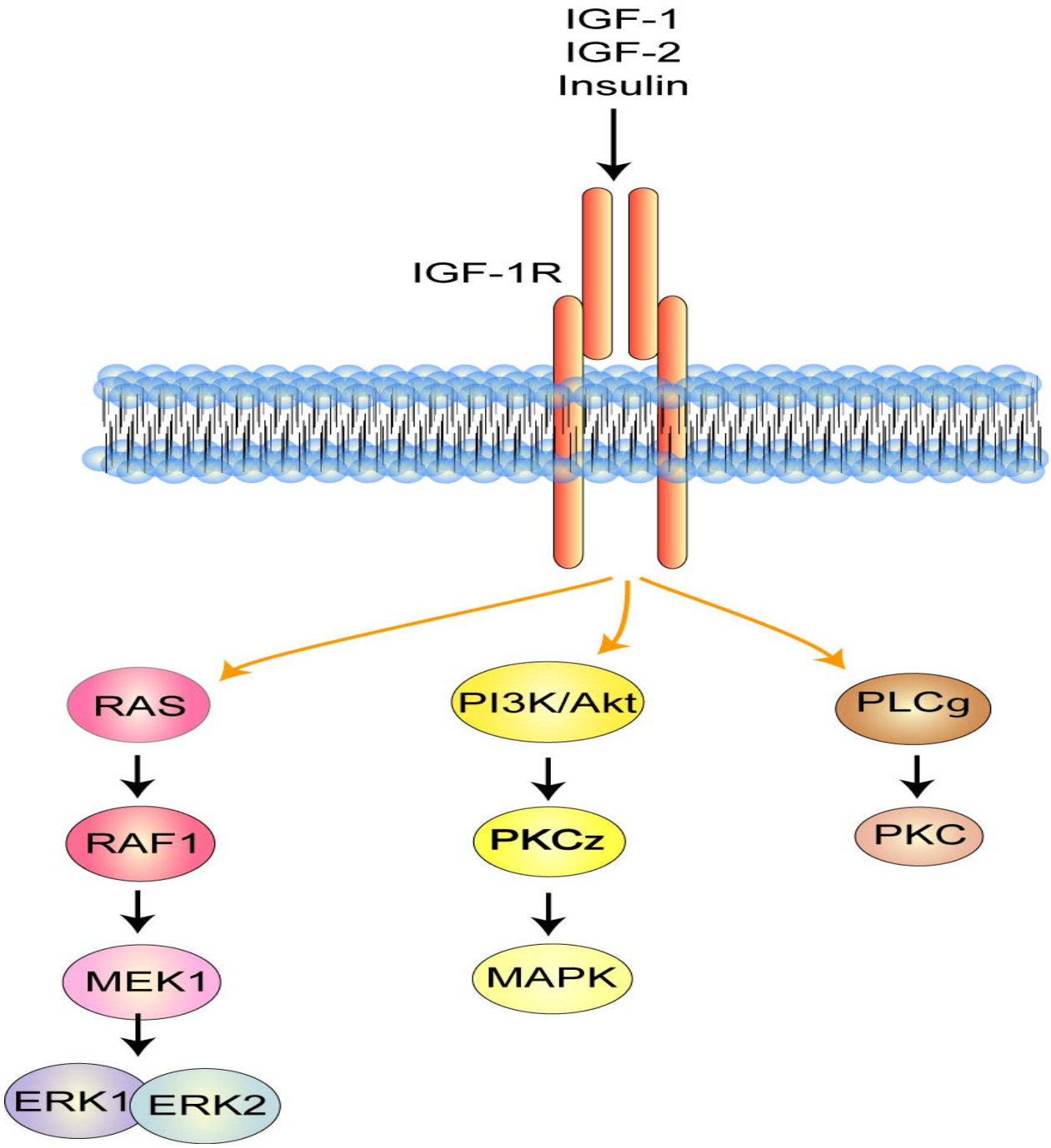


Figure 1-6. IGF-1R signaling pathway. IGF-1R is a tetramer consisting of 2 extracellular α-chains and 2 intracellular β-chains with the intracellular tyrosine kinase domain. The activation of IGF-1R signaling pathways induces numerous physiologic actions of IGF-1.

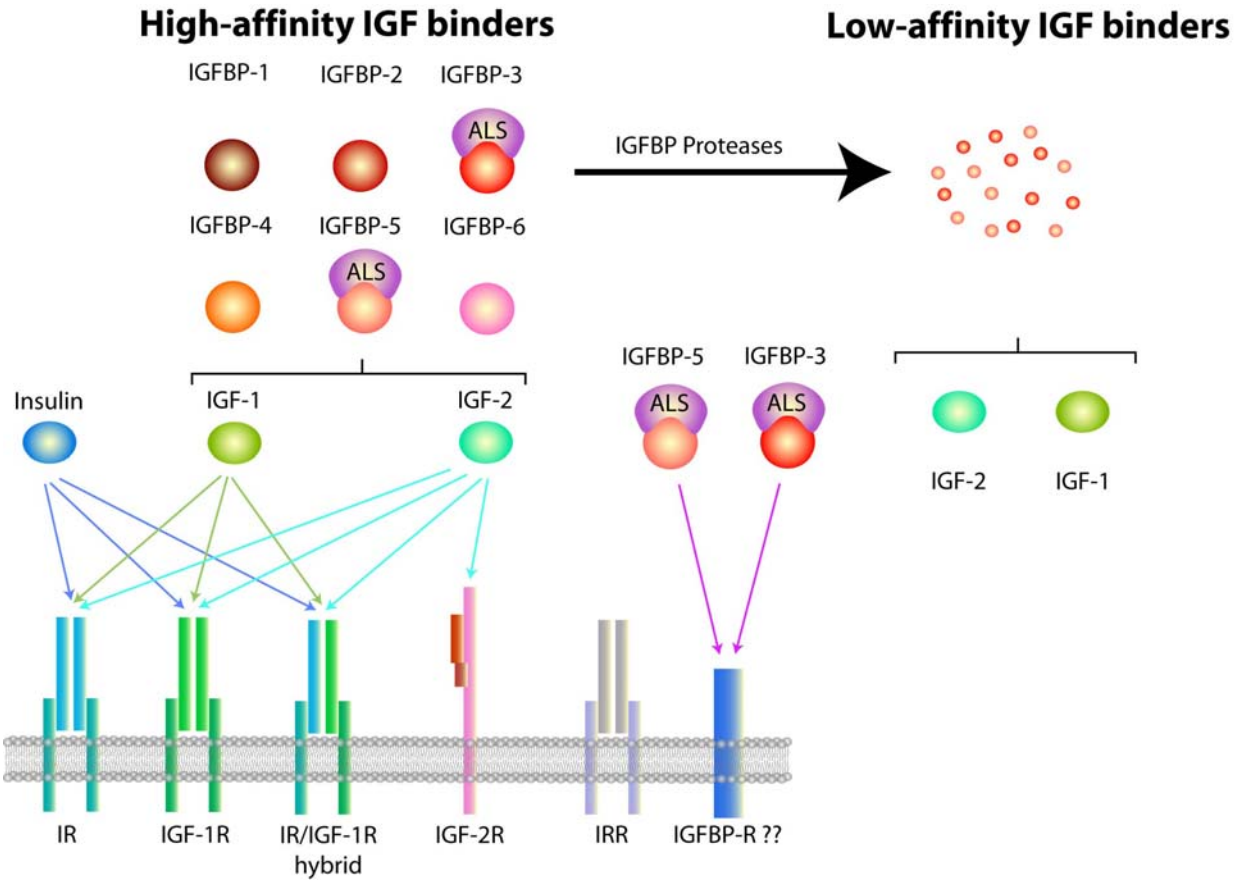


Figure 1-7. Schematic diagram of IGF system. Ligands (IGF-1, IGF-2 and insulin), IGFBPs (1 to 6) and receptors (IR, IGF-1R, hybrid IR/IGF-1R, IGF-2R, IRR and IGFBP-R) are represented. IGF-I interacts with IGF-1R, IR, hybrid IR/IGF-1R and IGFBPs; IGF-2 interacts with IR (mainly with the IR from lacking the exon 11 sequence), IGF-1R, hybrid IR/IGF-1R, IGF-2R and IGFBPs; insulin interacts with IR, IGF-IR and hybrid IR/IGF-IR. Some IGFBPs are known to be cleaved by IGFBP proteases releasing IGFBP proteolysed fragments, which have low-affinity for IGFs. IGFBP-related proteins (IGFBP-rPs) which have low affinity for IGFs also exist. IGFBP-3 and IGFBP-5 may act through their own receptor (IGFBP-R).

Cells.[111-119] Whereas an inhibitory role for IGFBP-3 has been widely reported in the field of cancer research, several other researchers have shown contradictory results that IGFBP-3 enhances angiogenic effects.[120,121] IGFBP-3 also induces differentiation of chondrocytes and human skeletal myoblasts.[123]

Liver and kidney are the main sources of IGFBP-3.[124,125] According to the study by Foulstone's group, skeletal muscle may be another source of autocrine tissue for production of IGFBP-3.[123] The level of IGFBP-3 in serum is modulated by not only its rate of synthesis but also post-translational modification and proteolysis. While normal individuals have minimal IGFBP-3 protease activity, IGFBP-3 protease activity is increased among individuals with pregnancy, acute catabolic illness, or diabetes.[116] IGFBP-3 proteases have been identified including plasmin, matrix metallproteases, kallikreins, prostate-specific antigen, and cathepsin D.[126]

IGFBP-3 concentration in serum is also regulated by other factors such as IGF-I, HIF-1, VEGF, NO, and TGF-β. IGF-I affects HIF-1 which upregulates VEGF and IGFBP-3. IGFBP-3 has been identified as one of the hypoxia induced factors.[71,127] Felser and colleagues demonstrated that IGFBP-3 doesn't contain an HRE within its promoter, and that IGFBP-3 gene expression was markedly reduced in HIF-1α-deficient cells under hypoxic conditions.[128] VEGF enhances upregulation of IGFBP-3 both HIF-1 dependent and independent ways.[129] High concentration of NO, induced by iNOS, decreases the levels of IGF-1 and IGFBP-3 by activating IGFBP-3 protelysis in serum.[130] TGF-β modulates IGF-independent IGFBP3 function.[131-133] TGF-β1 increases the secretion of IGFBP-3 in a variety of breast cancer cell lines and renal carcinoma cells.[122,134-136] IGFBP-3 is induced by TGF-β and is critical in mesenchymal cell

growth and podocyte apoptosis.[122,137,138] Although IGFBP-3 has widely been studied for decades, there are still many questions about the functions of IGFBP-3.

Sphingosine 1-phosphate

Sphingosine 1-phosphate (S1P) is a platelet derived sphingolipid that has been broadly implicated in angiogenesis, platelet activation, inhibition of apoptosis, cytoskeletal organization, adherens junction assembly, and morphogenesis.[139-141] Sphingosine kinase (SK) catalyzes the formation of S1P by phosphorylation of sphingosine. Basal levels of S1P in mammalian cells are generally low, but can increase rapidly or transiently when cells are exposed to mitogenic agents or other stimuli. These signals activate SK which is responsible for increased level of S1P. SK is an evolutionarily conserved lipid kinase which consists of five conserved domains. There are two isoforms of SK: the sphinosine kinase type 1 (SK1) and the sphinosine kinase type 2 (SK2). SK1 is mainly expressed in the cytosol, whereas SK2 is localized in the nucleus.[142] SK1 and SK2 have different functions. Overexpression of SK1 protects against apoptosis resulting in enhanced fibroblasts proliferation, tumor formation in NOD/SCID mice.[143] In contrast to pro-survival SK1, SK2 contains a functional putative BH3-only domain that induces the inhibition of cell growth. Maceyka, *et al*. showed that SK2 has catalytic activity to induce apoptosis.[142,144]

It is accepted that S1P is the ligand for plasma membrane localized G protein-coupled receptors (GPCR) referred to as endothelial differentiation gene (EDG) receptors or S1PRs. S1P binds to the five members of this receptor family: $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$ (previously referred to as EDG-1, 5, 3, 6, and -8). These receptors are highly specific and only bind S1P. The diverse biological processes that are triggered by S1P depend on the pattern of expression of S1P receptors in each cell type as well as coupled G proteins.[139] The study of $S1P_1$ null mice emphasized the importance of $S1P_1$ on endothelial cell-pericyte communication in vascular

maturation and angiogenesis.[145] For instance, Liu et al. demonstrated that $S1P_1$ null mice are embryonic lethal due to massive hemorrhage that is caused by incomplete vascular maturation in arteries and capillaries.[145,146]

S1P binds to the $S1P_1$ receptor which induces the activation of eNOS localized in caveolae which poses a sphingolipid enriched domain in the plasma membrane.[147,148] $S1P/S1P_1$ pathway acutely increases eNOS phosphorylation through PI3K and Akt activities in bovine aortic endothelial cells.[149]

In addition, S1P has been identified as a potent signal-transducing molecule that may exert diverse biological responses such as cellular differentiation, hypertrophy, proliferation and migration.[150-153]

S1P activates the small GTPases Rac and Rho, functioning as a chemoattractant for endothelial cells. S1P induced Rho-dependent integrin clustering into focal contact sites that modulate cell adhesion, spreading, and migration.[150] To activate cell migration, S1P enhances phosphorylation of protein kinase Akt in endothelial cells. S1P has been shown to have dual effects on migration of early lymphocytes. In low levels, S1P induced chemoattractant migration of CD4 and CD8 T cells and also enhanced chemotaxis to CCL-21 and CCL-5. However, at higher levels, S1P had the opposite effect, reducing the migratory responses.[154,155]

S1P acts extracellularly by binding to members of the S1P receptors and regulating cell movement. In addition, S1P also acts as a second messenger intracellularly to regulate calcium homeostasis and apoptosis.[88,141,156-158] However, the mechanism of S1P transport is unknown. Recent studies in yeast showed that a member of ABC family of protein might be involved in S1P translocation.[159,160]

Nitric Oxide

Nitric Oxide (NO) is a highly reactive, diffusible, and unstable radical and is involved in signaling in the cardiovascular, gastrointestinal, genitourinary, respiratory, and nervous systems. For instance, NO regulates cellular immunity, angiogenesis, neurotransmission, and platelet aggregation and also promotes synaptic transmission and cytostatic/cytotoxic actions in macrophages.[64,82,161,162]

NO is generated by NO synthases (NOS). NOS is a heme-containing enzyme that is linked to NADPH-derived electorn transport. NOS catalyzes the oxidation of L-arginine to L-citrulline and NO, with tetrahydrobioterin and NADPH as essential cofactors.[163]

Three NOS isoforms have been identified and named after the cell type or conditions in which they were first described: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible or inflammatory NOS (iNOS). Because free NO is a transient species with a half-life of about 5 seconds, many investigations of this gaseous molecule have largely relied on studies of NOS.

All three isoforms of NOS are found in different cell types in the eye.[164] nNOS is responsible for producing NO in photoreceptors and bipolar cells, whereas eNOS is present in vascular endothelial cells. iNOS, which is found in Muller cells and in retinal pigment epithelium, is involved in inflammatory process and phagocytosis of the photoreceptor outer segment. iNOS is also thought to be responsible for the pathogenesis of diabetic retinopathy.[161,164-167]

NO generation is important to maintain the vasculature in a relaxed state, inhibit the adhesion of platelets and white cells, and suppress the replication of smooth muscle cells.[168] eNOS derived NO diffuses into smooth muscle cells or pericytes then binds to the iron within the heme-group of guanylyl cyclase and produces a conformational change that leads to enzyme activation. NO has been observed to modulating vasculogenesis. NO has an important function

for the stem cell microenvironment in the bone marrow as a molecular mediator in controlling the stem cell niche. eNOS is also important as it promotes angiogenesis and regulates the expression of VEGF. eNOS deficient mice have an impaired capacity to mobilize cells from the bone marrow.[169] Guthrie, *et al*. found that NO/NOS pathway is a significant regulator of neovascularization and can modulate hemangioblast activity by dictating the size and branching characteristics of blood vessels that are formed in response to ischemic or chronic injury.[170]

Disordered NO generation has 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.[171,172] In patients with diabetes mellitus reduced NO bioavailability may result from altered NO metabolism. 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 (BH4), resulting in a specific loss of endothelial NO bioavailability.[173] The subsequent 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[174]. NO and its signaling mechanism modulate various physiological processes, however, relatively short half-life makes the study less feasible in this field.

Relatively stable NO donors with potential therapeutic value have been developed. Synthetic chemical reagents that release NO continuously over a period of time under physiological conditions have long been used in clinical management of cardiovascular diseases. NO donors developed with therapeutic value must be able to control the amount of NO released, produce byproducts with minimal side effects, and the release of NO should not be affected by common biochemical factors. NO decomposes rapidly into nitrite (NO_2) and nitrate (NO_3) in

biological solutions. These two stable compounds are indicators of NO activity *in vivo* and can be used as an alternative way to analyze NO concentration in serum.[175]

Carbon Monoxide

Endogenous CO is a signaling molecule that regulates physiological vascular functions. CO is generated from a family of heme oxygenases (HO), consisting of three isoforms. HO-1 is an inducible stress enzyme, while HO-2 and HO-3 are constitutively expressed proteins. HO-1 is inducible after the stimulation of cytokines, hypoxia and NO.[176] HO-1 (32 kDa) was first purified from the livers of $CoCl_2$ or heme-induced rats and from porcine spleen.[177-180] HO-1 catalyzes the rate-limiting step in the oxidative degradation of heme to generate CO, bilirubin (an antioxidant derived from biliverdin) and iron (sequestered by ferritin).[181-184]

Recent researchers have revealed that CO has profound effects on intracellular signaling processes such as anti-inflammatory, antiproliferative, antiapoptotic, and anticoagulative effects. Using a HO-1 knockout mice, Bak, *et al.* showed that CO generated from HO-1 has a protective role in cardiovascular system from ischemia/reperfusion induced damage.[176]

The physiological signaling effects of CO involve relatively few defined mechanisms. The modulations of PKG, PKA, and subsequent stimulation of cGMP or cAMP production are commonly observed in CO related signaling pathways. There are many similarities between CO and NO. Both gases are endogenously produced. Their synthetic enzymes, HO and NOS, are both oxidative enzymes that use NADPH as an electron donor. CO and NO have similar physiological functions (i.e., vasodilation, inhibition of platelet aggregation, and neurotransmission), and can act as second messenger.[185,186]

Similar to NO, CO binds directly to the heme iron of soluble guanylyl cyclase (sGC), leading to the stimulation of enzymatic activity. The vasoactive properties of CO rely on the stimulation of sGC and the subsequent elevation of cGMP levels.[187] CO-mediated activation of

sGC leads to the increase in cGMP production, with a potency of enzyme activation 30–100 times lower than that of NO.[187-189] Other signaling mechanisms of CO include the modulation of MAPK activation and the stimulation of Ca^{2+}-dependent K^+ channel activity.

HO-1 activity is correlated with angiogenic factors. VEGF activates HO-1 expression in endothelial cells.[190,191] In normal tissue, depending on the amount of NO, HIF-1 modulates HO-1 activity.[192] Deshane, *et al.* demonstrated that SDF-1 directly regulates HO-1 activity which promotes angiogenesis in different cell types including human and mouse aortic endothelial cells as well as mouse EPCs.[193]

A novel class of compounds, termed carbon monoxide-releasing molecules (CORMs), are stable carbonyl transition metal complex with the capacity of releasing CO in biological systems, and are becoming a useful research tool to explore the mechanism of which CO exerts its pharmacological activities.[194,195] Several experiments of CORMs have provided mechanistic insights in the behavior of CO in biological systems. CORM-1 (dimanganese decacarbonyl), CORM-2 (tricarbonyldichloro ruthenium(II) dimmer), and CORM-3 ((tricarbonylchloro(glycinato)ruthenium(II)) simulate the bioactivities of gaseous CO including vessel relaxation[196,197], protection against ischemia–reperfusion injury [194,198], and prevention of organ rejection following transplantation and inhibition of the inflammatory response.

Vasodilator Stimulated Phosphoprotein

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 includes the *Drosophila melanogaster* protein Enabled (Ena), its mammalian ortholog Mena, and the Ena-Vasp-like protein Evl.[199] 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 (EVH2) domain.[200] The structure of EVH1 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 the localization of Ena/VASP family to focal adhesions as well as to the periphery of protruding lamellipodia.[199,201,202] The central proline-rich region has binding sites for several SH3 and WW 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.[199,200,203]

VASP is a cytoskeletal actin filament promoting protein, which is involved in platelet activation, cell adhesion, and migration.[204,205] VASP mutant mice exhibit defects in the actin-dependent process of platelet aggregation.[206] The results from genetic approaches such as loss-of- function experiment, site directed mutation, or overexpression study are suggesting that the importance of Ena/VASP proteins in the developmental and physiological processes in various cell types. For instance, VASP modulates T cell activation, phagocytosis, and epithelial morphogenesis. It also induces migration of neutrophils, fibroblasts, and neurons.[207-212]

In mammalian cells, VASP is localized to focal adhesions and areas of dynamic membrane activity in actin-filament assembly. In endothelial cells, for instance, VASP functions in membrane ruffling, aggregation, and tethering of actin filaments during the formation of endothelial cell-substrate and cell-cell contacts. VASP expression is increased in endothelial cells during angiogenesis.[213]

Hypoxia has a direct influence on barrier function by decreasing VASP expression. Rosenberger, *et al.* showed that VASP transcription was reduced in a HIF-1-dependent manner (HIF-1α functioning as a transcriptional repressor). They further demonstrated hypoxia-dependent binding of HIF-1 to the human VASP promoter by functional studies using chromatin immunoprecipitation and site-directed mutagenesis. VASP expression during hypoxia is involved in tissue permeability.[214]

Vertebrate VASP was discovered and characterized as a common substrate for both PKA and PKG serine/threonine kinases.[215] Elevated guanosine 3', 5'-cyclic monophosphate (cGMP) or adenosine 3', 5'-cyclic monophosphate (cAMP) stimulates the phosphorylation of VASP. To date, three phosphorylation sites (Ser157, Ser239, and Thr278) have been identified. Phosphorylation of Ser 157 of VASP leads to a shift in apparent molecular mass in SDS-PAGE from 46 to 50 kDa, indicating phosphorylation causing a change in secondary structure of the molecule.[216] Phosphorylation of serine 239 in VASP is a useful marker for monitoring PKG activation as well as signaling pathway. Unlike phosphorylation of serine 157, it doesn't alter the electrophoretic motility of VASP.[217,218] During the subsequent cell moving, VASP becomes heavily phosphorylated.[219] Phosphorylated VASP has been localized to cell-cell junctions and could be co-immunoprecipitated with the tight-junction marker zoula-occludens-1 (ZO-1) protein from endothelial cells.[215] There is a need for more research to identify whether such phosphorylation reflects the overall phosphorylation of all VASP within the cell. The question of whether VASP at the leading edge or VASP at focal adhesions are differently phosphorylated also remains to be answered.[205]

Significance (Specific Aims)

The role of HSC and EPC in supporting postnatal vasculogenesis has been extensively studied regarding many physiological and pathological situations. Blood vessel development is a complex process, involving multiple proteins expressed by different cell types, all contributing to an integrated sequence of events.

The goal of this study was to highlight IGFBP-3 among other angiogenic factors involved in vessel development. IGFBP-3 regulates cell activity in various ways and exerts both pro-angiogenic and anti-angiogenic actions. Characterization of its putative receptor which initiates downstream cascade is thought to provide better description of IGF-independent effects. Numerous investigators have tried to determine the characteristics of IGFBP receptors; however, specific IGFBP receptors still remain unknown.

Here, we demonstrate that IGFBP-3 has a critical function in vessel development related with NO and SK/S1P signaling pathways.

The underlying hypotheses are; (1) IGFBP-3 has an angiogenic effect on HSCs as well as EPCs. (2) IGFBP-3 modulates EPC migration to participate in neovascularization by influencing NO generation and VASP redistribution. (3) The down stream pathway of IGFBP-3 on EPCs is related to SK signaling.

CHAPTER 2
METHODS AND MATERIALS

Cell Preparation

Mobilized peripheral blood derived human $CD34^+$ cells and $CD14^+$ cells were commercially purchased (Lonza Walkersville, Inc. Walkersville, MD). A vial of frozen cells were thawed in a 37℃ water bath then washed with hematopoietic progenitor growth medium (HPGM; Lonza Walkersville, Inc. Walkersville, MD) containing 10% fetal bovine serum (FBS) and 20 U/ml of DNase I (Sigma-Aldrich, St. Louis, MO). $CD34^+$ cells were cultured in HPGM supplemented with 25 ng/ml of human stem cell factor (SCF; R&D Systems, Inc. Minneapolis, MN), 50 ng/ml of human thrombopoietin (TPO; R&D Systems, Inc. Minneapolis, MN), and 50 ng/ml of human Flt/Flk2 ligand (FL; R&D Systems, Inc. Minneapolis, MN) for maintaining an undifferentiated state. $CD14^+$ cells were maintained an undifferentiated state in HPGM supplemented with 10% FBS.

To expand $CD34^+$ cells, defined serum free medium (StemSpan SFEM; StemCell Technologies, Inc. Vancouver, Canada) was used for culture. One ml of StemSpan SFEM with the addition of cytokines cocktail (100 ng/ml FL, 100 ng/ml SCF, 20 ng/ml interleukin-3, and 20 ng/ml interleukin-6; StemCell Technologies, Inc. Vancouver, Canada) and 50 ng/ml TPO (R&D Systems, Inc. Minneapolis, MN) enables 300,000 $CD34^+$ cells to proliferate and expand without differentiation. The number of cells was determined with a hemacytometer (Hausser Scientific, Horsham, PA) every 3 days when the medium was changed.

Cryo-preserved human lung derived microvascular endothelial cells (HMVEC-L) were commercially purchased (Lonza Walkersville, Inc. Walkersville, MD). Once the cells were thawed, endothelial cell basal medium-2 (EBM-2) supplemented with growth supplements (5% FBS, 0.04% Hydrocortisone, 0.4% hFGF-B, 0.1% VEGF, 0.1% IGF-1, 0.1% ascorbic acid, 0.1%

EGF, and 0.1% GA-100) (EGM-2-MV singleQuots) (Lonza Walkersville, Inc. Walkersville, MD) was used for optimal growth and proper maintenance. When plated cells were confluent the cells were washed twice with PBS then 0.025% trypsin and 0.01% EDTA mix (Lonza Walkersville, Inc. Walkersville, MD) was added. The plate was placed for 45 seconds at 37℃ in humidified 5% CO_2 incubator. The trypsin was neutralized using twice the volume of trypsin neutralizing solution (Lonza Walkersville, Inc. Walkersville, MD) and then cells were centrifuged at 1000 RPM in an Eppendorf CT 5810R. The pellet was resuspended with EBM-2 containing growth supplements for splitting into new plate.

Endothelial progenitor cells (EPC) were isolated from peripheral blood from healthy individuals or diabetic patients. The blood was collected into cell preparation tubes (CPT; BD Biosciences, San Jose, CA) and spun to obtain mononuclear cells. EPC was separated from the mononuclear fraction using a $CD34^+$ isolation kit (StemCell Technologies, Vancouver, CA). Mononuclear cells (2×10^7) were incubated with a $CD34^+$ selection cocktail for 15 minutes. 50 µl of nanoparticles were then added to the cells and incubated for a further 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 (Endocult; StemCell Technologies, Vancouver, Canada). These isolated EPCs were cultured for tube formation and differentiation assay using Endocult containing 20% Endocult supplement (StemCell Technologies, Vancouver, Canada).

Migration Assay

$CD34^+$ cells and $CD14^+$ cells were stained with calcein-AM (Molecular Probes, Eugene, OR), prior to loading them into the upper wells of a disposable chemotaxis chamber (Neuro

Probe, Gaithersburg, MD). The lower wells were filled with IGFBP3 at 0, 1, 10, and 100 ng/ml dissolved in HPGM (negative control) or HPGM supplemented with 20% FBS (positive control). The chamber was incubated at 37^{o}C, 95% humidity, 5% CO_2 for 4.5 hours. The number of migrating cells was determined by relative fluorescence of the lower chamber using a Synergy^{TM} HT (Bio-Tek Instruments, Inc. Winooski, VT) with an excitation of 485 ± 20 nm and emission of 528 ± 20 nm.

EPC Tube Formation

Peripheral blood was collected into CPT tubes with heparin (BD Biosciences, San Jose, CA) by routine venipuncture. The mononuclear cells were collected after centrifugation at room temperature in a swinging bucket rotor at 1,800g for 20 minutes. These cells were then cultured on fibronectin-coated culture dishes (BD Biosciences, San Jose, CA) with Endocult stem cell liquid media (Stem Cell Technologies, Vancouver, CA) per manufacturer's protocol. IGFBP3 was added to the cultures at day 3 at 0, 1, 10 and 100 ng/ml. Cells were imaged on day 5. Images were captured with a fluorescence microscope (Axiovert 135; Carl Zeiss, Thornwood, NY). The endothelial nature of the cells was confirmed by incorporation of Dil (1, 1'-dioctadycl-3, 3, 3'3'-tetramethyl-indocarbocyanin percholrate)-labeled acetylated-LDL (Molecular Probes, Eugene, OR) at 50µg/ml final concentration.

Cell Proliferation Assay

A high sensitivity cell proliferation kit (ViaLight Plus Kit: Lonza Rockland, Inc. ME) was used according to manufacture's protocol to assay for cell proliferation. Cell proliferation was measured based on the bioluminescent cytoplasmic ATP level. Cultured cells on 96 well plates were removed from the incubator and allowed to cool -to room temperature for about 5 minutes. 50µl of the cell lysis reagent (Lonza Rockland, Inc. ME) was added to each well to extract ATP

from metabolically active cells. The cell lysate (100 µl) was transferred to a white walled luminometer plate (Lonza Rockland, Inc. ME). Then ATP monitoring reagent plus (AMR PLUS: Lonza Rockland, Inc. ME) was added to each well to generate a luminescent signal. The luminometer (BioTek Instruments, Inc. Winooski, VT) was programmed to take one second integrated luminescence reading of each well.

Nitric Oxide Measurement

NO production was measured by the use of 4-amino-5-methylamino-2', 7'-difluorofluorescein diacetate (DAF-FM diacetate: Invitrogen , Carlsbad, CA). The cells were washed with PBS containing calcium, magnesium, and 1 mg/ml glucose to remove phenol red in the culture media. The cells were incubated with 5 µM DAF-FM diacetate for 1 hour on ice in the dark. Cells were washed with PBS containing calcium, magnesium, and 1 mg/ml glucose then transferred to black 96 well plate (Nunc International, Rochester, NY). NO fluorescence was measured using excitation and emission wavelengths of 488 ± 20 nm and 520 ± 20 nm respectively.

Western Blot Analysis

For western blot analysis 8 µg of total protein was loaded on a 10-20% gradient Criterion gel (BioRad, Richmond, CA). The samples were electrophoresed at 120 V for 20 minutes to allow for stacking the samples and then 140 V for 65 minutes to separate proteins. The proteins were transferred from the gel to a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA) using a semi dry membrane apparatus (Bio-Rad Laboratories, Inc., Hercules, CA) at 20 V for 40 minutes. The membrane was blocked in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1 hour at room temperature with gentle shaking. The blocked membrane was incubated with diluted primary antibody at 4^{o}C overnight in a cold room. The membrane was

washed 4 times for 5 minutes in PBS containing 0.1% Tween-20 (Fisher Scientific, Pittsburgh, PA) and then incubated with diluted fluorescently labeled secondary antibody for 1 hour at room temperature with gentle shaking. The membrane was washed 4 times for 5 minutes each with PBS containing 0.1% Tween-20 followed by final washing with PBS to remove excessive Tween-20. The membrane then was scanned in the appropriate channels using Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE). The same membrane was used to detect the internal protein control, cofilin (Sigma-Aldrich, St. Louis, MO).

In-cell Western Assay

HMVEC-L was grown in 96-well plate (Nalge Nunc International, Rochester, NY). When the cells reached 75-80% of confluence, cells were washed twice with PBS. Instead of serum starvation, cells were treated with IGFBP-3 in EBM-2 without cytokines during the treatment.

$CD34^+$ cells were cultured in defined serum free medium (StemSpan SFEM; StemCell Technologies, Inc. Vancouver, Canada) to obtain the optimal number of cells. StemSpan SFEM with the addition of cytokines cocktail (100 ng/ml FL, 100 ng/ml SCF, 20 ng/ml interleukin-3, and 20 ng/ml interleukin-6; StemCell Technologies, Inc. Vancouver, Canada) and 50 ng/ml TPO (R&D Systems, Inc. Minneapolis, MN) enables $CD34^+$ cells to proliferate and expand without differentiation. The number of cells was determined with a hemacytometer (Hausser Scientific, Horsham, PA). The cells were transferred into 96-well plate (10,000 cells per well: BD Falcon, San Jose, CA). After incubation with or without CO or NO donor, cells were fixed with 4% formaldehyde for 20 minutes at room temperature then pelleted by centrifugation. Fixing solution was removed then triton washing solution (PBS containing 0.1% triton X-100) was added to the cells for permeabilization. After permeablization, cells were blocked in LI-COR Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1.5 hour at room temperature with moderate shaking. Blocking buffer was removed by aspiration and the cells were incubated

with 50 µl of diluted primary antibody at 4°C overnight in a cold room. The cells were washed 4 times for 5 minutes in PBS containing 0.1% Tween-20 (Fisher Scientific, Pittsburgh, PA) and then incubated with diluted fluorescently labeled secondary antibody. After 1 hour the cells were washed 4 times for 5 minutes each with PBS containing 0.1% Tween-20 followed by a final washing with PBS to remove excessive Tween-20. The 96-well plate was scanned in the appropriate channels using Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE). Relative quantification was normalized and re-adjusted in cell number from well to well using DNA staining.

Quantitative Transcription Analysis with Real Time Polymerase Chain Reaction (RT PCR)

Total mRNA from human $CD34^+$ cells, human $CD14^+$ cells or retina from the mouse pups was isolated using the Total RNA Mini Kit (Bio-Rad Laboratories, Inc., Hercules, CA). The mRNA was transcribed using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA) and real-time PCR were performed using iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA). Primers for the PCR were designed using vector NTI (Infomax, North Bethesda, MD) and purchased (Intregrated DNA Technologies, Coralville, IA).

Immunohistochemistry

EPCs were cultured on 8-well tissue culture chamber slides coated with human fibronectin (BD Biosciences San Jose, CA) and treated with 100 µM diethylenetriamine/nitric oxide adduct (DETA-NO) (Sigma-Aldrich, St. Louis, MO), 10 µM CO donor (tricarbonyldichlororuthenium (II) dimer) (Sigma-Aldrich, St. Louis, MO), or 100 ng/ml IGFBP-3 (Upstate cell signaling solution, Lake Placid, NY) for 15 minutes or 4 hours. After treatment, medium was removed and fresh ice cold 4% paraformaldehyde (PFA) was added and the samples held overnight at 4°C. Cells were then washed in PBS and permeabilized with 0.1% Triton X-100 (Fisher Scientific, Pittsburgh, PA) for 30 minutes at room temperature. The cells were washed 3 times

with PBS and blocked in 10% normal goat serum (Jackson ImmunoResearch Labs, West Grove, PA) or 1% bovine serum albumin (BSA; Sigma-Aldrich, St.Louis, MO) at room temperature to block non specific antigens. After 1 hour the cells were incubated with 5 μg/ml mouse anti-VASP antibody (BD Biosciences, San Jose, CA) in 5% normal goat serum overnight at 4℃.

Specific secondary antibody, goat anti-mouse IgG1-fluorescein isothiocyanate (FITC; Southern Biotech, Birmingham, AL) was diluted and added to each chamber for 1 hour at room temperature. Then the cells were washed, dried, and mounted with Vectashield ® 4', 6-diamino-2-phenylindole (DAPI) (Vector Laboratories Inc., Burlingame, CA) for DNA labeling. Cells were examined using a fluorescence microscope (Nikon Eclipse TE200) using a Nikon plan-fluor 40 X 1.30 oil objective and a FITC-conjugated standard filter set (520 ± 2 nm). Pictures were captured using a SPOT™ digital camera 0.60X HRD060 - NIK (Diagnostic Instruments, Inc. Sterling Heights, MI) and processed using SPOT™ Advanced software Version 2.2.1 for Windows.

Flow Cytometry Analysis

Protein expression of VEGF receptor, phosphorylated eNOS and CD133 cell surface antigen in $CD34^+$ cells were evaluated using flow cytometry analysis. VEGF receptor expression in $CD34^+$ cells was examined after incubation for 0 or 15 minutes in 5% CO_2 at 37℃ with exposure to 100ng/ml IGFBP3 (Upstate cell signaling solution, Lake Placid, NY). Following treatment, the cells were permeabilized using a Cytofix/Cytoperm Kit (BD Bioscience, San Jose, CA). The cells were blocked with 10% normal human serum (Jackson Immuno Reserch labs, West Grove, PA) in PBS. Ten μg anti-VEGFR-1 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) or 10 μg anti-VEGFR-2 antibody (NeoMarkers, Fremont, CA) was added to the cells and subsequently incubated for 30 minutes on ice. The cells were washed with PBS and incubated

with 23 μg of FITC conjugated goat anti-mouse antibody (Jackson Immuno Research labs, West Grove, PA) in the dark for 30 minutes on ice. Cells were then washed and analyzed by flow cytometry. The isotype control for both VEGFR-1 and VEGFR-2 antibodies was anti-GFP (Molecular probes, Carlsbad, CA) antibody (15 μg). To measure the eNOS protein expression, the cells were incubated with 100 ng/ml of IGFBP-3 for up to 72 hours. IGFBP-3 was added every 24 hours. When the cells were collected for analysis, cells were permeabilized and blocked. Then 5 μg/ml of anti-eNOS antibody (BD Bioscience, San Jose, CA) was added for 30 minutes. As a secondary antibody, 23 μg of FITC conjugated goat anti-mouse lgG (Jackson ImmunoResearch Labs, West Grove, PA) was used. The surface expression of CD133 antigen was assessed using a phycoerythrin (PE) conjugated anti-CD133 antibody (Miltenyi Biotec Inc. Auburn, CA). $CD34^+$ cells were incubated with or without IGFBP-3 for up to 72 hours as above. Isotype control for this experiment was PE-conjugated mouse $IgG_{a,\kappa}$ immunoglobulin isotype control monoclonal antibody (BD Bioscience, San Diego, CA). Apoptotic dead cells were removed before analysis by 7-aminoactinomycin D (Sigma-Aldrich, St. Louis, MO) positive selection. Data were acquired with FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) and were analyzed with BD Cell Quest™ (BD Biosciences, San Jose, CA).

Hematopoietic Stem Cell (HSC) Transfection

Mouse HSCs were obtained from bone marrow isolated from homozygous transgenic gfp mice. Highly enriched gfp^+, $Sca1^+$ (stem cell antigen 1) and $c\text{-}kit^+$ HSCs were obtained by fluorescence-activated cell sorting (FACS). Cells were transfected with a recombinant adeno-associated virus (rAAV) vector encoding IGFBP-3. The rAAV vector was chosen for long term IGFBP-3 overexpression in the eye. Expression of IGFBP-3 was selectively increased in proliferating endothelial cell by a specific promoter composed of 7 x 46-mer multimerized

endothelin enhancer (ET) upstream of a human Cdc6 (cell division cycle 6) promoter. Gfp^+ HSCs were transfected using polyethylenimine (PEI) /plasmid complexes. 4.2 mg/ml stock PEI (Sigma-Aldrich, St. Louis, MO) solution was made in acidified distilled water (pH 5.0). The PEI /plasmid complexes were prepared by adding branched PEI to the plasmid DNA. 100 µl PEI /plasmid complex were composed of 150 mM sodium chloride (NaCl) 1 µg DNA, and 24 µl PEI stock solution. The complexes were then mixed by vortex mixer set on high for 10 seconds and incubated for 30 minutes at room temperature then added to the cells.

Experimental Animals

All animal procedures conducted in this study were in agreement with the National Institutes 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 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 Resources facilities.

Oxygen Induced Retinopathy (OIR) Mouse Model

The OIR mouse model was used to induce the formation of preretinal neovascularization in mouse pups. On postnatal day 7 (P7), the pups and the nursing dam were placed into an oxygen 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 75% O_2 simulates a hypoxic stimulus that initiates the formation of the preretinal vasculature. The mice were sacrificed at P12.5 and P17 and their eyes removed for analysis.

One cohort of animals (n=12) underwent the OIR model and were euthanized at P12.5 to examine the expression of VEGF-A and IGFBP-3 in combined posterior poles and neural retinas.

A second cohort of mice was intravitreally injected on P1 with either the plasmid expressing mouse IGFBP-3 under the control of the proliferating endothelial cell-specific promoter or the cloning vector as an injection control. For intravitreal injection into P1 mouse pups, ice-induced anesthesia was performed by placing the neonate on a plastic shield over a layer of crushed ice for 1 to 2 minutes. By utilizing a proliferating endothelial cell-specific promoter IGFBP-3 expression was targeted to areas of neovascularization. Selected animals were euthanized immediately upon removal from hyperoxia at P12.5. Eyes from mice injected with IGFBP-3 plasmid (n=9) were compared with eyes from mice injected with empty plasmid vector (n=9), or the uninjected eye of the same animal (contralateral eye). A third cohort of mice (n=18) was injected on P1 with mouse gfp^+ HSCs (5×10^3 cells per eye in 1 μl injection volume) transfected with the identical plasmid as described above. The mice were subjected to the OIR model and euthanized at P17. Data from these mice were compared with the uninjected eye of the same animal or with mice injected with mouse gfp^+ HSCs transfected with empty plasmid (n=18).

Retinal Flat Mounts

The mouse thoracic cavity was opened, the right atrium was punctured with a 27-gauge needle and the left ventricle was cannulated with a 22-gauge angiocatheter, and the mouse was perfused with rhodamine-labeled dextran for preparation of retinal flat mounts. One whole eye from the animal was removed and fixed in 10% PFA overnight. The retina was washed overnight in running water and then incubated for 3 hours at 37°C in 0.1M Tris buffer (pH 7.8) supplemented with DIFCO BACTO 1:250 Trypsin (Invitrogen, Carlsbad, CA). The incubation was terminated after the internal limiting membrane had been removed. All nonvascular cells were brushed away from the vasculature. The retina was then mounted onto a glass slide.

GS Isolectin and GFP Double Labeled Immunohistochemistry

Retinal whole-mounts were prepared as described above. *GS* isolectin B4 (Sigma-Aldrich, St. Louis, MO) and chicken anti-GFP or mouse anti-GFP/EGFP (Chemicon, Temecula, CA) were used to co-visualize gfp^+ cells with the vasculature. Retinal whole mounts (n=6) were permeabilized with 0.1% Triton X-100 (Fisher Scientific, Pittsburgh, PA) in PBS. Samples were then blocked in 1% BSA in PBS for 30 minutes and retina was incubated with biotinylated *GS* isolectin B4 (Sigma, St Louis, MO) overnight. The retinas were washed twice for 10 minutes in PBS supplemented with 0.1% Triton X-100 followed by a last 10 minute wash in PBS. The retinas were then incubated with streptavidin Cy-3 (1:100) for 2 hours at room temperature. Retinas were incubated overnight at 4°C with diluted anti-GFP antibody then washed with PBS supplemented with 0.1% Triton X-100. The retinas were allowed to incubate for 4 hours at room temperature with the appropriately diluted secondary antibodies followed by washing. Finally the whole retinas were flat mounted with the ganglion cell layer up in Prolong Anti-Fade (Molecular Probes, Eugene, OR).

Microscopy and Mapping

Retinal whole-mounts (n=6) were examined by both deconvolutional and confocal microscopy. Zeiss microscope (model Axioplan 2 attachment HBO 100; Carl Zeiss, Inc.Germany) and Axiocam HRm camera (Carl Zeiss, Inc, Germany) were used for deconvolutional analysis. Confocal microscopy was conducted with an argon-krypton laser (Leica Microsystems, Wetzlar, Germany) mounted on a Leica DMRBE epifluorescence photomicroscope. Alexa Fluor 488 and Cy3 fluorescence was excited sequentially at 488 and 550 nm respectively. Images were processed with Adobe Photoshop software (Adobe Systems, Inc. San Jose, CA).

Vascular Density Analysis and Statistical Analysis

To validate vascular density, morphological analysis needed to be changed to a quantitative measurement. After retina whole mounts were immunostained, vascularties in retina were then subject to vascular density analysis. The retina was divided into 3 representative fields of views from each of the central, mid-peripheral and peripheral retinas. Fields of view selected as peripheral retinas included regions of capillary sized vessels directly adjacent to radial arterioles. The area of central and mid-peripheral retina included the radial arteriole. Then each field was captured using the 40x objective. A 10 x 10 grid was superimposed onto the micrograph and the incidence of presence or absence of vessels at the intersection points of each grid was determined so that vascular density expressed as a number from 0 to 100. The mean vascular density incidence was determined for each area and compared with its control. The data is presented as means ± standard deviation (SD), and the statistical significance of differences among mean values was determined by one-way ANOVA and the Tukey HSD multiple comparison post hoc tests for the hyperoxia experiments, and a two-tailed t-test utilized for the hypoxic experiment. ANOVA statistical analysis was performed with SPSS 13.0 software (SPSS, Chicago, Illinois), and two-tailed t-test statistical analysis was performed with a P value of <0.05.

CHAPTER 3
RESULTS

IGFBP-3 Induces Migration of $CD34^+$cells and Endothelial Cells

$CD34^+$ cells are known to leave the circulation and migrate along a hypoxic gradient into sites of ischemia and it is believed that $CD34^+$cell trafficking is mainly regulated by hypoxia-regulated factors. To determine whether IGFBP-3 functions as a hypoxia-regulated factor, the effect of IGFBP-3 on migration of different cell types was examined. Migration assays for circulating cells were performed using modified Boyden chamber assay. Figure 3-1A demonstrates that IGFBP-3 stimulated the migration of circulating $CD34^+$ cells in a concentration-dependent manner, whereas circulating $CD14^+$ monocytes showed a blunt response to increasing concentration of IGFBP-3.

Human retinal endothelial cells (HREC) were also exposed to varying concentrations of IGFBP-3 for 12 hours using the Boyden chamber assay for adhered cells. As depicted in Figure 3-1B, primary cultured HREC migrated toward IGFBP-3, but the response was not as robust as seen with the circulating $CD34^+$population. These results (Figure 3-1A and B) demonstrate that IGFBP-3 has a cell-type specific chemotactic function.

Compared to mature endothelial cells, the response of $CD34^+$cell to IGFBP-3 was exceptionally sensitive even at the lowest concentration (1ng/ml) supporting that mobilization of bone marrow derived cells can be triggered by subtle changes in a hypoxia regulated factor such as IGFBP-3.

IGFBP-3 Increases Expression of VEGF Receptors on $CD34^+$cells

To examine possible was in which IGFBP-3 could modulate $CD34^+$cell response to hypoxia, we examined whether IGFBP-3 could influence VEGF and SDF-1 receptor expression on $CD34^+$cells. VEGF and SDF-1 are well known stem cell homing factors that are also

hypoxia-regulated factors. Exposure of $CD34^+$cells to varying concentrations of IGFBP-3 resulted in increased expression of VEGFR-1 (Figure 3-2A) and VEGFR-2 (Figure 3-2B) on $CD34^+$cells. By contrast, IGFBP-3 did not have an effect on CXCR-4 expression in these cells (Figure 3-2C). Figure 3-2 suggested that IGFBP-3 has a VEGF-dependent, SDF-1-independent function on $CD34^+$cells.

IGFBP-3 Promotes $CD34^+$cells Differentiation to Endothelial Cells

Exposure to IGFBP-3 for 72 hrs resulted in a reduction of CD133 surface expression in $CD34^+$cells (Figure 3-3A), which is associated with promoting differentiation of immature cells to a more committed phenotype. EPC grown on fibronectin showed a dose-dependent tube formation and acetylated LDL incorporation compared to control untreated cells (Figure 3-3B), supporting that IGFBP-3 can influence multiple steps that are relevant to angiogenesis.

IGFBP-3 Enhances $CD34^+$cells Proliferation

To determine whether IGFBP-3 modulates proliferation of $CD34^+$cells *in vitro*, $CD34^+$cells were cultured in the presence of 100 ng/ml IGFBP-3. After 24 hour, 3 days, and 5 days suspension culture, cytoplasmic ATP was detected. IGFBP-3 increased the proliferation of $CD34^+$cells by 36.11% (day 3) and 56.03% (day 5) compare to untreated control (Figure 3-4). Corroborated with the result observed in Figure 3-3B, IGFBP-3 enhances the proliferation of $CD34^+$cells as well as EPC.

Expression of Hypoxia-regulated Factors in Retina

Messenger RNA was extracted from posterior cups including neural retinas of mouse pups to check the expression level of IGFBP-3 in ischemic retina. Neonatal mice were divided into two groups. One group was exposed to high oxygen for 5 days then returned to normal oxygen tension and the other group was subjected to normoxia control. VEGF and IGFBP-3 mRNA levels were determined using reverse transcription on total mRNA followed by real time PCR on

the cDNA products using specific primer pairs for VEGF and IGFBP-3. As shown in Figure 3-5, the mRNA expression of VEGF and IGFBP-3 was significantly increased in hypoxic retina. The fold-change in IGFBP-3 expression was markedly greater that the fold-change in VEGF. It supports the importance of IGFBP-3 response following hypoxia.

IGFBP-3 Protects Neonatal Retinal Vessels from Oxygen Induced Vaso-obliteration

To validate the function of IGFBP-3 *in vivo*, mouse pups were injected with rAAV protein expression vectors expressing IGFBP-3 on postnatal day 1 (P1). The expression of the IGFBP-3 was driven by a proliferating endothelial cell specific promoter (ET/cdc6). The uninjected eyes (contra-lateral) of IGFBP-3 plasmid injected mice were used as one of the control conditions and empty plasmid injected eyes served as the other control. Vessels positive for *Griffonia simplicifolia* isoletin B4 (*GS* isolectin) show the changes in the vasculature following high oxygen exposure (Figure 3-6 A to L). The *GS* isoletin conjugated to HRP provided a low magnification view of the entire retinal vasculatures (Figure 3-6 M and N). The vessels in the IGFBP-3 treated eye had a more normal and mature vascular tree (Figure 3-6 A-D and M), than eyes treated with plasmid control. Vessel growth was shown in the IGFBP-3 injected eyes as evidenced by the presence of lectin positive vascular extensions migrating towards the avascular peripheral retina (arrows in Figure 3-6 D).

In contrast, massive oxygen-induced vaso-obliteration was shown in empty plasmid injected retina (Figure 3-6 E-H). The remaining vascular remnants, shown at higher magnification in Figure 3-6 F-H, lacked effective vascular perfusion and had a highly aberrant branching pattern (Figure 3-6 N). The morphology of vascular remnants in the peripheral retina of the uninjected eyes (the contra-lateral eye of the pups injected with IGFBP-3 containing plasmid) had regions with vascular abnormalities including reduced capillary density (Figure 3-6 I-L) and closure of capillary segments (arrow in Figure 3-6 K). As depicted in Figure 3-6, over-

expressing IGFBP-3 *in vivo* resulted in the maintenance of a vascular bed with a more normal morphology under hyperoxia condition.

Quantitative Analysis of Vascular Density in Vaso-obliteration Phase

To determine vascular density, morphological analysis was performed. Figure 3-7 shows representative retinas from IGFBP-3 expressing plasmid injected eyes (Figure 3-7 A-C), contra-lateral uninjected eyes (D-F), and control plasmid injected eyes (G-I). A 10 x10 grid was superimposed onto the 40 X objectives from each field of retina and the vessels found at intersection points of each grid were determined. The number counted as a vascular density was expressed as a percentage from 0 to 100 (bottom right corner of the image).

Figure 3-7 J summarizes quantitative measurement of vascular density results from central, mid peripheral, and peripheral regions of retinas in each group. As revealed in Figure 3-7 J, IGFBP-3 protected the retinal vasculatures from hyperoxia-induced vessel regression in mid peripheral and peripheral regions of the retina.

IGFBP-3 Decreases the Incidence of Pre-retinal Neovascularization

Abnormal vessel growth in pre-retinal region is a hallmark of proliferative retinopathies. Pre-retinal blood vessels grow outside the retinal inner limiting membrane into the vitreous space under pathological circumstances. Neovascularization in the eye of OIR model was evaluated by the average number of pre-retinal endothelial nuclei per H&E stained retinal section. Figure 3-8 shows reduced aberrant neovascularization by induction of IGFBP-3 in mouse retina.

IGFBP-3 Expression in Transfected HSC

To examine IGFBP-3 expression by the plasmid, HSC were transfected with IGFBP-3 expressing plasmid and total mRNA was isolated. The promoter (ET/cdc6) has been previously characterized both *in vitro* and *in vivo.* [220,221] Low molecular weight PEI was used for effective

transfection of HSC. Transfection efficiency of 40% was typically observed. The fold-change of IGFBP-3 in transfected cells was significantly increased compared to untransfected HSC (Figure 3-9).

Co-localized IGFBP-3 Expressing gfp$^+$HSC within the Vasculature Inhibit Neovascularization

To evaluate the effect of endogenous IGFBP-3 on HSC behavior in vivo, P1 mouse pups underwent intravitreal injection with IGFBP-3 plasmid-transfected HSC. The effect was observed by incorporation of gfp$^+$ HSC into the retinal vasculature (Figure 3-10 A-C). Gfp$^+$HSC localized vascular endothelial cells were evident in radial arterioles (Figure 3-10A) and hemangiomas (B). In addition, filopodial extensions were seen originating from the neovascular clump towards avascular retina (C). Eyes injected with IGFBP-3 transfected HSC showed less pathological neovascularization compared to uninjected eyes or eyes injected with control transfected HSC. Figures 3-10D and 3-10E demonstrate quantitative vascular density from the retinas during hyperoxia and hypoxia phase, respectively. IGFBP-3 overexpressing HSC inhibit abnormal neovascularization in hypoxia phase by protecting neonatal retinal vessels from oxygen induced regression in hyperoxia phase.

Hypoxia-regulated Factors and Nitric Oxide Signaling

To support hypothesis that hypoxia-regulated factors modulate EPC mobilization by increasing nitric oxide and activation of its downstream signaling pathways, we examined the downstream signaling of two well known EPC chemoattractant, VEGF and SDF-1. CD34$^+$cells were treated with either 25 ng/ml VEGF or 100 nM SDF-1. Anti-phoshporylated eNOS antibody was used to demonstrate whether these hypoxia-regulated factors activate eNOS in CD34$^+$cells. VEGF induced eNOS phosphorylation in 15minutes (Figure 3-11A), however SDF-1 showed no significant effect on eNOS phosphorylation in CD34$^+$cells (Figure 3-11B). SDF-1

mediated its effects through generation of CO rather than NO. SDF-1 increased HO-1 expression to generate CO which in turn triggers cell migration.[193]

NO and CO Promotes CD34$^+$cells Migration

Direct stimulation of CD34$^+$cells by NO has been reported.[170,222] To determine the effect of CO on cell motility, migration assay was performed with CO-treated CD34$^+$cells. Exposure to exogenous CO acutely increased cell migration in response to chemotactic stimulus, SDF-1 (Figure 3-12). The NO donor (DETA-NO) was compared to the CO donor ($Ru(II)Cl_2(CO)_3$ dimer) and were used for pretreatment of CD34$^+$cells. Figure 3-12 shows both NO and CO has an effect on CD34$^+$cells migration.

Different Phosphorylation Sites of VASP

VASP function is initiated by phosphorylation. VASP was originally characterized as a substrate of both PKA and PKG. PKA and PKG phosphorylate VASP on residues-serine 157 and serine 239, respectively. The effects of NO and CO on cell migration is shown in Figure 3-12. CD34$^+$ cells were incubated for 15 minutes in the presence of either the NO or CO donor. Both NO and CO exposure enhanced VASP phoshporylation, however, site of phoshporylation was different (Figure 3-13). As described in Figure 3-13, NO donor increased VASP phosphorylation at serine 239, whereas CO increased phosphorylation at serine157 in CD34$^+$ cells.

NO Increases VASP Phosphorylation in Diabetic CD34$^+$cells

CD34$^+$ cells from diabetic individuals have been shown to have reduced NO bioavailability. Previously it was found that exogenous NO administration could correct decreased EPC migratory response in diabetic CD34$^+$cells.[222] To evaluate this observation further and to determine whether this improvement was due to increased VASP phosphorylation, the level of phospho-VASP in diabetic CD34$^+$cells was measured by flow cytometry analysis.

Diabetic $CD34^+$cells were isolated from patients with type 1 and type 2 diseases and the cells were pretreated with the NO donor. There was considerable patient to patient variation in the level of VASP expression; however NO treatment resulted in stimulation of VASP phosphorylation at serine 239 in all the diabetic cells (Figure 3-14).

NO and CO Cause VASP Redistribution to the Leading Edge of the Cells

Phospho-VASP is localized to focal adhesions and areas of dynamic membrane activity. Redistribution of VASP to the leading edge of the endothelial cells in response to exogenous NO or CO was observed (Figure 3-15). As shown in Figure 3-15 A and D, VASP is evenly distributed within the cytoplasm under basal conditions. VASP was redistributed to the advancing edge of the cell following 15 minutes stimulation with NO donor (3-15 B and E). CO donor also causes VASP redistribution as same pattern as NO donor (3-15C and F).

IGFBP-3 Increases eNOS Phosphorylation

Like the other hypoxia-regulated factors, SDF-1 and VEGF, IGFBP-3 modulates $CD34^+$cells mobilization (Figure 3-1). We next examined whether IGFBP-3 stimutaled NO generation in $CD34^+$cells. Two different western blotting analyses were conducted to determine eNOS activity.

In-Cell western assay is a quantitative analysis that is extremely sensitive. This assay utilizes an infrared fluorescence antibody. However, the validity of the In-Cell western assay needs to be examined by comparing it to standard western blotting. Figure 3-16A shows the result of the In-Cell western assay and 3-16B illustrates the result of standard western blotting analysis. Both results support that IGFBP-3 increases eNOS phosphorylation at Ser 1177 in $CD34^+$cells.

IGFBP-3 Induces NO Production

$CD34^+$cells were exposed to IGFBP-3 for 30 minutes then intracellular NO was monitored with DAF-FM diacetate to confirm increased NO generation in IGFBP-3 treated cells. As shown in Figure 3-17, IGFBP-3 increased NO production in $CD34^+$cells. NO release from IGFBP-3 treated cells was 3.7 fold greater than untreated cells.

IGFBP-3 Modulates VASP Phosphorylation

Increased NO generation by IGFBP-3 subsequently induced phosphorylation of VASP (Figure 3-18). $CD34^+$cells were exposed to IGFBP-3 (100 ng/ml) for 0, 10, 30, and 60 minutes. To obtain whole cell lysate including cytoplasmic protein as well as membrane-bound protein, 2X SDS-PAGE buffer was added to the cells. Two anti-phospho-VASP (Ser 157 and Ser 239) antibodies were used to detect the different sites of phosphorylation on VASP. Phospho-VASP at serine 239 was significantly increased by IGFBP-3 treatment.

Inhibition of SK Activity Results in Reduced NO Production

Our preliminary data shows IGFBP-3 enhances SK activity in $CD34^+$cells (not shown here). To confirm SK is a downstream signaling mediator of IGFBP-3, the level of NO production from SK inhibitor pretreated cells was measured. $CD34^+$cells were exposed to IGFBP-3 following pretreatment of SK blocker, dimethylsphingosine (DMS) for 30 minutes. As depicted in Figure 3-19, NO generation was inhibited in SK blocked cells and it was not restored by addition of IGFBP-3. This result suggests that IGFBP-3 modulates SK and that the S1P/SK pathway is involved in IGFBP-3's modulation of $CD34^+$cells.

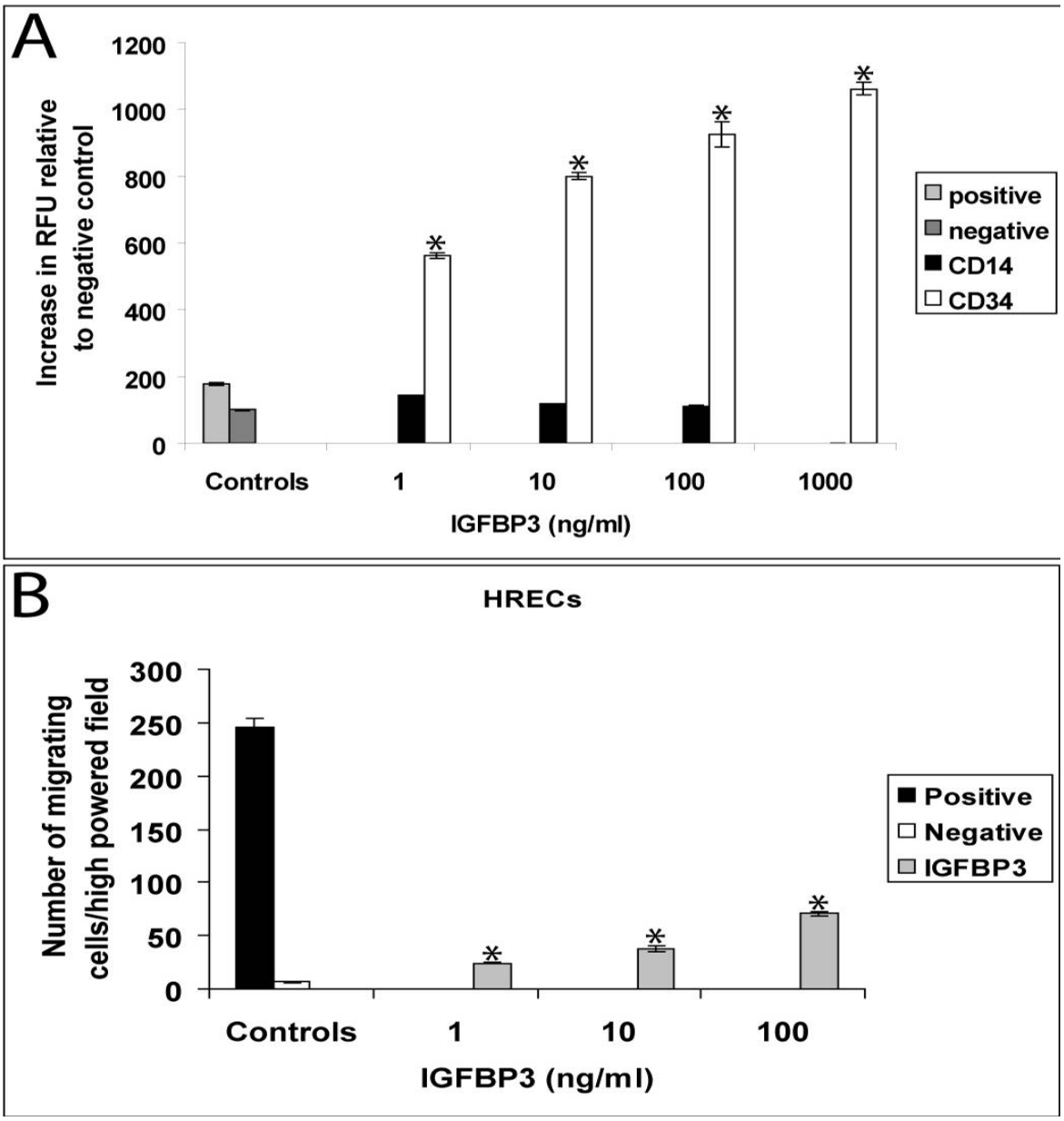


Figure 3-1. IGFBP-3 induces $CD34^{+}$cells and endothelial cells migration. Modified Boyden chamber assay was used for circulating cells (A) and the Boyden chamber assay was performed for adhered cells (B). Statistically significances were presented *$P < 0.05$ vs. negative control.

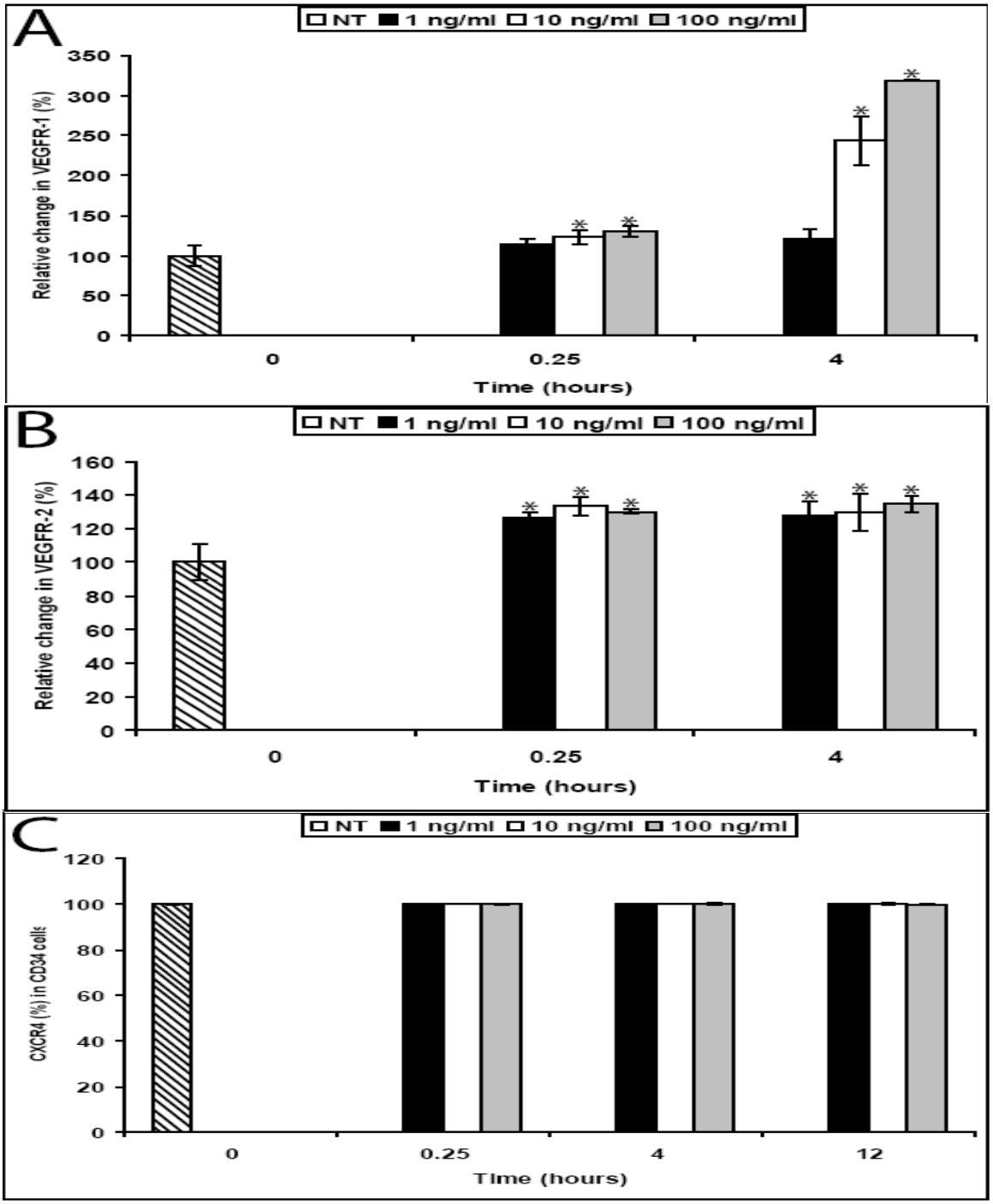


Figure 3-2. Receptor levels in $CD34^+$cells following IGFBP-3 exposure. $CD34^+$cells were exposed to IGFBP-3 for 15 min, 4 h, and 12 h (for CXCR4). A) VEGFR-1. *P < 0.05 vs. medium alone (non treatment, NT) for 15 min and *P <0.001 for 4 h. B) VEGFR-2. *P < 0.001 vs. NT for both 15 min and 4 h. C) CXCR-4.

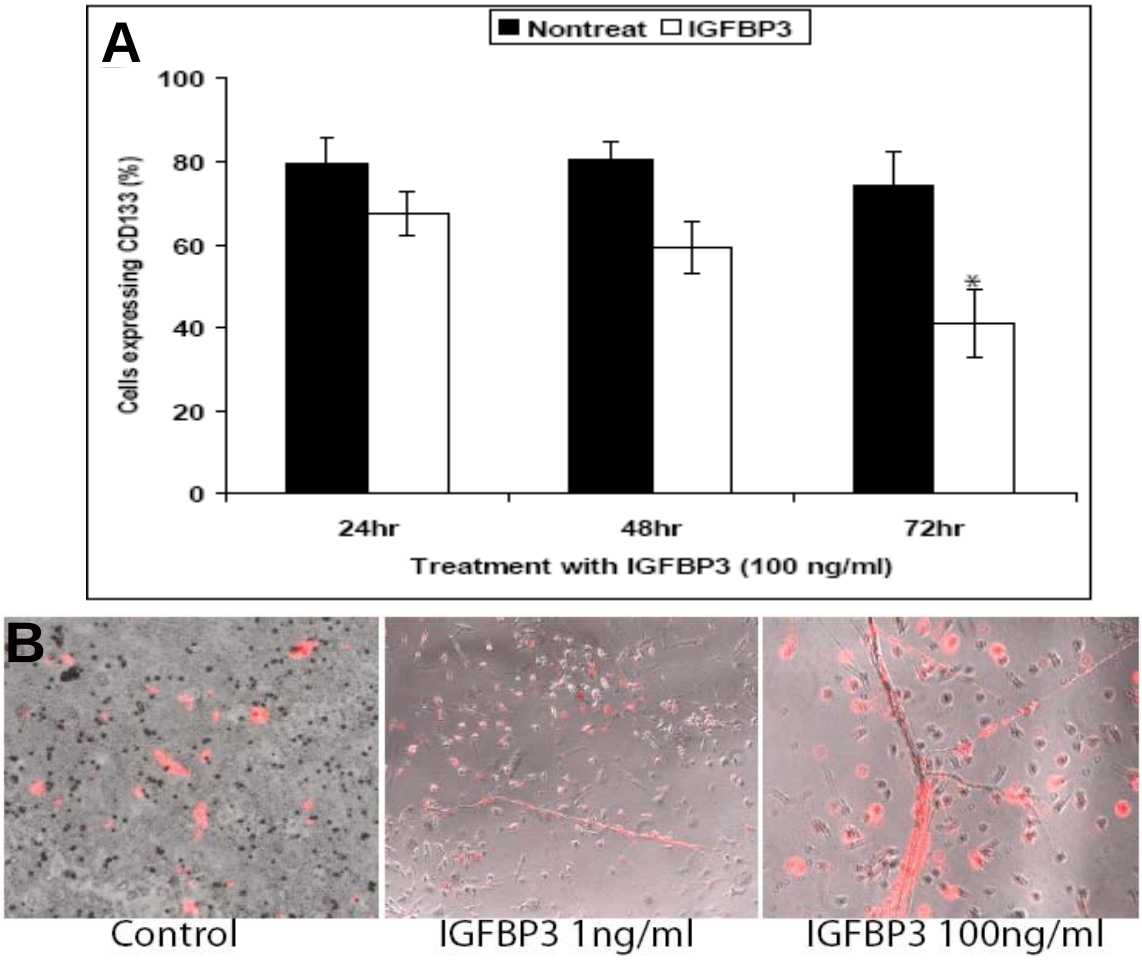


Figure 3-3. IGFBP-3 enhances $CD34^+$cells and EPC differentiation. A) $CD34^+$cell were exposed to IGFBP-3 (white bars) for 72 hours. Statistically significances were presented *P < 0.05 vs. control nontreated cells. B) Representative images for growing EPC in the presence of different concentration of IGFBP-3. Magnification: X100. Scale bars: 150 µm for left and center panel and 100 µm for right panel.

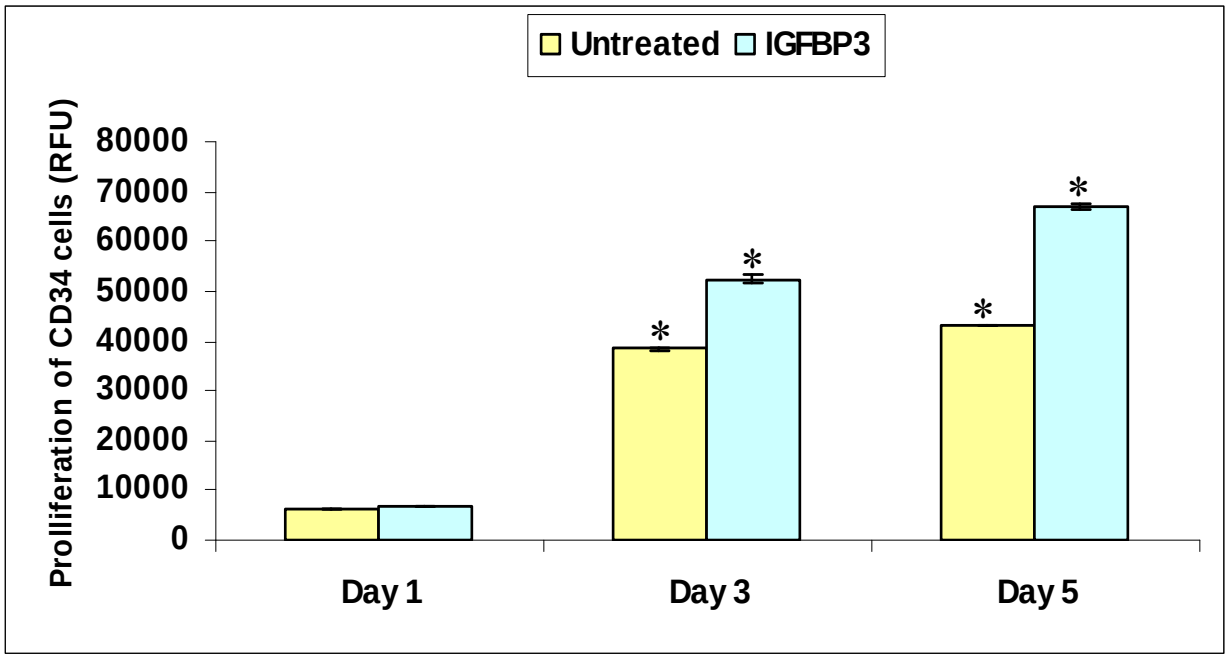


Figure 3-4. IGFBP-3 enhances $CD34^{+}$cells proliferation. Cells were cultured in StemSpan SFEM with addition of cytokines cocktail for 5 days. IGFBP3 signifies StemSpan SFEM with cytokines and 100 ng/ml IGFBP3. *$P < 0.001$ vs. Day 1 untreated cells and untreated cells at each time points.

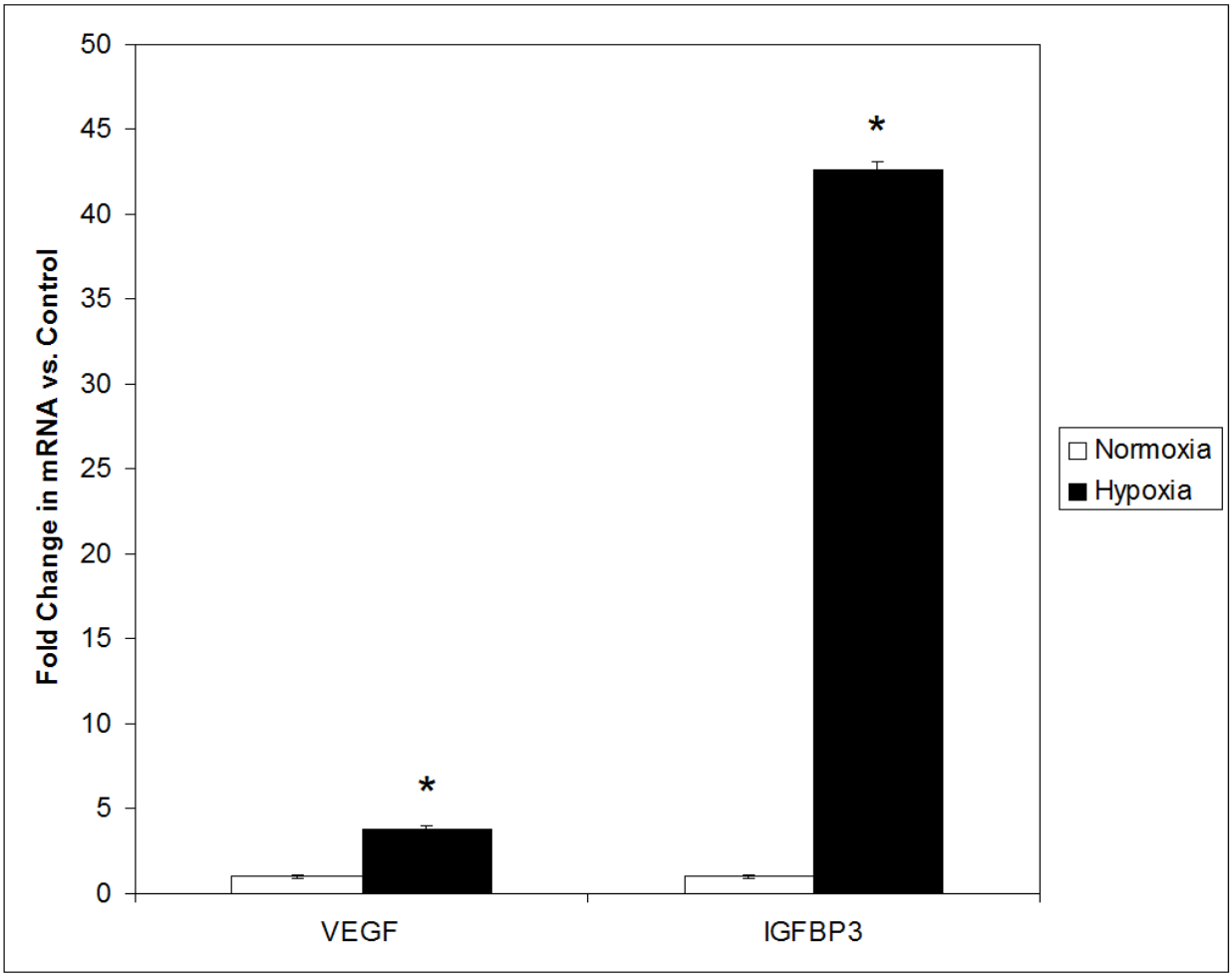


Figure 3-5. Hypoxia retina expresses IGFBP-3. Neonatal mouse pups (n=6 in each group) was euthanized at P12.5. Under hypoxic condition, both VAGF (*P < 0.05) and IGFBP-3 (*P < 0.0001) mRNA were significantly increased when compared to normoxia control retina

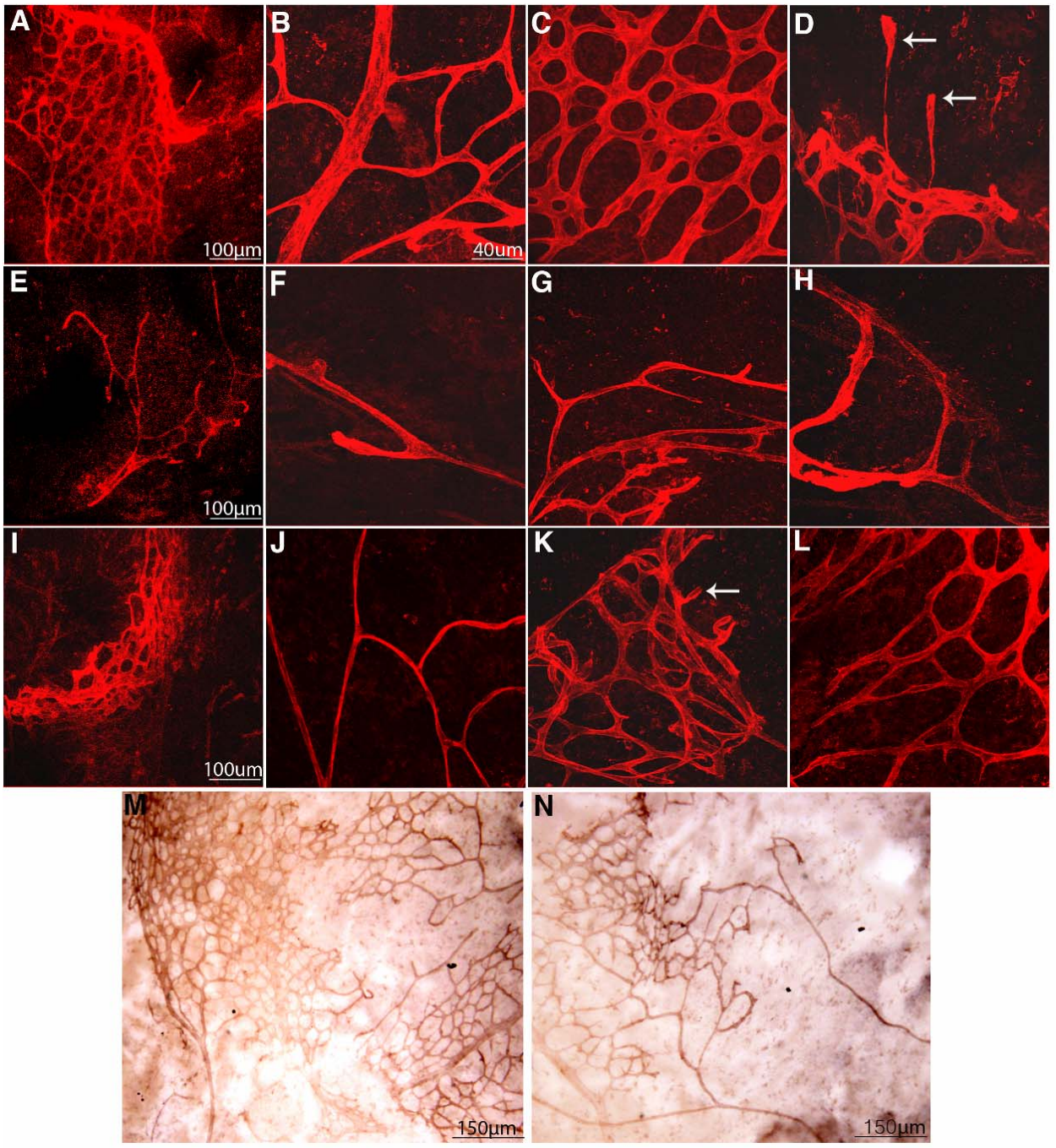


Figure 3-6. IGFBP-3 protects from hyperoxia-induced vascular regression. Retina from the eyes injected with IGFBP-3 expressing plasmid (n=9; A-D), control plasmid (n=9; E-H), and contra-lateral eyes (I-L). Low magnification views of *GS* isolectin HRP-labeled retinas from IGFBP-3 plasmid injected eyes (M) and control vector injected eyes (N).

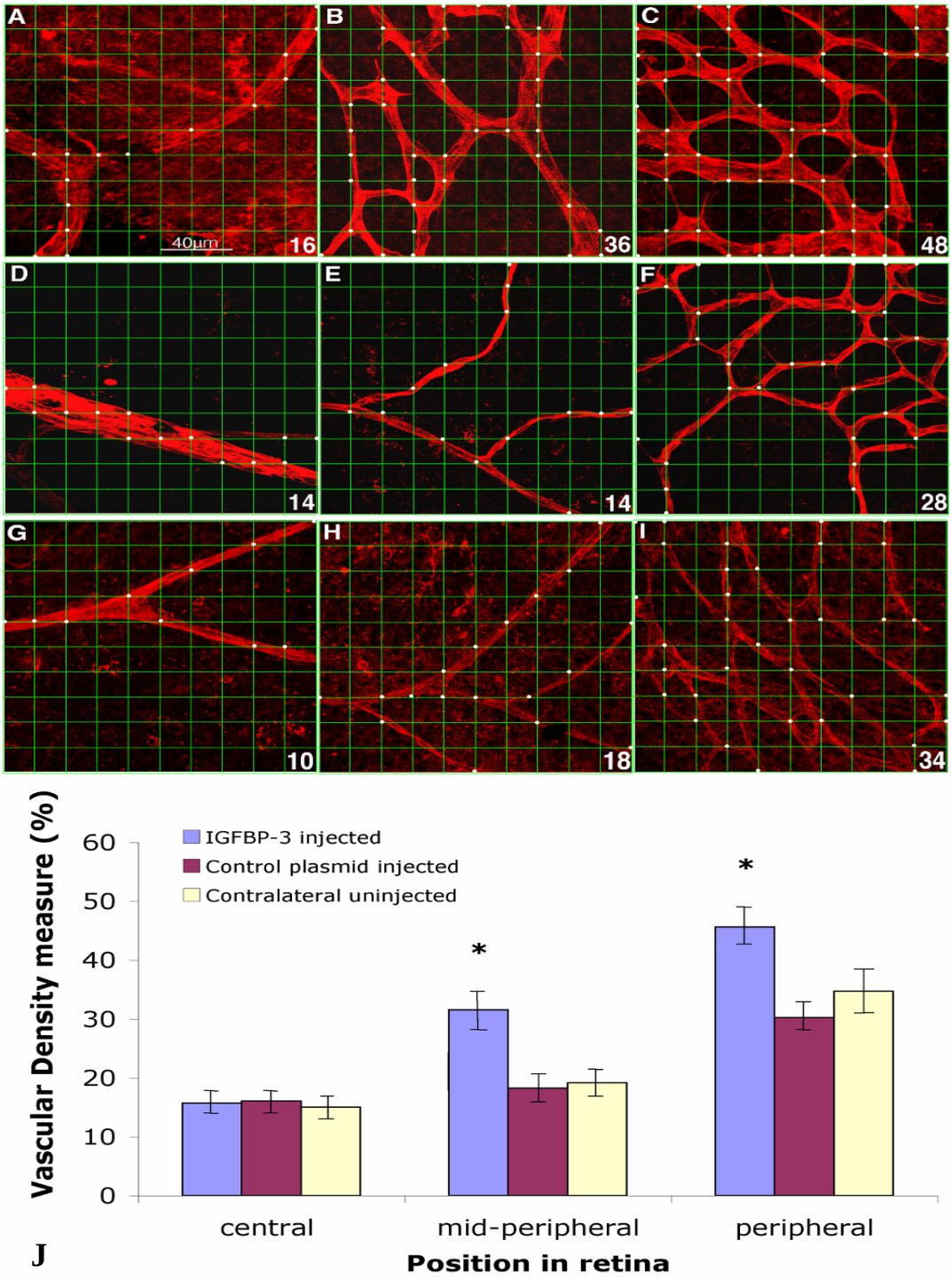


Figure 3-7. Quantitative analysis of vascular density. IGFBP-3 significantly protected the retinal vasculature from hyperoxia-induced vessel regression in mid peripheral (*P < 0.001) and peripheral regions (*P < 0.001), but did not have any significant effect on the central region of the retina (*P >0.05)

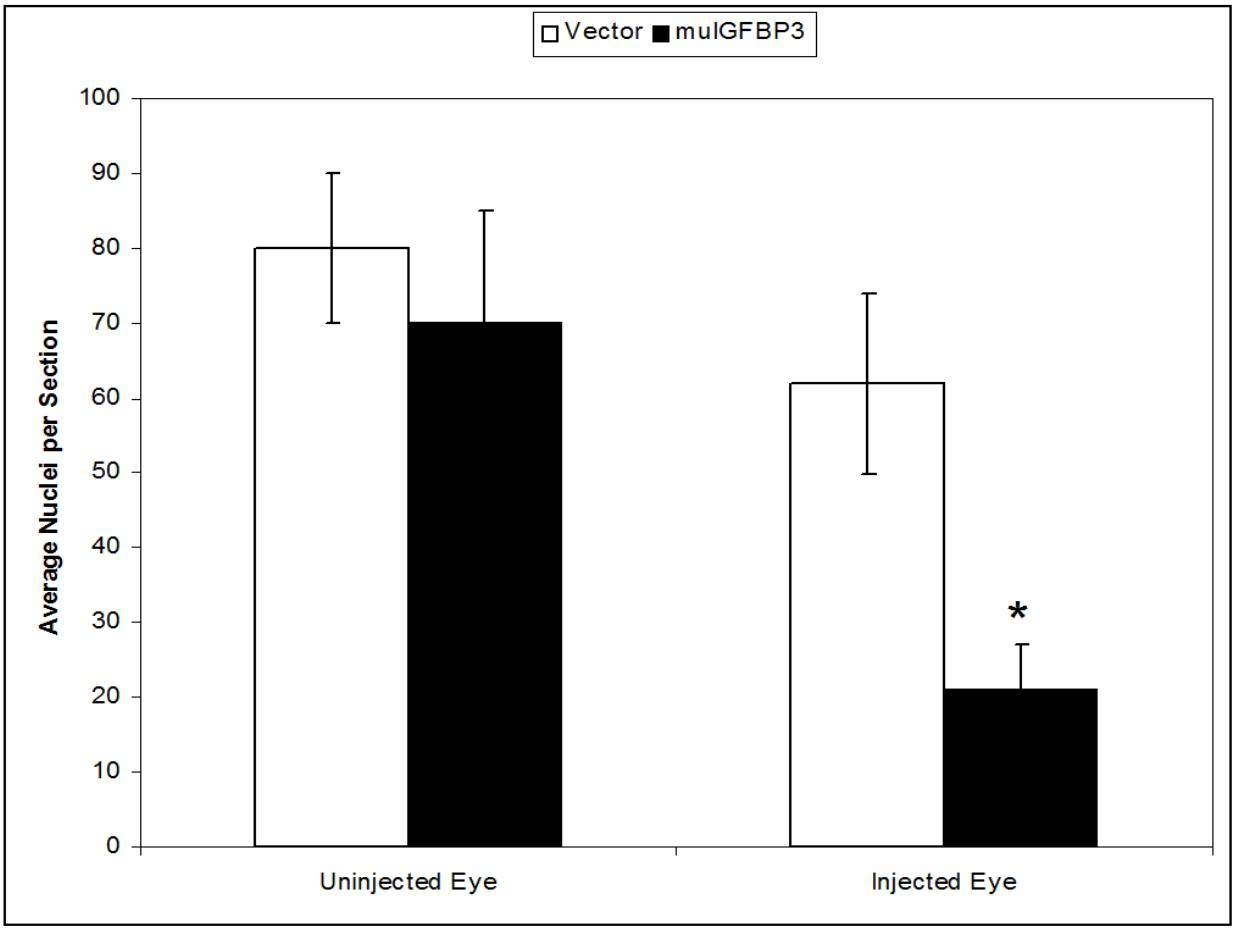


Figure 3-8. Reduced preretinal neovascularization by expression of IGFBP-3. IGFBP-3 expressing plasmid injected mice (n=9, black bars) and control plasmid injected mice (n=9, white bars) were subjected to the OIR model. Statistically significances were presented *P < 0.005 vs. control vector injected eyes.

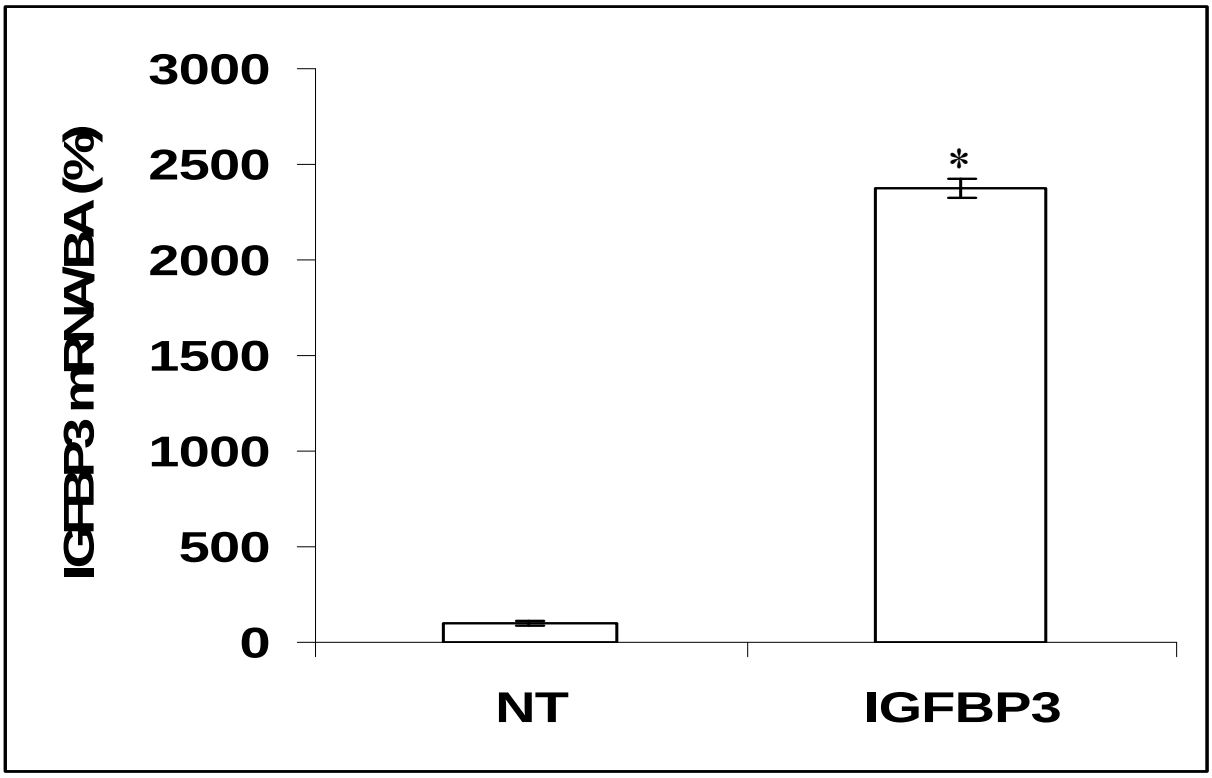


Figure 3-9. IGFBP-3 expression in plasmid transfected HSC. Transfection of GFP^{+}HSC with IGFBP-3 expressing plasmid results in a 25-fold increase in IGFBP-3 expression in vitro compared with nontransfected (NT) controls. *P= 0.02 vs. nontransfected HSC (NT).

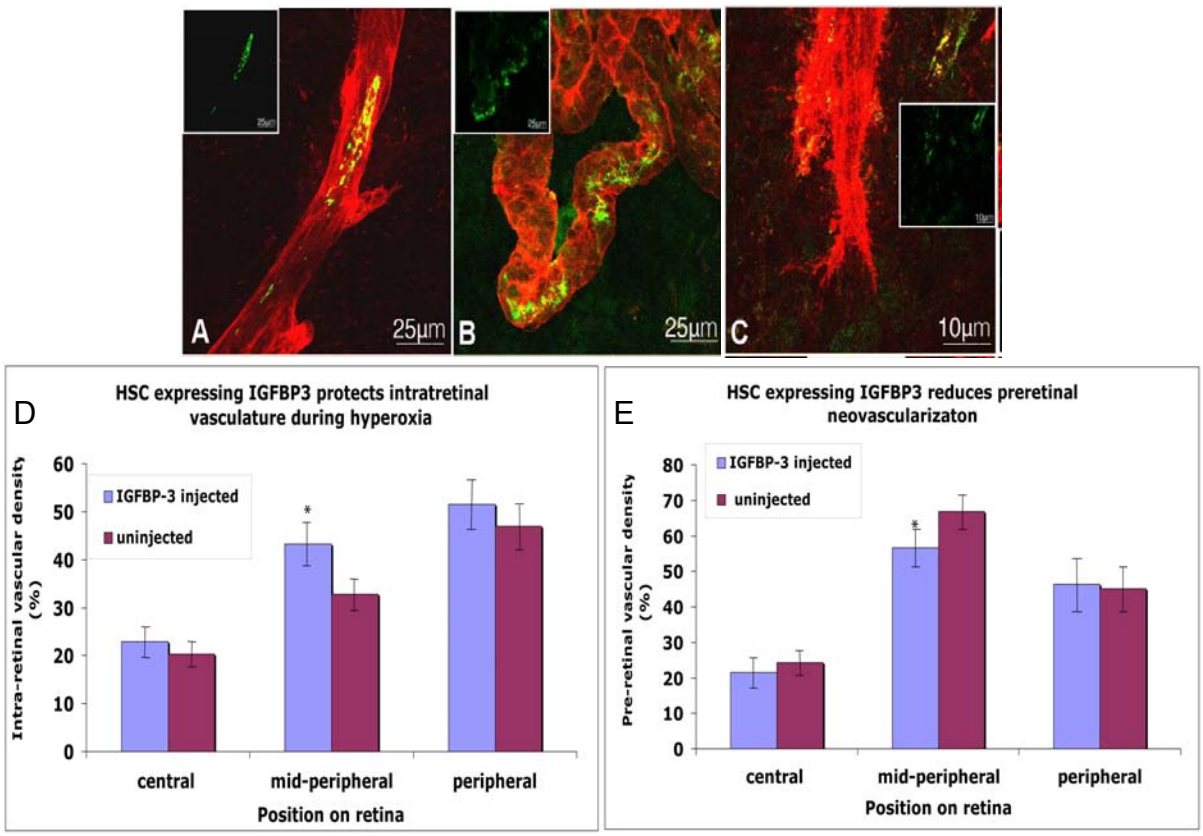


Figure 3-10. Localization of gfp+HSC expressing IGFBP-3 within the retinal vasculature. A) Representative image of redial arterioles. B) Hemangiomas. C) Filopodia. Merged green (gfp+HSC) and red (resident vasculature) channels represent incorporation of HSC in vasculature, indicating endogenously delivered IGFBP-3 in vivo. * P< 0.001 vs. uninjected controls in D. *P< 0.01 in E.

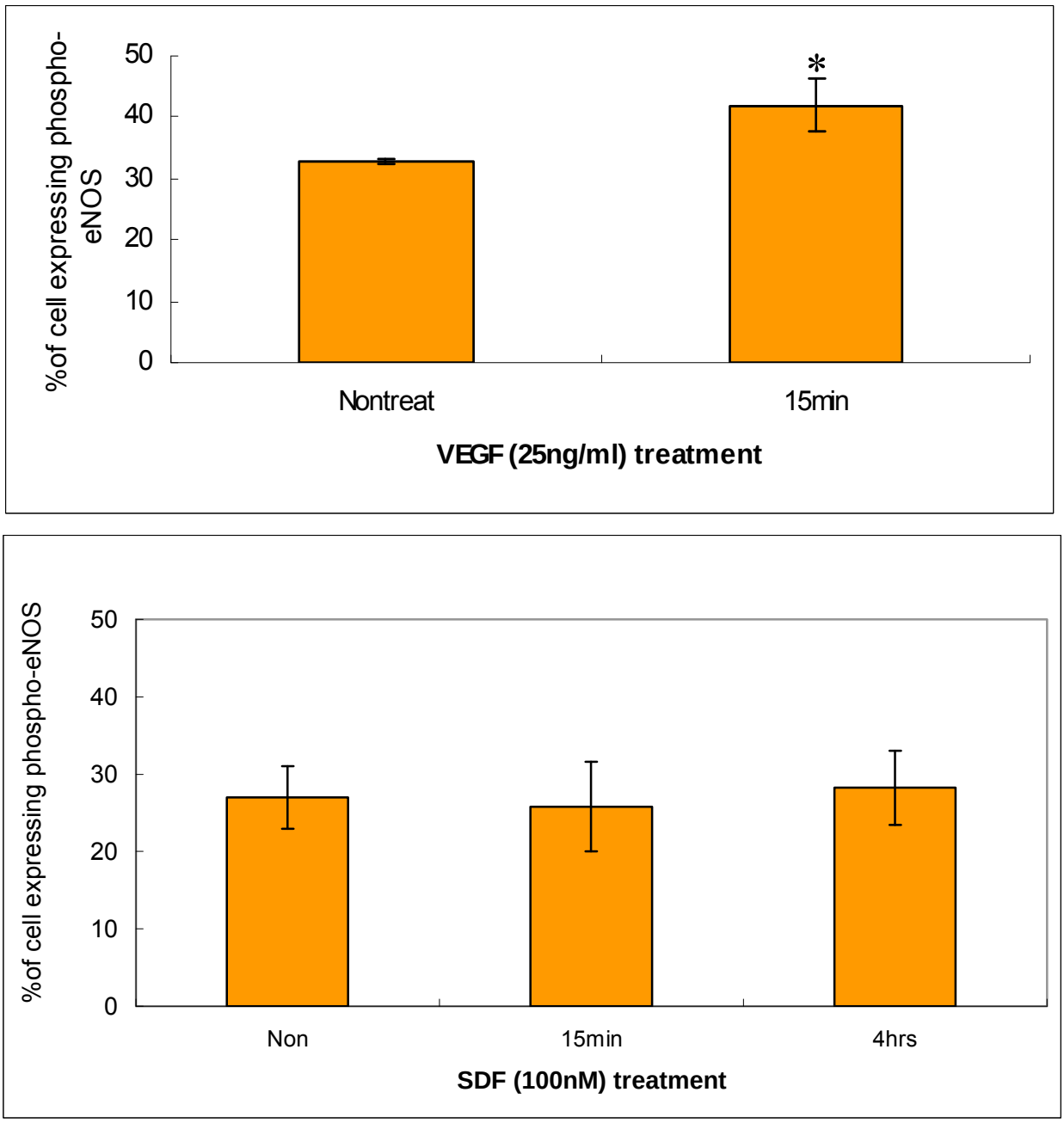


Figure 3-11. eNOS phosphorylation by hypoxia-regulated factor. $CD34^{+}$cells were treated with either 25 ng/ml VEGF or 100 nM SDF-1. Anti-phoshporylated eNOS (Ser 1177) antibody was used to examine the activation of eNOS in $CD34^{+}$cells. A) eNOS phosphorylation following exposure of VEGF (*P <0.05 vs. nontreated control cells). B) eNOS phosphorylation following exposure of SDF-1.

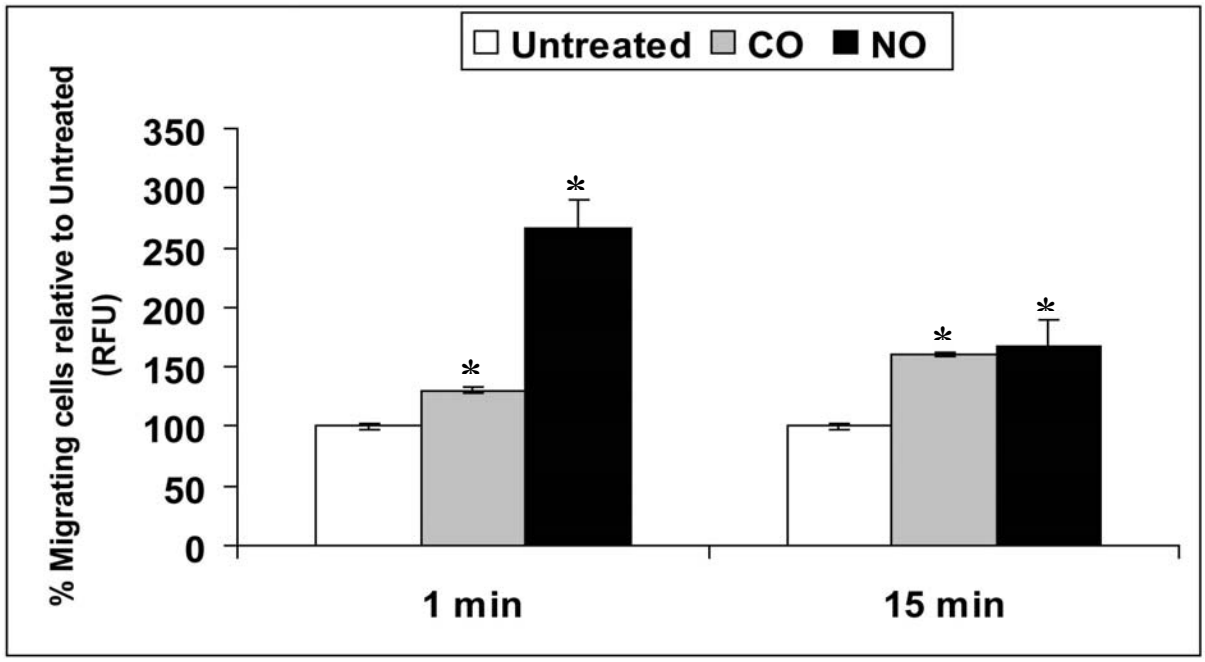


Figure 3-12. NO and CO stimulate $CD34^{+}$cells migration. Pretreatment with either the CO donor (Ru(II)Cl2(CO)3 dimer) or the NO donor (DETA-NO) for either 1 minute or 15 minutes increases the cells responsiveness to SDF-1. EPC were obtained from 3 healthy control subjects. Values represent means ±SD. *P<0.05 vs. untreated control.

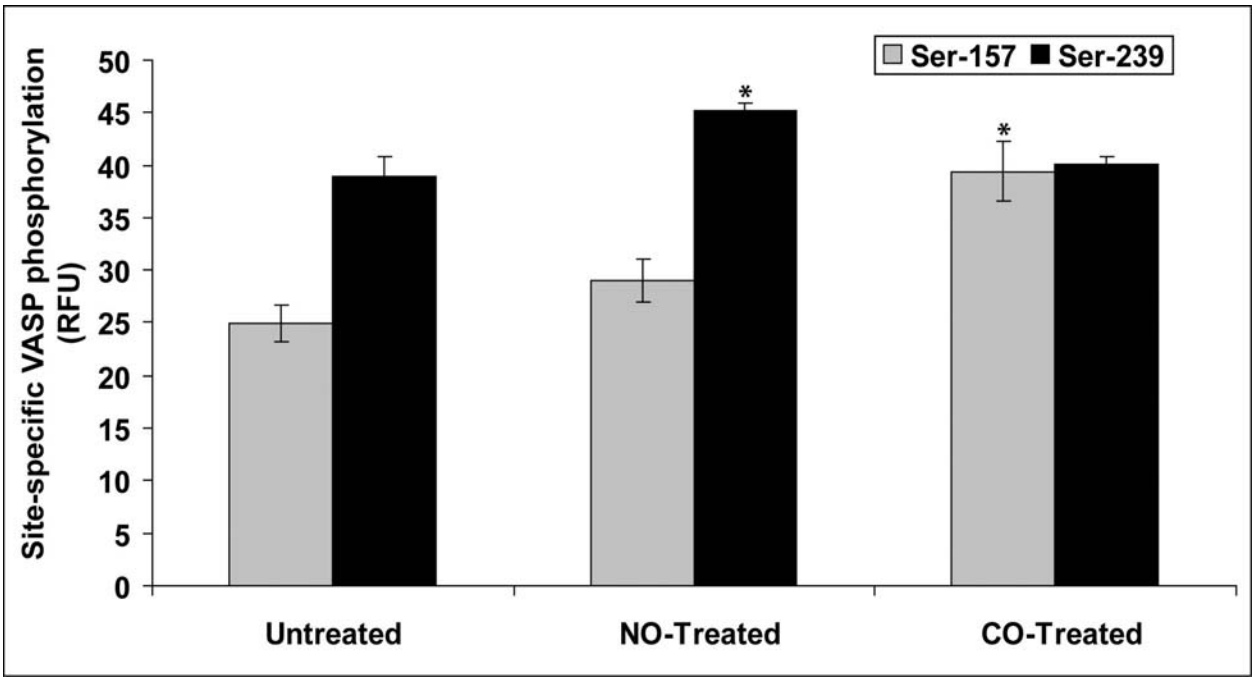


Figure 3-13. VASP, phosphorylated VASP 157, and 239 expression levels. VASP function is regulated by its phosphorylation on serine 157 and serine 239. $CD34^{+}$cells were incubated for 15 minutes in the presence of either the NO or CO donor. The NO donor increased the VASP phosphorylation at serine 239, whereas CO increased phosphorylation at serine157 in these cells. Values represent means ±SD. *P <0.05 vs. untreated control

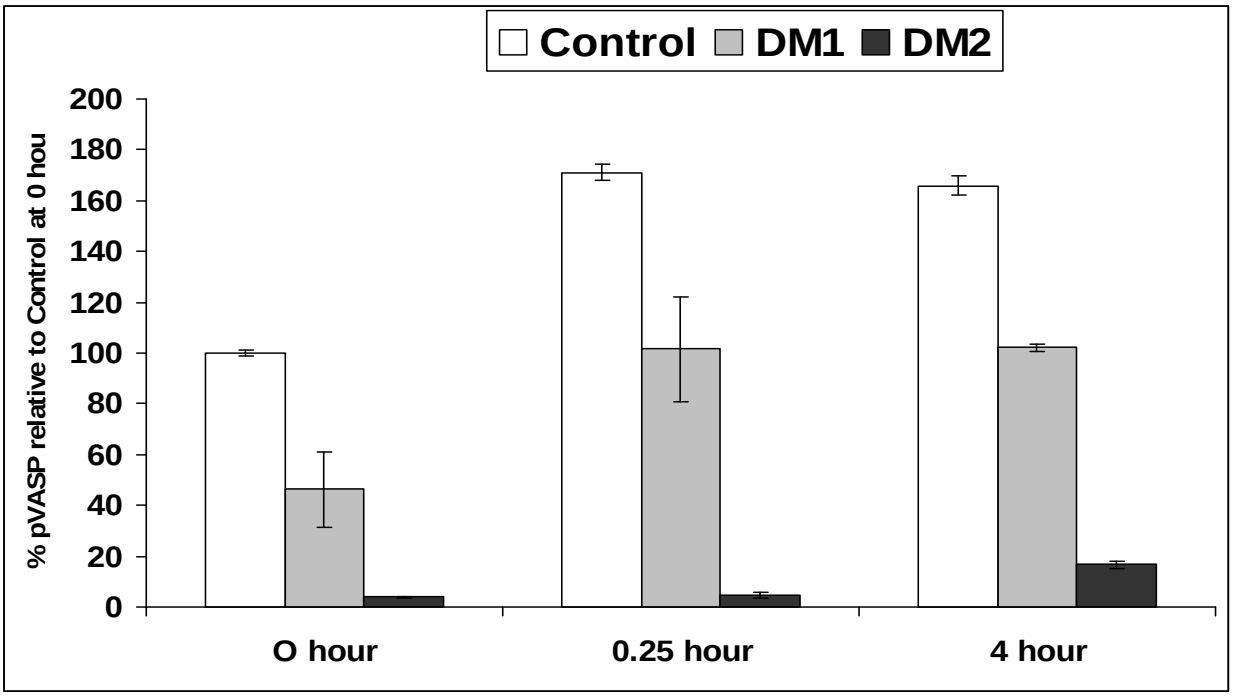


Figure 3-14. Diabetic $CD34^+$cells show increased VASP phosphorylation following exposure of NO donor. In $CD34^+$cells from two diabetic individuals one with type 1 and the other with type 2 disease, NO treatment resulted in stimulation of VASP phosphorylation at serine 239. $CD34^+$cells from diabetic individuals demonstrate reduced levels of pVASP but phosphorylation increases in response to NO exposure. Values represent means ±SD.

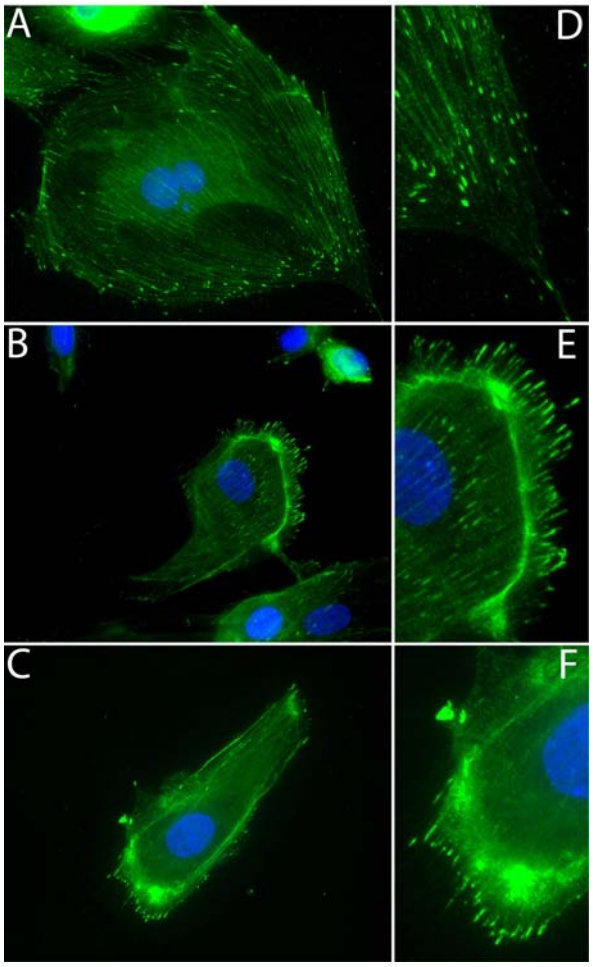
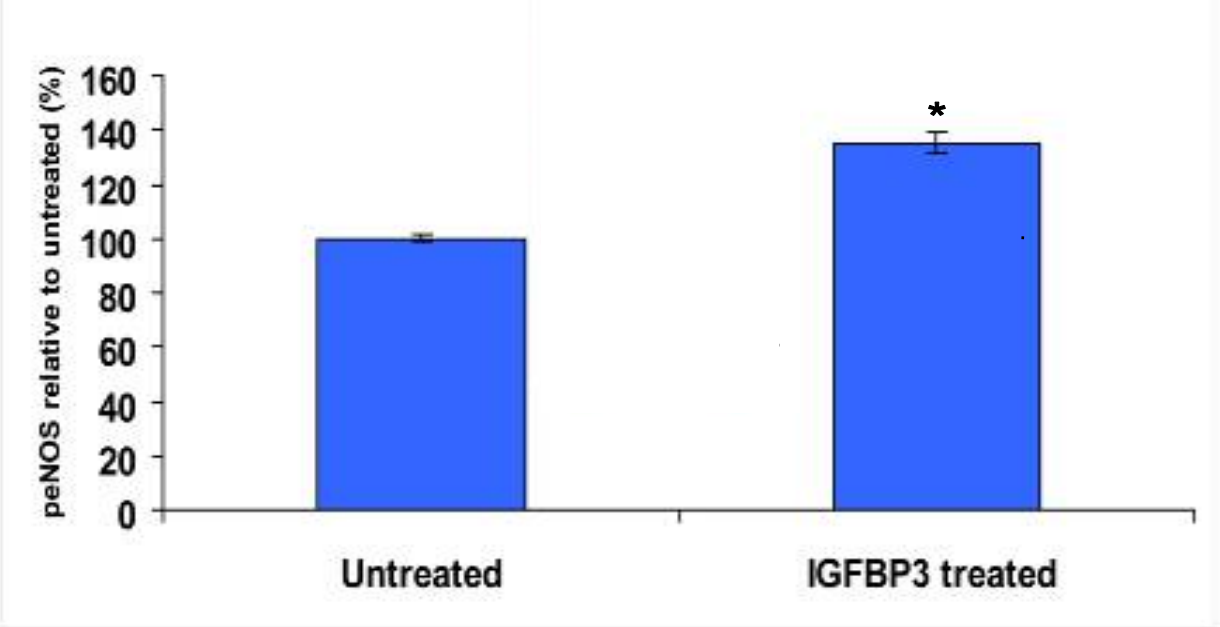


Figure 3-15. NO and CO mediates VASP redistribution within endothelial cells. A) Untreated cells showing equal VASP immunoreactivity throughout the cytoplasm. B) CO-induced redistribution of VASP to filopodia at the leading edge of the cells. C) NO-induced redistribution of VASP to filopodia. Green channel represents VASP redistribution. Blue channel shows DAPI stained nuclei. 100X magnification. D), E), and F) Details of A), B), and C), respectively.



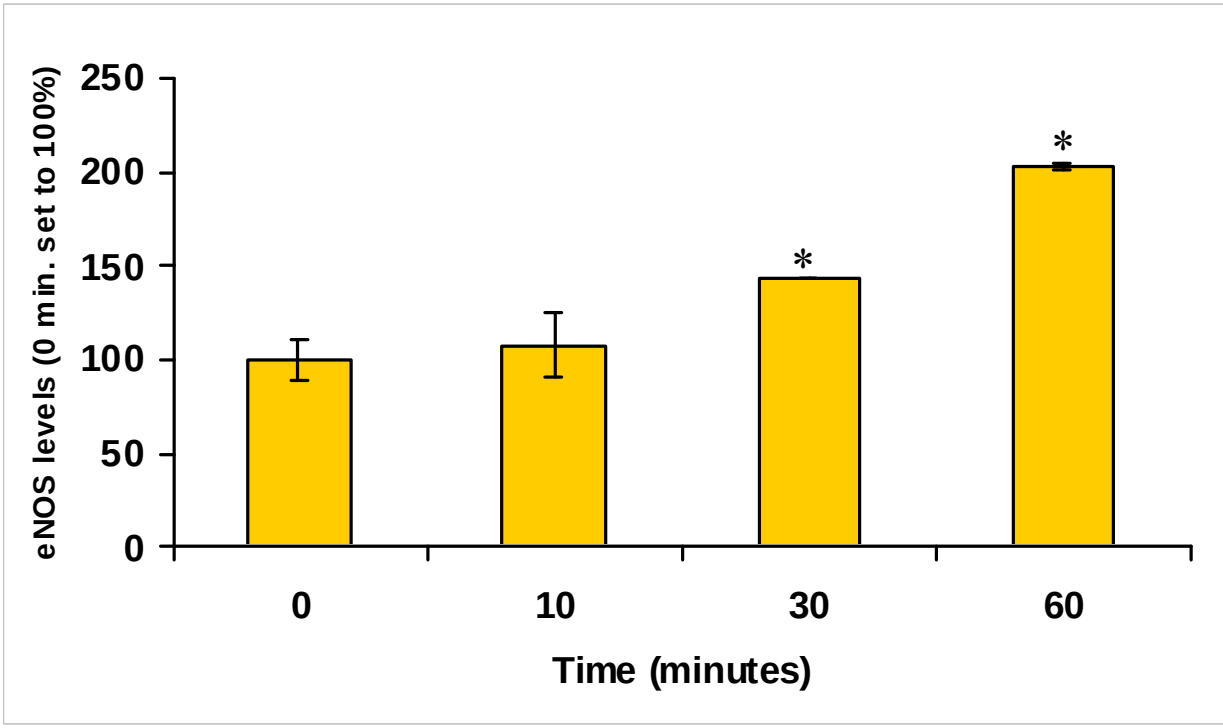


Figure 3-16. Phosphorylation of eNOS following exposure of IGFBP-3. Anti-phoshporylated eNOS (Ser 1177) antibody was used to examine the activation of eNOS in both analysis. A) In-Cell western analysis (*p=0.0006 vs. untreated cells). B) Western blotting analysis (*p<0.05 vs. 0 min treated cells). Values represent means ±SD.

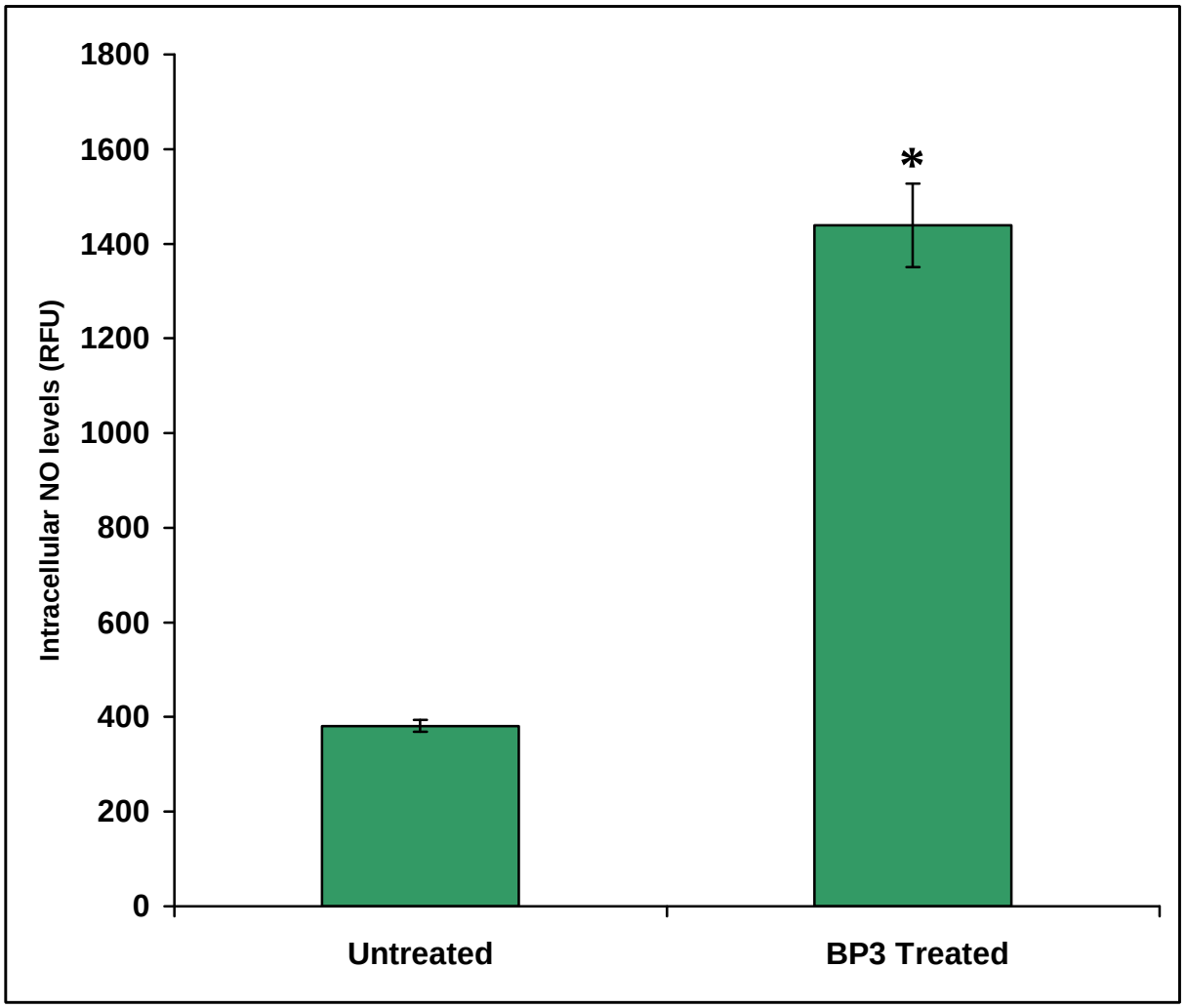
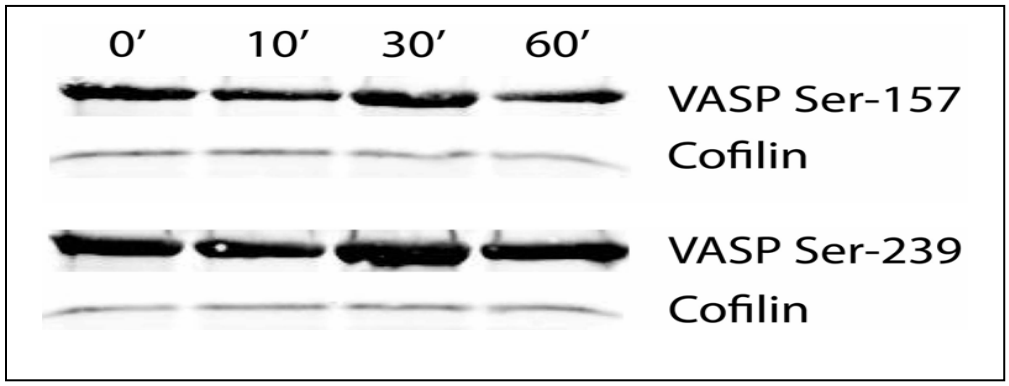


Figure 3-17. Increased intracellular NO production in $CD34^+$cells. $CD34^+$cells were exposed to IGFBP-3 for 30 minutes. 3.7-fold greater increase of intracellular NO in IGFBP-3 treated cells was confirmed by using DAF-FM diacetate. Values represent means ±SD. *$P < 0.05$ vs. untreated cells



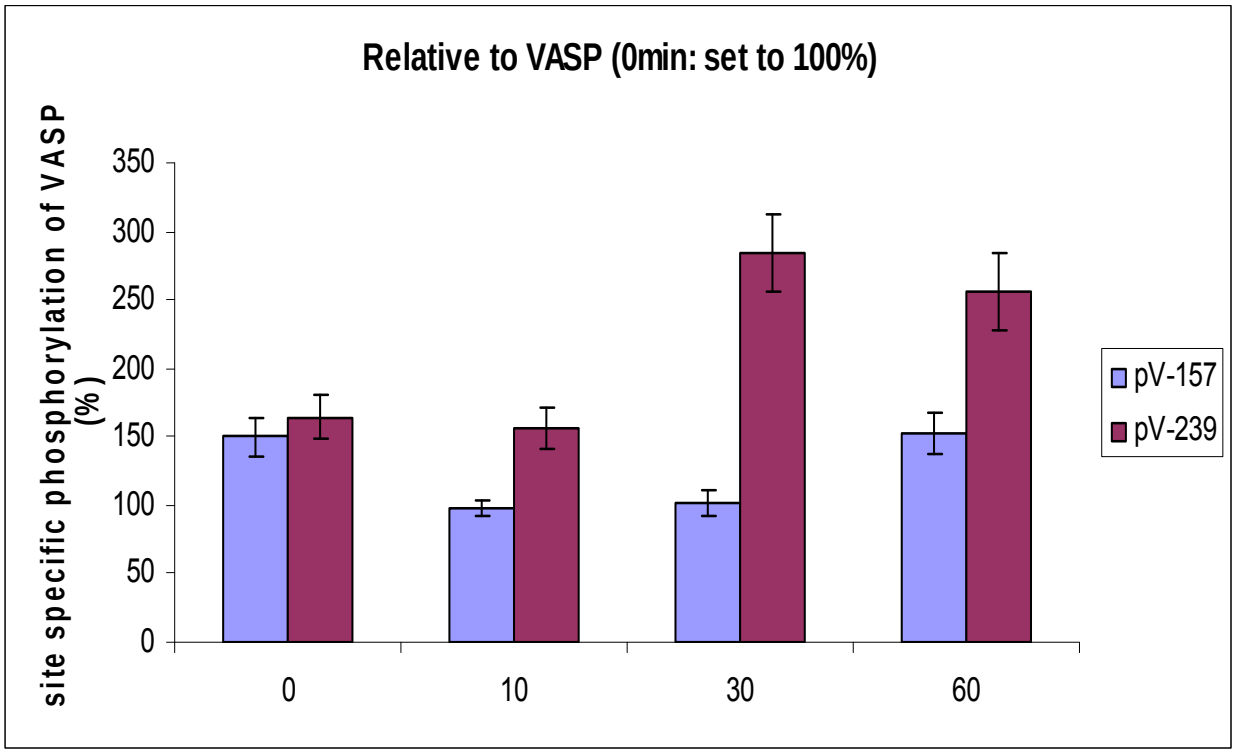


Figure 3-18. IGFBP-3 modulates site specific phosphorylation of VASP in $CD34^+$cells. IGFBP-3 (100 ng/ml) was exposed to $CD34^+$cells for 0, 10, 30, and 60 minutes. Two anti-phospho-VASP (Ser 157 and Ser 239) antibodies were used to detect the different sites of phosphorylation on VASP. Phospho-VASP at serine 239 was significantly increased by IGFBP-3 treatment. Values represent means ±SD.

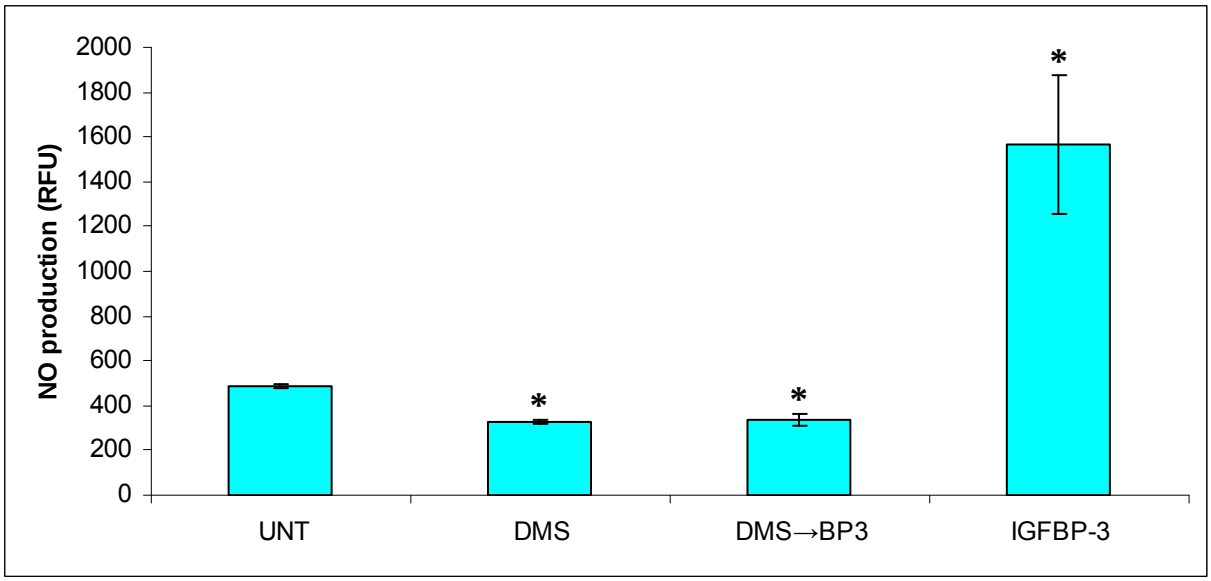


Figure 3-19. Inhibition of SK activity results in reduced NO production. DMS signifies $CD34^+$cells were incubated with 20 μM Dimethylsphingosine for 30 minutes. DMS→ BP3 signifies $CD34^+$cells were pretreated with 20 μM Dimethylsphingosine for 30 minutes followed by addition of 100 ng/ml IGFBP-3. Values represent means ±SD. *P<0.05 vs. untreated cells.

CHAPTER 4
DISCUSSION

This main purpose of this study was to address IGFBP-3 functions on EPC and its involved signaling pathways. The primary focus was depicting the effect of IGFBP-3 on stem cells and progenitor cells in retinal vasculatures.

Factors Influencing the EPC Studies

A difficulty for accomplishing this study was to obtain sufficient number of $CD34^+$cells from the peripheral blood to complete the experiments. These cells are an extremely rare population of cells representing less than 0.01% of cells in the circulation. Furthermore significant differences in circulating EPC numbers exist depending on the general health of the individual providing the cells. Pathological, pharmacological and physiological factors influence mobilization of $CD34^+$ EPC. Numbers of EPCs are inversely related to factors such as presence of coronary artery disease and endothelial dysfunction.[60] More specifically, increased levels of oxidative stress, inflammatory cytokines and asymmetric dimethylarginine, an endogenous inhibitor of endothelial nitric oxide synthase, have been linked to diminished mobilization and function of EPCs.[65] Indeed, cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), VEGF, SDF-1 and EPO, as well as therapeutic interventions with statins and estrogen are able to increase EPC numbers in circulation.[50] The contribution of BM derived cells to the endothelium of injured tissue or hypoxic areas ranges from 1% up to 26% of vessels[53,66]. The magnitude of recruitment of circulating endothelial cells is organ specific and dependent on the extent of vascular injury and remodeling.[53,66]

It is important to emphasize that identification of putative EPC is complicated due to not only small numbers of cells but also antigen plasticity, overlapping phenotype or antigen

expression among other subsets. Investigators have struggled to find an antigen or characteristic that is unique to EPC. Recently many studies have demonstrated that there is overlap in antigenic expression between EC and monocyte phenotypes. LDL uptake, lectin binding, and CD31/CD105/CD144 expression are inherent features of monocytes, making them often phenotypically indistinguishable from EPCs.[55] Furthermore, monocytes and their progeny can function as EPCs in various experimental models.[52,54,56]

The properties of these cells were examined using flow cytometry, a powerful technique for the analysis of multiple characteristics of a single cell. Flow cytometry was used to determine the characteristics of cells including cell size, granularity, and relative fluorescences. In this study, fluorescent antibodies were used to detect the densities of specific receptors and activities of specific enzymes.

We further attempted to overcome the lack of cells with In-Cell western assay, a quantitative immunofluorescence based technique. This technique uses infrared labeled secondary antibodies to directly detect protein in the cellular environment. One of the advantages in using infrared fluorescence is it reduces interferences caused by background autofluorescence from cell, culture environment, proteins and test compounds. Two separate detection channels (700 and 800 nm) result in more accurate evaluation. One channel can probe target protein of interest, the second channel can be used for normalization of cell number or protein concentration in these same cells. Quantification accuracy is maximized by normalization because adjustments can be made for differences in cell number from well to well. In order to test the suitability of In-Cell western assay in our system, we established a standard western blot analysis with same set of experimental groups. The results from In-Cell western and standard western blot analysis were showing both 44% increases in phospho-eNOS expression following

IGFBP-3 exposure (100 ng/ml, 30 min), supporting that In-Cell western represents a reliable way to tracking subtle changes of protein among small population of cells such as $CD34^+$cells.

More interestingly, this assay can quantify proteins in a 96-or 384-well microplate in less time and fewer proteins than a gel-based traditional western blot assay. The optimal cell number to run the In-Cell western was determined to be 10,000 cells per well. In contrast, traditional western blot analysis required 1,000,000 cells per lane.

IGFBP-3 as a Hypoxia-regulated Factor

EPCs support postnatal vasculogenesis by incorporating into the vascular lumen as well as delivering bioavailable angiogenic factors including VEGF, matrix metalloproteases (MMPs), and angiopoietins to new vessels.[92,223-225] Hypoxia especially HIF-1 either directly or indirectly regulates the cell type specific expression of multiple angiogenic growth factors and cytokines. Numerous hypoxia-regulated factors have been implicated in vasculogenesis associated with EPCs.

The mechanism of how HIF-1 modulates cellular response to hypoxia has been relatively well studied. HIF-1 is a heterodimeric transcription factor that consists of both HIF-1α and HIF-1β. The amino-terminal half of each subunit contains both basic helix-loop-helix (bHLH) and PAS (Per, ARNT, Sim) motifs that are required for dimerization and DNA binding.[74] HIF-1β is also known as aryl hydrocarbon receptor nuclear translocator (ARNT) because it dimerizes with the aryl hydrocarbon receptor as well as with HIF-1α. The carboxy-terminal half of HIF-1α mediates nuclear localization, protein stabilization, and transactivation.[74] Under hypoxic conditions, HIF-1α protein accumulates, the heterodimer translocates to the nucleus and binds to a family of genes containing a specific sequence motif (HRE).[73] The target genes are expressed in most cell types, such as those encoding glucose transporters, glycolytic enzymes, and VEGF,

as well as genes that are expressed in a cell type-specific manner, such as EPO, iNOS and IGF-2.[76]

VEGF is commonly expressed as one of the most potent regulator of both vasculogensis and angiogenesis. Compared to VEGF, the effects of IGFBPs on EPCs are greater. IGFBP-3 mRNA is abundantly expressed in hypoxia-related inflammatory angiogenesis.[226] Hypoxia-induced expression of IGFBP-3 was also validated by Northern blot analysis.[227] An anti-angiogenic role of IGFBP-3 has been reported in cancer research.[109,112,118,121] For instance, the growth-inhibitory effects of various anti-proliferative agents including TGF-β, retinoic acid, antiestrogens, vitamin D analogs, and TNF-α are associated with increases in IGFBP3 mRNA and protein expression.[122,132,133,135] IGFBP-3 has also been shown to stimulate angiogenesis, however prior to our studies the vasculogenic effects of IGFBP-3 had not been appreciated.

The expression level and various functional differences of IGFBP-3 are cell type specific and thus context dependent. We have previously demonstrated that mature human retinal endothelial cells express high levels of IGFBP-3. In contrast, $CD34^+$ cells released undetectable levels of IGFBP-3. This finding suggests that the immature $CD34^+$cells are more susceptible to changes in IGFBP-3 concentrations than mature endothelial cells. Furthermore, the concentrations of IGFBP-3 required to stimulate migration of $CD34^+$cells is extremely low. In support of our results, Liu et al. reported using in vitro cell proliferation assays and immunophenotype analysis that addition of nanomolar concentration of IGFBP-3 on human umbilical cord blood-derived $CD34^+$ $CD38^-$ Lin^- cells resulted in their proliferation.[228] These studies combining our findings have been conclusively proved that IGFBP-3 supports expansion of HSC in vitro. In our studies, we further extended these observations by showing that IGFBP-3

stimulates differentiation of $CD34^+$cells to endothelial cells by loss of immature HSC marker and tube formation assay.

Understanding of IGFBP-3 mechanism has been difficult due to its uncharacterized receptor. Several candidate receptors of IGFBP-3 have been described but their signaling functions are poorly understood. Granata et al. suggested possible interaction of IGFBP-3 signaling with SK related angiogenesis by endothelial cells.[120] Granata group suggested dual functions of IGFBP-3 on human endothelial cells. For instance, they revealed pro-apoptosis and anti-apoptosis action of IGFBP-3 by regulating intracellular ceramide levels. Our results also indicate that the possible relationship between SK/S1P and IGFBP-3. mRNA level of SK1 has been stimulated by IGFBP-3 whereas reduced mRNA level of SK2 was shown in $CD34^+$cells. Once SK activity was inhibited by SK blocker, dimethylsphingosine (DMS), IGFBP-3 was not able to exert its function on $CD34^+$cells. Based on our observations, together with the growing amount of published evidence, IGFBP-3 exerts its vasculogenic actions on EPC though SK/S1P signaling pathways.

Our in vivo studies have been showing the protective role of IGFBP-3 in vasculature following high oxygen stress with subsequent reduction of preretinal neovascularization. Thus, we postulate that IGFBP-3 expression may represent a physiological adaptation to ischemia and potentially a novel therapeutic target for treatment of ischemia conditions.

Corroborated with the work of Lofqvist et al, exogenous administration of IGFBP-3 may represent a novel approach for the treatment of conditions associated with pathological neovascularization such as retinopathy of prematurity or PDR[29]. However, there are potential obstacles for IGFBP-3 study: (1) The volume and the wide diversity of biological activity in which IGFBP-3 is involved, (2) IGFBP-3 is very closely related to IGF system, and (3) various

laboratories utilize different cell systems in which response to IGFBP-3 may not only be different but contradictory.

S1P: Possible Role in EPC Mobilization

Sphingosine was discovered by J.L.W. Thudichum in 1884. At that time, he suggested the name "sphingo" that were derived from the Greek mythology "Sphinx" for a new lipid due to its chemical nature containing both amine and alcohol groups, but insoluble in water.[229] Further investigations are required to examine intracellular targets of S1P, the mechanism of its transport in and out of the cells, and the modulation of S1P levels. Related to S1P receptors, future challenges include better characterization of the patho-physiological role of the various S1P receptors, what regulates their expression and their activity, and which genes are in turn regulated upon receptor activation.

SIP stimulates human umbilical vein endothelial cells in vitro and in vivo angiogenesis in the matrigel plug assay in mice.[230] Gene knock out studies of $S1P_1$ receptor in mice revealed that this receptor on endothelial cells has an important role in vessel stabilization during embryogenesis.[145] In addition, recent studies have expanded the sphingolipid signaling to the regulation of eNOS. eNOS pathway (eNOS induced endothelium-dependent vasodilation) has been suggested as a downstream target for the biological effects of S1P.[231] Furthermore, S1P-induced signaling in human lung EC resulted in cytoskeletal rearrangement (cortical F-actin) and barrier enhancement through PI3K.[232] Our immunohistochemistry result support these findings by showing that S1P rapidly induced VASP redistribution in human lung EC.

To be considered as a significant intracellular messenger, more experiments remain to be conducted. Transgenic models, gene targeting approaches, and in vivo use of small interference RNA will further help understand the physiological role of S1P and its receptors. We are currently exploring the underlying mechanism by which IGFBP-3 regulates EPC mobilization

and carefully examining the signal transduction cascades activated by IGFBP-3 will ultimately help understand the how it modulates EPC function .

VASP: New Perspectives and Open Questions

Hypoxia stimulates IGFBP-3 expression and also generates gaseous molecules, CO and NO by activating HO-1 and NOS respectively. In the current study, two major serine phosphorylation sites of VASP were evaluated on $CD34^+$cells in response to NO, CO, and IGFBP-3. We have observed that IGFBP-3 mediates increases in phosphorylation, and redistribution of VASP which subsequently supports EPC migration. To distinguish the difference of VASP phosphorylation induced by CO, NO, or IGFBP-3, we used two phosphospecific VASP antibodies targeting the Ser157 and Ser239 residue phosphorylation sites. We observed that IGFBP-3 (as well as NO) exposure to $CD34^+$cells induced VASP phosphorylation on serine 239. In contrast, VASP was phosphorylated through serine 157 by exogenous CO administration to $CD34^+$cells.

All vertebrate Ena/VASP proteins are substrates for the cyclic nucleotide-dependent kinases PKA/PKG.[203,206] Both PKA and PKG recognize and phosphorylate all three sites of VASP, but with different specificities and kinetics. Ser157 is the site preferred by PKA, whereas Ser239 is phosphorylated by PKG.[217] Thr238 is phosphorylated last by both PKA and PKG. Phosphorylation at additional sites in VASP might add additional levels of regulation, but it appears that the conserved N-terminal PKA/PKG site is the major site of phospho-regulation in vertebrate Ena/VASP proteins. Consistent with this, Loureuro *et al* proved that mutation of this N-terminal phosphorylation site in Mena was sufficient to block function in fibroblasts.[233]

Phosphorylation by different kinases has shown cell type specificity. Studies of platelets derived from knockout mice revealed a requirement for VASP in this cyclic-nucleotide-mediated signaling cascade, suggesting that VASP plays a key role in mediating this PKA-dependent

function.[206,234] Furthermore, PKA inhibitors reverse cGMP-induced inhibition of thrombin-induced platelet aggregation, whereas PKG inhibitors further enhance the inhibitory effect of cGMP analogs. Thus, PKA plays a predominant role in the cGMP-induced phosphorylation of VASP and platelet inhibition in human platelets.[235] PKA-dependent phosphorylation of Ena/VASP proteins correlates with changes in cell adhesion in fibroblasts.[233] Under basal conditions, the majority of VASP (more than 95%) is in the unphoshporylated state in human micro vascular endothelial cells. However, PKA induced VASP phosphorylation changed its localization to cell-cell junctions and regulated endothelial permeability.[215]

The role of NO/PKG/ pathway has been tested by many researchers. PKG plays an important role in smooth muscle cell relaxation, inhibition of platelet aggregation, retinal signal transduction and synaptic transmission.[211] In rat aorta phosphor-VASP ser239 correlates with relaxation of VSMC layer.[206] sGC is the intracellular mediator for the ubiquitous biological messenger NO.[163,187] However, NO-independent stimulators of sGC are very desirable as both pharmacological tools to proof the NO/cyclic GMP pathway and as potential therapeutic agents. Organic nitrates like GTN or ISDN have been used for decades as a treatment for coronary heart disease. However, the major drawbacks of this therapy are the development of tolerance and the negligible anti-platelet effect. This obstacle could now be overcome by the discovery of potent and specific NO-independent sGC stimulators. As discussed above, PKA/PKG phosphorylation plays a crucial role in various cell types and in numerous animal models. In this study, we suggested the role of PKA/PKG in the regulation of VASP function in human progenitor cells and EC. We found out NO and IGFBP-3 phosphorylates VASP-Ser239 through PKG whereas CO activates phosphor-VASP-Ser157 through PKA. As depicted in this study, both physiological gases and IGFBP-3 regulate human mature EC and progenitor cell dynamics by

VASP phoshporylation and localization. Thus, our findings may explain what initiates progenitor cell migration from BM and how EC migrates into ischemic tissues, although the kinetic analysis and detailed structural changes that were derived by different site of phosphorylation are still unknown.

Although a great deal of information about VASP proteins is available there are still a number of very important questions that remain unanswered. For example, a question concerning how VASP proteins interact with the barbed ends of actin filaments and what allows filament elongation need to be answered. Identification of whether such phosphorylation reflects the overall phosphorylation of all VASP within the cell will be the direction of future studies in our lab. The question of whether VASP at the leading edge or VASP at focal adhesions are differently phosphorylated also remains to be determined.

Conclusions

Circulating bone marrow-derived stem cells and progenitor cells home to areas of hypoxia and participate in vessel development and re-vascularization to facilitate vascular repair. In this study, we asked whether the hypoxia-regulated factor IGFBP-3 could serve as a homing factor for EPCs and stimulate their vasculogenic functions. We examined the effect of IGFBP-3 on NO generation, consequent VASP activation and redistribution. We also evaluated the role of SK in IGFBP-3 modulating EPC vasculogenesis.

Exposure of $CD34^+$ EPC population to nanomolar concentrations of IGFBP-3 resulted in rapid differentiation into endothelial cells, dose-dependent migration, and capillary tube formation. For in vivo study, a plasmid expressing IGFBP-3 under the control of a proliferating endothelial-specific promoter was designed. This plasmid was injected either alone or HSC transfected form into the vitreous of neonatal pups undergoing the oxygen-induced retinopathy

model. Endogeneously delivered IGFBP-3 resulted in reduced areas of vaso-obliteration, protection of the developing vasculature from hyperoxia, and reduction in preretinal neovascularization compared to control conditions.

This study supports that IGFBP-3 promotes EPC mobilization by cytoskeletal changes through NO signaling pathway related phosphorylation and redistribution of VASP. In EPCs, IGFBP-3 induced eNOS phoshporylation and NO generation. Similar to NO induced PKG related VASP phosphorylation, IGFBP-3 selectively phoshporylated VASP on serine 239. Granata group suggested pro-apoptosis and anti-apoptosis action of IGFBP-3 is regulated by intracellular ceramide/S1P levels. Our results also indicate that the possible relationship between SK/S1P and IGFBP-3. SK1 mRNA was upregulated by IGFBP-3. IGFBP-3 induced intracellular NO production in EPCs was significantly reduced by SK inhibitor. These findings provide a mechanism for EPC mobilization and angiogenic function of IGFBP-3. IGFBP-3 expression may represent a physiological adaptation to ischemia, and IGFBP-3 could be considered as a novel therapeutic target for treatment of ischemic conditions.

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BIOGRAPHICAL SKETCH

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