ANGIOGENESIS OF HUMAN RETINAL MICROVASCULAR ENDOTHELIAL CELLS: ROLE OF INSULIN-LIKE GROWTH FACTOR-1 AND HYPOXIA-INDUCIBLE FACTOR-1 ALPHA IN THE PHOSPHATIDYLINOSITOL 3-KINASE PATHWAY

by

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Abstract

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Retinal vascular formation is a complex process that requires a precise temporospatial regulation of various elements, including the growth factors. Insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) are ligands of specific receptor tyrosine kinases (RTKs), which, if activated, will initiate downstream pathways that ultimately promote cell survival, cell proliferation, vascular permeability and cell migration; all of which permit blood vessel development. Formation of blood vessels from pre-existing vessels may be normal (angiogenesis) or pathological (neovascularization) processes. Examples of required angiogenesis include wound healing and repair of the endometrium. In proliferative retinopathies, such as retinopathy of prematurity or diabetic retinopathy, there is a dysregulation of IGF-1 that results in the neovascular formation of abnormal retinal blood vessels from pre-existing vessels. In these proliferative retinopathies, neovascularization is intended to bring nutrients to tissues, with unfortunate risk of loss of vision. To add to the chemical mixture that promotes neovascularization, the transcription factor hypoxia-inducible factor (HIF) is important in inducing VEGF secretion in cells stimulated by either hypoxia or IGF-1. Therefore it is a convergence of several signaling pathways that ultimately leads to neovascularization. The goals of this thesis are to demonstrate the role of IGF-1 in the formation of retinal vasculature using

human retinal microvascular endothelial (HRMVE) primary cell line; and to study the *in vitro* effect of IGF-1 stimulation on HIF-1α in human retinal angiogenesis through the phosphatidylinositol 3-kinase (PI3K) pathway.

"Remembering that you are going to die is the best way I know to avoid the trap of thinking you have something to lose. You are already naked. There is no reason not to follow your heart".

Steve Jobs (1955 – 2011)

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LIST OF ABBREVIATIONS

^{o}C	degree Celsius
3D	three-dimensional
µm	micrometer
ADB	antibody dilution buffer
AMD	age-related macular degeneration
ARE	adenylate/uridylate-rich element
BCECF	2’-7’-bis(carboxyethyl)-5(6)-carboxyfluorescein
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CO_2	carbon dioxide
CSC	Cell Systems Corporation
DAG	1,2 diacylglycerol
DAPI	4’,6-diamidino-2-phenylindole
DMEM	Dulbecco’s modified Eagles medium
DR	diabetic retinopathy
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinases
FDA	food and drug administration
FGF	fibroblast growth factor
Flk	fetal liver kinase

Flt	fms-related tyrosine kinase
GDP	guanosine diphosphate
GH	growth hormone
GTP	guanosine-5’-triphosphate
GRB2	growth factor receptor-bound protein 2
HGF	hepatocyte growth factor
HIF	hypoxia-inducible factor
HIF-1α	hypoxia-inducible factor 1 alpha
HIF-1β	hypoxia-inducible factor 1 beta
HMDS	hexamethyldisilazane
HRE	hypoxia responsive element
HRMVEC	human retinal microvascular endothelial cell
Ig	immunoglobulin
IGF-1	insulin-like growth factor 1
IGFBP	insulin-like growth factor binding protein
IGFR	insulin-like growth factor receptor
IL	interleukin
IP3	inositol 1,4,5-triphosphate
IRES	internal ribosomal entry site
kDa	kiloDalton
KDR	kinase insert domain receptor
M	molar
MAP	microtubule-associated protein

MAPK	mitogen-activated protein kinase
MCSF	macrophage colony-stimulating factor
MEK	mitogen-activated protein kinase kinase
ml	milliliter
mM	millimolar
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
ng	nanogram
NGF	nerve growth factor
NGS	normal goat serum
nm	nanometer
nM	nanomolar
O_2	oxygen
ORP 150	oxygen-regulated protein 150
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDK1	3-phosphoinositide dependent protein kinase-1
PEDF	pigment epithelium-derived factor
PlGF	placental growth factor
PI3K	phosphatidylinositol 3-kinase or phophatidylinositide 3-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP3	phosphatidylinositol 3,4,5-triphosphate
PKB	protein kinase B

PKC	protein kinase C
PLCγ	phospholipase gamma
PTB	phosphotyrosine-binding
PRC	radial peri-papillary capillary
RNA	ribonucleic acid
ROP	retinopathy of prematurity
RPE	retinal pigment epithelium
RTK	receptor tyrosine kinase
SEM	scanning electron microscopy
SFK	Src family kinase
SH	Src homology region
SOS	son of sevenless
TGF-β	transforming growth factor beta
TIMP	tissue inhibitor of metalloproteinase
TNF-α	tumor necrosis factor alpha
tPA	tissue plasminogen activator
UTR	untranslated region
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

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Chapter 1: Background and Significance

Introduction

Blood vessel formation process can be classified into two types: (a) vasculogenesis, the formation of new blood vessels *de novo* from precursor mesenchymal origin cells, and (b) angiogenesis, the formation of new blood vessel sprouts from pre-existing blood vessels (Risau et al., 1988). The latter also has a connotation of a homeostatic response to maintain tissue health. The term 'neovascularization' usually pertains to formation of new blood vessels, usually in a pathological condition.

The human retinal vasculogenesis occurs along with the development of the primitive optic nerve head and results in the formation of the primary vasculature (Provis, 2001; Sandercoe, Madigan, Billson, Penfold, & Provis, 1999). From the primary vasculature, deep capillaries are formed by angiogenesis, and must occur within a given time and space to prevent vessels from blocking the photoreceptors (Chan-Ling et al., 2011). Hence, any angiogenesis that occurs after the formation of primary vasculature could be a pathological process that possibly results in visual impairment or blindness due to abnormal retinal vascularization. This is in contrast to what happens in other organs, such as the dermis, where new blood vessels formation events can occur physiologically during prenatal development and postnatal life (e.g. wound healing) or in pathological conditions such as in cancer or in inflammation reaction.

The complex process of retinal vasculature formation is tightly regulated in time and place by various pro-angiogenic and anti-angiogenic factors. The delicate balance of both factors at the right time and at the proper place determines the final outcome. Vascular endothelial growth factor (VEGF) is the major pro-angiogenic growth factor used for inducing the blood vessel formation. *In vitro* and *in vivo* studies confirm that normal vascular formation is

profoundly dependent upon VEGF expression in coordination with several other growth factors which include angiopoietin, platelet-derived growth factor (PDGF), IGF-1, basic fibroblast growth factor (bFGF or FGF2) (Klagsbrun, 1991), TNF-α (tumor necrosis factor-alpha) (Sunderkotter, Roth, & Sorg, 1990), TGF-β (transforming growth factor-beta) (Pepper, 1997), HGF (hepatocyte growth factor) (Rosen et al., 1997), IL8 (interleukin 8) (Rogala et al., 2001) and pro-angiogenic extracellular matrix (ECM) e.g. laminin (Kibbey, Yamamura, Jun, Grant, & Kleinman, 1994), tenascin-C (Higuchi, Ohnishi, Arita, Hiraga, & Hayakawa, 1993; Jallo et al., 1997).

VEGF alone is inadequate for normal vascular formation to occur (Lloyd, Prior, Li, Yang, & Terjung, 2005). Mice null for either angiopoietin-1 (*Ang-1*) or its *Tie-2* receptor die *in utero* with immature vascular tube formation, a lack of association of endothelial cells with the underlying matrix and an inability to recruit supporting cells (Sato et al., 1995; Suri et al., 1996). PDGF is important in recruiting pericytes to the outer walls of blood vessels that allows vessel stability and branching (von Tell, Armulik, & Betsholtz, 2006).

Some anti-angiogenic factors known to inhibit angiogenic process are endostatin (Beecken et al., 2004; Li et al., 2003), angiostatin (O'Reilly, Holmgren, Shing, Chen, Rosenthal, Cao, et al., 1994; O'Reilly, Holmgren, Shing, Chen, Rosenthal, Moses, et al., 1994), anti-angiogenic ECM molecules (e.g. thrombospondin (Good et al., 1990; Taraboletti, Roberts, Liotta, & Giavazzi, 1990), fibronectin (Saiki et al., 1990)), and pigment epithelium-derived factor (PEDF) (Ohno-Matsui et al., 2001). Transforming growth factor-beta (TGF-β) has unique characteristic in its role in angiogenesis that its pro-angiogenic or anti-angiogenic activity is dose dependent (Orlova, Liu, Goumans, & ten Dijke, 2011; Pepper, 1997). Angiogenesis process is influenced by the presence or absence of other factors such as oxygen level (Knighton, Silver, &

Hunt, 1981; Ohsaki, Fujimoto, & Miki, 1987), glucose level (Dubois et al., 2010; Maltepe, Schmidt, Baunoch, Bradfield, & Simon, 1997; Shweiki, Neeman, Itin, & Keshet, 1995), tissue perfusion (Gowdak et al., 2000) and pericytes (Hirschi & D'Amore, 1997; Nehls, Denzer, & Drenckhahn, 1992; Stratman & Davis, 2012).

Anatomy and Physiology of Human Retina

The retina is located at the posterior segment of the eye that captures light rays and converts them into electrical signals that eventually will be translated into comprehensible images by the visual pathway and the brain (**Figure 1**).

The retina covers the posterior eye on the innermost part, and it consists of the pars neural retina that contains photoreceptors, up to the irregular line of ora serrata, and continues as pars caeca retinae, the part of retina without photoreceptors.

The macula lutea, located close to the center of the retina, has a center, the fovea, where the largest concentration of cones is in the retina. This makes the macula with its fovea important for high-resolution vision (i.e. acuity vision), without its normal functioning, visual detail for our daily activities is hindered. The optic disc or optic nerve head is located at the nasal (medial) side of the retina, and is where the optic nerve and the blood vessels of central retina are coming into or out of the retina (**Figure 2**).

Human retina is composed of nerve cells (ganglion, bipolar, horizontal & amacrine cells), photoreceptor cells (rods & cones), retinal pigment epithelial (RPE) cells, and the supporting cells (Muller cells, astrocyte & microglia). The retina is distinctly divided into two parts (**Figure 3**):

1. Neural retina (pars optica retinae) that is composed of ten layers, from the anterior (= inner = superficial part) to posterior (= outer = deep part) retina, these layers are (Kolb H., 2011a):
    a. Inner limiting membrane layer: a basement membrane at the interface between the retina and the vitreous body; also is the inner limit of Muller cells processes, thus forming a barrier between the vitreous humor and neural retina.
    b. Optic nerve fiber layer: the layer of the non-myelinated axons of the ganglion cells, which will continue to be the myelinated optic nerve when it penetrates the collagen of the sclera through lamina cribrosa, and will synapse mainly at the lateral geniculate nucleus where its neuron will eventually send the axons to the primary visual cortex through the optic radiation.
    c. Ganglion cell layer: is the layer of ganglion cell bodies, which is the third neuron of the visual pathway. Ganglion cells collect visual information from the bipolar cells and amacrine cells.
    d. Inner plexiform layer: is the layer of cell processes and synapses of ganglion, bipolar and amacrine cells. Bipolar cells are considered as the second neurons of the visual pathway.
    e. Inner nuclear layer: is the layer of nuclei and cell bodies of bipolar, horizontal, amacrine and Muller's cells.
    f. Outer plexiform layer: is the layer of synapses between the processes of horizontal, bipolar and photoreceptor cells.
    g. Outer nuclear layer: a layer of rod and cones cell bodies. The photoreceptor is the first neuron on the visual pathway that captures the light photons.

h. Outer limiting layer: is a layer of zonula adherens between photoreceptor cell inner segments and the terminals of the Muller glial cells. This layer acts as the barrier between the subretinal space and the neural retina.
i. Layer of inner and outer segments of rod and cones.
j. Retinal pigment epithelium lies immediately on Bruch's membrane and acts as support and phagocytic functions for the photoreceptors.

2. The small marginal blind zone (pars caeca retinae) covers the iris and ciliary process. This is a continuation of the neural retina beyond the ora serrata. This part of the retina does not contain photoreceptors.

The neural retina is vascularized by two terminal arteries: retinal and choriocapillaris vasculatures (Chan-Ling, 2008). They both are the distal continuation of the ophthalmic artery, which is the branch of the internal carotid artery. The first branch of the ophthalmic artery is central retinal artery, which passes through the center of the optic nerve to enter the eye bulb at the optic disc and immediately branches into superficial and deep retinal plexi. Initially, the inner (superficial) vessels lie between the vitreous body and inner limiting membrane, but peripherally, they pass deeper within the inner retinal layers (Ross, 1989), beneath the inner limiting membrane, in the ganglion and nerve fiber layers. The deep capillary plexus, located in inner nuclear layer or on either side of the inner nuclear layer (Gariano, 2010; Henrikson, 1997). Both superficial and deep capillary plexi supply the inner retinal layers (i.e. inner limiting membrane, ganglion cell, inner plexiform, inner nuclear and outer plexiform layers). Normally, these capillary plexi do not extend into the outer plexiform layer (Anand-Apte, 2010)(**Figure 4**).

Further distally, after branching to the central retinal artery, the ophthalmic artery is branched into the posterior ciliary artery, long posterior ciliary artery and short posterior ciliary artery: all enter the sclera and, through the choriocapillaris plexi, supplying the outer layers of the retina (including photoreceptors and retinal pigment epithelium layer), choroid, ciliary body, iris and also the fovea and its small surrounding area on macula. An important branch of the short posterior ciliary artery is the radial peri-papillary capillaries (RPCs) located superficially in the nerve fiber layer surrounding the optic disc and vascularizes the nerve fiber bundles (Chan-Ling, 2008). Exchange of plasma contents and wastes from the photoreceptors is regulated in part by the RPE, which serves as the outer retinal blood barrier.

The processing of light rays captured by the retina is an intricate process that involves many different electrochemical and biological processes with the image forming light traverses from anterior to the posterior of retina and all these processes are dependent upon normal retinal and choroidal vasculatures for physiological retinal homeostasis. Due to the tight space and sensitive neurons and supportive cells surrounding the retinal and choroidal capillaries, any abnormal formation of new vascularization (neovascularization) either of retinal or choriocapillaris may result in dire consequences such as retinal detachment.

Histology of the Retinal Capillary

Both blood vascular and lymphatic vascular systems form the human circulatory system. Starting with the heart as the pump, the blood vascular system continues as blood vessels that carry blood through the arteries, arterioles, capillaries, venules, and veins; and the lymphatic vascular system carries the lymph fluids through the lymphatic capillaries from the tissues to the large veins.

The vascular wall consists of endothelium, muscular tissue, and connective tissue, which altogether vary in thickness, arrangement and size according the locations, physiological functions, blood pressure and the local metabolic needs. The capillaries and post-capillary venules, however, have a slightly different vascular wall arrangement: these vessels consist of one layer of endothelium, basal lamina and pericytes. The structure of capillary is permissive for its functions of metabolic exchange between blood and the surrounding tissues with the diameter varies between about 5 to 10 μm, and if cut transversely, the wall consists of one layer of one to three endothelial cells covered on the outside with basal lamina, which is produced by endothelium itself. The endothelium is polygonal shape and elongated in the direction of blood flow. Its nucleus causes the cell to bulge into the capillary lumen with its cytoplasm contains organelles, including a small Golgi complex, mitochondria, free ribosomes, and a few cisternae of rough endoplasmic reticulum (McPhee, 2007). Zonula occludens junctions are present between most endothelial cells and allow permeability of fluid and macromolecules that play a significant role in physiological and pathological conditions. At various locations along capillaries and venules are cells that are of mesenchymal origin, called pericytes, with long cytoplasmic processes that partly surround the endothelial cells. Pericytes are enclosed in their own basal lamina, which may fuse with that of the endothelium's basal lamina. The presence of myosin, actin and tropomyosin in pericytes suggests that these cells also have a contractile function. After tissue injury, pericytes proliferate and differentiate to form new blood vessels and connective tissue cells, thus participating in the repair process (McPhee, 2007).

Human Retinal Vascular Development

Human embryonic eye development starts when the lateral evaginations of diencephalon appear at about the third week gestation of embryonic stage that later on become the optic vesicles. Optic vesicles grow laterally, connect to the diencephalon through the optic stalk, and come in contact with surface ectoderm to induce the formation of lens placodes. The lens placode then invaginates and develops into lens vesicle, which later invaginates together with optic vesicle. Optic vesicle separates into a two-layer optic cup: the inner layer of future neural retinal and the outer pigmented layer of future retinal pigment epithelium. By the 25 week gestation of embryonic life, the neural retinal already consists of the nucleated layers, cell processes layers, the specialized neurons of rods, cones, bipolar cells, ganglion cells and supporting cells (Sadler, 2004).

The vasculature of human retina starts to develop at about 14 - 16 weeks of gestation with the development of superficial plexus. As astrocytes radiate from the optic nerve head (i.e. optic disc) across the surface of immature retina in a central to periphery fashion, they form a scaffold upon which primary vascular plexus is formed (Provis et al., 1997). This astrocyte patterning is guided by radially oriented axon bundles of ganglion cells that produce the platelet-derived growth factor (PDGF). By 32 weeks post-gestation, the superficial retina capillaries will reach its outer limit at the retinal periphery, leaving a narrow border of avascular tissue at the periphery of retina.

The deep vascular plexus development begins in the perifoveal region between 25 – 26 weeks' gestation in human, which coincides with peak period of eye opening and when the visually evoked potential, an indication of visual pathway and photoreceptor activity, are first detected in the human fetus (Chan-Ling, 2008). This vascular plexus develops from the pre-

existing superficial vessels starting in the perifoveal region of the retina via angiogenesis, a process that is driven by augmentation of metabolic demand of maturing retinal neuron; thus, it has the same topographical development as photoreceptor maturation. In humans, the deep vascular plexus reaches the edge of the retina at birth, but the pericytes and mural cells do not appear until 2 months after birth. Normally, the infant's retinal vasculature development reaches the ora serrata at birth and achieves the adult pattern of vascular network by the third month after birth, histologically, the retinal capillaries are still immature for several years postnatal (Park, 2006). Since retinal vascular maturation is incomplete at the time of birth, retinal vasculature remains subject to postnatal developmental abnormalities, and preterm infants who have had less than 37 weeks of development, are especially prone to have abnormal retinal vasculature development.

Angiogenesis involves inputs from different cell types (neurons, glial and endothelial cells), cytokines, growth factors, retinal oxygen tension, acid-base balance and extracellular matrix (ECM) proteins. The balance between pro-angiogenic and anti-angiogenic factors determines the end result of vascular formation in physiologic or pathologic conditions. Angiogenesis begins first with the dissolution phase (activation phase), which begins with induction of proteolytic activation of pre-existing vessels endothelial cells (Behzadian et al., 2003). This brings about the breakdown of cell junctions, degradation of the underlying basement membrane and the penetration of activated endothelial cells to the surrounding tissue. This last step includes the proliferation and migration of endothelial cells towards the source of stimuli, the stimulus majority being ischemia and resulting hypoxia. Endothelial cells become elongate and assemble into a meshwork of interlaced cords. This dissolution phase will be immediately followed by the resolution phase (differentiation phase) that is marked by cessation

of endothelial cell proliferation but an increase endothelial remodeling to form the required lumen, reconstruction of vascular basement membrane for vessel stabilization and pruning of redundant connections. The interconnecting tubes and tufts are reconfigured to the prototypical vessel arrangement with branching and bifurcation. Pericytes are recruited and wrapped around the vessels at this phase.

Although VEGF is not the only factor in angiogenesis, it has a central role in vascular endothelial cell proliferation and thus essential in angiogenesis and has been shown to be released by a variety of retinal cells: astrocytes (Stone et al., 1995), Müller cells (Brooks, Gu, Kaufmann, Marcus, & Caldwell, 1998), RPE (Spilsbury, Garrett, Shen, Constable, & Rakoczy, 2000) , pericytes (Darland et al., 2003), vascular endothelial cells (Simorre-Pinatel et al., 1994), and ganglion cells (Sandercoe, Geller, Hendrickson, Stone, & Provis, 2003). Two-thirds of the inner retina superficial plexus is formed through vasculogenesis, while the remaining one-third (in addition to the formation of deep retinal vasculatures) is formed through the angiogenesis process. Although superficial retinal plexus is already formed by 18 weeks gestation, VEGF mRNA is not detected in human retina by in situ hybridization until 20 week gestation (Provis et al., 1997). This shows that while angiogenesis is dependent upon the presence of VEGF, vasculogenesis probably is not. The findings that knocked out mice for VEGF still have developing endothelial cells but knocked out mice for VEGFR2 are lacked of developing endothelial cells, suggest that there are additional molecules, binding to VEGFR2 that determine endothelial cell fate (Conway, Collen, & Carmeliet, 2001).

Physiological intra-uterine hypoxia has been proposed to promote endothelial cell proliferation and migration that induces the formation of capillary network across the developing neural layer along the inner surface of retina (Hughes, Yang, & Chan-Ling, 2000; Provis et al.,

1997; Stone et al., 1995). The fetal blood in the ascending aorta that is leaving the left ventricle has oxygen saturation of about 65% (Kiserud, 2005), compared to 90% in 10-minute old newborns (Kamlin, O'Donnell, Davis, & Morley, 2006; Kratky et al., 2012; Rabi, Yee, Chen, & Singhal, 2006).

Most human retinal vascular development is nearly complete at term birth; in other species, including rodents, retinal vascular development largely occurs postnatally. These significant differences must be taken into consideration in any retinopathy of prematurity (ROP) study using animal models. In humans, VEGF expression at the inner retina is not observed until about the 20 week of gestation. This is opposed to the RPE layer that has VEGF expression since much earlier age. The centrifugal growth pattern of the retinal vasculature following the maturation of retinal photoreceptors suggests that VEGF presence in the neural retina milieu is regulated temporally and spatially.

Multiple processes are necessary in angiogenesis, all of which require the interaction between cells and the extracellular matrix (Chung, Lee, & Ferrara, 2010). The matrix metalloproteinases (MMPs), a family of proteases important in degrading ECM proteins, are crucial for cell migration and tube formation during angiogenesis. Two MMPs crucial in neovascular formation are the gelatinases MMP2 and MMP9. The gelatinases MMP2 and MMP9 are produced in the retina by retinal pigment epithelium and endothelial cells. Human MMP9 (gelatinase B) is a 92kDa (pro-MMP9) or 82 kDa (active forms) zymogen. Pro-MMP9 is secreted by endothelial cells or RPE and can be activated by MMP3, tissue plasminogen activator (tPA) or by certain bacterial proteinases. Human MMP2 (gelatinase A) is secreted as a 72 kDa pro-enzyme (pro-MMP2). Both MMP2 and MMP9 degrade gelatin (denatured collagen) and type IV collagen, which is the major component of basement membrane (Zucker, Pei, Cao,

& Lopez-Otin, 2003). Tissue inhibitors of MMPS, the TIMPs, in turn regulate MMP activities. For the gelatinases, TIMP1 is the most important in that it inhibits both the pro- and active MMP9 forms. Studies comparing oxygen-induced retinopathy in MMP2-knockout, MMP9-knockout or wild type mouse or rat models suggested that MMP2 appeared to play a predominant role in regulating retinal neovascularization (Barnett et al., 2007; Ohno-Matsui et al., 2003). Chapter 5 of this thesis will demonstrate the comparison of total MMP2 and total MMP9 released from HRMVECs stimulated by IGF-1 both without and with PI3K pathway blocker Wortmannin to demonstrate the PI3K pathway role in the release of these two MMPs.

Molecular Mechanisms of Retinal Angiogenesis

The complex process of retinal angiogenesis in hypoxic condition has been strongly correlated with transcription factor regulator hypoxia inducible factor (HIF-1), a heterodimer (α and β subunits) that regulates the expression of genes that mediates adaptive response (Das & McGuire, 2003; Semenza, 2011) and in turn will induce the synthesis of VEGF.

VEGF and IGF-1

VEGF receptors (VEGFRs) are receptor tyrosine kinases (RTKs) with about 750-amino-acid residue at the extracellular domain that functions as the binding domain and dimerization domain. A single transmembrane region connects the extracellular domain to the intracellular tyrosine kinase domain with a C-terminus tail. VEGFR activation requires dimerization and will lead to autophosphorylation. VEGFR2 is the main VEGF receptor for signal transduction that will leads to angiogenesis and mitogenesis of endothelial cells. Ligand binding to the VEGF tyrosine kinase receptor leads to formation of functional dimeric receptors that causes phosphorylation of tyrosine residue in the activation lip of the other kinase. These

phosphotyrosine residues formed in the activated RTKs serve as binding or docking sites for SH2 domains or PTB domains.

VEGF is a 36-46 kDa heparin-binding glycoprotein that acts via endothelial-specific receptor tyrosine kinases: VEGFR1 (Flt1), VEGFR2 (KDR/Flk1) and VEGFR3 (Flt4); and non-tyrosine kinase receptors: neurophilin-1 and neurophilin-2. VEGF family consists of VEGF-A, VEGF-B, VEGF-C, placental growth factor (PlGF), VEGF-F and the viral VEGF homologue VEGF-E. PlGF does not affect embryonic angiogenesis in mice although PlGF contributes to angiogenesis under pathological conditions (Carmeliet, et al., 2001). Studies have demonstrated that VEGF-B is important in embryonic angiogenesis while VEGF-C is important in lymphangiogenesis. VEGF-B, VEGF-C and VEGF-D have been implicated in angiogenesis, although the full extent of their involvement is not fully understood. VEGF-A (the most extensively studied isoform, typically just referred to as VEGF) has the dominant role in the development of normal or pathological angiogenesis, acts as a potent endothelial cell mitogen that stimulates proliferation, migration, tube formation and vascular permeability. Alternate splicing on proximal site of mRNA exon 8 of the VEGF gene results in several variants: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ (Plate & Warnke, 1997; Robinson & Stringer, 2001). Alternate splicing on exon 6 and 7 of the VEGF gene determines the heparin binding activity thus will determine the solubility on the matrix. Both $VEGF_{189}$, and $VEGF_{206}$ have strong heparin binding, so they will remain sequestered in the extracellular matrix, while $VEGF_{121}$ has no heparin binding capacity, so it is secreted and highly soluble. $VEGF_{165}$ has intermediate heparin binding capacity. The expression of VEGF has been demonstrated through the activation of VEGFR1 and VEGFR2; while the neurophilins act as the co-receptor for some of the isoforms. The role of VEGF in retinal angiogenesis is evident through several ways,

including the spatiotemporal changes in VEGF and VEGFR distribution during retinal development, differential effects of VEGF isoforms, and alternate transduction of VEGFR activation on different endothelial cells (Gariano & Gardner, 2005). Together with other pro- and anti-angiogenic factors, VEGF sculpts the retinal angiogenesis in a complex mechanism.

Under hypoxic conditions, VEGF expression increase is mediated by several ways (Penn et al., 2008):

1. Up regulation of Hypoxia-inducible Factor-1 (HIF-1). HIF-1 is a transcription factor, which is composed of 2 subunits: HIF-1α (produced under hypoxic condition and highly O_2 labile) and HIF-1β (produced constitutively). Under normoxic condition, the HIF-1α is degraded through hydroxylation, however under hypoxic condition, HIF-1α will form a complex with HIF-1β and translocate to the nucleus and activate the hypoxia responsive element (HRE) on the 5'flanking region of hypoxia –inducible genes (e.g. VEGF gene) and stimulate transcription (Arjamaa & Nikinmaa, 2006; Toffoli et al., 2009).
2. Post translational events:
    a. Increase VEGF mRNA stability. *In vitro* studies have demonstrated that in hypoxic condition, mRNA half-life increases by 2 – 3 folds compared to normoxic condition due to the presence of HuR binding protein, which is an mRNA stabilizing protein. HuR binds to the adenylate/uridylate-rich elements (AREs) in the 3' untranslated region of VEGF mRNA, preventing it from degradation due to HuR's ability to affect the half-life of the mRNA by governing VEGF mRNA's decay rate.
    b. Formation of alternatively spliced VEGF through the action of IRES (internal ribosomal entry site) in 5' UTR of VEGF provides the control over which VEGF isoforms to be generated.

3. Increase expression of ORP 150 (oxygen-regulated protein 150), which is a molecular chaperone to facilitate VEGF protein transport and secretion.

The different mechanisms of hypoxia-induced VEGF expression are crucial in the formation of blood vessel in physiologic or pathologic conditions. The retinal vascular formation is closely correlated with level of oxygenation *in utero*, and any disturbance to the delicate balance of oxygen blood level might affect the normalcy of retinal blood vessels formation. Beside oxygen levels, there are other factors that affect the vascular blood formation, such as the glucose level, blood pH and the presence or absence of other growth factors.

IGF receptors belong to the tyrosine kinase receptors and member of insulin receptor family. There is a 60% homology between IGF-1 receptor and the insulin receptor. IGF-1 receptor is a dimer receptor bound by disulfide bonds and consisting of two alpha subunits and two beta subunits. The α chains are located extracelullarly, while β subunits span the membrane and are responsible for intracellular signal transduction triggered by ligand stimulation. Mature IGF-1 receptor has a molecular weight of 320 kDa. Ligand binding to IGF-1 receptor, the alpha chains, will induce the autophosphorylation of the beta chains that will trigger a cascade of intracellular signaling that is cell type specific: mostly will lead to cell survival and cell proliferation. IGF-1 binds to at least two cell surface receptors: IGF-1 receptors (IGF-1R) and the insulin receptor. IGF-1 binds to IGF-1 receptor at a significantly higher affinity that to insulin receptor. Although IGF-1binds to insulin receptor100-fold less well compared to insulin, IGF-1 *in vivo* effects on insulin receptor activation is approximately 0.1 x the potency of insulin.

The actions of IGF-1 and IGF-2 are modulated, among others, by IGF binding proteins (IGFBPs) which functions to transport IGFs, regulate the IGFs' availability at the tissue, and IGF-receptor binding (Randolph, Yee, & Feldman, 1993). There are six distinct subgroups of

IGFBP: IGFBP-1 through IGFBP-6, based on conservation of gene (intron-exon) organization, structural similarity, and binding affinity for IGFs. It has been shown that IGFBP3 is the most abundant among the IGFBPs in the human circulation and serves as an important transporter of IGF-1 and IGF-2. Randolph et al. (1993) also provide evidence that primary culture of human retinal pigment epithelium express IGFBP3 and IGFBP6 genes and proteins.

IGF-1 primarily produced in the liver in adult life as a result of stimulation by the growth hormone, while in fetus and newborn babies, it is produced by the placenta and the amniotic fluid. Physiologic function of IGFs mainly is in the promotion of cell proliferation and inhibition of apoptosis. Almost every cell in the human body can be affected by the IGFs: kidney, liver, muscle, nerve, skin, lungs, cartilage and the formation of retinal vascularization. Hellstrom et. al. showed the critical role of the GH/IGF-1 system in human retinal vascularization by comparing the fundus photography of people with genetic defects in the GH/IGF-1 axis and control. They showed that patients with GH/IGF-1 axis defects had significantly low number of vascular branching points compared to controls, although there are significant differences in the tortuosity index for arteries or veins (Hellstrom et al., 2002).

Receptor Tyrosine Kinase Activation Pathways

Receptor tyrosine kinases (RTK) are class of enzyme-coupled receptors with intrinsic tyrosine kinase activity at the cytoplasmic domain; its activation will directly phosphorylate specific tyrosines on themselves and on a small set of intracellular signaling protein. Ligands for these receptors are growth factors, and include several of importance to the retina. These include epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), vascular

endothelial growth factor (VEGF), macrophage-colony-stimulating factor (MCSF), and nerve growth factor (NGF). RTK activation triggered by ligand-receptor binding initiates a cascade of intracellular events leading to activation of cytosolic kinases that will ultimately affect processes that are cell specific. Some of the cytosolic kinases move to the nucleus to phosphorylate one or more transcription factors (Alberts et al., 2007).

Ligands binding to the RTK receptors will stimulate the receptor conformational changes to form a functional dimeric receptor. Most RTKs are a monomeric (including VEGFR) while others are dimeric (including IGF-1 receptor and the ligand binding will activate these disulfide-linked dimers), but regardless of the mechanism through which ligand binds RTK into a functional dimeric state, the downstream activity is universal. The receptor dimerization will bring two intrinsic kinases together and phosphorylate each other on the cytosolic tyrosine residues of the receptors, and the activated kinase the will phosphorylate other tyrosine residues to form phosphotyrosine, this is known as trans-autophosphorylation. The trans-autophosphorylation will trigger the cascade of signaling protein activations which combinations are dependent upon the type of RTK activated and thus will lead to different pathways and responses. Thus autophosphorylation of the activated RTKs will lead to two important steps: first, phosphorylation of tyrosine in the kinase domain that enhances the kinase activity of the receptor, and second, phosphorylation of tyrosine outside the kinase domain that will create high affinity docking sites for signal transduction proteins (e.g. SH2 domain-containing adapter proteins such as GRB2) that will then trigger the downstream signaling pathway (Lodish et al., 2007).

The adapter protein GRB2 binds to a specific phosphotyrosine on an activated RTK through its SH2 domain; and to SOS protein through its SH3 domains to bring a GTPase switch

protein, Ras, closer to be activated (inactive Ras-GDP to an active Ras-GTP). The Ras activation promotes the formation of three sequentially acting protein kinases (Raf, MEK and MAP kinase) that are associated with a scaffold protein located at the membrane of signaling complexes that culminates in the activation of MAPK, a serine/threonine kinase also known as ERK.

Activated MAPK then translocates either directly to the nucleus or through the activation of other kinase (e.g. $p90^{RSK}$), which then moves to the nucleus. Both the MAPK and the activated kinase $p90^{RSK}$ will phosphorylate certain transcription factors (e.g. ternary complex factor (TFD) and serum response factor (SRF)) and stimulate the gene transcription (e.g. c-Fos gene). In this example, c-Fos gene induces expression of proteins necessary for cells to progress through the cell cycle. The scaffolding proteins that prevent the activation of other pathways' same-shared components activated by different ligands stabilize ras-MAPK cascade-specific components. Scaffold proteins for MAPK pathways are well documented in yeast, fly and worm cells but not in mammalian cells. The dose-dependent ERK/ MEK signaling pathway activation by VEGF has been suggested to be important in bovine retinal endothelial angiogenesis process through gene expression and cell proliferation, but not in tube formation (Bullard, Qi, & Penn, 2003).

Activated RTKs and cytokine receptors can also initiate signaling pathways that involve membrane-bound phosphorylated inositol lipids (phosphoinositides). Inositol 1,4,5-triphosphate (IP3)/1,2 diacylglycerol (DAG) signaling pathway is activated when the SH2-domain containing phospholipase γ (PLC γ) is bound to activated RTKs. Enzyme PLC γ is thus brought closer to its membrane-bound substrate PIP2 (phosphatidylinositol 4,5-bisphosphate) which then cleaved to generate two second messengers: DAG and IP3. The IP3/DAG pathway leads to the increase of

cytosolic calcium and protein kinase C (PKC) activation respectively. Cytosolic calcium will increase prostaglandin production and thus increasing vascular cell permeability; while PKC activation was hypothesized to be involved in the activation of Raf, MEK and MAPK pathway that leads to gene expression and cell proliferation in angiogenesis process. Another phosphoinositides pathway can be initiated by the binding of phosphoinositides-3 kinases (PI3K) through its SH2 domain to the phosphotyrosine residues in the activated RTKs, leading to the formation of phosphatidylinositol 3-phosphates (= PI 3-phosphates) including phosphatidylinositol 3,4-bisphosphate (PIP2) or phosphatidylinositol 3,4,5-triphosphate (PIP3). PI 3-phosphates function as the docking site for various signal-transducing proteins, especially protein kinase B (PKB), also known as Akt. Full activation of PKB requires recruitment of another kinase: PDK1, and will lead the dissociation of PKB from the plasma membrane and phosphorylation of many target proteins at the plasma membrane, cytosol or nucleus. The main final effects of PKB are promoting cell survival (anti-apoptotic) and cell growth. A selective PI3K inhibitor exerts anti-angiogenic effects in a dose-dependent manner in zebrafish retinal angiogenesis (Alvarez et al., 2009). PI3K has been shown to influence the PLCγ activation in a cell type- and primary activating event strength-dependent manner (Carpenter & Ji, 1999).

Src family kinases (SFKs) are membrane-attached non-receptor protein tyrosine kinases that link a variety of extracellular cues to intracellular signal pathways. SFKs are 52 – 62 kDa proteins. *In vivo* study on Sprague-Dawley ROP model showed that SFKs were highly phosphorylated at the activation loop Tyr^{416}, a phosphorylation site of Src family tyrosine kinase, in retinas developing pathologic angiogenesis but not in those undergoing physiological intraretinal vascularization. Thus SFK activation through Tyr^{416} dependent mechanism may be an important factor in the pathogenesis of retinal neovascularization (Werdich & Penn, 2006).

Proliferative Retinopathies

In proliferative retinopathies, abnormal retinal vascular formation has been found to be an important feature; therefore the understanding of the specific factors that contribute to the disease might be important for the prevention, treatments or establishment of potential treatments. Retinopathy of prematurity (ROP), diabetic retinopathy (DR) and age-related macular degeneration (AMD) are common conditions marked with abnormal retinal blood vessels formation that could lead to retinal detachment and blindness.

Retinopathy of prematurity (ROP) is the leading cause of blindness among newborns in developed countries. This visual morbidity is associated with premature birth (gestation age less than 37 weeks) and very low-birth weight (less than 1.5 kg). An epidemiological study by Lad, et al. (2009), found that from 34 million live birth babies from 1997 to 2005 in the US: 0.17% of overall and 15.58% of premature infants with length-of-stay of more than 28 days were found to have ROP. Since birth weight correlates strongly with gestational age at the time of birth, it has been also found to be a strong risk factor for ROP. According to the National Eye Institute (NEI), from approximately 28,000 babies born with a birth weight less than 1.25 kg in the United States each year, about 14,000 – 16,000 of them will develop some degree of ROP. Although about 90% of these ROP infants will develop the mild form that does not require treatment, 1,100 to 1,500 of them will develop the severe form of ROP that require medical treatment and 400 – 600 will become blind from ROP (NEI, 2009). Successful standard treatment modalities for active ROP are retinal ablation of avascular retina using laser photocoagulation or cryotherapy. The Multicenter Trial of Cryotherapy for ROP (CRYO-ROP) study showed long-term benefit of cryoablation treatment of threshold disease of ROP compared to no treatment (NEI, 2003). However, this study also found that even with cryoablation treatment, 44.4% of

treated eyes had a visual acuity of 6/60 or worse at 10 year follow up. Some infants still progress to retinal detachment despite timely and thorough ablation, requiring additional surgical treatment. A new treatment option that has been used in a small group of patients is anti-VEGF injection either intravitreal or into the anterior chamber of the eye. This treatment still needs to be fully studied, but currently may be used in advanced case patients with laser therapy or in patients with contraindications for laser treatment. Gene therapy by using gene transfer via an intravitreal injection of a control vector carrying the appropriate gene to express anti-VEGF locally has been reported to have promising results in animal studies (Clark & Mandal, 2008).

Pathogenesis of ROP is a complex process that begins with the disruption of retinal vascular maturation. This disruption is due to the enhancement of fetal blood oxygen level from intra- and extra-uterine environments as the fetus born prematurely (that could be exaggerated by high oxygen gas supplement). Since the maturation of retinal vasculature requires a physiological hypoxic environment *in utero*, the premature birth will disrupt the normal retinal vascularization maturation process. Other confounding factors that contribute to ROP are the premature withdrawal of maternally derived factors such as insulin-like growth factor-1 (IGF-1) produced by the placenta and amniotic fluid or ω-3 polyunsaturated fatty acids (PUFAs) (Heidary, Vanderveen, & Smith, 2009). IGF-1 serum levels in age-matched premature babies are directly correlated with the severity of clinical ROP and appear to be a strong determinant of risk for ROP together with gestation age at birth and birth weight. However, the mechanism(s) of IGF-1 in exerting its action to affect neovascularization is not fully established. Smith et al. (1999) suggested that IGF-1 regulation of VEGF action is mediated at least in part through the control of VEGF activation of p44/42 mitogen-activated protein kinase (MAPK). Another study demonstrated that low levels of IGF-1 prevent VEGF-induced activation of protein kinase B

(Akt) (Hellstrom et al., 2003). Both MAPK and Akt pathways are important signaling mechanisms for endothelial cell proliferation and survival; hence they are essential for angiogenesis. These findings demonstrate that while the role of IGF-1 in VEGF-dependent angiogenesis is crucial and recognized clinically, its molecular pathway in angiogenesis process is not yet clear. This thesis is designed to elucidate the mechanism of IGF-1 in affecting the VEGF-dependent angiogenesis process. The understanding of IGF-1 mechanism affecting the VEGF-dependent ROP neovascularization might eventually lead to the prospect of manipulating IGF-1 activity for ROP treatment purposes.

Diabetic retinopathy (DR) is the main cause of blindness in diabetic patients especially in the group with uncontrolled glucose levels. The prevalence of DR in diabetic patient and general US populations age 40 or older are about 28.5% and 3.8% respectively (Zhang et al., 2010). The pathophysiology of DR is assumed to be because of the dropping off of the pericyte cells surrounding the capillaries of the retina causing the retinal vessels to be porous and causing the vessels to be leaking, retinal capillaries occlusion, blot hemorrhages of blood, macular edema and at the later stage, neovascularization to occur (Antonetti, Klein, & Gardner, 2012). The pathophysiology of DR is a combination of pathologic vascular abnormalities, neuronal dysfunction, and the inflammatory reaction all due to abnormal glycemic environment (Antonetti et al., 2012). Several mechanisms have been found to contribute to the increase vascular permeability. Hyperglycemia-induced expression of protein kinase C will induce the Src-homology 2 domain-containing tyrosine phosphatase 1, that will inhibit the platelet-derived growth factor (PDGF) β signaling that will contribute to pericyte cell death (Geraldes et al., 2009). The hypoxic environment stimulates the up-regulation of angiogenic factors especially VEGF that will also contribute to the increase vascular permeability in retinal capillaries. VEGF

will activate protein kinase C that in turn will lead to ubiquitin-mediated endocytosis of tight junction components, occludin, and increase vascular permeability (Murakami, Felinski, & Antonetti, 2009). Another target of VEGF is the activation of tyrosine kinase Src that will likewise induce retinal vascular permeability (Scheppke et al., 2008). Plasma kallikrein system that leads to bradykinin-receptor activation (Gao et al., 2007), urokinase plasminogen activator (Yang, Duh, Caldwell, & Behzadian, 2010), and matrix metalloproteases 2 and 9 (Navaratna, McGuire, Menicucci, & Das, 2007) have been identified to contribute to induce vascular permeability and thus diabetic retinopathy.

Although the blood levels of IGF-1 in diabetic patients have been found to be clinically inconsistent, there has been evidence that local retinal tissue level of IGF-1may be more relevant in the pathogenesis of DR (Wilkinson-Berka, Wraight, & Werther, 2006). IGF-1 and IGF binding protein (IGFBP) have been found in all the cellular components of the retina in response to hyperglycemia or hypoxia, and IGF-1 has been shown to increase in the vitreal fluid in patients with proliferative DR (Boulton et al., 1997; Grant, Russell, Fitzgerald, & Merimee, 1986). Moriarty, et al have suggested that IGF-1 has an autocrine and paracrine roles in retinal pericytes that high glucose level will induce the pericytes to overproduce IGF-1 that will activate the IGF-1 receptors on pericytes themselves and the final effect is detrimental to the pericyte survival (Moriarty, Boulton, Dickson, & McLeod, 1994; Ruberte et al., 2004).

Age-related macular degeneration (AMD) is a progressive chronic disease of the central retina and a leading cause of vision loss worldwide (Lim, Mitchell, Seddon, Holz, & Wong, 2012). The dry form of the disease is characterized by macular drusen and alterations in the pigmented epithelium of the retina, while the intermediate to severe cases of the dry form are characterized by larger drusen and geographic atrophy, which can cause severe vision loss if the

fovea is involved (Noble & Chaudhary, 2010). The wet type age-related macular degeneration characterized by neovascularization beneath the retinal macula culminates in blood leakage resulting in irreversible damage to the photoreceptors. Most visual loss occurs in the late stages of the disease due to one of two processes: neovascular (wet type) AMD and the geographic atrophy (late dry type) (Lim et al., 2012). Although only about 10% of people with AMD have the wet type (characterized by choroidal neovascularization) compared to about 90% of dry type AMD (without choroidal neovascularization), the rapid visual loss when the neovascularization appears at the macula has recently clinically drawn the attention to VEGF as a potential therapeutic target (Lim et al., 2012). A recent FDA approved two-year study of wet AMD patients in which monthly versus as needed intravitreal injection with anti-VEGF [ranibizumab (Lucentis®) and the off-label use of anti-VEGF bevacizumab (Avastin®)] show significant visual acuity improvement. Both ranibizumab and bevacizumab are monoclonal antibodies targeted at VEGF A. These results are superior clinically compared to the more traditional treatments of wet AMD such as steroid or photodynamic therapy (Group et al., 2011). The current standard of care for subfoveal choroidal neovascular membranes secondary to AMD is intravitreal injection of an anti-vascular endothelial growth factor medication, which has been shown to stabilize visual acuity in 90% of patients, and up to 40% substantial improvements in vision (Noble & Chaudhary, 2010). Patients with extrafoveal choroidal neovascular membranes may benefit from argon laser photocoagulation (Noble & Chaudhary, 2010). On the other hand, the clinical results of anti-VEGF therapy on retinopathy of prematurity (ROP) are not as impressive, probably due to the complex pathogenesis of ROP, although the results are reported as encouraging, but unlike for AMD, the recommendation broad adoption use anti-VEGF treatment on ROP is not still not recommended (Recchia, 2011).

Unanswered Questions from the Literature

Although the angiogenesis mechanism has been studied intensively, the correlation molecular mechanisms of IGF-1 in angiogenesis or proliferative retinopathy have not been completely elucidated. Clinical evidence of strong correlation between the low blood level of IGF-1 in premature babies and the worse outcome of retinopathy of prematurity has been documented without any sufficient molecular explanation. The successful treatment of age-related macular degeneration (AMD) with anti-VEGF shadowed by the not so successful treatment of retinopathy of prematurity with the same approach (Recchia, 2011) probably could be explained by the importance of other factors besides VEGF, such as IGF-1 that play a role in blood vessel formation, at least in retinopathy of prematurity.

Specific Aims

To study the roles of IGF-1on blood vessel formation in human retinal microvascular endothelial cells (HRMVECs) by:

1. visualizing the effects of IGF-1 on the tube formation on HRMVECs
2. determining the effects of IGF-1 on the expression of HIF-1α on HRMVECs
3. determining the roles of PI3K component of the tyrosine kinase pathway and HIF-1α with stimulation of IGF-1 on HRMVECs

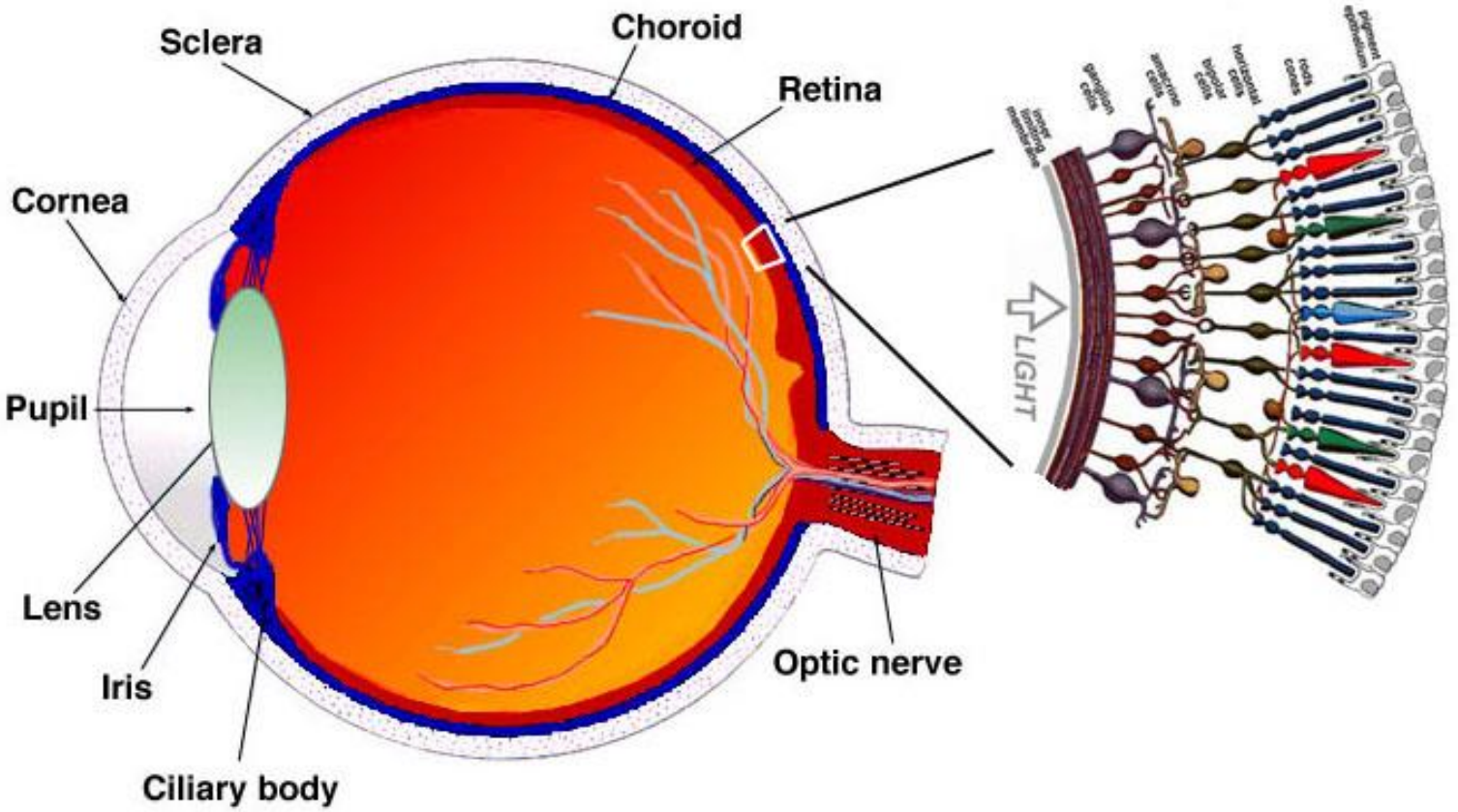

Figure 1: Cross section of the human eye with the enlarged image of retinal layers.
Source: Webvision, http://webvision.med.utah.edu/(Kolb H., 2011b)

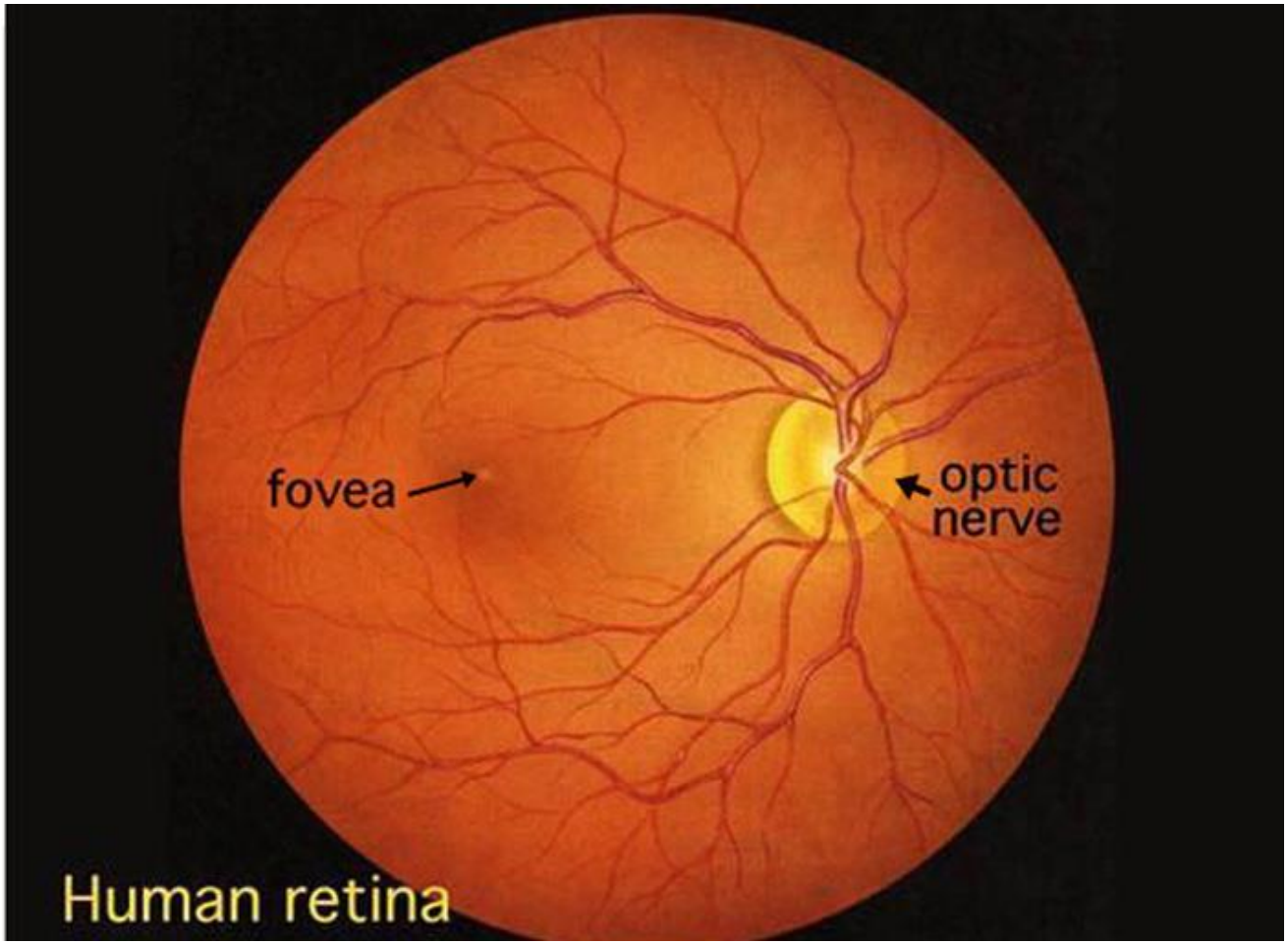

Figure 2: The retinal funduscopic photo of the right eye.
The central retinal blood vessels emerge from the optic disc on the medial (nasal) side of the retina. The fovea within the macula is shown in proximity of the center of the retina, lateral (temporal) from the optic disc.
Source: Webvision, http://webvision.med.utah.edu/(Kolb H., 2011b)

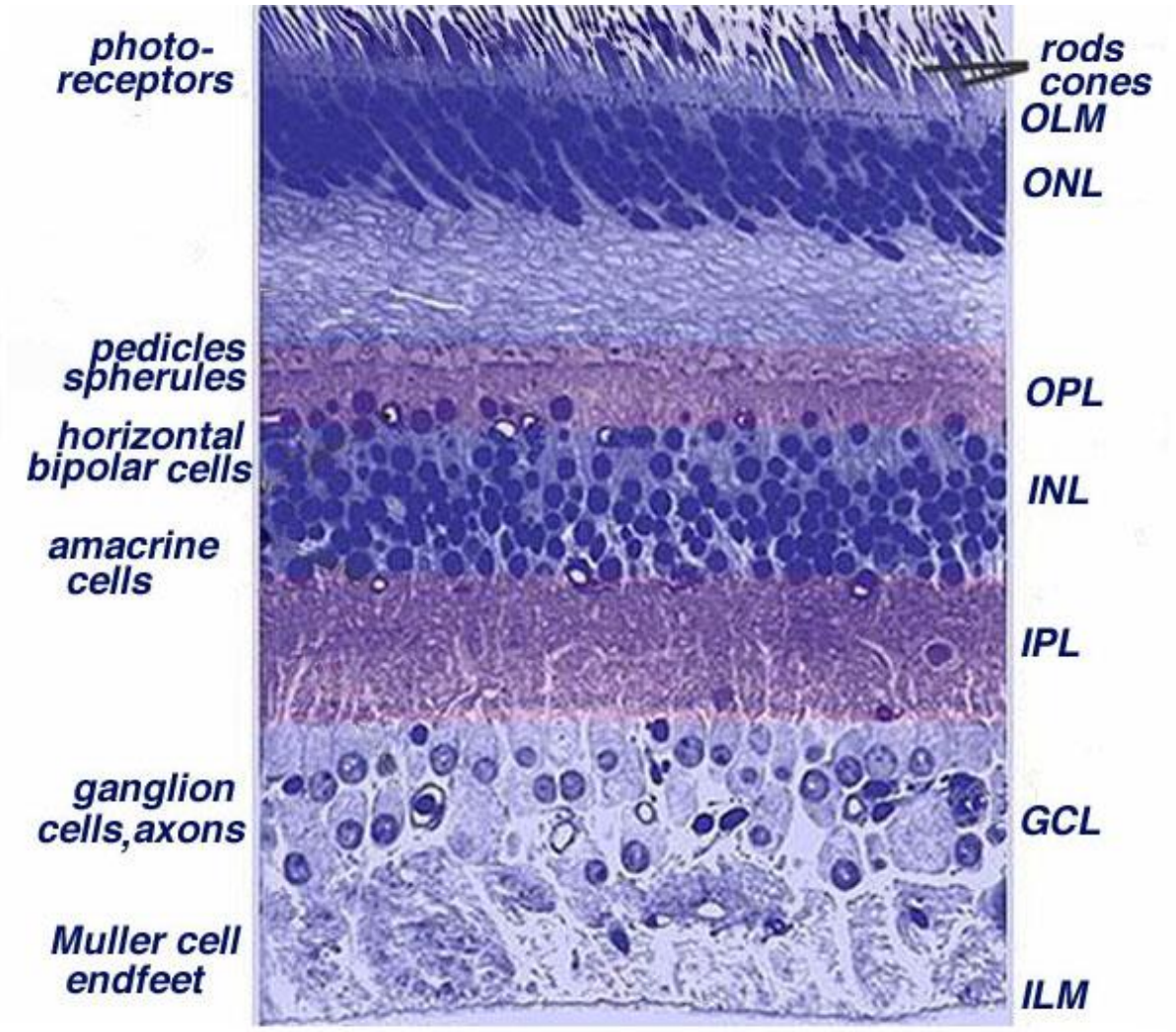

Figure 3: Layers of human retina, sectioned vertically through central part.
ILM = inner limiting membrane; GCL = ganglion cell layer; IPL = inner plexiform layer; INL = inner nuclear layer; OPL = outer plexiform layer; ONL = outer nuclear layer; OLM = outer limiting membrane.
Source: Webvision, http://webvision.med.utah.edu/(Kolb H., 2011b)

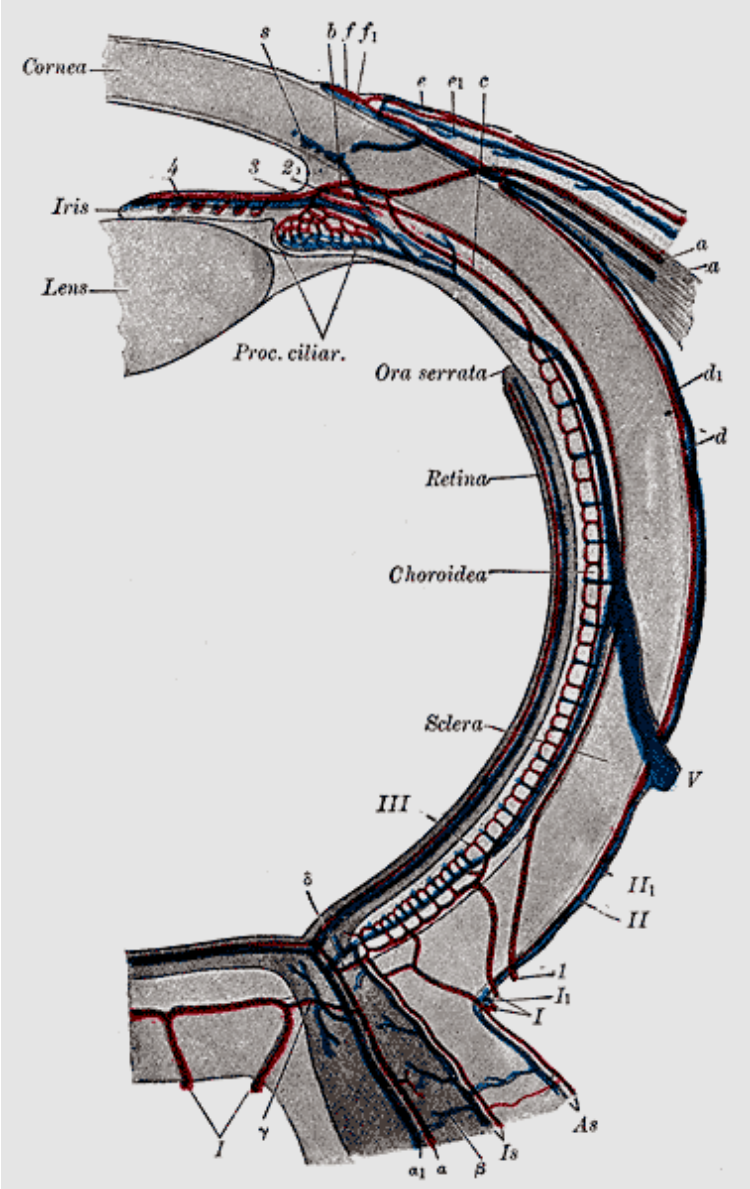

Figure 4: Horizontal section of the eye with its blood vessels.
Course of vasa centralis retina: *a.* Arteria. *a.1* Vena centralis retina. B. Anastomosis with vessels of outer coats. C. Anastomosis with branches of short posterior ciliary arteries. D. Anastomosis with choroidal vessels.
Course of vasa short ciliaris posterior: I. Arteriæ, and I1. Vena short ciliaris posterior II. Episcleral artery. II1. Episcleral vein. III. Capillaries of lamina choriocapillaris.
Course of vasa long ciliaris posterior: 1. a. ciliaris posterior. 2. Circulus iridis major cut across. 3. Branches to ciliary body. 4. Branches to iris.
Course of vasa ciliaris anterior: *a.* Arteria. *a*1. Vena ciliaris anterior. *b.* Junction with the circulus iridis major. *c.* Junction with lamina choriocapillaris. *d.* Arterial, and *d*1. Venous episcleral branches. *e.* Arterial, and *e*1. Venous branches to conjunctiva sclera. *f.* Arterial, and *f*1. **Venous branches to corneal border**. *V.* Vena vorticosa. *S.* Transverse section of sinus venosus sclera. Source: modified from Gray's Anatomy (Gray, 1918)

Chapter 2: IGF-1 Induces Upregulation of VEGFR2 in ARPE-19 Cells

Introduction

VEGF has a central role in angiogenesis (also called neovascularization), and its production by retinal pigment epithelium (RPE). VEGF is likely the main stimulator of choroidal angiogenesis during normal embryonic development. It is also implicated in certain retinopathies such as the wet type age-related macular degeneration (AMD), during which neovascularization beneath the retinal macula culminates in blood leakage resulting in irreversible damage to the photoreceptors. Although only about 10% of people with AMD have the wet type (characterized by choroidal neovascularization) compared to about 90% of dry type AMD (without choroidal neovascularization), the rapid visual loss when the neovascularization appears at the macula has recently clinically drawn the attention to VEGF as a potential therapeutic target (Lim et al., 2012). A recent FDA approved two-year study of wet AMD patients in which monthly versus as needed intravitreal injection with anti-VEGF [ranibizumab (Lucentis®) and the off-label use of anti-VEGF bevacizumab (Avastin®)] showed significant visual acuity improvement. Both ranibizumab and bevacizumab are monoclonal antibodies targeted at VEGF A, which irreversibly bind vitreal VEGF and make it ineffective. These results are superior outcomes compared to the more traditional treatments of wet AMD such as photodynamic therapy (Group et al., 2011). On the other hand, the clinical results of anti-VEGF therapy on retinopathy of prematurity (ROP) are not as impressive, probably due to the complex pathogenesis of ROP. Although the results are reported as encouraging but, unlike for AMD, broad adoption use anti-VEGF treatment on ROP as primary or adjunctive treatment is still not recommended (Recchia, 2011).

The role of VEGF and IGF-1 in ROP is significant (Chen & Smith, 2007): low postnatal IGF-1 levels in preterm infants also correlates with brain development and abnormal retinal function in ROP (Lofqvist et al., 2006) and directly correlates with the severity of clinical ROP (Hellstrom et al., 2001; Lofqvist et al., 2006; Smith et al., 1999). *IGF-1* null mice have a slower retinal vascular growth compared to wild type control, suggesting the permissive effect of IGF-1 in VEGF-dependent vascular formation and the maximum VEGF activation will not happen without the binding of IGF-1 ligand to its cognate receptor (IGFR) (Hellstrom et al., 2003). IGF-1 is also associated with the production of VEGF in by a RPE cell line (Punglia et al., 1997; Slomiany & Rosenzweig, 2004).

The VEGF receptor VEGFR2 (flk-1/KDR) is expressed on the blood vessel and lymphatic endothelial cells and the binding of its ligands (VEGF-A, VEGF-C, VEGF-D, and VEGF-E) is important in proliferation, migration, permeability and survival in vasculogenesis and angiogenesis (Holmes, Roberts, Thomas, & Cross, 2007). IGF-1 induces the release of VEGF through HIF-1α in ARPE-19 cell lines (Slomiany & Rosenzweig, 2006).

This study was designed to demonstrate the upregulation of VEGFR2 (flk-1/KDR) in ARPE-19 cells to serve as a cellular model for the induction of angiogenesis by IGF-1. Data from these studies could be applied to the choroidal layer of the eye or to the retinal capillaries.

Materials & Methods

ARPE-19, a spontaneously arising human retinal pigment epithelium cell line (ATCC, Manassas, VA) were cultured in two 4-well chamber slides using a Dulbecco's modified Eagles medium (DMEM)/Ham's F12 medium supplemented with HEPES buffer containing 20% fetal bovine serum, 56 nM final concentration sodium bicarbonate, and 2 mM L-glutamine. The

slides were incubated in a 37^0C incubator with 5% CO_2 and ambient O_2 until about 80% confluent. Afterwards, media in each chamber were replaced with serum free media for 24 hours, followed by treatment with 25 ng/ml human recombinant human recombinant IGF-1 (Abcam, Cambridge, MA) for 2 hours incubated in the same environment. Media were removed and cells were rinsed in phosphate buffered saline (PBS, pH 7.2), fixed in 4% paraformaldehyde in PBS for 15 and repeatedly rinsed in several washes with PBS. Non-specific antigenic sites were blocked in blocking buffer (10% normal goat serum (NGS), 2% bovine serum albumin (BSA), and 0.05% Triton X-100 in PBS at room temperature with gentle agitation. Primary antibodies (rabbit anti-VEGFR2 from Upstate or rabbit anti-HIF-1α from Millipore) were diluted in an antibody dilution buffer (ADB) containing 2% NGS plus 2% BSA in PBS. The dilutions were 1:200 for the anti-VEGFR2and 1:250 for the anti-HIF-1α. Cells were incubated in the primary antibody for 2 hr at room temperature with gentle agitation, rinsed in PBS, and then incubated in secondary antibodies diluted in ADB at room temperature for 1 hour with gentle agitation. The secondary antibody (Invitrogen®) was goat anti-rabbit IgG conjugated with Alexa Fluor 635 (1:200 dilution). Cultures were rinsed thrice with PBS (15 min each), then treated with 10 mM $CuSO_4$ in 50 mM ammonium acetate (pH 5.0) for 30 minutes to reduce autofluorescence. The cells were then washed with 50 mM ammonium acetate for 30 seconds 3 times. The cultures were then counterstained with Prolong® Gold antifade reagent with DAPI (Invitrogen) and covered (#1.5 cover glass), and finally the cover slips were sealed with nail polish. The cultures were examined by confocal microscopy (Leica SP2 AOBS) using a 405 nm excitation for DAPI and 635 nm excitation for the Alexa Fluor 635. Relative intensities for regions of interest were examined for both control and treated cells using the Leica software.

Data were then analyzed using t-test employing Statistica software with statistical precision set at a p value of < 0.05.

Results

The data obtained confirmed a significant up-regulation of VEGFR2 expression (**Figure 5**, **Figure 6** and **Figure 7**; $p<0.0001$) in IGF-treated cultures compared to untreated controls that suggested the upregulation of VEGF release. There was also significant increase in the expression of HIF-1α in the IGF-1-treated cells compared to controls (**Figure 8**, **Figure 9** and **Figure 10**; $p < 0.0001$). These results suggested that IGF-1 stimulation of ARPE-19 induces the release of VEGF from the RPE through the upregulation of HIF-1α transcription factor. The release of VEGF from the RPE further up-regulates expression VEGFR2.

Discussion

These data indicate that ARPE-19 cells treated with IGF-1 induce the up-regulation of VEGFR2, and thus support the idea that the induction of angiogenesis the retina by IGF-1 is VEGF-dependent, through the up-regulation of VEGF. As Hellstrom et al (2001) and Smith et al (2002) suggest that initially IGF-1 is low in early months of life in premature babies resulting in the lack of retinal development at the retinal periphery causing hypoxic microenvironment in the particular retinal area. When the IGF-1 increases to a normal level later in life, they suggest that there is a potentiation of VEGF-induced retinal vascularization that leads neovascularization of retinopathy of prematurity. Data from our study suggest that the induction of IGF-1 *in vitro* leads to the up-regulation of VEGFR2, which is the dominant VEGF receptor for angiogenesis,

and the permissive role of IGF-1 in angiogenesis has the possibility to potentiate the effect of VEGF-dependent angiogenesis.

HIF-1 transcription factor plays a crucial role in angiogenesis by regulating the expression of VEGF. In low oxygen environment, degradation of HIF-1α is inhibited leading to its accumulation and dimerization with the constitutively produced-HIF-1β, and translocate to the nucleus to activate the transcription of target genes. In normoxic environment, HIF-1α expression can be regulated by growth factors and hormones. In ARPE-19 cells, it has been shown that IGF-1 regulates HIF-1α expression through both PI3K/mTOR (Treins, Giorgetti-Peraldi, Murdaca, Semenza, & Van Obberghen, 2002), and MAPK (Treins, Giorgetti-Peraldi, Murdaca, Monthouel-Kartmann, & Van Obberghen, 2005) dependent pathways. Thus data from this *in vitro* experiment supports the permissive role of IGF-1 in inducing the VEGF-dependent angiogenesis through the up-regulation of HIF-1α in normoxic environment. This understanding might be crucial in understanding the basis of less than successful VEGF suppressive therapeutic studies of anti-VEGF treatment of retinopathy of prematurity

Summary

This study has been shown that IGF-1significantly upregulates VEGFR2 (flk-1/KDR) and HIF-1α in ARPE-19 cells *in vitro* that suggested the VEGF release from retinal pigment epithelium cells with IGF-1 stimulation is mediated by HIF-1α in normoxic environment. Since recent clinical trial results of the use of anti-VEGF on ROP have not been shown to be successful, we speculated that this is because of the role of IGF-1 that has been shown to be crucial in the pathogenesis in ROP needs to be addressed for the treatment of ROP.

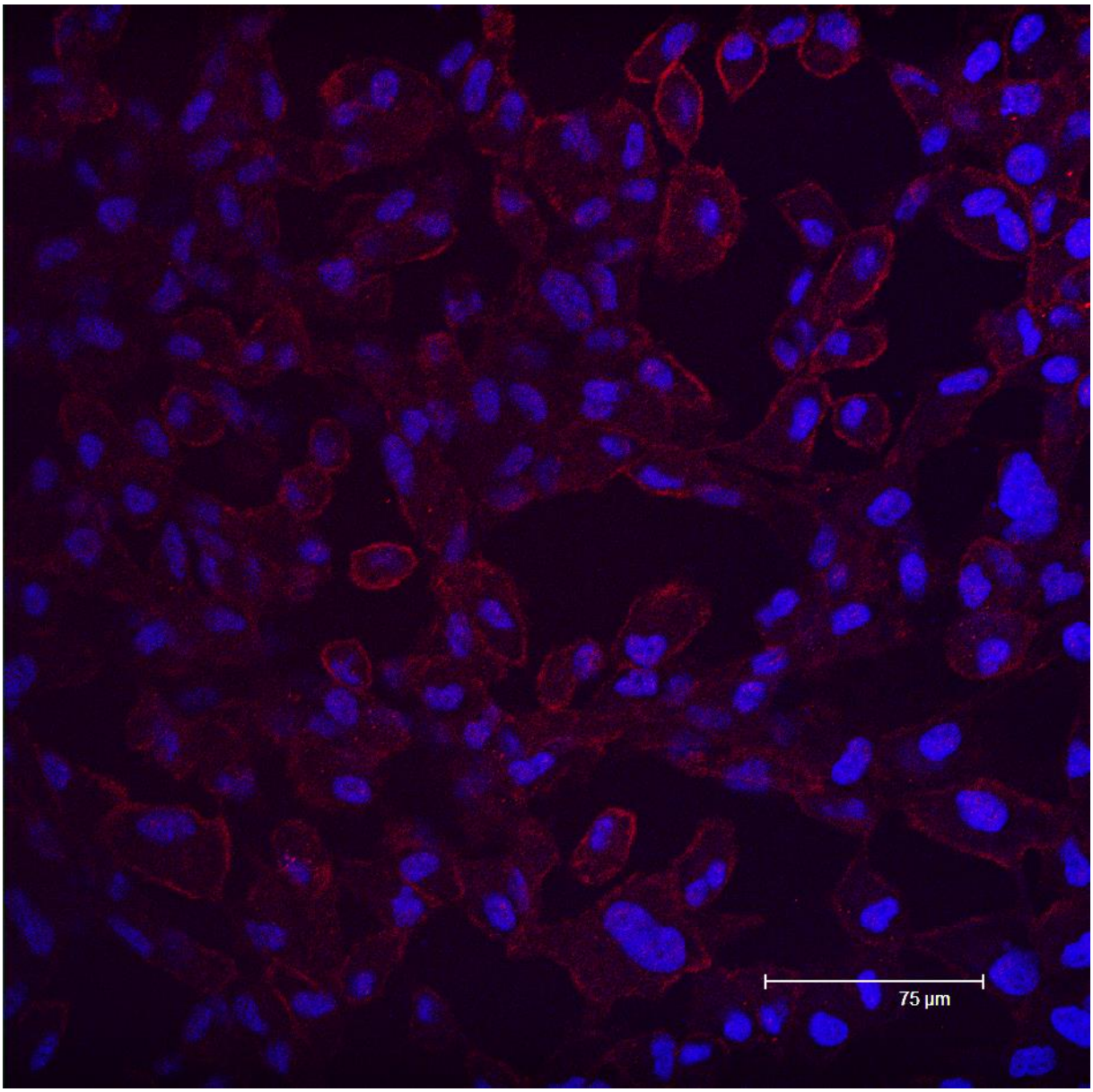

Figure 5: Confocal image of control ARPE-19 cells stained with anti-VEGFR2 antibody (red) and DAPI (blue)

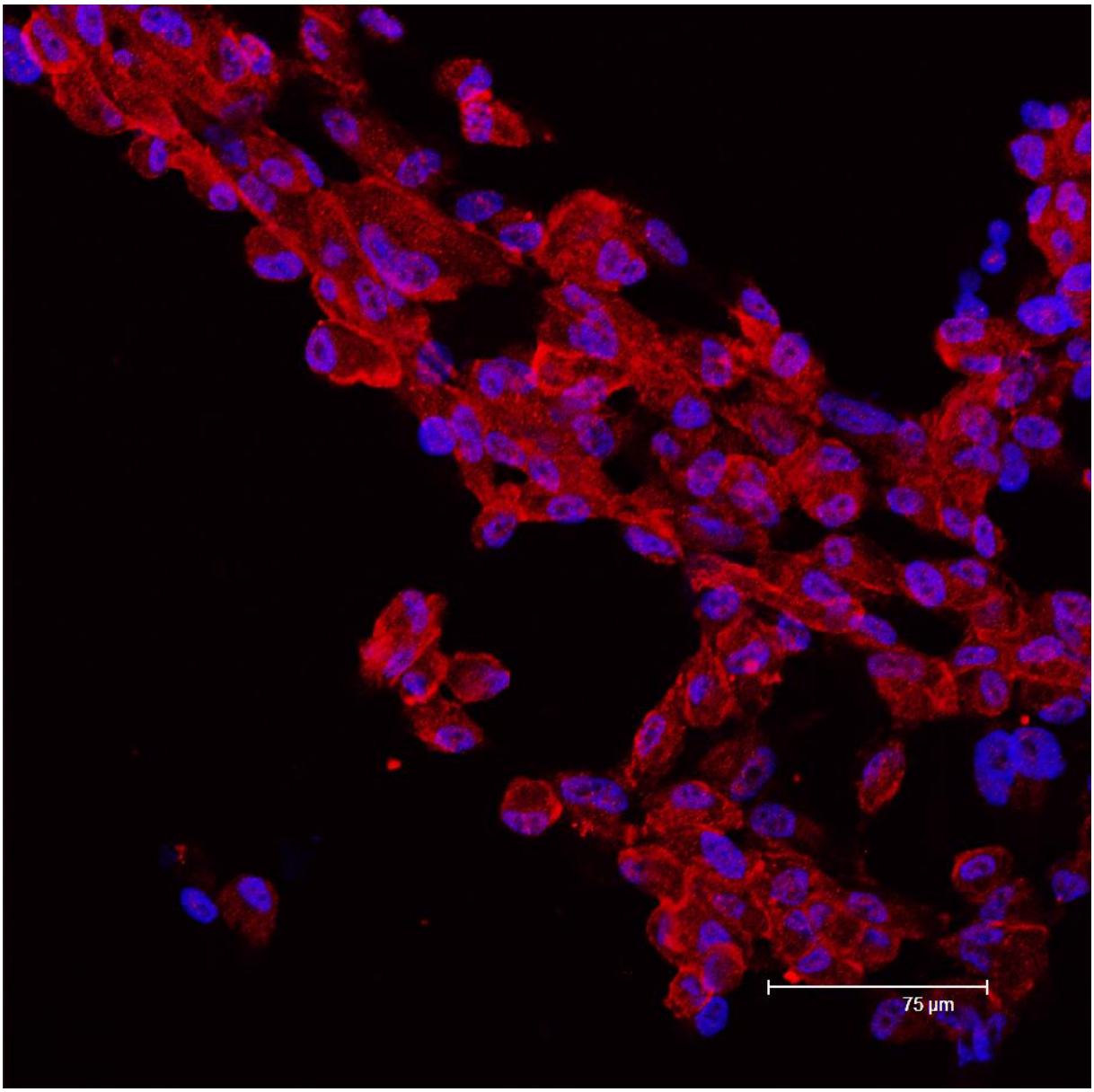

Figure 6: Confocal image of ARPE-19 cells treated with 25 ng/ml IGF-1 stained with anti-VEGFR2 antibody (red) and DAPI (blue)

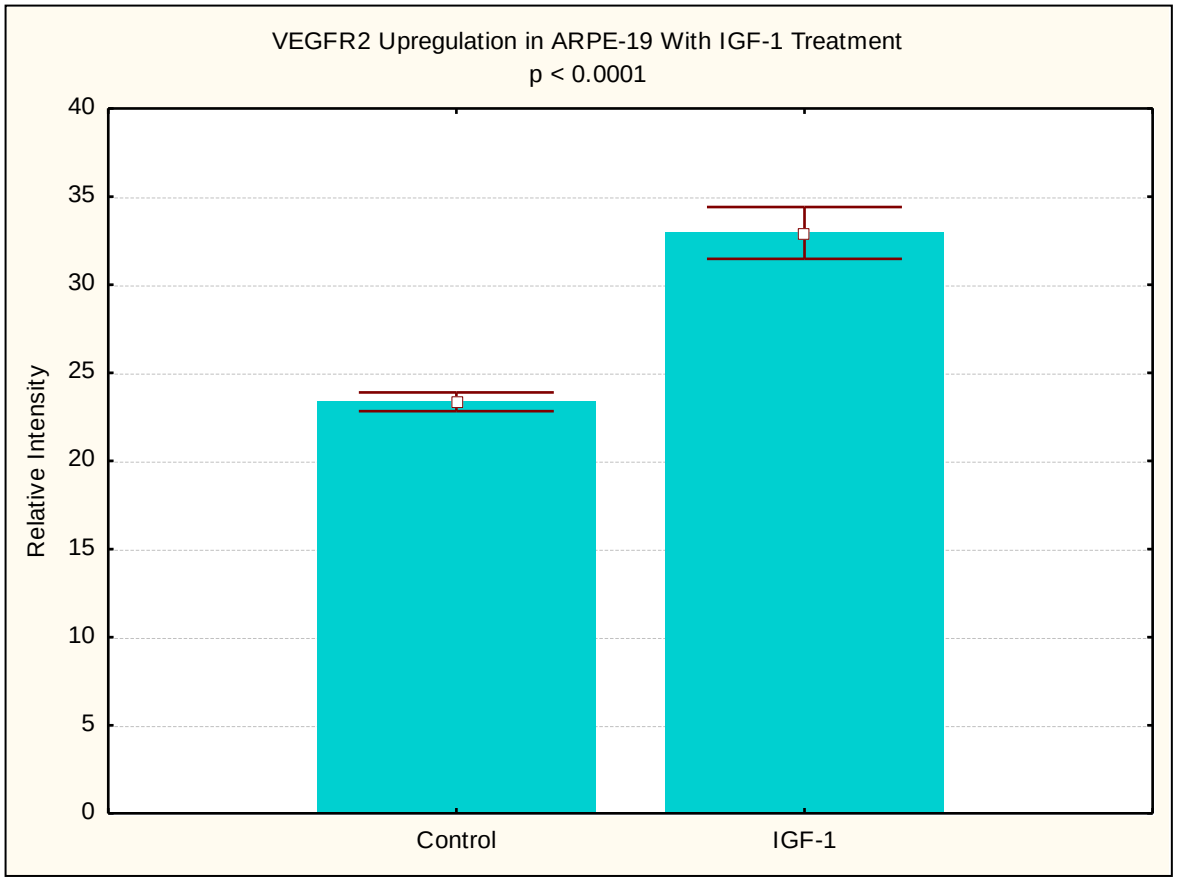

Figure 7: Quantitative graph comparing confocal relative absorbance of VEGFR2 between ARPE-19 cells control and treated with IGF-1

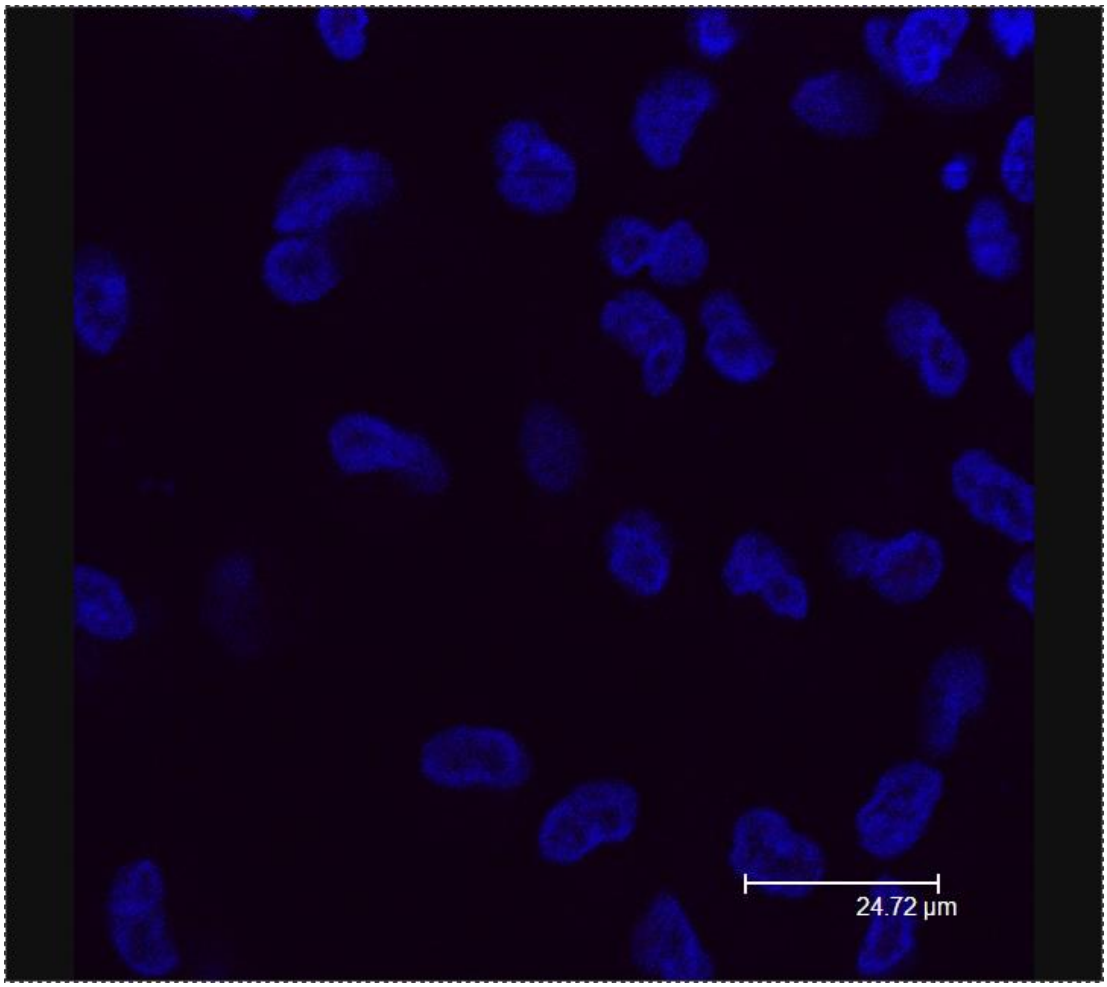

Figure 8: Confocal image of control ARPE-19 cells stained with anti-HIF-1α antibody (red) and DAPI (blue)

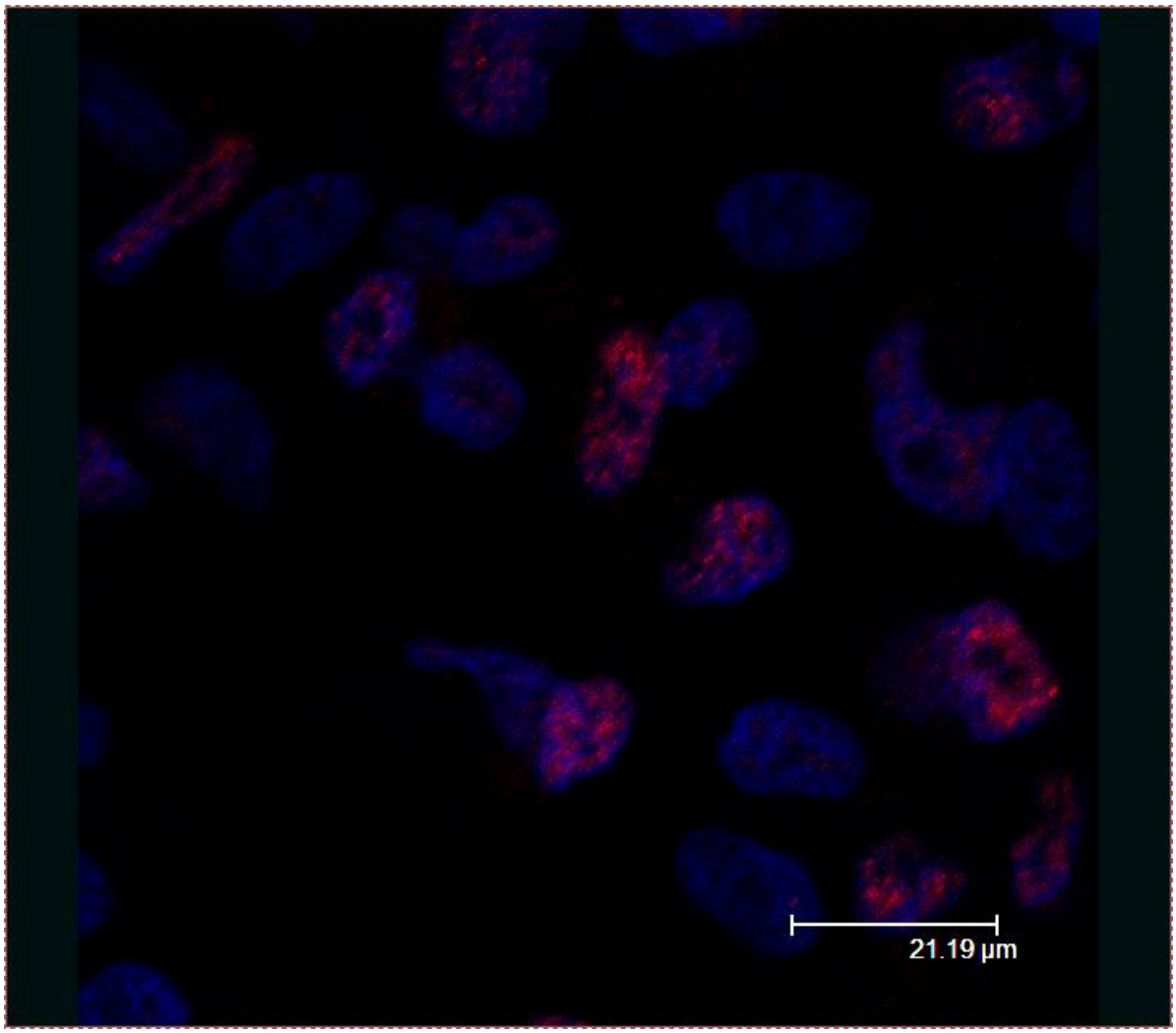

Figure 9: Confocal image of ARPE-19 cells treated with 25 ng/ml IGF-1 stained with anti-HIF-1α antibody (red) and DAPI (blue)

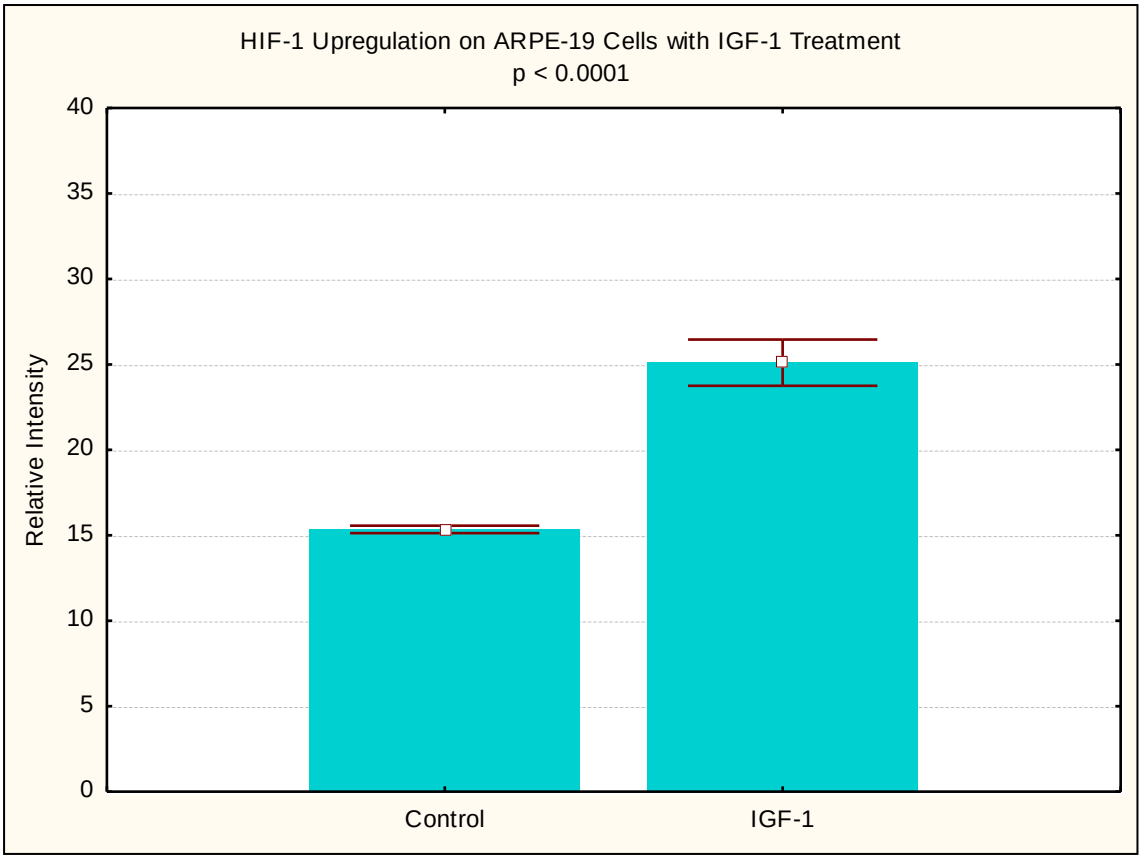

Figure 10: Quantitative graph comparing confocal relative absorbance of HIF-1α between ARPE-19 cells control and treated with IGF-1

Chapter 3: IGF-1 Induces Up-regulation of HIF-1α in HRMVEC

Introduction

The role of IGF-1 in the development of blood vessels in the retina was clinically shown with the correlation of low blood level of IGF-1 and the severity of retinopathy of prematurity in premature babies and babies born with very low birth weight. The clinically negative correlation effect has been used as the basis to hypothesize that IGF-1has an important role in the formation of normal blood vessels in the retina besides the importance of VEGF. Thus, we can imply that VEGF is important but not sufficient for normal blood vessel formation, since there are growth factors that are also necessary for a normal blood vessel formation in the retina.

Other related studies have found that IGF-1 has the capability to induce VEGF release from NIH3T3 mouse embryonic fibroblast cells and HCT116(h) colon cancer cells in PI3K- and MAPK-dependent manner (Fukuda et al., 2002; Miele, Rochford, Filippa, Giorgetti-Peraldi, & Van Obberghen, 2000), also in ARPE-19 cells in a PI3K-dependent pathway (Slomiany & Rosenzweig, 2006). These studies proposed that IGF-1 induces the up-regulation of VEGF through the receptor tyrosine kinase (RTK) pathways, thereby inducing angiogenesis.

The purpose of this study is to demonstrate that IGF-1 can induce the formation of VEGF and VEGFR2, and thus also angiogenesis. We use the human retinal endothelial primary culture cells as a model of the angiogenesis in the human retina. As far as we are aware, this experiment is the first to demonstrate directly the effects of IGF-1 on human retinal endothelial primary culture cells to determine the positive correlation of IGF-1 induction and the up-regulation of VEGFR2 through the PI3K pathway which is one of the receptor tyrosine kinase (RTK) pathways that are activated by IGF-1 and VEGF.

Materials & Methods

Cell culture. Human retinal microvascular endothelial cell (HRMVECs) primary cell line culture (ACBRI181) were obtained from Cell systems (Kirkland, WA) maintained in Cell Systems Corporation (CSC) complete media (Cell Systems, Kirkland, WA) which is a modified DMEM/F12 (1:1) supplemented with elevated pyruvate, glutamine, bovine serum albumin, and vitamins/amino acids. This media is activated with CSC culture boost-R (Cell Systems, Kirkland, WA), which contains human recombinant growth factors: FGF and EGF. The media is also treated with antibiotic treatment Bac-Off® (Cell Systems, Kirkland, WA), which contains fluoroquinone class antibiotic ciprofloxacin. The cells were cultured in a T25 flask that has its tissue culture surface freshly coated with Attachment Factor (Cell Systems, Kirkland, WA), which is an extracellular matrix product that promotes cell attachment to tissue culture surface and encourages correct polarity and cytoskeletal organization. The cells were incubated at 37^0C, 5% CO_2, room air O_2 and 100% humidity. The cells were fed 12-24 hours after seeding, and at least every 48 hours thereafter until 70 – 80% confluent. For this dissertation, HRMVECs were used before the passage 8, because according the HRMVEC's manufacturer manual after the 10^{th} passage the endothelial cells undergo morphological and functional changes that make them unsuitable for angiogenesis assessment.

Confocal microscopy imaging. HRMVECs were seeded at 5000 cells/cm^2 to a Lab-Tek II 8-chamber slides until 70-80% confluent, made quiescent with CSC complete serum free media for 18 hours and then treated for 120 minutes with 25 ng/ml human recombinant IGF-1 (Abcam, Cambridge, MA). After several washes with 1x PBS (Sigma-Aldrich, St. Louis, MO), fixation with 4% paraformaldehyde for 15 minutes followed with another set of 1x PBS washes, the cells were treated with primary antibody rabbit anti-HIF-1α (Millipore, Billerica, MA) in a

shaker for 2 hours at room temperature and then secondary antibody goat anti-rabbit Alexa Fluor 635 (Invitrogen, Carlsbad, CA) in a shaker for 1 hour at room temperature. All the primary and secondary antibodies are diluted in ADB. Chambers then washed with 10 mM $CuSO_4$ solution in 50 mM ammonium acetate (pH 5.0) for 30 minutes to reduce autofluorescence. After buffer wash, the nuclei were stained with DAPI (SlowFade® Gold; Invitrogen). The slides were examined by confocal microscopy (Leica SP2 AOBS) using a 405 nm and 635 nm excitation to observe the nuclei and HIF-1α respectively. Relative intensities for regions of interest were examined for both control and treated cells using the Leica software. Data were then analyzed using t-test employing IBM Statistica® software with statistical precision set at a *p* value of < 0.05.

Results

With the treatment of IGF-1 in the HRMVECs stained for HIF-1α using confocal microscopy, the relative intensities of the HIF-1α absorbance were compared between the control and IGF-1 treated. The HIF-1α is upregulated in the treated cells (**Figure 11** and **Figure 12**). The normalized relative intensities of the regions of interest of both the control and treated HRMVECs were shown to be significantly different with *p* value of < 0.05 (**Figure 13**). The HIF-1α relative intensities were normalized against DAPI-stained nuclei relative intensities.

Discussion

The induction of HRMVECs with IGF-1 has been shown to upregulates HIF-1α in several cell lines, including in ARPE-19 cells. In this experiment, it has been shown that IGF-1 will induce the upregulation of HIF-1α in HRMVECs that is in line with the proposed hypothesis

that the IGF-1 will induce the angiogenesis through the upregulation of VEGF even in the normoxic environment that is through the upregulation of HIF-1α. This *in vitro* experiment needs to be further shown in *in vivo* experiment since the release of HIF-1α might be a process that is dependent on several cells in the retina, including retinal pigment epithelium, astrocytes, and retinal pigment epithelium cells. The pathways that are important in this condition are the tyrosine kinase pathways. The PI3K pathway inhibition experiment in this case has been shown to be inconclusive due to technical reasons that should be pursued in the future.

Summary

The stimulation of HRMVECs with the IGF-1 induces the release of HIF-1α which is important in the process of angiogenesis. This has been proposed as the way angiogenesis happened in the normoxic environment because it will induce the release of VEGF in the developing or probably also in the pathologic conditions such as proliferative retinopathies.

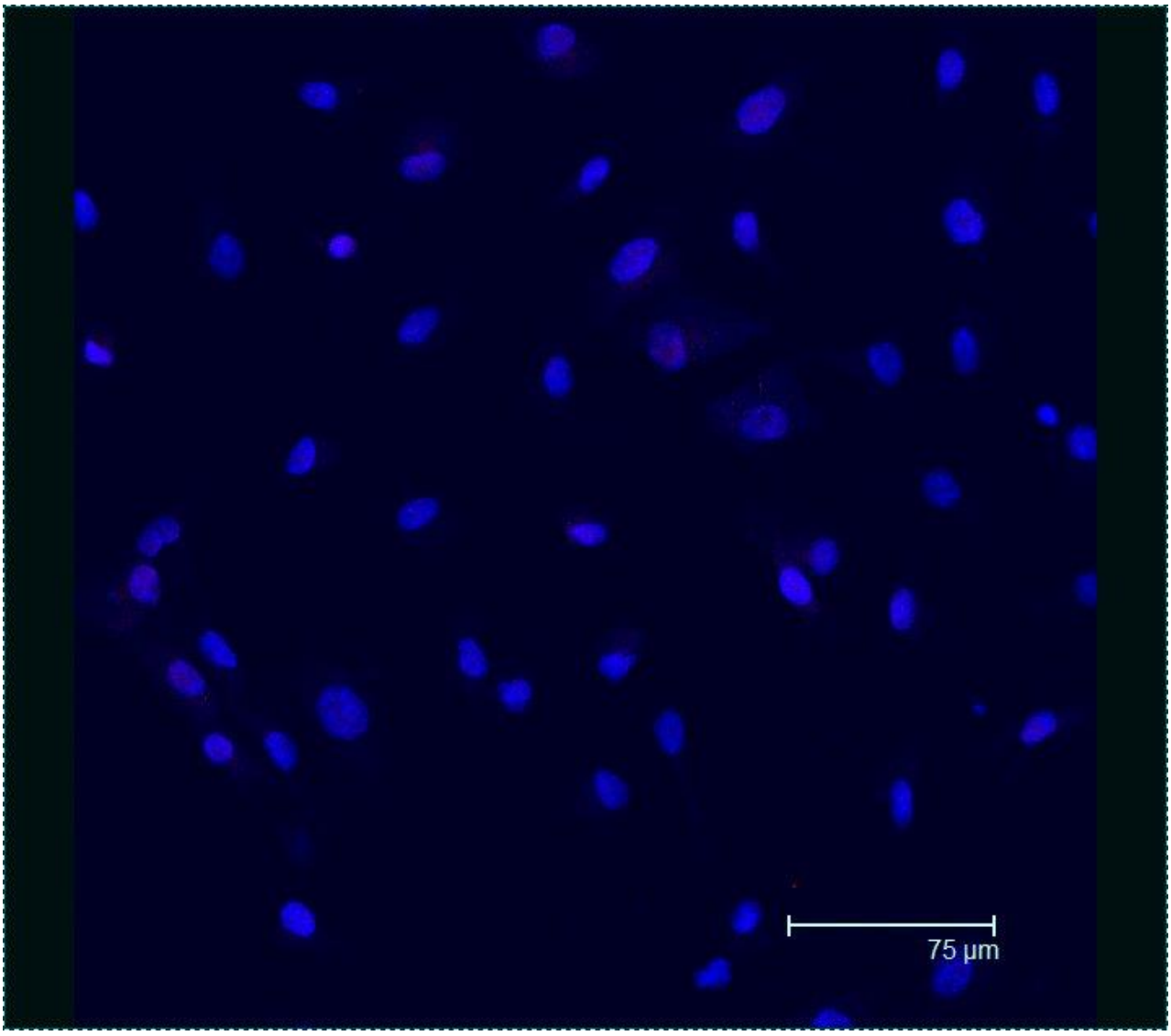

Figure 11: Confocal image of control HRMVECs stained with anti-HIF-1α antibody (red) and DAPI (blue)

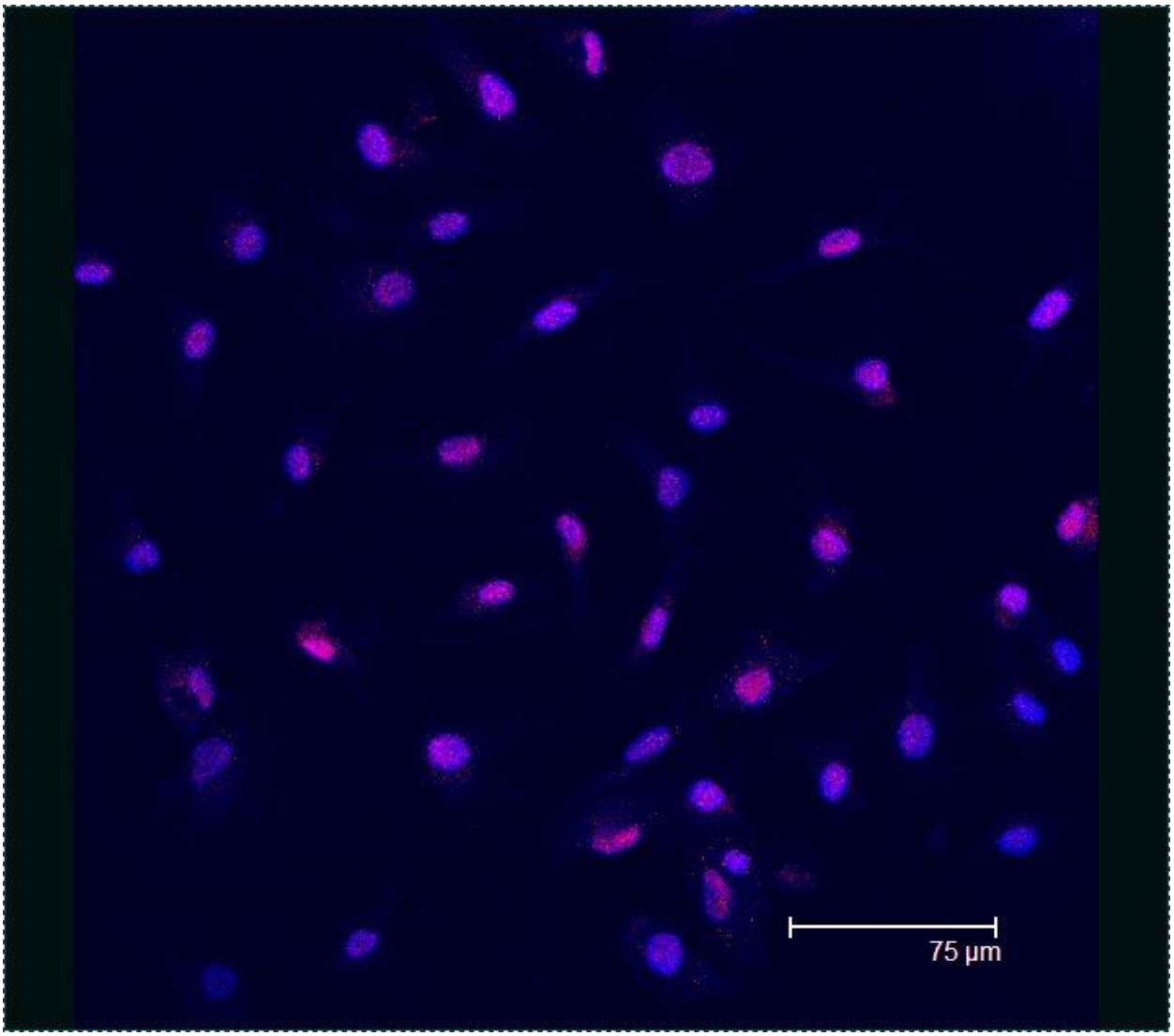

Figure 12: Confocal image HRMVECs treated with 25 ng/ml IGF-1 stained with anti-HIF-1α antibody (red) and DAPI (blue)

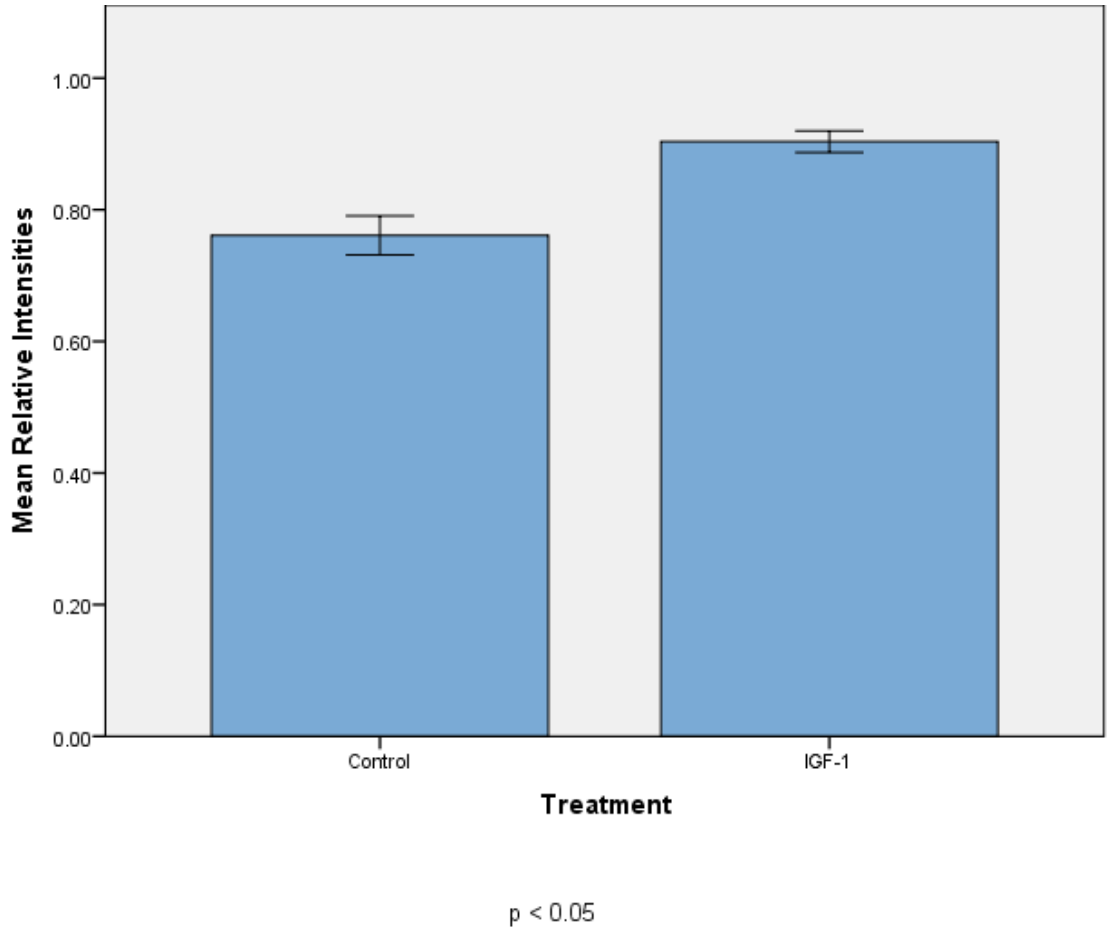

Figure 13: Quantitative graph comparing confocal normalized relative intensities of HIF-1α antibody between HRMVECs control and treated with IGF-1 25 ng/ml.
(Relative intensities of HIF-1α are normalized against the relative intensities of DAPI-stained nucleus on each cell in region of interests)

Chapter 4: Roles of IGF-1 in *in vitro* HRMVECs 3D Tube Formation

Introduction

The retinal vascular formation is a complex process that involves several cell types and a plethora of cytokines and growth factors. The balance between the pro-angiogenic and anti-angiogenic factors eventually results in the orderly formation of the retinal capillary network. The pro-angiogenic factors include vascular endothelial growth factor (VEGF), angiopoeitin-1 , basic fibroblast growth factor (b-FGF or FGF-2) , tumor necrosis factor alpha (TNFα), insulin-like growth factor-1 (IGF-1), and platelet derived growth factor (PDGF). The anti-angiogenic factors include endostatin, angiostatin, tissue inhibitors of metalloproteinases (TIMP), and pigment epithelial derived factor (PEDF) (Behzadian et al., 2008). Under physiologic conditions, angiogenesis proceeds in three distinct phases. Phase 1 starts with the resolution phase (activation phase) that is marked by the elongation of pre-existing vessels endothelial cells and is accompanied by the induction of proteolytic enzymes (name the matrix metalloproteinases or MMPs), followed by the breakdown of cellular junctions, increases in paracellular permeability, and degradation of the underlying basement membrane. These events are necessary so that proliferating endothelial cells are free to penetrate surrounding tissue by chemotactic migration along chemical concentration gradients towards the source of angiogenic stimuli. The major initial stimuli may include ischemia or hypoxia in developing retinas, which in turn stimulates the release of pro-angiogenic modulators into the microenvironment (Behzadian et al., 2008). The second phase, known as the resolution phase (differentiation phase), follows and is marked by the halting of endothelial proliferation, the onset of lumen formation, reconstruction of the vascular basement membrane, and the pruning of redundant connections that involves apoptosis.

Stage classification of *in vitro* angiogenesis of microvascular endothelial cells isolated from the mature and regressing bovine corpus luteum according to Bahramsoltani, Plendl (2004) and Lienau et al. (2005). These stages are as follows (Hirschberg, Sachtleben, & Plendl, 2005):

Stage 1. Confluent monolayer. After initial proliferative stage after cell seeding, the endothelial cells form a confluent monolayer.

Stage 2. Early phase sprouting. Less than half of the endothelial cells within the examined field of sight display long cellular projections (invapodial structures). The cell shape changes from polygonal to elongate.

Stage 3. Late phase sprouting. More than half of the endothelial cells within the examined field of sight display long cellular projections (invapodial structures).

Stage 4. Early phase linear and circular alignment: after development cellular projections, the cells from different areas of the culture dish align linearly or circularly. Less than half of the endothelial cells within the examined field of sight display alignment.

Stage 5. Late phase liner and circular alignment: more than half of the endothelial cells within the examined field of sight display alignment.

Stage 6. Network formation. Via elongation and alignment of cells, a fine network of cells is established. The network is comprised mainly of rows of single cells. In some areas, the cells involved in network formation are detached from the bottom of culture dish.

Stage 7. Early stage of three-dimensional organization. Formation of lumen structure of less than half of the culture dish bottom within the examined field of view is covered by attached confluent cells

Stage 8. Late stages of three-dimensional organization. The cellular strand within the capillary-like network grow in diameter, hardly any cells attached to the bottom of the well are detected within the examined field of vision.

The development of retinal vascularization is correlated to the level of VEGF in the microenvironment of the retina. VEGF sculpts the developing retinal vasculature in spatiotemporal changes of VEGF and its cognate receptor (VEGFR) distribution. To complicate the process, there are different VEGF isoforms, each with distinct spatial ranges of actions and guidance functions; there are alternating transductions of VEGFR activation on different endothelial cells, and we have to consider the interplay between VEGF and other pro-angiogenic and anti-angiogenic factors. This complex interplay provides opportunities to generate complex vascular networks.

VEGF isoforms are formed by alternative splicing of an eight axon *VEGF* gene and results in at least six forms. These six isoforms are designated by their amino acid length and include $VEGF_{121}$, $VEGF_{121}b$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{165}b$, $VEGF_{189}$, and $VEGF_{206}$. Interestingly, the two isoforms $VEGF_{121}b$ and $VEGF_{165}b$ reported to be anti-angiogenic may in fact not exist (Harris et al., 2012). Although VEGF is widely expressed in developing retina, its receptors (flk-1, flt-1, neurophilin 1 and 2) appear in a temporally and spatially distinct fashion. These receptors mediate discrete functions: proliferation, migration, guidance, survival, and permeability within the areas of VEGF expression; each receptor's action is limited by emergence of receptors and ligand availability and subtypes.

The effects of IGF-1 on angiogenesis is by directly influencing endothelial cell growth (differentiation, proliferation, protection from apoptosis, cellular transformation, and also in cancer progression) (Sehat, Andersson, Vasilcanu, Girnita, & Larsson, 2007) and also through

interaction with locally produced VEGFs (as permissive factor for maximum VEGF stimuli of angiogenesis). This has been shown by the correlation between low IGF-1 levels in serum with poor initial vascular development that leads to retinopathy of prematurity.

The activation of receptor tyrosine kinase by IGF-1 will lead to its receptor autophosphorylation and subsequent phosphorylation of intracellular proteins that will stimulate multiple signaling cascades including the phosphatidylinositol 3-kinase (PI3K) pathway. Inositol 1,4,5-triphosphate (IP3)/1,2 diacylglycerol (DAG) signaling pathway is activated when the SH2-domain containing phospholipase γ (PLC γ) is bound to activated RTKs. Enzyme PLC γ is thus brought closer to its membrane-bound substrate PIP2 (phosphatidylinositol 4,5-bisphosphate) which then cleaved to generate two second messengers: DAG and IP3. The IP3/DAG pathway leads to the increase of cytosolic calcium and protein kinase C (PKC) activation respectively. Cytosolic calcium will increase prostaglandin production and thus increasing vascular cell permeability; while PKC activation was hypothesized to be involved in the activation of Raf, MEK and MAPK pathway that leads to gene expression and cell proliferation in angiogenesis process. Another phosphoinositide pathway can be initiated by the binding of phosphoinositides 3 kinases (PI3K) through its SH2 domain to the phosphotyrosine residues in the activated RTKs, leading to the formation of phosphatidylinositol 3-phosphates (= PI 3-phosphates) including phosphatidylinositol 3,4-bisphosphate (PIP2) or phosphatidylinositol 3,4,5-triphosphate (PIP3). PI 3-phosphates function as the docking site for various signal-transducing proteins, especially protein kinase B (PKB), also known as Akt. Full activation of PKB requires recruitment of another kinase: PDK1, and leads to the dissociation of PKB from the plasma membrane and phosphorylation of many target proteins at the plasma membrane, cytosol or nucleus. The main final effects of PKB are promoting cell survival (anti-apoptotic)

and cell growth, thus leads to angiogenesis. A selective PI3K inhibitor exerts anti-angiogenic effects in a dose-dependent manner in zebra fish retinal angiogenesis (Alvarez et al., 2009). PI3K influences PLCγ activation in a cell type- and primary activating event strength-dependent manner (Carpenter & Ji, 1999). IGF-1 has been shown to stimulate HIF-1α accumulation, nuclear localization, HIF-1 activation and VEGF expression through a PI3K/mammalian target of rapamycin (mTOR) and MAPK-dependent pathways in ARPE-19 cells (Treins et al., 2005).

In these studies, we designed the experiments to demonstrate the effects of IGF-1 stimulation on HRMVECs 3D vascular network branching and tube formation *in vitro*.

Materials & Methods

Cell culture. Human retinal microvascular endothelial cell (HRMVECs) primary cell line culture (ACBRI181) were obtained from Cell Systems (Kirkland, WA) maintained in Cell Systems Corporation (CSC) complete media (Cell Systems, Kirkland, WA) which is a modified DMEM/F12 (1:1) supplemented with elevated pyruvate, glutamine, bovine serum albumin, and vitamins/amino acids. This media was activated with CSC culture boost-R (Cell Systems, Kirkland, WA), which contained human recombinant growth factors: FGF and EGF. The media was supplemented with the proprietary antibiotic Bac-Off® (Cell Systems, Kirkland, WA), which contained the fluoroquinone class antibiotic ciprofloxacin. The cells were cultured in a T25 flask whose culture surface freshly was coated with Attachment Factor (Cell Systems, Kirkland, WA), an extracellular matrix product that promotes cell attachment to tissue culture surface and encourages correct polarity and cytoskeletal organization. The cells were incubated at 37^0C, 5% CO_2, 21% O_2 of room air and 100% humidity. The cells were fed 12-24 hours after seeding, and at least every 48 hours thereafter until 70 – 80% confluent. For this dissertation,

HRMVECs were used before the passage 8, because after passage 10, the endothelial cells undergo morphological and functional changes that make them unsuitable for angiogenesis assessment.

3D Tubule formation assay. HRMVECs made quiescent in CSC complete serum free media (Cell Systems, Kirkland, WA) for 18 hours were seeded to solid matrigel-coated Lab-Tek II 8-chamber slides at ~5000 cells/cm^2 density. The growth factor-reduced matrigel matrix was obtained from BD Biosciences (Bedford, MA). HRMVECs were treated with 25 ng/ml human recombinant IGF-1 (Abcam, Cambridge, MA). The HRMVECs positive controls were grown in activated CSC complete media (Kirkland, WA), while the negative control was grown in CSC complete serum free media (Kirkland, WA). In separate experiments, inactivation of the PI3K pathway was achieved by pretreating quiescent HRMVECs with 100 nM Wortmannin (Sigma, St. Louis, MO) in medium for 2 hours before they were plated. Plating of cells and subsequent treatment with IGF-1 was as above. The cultures were incubated 37^0C, 5% CO_2, 21% O_2 of room air and 100% humidity for 16 hours. Tubular development was observed by an inverted light microscope with 40x objective and digitally photographed; the branching point quantification was done manually at the end of 16 hours. The manual counting was done by counting the branching of the tubes for each unit area of field of view; branches that were already counted were marked manually to prevent double counting. Statistical calculation was done using One-Way Anova with IBM SPSS statistics software.

Live Imaging of 3D Tubule formation in matrigel. Cultures were also prepared as above for long-term imaging. Tubular development was observed using a Live Cell Imager (Carl Zeiss) with brightfield, 10x objectives, 37.6^oC and room air O_2 incubation chamber temperature, at image acquisition interval of 1 hour for 24 hours. At the end of the 24-hour, the tube

formation was stained using the physiological fluorescent agent BCECF (2'-7'-bis(carboxyethyl)-5(6)-carboxyfluorescein) which gave contrast to the HRMVEC cytoplasm to visualize the tube formation by taking the stack snap shot images and compiling the images into a movie file.

Scanning electron microscopy. This experiment was designed to visualize the physical HRMVEC tube formation. HRMVECs were also prepared as above for observation by scanning electron microscopy (SEM), with the exception of plating of cells onto glass coverslips. The glass coverslips were placed in a sterile 24 well plate (Falcon, Franklin Lakes, NJ) with CSC complete serum free media (Cell Systems, Kirkland, WA) supplemented with 25 ng/ml human recombinant IGF-1 (Abcam, Cambridge, MA). The HRMVECs positive controls were grown in activated CSC complete media (Kirkland, WA), while the negative control was grown in CSC complete serum free media (Kirkland, WA). Separate experiments were done with the HRMVECs pretreated with 100 nM Wortmannin for 2 hours to block the PI3K pathway. The chamber slides were incubated 37^{o}C, 5% CO_2, 21% O_2 of room air and 100% humidity for 16 hours to give the chance for the cells to attach to the cover slip surface and forming the tubes. Afterwards, the media were suctioned from the wells and the cells were fixed with Karnovsky's fixative (4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) and 1% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in 0.1M cacodylate buffer) for 2 hours, and then rinsed in 0.1M cacodylate buffer solution (Electron Microscopy Sciences, Fort Washington, PA) three times for 10 minutes each time. The specimens were then dehydrated in ascending ethanol series (diluted from 200 Proof, absolute, anhydrous ACS/USP grade (Pharmco-AAPer, Brookfield, CT)) (50%: 15 minutes; 75%: 15 minutes; 95%: 15 minutes twice; 100%: 30 minutes twice), before finally incubated in solution of 100% ethanol and

hexamethyldisilazane reagent (HMDS) (Electron Microscopy Sciences, Fort Washington, PA) with 1:1 ratio (v/v) for 5 minutes, and then 100% HMDS for 10 minutes. HMDS was decanted; the cells were then drizzled with one drop of HMDS, and afterwards left under the extractor hood for air-drying. The glass coverslips were removed from the 24-well plate well with fine tip tweezers, and mounted onto aluminum stubs using double-sided tape. The stubs with the samples on it were sputter-coated with gold palladium (10 nm) and SEM images were obtained with Amray1910 field emission scanning electron microscope.

Results

The 3D tube formation assay experiments results are shown on **Table 1**. The statistical calculation using One-way ANOVA with IBM SPSS 20 statistic demonstrates that there were no significant differences statistically in the branching point formations among treatment groups except for the negative control without the Wortmannin. The Tukey HSD post hoc test was used to make pairwise comparisons among the individual treatment means, with the family wise significance level set at 0.05. The test confirmed the differences between the negative control without Wortmannin mean and other groups of HRMVECs with and without Wortmannin. The negative control without Wortmannin group is the only group with significant difference compared with the other groups. The conservative *p-values* for the differences between the negative control without Wortmannin group and those for positive control without Wortmannin, IGF-1 without Wortmannin, positive control with Wortmannin, negative control with Wortmannin, and IGF-1 with Wortmannin groups are < 0.0001, < 0.011, 0.0001, 0.001, and 0.0001, respectively. The mean branching point formation in the negative control without

Wortmannin is lower than the means of other treatments with statistical significance of $p < 0.05$ (**Figure 20**).

From the 3D live imaging experiment, the positive control montage is shown on **Figure 21**, it shows the stages of network formation of HRMVECs until the formation of point branches and honeycomb appearance. Since the lowest available live imager magnification available was the 10X, it was impossible to view the live network formation in a larger field of view. BCECF was used as a cytoplasm fluorescent contrast to view the tube formations. Digital image stacks of BCECF-stained HRMVECs' cytoplasm were obtained from different level of depths and then reconstructed into an animation movie. The positive control shows the complete tube formation compared to the negative control and also to the IGF-1 treated group (**Figure 22**, **Figure 23** and **Figure 24**). The movie animations of HRMVECs 3D live imaging experiments are recorded on the attached DVD (best viewed with QuickTime Player).

The scanning electron microscopy results demonstrate the branching and tube formations. The tube formation and branching formation in the positive control without Wortmannin appears to be smooth and well organized compare with other treatment groups, except in the negative control with Wortmannin group that appears with 'broken' or incomplete branches and cord connections between the honeycombs on top on incomplete tubing formations. The tube formation can be better observed on the higher magnification on the right side of the figures. On the positive control group, the tube formation can be seen forming an almost perfect tube form compared to other groups. The web formation on the positive control appears to be a perfect "honeycombed" appearance (**Figure 25**), as opposed to the other groups that form a less than perfect "honeycombed" appearance or some broken strings of web connections (**Figure 26**, **Figure 27**, **Figure 28**, **Figure 29** and **Figure 30**). The strings that connect between the

honeycombs from the positive control group also appear thicker and well defined compared to other groups, with the negative control with Wortmannin appeared to be the poorest.

Discussion

The data from this thesis showed that the presence of fully activated media, which is supplemented with the hormonal level of fibroblast growth factor (FGF) and epidermal growth factor (EGF), both are activating tyrosine kinase pathway, and angiogenic in nature. The supplementation is necessary for the endothelial cells to survive *ex vivo*. In this way, the positive control of this thesis experiments show the closest condition to *in vivo* environment as comparison. The negative control and the IGF-1 treatments are using the serum free media without any growth factors supplementations. From the branching point (tube formation assay) experiment, the IGF-1 treated group seems to be able to produce the branching points in the same level quantitatively as the positive control group, but at significantly different from negative control, and it seems like it is not PI3K pathway-dependent (**Figure 20**). This might be explained that the HIF-1α and VEGF up-regulation from IGF-1 stimulation might also happen through the MAPK pathway at least it has been shown in ARPE-19 cells (Treins et al., 2005). From the scanning electron microscopy experiment, the data showed that the network and tube formation in the positive control group has the finest formation which showed with the complete formed honeycomb appearance, and the thicker connecting endothelial projections (invapodial structures), and more a lumen-like formation compared to other groups. In other groups, the honeycomb appearance and the tube formation appears to be more "incomplete" compared to the positive control groups including the IGF-1 groups without Wortmannin that appeared to be able to form the honeycomb appearance, branching, and tube formation, but not as complete as the

positive control without Wortmannin. Furthermore, the negative control with Wortmannin appeared to have the poorest tube formation, cord connections between the honeycombs.

The 3D live imaging results with the BCECF staining were significant in showing the visual image of the tube formation. It appears that the positive control is the only one of the treatment group that shows a more complete tube-like formation, as seen from the picture montage of the video. As for the 3D live imaging without the BCECF, the results were limited with the available objective magnification of 10X that could not show larger field of view so that comparison between different treatment groups were inconclusive from the experiment. From these experiments, it showed that although IGF-1 treated HRMVEC group can form the same amount of branching, honeycomb network appearance, and tube formation, at least to the same level quantitatively as the positive control group, but qualitatively, they appeared to be not equal. Compared to the negative control, HRMVECs treated by IGF-1 appear to be significantly affected by the growth factor stimulation shown by the significant increase of branching point formation and the completeness of tube formation. This might establish a conclusion that IGF-1 would not be able to stimulate a sufficient functional vascular formation on its own without the contribution of other factors. VEGF that is assumed to be up-regulated with IGF-1 stimulation, in this case, probably is not sufficient to induce a normal quality vascular formation. This study might be further concluded that IGF-1 has crucial role in HRMVEC *in vitro* angiogenesis at the early stages of angiogenesis when the formation of honeycomb or branch network formation occurs, but at the later stage of *in vitro* angiogenesis, IGF-1 alone will not be sufficient to promote a normal tube formation.

The PI3K pathway seems to be not significant in the formation of branching points in HRMVECs angiogenesis, since the branching point formation in the 3D tube formation assay

study shows that there is no significant difference among the groups, except in the negative control without Wortmannin group, which has significantly lower branching points compared to other treatment groups. IGF-1 appears to be able to stimulate the formation of branching points without statistical significance compared to positive control group. This might be explained by the role of MAPK (Treins et al., 2005) in IGF-1 stimulation in angiogenesis. On the live imaging and scanning electron microscopy experiments, the PI3K inhibitor appears to have a role in the formation a complete tube formation. PI3K pathway seems has a dominant role in the later stage of angiogenesis, which is the tube formation stage, but it has a less importance in the early stage of angiogenesis.

Summary

The role of IGF-1 in angiogenesis is crucial that it is shown in this *in vitro* study to be able to induce the network, branching and tube formation quantity in HRMVECs that significantly higher to a growth factor-free environment-treated HRMVECs but equal to the positive control HRMVECs. Nevertheless, the qualitative difference in tube formation that is formed by IGF-1 stimulation alone is not sufficient to induce a normal tube formation. This might be interpreted that the importance of IGF-1 stimulation in the formation of fully functioning blood vessels is more crucial at an earlier stage of angiogenesis of branch formation, but not so much at the later stage of angiogenesis where tube formation occurs when the presence of other cytokines and growth factors are necessary. Furthermore, this HRMVECs angiogenesis might be a PI3K-dependent event especially during the tube formation; however, this pathway seems not the dominant pathway in the earlier stage of angiogenesis of honeycomb and branching point formation.

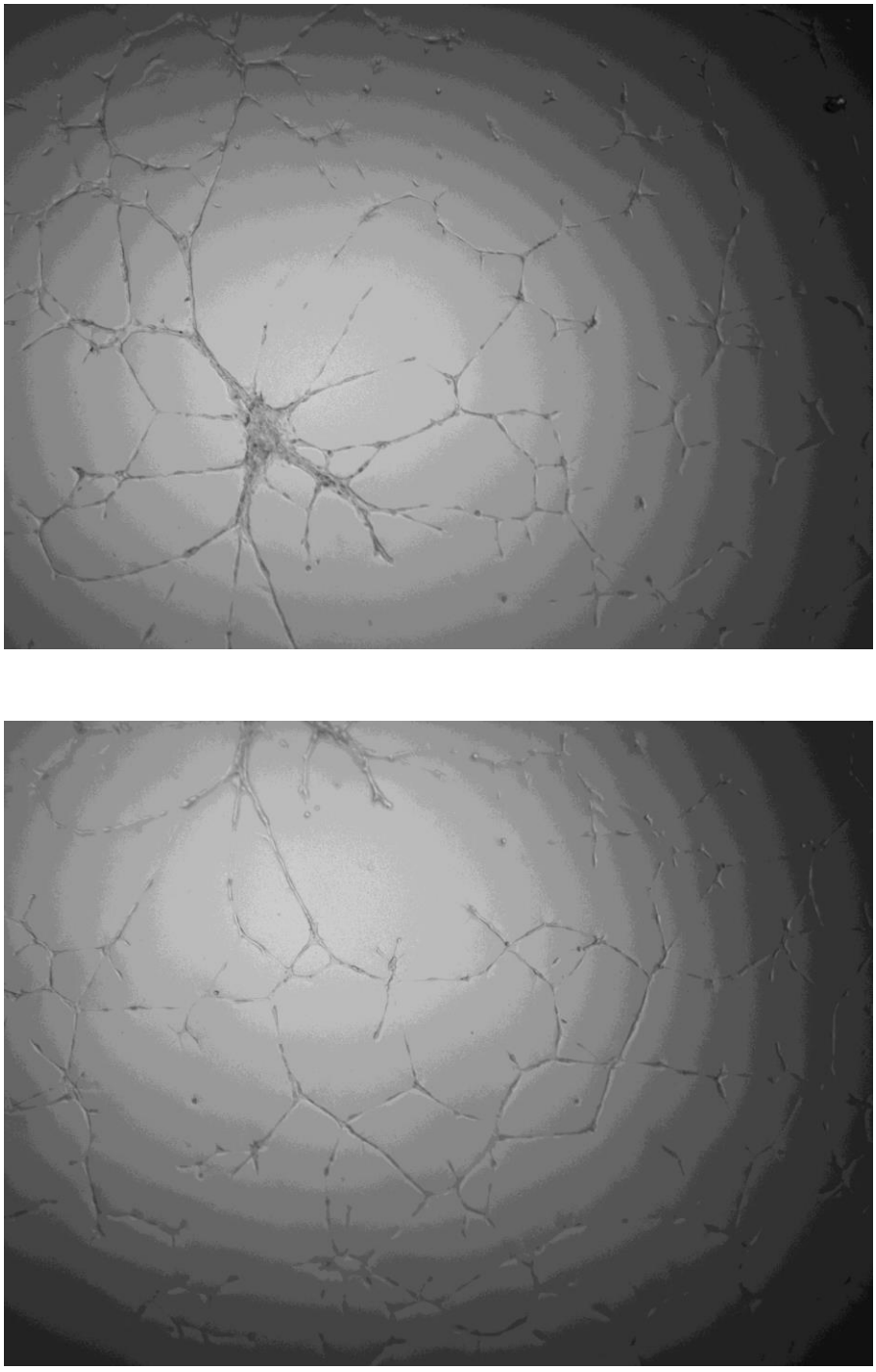

Figure 14: 3D tube formation assay positive control without Wortmannin
Photographed with 40X objective lens of inverted light microscope

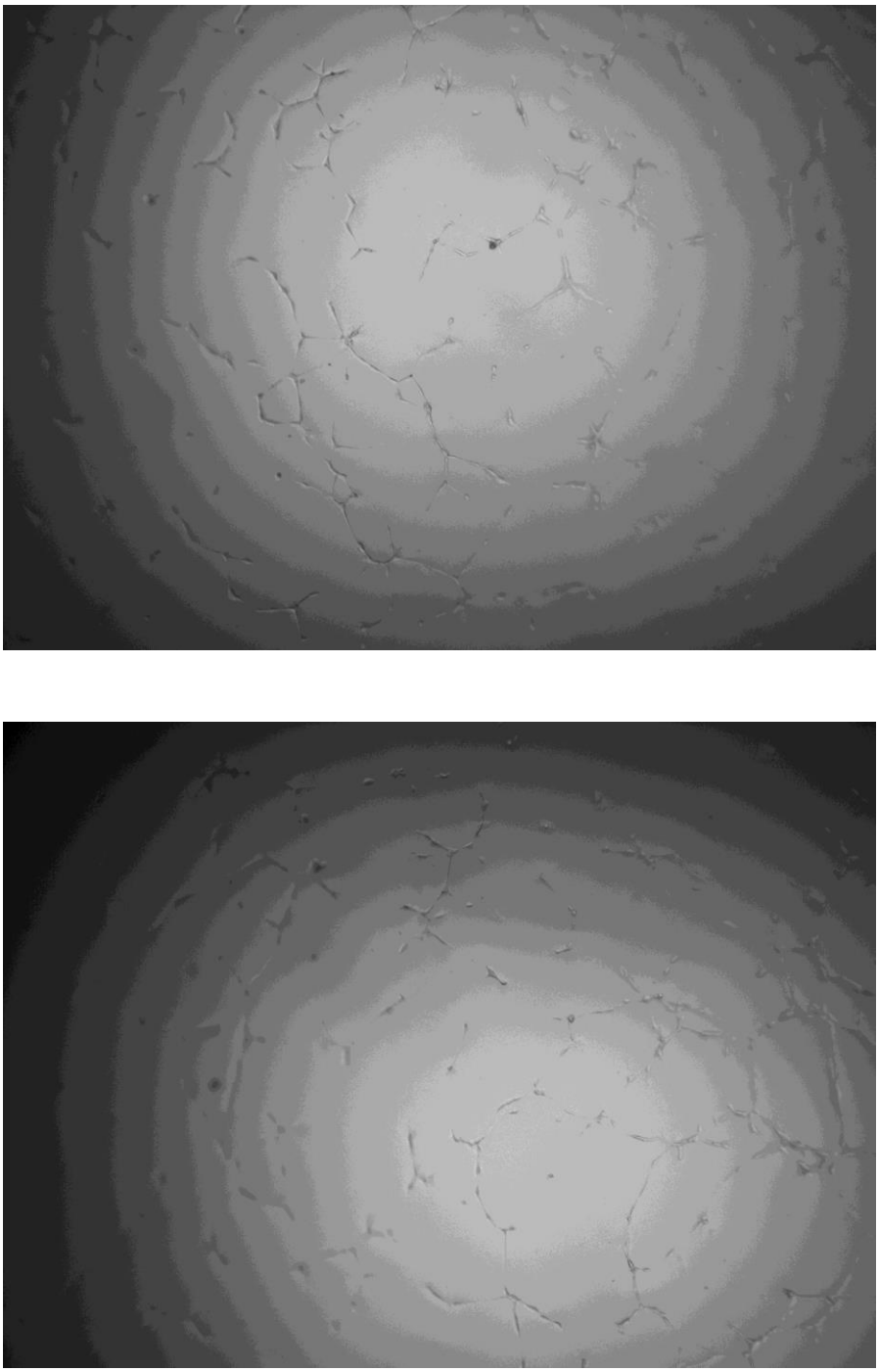

Figure 15: 3D tube formation assay negative control without Wortmannin
Photographed with 40X objective lens of inverted light microscope

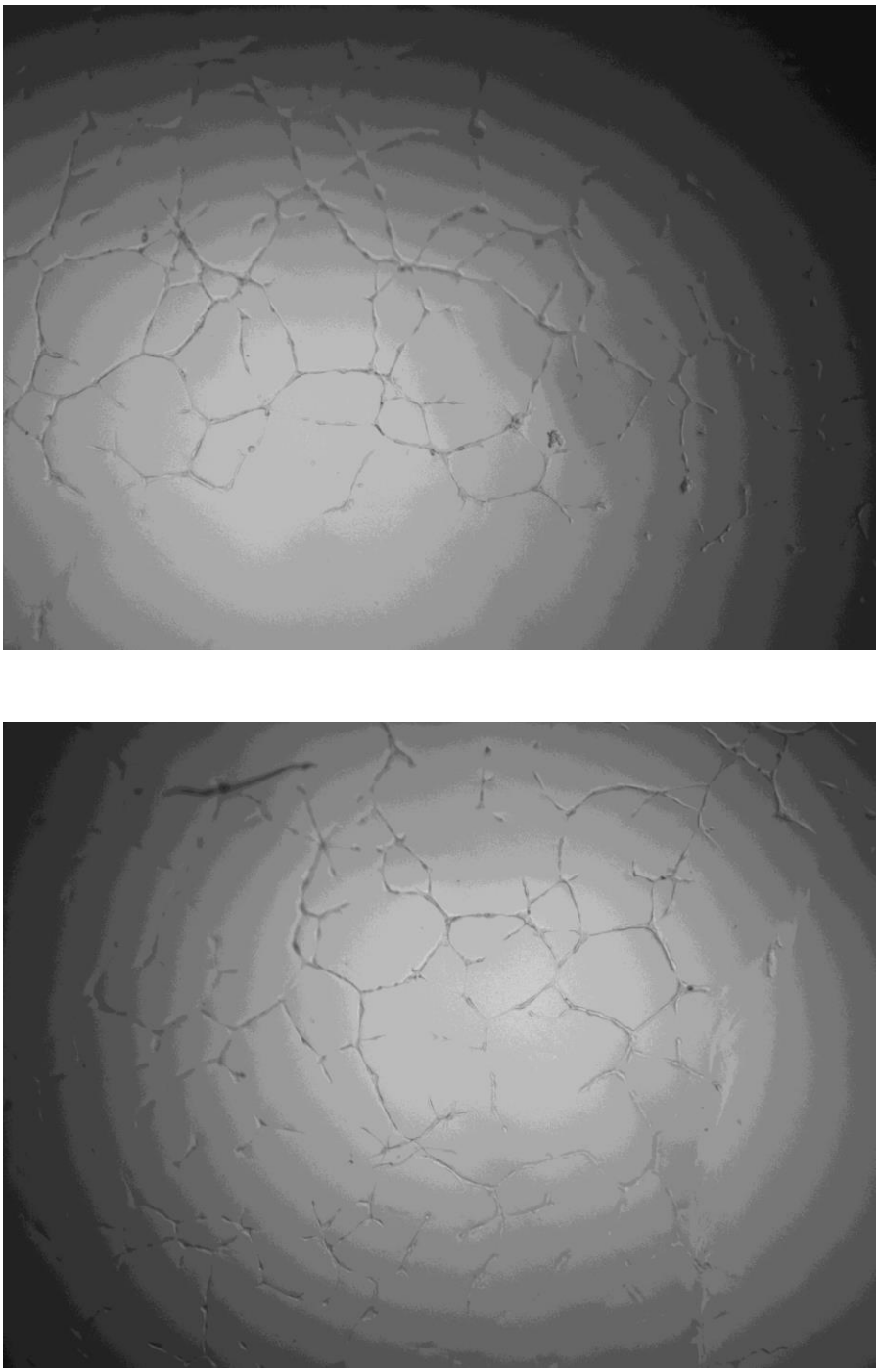

Figure 16: 3D Tube formation assay IGF-1 treated without Wortmannin
Photographed with 40X objective lens of inverted light microscope

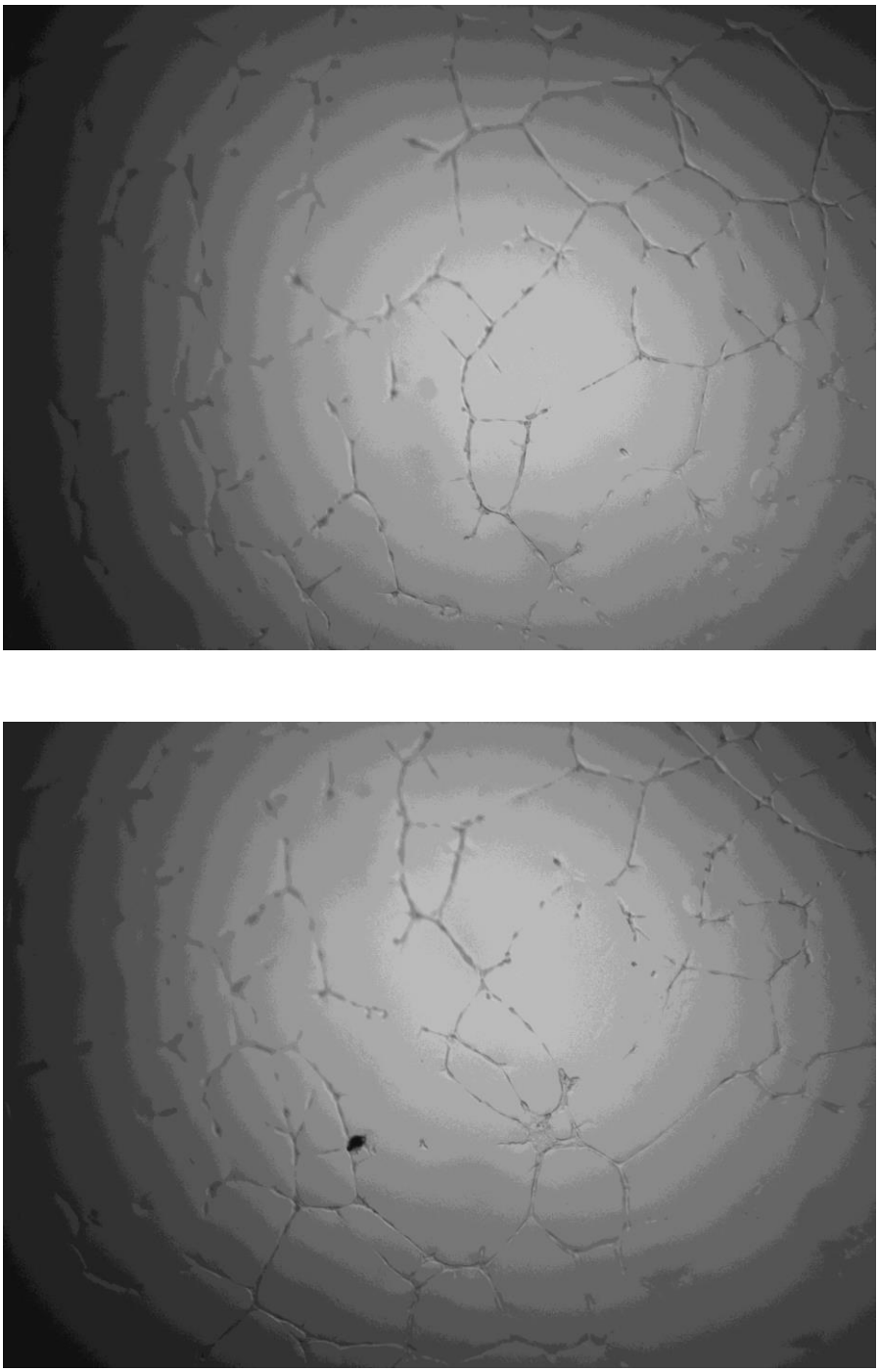

Figure 17: 3D tube formation assay positive control with Wortmannin
Photographed with 40X objective lens of inverted light microscope

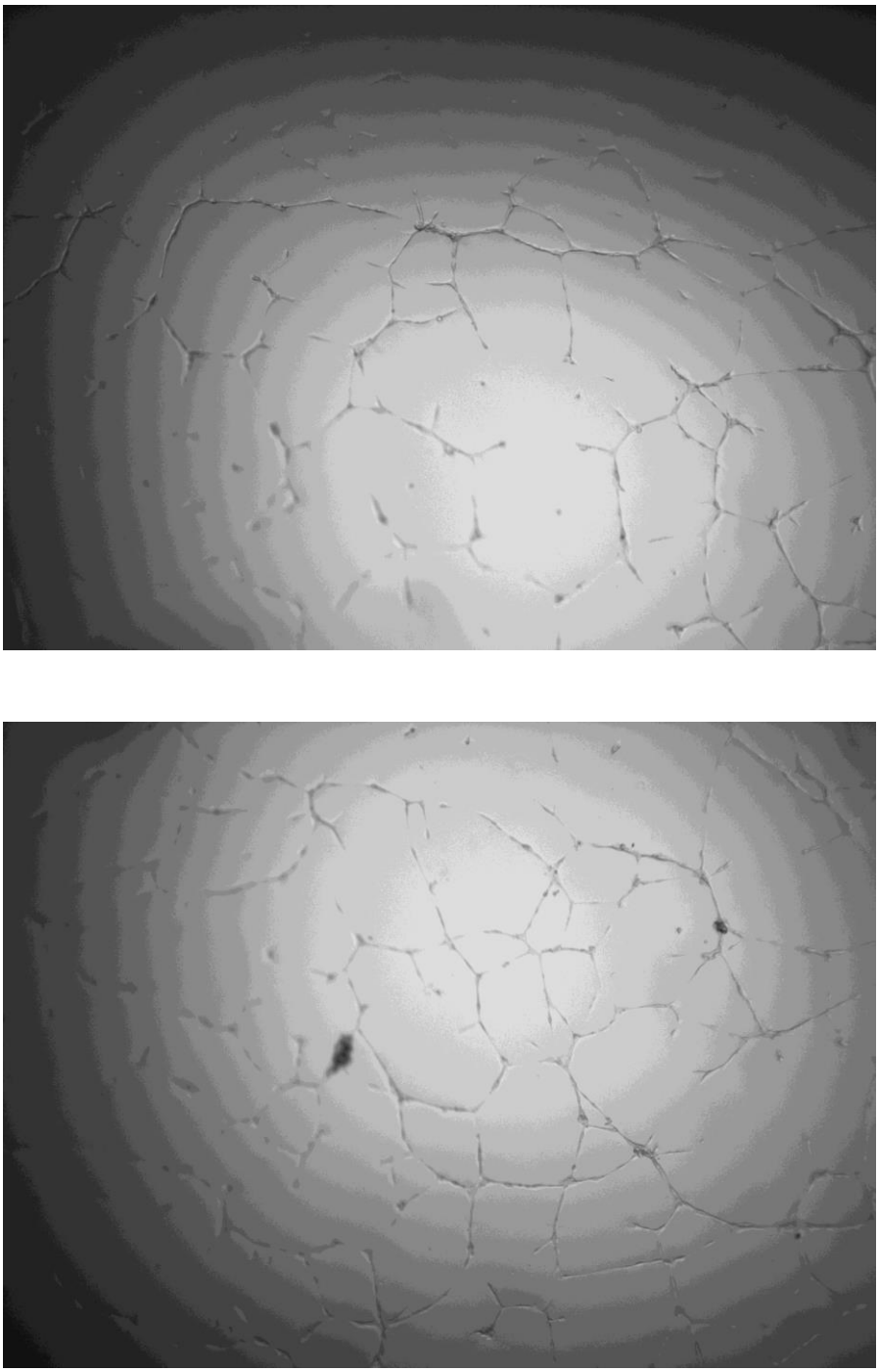

Figure 18: 3D tube formation assay negative control with Wortmannin
Photographed with 40X objective lens of inverted light microscope

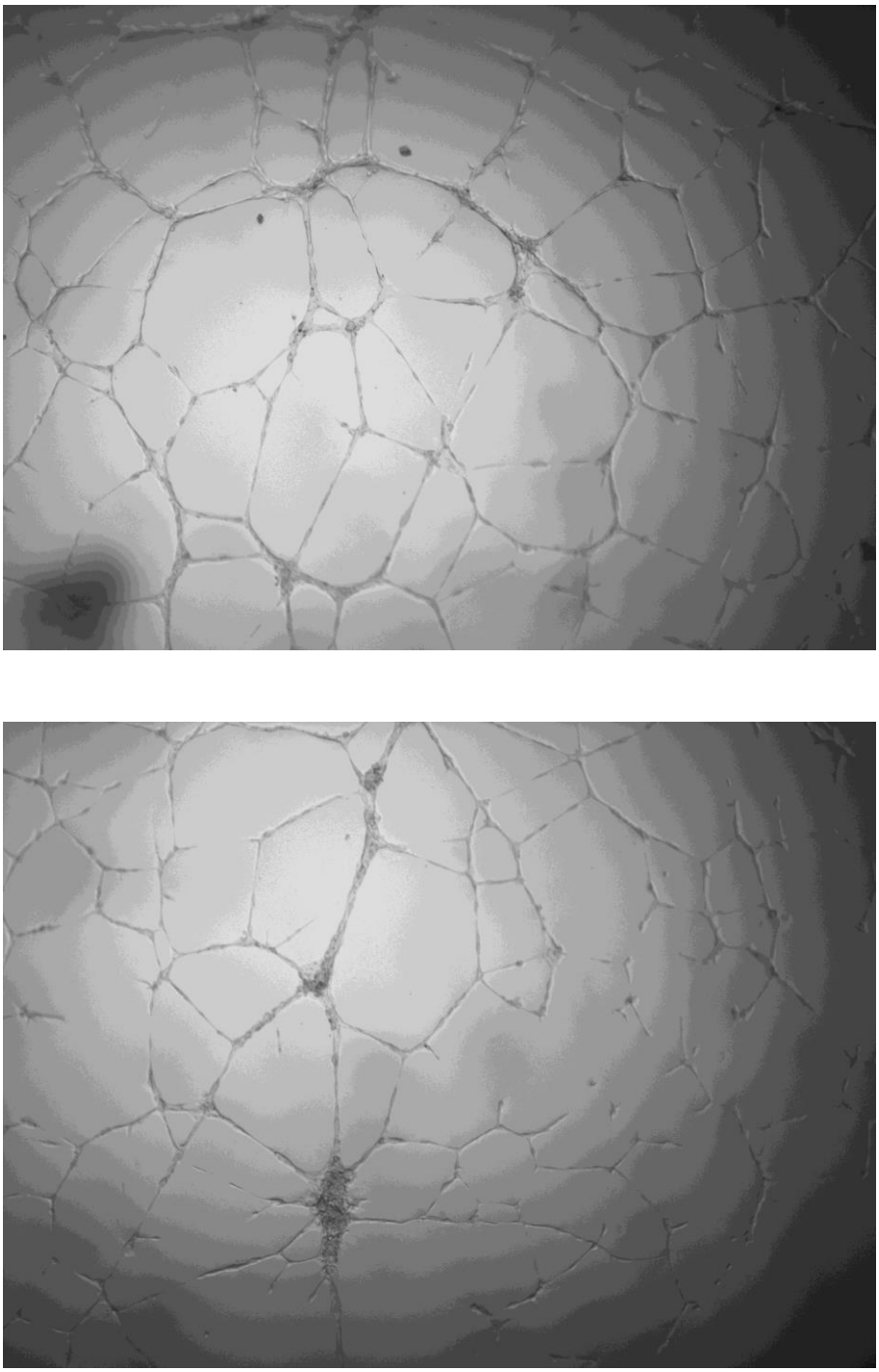

Figure 19: 3D tube formation assay treated with IGF-1 and Wortmannin
Photographed with 40X objective lens of inverted light microscope

Treatments		Branching points / unit area of field of view																					
No Wortmannin	Positive Control	49	55	50	61	52	52	61	56	46	50	46	58	53	49								
	Negative Control	33	30	24	44	52	31	28	13	25	26	38											
	IGF-1	51	50	53	40	53	38	38	38	44													
With Wortmannin	Positive Control	63	49	75	75	43	60	52	42	58	65	39	40	53	58	47	53	51	51	57	56	49	53
	Negative Control	36	39	58	55	47	27	44	37	60	60	45	35	40	49	51	52						
	IGF-1	48	38	54	49	44	50	61	40	57	38	60	63	56	63								

Table 1: Branching point quantification of 3D tube formation assay counting results

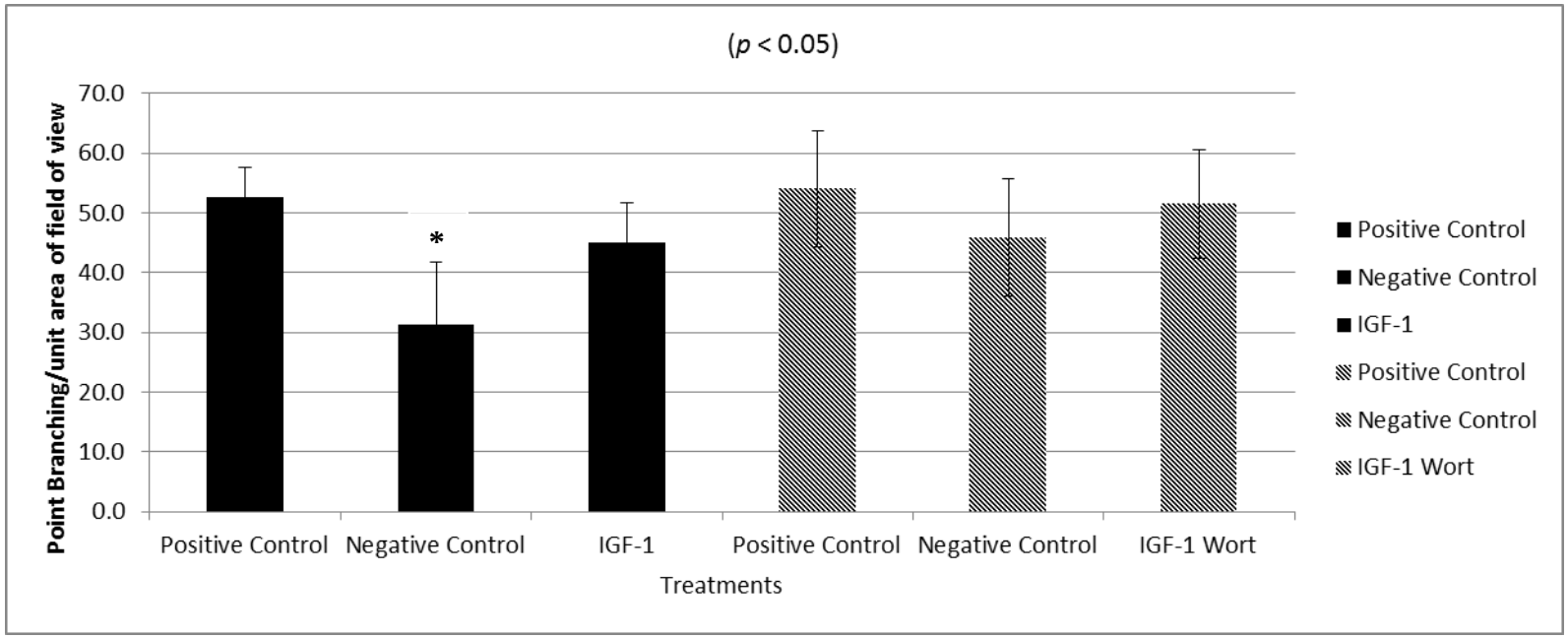

Figure 20: Statistical calculation of branching points of HRMVECs 3D tube formation assay
(* Statistically significant with $p < 0.05$)

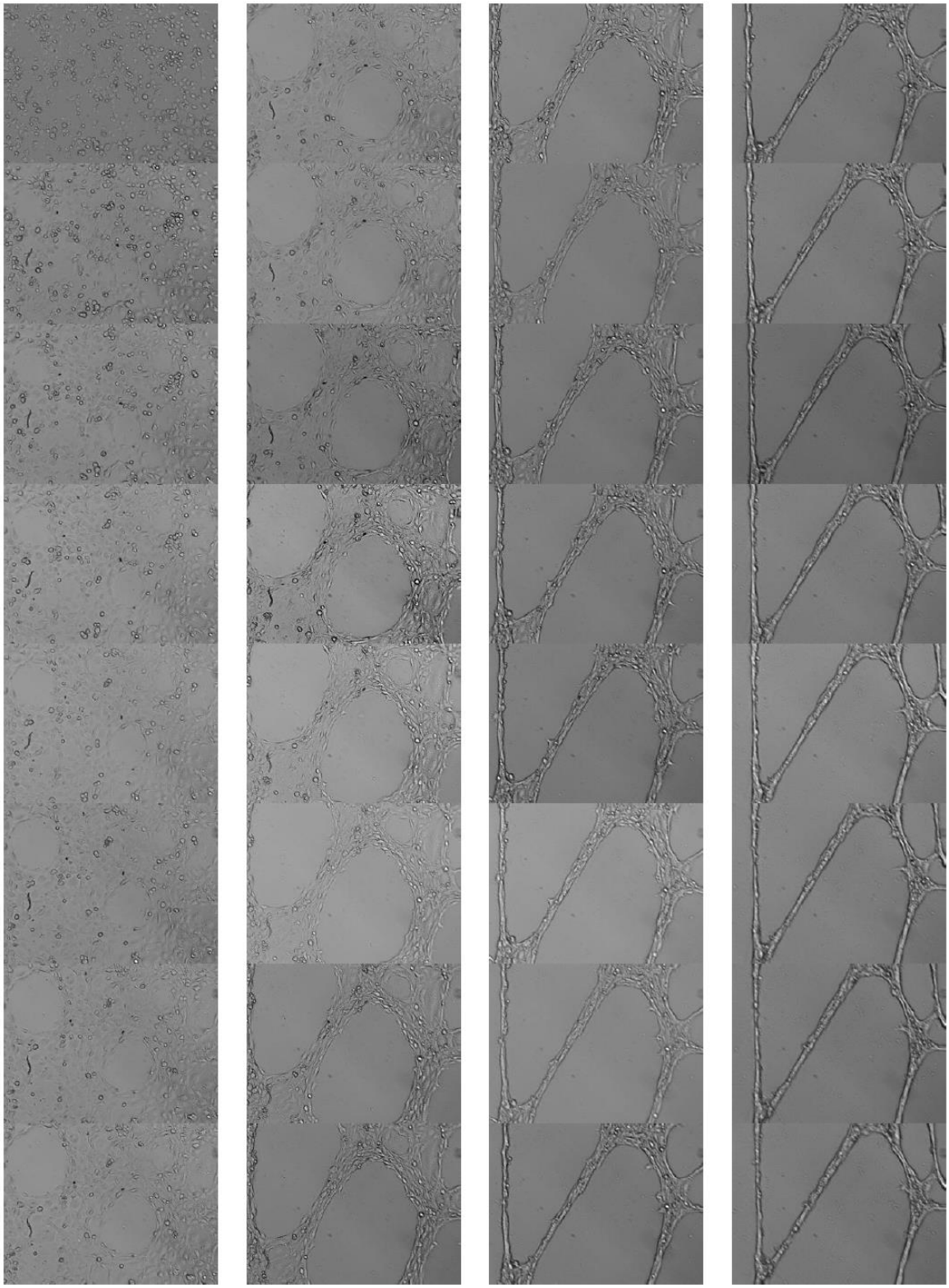

Figure 21: 3D live imaging montage of positive control HRMVECs.
(Complete movie is in the attached DVD)

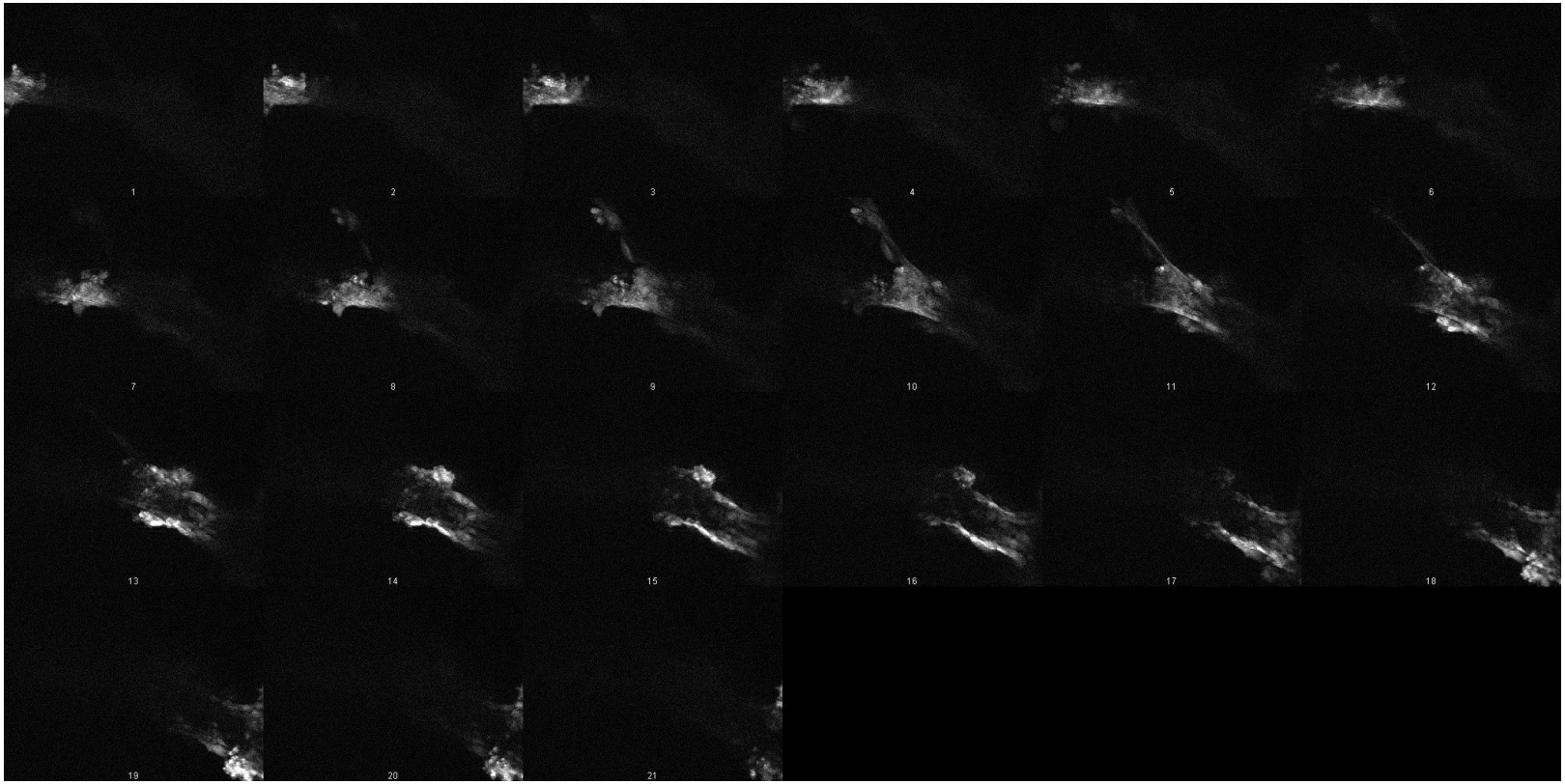

Figure 22: 3D live imaging montage on HRMVECs positive control with BCECF
(Complete movie is in the attached DVD)

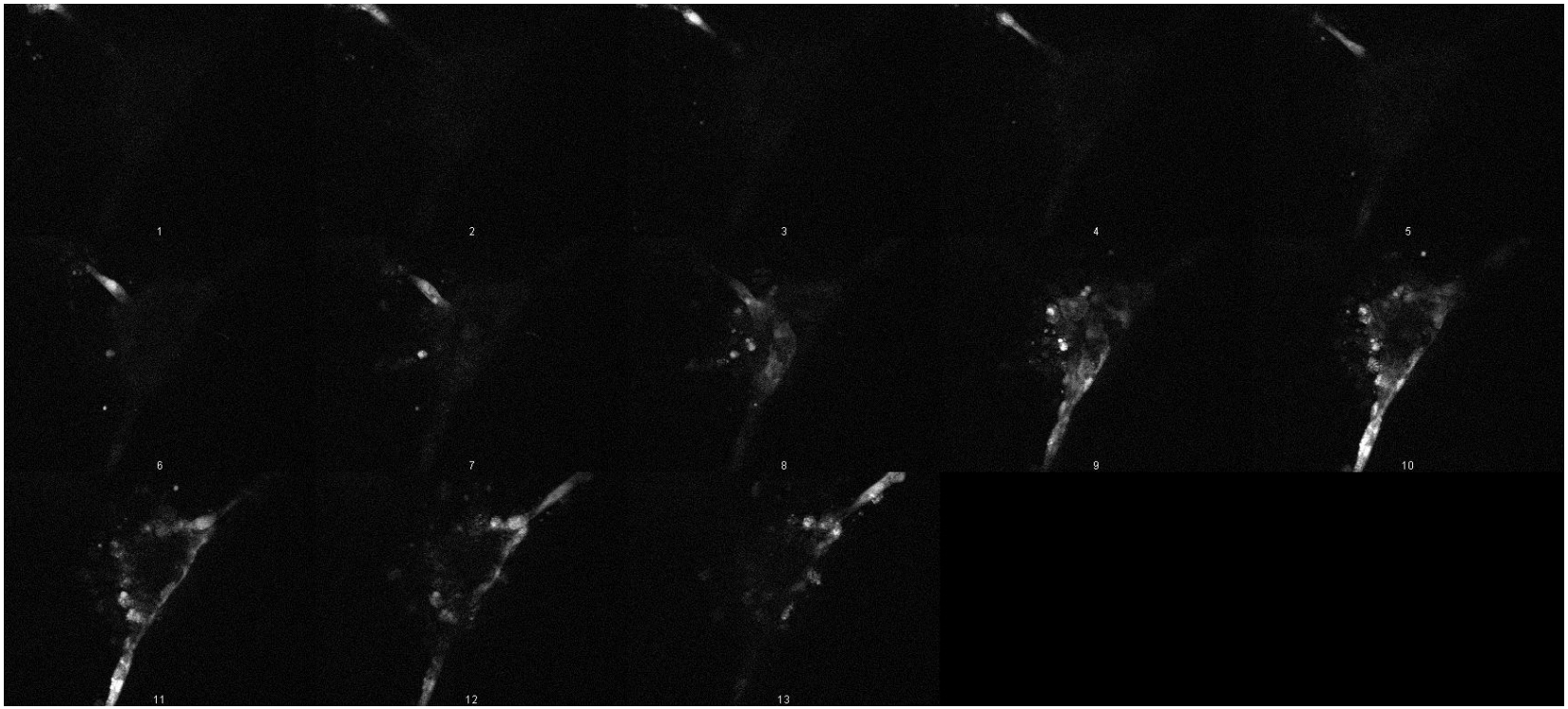

Figure 23: 3D live imaging montage on HRMVECs negative control with BCECF
(Complete movie is in the attached DVD)

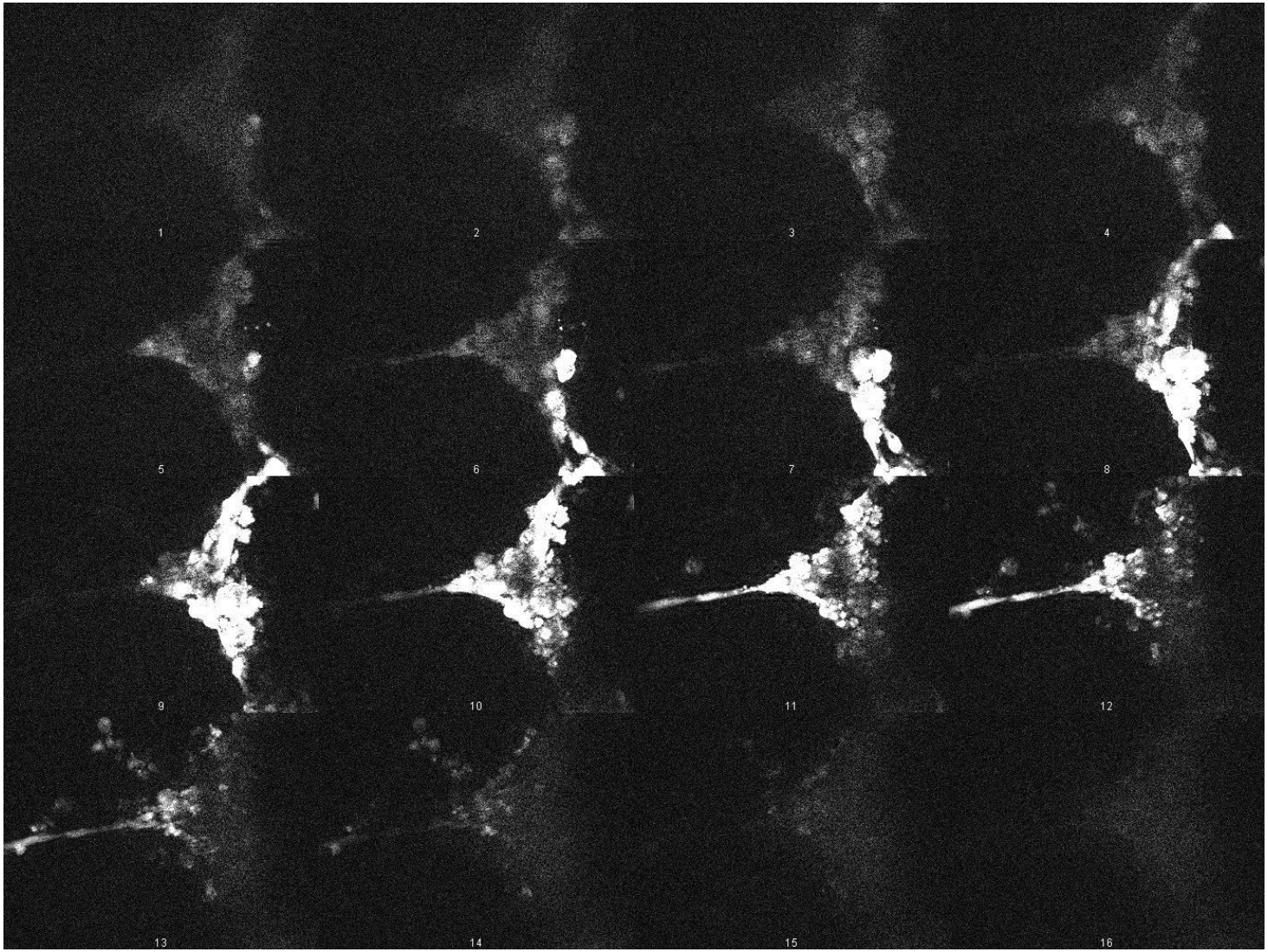

Figure 24: 3D live imaging montage of HRMVECs treated with 25 ng/ml IGF-1 with BCECF
(Complete movie is in the attached DVD)

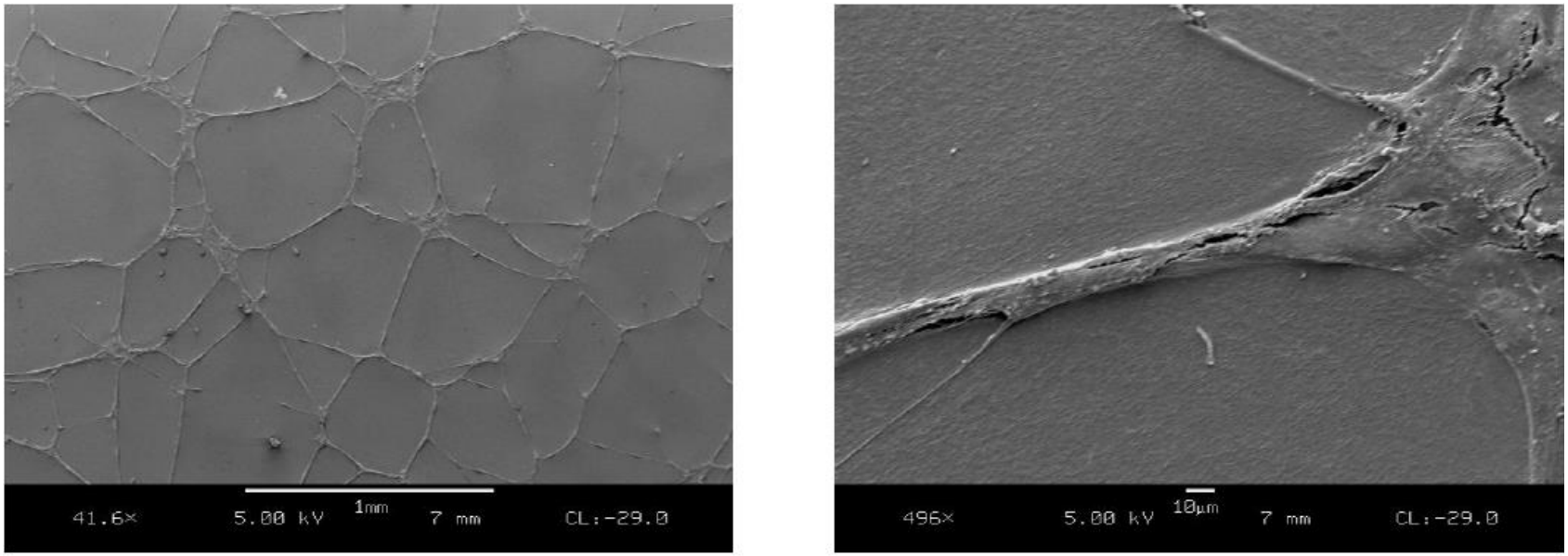

Figure 25: Scanning electron microscopy of HRMVECs positive control without Wortmannin

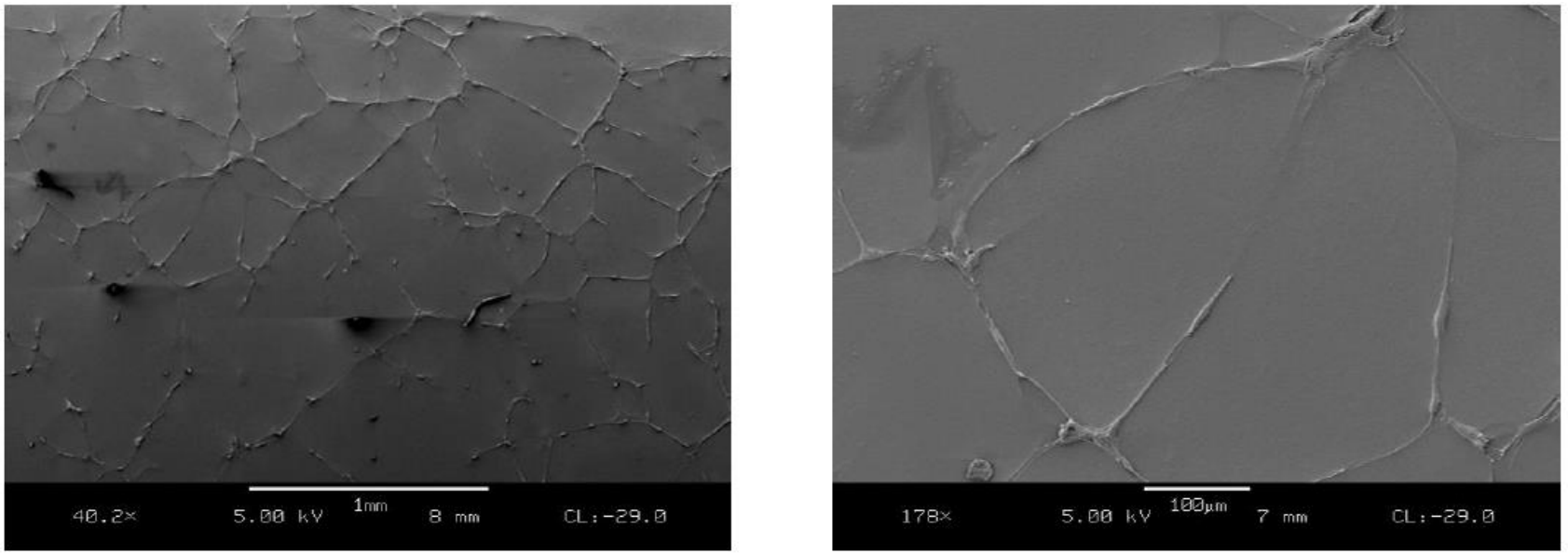

Figure 26: Scanning electron microscopy of HRMVECs negative control without Wortmannin

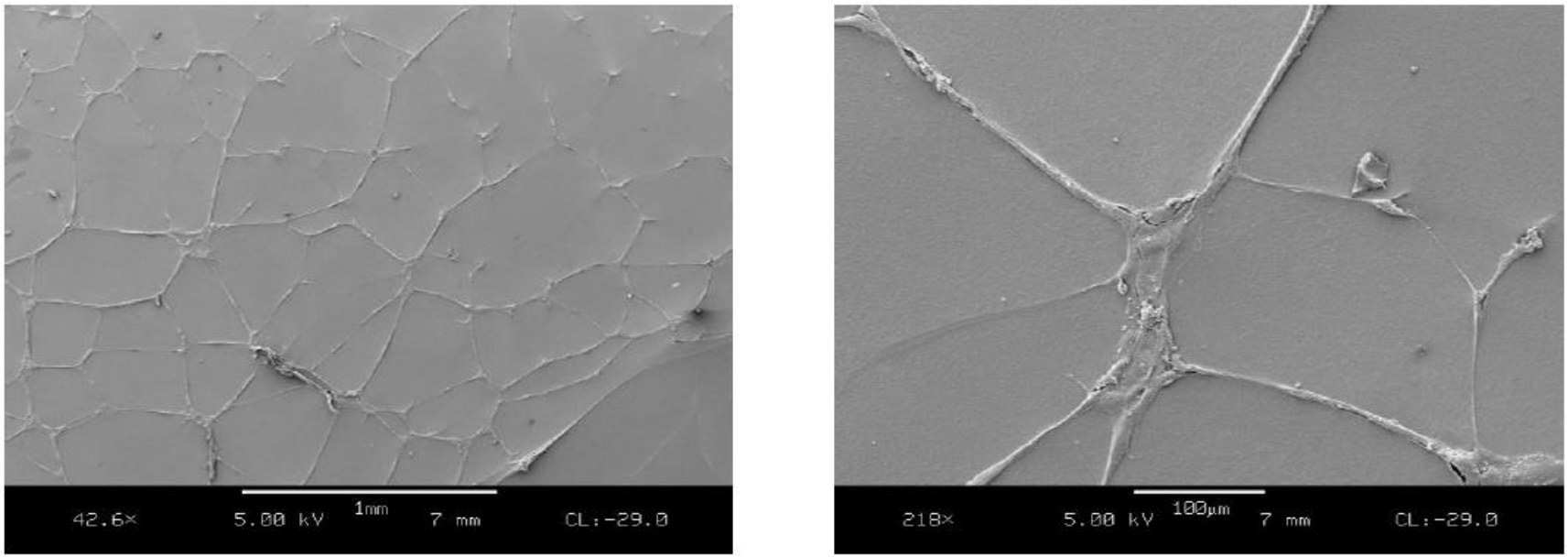

Figure 27: Scanning electron microscopy of HRMVECs treated with 25 ng/ml IGF-1 for 2 hours without Wortmannin

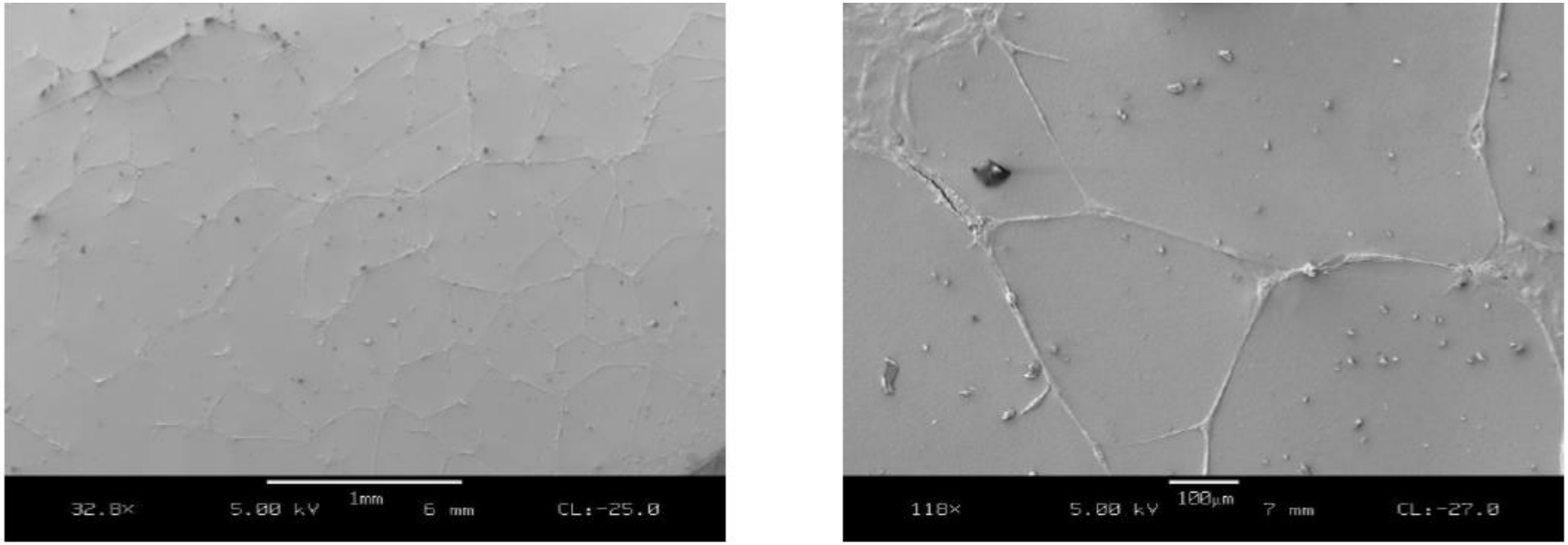

Figure 28: Scanning electron microscopy of Wortmannin-pretreated-HRMVECs positive control

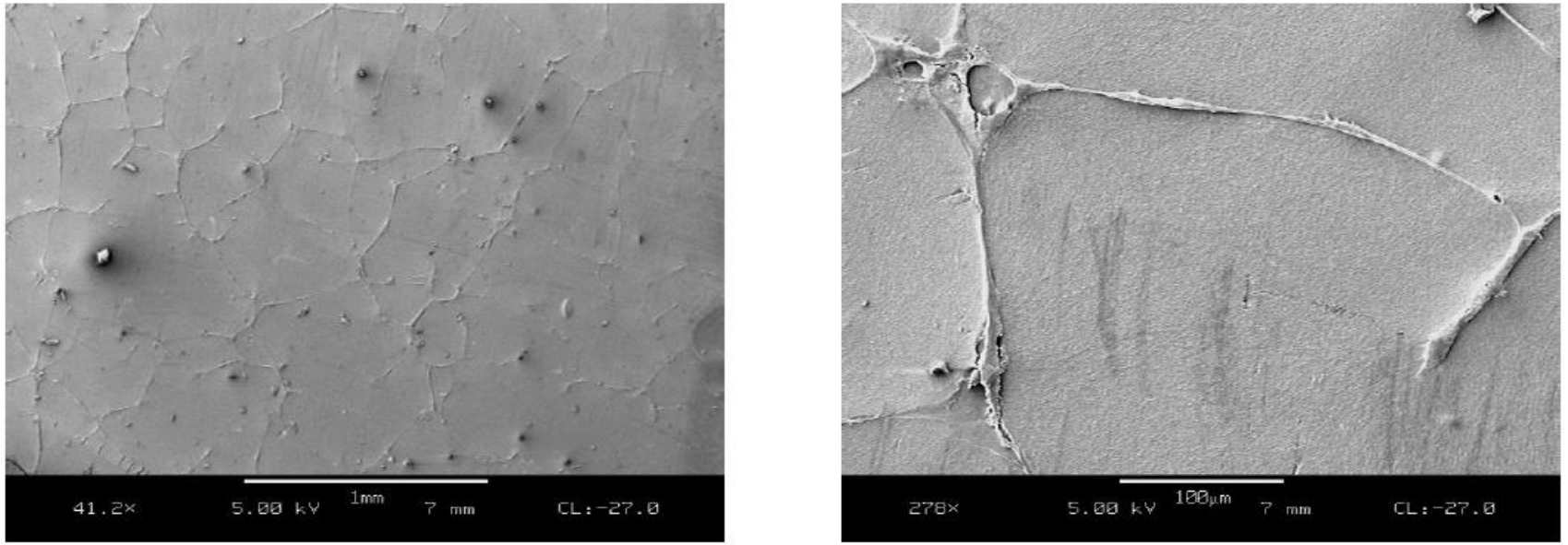

Figure 29: Scanning electron microscopy of Wortmannin-pretreated-HRMVECs negative control

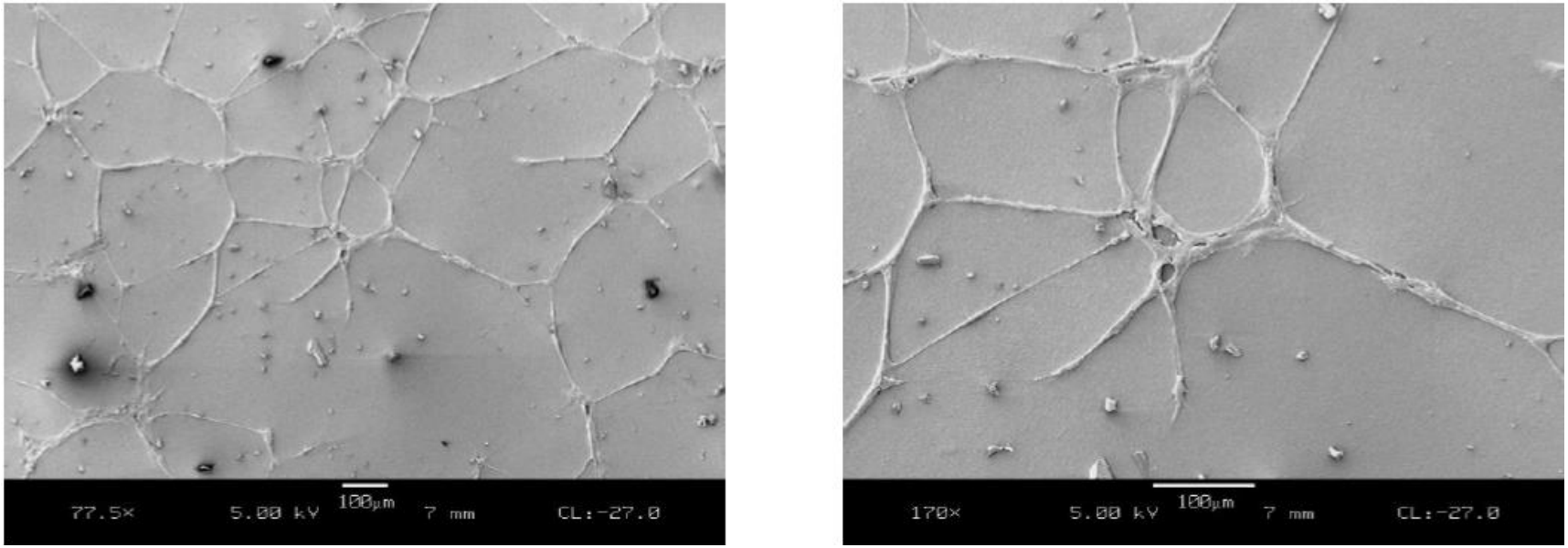

Figure 30: Scanning electron microscopy of Wortmannin-pretreated-HRMVECs stimulated with 25 ng/ml IGF-1

Chapter 5: Total MMP2 and Total MMP9 Released by IGF-1-Stimulated HRMVECs

Introduction

Increase vascular permeability is the first step in angiogenesis that allows the extravasation of plasma proteins that provides the favorable microenvironment. The key role in this process is the activation of plasminogen and matrix metalloproteinases (MMPs) that leads to the extracellular matrix (ECM) degradation and the release of growth factors, which stimulates the migration proliferation of endothelial cells (Behzadian et al., 2008; Pepper, 2001). MMPs are a family of at least 20 extracellular proteolytic zinc ion-containing enzymes that are mainly involved in tissue remodeling (Behzadian et al., 2008). The substrates of MMPs include all forms of collagen, elastin, laminin, fibronectin, proteoglycans and other ECM components (Siefert & Sarkar, 2012). Cytokines, growth factors, oncogenes, chemical agents activation or mechanical stress on cells can lead to increase processing of MMPs from inactive zymogens to the active enzymes (Nagase & Woessner, 1999), but cytokines and their receptors can also be the substrates for MMP actions, which may be important in down-regulation of cell surface receptors.

After activation, MMP activity is regulated by endogenous inhibitors: tissue inhibitors of metalloproteases (TIMPs) are main MMP inhibitors found in tissue fluids with variable affinities to different MMPs. In plasma, the predominant MMP inhibitor is the α2-macroglobulin, which will inhibit MMP irreversibly because the α2-macroglobulin/MMP complexes will be cleared by endocytosis later on (Siefert & Sarkar, 2012; Sternlicht & Werb, 2001). Based on their targets of the proteolysis, MMPs can be classified as collagenases, gelatinases and stromelysins (Siefert & Sarkar, 2012; Tallant, Marrero, & Gomis-Ruth, 2010). Collagenases cut collagen at a single site and include MMP1, MMP8, and MMP13. Gelatinases degrade both collagens and gelatins and

include MMP2 and MMP9. Stromelysins have a broader range of targets and include MMP3, MMP10, and MMP11. Although a broad range of MMPs (except MMP9) has not been shown to be crucial in blood vessel formation during embryonic life, MMP2, MMP9 and MMP14 (=MT1-MMP) might have been specific roles in postnatal vascular remodeling and angiogenesis (Page-McCaw, Ewald, & Werb, 2007; Siefert & Sarkar, 2012). This might be contributed to the relative absence of type I collagen in embryo and the study that shows defective vascular formation with extremely small lumen in double mutant of *MMP2* and *MMP14* which might be enough to support embryonic life and development but not for postnatal survival (Page-McCaw et al., 2007). Endothelial cells from various tissues have been described to produce MMP1, MMP2, MMP9, and MT1-MMP, all implicated in the regulation of angiogenesis (Taraboletti et al., 2002). A study with human umbilical vein endothelial cells shows the release of both active and proenzyme forms of MMP2 and MMP9-containing vesicles after stimulation with basic fibroblast growth factor (FGF-2) and VEGF (Taraboletti et al., 2002). The vesicles also contain the endogenous MMP inhibitors: TIMP1 and TIMP2 which are the regulators of MMPs activities; and MT1-MMP which mediates the activation of pro-MMP2 through formation of ternary complex with TIMP2 (Taraboletti et al., 2002).

In pathologic conditions of the retina, MMP activity has been associated with numerous diseases conditions, including age-related macular degeneration, proliferative diabetic retinopathy, glaucomatous optic nerve damage, vitreoretinopathy, and others (Behzadian et al., 2008). MMP2 has been shown to be essential in the regulation of retinal neovascularization, compared to MMP9 in C57Bl/6 mice. This study compared the oxygen-induced retinal neovascularization in C57Bl/6 mice null of *MMP2*, *MMP9*, or wild type; mice with null *MMP9* and wild type show significantly higher neovascularization compared to the *MMP2* null mice (Ohno-Matsui et al.,

2003). In this study, we designed the experiments to elucidate the MMP2 and MMP9 profiles in the HRMVECs with IGF-1 stimulation, and to observe the PI3K pathway associated with the stimulation.

Materials & Methods

Cell culture. Human retinal microvascular endothelial cell (HRMVECs) primary cell line culture (ACBRI181) were obtained from Cell systems (Kirkland, WA) maintained in Cell Systems Corporation (CSC) complete media (Cell Systems, Kirkland, WA) which is a modified DMEM/F12 (1:1) supplemented with elevated pyruvate, glutamine, bovine serum albumin, and vitamins/amino acids. This media is activated with CSC culture boost-R (Cell Systems, Kirkland, WA), which contains human recombinant growth factors: FGF and EGF. The media is also treated with antibiotic treatment Bac-Off® (Cell Systems, Kirkland, WA), which contains fluoroquinone class antibiotic ciprofloxacin. The cells were cultured in a T25 flask that has its tissue culture surface freshly coated with Attachment Factor (Cell Systems, Kirkland, WA), which is an extracellular matrix product that promotes cell attachment to tissue culture surface and encourages correct polarity and cytoskeletal organization. The cells were incubated at 37^0C, 5% CO_2, room air O_2 and 100% humidity. The cells were fed 12-24 hours after seeding, and at least every 48 hours thereafter until 70 – 80% confluent. For this dissertation, HRMVECs were used before the passage 8, because after passage 10, the endothelial cells undergo morphological and functional changes that make them unsuitable for angiogenesis assessment.

Sample Preparation. For this experiment, confluent HRMVEC cells were made quiescent with CSC complete serum free media for 18 hours and then treated with 25 ng/ml IGF-1 (Abcam, Cambridge, MA) diluted in CSC complete serum free media for 120 minutes. The

positive control was treated with activated CSC complete media with serum (Cell Systems, Kirkland, WA) and the negative control cells were treated with CSC complete serum free medium (Cell Systems, Kirkland, WA). The media supernatants were suctioned off and placed in labeled Ependorf tubes after 120 minutes treatment. On separate experiments, quiescent HRMVECs were pretreated with 100 nM Wortmannin diluted in CSC complete serum free media for 2 hours to block the PI3K pathway before the treatment groups were treated with 25 ng/ml IGF-1 (Abcam, Cambridge, MA), activated CSC complete media with serum (Cell Systems, Kirkland, WA) as positive control and CSC complete serum free media (Cell Systems, Kirkland, WA) for negative control. As with the previous sample preparation, the media supernates were then suctioned and placed in labeled Ependorf tubes after 120 minutes treatment.

Total matrix metalloproteinase-9 (MMP9) quantification ELISA. The ELISA kit used was the Quantikine Human MMP9 total (R&D Systems, Minneapolis, MN). All reagents and samples are brought to room temperature before use. After adding standards, positive control, negative control, IGF-1 samples to the assay diluent in the wells, the microplate was placed on a shaker set at 500 rpm. After aspirating, the wells was washed four times using wash buffer. Afterwards, the wells were filled with MMP9 conjugate, and were incubated for 1 hour at room temperature on the shaker. Repeat aspiration and washing four times, and add the substrate solution to each well and incubated for 30 minutes at room temperature and protected from light. Afterwards, add the Stop solution to each well, and gently tap the microplate to ensure thorough mixing, and the optical density was read within 30 minutes, using microplate reader set to 450 nm.

Total matrix metalloproteinase-2 (MMP2) quantification ELISA. The ELISA kit used was the Quantikine Human/Mouse/Rat MMP2 total (R&D Systems, Minneapolis, MN). All reagents and samples are brought to room temperature before use. After adding standards, positive control, negative control, IGF-1 samples to the assay diluent in the wells, the microplate was placed on a shaker set at 500 rpm. After aspirating, the wells was washed four times using wash buffer. Afterwards, the wells were filled with MMP2 conjugate, and were incubated for 2 hour at room temperature on the shaker. Repeat aspiration and washing four times, and add the substrate solution to each well and incubated for 30 minutes at room temperature and protected from light. Afterwards, a stop solution was added to each well, the microplate to gently agitated to ensure thorough mixing, and the optical density was read within 30 minutes, using a microplate reader set to 450 nm.

Results

MMP2 was observed to be stimulated by IGF-1 stronger than MMP9 in HRMVECs, in both conditions of the presence of PI3K inhibitors Wortmannin and without (**Table 2** and **Table 3**). The comparison of each absolute level of MMP2 and MMP9 with positive control without Wortmannin shows that the levels of both MMP2 and MMP9 stimulated with IGF-1 are less than positive control without blocking the PI3K pathway (**Figure 31**). The MMP2 production appeared to be

Discussion

With Elisa experiment on HRMVECs, it is demonstrated MMP2 level is upregulated higher than MMP9 in both with or without the presence of PI3K blocker Wortmannin. Both

MMP2 and MMP9 productions are growth factor-dependent with MMP2 affected more by the presence of growth factors in the media, since in the positive control groups, the media are supplemented with FGF and EGF. The absence of these two growth factors causes reduction in MMP2 production more than the reduction in MMP9 production. The stimulation of IGF-1 diluted in CSC complete serum free media shows up-regulation more of MMP2 than MMP9, almost to the same extent of the upregulation in positive control. This might be interpreted as the dependency of MMP2 release by FGF and EGF stimulation (present in the positive control group) and IGF-1 (the IGF-1 treated group) more than the dependency of MMP9's. IGF-1 appears to recover the absence of growth factors in the negative control groups although not causing the up-regulation of MMP9 to the same extent as to MMP2. PI3K blockade with Wortmannin appears to reduce both MMPs production with MMP9 production affected more than MMP2's. The inhibition of PI3K pathway with Wortmannin on HRMVECs appears to blunt the effects of growth factors in the positive control treated (FGF and EGF) and on the IGF-1 stimulation on the up-regulation of MMPs, so it appears that IGF-1 stimulated the MMPs is PI3K dependent, especially on MMP2 more than MMP9 productions, therefore, the stimulation of IGF-1 does not recover the production so MMPs when PI3K is blocked. The PI3K-dependent pathway seems to be the common denominator pathway for growth factors, at least for FGF, EGF and IGF-1, for HRMVECs' MMP2 and MMP production. There are other pathways that we need to study further, such as the MEK-MAPK pathway, in human breast cancer cells, IGF-1 has been shown to stimulate the release of MMP2 via a MEK-MAPK pathway-VEGF release in an unpublished study (Thant, 2005).

Summary

The stimulation of HRMVECs with IGF-1 was shown to induce the release of MMP2 more than MMP9. IGF-1 appears to stimulate the release of MMP2 in PI3K dependent pathway, probably through the release of VEGF. On top of IGF-1, MMPs release also appears to be stimulated by the presence of FGF and EGF, MMP2 more than MMP9 in PI3K pathway-dependent, showed by the blunt effects of IGF-1 stimulation for the release of MMPs in the presence of PI3K blockade. It appeared that PI3K is a common denominator pathway for FGF, EGF and IGF-1 for HRMVECs in regards to MMP2 and MMP9 productions.

		MMP2 (ng/ml)	Ratio to Pos control MMP2 Levels
Without Wortmannin	Positive Control	2.414	1.000
	Negative control	1.081	0.448
	IGF-1	2.121	0.879
With Wortmannin	Positive Control	2.111	0.875
	Negative control	1.899	0.787
	IGF-1	1.879	0.778

Table 2: MMP2 levels measured by Elisa method on HRMVECs

		MMP9 (ng/ml)	Ratio to Pos control MMP 9 Levels
Without Wortmannin	Positive Control	2.270	1.000
	Negative control	1.601	0.705
	IGF-1	1.635	0.720
With Wortmannin	Positive Control	1.546	0.681
	Negative control	1.506	0.664
	IGF-1	1.452	0.640

Table 3: MMP9 levels measured by Elisa method on HRMVECs

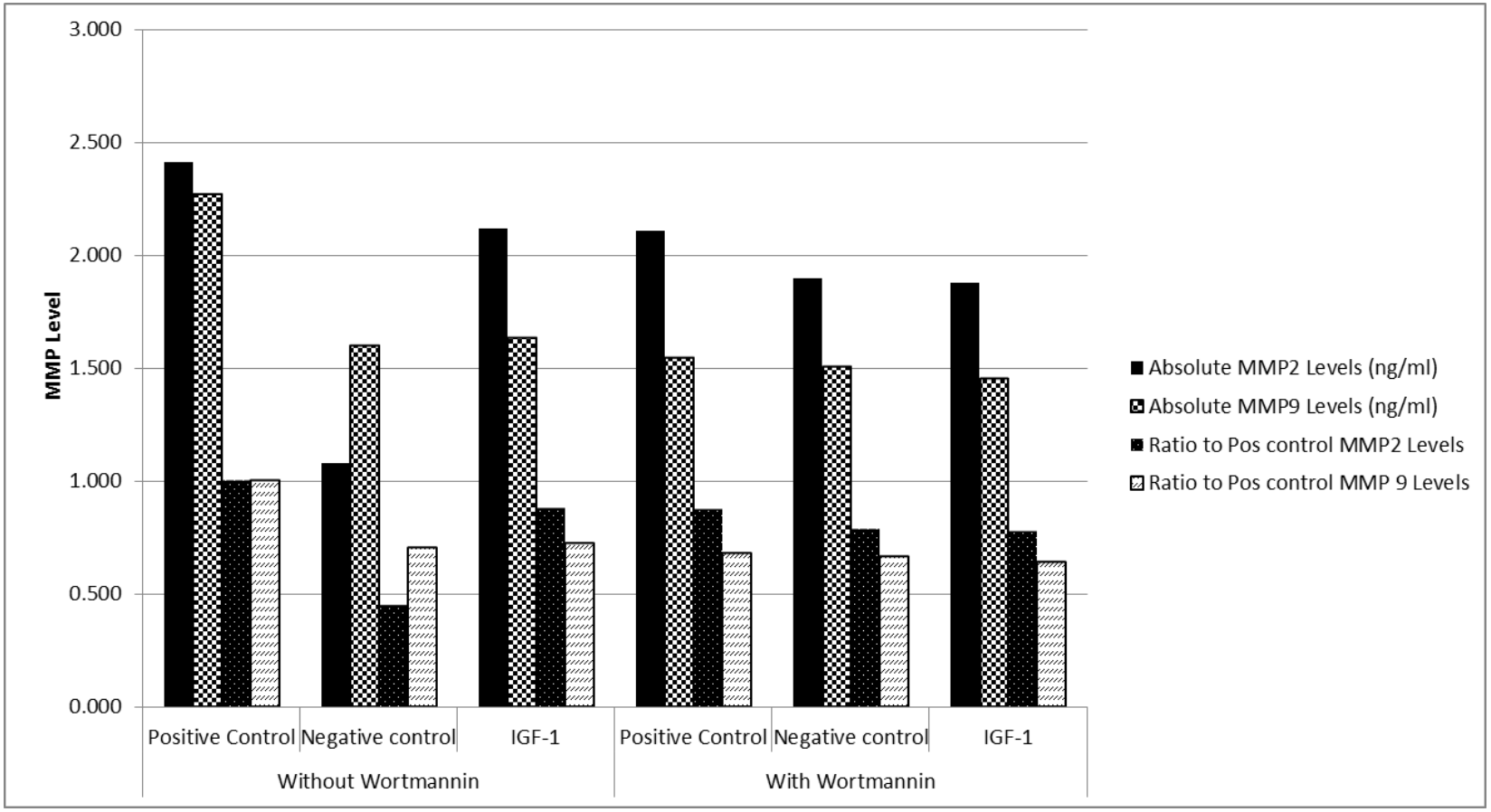

Figure 31: Quantitative graph comparing the absolute and relative to positive control of MMP2 and MMP9 levels on HRMVECs

Chapter 6: Conclusions and Future Directions

This thesis has established the role of IGF-1 in HRMVEC angiogenesis through the upregulation of HIF-1α in a PI3K-dependent pathway, although the role of other tyrosine kinase pathways still could not be ruled out. The presence of IGF-1 in HRMVEC angiogenesis is crucial, especially during the early stage of angiogenesis when the formation of branching networks is happening. This stage appears to be not so much dominated by PI3K pathway, probably other tyrosine kinase pathways has more dominant roles, such as MAPK or Src pathways. However, the later stage of HRMVECs angiogenesis appears to be dependent on more other cues from cytokines or growth factors in the tube formation, and this stage appears to be more PI3K pathway dominant.

PI3K pathway seems to be the common denominator pathway for IGF-1, besides the FGF and EGF that are present in the experiment media by default, for HRMVECs in regards to MMP2 and MMP9 productions.

The study of the roles of IGF-1 in the HRMVEC angiogenesis of this thesis has led to unanswered questions: 1) whether the process of HRMVEC angiogenesis is affected by the VEGF released by the retinal pigment epithelial cells by IGF-1 stimulation. 2) Whether IGF-1 affects the HRMVEC angiogenesis dependent on MAPK or Src pathway besides the PI3K pathway. 3) What IGF-1 effects on the HRMVEC angiogenesis *in vivo.*

1) VEGF from retinal pigment epithelial cells by IGF-1 stimulation effects on retinal endothelial angiogenesis.

   The deeper retinal layers of the retina (photoreceptor layers) and choroid are vascularized by the choroidal capillary plexus. And since the retinal pigment epithelial layer is located in between the photoreceptor layer and the choroid, the release of VEGF from the retinal

pigment epithelium will most likely affects the choroidal capillary plexus angiogenesis. The complexity of retinal angiogenesis in vivo is multiplied by the possibility that VEGF has also been shown to be released from astrocytes, Muller cells and ganglion cells in hypoxic environment.

2) IGF-1 stimulation has been known to induce several pathways, and some of them probably are important in angiogenesis. PI3K and MAPK has been known to be important in this process, but other pathway such as Src pathway might also be important in HRMVECs angiogenesis stimulated by IGF-1.
3) The importance of IGF-1 in stimulating angiogenesis through PI3K pathway *in vitro* has been shown, but to see the effects of IGF-1 on PI3K pathway *in vivo* still need to be done.

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