DIFFERENTIAL MODULATION OF ARTERIAL VS VENOUS SMOOTH MUSCLE CELL PROLIFERATION AND MIGRATION BY HYPOXIA AND HYPOXIA INDUCIBLE ENDOTHELIAL CELL GROWTH FACTORS

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This dissertation is dedicated to my son Adedeji Tendai Alimi

Abstract

Despite intensive research studies, theories have yet to focus on the contribution of hypoxia to patency differences observed clinically between arterial vs. venous grafts. This study investigates the differential hypoxic response of endothelial cell (EC) derived growth factor on modulation of smooth muscle cell (SMC) proliferation and migration under hypoxia and its contribution to graft patency.

Our study shows differential regulation of arterial vs venous smooth muscle cell proliferation and migration under hypoxia. Initiation of SMC proliferation under hypoxia (<5% O_2) occurred only after incubation with hypoxic endothelial cell conditioned media (H-ECM). After investigating several possible growth factors in the H-ECM that may be responsible for SMC proliferation, the greatest difference was observed in vascular endothelial growth factor (VEGF-A) & platelet derived growth factor homodimer B (PDGF-BB) expression. Under Hypoxia 2 fold increase in VEGF-A was observed in arterial derived SMC (A-SMC) in comparison to venous derived (V-SMC) which showed no significant change. V-SMC showed higher VEGFR-2 expression under hypoxia, while A-SMC had significantly lower ($p<0.05$) receptor expression. Incubation with VEGFR-2 neutralizing antibody / PDGFR antagonist in V-SMC prior to addition of H-ECM resulted in decreased proliferation. A-SMC proliferation under hypoxia did not decrease with incubation of VEGFR-2 neutralizing

antibody but did decrease upon PDGFR antagonist incubation. Proliferation in the both SMC was regulated by an ERK1/2 dependant mechanism with V-SMC showing greater ERK1/2 expression under hypoxia upon addition of hypoxic endothelial cell conditioned media.

Our work was also focused on investigating the impact of hypoxia on smooth muscle cell migration. In summary we determined that SMC migration was occurring via an autocrine and paracrine mechanism under hypoxia. Migration in V-SMC under hypoxia was regulated via VEGFR-1 since there was an induction in VEGFR-1 expression under hypoxia and migration in V-SMC decreased upon neutralization with a VEGFR-1 antibody. V-SMC migration was also regulated by PDGF-BB since there was a partial reduction in V-SMC migration under hypoxia upon neutralization with PDGF-BB antibody. Migration in A-SMC was induced more by PDGF-BB since neutralization with PDGF-BB antibody decreased A-SMC migration to basal levels.

In conclusion our studies illustrate that origin of cellular growth factors play an important role in how the pathology of a disease develops. Therefore combination therapies focusing on the use of anti-VEGFR-1 antagonist to combat migration and anti VEGFR-2 and PDGFR β to combat proliferation would work best to help alleviate intimal hyperplasia a result of smooth muscle cell migration and proliferation in venous derived grafts and surgical interventions.

Key words: Hypoxia, vascular smooth muscle cells, VEGF-A, PDGF-BB, graft patency

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CHAPTER 1
BACKGROUND-LITERATURE REVIEW

INTIMAL HYPERPLASIA

Intimal hyperplasia leading to restenosis is the major process that limits the success of cardiovascular intervention (Patel and others 2010). Recurrent arterial narrowing (restenosis) is the major complication limiting the success of revascularization procedures. It can occur after coronary and peripheral angioplasty, bypass grafting, endarterectomy, and arteriovenous fistula formation (Patel and others 2010). In the short term it may be caused by elastic recoil of the vessel wall, thrombus formation at the site of injury, and variations in operative technique that lead to a smaller anastamosis or kinking of the vessel (Patel and others 2010). Long-term patency over the years is limited by intimal hyperplasia, involving the proliferation and migration of intimal smooth muscle cells (Patel and others 2010).

The innermost, intimal layer of normal artery wall is lined with endothelial cells that reside on a thin basement membrane bounded by an internal elastic lamina (Raines 2004). Occasionally, SMC are also found in this layer, but the majority of SMC reside beneath the internal elastic lamina in the medial layer that contains many layers of SMC surrounded by a collagen- and elastic-rich extracellular matrix bounded on the abluminal side by an external elastic lamina. The outermost layer of the artery is

composed of loose connective tissue containing fibroblasts, small vessels and autonomic nerve endings (Raines 2004). This structure of the vessel is illustrated in figure 1.1.

Figure 1.1

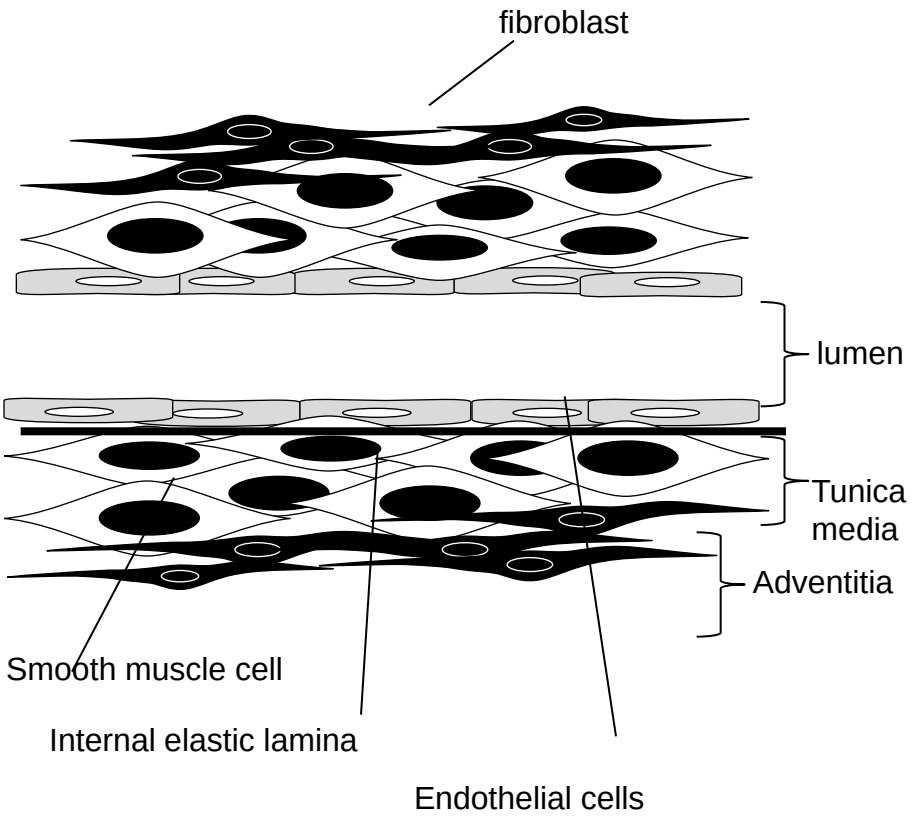


Figure 1.1 Diagram showing structure of a vessel. The figure represents the structure of a vessel with the inner layer (lumen/ tunica intima) comprising of endothelial cells , the tunica media of smooth muscle layer and the outer layer of fibroblast

Preventing intimal hyperplasia is an important therapeutic target and strategies include not only continued development of stent design and coating materials, but also manipulation of the cellular response to vascular injury (Patel and others 2010). In addition to regulating vascular tone and permeability the endothelium is emerging as a key modulator of the cellular response to vessel wall injury (Patel and others 2010). Accelerated re-endothelialization following arterial injury inhibits SMC proliferation and therefore has the potential to prevent or reduce intimal hyperplasia (Patel and others 2010).

Clinical impact of IH

The overall incidence of restenosis is approximately 30% a year after coronary angioplasty and bare metal stenting (Roiron and others 2006) and there is a similar incidence following angioplasty and for peripheral arterial disease (Muradin and others 2001). IH leading to restenosis leads to severe morbidity and mortality, more physical limitations, repeated hospitalizations and reduced quality of life (Roiron and others 2006). The economic impact of repeated revascularization procedures is also considerable (Ryan and others 2009).

Mechanism of Intimal Hyperplasia

Mechanism of IH can be divided into three phases, an inflammatory phase, cellular proliferation and migration and phase of remodeling of the extracellular matrix (Patel and others 2010). The purpose of this

investigation is to determine the role hypoxia plays in the proliferative and migratory stages of IH since hypoxia has been implicated in IH. Figure 1.2 illustrates summary of intimal hyperplasia mechanism/s.

Figure 1.2

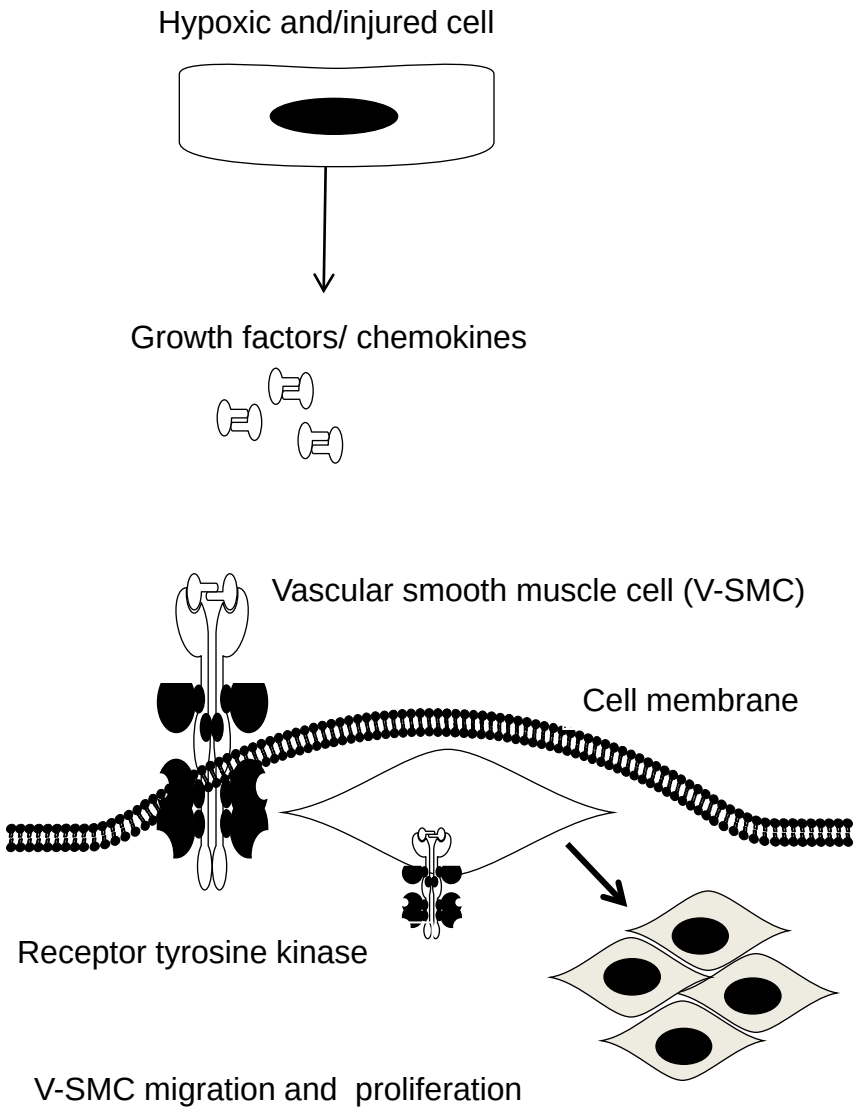


Figure 1.2 Summary of intimal hyperplasia. Following vessel injury and/ hypoxia endothelial cells release growth factors that bind to tyrosine kinase receptors on VSMC and induce smooth muscle cell migration and proliferation

Inflammatory stage

Platelet activation

Platelet activation occurs as one of the earliest “response to injury” events and is proportional to the degree of intimal damage. It is a three-phase effect consisting of adhesion, aggregation and activation (Mitra, Gangahar, Agrawal 2006). Endothelial denudation exposes the subendothelial matrix and leads to platelet adhesion and aggregation (Mitra, Gangahar, Agrawal 2006). Platelet adhesion involves a platelet receptor Gplb, plasma von Willebrand factor and fibronectin, whereas platelet aggregation requires tissue factor, fibronectin, von Willebrand factor, fibronectin and the platelet receptor GpIIb-IIIa. The adhered platelets release adenosine diphosphate and activate the arachidonic acid synthesis pathway to produce thromboxane A_2, a potent chemoattractant and SMC mitogen (Mitra, Gangahar, Agrawal 2006). The activated platelets then release numerous bioactive substances, which initiate the SMC proliferative mechanisms. These substances include growth factors, platelet-derived growth factor (PDGF), TGF-β, cytokines IL-1, IL-6 and IL-8, and thrombin. Platelet activation also leads to thrombus generation and tissue factor expression through CD40L, a transmembrane protein found in platelets (alpha granules) (Mitra, Gangahar, Agrawal 2006). CD40L is also responsible for the expression

of the various adhesion molecules, which adhere the leukocytes on the endothelial surface (Mitra, Gangahar, Agrawal 2006). Events take place on the platelet surface, which occur subsequent to or simultaneously with platelet activation. P-selectin, an alpha granule adhesion molecule, binds to P-selectin glycoprotein ligand-I (PSGL-1) on the surface of leukocytes and leads to leukocyte activation (Mitra, Gangahar, Agrawal 2006).

Leukocyte recruitment

Leukocyte recruitment results in chronic inflammation, the hallmark of the restenosis process (Mitra, Gangahar, Agrawal 2006). Initially leukocytes attach loosely and roll along adhered platelets mediated by PSGL-1 and platelet P-selectin. The activated leukocytes subsequently undergo trans-platelet migration and vessel wall invasion, which is mediated by the leukocyte integrin mac-1 and the platelet-localized receptors GPIba and ICAM-2 (Mitra, Gangahar, Agrawal 2006). Leukocyte recruitment is driven by chemo attractant cytokines and chemokines secreted by SMC, EC and inflammatory cells themselves (Mitra, Gangahar, Agrawal 2006). IL-8 induces neutrophil recruitment, whereas other cytokines (IL-1, IL-6) and TNF-α in association with reactive oxygen species, proteolytic enzymes and growth factors play a significant role in modulating the inflammatory response (Mitra, Gangahar, Agrawal 2006). It has been observed that the inflammatory response is proportional to the extent of

the injury. Intravascular stenting produces the most profound injury accompanied by the most marked aggregation of inflammatory cells rich in neutrophils and monocytes/ macrophages (Mitra, Gangahar, Agrawal 2006). Adventitial inflammation also plays a role in IH development, and this has been reported to involve E-selectin, which controls adventitial inflammation through leukocyte recruitment. mAb directed against E-selectin significantly reduced this inflammatory response (Mitra, Gangahar, Agrawal 2006).

Activation of coagulation cascade

Vascular injury causes exposure of tissue factor (TF) to the circulating blood, which triggers the coagulation cascade resulting in thrombin generation. Tissue factor (TF), a glycoprotein of the vessel wall, and monocytes initiates the coagulation cascade by binding factor VII/VIIa (Mitra, Gangahar, Agrawal 2006). Inhibition of TF activity markedly reduces the thrombogenic response and the subsequent IH formation (Mitra, Gangahar, Agrawal 2006). Circulating TF becomes incorporated into the thrombus, mediated by PSGL-1 and P-selectin binding, between the microparticle and the activated platelet (Mitra, Gangahar, Agrawal 2006). The generated thrombin stimulates SMC proliferation both directly and indirectly by inducing platelet release of PDGF, which is a potent mitogen of SMC (Mitra, Gangahar, Agrawal 2006). In vein graft

occlusion, atherosclerosis plays a very significant role, particularly on late-stage graft occlusion. Connecting the vein to the artery causes endothelial dysfunction as a result of altered haemodynamic factors, and inflammatory response elicited at such sites (Mitra, Gangahar, Agrawal 2006).

Intimal hyperplasia is initiated by factors which include ischemia caused by damage to vasovasorum (Corson and others 1985) and haemodynamic changes which result in endothelial cell damage and loss (Kennedy and others 1989). This is followed by the deposition of a layer of platelets and fibrin at the site of injury and occasionally thrombus formation. Leukocyte migrate into the vessel wall, driven by chemical gradients of chemotactic cytokines released from SMC`s and leukocytes resident in the medial layer (Patel and others 2010).

Cellular proliferation phase

This phase is marked by release of growth and chemotactic factors from activated platelets, leukocytes, surrounding endothelial cells and medial smooth muscle cells (Patel and others 2010). These include platelet derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), interleukin-1 growth factor (IGF), Interleukin-1(IL-1), IL-6, IL-8 and vascular endothelial growth factor (VEGF) (Patel and others 2010). The growth factors stimulate the migration and proliferation

of SMC`s from the media into the neointima. These growth factors also stimulate the proliferation and migration of surrounding endothelial cells into the denuded area (Patel and others 2010). The neointima consists predominantly of SMC`s, endothelial cells, ECM and macrophages (Patel and others 2010). This phase is the primary focus of our studies and information on factors contributing and regulating this phase will be elaborated on further in the studies.

Remodeling phase

The remodeling phase occurs over a longer period of time and involves ECM protein degradation and resynthesis. There is a shift to fewer cells and an increase in ECM (Patel and others 2010). The endothelium inhibits SMC growth by acting as a selectively permeable barrier which protects against circulating growth factors (Inoue and Node 2009). EC`s also secrete NO, transforming growth factor-b (TGF-β), heparin and heparin-like molecules, which maintain SMCs in a quiescent state (Patel and others 2010).

Bypass grafts

Clinically it has been observed that arterial grafts have greater patency than venous grafts (Kobayashi 2009; Mehta and others 1997), (Mehta and others 1997). Improved outcome after coronary bypass surgery over

the last decade has been attributed to the increasing use of arterial conduits and their superior patency rates over that of venous derived grafts (Mehta and others 1997). In spite of this trend, autologous saphenous vein has remained an important and convenient conduit for a variety of operative scenarios, and is still used for more than 70% of grafts (Mehta and others 1997). As a result, vein graft failure continues to represent a significant clinical and economical burden upon the healthcare services. Between 15 to 30% of saphenous vein grafts occlude within the first year of surgery, increasing to over 50% after 10 years. By this time, more than 10% of patients will require further intervention to alleviate symptoms arising from occluded grafts (Mehta and others 1997). A lot of steps have been taken to prevent IH in vein derived grafts including avoiding damage at the time of implantation, meticulous surgical technique, and pharmacological intervention; however none of these methods has been shown to prevent vessel occlusion especially in venous derived grafts. It is therefore essential to investigate the differences in response between venous and arterial derived grafts following injury and could these differences on a cellular and molecular level offer some insight to the clinical differences observed that show arterial grafts having a greater patency than venous derived grafts.

Contribution of hypoxia to Intimal hyperplasia

The function of vascular smooth muscle cells (SMC), is known to be altered by hypoxia (Lee and others 2000), SMC are also known to function in a similar manner when they are recovered from lesions in IH (Lee and others 2000) . The anoxemia theory of artery wall pathology in atherosclerosis was first suggested in 1944 and since then several investigators have developed techniques to directly measure artery wall *p*O2 levels (Zemplenyi, Crawford, Cole 1989). Zemplenyi *et al.* describe a microcathode puncture apparatus to measure iliofemoral arteries *in vivo* (Zemplenyi, Crawford, Cole 1989). Niinikoski *et al.* ,Santilli *et al.* , and Buerk and Goldstick all describe techniques to directly measure artery wall oxygen tension levels *in vivo* and have found depressed *p*O2 levels in the artery wall (Lee and others 2000), (Bjornheden and others 1999), (Buerk and Goldstick 1982). Bjornheden *et al.* also describes a hypoxia marker, 7-(49-(2-nitroimidazol-1-yl)-butyl) theophylline, that was found in atherosclerotic plaque, further confirming the role of anoxemia in IH and atherosclerosis (Bjornheden and others 1999; Lee and others 2000). At the time of surgery, the vasa vasorum is dissected away from the adventitia, preventing diffusion of blood from the adventitial surface. In addition, arterial suturing further contributes to decreased diffusion of arterial blood from the luminal side (Lee and others 2000). Understanding how vascular endothelial cells respond to hypoxic

conditions might offer some insight on how communication between the endothelial cells and smooth muscle cells initiate IH.

Hypoxia and regulation of cells under hypoxia by Hypoxia Inducible factor-1 alpha (HIF-1α)

Cells in mammalian tissues typically experience oxygen concentrations in the 40-60 mmHg range (Kaluz, Kaluzova, Stanbridge 2008). Hypoxia, in this paper is defined as a state of reduced oxygen levels below normal levels, which occur under pathological conditions with IH being observed as an end point. In nucleated cells the primary transcriptional response to hypoxic stress is mediated by the hypoxia-inducible factors (HIF) (Majmundar, Wong, Simon 2010).

HIF is a heterodimer that consists of a hypoxia stabilized HIF1-α subunits and the constitutively expressed HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator or ARNT) (Kaluz, Kaluzova, Stanbridge 2008). Both α and β subunits belong to the family of the basic helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) domain-containing transcription factors. bHLH and PAS domains mediate DNA binding and dimerization; the other domains in the α subunit includes a unique oxygen dependant degradation domain (ODDD) and two transactivation domains: the N-terminal activation domain (NAD) and C terminal activation domain (CAD)(Kaluz, Kaluzova, Stanbridge 2008). Three structurally closely related α subunits (HIF-1α, HIF-2α, and HIF-3α) have been identified to

date. In addition to these three isoforms, their spliced variants also have been described. Inhibitory PAS protein preferentially dimerizes with HIF-1 α preventing the formation of active HIF-1α/ HIF-1β heterodimers.(Kaluz, Kaluzova, Stanbridge 2008). Figure1.3 illustrates summary of HIF1-alpa structural features.

Figure 1.3

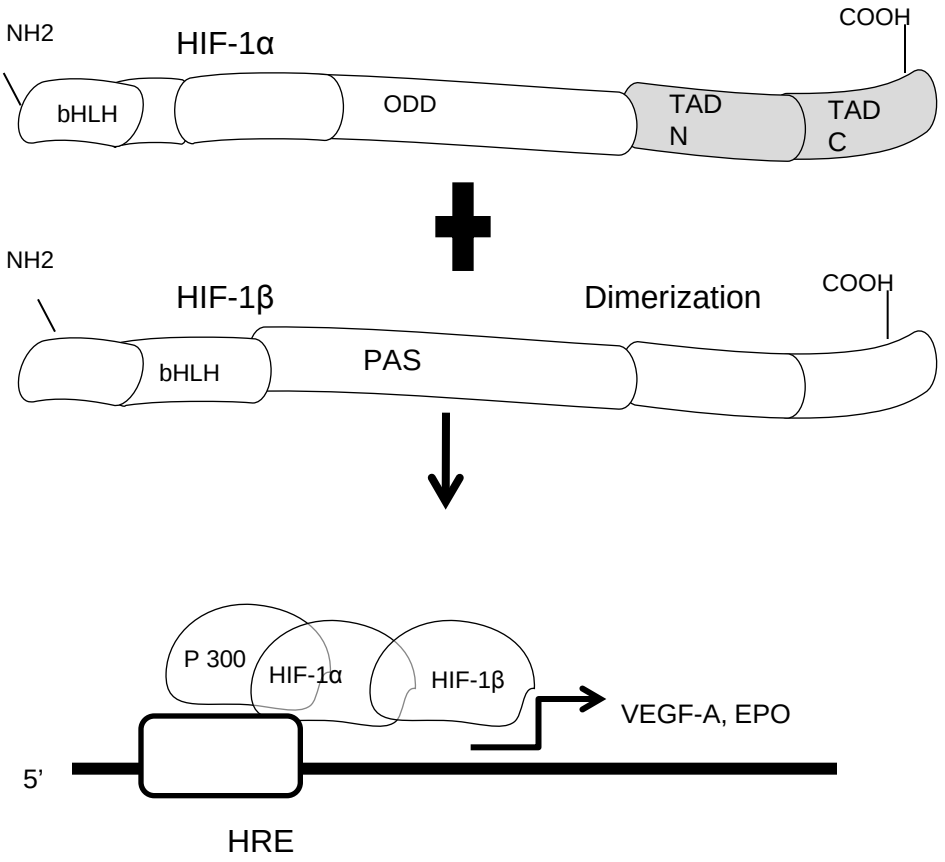


Figure 1.3 Structure of HIF1-alpha. Under normoxic conditions HIF-1α is degraded by PHD`s. Under hypoxic conditions HIF-1alpha is stabilized binds with HIF-1β and co-activators p300 binds to Hypoxia response element (HRE) and results in transcription of genes like VEGF

Levels of HIF1-alpha are regulated by the prolyl hydroxylase domain-containing enzymes (PHDs), factor-inhibiting HIF-1alpha (FIH1) and metabolites. HIF1-α subunits are hydroxylated at conserved proline residues (Majmundar, Wong, Simon 2010). These modifications are mediated by PHDs, whose activities are regulated by oxygen availability (Majmundar, Wong, Simon 2010). Hydroxylated HIFα is, in turn, recognized and marked for proteosomal destruction by the E3 ubiquitin ligase, the von Hippel-Lindau protein (pVHL) complex (Majmundar, Wong, Simon 2010). Under hypoxic conditions PHD activity is diminished, and stabilized HIFα proteins can induce transcription of genes with adaptive functions. PHDs have been proposed to be oxygen sensors linking cellular oxygen concentration to HIF molecular responses (Majmundar, Wong, Simon 2010). HIFα subunits are also substrates for an asparaginylhydroxylase; factor inhibiting HIF1α (FIH) (Majmundar, Wong, Simon 2010). This enzyme is oxygen dependant and thus represents another component of the oxygen-sensing machinery (Majmundar, Wong, Simon 2010). Hydroxylation by FIH disrupts a critical interaction between HIFα and coactivators p300/CBP, impairing HIF transcriptional activity (Majmundar, Wong, Simon 2010). In response to hypoxia HIF-1alpha and HIF-2 alpha regulate angiogenenic genes.(Majmundar, Wong, Simon 2010). Both alpha subunits are highly

conservative at protein levels and share similar domain structure and heterodimerize with HIF-1 beta, and bind to the same DNA sequence, hypoxia responsive element (HRE) (Loboda, Jozkowicz, Dulak 2010).

Hypoxia is known to induce growth factors like VEGF-A and PDGF-BB in endothelial cells (Faller 1999). VEGF-A has been shown to induce smooth muscle cell proliferation and migration through a vascular endothelial cell growth factor receptor 1 [(Banerjee and others 2008a).

Growth factor and receptor signaling under hypoxia

Vascular endothelial growth factor (VEGF) is a heparin binding angiogenic growth factor displaying high specificity for endothelial cells (Neufeld and others 1999). The human VEGF-A is organized in eight exons, separated by seven introns (Ferrara, Gerber, LeCouter 2003). VEGF exists as one of four different molecular species 121, 165, 189, and 206 (Ferrara, Gerber, LeCouter 2003). It is well established that alternative exon splicing of a single VEGF gene is the basis for the molecular heterogeneity (Ferrara, Gerber, LeCouter 2003). VEGF165 is the predominant molecular species produced by a variety of normal and transformed cells (Ferrara, Gerber, LeCouter 2003). Transcripts encoding VEGF121 and VEGF189 are detected in the majority of cells and tissues expressing the VEGF gene (Ferrara, Gerber, LeCouter

2003). VEGF121 is a freely soluble protein; VEGF165 is also secreted although a significant fraction remains bound to the cell surface and the extracellular matrix. In contrast, VEGF189 and VEGF 206 are almost completely sequestered in the extracellular matrix (Ferrara, Gerber, LeCouter 2003). Factors that can potentiate VEGF production include fibroblast growth factor (FGF), PDGF, tumor necrosis factor (TNF), transforming growth factor b (TGF-b), keratinocyte growth factor (KGF), insulin growth factor (IGF), interleukin 1b (IL-b), and IL-6 (Neufeld and others 1999).

Several mechanisms have been shown to participate in the regulation of VEGF gene expression. The focus of this study was to elucidate the role hypoxia plays in modulation of VEGF on smooth muscle cells therefore a more detailed analysis of the role of hypoxia on VEGF secretion will be represented. Oxygen tension has been shown to play a major role, both *in vitro* and *in vivo* on VEGF regulation (Ferrara, Gerber, LeCouter 2003). VEGF mRNA expression is rapidly and reversibly induced by exposure to low pO2 in a variety of normal and transformed cells (Ferrara, Gerber, LeCouter 2003). Ischemia caused by occlusion of the left anterior descending coronary artery results in a dramatic increase in VEGF mRNA levels suggesting that hypoxia through a VEGF mediated pathway modulates the vascular pathologies that occur during vessel occlusion (Ferrara, Gerber, LeCouter 2003). mRNA stability has been

identified as an important posttranscriptional component (Ferrara, Gerber, LeCouter 2003).

Two VEGF receptor tyrosine kinases (RTKs) have been identified (Ferrara, Gerber, LeCouter 2003). The Flt-1 (fms-liketyrosine kinase) and KDR (kinase domain region) receptors bind VEGF with high affinity (Ferrara, Gerber, LeCouter 2003). Flt-1 and KDR/Flk-1 have seven immunoglubin (Ig)-like domains in the extracellular domain a single transmembrane region and a tyrosine kianse sequence interrupted by a kinase insert domain (Ferrara, Gerber, LeCouter 2003). Flt-1 has the highest affinity for VEGF165 with KDR having a lower affinity. An alternative spliced soluble form of Flt-1 (sFlt-1), lacking the seventh Ig-like domain, transmembrane sequence and the cytoplasmic domain is able to bind to VEGF with a high affinity and is able to inhibit VEGF-induced mitogenesis, making it a physiological negative regulator of VEGF action (Ferrara, Gerber, LeCouter 2003). Neuropilin-1 (NRP-1), a type 1 transmembrane protein and coreceptor of VEGF-A165 has been shown to participate in VEGF-A induced signaling by binding with VEGFR-2 (Banerjee and others 2008a).

VEGF Growth receptor signaling under normoxia and under hypoxia

VEGFR-1 blocking antibodies have been shown to prevent endothelial

cell migration in response to VEGF-A (Takahashi and Shibuya 2005). VEGFR-1 modulates the reorganization of actin via p38 MAPK (mitogen-activated protein kinase) whereas VEGFR-2 contributes to the re-organization of the cytoskeleton by phosphorylating FAK (focal adhesion kinase) suggesting a different contribution of the two receptors to the chemotactic response (Takahashi and Shibuya 2005). VEGFR-1 signaling is also involved in the migration of monocytes/macrophages and in the reconstitution of haematopoiesis by recruiting haematopoietic stem cells (Takahashi and Shibuya 2005).

VEGFR-2 is expressed in vascular and lymphatic endothelial cells and other cell types such as smooth muscle cells and haematopoietic stem cells (Takahashi and Shibuya 2005). Binding of VEGF on VEGFR-2 results in autophosphorylation of the tyrosine residues (Takahashi and Shibuya 2005). Tyrosine autophosphorylation allows for recruitment of adaptor proteins such as SH2 and grb. VEGFR-2 is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF (Takahashi and Shibuya 2005). Survival signaling for VEGFR-2 involves PI3K (phosphoinositide 3-kinase)/ Akt pathway(Takahashi and Shibuya 2005). Autophosphorylation of tyrosine 1175 is crucial for VEGF dependent proliferative effects via the PLC-*γ* /PKC/Raf/MEK [MAPK/ERK (extracellular-signal-regulated kinase) kinase]/ERK pathway (Takahashi and Shibuya 2005).

Signal transduction under hypoxia

MAP kinases have been reported to modulate transcriptional activity of HIF (Majmundar, Wong, Simon 2010). ERK1/2 and p38 MAP kinases can stimulate HIF1- alpha activity without affecting its stability although MEK1 and p38 inhibitors have been shown to mediate gene expression, the significance of MAP kinases, with respect to influencing HIF function is most likely cell-type specific (Majmundar, Wong, Simon 2010). ERK1/2 and p38 MAP kinases have been shown to phosphorylate HIF-alpha *in vitro*.

Hypoxia has been shown to cause smooth muscle cell replication and extracellular matrix accumulation resulting in vessel wall remodeling (Kourembanas and others 1997). The cellular responses to hypoxia involve complex cell-cell interactions mediated by the release of growth factors, cytokines and biological messengers. Several growth factors have been implicated to modulate IH; however controversy surrounds the exact initiating factors involved in SMC migration and proliferation (Pak and others 2007). Studies by Bentinz et.al (Benitz and others 1986) have demonstrated that hypoxia does not induce smooth cell proliferation contrary to studies by Pak et al which state that hypoxia induces smooth muscle cell proliferation (Pak and others 2007). Studies conducted by Mayuko et.al have demonstrated that VEGF induces smooth muscle cell proliferation in an autocrine manner (Osada-Oka and

others 2008). Studies determining the exact initiating factor for smooth muscle cell proliferation under hypoxia and whether its autocrine and paracrine mechanism are still yet to be elucidated. Mayuko et.al has demonstrated that under hypoxic conditions (5% CO2 and 1% O2), the mRNA and protein levels of VEGF, and the mRNA level of VEGF receptor-1 (VEGFR-1) increased with an increase in HIF-1alpha in coronary artery smooth muscle cells (Osada-Oka and others 2008). Hypoxia enhanced the incorporation of thymidine which was completely inhibited by a neutralizing antibody against VEGF (Osada-Oka and others 2008). Studies by Sternmark K.R et al (Stenmark and others 1999) suggest that the medial layer is made up of different subpopulations of smooth muscle cells. In response to hypoxia there are remarkable differences in the proliferative and matrix producing responses of these cells to the hypoxic environment. Some cell populations proliferate and increase matrix protein synthesis, while in other cell populations no apparent change in the proliferative or differentiation state of the cell. Stermark et al also claims that in other cases in response to hypoxia the adventitial layer composed mostly of fibroblasts rather than the medial layer is what proliferates. There is accumulating evidence which suggests that the unique response exhibited by specific cell types of hypoxia *in vivo* can be modeled *in vitro*. Isolated smooth muscle cells from these layers demonstrate significant

increases in proliferation in response to hypoxia, and others which exhibit no change or, in fact, a decrease in proliferation under hypoxic conditions (Stenmark and others 1999). These authors suggest that only certain fibroblast populations are capable of responding to hypoxia with an increase in proliferation. Signaling pathways activated in these cell populations exhibit proliferative responses to hypoxia. In the absence of serum or mitogens, hypoxia specifically activates select members of the protein kinase C isozyme family, as well as of the mitogen activated protein kinase (MAPK) family of proteins. This selective activation appears to take place in response to hypoxia only in those cells exhibiting a proliferative response, and antagonists of this pathway inhibit the response (Stenmark and others 1999).

As important as determining the initiating events, the identification and characterization of key factors that are functionally important in propagation of IH is needed since these factors could be potential targets for therapeutic intervention. Hypoxia increases the expression of a number of genes encoding vascular cell mitogens produced by endothelial cells: platelet-derived growth factor B (PDGF-B) and vascular endothelial growth factor (VEGF) (Kourembanas and others 1997). The summary of VEGFR signaling pathway is illustrated in figure 1.4

Figure 1.4

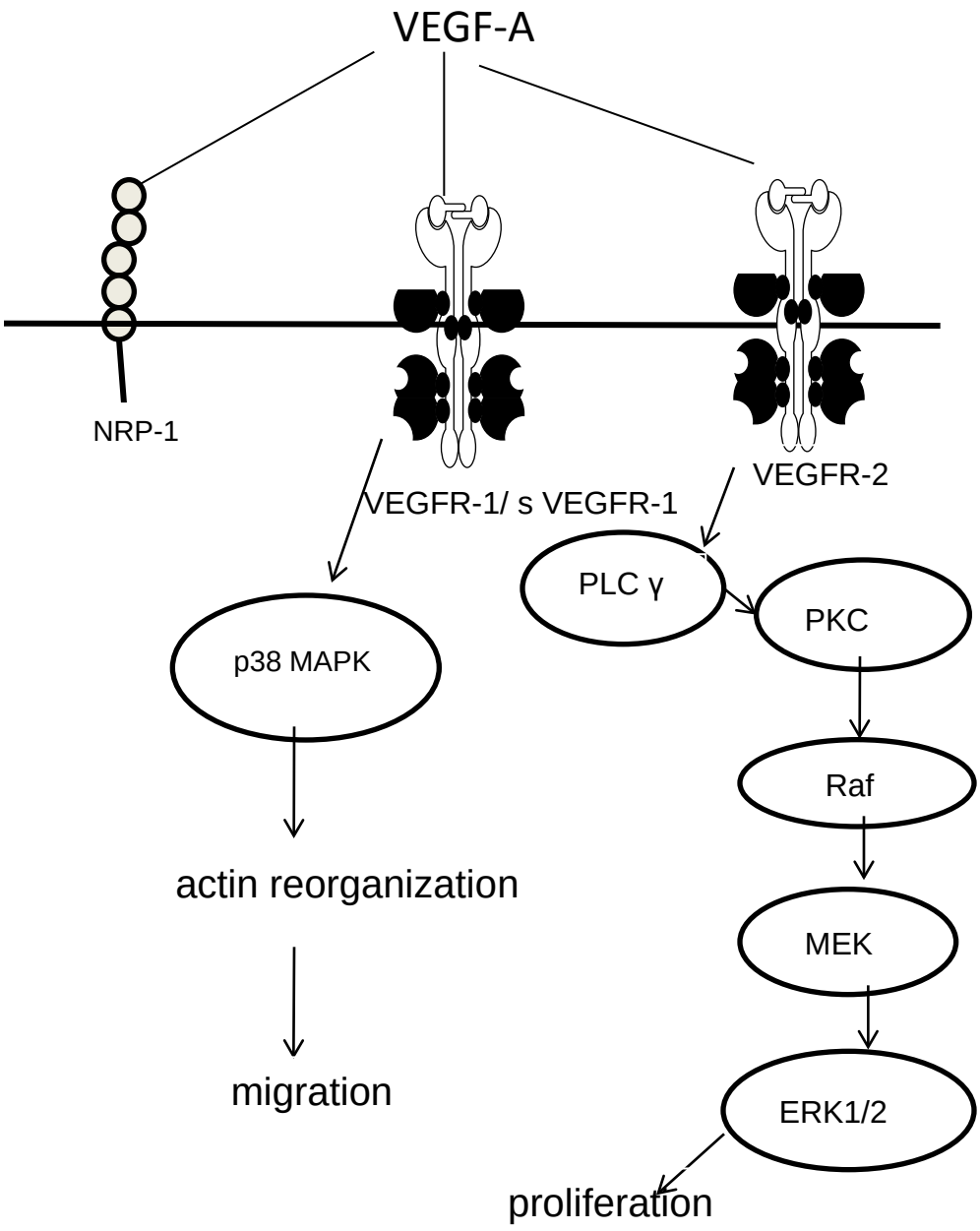


Figure 1.4 VEGF-A and VEGF Receptor downstream signaling. The figures showing VEGF-A and VEGFR1, R2 and the downstream signaling pathways activated. ERK1/2 is activated mainly during proliferative responses and p38 mainly during migrational responses

PDGF-BB

Platelet- derived growth factor (PDGF) represents one of the strongest mitogens for smooth muscle cells (ten Freyhaus and others 2011). Endothelial cells have been shown to secrete PDGF-BB under hypoxia via a HIF-1alpha dependent mechanism. PDGF exerts its actions via two transmembrane receptor subtypes alpha and beta, which belong to the family of receptor tyrosine kinases (ten Freyhaus and others 2011). Of the two receptor subtypes, PDGF beta receptor (PDGFR-β) mediated signals are particularly important for vascular development and remodeling (ten Freyhaus and others 2011).

On ligand binding, PDGFR β autophosphorylates on tyrosine residues and subsequently recruits and activates SH-2 domain-containing signaling molecules, which associate with the receptors at specific binding sites (ten Freyhaus and others 2011). Inhibition of PDGF signaling by tyrosine kinase inhibitor Imatinib was recently shown to reverse vascular arterial cells remodeling and to improve survival in animal models of pulmonary hypertension (ten Freyhaus and others 2011). These data highlight the importance of PDGF signaling in the vascular pathophysiologies (ten Freyhaus and others 2011).

PDGF-BB is widely expressed in normal and transformed cells and is produced by monocytes, macrophages; vascular endothelial and smooth muscle cells (Aversa and others 2001). There are three PDGF isoforms

(AA, AB and BB) that exert their biological actions via binding to cell surface receptors alpha and beta. PDGF mediated events, which include chemo-attraction, activation of inflammatory cells; vasoconstriction and influence on the synthesis or degradation of matrix constituents are exerted locally in an autocrine or paracrine manner and are involved in normal as well as pathological processes such as atherosclerosis and intimal hyperplasia (Aversa and others 2001).

Aversa A et al found out that cytoplasmic localization of PDGF peptides under hypoxia was focal in the endothelium and diffuse in the smooth muscle cell (Aversa and others 2001).

Following vascular injury, VSMCs respond to soluble factors including basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), transforming growth factor beta-1(TGF) and platelet-derived growth factor. These factors are secreted by injured endothelial cells and smooth muscle cells as well as platelets and macrophages (Kingsley and others 2002). In response, vascular smooth muscle cells secrete proteolytic enzymes that digest the extracellular matrix proteins in the tunica media and deposit new ECM that support smooth muscle cell migration and proliferation (Kingsley and others 2002). The precise contributions of growth factors and specific ECM proteins to the development of neointima as well as molecular mechanisms linking the growth factor signaling to smooth muscle cell migration and proliferation

are yet to be elucidated (Kingsley and others 2002). Studies conducted by Kingsley K et al have demonstrated that VSMC proliferation was enhanced in the presence of PDGF-BB when plated on laminin-5, and that bFGF, EGF, or TGF-b1 had no drastic effects on SMC proliferation. The effect was blocked by PD98059, a specific inhibitor of the MEK1/2 members of the MAP kinase signaling pathway (Kingsley and others 2002).

All PDGFs can function as homodimers, but only PDGF-A and PDGF-B form heterodimers. PDGF-BB is able to bind to both the PDGFR alpha and PDGFR beta and therefore can bind both heterodimers and homodimers of the receptors (Raines 2004). In contrast PDGF-AA can only bind to PDGFR alpha homodimers and PDGF-AB binds both homodimers of the PDGFR alpha and beta and the heterodimer (Raines 2004).

Transforming growth factor beta 1 (TGF-β1)

TGF-β1 is among the most potent soluble growth factors that regulate smooth muscle cell differentiation (Long and Miano 2011). Members of the TGF-β1 superfamily transmit signals through both SMAD dependent and SMAD-independent pathways (Long and Miano 2011). The classic pathway is through transmembrane serine-threonine kinase receptors, which mediate the phosphorylation of receptor-specific SMAD2 and

SMAD3 (Long and Miano 2011). The phosphorylated SMAD2/SMAD3 complex then interacts with the common SMAD4 to form a heteromeric complex which translocates to the nucleus and binds to smad-binding elements (SBE) located in the regulatory region of a number of target genes (Long and Miano 2011). SMAD-independent pathways, such as MAPK and PI3 kinase, can also be triggered by TGF- β to initiate signal transduction and gene regulation (Long and Miano 2011). Hypoxia has been shown to stimulate the production of TGFβ-1 in cultured smooth muscle cells (Kingsley and others 2002). TGFβ-1 is known to induce extracellular matrix expression and inhibit growth and proliferation of vascular smooth muscle cell (Kingsley and others 2002). Kingsley K et. al demonstrated that TGFβ-1 inhibited cellular proliferation compared to control (Kingsley and others 2002). TGFβ-1 induced apoptosis in vascular smooth muscle cells at high concentrations (Kingsley and others 2002). TGFβ-1 is known to exert a cytostatic effect at low concentrations and reduces cell numbers at higher concentrations (Kingsley and others 2002).

FGF-2

FGF-2 proteins constitute one of the largest families of growth factors, comprising 18 members and four homologues in humans named FGF1

to FGF23 (Bosse and Rola-Pleszczynski 2008). The cytoplasmic low molecular weight isoform of FGF2 is a single chain non glycosylated protein (Bosse and Rola-Pleszczynski 2008). FGF-2 is also found anchored to ECM components at the extracellular surface of the plasmalemma and within the basement membrane of different tissues (Bosse and Rola-Pleszczynski 2008) suggesting that it can be released from a cell. FGF-2 is actually the main growth factor stored in the basement membrane.

FGF-2 can bind to four high affinity receptors, named FGF receptor (FGFR) 1 through FGFR4. These receptors are type I transmembrane proteins with 3 immunoglobin-like domains (D1-D3) and a cytoplasmic tail bearing an intrinsic tyrosine kinase domain (Bosse and Rola-Pleszczynski 2008). Several alternative splicing events take place following transcription of FGFR1, FGFR2 and FGFR3 and give rise to different isoforms with distinct functional properties. FGFR dimerization is a prerequisite for the activation of downstream signaling pathways (Bosse and Rola-Pleszczynski 2008). FGF- induced FGFR dimerization brings the two chains of the receptor into sufficient proximity to permit the protein tyrosine kinase (PTK) domain of each chain to transphophorylate several tyrosine residues on the other chain (Bosse and Rola-Pleszczynski 2008). Phosphorylated tyrosines then serve as docking sites for proteins containing src hology (SH)2 or phosphotyrosine-binding

(PTB) domains. Adaptor proteins, such as shc, shb and crk, bind directly to the activated receptor (Bosse and Rola-Pleszczynski 2008). This initiates signaling transduction pathways involved in mitogenesis, such as the Ras/mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K)/Akt pathways. FGFR dimerization also leads to the activation of phospholipase (PL)Cg, which catalyses phosphatidylinositol4,5-diphosphate into diacylglycerol (DG) and inositol 3- phosphate (IP3). These second messengers (DG and IP3) subsequently activate conventional and novel forms of protein kinase (PK)C and increase the intracellular concentration of Ca^{2+} by binding to IP3 receptors on the endoplasmic reticulum, respectively. These enumerated pathways are the ones that ensure the signal transduction of activated FGFR and thus are responsible for the altered behavior adopted by a particular cell type in response to FGF stimulation. However, their respective contribution, as well as the role that other signaling pathways might have in the elaboration of this altered cellular behavior, is hard to predict owing to the cell type-specific nature of these responses (Bosse and Rola-Pleszczynski 2008). However, the detailed mechanisms regulating FGF2 expression, secretion, tissue distribution and localization, as well as the operational mechanisms leading to the elaboration of a functional receptor complex and the transduction of productive intracellular signaling pathways will require further elucidation

(Bosse and Rola-Pleszczynski 2008). FGF-2 induces smooth muscle cell proliferation *in vitro.* FGF-2 synergizes with platelet-derived growth factor (PDGF)-AA and PDGF-AB, as well as with other receptor tyrosine kinase acting ligands, such as insulin (Bosse and Rola-Pleszczynski 2008). The synergism with some of the members of PDGF family was attributed to its ability to induce the expression of PDGF receptor a-chain (PDGFRa) (Bosse and Rola-Pleszczynski 2008).

Role of MicroRNAs in IH

Over the past decade, microRNAs (miRs) have emerged as key regulators of many physiological and pathological cellular processes (Crosby and others 2009). They are synthesized as primary transcripts, which undergo stepwise processing by two ribonuclease III enzymes, DROSHA and DICER (Crosby and others 2009). This leads to the formation of 19-24 nucleotide long mature duplexes. Selective transfer of one of the strands to the RNA-induces silencing complex (RISC) is followed by base pairing to partially complementary sites, which occur primarily within the 3` untranslated regions (UTRs) of target genes (Crosby and others 2009). Aberrant expression of miRs frequently leads to abnormalities in development and the pathogenesis of disease and a growing number of miRs have been discovered as powerful modulators in cardiovascular biology. For example, miR21 has been linked to the SMC proliferative phenotype in a ballon-injured rat carotid artery model

(Long and Miano 2011).

Vascular smooth muscle cells, the predominant cells in tunica media of artery, are highly specialized cells that regulate blood pressure through the regulation of blood vessel tone (Mitra, Gangahar, Agrawal 2006). In contrast to terminally differentiated muscle cells, vascular smooth muscle cells retain remarkable plasticity and can switch between differentiated and dedifferentiated phenotypes in response to physiological and pathological cues such as vascular injury, hypertension and atherosclerosis (Mitra, Gangahar, Agrawal 2006). Differentiated vascular smooth muscle cells demonstrate a very low rate of proliferation, appropriate contractility to contractile cues and express SMC specific genes, such as smooth muscle alpha-actin, smooth muscle myosin heavy chain (SMHC) SM22alpha, and calponin (Mitra, Gangahar, Agrawal 2006).

In response to vascular injury or growth factor signaling, VSMCs dedifferentiate and adopt a synthetic phenotype, which is characterized by increased proliferation, migration, enhanced production of collagens and matrix metalloproteinases, and diminished expression of SMC-specific contractile markers (Song and Li 2010). Many miRNAs are highly expressed in vascular system and involved in the control of proliferation and differentiation of VSMCs (Song and Li 2010). Several recent reports demonstrate that miR-143 and miR-145 are enriched on

VSMCs and play a significant role in regulating the phenotype switching of VSMCs (Song and Li 2010). *In vitro* over expression of miR-145 or miR-143 was sufficient to promote differentiation and proliferation of cultured VSMCs (Song and Li 2010). In contrast, miR-143 and miR-145 deficient VSMCs were absent of contractile abilities to vasopressive stimuli and maintained in the synthetic state (Song and Li 2010). They also indicated a significant increase in the ability to migrate toward PDGF. *In vivo,* miR-143/miR-145 mutant mice revealed a significant decrease in the number of contractile VSMCs and a remarkable increase in the number of synthetic VSMCs in the aorta and the femoral artery of miR-143/miR-145 mutant mice, with a reduced media thickness (Song and Li 2010). VSMCs within miR-143/145 mutant artery showed a pro-synthetic morphological features and a significant downregulation in the expression of SMC-specific differentiated markers (Song and Li 2010). Taken together these data suggest that miR-143/145 play a pivotal role in maintaining the differentiated phenotype of VSMCs since deficiency of miR-143 and miR-145 leads to VSMCs phenotypic switching from contractile state (Song and Li 2010).

CHAPTER 2: Growth Factor Induced Proliferation in Venous vs. Arterial Smooth Muscle Cell is Differentially Regulated Under Hypoxia

Introduction

Intimal hyperplasia (IH) occurs when smooth muscle cells (SMC) migrate and proliferate from the tunica media into the tunica intima of a vessel due to hypoxia following injury (Muto and others 2007) IH occurs in most vascular surgeries including vascular grafts and by-pass surgeries which ultimately leads to vessel occlusion and graft failure (Vorp and others 2001). Studies conducted on graft patency have shown that arterial grafts have a higher patency than vein grafts. Almost 90% occlusion occurring in venous grafts after ten years as compared to only 50% occlusion in arterial grafts (Cheng and others 2010).

IH is a growing concern because the effective treatment for the prevention of IH in clinical practice still continues to elude vascular surgeons (Hulten and Levin 2009). This is of great concern considering that there is an overall increase in coronary arterial disease incidences in America (Jo and others 2006). Surgical bypass of arterial occlusions using autogenous vein provides an effective treatment for many patients with advanced coronary atherosclerosis (Jo and others 2006), (Abraham and Distler 2007). Conventional pharmacotherapy has limited impact on graft failure (Abraham and Distler 2007). Therefore it is necessary to

investigate the mechanism by which IH occurs so as to identify novel therapeutic targets that can inhibit IH.

Several growth factors have been implicated to modulate IH; however controversy surrounds the exact initiating factors involved in SMC migration and proliferation(Ferrara, Winer, Burton 1991)(Ferrara, Gerber, LeCouter 2003). As important as determining the initiating events, the identification and characterization of key factors that are functionally important in propagation of IH is needed since these factors could be potential targets for therapeutic intervention (Ferrara, Winer, Burton 1991; Lee and others 2000). One theory states that hypoxia alone in an autocrine mechanism acts as a stimuli on SMC to initiate proliferation (Pak and others 2007; Schultz, Fanburg, Beasley 2006; Shibuya and Claesson-Welsh 2006; Shibuya and Claesson-Welsh 2006). Another theory suggests that the combined action of growth factors, proteolytic agents, and extracellular matrix proteins that are produced by a dysfunctional endothelium following injury or hypoxia, induce proliferation and migration of resident SMCs from the media into the intima (Helfrich and others 2010)). However most theories agree that hypoxia plays a pivotal role in smooth muscle cell migration and proliferation. Studies that have been conducted to show the interaction between SMC and endothelial cell (EC) under hypoxia have been inconclusive and

controversial (Growcott, Banner, Wharton 2005; Helfrich and others 2010; Pak and others 2007).

Hypoxia induces the secretion of growth factors like Vascular Endothelial Growth Factor (VEGF), and Platelet Derived Growth Factor-BB (PDGF-BB) (Helfrich and others 2010).These growth factors under normoxic condition have been shown to induce SMC de-differentiation and enhance proliferation(Helfrich and others 2010). VEGF is a homodimeric glycoprotein, produced by all nucleated cells including endothelial cells (Cogo and others 2003). VEGF gene expression is induced by hypoxia and returns back to base levels upon re-oxygenation in endothelial cells. VEGF is known to bind to three receptor tyrosine kinases, flt-1 (fms-like tyrosine kinase, VEGFR-1), Flk-1/KDR (fetal liver kinase 1- murine homologue/Kinase insert Domain containing Receptor-human homologue) VEGFR-2 and flt-4 (VEGFR-3)(Murray, Kulkarni, Ezzati 2006). Among these VEGFR-2 is the primary mediator of VEGF signaling and is responsible for the proliferative effects observed with VEGF (Murray, Kulkarni, Ezzati 2006). PDGF-BB, a mitogenic peptide produced by endothelial cells. is known to cause proliferation of vascular cells(Schultz, Fanburg, Beasley 2006; Shibuya 2010b). Hypoxic conditions have been shown to increase PDGF mRNA levels in EC (Patel and others 2010; Schultz, Fanburg, Beasley 2006)(). PDGF-BB signals via PDGFR β which is a tyrosine kinase.

In this study we investigated the interaction of endothelial cells with smooth muscle cells under hypoxia as a potential mechanism for initiation of intimal hyperplasia. In addition we elucidated the different responses observed between venous and arterial derived smooth muscle cells under hypoxia and what role hypoxic insult played in contributing to the differences in patency observed clinically between venous and arterial derived grafts.

Our findings showed that venous vs arterial derived cells under hypoxia showed differences in growth factor profiles (VEGF-A and PDGF-BB) as well as their receptor expression. These differences play a crucial role in causing IH thus differences in graft patency. Therefore differential therapies should be used for venous derived vs. arterial derived grafts to improve patency of each graft.

METHODS

Cell culture

Human umbilical vein smooth muscle cell (V-SMC) were obtained from ScienceCell Reseach Laboratories (Carlsbad, CA) and maintained in SmGm-2 (Lonza, Walkersville, MD). Human aortic smooth muscle cell (A-SMC) and human aortic endothelial cell (A-EC) obtained from Lonza were maintained in SmGM-2 and EGM-2 (Lonza) medium respectively.

Human umbilical vein endothelial cells (V-EC), (kind gift from Dr Ramakrishnan, University of Minnesota) were also maintained in EGM-2 media.

Reagents: p-ERK1/2, total-ERK1/2 primary antibodies; anti-rabbit IgG and anti-mouse IgG secondary antibodies were purchased from Cell signaling Technology (Beverly, MA). VEGFR-2, α- tubulin, β-actin, Ephrin B2 and PDGFR-β primary antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, California); anti-human VEGFR2 (PE), anti-human VEGFR-2 neutralizing antibody, anti-human PDGF-BB neutralizing antibody, Ephrin B4 (PE) were purchased from (R&D Systems, Minneapolis, MN) ; anti-rabbit secondary antibody (FITC) was acquired from BD bioscience (New Jersey, USA). Imatinib mesilate (PDGFR antagonist), VEGFR-2 tyrosine kinase inhibitor was purchased from Calbiochem (New Jersey, USA).

Treatment: Cells were serum starved for 24hr in 1% FBS + (EBM-2 and SmBm) and then subjected to normoxia and hypoxia for different time points depending on assay conducted. 3hrs treatment was used for isolation of total mRNA in cells. 24hrs for proliferation assay, growth factor and growth factor receptor analysis.

Normoxic conditions (21% oxygen), is defined here as atmospheric pressure and temperature air in a 5% CO_2, 37°C cell culture incubator.

To achieve hypoxia (3-5% O_2), cells were placed in a modular chamber (Billups Rothenberg, Inc., Del Mar, CA) and flushed with a mix of 0% O_2, 5%CO_2, and 95% N_2 at 10L/min for 15 minutes. Chambers remained tightly sealed and placed in a 5% CO_2, 37°C incubator. This method achieves pO_2 levels less than 35mmHg as determined from cell culture medium analyzed using a blood gas analyzer, Rapid Lab248 (Chiron Diagnostics Tarrytown, NY); pO_2 levels of culture supernatant from cells grown under normoxic conditions was 150–160 mmHg.

BrdU cell proliferation assay

BrdU ELISA from Roche diagnostics (Madison, WI) was used to measure cell proliferation. SMC`s ($1x\ 10^3$) were plated in a 96 well plate after serum starvation for 24hrs. Cells were then placed under hypoxia for 24hrs. At 24 hrs BrdU (100μM) was added for 4 hrs; which is incorporated into the newly synthesized DNA of the replicating cells. For experiments determining effect of conditioned endothelial cell media on SMC proliferation 100ul of conditioned media was added to each well of the 96 well plates prior to hypoxia treatment for 6, 12, 24 and 48hrs, and then pulse labeled with BrdU. Only data for 24hr hypoxia treatment is shown since we observed the greatest increase in proliferation at this time point. To determine effect of VEGF dose response, ($5\ x\ 10^3$) cells (A-SMC<, V-SMC and V-EC) were plated in a 48 well tissue culture plate and placed under hypoxia for 24 hrs in the presence of 0, 5, 10 and 15

ng/mL of VEGF. BrdU incorporation in these cells was used as an indicator of cell proliferation. Cells were fixed with FixDenature solution (Roche Diagnostics) and then were exposed for immunodetection with a peroxidase-conjugated anti-BrdU antibody. Absorbance was measured at a wave length of 370nm using a Fluostar Omega BMG Labtech (Cary, NC) microplate reader.

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT assay)

To determine cell viability an MTT assay was conducted. In this assay SMC`s (1x 103) were plated in 96 well plates, serum starved and then placed under hypoxic and normoxic conditions for 24hrs. The cells were then incubated with MTT (2.5mg/mL) for 4 hrs. Cells were solubilized with 100% DMSO (Sigma Aldrich, St. Louis, MO) and absorbance at 450 nm was measured using a plate reader (Omega BMG Labtech, Cary, NC). % change in cell viability was determined normalized to control (cell viability under normoxia).

Quantitative analysis of VEGF-A and PDGF-BB secretion

Protein levels of VEGF-A and PDGF-BB secreted in cell culture media was determined using a human PDGF-BB and VEGF-A (ELISA) kit as per manufacturer's instructions. SMC and EC cells (5 $x10^3$) were plated

in a 48 well plate, serum starved and placed under hypoxia for 24hrs. 100ul of cell culture supernatants was collected and analyzed for VEGF-A and PDGF-BB secreted. (R&D Systems, Minneapolis, MN).

Angiogenesis assay for growth factor secretion

An angiogenesis ELISA strip II for analysis of cytokines from Signosis (Sunnyvale, CA) was used to compare levels of PDGF-BB, IGF-I, FGF-b and TGF-β growth factors in endothelial cells. Endothelial cells (5 $x10^{3)}$ were plated in a 48 well plate, serum starved and placed under normoxic or hypoxic conditions for 24hrs. 100ul of the supernatant was collected and tested for the growth factors described above according to the manufacturer's instructions. Absorbance was measured at 560nm using a microplate reader.

qRT-PCR and RT-PCR

RNA isolation and cDNA preparation

RNA was extracted from cells (SMC and EC) using 1ml TRIZOL reagent from Invitrogen (Carlsbad, CA). Total RNA (1ug) of each sample was reverse transcribed using an oligo d (T) primer and RNase MMLV reverse transcriptase, according to the manufacturer`s protocol, Promega (Madison , WI). cDNA (100ng) was used for real time PCR and gel based PCR to study the genes listed below.

Sense and antisense oligonucleotide primers for the growth factors (VEGF, PDGF-BB, FGF-2, TGF-β) and their receptors (PDGFRβ, TGFβ R-1, TGFβ R-2) and β-actin were designed for RT-PCR using DNA sequence information obtained from the Genome Database (National Center for Biotechnology Information) and were synthesized at Bio-Medicine Genomic Facility at the University of Minnesota and miR-125b primers were purchased from Qiagen (maryland, USA).

The following specific primers were used:

VEGF-A sense: 5`ATC ATG CGG ATC AAA CCT CA-3`, antisense: 5` CAA GGC CCA CAG GGA TTT TC-3`

PDGF-BB 5` CGA GTT GGA CCT GAA CAT GA-3` antisense 5' GTC ACC GTG GCC TTC TTA AA-3' (Osada-Oka M, et al)

PDGFRβ sense: 5` TGC TCA TCT GTG AAG GCA AG-3`, antisense 5` TGG CAT TGT AGA ACT GCT CG -3`

FGF-2 sense: 5` AGA GCG ACC CTC ACA TCA AG-3`, antisense 5` ATA GCT TTC TGC CCA GGT CC-3`

TGF-β sense: 5` TAT CGA CAT GGA GCT GGT GA-3` , antisense 5` CAC GTG CTG CTC CAC TTT TA-3`

TGFβ R-1 sense: 5` ACA GAT GGG CTC TGC TTT GT-3` antisense 5` AGG GCG ATC TAA TGA AGG GT-3`

TGFβ R-2 sense: 5` TGC CCC AGC TGT AAT AGG AC-3`, antisense GGA GAA GCA GCA TCT TCC AG- 3`

β-actin sense 5` GAT CAT TGC TCC TCC TGA GC-3` antisense 5' CAC CTT CAC CGT TCC AGT TT-3`

The real-time PCR analysis was performed using SyBR-Green mix (Applied Biosystems, Carlsbad, CA) on a 7500 Real Time PCR station (Applied Biosystems, Carlsbad, CA). Transcript levels of RNU6B were used as endogenous control for mir-125b levels for other QT-PCR reactions β-actin was used as an internal control. The results for Real-Time PCR were calculated by using the ΔcT- ΔcT method and were expressed as fold change. Image J software was used to quantify intensity of the bands obtained after running PCR product on a 1% agarose gel for gel based PCR.

Western blot analysis (WB)

SMC and EC (1 X 10^6) cells were plated in a cell culture flask and placed under normoxia and hypoxia for 24hrs. Cells were then lysed with 500uL lysis buffer from Sigma (St. Louis, MO) according to manufacturer's instructions. Total protein concentration of the supernatants was determined using Bio-Rad DC protein assay from Bio-Rad (Hercules, CA). 30ug of total protein was loaded for p-ERK1/2 WB, 100ug for HIF1-alpha WB, 50ug for VEGFR-2 and PDGFR β WB analysis and their respective loading controls. The samples were electrophoresed in a 7% discontinuous SDS-PAGE. The resolved proteins were transfered to a

PVDF membrane from Bio-Rad (Hercules, CA) which was then blocked for 1hr with 5% non-fat milk at room temperature. The membrane was incubated with (1:500) primary antibody concentration (PDGFR β, α-tubulin, β actin, ERK1/2 and VEGFR-2) overnight at 4^{o}C. Membrane bound primary antibodies were detected using anti mouse or rabbit IgG secondary antibodies (1:1000) conjugated with horseradish peroxidase. Immunoblots were detected with UltraQuant 6.0 Ultralum (Claremont, CA) using enhanced chemiluminescence technique (Immobilon Western HRP Substrate; Millipore) (Billerica, MA). Quantification of bands was performed using UltraQuant 6.0 (Claremont, CA).

Flow cytometry

SMC`s (1 X $10^{6)}$ were plated in a tissue culture flask and placed under normoxia and hypoxia for 24hrs. Cells were trypsinized using trypsin (5%) and centrifuged at 1200 rpm for 5 mins. Pelleted cells were washed twice with staining buffer (100ul PBS + 0.5% bovine serum albumin (BSA) + 0.02% sodium azide) and incubated at 4°C for 30mins with 1ug anti-human VEGFR-2 (PE). To check the purity of the SMCs, we verify the appropriate phenotypic marker: Anti-human Eph-B4 (PE) (R& D Systems, Minneapolis, MN; 1ug) and anti-human Ephrin B2 (primary antibody) with anti rabbit PE conjugated (secondary antibody; 1:1000) for V-SMC and A-SMC respectively was used. Appropriate isotype

control antibody was included in all the experiments. After washing, stained cells were resuspended in staining buffer (250ul) and acquired (10,000 events) in FACSCanto flow cytometry (BD Biosciences, NJ). Acquired data were analyzed using FlowJo software (Tree star, Ashland, Oregon).

Statistics

Data are expressed as mean ± SD. Each experiment was performed at least 3 independent times. Interassay and interarray variation was accounted for all experiments. Statistical computer package STATVIEW (SAS Institute, Cary, NC) was used for the analysis of variance (ANOVA) and Post-hoc Bonferroni test were performed to know the effects of each variable and to reveal the statistical significance. The confidence level of the study was proposed to be 95%; hence a $p<0.05$ has been considered significant.

Results

To confirm the arterial and venous phenotype of the SMC, surface expression of ephrin B2 (an arterial cell marker) and eph-B4 (a venous cell marker) was determined. Arterial derived SMC were found to

exclusively express ephrin B2 receptor whilst venous derived SMC expressed eph-B4 receptor **(Figure 2.1).**

To determine the different proliferative abilities of A-SMC compared to V-SMC cells under hypoxia, BrdU ELISA was used as an indicator of cell proliferation. Our results as illustrated in **Figure 2.2** showed that hypoxic treatment of arterial and venous SMCs resulted in a significant reduction in BrdU incorporation when compared to cells under normoxic conditions. To further determine that the reduction in cell proliferation was not due to cell viability, MTT cell viability assay was done. The assay confirmed that there was no difference in viability under hypoxia **(Figure 2.2B)**. From this result we concluded that hypoxia alone does not initiate smooth muscle cell proliferation, but in fact both arterial derived and venous derived proliferation significantly ($p<0.05$) decreased under hypoxia. Since our data in **Figure 2.2** showed that hypoxia alone does not initiate smooth muscle cell proliferation next we investigated if SMC proliferation leading to IH *in vivo* acts through a paracrine mechanism.

To test the paracrine mechanism, conditioned media from hypoxic EC (A-EC and V-EC) was incubated with A-SMC and V-SMC and subjected to hypoxia for 24hrs. BrdU incorporation was used to determine proliferation of SMCs. Our results indicated that there was a significant ($p< 0.05$) increase in both A-SMC and V-SMC proliferation

under hypoxia when incubated with hypoxic EC conditioned media (**Figure 2.3A**). We concluded that SMC proliferation under hypoxia occurs via a paracrine mechanism and is initiated by hypoxic conditioned media. To further confirm that hypoxic EC conditioned media was the initiator in SMC proliferation, V-EC and A-EC were placed under hypoxia for 24hrs and conditioned media was collected. The media in the same cells was replenished and the cells were then placed under normoxia for a further 24hrs and their conditioned media collected (reperfusion). EBM, conditioned EC media under normoxia and reperfused media under normoxia were used as controls. The results showed significant ($p < 0.05$) reversibility in SMC proliferation that had been initiated by hypoxic EC conditioned media **(Figure 2.4).** This result further confirmed that it was indeed the hypoxic EC conditioned media that was initiating SMC proliferation.

Hypoxia induces significant VEGF mRNA and protein expression in A-SMC, A-EC, and V-EC but not in V-SMC

To investigate the growth factors present in the hypoxic EC conditioned media that induced SMC proliferation, arterial and venous derived SMC and EC were placed under hypoxia for 3hrs. EC mRNA was quantified for FGF-2 and TGF-β expression levels and SMC mRNA was quantified for FGF-2, TGF-β; TGFβR1 and TGFβR2 expression levels under both normoxic as well as hypoxic conditions (**Figure 2.5A,**

B &C). An angiogenesis ELISA was used to quantify growth factors PDGF-BB, IGF-I, VEGF-A, FGF-b and TGF-β protein levels (**Figure 2.5D**). To establish the growth factors of interest we looked at which growth factors were commonly increasing significantly between V-EC and A-EC and had the greatest fold change as well. Although there was a significant change in IGF, FGF-b and TGF-B levels, these changes were not common to both EC types and the fold changes were not as dramatic compared to changes in VEGF-A mRNA and protein levels. Analysis of the different growth factors showed that VEGF-A protein expression levels had the greatest induction (15 fold) under hypoxia. Therefore to further investigate VEGF-A protein levels in SMC and EC under hypoxia a VEGF-A ELISA was used. Results showed a significant increase ($p<0.001$) in VEGF-A mRNA levels in A-EC (4 fold), V-EC (5 fold) and A-SMC (6 fold) under hypoxia **(Figure 2.5E&F).** There was also a significant ($p< 0.001$) increase in A-SMC (1.6 fold) VEGF-A protein levels and a significant ($p<0.001$) increase in A-EC (35 fold) and V-EC (15 fold) VEGF-A protein levels under hypoxia **(Figure 2.5G& H)**. In contrast V-SMC showed non-significant changes in VEGF-A mRNA and protein expression under hypoxia **(Figure 2.5F & G respectively)**

HIF1-alpha stabilization under hypoxia is greater in V-SMC compared to A-SMC

To investigate whether the lack of VEGF-A increase in V-SMC was due to lack of HIF1-alpha stabilization under hypoxia we looked at HIF1-alpha levels. HIF1-alpha is stabilized under hypoxia and translocates to the nucleus where it is responsible for transcription of hypoxia regulated genes like VEGF-A. V-SMC and A-SMC were placed under hypoxia and HIF1-alpha levels determined using WB. The results **(Figure 2.6)** showed greater HIF1-alpha stabilization (3 fold) in V-SMC under hypoxia when compared to A-SMC. Based on this result we concluded that the lack of VEGF-A expression in V-SMC under hypoxia was not due to lack of HIF1-alpha stabilization. A possible explanation for lack of VEGF-A protein expression in V-SMC under hypoxia was that there could be an increase in microRNA`s that target VEGF-A mRNA. A micro-RNA array was used to screen for microRNA`s that are regulated under hypoxia in A-SMC and V-SMC. Of particular interest mir-125b, 29a and 29b showed VEGF-A as a target gene (unpublished data). Mir-125b levels in A-SMC and V-SMC under hypoxia were further confirmed by QT-PCR **(Figure 2.6B)**. Under hypoxia, V-SMC showed a 15 fold increase in mir-125b expression at 6 hrs as compared to A-SMC which did not show a significant change. Based on this data we hypothesized

that up regulation of mir-125b in V-SMC might be a possible reason why V-SMC do not express significant amounts of VEGF-A under hypoxia.

Based on our results in **Figure 2.5** we concluded that there may be other growth factors in the hypoxic endothelial cell culture supernatant that were initiating SMC proliferation especially in arterial derived SMC since they expressed VEGF-A under hypoxia but yet failed to proliferate. To investigate the lack of A-SMC proliferation under hypoxia even in the presence of significant VEGF-A amounts we investigated VEGFR-2 expression levels on these cells.

VEGFR-2 is differentially expressed under hypoxia in SMC

VEGFR-2 is implicated in the proliferative abilities of VEGF-A (Murray, Kulkarni, Ezzati 2006). To investigate if the inability of A-SMC to proliferate under hypoxia in the presence of VEGF-A was due to lack of receptor expression, we looked at VEGFR-2 levels on these cells under hypoxia using VEGFR-2 antibody. Fluorescence activated cell sorter (FACS) was used to determine receptor levels on these cells under hypoxic conditions. Under hypoxia VEGFR-2 expression is 62.3% in V-EC, 43.9% in V-SMC and 4.2% in A-SMC **(Figure 2.7A)**. This observation was further confirmed by western blot analysis. The results from the western blot showed 2 fold higher VEGFR-2 expression levels in V-SMC when compared to A-SMCs under hypoxia **(Figure 2.7B)**.

From this result we concluded that A-SMC lacked significant VEGFR-2 expression under hypoxia.

V-SMC proliferation but not A-SMC is partially mediated through VEGFR2 under hypoxia

The role of VEGF-A in SMC proliferation under hypoxia was determined by conducting a VEGF-A (0, 5, 10 and 15 ng/mL) dose response experiment in V-EC, A-SMC and V-SMC. Results showed that VEGF-A induced V-EC proliferation under hypoxia **Figure 2.7C**. VEGF only induced V-SMC proliferation at higher doses (10 & 15 ng/mL) **Figure 2.7C**. VEGF treatment did not have an effect on A-SMC proliferation. To further validate the role VEGF-A plays in inducing SMC proliferation under hypoxia we used VEGFR-2 neutralizing antibody. Neutralization of VEGFR-2 did not result in any proliferative changes for the A-SMC, but caused a significant decrease ($p<0.05$) in V-SMC proliferation upon addition of hypoxic endothelial cell conditioned media under hypoxia **(Figure 2.8).** Based on these data we concluded that VEGF-A was not the main cause of A-SMC proliferation but contributed to V-SMC proliferation. Based on our data in (**Figure 2.9A)** which showed greater than 5 fold induction in PDGF-BB expression under hypoxia and current literature that states that EC under hypoxia produced PDGF-BB, we decided to look at PDGF-BB levels in EC under hypoxia.

PDGF-BB initiates A-SMC proliferation under hypoxia

To determine other possible growth factors that were in the hypoxic endothelial cell culture media that were causing SMC proliferation, especially A-SMC proliferation we measured PDGF-BB levels in A-SMC, V-SMC, V-EC & A-EC. Our results showed that there was a significant ($p< 0.001$) 3-6 fold increase in PDGF-BB mRNA levels in A-EC and V-EC under hypoxia **(Figure 2.9A).** There was an increase in PDGF-BB protein levels under hypoxia in V-EC (2 fold) and although the increase in A-EC was not significant, basal level expressions of PDGF-BB were still high (**7 fold higher**) when compared with PDGF-BB levels in SMC under hypoxia **(Figure 2.9B**).

We next investigated PDGFR β mRNA and protein expression under hypoxia. A-SMC expressed greater PDGFR β mRNA and protein levels under hypoxia when compared to V-SMC **(Figure 2.9C and Figure 2.9D)** respectively. To confirm PDGF-BB contribution to A-SMC proliferation we pre-incubated the hypoxic EC conditioned media with PDGF-BB neutralizing antibody and placed the SMC under hypoxia. There was a significant decrease ($p< 0.001$) in A-SMC and V-SMC proliferation back to basal level proliferation (**Figure 2.9E**). To further confirm the role of PDGF-BB in SMC proliferation induced by paracrine factors we used imatinib mesilate (a relatively selective inhibitor of protein tyrosine kinases including PDGFR). Imatinib mesilate (1μM) was

pre-incubated with SMC before hypoxic EC conditioned media was cultured with hypoxic SMC. Our results showed a decrease in both A-SMC and V-SMC proliferation **(Figure 2.9F)**.

V-SMC exhibit greater ERK1/2 activation than A-SMC in the presence of hypoxic conditioned media under hypoxia.

Extracellular signal-regulated kinase (ERK) 1/2 signaling mediates communication between growth factor receptors and the cell nucleus and is known as a key regulator in cell proliferation (Ray and others 2008).To determine the molecular mechanism involved in induction of SMC proliferation by hypoxic conditioned media the following experiment was done. V-SMC and A-SMC were incubated with hypoxic V-EC conditioned media under hypoxia and ERK1/2 phosphorylation determined using WB. V-EC hypoxic conditioned media was selected in this experiment because there was a trend showing V-EC conditioned media inducing greater proliferation in SMC when compared to A-EC conditioned media **(Figure 2.3)**. Our results as indicated in **Figure 2.10** shows that V-SMC exhibit significantly ($p<0.05$) greater ERK1/2 phosphorylation under hypoxia (2 fold) and upon addition of hypoxic EC conditioned media when compared to ERK1/2 phosphorylation in A-SMC (1 fold). A-SMC also show an increase in ERK1/2 activation under hypoxia with the response less drastic in comparison to the response observed in V-SMC.

The data showed that hypoxia alone does not initiate SMC proliferation in an autocrine manner. SMC proliferation under hypoxia occurs via a paracrine mechanism and is initiated by hypoxic endothelial cell derived growth factors (PDGF-BB & VEGF-A) in V-SMC. PDGF-BB plays a more dominant role in causing A-SMC proliferation. VEGF-A only initiates SMC proliferation in V-SMC and does not directly initiate proliferation in A-SMC due to lack of VEGFR-2. ERK1/2 activation further illustrates these observations with our results showing greater ERK1/2 activation in V-SMC when compared to A-SMC under hypoxia upon incubation with hypoxic conditioned media.

Discussion

Despite intensive research for more than two decades, failure of venous derived grafts continues to be a major clinical problem for which there is no effective preventative strategy (Vorp and others 2001). Theories explaining why graft stenosis is more prevalent in the venous derived graft than the arterial derived graft have focused upon the handling and preparation of the graft, surgical trauma, and altered hemodynamics in the arterial circulation (Li and others 2006). The biggest limitation in these studies is that the role of hypoxia as an insult in cell modulation is neglected, although extensive research has shown that hypoxia is a common insult in most of the vascular pathologies that

inevitably lead to intimal hyperplasia (Achouh and others 2010; Turner and others 2007). Our study is one of the few reports that identify how hypoxia contributes to the underlying mechanism behind the differences observed clinically in arterial vs. venous derived grafts. We provide evidence showing how hypoxia induced endothelial cell derived growth factors modulate and interact with venous and arterial smooth muscle cells and their respective receptors under hypoxia to initiate IH.

Our study supports the hypothesis that hypoxia induces IH via a paracrine mechanism. We further established that venous derived grafts are less patent when compared to arterial grafts because V-SMC proliferation under hypoxia is initiated predominantly by VEGF-A and PDGF-BB via a VEGFR-2 and PDGFR β respectively. In contrast, proliferation in A-SMC under hypoxia is initiated only by PDGF-BB through a PDGFR β dependant mechanism. This observation was further supported by greater activation of ERK1/2 (a kinase that is known as a key regulator in cell proliferation) activation (3 fold) in V-SMC when compared to A-SMC; upon addition of conditioned media under hypoxia.

Critical issues that still need to be addressed are the effects of VEGF-A and PDGF-BB signaling in SMC and disease pathogenesis and successful, incorporation of inhibitors into therapeutic strategies (Bjornheden and others 1999; Byrne, Bouchier-Hayes, Harmey 2005; Faller 1999).Although many strategies have been developed to inhibit

SMC proliferation and reduce IH including drug eluting stents, most of the drugs tested thus far have not been successful in completely reducing IH(Bjornheden and others 1999). Therapies using anti-VEGFR-2 methods have not produced optimal outcomes in reducing IH(Byrne, Bouchier-Hayes, Harmey 2005).Therefore, in depth understanding of mechanisms involved in IH are necessary and can help adapt therapy towards specific procedures depending on the origin of graft used.

Consistent with the literature, we demonstrated induction of VEGF mRNA and protein levels in EC and A-SMC under hypoxia (Brogi and others 1994; Growcott, Banner, Wharton 2005; Lee and others 2000). Contrary to studies performed by Pancholi S et al, venous derived smooth muscle cells did not show a significant increase in VEGF-A induction under hypoxia (26)**.** Interestingly, Pancholi`s studies showed approximately a 1.5 fold increase in VEGF-A mRNA which is consistent with our observations for (Growcott, Banner, Wharton 2005; Pancholi and Earle 2000) VEGF-A mRNA levels in V-SMC; however our data showed an insignificant decrease in VEGF-A protein levels under hypoxia (Growcott, Banner, Wharton 2005; Pancholi and Earle 2000). A possible explanation for this observation was that HIF1-alpha stabilization is inhibited in V-SMC when compared to A-SMC, but our results showed us that V-SMC showed a 3 fold increase in HIF1-alpa stabilization when compared to HIF1-apha stabilization in A-SMC. A

hypothesis generated from our data suggested that V-SMC express microRNA 125b that degrades and prevents translation of VEGF-A mRNA to protein under hypoxia. When comparing the differences in VEGF-A induction under hypoxia in arterial vs venous derived cells our results clearly show that the arterial derived EC and SMC have significantly higher amounts of VEGF-A produced; 35 fold in A-EC and 1.5 fold in A-SMC compared to approximately 15 fold induction for V-EC and insignificant 0.8 fold change in V-SMC. We found it surprising that even in the presence of VEGF-A the arterial derived smooth muscle cells did not proliferate in an autocrine manner. Studies conducted by Ferrara et al, also demonstrated that arterial derived smooth muscle cells were producers of VEGF. We also observed that these cells did not seem to proliferate in an autocrine manner in the presence of VEGF(Growcott, Banner, Wharton 2005). We went on to further demonstrate that reduced proliferation in A-SMC despite presence of VEGF is due to a significantly lower expression of VEGFR-2 , the receptor responsible for the proliferative effects of VEGF-A(Osada-Oka and others 2008). As expected, incubation with VEGFR-2 neutralizing antibody prior to addition of hypoxic endothelial conditioned media did not have a significant effect on A-SMC proliferation. We proposed a hypothesis that VEGF-A released by A-SMC may play a paracrine role in the maintenance of endothelial lining integrity and lead to increase in

angiogenesis. An increase in angiogenesis will reduce hypoxia in SMC and promote wound healing; ultimately resulting in a decrease in hypoxic derived EC growth factors which are initiators in SMC proliferation. We considered this as a possible explanation as to why arterial derived grafts are more patent when compared to venous derived grafts.

Venous derived smooth muscle cells showed VEGFR-2 expression under hypoxia and were responsive to VEGF-A induced proliferation, contrasting A-SMC response. VEGFR-2 antagonist therapies might be better suitable for treatment of venous derived grafts since venous derived smooth muscle cells express 3 fold greater VEGFR-2 under hypoxia.

PDGF-BB is reported to stimulate the proliferation of vascular smooth muscle cells and to be involved in vascular modeling through a HIF-1 dependent mechanism (Abedi and Zachary 1995; Muto and others 2007). Our findings suggest the involvement of PDGF-BB in the proliferation of cells exposed to hypoxia; however, it is still unclear whether hypoxia elicits PDGF-BB induced proliferation via a paracrine or autocrine loop in SMC. We determined that EC produce 100 fold more PDGF-BB when compared with SMC upon hypoxic insult (Lee and others 2000). In vascular smooth muscle cell proliferation that occurs in IH through PDGF-BB is mediated by the PDGF-β receptor(Sirois and others 1993). Our data showed PDGFR-β expression in both venous

and arterial derived smooth muscle cells under hypoxia with increased receptor expression under hypoxia in A-SMC. An important finding in this present study was that A-SMC under hypoxia expressed significantly higher (3 fold) PDGFR- β when compared to V-SMC. Based on this result we concluded that SMC proliferation under hypoxia is initiated by PDGF-BB via a paracrine mechanism. Of particular interest was the contribution of PDGF-BB in arterial derived smooth muscle cell proliferation, because upon incubation with PDGF-BB neutralizing antibody, proliferation decreased to basal levels. This supported the idea that PDGF-BB is the major player in induction of A-SMC proliferation under hypoxia. Studies looking at differential effects of imatinib (PDGFR antagonist) on PDGF-Induced proliferation and PDGF receptor signaling in human arterial and venous smooth muscle cells concluded that imatinib was more efficacious towards inhibiting arterial SMC proliferation supporting our findings that PDGF-BB plays a more significant role in arterial derived smooth muscle cell proliferation (Hacker and others 2007). In summary, we have demonstrated that IH is a paracrine event initiated by hypoxia derived endothelial cell growth factors. V-SMC`s were shown to express VEGFR-2 under hypoxia while A-SMC had significantly lower expressions under hypoxia. On the other hand the A-SMC expressed higher PDGFR-β which increased under hypoxia. V-SMC expressed PDGFR- β but at lower levels when

compared to A-SMC. Based on our findings we concluded that anti-PDGF therapies are most efficient in prevention of IH in arterial derived pathologies because A-SMC express predominantly PDGFR-β. Reduction in venous IH can be achieved through utilization of VEGFR-2 and PDGFR- β antagonists since V-SMC express both VEGFR-2 and PDGFR- β under hypoxia. Our working model is illustrated in **Figure 2.11**. Clinically, therapy with PDGFR antagonists plus anti-VEGFR-2 may prove to be efficacious in managing IH in venous derived grafts. Therapy with PDGFR antagonist would be more effective in managing IH in arterial derived grafts. These different therapies can be adapted towards the type of graft used for optimal results.

Figure 2.1:

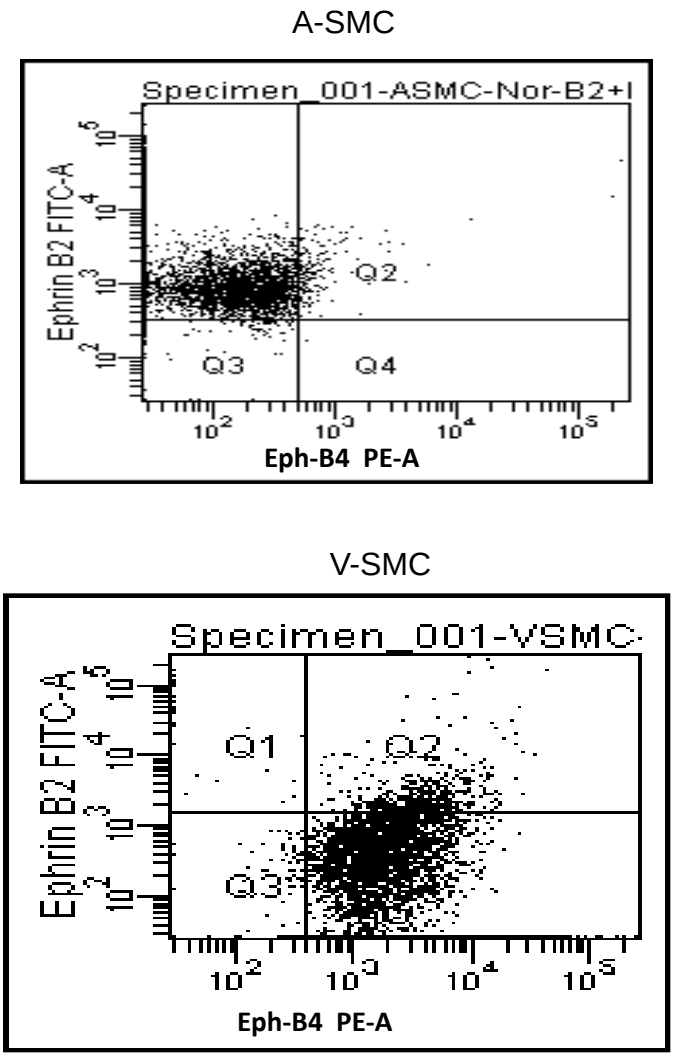


Figure 2.1 V-SMC express Eph-B4 and A-SMC Ephrin B2 under hypoxia. V-SMC and A-SMC were incubated under hypoxia for 24hrs. To confirm the phenotype of V-SMC and A-SMC; Eph-B4 (PE) and Ephrin B2 (FITC) surface protein expression levels were determined using FACS analysis prior hypoxia treatment. The data shown is a representation of at least three different cell preparations.

Figure 2.2 A &B

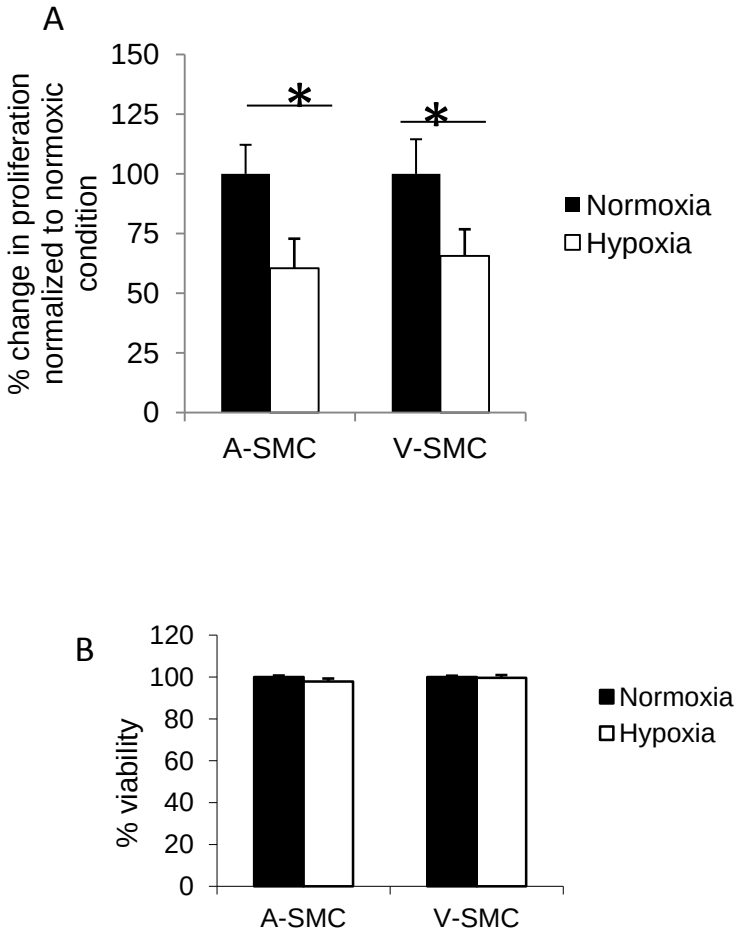


Figure 2.2. Hypoxia reduces smooth muscle proliferation A-SMC and V-SMC were exposed to hypoxia (30 mm Hg PO2) for 24 hours. **A)** BrdU incorporation was used as an indicator of SMC proliferation. **B)** Cell viability under hypoxia was determined using MTT. Data is expressed as percentage (%) change in SMC proliferation/viability normalized to control (under normoxia). Data was considered significant at *p<0.05. Data are the mean ± SD of at least three different cell preparations

Figure 2.3 A

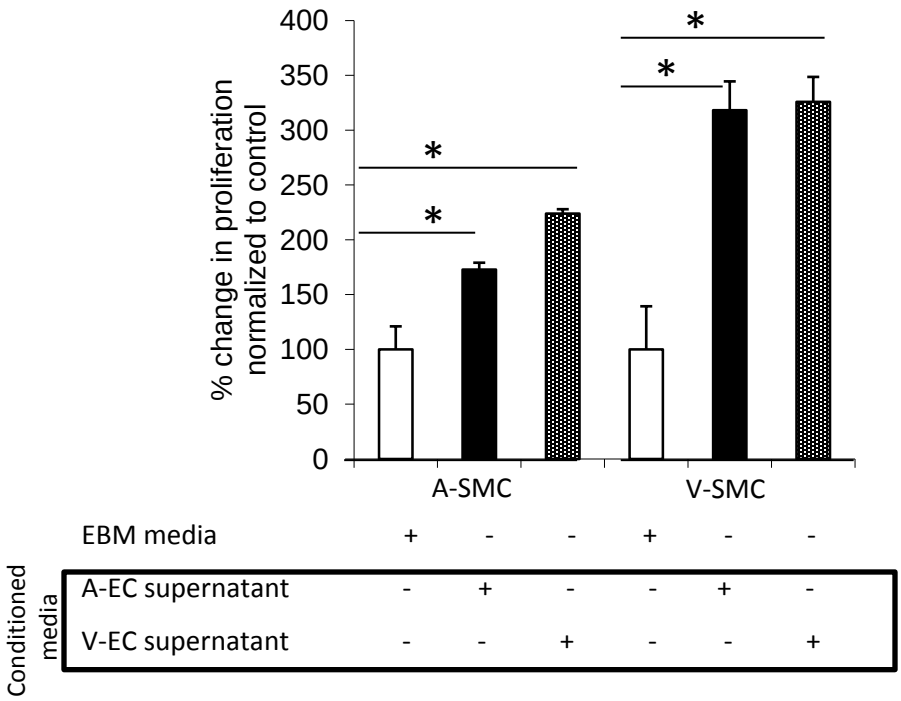


Figure 2.3A : Hypoxic EC conditioned media (H-ECM) induces SMC proliferation under hypoxia

H-ECM (100µl) derived from A-EC and V-EC was incubated with A-SMC and V-SMC and subjected to hypoxia for 24 hours. BrdU incorporation was used as an indicator of SMC proliferation under hypoxia. Data is expressed as % change in SMC proliferation normalized to control (EBM-2).

Figure 2.4

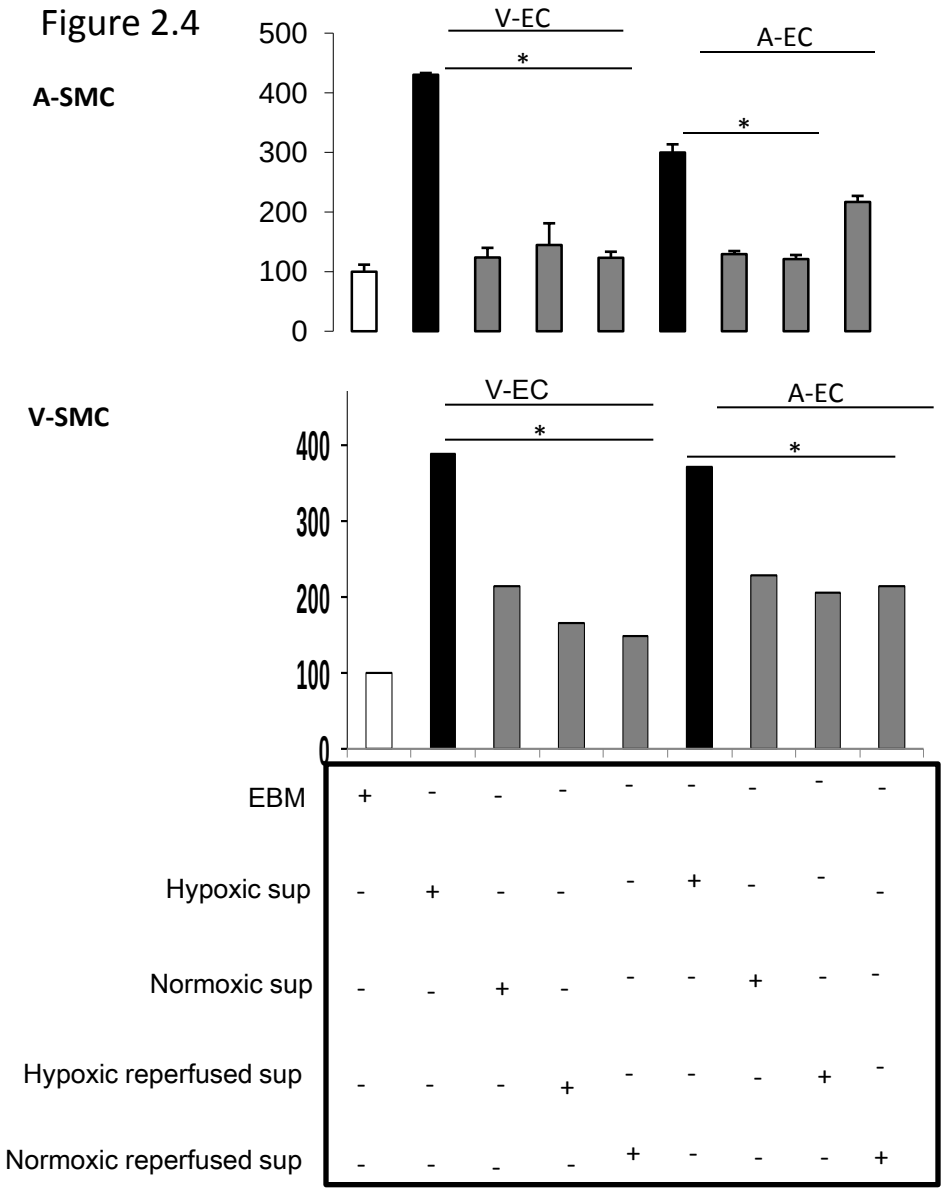


Figure 2.4 :Conditioned media from reperfused EC reverses SMC proliferation induction: V-EC and A-EC were placed under hypoxia for 24hrs and conditioned media was collected. The media in the same cells was replenished and the cells were then placed under normoxia for a further 24hrs and their conditioned media collected (reperfusion). The data shown represents A-SMC and V-SMC proliferation under hypoxia with hypoxic EC conditioned media and reperfused EC media using BrdU as an indicator of prolfieration. EBM, conditioned EC media under normoxia and reperfused media under normoxia were used as controls, Data was considered significant at *$p<0.05$. Data are the mean ± SD of at least three different experiments.

Figure 2.5 A, B & C

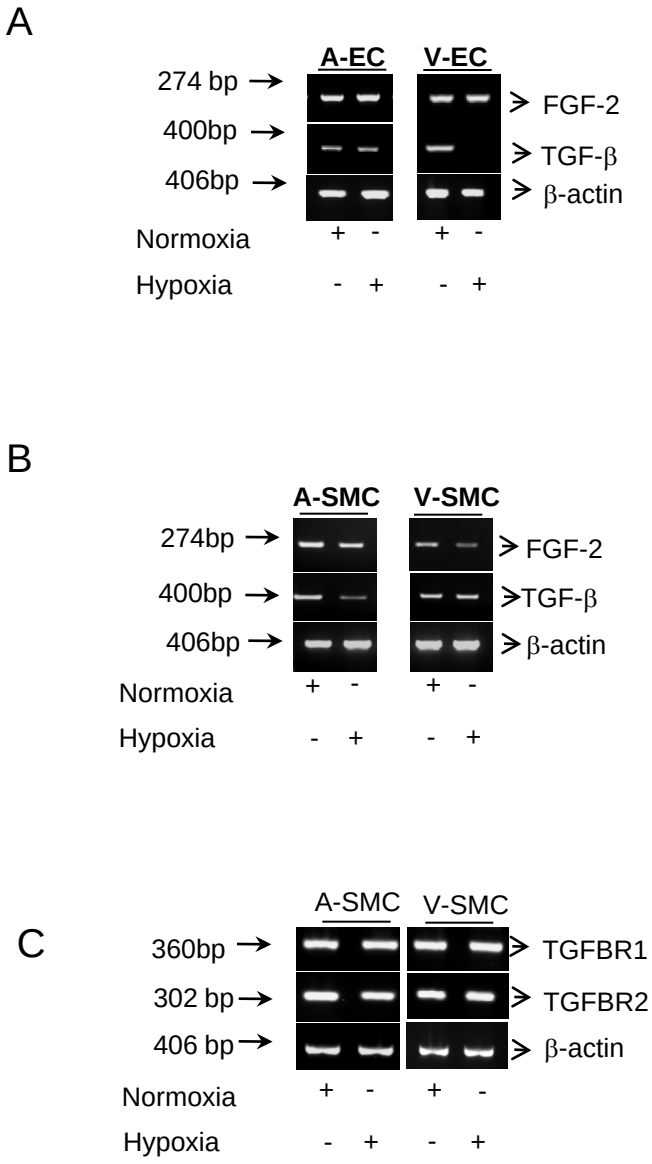


Figure 2.5: Hypoxia induces VEGF-A expression in A-SMC, A-EC, and V-EC but not in V-SMC

A-EC, V-EC, A-SMC and V-SMC were placed under hypoxia (3hrs) and mRNA isolated. Samples were analyzed for growth factor levels and their respective receptors. **A** FGF-2 & TGF-β mRNA expression in A-EC and V-EC. **B & C** FGF-2, TGF-β, PDGFR β, TGFBR1 mRNA expression in A-SMC and V-SMC.

Figure 2.5 D

D

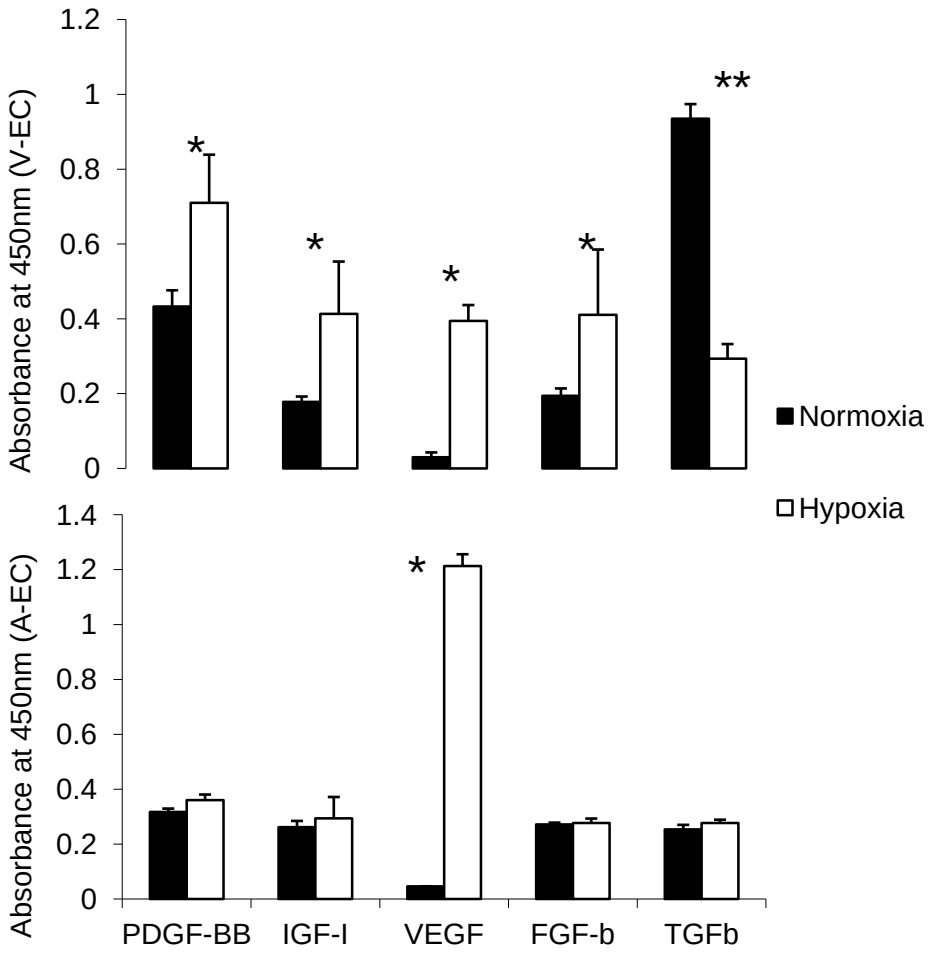


Figure 2.5: Hypoxia induces VEGF-A expression in A-SMC, A-EC, and V-EC but not in V-SMC

Culture supernatant from A-EC and V-EC was collected and an angiogenesis ELISA (signosis) was used to analyze the effect of hypoxia on growth factor protein levels PDGF-BB, IGF-I, VEGF, FGF-2, TGF- β. Data was considered significant at *$p<0.05$, **$p<0.001$. Data are the mean ± SD of at least three independent experiments.

Figure 2.5 E& F

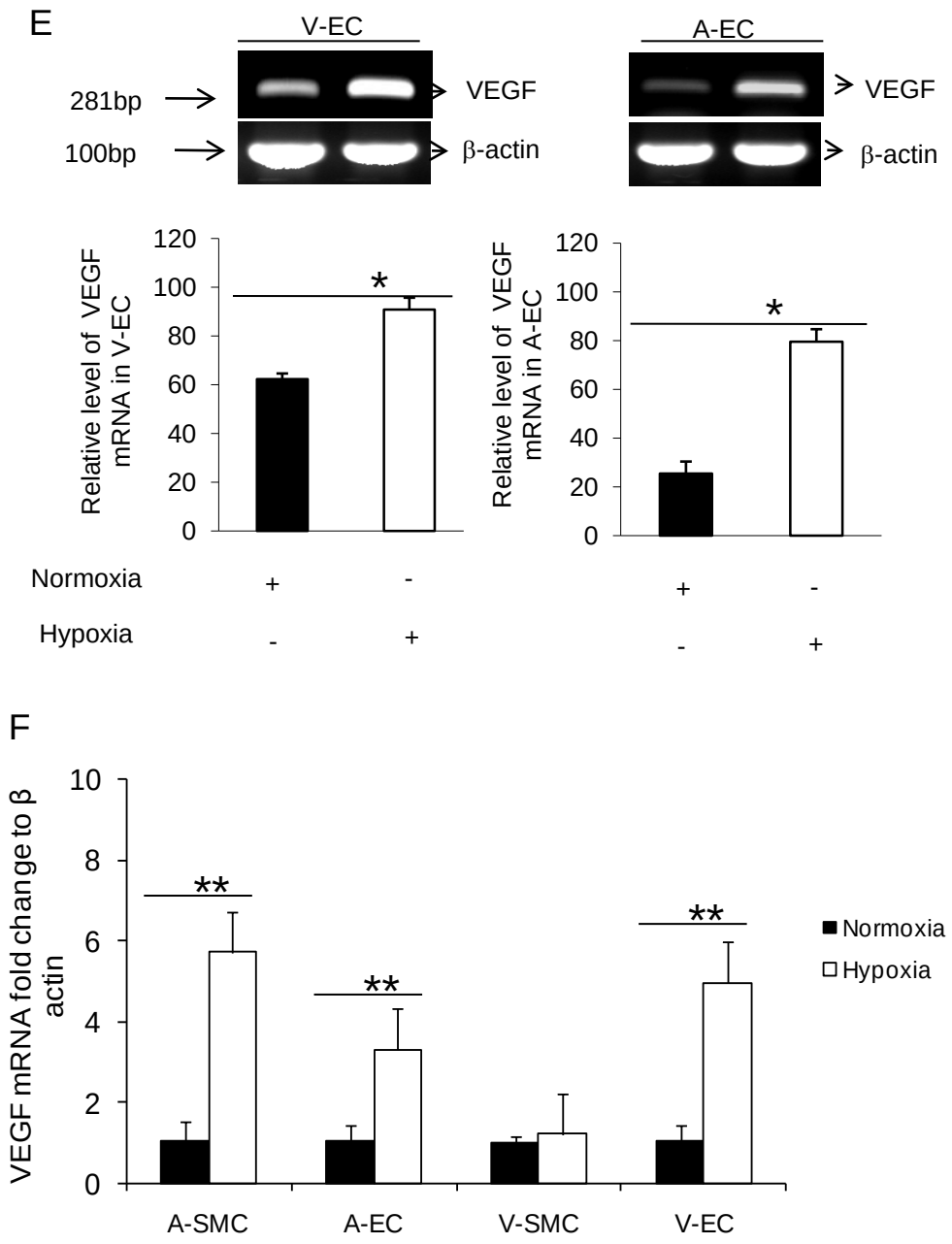


Figure 2.5: Hypoxia induces VEGF-A expression in A-SMC, A-EC, and V-EC but not in V-SMC. VEGF mRNA was analyzed using: **E)** RT-PCR in A-EC & V-EC and **F)** QT-PCR in A-EC, V-EC, A-SMC & V-SMC after 3hr exposure to hypoxia Data was considered significant at *p<0.05, **p<0.001. Data are the mean ± SD of at least three independent experiments.

Figure 2.5 G,H

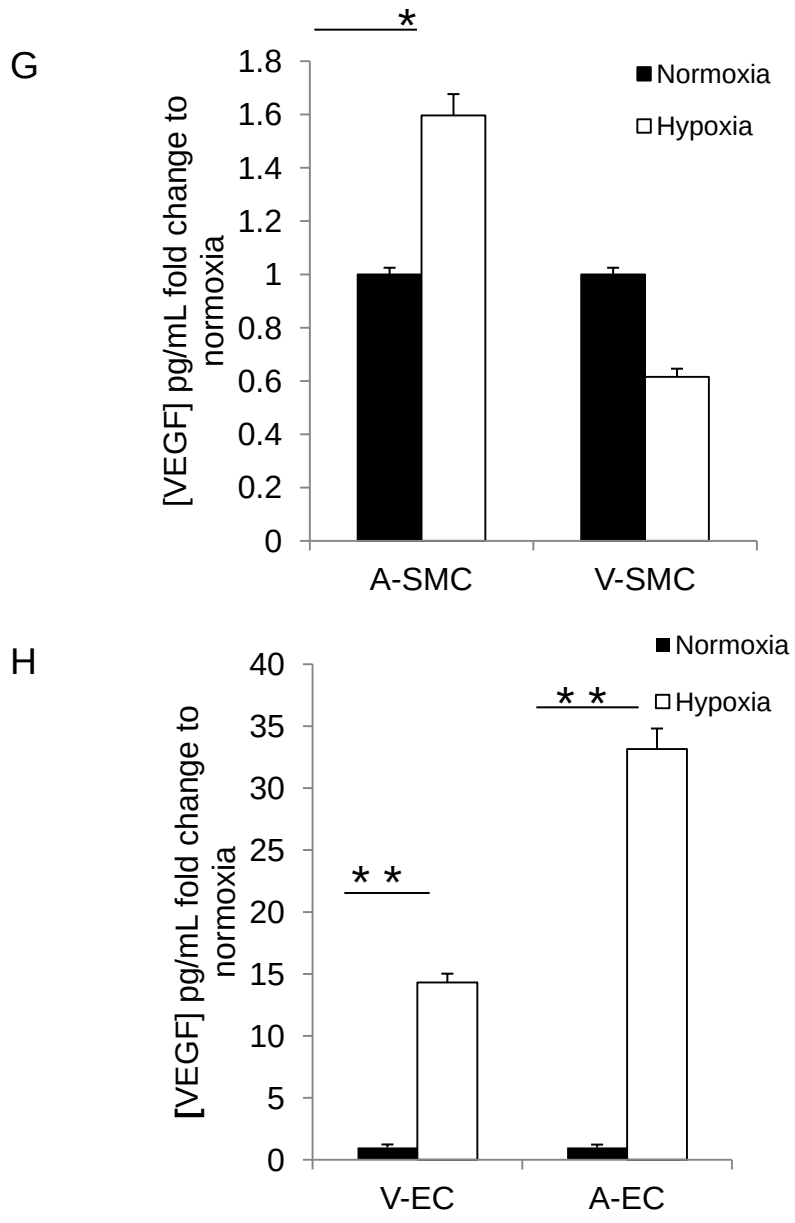


Figure 2.5: Hypoxia induces VEGF-A expression in A-SMC, A-EC, and V-EC but not in V-SMC. VEGF protein levels for A-SMC, V-SMC, V-EC and A-EC were determined using VEGF ELISA after 24 hrs under hypoxic conditions. Data was considered significant at *p<0.05, **p<0.001. Data are the mean ± SD of at least three independent experiments.

Figure 2.6 A

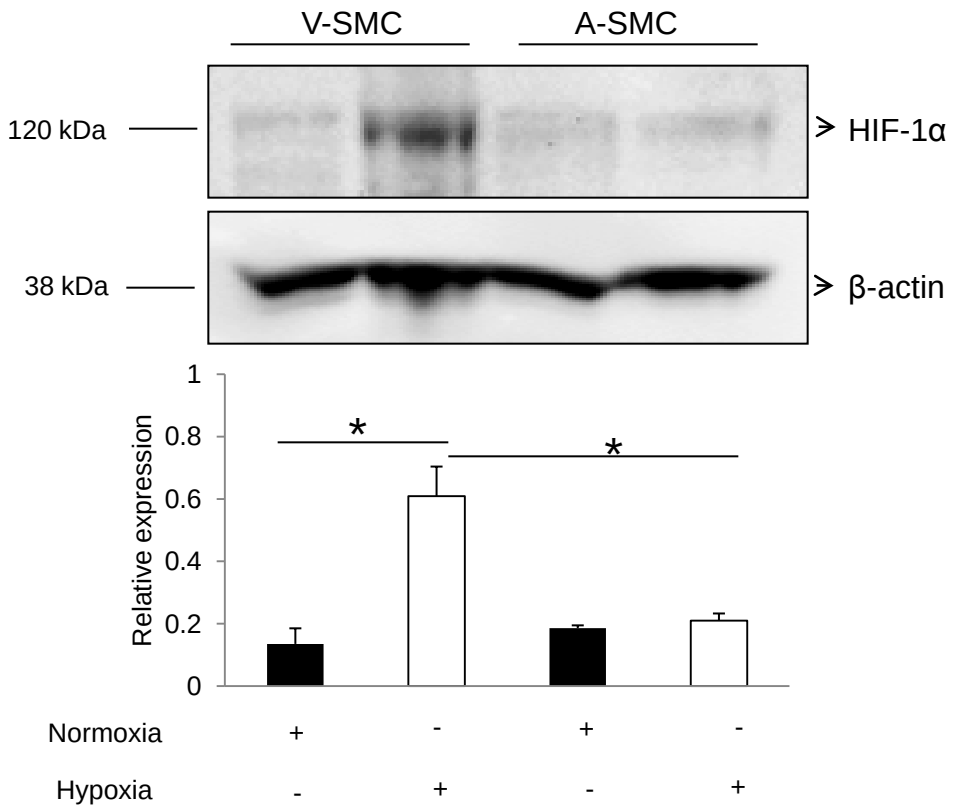
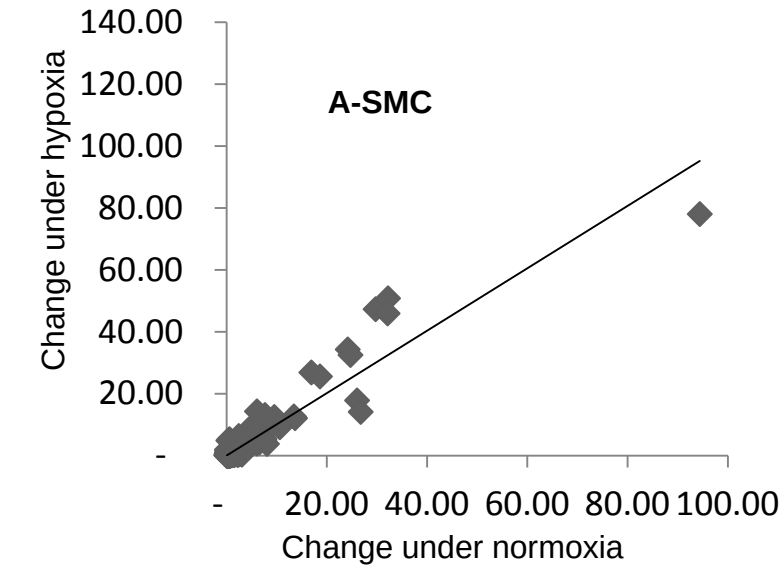


Figure 2.6A: V-SMC exhibit greater HIF1-alpha stabilization under hypoxia V-SMC and A-SMC were incubated under hypoxia for 24hrs. HIF1-alpha protein levels expression was analyzed using western blot. Significance was determined at *p<0.05. The bar graphs represent the mean ± SD of at least three different cell preparations.

Figure 2.6 B



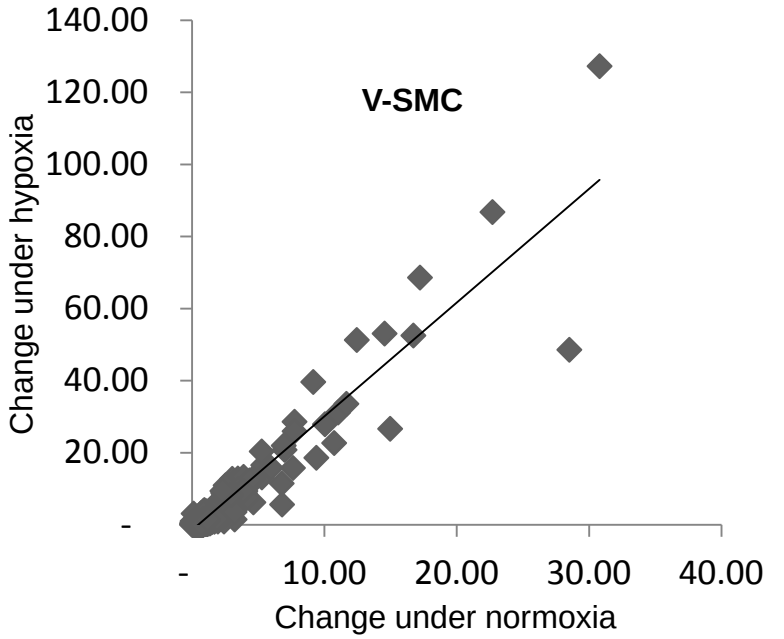


Figure 2.6B: V-SMC have greater miRNA regulation under hypoxia. A micro-RNA array was used to screen for microRNA`s that are regulated under hypoxia in A-SMC and V-SMC .Data is representing microRNA regulated under hypoxia with V-SMC showing more microRNA being regulated under hypoxia. Post-hoc Bonferroni test were performed to know the effects of each variable and to reveal the statistical significance. Data was considered significant at *p<0.05

Figure 2.6 C

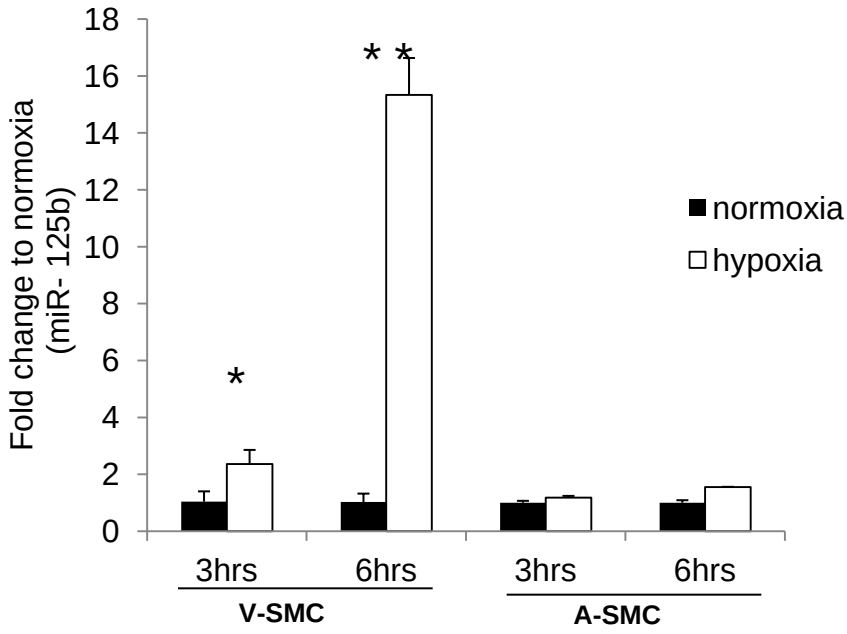


Figure 2.6C: V-SMC have greater miRNA 125b regulation under hypoxia mir-125b levels in A-SMC and V-SMC under hypoxia was determined by QT-PCR (3 and 6hrs). Significance was determined at *p<0.05, **p<0.001. The bar graphs represent the mean ± SD of at least three different cell preparations.

Figure 2.7 A

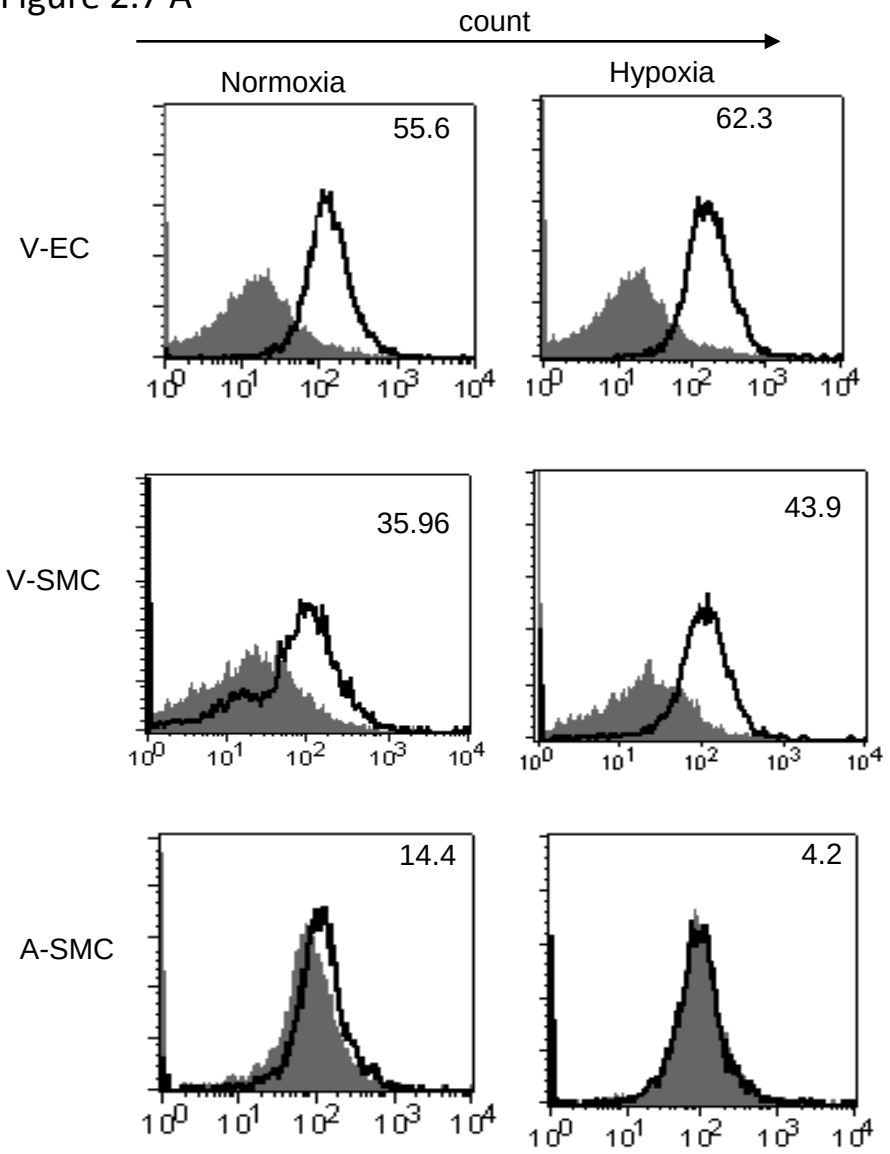


Figure 2.7A VEGFR-2 is differentially expressed under hypoxia in SMC V-EC, V-SMC and A-SMC were incubated under hypoxia for 24hrs. VEGFR-2 protein expression levels were determined using **A)** FACS analysis with a PE-conjugated antibody. Data are the representative of at least three independent experiments.

Figure 2.7

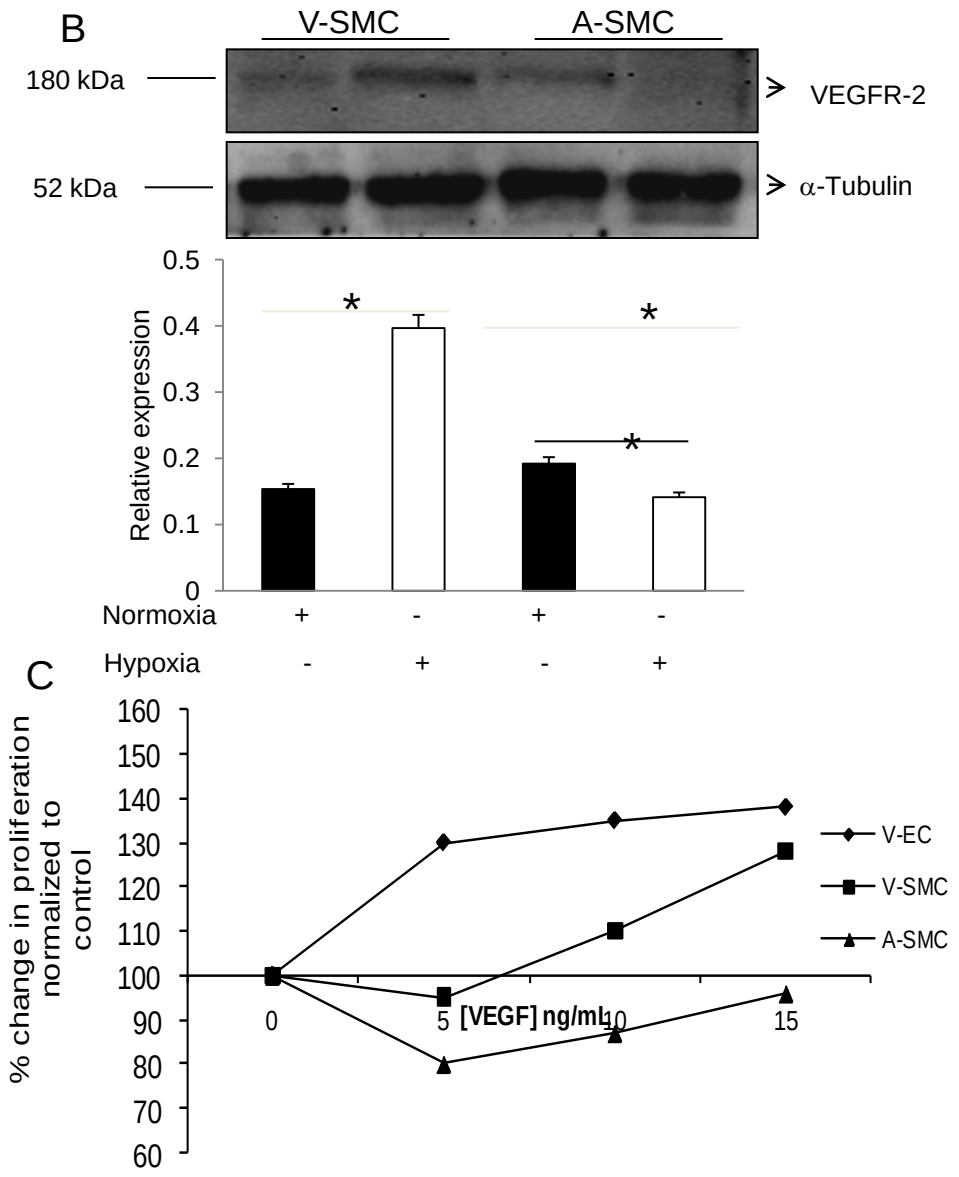


Figure 2.7: VEGFR-2 is differentially expressed under hypoxia in SMC V-EC, V-SMC and A-SMC were incubated under hypoxia for 24hrs. VEGFR-2 protein expression levels were determined using **B)** Western blot **C)** VEGF dose response on V-EC, V-SMC and A-SMC proliferation was determined using the following VEGF concentration (0, 5, 10, and 15 ng/mL) for 24hrs under hypoxia. In these experiments (A, B and C) V-EC was used as a positive control. Significance for differences in VEGFR-2 expression levels was determined at *$p<0.05$. The bar graphs represent the mean ± SD of at least three different cell preparations.

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Figure 2.8

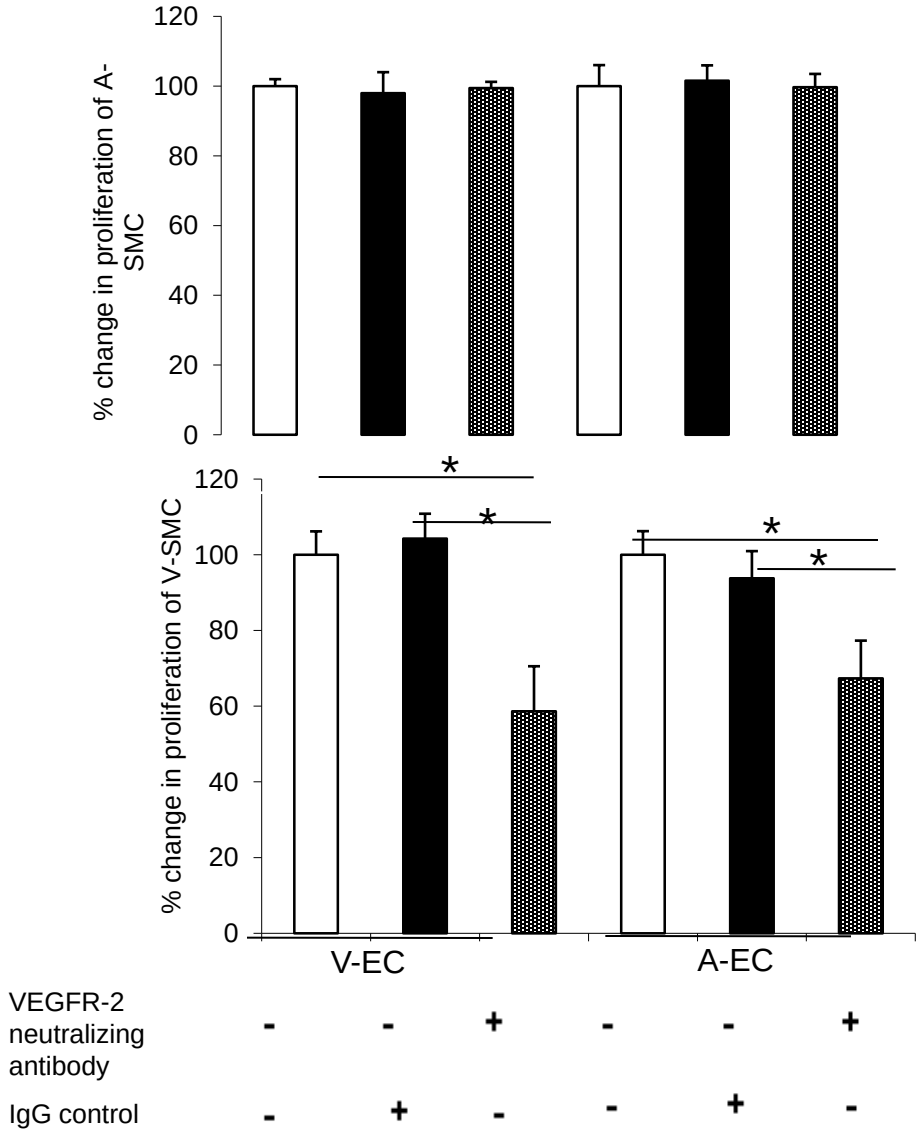

Figure 2.8: V-SMC proliferation but not A-SMC is partially mediated through VEGFR-2 under hypoxia A-SMC and V-SMC proliferation after addition of A-EC and V-EC derived H-ECM with pre-incubation of VEGFR-2 neutralizing antibody. Data is expressed as % change in SMC proliferation normalized to control (EBM-2). Significance was determined at *$p < 0.05$. Data are the mean ± SD of at least three different cell preparations.

Figure 2.9

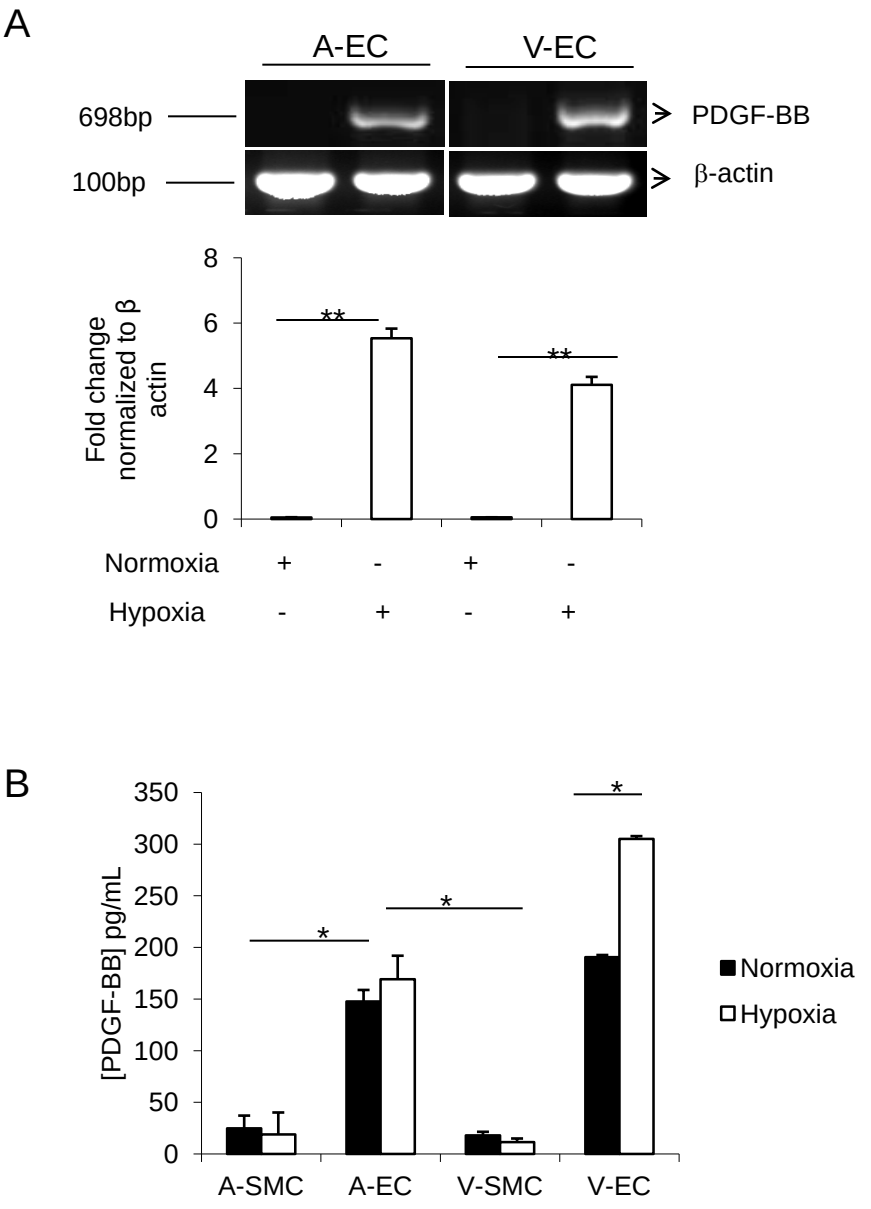

Figure 2.9: PDGF-BB mRNA and protein levels are highly induced under hypoxia in EC A) representative PDGF-BB mRNA levels in A-EC and V-EC; **B)** PDGF-BB protein levels in A-EC, V-EC, A-SMC & V-SMC under hypoxia using ELISA. Data was considered significant at *p<0.05, **p<0.001. Data are the mean ± SD of at least three independent experiments.

Figure 2.9

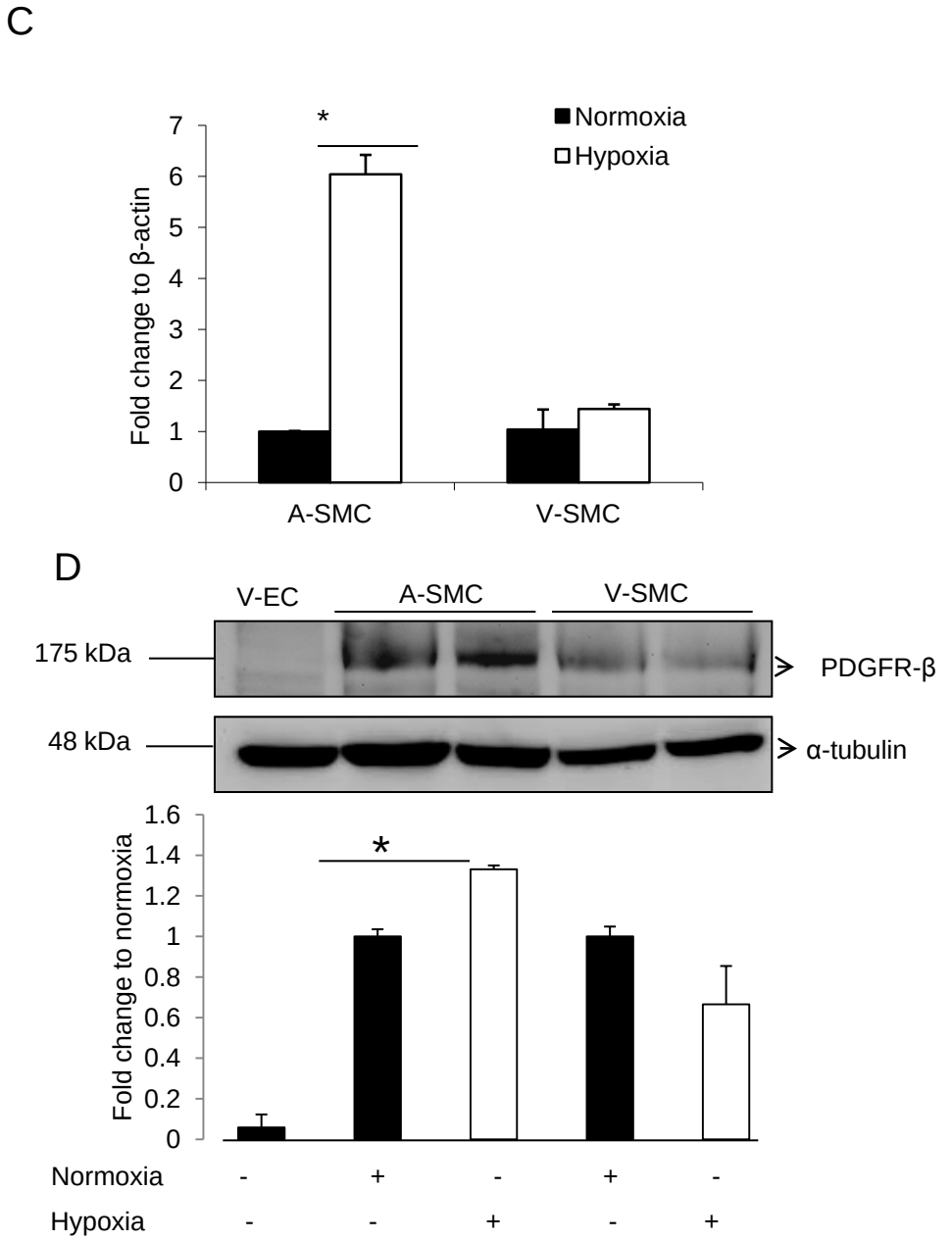

Figure 2.9: Hypoxia induces PDGFR-β expression in A-SMC. Cells (A-SMC, V-SMC) were exposed to hypoxia for 6 hours**. C)** PDGFR β mRNA and B-actin for A-SMC and V-SMC were analyzed using QT-PCR. **D)** PDGFR β protein expression in SMC using WB . Data was considered significant at *p<0.05, **p<0.001. Data are the mean ± SD of at least three independent experiments.

Figure 2.9 E

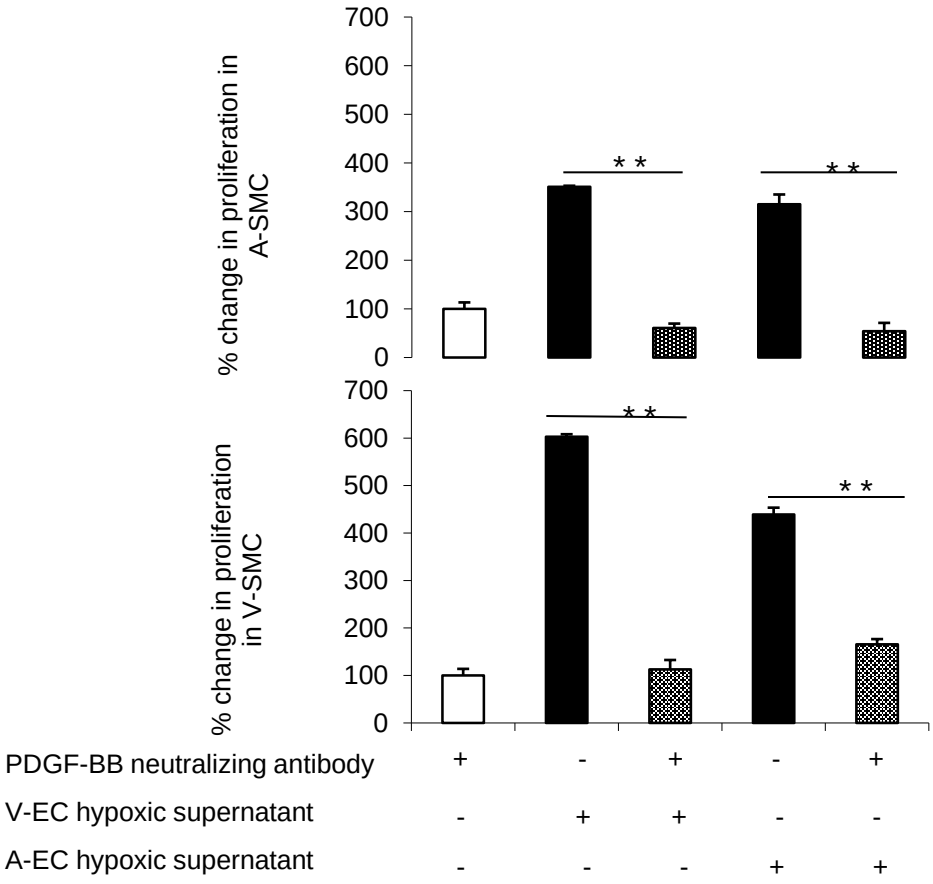

Figure 2.9E: Proliferation in A-SMC and V-SMC is regulated by PDGF-BB under hypoxia A-SMC and V-SMC proliferation after addition of (A-EC and V-EC) derived H-ECM with pre-incubation of PDGF-BB neutralizing antibody. BrdU incorporation using an ELISA was used as an indicator of SMC proliferation. Data was considered significant at *$p<0.05$, **$p<0.001$. Data are the mean ± SD of at least three independent experiments.

Figure 2.9F

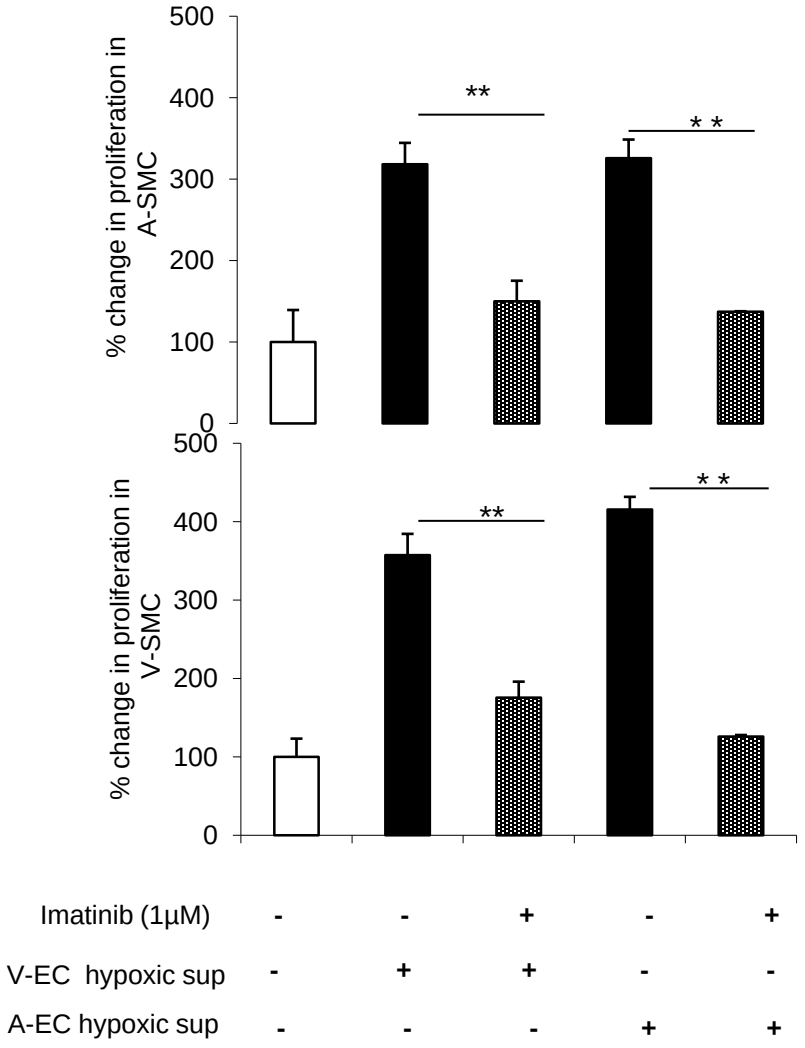

Figure 2.9F: Proliferation in A-SMC and V-SMC is regulated by PDGFR under hypoxia A-SMC and V-SMC proliferation after addition of (A-EC and V-EC) derived H-ECM with pre-incubation of PDGFR antagonist (imatinib (1µM)). BrdU incorporation using an ELISA was used as an indicator of SMC proliferation. Data was considered significant at *p<0.05, **p<0.001. Data are the mean ± SD of at least three independent experiments.

Figure 2.10

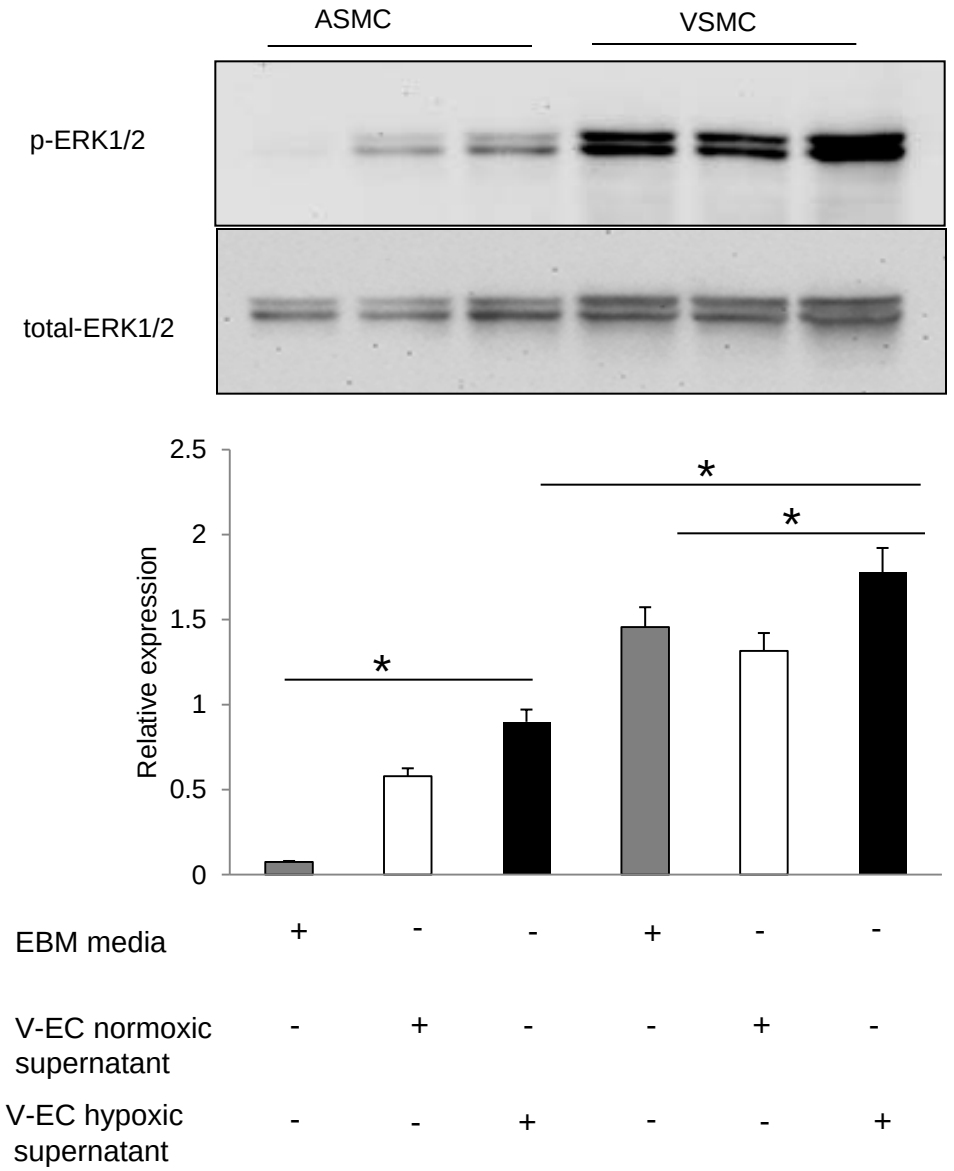

Figure 2.10: Hypoxic EC conditioned media induces greater ERK1/2 activation in V-SMC than A-SMC under hypoxia V-SMC and A-SMC were incubated with hypoxic EC conditioned media under hypoxia for 24hrs. ERK1/2 phosphorylation was determined using Western blot analysis. Significance was determined at *$p < 0.05$. The bar graph represents the mean ± SD of at least three different cell preparations.

Figure 2.11

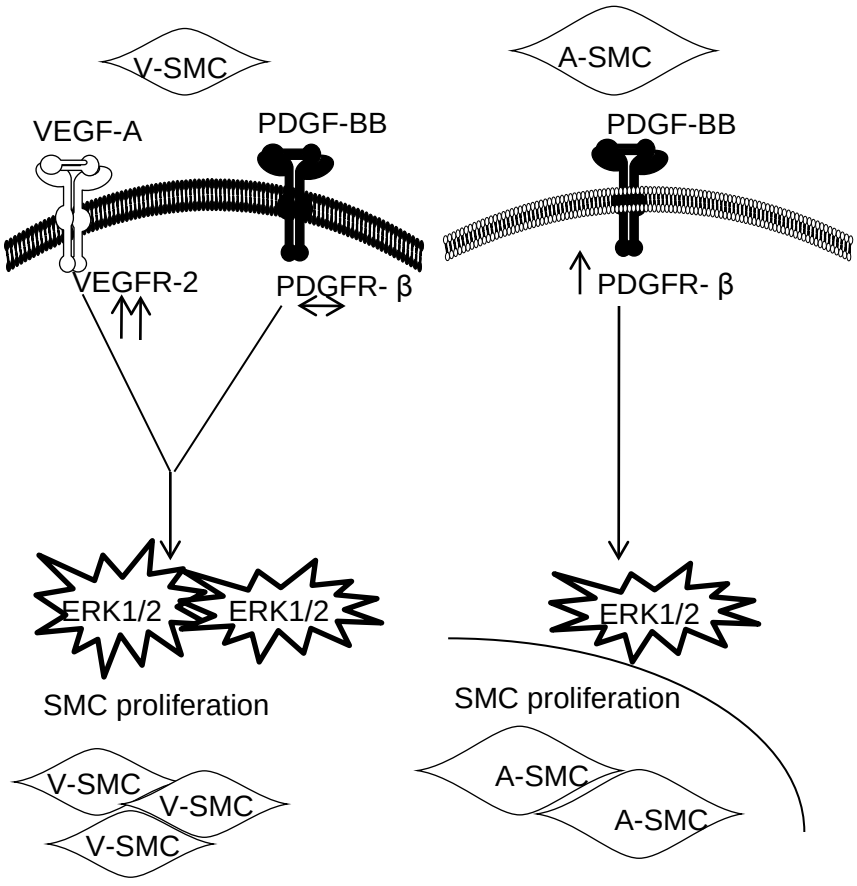

Figure 2.11: Summary of working model. Diagram shows how hypoxia differentially regulates VEGFR-2 and PDGFR expression which results in different proliferative effects between A-SMC and V-SMC with ERK1/2 playing a role

CHAPTER 3: HYPOXIA INDUCES SMC MIGRATION THROUGH A GROWTH FACTOR DEPENDANT AND INDEPENDENT MECHANISM

INTRODUCTION

The molecular interactions between cells are indispensable measures of the maintenance of physiological and pathophysiological processes. The malformation and malfunction of vascular endothelial cells (VECs) has been identified as one of the major pathophysiological mechanisms contributing to cardiovascular diseases (Banerjee and others 2008a). The endothelium is located within the blood vessel wall and acts as a barrier between the blood and the vascular smooth muscle cells (VSMCs)(Wu and others 2011). The migration of vascular smooth muscle cells from the media to intima and their subsequent proliferation are critical causes of vessel wall thickening in pathophysiological conditions like atherosclerosis and aortic aneurysms and intimal hyperplasia (IH) (Osada-Oka and others 2008). In vascular surgeries and procedures like bypass grafts, IH, a vessel response to injury has been observed and is the leading consequence of graft failure.

Studies conducted on graft patency have shown that arterial grafts have a higher patency than vein grafts (Kobayashi 2009; Zegdi and others 2009). Almost 90% occlusion occurring in venous grafts after ten years as compared to only 50% occlusion in arterial grafts (Zegdi and others

2009)**.** Due to the impact upon quality of life, medical costs, and the potential loss of life, studies examining the causes and mechanism/s of IH are essential and clinically relevant (Lee and others 2000).

Researchers consider that the injury and the malfunction of VECs are closely related to atherosclerosis and intimal hyperplasia (Banerjee and others 2008b; Wu and others 2011). Therefore understanding the functional integrity of the endothelial monolayer is essential to prevent vascular leakage and formation of neointimal hyperplasia. Despite several efforts to control IH, a suitable treatment modality to control this process is elusive due to an incomplete understanding in the mechanisms responsible for IH as well as the differential response in smooth cell migration observed in arterial and venous derived smooth muscle cells (Lee and others 2000). The function of vascular smooth muscle cells (SMC), is known to be altered by hypoxia (Lee and others 2000), SMC are also known to function in a similar manner when they are recovered from lesions in IH (Lee and others 2000) . The anoxemia theory of artery wall pathology in atherosclerosis was first suggested in 1944 and since then several investigators have developed techniques to directly measure artery wall *p*O2 levels (Zemplenyi, Crawford, Cole 1989). Zemplenyi *et al.* describe a microcathode puncture apparatus to measure iliofemoral arteries *in vivo* (Zemplenyi, Crawford, Cole 1989). Niinikoski *et al.* ,Santilli *et al.* , and Buerk and Goldstick all describe

techniques to directly measure artery wall oxygen tension levels *in vivo* and have found depressed *p*O2 levels in the artery wall (Bjornheden and others 1999; Buerk and Goldstick 1982; Lee and others 2000). Bjornheden *et al.* also describes a hypoxia marker, 7-(49-(2-nitroimidazol-1-yl)-butyl) theophylline, that was found in atherosclerotic plaque, further confirming the role of anoxemia in IH and atherosclerosis (Bjornheden and others 1999; Lee and others 2000). At the time of surgery, the vasa vasorum is dissected away from the adventitia, preventing diffusion of blood from the adventitial surface. In addition, arterial suturing further contributes to decreased diffusion of arterial blood from the luminal side (Lee and others 2000). All these studies above have implicated the involvement of hypoxia as a contributing factor to IH and yet limited studies have been conducted to focus on the effects hypoxia has on SMC migration in the presence of growth factors and whether the differences observed clinically in arterial vs venous grafts could be due to differences in response of these cells to hypoxia. Therefore understanding how vascular cells respond to hypoxic conditions might offer some insight on how communication between the endothelial cells and smooth muscle cells initiate IH.

Hypoxia is known to induce growth factors like VEGF-A and PDGF-BB in endothelial cells (Faller 1999). VEGF-A has been shown to stimulate smooth muscle cell migration through a vascular endothelial cell growth

factor receptor 1 mediated pathway (Banerjee and others 2008a). Our study will go on to further investigate the effect of hypoxia on VEGFR1 expression in SMC and if differences in expression could offer a possible explanation why venous derived smooth muscle cells migrate greater than arterial derived smooth muscle cells. PDGF-BB has also been shown to induce smooth muscle cell migration via Platelet derived growth factor receptor β and therefore its contribution to ASMC vs VSMC migration will be evaluated (Millette and others 2006).

Protein kinases are activated by a range of stresses, such as inflammatory cytokines, growth factors, ultraviolet radiation and various forms of oxidative stress, leading to changes in cell cycle or expression of key genes, including cytokines, chemokines, and growth factors (Chung 2011). The mitogen-activated protein kinase (MAPK) family includes three distinct stress-activated protein kinase pathways: p38, c-Jun N-terminal kinase (JNK), and extracellular regulating kinase (ERK)(Chung 2011). The ERK pathway is predominantly activated in cases of proliferative stimuli, whereas the JNK and p38 MAPK pathways respond to environmental stresses (Chung 2011; Griendling and others 2000). p38MAPK has been implicated in cell migration and hypoxia is known to modulate p38 MAPK via ROS production (Griendling and others 2000) we therefore hypothesized that migration in A-SMC and V-SMC was through a p38MAPK dependant mechanism.

This current study was designed to evaluate the effect of hypoxia on cellular migration and investigate whether hypoxia has differential effects on arterial vs venous derived smooth muscle cell migration. These differences would offer some insight as to why arterial grafts are more patent compared to venous grafts and these differences can be manipulated in a clinical setting to maximize therapy against IH.

METHODS

Cell culture

Human umbilical vein smooth muscle cell (V-SMC) were obtained from ScienceCell Reseach Laboratories (Carlsbad, CA) and maintained in SmGm-2 (Lonza, Walkersville, MD). Human aortic smooth muscle cell (A-SMC) and human aortic endothelial cell (A-EC) obtained from Lonza were maintained in SmGM-2 and EGM-2 (Lonza) medium respectively. Human umbilical vein endothelial cells (V-EC), (kind gift from Dr Ramakrishnan, University of Minnesota) were also maintained in EGM-2 media.

Reagents: p-38MAPK, total-p38 MAPK primary antibodies; anti-rabbit IgG and anti-mouse IgG secondary antibodies were purchased from Cell signaling Technology (Beverly, MA). β-actin and VEGFR-1 primary antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, California); anti-human VEGFR-1 neutralizing antibody, anti-human

PDGF-BB neutralizing antibody were purchased from (R&D Systems, Minneapolis, MN). Imatinib mesilate (PDGFR antagonist), VEGFR-2 tyrosine kinase inhibitor was purchased from calbiochem (New Jersey, USA).

Treatment: Cells were serum starved for 24hr in 1% FBS + (EBM-2 and SmBm) and then subjected to normoxia and hypoxia for different time points depending on assay conducted. 3hrs treatment was used for isolation of total mRNA in cells. 24hrs for migration assay, growth factor and growth factor receptor analysis.

Normoxic conditions (21% oxygen), is defined here as normal room air in a 5% CO_2, 37°C cell culture incubator. To achieve hypoxia (3-5% O_2), cells were placed in a modular chamber (Billups Rothenberg, Inc., Del Mar, CA) and flushed with a mix of 0% O_2, 5%CO_2, and 95% N_2 at 10L/min for 15 minutes. Chambers remained tightly sealed and placed in a 5% CO_2, 37°C incubator. This method achieves pO_2 levels less than 35mmHg as determined from cell culture medium analyzed using a blood gas analyzer, Rapid Lab248 (Chiron Diagnostics Tarrytown, NY); pO_2 levels of culture supernatant from cells grown under normoxic conditions was 150–160 mmHg.

Scratch assay

SMC`s were plated on a cover slip until 80% confluent. A wound was placed across the center of the cover slip using a 1000uL pipette tip. Cells were placed under hypoxia and normoxia. After 24hrs the cells were fixed using 4% parafomaldehyde and stained with 4',6-diamidino-2-phenylindole (DAPI). A zeiss microscope at 40x magnification was used to capture several images in different fields of the scratched area to determine the number of cells that had migrated into the scratch area. Average distance of cells migrated into the scratch area was measured to determine effect of the treatment on migration. Treatments were performed in triplicate.

ECIS migration assay

SMC`s were placed in a 8 well chamber and exposed to hypoxia for 24 hours. The cells were pre-treated with neutralizing antibody prior to injury and placement of endothelial cell hypoxic conditioned media. To form the wounded area, AC current was delivered to the small active electrodes located on the central part of each well, and then the cells located on the electrodes were killed and detached. Thereafter, the medium was replaced with new medium containing vehicle or associated treatment. The wounded areas of each well were gradually healed by the migration of the viable cells surrounding the small electrodes, and the migratory

response could be measured in real-time by recording the recovery of electrical impedance. Transcellular electric resistance (TER) of cells was recorded using Electric cell-substrate impedance sensing system (ECIS) (Applied Biophysics). The data shown is real-time representative tracings obtained showing the effect of a particular treatment on cell migration.

Boyden chamber migration

A Boyden chamber was used to evaluate SMC migration under hypoxia. A-SMC and V-SMC were plated on a 8micron membrane in the upper chamber and normoxic. Hypoxic endothelial cell conditioned media was placed on the bottom chamber. SMC migrating through the pores were stained using crystal violet and absorbance determined calorimetrically. Data represented as % increase in migration after 6hrs hypoxia.

RNA isolation and cDNA preparation

RNA was extracted from cells (SMC and EC) using 1ml TRIZOL reagent from Invitrogen (Carlsbad, CA). Total RNA (1ug) of each sample was reverse transcribed using an oligo d (T) primer and RNase MMLV reverse transcriptase, according to the manufacturer`s protocol, promega (Madison , WI). cDNA (100ng) was used for real time PCR and gel based PCR to study the genes listed below.

Sense and antisense oligonucleotide primers for VEGFR-1 and β-actin were designed for RT-PCR using DNA sequence information obtained from the Genome Database (National Center for Biotechnology Information) and were synthesized at Bio-Medicine Genomic Facility at the University of Minnesota.

The following specific primers were used:

VEGFR-1 sense: 5-`CAT CAA CCT CCC CAC CAC-3`, antisense: 5`-TAT TTT TTC AGT CCC ACA GTT AGC-3`

β-actin sense 5` GAT CAT TGC TCC TCC TGA GC-3` antisense 5' CAC CTT CAC CGT TCC AGT TT-3`

Image J software was used to quantify intensity of the bands obtained after running PCR product on a 1% agarose gel for gel based PCR.

Western blot analysis (WB)

SMC (1 X 10^6) were plated in a cell culture flask and placed under normoxia and hypoxia for 24hrs. Cells were then lysed with 500uL lysis buffer from Sigma (St. Louis, MO) according to manufacturer's instructions. Total protein concentration of the supernatants was determined using Bio-Rad DC protein assay from Bio-Rad (Hercules, CA). 60ug of total protein was loaded for p-38 MAPK, p-AKT WB, 50ug for VEGFR-1 WB and their respective loading controls. The samples were electrophoresed in a 7% discontinuous SDS-PAGE. The resolved

proteins were transferred to a PVDF membrane from Bio-Rad (Hercules, CA) which was then blocked for 1hr with 5% non-fat milk at room temperature. The membrane was incubated with (1:500) primary antibody concentration (VEGFR-1, β actin, and p38MAPK) overnight at 4^{o}C. Membrane bound primary antibodies were detected using anti mouse or rabbit IgG secondary antibodies (1:1000) conjugated with horseradish peroxidase. Immunoblots were detected with UltraQuant 6.0 Ultralum (Claremont, CA) using enhanced chemiluminescence technique (Immobilon Western HRP Substrate; Millipore) (Billerica, MA). Quantification of bands was performed using UltraQuant 6.0 (Claremont, CA).

RESULTS

Hypoxia induces Smooth Muscle Cells migration via an autocrine manner

Controversy still exists as to whether effects of hypoxia on SMC migration are autocrine or paracrine (Banerjee and others 2008a; Ferrara, Winer, Burton 1991; Osada-Oka and others 2008; Patel and others 2010). To elucidate the role of hypoxia on SMC migration and if the effect was autocrine or paracrine the following experiment was performed using real-time cell monitoring of impedance. A-SMC and V-

SMC were placed in a 6 well plate at 80% confluence. An electric voltage was used to generate a wound and real time tracings of the cells migrating towards the generated wound area observed under hypoxia over a period of 24hrs. **Figure 3.1** is an illustration showing a summary of how the assay was performed. The tracings shown in **figure 3.2A** represent cell movement over a period of 15hrs. The slope of the tracings showed that V-SMC migrated at a more significant ($p<0.05$) speed of 1.1 micron/hr as compared to A-SMC which migrated at a speed of 1.02micron/hr. **Figure 3.2B** is a graphical representation of migration after 5hrs. The results were further confirmed using the scratch assay. Arterial and venous derived smooth muscle cells were plated on a glass slide until 80% confluent; a scratch assay was performed and then placed under hypoxia for 24hrs. The cells were stained with DAPI and their migration towards the scratched/wounded area investigated. Our results showed smooth muscle cells migrating towards the wounded area in both V-SMC and A-SMC. V-SMC cells showed a greater number of cells migrating towards the center of the scratch assay when compared to migration in A-SMC **(figure 3.2C)**. Based on this data we concluded that hypoxia induces smooth muscle cell migration in both V-SMC and A-SMC via an autocrine manner.

In our next set of experiments we looked at the role paracrine factors play in SMC migration under hypoxia.

Hypoxic Endothelial Cell conditioned media induces Smooth Muscle Cells migration under hypoxia

To test whether there was a paracrine mechanism the following assay was conducted. A-SMC and V-SMC were placed in a 6 well plate at 80% confluence. An electric voltage was used to generate a wound. The media in the wells was replaced with conditioned media from hypoxic EC (HECM) and media from EC under normoxia (NECM) and incubated with A-SMC and V-SMC and subjected to hypoxia for 24hrs. Real-time cell impedance generated by the migrating cells was monitored under hypoxia over a period of 24hrs. The tracings shown in **figure 3.3 A& B** represent cell movement over a period of 5hrs. The slope of the tracings showed that V-SMC migrated at a more significant ($p<0.05$) rate upon addition of HECM when compared to addition of normoxic endothelial cell conditioned media (NECM). Representative data of the scratch assay under similar conditions in V-SMC is shown in **Figure 3.3C.** This result was further confirmed using the classical Boyden chamber assay. In this assay HECM was placed in the bottom chamber of the well and SMC placed on top of an 8uM semi-permeable membrane. The chamber was placed under hypoxia and the number of SMC migrated over 5hrs stained using a crystal violet stain. The stain was dissolved using DMSO and a calorimetric reading obtained. The data is represented as %

change in migration based on control which is change in migration when EBM-2 media (endothelial cell media without growth factors) was placed in the bottom chamber **Figure 3.3D & E**. Based on this data we concluded that hypoxic endothelial cells act through a paracrine manner to induce SMC migration. Based on our data in **Figure 2.5A &B** we determined that VEGF-A and PDGF-BB as the two main growth factors that were greatly induced under hypoxia in endothelial cells. VEGF-A has been shown to induce smooth muscle cell migration through a vascular endothelial cell growth factor receptor 1 (Banerjee and others 2008a). To determine if VEGFR-1 and PDGF-BB played a role in the differential effects of hypoxia on SMC migration the following studies were conducted.

VEGFR-1 and PDGF-BB play a role in SMC migration

In this experiment VEGFR-1 and PDGF-BB neutralizing antibodies were pre-incubated with SMC prior to treatment with HECM. A-SMC and V-SMC were placed in a 6 well plate at 80% confluence and incubated with HECM, and NECM as a control. An electric voltage was used to generate a wound and real time tracings of the cells migrating towards the generated wound area observed under hypoxia over a period of 24hrs **(figure 3.4A & B)**. The tracings shown in **figure 3.4A & B** represent cell movement over a period of 5hrs. The slope of the tracings showed that V-SMC migrated greater upon addition of HECM when

compared to addition of normoxic endothelial cell conditioned media (NECM). Upon addition of PDGF-BB neutralizing antibody there was partial neutralization of V-SMC migration. Upon addition of VEGFR-1 neutralizing antibody there was almost complete neutralization of V-SMC migration. Based on this data VEGFR-1 plays more of a significant role in SMC migration when compared to the role PDGF-BB does. The slope of the tracings in A-SMC indicated similar results in increase of migration upon addition of HECM but at a slower rate since the slope of the tracings was less compared to slope determined upon addition of HECM in V-SMC under hypoxia. Upon neutralizing with PDGF-BB there was complete neutralization of cell migration in A-SMC contrary to the results obtained upon PDGF-BB neutralization in V-SMC. Neutralization with VEGFR-1 showed a partial decrease in A-SMC migration under hypoxia. **Figure 3.4C&D** represent images of the wounded areas electrode after 5hrs which also exhibit similar trends as the results discussed above where VEGFR-1 plays a more dominant role in V-SMC migration when compared to migration in A-SMC under hypoxia upon addition of HECM. To determine further why VEGFR-1 played a more dominant role in V-SMC migration compared to A-SMC migration under hypoxia upon addition of HECM we investigated VEGFR-1 levels in these cells under hypoxia.

V-SMC expresses greater VEGFR-1 mRNA and protein levels under hypoxia

Since VEGFR-1 is implicated in SMC migration and our data in **figure 3.4B** showed that there was a partial decrease in A-SMC migration under hypoxia whilst there was a complete decrease in migration in V-SMC **(figure 3.4A)** we investigated the levels of VEGFR-1 mRNA and protein levels in V-SMC and A-SMC under hypoxia. Our results showed us that both V-SMC and A-SMC expressed VEGFR-1 mRNA under normoxia with V-SMC showing an induction under hypoxia (1.5 fold) compared to A-SMC which showed a 0.8 fold decrease in VEGFR-1 expression under hypoxia **(figure 3.5A)**. A western blot was conducted to determine the protein levels and similar trends were observed showing the V-SMC to be expressing greater VEGFR-1 under hypoxia **(figure 3.5B)**. Since p38 MAPK has been implicated in SMC migration the role p38 plays in V-SMC vs A-SMC migration was determined (Chung 2011; Rao 1996).

Hypoxic endothelial cell conditioned media induce p-38 MAPK expression in SMC under hypoxia

V-SMC and A-SMC cells were plated and placed under hypoxia for 3hrs. The cells were collected and p38MAPK protein levels determined using WB. Our data showed an induction in p38 MAPK levels under hypoxia in

both A-SMC and V-SMC upon addition of HECM. V-SMC showed a greater p38 MAPK induction **(figure 3.6)**. We investigated if SB a well known p38 MAPK inhibitor inhibited SMC migration.

SMC migration occurs via a p38 MAPK dependant mechanism

EXcelligence real-time monitoring of SMC under hypoxia was used to monitor the effects of SB on SMC migration upon addition of HECM. The experiment was conducted as stated above with the exception of pre-incubation of the SMC with SB (a well known p38 MAPK inhibitor). Our data indicated **(figure 3.7)** that there was a decrease in both A-SMC and V-SMC migration under hypoxia with SB incubation prior to addition of HECM implicating a p38MAPK dependant pathway in SMC migration.

Discussion

This study is one of the few studies that have investigated the effect of hypoxia on smooth muscle cell migration using real-time monitoring, as well as compare the differences in migration between arterial and venous derived smooth muscle cells. We demonstrate that hypoxia via an autocrine manner contributes to smooth muscle cell migration. This was confirmed by an increase in smooth muscle cell migration towards the scratch area under hypoxia, as well as an increase in smooth muscle cell migration towards the wounded area with EXcelligence real time monitoring of the smooth muscle cells. VEGF-A induced vascular smooth

muscle cell migration through the activation of VEGFR-1 –p38MAPK signaling cascade as well as through a PDGFR β- p38MAPK pathway. Moreover, the proposed pathway showed that there was differential regulation in smooth muscle cell migration between arterial and venous smooth muscle cell illustrating why clinically venous grafts are more patent than arterial grafts. Migration in V-SMC was predominantly VEGFR-1 dependant as compared to A-SMC which was more PDGF-BB dependant evident by complete reduction in V-SMC migration upon pre-incubation with VEGFR-1 neutralizing antibody. Studies by Parenti et. al have also demonstrated that hypoxic insult results in an increase in smooth muscle cell migration via a VEGFR-1 mediated pathway (Parenti and others 2002). Parenti et al investigated the influence of hypoxia and endothelial loss has on the responsiveness of vascular smooth muscle cells (VSMCs) to vascular endothelial growth factor (VEGF-A). The study used rat aortic rings deprived of endothelium and cultured them in three-dimensional fibrin gels. The authors observed an increase in sprouting of tubular structures in response to VEGF-A which was observed only under hypoxia. Western blot and immunohistochemistry of these endothelium-deprived preparations exposed to hypoxia showed VEGFR-1 receptor expression in all medial cells (SMC). Our study went on to further elaborate on the differential responses between venous and arterial derived smooth muscle cells and their response to hypoxia. Our

study investigated effects of hypoxia on VEGFR-1 expression in arterial vs venous smooth muscle cells. VEGFR-1 is a tyrosine kinase receptor that has been shown to be responsible for cell migration (Parenti and others 2002). VEGFR-1 knockout mice have been shown to be important for the promotion of the lung-oriented metastasis of a lewis lung carcinoma via premetastic induction of matrix metalloproteinase 9 (MMP9) in the lung (Shibuya 2010a). Similar experimental model demonstrated that VEGFR-1 positive hematopoietic bone marrow progenitors initiate the premetastatic niche in the lung and other tissues (Shibuya 2010a). Our data showed that hypoxia induced VEGFR-1 in venous smooth muscle cells whilst showing a non-significant effect on VEGFR-1 expression in arterial derived smooth muscle cell. This result led us to hypothesize that greater VEGFR-1 expression in VSMC contributed to their ability to migrate more under hypoxia when compared to ASMC. This increase in migration is what ultimately contributes to the pathophysiology of IH observed with most vascular surgeries including bypass grafts. Based on this observation VEGFR-1 antagonist could be used clinically to help alleviate IH after vascular surgeries.

Hypoxia has been shown to regulate phosphorylation of MAP Kinases (Banerjee and others 2008b). For example p38 MAPK and PI3K/AKT kinase are activated by various stimulants including reactive oxygen species (ROS). In addition growing evidence indicates that the

epidermal growth factor receptor (EGF-R) and the PDGF receptor (PDGF-R) serve not only as receptors for EGF and PDGF, respectively, but also as a scaffold for assembly of signaling complexes by G protein-coupled receptors such as those for Ang II (Griendling and others 2000) and transactivation of both these growth factor receptors is redox sensitive. In SMCs, hydrogen peroxide induces tyrosine phosphorylation of the EGF-R and stimulates its association with Shc (src homology complex)-Grb2(growth factor receptor- bound protein 2)- Sos(son-of-sevenless) complexes to activate subsequent signaling cascade (Rao 1996). ROS activates Ras via an oxidative modification of cysteine-118, leading to inhibition of the GDP-GTP exchange (Lander and others 1997; Rao 1996) in addition, ROS-triggered Ras activation induces recruitment of phophatidylinositol 39-kinase to Ras, an event that is required for activation of downstream signals such as Akt and MAPK (Deora and others 1998). In endothelial cells, hydrogen peroxide activates p38MAPK and its downstream target (Griendling and others 2000) and p38MAPK is known to be involved in cell migration. Our studies demonstrated that pre-incubation with SB a well known p38MAPK inhibitor upon addition of hypoxic endothelial cell conditioned media reduces both A-SMC and V-SMC migration to basal levels. Based on this data we concluded that p38MAPK plays a role in both A-SMC and V-SMC migration.

In summary, we demonstrate that VEGF-A enhances smooth muscle cell migration under hypoxia differentially in A-SMC when compared to V-SMC. V-SMC migration under hypoxia was predominantly through a VEGFR-1 dependant mechanism since upon neutralization with VEGFR-1 antibody migration of these cells returned to basal levels. On the other hand migration in A-SMC was partially dependent on PDGF-BB and the use of PDGF-BB neutralizing antibody resulted in a decrease in A-SMC migration to basal levels. VEGFR-1 neutralizing antibody also resulted in a partial decrease in A-SMC migration. Further experiments focusing on the exact mechanism employed by PDGF-BB and VEGF-A in A-SMC vs. V-SMC migration needs to be conducted in order to determine whether the two growth factors work synergistically or not. **Figure 3.8** represents our working model. Differences elaborated in this study can facilitate the development of new drug therapies that are specific to smooth muscle cell origin to help manage intimal hyperplasia in vascular surgeries.

Figure 3.1

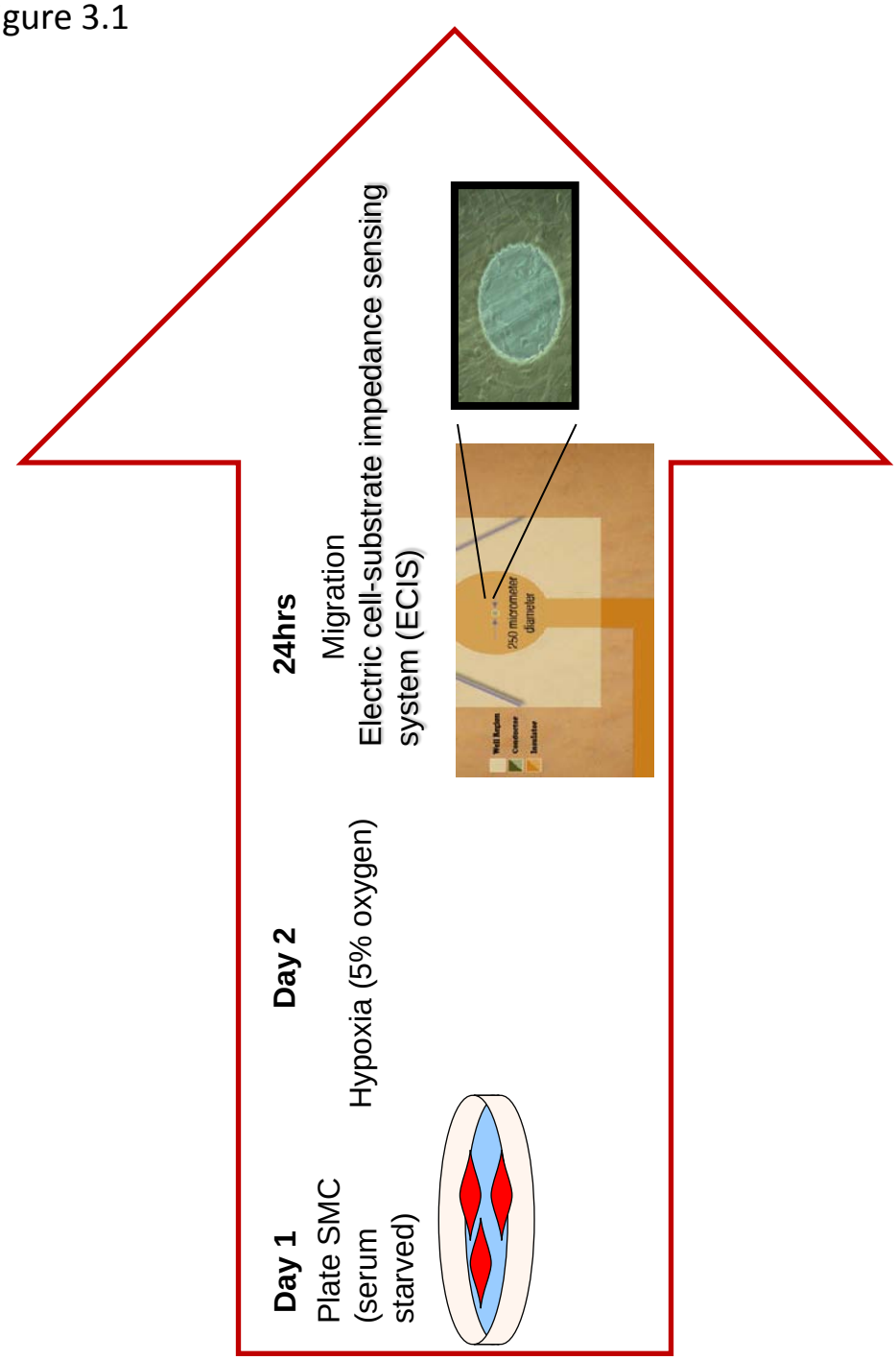

Figure 3.1 Summary of ECIS assay The diagram shows how the ECIS assay was used to determine cell migration under hypoxia

Figure 3.2

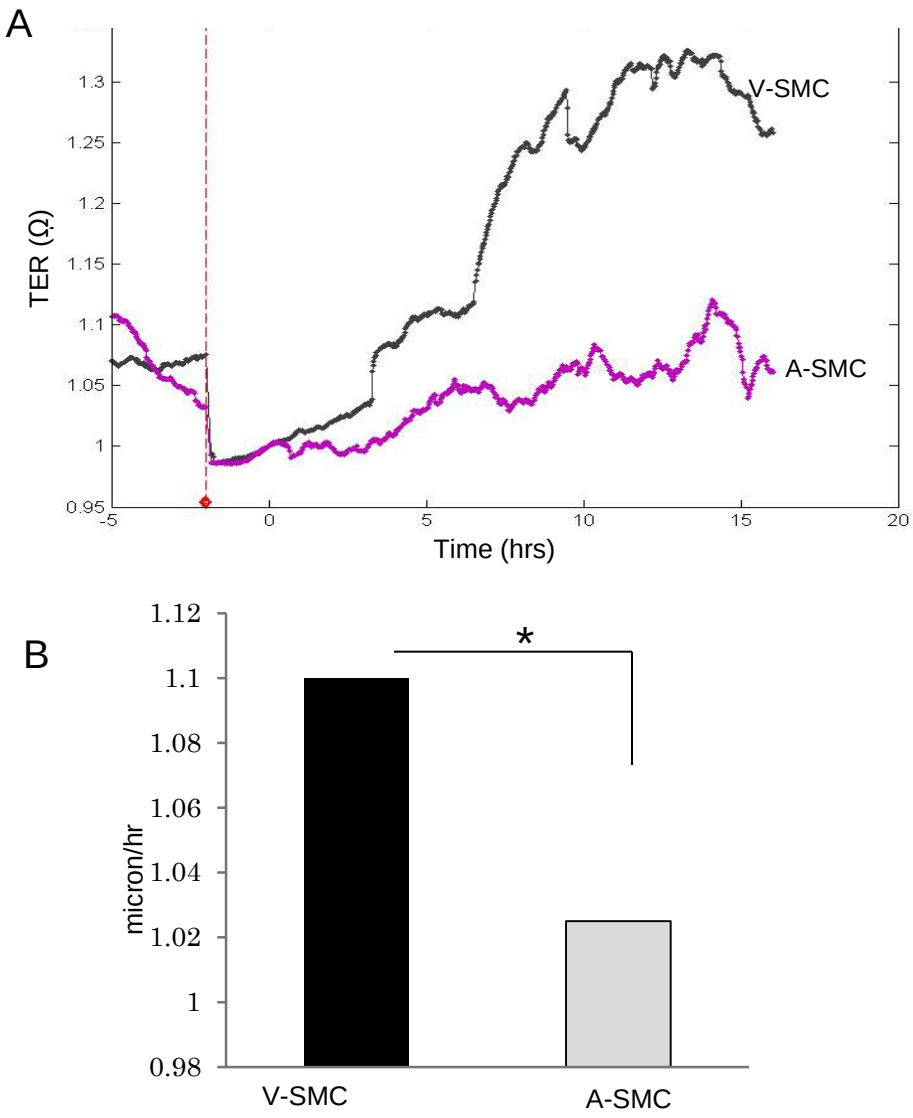

Figure 3.2. Hypoxia induces significantly greater migration in V-SMC when compared to A-SMC. A-SMC and V-SMC were plated in a 6 well chamber and exposed to hypoxia for 24 hours. Confluent cells were wounded and transcellular electric resistance (TER) of cells were recorded using an ECIS system (Applied Biophysics). **B)** the figure is a representation of average SMC migration at 6hrs. *p<0.05.

Figure 3.2

C

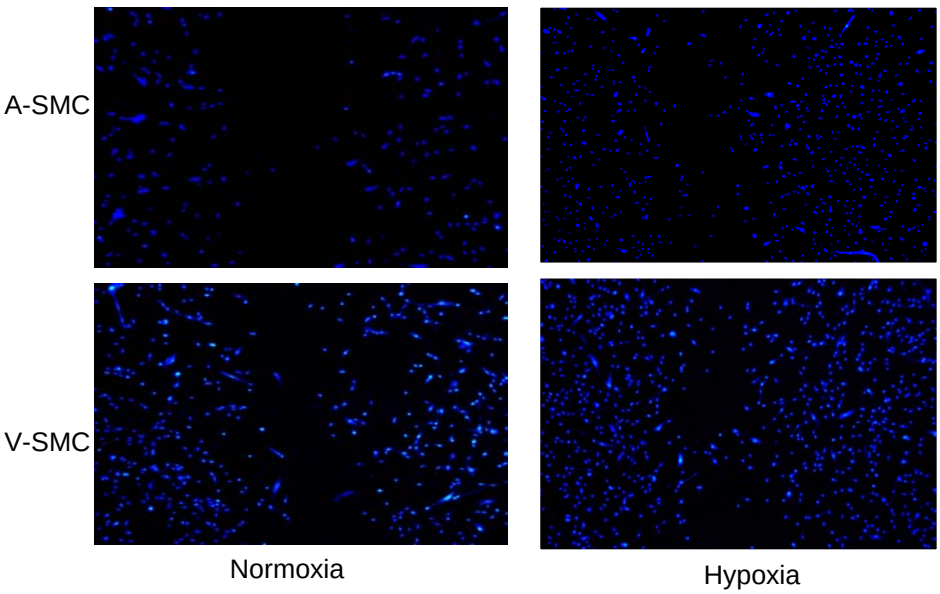

Figure 3.2C: Hypoxia induces significantly greater migration in V-SMC when compared to A-SMC . A-SMC and V-SMC were plated on a coverslide until 80% confluent. A scratch was made in the center of the slide using a 1000uL pipette tip and placed under hypoxia for 24hrs. The cells were stained with DAPI and visualized at 40X magnification under a microscope. Representative data is shown above.

Figure 3.3

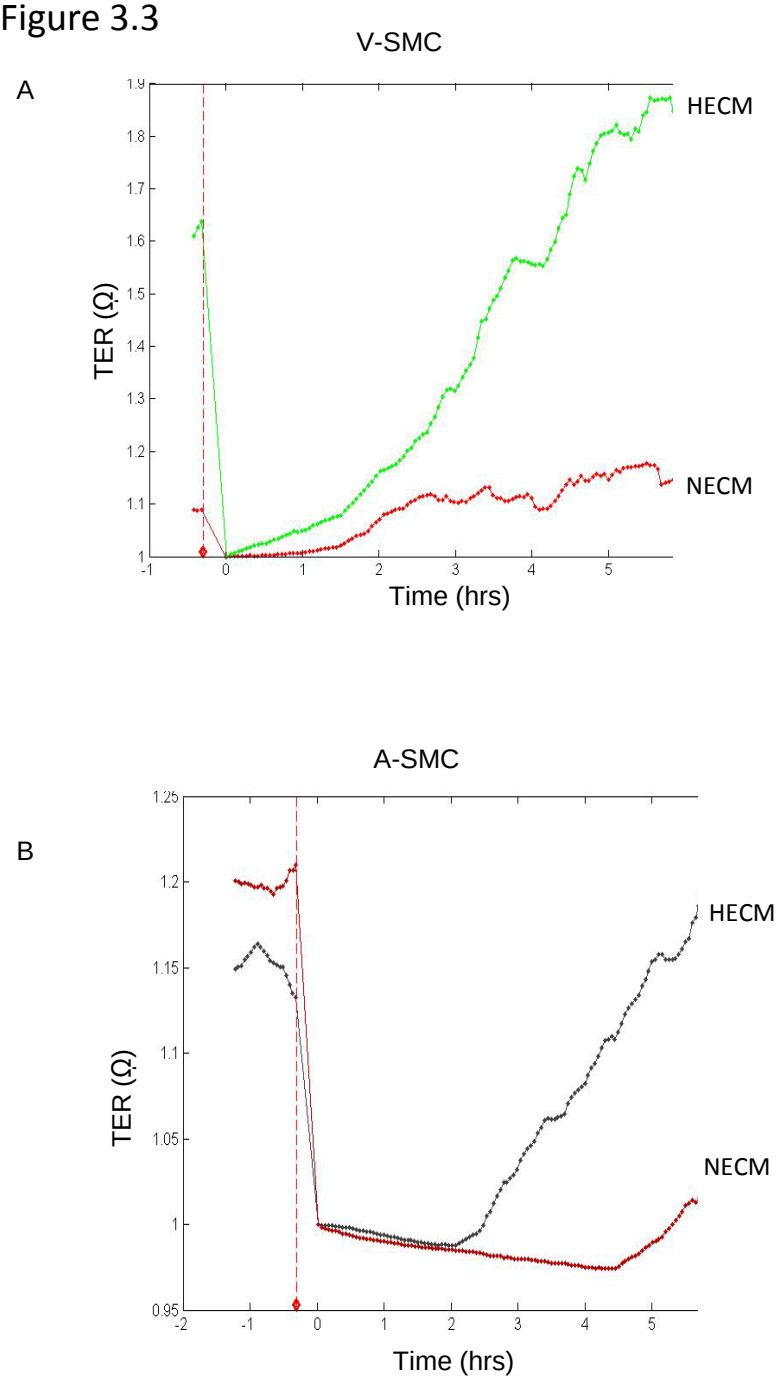

Figure3.3. Hypoxic EC conditioned media induce further migration in SMC with V-SMC exhibiting greatest migration. EC were placed under hypoxia for 24hrs. Conditioned media from EC under normoxia (NECM) and EC under hypoxia (HECM) was collected and 300uL was placed on **A)** V-SMC and **B)** A-SMC under hypoxia. Confluent cells were wounded and transcellular electric resistance (TER) of cells were recorded using an ECIS system (Applied Biophysics).Data is representative of three independent experiments

Figure 3.3

C

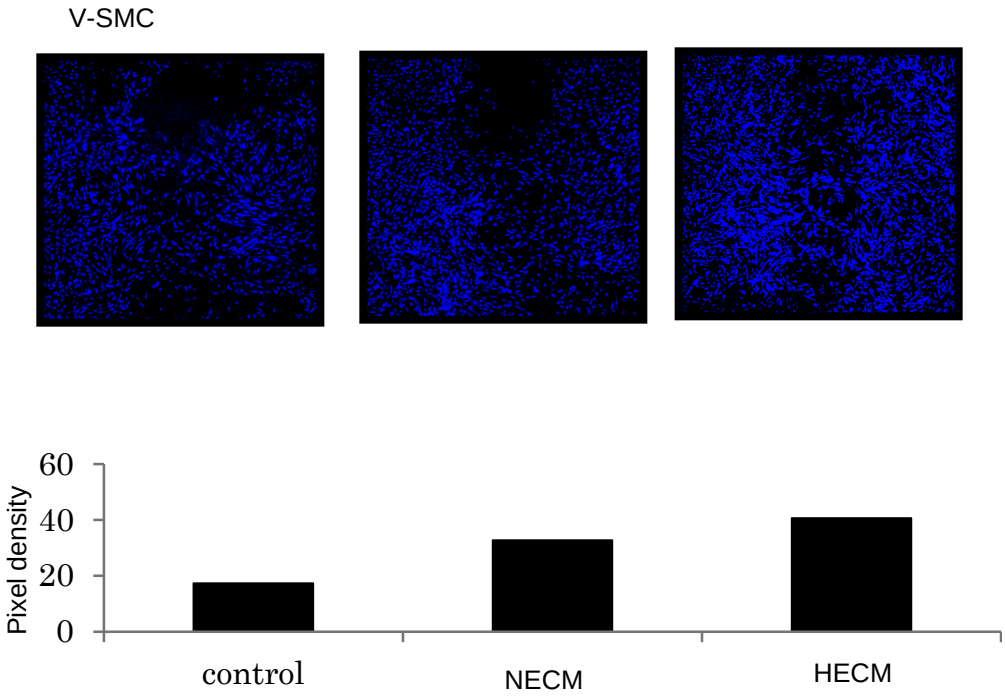

Figure 3.3C. Hypoxic EC conditioned media induce further migration in SMC with V-SMC exhibiting greatest migration. V-SMC were plated on a coverslide until 80% confluent. A scratch was made in the center of the slide using a 1000uL pipette tip and conditioned media from EC under normoxia (NECM) and EC under hypoxia (HECM) was collected and incubated with V-SMC under hypoxia. The cells were stained with DAPI and visualized at 40X magnification under a microscope. Representative data is shown above..

Figure 3.3

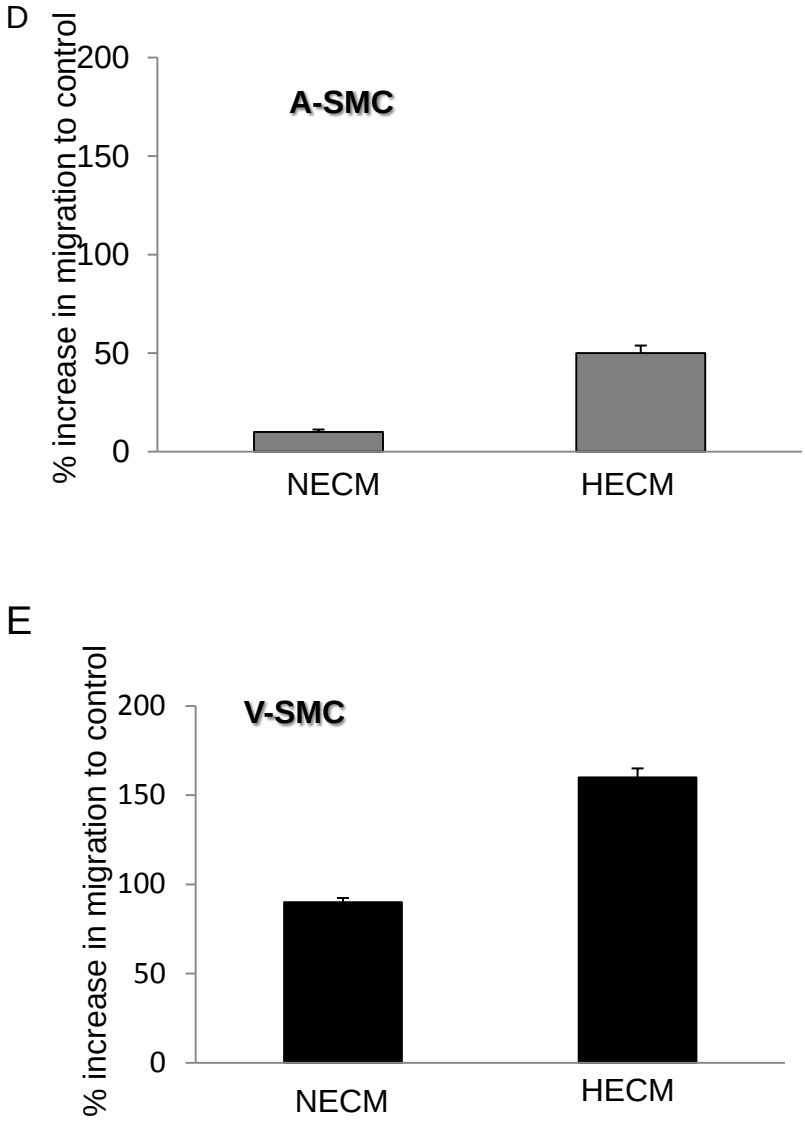

Figure 3.3D&E: Hypoxic EC conditioned media induce further migration in SMC with V-SMC exhibiting greatest migration. A Boyden chamber was used to evaluate SMC migration under hypoxia. A-SMC and V-SMC were plated on a 8micron membrane in the upper chamber and NECM and HECM were placed on the bottom chamber . SMC migrating through the pores were stained using crystal violet and absorbance determined colorimetrically. Data represented as % increase in migration after 6hrs hypoxia. *p<0.05

Figure 3.4

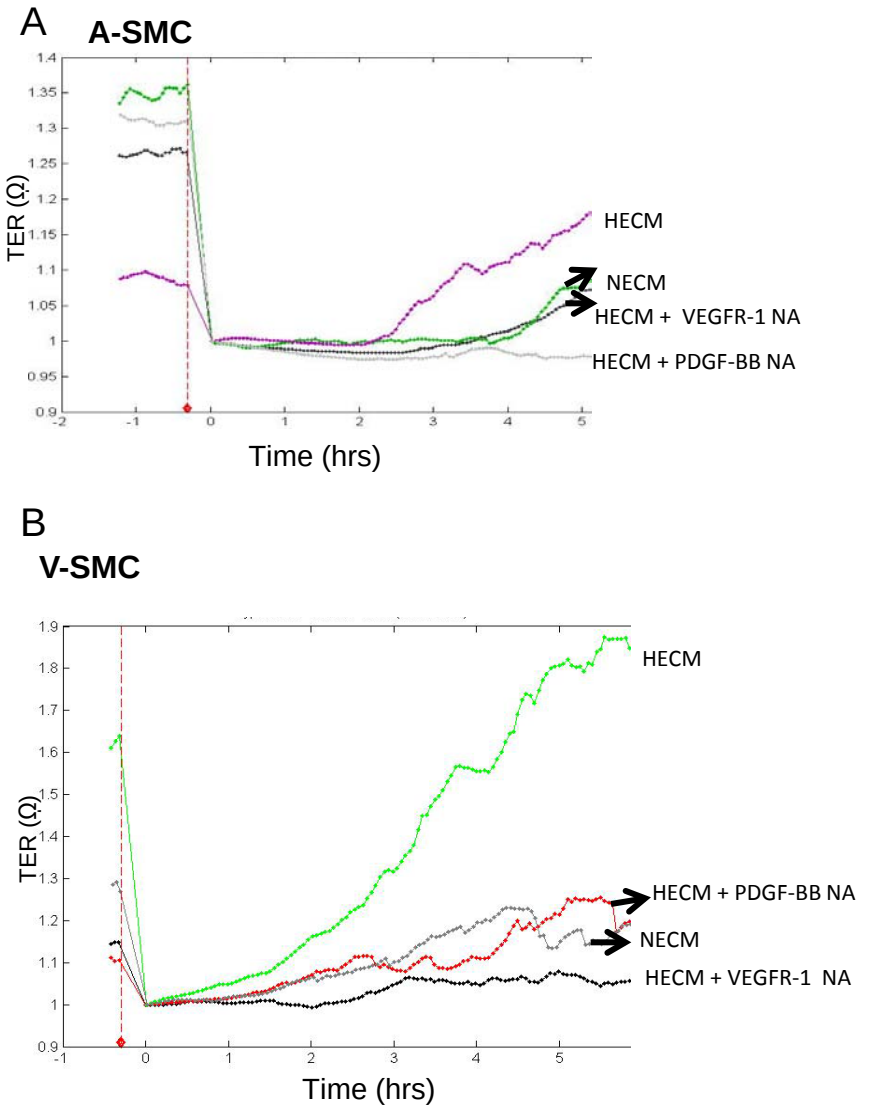

Figure 3.4 VEGFR-1 and PDGF-BB play a role in SMC migration Conditioned media from EC under normoxia (NECM) and EC under hypoxia (HECM) was collected and 300uL was placed on A-SMC and V-SMC under hypoxia. SMC were pre-incubated with VEGFR-1 neutralizing antibody and HECM with PDGF-BB neutralizing antibody prior to wounding. Confluent cells were wounded and transcellular electric resistance (TER) of cells were recorded using an ECIS system (Applied Biophysics). The figure shown above is a representation of three independent experiments

Figure 3.4

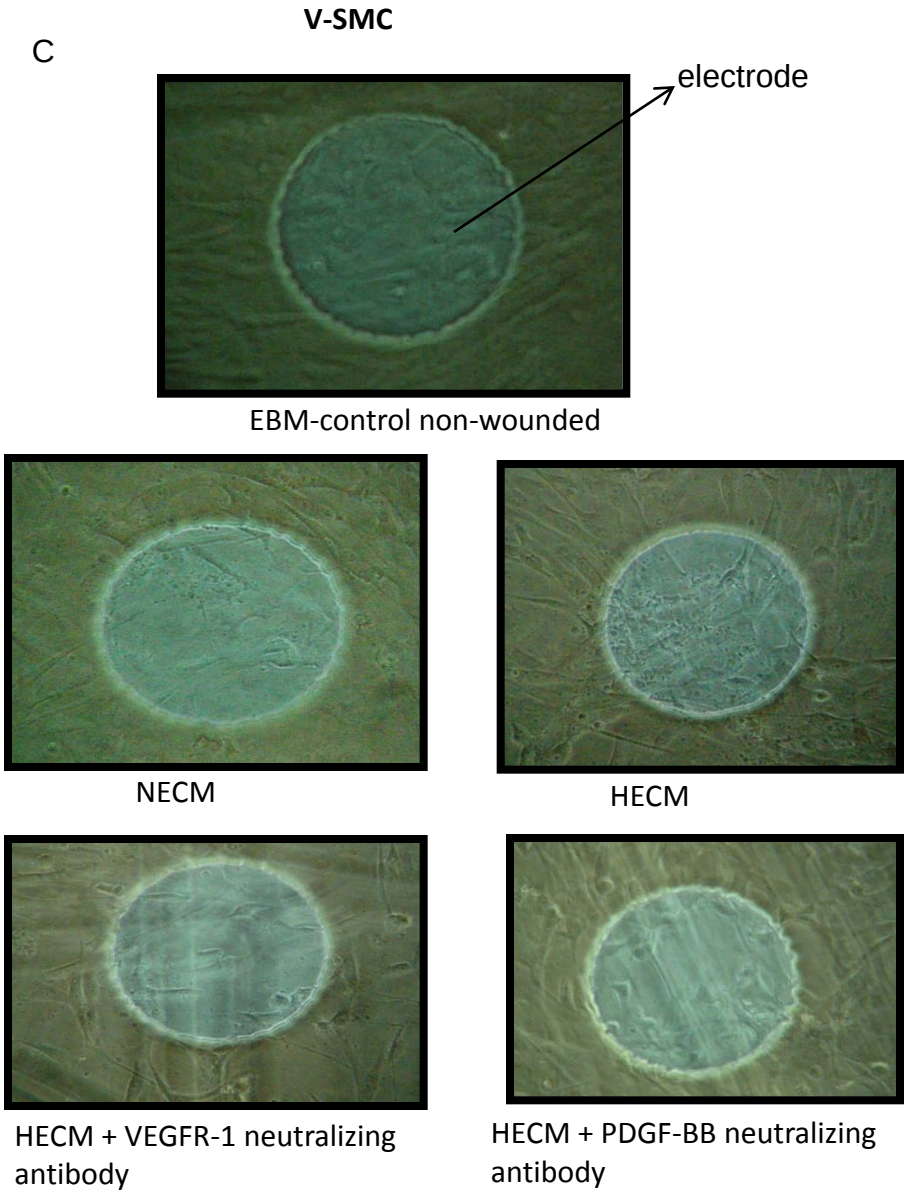

Figure 3.4C VEGFR-1 and PDGF-BB play a role in SMC migration Conditioned media from EC under normoxia (NECM) and EC under hypoxia (HECM) was collected and 300uL was placed on V-SMC under hypoxia. SMC were pre-incubated with VEGFR-1 neutralizing antibody and HECM with PDGF-BB neutralizing antibody prior to wounding. Confluent cells were wounded and after 5hrs wounding a microscope at 20X magnification was used to capture images in each well. The figure shown above is a representation of three independent experiments

Figure 3.4

D

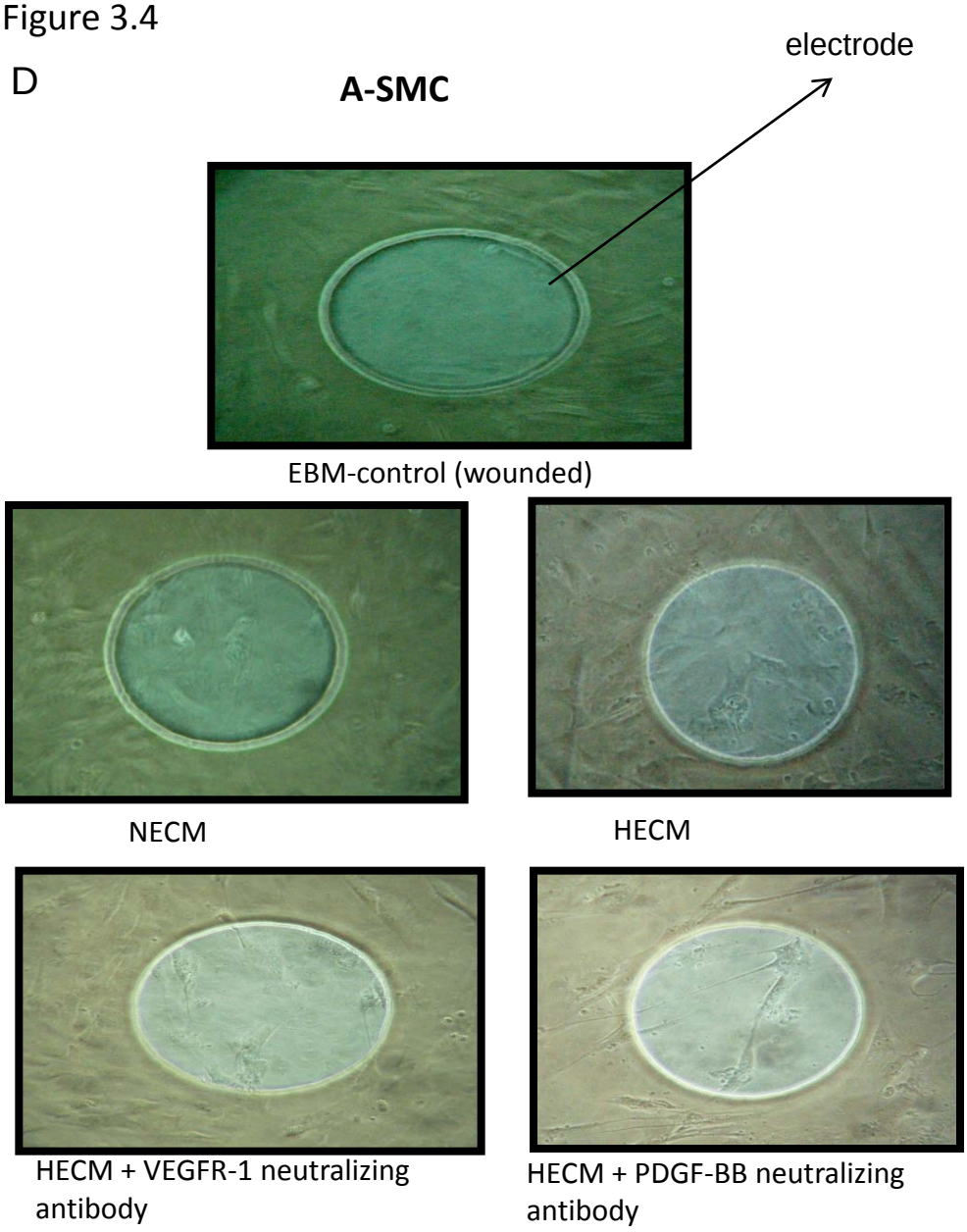

Figure 3.4D VEGFR-1 and PDGF-BB play a role in SMC migration Conditioned media from EC under normoxia (NECM) and EC under hypoxia (HECM) was collected and 300uL was placed on A-SMC under hypoxia. SMC were pre-incubated with VEGFR-1 neutralizing antibody and HECM with PDGF-BB neutralizing antibody prior to wounding. Confluent cells were wounded and after 5hrs wounding a microscope at 20X magnification was used to capture images in each well. The figure shown above is a representation of three independent experiments

Figure 3.5

A

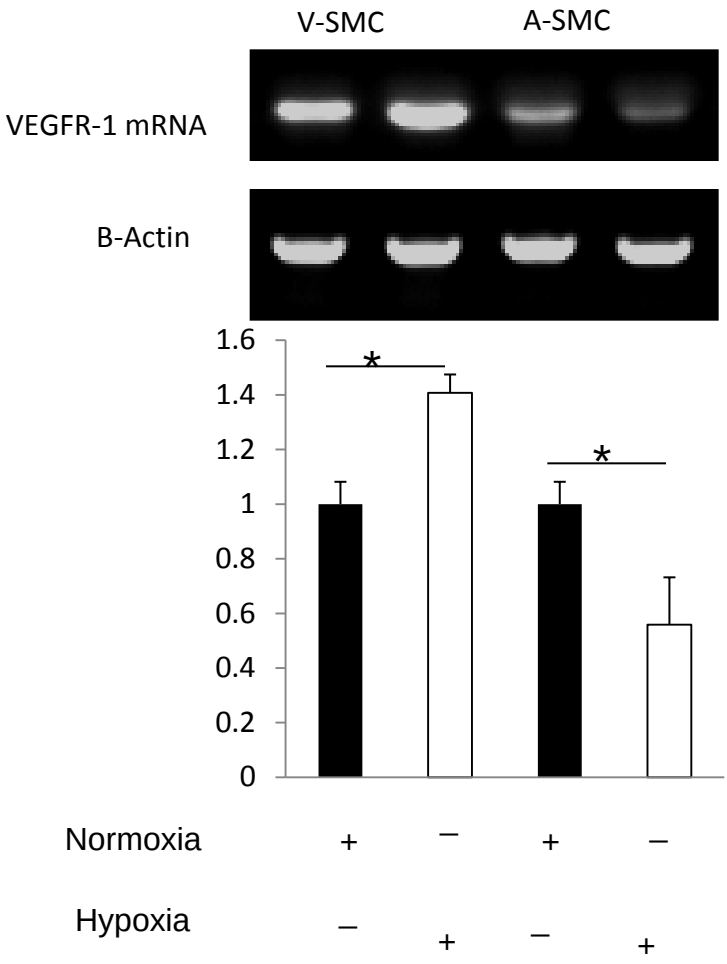

Figure 3.5 V-SMC express greater VEGFR-1 mRNA and protein levels under hypoxia SMC`s were placed under hypoxia for 3hrs. VEGFR-1 mRNA was analyzed using: **A)** RT-PCR in A-SMC & V-SMC *p<0.05 Data are the mean ± SD of at least three independent experiments.

Figure 3.5

B

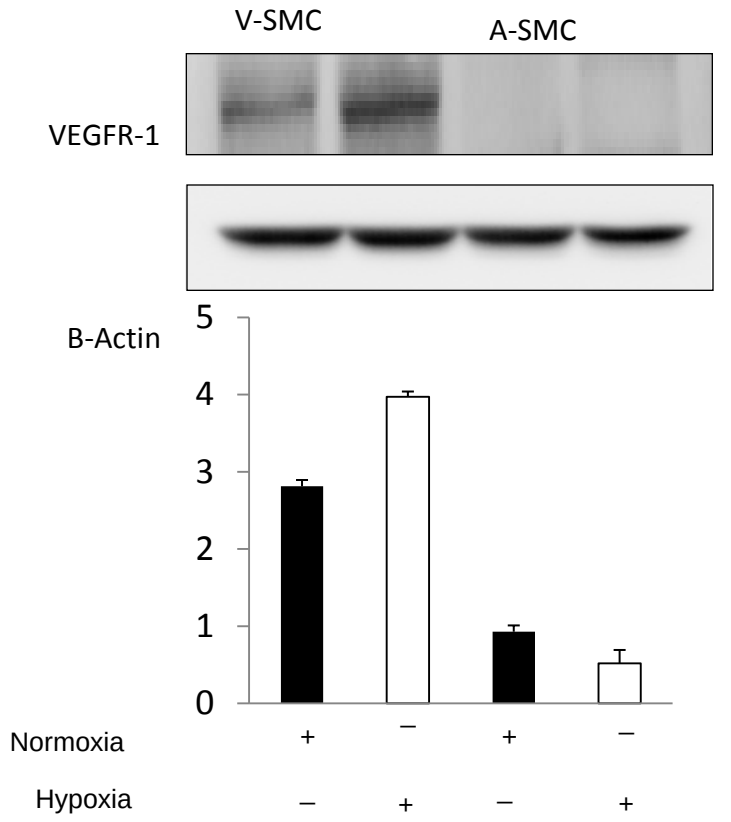

Figure 3.5B V-SMC express greater VEGFR-1 protein levels under hypoxia SMC`s were placed under hypoxia for 24hrs. VEGFR-1 protein levels were analyzed using WB: **B)** protein levels in A-SMC & V-SMC *p<0.05 Data are the mean ± SD of at least three independent experiments.

Figure 3.6

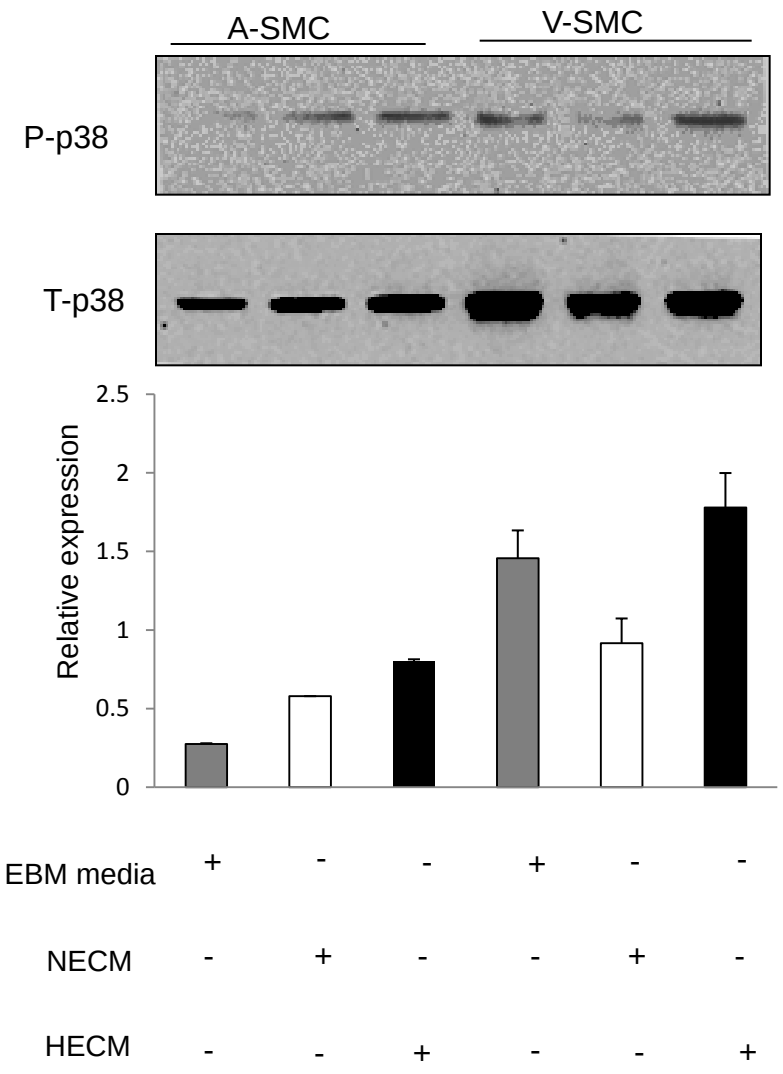

Figure 3.6 A: A-SMC and V-SMC were exposed to hypoxia for 3 hours. Cell culture supernatant from hypoxic and normoxic EC was collected and incubated with the SMC under hypoxia. p38MAPK protein levels were analyzed using Western blot analysis. Significance was determined at *p < 0.05. The bar graph represents the mean ± SD of at least three different cell preparations.

Figure 3.7

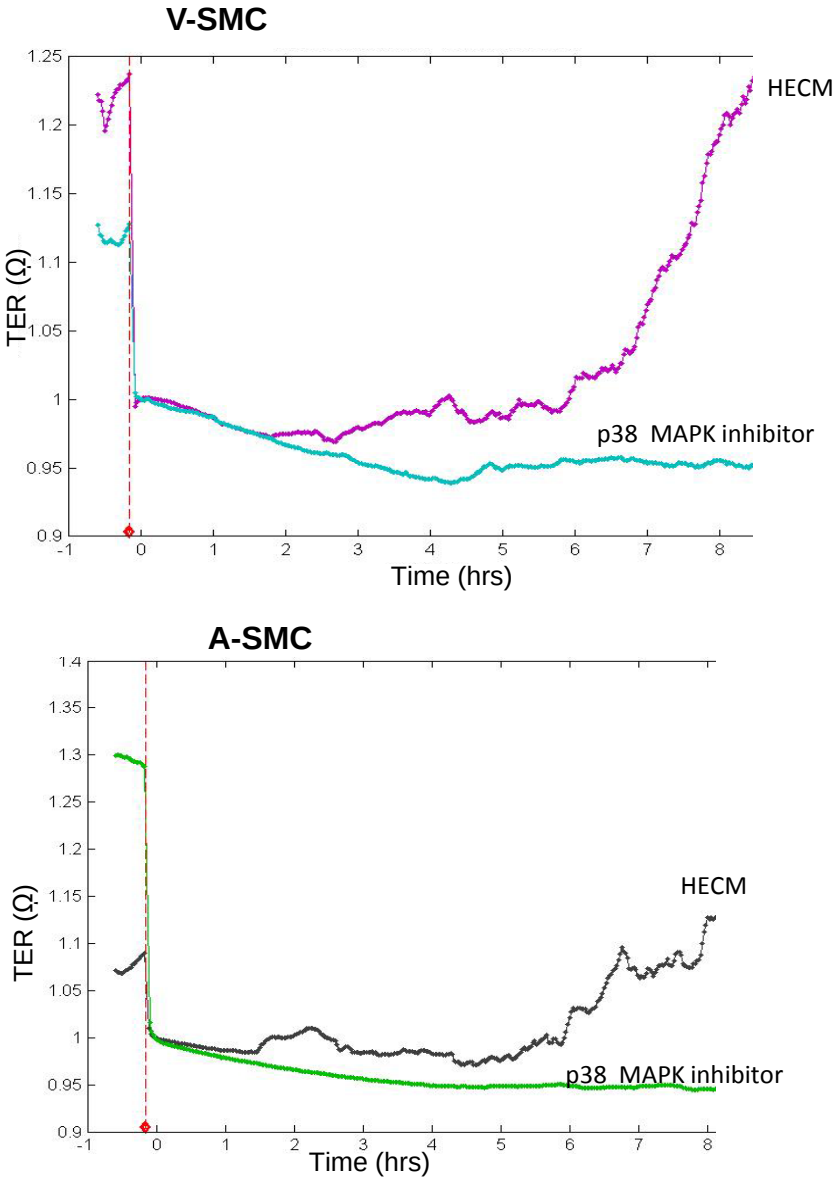

Figure 3.7: S**MC migration occurs via a p38 MAPK dependant mechanism** EC were placed under hypoxia for 24hrs. Conditioned media from EC under normoxia (NECM) and EC under hypoxia (HECM) was collected and 300uL was placed on A-SMC and V-SMC under hypoxia with pre-incubation with p38MAPK. Confluent cells were wounded and transcellular electric resistance (TER) of cells were recorded using an ECIS system (Applied Biophysics. Data shown is representative of three independent experiments

Figure 3.8

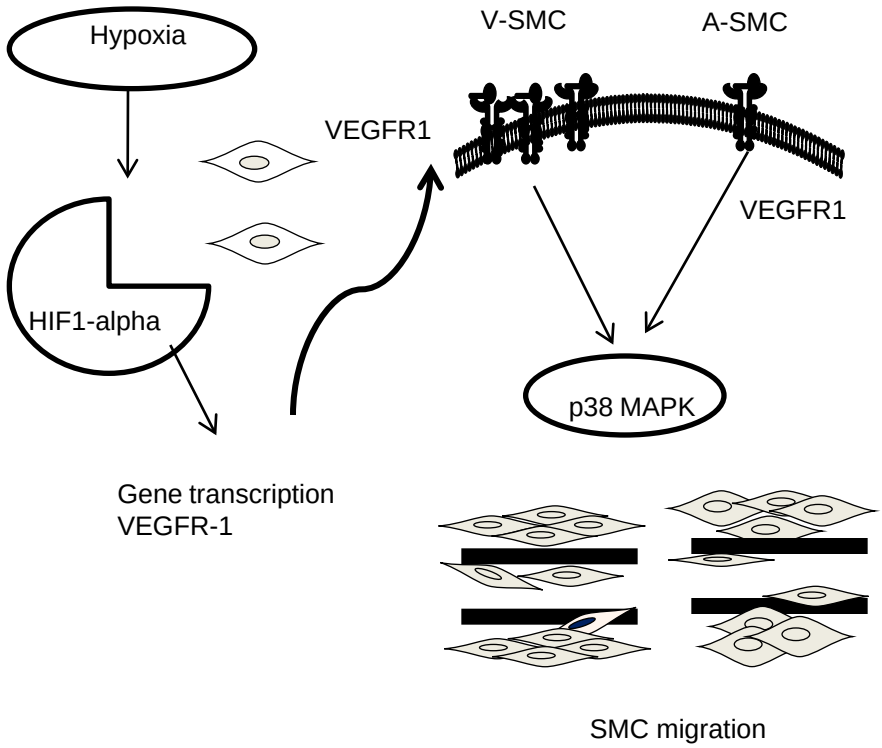

Figure 3.8: Summary of mechanism involved in SMC migration under hypoxia The diagram shows an increase in VEGFR-1 under hypoxia which results in phosphorylation of p38MAPK, ultimately resulting in an increase in SMC migration

CHAPTER 4
SUMMARY AND CONCLUSIONS

In summary our studies have shown that there is differential regulation of arterial vs venous smooth muscle cell proliferation and migration under hypoxia. We show that A-SMC secrete significant amounts of VEGF-A when compared to V-SMC. These findings were also supported by Ferrara N et.al who also demonstrated that cultured A-SMC under normoxic conditions express and secrete VEGF-A (Ferrara, Gerber, LeCouter 2003). Our studies went on to further show that hypoxia regulates this induction of VEGF-A in A-SMC through a HIF1-alpha independent mechanism since there was an insignificant increase in HIF1-alpha expression in A-SMC when cultured for 24hrs under hypoxia. Our study also showed an induction in HIF1-alpha in V-SMC under hypoxia with a corresponding increase in VEGF-A mRNA but an insignificant increase in VEGF-A protein levels. We hypothesized that this discrepancy in lack of translation of VEGF-A was attributed to an increase in miR 125b which is up regulated in V-SMC and down regulated in A-SMC under hypoxia. miR 125b has VEGF-A mRNA as a target and thus prevents translation of VEGF-A mRNA synthesized by the V-SMC under hypoxia.

To determine the role of paracrine factors in SMC proliferation and migration under hypoxia we investigated the growth factors regulated in

endothelial cells under hypoxia. From our findings we concluded that VEGF-A and PDGF-BB where the most significant growth factors in our study induced in V-EC and A-EC under hypoxia with V-EC having a greater induction of growth factors (IGF, FGF-2) across the panel when compared to A-SMC. We observed that upon addition of hypoxic endothelial cell culture supernatant there was an increase in both A-SMC and V-SMC proliferation and we attributed this to an increase in growth factors VEGF-A and PDGF-BB under hypoxia. V-SMC were more responsive to induction in proliferation from both VEGF-A and PDGF-BB. On the other hand A-SMC were only responsive to proliferation induction by PDGF-BB since in our studies we determined that A-SMC did not express VEGFR-2 under hypoxia. VEGFR-2 expression is essential for induction of VEGF-A proliferative effects (Neufeld and others 1999) .

Both these growth factor induced effects in prolfieration were regulated via an ERK1/2 mechanism with V-SMC showing greater ERK1/2 expression under hypoxia upon addition of hypoxic endothelial cell conditioned media.

Our work was also focused on investigating the impact of hypoxia to smooth muscle cell migration. In summary we determined that SMC migration was occurring via an autocrine and paracrine mechanism under hypoxia. Migration in V-SMC under hypoxia was regulated via VEGFR-1 since there was an induction in VEGFR-1 expression under

hypoxia and migration in V-SMC decreased upon neutralization with a VEGFR-1 antibody. V-SMC migration was also regulated by PDGF-BB since there was a partial reduction in V-SMC migration under hypoxia upon neutralization with PDGF-BB antibody. Migration in A-SMC was induced more by PDGF-BB since neutralization with PDGF-BB antibody decreased A-SMC migration to basal levels.

In conclusion our studies illustrate that origin of cells play an important role in how the pathology of a disease develops. As mentioned earlier, clinically it has been observed that arterial grafts have a greater patency than venous grafts (Kobayashi 2009; Mehta and others 1997). Therefore combination therapies focusing on the use of ant-VEGFR-1 antagonist to combat migration and anti VEGFR-2 and PDGFR β to combat proliferation would work best to help alleviate IH in venous derived grafts and surgical interventions.

FUTURE DIRECTIONS

Redox involvement

Most of the work in our studies neglected to look at the influence reactive oxygen species (ROS) play in the regulation of smooth muscle cell migration and proliferation. Much remains to be learned concerning the signaling pathways and genes that are regulated by ROS.

Redoxsensitive response appear to be cell specific, it will be important to identify the sources of oxidant stress in each cell and whether the differences observed between arterial and venous smooth muscle cell redox regulation could contribute to the differences in graft patency observed clinically. Identifying the sources of oxidant stress in each cell, the mechanism of regulation of antioxidant enzymes, and the effect of ROS on signaling pathways specific to the function of that particular cell would help gain further insight into the physiological responses affected by oxidant stress (Griendling and others 2000). Understanding of these events will enable us to devise therapeutic strategies to target specific cellular events contributing to vascular disease.

Endothelial dysfunction is a hallmark of multiple vascular diseases, including atherosclerosis. Impaired endothelial function has several consequences, the most important of which is decreased endothelium – dependant vasodilation. The endothelial cell redox rheostat I primarily regulated by the dynamic production of and interaction between nitric oxide and oxygen (Griendling and others 2000).

The Inflammatory and immune response

Another consequence of endothelial dysfunction and SMC activation is increased monocyte adhesion , foam cell formation, and thrombosis. Ang II and TNF-a induce the expression of proinflammatory molecules such

as VCAM-1, MCP-1, and the thrombin receptor (Griendling and others 2000). Each of these molecules is in turn redox sensitive and in the case of MCP-1 and the thrombin receptor, a role for ROS in Ang II-mediated gene expression has been demonstrated. Theories implicating immune cell function as a contributing factor to IH are also proposed therefore investigating the effect recruited immune cells and the cytokines they secrete have on IH will also be an important measure to help combat IH. Rac and Rho GTPases are also knowm to regulate migration. Investigating levels of these kinases would also help expand these studies.

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