EPAS1 Promotes Endothelial Cell Migration in an IRX3-Dependent Manner

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ABSTRACT

BIOLOGICAL SCIENCES

BARNETT, PETRINA K. B.S. SPELMAN COLLEGE, 2008

EPAS1 PROMOTES ENDOTHELIAL CELL MIGRATION IN AN IRX3-DEPENDENT MANNER

Committee Chair: Valerie Odero-Marah, Ph.D.

Dissertation dated May 2016

Iroquois homeobox gene 3 (Irx3) is a transcription factor belonging to the Iroquois family of homeobox genes that is expressed in the embryonic organs of multiple organisms. Although much is known about Irx3 during embryogenesis, the cross talk of expression and function of Irx3 in peripheral vascular disease (PVD) remains to be investigated. Herein, we demonstrate that Endothelial PAS Domain 1 (EPAS1), which is involved in vasculogenesis and diseases associated with hypoxia, is an upstream regulator of Irx3, and this interaction contributes to the cellular movement of human microvascular endothelial cells (HMVECs). Genetic EPAS1 loss-of-function (LOF) studies in HMVECs resulted in a reduction of Irx3 mRNA, and Irx3-mediated endothelial cell migration in wound healing. In contrast, the effects of EPAS1 Gain-of-function (GOF) in HMVECs showed increased Irx3-mediated endothelial cell migration in wound healing. Taken together, these results reveal that Irx3 is an important regulator of cellular movement as a downstream target of EPAS1 signaling in HMVECs, which regulates migration.
EPAS1 PROMOTES ENDOTHELIAL CELL MIGRATION IN AN IRX3-
DEPENDENT MANNER

A DISSERTATION

SUBMITTED TO THE FACULTY OF CLARK ATLANTA UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

PETRINA K. BARNETT

DEPARTMENT OF BIOLOGICAL SCIENCES

ATLANTA, GEORGIA

MAY 2016
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<th>Description</th>
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<tbody>
<tr>
<td>Ad.</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>ADT</td>
<td>Androgen deprivation therapy</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>ALK5</td>
<td>Activin receptor-like kinase 5</td>
</tr>
<tr>
<td>APP</td>
<td>Aβ precursor protein</td>
</tr>
<tr>
<td>ARNT</td>
<td>Aryl Hydrocarbon Nuclear Translocator</td>
</tr>
<tr>
<td>Arp2/3</td>
<td>Actin-related protein 2/3</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BHLH</td>
<td>Basic helix-loop-helix</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>BOA</td>
<td>Biology on arrays</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CITED2</td>
<td>Cbp/P300-Interacting Transactivator, With Glu/Asp-Rich Carboxy-Terminal Domain, 2</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>COX2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td><strong>De novo</strong></td>
<td>From the beginning</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DU-145</td>
<td>Moderately aggressive prostate cancer cell line isolated from a lesion in the brain of a patient with metastatic carcinoma of the prostate and a 3-year history of lymphocytic leukemia</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td><strong>EPAS1</strong></td>
<td>Endothelial Per-Arnt-Sims Domain 1, also known as HIF2α</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial progenitor cell</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>ESC</td>
<td>Endometrial stromal cells</td>
</tr>
<tr>
<td><em>Et. al.</em></td>
<td>Latin meaning “and others”</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GOF</td>
<td>Gain of Function</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HD</td>
<td>Homeodomain</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>HIF1α</td>
<td>Hypoxia-inducible factor 1 alpha</td>
</tr>
<tr>
<td>HIF2α</td>
<td>Hypoxia-inducible factor 2 alpha, also known as EPAS1</td>
</tr>
<tr>
<td>HMVEC</td>
<td>Human microvascular endothelial cell</td>
</tr>
<tr>
<td>HOX</td>
<td>Homeobox</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxia response element</td>
</tr>
<tr>
<td>HuR</td>
<td>Human antigen R, also known as ELAV-like protein</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IHD</td>
<td>Ischemic heart disease</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>IL-17</td>
<td>Interleukin-17</td>
</tr>
<tr>
<td><strong>In situ</strong></td>
<td>In the original position</td>
</tr>
<tr>
<td><strong>In vitro</strong></td>
<td>In an artificial environment outside the living organism</td>
</tr>
<tr>
<td><strong>In vivo</strong></td>
<td>Within a living organism</td>
</tr>
<tr>
<td>Irx</td>
<td>Iroquois homeobox</td>
</tr>
<tr>
<td><em>Irx3</em></td>
<td>Iroquois homeobox gene 3</td>
</tr>
<tr>
<td>ISH</td>
<td>In-situ hybridization</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>KRAS</td>
<td>Kirsten rat sarcoma viral oncogene homolog</td>
</tr>
</tbody>
</table>
LNCaP  Lymph Node Carcinoma of the Prostate, androgen sensitive prostate cancer cell lines
MAPK  Mitogen-activated protein kinase
mir  Micro-ribonucleic acid
mir-133  Micro-ribonucleic acid 133
mir-143  Micro-ribonucleic acid 143
mir-145  Micro-ribonucleic acid 145
mir-146  Micro-ribonucleic acid 146
miRNA  Micro-ribonucleic acid
miRNA-125  Micro-ribonucleic acid 125
MMP  Matrix metallo-proteinase
MOPS  3-(N-Morpholino)propanesulfonic acid
mRNA  Messenger ribonucleic acid
MSC  Mesenchymal stem-like cells
NF-κB  Nuclear factor kappa-light-chain-enhancer of activated B cells
O₂  Oxygen
PC-3  Androgen insensitive prostate cancer cells derived from bone metastasis of grade IV of prostate cancer
PCCs  Prostate Cancer Cells
PCR  Polymerase chain reaction
PMSF  Phenylmethanesulfonyl fluoride
PTN  Pleiotrophin
qRT-PCR  Quantitative real-time PCR
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>Rac1</td>
<td>Ras-related C3 botulinum toxin substrate 1</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homolog gene family, member A</td>
</tr>
<tr>
<td>RhoC</td>
<td>Ras homolog gene family, member C</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RWPE-1</td>
<td>Prostate epithelial transformed by Human Papilloma virus</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate - polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>TALE</td>
<td>Transcription activator-like effectors/ Three amino acid loop extension</td>
</tr>
<tr>
<td>TIE2</td>
<td>Endothelial Tyrosine-Protein Kinase Receptor</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGF-AR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
</tbody>
</table>
CHAPTER I
INTRODUCTION

Angiogenesis, the process of new blood vessel growth from pre-existing blood vessels, is a natural event that occurs under both normal and pathological conditions.\textsuperscript{1} The resulting blood vessel delivers oxygen (O\textsubscript{2}) and nutrients to all cells within the body.\textsuperscript{2} Thus, O\textsubscript{2} levels serve as a key player in the development and subsequent function of angiogenesis.\textsuperscript{3} There are several families of transcription factors that have been implicated in angiogenesis regulation, such as the hypoxia inducible factors (HIFs) which are stabilized mainly in response to decreased O\textsubscript{2} availability, and are responsible for most of the transcriptional responses to low O\textsubscript{2}.\textsuperscript{4} Hypoxia-inducible factor (HIF) is a transcription factor that responds to changing intracellular oxygen concentration.\textsuperscript{5} Under typical oxygen levels (normoxia), HIF is hydroxylated and acetylated, modifications that target the transcription factor for VHL-mediated ubiquitin degradation.\textsuperscript{5} During hypoxia, HIF accumulates and is transported to the nucleus where it induces expression of numerous target gene products.\textsuperscript{5} Secreted growth factors such as Vascular endothelial growth factor (VEGF) induce signaling pathways including Phosphoinositide phospholipase C gamma, Phosphoinositide 3-kinase, Proto-oncogene tyrosine-protein kinase Src, and mothers against DPP homolog 1 (Drosophila) signaling that result in endothelial cell proliferation, increased vascular permeability, and cell migration.\textsuperscript{6} In addition to hypoxia, the PI3K and Ras pathways can increase HIF expression by...
promoting HIF translation.\textsuperscript{5} Pericytes are also a major player in the angiogenesis signaling pathway because they provide structural support for newly formed blood vessels, promote endothelial cell survival, guide sprouting vessels, and regulate vasoconstriction and dilation.\textsuperscript{7} This is done through a reciprocal signaling mechanism in which PDGF-BB secreted into the matrix by endothelial cells acts as a ligand for PDGF receptor-β located on the pericyte membrane. In return, pericytes produce and secrete VEGF that signals through the endothelial VEGF receptor. Extracellular matrix proteases and regulators induce tissue matrix remodeling in preparation for migration of endothelial cells from existing vessels to form new tubing.\textsuperscript{8} Thus, discoveries that target angiogenesis signaling can bring insight to VEGF signaling, the clinical efficacy of VEGF-targeting therapies, and potential mechanisms of resistance. Recent studies have identified HIF pathways as master regulators of angiogenesis.\textsuperscript{9} This is due, in part, to low O\textsubscript{2} levels, which promotes vessel growth by modulating multiple pro-angiogenic signaling pathways that mediate key aspects of endothelial cell function.\textsuperscript{10}

Central to the process of angiogenesis is endothelial cell migration. This process is regulated by a stringent equilibrium between pro- and anti-angiogenic molecules.\textsuperscript{11} Before propagation of endothelial cells from preexisting vessels can occur the following must first take place: the mural cells must be removed; the endothelial cell basement membrane must be degraded; and remodeling of the perivascular stroma must take place.\textsuperscript{12} Essential mediators in these processes include Tie2-ligand, serine protease, and matrix metallo-proteinases (MMPs).\textsuperscript{13} Pro-angiogenic factors such as vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) induce migration of
endothelial cells. VEGF is the most well-studied growth factor in angiogenesis. It is a dimeric chemokine protein, and has membership in a family that includes VEGF-A, -B, -C, -D, -E, and placenta growth factor (PIGF). There are six molecular isoforms of VEGF in humans; VEGF121, -145, -165, -189, -206 (alternatively spliced isoforms of VEGF-A), VEGF-167, and -186 (alternative splice forms of VEGF-B). VEGF121 is the most frequent, VEGF165 is the most potent and VEGF189 is the most abundant isoform, making VEGF-A, the best characterized member of homodimeric glycoprotein family.

The various isoforms and isotypes of VEGF allows for tissue specific regulation of these cellular processes in vivo. VEGF-A is a critical component of early vascular development. In collaborative studies, it has been shown that mice lacking one VEGF-A allele for its receptor have malformed blood vessels leading to embryonic death at day 10. VEGF-A acts in a dose-dependent fashion to sustain angioblast differentiation and survival during early development. After birth however, the vasculature becomes independent from VEGF-A. The vasculature continues to remain relatively quiescent during adulthood, exceptions to this can be seen in the cases of the female reproductive cycle, would healing, and cancer. In the case of cancer, upon signaling from VEGF-A, the once dormant cells begin the angiogenic process thus supporting cancer progression. During this process, stimulation by a VEGF gradient, results in the specification of a leading endothelial cell (EC) called a ‘tip,’ followed by a ‘stalk’ cell that trails behind the tip cell; both of which maintain connectivity with the preexisting vessel. The constant remodeling of the actin cytoskeleton within endothelial cells into lamellipodia, stress fibers, and filopodia is critical to their ability to migrate.

Lamellipodia are cytoplasmic protrusions that form at the leading edge of migrating cells
and are associated with the small hydrolyzed guanosine triphosphate (GTPase) Rat sarcoma (Ras)-related C3 botulinum toxin substrate 1 (Rac1) and Actin-related protein 2/3 (Arp2/3) complexes. Stress fibers are actin filaments of opposite polarity connected by α-actin. Filopodia are membrane projections made up of parallel actin filament organized into tight bundles that act as a sensor to motile stimuli. Actin-based endothelial movement involves protuberance of lamellipodia leading to forward extension; the sensing of signals by the filopodia; attachment of protrusions to the extracellular matrix (ECM); along with stress-fiber mediated contraction of the cell body. Once endothelial cells reach an area of decreased vessel density, they arrange in a monolayer forming tube-like structures. Lastly, mesenchymal cells in the surrounding tissue migrate and proliferate to the adluminal space of the nascent vessels. The mesenchymal cells then differentiate into pericytes that stabilize the newly developed vessel.

Endothelial PAS Domain 1 (EPAS1), also known as Hypoxia inducible factor 2 alpha (HIF2α), has been well reported as a proangiogenic mediator. HIFs require heterodimers composed of single copies of α and β-subunits, the latter of which is also called the aryl hydrocarbon receptor nuclear translocator (ARNT). The mammalian genome contains three HIF-genes, HIF-1, HIF-2(also called endothelial Per/ARNT/Sim (PAS) domain protein 1 (EPAS1)), and HIF-3, that share similar domain structures but likely serve non-overlapping physiological roles. Loss of HIF-1α eliminates all oxygen-regulated transcriptional responses analyzed, suggesting that HIF-1α is dispensable for hypoxic gene regulation. In contrast, EPAS1 has been shown to regulate some hypoxia-inducible genes in transient transfection assays and during embryonic development in the
lungs and other tissues. Disruption of the HIF-1α gene induced prominent cardiovascular malformations, a lack of organization of the vascular network, and neural tube defects in homozygous mice resulting in early embryonic death, whereas inactivation of the HIF-2α gene was also lethal but attributed to bradycardia due to a defect in fetal catecholamine synthesis or improper remodeling of the primary vasculature during development. The abundant mRNA expression of EPAS1 in highly vascularized tissues such as the heart, lung, placenta, and liver and its hypoxic protein induction suggested a major role in the regulation of angiogenesis. Hypoxia inducible factor 1 alpha (HIF1α) transactivates the EPAS1 gene and encodes a transcription factor involved in the induction of genes regulated by local tissue oxygen levels. The EPAS1 protein contains a basic-helix-loop-helix (bHLH) domain, a protein dimerization domain, as well as a common hypoxia response element (HRE) sequence found in proteins coding for signal transduction pathways that respond to oxygen levels. Previous reports demonstrate that EPAS1 transactivates specific hypoxia-inducible genes such as vascular endothelial growth factor-A (VEGF-A). EPAS1 forms a heterodimeric complex with the aryl hydrocarbon nuclear translocator (ARNT) prior to transcriptional activation of target genes such as VEGF-A. EPAS1 expression is preferentially expressed in endothelial cells and, unlike HIF1α, is capable of specifically activating the transcription of the endothelial tyrosine kinase gene Tie2. Although previous reports demonstrate EPAS1 is a master regulator of hypoxia-regulated genes, there are still unknown downstream targets that have not yet been investigated. To date, there have been few studies that have linked Homeobox (Hox) genes to hypoxia. Hox genes are critical for morphogenesis, organogenesis, and
differentiation, while emerging data has identified the role of Hox genes in cardiovascular development. The Iroquois homeobox proteins (IRX 1-6) are a class of the Homeobox three amino acid loop extension (TALE) family and are essential in early patterning of many embryonic tissues in a spatially and temporally restricted manner. IRX3 has been shown to be involved in the development of the pronephric kidneys, the purkinje fibers of myocardium, neurons, lung, and vertebrate kidney. Most interesting, Irx3 has been identified to be involved in wing development and vein formation in drosophila. This discovery was the first to suggest that IRX3 may be involved in endothelial cell migration, which was confirmed by our own studies that revealed IRX3 is responsive to vascular endothelial cell growth factor (VEGF-A) in microvascular endothelial cells.

Here, we report the novel observation that EPAS1 directly regulates IRX3 function under proangiogenic conditions in HMVECs. Chromatin immunoprecipitation (ChIP) studies confirm EPAS1 binds to a distal and a proximal site located in the Irx3 upstream promoter region. Additionally, EPAS1-mediated Irx3 expression potentiates endothelial cell migration in wound healing assays. Strategies to pharmacologically regulate IRX3 function may provide new therapies to inform ischemic cardiovascular patient treatment decisions as well as provide insight into the cancer models that also use angiogenesis to metastasize.

Ischemic Heart Disease (IHD) is a broad term for complications caused by narrowed arteries and it is the leading cause of death in the Western world. Angiogenesis is the predominant mechanism for increasing collateral blood flow. Traditionally, patients with IHD requiring revascularization have undergone coronary artery bypass
surgery, or percutaneous transluminal balloon angioplasty. However, in patients with severe diffuse coronary heart disease, revascularization is nearly impossible. Thus before successful therapeutics can be developed, further elucidation of the molecular mechanisms underlying angiogenesis is necessary. Angiogenesis, which is one of the seven hallmarks of cancer is required for invasive tumor growth and metastasis, also constitutes an important point in the control of cancer progression. Cancer cells initially lack angiogenic ability, limiting their aptitude to expand. When a tumor begins to expand to a point where it needs increased supply of nutrients and oxygen, this creates a hypoxic environment. Hypoxia triggers the tumor to release pro-angiogenic factors that result in the growth of new blood vessels towards and into the tumor. The formation of new blood vessels from pre-existing blood vessels (angiogenesis) allows the tumor to increase in size. Tumors frequently over express pro-angiogenic factors such as vascular endothelial growth factor (VEGF), which allows them to make the switch to the angiogenic phenotype; making tumor angiogenesis an important therapeutic target. Limitations of traditional cancer therapies are that they do not cause regression of tumors that have metastasized to distant cites. The advantage of this study is that anti-angiogenic agents can cause production of endothelial cells prove to also cause regression of metastatic prostate cells and also prevent their dissemination.

**Purpose of the Study**

The purpose of this study is to investigate the role of *EPAS1* as a positive transcription regulator of *Irx3* expression to promote pro-migratory phenotypes *in vitro*. To elucidate the role of *EPAS1* and *Irx3* in the context of angiogenesis we will: (1) define
the temporal expression pattern of *EPAS1* and *Irx3* in human endothelial cells in response to pro- and anti-angiogenic factors such as hypoxia, VEGF-A; (2) utilize both gain and loss of function approaches to determine the mechanism by which *EPAS1* regulates *Irx3*; and (3) examine the molecular and cellular mechanistic effects of *EPAS1* on *Irx3* induced cellular migration *in vitro*. Our studies will contribute to the development of therapeutics that reveal a novel and unique way in which transcription factors may be utilized in human endothelial cells to modulate angiogenesis.

**Statement of the Problem**

Normal angiogenesis is an essential process required for wound healing, restoring normal blood flow after injury, and early embryonic development whereas deregulation of angiogenesis contributes to the development of many diseases such as ischemia, cancer, and arthritis. To date, the Iroquois homeobox transcription factors have not been implicated in the angiogenic process and few transcription factors have been identified that respond to vascular endothelial growth factor. Herein, for the first time, we will demonstrate that Iroquois homeobox 3 in crosstalk with Endothelial PAS domain 1 effectively modulates angiogenesis in endothelial cells in the presence and absence of vascular endothelial growth factor A. Further analysis of *EPAS1* revealed it is selectively expressed in endothelial cells (EC) and is also involved in angiogenesis and is activated by hypoxic conditions. The mechanistic cross talk between the *EPAS1* and *Irx3* transcription factors remains to be defined.
CHAPTER II
LITERATURE REVIEW

Migration and Maladies

Peripheral Vascular Disease

Peripheral vascular disease (PVD) is a condition in which the blood vessels in the lower extremities are narrowed, restricting blood flow. Development of PVD is characterized by narrowing and occlusion of arterial vessels and eventual reduction in distal perfusion by buildup of plaque in blood vessels. This plaque is composed of fat, cholesterol, calcium, fibrous tissue, and other substances in the blood. When plaque builds up in the body's arteries, the condition is called atherosclerosis. Over time, atherosclerosis can cause hardening and narrowing of the arteries. Critical limb ischemia is the end stage of lower extremity PVD in which severe obstruction of blood flow results in ischemic pain at rest, ulcers, and a significant risk for limb loss causing this disease to be a significant contributor to morbidity and mortality. Individuals with diabetes, high blood pressure, high cholesterol, advanced age, those who smoke or are inactive, are at risk for developing PVD. A less common cause of PVD is Buerger disease seen in younger individuals. Approximately eight to twelve million people in the United States currently have peripheral vascular disease. The effects of this disease continue to prove detrimental to patient quality of life.
Current treatment goals for PVD should not only focus on disease progression and short-term relief but also on the improvement of patient long-term survival. The standard therapy for severe, limb-threatening ischemia is revascularization aiming to improve blood flow to the affected extremity.\textsuperscript{55} If revascularization has failed, or is not possible, amputation is often necessary.\textsuperscript{56} Migration, proliferation, and stabilization of progenitor endothelial cells that make up the arteries can lead to PVD treatment alternatives. The mechanism underlying arteriogenesis includes the addition and successful migration of bone marrow derived monocytes to the perivascular space.\textsuperscript{57} Proper cellular migration is necessary for this form of treatment to be successful. The rationale of hematopoietic stem cell/bone marrow cell therapy in PVD is to induce arteriogenesis by enhancing the biological restoration process.\textsuperscript{58} This requires large numbers of functionally active precursor cells transferred from the same individual's body, and subsequently, a large quantity of bone marrow cells.\textsuperscript{59} In order to suggest the use of hematopoietic stem cell/bone marrow cell therapy in PVD, the bone marrow-derived monocytes must adhere to and invade the collateral vessel wall to induce arteriogenesis by boosting the physiological repair processes.\textsuperscript{60} Cellular migration of bone marrow cells is currently being investigated as a viable treatment for PVD.\textsuperscript{61}

**Rheumatoid Arthritis**

Migration is a key contributor to the onset of many unfavorable conditions such as rheumatoid arthritis (RA). In patients with RA, the synovium is a point of interest. The synovium is a thin piece of tissue lining the diarthrodial joint. Under normal conditions, the synovium consists of a lining of cells and a sublining layer of loose connective tissue
with blood vessels sparsely distributed throughout. In RA patients, the structure of the synovium drastically changes. The synovium in RA becomes inflamed and increases in mass due to hyperplasia of the lining of cells. Synovial inflammation in RA is partially dependent on migration of inflammatory cells, their retention at the inflammation site, and insufficient apoptosis of chronic inflammatory and stromal cells.

Proinflammatory cytokines, such as interleukins (ILs) are also key contributors to the progression of RA. IL-17 has pleiotropic effects on many cell types, induces migration of innate immune cells, increases production of cytokines, chemokines, and matrix metalloproteases, all of which contribute to the initiation and inflammatory phases of RA. Knowledge of these role of migration in RA could lead to a better understanding of the mechanism by which the systemic immune response causes local joint disorders and will help to provide a molecular basis for therapeutic strategies.

**Endometriosis**

The migration of human endometrial stromal cells (ESCs) is increasingly recognized to contribute to the intense tissue remodeling associated with embryo implantation, trophoblast invasion, and endometrial regeneration. Coordinated migration of ESCs in response to embryonic and trophoblast signals are key to successful implantation. Migration of ectopic endometrial cells to the eutopic endometrium has been documented in an endometriosis mouse model. The migrated cells relocated primarily to the stroma in the basal layer of the endometrium and their expression profile revealed characteristics of cells having undergone epithelial-to-mesenchymal transition. Similarly, Interleukin 1β (IL-1β)-induced migration and invasiveness of mesenchymal
stem-like cells (MSCs) from ovarian endometriomas involves up-regulation of cyclo-oxygenase 2 (COX-2). Interestingly, enhanced Matrix metallo-proteinase (MMP) expression, migration and invasion of ectopic ESCs in response to IL-1β can be antagonized with an antioxidant and potent inhibitor of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Migration of ESCs also serves to support blastocyst implantation and embryo selection through discriminate motile responses directed by embryo quality. Harnessing the migratory capacity of progenitor mesenchymal stem cells in the endometrium may offer new therapeutic opportunities in regenerative medicine.

**Autism**

Migration is also vital for normal brain development. Dysregulation of migration is suggested to be a contributor of autism. Autism is characterized by delayed speech development, impaired socialization, and rigid behavior including stereotypic movements. In animal models, full-length Aβ precursor protein (APP) functions in normal migration of neuronal precursors into the cortical plate during brain development. Knockdown of APP inhibits neuronal migration from the cortical ventricular zone to the cortical plate in mice. Conversely, overexpression of APP accelerates migration of neuronal precursor cells into the cortex. Therefore, the location of APP at the synapse and its developmental function in migration and suppression of cell adhesion support the notion that dysregulated levels of APP contribute to brain growth without guidance as seen in autism. Proliferation, migration, differentiation, myelination, and synaptogenesis are all steps involved in the generation of a mature neuron. Notable, some
of the known functions of APP in these processes include promotion of proliferation, cell–cell adhesion and migration.\textsuperscript{77}

\textbf{Angiogenesis}

\textbf{Endothelial Cell Migration}

Angiogenesis is a process complementary to vasculogenesis during development.\textsuperscript{78} However unlike vasculogenesis, which is the \textit{de novo} formation of blood vessels, angiogenesis is the sprouting of new vessels from preexisting vessels.\textsuperscript{79} It prunes and refines the embryonic vascular network and prompts vessel extension and branching to form arterioles, venules, and the broad network of capillaries.\textsuperscript{80} Central to the process of angiogenesis is endothelial cell migration. This process is regulated by a stringent equilibrium between pro- and anti-angiogenic molecules.\textsuperscript{11} Before propagation of endothelial cells from preexisting vessels can occur the following must first take place: the mural cells must be removed;\textsuperscript{12c} the endothelial cell basement membrane must be degraded;\textsuperscript{12b} and remodeling of the perivascular stroma must take place.\textsuperscript{12a} Key players in these processes include Tie2-ligand,\textsuperscript{13a} serine protease,\textsuperscript{13b} and matrix metalloproteinases (MMPs).\textsuperscript{13c} Pro-angiogenic factors such as vascular endothelial growth factor (VEGF-A), and fibroblast growth factor (FGF) induce migration of endothelial cells.\textsuperscript{14,81} During this process, stimulation by a VEGF-A gradient, results in the specification of a leading endothelial cell (EC) called a ‘tip,’ followed by a ‘stalk’ cells that trails behind the tip cell; both of which maintain connectivity with the preexisting vessel.\textsuperscript{24} The constant remodeling of the actin cytoskeleton within endothelial cells into lamellipodia, stress fibers, and filopodia is critical to their ability to migrate.\textsuperscript{82} Lamellipodia are
cytoplasmic protrusions that form at the leading edge of migrating cells and are associated with the small GTPase Ras-related C3 botulinum toxin substrate 1 (Rac1) and Actin-related protein 2/3 (Arp2/3) complexes. Stress fibers are actin filaments of opposite polarity connected by α-actin. Filopodia are membrane projections made up of parallel actin filament organized into tight bundles that act as a sensor to motile stimuli. Actin-based endothelial movement thus involves protuberance of lamellipodia leading to forward extension; the sensing of signals by the filopodia; attachment of protrusions to the ECM; along with stress-fiber mediated contraction of the cell body. Once endothelial cells reach an area of decreased vessel density, they arrange in a monolayer forming tube-like structures. Lastly, mesenchymal cells in the surrounding tissue migrate and proliferate to the adluminal space of the nascent vessels. The mesenchymal cells then differentiate into pericytes that stabilize the newly developed vessel.

Migration in Tumor Progression

Migration in Lung Cancer

Lung cancer is the most common cause of cancer death, both in the United States and worldwide. According the National Institute of Health, Surveillance, Epidemiology, and End Results Program fact sheet on lung cancer, once lung cancer is diagnosed, outcomes are poor, with less than 20% of patients surviving 5 years after diagnosis. Pleiotrophin (PTN) is a heparin-binding growth factor that is highly expressed in certain solid cancers, such as in breast and lung cancers. PTN activates its cell surface receptors, regulating multiple functions including cell adhesion, cell migration, cell proliferation and cytoskeletal stability. Researchers suggest that focusing on reversing
migration of lung cancer cells may be an effective method of treating this disease. One method to reverse migration in lung cancers is the use of the pharmalogical therapeutic Tamoxifen® which is the most thoroughly studied example to date. Although an anti-estrogen medication used for breast cancers, Tamoxifen® has also been shown to lower lung cancer mortality. Bouchardy C., et al observed that compared with expected outcomes in the general population, lung cancer patients receiving the anti-estrogen treatment Tamoxifen® had lower lung cancer mortality. This study further supports the hypothesis that estrogen therapy modifies lung cancer prognosis by limiting migration of the lung cancers cells.  

Migration in Breast Cancer

According to the American Cancer Society, an estimated 231,840 new cases of female breast cancer were diagnosed in 2015. These diagnoses represent 29% of all new cancer cases among women. Regrettably, there will also be an estimated 40,290 deaths from breast cancer, which represent 15% of all cancer deaths among women. As in the case with most cancers, breast cancer becomes lethal once it has metastasized to other parts of the body. Therefore, reversing migration of breast cancer cells is also an effective therapeutic strategy for this disease. Researchers have discovered several molecules, proteins and environmental factors that contribute to migration of breast cancer cells. For example, stress fibers are associated with non-motile cells, while the absence of stress fibers is associated with cell migration and tumor invasion. Ras homolog gene family, member A (RhoA) and Ras homolog gene family, member C (RhoC) are equally capable of mediating stress fiber formation and generating contractile force needed for retraction
of the trailing edge during migration.\textsuperscript{89} In addition to the Ras homolog gene family, MicroRNAs (miR), such as miR-125, also been implicated in migration of breast cancer cells. Mir-125 has two known isoforms in humans: miR-125a and miR-125b. Altered expression of miR-125 has been observed in several malignancies, including breast cancer\textsuperscript{90}. The miR-125a and miR-125b were both found to be significantly downregulated in breast cancer patients.\textsuperscript{90b} Guo et al explored the role of miRNA-125 in breast cancer cells. Overexpressing miR-125a led to decreased in Human antigen R (HuR) protein levels, suppressed cell growth, and reduced cell migration and proliferation.\textsuperscript{91} These results suggest that miR-125a can potentially aid in tumor suppression in breast cancer by utilizing HuR as a direct and functional target to reduce cell migration of breast cancer cells.

**Migration in Prostate Cancer**

A number of miRNAs have been shown to influence key cellular processes involved in prostate tumorigenesis; including cell proliferation, apoptosis-avoidance, and migration via the androgen-signaling pathway. MiRNAs have shown differential expression in the serum of patients with prostate cancer of varying stages, compared with unaffected individuals. Downregulation of miR-145 for example, has been suggested to lead to enhanced cell proliferation, migration and invasion in prostate cancer. Fuse and colleagues demonstrated that the capacity of PC3 and DU145 prostate cancer cells to proliferate, migrate, and invade was impaired by transfection with miR-145.\textsuperscript{92} MiR-143 is also downregulated in prostate cancer, and has been shown in vitro to inhibit proliferation and migration of prostate cancer cells by suppressing Kirsten rat sarcoma viral oncogene
homolog (KRAS) expression, thereby inhibiting the EGFR/RAS/mitogen-activated protein kinase (MAPK) pathway.\textsuperscript{93} MiR-133\textsuperscript{94} and miR-146a\textsuperscript{95} have also been shown to produce similar anti-proliferative, anti-migratory effects in androgen-insensitive prostate cancer cell lines \textit{in vitro}.
CHAPTER III

METHODOLOGY

Human Microvascular Endothelial Cell Culture

Human microvascular endothelial cells (HMVECs) are an immortalized human microvascular endothelial cell line. These cells have been immortalized by transfecting them with a PBR-322-based plasmid containing the coding region for the large T antigen, the coding region for the simian 40 A gene product. These cells were cultured in MCDB-131 Medium (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 0.2% epidermal growth factor (EGF), and 0.1% hydrocortisone. Cultures were incubated in 5% humidified CO2 at 37°C in 100-mm tissue culture plates. To passage cells, media was aspirated and the monolayer was washed once with 1X phosphate buffered saline (PBS). Cells were trypsinized at 37°C until detachment was observed. Fresh medium was added to cells to inactivate the trypsin. Cells were resuspended and centrifuged for 5 minutes at 2,000 rpm. Cells were washed once in 1X PBS and centrifuged again for 5 minutes at 2,000 rpm. Cells were passaged every 5 days at 1:3.

Prostate Cancer Cell Culture

RWPE-1, LNCaP, DU-145, and PC-3 cells were obtained from the American Type Culture Collection. LNCaP, DU-145, and PC3 cells were maintained in complete media (RPMI 1640 containing 10% fetal bovine serum (FBS), 1% non-essential amino
acids, 1 % antibiotic-antimitotic and 1 % L-glutamine) at 37°C in 5 % CO2 (MediaTech) or starvation media (phenol-free RPMI containing 1 % L-glutamine) for 48-hours. RWPE-1 was maintained in keratinocyte serum-free medium with supplements (Life Technologies) All cells were maintained between 60 % to 80 % confluency. Cultures were incubated in 5% humidified CO2 at 37°C in 100-mm tissue culture plates. To passage cells, media was aspirated and the monolayer was washed once with 1X phosphate buffered saline (PBS). Cells were trypsinized at 37°C until detachment was observed. Fresh medium was added to cells to inactivate the trypsin. Cells were resuspended and centrifuged for 5 minutes at 2,000 rpm. Cells were washed once in 1X PBS and centrifuged again for 5 minutes at 2,000 rpm. Cells were passaged every 5 days at 1:3.

293A Cell Culture

A subclone of the 293 cell line (human embryonic kidney cells), 293A cells are used to facilitate the production, amplification and tittering of replication incompetent adenovirus. Minimal essential medium (MEM)-alpha medium (Life Technologies, Carlsbad, CA) supplemented with 10% FBS was used to culture the 293A cells. Cells were incubated and passaged as described for HMVECs, except cells were passaged every 3 days at 1:10.

Propagation of Cells from Frozen Stocks

To propagate HMVEC and 293A from frozen stocks, cryotubes were removed from liquid nitrogen storage and rapidly thawed in a 37°C water bath. Thawed cells were added to a 100-mm cell culture plate containing pre-warmed MCDB-131 medium or
MEM-alpha medium. Cells were incubated in 5% humidified CO2 at 37°C and passaged as previously described.

**Generation of Frozen Cells for Long Term Storage**

To prepare frozen HMVEC, RWPE-1, DU-145 or 293A cells, cells were grown to 80% confluency in 100-mm cell culture dishes. They were harvested by trypsinization and washed in 1X PBS. The cells were resuspended in Freezing Medium (Life Technologies, Carlsbad, CA) and aliquoted into cryotubes. Cells were frozen at -80°C for 24 hours then transferred to liquid nitrogen for long-term storage.

**Addgene Plasmids**

HA-HIF2alpha-pcDNA3 (EPAS1\(^{WT}\)) (Addgene plasmid # 18950)\(^97\) and HA-HIF2alpha-P405A/P531A-pcDNA3 (EPAS1\(^{P405A/P531A}\)) (Addgene plasmid # 18956)\(^98\) were gifts from William Kaelin. Plasmids were transfected into HMVECs using Xfect Transfection Reagent (Clontech Laboratories, Inc, Mountain View, CA). 24 hours prior to transfection, HMVECs were plated in 1 ml of complete growth medium and were 50–70% confluent at the time of transfection. Xfect Polymer was thawed at room temperature and thoroughly vortexed before use. In a microcentrifuge tube, 5 μg of plasmid DNA was diluted with Xfect Reaction Buffer to a final volume of 100 μl.

Contents were mixed well by vortexing for 5 seconds at high speed. Plate was incubated for 10 min at room temperature to allow nanoparticle complexes to form. Contents were spun down for 1 second to collect the contents at the bottom of the tube. The entire 100μl of nanoparticle complex solution was added dropwise to the cell culture medium. The 6-well plate was gently back and forth to mix. The plate was incubated at 37°C for 6 hours.
After 6 hours, the nanoparticle complexes were removed from cells by aspiration and replaced with 2 ml fresh complete growth medium, and returned to the 37°C incubator.

**Adenovirus Vectors**

Adenovirus vectors Ad.CMV.Irx3-V5, Ad.CMV.Xnull-V5, Ad.CMV.mirIrx3-eGFP, and Ad.CMV.mirNeg-eGFP were grown in 293A cells, and express high levels of the adenovirus type 5 E1A and EB proteins. All constructs were under the control of the human cytomegalovirus (CMV) major immediate-early promoter.

**Growth of Viral Stocks**

Adenoviral vectors were grown in 293A cells in Invitrogen’s MEM-Alpha medium supplemented with 10% FBS. 293A cells were infected at multiplicity of infection (MOI) of 20 and harvested by centrifugation at the time of maximal cytopathic effect. Virus was released from 293A cells by lysing with three cycles of freezing in dry ice and 90% ethanol and thawing in a 37°C water bath. Cell debris was pelleted by centrifugation and the supernatant was saved and frozen at -80°C.

**Transduction of Prostate Cancer Cells**

Prior to transduction with adenoviral vectors, DU-145 or RWPE-1 cells were grown to 80% confluency in RPMI complete medium or Keratinocyte complete medium, respectively, in 100-mm cell culture dishes. On the day of transduction medium was removed and cells were rinsed with 1X PBS. PBS was removed and 5-ml medium was added back to each 100-mm culture dish. Adenoviral lysate was added to each 100-mm dish at a concentration of 20 MOI. DU-145 or RWPE-1 cells were incubated with the virus for 3 hours at 37°C. After 3 hours, an additional 5-ml of medium was added to each
100-mm culture dish. DU-145 or RWPE-1 cells were incubated with viral lysate overnight at 37°C. Transduced DU-145 or RWPE-1 cells were used the next day for experiments.

**Transduction of Human Microvascular Endothelial Cells**

Prior to transduction with adenoviral vectors, HMVECs were grown to 80% confluency in MCDB-131 medium in 100-mm cell culture dishes. On the day of transduction medium was removed and cells were rinsed with 1X PBS. PBS was removed and 5-ml medium was added back to each 100-mm culture dish. Adenoviral lysate was added to each 100-mm dish at a concentration of 20 MOI. HMVECs were incubated with the virus for 3 hours at 37°C. After 3 hours, an additional 5-ml of medium was added to each 100-mm culture dish. HMVECs were incubated with viral lysate overnight at 37°C. Transduced HMVECs were used the next day for experiments.

**Transfection of Human Microvascular Endothelial Cells**

Prior to transfection with adenoviral vectors, HMVECs were grown to 80% confluency in MCDB-131 medium in 6-well cell culture dishes. On the day of transduction medium was removed and cells were rinsed with 1X PBS. PBS was removed and 3-ml medium was added back to each culture dish. HMVECs were transfected with *EPAS1* wild-type plasmid (Addgene plasmid #18950) or An *EPAS1* mutant plasmid, *EPAS1/P405A/P531A-pcDNA3* (Addgene plasmid #18956), using Clonetech® X-fect transfection reagent following manufacture’s protocol. After a six hour incubation experiments were used for experimentation.
Cell Counting

HMVECs and PCCs were counted prior to experiments using the Guava Viacount assay (Millipore; Billerica, MA).

Qiagen® Purification of Total RNA

Total RNA from HMVECs treated with vehicle or 20 ng/ml VEGF-A was isolated from HMVECs at various time points using the Qiagen RNeasy® Kit (Valencia, CA) following the manufacturer’s protocol. Cell culture medium was aspirated and cells were gently washed in 1X PBS. Lysis was performed directly in the 100-mm culture dishes using 500 ml of Cell Lysis Buffer. Cells were scraped and the lysate was transferred to a QIAshredder spin column and placed in a 2 ml collection tube. The tube was centrifuged for 2 minutes at full speed. One volume of 70% ethanol was added to the lysate and mixed by pipetting up and down, then 700 µl of the sample was transferred to an RNeasy spin column on a vacuum manifold. The vacuum was switched on allowing complete transfer of the lysate. This step was repeated until the remaining volume of each sample was added to the RNeasy spin column and then 700 µl of Buffer RW1 was added to each RNeasy spin column. The vacuum manifold was switched on allowing complete transfer of the buffer and 500 µl of Buffer RPE was added to each RNeasy spin column. The vacuum was applied allowing complete transfer of the solution. The RNeasy spin column were removed from the vacuum manifold and placed inside 2 ml collection tubes. The tubes were centrifuged at full speed for 1 minute. Each RNeasy spin column was then placed in a new 1.5 ml collection tube and 52 µl of RNase-free water was added to
the spin column membrane. The tubes were centrifuged for 1 minute at > 8,000 x g to elute the RNA. RNA was stored at -80°C.

**Nanodrop Quantification of Isolated RNA**

The concentration of total RNA was determined by measuring the optical absorbance at 260 nm using a Nanodrop apparatus. Sample RNA was used for quantification, and DNase/RNase free water was used as a reference.

**Reverse Transcription of Isolated RNA**

RNA was reverse transcribed using Clonetech’s (Mountain View, CA) cDNA EcoDry Premix. Briefly, 1 µg of total RNA from each sample was diluted to a final volume of 20 µl. Each sample of diluted RNA was added to a tube of EcoDry Premix. The samples were mixed by pipetting up and down several times to dissolve the pellet. Samples were incubated at 42°C for 60 minutes. The reaction was stopped by heating samples at 70°C for 10 minutes. cDNA was stored at -20°C.

**Primer and Oligonucleotide Design**

PCR primers were designed using the Real Time PCR Assay Design Center by Roche Applied Science. Primer and oligonucleotides were manufactured by Eurofins EWG Operon (Huntsville, AL).

Real-time PCR sequences used (showed 5’ to 3’):

1. *Irx3* forward primer: CTCTCCCTGCTGGGCTCTCT
2. *Irx3* reverse primer: CAAGGCACTACAGCGATCTG
3. *EPAS1* forward primer: GAACATTTGGGAAATCTCTTGC
4. *EPAS1* reverse primer: CGGAAGAACAATGTAGTCTTTGC
5. *Tie2* forward primer: GAACATTTGGGAAATCTCTTGC
6. *Tie2* reverse primer: CGGAAGAACAATGTAGTCTTTTC
7. 18s forward primer: GGAAGGGCACCACCAGGAGT
8. 18s reverse primer: TGCAGCCCCGGACATTCTAAG

**Quantitative Real Time Polymerase Chain Reaction**

qRT-PCR was performed using the SYBR Green I Master Kit (Roche Applied Science) with 2.5 µl of cDNA added to 7.5 µl of reaction volume containing 1X master mix, 10 µM of forward and reverse primers, and water. Quantitative PCR results from each primer pair was normalized to results obtained from the 18s cDNA internal control. A linear standard curve was constructed and each sample was run in triplicate. Quantification was performed at the log-linear phase, plotted against a serial dilution of human reference cDNA with an internal control transcript of 18s cDNA. To determine statistical significance a one-way ANOVA and Newman-Keuls test was performed comparing treatment groups at each time point to the corresponding treatment at 0 hours post-VEGF-A treatment.

**Protein Extraction**

Cells were cultured to 70% confluency in 100-mm cell culture dishes before treatment with 20 ng/ml VEGF-A over a 48-hour time course. At each time point media was removed from cells and cells were rinsed with 1X PBS and then 150 µl of Mammalian Protein Extraction Reagent (Thermo Scientific, Waltham, MA) was added to each culture dish which were then placed on a shaker for 5 minutes at room temperature. Cells were scraped and collected in a 1.5 ml Eppendorf tube and placed in a mixture of
70% ethanol and dry ice for freezing. Once frozen, cells were placed in a 37°C water bath to thaw. A total of three freeze-thaw cycles were completed before cells were centrifuged at 14,000 x g to pellet debris. The supernatant containing protein was then transferred to a new 1.5 ml Eppendorf tube and stored at -80°C.

**Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis**

Protein (13-25 µg) samples were combined with 1 µl of NuPAGE (Life Technologies, Carlsbad, CA), Reducing Agent and 2.5 µl of NuPAGE LDS Sample Buffer. The volume of each sample preparation was brought to a total volume of 25 µl with RNase-free water. Samples were heated at 70°C for 10 minutes in a heat block before loading into a pre-cast NuPage 4-12% Bis-Tris gradient gel with SeeBlue Pre-stained Protein Standard (Life Technologies, Carlsbad, CA). The running buffer used was 1X 3-(N-morpholino)propanesulfonic-acid (MOPS) buffer at 200 V for 45 minutes.

**Western Immunoblotting**

Protein transfer from precast 4-12% Bis-Tris gels to PVDF membranes was performed using the iBlot (Life Technologies, Carlsbad, CA) apparatus according to the manufacturer’s protocol. Membranes were blocked for 1 hour at room temperature on a rocker with Invitrogen’s Western Breeze Blocking Reagent (Life Technologies, Carlsbad, CA) and incubated with a primary antibody solution overnight at 4°C on a rocker. The next day, membranes were washed four times with Invitrogen’s Antibody Wash Solution and incubated with the Western Breeze anti-species Secondary Antibody Solution for 1 hour at room temperature on a rocker. Protein signals were developed by
chemiluminescence and exposed to X-ray film for various lengths of time dependent on
the protein of interest.

Antibodies used for Western immunoblot:

1. Iroquois homeobox protein 3 rabbit polyclonal antibody (Santa Crus, Santa
   Cruz, MA), 1:200

2. Endothelial PAS Domain 1 mouse monoclonal antibody (Novus Biologicals;
   San Diego, CA), 1:1000

3. Phosphor-vascular endothelial growth factor receptor 2 (Tyr1175) rabbit
   monoclonal antibody (Cell Signaling Technology, Danvers, MA), 1:1000

4. Phospho-Phosphoinositide-specific phospholipase C gamma 1 (Ser1248)
   rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA),
   1:1000

5. Phospho-Src protein tyrosine kinase (Tyr416) rabbit monoclonal antibody
   (Cell Signaling Technology, Danvers, MA), 1:1000

6. GAPDH antibody (Santa Cruz; Dallas, TX), 1:5000

The membranes were scanned and the photos were scanned and analyzed with National
Institute of Health Image J® software in order to obtain densitometry for quantitative

**Prostate Cancer Wound Healing Assay**

RWPE-1 and DU-145 cells were seeded at a high density (1.0X10^6 cells/ml) in 6
wells of a 6-well cell culture plate and allowed to adhere overnight in complete medium.
Cells were then transduced as previously described at a concentration of 20 MOI with the
following adenoviral vectors: Adenovirus vectors Ad.CMV.Irx3-V5, Ad.CMV.Xnull-
V5, Ad.CMV.mirIrx3-eGFP, and Ad.CMV.mirNeg-eGFP. PCAs were incubated with virus overnight at 37°C. The following day, a p-10 pipet tip was used to create a wound across each well by scraping the tip from end-to-end. Floating cells were removed by washing each well with 1X PBS after scraping. Fresh complete medium was added to each well. The wound was monitored over a 48-hour period, with phase contrast and fluorescent images taken every 24 hours and rate of closure was quantified by measuring the wound area using ImagePro Plus software.

Human Microvascular Endothelial Cells Wound Healing Assay

HMVEC cells were seeded at a high density (1.0X106 cells/ml) in 6 wells of a 6-well cell culture plate and allowed to adhere overnight in complete medium. Cells were then transfected and transduced as previously described at a concentration of 20 MOI. HMVECs were incubated with virus overnight at 37°C. The following day, a p-10 pipet tip was used to create a wound across each well by scraping the tip from end-to-end. Floating cells were removed by washing each well with 1X PBS after scraping. Fresh complete medium was added to each well. The wound was monitored over a 48-hour period, with phase contrast and fluorescent images taken every 24 hours and rate of closure was quantified by measuring the wound area using ImagePro Plus software.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using the Active Motif (Carlsbad, CA) ChIP-It Express Kit and the Covaris Sonicator (Woburn, MA). To prepare chromatin, cells were grown to 70-80% confluency and treated with VEGF-A or vehicle. Cells were harvested at 0 hours and 12 hours post treatment. At each time point,
medium was aspirated off the cells and cells were rinsed with 1X PBS. To fix cells 20 ml of Fixation Solution containing 37% formaldehyde was added to each plate for 10 minutes. The fixation reaction was stopped using 10 ml of Glycine Stop-Fix solution. Cell Scraping Solution containing 100mM PMSF was added and cells were scraped and transferred to 1.5ml Eppendorf tubes. Cells were pelleted by centrifuging for 10 minutes at 2,500 rpm then resuspended in 1 ml of ice cold Lysis Buffer and incubated on ice for 30 minutes. Cells were transferred to a dounce homogenizer and dounced on ice with 10 strokes to release nuclei. Cells were transferred to a 1.7 ml siliconized microcentrifuge tube and centrifuged at 2,400 rpm for 10 minutes at 4°C to pellet the nuclei. The supernatant was carefully removed and discarded. The pellets were then resuspended in 1.0 ml of Covaris Wash Buffer and incubated for 10 minutes at 4°C. The microcentrifuge tubes were then centrifuged at 1,700 x g for 5 minutes at 4°C. The supernatants were removed and the sides of the microcentrifuge tube gently rinsed with 1 ml of Covaris Shearing Buffer. The microcentrifuge tubes were again centrifuged at 1,700 x g for 5 minutes at 4°C. The supernatant was carefully removed by pipetting and the pellet was resuspended in 520 µl of Covaris Shearing Buffer. Each sample was then separated into four 130 µl aliquots containing approximately 3 million cells each.

Chromatin was sheared using the Covaris Sonicator for 8 minutes per sample according to the manufacturer’s operating protocol. After shearing, 10 µl of chromatin was removed from each sample (total input) and stored at -20°C.

The ChIP reaction was set up by adding protein G beads, sheared chromatin, phosphatase inhibitor compound (PIC), primary antibody, and dH2O to a siliconized 1.7
ml microcentrifuge tube. For this experiment the primary antibodies used were a *EPAS1* mouse monoclonal antibody (Novus Biologicals; San Diego, CA), an RNA Polymerase II control antibody included in the Active Motif ChIP It Express Kit, and IgG2A negative control antibody. Tubes were capped, mixed thoroughly and incubated on a rolling shaker overnight at 4°C. Tubes were then placed on a magnetic stand to pellet the beads and the supernatant was carefully removed and discarded. Magnetic beads were washed four times with ChIP Buffer 1, and two times with ChIP Buffer 2. The magnetic beads were resuspended in Elution Buffer AM2 and incubated for 15 minutes at room temperature on a rotator, and then 50 µl of the Reverse Cross Link buffer was added to elute chromatin from the magnetic beads. Chromatin containing supernatant was transferred to a fresh tube. The isolated chromatin, as well as the input DNA was incubated at 94°C for 15 minutes. Tubes were returned to room temperature and 2 µl of Proteinase K were added to the tubes. Tubes were incubated at 37°C for 1 hour. Tubes were then returned to room temperature and 2 µl of Proteinase K Stop Solution was added to each tube. Precipitated DNA was then amplified by quantitative PCR using the SYBR Green I Master Kit as previously described. The *Irx3* primer sequences used were as follows (5’-3’):

*EPAS1* binding sites on *Irx3* Promotor (see Table 1).
Table 1: EPAS1 Binding Sites on Irx3 Promotor

<table>
<thead>
<tr>
<th>DISTANCE FROM TRANSCRIPTIONAL START SITE</th>
<th>5’ FLANK</th>
<th>CONSENSUS SEQUENCE</th>
<th>3’ FLANK</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,653 NT</td>
<td>5’-</td>
<td>GCGTG</td>
<td>TGCGA</td>
</tr>
<tr>
<td></td>
<td>GCACACGTGGGCA</td>
<td></td>
<td>TGGACAGACA</td>
</tr>
<tr>
<td></td>
<td>GCCGTTCCCCTGTGC</td>
<td></td>
<td>CACTCTCCCA</td>
</tr>
<tr>
<td></td>
<td>GCATCCGGCGCACG</td>
<td></td>
<td>AACTTCCCACG</td>
</tr>
<tr>
<td></td>
<td>CGCGCCCAA</td>
<td></td>
<td>TGCAT-3’</td>
</tr>
<tr>
<td>2,518 NT</td>
<td>5’-</td>
<td>GCGTG</td>
<td>AAGCG</td>
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<td></td>
<td>CTTGTGGGTGGG</td>
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<td>CGTTCGTGCG</td>
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<td></td>
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<td></td>
<td>GCAAGATGATGGAG</td>
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<td>AAGCTTCTCT</td>
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<td>TTCTTGTCGAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CTGCT-3’</td>
</tr>
</tbody>
</table>

**Statistical Analysis**

Data expressed is the average of three technical and/or independent experiments. Statistical significance between two groups was calculated using the Student’s T-Test. Analyses of more than two groups was calculated using a one-way analysis of variance (ANOVA) and a Newman-Keuls post hoc test. A confidence level of <0.05 or <0.001 was used for statistical significance.
CHAPTER IV

EXPERIMENTAL DESIGN

Hypothesis

We hypothesize that $EPAS1$ via $Ir\times 3$ will act as a pro-migratory marker in human microvascular endothelial cells (HMVECs).

Specific Aim 1

To identify and validate transcriptional regulators of $Ir\times 3$ under pro-angiogenic conditions.

Working Hypothesis for Specific Aim 1

$Ir\times 3$ is regulated by transcription factors in response to $EPAS1$ that alters the signaling pathways involved in migration. Furthermore, once activated in response to VEGF-A, $Ir\times 3$ responds to upstream target genes involved in migration such as $EPAS1$.

Rationale

Data from our lab have demonstrated that $Ir\times 3$ is responsive to VEGF-A; therefore, in this aim we will show that $Ir\times 3$ is expressed in response to other pro-angiogenic conditions such as hypoxia and this response is essential to EC migration. Additionally, in Aim 1 we proposed that in response to VEGF-A, there is increased transcriptional $EPAS1$ activity on the $Ir\times 3$ promoter.
Specific Aim 2

To define the temporal expression pattern of Irx3 in human microvascular endothelial cells in response to pro-angiogenic conditions.

Working Hypothesis for Specific Aim 2

Irx3 is expressed in endothelial cells in vitro. The expression of Irx3 is modulated in a temporal manner in response to pro-angiogenic factors, such as VEGF-A and hypoxia.

Rationale

Genetic mapping studies in Drosophila indicate that disruption of caupolican, an orthologue of Irx3, results in disorganized wing vein development. Furthermore, our preliminary data demonstrates: (1) Irx3 expression in human ECs and in mouse ECs in the cerebral microvasculature and (2) elevated Irx3 expression in response to the pro-angiogenic factor VEGF-A. We therefore rationalize that Irx3 is expressed in ECs and the expression of Irx3 is regulated by EPAS1 and other factors that govern migration.

Specific Aim 3

Use genetic gain- or loss- of EPAS1 function to test the role of Irx3 on the migratory potential of endothelial cells.

Working Hypothesis for Specific Aim 3

We hypothesize that Irx3 expression is upregulated in the presence of EPAS1 modulators and is required for endothelial cell migration, a key step in the angiogenic cascade. Transient knockdown of EPAS1 will limit Irx3 EC migration, while forced expression of EPAS1 on Irx3 will confer upon ECs a migratory advantage.
Rationale

It is anticipated that Aim 1 will establish that *Irx3* is expressed in human ECs, therefore in Aim 3 we will perform functional assays to demonstrate that *Irx3* expression is not only upregulated in response to VEGF-A and hypoxia, but that *Irx3* upregulation results in increased EC migration by modulation of *EPAS1* expression.

Specific Aim 4

Investigate the role of *Irx3* in DU-145 prostate cancer cells.

Working Hypothesis for Specific Aim 4

We hypothesize that *Irx3* expression is required for prostate cell migration, a key step in the angiogenic cascade. Transient knockdown of *Irx3* will promote cell migration, while forced expression of *Irx3* will inhibit prostate cancer migratory advantage.

Rationale

It is anticipated that Aim 1 will establish that *Irx3* is also expressed in prostate cancer cells, therefore in Aim 4 we will utilize genetic *Irx3* modifications to perform functional assays to demonstrate that *Irx3* modulates cell migration.

Specific Aim 1: Identify and Validate Transcriptional Regulators of *Irx3* in Human Microvascular Endothelial Cells

We utilized a siRNA screening approach to identify potential upstream transcription regulators of *Irx3* expression in HMVECs in response to 12 hours of VEGF-A stimulation. HMVECs were reverse-transfected in multiwell plates containing two validated siRNAs for a single transcription factor. Next, cells were treated with VEGF for a period of twelve hours. Total RNA was harvested, and quantitative RT-PCR was
performed. Analysis of the high-throughput siRNA screen revealed a -30.99 fold decrease in \(Ir\overline{x}3\) expression upon siRNA silencing of \(EPAS1\) compared with vehicle-treated negative siRNA control wells (see Table 2). These results suggest that \(EPAS1\) functions as an upstream positive regulator of \(Ir\overline{x}3\) expression in response to VEGF treatment.

Table 2: \(EPAS1\) Identified as an Upstream Regulator of \(Ir\overline{x}3\)

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>HMEC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Number/well</td>
<td>20000</td>
</tr>
<tr>
<td>Treatment</td>
<td>VEGF</td>
</tr>
<tr>
<td>Concentration</td>
<td>20ng/ml</td>
</tr>
<tr>
<td>Duration</td>
<td>12hr</td>
</tr>
<tr>
<td>Gene Target</td>
<td>(Ir\overline{x}3)</td>
</tr>
<tr>
<td>Treatment Induction</td>
<td>2.16</td>
</tr>
</tbody>
</table>

To validate the siRNA screening results, we investigated the effect of gain- or loss of function of \(EPAS1\) on endogenous \(EPAS1\) and \(Ir\overline{x}3\) mRNA expression. HMVECs were transfected with \(EPAS1\) wild-type plasmid (\(EPAS1^{WT}\)), or an \(EPAS1\) transactivation mutant plasmid, \(EPAS1^{P405A/P531A}\)-pcDNA3 (\(EPAS1^{P405A/P531A}\)) for twenty-four hours under...
normoxic conditions. Total RNA was harvested, and quantitative RT-PCR was performed to determine relative EPAS1 or Irx3 mRNA abundance. Our results (see Figure 1, #A) indicate that compared to the control, the wild type plasmid, EPASI^WT^ demonstrate a significant fold increase for HMVECs mRNA of EPASI (20.93-fold ± 0.408, p=0.001). In contrast, HMVECs transfected with EPASI^[P405A/P531A] resulted in less EPASI mRNA expression than the EPASI^[WT] of mRNA of EPASI (13.44-fold ± 0.594, p=0.001) compared to the control group. Our results also indicate EPASI^[WT] transfected HMVECs resulted a significant increase of Irx3 mRNA (16.44-fold ± 0.108, p=0.002) compared to control conditions. In contrast, HMVECs treated with EPASI^[P405A/P53A] resulted in a significant decrease of Irx3 mRNA (1.6-fold ± 0.019, p=0.001) compared to the control. These results demonstrate that our transient transfections of EPASI successfully modulated mRNA of EPASI and Irx3 expression in HMVECs.

EPASI bHLH proteins are well known to function as transcription factors involved in the induction of genes regulated by oxygen that bind to core DNA sequence 5’-A/GCGTG-3’ within the hypoxia response element (HRE) of target gene promoters. We identified two putative EPASI consensus binding sites; 5’-

AAGCTTCTGGCCTGAAAGCGCGT-3’ located at -2518 nt (proximal); and 5’-

CGCGCCAAGGCGTTGGCGATGG-3’ at -2,653nt (distal) relative to the transcriptional start site (see Figure 1, #B). To confirm whether EPASI binds the Irx3 promoter directly or through an intermediate cofactor, we performed chromatin immunoprecipitation (ChIP) experiments to determine EPASI binding dynamics in response to VEGF-A (see Figure 1, #C).
Figure 1: EPAS1 is a Positive Regulator of Irx3.
(A) EPAS1 and Irx3 were successfully knocked down in HMVECs using EPAS1 WT plasmid or EPAS1P405A/P531A plasmid and confirmed by QRT-PCR analysis of EPAS1 or Irx3. (B) Schematic of the predicted Irx3 binding sites on EPAS1 regulatory regions. (C) Quantitative analysis of EPAS1 binding on Irx3 promoter region.
MVECs were harvested at 12 hours after VEGF treatment and subjected to protein-DNA cross-linking. After immuno-precipitation with anti-EPAS1 or an IgG isotype control, DNA fragments were reverse-cross-linked and PCR-amplified with primers sets flanking the predicted EPAS1 binding sites in the upstream regulatory region of the Irx3 promoter. Primers designed to amplify the sequences for the proximal and distal binding sites were used in the quantitative RT-PCR confirmation analysis. Additionally, primers designed in an intronic region between exons 3 and 4 of EPAS1 was used as a negative control. The results indicate that at 12 hours under vehicle conditions, chromatin-fold enrichment at the proximal site was 14.16 ± 0.368-fold and chromatin-fold enrichment at the distal site was 13.65 ± 0.241-fold, respectively. Cells treated for 12 hours with VEGF resulted in alleviated EPAS1 binding of the Irx3 promoter with significantly more enrichment (31.79- ± 0.640-fold) at the proximal Irx3 binding site (p = 0.0028) and 31.08- ± 0.325-fold enrichment at the distal site (p = 0.001) compared with vehicle controls. The intronic region of Irx3 showed no significant change in fold enrichment under either condition. Taken together, these results indicate that EPAS1 is a positive regulator of Irx3 in the presence of VEGF and binds directly to the Irx3 promoter. Furthermore, absence of the VEGF ligand partially alleviates EPAS1-mediated repression of Irx3 in HMVECs.

Specific Aim 2: To Define the Temporal Expression Pattern of Irx3 in Human Microvascular Endothelial Cells in Response to Pro-Angiogenic Conditions

To determine the effect that pro-angiogenic conditions such as VEGF-A and hypoxia, had on Irx3 and EPAS1 gene expression, we treated HMVECs with VEGF-A or hypoxia over a period of 48 hours and then isolated total RNA at various time points
for quantitative RT-PCR. The results indicate that at 12 hours, *Ir3* (see Figure 2, #A) and *EPAS1* (see Figure 2, #C) expression increased significantly 13.04 fold (*p* = 0.0036) and 22.10 fold (*p* < 0.0001), respectively, post-VEGF-A treatment. *Ir3* and *EPAS1* expression increased in a temporal manner from 8–48 h post-VEGF-A treatment compared with time-matched vehicle controls. These results also show a reduction in *Ir3* and *EPAS1* expression after 48 hours, which is likely due to cells reaching confluency. Additionally, endogenous *Ir3* expression is elevated in HMVECs in response to VEGF-A treatment in a temporal manner and peaks in expression at 12 h. Furthermore, *Ir3* and *EPAS1* were decreased as HMVECs reached confluency 48 hours post-VEGF-A treatment. Our results also indicate by 48 hours, *Ir3* and *EPAS1* expression increased significantly, 13.83-fold (*p* = 0.064) and 32.69-fold (*p* < 0.0001), respectively, post-hypoxia treatment with expression as early as 8 hours. *Ir3* (see Figure 2, #B) and *EPAS1* (see Figure 2, #D) expression increased in a progressive manner from 8–48 h post-hypoxia treatment compared with time-matched vehicle controls. These results also show a positive correlation between time and hypoxia. Endogenous *Ir3* and *EPAS1* expression are elevated in HMVECs in response to hypoxia treatment in a progressive manner with dramatic expression at 48 h. Herein, we are also the first to demonstrate that *Ir3* is stimulated under hypoxic conditions *in vitro* and mimics the expression pattern of *EPAS1* under pro-angiogenic conditions.
Figure 2. *Irx3* and *EPAS1* mRNA Expression in Response to VEGF-A and Hypoxia.

Specific Aim 3: Test the Role of *Irx3* on the Migration of Human Microvascular Endothelial Cells

To demonstrate effective gain of function of *EPAS1* expression, HMVECs were transfected with *EPAS1* plasmids as previously described. To study the effect of *EPAS1* gain of function on *Irx3*-induced cell migration, HMVECs were transfected with *EPAS1* wild-type plasmid once they were~80% confluent in a six well plate. After a six hour incubation, ECs were placed in complete media overnight. Cells were then transduced
with recombinant adenoviral vectors containing a CMV immediate-early promoter (CMV-IE), human Irx3 cDNA (BC023667.2)/tag on demand V5 epitope (Ad.CMV.Irx3-v5 and Ad.CMV.Xnull-v5), CMV-IE/mirIrx3/eGFP, or synthetic scrambled control miRNA (Ad.CMV.miIrx3-eGFP and Ad.CMV.miNeg-eGFP) (m.o.i. = 20) for 12 hours in complete media. A wound was made in each well and cells were allowed to migrate for 12 hours. Results indicate that cells transfected with EPAS1 WT and transduced with Ad.CMV.Irx3-v5 resulted in increased wound healing as early as 12 hours compared with the Ad.CMV.Xnull-v5 control vector. In contrast, HMVECs transfected with EPAS1 WT and transduced with Ad.mirIrx3-eGFP exhibited markedly reduced migration at 12 hours compared with the negative control vector Ad.mirNeg-eGFP (see Figure 3, #A). Temporal assessment of the wound area in HMVECs, transfected with EPAS1 WT and transduced with the Ad.mirIrx3-eGFP, indicate an area of 32.50 ± 0.707% at 12 hour versus control vector Ad.mirNeg-eGFP-transduced cells (53.5 ± 2.121%). Additionally, cells transfected with EPAS1 WT and transduced with Ad.CMV.Irx3-v5 resulted in increased wound healing as early as 6 hours compared with the Ad.CMV.Xnull-v5 control vector (see Figure 3, #B).
Figure 3: Gain of Function of *EPAS1* Modulates the *Irx3* Endothelial Cell Migratory Potential.

(A) Closure of wound area of HMVEC using HMVECs transfected with *EPAS1*<sup>WT</sup> Plasmid and *Irx3* viral vectors. (B) Quantitative analysis of HMVEC migration obtained from Figure 3A.
To demonstrate effective loss of function of \textit{EPAS1} expression, HMVECs were transfected with \textit{EPAS1} plasmids as previously described. To study the effect of \textit{EPAS1} loss of function on \textit{Irx3}-induced cell migration, HMVECs were transfected with \textit{EPAS1}^{P405A/P531A} and transduced with \textit{Irx3} adenoviral vectors as previously described. A wound was made in each well and cells were allowed to migrate for 12 hours. Results indicate that cells transfected \textit{EPAS1}^{P405A/P531A} and transduced with Ad.CMV.\textit{Irx3-v5} resulted in decreased wound healing as early as 12 hours compared with the Ad.CMV.Xnull-v5 control vector. Additionally, cells transfected with \textit{EPAS1}^{P405A/P531A} and transduced with Ad.mir\textit{Irx3-eGFP} exhibited markedly reduced migration at 12 hours compared with the negative control vector Ad.mirNeg-eGFP (see Figure 4, #A).

Temporal assessment of the wound area in HMVECs, transfected with \textit{EPAS1}^{P405A/P531A} and transduced with all of the \textit{Irx3} recombinant viral vectors Ad.mir\textit{Irx3-eGFP} did not result in closure of the wound even after 12 hours versus control vector. This data indicate that \textit{EPAS1} gain of function or loss of function can regulate \textit{Irx3} modulation of wound closure rates in HMVECs and suggest a functional role of \textit{EPAS1} in mediating \textit{Irx3}-induced EC migration (see Figure 4, #B).
Figure 4. Loss of Function of EPAS1 Modulates the Irx3 Endothelial Cell Migratory Potential.

(A) Closure of wound area of HMVEC using HMVECs transfected with EPAS1P405A/p531A plasmid and Irx3 viral vectors

(B) Quantitative analysis of HMVEC migration obtained from Figure 4, #A.
Specific Aim 4: Investigate the Role of Irx3 in Du-145 Prostate Cancer Cells

Cistrome studies from Specific Aim 1 identified transcription factors involved in cellular death, survival, and movement in DU-145 cells. CITED2 is identified as a downstream target of Irx3 in DU-145 cells (see Table 3).

Table 3. CITED2 Transcriptional Regulator Identified to Interact with Irx3 in DU-145 Cells

A previous study identified hypermethylation of the 5' CpG island region of the homebox gene Irx3 in TRAMP. The aforementioned study utilized LNCaP cells. Table 4 compares several prostate cancer cell lines which includes their metastatic behavior. Herein, we observe that LNCaP does not metastasize to the bone whereas other cell lines, such as DU-145, do have metastatic potential. We therefore rationalize that Irx3 is differentially expressed in prostate cancer cell lines and the expression of Irx3 may regulate migration.
To investigate the expression patterns of \textit{Irx3} in prostate cancer cell lines, western blotting was performed using protein obtained from primary cultures of human prostate epithelial cells, RWPE-1, and human prostate cancer cells LNCaP, DU-145, and PC-3 (see Figure 5). Previous data from our lab identified that human microvascular endothelial cells (HMVECs) expressed high levels of \textit{Irx3} when exposed to 12 hours of VEGF-A\textsuperscript{41} therefore we used HMVECs as a positive control for \textit{Irx3} expression in this panel. Elevated protein expression of \textit{Irx3} can be seen in HMEC-1, RWPE-1 and DU-145 cell lines as compared to LNCaP and PC-3 (see Figure 5).
Fold Change of Relative Density of Irx3/GAPDH

<table>
<thead>
<tr>
<th></th>
<th>HMVEC 12H</th>
<th>RWPE-1</th>
<th>LNCaP</th>
<th>DU-145</th>
<th>PC-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-A</td>
<td>1</td>
<td>**</td>
<td>0.5</td>
<td>1</td>
<td>0.8</td>
</tr>
</tbody>
</table>

**Figure 5.** Irx3 Protein is differentially expressed in panel of prostate cancer cell lines.

Densitometry data from the western blot reveals that Irx3 protein levels are significantly decreased in LNCaP (0.2-fold ±0.087, p=0.002) and PC3 (0.3-fold ±0.093, p=0.005) cells compared to our control group HMVECs (see Figure 6).

**Figure 6.** Densitometry of Figure 5 Irx3 protein is differentially expressed in panel of prostate cancer cell lines.
Complimentary DNA obtained was analyzed using Quantitative Polymerase Chain Reaction. Analysis of mRNA from HMEC-1, RWPE-1, LNCaP, DU-145, and PC-3 reveal that expression of *Irx3* is significantly decreased in LNCaP and PC-3 cells compared to RWPE-1 (see Figure 7).

**Figure 7.** *Irx3* mRNA is differentially expressed in a panel of prostate cancer cell lines.

RT-PCR was performed with the cDNA obtained from Figure 3, which reveals that genomic DNA levels are elevated in HMVEC, RWPE-1 and DU-145 cell lines (see Figure 8).

**Figure 8.** *Irx3* Genomic DNA is differentially expressed in a panel of prostate cancer cell lines.
Recombinant adenoviral vectors were designed as previously described\textsuperscript{41}. RWPE-1 cells were transduced in normal growth medium with the indicated adenovirus for 12 hours. Whole cell lysate were collected and ran on a western blot which reveals that there is higher expression of \textit{Irx3} transduced with Ad.CMV.\textit{Irx3-v5} and there is less expression of \textit{Irx3} in both the Ad.mir\textit{Irx3}-eGFP and its control Ad.mirNeg-eGFP in RWPE1 (see Figure 9). Densitometry data from the western blot reveals that \textit{Irx3} levels significantly increase in cells treated with Ad.CMV.\textit{Irx3-v5} and significantly decrease upon treatment with Ad.mir\textit{Irx3}-eGFP and its control Ad.mirNeg-eGFP in RWPE-1 (see Figure 10).

\begin{center}
\begin{tabular}{lcccc}
\hline
\textit{Irx3} & 61 kDa & & & \\
\hline
GAPDH & 37 kDa & & & \\
\hline
\end{tabular}
\end{center}

\begin{center}
\begin{tabular}{lcccc}
\hline
Ad.CMV.Xnull-v5 & - & + & - & - \\
Ad.CMV.Irx3-v5 & - & - & + & - \\
Ad.CMV.miNeg-eGFP & - & - & - & + \\
Ad.CMV.mir\textit{Irx3}-eGFP & - & - & - & + \\
\hline
\end{tabular}
\end{center}

\textbf{Figure 9.} \textit{Irx3} successfully transduced in RWPE-1 cell lysates.
Figure 10. Densitometry of Figure 9 *Irx3* successfully transduced in RWPE-1 cell lysates.

Similarly, DU-145 cells were transduced in normal growth medium with the indicated adenovirus for 12 hours. Whole cell lysates were collected and ran on a western blot which reveals that there is higher expression of *Irx3* transduced with Ad.CMV.*Irx3*-v5 and there is less expression of *Irx3* in both the Ad.mir*Irx3*-eGFP and its control Ad.mirNeg-eGFP in DU-145 (see Figure 11). Densitometry data from the western blot reveals that *Irx3* levels significantly increase in cells treated with Ad.CMV.*Irx3*-v5 and significantly decrease upon treatment with Ad.mir*Irx3*-Egfp and its control Ad.mirNeg-eGFP in DU-145 (see Figure 12).
**Table**

<table>
<thead>
<tr>
<th></th>
<th>Ad.CMV.Xnull-v5</th>
<th>Ad.CMV.Irx3-v5</th>
<th>Ad.CMV.miNeg-eGFP</th>
<th>Ad.CMV.miIrx3-eGFP</th>
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<tbody>
<tr>
<td><strong>Fold Change of Relative Density of Irx3/GAPDH</strong></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Figure 11.** *Irx3* successfully transduced in DU-145 cell lysates.

**Figure 12.** Densitometry of Figure 11 *Irx3* successfully transduced in DU-145 cell lysates.
Complimentary DNA obtained from RWPE-1 and DU-145 treated with the gain and loss-of-function \textit{Irx3} and their controls reveals that there is a ~20 fold increase of \textit{Irx3} in cells treated with Ad.CMV.\textit{Irx3-v5} compared to its control Ad.CMV.xnull-v5 (see Figure 13, #A). There is a ~0.5 fold decrease of \textit{Irx3} expression in Ad.mir\textit{Irx3-eGFP} compared to its control Ad.mirNeg-eGFP (see Figure 13, #B).

\textbf{Figure 13:} Quantitative RT-PCR analysis of \textit{Irx3} expression after transduction with the indicated (A) gain of function or (B) loss of function Adenoviral Vector at 12 hours.
The effect of *Irx3* gain- and loss-of function on cell motility was assessed by wound healing assays RWPE-1 (see Figure 14) and DU-145 (see Figure 15) were transduced with recombinant adenoviral vectors. Twenty-four hours after the wound, the non-transduced RWPE-1 cells had an open wound area of 57%, Ad.CMV.*Irx3*-v5 had an open wound of 76% and Ad.*mirIrx3*-eGFP 61%. At 72 hours non transduced RWPE-1 cells had an open wound area of 2%, Ad.CMV.*Irx3*-v5 had an open wound of 11% and Ad.*mirIrx3*-eGFP 1%.

<table>
<thead>
<tr>
<th>Vector</th>
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<th>72 hours</th>
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<td>Ad.CMV.Xnull-v5</td>
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<td>-</td>
</tr>
<tr>
<td>Ad.CMV.Irx3-v5</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ad.CMV.miNeg-eGFP</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ad.CMV.miIrx3-eGFP</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**Figure 14:** Wound Healing Assay of RWPE-1 cells transduced with *Irx3* Adenoviral Vectors.

**Figure 15:** Quantitative data of Wound Healing Assay of RWPE-1 cells transduced with *Irx3* Adenoviral Vectors.
This similar trend is seen in DU-145 cells. At 24 hours the non-transduced DU-145 cells had an open wound area of 29%, Ad.CMV.\textit{Ir}x3-\textit{v}5 had an open wound of 52% and Ad.\textit{mir}\textit{Ir}x3-\textit{eGFP} 28%. At 72 hours non-transduced DU-145 cells had an open wound area of 3%, Ad.CMV.\textit{Ir}x3-\textit{v}5 had an open wound of 16% and Ad.\textit{mir}\textit{Ir}x3-\textit{eGFP} 1%. Transduction of RWPE-1 and DU-145 with gain-of-function \textit{Ir}x3 did not completely close the wound area even after 72 hours. In contrast, the cell lines transduced with loss of \textit{Ir}x3 function closed the wound area at a faster rate in RWPE-1 (see Figure 16) and DU-145 (see Figure 17) cell lines over a 72 hour period. These data indicates that \textit{Ir}x3 loss-of-function can modulate wound closure rates of RWPE-1 and DU-145 cells and suggest a functional role in mediating prostate cell migration.

<table>
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<th>24 hours</th>
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<td>Ad.CMV.Xnull-\textit{v}5</td>
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<td>Ad.CMV.\textit{Ir}x3-\textit{v}5</td>
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\textbf{Figure 16}: Wound Healing Assay of DU-145 Cells transduced with \textit{Ir}x3 Adenoviral Vectors.
Figure 17. Quantitative data of Figure 16 Wound Healing Assay of RWPE-1 transduced with *IrX3* Adenoviral Vectors.

**Discussion**

Migration of endothelial cells is a key component in the series of molecular and cellular events that occur during angiogenesis.\(^{104}\) Many previous studies illustrate essential signaling pathways that are essential for angiogenesis, there remains a lack of insight regarding transcription regulatory networks that govern EC migration and cell fate.\(^{105}\) Homeobox genes are characterized as early mediators of fetal patterning and development.\(^{106}\) IRX3 functions as an early facilitator of transcription signaling which targets specific genes during normal embryogenesis.\(^{107}\) In the context of angiogenesis, most transcriptional responses to low O\(_2\) are mediated by hypoxia-inducible factors
Our siRNA screening studies identified EPAS1 as a transactivator of Irx3 expression in response to VEGF-A treatment. Activation of VEGF receptors contribute to ECs migration and proliferation during angiogenesis and lymph-angiogenesis. It has been demonstrated that genetic or pharmacological strategies to manipulate EPAS1 expression can severely disrupt angiogenesis via the VEGF receptor. It is also important to note that VEGF does not directly activate EPAS1, but instead EPAS1 transactivates the VEGF ligand promoter under hypoxia via HIF-1-binding and is enhanced markedly by cotransfection with ARNT.

Our ChIP studies confirmed the results of our siRNA screen, demonstrating that EPAS1 binds to the Irx3 promoter with increased -fold enrichment in the presence of VEGF. In response to VEGF ligand, we observed binding enrichment at the classical bHLH EPAS1 binding sites located in the upstream regulatory region. EPAS1 is a known trans-activator of hypoxic-inducible genes that promote angiogenesis. Our data suggest that EPAS1 transactivates Irx3 by binding the classical bHLH EPAS1 binding site that may also involve HIF-dependent cofactor association in the Irx3 promoter region. Future experiments to decipher the mechanism through which EPAS1 directly regulates Irx3 expression during angiogenesis and the interaction with Notch signaling mediators, and cofactors are required.

After we confirmed that EPAS1 is a positive regulator of Irx3 we investigated the temporal expression of Irx3 in endothelial cells in response to known EPAS1 effectors; VEGF and hypoxia stimulation. Our findings indicate that Irx3 and EPAS1 are expressed in HMVECs, that it is temporally regulated by VEGF as early as 8 h after VEGF-A
treatment, and that it is maintained over a period of 48 h. This correlates to a decrease in
Ir3 and EPAS1 mRNA expression when cultured HMVECs become confluent 48 h after
VEGF-A treatment. Our results also indicate by 48 hours, Ir3 and EPAS1 expression
increased significantly post-hypoxia treatment with expression as early as 8 hours. Ir3
and EPAS1 expression increased in a progressive manner from 8–48 h post-hypoxia
treatment compared with time-matched vehicle controls. These results also show a
positive correlation between time and hypoxia. These results demonstrate that
endogenous Ir3 and EPAS1 expression are elevated in HMVECs in response to hypoxia
treatment in a progressive manner with dramatic expression at 48 h. Our results agree
with others that EPAS1 is expressed in endothelial cells.31a Although Ir3 behaved in a
similar manner as EPAS1, greater insight is needed to identify downstream Ir3
transcription targets in the endothelium of the vasculature and perform metadata analyses
to decipher common and specific gene targets of IRX proteins in endothelial cells in
response to hypoxia in a strict manner.

IRX genes have been identified and conserved in human, mice and an array of
other species.112 Synonymous IRX proteins have been identified in drosophila. Early
reports in Drosophila describe the requirement of the IRX proteins Araucan (Ara) and
Caupolican (Caup) for proper wing vein formation. Loss of L1-L5 wing vein formation
was observed when the loci of the Ara and Caup were disrupted.40,113 We previously
reported that transient knockdown of endogenous Ir3 in HMVECs suppressed VEGF-
induced cell migration during wound closure and chemotactic migration, whereas
enforced expression of Ir3 resulted in accelerated migration in wound closure and
chemotaxis. These data demonstrate that in the presence of EPAS1, IRX3 accelerated migration of HMVECs, whereas, a transactivation mutation of EPAS1 partially abrogated the ability of IRX3 to promote cell migration. The EPAS1 transactivation mutant contains mutations at Proline 405 to Alanine and Proline 531 to Alanine, which leads to loss of DNA-binding capability. The results of the migration assays are particularly interesting because directional migration is critical for angiogenesis during fetal development.

Here, we show that EPAS1 is an upstream proangiogenic transcription regulator of Irx3 and functions to promote endothelial cell migration s essential to productive angiogenesis.

We demonstrate that IRX3 may be utilizing the Src signaling pathway. Herein, we report that IRX3 partially promotes EC migration via activation of the Src signaling pathway. Previous reports have indicated that Src activation promotes survival, migration, and invasion pathways. Many transmembrane proteins have been identified that can activate the Src pathway such as adhesion receptors, receptor tyrosine kinases, G-protein coupled receptors and cytokine receptors. We posit that in our model system transcription activation of Irx3 promotes EC migration via the Src pathway that can be accentuated by EPAS1, however rigorous studies need to be performed to delineate the precise mechanism.

In addition to the work we conducted using endothelial cells our ChIP-on-ChIP data also revealed that Irx3 may also play a role in prostate cancer cell migration. Prostate cancer is the second most frequently diagnosed cancer in men worldwide, the sixth leading cause of cancer death in men and the fifth most common cancer globally and
remains an important public health concern in Western countries and an emerging malignancy in developing nations. Relative high rates of prostate cancer has also been found in developing areas such as the Caribbean, South America and sub-Saharan Africa, while the lowest rates are found in South-Central Asia. Prostate cancer is the leading cause of cancer and the second leading cause of morbidity in men in North America. African American men have the highest incidence and mortality rates as compared with Caucasian men and present with more aggressive disease at the time of diagnosis. However, the information is very limited on the role of Irx3 in prostate cancers.

Extensive scientific evidence indicates that hox genes are critical for morphogenesis, organogenesis and differentiation, while emerging data has identified the role of hox genes in tumor development. Iroquois homeobox genes (Ir) are a member of the Homeobox (HOX)-Three Amino acid Loop Extension (TALE) family of genes and are essential in early patterning of many embryonic tissues in a spatially and temporally restricted manner. Several Ir members have shown to be involved in carcinogenesis. For example, Irx1 acts as a tumor suppressor gene in gastric cancers; Irx3 has been shown to be down-regulated in LNCaP cells; Irx4 has been shown to suppress prostate cancer growth in benign prostatic hyperplasia and prostate cancer genome wide association studies; and Irx5 regulates cell cycle and apoptosis in human prostate cancer patient samples. One study has reported that hypermethylation of the 5′ CpG island region of the homeobox gene Irx3 in TRAMP is associated with reduced gene expression which allowed TRAMP to have conditions reminiscent of human prostate cancer. There is very limited information of the role of Irx3 on prostate
cancer progression and the expression and function of Irx3 in human cancers remains to be investigated.

Herein we have shown the expression profile of Irx3 in normal and prostate cancer cell lines reflecting its expression in normal and disease states of varying stages. We report that significant expression of Irx3 is present in normal prostate epithelial cells as well as moderately aggressive prostate cancer cell, DU-145. LNCaP cells have been categorized as a prostate cancer cell line that is androgen sensitive with low tumorigenicity,\textsuperscript{123} whereas DU-145 and PC-3 are both androgen sensitive with moderate and high tumorigenicity, respectively.\textsuperscript{124} Our findings agree with others that Irx3 has low expression in LNCaP cells, however we are the first to identify the protein and mRNA levels of Irx3 in RWPE-1, DU-145 and PC-3 cell lines. Further studies will need to be conducted to identify the potential relationship between Irx3, androgen and its receptor.

Prostate cancer metastasis is a complex and vigilant process of events of which cellular migration is involved.\textsuperscript{125} Numerous studies have identified the importance of migration in prostate cancer metastasis,\textsuperscript{126} however greater insight is needed on the role of Hox genes that regulate prostate cancer migration. Therefore, we investigated the functional significance of Irx3 in DU-145 prostate cancer cells. Upon successful transduction of gain- and loss- of Irx3 function in the cells, our findings Irx3 plays a role in cell migration. Gain of Irx3 function decreased cell migration in both RWPE-1 and DU-145 cell lines. Once Irx3 function was knocked down, cells migrated at a faster rate than compared to the non-transfected cell lines. Other reports in Drosophila have identified Irx proteins to be necessary for wing vein development, specifically involved
with migration. In the present study we have also identified that *Irx3* plays a vital role in DU-145 and RWPE-1 cellular movement.

It is believed that part of the reason prostate cancer is so widespread in developed countries is due to increased practice of prostate specific antigen (PSA) testing and subsequent biopsies. Recent discoveries have attributed cancer therapeutics to an onset of cardiovascular disease. For example, a recent report indicated that androgen deprivation therapy (ADT), which is suggested to prostate cancer patients to reduce levels of male hormones, was linked to triple the risk of heart-related death in men with heart failure or in those who had a previous heart attack.

In summary, the present study has provided direct evidence of the vital role of *Irx3* in DU- migration of these cells. The varying levels of *Irx3* in endogenous protein and mRNA levels of our prostate cell panel also supports the inclusion of *Irx3* in a tissue- or urine based cancer biomarker panel to inform prostate cancer patient treatment decisions. *Irx3* over expression has been shown to decrease prostate cell migration while knock down of *Irx3* had opposite effects. Strategies to pharmacologically regulate *Irx3* function in moderately metastatic prostate cancer cells may provide for new therapies.
CHAPTER V

CONCLUSION

The present study has provided direct evidence of the vital role of *Irx3* in prostate cancer and endothelial cell migration of these cells. The varying levels of *Irx3* in endogenous protein and mRNA levels of various cell lines also supports the inclusion of *Irx3* in a tissue- or urine based biomarker panel to inform cancer or cardiovascular patient treatment decisions. *Irx3* over expression has been shown to decrease prostate cell migration and while knock down of *Irx3* had opposite effects. Strategies to pharmacologically regulate *Irx3* function in moderately metastatic prostate cancer cells may provide for new therapies. Additionally, our findings describe a novel functional role of *Irx3* as a proangiogenic mediator of *EPAS1* and hypoxia signaling by promoting EC migration. Taken together under hypoxic condition these results indicate that *Irx3* is a downstream target of *EPAS1* signaling to promote migration in HMVECs or ablate migration of DU-145 cells via CITED2 (see Figure 18). Therefore, strategies to pharmacologically regulate *Irx3* function may provide new therapies to inform prostate cancer or ischemic cardiovascular patient treatment decisions.
**Future Directions**

In this study we elucidated a mechanism by which Iroquois homeobox gene 3 (Irx3) regulates angiogenesis through EPAS1 signaling in response to VEGF-A and hypoxic conditions. Each experiment discussed in this study was performed *in vitro* using human prostate epithelial, transformed prostate epithelial, or microvascular endothelial cells in culture. Thus, the future directions of this project involve the expansion of this work into *in vivo* model systems. *In vivo* studies would be more insightful in showing migration in response to the number of cellular and molecular interactions involved in this process.
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