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Novel Vascular Endothelial Growth Factor Signaling in Heart Development and Endothelial Homeostasis

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Novel Vascular Endothelial Growth Factor Signaling in Heart Development and Endothelial Homeostasis

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular, Cell, and Developmental Biology

by

Courtney Kay Domigan

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ABSTRACT OF THE DISSERTATION

Novel VEGF Signaling Pathways in Embryonic Heart Development
and Adult Endothelial Homeostasis

by

Courtney Kay Domigan
Doctor of Philosophy in Molecular, Cell and Developmental Biology
University of California, Los Angeles, 2014
Professor Luisa Iruela-Arispe, Chair

Vascular Endothelial Growth Factor (VEGF) is a potent endothelial cytokine and mitogen that directs the development of vasculature. Canonical VEGF signaling is a well described phenomenon whereby hypoxic tissues secrete VEGF, which then diffuses to surrounding VEGF-specific receptors. Interaction of VEGF and its receptors stimulates growth of the vascular system, through vasculogenic and angiogenic processes. Once formed, the circulatory system allows for the delivery of oxygen and nutrients to all tissues in the body. Blockade of VEGF signaling is used in the clinic to impede angiogenesis in some cancer settings, which suppresses tumor load by limiting the available blood supply required for survival and metastasis. However, patients treated with anti-VEGF therapy are beset by unexpected cardiovascular side-effects that suggest other non-canonical functions of VEGF protein. Here we identify several novel non-canonical roles for VEGF in the developing embryo heart and in the adult endothelium.

In embryogenesis, VEGF receptors are expressed in diverse tissues apart from the inner layer of the vasculature, which indicates that these tissues respond to VEGF in a non-canonical manner. Using a chimera approach, we found evidence that VEGF takes part in cardiac chamber specification of the ventricles, which was further demonstrated with VEGF gain- and loss-of-function genetic models. Because VEGF is implicated in early cardiomyocyte differentiation, we investigated the expression patterns of VEGF's major receptor (VEGFR2) throughout cardiogenesis with a reporter mouse model. We identified subsets of cardiomyocytes that express VEGFR2 in the atrial septum and the primordial conduction system.

VEGF is expressed by the adult endothelium, and is essential for a non-canonical pathway that promotes homeostasis via autocrine signaling. Using mouse models and primary human cell culture, we were able to demonstrate that endothelial VEGF maintains cell survival through constitutive suppression of the transcription factor Foxo1. In the absence of VEGF, cellular Foxo1 levels increase and causes deregulation of cell metabolism and autophagy, which ultimately induces cell death.

Together, this work describes several novel non-canonical roles for VEGF in the heart, and further elucidates the molecular mechanisms behind autocrine VEGF signaling in the endothelium. These findings expand our understanding of the basic biology of VEGF and expose potential limitations for the use of anti-VEGF blockade in human patients.

The dissertation of Courtney Kay Domigan is approved.

Utpal Banerjee

William Lowry

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University of California, Los Angeles

2014

DEDICATION

To my parents who taught me to look at the world with curiosity.

And to my husband, who laughed long and hard with me along this long and winding road.

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Chapter 1 - Introduction

VEGF signaling pathways:

Developmental and Homeostatic Roles

1.1 - Vasculogenesis, Angiogenesis and Vascular Endothelial Growth Factor

The formation of the vascular system occurs through two independent processes: vasculogenesis and angiogenesis. **Vasculogenesis** is the *de novo* formation of blood vessels from endothelial progenitors in the embryo. Endothelial cells, which comprise the inner lining of the vasculature, emerge concurrently from several regions within the embryo and form “blood islands” within the yolk sac. These so-called blood islands are temporary structures that give rise to the initial vasculature plexus in development. Mesodermal precursor cells migrate together to form blood islands and eventually differentiate into two pools: the inner pool of cells become hematopoietic cells, while the outer layer develops into endothelial precursors. The endothelium forms lumenized sacs that expand into a primitive vascular plexus in multiple areas within the embryo and later connect to each other through a process called anastomosis. Prior to the first heartbeat, while the embryo can still survive via oxygen diffusion, the primitive vasculature connects to the developing heart tube thus forming a complete cardiovascular circuit [1]. This initial vascular supply, however, lacks complexity and hierarchy needed for mature vascular function.

The growth of blood vessels from existing vasculature is referred to as **angiogenesis**, a well-studied process responsible for vascular expansion in development and in adult vertebrates. In the adult, angiogenesis occurs in select physiological settings, such as during the menstrual cycle and wound healing, although under homeostatic conditions little vascular turnover is thought to occur. More interesting to clinicians, is angiogenesis associated with pathological conditions. These include ocular neovascular diseases where vessel overgrowth is a direct cause of blindness, or in cancer where blood vessel expansion is needed to support the nutritional needs of the growing tumor.

Vascular Endothelial Growth Factor (VEGF) is the major regulator of vasculogenesis and angiogenesis in most known settings. A significant body of literature exists detailing the effects

of this growth factor on vascular growth and development. This dissertation aims to expand on existing knowledge by exploring several new facets of cardiovascular development, and VEGF signaling specifically. However, the goal of this introductory chapter is to provide a solid background on the concepts that have been well established in the vascular field, and to highlight gaps in the literature that this dissertation addresses directly.

1.2 - VEGF in the context of embryo development

Discovery of VEGF signaling pathway components

In the early days of molecular biology, it was found that supernatants taken from the cell media of tumor cells induced strong vascular permeability, and thus the unknown factor responsible for this effect was named vascular permeability factor (VPF) [2]. This growth factor was later purified from bovine pituitary follicular cells and re-named Vascular Endothelial Growth Factor (VEGF) due to its specific pro-mitotic effect on the endothelium [3]. It was suspected that VEGF was important for angiogenesis in the embryo based on its expression pattern in relation to active vessel development [4]. However, it was only until the inactivation of this gene in the germ line that the relevance of this growth factor as chief regulator of vascular growth was fully acknowledged [5] [6]. Today, VEGF is known to be an essential director of vasculogenesis as well as the most potent angiogenic factor yet identified.

VEGF's major receptor VEGFR2 was found to be equivalent to fetal-liver kinase 1 (Flk1) when sequenced from a cDNA clone abundantly expressed in developing embryos and in proliferating endothelial cells [7] [8]. Early experiments exploring the developmental interplay between ligand and receptors revealed expression of VEGF as early as E7.0, with the receptors detected soon afterwards in endothelial precursors. Expression of ligand and receptor was generally observed in complementary tissues, supporting the hypothesis that VEGF is secreted by the tissues requiring vascularization, and the endothelium responds by proliferation and migration towards this chemokine [9] [10] [11].

Mutating the VEGF pathway in the mouse

Before much was known about VEGF itself, the developmental roles of VEGF's major receptors were investigated using knockout animals. The importance of VEGFR2 was made abundantly clear in the VEGFR2 KO mouse. Homozygous ablation resulted in early embryonic lethality between E8.5-9.5. Blood-island formation was almost completely ablated, indicating VEGFR2 is crucial not only for angiogenesis, but for hematopoiesis [12]. Ablation of VEGFR1 resulted in embryonic lethality around E9.0 as well, but with a distinct phenotype. Endothelial cells and blood cells were identified in these animals by several markers, indicating VEGFR1 is dispensable for differentiation of these cell types. However, the vasculature was severely disorganized, with abnormal lumenization and general thickening of the vasculature due to an increase in the number of endothelial layers [13]. These findings indicated that VEGFR1 provided signals for the differentiation of hematopoietic cells and regulated excessive proliferation of endothelial cells by functioning as a "decoy" receptor.

More severe than either of the receptor KO animals which are only lethal when homozygous mutant, the genetic ablation of a single allele of VEGF was found to be lethal between E10-E12. Endothelial and blood cells are present in these heterozygous mutants, though they are fewer and disorganized, suggesting that hemogenic endothelial differentiation is strictly dependent on wild type levels of VEGF. The dorsal aorta was not detected in most embryos, and general disorganization of the vessels revealed no connection between the heart and the vascular system. Unsurprisingly, heterozygous VEGF-KO embryos are underdeveloped in the forebrain, heart and limb bud, and apoptosis and necrosis are evident to a much higher degree than in WT counterparts. Embryonic demise occurs no later than E12.5 [5] [6].

1.3 - Diverse roles for VEGF in cardiac development

Brief overview: Heart development

A functional beating heart is required early in development when the embryo reaches a size that can no longer be sustained by diffused oxygen. Heartbeat can be observed before the heart has developed into its familiar four-chambered morphology, when the primordial heart is a simple heart tube at E8.0 **(Figure 1.1)**. The heart tube is comprised of two layers: an outer myocardium and the inner endocardium, separated by cardiac jelly. The endocardium is highly similar to vascular endothelium, and thought to be partially derived from endothelial precursors [14].

By E8.5, the heart tube begins looping to the right while growing in length, expanding primarily from the recruitment of cardiac progenitor cells originating from a population called the second heart field **(Figure 1.1)**. By mid-gestation, most proliferation occurs within the heart rather than by recruitment, and particular transcriptional programs are enacted that specify the unique characteristics of each chamber and cell type. Finally, the specialized structures of cardiac septa and valves are formed by a combination of inter-heart differentiation and intra-heart cell recruitment from the neural crest [15].

The molecular events coordinating heart development are well-studied, but not entirely understood. The cacophony of closely-related yet unique cell populations, coupled with fast-changing topological relationships makes the developing heart a difficult organ to study. One factor among many that orchestrates cardiac morphogenesis is VEGF.

Expression patterns of VEGF throughout heart development

Early immunohistochemistry and Northern blot analysis suggested that VEGF is highly expressed in the developing heart compared to expression by homeostatic adults [16]. As antibodies used to detect VEGF are notoriously unreliable, a VEGF-LacZ reporter mouse was engineered that significantly improved sensitivity for detection of VEGF expression in the mouse [17].

A cursory look at VEGF expression through heart development showed strong VEGF expression in several early cardiac populations **(Figure 1.1)**. The myocardium strongly stained

for the B-gal reporter, indicating strong activity of the VEGF promoter at E8.5. Strong endocardial reporter activity was also observed until E9.5, after which it was only observed in the cells lining the outflow tract **(Figure 1.1A-F)**. The endocardial cells lining the heart cushions are highly positive for VEGF expression, but lose expression as soon as they undergo differentiation to form the cardiac valves, a process discussed in more detail below. Later in development, by E14.5, strong X-gal staining was seen in the trabecular myocardium of the ventricle, with lower levels observed in the atrial myocardium **(Figure 1.1G-I)**. VEGFR-2 expression was by-and-large complementary to that of VEGF, with higher levels observed in the endocardium of the ventricles than that of the atria [17].

Embryo models of VEGF over-expression

Soon after VEGF was identified and cloned, the effects of exogenous VEGF addition were studied in a multitude of biological systems, including the embryo. Application of VEGF to developing quail embryos resulted in dilated vessels, and most strikingly, heart defects. Overgrowth of the endothelial-like endocardial cells was observed, as well as an overall enlargement of the heart, primarily in the atria [18].

In an attempt to generate VEGF reporter lines, Nagy and colleagues developed a number of knock-in LacZ transgenics. The location of the LacZ within different regions of the 3' UTR resulted in alterations of expression due to changes in VEGF mRNA stability. In this manner, hypermorph and hypomorph alleles were developed. These were used in studies to evaluate systemic increases or decreases in VEGF protein levels. A slight over-production of VEGF from the VEGF-LacZ hypermorphic allele results in 1.5-2 times wild type protein levels, and causes embryonic lethality due to severe heart defects [19]. Early vascular and cardiac development of these embryos was normal, but by E12.5 they began to die of congestive heart failure and vascular leakage. Hearts were grossly enlarged but displayed a thinning of the compact layer of the ventricular wall. Interestingly, these hearts had excessive elaboration of the trabecula, suggesting a pro-trabeculation role for VEGF in cardiomyocyte development [19].

VEGF contributes to cardiomyocyte differentiation

To better understand the importance of VEGF in cardiomyocyte differentiation and cardiac morphogenesis, VEGF was deleted in ventricular myocardium using MLCv promoter [20]. While 60% of these mice were viable, they had severe heart defects including thinner ventricular walls and diminished cardiac function. The authors speculated that these defects were largely the result of a diminished capacity of the myocardium to recruit microvessels [20]. However, these experiments did not rule out a cell-autonomous function for VEGF in the myocardium.

Several stem cell models have been used to aid our understanding of VEGF effects on cardiomyocyte differentiation. The addition of exogenous VEGF promotes cardiomyocyte differentiation from embryonic stem cells [21]. In another system, VEGF expression by adipose-derived stem cells was required for cardiomyocyte differentiation, an effect which was blocked by the addition of anti-VEGF antibodies [22]. To clarify the stage at which VEGF was required for in vitro cardiomyocyte differentiation, an inducible VEGF construct was transduced into embryonic stem cells. Induction of VEGF during a brief time window did not enhance production of cardiomyocytes from embryoid bodies. However, cells that were induced to express VEGF during differentiation were shown to enhance cardiac function after injection of into an animal model of myocardial infarction [23]. Together, these experiments portray VEGF as a pro-myogenic factor during cardiogenesis **(Figure 1.1 A-D)**.

Specific roles for VEGF in valve development

Effective flow of blood through the heart requires proper formation of the cardiac valves, which act to ensure unidirectional flow and block regurgitation of blood. Heart valve formation is initiated in development by the delamination of the endocardium lining the endocardial cushions (a process referred to as endocardial-to-mesenchymal transformation) and migration of these cells into the cushion where they differentiate and begin to express alpha-smooth muscle actin

[24]. Several studies, detailed below, indicate that this specialized differentiation process is tightly controlled by cardiac VEGF expression **(Figure 1.1F)**.

Artificial over-expression of VEGF in the myocardium at E9.5 revealed that VEGF inhibits the formation of endocardial cushions by blocking the necessary endocardial-to-mesenchymal transformation needed to form the valves [25]. Although this work began by exploring the potential ways by which hypoxia contributes to fetal heart defects, they proposed that endogenous VEGF acted in development to limit the area of EMT in the heart [26].

While these studies provided ample evidence that VEGF was required for valve formation in the embryo, most heart valve defects are not apparent until adulthood. In one cell culture model it was shown that adult valves can be induced to undergo EMT upon addition of TGF-Beta. In agreement with the aforementioned reports, this model was sensitive to the addition of VEGF which inhibits the EMT process through VEGFR-2 [27].

Conflicting experiments showed that VEGF's role in developmental EMT was not as straightforward as "inhibitory". Using a model of hyperglycemia that results in decreased VEGF expression, it was found that low levels of VEGF can actually inhibit EMT of cells into the endocardial cushion, an effect that was reversed by addition of exogenous VEGF [28].

To address disagreements in the literature, more detailed experiments were performed using a variety of mouse models to inactivate VEGF and its receptors at different stages of valve development. It was found that VEGF signals positively regulate EMT in the outflow tract, but not the atrioventricular canal, in early stages of development. At later time points, VEGF was required again to mature the atrioventricular canal leaflets [29].

It is clear from these conflicting reports in the literature that the effect of VEGF on this specialized developmental process cannot be understood until the spatial and temporal patterns of VEGF are better known. In the fast changing landscape of the developing heart, it is not entirely clear which cell types contribute to localized levels of VEGF, and how VEGF levels correlate to each step in valve development **(Figure 1.1 F)**.

VEGFR2 receptor in heart development

Derived from endothelial precursors, endocardial cells are known to express high levels of VEGFR2 [14]. Somewhat surprisingly, a subset of cardiac muscle was shown to have a contribution from the VEGFR-2 lineage, suggesting the existence of a common endothelial and cardiac precursor [30] **(Figure 1.1A-B)**. A VEGFR2-LacZ knock-in mouse model highlighted angioblast and endothelial expression, but reported incomplete coverage of B-gal expression in tissues where VEGFR2 could be detected immunologically [31]. Refinement of the VEGFR2-LacZ allele was achieved by crossing VEGF-LacZ lines with a constitutive Cre strain, effectively deleting a PGK-neo cassette that was used to produce VEGFR2 knock-in stem cells. Without interference from the cassette, B-gal analysis revealed that early VEGFR2 progenitors *can* give rise to the cardiac lineage, and that in fact the gene is widely expressed in cardiomyocytes of the E8.5 heart [32].

In studies where ESCs were differentiated into embryoid bodies, a likely common progenitor of cardiac, hematopoietic and endothelial cells was identified with a VEGFR-2+ CD31- VE-cadherin- identity [33]. Further evidence of a VEGFR2+ cardiovascular precursor cell with myocyte, endothelial and vascular smooth muscle potential was found in stem cell differentiation experiments, with collaborating evidence in the embryo [34]. This precursor cell population was further defined as a Isl1+/Nkx2.5+/VEGFR2+ [35]. Finally, a VEGR2+ cardiovascular precursor was identified in human stem cell differentiation cultures, suggesting a similar process occurs in human differentiation [36]. These experiments suggests that VEGF has a direct influence on early cardiomyocyte development, most likely by way of the receptor VEGFR2.

1.4 - Canonical VEGF signaling

Brief overview: Canonical VEGF signaling

To better understand its mechanism of action, cellular and biochemical experiments have aimed to parse apart the downstream effects of VEGF on endothelium. A body of literature has developed over several decades to explain the mechanisms by which VEGF binds to its receptors and mediates phosphorylation. This process has been confirmed by multiple labs and became known as the “canonical” process of VEGF signaling. Canonical signaling is responsible for the most obvious effects of VEGF during developmental and pathological settings.

Canonical VEGF signaling is best exemplified by the following scenario **(Figure 1.2A)**: In a given tissue that requires vascularization, hypoxia (presumably due to lack of blood flow) stabilizes the transcription factor HIF1 which travels to the nucleus and directly drives expression of VEGF through its promoter’s hypoxia responsive element. VEGF, a secreted factor, diffuses from the cell of origin, effectively forming a cytokine concentration gradient. Upon encountering the endothelium, VEGF binds to its receptor tyrosine kinases, inducing dimerization and auto-phosphorylation at the cell surface. Phosphorylation of the receptor triggers a mitogenic and migratory signaling cascade, that results in proliferation and chemotaxis toward the origin of secreted VEGF. Most publications agree that it is through canonical VEGF signaling that hypoxic tissues recruit blood vessels, in the case of normal development and in tumor settings [37] [38] [39].

Mounting evidence for the central role of the VEGF signaling pathway for vascular development prompted a flurry of experimentation that aimed to delineate its molecular mechanism of action [2]. This section will outline some of the crucial findings that reinforced the canonical signaling model, and some findings that build the complexity of the model without abandoning the basic canon.

VEGF protein overview

VEGF is now known to be a small secreted glycosylated homodimer that is a member of the VEGF family. “VEGF” was later renamed VEGF-A after the discovery of its homologues:

PlGF, VEGF-B, VEGF-C, VEGF-D and VEGF-E (a viral homologue). Early structural studies established that VEGF must be dimerized to bind to its receptor [40], but generally VEGF can stimulate VEGFR-2 with or without the glycosylation modification, allowing use of recombinant bacterial VEGF as the source of recombinant ligand.

VEGF is expressed as several isoforms that differ primarily in degree of extracellular matrix binding capacity. The major isoforms found in physiological conditions are VEGF121, VEGF165 and VEGF189. Because it excludes the introns containing heparin-binding domains, VEGF121 has the least extracellular matrix (ECM) binding capacity, while VEGF189 binds the ECM most tightly. It has been found that alteration of the ECM binding domain alters distribution in tissues, and even receptor responses [41].

VEGF is a ligand of the receptor tyrosine kinases VEGFR1 and VEGFR2, and co-receptor Neuropilin-1

Around the time that VEGF was identified, the two major VEGF receptors VEGFR1 and VEGFR2 were characterized. These receptor trysine kinases contain seven immunoglobulin-like repeats in the extracellular domain, and a cytoplasmic kinase catalytic domain consistent with other receptor tyrosine kinase sequences [42] After determining the endothelial specificity of these receptors, it was found that VEGF bound to them with high affinity promoting dimerization, and was able to induce cellular effects, such as calcium release, in receptor-expressing cells [43]. [7,8,44].

As expected in a receptor tyrosine kinase/ligand interaction, VEGF indeed stimulates phosphorylation of VEGFR1 and VEGFR-2 in bovine aortic endothelial cells through their ability to auto-phosphorylate [45]. Although several experimental systems demonstrate VEGF-induced phosphorylation of VEGFR-1, the kinase activity of this receptor is reported to be one tenth that of VEGFR2, although it has much greater affinity for VEGF ligand [46]. Perhaps unsurprisingly, an animal mutant lacking VEGFR1's intracellular kinase domain developed normal vasculature, suggesting its major function is through its extracellular domain [47]. Although the role of VEGFR1

is less well understood (likely because it lacks a robust biochemical signaling response to VEGF) it is now thought that VEGFR-1 acts primarily as a VEGF ligand-trap or “decoy receptor” that negatively regulates or modulates access of VEGF to VEGFR-2 [39].

A third major receptor for VEGF was identified to be Neuropilin-1, which is largely considered to be a “co-receptor”. Although conflicting details of Nrp1 function have been identified in different experimental systems, most literature is in agreement that Nrp1’s major function is to enhance VEGFR2 signaling response to VEGF [48] [49].

VEGFR2 signaling overview

The strongest contribution to VEGF’s drastic effect in endothelial cells is through the activation of VEGFR2. Ligand-binding induces VEGFR2 dimerization and auto-phosphorylation at several tyrosine residues within the cytoplasmic region. Phosphorylation of these tyrosines serve as docking sites for multiple proteins that mediate several signaling cascades, usually characterized by the presence of an SH2 domain. Important direct binding partners for signal transduction include PI3K, PLC-gamma, Src tyrosine kinases, Ras GAP, protein kinase C, ERK and MAPK [39].

The major cellular outputs of VEGFR2 signaling are those endothelial responses originally observed in VEGF treatments: proliferation, migration and survival [50].
Proliferation is primarily mediated by PLC-gamma binding to phosphorylated tryosine 1175, which regulates a PKC-Raf-MEK-MAPK cascade and results in increased DNA synthesis [51].
Migration phenotypes appear to be regulated through phospho tyrosine 951, which is bound by T cell-specific adaptor (TSAd) which mediates the migratory response of endothelial cells to VEGF, likely through downstream Src signaling [52,53].

Signaling through VEGFR2 has also been shown to enhance cell survival in stimulated endothelial cells, an effect mediated by the PI3-kinase/Akt pathway [54]. Resistance to apoptosis is further enhanced by an induction of expression of the anti-apoptotic proteins Bcl2, [55] Survivin and X-chromosome-linked Inhibitor of apoptosis (XIAP) [56].

1.5 - VEGF and Foxo transcription factors

Foxo1 background

Forkhead box O (Foxo) proteins are a family of transcription factors that regulate a multitude of developmental and homeostatic expression programs having to do with cell cycle, apoptosis and metabolism [57]. In multiple systems and organisms, Foxos have been shown to be negatively regulated by pro-survival growth factors [58]. Because Foxos share a common DNA-binding domain, they are functionally redundant in many contexts, though this is limited by individual Foxo expression patterns [59].

In most homeostatic contexts, Foxo proteins are actively suppressed by hyper-phosphorylation by regulating pathways, which causes shuttling of Foxo out of the nucleus. In the context of a strong growth factor input, cytoplasmic Foxos are then polyubiquitinated and degraded over time [60]. Foxo transcription factors are known to be responsive to several survival pathways in vitro and in vivo, in particular to the Akt pathway. In fact, in the presence of survival factors, Foxo3 is a direct phospho-target of Akt which suppresses Foxo3 pro-apoptotic transcriptional activity [61]. In this way pro-survival cellular inputs suppress pro-apoptotic Foxo transcriptional programs.

Effects of VEGF on Foxo

Given that Akt is one of the many downstream pathways triggered by VEGF, and the Foxo1 mutant mouse dies of a vascular phenotype [62], early experiments studied the effects of VEGF stimulation on Foxo transcription factor in endothelial cells. It was found that VEGF stimulation induces phosphorylation of forkhead transcription factors, which suppresses their activity and results in a pro-survival and mitogenic phenotype **(Figure 1.3A)** [63].

Phosphoproteomic analysis of VEGF-stimulated HUVECs revealed again that Foxo1 is phosphorylated in response to VEGF as expected, and silencing of Foxo1 heightens endothelial migration and proliferation responses in the presence of VEGF [64]. These results suggest that

homeostatic presence of Foxo1 acts as a “breaks” mechanism that opposes out-of-control VEGF signaling, while VEGF induces its degradation.

Effects of Foxo on VEGF signaling

Because Foxos are generally found to be degraded when cells are inundated by growth factors, it is somewhat surprising that the removal of Foxos can actually disrupt endothelial cascades in response to VEGF **(Figure 1.3)**. One study reports that VEGF signaling induces the expression of the mitochondrial antioxidant manganese superoxide dismutase (Mn-SOD) [65]. Paradoxically, although VEGF is known to degrade and depress Foxo protein, the addition of constitutively-active Foxo3 actually enhances Mn-SOD induction due to VEGF. This study suggests that in certain circumstances, Foxo-dependent transcription events may actually be increased upon addition of growth factor [65].

Another instance where Foxos mediates unexpected outcomes in the endothelium was revealed through gain- and loss-of-function studies in the presence or absence of VEGF [66]. In these studies, endothelial cells were treated with a constitutively-active Foxo1 (Foxo1-CA) or siRNA against Foxo1 (KD-Foxo1) and then stimulated with VEGF. As expected, a group of genes were inhibited by VEGF addition (or by KD-Foxo1) but increased with Foxo1-CA These genes fit a straightforward model where the presence of Foxo1 induces their expression, and when VEGF signaling degrades Foxo1, they are no longer expressed **(Figure 1.3A)**.

Unexpectedly, a second group of genes behaved in an opposite manner: VEGF *induces* their expression, and this induction requires the *presence* of Foxo1 **(Figure 1.3B)**. These genes were induced by VEGF, super-induced by VEGF and Foxo1-CA, and repressed in KD-Foxo1 conditions. This expression pattern is consistent with VEGF-induced Foxo1 transcriptional activity, an unexpected outcome when the majority of literature describes Foxo repression upon growth factor stimulation [66].

Alternatively it is possible that a group of transcription factors is at play. For example, the expression of several endothelial-specific genes is controlled by synergistic binding of Foxo and

Ets transcription factors to the “FOX:ETS” enhancer motif during development [67]. Because developing embryos express high levels of VEGF to direct blood vessel development but also depend on Foxo1 for the FOX:ETS transcriptional program, VEGF and Foxo cannot be purely antagonistic factors in all contexts.

These results suggest that the role of Foxos in endothelium is more nuanced than anticipated, and that they serve another important purpose besides “getting out of the way” of VEGF signaling. The results described in Chapter 5 will provide a slightly new understanding of this interplay between Foxos and VEGF. In this chapter, we find a small amount of autocrine VEGF acts to constitutively suppress Foxo1 levels which affects cytoplasmic signaling, but does not appear to directly influence Foxo1 transcriptional targets.

1.6 - Clinical VEGF blockade outcomes

In 1971, after several ground-breaking studies that showed tumors require active blood flow for growth, Judah Folkman published a hypothesis that added fresh urgency to the angiogenic field. He postulated that tumors require blood flow to survive, and by blocking neo-angiogenesis in the body, tumors can be effectively starved to death [68]. VEGF is the most potent and specific stimulator of the endothelium, and so became a natural target for anti-angiogenic therapy in the cancer setting.

Successes of anti-VEGF therapies

Numerous *in vivo* animal model studies suggested that anti-VEGF therapy should be an effective treatment in conjunction with conventional chemotherapeutics [69]. Treatment of metastatic colorectal cancer with humanized anti-VEGF antibody (Bevacizumab) along with bolus-IFL (irinotecan, 5FU, leucovorin) chemotherapy resulted in a significant survival benefit to patients, thus allowing the first FDA approval of an anti-VEGF drug in the cancer setting [70]. As of now, Bevacizumab therapy is approved for metastatic colorectal cancer, metastatic renal cell carcinoma, non-small cell lung cancer and recurrent glioblastoma in the United States [71]. This

approach was soon followed by tyrosine kinase inhibitors sorafenib, sunitinib or pazopanib that act on downstream VEGF signaling- as well as on other tyrosine kinase pathways in cancer and the endothelium. Sunitinib is currently approved for treatment of gastrointestinal stromal tumors, metastatic renal cell carcinoma, and pancreatic neuroendocrine tumors [71].

Serious side-effects for anti-VEGF therapy

In light of the Folkman hypothesis that angiogenesis blockade will specifically affect tumor vasculature, it was somewhat surprising that anti-VEGF therapy resulted in rare, but serious, side effects. Most commonly observed side-effects include hypertension, proteinuria and problems with wound healing [72]. More serious thromboembolic events, gastrointestinal perforations, congestive heart failure and life-threatening bleeding were also observed in different clinical settings [73]. Meta-analysis of patients treated with RTK inhibitors found slightly more severe effects than those treated with bevacizumab or other VEGF-traps [71]. The global side effects observed in clinical VEGF-blockade tells us that VEGF is needed for far more than neo-angiogenesis in the adult body.

Another criticism of anti-VEGF therapy is that although it significantly prolongs progression-free survival, it does not significantly increase overall survival in nearly all "successful" phase III trials [71]. The overall benefit of anti-VEGF therapy for patients is in question, as the worst months in terms of quality-of-life may be extended for a patient, and only at great financial cost. One course of bevacizumab may cost close to $100,000.00, leaving patient's surviving relatives with false hope, and ultimately fewer resources moving forward [71].

1.7 - VEGF in the adult

What is VEGF doing besides directing angiogenesis?

Despite the consensus among vascular biologists that little angiogenesis occurs in the adult, VEGF is expressed in adult homeostatic conditions. VEGF mRNA was observed in significant amounts in several tissues, the highest of which were the lung alveloli, glomeruli of

the kidney and in cardiac myocytes [74]. VEGF expression was also observed in macrophages, which are now thought to aid in the angiogenic process [74].

Using the VEGF-LacZ mouse model, more detailed analysis of homeostatic VEGF expression was established [75]. VEGF was found highly-expressed in cells adjacent to fenestrated blood vessels (podocytes, choroid plexus epithelium and hepatocytes), as well as in cardiac and skeletal myocytes and several other tissues. Several tissues were found to have constitutive VEGFR-2 phosphorylation, indicating activity of VEGF through this receptor even in a homeostatic setting [75].

Autocrine VEGF is required for endothelial cell maintenance

Interestingly, VEGF expression can be observed in stable adults in the aortic endothelium [75], in larger vessels of the lung and in smaller vessels of the intestine [76], albeit in a non-uniform expression pattern. To investigate the role of endothelial VEGF in homeostatic animals, VEGF was specifically excised in the endothelial compartment, creating VEGF-ECKO mice. These mice display a 30% embryonic lethality, and then continue to die throughout adulthood. By six months of age, only 44% of the mendelian predicted mutants survived [76], all others dying either *in utero,* or from sudden death associated with organ failure.

Histological analysis of VEGF-ECKO revealed multiple hemorrhagic events, endothelial cell rupture and other signs of vascular degradation that contribute to organ failure [76]. Because the gross levels of VEGF in the animal were unchanged, or even increased in some tissue beds, it can be assumed the endothelial defect does not originate from a lack of extracellular VEGF, but a lack of intracrine/intracellular VEGF. This was confirmed in cell culture experiments where co-culture of purified and labeled WT and ECKO endothelial cells could not rescue cell death of the VEGF-ECKO endothelium, suggesting that VEGF's role is indeed cell-autonomous [76]. These results radically depart from canonical VEGF signaling, and raise new questions in the vascular field.

1.8 - Summary of upcoming chapters

The goal of this dissertation is to expand on existing knowledge of cardiovascular development and, specifically, VEGF signaling. Evidence for a cell-autonomous signaling loop is expanded here with VEGF-KO chimeric embryos, which reveals differences in VEGF expression between cardiomyocytes of the atria and ventricle. Over-expression of VEGF in the atria is found to modulate Notch signaling which is an important regulator of cardiac trabeculation in development. Finally, new evidence will be provided that indicates cell-autonomous VEGF in the endothelium is important for metabolic homeostasis and staving off autophagic cell death.

Recent Advances in Vascular Development

This review summarizes emerging literature aiming to understand several different aspects of vascular development, including the specification of hemogenic endothelium, the origin of vascular disorders of the brain, and refinement of endothelial signaling pathways.

Cell-autonomous VEGF in Development: VEGF directs Notch-1 mediated ventricular trabeculation

The discovery that autocrine VEGF plays such an important role in maintenance of the endothelium prompted the question: Is autocrine VEGF important for development of the endothelium? And further, is autocrine VEGF important for the differentiation of other tissues in the embryo? To address this question, we used a chimera approach.

The creation of chimeric embryos comprised of VEGF-KO and WT cells revealed that cell-autonomous VEGF is dispensable in most cell types. Unexpectedly, VEGF-KO cells are recruited in higher numbers into the atrial chamber of the heart, but not the ventricles. This pattern is reflected in the pattern of endogenous VEGF expression in the heart, which is very low in the atria while quite high in the ventricles. We hypothesized that the high VEGF

expression observed in the ventricle contributes to the compact, trabeculated phenotype of ventricular cardiomyocytes while a lack of VEGF results in the smoother, less trabeculated cardiomyocytes of the atria.

To investigate the effect of different endogenous VEGF levels in the atria and ventricle, a mouse model of VEGF over-expression in the early heart was created which mis-expresses VEGF in the atria at early developmental time points. These animals suffer from severe heart defects including an over-growth of atrial cardiomyocytes. Expression analysis shows that the atria increases production of Neuregulin1, BMP10 and Notch1, all members of a pro-trabeculation molecular pathway. These experiments suggest that endogenous ventricular VEGF influences the compact trabecular morphology of this tissue, while a lack of VEGF in the atrium results in fewer trabecula required by this chamber.

Novel VEGFR2 populations in cardiac development

While cell culture experiments have shown that VEGF and its receptor VEGFR2 is crucial for the development of cardiac precursors, the anatomical location and persistence of these precursors is not known. Using a VEGFR2-LacZ mouse model, we investigated spatial-temporal VEGFR2 expression in the heart throughout cardiogenesis. We found several surprising populations, including VEGFR2+ cardiomyocytes localized to the atrial septum, which were persistent into late development.

Autocrine VEGF signaling maintains energy metabolism and survival in the endothelium

Genetic models have shown that cell-autonomous VEGF is essential for homeostasis of the endothelium, however it is not known if this is due to a failure in differentiation rather than a need for continuous signaling. Here we found that induced deletion of VEGF in the fully-differentiated adult endothelium results in premature lethality, demonstrating that continuous VEGF expression is needed for survival.

Previous work on cell-autonomous VEGF has described, but not explained, the mechanism by which VEGF supports cellular viability. Here we show that siRNA depletion of VEGF in endothelial cell culture induces suppression of glucose metabolism and mitochondrial respiration, and non-apoptotic cell death which is partially rescued by blockade of autophagy. The transcription factor Foxo1 is found to be responsible for the cell death phenotype, as silencing of Foxo1 in VEGF-depleted cells completely rescues the cell death phenotype.

Conclusions: Emerging Modes of VEGF signaling

While “canonical VEGF signaling” provides a robust model that helps us understand the most obvious effects of VEGF on the endothelium, recent experiments have pushed the boundaries of this model into new territory. This chapter summarizes recent unexpected findings, from this dissertation as well as published literature, that support several different modes of non-canonical signaling. This includes an expansion of our understanding of autocrine VEGF signaling, ligand-independent signaling through VEGF receptors, and modifications to the VEGF pathway by unanticipated co-receptors.

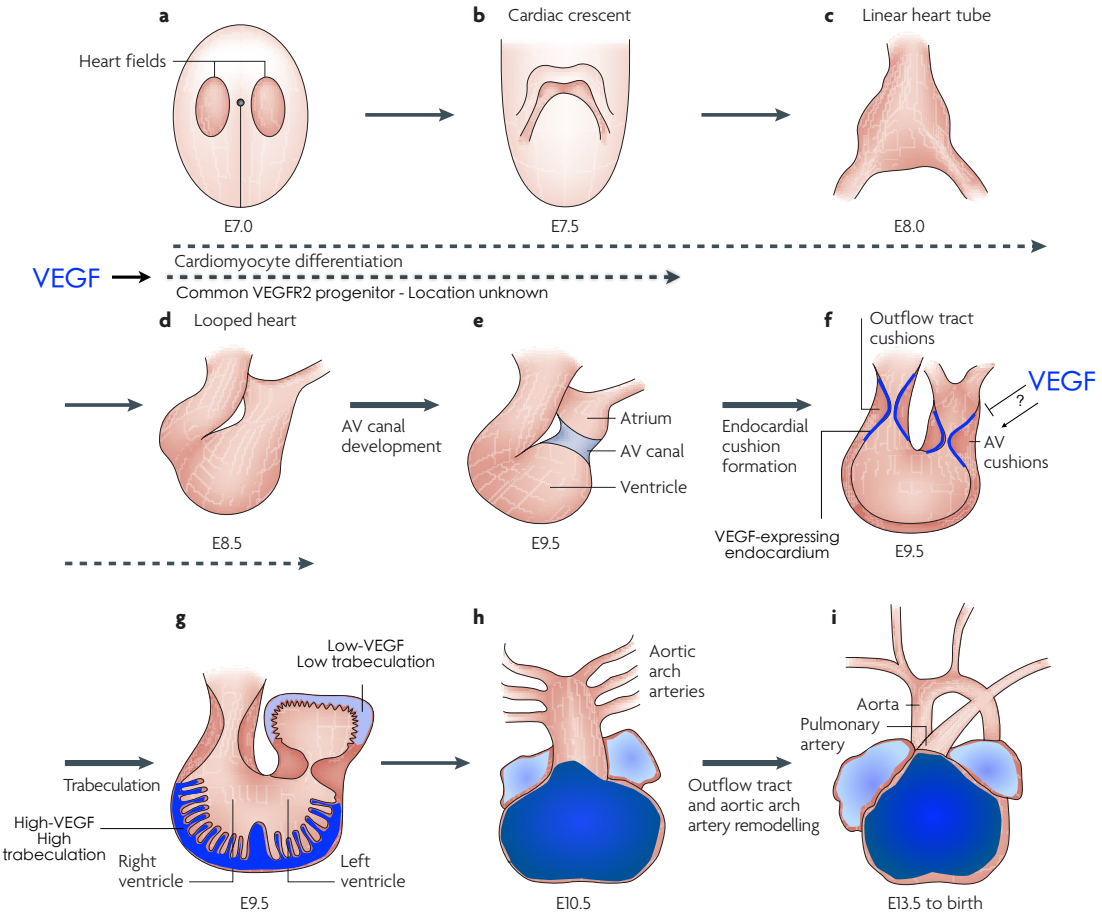
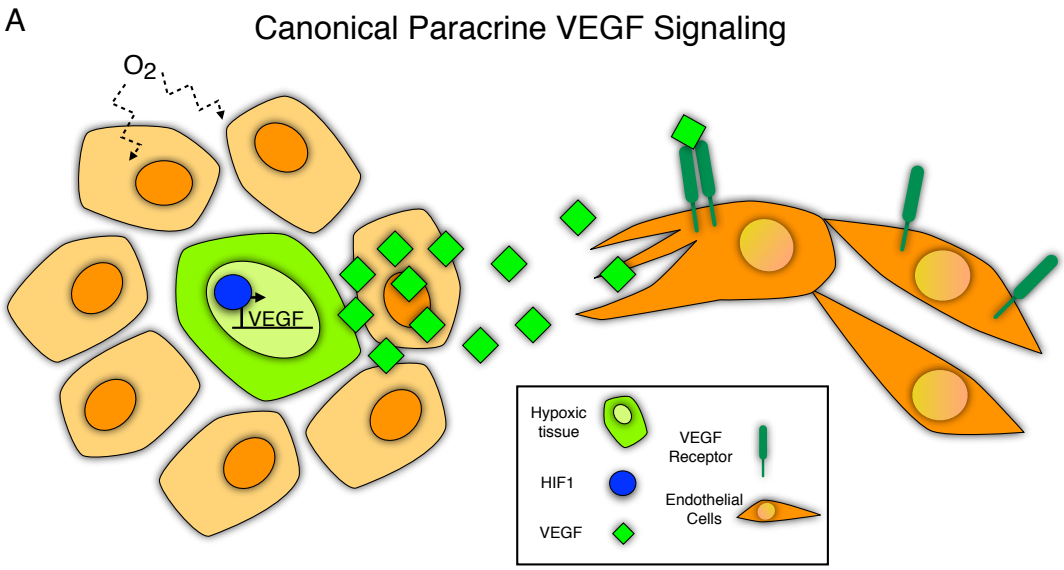


Figure 1.1 Varied roles for VEGF in cardiac development
Stem cell differentiation experiments suggest that VEGF is crucial for early cardiomyocyte differentiation in the embryo, and most likely acts on early VEGFR2+ cardiac progenitors. A) Heart development begins with early cardic specification of the heart fields which merge into B) the cardiac crescent which is then transformed into C) the linear heart tube comprised of inner endocardium and external myocardium. D) The heart then loops rightward and E) chamber balooning morphogenesis is apparent. F) Valve morphogenesis begins at E9.5 when VEGF+ endocardial cells lining the AV and OFT cushions begin EMT transition and loose VEGF expression. Conflicting reports exist suggesting positive and negative roles for VEGF on valve formation. G) Trabeculation is underway by E9.5 when differences in VEGF expression between the atria and ventricle are apparent. VEGF is highly expressed by the highly-trabeculated myocardium of the ventricle, while the less trabeculated atrium expresses lower VEGF levels. This pattern is maintained between H) E10.5 to I) E13.5 after which VEGF expression increases in the atria into adulthood. Figure adapted with permission from *The multifaceted role of Notch in cardiac development and disease. Nature Reviews Genetics, 2008.*



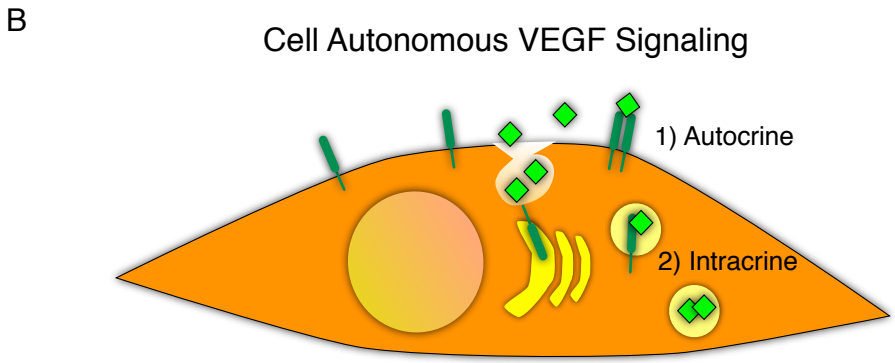


Figure 1.2 Canonical Paracrine vs. Cell-autonomous VEGF Signaling
A) Paracrine VEGF signaling is well-studied phenomenon where, typically, hypoxic tissues are induced to express VEGF by the oxygen-responsive transcription factor HIF1. VEGF is secreted and diffuses from the cell of origin, and interacts with specific receptor tyrosine kinases on endothelial cells. Signaling cascades within the endothelium results in migration, proliferation, and extension of the vasculature, thus providing oxygen to they hypoxic tissue.
B) Emerging evidence shows that VEGF has important signaling roles cell-autonomous to the endothelium. Two modes of signaling may be at work: 1) Autocrine signaling where VEGF secreted from the endothelium acts directly on its own cell-surface receptors or 2) Intracrine signaling where VEGF co-localizes with its receptor in sub-cellular compartments and induces an intracellular signaling cascade. While direct evidence for autocrine or intracrine have not solidified, genetic and cellular expreiments clearly demonstrate that endothelial VEGF is crucial for endothelial viability.

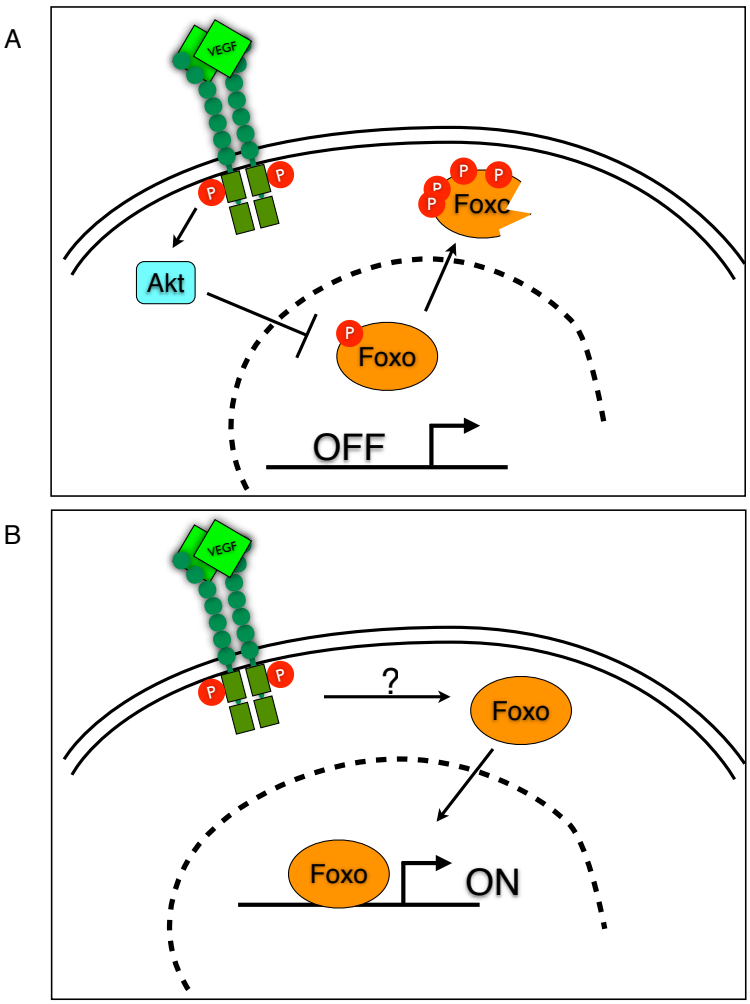


Figure 1.3 Conflicting interactions between VEGF and Foxo
A) High doses of VEGF induces Akt activation which phosphorylates Foxo transcription factors. Foxo is then shuttled out of the nucleus and degraded, and so is absent from transcriptional targets.
B) Experiments studying transcriptional responses to VEGF have shown that the presence of Foxos are crucial for downstream expression of some genes.

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Chapter 2

Recent advances in vascular development

Abstract

Purpose of Review

This review offers a concise summary of the most recent experimental advances in vascular development using the mouse as a model organism.

Recent Findings

Recent mouse studies have revealed a spread of phenotypic diversity between endothelia of distinct developmental origins and organs. For example, expression of unique transcription factors distinguishes hemogenic from non-hemogenic endothelium within the same vessel. Vasculature of the brain is particularly susceptible to endothelial malformations due to combinatorial germline and somatic mutations; surprisingly these mutations can afflict the endothelium by either cell autonomous or paracrine effects. Mutant mice have been used to understand how multiple signaling pathways integrate and refine cellular responses. In particular, we learned how VEGFR3 regulates Notch signaling and EphrinB2 coordinates VEGFR2 responses. The regulation of Prox1 by miR181 highlighted the contribution of microRNAs in the induction of lymphatic endothelium. Information gained on heterotypic interactions has further clarified the influence of blood vessels on the morphogenesis of parenchyma and contributed to our understanding of organ-specific endothelial differentiation. Finally, mouse models have uncovered endothelial cell polarity as a keystone for successful vascular lumenization.

Summary

Our understanding of the process of vascular development has gained significant refinement in the last two years and has clarified the origin of several disorders rooted in development.

Keywords

Alagille syndrome, cerebral cavernous malformations, hemogenic endothelium, macrophage, pericyte, vascular development, vascular lumen.

Key Points

* Hematopoietic stem cells are derived from hemogenic endothelium. Hemogenic potential of the endothelium relies on expression of Runx1 and suppression of HoxA3.
* Cross-interactions of distinct signaling pathways such as Ephrin-B2/VEGF or VEGF/Erk1/2, can significantly change cellular output.
* Endothelial cell polarity has emerged as a necessary pre-condition for vascular lumen formation.
* Vascular diseases that first present in adulthood, such as cerebral cavernous malformations and brain arteriovenus malformations, often have origins in developmental defects.
* Heterotypic cell interactions have emerged as critical regulators of vascular morphogenesis and organ specific differentiation.

Introduction

Efforts in experimental research during the last two years have continued to seek molecular information to better explain the morphogenesis of the vasculature, its plasticity and ability to provide unique functions to specific organs. We have identified five areas where the use of the mouse model has particularly excelled our understanding of vascular development. In this review, we summarize the highlights in these areas and place the latest advancements in the context of existent knowledge.

Hemogenic endothelium: the origin of definitive hematopoietic cells

Endothelial and hematopoietic lineages are spatially and chronologically linked at the onset of development[1]. This close relationship has long prompted the hypothesis that hematopoietic and endothelial cells emerge from a common progenitor: the hemangioblast[2]. However, blood emergence has been observed in close contact or “budding” from endothelium in a number of vascular beds including the dorsal aorta, the aorta-gonad-mesonephros region (AGM), the vitelline artery and the placenta[3]. These observations elicited the historical speculation that a subset of endothelial cells hold hematopoetic potential, (hemogenic endothelium). During the last four years, a preponderance of experimental evidence supports the notion that definitive hematopoietic stem cells (HSC) do in fact originate from a specialized endothelium with temporally restricted hemogenic capacity[3-7]. *In vitro* work further verifies that hematopoietic cells originate from an endothelial intermediate. Live culture imaging of mouse Flk-1+ hemangioblasts derived from embryonic stem cells demonstrate an endothelial stage prior to HSC emergence. Cultured hemangioblasts first generate phenotypically defined endothelial cells which then begin to express hematopoietic transcription factors, such as Runx1. Subsequent culture of these Runx1+ endothelial cells results in the emergence of rounded cells with hematopoietic markers[6]. Importantly, in human tissues, hematopoietic cells in the AGM were found to co-express Vascular Endothelial (VE) cadherin and CD45 showing concurrent endothelial and hematopoietic traits[5].

Curiously, the hemogenic capacity of endothelial cells is limited to specific vascular beds, and temporally restricted to a narrow developmental window. This begs the question: How is this endothelium specified to such a remarkable function? While the full answer to this question remains incomplete, lineage tracing experiments revealed that all hemogenic endothelium of the embryo originates from the lateral plate mesoderm (Hox6B positive); while non-hemogenic endothelium does not express Hox6B[*8]. Furthermore, hemogenic endothelium lineage restriction has been shown to rely on the expression of Runx1 and absence of HoxA3 **(Figure 2.1)**[**9]. HoxA3 is expressed in embryonic vasculature in a mutually exclusive pattern to Runx1. Induction of HoxA3 expression in embryonic stem cells and *ex vivo* cultures repressed hematopoiesis, but not endothelial fate, indicating that expression of HoxA3 limits areas of hemogenic endothelium[**9]. Interestingly, Runx1 is able to "rescue" hematopoiesis and de-regulate endothelial markers, which corresponds to the transient Runx1 expression on budding hematopoietic cells found at hemogenic sites.

Coordinated signaling of multiple pathways in endothelial cells

Understanding the regulation of concurrent operational signaling pathways has been a problem challenging to tackle as the integration of signaling inputs and outputs is difficult to trace. Nonetheless, recent advances have shed light on how signaling interplay is able to finely tune cellular output.

Ephrins are transmembrane ligands that interact with Eph receptors and mediate bidirectional signaling: "reverse" signaling via the cytoplasmic PDZ domain of ephrin and "forward" signaling into the Eph receptor tyrosine kinase. EphrinB2 is specific to arterial endothelium and required for developmental angiogenesis, though its mechanism of action has been mysterious. Mutations in the PDZ-domain of ephrinB2 result in a reduction of vascular sprouting and filopodia extensions; while overexpression of ephrinB2 increases filopodia and motility to such an extent that mature vasculature formation is compromised[10-11]. EphrinB2 protein was found to be concentrated on filopodia of tip cells[11]. This is consistent with the

hypothesis that EphrinB2 regulates the directional response to a growth factor gradient, such as VEGF. The effects of ephrinB2 on VEGFR2 and VEGFR3 were assessed *in vitro* and *in vivo*. It was found that when ephrinB2 is compromised (by absence or disruption of PDZ signaling) VEGF receptors are not properly endocytosed, which in turn reduces signaling output[10-11]. These experiments suggest that because of its location, ephrinB2 may be critical in offering precise spatial control of VEGF receptor activity.

Induction of collateral arterial growth remains a critical problem in heart and limb ischemia, particularly since these vessels are poorly responsive to growth factor stimulation. It has been proposed that defective activation of ERK might be the common denominator of this impaired arteriogenesis[**12]. This conclusion was reached by following signaling pathways downstream in a synectin-null mouse model that displays defects in arterial morphogenesis[13]. Lack of synectin is accompanied by a reduced response to VEGF and ERK1/2 activation. Furthermore, it was found that arterial defects due to either synectin inactivation or LDL-R and ApoB48 mutations can be almost completely corrected by enhancing ERK1/2 activation *in vitro* or *in vivo*[**12]. This work demonstrates that ERK1/2 signaling is a key regulator of arterial growth, and that growth factor-resistant vessels can instead respond to downstream enhancement of ERK1/2.

Notch-Delta signaling has been shown to coordinate the ratio of tip and stalk cells during vascular sprouting. More recently, VEGFR3 was found to positively-regulate Notch signaling[14]. Endothelial ablation of VEGFR3 resembles a Notch inactivation phenotype that is typified by overabundance of tip-cells[14]. This phenomenon is due to a passive VEGFR3 signaling mechanism, as antibody blockade of VEGFR3 or ligand ablation does not recapitulate the knockout phenotype[14].

The contribution of microRNAs (miRs) to the regulation of vascular development is largely unknown and in fact, the prevalence of miR up-regulation in disease states implies that these molecules are early responders triggered to maintain homeostasis. Nonetheless, novel contributions of miRs during the neo-angiogenic process have been revealed in the past few

years[15-18]. More recently, miR-181 was found to regulate Prox1, a transcription factor required for lymphatic endothelial cell specification. miR-181 is highly expressed in the vasculature, but significantly reduced in lymphatic endothelial cells, reciprocally to Prox1 expression[**19]. Forced expression of miR-181 in embryonic lymphatic endothelium reduced lymphatic markers and induced vascular endothelial markers. This data suggests miR-181 is crucial for lymphatic/ endothelial lineage divergence[**19]. Although endogenous miR-181 expression was examined *in vivo*, a miR-181-null mouse model would conclusively reveal the biological relevance of the miR regulation to vascular and lymphatic development.

Lumen Formation

Successful lumenization of vessels requires concurrent changes in endothelial cytoskeleton, cell-cell adhesion and cell-matrix adhesion. Current findings suggest these events hinge upon the formation and maintenance of endothelial cell polarity.

Beta1 integrin has emerged as a novel regulator of endothelial cell polarity and lumen formation in the developing vasculature[*20-21]. Conditional loss of Beta1 integrin in endothelial cells generated lumenal obstructions in multiple vascular beds of the mouse embryo. Cell tracing experiments revealed that the occlusions consisted of cuboidal endothelial cells that were aberrantly adherent to neighboring cells in all directions. The phenotype ultimately resulted in embryonic lethality between E14.5-17.5[**19]. Supporting these findings, the cell-polarity protein Par3 was down-regulated in the absence of Beta1 integrin. Multiple adhesion proteins including VE-cadherin, CD99 and PECAM were found to be mis-localized on those endothelial cells lacking the integrin[**19]. The findings are consistent with a previously considered role for Beta1 in the cell polarity of epidermal cells[22-23].

Further insight on lumen formation was gained from research on the novel endothelium-restrictive small GTPase Rasip1[24]. Genetic inactivation of Rasip1 results in lethality by E10.5 [**25] with phenotypes that strikingly resemble the polarity defects observed in Beta1 Integrin deletion mutants[*20]. Rasip1 mutants exhibited severe vascular remodeling defects and displayed poor

blood flow due to occluded lumens in multiple vascular beds[**25]. Rasip1 and its newly described binding partner Arhgap29 affected the activation, though not levels, of Beta1 integrin[**25]. The data indicates that Rasip1 is upstream the Beta1 integrin pathway and in this way contributes to the regulation of endothelial polarity **(Figure 2.2)**.

In addition to molecular signaling events, mechanical forces are important for launching successful lumenization. For a brief period of time before lumenization, cords of endothelial cells form transient apical cell-cell contacts where the lumen eventually emerges. It has been observed that in the time preceding lumenization, the CD34 sialomucin (Podocalyxin/gp135) accumulates on this apical/cell-cell contact face in the mouse aorta[*26]. Because the CD34 sialomucin is decorated with a large number of negatively charged sialic acids, it was hypothesized that lumenization depends on CD34-driven electrostatic repulsion between the adjacent membranes **(Figure 2.2)**. Enzymatic removal of the negative charge and subsequent whole-embryo culture yield failure of aorta luminization, while restoration of the negative charge by injection of dextran sulfate rescued lumenization [*26]. This study provides a mechanical explanation for the breakdown of cell-cell adhesion in the emerging cord, though it is surprisingly not sufficient for lumenization. Xu et al. observed that, despite failure to lumenize, CD34 is correctly localized between endothelial cells in Rasip1 mutant mice[**25]. This suggests that the electrostatic force generated by CD34 localization is necessary, but not sufficient to drive lumen formation.

The formation of lumenized vessels requires a 3 dimensional matrix that appears to trigger signaling events which initiate the process of tubulogenesis[27]. Furthermore, it has long been recognized that fibroblasts are required in culture to drive vascular lumen formation, however the specific molecules responsible were only identified recently[28]. Fibroblast-secreted factors were shown to significantly stiffen the matrix gel surrounding sprouting endothelial cells; this is predicted to facilitate lumen formation by providing mechanical strength to endothelial cells necessary for adherence[28].

Closely regulating the size and shape (if not directly the genesis) of lumens, Notch has been shown to act cell-autonomously in endothelial cells[29]. Notch was linked to lumen size and vessel patterning in gain- and loss-of-function mutants. Over-expression of activated Notch4 in the endothelium leads to dilated vessels and death at E10.5. Conditional inactivation of endothelial Notch4 or Notch1 results in smaller lumens and mortality at E9.5[30]. Similar findings were also documented more recently in an endothelial-specific RBPJ knockout model[31].

Molecular basis of vascular diseases that hold developmental roots

Cerebral cavernous malformations (CCMs) are vascular lesions located in the central nervous system and are characterized by an abundant number of vascular tufts with extremely thin walls[32]. Using standard linkage analysis, three genes (CCM1, 2 and 3) were initially identified as hereditary causative components for the disease, albeit functional information on these proteins was absent[33]. More recently, somatic mutations have been shown to cooperate with germline CCM mutations to result in loss of heterozygosity and, ultimately, manifestation of the disease[34-35]. These discoveries together with the generation of a homozygous mutant mouse and mutant cells have shed light into how CCM proteins function to provide structural stability to the endothelial lining.

Inactivation of both copies of either CCM1 or CCM2 in mice results in early embryonic lethality; though heterozygous animals show no phenotype, possibly because mice do not live long enough to experience a second somatic mutation[36]. To model the two-hit mutation hypothesis, McDonald and colleagues crossed CCM1 heterozygotes with a mismatch repair-deficient background. Imposition of genetic instability was sufficient to facilitate a second hit mutation and resulted in animals that recapitulated most hallmarks of CCM disease[**37]. An alternative mouse model for CCM was created by inducing deletion of CCM at several stages post-birth. Tamoxifen-induced deletion at P1 generates CCM-like lesions in the cerebellum and retina a week post-induction[38]. Interestingly, deletion in adult animals did not yield CCM lesions.

This suggests that CCM disease progression may, in fact, be dependent on active angiogenesis, which in humans could occur due to age-related hypoxic events[38].

Although endothelial depletion of CCM proteins has been shown to cause lesions, an endothelial non-autonomous effect of CCM3 deletion was also reported. Deletion of CCM3 in various non-endothelial cell types of the central nervous system yield broadly spread embryonic lethality[39]. Gfap-cre ablation of CCM3 led to an abundance of astrocytes accompanied by dilated, simplified vessels in the brain. About two-thirds of these mice developed CCM-like lesions. Together, the data favors an alternative mechanism of CCM formation based on paracrine signaling.

Another recent development that has contributed to our understanding of hereditary vascular disorders has been in the area of brain arteriovenus malformations (BAVMs). BAVMs are often lethal vascular lesions composed of abnormal arteries and veins. Because BAVMs exhibit no clinical manifestation until adulthood, it is unclear whether they represent a developmental defect or an adult pathology. Abnormal TGF-beta signaling has been associated with BAVMs as mutations in this pathway are correlated with AVMs in multiple organs in human patients[40]. It was found that the TGF-beta-activating integrin Beta8 has reduced expression in human BAVMs[41]. Local deletion of Beta8 in the brain reduces TGF-beta activation and induces enlarged dysplastic capillaries similar to BAVMs. Furthermore, certain Beta8 SNPs in humans were found to correlate with disease progression, suggesting that lack of Beta8 contributes to BAVMs[41].

The Notch pathway has been implicated in a variety of hereditary disorders including Alagille syndrome and patent ductus arteriosus. Alagille syndrome is a multi-symptomatic disorder primarily characterized by a paucity of intrahepatic bile ducts in the liver. This disease has been linked to mutations in Jagged1 (95% of the cases) and in Notch2 (5% of the cases)[42] . The onset and cellular origin of the disease was not well understood until recently. Interestingly, deletion of Jag1 in vascular smooth muscle cells (SMCs) recapitulates Alagille's paucity of bile ducts[*43]. It was found that biliary progenitors were unable to organize into tubes in mutant

animals, and instead remained in a single layer adjacent to the portal vein*[43]. This unexpected finding demonstrates an instructive role of the vasculature in liver / biliary duct morphogenesis, and provided a clear understanding as to the cellular origin of the disease.

Patent ductus arteriosus, the most common congenital heart defect found in humans may also be caused by aberrant Notch signaling. Feng et al. developed a mouse model of patent ductus arteriosus by deletion of the Notch ligand Jag1 in vascular SMCs that results in lethality at P2*[44]. Immunofluorescence analysis shows that these mice have defects in SMC differentiation, particularly in the ductus arteriosus. The authors found that injection of idomathacin (an anti-inflammatory drug used to inhibit prostanoids) rescues ductus arteriosus closure in approximately 50% of the SMC KO mice*[44]. This study suggests that Jag1 is crucial for SMC differentiation that allows closure of the ductus arteriosus by SMC contraction.

Heterotypic cell interactions in vascular development

Vascular morphogenesis requires the collaborative effort of multiple cell types. Supporting cells from many different lineages have been shown to enhance and direct vessel development in specific organ beds. Individual organs have distinct cell types that impose unique phenotypic characteristics to the endothelium, however the molecular underpinnings are far from clear.

Recent literature highlights the contribution of macrophages to angiogenesis. Specialized macrophages called tumor-associated macrophages (TAMs) and Tie-2 expressing monocytes (TEMs) promote pathological tumor angiogenesis[45]. The discovery of pro-angiogenic macrophages launched interest in the role of these cells during normal developmental angiogenesis. Embryonic Tie2-positive monocytes were observed at active sites of angiogenesis in developing embryos, suggesting an active contribution to angiogenesis[46]. Furthermore, expression profiles of TEMs sorted from mammary tumors were compared to embryonic TEMs, which showed a significant overlap in expression[46].

While the above studies suggested a vague role for macrophages in vascular development, Fantin and colleagues determined that tissue macrophages specifically promote anastamosis. Yolk sac-derived macrophages in the developing hindbrain are found at points of bridging endothelial tip cells. They accumulate at highest numbers when the vasculature is organizing a network at E11.5, suggesting that macrophages aid in the bridging of tip cells[**47] **(Figure 2.3)**. Absence of yolk-sac macrophages in the PU.1-null mouse results in a reduced number of vascular intersections rather than number of tip-cells. Furthermore, no reduction in VEGF levels was detected. Macrophage depletion shows a phenotype distinct from that of VEGF gradient defects, which reinforces the idea that macrophages aid EC anastamosis rather than promote sprouting[**47].

It has been proposed that lymphatic endothelium incorporates trans-differentiated macrophages into the endothelial wall. Gordon and colleagues showed that although intimately localized, a macrophage/lymphatic transition cell was never observed and historical macrophage tracing never yielded lymphatic endothelium that was positive for the tracer. Macrophages do, however, influence lymphatic development. Loss of macrophages results in hyperplastic lymphatic vessels due to overproliferation of dermal lymphatic endothelium[48]. These results indicate a directing role for macrophages in the size, shape and complexity of the developing vasculature.

Endothelial cells vary phenotypically from tissue to tissue due to their interactions with distinct cell types. Strikingly, the blood-brain barrier (BBB) requires endothelial cells to act as the major impediment to the passage of solutes from the blood; a process that depends on specialized, complexed tight junctions and a lower rate of vesicular transport. Armulik and colleagues determined that this endothelial behavior is imposed, in part, by pericytes. Using two pericyte-deficient mouse models, it was found that a lack of pericytes was correlated with increased water content and accumulation of BBB-impermeable dyes in the brain[*49] **(Figure 2.3)**. The increased BBB permeability was attributed to a defect in transcytosis of the endothelial cells. Analysis of microvascular fragments indicated that in the absence of endothelial-pericyte

contact, lower levels of several endothelial BBB markers were noted*[49]. This data indicates for the first time that pericyte contact with endothelial cells induces changes in expression that induce specialized BBB endothelial phenotype.

Relationships between vessels and the organs they inhabit are reciprocal; just as parenchyma informs endothelial development, so too can endothelial cells direct organ differentiation. As previously mentioned, perturbation of vessel function by deletion of Jag1 in vascular smooth muscle cells disrupts the differentiation of bile ducts in the liver*[42]. Correspondingly, adipose tissue development, as studied in the postnatal epididymal adipose tissue (EAT) model, is dependent on preceding angiogenesis. When angiogenesis in EAT is blocked by injection of a VEGF-Trap, adipocytes are of much smaller size and fail to cover the entire vascular network[50] **(Figure 2.3)**. This research points to an important role held by the vasculature in the regulation of tissue growth and reciprocal instructive interactions.

Conclusion

During the last two years, a significant number of advancements have been achieved using mouse models. In particular, signaling experiments have left the culture dish to find a home in a whole-organism. However, weaving together a multitude of signaling pathways to a point where a complicated input translates to a known cellular output will progressively impose additional challenges. Yet, it is remarkable that using the mouse model, major strides were made towards understanding vascular lumen formation. Future progress in this field also hinges on the development of cell culture systems that are amenable to concurrent live imaging and flow.

Vascular diseases often present no symptoms until adulthood, although the pathology can originate from a developmental defect or genetic incident. Understanding the molecular and cellular origins of vascular diseases will expand the toolbox available for diagnosis and treatment of affected patients. Equally important, this research is critical to our understanding of fundamental developmental processes. In particular, the vasculature appears to hold the ability

to impart inductive signals to the adjacent parenchyma and directly contribute to the ultimate 3D architecture of an organ.

Finally, the discovery that certain regions of endothelium have hemogenic potential has opened a pandora's box: what exactly are the elements that specify hemogenic capacity? Can this capacity be regained? Recent findings suggest a specialized transcription machinery that is distinct to hemogenic endothelium. Reproduction of this capacity *ex vivo* holds significant therapeutic value.

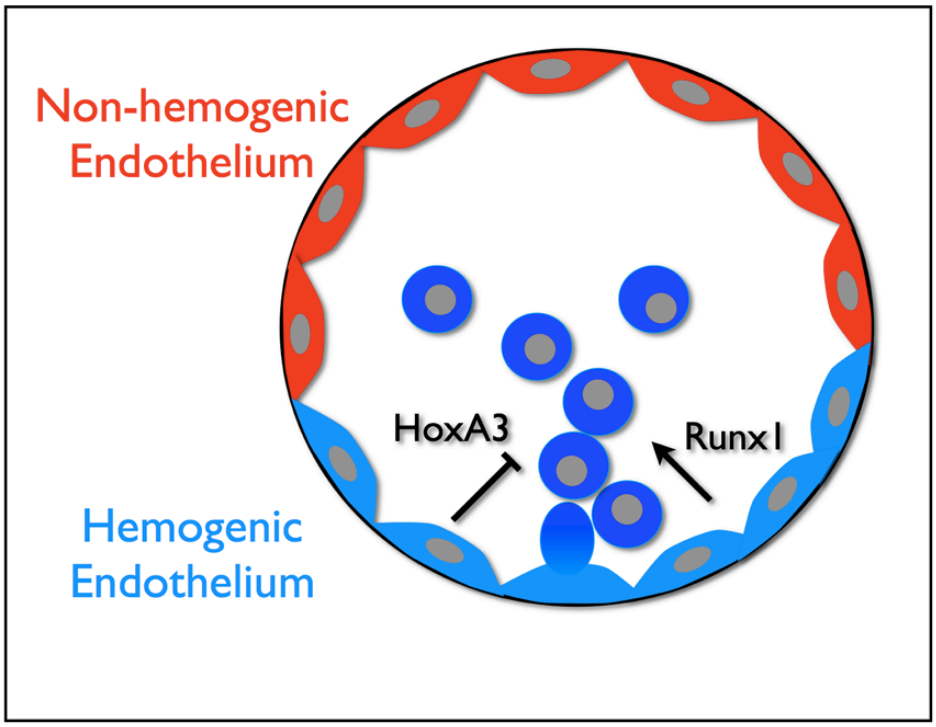

Figure 2.1 Hemogenic potential of the dorsal aorta
The endothelium of the dorsal aorta is derived from the lateral plate mesoderm. This endothelium, however, contains two functionally distinct regions: the hemogenic endothelium (ventral) and non-hemogenic endothelium (dorsal). HoxA3 expression suppresses the hemogenic capacity of endothelium, while the hematopoietic transcription factor Runx1 induces hematopoiesis.

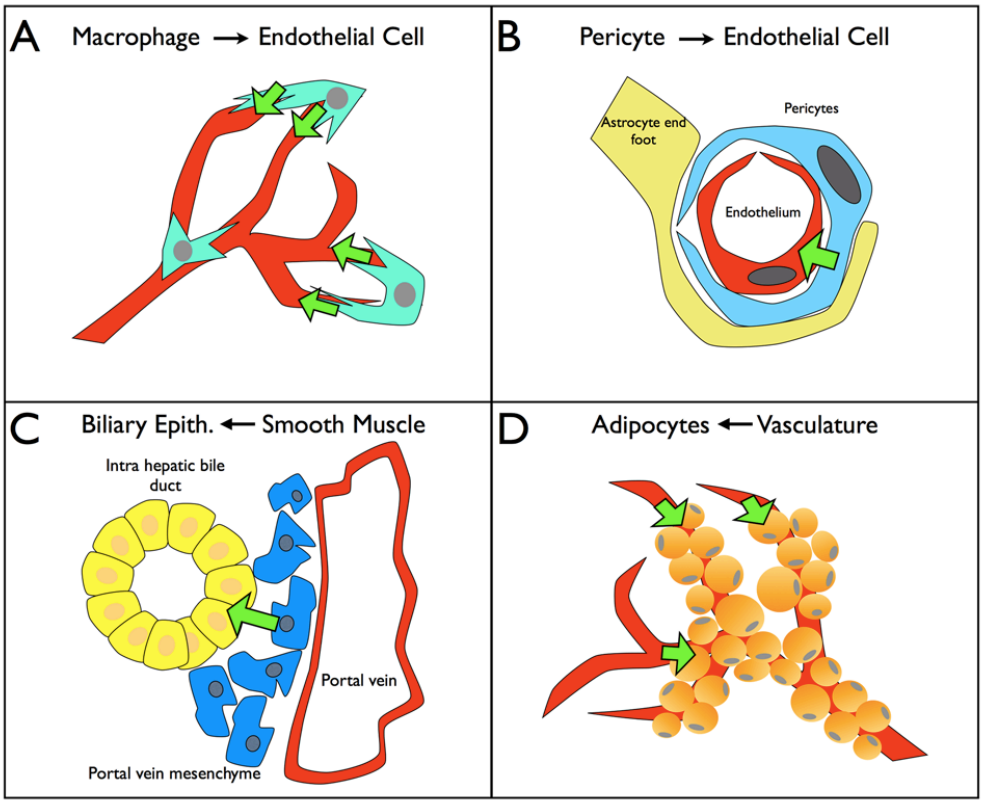

Figure 2.2 Heterotypic cell-cell interactions in vascular development
A) Macrophages mediate bridging and connection between tip-cells in the retina and central nervous system. B)Pericyte contact with endothelial cells in the CNS induces cellular changes that allow proper blood brain barrier function. C) Deletion of Jag1 in the portal vein mesenchyme disturbs the development of intrahepatic bile ducts. This is an example of instructive signaling of vascular cells to the adjacent parenchyma. D) Preceding angiogenesis is required for proper adipocyte differentiation.

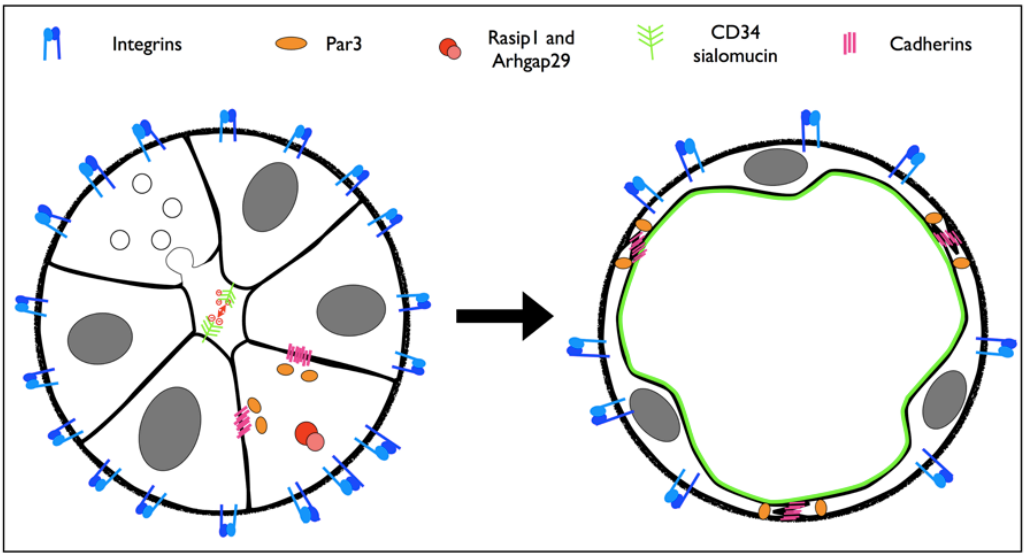

Figure 2.3 Molecular regulation of vascular lumen emergence
Lumen formation requires the concurrent efforts of many molecules. Beta1 integrin binds to the extracellular matrix and initiates the process of endothelial cell polarization. Recent studies indicate that adequate levels of Beta1 integrin are necessary for expression and localization of the intrinsic polarity protein Par3, and other cell-cell interaction proteins such as VE-cadherin, PECAM-1. Rasip1 is a small GTPase necessary for vascular lumenization. Rasip1 interacts with binding partner Arhgap29 and is needed to activate Beta1 integrin, suggesting an upstream role. Vascular lumenization relies on the concerted exocytosis of large vacuoles which join to become the common lumen, a process disrupted by the deletion of Beta1 integrin. Negatively-charged CD34 sialomucins provides electrostatic repulsion between opposing endothelial faces poised to lumenize. In mature vessels, CD34 is a marker for the apical surface of endothelial cells.

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Chapter 3

Cell-autonomous VEGF in the developing embryo

Abstract

Vascular Endothelial Growth Factor (VEGF) is a secreted factor that is known to act in a paracrine manner to direct developmental angiogenesis. However, emerging reports demonstrate that VEGF plays autocrine roles as well, though the extent of this function is not fully explored. Here we show, using an unbiased chimeric VEGF KO mouse model, that autocrine VEGF is dispensable in many tissues except regions of the brain and small capillaries under the skin. In a phenomenon that has not previously been described in the literature, we found that VEGF KO cells were preferentially recruited to the atrium of the heart but not the ventricles. To explore the biological relevance of this finding, we determined that VEGF is not expressed in the atrium but is highly expressed in the ventricle in a specific developmental window. We hypothesized that cell-autonomous expression of VEGF might be a determinant of atrial vs. ventricular cardiomyocyte cell fate, and so induced VEGF expression in the atrium using a Sarcolipin-Cre (Sln::Cre) which would directly test this hypothesis. However this approach did not yield any information due to the time-sensitive expression window of Sarcolipin after recruitment of the secondary heart field. Induction of VEGF at an earlier time point was achieved with SM22-Cre (SM22::Cre). Indeed, findings from this cross resulted in a phenotypically and molecularly ventricularized atria. These results suggest that VEGF is a crucial determinant of a cardiomyocyte's ventricular or atrial fate in a small developmental time window.

Introduction

Vascular endothelial growth factor (VEGF) was initially identified as a secreted protein that acts in a paracrine manner on endothelium and other cell types that express VEGF receptors, namely VEGFR2, VEGFR1 or Nrp1 [1,2]. VEGF has been well defined as the key cytokine required for vessel growth and development, and much of the existing literature supports this “canonical” function. However, “non-canonical” roles for VEGF have been emerging suggesting an autocrine signaling function that, though poorly-described, is critical for endothelial survival and homeostasis [3].

It has been well established that VEGF is crucial for embryonic development, as it was in fact the first heterozygous lethal mutant described [4,5]. Heterozygous VEGF-KO mice die between E11 and 12 suffering from several intertwined phenotypes that broadly affect the cardiovascular system. VEGFR2+ (VEGF’s major receptor and endothelial cell marker) cells are present indicating some level of endothelial cell differentiation, but capillary morphogenesis is sparse and grossly disorganized [5]. Other phenotypes include delayed positioning and segmentation of forelimb buds, underdevelopment of the forebrain, and increased apoptosis throughout the body. Interestingly, the heart displays developmental delay as well as a thinner cardiomyocyte layer, particularly in the ventricular wall [5].

While the VEGF KO phenotypes observed in many organs are often dismissed as secondary to vascular defects, they do not entirely overlap with the phenotypes of other mutants with abrogated vasculature or blood-flow defects. The complete VEGFR2-KO is lethal at E9.5-10, appears to entirely lack blood vessels and even blood islands [6]. However, these mutants have a similar number of somites to their WT counterparts and gross organization of the body is intact.

To phenotypically separate simple blood delivery from other vascular functions, one may examine a mouse model with normal vascular development, but defective heart beat and thereby a lack of blood-flow. Ablation of the Na+/Ca+ exchanger Nkx1 abolishes cardiac heart beat and provides such a system to study the effects of blood flow (and therefore oxygen and

nutrient delivery) on embryogenesis [7]. Evaluation of this mouse mutant provides a critical control to the VEGF-KO. In the case that the VEGF-KO phenotype was simply the result of oxygen and nutrient deprivation due to malformed vasculature then one would expect Nkx1-KO and VEGF-KO to phenocopy each other. However, unlike the VEGF-KO, Nxk1 mutants develop limb buds, neural tube and somites at a similar pace to their WT counterparts up until E9.5, after which they die from lack of circulation [7].

These results suggest a subtle role that is distinct from the “canonical” function where VEGF activates endothelium for angiogenesis. Because vessels permeate and are crucial for the function of virtually all organs, it has been incredibly difficult to distinguish between direct action of VEGF on an organ and indirect action on the vasculature of that organ. Several groups have identified direct cell-autonomous action of VEGF on non-endothelial populations by genetic ablation, comparing blockade of extracellular and intracellular VEGF and cell-culture systems. Using these techniques, cell-autonomous VEGF has been shown to play a role in hematopoiesis [8], maturation of megakaryocyte precursors [9], podocyte function [10] as well as maintenance of several tumor populations [11,12].

Further exploring “non-canonical” (non-angiogenic, non-paracrine) roles for VEGF, it was found that ablation of VEGF from the endothelial compartment results in vessel deterioration and sudden death in adult mice [3]. This unexpected result demonstrates that continuous low-level VEGF expression within the endothelium is crucial for normal vascular homeostasis, and further indicates a cell-autonomous role for VEGF.

To investigate possible cell-autonomous requirements for VEGF in an unbiased way, we examined the developmental potential of embryonic stem cells (ESCs) lacking VEGF in a WT embryo environment. Several chimeras were recovered and it was found that an approximate chimerism of 25% was tolerated in these viable embryos. We found that cell-autonomous VEGF is not an absolute necessity for endothelial development of large vessels but may in fact be more important for microvascular integrity.

Surprisingly, we observed that VEGF KO cells seemed to be specifically recruited into the atrium of the heart but not the ventricles. This result was reflected in the observation that VEGF is expressed at a very low level in the atrium and higher in the ventricle over a specific developmental window. Finally, VEGF loss- and gain-of-function was performed within the atrial compartment to explicate the importance of VEGF expression on its development. Forced expression of VEGF in the atrium induced “ventricularization” of the atrial wall when induced in this compartment at an early differentiation time point (E9) but had no effect when induced later (E12.5).

Materials and Methods

Stem cell culture

Embryonic Stem Cells (ESCs) were grown on irradiated DR4 MEFs. Cells were fed every other day and passaged when they reached 70% confluency. ESCs lacking VEGF and control cells were a gift of Andras Nagy (Samuel Lunenfeld Research Institute; Toronto, Canada) VEGF KO stem cell generation is previously described [5]. VEGF KO stem cells were further targeted for homologous recombination with a Rosa26::EGFP construct, single colonies were picked, expanded and tested by Southern blot for integration. To analyze expression of VEGF in KO ESCs, embryoid bodies were formed by sorting ESCs from MEFS that were distinguished by virtue of the GFP marker. Cells were resuspended in ESC media without LIF and grown on low-adhesion plates for 6 days, then lysed for RNA extraction.

Generation of chimeras

Embryos were collected at day E2.5 from WT superovulated B6 dsRed or non-transgenic female mice. Embryos were cultured in incubator-equilibrated KSOM media under mineral oil overnight to blast stage. ESCs were treated with trypsin and left on gelatin-coated plate for 1hr to select out MEFs. Media containing stem cells was centrifuged to collect cells and resuspended in minimal media. Blastocysts and late-morula were placed on injection plate in

M2 media and ESCs were pipetted on top in their own media. 10-12 stem cells were collected in a glass pipette and then injected into each blast cavity. Blasts were allowed 1hr recovery in KSOM. Injected blasts were transferred into pseudopregnant females by “NSET” non-surgical embryo transfer device. Embryos were harvested from pregnant females 13 days after implantation and processed for confocal microscopy. Embryos were vibratomed into 200-300uM transverse sections throughout the body and fixed in Karnovsky’s fixative overnight. Sections were analyzed confocal microscopy.

Animals

B6 dsRed transgenic mice (B6.Cg-Tg(CAG-DsRed*MST)1Nagy/J) and WT B6 mice were obtained from Jackson Laboratory and used as embryo donors for stem cell injections. CD-1 (Charles River) females were used as pseudopregnant recipients. VEGF-IRES-LacZ mice [13], Sln::Cre mice [14], R26::VEGF mice [15], SM22::Cre [16], and floxed-VEGF mice were previously described [17].

PCR

To genotype VEGF KO cells, ESCs were sorted from MEFS by FACS and genotyped with primers in the intron between exon 3 and 4. VEGF F 5’ AAG AAG ATG TCT GCT CTG CGC TCT-3’, VEGF R 5’- TAT AAT GCC GTG GAA GGG CAC ACT- 3’. cDNA was created using SuperScript III, and VEGF mRNA amplified as previously described [3].

LacZ staining and histology

LacZ staining carried out as previously described [18]. P0 hearts were perfused briefly with 4% PFA, dissected and fixed overnight. Samples were embedded in paraffin, sectioned and H&E stained by UCLA Tissue Procurement Core Laboratory.

Results

Characterization of VEGF KO embryonic stem cells and chimera technique

To establish chimeric embryos, we first validated VEGF KO ESCs. VEGF KO ESCs were found to form colonies similar to WT controls when plated on MEFs in standard culture conditions (Figure 3.1A). VEGF KO stem cells were established by ablation of exon 3 by a neomycin cassette that includes a stop codon [5]. A genomic PCR targeting the intron between exon 3 and 4 verified disruption of WT VEGF allele as previously described (Figure 3.1A-B) [5]. VEGF -/- ESCs were differentiated into embryoid bodies and then lysed for RNA extraction, where VEGF mRNA was found to be strongly decreased in VEGF KO cells (Figure 3.1C). As expected, we observed a nominal amount of VEGF mRNA contamination from wild-type "feeder" embryonic fibroblasts which are required for the culture of ESCs.

Day 2.5 8-cell-stage host embryos were collected from super-ovulated dsRed WT B6 females and cultured overnight to blastocyst stage. Validated VEGF -/- ESCs were trypsinized into single-cell suspension, picked up in groups of 10-12 by microinjection pipette, and injected into the cavity of blastocyst-stage embryos (Figure 3.1D). Recipient dams were plugged by vasectomized males and used at a pseudo-pregnant stage of day 2.5. Successfully injected embryos were implanted into host dams by non-surgical embryo transfer (NSET). Embryo harvests were initiated 13 days after NSET, when embryos were expected to be E15.5 (Figure 3.1F). Interestingly, embryos recovered appeared to be delayed, closer to a developmental stage matching E14.5 (Figure 3.1E).

Very few live pups were recovered from recipient dams. In fact, only 5% and 7% of total embryos implanted were recovered from embryos injected with WT ESC and VEGF KO ESC respectively (Table 3.1). In total, out of 218 WT and 390 VEGF KO injected embryos, only 2 WT and 9 VEGF KO embryos with detectable chimerism were collected (Table 3.1). The low success rate of the chimera technique severely limited the sample size.

Because autocrine VEGF has been shown to be important for endothelial homeostasis in the adult [3], we analyzed vascular beds throughout the embryo to assess the requirement of

cell-autonomous need for VEGF in endothelial cell differentiation. Interestingly, endothelial cells of the large vessels take up VEGF KO cells at a rate similar to background chimerism of the animals (arrowheads, Figure 3.2A). Smaller vessels in close proximity to the skin appeared to have a stronger requirement for WT VEGF cell incorporation (Figure 3.2 B). The microvasculature imaged entirely excluded VEGF KO cell incorporation, suggesting a strong cell-autonomous VEGF requirement in this vascular bed. Interestingly, larger lymphatic vessels were found to incorporate VEGF KO cells (asterisk, Figure 3.2B).

Embryonic chimeras were surveyed for chimerism rates in many organs throughout the body, identified by histology from transverse sections. The limb (containing developing skeletal muscle and cartilage) displayed varying degrees of chimerism up to a very high incorporation of VEGF KO (Figure 3.2C, H), indicating that cell-autonomous VEGF is not required in a cell-autonomous manner for skeletal muscle development.

Developing cartilage of various tissues such as the cartilage primordium of the nasal septum (Figure 3.2D) or the limb (Figure 3.2C) displayed variable acceptance of VEGF KO cells. The nasal septum appears to exclude VEGF KO cells to a large extent, whereas cartilage developing in the limb incorporated a large number of VEGF KO cells. The skin of the digital inter-zone accepted a large number of VEGF KO cells (Figure 3.2E) indicating no need for cell-autonomous VEGF in this tissue bed. Regions of the brain excluded VEGF KO cells (Figure 3.2F) although closer analysis was not possible as many regions were either highly auto-fluorescent or did not clearly express either dsRed or GFP. Another region lacking dsRed or GFP expression was in the epithelium of the lung (Figure 3.2G), which made analysis of cell-autonomous VEGF requirement impossible. This was likely due to an unanticipated silencing of the promoters driving the fluorescent reporter genes.

VEGF KO cells are recruited to the atrium of the heart

Typically, chimeras reveal cell-autonomous functions in those tissues that require the gene because those tissues will be made up entirely of WT cells. Surprisingly, VEGF KO cells

were recruited preferentially to VEGF WT cells in the atrium of the E15.5 heart (Figure 3.3A, B). This trend appeared to be significant in the two chimeric embryos with images vibrant enough to be analyzed by cell counting. Confocal images were obtained from dissected vibratomed heart sections, or from other randomized tissue beds in the same embryo (Figure 3.3B).

To see if the observed recruitment of VEGF KO cells to the atria is maintained throughout development, chimeric hearts were harvested in neonates (P0) (Figure 3.3C). The atria, particularly the right atria (RA), clearly incorporated a high percentage of VEGF KO cells as observed in whole-mount fluoroscopy (Figure 3.3C). Confocal microscopy confirmed the enhancement of VEGF KO cells specifically in the atrium of this animal.

One chimera was allowed to mature to adulthood where it was observed to be chimeric by coat color and by all appearances healthy. This chimera was found to maintain the VEGF KO enhancement of the right atrium (Figure 3.3D), suggesting that atrial VEGF was not required cell-autonomously even in later developmental stages.

Blastocyst injections of WT ESC into WT host embryos were not successful enough to serve as good controls for these experiments (Table 3.1, Figure 3.3E). Because the WT stem cells used were from a different stock of ESCs, we speculate that they were partially differentiated or somehow changed in culture and thus did not incorporate readily into chimeras (Figure 3.3E).

Endogenous expression patterns of VEGF in the heart

Current literature does not report in detail on levels and distribution of WT expression of VEGF in the atrium and ventricles during development. To explore the physiological relevance VEGF KO recruitment to the atrium, we first investigated VEGF expression levels in the atria and ventricles using a LacZ-tagged VEGF allele (VEGF-IRES-LacZ) [13]. At early heart tube stage (E8) we observed low expression levels of VEGF throughout the heart (arrowhead, Figure 3.4A). By E9.5, the ventricular myocardium began to strongly express VEGF while the atrial tissue maintained low expression. The pattern of high ventricular and low atrial expression

continued through E14.5, but by E17.5 VEGF was present in both the atria and the ventricles at equivalent levels (Figure 3.4A). Interestingly, VEGF expression was very high in the atrium by neonatal stages and appears to be maintained through adulthood. Geriatric VEGF-IRES-LacZ mice showed a lower expression level in general, with atrial and ventricular tissues expressing similar levels of VEGF (Figure 3.4A). During a restricted developmental window (E12-E15), VEGF was not expressed by atrial tissue except in an un-identified “stripe” of tissue in the right atria (arrows, Figure 3.4A). While we could not identify this structure as a specific anatomical entity, this expression pattern was consistent among litters.

To confirm VEGF levels indicated by the VEGF-IRES-LacZ model with WT embryos, we analyzed expression by RT-PCR from carefully dissected atria and ventricles. We observed that while levels of VEGF in the atria and ventricle were similar from E9-E10, by E11 the ventricles express almost double the VEGF of the atria (Figure 3.4B). The trend of VEGF expression being higher in the ventricles persists and was even more apparent by E15, which reflects the trends observed in VEGF-IRES-LacZ histology.

Genetic models to induce VEGF mis-expression in the atrium at mid-gestation

To investigate the effect of atrial selection against VEGF expression, we initially created two genetic models to mis-express VEGF in the atria. Based on the selective down-regulation of VEGF in the atria, we hypothesized that artificially inducing VEGF levels was likely to reveal important biology associated with the role of VEGF in that tissue.

First, VEGF was “knocked-out” in the atrium using a floxed VEGF allele (VEGF-flox) [17] crossed to Sarcolipin::Cre (Sln::Cre) animals to create Sln-VEGF-KO [14] which is expressed in the atrium starting at E10.5, but not in other areas of the heart (Figure 3.4C, E). A “knock-in” of VEGF was created by crossing to a conditional Rosa26-lox-stop-VEGF164 (R26::VEGF) allele [15] to Sln::Cre to create Sln-VEGF-KI (Figure 3.4C).

Animals double heterozygous for both VEGF-flox and Sln::Cre were crossed and progeny were genotyped at time of weaning (P16-P20). To determine lethality resulting from

VEGF excision in the atrium we examined a large cohort of heterozygous crosses and recorded their genotypes (Table 3.2). While the VEGF-flox allele was observed at slightly lower frequencies than expected in the presence of Sln::Cre, several viable and macroscopically normal progeny were produced. The hearts of these mutants appeared entirely normal when compared to their WT littermates (data not shown). This phenotype matched our expectations that the Sln-VEGF-KO would be compatible with life as observed in our P20 chimera (Figure 3.3D).

Similar heterozygous crosses with the knock-in mutant were produced, genotyped, and an embryonic lethality was observed when both alleles of R26::VEGF were present in any combination with Sln::Cre (Figure 4D). Homozygous Sln-VEGF-KI were not observed as early as embryonic day E10.5 (data not shown). This result is surprising because Sln::Cre excision begins at E10.5 in the atrium and is not strongly penetrant until E12.5 [14]. Accordingly, the observed early embryonic lethality cannot be explained by an atrial defect alone.

We examined expression of Sln::Cre in early embryos to assess whether VEGF was over-expressed in embryonic regions not previously reported (Figure 3.4E). We observed that Sln::Cre is strongly expressed in the somites, which creates a situation where VEGF is over-expressed strongly in a large region of the animal. Because it has been reported that even a modest increase of VEGF levels is embryonically lethal [19], we expected that this off-target effect was going to result in early lethality in the Sln-VEGF-KI before an atrial phenotype could be observed.

Heterozygous Sln-VEGF-KI mutants were analyzed (Sln::Cre X R26::VEGF) for “ventricularization” of the atria as predicted by our chimeras (Figure 3.4F). However, we did not observe any gross developmental differences between the atria of the knock-ins and their WT litter-mates in H&E histological sections.

Early induction of VEGF expression in cardiomyocytes induces ventricularization of the atria

Given the lack of a phenotype in the gain and loss-of function studies with Sln-VEGF, we considered that perhaps induction of atrial VEGF with Sln-Cre (expression penetrant at E12.5) may occur too late in heart differentiation to result in morphological changes in the atrial compartment. We therefore created another model of VEGF “knock-in” into the heart at an earlier time point of E8.5 using the SM22::Cre. A caveat of this model is the broader targeting of the cre that expands into the entire heart and vascular smooth muscle. Thus, we crossed the conditional R26::VEGF mouse to the SM22::Cre mouse [16] creating SM22-VEGF which induces Cre expression throughout the myocardium by E8.5 [20] (Figure 3.5A).

To investigate the effects of VEGF gain-of-function over time, mutant embryos were collected at different developmental time points (Figure 3.5B). SM22-VEGF mice were found at mendelian ratios and were by all appearances normal at E10.5, including heart morphology (data not shown). Two clear death-windows were identified, the first lethality observed at E14.5, with numbers stabilizing until complete lethality which was noted at P0 (Figure 3.5B). Embryos harvested at E13.5 displayed multiple hemorrhages with a penetrance of approximately 2/3 of mutants (Figure 3.5B).

Although no SM22-VEGF mice were viable after P0, we observed irregular phenotypes at different time points. To investigate if variability was due to molecular differences in VEGF expression levels. Thus, we evaluated VEGF by RT-PCR in WT and mutant atria. It was found that mutants collected at E15.5 could be separated into two groups, likely based on Cre penetrance: 1) VEGF Low and 2) VEGF High (Figure 3.5D). It is reasonable to assume that that those animals that survived to P0 fall into the category of VEGF Low.

P0 SM22-VEGF hearts displayed a malformed ventricular shape as well as bulbous atrial tissue when compared to their WT littermates (Figure 3.5E). Histologically, the atrial appendage typical of WT animals exhibits an extremely thin and delicate wall (Figure 3.5E bars). In contrast, SM22-VEGF animals show areas with thickened atrial walls that closer resemble a ventricular tissue rather than WT atria (Figure 3.5E bars). The atrial overgrowth in SM22-VEGF animals was primarily composed of cardiomyocytes, as they stain for the

cardiomyocyte marker TroponinT (Figure 3.5E white arrowhead). Heart size was quantified, and it was found that P0 SM22-VEGF hearts were increased in total size (Figure 3.5F), ventricular thickness (Figure 3.5G), atrial area (Figure 3.5H) and atrial density (Figure 3.5I).

To determine at which point in development this phenotype begins, mid-gestation hearts were examined for atrial thickening. Subtle thickening was seen at E14.5 (Figure 3.6A), with more obvious atrial overgrowth apparent at E15.5 (Figure 3.6B asterisk).

SM22-VEGF atria were examined for atrial and ventricular markers, and were found to have a qualitative decrease in the atrial markers MLC1a and MLC2a (Figure 3.6C). Interestingly, mutant atria had a significant increase in MLCV1 (Figure 3.6D). Collectively, at the molecular level, these results suggest a switch from atrial to ventricular phenotype.

Because ventricles are heavily trabeculated, and trabeculation is known to be regulated by Notch1, we investigated the expression of different components of the Notch/trabeculation pathway in E15.5 VEGF High atria (Figure 3.6 H). Although unchanged in VEGF-Low mutants, Notch1, BMP10 and Neuregulin1 were all significantly up-regulated in VEGF-High animals (Figure 3.6 E-G).

Discussion

It is abundantly clear that VEGF levels in developing embryos must be precisely regulated. Models inducing slightly higher amounts of VEGF result in embryonic lethality [19]. The identical outcome is also found in models producing too little VEGF, such as the ablation of a single VEGF allele [4,5]. To circumvent the systemic lethality that results from ablation of the secreted growth factor VEGF, we attempted to understand the cell-autonomous role of VEGF by producing VEGF KO/WT chimeras. We hypothesized that a high percentage of VEGF chimerism would not be tolerated by embryos, and we in fact found that several viable embryos were recovered with a consistent chimerism of approximately 25%. Chimeras produced from VEGF-KO ESCs were in fact slightly more successful than those produced from a single WT ESC line, which indicates the need to repeat these experiments with additional ESC clones.

VEGF was reported to signal in an autocrine manner to maintain homeostasis within the endothelial compartment [3]. As the VEGF endothelial-KO mouse displays a 31.6% prenatal lethality, it is somewhat surprising that we did not observe a selection against VEGF-KO cells within the endothelium of the major vessels in VEGF-KO chimeras. We did observe that smaller vessels tended to exclude VEGF-KO cells, and in fact we did not see any VEGF-KO cells in this compartment within the images collected. This is somewhat contradictory to recent reports that found adult endothelial VEGF expression to be exclusively arterial, and not expressed in veins or capillaries [21]. This discrepancy may highlight a developmental necessity for autocrine VEGF in the microvasculature. This might no longer be required in the adult in these smaller vessels, but instead needed in large arteries.

VEGF has found to be important for the development of many organs in a variety of models. This conclusion has been reached from conditional ablations of VEGF compartment by compartment [22]. Results of these experiments are obscured by VEGF's role as a secreted factor acting on endothelium; phenotypes may be attributed to vascular defects rather than a direct effect on the cell type of interest. Our chimeric approach is unique in that it does not disrupt normal vascular development and will instead reveal any cell-autonomous need for VEGF within the organ compartment itself. This technique revealed that most organs do not require cell-autonomous VEGF for organogenesis (skeletal muscle, cartilage, skin, lung), though some areas of the brain may have excluded VEGF-KOs to a higher degree than the background of the organism. More careful analysis of these organs is required to make further conclusions.

VEGF has been implicated in many aspects of heart development. Ablation of VEGF from cardiomyocytes was carried out using the MLCV2 promoter driving Cre expression [23]. Hearts from the mutant embryos displayed thinned ventricular walls, decreased microvasculature and contractile dysfunction. While this phenotype was largely attributed to decreased coronary vasculature, a cell-autonomous role for VEGF cannot be ruled out. In fact, VEGF does improve differentiation of stem cells towards cardiomyocyte fate *in vitro* [24]. VEGF is essential for other processes in cardiogenesis as well. For example, VEGF signaling has been

shown to be crucial for valve development in the heart, required for EMT in the outflow tract, and responsible for the atrioventricular canal differentiation into valve leaflets [25]. We found that cell-autonomous VEGF expression was not crucial for cardiomyocyte development based on an uptake of VEGF-KO cells similar to the background chimerism of the animal.

We were perplexed by a strong enhancement of VEGF-KO cells in the atrium of the heart. To our knowledge, he phenomenon of an increased recruitment of a KO cell population has not been reported. One explanation for a tissue selecting for the *absence* of a genetic factor, is that the down-regulation of that factor is a prerequisite for differentiation into that tissue type. In this case, it would seem that for a cell to differentiate into an atrial cardiomyocyte, it must first down-regulate VEGF. Or, that from a large population of committed cardiomyocytes, those that have down-regulated VEGF are selected for atrial specification.

VEGF expression in the heart has been described in the literature with little detail [13]. Here we characterize dynamic changes in cardiac VEGF expression, focusing on differences between the atrial and ventricular cardiomyocytes. Reflective of our chimera results, we found that VEGF levels are indeed distinct between the two tissues throughout a large portion of development, with the atria expressing very low and the ventricles expressing very high levels. This coupled with our chimera results led us to believe that VEGF expression is a meaningful determinant of cardiomyocyte differentiation into the atrial or ventricular lineage.

Much is known about the molecular signatures of chamber specification in the heart, especially at the transcription factor level [26]. Growth factors have also been reported to influence atrial and ventricular specification and function as seen in the zebrafish model of type 1 BMP receptor ablation [27]. This report demonstrates a specific reduction in atrial size when gene alk8 was ablated, and went on to show that alk8 had a cell-autonomous role in this process. In this light, we hypothesized that myocardial VEGF expression would play a similar role in ventricular specification. By forcing expression of VEGF in the atrium, we expected to observe an ectopic induction of ventricular phenotype that would be reflected by dense myocardium, increased trabeculation and size.

While our initial genetic atrial VEGF knock-in model using Sarcolipin-Cre did not demonstrate a “ventricularization” of the atria phenotype, this was likely the result of missing the critical time-window when this compartment is sensitive to VEGF. Sln::Cre begins peppering the atrium at E10.5 and strongly labels the tissue by E12.5 [14]. Our data on VEGF expression levels, however, notes differences in atrial and ventricular VEGF profile as early as E9.5. To resolve this issue, we created another genetic model that targets the heart at an earlier time point.

SM22-alpha is transiently expressed in the myocardium between E8 and E12 [28]. Using SM22::Cre to induce VEGF expression in the heart at an earlier time point, we observed a cardiac phenotype consistent with our hypothesis. The atria of SM22-VEGF have thickened walls of a histological consistency similar to that of the compact layer of the ventricle, and furthermore show a decrease in atrial marker expression, and an increase in the expression of ventricular MLCV1. This finding suggests that high VEGF expression perturbs cardiomyocyte development towards compact muscular ventricular tissue phenotype. Conversely, a lack of VEGF expression allows atria to develop into thin-walled structures with delicate trabeculations typical of WT animals.

Little has been described in the literature regarding the relative lack of trabeculation in the atrium. However, ventricular chamber morphogenesis is first characterized by trabeculae formation, a phenomenon that is highly-regulated by interactions between the endocardium and the myocardium. Central to this phenomenon is the Neuregulin-1 (Nrg1)-ErbB pathway. Nrg1 is a growth factor expressed in as many as 15 different isoforms which can be secreted or membrane-anchored, and is the ligand for the erbB family of receptor tyrosine kinases. Nrg1 is present in the endocardium, and in this manner it is complementary to ErbB receptors in the myocardium. Disruption of Nrg1 [29], ErbB2 or ErbB4 results in a remarkably similar cardiac phenotype marked by a lack of ventricular trabeculation [30].

More recent work has connected Notch1 signaling to ventricular trabeculation. After observing that Notch1 activity increases in areas of trabeculation, the Notch1-KO was found to

have reduced trabeculation, accompanied by decreased expression of Nrg1 and BMP10, which are crucial regulators of myocardial proliferation [31].

Because VEGF has been reported to induce the expression of Notch1 and its ligand Dll4 in arterial endothelial cells [32], we investigated the Notch1-trabeculation pathway in VEGF over-expressing hearts. The molecular signature we observed is consistent with upregulation of the Notch1-trabeculation pathway, with elevated expression levels of Notch1, BMP10 and Nrg1 in VEGF-High mutant atria. Perhaps unsurprisingly, over-expression of Notch-intracellular domain in the atria results in atrial hyperplasia quite similar to the SM22-VEGF phenotype reported here [33], which is further evidence for activation of this pathway.

Our investigations into the heart revealed a specific and dynamic pattern of VEGF expression in development, with high expression throughout the ventricles and very low or no expression in the atria. We found that over-expression of VEGF around E8-9, but not later, was able to induce a ventricular, hyper-trabeculated molecular phenotype in the atria. These data suggest that the endogenous VEGF highly-expressed in the ventricles is a determinant of ventricular cardiomyocyte fate, while myocardium that does not express VEGF will by default adapt an atrial phenotype.

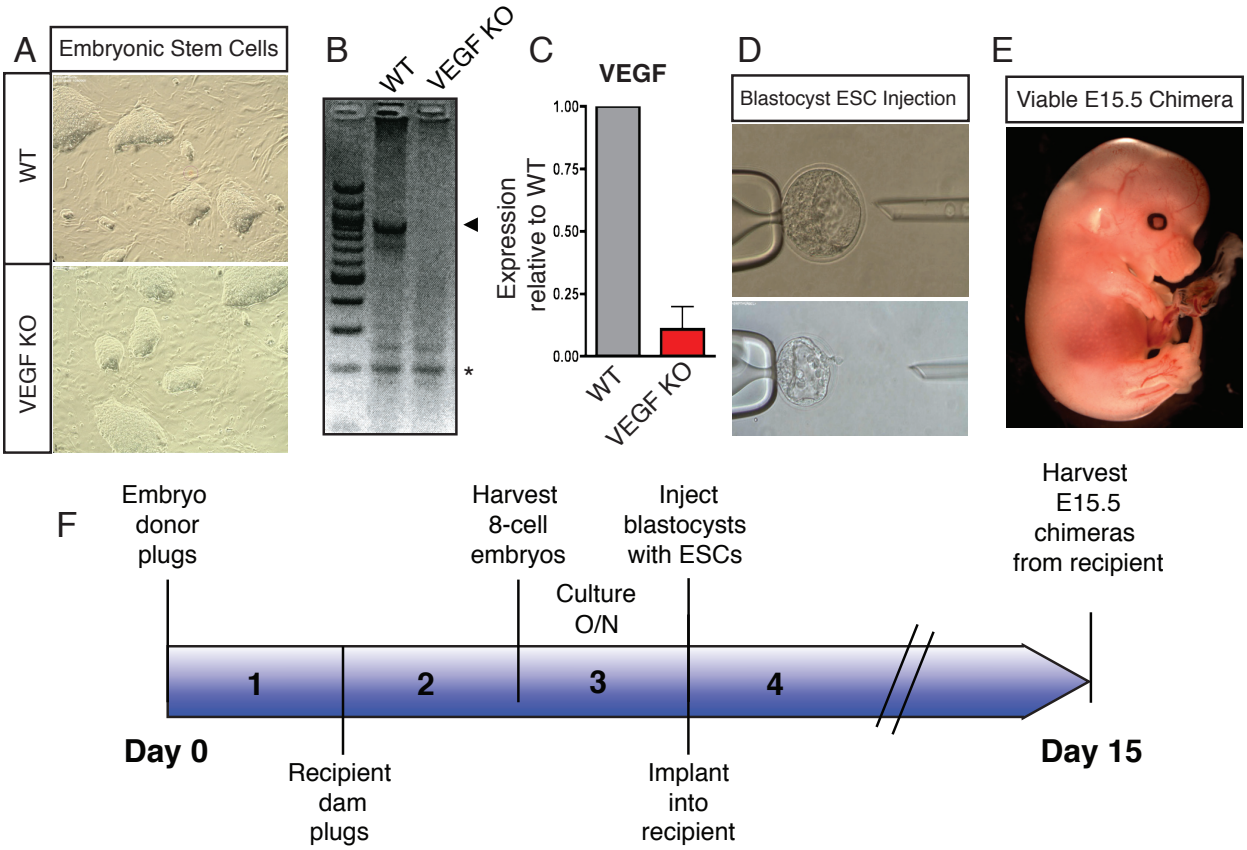

Figure 3.1 Characterization of VEGF KO ESCs and chimera technique
A) VEGF KO stem cells form morphologically normal stem cell colonies when grown on MEFs. B) Validation of genetic ablation of VEGF by PCR of intron between exon 3 and 4, band indicated by arrow. Non-specific band indicated by star. This region is ablated by a neomycin cassette and results in loss of VEGF transcript and protein. C) VEGF expression of day 6 embryoid bodies derived from sorted WT and VEGF KO stem cells as determined by RT-PCR. D) 8-cell embryos were harvested from E2.5 dsRed or WT superovulated females and cultured overnight to blast stage. Blasts were injected with stem cells. E) Viable chimeras were harvested at E15.5 F)Timeline of chimera generation.

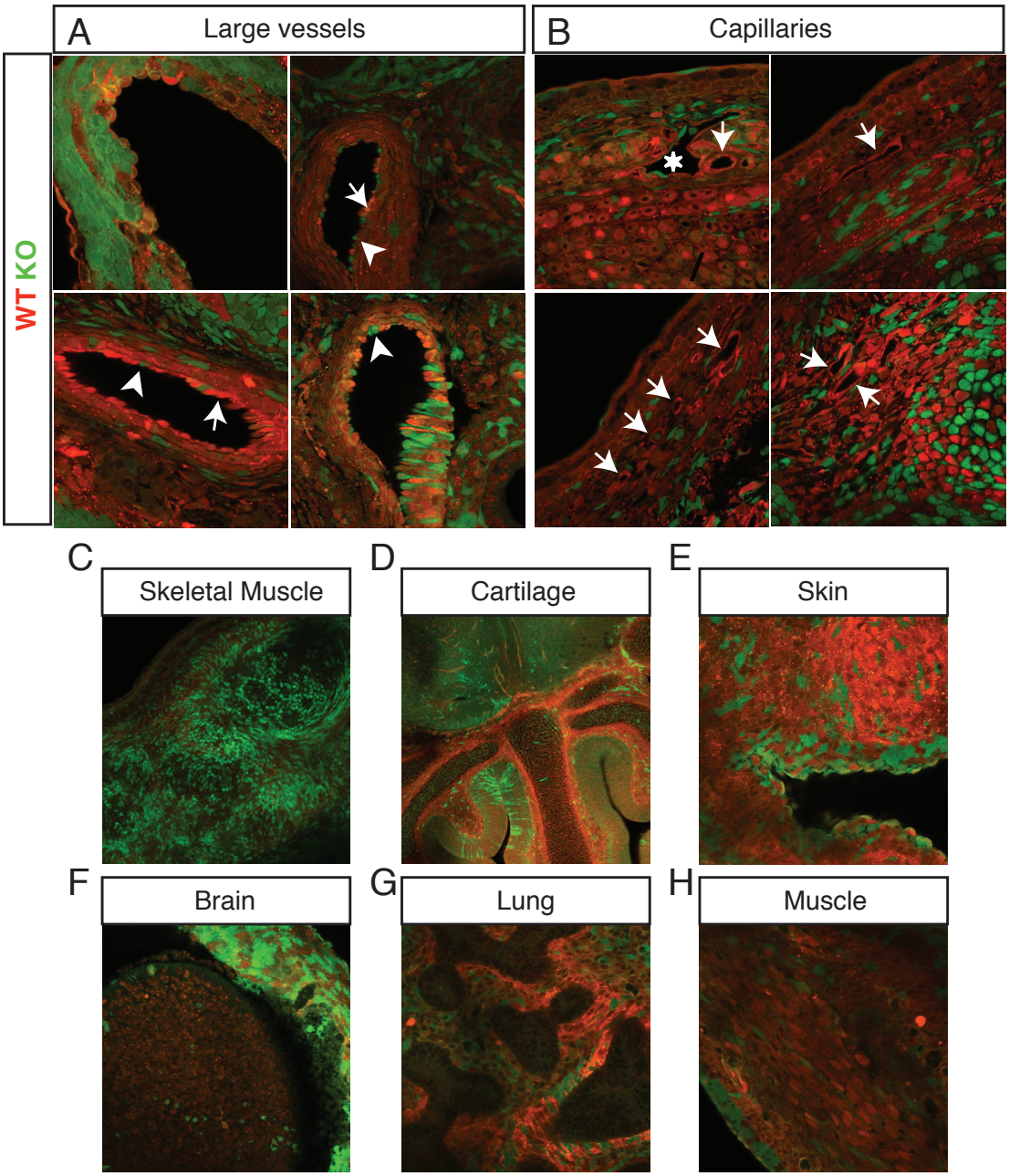

Figure 3.2: Chimerism of VEGF KO cells in vessels and many organs

A) Confocal analysis of embryos harvested at E15.5. WT host embryos express dsRed, VEGF KO ESCs express EGFP. Arrowheads show examples of KO incorporation into endothelium, arrows show WT incorporation. B) Analysis of small vessels. Arrows indicate small vessels that exclude KO cells, star shows a lymphatic vessel that has incorporated KO cells into the endothelial layer. C) High chimerism observed in limb. D) Cartilage primordium of the nasal septum E) Skin of interdigital region F) Low integration of VEGF KO cells observed in the brain G) Epithelium

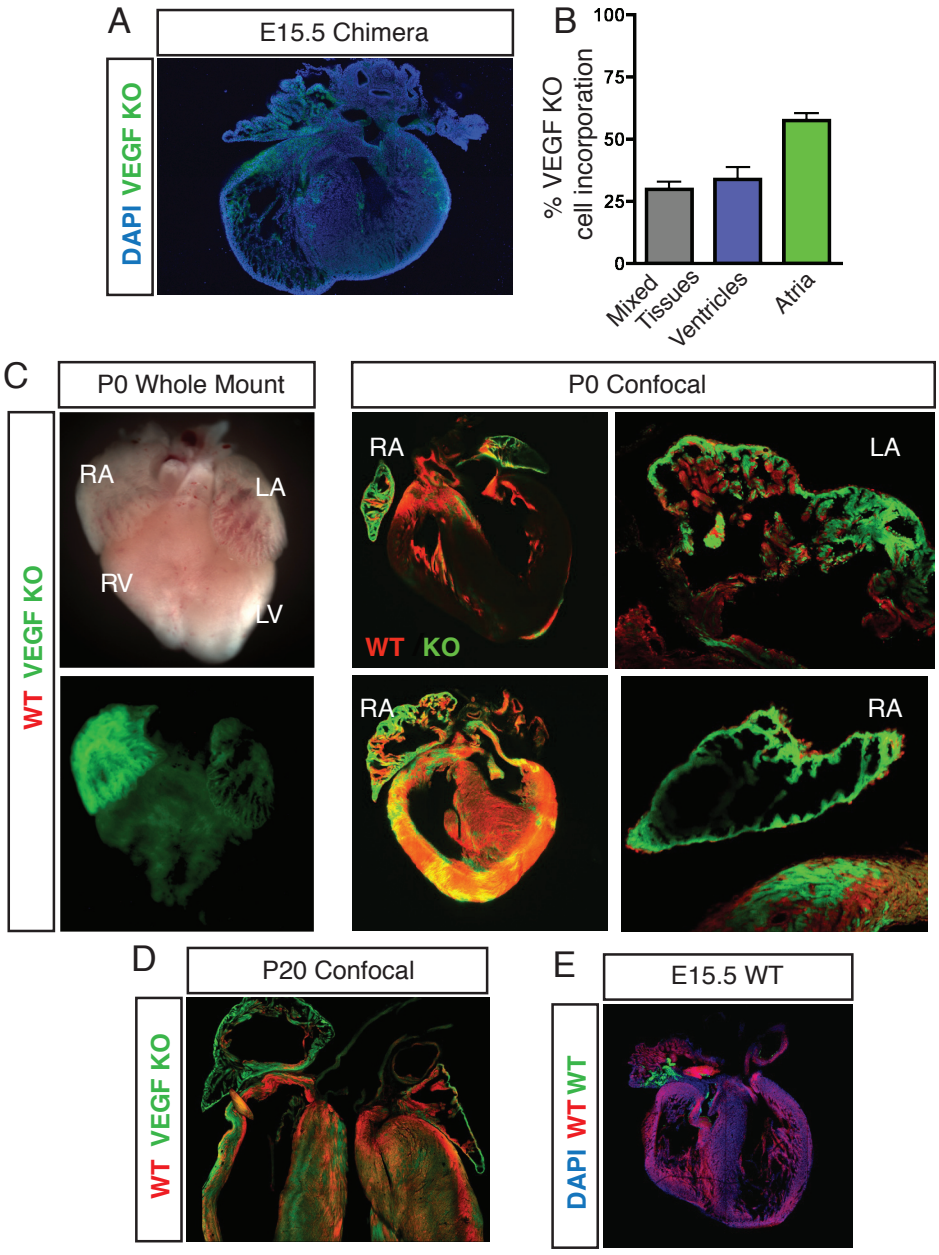

Figure 3.3: VEGF KO Chimerism in the heart

A) Confocal microscopy analysis of vibratomed heart dissected from an E15.5 chimera of VEGF KO GFP ESCs injected into WT non-fluorescent host. B) The integration of VEGF KO ESCs into WT host embryos was quantified by cell counting of random images (n=2 chimeras). C) Whole-mount fluorescent images were obtained from the heart of a neonatal P1 chimera. This heart was further processed by vibratome and analyzed by confocal microscopy in the right panel. D) P20 chimera analyzed for VEGF KO chimerism in the heart. E) Control embryo was obtained by injecting WT GFP ESCs into WT

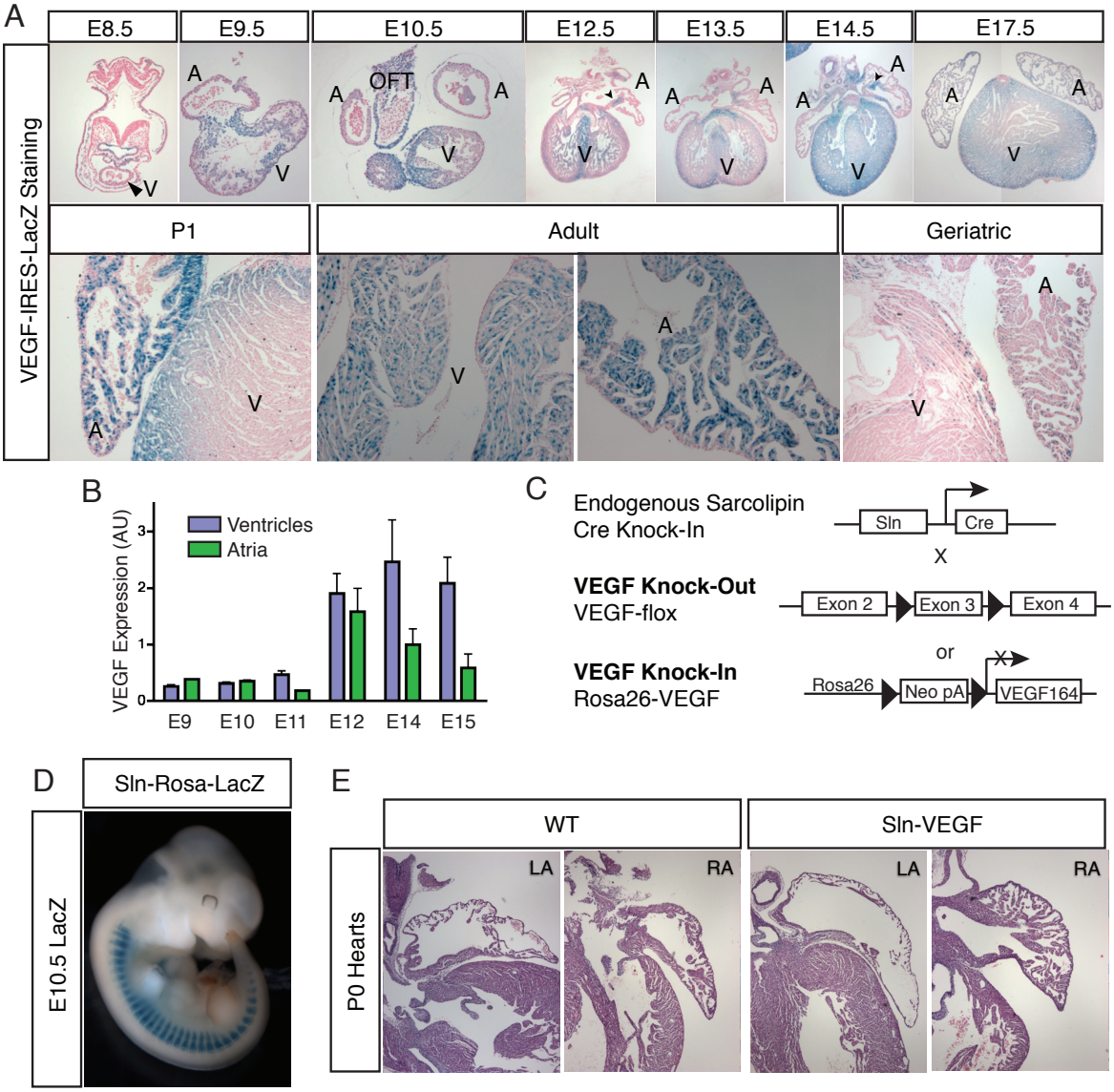

Figure 4: Normal VEGF expression in the heart and mis-expression in atrium by Sln Cre
A) Expression of LacZ reporter driven by the VEGF promoter observed in the heart at embryonic stages E8.5-E17.5 shows high expression in the ventricular myocardium but low expression in the atria. Neonatal (P1), adult and geriatric animals in lower panels show approximately equal atrial and ventricle VEGF expression. B) Developmental VEGF expression is higher in ventricles as observed by RT-PCR of VEGF mRNA. C) Schematic of transgenes used to create “Knock-Out” (VEGF-flox) and “Knock-In” (R26::VEGF) of the atrium (Sln::Cre). D) Sln::Cre expression pattern indicated by a cross to Rosa-LacZ. High somite expression is evident at E10.5. E) H&E of neonatal hearts harvested from R26::VEGF heterozygotes show left and right atria that are apparently normal when compared to WT litter mates.

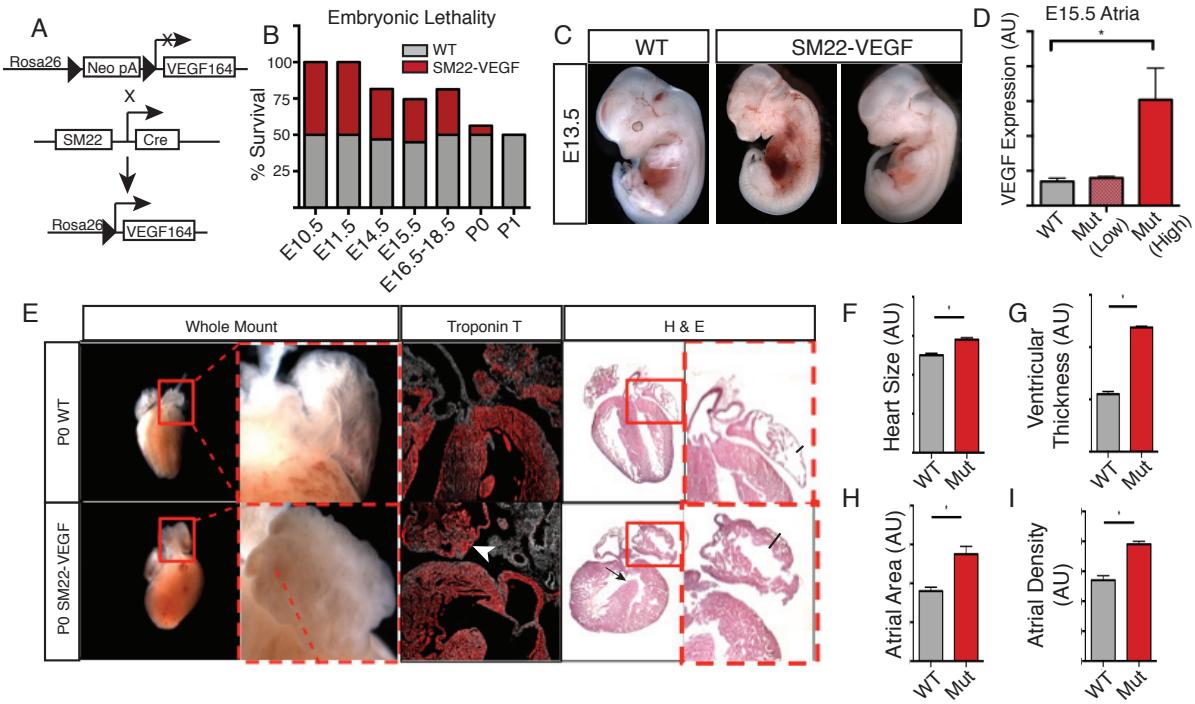

Figure 3.5 SM22-VEGF results in embryonic lethality and heart defects

A) Schematic of crosses used to induce VEGF in the heart with the SM22 promoter. B) Lethality of SM22-VEGF mutants starts at E14.5, penetrant by P0-P1. C) Embryos collected from R26::VEGF x SM22::Cre crosses at E13 showed a bloody, hemorrhaging phenotype with approximately 2/3 mutant penetrance. D) Mutants harvested at E15.5 can be separated into Low and High VEGF expressors. E) At P0, enlarged atria found to have overgrowth of TroponinT+ cardiomyocytes. White arrowhead indicates troponinT+ atrial overgrowth. Arrow points to ventricular septal defect. F) Quantification of heart size G) ventricular thickness H) atrial area and I) atrial density.

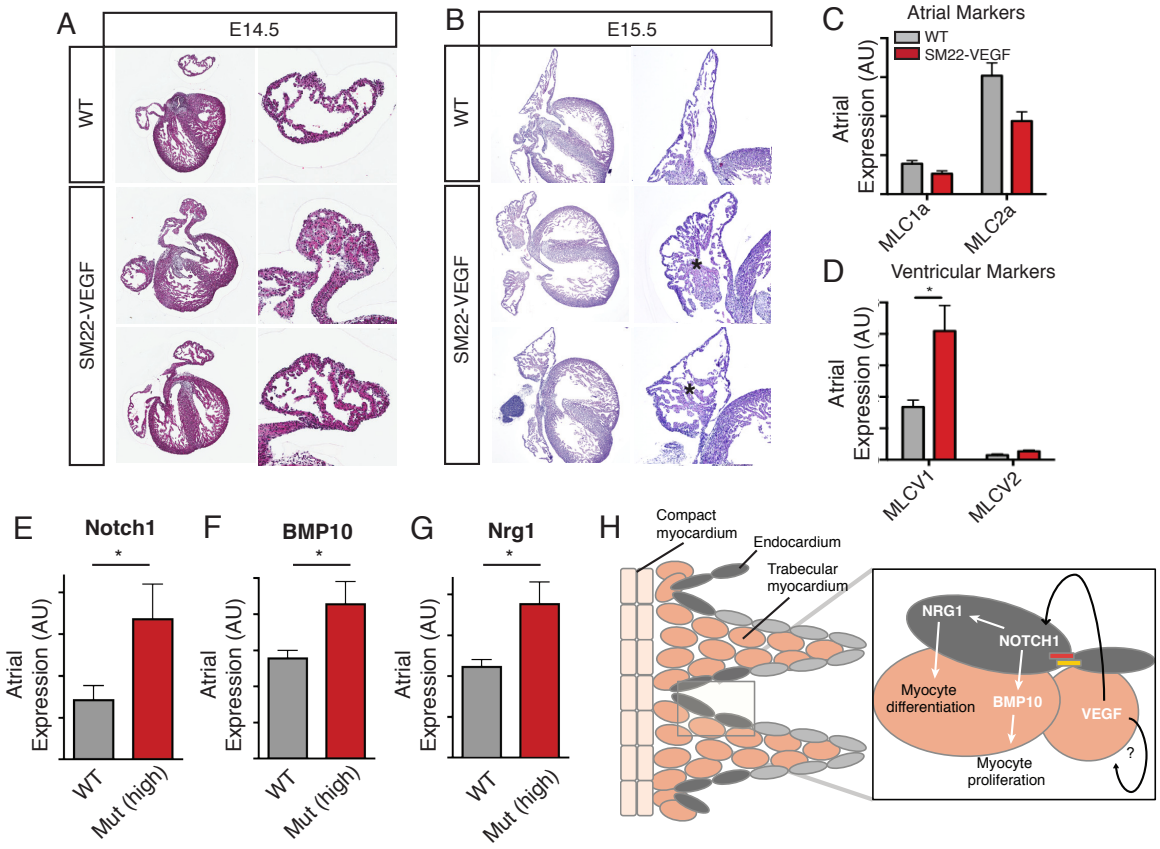

Figure 3.6 SM22-VEGF induces trabecular phenotype in the atrium
A) Slight thickening of the atria observed at E14.5. B)Atrial overgrowth apparent by E15.5. C) Qualitiative decrease in expression of atrial markers MLC1a and MLC2a are observed in P0 SM22-VEGF atria. D) Quantitative increase in ventricular marker MLCV1 is observed in the atria of P0 SM22-VEGF. E) Increased expression of Notch1, F) BMP10, G) Nrg1 observed in atria of E15.5 SM22-VEGF High expressing population. H) Interactions between endocardium and myocardium regulate myocyte differentiation and proliferation to form trabeculae. Notch1 signaling in the endocardium upregulates Nrg1, which induces myocyte differentiation and BMP10, which is important for proliferation. We show that over-expression of VEGF upregulates Notch1 expression, potentiating this pro-trabeculation pathway.

Table 3.1
Wild-type and VEGF-KO chimera outcomes

Chimera type (stem cell/host embryo)	Injected blastocysts	E15.5 did not implant	E15.5 reabsorbed	E15.5 viable	E15.5 chimeric
WT/B6	218 (100%)	188 (86.2%)	19 (8.7%)	11 (5.0%)	2 (0.9%)
VEGF KO/B6	390 (100%)	304 (78.0%)	56 (14.4%)	30 (7.7%)	9 (2.3%)

Table 3.2
Lethality of Sarcolipin VEGF-KO and VEGF-KI

Sln-VEGF-KO Progeny

VEGF-flox X Sln::Cre

73 Total Animals	**FF SS**	**FF S+**	**FF ++**	**F+ SS**	**F+ S+**	**F+ ++**	**++ SS**	**++ S+**	**++ ++**	Average Litter Size
Observed	2	6	2	2	25	7	5	19	5	5.88
Expected	4.56	9.13	4.56	9.13	18.25	9.13	4.56	9.13	4.56	

Sln-VEGF-KI Progeny

Rosa-VEGF X Sln::Cre

111 Total Animals	**RR SS**	**RR S+**	**RR ++**	**R+ SS**	**R+ S+**	**R+ ++**	**++ SS**	**++ S+**	**++ ++**	Average Litter Size
Observed	0	0	4	8	28	15	13	35	9	6.87
Expected	6.94	13.88	6.94	13.88	27.75	13.88	6.94	9.13	4.56	

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Chapter 4

Novel VEGFR2 populations in cardiac development

Abstract

Our understanding of the contribution of VEGFR2 to early cardiogenesis has been restricted to information generated by either stem cell differentiation experiments *in vitro* and mouse models *in vivo*. Cell culture differentiation experiments have clearly demonstrated an important role for VEGFR2 in cardiac progenitors, but mouse models have not yet identified where these cells reside in the developing heart. Using a VEGFR2-LacZ expression model, we mapped VEGFR2 expression throughout heart development and find that VEGFR2 is expressed by several novel populations including the dorsal mesenchymal protrusion (DMP), the atrial septum and transiently on the sinoatrial node (SAN).

Introduction

Heart development overview

The precardiac mesoderm of the vertebrate heart is specified at gastrulation to form what is referred to as the primary heart field, which migrates in an orderly fashion to form the cardiac crescent and eventually becomes the primary heart tube [1]. The primary heart tube is comprised of two major cell types that persist throughout heart development: the inner endocardium and the outer myocardium. Further cellular contributions to the heart occur at either pole from a population referred to as the secondary heart field, which continues to contribute to the heart as it loops to the right and begins differentiation into distinct chamber and valve components. The inflow region of the heart tube balloons to form a common atrial chamber, which is separated from the left ventricle by the atrio-ventricular canal [2]

Atrial septation

To form a four-chambered heart from a looped tube, septa must develop splitting the common atria and common ventricle. The atrial septum is made up of several cell populations. The septum primum emerges from the roof of the common atrium and descends into the atrium towards the endocardial cushions, eventually becoming myocardialized [3]. The septum secundum develops from the cranial margin rather late in development. This is a structure that acts as a seal to the flap valve, allowing complete partitioning the atrial chambers and can be found on the roof of the atria as a small muscular protrusion [2].

Dorsal mesenchymal protrusion

The dorsal mesenchymal protrusion (DMP) is an extension of the splanchnic mesenchyme into the atria that is important for the formation of the atrial septum. This population is Isl1 positive, which indicates secondary heart field origin [4], but Isl1 has also been shown to mark a common cardiac, smooth muscle and endothelial progenitors suggesting some amount of plasticity in this cell population [5]. The DMP enters the atrium continuous with the

septum primum, and bridges the AV cushions to the septum where they fuse. Around E13.5, the DMP undergoes mesenchymal-to-myocardial differentiation, associated with a decrease in Isl1 expression, and increased expression of myocardial markers.

Coronary Vasculogenesis

The heart itself must become vascularized to sustain myocardial function, and so coronary arteries begin to form around E10.5. Several (somewhat contradictory) cellular origins for coronary vasculature have been described. The proepicardial organ (PEO) is an extra-cardiac source that populates both the epicardium as well as some coronary endothelial cells [6]. Coronary arteries have also been shown to derive from sprouts out of the sinus venosus, which is the inflow tract of the embryonic heart [7]. These reports and others highlight the vascularization of the highly-muscular ventricular compartments of the heart, however surprisingly little information exists describing the vascularization of the atria.

Sinoatrial Node Development

The conduction system of the heart is a specialized set of tissues that coordinate heartbeat so that blood flows in a uni-directional pattern. The conduction system is comprised of three major parts: the Sino-Atrial Node (SAN), the atrioventricular node and the Purkinje fiber system. The SAN is anatomically located in the right atrium, and acts as the pacemaker of the heart, the initiator of conductive signaling [8]. The SAN is derived from primordial heart-tube myocardium which have not fully differentiated into chamber myocardium [9].

Involvement of VEGFR2 in heart development

Although often used as an “endothelial marker” in adults, VEGFR2 is expressed in non-endothelial populations during development, several of which significantly contribute to cardiac development. Stem cell differentiation experiments have shown that VEGFR2 is an important marker of mesenchymal precursors common to cardiomyocytes, endothelial cells and

hematopoietic cells [10] [11]. However, stem cell culture experiments leave it unclear where these precursors reside within the embryo, and how long they may persist.

Cardiomyocytes may be directly influenced by VEGFR2 expression as lineage tracing experiments confirmed that VEGFR2 mesodermal progenitors can differentiate into muscle lineages, including cardiomyocytes [12]. Furthermore, a modified VEGFR2-LacZ allele showed fairly penetrant VEGFR2 expression throughout the heart at E8 [13]. Knock-out of VEGFR2's ligand (VEGF) in cardiomyocytes results in a thinner layer of cardiomyocytes and decreased cardiac function, an effect attributed to a decreased microvasculature [14]. However, it is not known what direct effect VEGF may have on early myocardiogenesis, as the precise expression pattern of VEGFR2 has not been well described.

Here we examine the expression pattern of VEGFR2 using a VEGFR2-LacZ model as well as anti-VEGFR2 immunohistochemistry, and report several unexpected VEGFR2 populations throughout cardiogenesis. We find that VEGFR2, contrary to other vascular markers, reveals some of the earliest coronary arteries to populate the atria. Strong expression of VEGFR2 is observed in the DMP, in the descending septum primum of the atrium, and in the septum secundum later in development. We also observed transient expression of VEGFR2 in the SAN of the developing animal. These populations suggest a broader and more nuanced role for VEGFR2 in cardiac development beyond the production of cardiogenic stem cells in culture.

Materials and Methods

Animals

VEGF-LacZ animals were previously described [15]. WT B6 animals were used for all immunohistochemistry experiments.

LacZ staining

LacZ staining was carried out as previously described [16]. P0 hearts were perfused briefly with 4% PFA, dissected and fixed overnight. Samples were embedded in paraffin, sectioned and H&E stained by UCLA Tissue Procurement Core Laboratory.

Immunohistochemistry

Whole embryos were collected from timed pregnant B6 female dams, fixed in 2% PFA overnight, and then stepped into a 30% sucrose gradient before freezing in OCT media. Frozen hearts were kept at -80C and sectioned with a cryostat into 8um sections.
VEGFR2 staining on frozen sections was done using Invitrogen Molecular Probes TSA Detection Kit (cat# T-20932) and BD Pharmingen rat polyclonal anti-Flk1 antibody (cat# 550549) according to the manufacturer's direction. HCN4 and TroponinT were used on frozen sections with Vector M.O.M. Immunodetection Kit (BMK-2202). anti-HCN4 polyclonal rabbit antibody (Cat# ab69054) and anti-Cardiac TroponinT antibody [1F11] (Cat# ab10214) were obtained from Abcam. Nuclei were counterstained using DAPI.

Confocal Microscopy

All slides were mounted in Mowiol® 4-88. Carl Zeiss LSM 510 META Laser Confocal Microscope was used to acquire 40X images with 0.80 aperture at room temperature. ZEN® 2009 was used as the acquisition software.

Results

Development of VEGFR2+ coronary arteries

To investigate VEGFR2 expression throughout cardiac development, we employed a VEGFR2-LacZ reporter model (Figure 4.1A) [15] and stained hearts at several different timepoints. VEGFR2 expression was expected in both the coronary vasculature as well as in the endocardium, so we examined whole-mount preparations to help visualize superficial vessels.

We observed angiogenic sprouting from the sinus venosus, or inflow tract, starting at E11.5 (Figure 4.1B arrowheads) which is largely in agreement with current coronary angiogenesis models [6]. In contrast to the apelin-lacZ knock-in mouse, which specifically labels coronary endothelial cells, but not endocardium [7], we observed the emergence of enlarged VEGFR2+ atrial coronary vessels as early as E12.5 (Figure 4.1C arrowhead). At this time a caudal expansion of the vascular plexus had begun to enmesh the ventricle, which appear further matured by E14.5 (Figure 1.D).

Novel VEGFR2+ populations in the heart

Although in vitro cell culture experiments strongly suggest the presence of a VEGFR2+ multipotent endothelial and cardiac progenitor, the location and persistence of this cell population has not been previously described.

At E10.5, the DMP is seen attached to the atria after dissection (Figure 4.1E, asterisk). The DMP was found to be strongly VEGFR2+ throughout the undifferentiated mesenchymal population pushing into high-VEGFR2 expressing atrial endocardium. At this time early in cardiac development, a small number of cells in the cardiac cushion appear to be VEGFR2+ as well (Figure 4.1E arrowhead).

We observed a population of cells in atrial septum that strongly express VEGFR2. The development of this population closely resembles the progression of the DMP at E11.5, and

possibly the septum primum from E12.5 to E13.5 (Figure 4.1F-H). Extremely high expression of VEGFR2 is no longer observed in the septum at E17.5, although it does maintain some degree of VEGFR2 positivity (Figure 4.1J arrow). We also observed a region of high VEGFR2 expression in the anatomical position of the SA-Node at E17.5 (Figure 4.1J, white arrowhead). A table summarizing our findings of VEGFR2-LacZ staining in the heart over time (based on histological and anatomical localization) can be found in Table 4.1.

VEGFR2+ cardiomyocytes in the atrial septum

The cardiac VEGFR2-LacZ staining suggested the existence of a novel VEGFR2+ cardiomyocyte population in the DMP and atrial septum. To investigate the myocardial status of these cells, we performed immunohistochemistry on wild-type embryos at several different timepoints, co-staining for VEGFR2 and cardiomyocyte marker Cardiac Troponin T (Figure 4.2).

At the earliest timepoint investigated, E10.5, we observed several clumps of VEGFR2+ TroponinT+ within the atrial chamber (Figure 4.2A, arrows). The positioning of these cells coincides with the primordial atrial septum. Adjacent to these VEGFR2+TroponinT+ cells we observed a VEGFR2+TroponinT- population (Figure 4.2A, open arrowhead). This population has not been described to our knowledge, and may be the primordial endocardium that has been reported to derive from VEGFR2+ cardiovascular progenitors in stem cell experiments [17].

By E12.5, the atrial septum has joined to the AV cushions and some regions have begun to myocardialize. We observe maintained VEGFR2 expression throughout the atrial septum, strongly co-localizing with TroponinT in this section (Figure 4.2B, arrowhead).

The atrial septum continues to develop through E14.5, where we observed fairly robust VEGFR2 staining throughout the septum primum (Figure 4.2C, arrhowhead). This region does not stain for myocardial markers, suggesting it is a developing region of the septum primum. By

E14.5, the septum secundum is apparent on the roof of the atrium, and co-stains for VEGFR2 and TroponinT (Figure 4.2C, asterisk).

Transient expression of VEGFR2 in the SA-Node

The SA-Node develops from the myocardium of the sinus venosus at E9.5 [18], becoming the major pacemaker of the heart. High expression of HCN4, hyperpolarization-activated, cyclic nucleotide gated-ion channel 4, is characteristic of cardiac pacemaker tissues and so serves as a good marker for conduction tissues in the heart. HCN4 is more widely expressed in early development of the heart, and becomes increasingly restricted to conductive tissue over time [19].

Because we observed high expression of VEGFR2-LacZ in the anatomical location of the SAN, we performed immunohistochemistry on embryos at several different time points looking for co-localization of VEGFR2 with HCN4 (Figure 4.3). We observed an early cluster of HCN4+VEGFR2+ cardiomyocytes near the DMP/atrial septum at E10.5 (Figure 4.3A, arrowheads). The SAN, more clearly defined at E12.5, continued to express VEGFR2 at this timepoint (Figure 4.3B, arrowheads). By E14.5, we no longer observed strong VEGFR2 staining in the SAN, suggesting VEGFR2-positivity is a transient property of the conduction system (Figure 4.3C, arrows).

Discussion

Although VEGFR2 and its ligand VEGF are highly-implicated in several aspects of heart development, it is not known precisely which cellular subsets they act on throughout cardiogenesis. In the work presented here, close temporal analysis of VEGFR2 expression in the heart reveals several novel VEGFR2-positive populations, including early atrial coronary arteries, the DMP with the associated atrial septum, and the early SAN.

We found that VEGFR2-positive cardiomyocytes could be identified, not only in early development when cardiac precursors are less differentiated, but also at later developmental time points when precursor populations are not thought to be as prevalent. This may not be entirely surprising as addition of VEGF to cell culture models of cardiomyocyte differentiation has been shown increase myogenic outcomes [20,21]. These experiments suggest a direct role for VEGF in myocardiogenesis, although the receptor through which this action takes place is not addressed.

Isl1 expression is highly-associated with cardiac progenitors in the embryo as well as the adult, and has similar cardiac expression patterns to that of VEGFR2 described in this paper, namely in the atrial septum and around the pulmonary veins [22]. Although we were unable to visualize Isl1/VEGFR2 co-staining in our experiments, the coincident patterns of expression suggest that VEGFR2 may be a co-marker of this Isl1+ progenitor population. In this light, it may not be too surprising that VEGFR2 was also observed in the early SAN, which is considered closely related to primordial myocardium that has been “left behind” developmentally.

Altogether, these data reveal an active role for VEGFR2 in cardiogenesis in addition to simply providing mesenchymal cardiac progenitors. More refined animal model experiments are required to fully understand the importance of VEGFR2 expression throughout cardiogenesis, in particular its possible role in maintaining cardiac potential into adulthood.

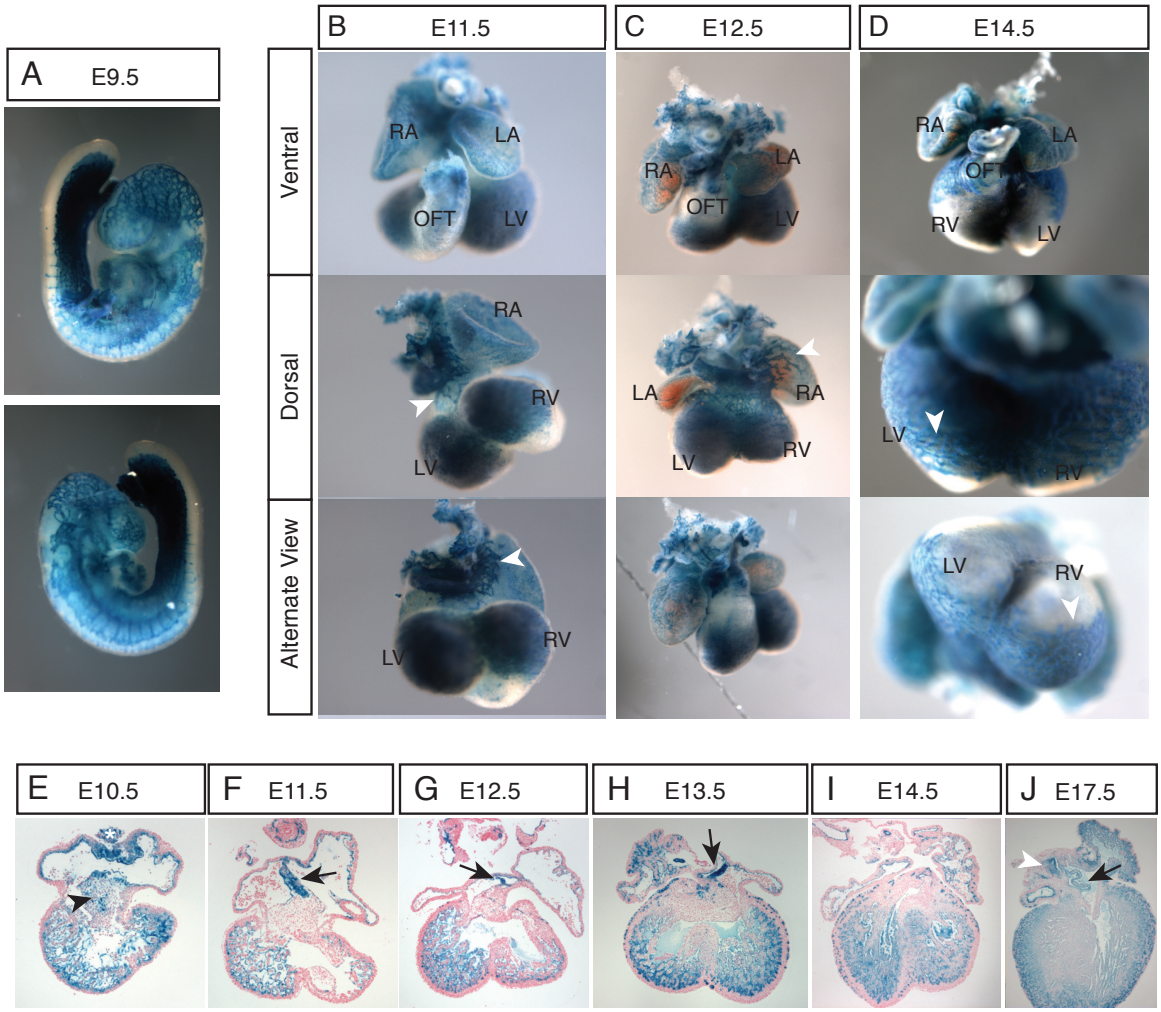

Figure 4.1 Cardiac VEGFR2 expression in embryogenesis.
A) LacZ staining of whole-mount E9.5 embryos. Whole mount LacZ staining of dissected hearts at B)E11.5 C)E12.5 D) E14.5 showing anterior, posterior, and alternatve views of interest. RA, right atrium; LA, left atrium, RV, right ventricle; LV, left ventricle; OFT, outflow tract. Histological sections of whole mount LacZ stained hearts from E) E10.5 F) E11.5 G) E12.5 H) E13.5 I) E14.5 and late embryonic stage J) E17.5. Black arrowhead marks VEGFR2+ staining in cardiac cushion. White astrisk marks VEGFR2+ dorsal mesenchymal protrusion. Arrows indicate VEGFR2+ atrial septum. White arrowhead indicates VEGFR2+ area of SA node.

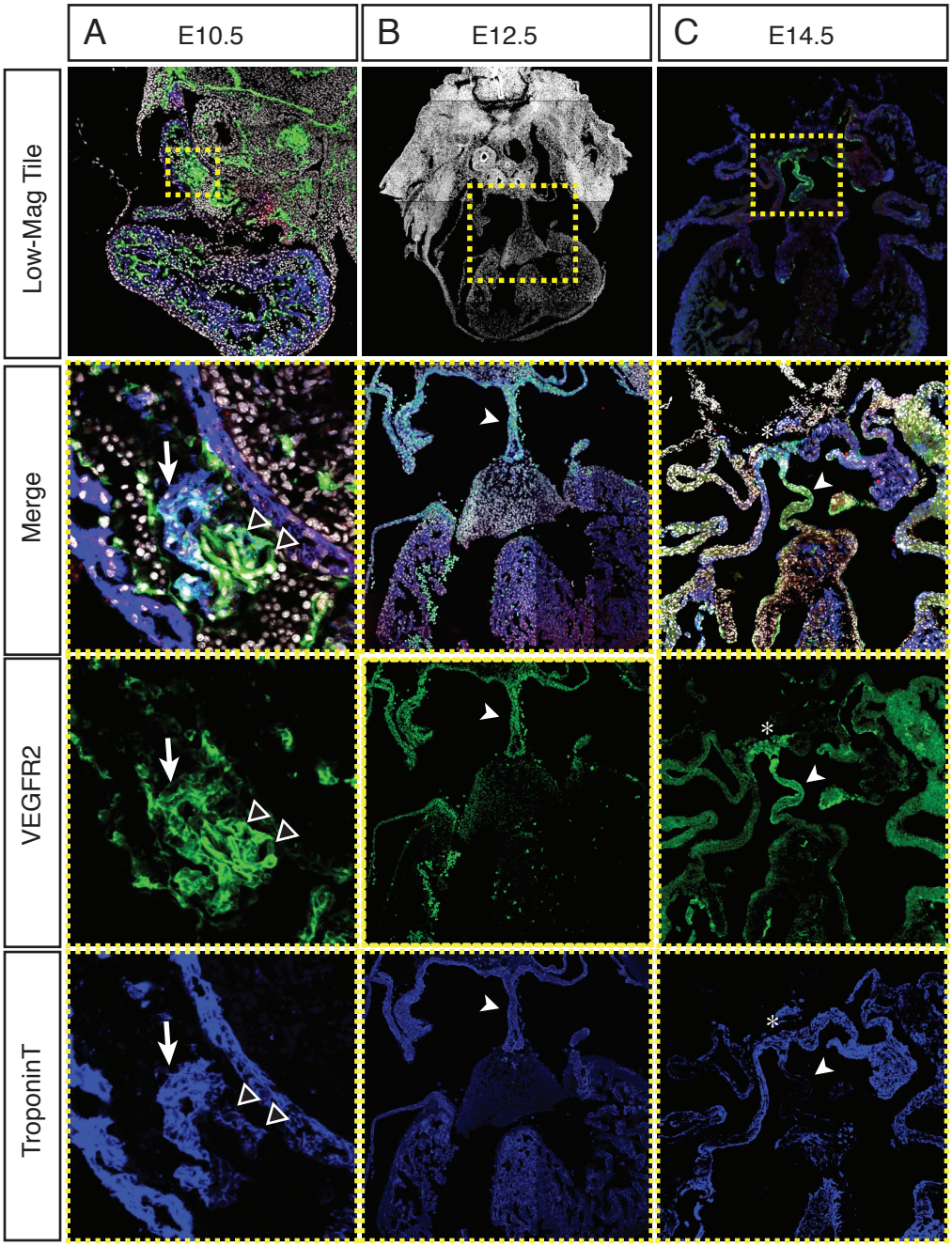

Figure 4.2 Atrial Septum contains VEGFR2+ cardiomyocytes
Immunohistochemistry colocalizing VEGFR2 with cardiomyocyte marker TroponinT shows double-positive cells localized to the atrial septum at A) E10.5 B) E12.5 and at C) E14.5. Arrow indicates early VEGFR2+TropT+ clump of cells near the primordial atrial septum. Open arrowheads indicate VEGFR2+TroponinT- cell population. Arrowheads indicate the atrial septum. Asterisk indicates small VEGFR2+TropT+ population remaining in the most anterior region of the atrial septum.

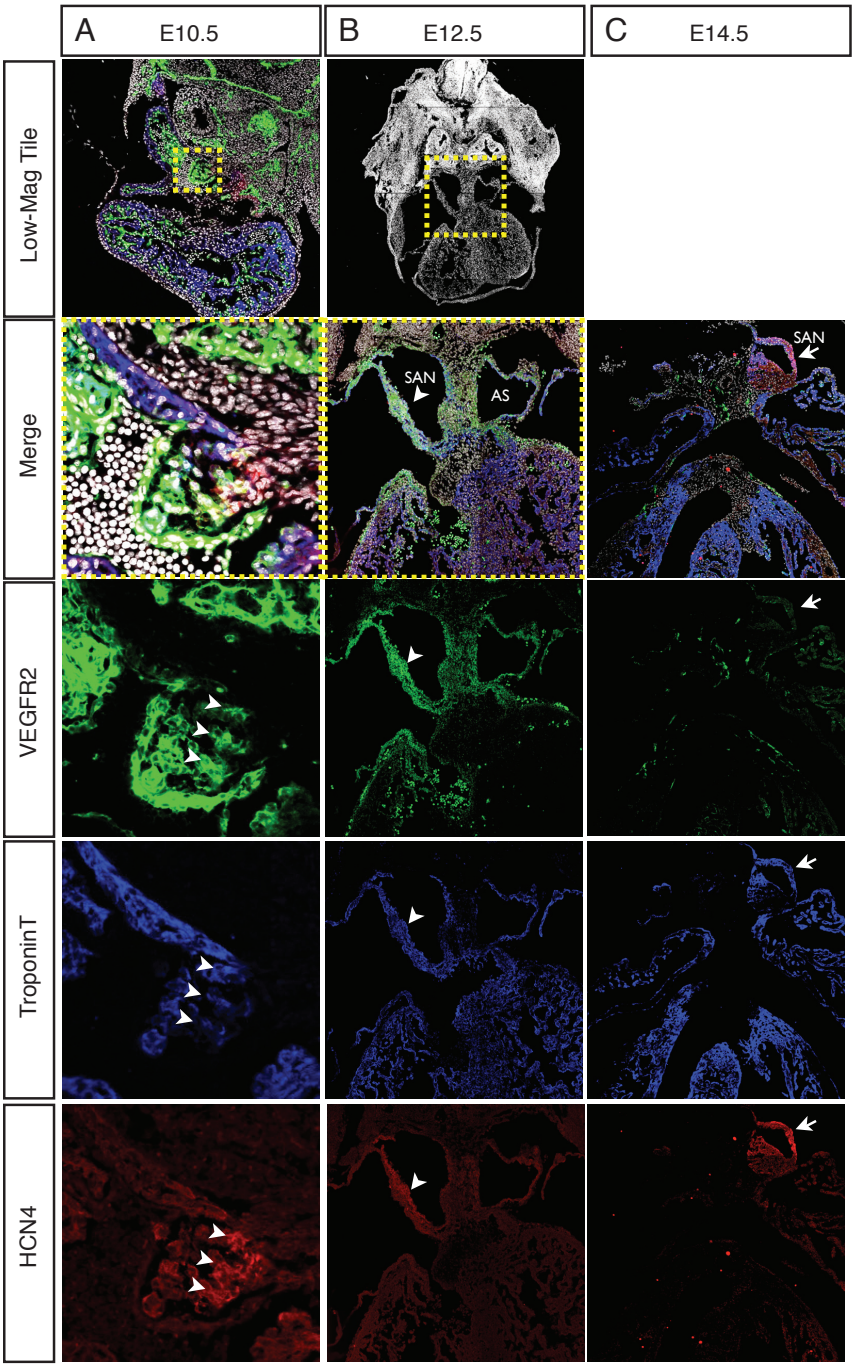

Figure 4.3 Transient expression of VEGFR2 on primordial conduction system

A) E10.5 embryos have small VEGFR2+TroponinT+HCN4+ population in atrium. B) The SAN of E12.5 embryos stains VEGFR2+. C) By E14.5 the SAN no longer expressed VEGFR2. Arrowheads indicate VEGFR2+ TroponinT+ HCN4+ populations. Arrows indicate VEGFR2-TroponinT+HCN4+ populations. SAN, sino-atrial node; AS, atrial septum.

Table 4.1
VEGFR2 expresion in cardiac populations over development

VEGFR2-LacZ Expression							
Regions of Heart	**Cell Types**	**E10.5**	**E11.5**	**E12.5**	**E13.5**	**E14.5**	**E17.5**
Dorsal Mesoderm		***	N/A	N/A	N/A	N/A	N/A
Dorsal Mesocardium		***	N/A	N/A	N/A	N/A	N/A
Outflow Tract	Endothelium	**	***	***	***	***	***
	Wall	*	**	***	***	***	***
	Valve					-	-
Ventricle	Endocardium	***	***	***	***	**	**
	Coronary	N/A	**	**	***	***	***
	Cardiomyocytes	**	-	-	-	-	-
	Septum	*	-	-	-	-	-
	Valve						-
Endocardial Cushion		*					
Atrium	Endocardium	***	***	***	***	***	***
	Coronary	N/A	**	**	**	**	**
	Cardiomyocytes	**	-	-	-	-	-
	Septum	***	***	***	***		*
	Venous valve		***	***	***		***
	SA-Node		Likely		Likely		Likely

Table Key	- No expression observed	* Light/scattered expression	** Moderate expression	** High expression	N/A Developmental structure no longer exists

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Chapter 5

Autocrine VEGF signaling maintains energy metabolism and survival in the endothelium

Abstract

Autocrine VEGF is necessary for endothelial survival, although the cellular mechanisms supporting this function are unknown. Here we show that continuous expression of endothelial VEGF is needed to sustain vascular integrity in adults vessels, and cellular viability under homeostatic conditions. Endothelial cell death due to VEGF depletion is accompanied by suppression of glucose metabolism, mitochondrial respiration and an increase in autophagy. Gene expression profiling showed that endothelial VEGF regulates cell cycle and mitochondrial gene clusters, as well as several, but not all, targets of the transcription factor Foxo1. Furthermore, VEGF-deficient endothelium *in vitro* and *in vivo* showed increased levels of Foxo1 protein in the nucleus and cytoplasm. Silencing of Foxo1 in VEGF-depleted cells reversed expression changes of several of the gene clusters that were de-regulated in VEGF knockdown, and furthermore rescued cell death and autophagy phenotypes. Our data suggest that endothelial VEGF maintains vascular homeostasis through constitutive Foxo1 suppression, thereby ensuring physiological metabolism and cell survival.

Introduction

Vascular Endothelial Growth Factor (VEGF) is critical for proper differentiation and development of vasculature [1] [2]. Under culture conditions, the addition of exogenous VEGF elicits a multitude of cellular responses in addition to its well known role in angiogenesis. These include endothelial survival [3] and protection from apoptosis [4]. While the response of the vasculature to high levels of exogenous VEGF is widely recognized, it has also been observed that the endothelium itself expresses VEGF in response to hypoxia *in vitro* [5] and in several vessel beds *in vivo* [6] [7] [8]. In fact, cell-specific genetic inactivation of VEGF in the endothelial compartment causes vascular degeneration, ultimately resulting in a 55% mortality in mutant mice [7]. However, despite its biological impact, the mechanisms at fault that result in this phenotype have not been determined.

Strikingly, cell death in VEGF-deficient endothelium cannot be rescued by addition of exogenous VEGF, or even by co-culture with WT endothelium which could presumably supply endothelial-derived VEGF to neighboring cells [7]. These experiments suggest that VEGF, in a cell-autonomous manner, contributes to cell survival independent of surface ligand/receptor interactions. These cell-autonomous responses are not exclusive to the endothelium and have been also shown in hematopoietic cells [9] and in some tumor cells [10] [11].

Anti-VEGF therapy is used in several clinical settings to abrogate neo-angiogenesis. The approach is extremely successful in the treatment of opthalmic diseases where anti-VEGF drugs are administered by direct intravitreal injection [12]. In addition, systemic blockade of VEGF has been used in the context of cancer [13]. This approach has resulted in the unpredicted occurrence of several side effects. Most common are hypertension, proteinuria, intestinal perforations, hemorrhage and cardiac impairment [14], indicating that long-term VEGF blockade has consequences to normal physiology. Although these side effects do not preclude use of anti-VEGF drugs as therapy, they indicate a homeostatic need for VEGF in adult settings and

call into caution the implementation of long-term treatments. Additional modalities of systemic VEGF blockade, particularly through small molecular inhibitors that target VEGFR-2 and penetrate the cell, have the potential to impact both the well-understood paracrine signaling that drives pathological neo-angiogenesis, but also the less-understood autocrine, and possibly intracrine, pathway that is required for normal vascular maintenance. As stronger anti-VEGF therapies make their way to clinical trials, a better understanding of physiological autocrine VEGF signaling must be developed.

Stimulation of endothelium with high doses of VEGF produces a metabolic signature characterized by a high flux of glucose and increased glycogen metabolism that can be suppressed with inhibitors of VEGFR-2 [15]. Recent studies focusing on the role of glycolysis in angiogenesis have revealed that VEGF stimulation increases expression of the potent glycolytic activator PFKFB3, which is crucial for vessel sprouting [16]. Furthermore, exposure of brain endothelial cells to exogenous VEGF enhances Glut1 expression thereby promoting glycolytic energy production from increased glucose influx [17]. Although glycolysis is the major contributor to ATP production in the endothelium, VEGF treatment has also been shown to increase fatty acid uptake through AMPK [18] and by upregulation of the fatty-acid transporter FABP4 [19]. While it is clear that a spike in VEGF signaling has profound effects on endothelial metabolism to allow the proliferation and migration involved in angiogenesis, it is not known what role VEGF may play in maintaining normal metabolic homeostasis and at times of energy crisis.

Results

Autocrine VEGF is required for adult vascular homeostasis

The need for cell-autonomous VEGF signaling in endothelial cell homeostasis was previously demonstrated using a constitutive deletion model that results in sudden death of a large proportion of adult animals [7]. Because the sudden death phenotype was not completely penetrant, we postulated that VEGF may be required for protection from certain types of stress that may occur incidentally in the adult. As VEGF is increased under hypoxia [5], we tested the effects of low oxygen levels on Cre negative (controls) and VEGF-Endothelial Cell Knock Out (ECKO) animals. At baseline, histological analysis of VEGF-ECKOs with induced deletion revealed intestinal perforations, brain hemorrhage and heart fibrosis indicative of a loss of vascular integrity **(Figure 5.1A)** as previously reported for the constitutive deletion [7]. However, the severity and penetration of the injuries was highly increased under hypoxic conditions. Evaluation of control (n=19) and VEGF-ECKO (n=24) mice under both conditions showed that while incidence of at least one of those pathologies was noted in 64.3% of VEGF-ECKO mice in normoxia, 90% of this group were affected in hypoxic conditions. Control animals were entirely protected from injury in the presence of endogenous VEGF in both normoxia and hypoxia **(Figure 5.1A)**. Interestingly, we found that although lesions in all three organs were increased upon exposure of VEGF ECKO mice to hypoxia, the cardiac vascular beds were particularly susceptible to removal of VEGF **(Figure 5.1B)**.

A caveat of the VEGF-ECKO model of constitutive deletion was the intrinsic difficulty to discern whether VEGF excision from the onset of endothelial specification carried developmental rather than homeostatic consequences to adult mice. Hypothetically, VEGF may be required in a small developmental time window for endothelial differentiation, after which it becomes no longer necessary. Thus, we performed additional experiments, using an inducible model to directly address this question. Tamoxifen-inducible deletion of VEGF in endothelial

cells was accomplished by generating mice (referred to as VEGF-iECKO) that carried an inducible VE-CAD Cre transgene in the background of VEGF lox/lox. VEGF deletion in young adult mice resulted in sudden death of 33% of these animals by 50 weeks, indicating that autocrine VEGF is indeed required for homeostatic function of the adult endothelium **(Figure 5.1C)**.

Intracellular endothelial VEGF is critical for maintenance of endothelial cell viability and survival

A relatively trivial explanation to the findings is that loss of VEGF from the endothelial compartment decreased the local supply of VEGF available to the cell and thus restricted signaling of surface receptors. To determine whether loss of autocrine VEGF could be rescued by simple supplementation of exogenous VEGF, we assessed cell viability *in vitro* with endothelium isolated from VEGF-ECKO adults in the presence and absence of exogenous VEGF. Interestingly, the cell viability defect of VEGF-ECKO could not be rescued by addition of exogenous recombinant VEGF **(Figure 5.1D)**. In contrast, infection of VEGF-ECKO cells with adeno-VEGF increased viability to WT levels **(Figure 5.1D)**. This suggests that rescue could only occur if VEGF was delivered intracellularly and that an endothelial source of VEGF, rather than just the total amount, was essential. To separate the historical effects of autocrine VEGF on embryonic differentiation versus its role in adult homeostasis, we isolated endothelium from floxed-VEGF adults, and then ablated VEGF in culture by adeno-Cre exposure **(Figure 5.1E)**. We found that even after normal differentiation in a phenotypically WT environment (VEGF lox/lox Cre-negative), ablation of VEGF in adult endothelium also resulted in decreased cellular viability **(Figure 5.1E)**.

Because these data suggest that endothelial viability depends on an intracellular source of VEGF, we exposed HUVECs to internal and external blockade of VEGF. Anti-VEGF antibody

was used to block extracellular VEGF, whereas internal / external VEGF signaling was blocked with a small molecule inhibitor to VEGFR2 (SU4312). HUVECs treated with a high dose of anti-VEGF antibody remained similarly viable and equivalent to non-treated cells, while SU4312-treated cells cultures showed a drastic cell reduction **(Figure 5.1F)**. Cells were treated with saturating doses of inhibitors sufficient to block the signaling effects of even large amounts of exogenous rVEGF, far in excess of endogenous VEGF levels **(Figure 5.1G)**. These results suggest that endothelial cells were dependent on an endogenous intracellular source of VEGF for their survival.

Lack of VEGF results in cell death, increased autophagy and metabolic collapse

The determination of cell viability could not distinguish between decrease in proliferation or promotion of cell death, thus we sought to investigate the rate of cell death in a confluent monolayer that had lost VEGF expression. We subjected HUVECs to siRNA knock-down of VEGF (KD-VEGF) and found that a reduction in approximately 50% of VEGF transcript resulted in a severe loss of cells compared to controls after 3 days **(Figure 5.2 A-C)**. These findings were confirmed using endothelial cells derived from VEGF lox/lox mice exposed to adeno-Cre *in vitro* to generate VEGF null (KO-VEGF IMEC) and relative control (WT IMEC) endothelium. Cell death was assessed with a cell-impermeant DNA dye in two hour increments over 24hrs in real time and we observed that KO mouse endothelial cultures had a high incidence of cell death under serum-starvation when compared to cultures with wild-type levels of VEGF **(Figure 5.2D-E)**. To test whether extracellular VEGF could rescue the this phenotype, KO murine endothelial cells were further treated with exogenous recombinant VEGF (rVEGF) which did not significantly alter viability **(Figure 5.2D)**. However, infection of KO IMECs with lentiviral-VEGF resulted in a clear rescue and decrease in cell death with numbers that now approached WT

levels **(Figure 5.2D-E)**, suggesting that VEGF must be directly expressed by the endothelium itself and have cell-autonomous effects.

To further clarify the specific mechanism of cell demise, we first evaluated apoptosis. Interestingly, in cell culture, classic indicators of apoptosis such as cleaved-Caspase 3 and cleaved PARP were not easily detected in KD-VEGF cells, although they could be induced by treatment with staurosporine as a positive control **(Supplemental Figure 5.1A-B)**. We additionally performed TUNEL staining on KD-VEGF and control cells, and while we saw a few positive cells, were unable to observe a number TUNEL-positive nuclei that would be consistent with the number of cells lost in the absence of VEGF **(Supplemental Figure 5.1C)**.

We then evaluated the metabolic status of WT and KO endothelial cells. Absence of VEGF resulted in a reduction of metabolic activity in that glucose uptake, lactate production and triglyceride synthesis were all decreased **(Figure 5.3A-B, E)**. Suspecting additional metabolic effects, we investigated cellular oxygen consumption and mitochondrial respiratory capacity. Our findings indicate an overall depression of mitochondrial function **(Figure 5.3C-D)**. Furthermore, except for the case of triglyceride synthesis, all of these phenotypes were manifested under both serum-starvation and in the presence of serum which, frequently, but not in this case, can compensate for small metabolic defects with an abundance of nutrients **(Figure 5.3A-E)**. Together, these results suggest that autocrine VEGF maintains normal metabolism in endothelial cells, without which the endothelium is challenged to survive.

A common compensatory mechanism in dying cells attempting to mitigate life-threatening metabolic needs is to obtain nutrients through increased autophagy. We observed a marked increase in autophagic vacuoles in KD-VEGF HUVECs suggesting over-activation of autophagy **(Figure 5.4A-B)**. Because increased autophagy contributes to cell death in some contexts [20], we inhibited the autophagic pathway and checked for rescue of KD-VEGF cell death. We found that both silencing of the Atg7 gene and pharmacological blockade with

chloroquine (which inhibits lysosome acidification and fusion with autophagosomes) resulted in a significant rescue of KD-VEGF cell death **(Figure 5.4C-D)**. Thus, disabling autophagy significantly increases cell survival in mutant cells, suggesting that autophagic cell death contributes to the KD-VEGF phenotype.

To determine whether this increased in autophagy occurred *in vivo*, we examined the ultrastructure of WT and VEGF-ECKO littermate control endothelium and compared the prevalence of autophagic vacuoles. VEGF-ECKO endothelium had a higher incidence of autophagic vesicles that were not readily observed in WT endothelium **(Figure 5.4E)**.

Depletion of VEGF induces activation of the transcription factor Foxo1

Forkhead transcription factors have been linked to autophagy, metabolic regulation and exogenous VEGF signaling [21]. Therefore, the perturbation of these pathways prompted us to directly investigate the status and activity of Foxo1 in the absence of VEGF. Increased total Foxo1 protein levels were indeed observed in KD-VEGF cells by Western blot (Figure 5A-B) and by immunofluorescence **(Figure 5.5D-G)**, although little change was observed in the levels of Foxo1 transcripts **(Supplemental Figure 5.3D)**. Suppression of VEGF-R2 signaling by treatment with SU4312 also resulted in an increase of Foxo1 protein levels **(Figure 5.5C)**. Transcriptionally active Foxo1 is found translocated to the nucleus, but Foxo1 can also directly contribute to autophagy by cytoplasmic localization [20]. We quantified Foxo1 localization in fluorescent images and observed an increase in both nuclear and cytoplasmic localization in KD-VEGF cells, with the larger proportion of Foxo1 observed cytoplasmically **(Figure 5.5D,E)**.

We assessed the transcriptional activity of Foxo1 through evaluation of its target genes by microarray and RT-PCR analysis. The findings indicate that a subset of direct targets of Foxo1 were increased in KD VEGF cells, including CITED2, SOD2 and SEPP1 **(Supplemental Figure 5.2D)**. However, other previously-reported Foxo1 target genes, such as ID2 and CCNB2,

were not affected in the KD-VEGF condition **(Supplemental Figure 5.3F)**. This result may reveal a context-dependent Foxo1 transcriptional program, or alternatively that the contribution of Foxo might be cytoplasmic rather than genomic.

The findings prompted us to investigate the status of Foxo1 in VEGF-ECKO animals which have developed in the prolonged absence of VEGF. While we found constitutive expression of Foxo1 in the smooth muscle layer surrounding vessels in the lung **(Figure 5.5H arrows)** of wild-type (WT) mice, the transcription factor was conspicuously absent from the endothelium **(Figure 5.5H, asterisk)**. However, in VEGF-ECKO mice, Foxo1 was distinctly expressed by the endothelial layer in addition to its presence in smooth muscle cells **(Figure 5.5H, arrowheads)**, again in sharp contrast to WT littermates **(Figure 5.5H, asterisk)**.

Cell death due to VEGF depletion can be rescued by removal of Foxo1

Because the absence of VEGF in the endothelial compartment induces Foxo1 activity, we assessed whether Foxo1 contributed in any way to the KD-VEGF cellular phenotype. To test this hypothesis, we performed a double knock-down targeting both VEGF and Foxo1 (KD-VEGF +Foxo1) in confluent HUVECs and assessed cell number as a readout for survival **(Figure 5.6A-B)**. Depletion of Foxo1 indeed significantly rescued the cell death observed in KD-VEGF alone **(Figure 5.6B)**. Double knock-down of VEGF and Foxo1 did not interfere with VEGF suppression **(Supplemental Figure 5.3A)**.

To test if depletion of Foxo1 increased cell survival in a parallel pathway, we also evaluated single KD-Foxo1 and observed no significant effect compared to KD-Scr condition **(Figure 5.6B)**. Interestingly, incorporation of a constitutively-active Foxo1 (Ad-Foxo1CA) to KD-Scr causes only a modest decrease of approximately 20% in cell survival, while similar treatment to KD-VEGF results in severe cell death, resulting in approximately 60% cell demise **(Figure 5.6C)**, suggesting that Foxo1's activity requires a KD-VEGF background.

Because the microarray and cellular readouts indicate a reversal of the metabolic and cell death defects seen in KD-VEGF conditions, we next determined whether overactive autophagy was reversed. To assess the role of Foxo1 in KD-VEGF autophagy, we measured autophagic vacuoles in the rescue condition. We found that KD-VEGF/Foxo1 did indeed recover the levels of autophagic vacuoles in KD-VEGF to WT levels **(Figure 5.6D)**.

To identify the transcriptional relationships between Foxo1 and VEGF, expression microarrays were performed on control, KD-VEGF and double-knock down KD-VEGF+Foxo1 HUVECs. We found that a subset of genes were restored to wild type levels specifically in two patterns: Expression Pattern 1) Genes that are up-regulated in KD-VEGF and then down-regulated in KD-VEGF+Foxo1 **(Figure 5.6 E-F; Supplemental Table 5.3)** or Expression Pattern 2) Genes that are down-regulated in KD-VEGF and then up-regulated in KD-VEGF+Foxo1 **(Figure 5.6G-H; Supplemental Table 5.4)**. DAVID analysis of genes that follow Expression Pattern 1 revealed rescue of the highly-represented mitochondrial cluster as well as a reversal of cell-cycle cluster expression patterns **(Figure 5.6I,J)** indicating a reversal of cell cycle arrest and metabolic distress.

Discussion

The present study demonstrates that endothelial cell autonomous VEGF is essential for normal metabolic functions and survival of the endothelium. Our data further indicates that VEGF in the endothelium is required to maintain low Foxo1 protein levels and keep Foxo1 activation status under check. In the absence of endogenous VEGF, Foxo1 is increased in the nucleus and the cytoplasm, depressing endothelial metabolism and increasing autophagic activity. These findings were further substantiated with transcriptional data that show widespread changes in mitochondrial function and cell cycle that were corrected when Foxo1 levels were decreased in KO cells. In addition, endothelial cell death in the absence of cell-autonomous VEGF was also rescued through reduction of Foxo1. Overall the data suggest an important biological link between these two pathways in the maintenance of endothelial cell metabolism and survival.

It has been previously shown that exposure of endothelial cultures to VEGF results in deactivation of Foxo1 [22], one of the forkhead family of transcription factors which play important roles in the suppression of cell cycle progression, reduction of metabolism and response to cell stress [21]. Although high amounts of VEGF signaling ultimately leads to its degradation, the presence of Foxo1 modulates endothelial responses to VEGF. Foxo1 is in fact crucial for proper endothelial signaling cascades in response to VEGF [23], and contributes to the expression of a set of genes induced by VEGF [24]. In homeostatic conditions *in vitro*, Foxo1 regulates a distinct set of vascular angiogenic genes that suppress the angiogenic response of the endothelium [25]. *In vivo*, Foxo1-deficient mice die at E11 from vascular defects [26] and fail to respond properly to treatment with exogenous VEGF. However a role for this pathway in the maintenance of endothelial cell homeostasis and metabolism in the adult had not been suspected.

Mechanistically, we showed that absence of VEGF results in an increase in total and activated FOXO1. Moreover, we found that the endothelium of VEGF ECKO mice expresses

high levels of FOXO1 *in vivo* indicating an important regulatory loop. The cell demise was rescued in VEGF-ECKO cells by simply decreasing the levels of FOXO1, indicating that this is the main event that triggers cell death downstream VEGF.

Foxo transcription factors are widely studied as regulators of mitochondrial function [27], cell metabolism [28] and autophagy [29] [30] in several cell types, but these functions have not been well-linked to VEGF signaling in the endothelium. Foxo1 acts as a major regulator of physiological glucose homeostasis throughout the body, and is well known as a target of insulin signaling that suppresses its activity via the PI3K/Akt pathway [31]. At a cellular level, Foxo1 regulates the opposing metabolic processes of glycolysis and glucogenesis [28], and targets a myriad of genes directly involved in metabolic processes such as PDK4 [32], glucose-6-phosphatase and phosphoenolpyruvate carboxykinase [33]. In this study, we found that depletion of cell-autonomous VEGF increased Foxo1 activation and suppressed cellular metabolic flux in terms of glycolysis, mitochondrial respiration, and fatty acid synthesis.

Foxo transcription factors have been shown to induce autophagy promoting survival during metabolic stress in hematopoietic stem cells [34], and in cardiomyocytes [35]. However, autophagic cell death was also observed as a result of Foxo signaling in pathological settings such as muscle atrophy [36]. Non-canonical (not transcriptional) functions of Foxo1 in autophagy have also been described. In particular, cytoplasmic Foxo1 has been shown to induce autophagic death through direct binding of the autophagic protein Atg7 in cancer cells-- a function entirely independent of DNA-binding ability [20]. We observed an increase in both cytoplasmic and nuclear levels of Foxo1 in KD-VEGF cells, which allows both direct cytoplasmic pro-autophagy signaling as well as transcriptional effects. In our studies, Foxo1 suppressed the autophagic phenotype exhibited by VEGF KO cells, but constitutively active Foxo1 alone does not cause cell demise. Therefore, VEGF promotes additional changes in the biology of the endothelium that now sensitize the endothelium to alterations in Foxo1 levels.

It should be stressed, however, that our findings strongly suggest alternative effects of VEGF in targets other than FOXO1. In fact, our transcriptional data indicates that other genes are deregulated upon VEGF decrease and some of these were not rescued by decrease of FOXO1. Furthermore, as previously discussed, while FOXO1 reduction rescues VEGF depletion, we are unable to induce cell death to the same degree as observed in VEGF null endothelium. Indicating that VEGF alters additional pathways that control survival, and that FOXO1 is a critical trigger but only in the context of a previously-sensitized endothelium.

Overall the findings presented here indicate that cell-autonomous VEGF is required for regulation of endothelial cell metabolism and autophagy in fully differentiated cells. This function adds to the long list of contributions of this signaling pathway to endothelial biology and provides a call to caution to pharmacological treatments that promote long-term suppression to the VEGF signaling pathway.

Materials And Methods

Materials

hVEGF165 and B20 anti-VEGF antibody [37] was provided by the NIH Repository, Genentech (San Francisco, CA) and used as “rVEGF” at 100ng/mL unless otherwise indicated. Anti-human VEGFR2, phospho-VEGFR2 Tyr1175, Cleaved and whole Caspase 3, GAPDH, Foxo1 were from Cell Signaling. SU4312 was obtained from Sigma-Aldrich. Cell viability was determined using cell counting kit-8 (CCK-8 kit, Dojindo Molecular Technologies) as in [7]. Cyto-ID Autophagy detection kit was used to label autophagic vesicles (Enzo Life Sciences).

Generation of mice and cre induction

Generation of floxed-VEGF [38] and inducible VE-Cadherin-cre (VE-Cad-iCre) have been previously described [39]. These mouse lines were interbred to produce homozygous floxed-VEGF/VE-Cad-iCre mice which were induced to excise endothelial VEGF by treatment with tamoxifen. Tamoxifen was administered for 6 days by daily gavage and injections starting at 10 weeks of age and mice were monitored for lethality.

Hypoxia treatment of mice

Prior to treatment, mice were housed in standard polypropylene cages in a room maintained at 25oC and allowed full access to food and water. All animals were housed in a pathogen- free environment in an AAALAC-approved vivarium at UCLA, and experiments were performed in accordance with the guidelines of the Committee for Animal Research.

The mice were randomly assigned to two the experimental groups per genotype: (1) control group (10 Cre negative mice and 7 Cre positive mice) maintained under normoxic conditions; (2) experimental group (15 Cre negative mice and 15 Cre positive mice) exposed to hypoxia for 1 week followed by normonia for 2 weeks and additional hypoxia for 2 weeks. Humidity in the

chamber was maintained at 40–50% and temperature at 22–24 °C. All the groups were induced in custom-built chambers (Oxycycler model X, Biospherix, Redfield, NY) connected to a supply of O2 and N2 gas. A computer was programmed to control the inflow of PO2 at 8%. The intermittent hypoxia procedure used in the study reduced oxygen saturation by 60%. For the treatment, the PO2 gradually decreased to reach the target level. After exposing the animals to the decreased PO2 for 1w, the PO2 was gradually increased to reach the normal level in about 4h. The chambers were cleaned daily. For the normoxic control, animals were kept in the chamber circulated with room air for the identical corresponding period.

Cell Culture

Human umbilical vein endothelial cells (HUVECs; VEC Technologies) were cultured in complete MCDB131 medium (VEC Technologies). Primary mouse endothelial cells were isolated from adult VEGF-ECKO as described in [7]. IMECs were derived from homozygous flox-flox VEGF mice crossed with Immortomouse line (Charles River Labs) harvested from the lung as described in [40]. Floxed-VEGF was excised by andenoviral delivery of Cre protein as in [41], and then checked for complete excision by genomic PCR. IMECs were cultured in immortalized conditions in DMEM supplemented with 100U interferon-gamma at 33C. After expansion, IMECs were split into EC conditions (37C in MCDB131 with 10% FBS) to inactivate the immortalization promoter then used at passage 1-4 for experiments.

Incucyte Live Cell Imaging

For cell death assays, IMECs were grown to confluence in 96-well plates then treated with 0.1uM YOYO-1 (Essen Bioscience) in serum-free media as per the manufacturer's instructions with images collected at 2hr increments. Confluence assays were performed in 6-well plates

with images collected at 1hr increments. The mean of the data was calculated and plotted by ESSEN Incucyte software.

siRNA Transfection

Confluent HUVECs were transfected twice (on day 1 and day 3) with 20nmol of siRNA oligonucleotides using siPORT Amine transfection reagent (cat #AM4503; Ambion) in serum-free medium (optiMEM I; Invitrogen). Cells were analyzed by western, RT-PCR, microarray or for cell count on day 5 unless otherwise indicated. Oligonucleotides used: siGENOME Human VEGFA (7422) siRNA, ON-TARGETplus Human FOXO1 (2308) siRNA - SMARTpool, ON-TARGETplus Non-targeting siRNA #1 (KD-Scr) (Thermo Scientific).

Real-Time RT-PCR

RNA was isolated and amplified as in [40]. Primers used: VEGFA: Hs00900055_m1, FOXO1: Hs01054576_m1, CITED2: Hs01897804_m1, FOXO1: Hs01054576_m1, FOXO3: Hs00921424_m1, FOXO4: Hs00172973_m1, CCNB2: Hs00270424_m1, SOD2: Hs00167309_m1, SPRY2: Hs01921749_s1, KDR: Hs00911700_m1, CITED2: Hs01897804_m1, CCND1:Hs00765553_m1, MMP7: Hs01042796_m1, SEPP1: Hs01032845_m1, DCN: Hs00370384_m1, ID2: Hs04187239_m1 (Applied Biosystems). Primers used to verify microarray: Primers used to verify microarray: CCL23 5‘ctcctacaccccacgaagcat-3; 5’-ttcttcctggtcttgatccgt g-3’ [42]; NDRG4 5’-ggccttctgcatgtagtgatccg-3’, 5’-ggtgatctcctgcatgtcctcg-3’ [43]; ESM1 5’-aaggctgctgatgtagttc-3’ 5’-gctatttatggaagtgtatgtgttt-3’ [44]; TAGLN 5’-aagaatgatgggcactaccg-3’ 5’-actgatgatctgccgaggtc-3’[45].

Viral Transduction

Cells were infected overnight with Lentiviral-VEGF164 or control Lentiviral-YFP at a p24 titer of 0.16ug in serum-free media with protamine sulfate (4ug/mL). Adenoviral infection of was performed as in [41].

Western Blots

ECs were preincubated for 5min with 100uM Na3VO4 to inhibit phosphatase activity and then harvested for total protein in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycolate, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM glycerophosphate, and protease inhibitors 1 mM phenyl-methanesulfonylfluoride (PMSF), Complete EDTA-free protease inhibitor tablet (Roche)). Cell lysates were scraped into tubes, incubated under agitation at 4C for 30min, then centrifuged at 20,000 g for 20 min at 4°C. Equivalent levels of protein, determined using the DC protein assay reagent (Bio-Rad Laboratories) were separated by SDS-PAGE, transferred to nitrocellulose membranes (BioRad), probed with indicated antibodies, detected by Supersignal West Pico Chemiluminescent Substrate (Pierce Biotechnology), and quantified by densitometry using Image Lab.

Metabolic measurements

Glucose consumption and lactate production were measured using the Bioanalyzer 4 (Nova Biomedical). Cells were seeded at 150,000 cells per well in triplicate. Fresh media was added to the wells 24 hours after seeding, and glucose depletion from and lactate addition to the culture media over the following 24 hour period were determined. Values were normalized to cell number and time interval.

Basal oxygen consumption rates and mitochondrial respiratory capacity were measured using the XF24 Extracellular Flux Analyzer (Seahorse Bioscience). Cells were seeded at 62,500 cells per well in a 24-well plate 48 hours prior to assay, and media was renewed plus or minus serum

24 hours prior to assay. Values were normalized to total protein content using the BCA Protein Assay Kit (Pierce).

Microarray Analysis

Illumina gene expression data were normalized using quintile normalization method. Normalization and quality control were performed using “lima” R package (Smyth, 2005). We filtered out noise range probes by requiring that probes have at least 2 significant ($p<0.05$) detections within the triplicates of at least one experiment group. This resulted in retaining 20003 probes out of 47217 for the inferential analysis. The empirical Bayes moderated t-tests [46] were performed to compare the differences in gene expression between control and KD-VEGF genes. Z scores of expression values were generated for each gene and group means were plotted.

DAVID analysis was performed only on significant genes (p-value <0.05) with log-fold changes >1 between KD-Scr and KD-VEGF to create “upregulated” GO clusters. Similarly, DAVID analysis was performed on significant genes with log-fold changes <1 to create “downregulated” GO clusters.

To pick gene clusters fitting expression pattern 1, DAVID analysis was performed on the set of genes that were significantly upregulated in KD-VEGF compared to control, and significantly downregulated in KD-VEGF+Foxo1 compared to KD-VEGF. The list of genes that fit pattern 2 was created using the opposite expression patterns. DAVID analysis was performed on these lists of genes and GO clusters were made into heatmaps using z-scores of expression values.

Statistical Analysis

Differences between groups were evaluated wtih one-way ANOVA and followed with Tukey’s post-hoc multiple comparison test. Selected biologically interesting p values are reported. Pairs

of data were compared using unpaired two-tailed Student's t test. Survival curves were constructed using the Kaplan Meier method and were compared between groups with the log rank test. All analysis was performed using Prism (v4.0c; GraphPad Software) unless otherwise indicated, and values of <0.05 were considered significant.

Immunocytochemistry and confocal microscopy

ECs were grown to confluence on coverslips in 6-well plates and transfected as above. For autophagy, wells were treated with Green Autophagy Detection reagent and Hoechst 33342 Nuclear Stain (Cyto-ID Autophagy detection kit; Enzo Life Sciences) for 30 minutes at 37°C. The cells were then washed and fixed in 3% PFA for 15 minutes. For Foxo1 cell staining, cells grown on coverslips were fixed for 15 minutes in 3% PFA. The coverslips were incubated overnight in polyclonal anti-FOXO1 antibody followed by an incubation of anti-rabbit Alexa Flour® 488 for one hour. Nuclei were counterstained using DAPI. Adult lungs were fixed overnight in 2% PFA and embedded in paraffin. Antigen retrieval was achieved by a combination of proteinase K treatment and Tris-EDTA epitope retrieval. Sections were incubated overnight in polyclonal anti-FOXO1 antibody followed by HRP-conjugated anti-rabbit for one hour. Signal amplification was achieved using Molecular Probes® TSATM Kit #22. Nuclei were counterstained using DAPI. All slides were mounted in Mowiol® 4-88. Carl Zeiss LSM 510 META Laser Confocal Microscope was used to acquire 40X images with 0.80 aperture at room temperature. ZEN® 2009 was used as the acquisition software. Exported files were quantified using ImageJ®. P-values were obtained in a Student's t test from the average percent area, normalized to WT samples.

Summary of Supplemental Figures

Figure S1 shows a lack of apoptosis by several methods: western blots detecting cleaved-Caspase 3 and cleaved-PARP, and TUNEL staining for nicked DNA. Figure S2 shows representative gene clusters from DAVID analysis of genes down-regulated in KD-VEGF (blood vessel, Golgi) and down-regulated (mitochondria) as well as a "Foxo1 target" cluster assembled from the literature. Figure S3 shows microarray validation and changes in Foxo1 target genes in KD-VEGF conditions by RT-PCR. Table S1 and S2 provide a detailed list of the top ontological clusters derived from the list of genes most changed between KD-VEGF and KD-Scr HUVECs in an RNA microarray. Table S3 and S4 provide the top clusters derived from the list of genes responding to Foxo1 rescue following "Expression Pattern 1" (Table S3) and "Expression Pattern 2" (Table S4).

Abbreviations List

HUVEC Human Umbilical Vein Endothelial Cells

VEGF Vascular Endothelial Growth Factor

VEGFR-2 Vascular Endothelial Growth Factor Receptor 2

Foxo1 Forkhead box protein O1

WT Wild Type

KO Knock-out

ECKO Endothelial Cell Knock-Out

iECKO Inducible Endothelial Cell Knock-Out

rVEGF Recombinant VEGF

KD siRNA Knock-down

IMEC Immortalized Endothelial Cells

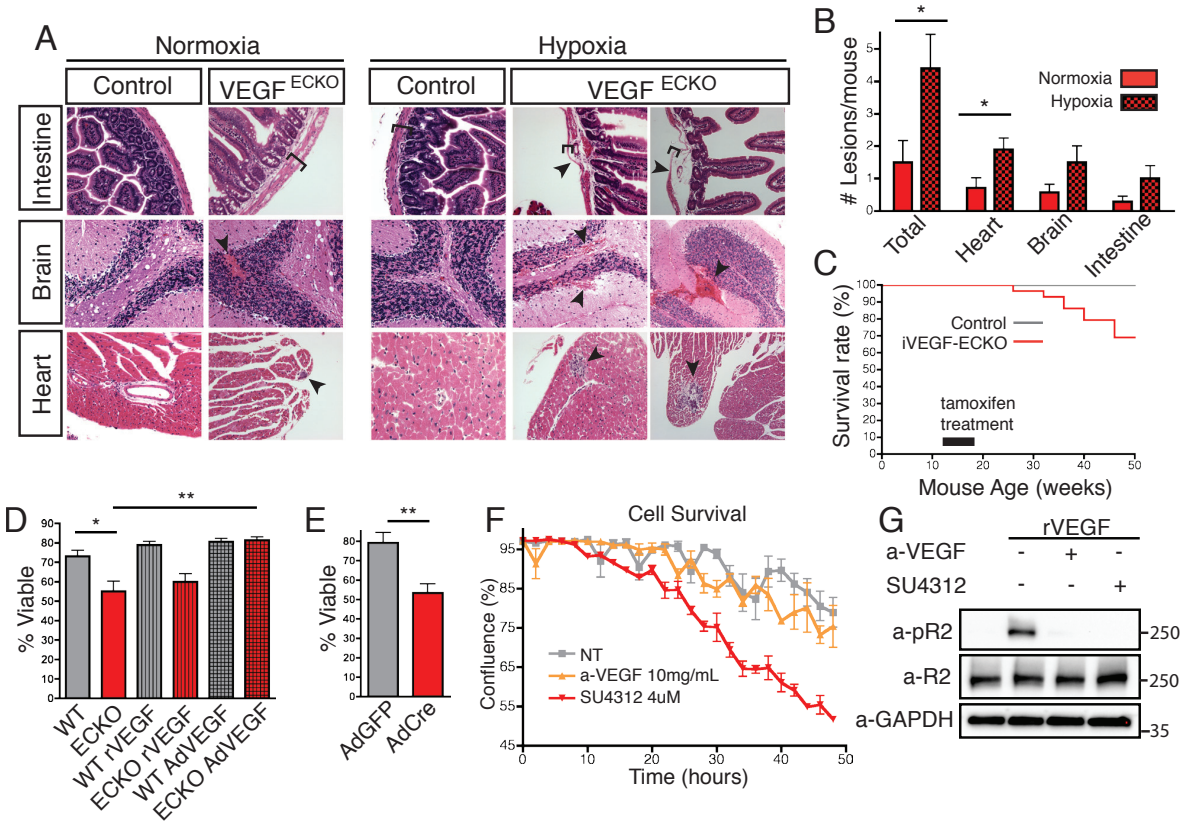


Figure 5.1: Autocrine VEGF is required for endothelial cell survival

(A) H&E stained histological sections of VEGF-ECKO mice after hypoxia treatment. Arrows indicate areas of hemorrhage. Brackets indicate thickness of outer intestinal wall. (B) Quantification of A) showing average number of lesions per VEGF-ECKO mouse in normoxia and hypoxia. No lesions were observed in control mice (VEGF-ECKO n=14 normoxia, n=10 hypoxia; Control n=9 normoxia, n=10 hypoxia). (C) Survival analysis of iVEGF-ECKO mice after tamoxifen injection at 10 weeks. Sudden death starting 15 weeks post-injection (Control n=25, iVEGF-ECKO n=30). (D) Viability of endothelial cells isolated from adult WT or VEGF-ECKO mice treated with exogenous rVEGF (100ng/mL) or with adenoviral-VEGF (n=10). (E) Viability of endothelium isolated from the liver of adult floxed-VEGF mice treated in culture with adenovrial-GFP (Ad-GFP) or Cre (Ad-Cre)(n=6). (F) Quantification of HUVEC survival over time when blocking endogenous VEGF extracellularly (B20 10mg/mL) or intracellular/extracellular (SU4312 4uM). (G) anti-VEGF antibody (B20 10mg/mL) or by cell-permeant VEGF-R2 inhibitor (SU4312 4uM) blockade of rVEGF (100ng/mL) induced phosphorylation of VEGF-R2.

*, P<0.05; **, P<0.005

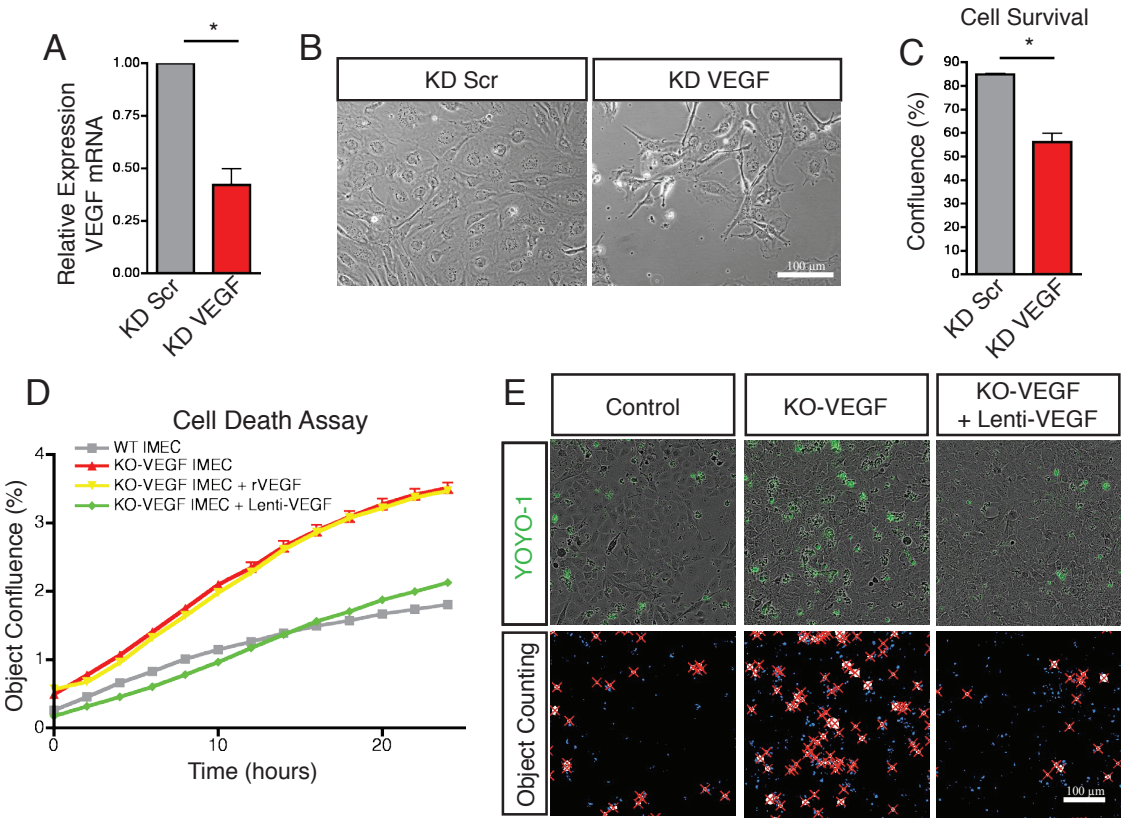


Figure 5.2: Reduction or inactivation of endothelial VEGF results in cell demise

(A) siRNA targeting of VEGF results in over 50% decrease in VEGF transcript (n=3) (B) KD-VEGF reduces cell viability. Images were collected of cell layer 5 days after first transfection. Bar, 100um. (C) Quantification of cell confluence from (B). (n=4) (D) Cell death was assessed in WT or KO-VEGF murine endothelium untreated, treated with rVEGF (100ng/mL) or infected with Lentiviral-VEGF. (n=9) (E) Representative images of (D) showing fluorescent signal of Yoyo-1 and object counting used for quantification (red x's). Bar, 100um.

*, $P<0.05$

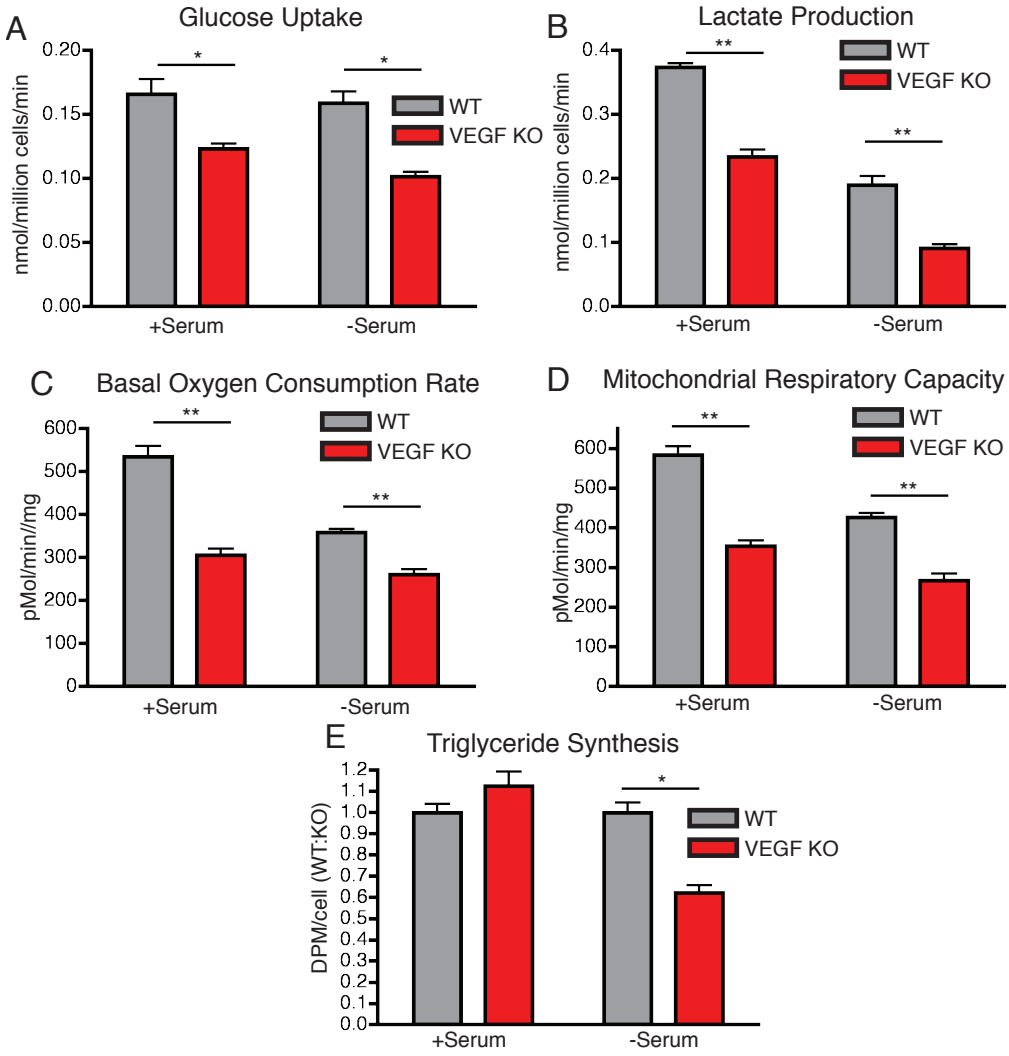


Figure 5.3: Inactivation of endothelial VEGF results in metabolic defects
KO-VEGF endothelial cells display decreased (A) glucose uptake (B) lactate production (C) basal oxygen consumption and (D) mitochondrial respiratory capacity measured in the presence or absence of serum. (n=6) (E) Triglyceride synthesis is depressed in KO-VEGF cells only in serum starvation conditions. (n=4)
*, P<0.05; **, P<0.005

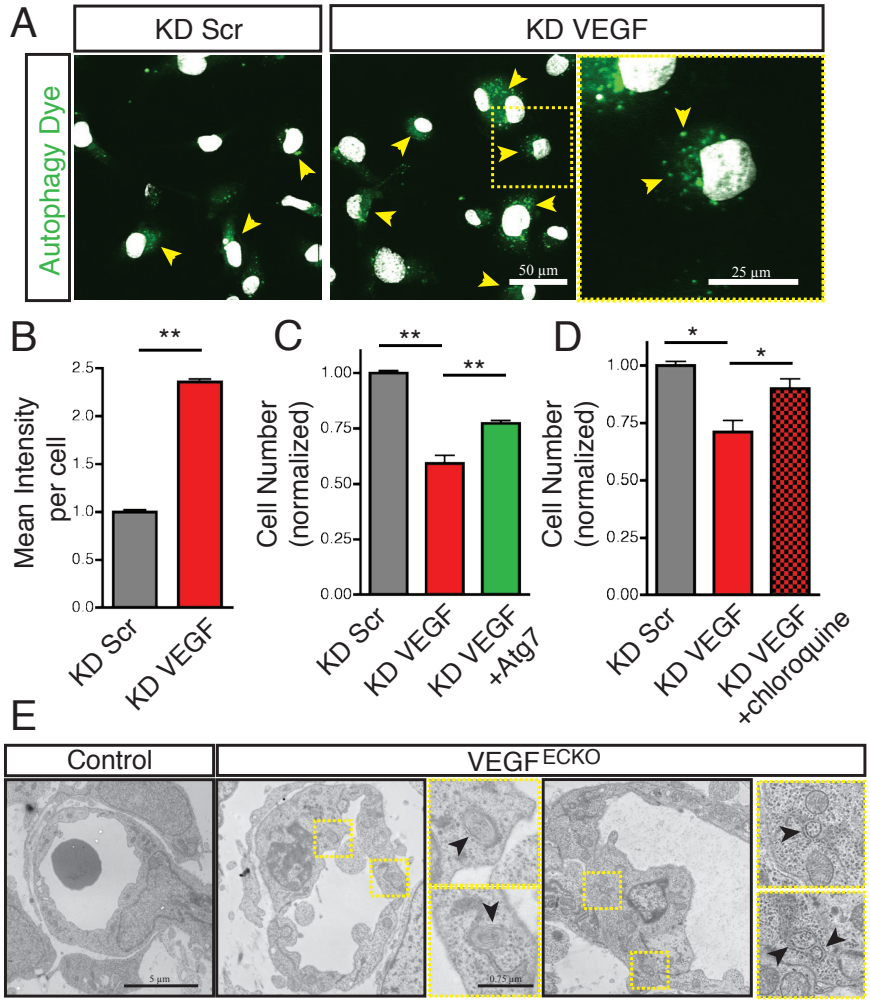


Figure 5.4: Increased autophagy in VEGF-depleted endothelium contributes to cell death phenotype
(A) Autophagic vacuoles (arrowheads) visualized with Cyto-ID autophagy detection kit and analyzed with confocal microscope. Bar, 50um. (B) Quantification of fluorescence in (F). (n=3) (C) Quantification of cell confluence as a measure of viability in siRNA targeting of VEGF or VEGF + Atg7 (n=6) (D) Quantification of cell confluence in KD HUVECs under autophagy blockade with 10uM chloroquine (n=3). (E) VEGF-ECKO adult animals display an abundance of double-membraned autophagic vacuoles (arrowheads) by electron microscopy. Bar, 5µm.*, P<0.05; **, P<0.005

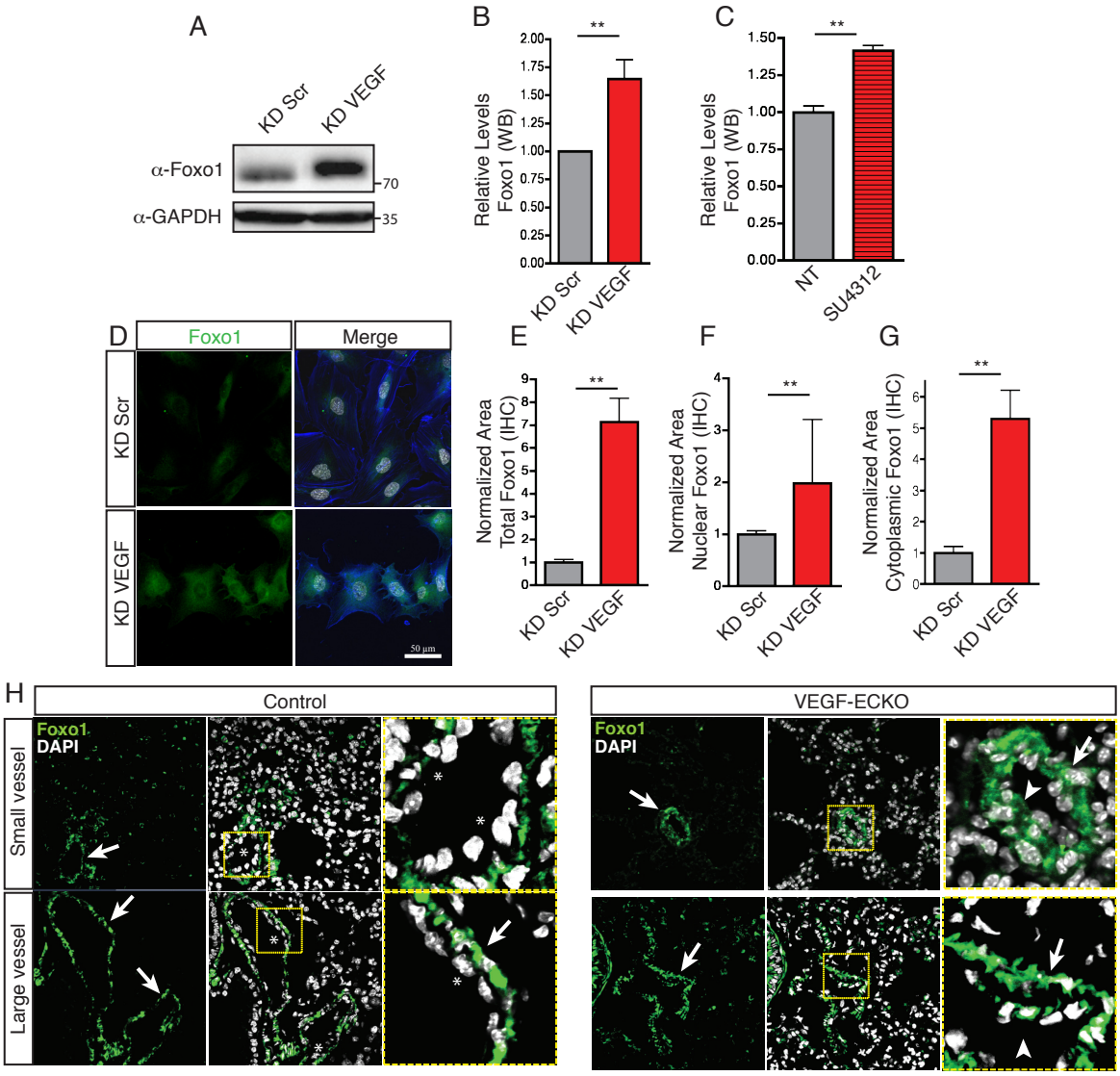


Figure 5.5: Activation of Foxo1 is a major consequence of silencing endothelial VEGF
(A) Western blot showing increased Foxo1 protein levels in KD-VEGF. (B) Quantification of Foxo1 protein levels (n=10). (C) Increased Foxo1 protein levels in HUVECs treated with SU4312 (4uM) (n=3). (D) IF for Foxo1 in KD-VEGF HUVECs shows increased Foxo1 levels. (E) Quantification of total Foxo1 fluorescence from B) (n=3). (F) Quantification of Foxo1 localization to nucleus (n=3). (G) Quantification of Foxo1 localization to cytoplasm (n=3). (H) Endothelial Foxo1 is expressed in the absence of VEGF in vivo, but this is not observed in WT endothelial cells. Arrowheads denote endothelium positive for Foxo1 in VEGF-ECKO. Asterisk indicates endothelial layer lacking Foxo1 in WT animals. Foxo1 staining detected in vascular smooth muscle cells (arrows) of both WT and VEGF-ECKO (n=2 animals). *, P<0.05; **, P<0.005

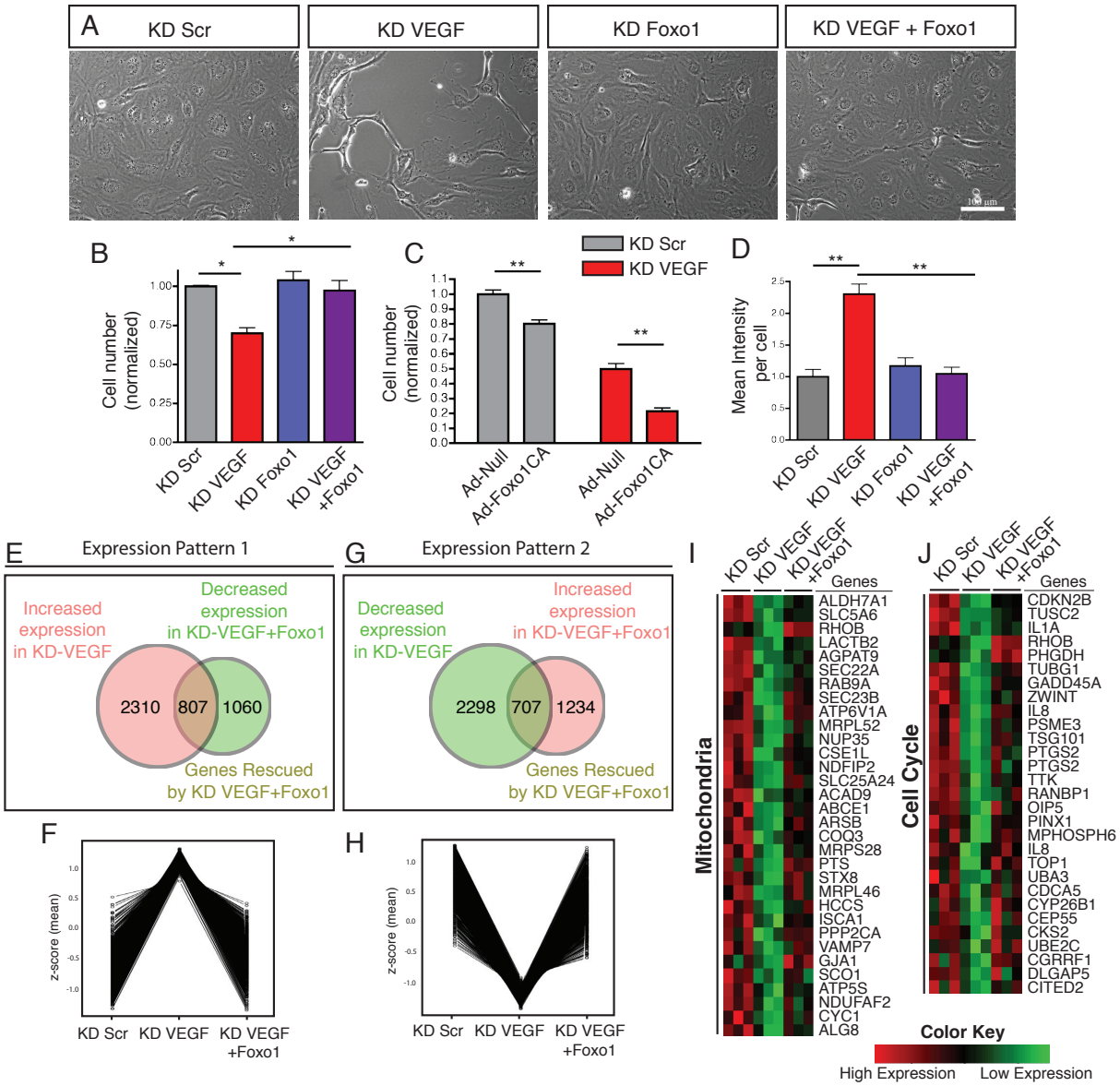
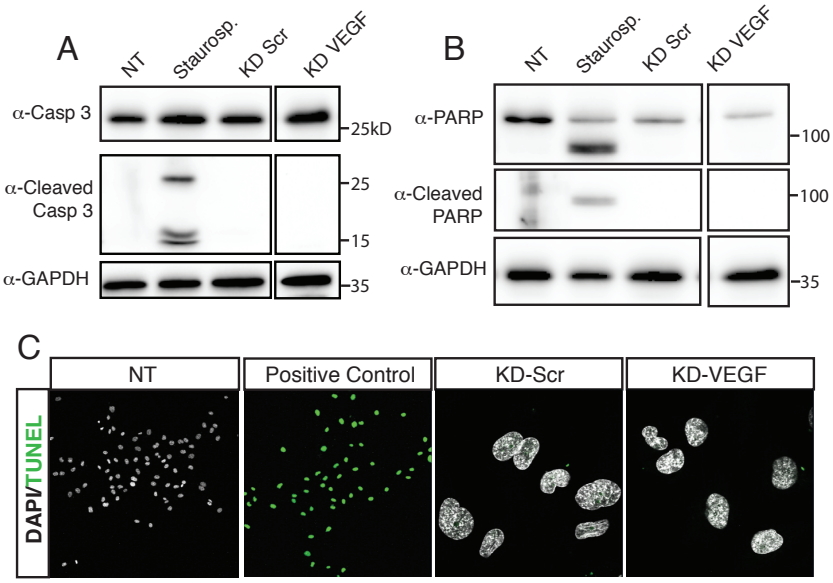
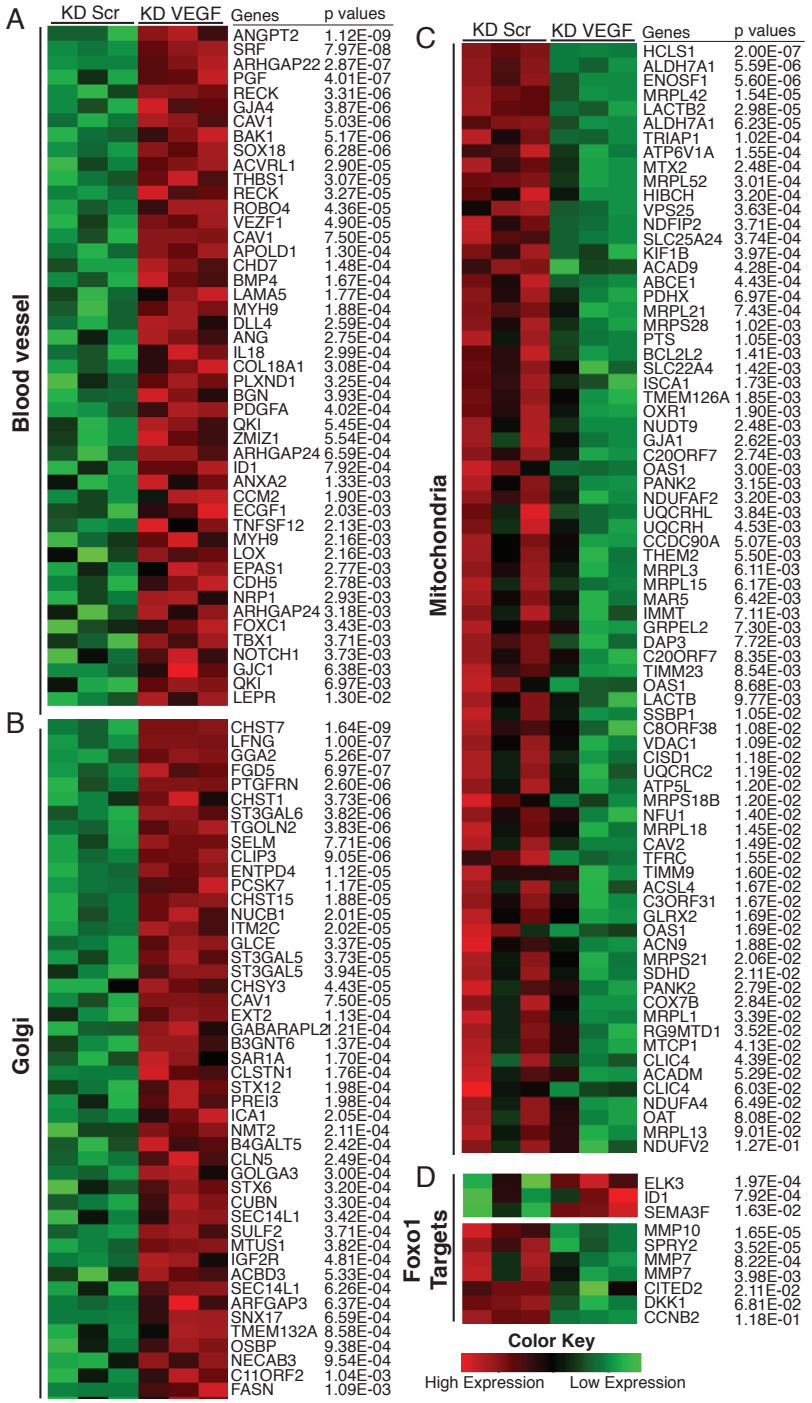


Figure 5.6: Foxo1 is essential to trigger cell death and autophagy in the absence of VEGF
(A) Images show KD-Foxo1 rescues KD-VEGF-induced cell death on day 4 after first transfection (Bar, 100µm). (B) Quantification of cell numbers found in A). (n=3) (C) Constitutively active Foxo1 (Ad-Foxo1CA) reduces cell viability of control and KD-VEGF cells compared to empty vector (Ad-Null) on day 5. (n=4) (D) Increased autophagic vacuoles were visualized with Cyto-ID autophagy detection kit when analyzed by confocal microscopy. (n=3) Z scores of microarray expression values were generated for each gene, the group means were plotted to explore the trend of changes in expression for KD-VEGF and KD-VEGF+Foxo1 rescue from controls. Among these probes that were significantly differentially expressed between groups, we identified probes with (E-F) increased expression in KD-VEGF and decreased expression in KD-VEGF+Foxo1 (pattern 1), and (G-H) probes with decreased expression in KD-VEGF and increased expression in KD-VEGF+Foxo1 (pattern 2). Venn diagrams were generated to reflect these patterns (F, H). Significantly rescued genes were subject to DAVID analysis, and heatmaps were generated from selected GO clusters for (I) mitochondria (J) cell cycle genes.
*, P<0.05; **, P<0.005

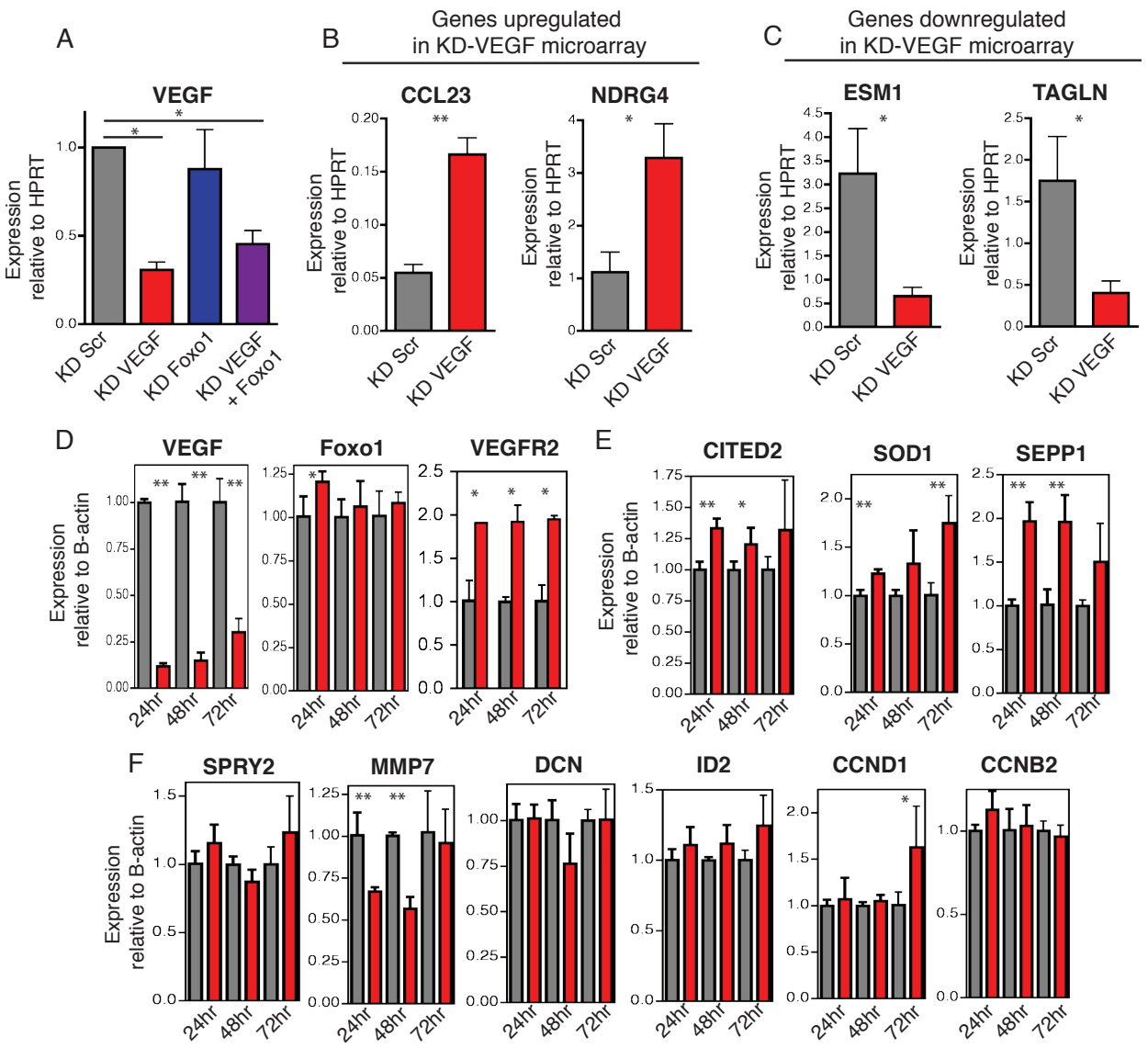


Supplemental Figure 5.1: Apoptosis was not detected in KD-VEGF HUVECs

(A) Total and cleaved Caspase 3 were detected by western blot in 25ug of protein lysate from KD-VEGF and KD-Scr HUVECs. (B) Total and cleaved PARP were analyzed as above. Positive and negative apoptosis controls were provided by non-treated and staurosporine (1hr 1uM) treated HUVECs. (C) TUNEL staining was performed on KD-VEGF and KD-Scr cells. Out of approximately 50,000 cells, 4 were found to be TUNEL positive in the KD-VEGF condition, while none were TUNEL positive in KD-Scr. Nuclease treatment was used to create nicked DNA as a positive control.



Supplemental Figure 5.2: Autocrine VEGF is required to maintain endothelial identity, mitochondrial and Golgi transcriptome
HUVECs were transfected with siRNA targeting VEGF and total RNA was isolated on day 4 after first transfection (n=3). Gene expression profile was assessed with Illumina human gene chip expression assay. GO clusters extracted from genes significantly up- and down-regulated in KD-VEGF analyzed by DAVID. Heatmaps highlight a subset of two down-regulated GO clusters: (A) blood vessel and (B) Golgi. (C) Mitochondrial genes were upregulated. (D) Select Foxo1 targets significantly up- or down-regulated.



Supplemental Figure 5.3: Microarray validation and further analysis of Foxo1 target genes

(A) A significant decrease in VEGF levels was maintained in double-knockdown conditions. Two of the top genes found (B) increased (CCL23 and NDRG4) and (C) decreased (ESM1 and TAGLN) in the KD-VEGF condition of the microarray were validated by RT-PCR. (D) RT-PCR analysis of several genes of interest showed that decrease in VEGF levels was maintained over several days, Foxo1 levels remained largely unchanged, and VEGFR-2 levels were drastically increased. Several previously-described Foxo1 targets were found to be (E) increased as expected in the KD-VEGF condition by RT-PCR (CITED2, SOD2, SEPP1) or (F) found to be unchanged or regulated contrary to previous reports (SPRY2, MMP7, DCN, ID2, CCND1, CCNB2).

Table 5.1 DAVID analysis of genes significantly downregulated in KD-VEGF

GO Cluster	Term	Count	Benjamini	Fold Enrichment
Cluster 1 - Enrichment Score: 9.02				
	golgi apparatus	128	8.53E-09	1.78
	GO:0005794-Golgi apparatus	175	4.96E-07	1.54
	GO:0044431-Golgi apparatus part	75	1.88E-06	1.96
Cluster 2 - Enrichment Score: 6.88				
	GO:0001568-blood vessel development	65	3.66E-05	2.08
	GO:0001944-vasculature development	65	5.01E-05	2.03
	GO:0048514-blood vessel morphogenesis	54	8.71E-04	2.01
	GO:0001525-angiogenesis	41	2.17E-03	2.17
Cluster 3 - Enrichment Score: 6.21				
	endoplasmic reticulum	146	2.91E-08	1.67
	GO:0005783-endoplasmic reticulum	179	2.75E-05	1.43
	GO:0044432-endoplasmic reticulum part	64	6.67E-02	1.42
Cluster 4 - Enrichment Score: 5.12				
	IPR001849:Pleckstrin homology	65	2.11E-03	1.86
	domain:PH	56	2.59E-03	1.92
	SM00233:PH	65	1.79E-03	1.76
	IPR011993:Pleckstrin homology-type	60	2.22E-01	1.57
Cluster 5 - Enrichment Score: 4.07				
	nucleotide-binding	278	5.93E-06	1.35
	atp-binding	226	8.02E-06	1.39
	transferase	232	3.14E-05	1.36
	kinase	129	3.80E-05	1.53
	GO:0000166-nucleotide binding	356	4.81E-03	1.24
	GO:0001882-nucleoside binding	261	1.56E-02	1.26
	GO:0001883-purine nucloside binding	259	1.18E-02	1.26
	GO:0030554-adenyl nuclotide binding	255	1.05E-02	1.26
	GO:0005524-ATP binding	240	1.12E-02	1.27
	GO:0032559-adenyl ribonucleotide binding	242	1.24E-02	1.26
	GO:0006793-phosphorus metabolic process	165	2.43E-02	1.33
	GO:0006796-phosphate metabolic process	165	2.43E-02	1.33
	GO:0004672-protein kinase activity	111	1.60E-02	1.43
	binding site:ATP	98	6.76E-02	1.47
	GO:0017076-purine nucleotide binding	299	1.42E-02	1.21
	GO:0032555-purine ribonucleotide binding	286	1.72E-02	1.21
	IPR017441:Protein kinase, ATP binding site	85	1.72E-02	1.48
	active site:Proton acceptor	113	2.07E-01	1.40
	domain:Protein kinase	84	1.35E-01	1.46
	IPR000719:Protein kinase, core	86	1.90E-01	1.43
	IPR002290:Serine/threonine protein kinase	52	2.30E-01	1.59
	nucleotide phosphate-binding region:ATP	152	2.63E-01	1.28
	serine/theronine-protein kinase	69	3.41E-01	1.48
	GO:0016310-phosphorylation	132	2.82E-02	1.29
	GO:0006468-protein amino acid phosphorylation	112	1.30E-01	1.32
	GO:0004674-protein serine/threonine kinase	77	1.58E-01	1.39
	SM00220:S_TKc	52	1.15E-01	1.51
	IPR008271:Serine/threonine protein kinase	64	2.05E-01	1.43
	IPR017442:Serine/threonine protein kinase-rela	64	4.99E-01	1.41

Table 5.2 DAVID analysis of genes significantly upregulated in KD-VEGF

GO Cluster	Term	Count	Benjamini	Fold Enrichment
Cluster 1 - Enrichment Score: 18.55				
	Mitochondrion	200	3.39E-21	2.03
	GO:0005739-mitochondrion	243	1.36E-20	1.85
	transit peptide	125	4.79E-16	2.22
	transit peptide:Mitochondrion	123	2.66E-14	2.22
	GO:0044429-mitochondrial part	134	3.25E-11	1.86
Cluster 2 - Enrichment Score: 13.68				
	GO:0031974-membrane-enclosed lumen	352	2.15E-18	1.57
	GO:0070013-intracellular organelle lumen	339	4.91E-18	1.57
	GO:0043233-organelle lumen	342	2.61E-17	1.55
	GO:0031981-nuclear lumen	272	7.67E-13	1.55
	GO:0005654-nucleoplasm	175	6.55E-10	1.64
	GO:0005730-nucleolus	131	6.49E-06	1.55
	GO:0044451-nucleoplasm part	106	3.60E-05	1.58
Cluster 3 - Enrichment Score: 8.86				
	GO:0030529-ribonucleoprotein complex	142	1.20E-19	2.28
	ribonucloprotein	90	4.56E-17	2.73
	ribosomal protein	65	9.91E-14	2.92
	GO:0006412-translation	95	3.14E-11	2.27
	GO:0005840-ribosome	69	1.55E-12	2.65
	GO:0033279-ribosomal subunit	48	3.28E-11	3.10
	GO:0003735-structural constituent of ribosome	57	4.70E-10	2.76
	protein biosynthesis	48	1.87E-05	2.16
	GO:0015934-large ribosomal subunit	25	1.30E-05	3.08
	hsa03010:Ribosome	30	3.46E-04	2.53
	GO:0006414-translational elongation	31	6.85E-04	2.43
	GO:0022626-cytosolic ribosome	25	4.13E-04	2.55
	GO:0044445-cytosolic part	37	9.77E-04	2.01
	ribosome	22	1.81E-03	2.55
	GO:0022625-cytosolic large ribosomal subunit	14	5.09E-03	3.04
	GO:0022627-cytosolic small ribosomal subunit	11	1.71E-01	2.27
	GO:0005198-structural molecule activity	89	8.50E-01	1.14
Cluster 4 - Enrichment Score: 8.85				
	GO:0000278-mitotic cell cycle	97	2.15E-09	2.07
	GO:0007049-cell cycle	167	4.24E-08	1.65
	mitosis	54	1.87E-08	2.50
	GO:0022403-cell cycle phase	98	3.96E-07	1.87
	cell cycle	101	5.33E-08	1.85
	GO:0022402-cell cycle process	123	4.49E-07	1.72
	GO:0048285-organelle fission	64	4.05E-07	2.21
	GO:0000087-M phase of mitotic cell cycle	62	1.03E-06	2.19
	cell division	66	2.72E-07	2.11
	GO:0000279-M phase	80	2.70E-06	1.92
	GO:0007067-mitosis	60	2.91E-06	2.16
	GO:0000280-nuclear division	60	2.91E-06	2.16
	GO:0051301-cell division	68	2.25E-04	1.82

Table 5.3 DAVID analysis of genes following Expression Pattern 1

GO Cluster	Term	Count	Benjamini	Fold Enrichment
Cluster 1 - Enrichment Score: 3.67				
	GO:0022402-cell cycle process	43	6.85E-03	2.17
	GO:0022403-cell cycle phase	34	9.64E-03	2.34
	GO:0000278-mitotic cell cycle	31	1.19E-02	2.39
	GO:0007049-cell cycle	50	2.16E-02	1.84
	GO:0000279-M phase	27	3.91E-02	2.34
	GO:0000087-M phase of mitotic cell cycle	20	9.66E-02	2.55
	GO:0048285-organelle fission	20	1.12E-01	2.49
	GO:0000280-nuclear division	19	1.59E-01	2.46
	GO:0007067-mitosis	19	1.59E-01	2.46
	cell cycle	30	4.49E-02	1.97
	GO:0051301-cell division	22	2.74E-01	2.13
	cell division	19	1.16E-01	2.18
	mitosis	15	1.07E-01	2.48
Cluster 2 - Enrichment Score: 3.10				
	ribonucleoprotein	27	4.60E-04	2.93
	ribosomal protein	19	5.86E-03	3.06
	GO:0005840~ribosome	20	1.58E-02	2.75
	GO:0030529~ribonucleoprotein complex	34	2.37E-02	1.95
	GO:0033279~ribosomal subunit	14	2.37E-02	3.23
	GO:0003735~structural constituent of ribosome	16	4.19E-01	2.75
	GO:0006412~translation	23	3.17E-01	1.98
	hsa03010:Ribosome	8	9.95E-01	2.24
	GO:0005198~structural molecule activity	23	9.94E-01	1.05
Cluster 3 - Enrichment Score: 2.61				
	GO:0005739~mitochondrion	65	2.75E-03	1.77
	GO:0044429~mitochondrial part	42	2.08E-03	2.08
	mitochondrion	48	1.79E-02	1.74
	transit peptide:Mitochondrion	30	7.81E-01	1.93
	GO:0031090~organelle membrane	57	5.12E-02	1.54
	transit peptide	30	5.81E-02	1.91
	GO:0031966~mitochondrial membrane	25	1.07E-01	1.87
	GO:0005740~mitochondrial envelope	26	1.01E-01	1.83
	GO:0031980~mitochondrial lumen	17	9.89E-02	2.21
	GO:0005759~mitochondrial matrix	17	9.89E-02	2.21
	GO:0031967~organelle envelope	33	1.68E-01	1.57
	GO:0031975~envelope	33	1.67E-01	1.57
	GO:0005743~mitochondrial inner membrane	17	4.22E-01	1.64
	GO:0019866~organelle inner membrane	17	5.50E-01	1.53
	mitochondrion inner membrane	11	6.46E-01	1.72
Cluster 4 - Enrichment Score: 2.34				
	GO:0051726~regulation of cell cycle	26	8.71E-02	2.24
	GO:0010564~regulation of cell cycle process	12	2.96E-01	3.00
	GO:0007346~regulation of mitotic cell cycle	14	3.29E-01	2.63
	GO:0007093~mitotic cell cycle checkpoint	7	3.48E-01	4.65
	GO:0000075~cell cycle checkpoint	9	5.34E-01	2.82
	GO:0031575~G1/S transition checkpoint	3	9.15E-01	5.04
Cluster 5 - Enrichment Score: 2.32				
	GO:0005761~mitochondrial ribosome	8	5.48E-02	4.92
	GO:0000313~organellar ribosome	8	5.48E-02	4.92
	GO:0005763~mitochondrial small ribosomal	4	2.35E-01	6.56
	GO:0000314~organellar small ribosomal	4	2.35E-01	6.56

Table 5.4 DAVID analysis of genes following Expression Pattern 2

GO Cluster	Term	Count	Benjamini	Fold Enrichment
Cluster 1 - Enrichment Score: 5.70				
	GO:0048514~blood vessel morphogenesis	23	1.00E-03	3.60
	GO:0001568~blood vessel development	24	1.75E-03	3.23
	GO:0001944~vasculature development	24	1.77E-03	3.15
	GO:0001525~angiogenesis	17	5.94E-03	3.79
Cluster 2 - Enrichment Score: 4.33				
	GO:0016477~cell migration	24	5.16E-03	2.87
	GO:0048870~cell motility	24	2.30E-02	2.58
	GO:0051674~localization of cell	24	2.30E-02	2.58
	GO:0006928~cell motion	31	3.71E-02	2.15
Cluster 3 - Enrichment Score: 2.23				
	hsa04360:Axon guidance	14	1.13E-01	2.94
	domain:Sema	6	5.97E-01	6.50
	IPR002165:Plexin	6	6.38E-01	6.36
	IPR001627:Semaphorin/CD100 antigen	6	6.38E-01	6.36
	IPR003659:Plexin/semaphorin/integrin	7	4.97E-01	5.11
	SM00630:Sema	6	5.93E-01	5.60
	SM00423:PSI	7	3.89E-01	4.50
	IPR015943:WD40/YVTN repeat-like	15	9.47E-01	1.64
	domain:Ig-like C2-type	5	1.00E+00	1.63
Cluster 4 - Enrichment Score: 1.83				
	IPR013761:Sterile alpha motif-type	10	3.81E-01	4.27
	IPR001660:Sterile alpha motif SAM	9	8.11E-01	3.18
	SM00454:SAM	9	6.85E-01	2.80
	domain:SAM	7	9.59E-01	2.90
	IPR011510:Sterile alpha motif homology 2	3	9.98E-01	2.40
Cluster 5 - Enrichment Score: 1.74				
	GO:0030054~cell junction	26	7.19E-01	1.67
	cell junction	21	3.46E-01	1.78
	GO:0005911~cell-cell junction	12	6.30E-01	2.10
Cluster 6 - Enrichment Score: 1.73				
	actin-binding	16	2.07E-01	2.19
	GO:0008092~cytoskeletal protein binding	26	7.74E-01	1.67
	GO:0015629~actin cytoskeleton	16	6.39E-01	1.97
	GO:0003779~actin binding	16	9.44E-01	1.59
Cluster 7 - Enrichment Score: 1.63				
	GO:0001667~ameboidal cell migration	6	6.73E-01	5.35
	GO:0014032~neural crest cell development	5	7.91E-01	5.00
	GO:0014033~neural crest cell differentiation	5	7.91E-01	5.00
	GO:0060485~mesenchyme development	6	8.10E-01	3.81
	GO:0001755~neural crest cell migration	4	8.08E-01	5.74
	GO:0014031~mesenchymal cell development	5	8.54E-01	3.23
	GO:0048762~mesenchymal cell differentiation	5	8.54E-01	3.23

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Chapter 6 - Conclusion

Breakthroughs in non-canonical VEGF signaling

Abstract

To conclude this work, the novel findings detailed in Chapter 3, 4 and 5 are summarized and put into context within the recent literature. Combined, the efforts in this thesis have advanced the field of non-canonical VEGF signaling and revealed a novel contribution of this signaling pathway to cardiac morphogenesis. Decades of previous work have demonstrated that VEGF acts on non-endothelial cell types, particularly during embryogenesis. Cardiac development is highly dependent on VEGF signaling for valve development, and work discussed in Chapter 3 and 4 demonstrates a key role in chamber specification and cardiomyocyte progenitor production. Autocrine VEGF signaling is a rapidly-expanding field, and is essential for the function of several cell types found in pathological, developmental and homeostatic conditions. Summarizing chapter 5, we demonstrate that autocrine VEGF is required for maintenance of endothelial metabolism and autophagic flux by way of the transcription factor Foxo1. New literature shows that VEGF receptors are found to activate in the absence of VEGF through several non-canonical mechanisms. Finally, canonical VEGF signaling is further refined upon examination of signaling outputs due to changes in ECM, novel receptor complexes, and interactions of the endothelium with diverse cell types.

6.1 - VEGF acting on non-endothelial cell types: Cardiogenesis

VEGF plays integral role in cardiac valve development

Although vascular endothelium is highly responsive to VEGF, literature continues to emerge describing the effects of VEGF on the differentiation of non-vascular tissue. VEGF has a developmental influence on cardiac populations in particular, and has especially well-documented effects on cardiac valve development [1]. For heart valves to develop, specialized cardiac cushions form at the atrial-ventricular (AV) junction as well as in the outflow tract (OFT). Endocardial cells lining these regions undergo endocardial-to-mesenchymal transformation, migrate through the cushions and differentiate into valve leaflets [1]. This EMT process is highly-regulated by VEGF which has been shown to trigger the initial EMT, but after which promotes suppression, and is thought to be responsible for terminating the process [2].

Recent work shows that VEGF also plays a role in the development of the OFT valves. Inactivation of VEGF by inducing over-expression of a soluble VEGFR1 ligand trap inhibited EMT differentiation in the OFT, but not the AV cushion [3]. For valves to fully develop, there must be a heart valve elongation step after termination of EMT. Inhibition of VEGFR2 by induction of a dominant-negative receptor inhibits valve elongation at this later step [3]. Inactivation of Bmp signaling in the secondary heart field was found to up-regulate VEGF through regulation of the miR-17-92 cluster. In this system, VEGF suppressed EMT in the OFT cushions [4]. From the contradictory results observed in slightly different systems, one can gather that VEGF is highly-important for valve development, but it is clear that VEGF manipulation must be more finely-tuned in a time and spatial-specific manner that current genetic models allow.

VEGF influences cardiomyogenesis and chamber specification

Circumstantial evidence suggests that cardiomyocyte differentiation is directly influenced by VEGF signaling. *In vitro* stem cell culture experiments have demonstrated that exposure of differentiating cells to VEGF increases yield of cardiomoycytes [5] [6]. This is supported by observations that VEGFR2 is an important marker of multipotent cardiac, hematopoietic and vascular progenitors derived from embryonic stem cell culture [7] [8].

In this work, we implicate VEGF signaling in several distinct cardiogenic processes. Using a chimeric approach (Chapter 3), we found that VEGF-KO cells were preferentially recruited to the atrium. This finding was supported by examination of endogenous VEGF expression using a VEGF-LacZ knock-in model, where we found high ventricular VEGF in contrast to extremely low atrial VEGF levels. This suggested to us a novel hypothesis: VEGF expression in cardiomyocytes induces a ventricular phenotype with high levels of trabeculation and a thick compact layer, while lack of VEGF enables cardiomyocytes to adapt an atrial phenotype with low trabeculation and thin chamber walls.

To test this hypothesis, we induced VEGF expression in the atrium at an early time point using the SM22 promoter, and found a qualitative and quantitative “ventricular switch” in the atrial chamber. SM22-VEGF animals displayed hyperplasia of the atria, with a muscular overgrowth of cardiomyocytes histologically similar to ventricular tissue. Examination of chamber specific markers showed a significant increase in the ventricular marker MLCV1 within mutant atria, with a slight decrease of atrial markers MLC1a and MLC2a.

One of the hallmarks of ventricular formation is the emergence of trabeculae. Several publications have linked Notch1 signaling to ventricular trabeculation [9]. In fact, induction of activated Notch1 in the atria induced a remarkably similar phenotype to that of the SM22-VEGF marked by atrial-hyperplasia [10]. We found up-regulation of the Notch1/trabeculation pathway in the SM22-VEGF mutant, with significant increases in the expression of Nrg1 and BMP10.

Cardiac VEGFR2 expression suggests VEGF signaling in cardiac progenitors

VEGF is known to act through several receptors, but VEGFR2 activation elicits the strongest signaling response and is directly implicated in early cardiomyocyte development [8]. In Chapter 4, we closely examined endogenous VEGFR2 expression in the nascent heart using a VEGFR2-LacZ knock-in. At early time points, we observed strikingly high expression of VEGFR2 in the dorsal mesenchymal protrusion (DMP), a developmental structure that is essential for bridging the primary atrial septum to the AV cushion for complete atrial septation.

This population continues to express VEGFR2 through its migration and fusion with the AV cushion, after which expression of VEGFR2 subsides.

We do, however, observe VEGFR2 expression in the septum primum, even as it becomes myocardialized. The septum secondum arrives later in development as a small muscular protrusion from the roof of the atrial chamber. In as late as E14.5 animals, we observed co-localization of VEGFR2 and cardiomyocyte markers in this region. It is in the atrial septum, therefore, where we observe the largest population of VEGFR2+ cardiomyocytes that persist in late stages of heart development.

The discovery that VEGFR2 is expressed on cardiac progenitors [8], as well as anatomical coincidence with cardiac stem cells identified by Isl1 [11] suggests that VEGFR2 is a good marker for cardiac stem cells. This hypothesis is further supported by scattered reports that the treatment of cardiac stem cells with VEGF improves cardiac recovery after infarction upon injection of these stem cells [12] [13] [14]. Further examination of this population and its response to VEGF may yield not only a more complete understanding of septal development, but better treatment options for patients with damaged cardiac tissue.

6.2 - Autocrine VEGF signaling

“Canonical” paracrine signaling by VEGF occurs when VEGF is secreted by a hypoxic tissue, and is detected by receptors on nearby endothelium which induces proliferation and migration of the vasculature. As the field of VEGF study progresses, it has become apparent that VEGF is also capable of signaling in an autocrine manner, where tissues co-express VEGF and its receptor, and even in an intracrine manner, where VEGF signaling occurs within intracellular organelles.

Autocrine VEGF signaling in cancer

The phenomenon of autocrine signaling has been well described in several types of cancer which rely on co-expression of VEGF and its receptors for growth and survival. Early

histological examination of breast cancer in human patients showed high expression of VEGF in aggressive tumors [15]. Although it was assumed that VEGF acted primarily as a paracrine signal on surrounding vasculature, *in vitro* experiments on metastatic breast carcinoma cells demonstrated that VEGF is a pro-survival factor outside a vascular context, and in fact acts through its receptor Nrp1 in an autocrine manner [16]. Reports continue to emerge to indicate that autocrine VEGF enhances tumor cell function in colorectal cancer [17], epithelial cancer [18] and precursor lesions to esophageal cancer [19].

Autocrine VEGF signaling in hematopoiesis

Several types of acute leukemia that express both VEGF and its receptor VEGFR2 are dependent on autocrine VEGF signaling for survival [20] [21]. Perhaps unsurprisingly, physiological hematopoiesis (the formation and differentiation of blood cells) is also dependent on autocrine VEGF signaling. When VEGF was genetically excised from purified hematopoietic stem cells (HSCs) and then implanted back into irradiated mice, it was found that VEGF-KO HSCs were unable to reconstitute hematopoietic function [22]. Follow-up in vitro experiments treated HSCs with a small-molecule inhibitor of VEGF-receptors and found a defect in colony formation. However, this defect was not recapitulated with extracellular anti-VEGF treatment [22]. These experiments suggest that VEGF's effect on HSCs is not only autocrine, but depends on an intracellular intracrine pathway.

More detailed investigation of VEGF and VEGF receptor expression in developing hematopoietic cell lineages showed that VEGFR2 is expressed in early HSCs and is down-regulated upon differentiation. VEGFR1 is specifically upregulated in precursors of megakaryocytic and monocytic lineages, and autocrine VEGF is required for complete megakaryocyte polyploidization [23].

Autocrine VEGF signaling is observed only in select compartments

Besides the hematopoietic compartment, co-expression of VEGF and its receptors is restricted to a small number of tissues, which originally suggesting a limited role for autocrine VEGF signaling in physiological settings. VEGF and VEGFR2 expression takes place by and large in complementary opposing tissues throughout embryonic development, with only the endocardium lining the cardiac cushion of the outflow tract found to robustly express both VEGF and its receptor [24]. Podocytes, specialized epithelium of the kidney, express both VEGF and the receptor Nrp1, and have been shown to use autocrine VEGF for survival and calcium homeostasis [25].

In the embryo, we investigated the developmental role of autocrine VEGF in an unbiased manner by creating VEGF-KO/WT chimeras (Chapter 3). Superficial analysis of these embryos at mid-gestation suggested that select tissues require cell-autonomous VEGF expression, such as the cartilage primordium of the nasal septum and some regions of the brain, while most tissues accepted VEGF-KO cells in an unbiased manner. Contrary to our expectations based on VEGF expression in the adult aorta [26], the endothelium of larger vessels readily incorporated VEGF-KO cells, which indicates cell-autonomous VEGF is not essential for their development. Smaller vessels and capillaries however, were not observed to incorporate VEGF-KO cells, and so appear to require cell-autonomous VEGF for differentiation.

6.3 - Autocrine VEGF signaling in the endothelium

The finding that autocrine VEGF is needed for the formation of capillary endothelium in the embryo is not unprecedented. Strong evidence for autocrine endothelial signaling is found in aortic endothelial cells which both express VEGF, and phospho-VEGFR2 can be observed [26]. Besides the aorta, arterial endothelia of the adult has been shown to express VEGF in scattered cells of larger vessels [27].

Deletion of VEGF from the endothelial compartment

The most striking evidence that cell-autonomous VEGF is required for endothelial cell function is by way of the VEGF-ECKO genetic model, where floxed-VEGF is excised from the endothelial compartment with VE-Cad::Cre [28]. Degeneration of the endothelia was observed in multiple tissue beds, resulting in organ failure that preceded a sudden death phenotype. By 25 weeks of age, the VEGF-ECKO genotype was 55% lethal [28].

Because VE-Cad::Cre is expressed at very early embryonic time points, it could not be ruled out that VEGF excision disrupted endothelial differentiation in a manner that resulted in adult vascular degeneration. To determine if autocrine VEGF was required continuously rather than in a developmental time window, we induced ECKO deletion in the adult (Chapter 5). We observed a similar sudden death phenotype to that of the VEGF-ECKO, with of 33% lethality of animals by 50 weeks. Cell culture experiments support this finding, where pseudo-WT adult floxed-VEGF endothelial cells showed decreased viability upon treatment with viral-Cre.

The case for intracrine VEGF signaling

The VEGF-ECKO model provides several compelling arguments for cell-autonomous intracrine VEGF signaling in the endothelium. It was observed that gross levels of VEGF were unchanged, or even elevated in several tissues [28]. This result suggested that normal levels of paracrine VEGF are available to the ECKO endothelium but were unable to compensate for a cell-autonomous function. Endothelial cells were purified from VEGF-ECKO and WT mice, and cell survival was examined individually and in co-culture conditions. Individual culture showed a severe survival defect of VEGF-ECKO, where after 5 days they were found at only approximately 7% of WT cell numbers. Theoretically, if endothelial VEGF signaling were autocrine rather than cell-autonomous, adjacent WT cells could supplement VEGF-ECKO cells with a supply of autocrine VEGF and ameliorate the death phenotype. This was found not to be the case in co-culture experiments which allowed labeled WT and VEGF-ECKO cells to intermingle; the endothelial VEGF produced by neighboring cells was unable to rescue VEGF-ECKO death phenotype [28].

We further strengthen the argument that intracellular VEGF is key for endothelial survival in a series of experiments comparing viability of VEGF-ECKO cells in different conditions (Chapter 5). We found that the cell viability defect of VEGF-ECKO cells could not be rescued by addition of extracellular VEGF, but could be rescued by infection with a VEGF virus. Similarly, exposure of normal WT human endothelial cells to extracellular anti-VEGF antibodies did not affect viability, while treatment with cell-penetrating inhibitors of VEGF signaling resulted in cell death. Together, these experiments strongly suggest that autocrine VEGF signals intracellularly within endothelial cells.

Metabolic mechanisms of cell death in VEGF-deficient endothelium

Removal of VEGF by genetic excision, or by siRNA knock down (producing "KD-VEGF" cells) resulted in cell death and a loss of cell confluence (Chapter 5). Based on several assays, the majority of cell death was not due to apoptosis, however we did notice a strong perturbation of cellular metabolism. VEGF-deficient endothelium was overall metabolically repressed, with decreased glucose uptake, lactate production and triglyceride synthesis. These effects were apparent even in the presence of serum which can often compensate for metabolic defects.

Deficiencies in nutrient uptake are often associated with an increase in autophagy, a process by which cells promote lysosome degradation of their own organelles in a metabolic crisis or other cell stress [29]. Increased autophagic flux can promote survival during cell stress in some contexts [30], but in others, autophagy itself actually induces cell death [31]. We observed an increase of autophagic vacuoles in VEGF-ECKO endothelium in the mouse, and also in VEGF-deficient human endothelium. Inhibition of autophagy by siRNA ablation of autophagic machinery or by pharmaco-inhibition partially rescued observed cell death. Therefore we conclude that, in the context of endothelial VEGF-deficiency, excessive autophagy is partially at fault for cell death.

Molecular players in VEGF-deficient endothelium: Foxo1 mediates cell death

Of the factors regulating metabolism and autophagy, we were immediately interested in the Foxo family of transcription factors, which both regulate metabolic homeostasis and are

highly reactive to growth factor signaling [32]. Foxo1 in particular is expressed highly in the endothelium, and unlike other Foxo knockouts, the Foxo1-KO mouse dies at E11.0 from severe vascular defects [33]. Furthermore, Foxo1 is considered a pro-death transcription factor that has been shown to degrade upon addition of exogenous VEGF to endothelial cells [34], making it a good candidate as a molecular effector of autocrine VEGF signaling.

In the absence of VEGF, Foxo1 protein levels were increased in endothelial cells, both *in vitro* and in the *in vivo* VEGF-ECKO animal model (Chapter 5). Foxo1 is a transcription factor that coordinates expression of several pro-cell death pathways, but to be transcriptionally "active" it must localize to the nucleus. Along these lines, we were surprised to find that a microarray of KD-VEGF cells did not reveal a Foxo1 transcriptional signature, nor did we see Foxo1 targets significantly altered by RT-PCR. Immunohistochemistry showed Foxo1 level increases in both the cytoplasm and the nucleus, alerting us to other possible Foxo1 signaling functions at work. Indeed, Foxo1 has been shown to directly contribute to autophagy through a non-translational cytoplasmic signaling function [31].

To assess if Foxo1 up-regulation in KD-VEGF cells was in any way responsible for the increased autophagy and cell death, we performed a double knock-down, producing KD-VEGF +Foxo1. Silencing of Foxo1 significantly rescued KD-VEGF cell death to WT levels, indicating that Foxo1 is the major effector of cell death downstream of KD-VEGF. Furthermore, we assessed microarrays of WT, KD-VEGF and KD-VEGF+Foxo1. We found that the absence of Foxo1 in KD-VEGF cells did reverse transcriptional changes in mitochondrial and cell-cycle gene clusters, though not in direct Foxo1 targets. We hypothesize that other Foxo1-interacting transcription factors may be de-regulated in the absence of VEGF and so contribute to a unique transcriptome.

Together, these results give important clues about the pathways regulated by autocrine VEGF in the endothelium. By keeping Foxo1 levels in check, autocrine VEGF maintains endothelial metabolism and autophagic flux to appropriate physiological levels. In the absence of autocrine VEGF, endothelial cells reduce their ability to consume glucose and affected normal

levels of cellular respiration. This metabolic collapse is accompanied by an increase in compensatory autophagy, which kills the cell, the vasculature and ultimately, the organism.

6.4 - VEGF-independent VEGF-receptor signaling

Autocrine signaling is not the only non-canonical VEGF pathway to be uncovered. Recent studies expand our understanding the realm of “VEGF” signaling by discoveries that included alternative ligands, and ligand-independent activation pathways of VEGF receptors.

Alternative “ligands”: Galectin binding to VEGF receptors

VEGF receptors, and nearly all cell surface proteins, must be glycosylated for proper function on endothelial cells [35]. Once dismissed as a simple chaperone for protein-folding, glycosylation is an often underestimated post-translational modification that in fact regulates a wide variety of biological functions. Glycosylation alters biological function of proteins in three major ways: 1) Stabilization of protein folds and ECM interactions, 2) Direct modulation of protein function and 3) Provision of binding sites for glycan-binding proteins [36]. It is this third function that is most relevant to VEGF signaling, where glycan-binding proteins themselves can act as alternative “ligands” to receptor tyrosine kinases.

Addition of the glycan-binding protein Galectin-1 (Gal-1) to endothelial cells induces cellular effects associated with growth factor stimulation, namely proliferation and migration. Bindings studies showed that Gal-1 binds directly to VEGF co-receptor Neuropilin-1 (Nrp1) in tumor-associated endothelial cells, and Gal-1-induced migration is due to this Nrp1 interaction [37]. Addition of exogenous Gal-1 and Gal-3 to endothelium induces angiogenic effects, phosphorylation of VEGFR1 and VEGFR2, and changes in receptor endocytosis-- all in the absence of VEGF ligand [38].

These findings are of particular interest in a cancer setting, where anti-VEGF therapies benefit some patients, while others are not affected or become resistant over time. It is thought that alternative compensatory angiogenic pathways are invoked in these cases, but the

mechanisms at work regulating this switch are not well known. A recent breakthrough work linked Gal-1 VEGFR2 binding to anti-VEGF resistance in a tumor setting [39]. The authors found that the “glycosylation signature” of endothelial cells is fundamentally changed upon treatment with physiologically relevant stimuli, and therefore altering the affinity of Gal-1 to the N-glycans of VEGFR2. In the tumor context, which is hypoxic and immunosuppressive, the N-glycans of VEGFR2 are altered in such a way that Gal-1 binding induces dimerization and activation of its pro-angiogenic signaling pathway [39]. It is therefore through this alternative-ligand pathway that tumors exposed to anti-VEGF therapy can continue to induce vessel growth, even in the absence of the most potent angiogenic factor, VEGF.

Ligand-independent VEGFR2 signaling

Other disease contexts result in aberrant VEGF signaling. In particular, diabetes is characterized by endothelial dysfunction that is in part due to hyperglycemia-induced reactive oxygen species (ROS) [40]. To some extent, ROS are a necessary part of normal signaling as they are known to be released upon receptor tryosine kinase activation and transiently inhibit phosphatase activity [41]. However, in hyperglycemic diabetic conditions, intracellular ROS pathways are elevated long-term and wreak havoc on normal signaling pathways. Recent investigations clarify the connection between the elevated ROS that occurs in diabetes and its effects on VEGF signaling in the endothelium.

Diabetes is marked by an inability to heal wounds, which is partially mediated by a failure of neoangiogenesis to populate damaged tissue with functional vessels [42]. Diabetic mouse models are unable to respond properly to VEGF in functional and signaling assays. In support of this finding, *in vitro* endothelial models exposed to high glucose also showed impaired VEGFR2 signaling response to exogenous VEGF addition [43]. Experiments treating endothelium with either high glucose or directly with ROS revealed that excessive ROS induces phosphorylation and degradation of VEGFR2, even in the absence of VEGF ligand [43].

The ligand-independent ROS signaling effect does not require the catalytic activity of VEGFR2 auto-phosphorylation, but it is instead mediated by Src family kinases localized to the

Golgi membrane [43]. A similar model of ligand-independent, Src-mediated receptor tyrosine kinase activation was observed in the EGFR receptor upon H_2O_2 treatment [44]. However, in the endothelium, ROS-mediated VEGFR2 phosphorylation occurs in the Golgi compartment, not at the cell surface [43]. The discovery that VEGFR2 can be phosphorylated in this distinct compartment increases the potential complexity of the VEGF pathway, as an entirely different set of downstream signaling effectors is available for interaction that may not be at the surface membrane.

6.5 - Refinement of canonical signaling

Canonical VEGF signaling is constantly being refined by discoveries that add increasing levels of complexity to an otherwise simplistic paracrine signaling cascade. VEGF receptors are shown to have altered signaling depending on their interactions with extracellular matrix (ECM) and surface receptors, engagement in heterodimerization, and their proximity to receptors on adjacent cells.

Interactions at the cell surface that influence VEGF signaling output: Receptors and Extracellular matrix components

VEGFR2 has the potential to interact with a multitude of factors on the cell surface that affect its response to VEGF-ligand binding, including endothelial cell receptors and ECM components. Early angiogenesis experiments showed that addition of ECM fragments modulated blood vessel development, an effect later attributed to integrins [45]. Integrins are a family of ECM-binding receptors, that upon ligand engagement, induce angiogenic signaling and survival pathways within the endothelium [46].

The activation of the integrin family of ECM-binding receptors is tightly-associated with VEGFR2 responses to VEGF ligand. Addition of VEGF induces physical association of VEGFR2 with integrin subunit Beta-3, and when integrin function is blocked, VEGFR2 cannot be fully phosphorylated [47]. Cross-talk between these two receptors has been demonstrated in several models, where activation of either receptor stimulates binding and activation of the other [46].

VEGF is spliced into at least nine different isoforms, which vary primarily in their ability to bind to the ECM or diffuse freely in a soluble form. Although multiple studies have shown that different VEGF isoforms elicit unique vascular responses, the signaling responsible for these effects was not well elucidated. Recent experiments investigated in detail the endothelial response to soluble VEGF compared to ECM-bound VEGF [48]. The kinetics of VEGFR2 signaling in the presence of bound-VEGF were significantly altered. Bound-VEGF induced prolonged VEGFR2 activation, which extended the downstream kinetics of the p38/MAPK pathway and altered VEGFR2 localization. The changes observed in bound-VEGF conditions were found to depend on association of VEGFR2 with the integrin Beta-1 [48]. Together these results indicate that the ECM context of the endothelium affects not only direct activation of integrins, but also interactions between multiple receptors.

Progressively complicated endothelial receptor clusters are being discovered which fine-tune angiogenic response. CD63 is a transmembrane tetraspanin expressed in endothelial cells that, when silenced, results in abrogated angiogenic response to VEGF and other growth factors [49]. CD63 binds both VEGFR2 and Beta1, and ablation of CD63 was found to disrupt VEGFR2-Beta-1 integrin complex formation. This and other signaling experiments demonstrate that CD63 is essential for coordinating integrin and VEGFR2 signaling in response to VEGF [49]. Another novel VEGFR2 complex important for conveying VEGF signaling requires coordination by syndecan-1 (Scd1). Scd1 organizes a complex of VEGFR2, VE-Cadherin, and alpha-V beta-3 integrin, without which the endothelium cannot respond to VEGF or VE-Cadherin engagement [50].

Non-canonical heterodimers in VEGF signaling

Canonical VEGF signaling is generally thought to be an interaction between homodimeric VEGF ligand and two homodimers of the receptor tyrosine kinase of interest. However, experiments exploring the heterodimerization of VEGF signaling components have uncovered theoretical and observed interactions between heterodimeric ligands and heterodimeric receptors.

Placental Growth Factor (PlGF) is a VEGF family member that interacts exclusively with VEGFR1, and is expressed by human endothelium. Due to their close homology, when expressed in the same cell PlGF and VEGF are able to form heterodimers which are mitotically active [51]. The fact that PlGF and VEGF can heterodimerize when co-expressed was exploited in a tumor model where over-expression of a dysfunctional PlGF essentially sequestered active VEGF and therefore suppressed tumor angiogenesis [52].

The ability of PlGF and VEGF to heterodimerize was further exploited as a tool to study the phsyological function of endogenous VEGFR1/VEGFR2 heterodimers [53]. A synthetic ligand specific to VEGFR1/2 heterodimers was created by co-expressing VEGFR2-specific ligand VEGF-E (a viral VEGF mimetic protein) and the VEGFR1-specific ligand PlGF1. Application of this ligand to endothelial cells induced several angiogenic responses such as VEGFR2 phosphorylation, migration and tube formation. However VEGFR1/2 activation did not induce proliferation, ERK signaling and other VEGFR2 functions, suggesting the heterodimer has a unique signaling function of its own [53].

Endogenous VEGF receptor heterodimers have been detected more directly with immunoprecipitation and *in situ* proximity ligation assays, both of which rely on close physical binding of two disparate proteins to produce signal [54]. In this case, VEGFR2 and VEGFR3 are found to be co-expressed in developing blood vessels. Heterodimers were observed at tip cell filipodia in angiogenic sprouts, and when VEGFR3 was blocked by antibodies, sprouting was decreased, suggesting that VEGFR2/3 heterodimers contribute to VEGF response [54].

Three-dimensional cellular interactions

Interactions between receptors, ligands, ECM and intracellular signaling machinery are further muddied by the fact that these interactions occur in a three-dimensional environment. In a cancer setting, VEGF receptors are often expressed both on neoangiogenic endothelium as well as on the tumors themselves, and so have an opportunity to interact with ligand and each other in opposing cell types (referred to as *trans* interactions). Upon VEGF stimulation, VEGFR2 and its co-receptor Nrp1 were found to form complexes in trans at the cell-cell interface between

co-cultured cells expressing either single receptor [55]. These complexes produce distinct signaling cascades in endothelial cell models, in part due to improper internalization of VEGFR2. In mouse tumor models, *trans* expression of Nrp1 suppressed angiogenesis and tumor growth by arresting VEGFR2 internalization and therefore downstream signaling [55]. These findings further expand the circumstances that must be taken into account when studying angiogenic signaling pathways. Realistically, a two-dimensional monoculture can only reveal so much about the nuanced biology at work in a human patient.

6.6 - Summary

VEGF is well established as a potent mediator of angiogenesis, but this description merely scratches the surface of its biological power. VEGF expression begins several days before vascular morphogenesis to direct mesodermal differentiation in the embryo, and in the adult it is required continuously to maintain vitality of the endothelium. This thesis contributes to our understanding of VEGF in these non-angiogenic settings, and further establishes a more nuanced view of this crucial growth factor.

6.6 - References

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