Role of Vascular Endothelial Growth Factor-A in Collateral Growth and Development

Jason A. Clayton

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Cell and Molecular Physiology.

Chapel Hill
2008

Approved by: Dr. James E. Faber Advisor

Dr. Robert Sealock Reader

Dr. Eleni Tzima Reader

Dr. Mark Majesky Reader

Dr. Victoria Bautch Reader

ABSTRACT

Jason Allen Clayton: Role of Vascular Endothelial Growth Factor-A in Collateral Growth and Development
(Under the direction of Dr. James E. Faber)

Ischemic vascular disease is the leading cause of morbidity and mortality in the United States. Current therapies rely on invasive surgical procedures. Arteriogenesis, or collateral artery growth, has the potential to greatly improve the outcome of patients with ischemic vascular disease by providing an endogenous bypass circulation. However, the underlying biology of arteriogenesis remains poorly understood. There are conflicting animal and clinical reports of VEGF administration improving outcomes or increasing adverse effects. Because of systemic VEGF's pleiotropic functions, we hypothesized that local endogenous VEGF directly promotes the growth of collaterals. In addition, because of its role in vascular branching morphogenesis we hypothesized that VEGF may also play a role in the embryonic development of collaterals. To test these hypotheses, we used mouse models genetically targeting the VEGF pathway ($VEGF^{hi/+}$, $VEGF^{lo/+}$, $VEGFR\text{-}1^{+/-}$, and $VEGFR\text{-}2^{+/-}$) and local inhibitory approaches combining plasmid electroporation of Cre recombinase in $VEGF^{loxP/loxP}$ mice and FltIgG (VEGF-trap) in wild-type mice. We have found that VEGFR-1 is the principle VEGF receptor responsible for mediating arteriogenesis through recruitment of circulating monocytes. We also

found impaired arteriogenesis in mice genetically expressing low levels of VEGF ($VEGF^{lo/+}$). In addition, Cre-mediated deletion of VEGF in the adductor led to impaired plantar perfusion recovery, increased ischemic appearance, and impaired limb use. Expression of FltIgG in the adductor also increased ischemic appearance and impaired limb use. There was also evidence of impaired collateral perfusion despite normal plantar perfusion. We found a VEGF genotype – collateral density relationship, such that $VEGF^{hi/+}$ mice had greater collaterals while $VEGF^{lo/+}$ mice had fewer. Developmentally, we found that $VEGF^{hi/+}$ and wild-type mice were born with more collaterals than $VEGF^{lo/+}$. Interestingly in wild-type and $VEGF^{lo/+}$ mice collateral density declined over the first 3 weeks of life at which time the adult density was attained, while $VEGF^{hi/+}$ mice retained their collaterals from birth. These data suggest that VEGF levels must be maintained high enough to establish optimal collateral density and to ensure maturation of nascent collaterals during development. We identified a key role for VEGFR-1 in mediating collateral enlargement in ischemia. In addition, we have identified the first molecule, VEGF, responsible for the formation of native collaterals in healthy tissue.

.

ACKNOWLEDGEMENTS

There are many people that I must thank; people that made my time spent in the lab more fun, saner, and more endurable.

First, I want to thank Jim Faber for being an excellent and enthusiastic mentor. He always knew when he needed to prod and when he needed to give me space. I appreciate his candidness and his zeal for science. His enthusiasm is contagious and it has helped me through experimental ruts and intellectual droughts. He has been a mentor in the lab and friend outside the lab and is my role model for what a scientist should be.

I would also like to thank other members of the lab. Dan Chalothorn joined the lab shortly before I did and set the standard as to how I should work in the lab. I only hope that I am as dedicated to my work as he is to his. Hua Zhang has been a second mentor. My surgical skills pale in comparison to his and I hope to be as efficient and careful with my experiments as he is. Gang Cheng was always a source of wry humor and helped lighten things up in the lab. He provided valuable advice on how to have a clinical and research oriented career.

Thanks also go to my committee members, Mark Majesky, Robert Sealock, Eleni Tzima, and Vicki Bautch. I was fortunate to have experts in the

field offer valuable insights and fruitful discussions about my research. They especially need to be commended for enduring marathon committee meetings.

Pursuing an advanced degree can be very difficult and stressful. However, I suffered minimal stress during my research, thanks in large part to the support of my family, especially my wife, Kathleen Christine. Having someone else in science has helped tremendously especially, when late nights at the lab became a routine. None of this could have been accomplished without her support and understanding.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
bFGF	basic fibroblast growth factor
CAD	coronary artery disease
CCR2	chemokine (c-c motif) receptor-2
CWS	circumferential wall stress
EC	endothelial cell
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cell
EPO	erythropoietin
eQTL	expression quantitative trait locus/loci
GRAN	granulocyte
HGF	hepatocyte growth factor
HIF-1	hypoxia inducible factor-1
HIF-2	hypoxia inducible factor-2
ICAM-1	intercellular adhesion molecule-1
IRES	internal ribosomal entry sequence
LDPI	laser Doppler perfusion imaging
LNA	locked nucleic acid
LYMF	lymphocyte
M-CSF	monocyte colony stimulating factor

MCP-1	monocyte chemoattractant protein-1
MCS	multiple cloning site
MMP-2	matrix metalloprotease-2
MONO	monocyte
mRNA	messenger ribonucleic acid
PAD	peripheral artery disease
PBS	phosphate buffered saline
PDGF-BB	platelet derived growth factor-B chain
PFA	paraformaldehyde
PlGF	placental growth factor
qRT-PCR	quantitative reverse transcription polymerase chain reaction
ROI	region of interest
RT-PCR	reverse transcription polymerase chain reaction
SEM	standard error of the mean
SMC	smooth muscle cell
VCAM	vascular adhesion molecule-1
VEGF	vascular endothelial growth factor
VEGFR-1	vascular endothelial growth factor receptor-1
VEGFR-2	vascular endothelial growth factor-2
VPF	vascular permeability factor (see VEGF)
VSMC	vascular smooth muscle cell
WBC	white blood cell

CHAPTER 1

INTRODUCTION

Ischemic vascular disease, including coronary artery disease (CAD), stroke, and peripheral artery disease (PAD), is the leading cause of morbidity and mortality in the United States. PAD affects 8-12 million Americans each year. Patients with PAD face a six to seven times increased risk for heart attack or stroke. Many patients are unsuitable candidates for the current treatments, which include arterial bypass surgery or stent placement, because of complicating co-morbidities, such as multiple vessel occlusions. These patients will often progress to gangrene or limb amputation. Therefore, it is extremely important to develop alternative therapies. One such alternative therapy is termed Therapeutic Angiogenesis. The goal of this therapy is to promote blood vessel growth in order to re-supply ischemic tissue with oxygenated blood.

The term Therapeutic Angiogenesis is a misnomer because angiogenesis (capillary growth), by itself, is incapable of restoring blood flow to ischemic tissue. Arteriogenesis is a unique process of vascular growth first described by Schaper and colleagues, which results in the growth of small arteriole-to-arteriole anastomoses (collateral arteries) into large conducting arteries that bypass a site of stenosis or complete blockage.[1] Arteriogenesis requires the proliferation and migration of endothelial cells (EC), vascular smooth muscle cells (VSMC) and

pericytes; recruitment of circulating monocytes and T-lymphocytes; and remodeling of the extracellular matrix.[2] While the mechanical and cellular mediators of arteriogenesis are well defined, the molecules that promote collateral artery growth are less well understood. Thus, the **purpose** of this dissertation is to examine the role of one of these molecules, vascular endothelial growth factor-A (VEGF-A), in collateral growth in ischemic disease. In addition, no studies have investigated the de novo formation of native collaterals in developing embryos. We thus wanted to examine the developmental time course of collateral formation in healthy tissue.

ARTERIOGENESIS

There are three types of vascular growth. In the embryo, "vasculogenesis" is the process whereby, endothelial progenitor cells migrate and organize to form a primary vascular plexus. While vasculogenesis is typically restricted to embryonic vessel growth, the term has been used in the adult to describe the incorporation of circulating endothelial progenitor cells (EPC) into growing blood vessels.[3, 4]

After development of the primary vascular plexus, a process termed "embryonic angiogenesis" remodels the vessels into distinct tree-like structures that presage the final vascular architecture. In the adult, "angiogenesis" describes the formation of new capillaries from pre-existing capillaries. This can occur via sprouting of endothelial cells from pre-existing capillary tubes or intussusception, whereby capillary endothelial cells extend projections into the

lumen to divide the capillary into multiple new vessels.[5, 6] The capillary network, which connects proximal arterioles to distal venules is the site of nutrient and gas exchange.

Arteriogenesis is a third type of vascular growth involving the enlargement, in diameter and length, of vessels connecting two arterioles from different, but parallel, arterial tress or conduits. These unique vessels are called collateral arteries in that they provide an alternative, or collateral, blood supply.[1, 7-14] They are rare in number and are thought to provide minimal nutritive flow in normal tissue. However, they are extremely important when a major supply artery is gradually or suddenly occluded. In diseases such as atherosclerosis, which progressively narrows the lumen, coronary collaterals can significantly enlarge 2–to–10 fold to effect an endogenous bypass of the partially occluded vessel.[15] Diseases and environmental conditions that put patients at higher risk for ischemic vascular disease, such as hypercholesterolemia, hypertension, diabetes mellitus, smoking, and aging, all negatively affect arteriogenesis.[16-20]

The severity of tissue injury, ischemia and cell death following arterial occlusion is largely dependent on the number and diameter of pre-existing collaterals and their ability to enlarge over time (arteriogenesis). Angiogenesis also contributes to tissue vascular adaptation to ischemia but can only function to better distribute whatever blood flow is provided by collaterals. Arteriogenesis has been extensively studied, and mechanisms that have been defined will be discussed in subsequent sections of this chapter. However, there are no data investigating the factors that regulate the number of collaterals that form in a

given tissue or individual. It has been shown that the number of collaterals in a tissue is, at least in part, genetically determined. Two inbred mouse strains have been identified that lie at opposite ends of the spectrum with respect to collateral number.[21-23] Compared to BALB/c mice, C57BL/6 show a greater number of collaterals in skeletal muscle[22, 23] and intestine.[23] In addition, in the pial circulation that supplies the cerebral cortex, C57BL/6 have more collaterals than BALB/c, which have almost none.[23] Genetic determination of collateral number (collateral density) also extends to humans. Wustmann and colleagues examined 51 individuals with angiographically normal coronary arteries and no evidence of cardiovascular disease. Upon transient experimental coronary artery balloon occlusion, they found a remarkably wide 10 fold variation in coronary collateral conductance wherein 20% - 25% of individuals showed high conductance and no evidence of myocardial ischemia whereas an equal percentage had low conductance and evidence of induced ischemia. Despite the genetic evidence, nothing is known about the molecules that regulate collateral density. One of the aims of this dissertation is to analyze VEGF-A as a possible regulator of collateral density.

MECHANICAL MEDIATORS OF ARTERIOGENESIS

Due to the unique position of collaterals in the circulation (ie, connecting two arterioles from adjacent arterial trees), these vessels normally exhibit little or no net directional flow. While having not been directly measured, the pressures on either side of the collateral are presumed to be equal, thus providing no

pressure gradient to drive unidirectional flow. Interestingly, reduced or disturbed flow is a hallmark of endothelial dysfunction, and vessels maintained in such an environment are prone to atherosclerosis and/or regression.[24] However, despite their very altered flow environment, collaterals persist and remain patent. The mechanisms that assure their persistence in normal healthy tissues are unknown.

Occlusion of an artery produces a decrease in perfusion pressure distal to the blockage due to metabolic and myogenic dilation of distal vessels. The decrease in distal pressure generates a pressure gradient across the collaterals that provides the driving force for unidirectional flow. The increase in flow causes an increase in fluid shear stress, which is detected by endothelial cells and induces their activation. Activated endothelial cells express adhesion molecules that bind circulating leukocytes.[1, 7-12, 24-28] Leukocytes, upon diapedesis and migration to a peri-collateral position, then secrete growth factors, cytokines, and proteases to coordinate the growth of collateral arteries.[1, 2, 7, 8, 10, 13, 25, 29-31]

Evidence for the importance of shear stress as the primary initiator of arteriogenesis comes from two studies in which blood flow across the collateral circulation was shunted to the venous circulation, producing chronically greater collateral shear stress.[27, 28] In both models, an anastomosis was created between the femoral artery, distal to the occlusion, and the femoral vein. This created an even greater increase in flow and shear across the collaterals than would otherwise have been observed with femoral ligation alone. Both studies saw a greatly enhanced arteriogenic response such that conductance was

normalized. While these studies suggest that shear stress is the limiting factor in arteriogenesis, the anastomoses created in these experiments also induced greater ischemia in the leg, which will increase circulating VEGF and thus complicates the interpretation of these studies.

In addition to elevations in flow causing increases shear stress, elevations in blood viscosity can also directly increase shear stress. This principle was demonstrated by studying mice overexpressing erythropoietin (EPO).[32] Transgenic EPO mice have a hematocrit of up to 90%, almost double that of wild-type, and an increase of blood viscosity of 4-fold.[33] Ligation of the femoral artery, resulted in enhanced arteriogenesis relative to wild-type littermates.[32] Together, the above studies provide strong evidence that elevated shear stress is the primary stimulus of arteriogenesis.

Despite the importance of fluid shear stress in promoting the growth of collaterals, it has been shown that collateral conductance and recovery of blood flow distal to the site of ligation never reaches pre-ligation measures.[1, 9-11, 21, 23, 32, 34] It has been proposed that circumferential wall stress (CWS) or tangential wall stress is a force that causes the premature cessation of collateral growth.[30, 35] Immediately after ligation, due to dilation of distal vessels and a decrease in downstream resistance, intravascular pressure drops in the collateral. This would tend to decrease wall tension and cause reductions in wall thickness. However, the upstream arteries that feed the collaterals may undergo flow mediated dilation, which will increase the flow and input pressure to restore perfusion to ischemic tissues. This will tend to counteract the decrease in wall

tension due to the drop in distal pressures. Therefore, it is likely that CWS will change minimally as a consequence of large vessel occlusion.

It is more probable that the normalization of fluid shear stress in the collateral provides the signaling break on collateral growth. Since CWS is directly proportional to vessel radius and shear stress is indirectly proportional to the cube of the vessel radius, small increases in vessel diameter lead to more rapid normalization of shear stress than CWS.

CELLULAR MEDIATORS OF ARTERIOGENESIS

Schaper first described the presence of monocytes attached to the activated endothelium of collateral vessels.[36] It has since been demonstrated that leukocytes are essential to collateral growth.[37] Arteriogenesis is severely impaired in animal models chemically depleted of leukocytes. This impairment of arteriogenesis can be rescued by simultaneous injection of purified isolated monocytes.[38] Similar to the pharmacological depletion of monocytes[38], genetic mouse models of monocyte depletion also have impaired arteriogenesis.[39] Osteopetrotic mice (*op/op*) have a point mutation in monocyte colony stimulating factor (M-CSF) resulting in secretion of non-functional M-CSF.[40-42] These mice have monocytopenia, lymphopenia, and granulocytosis with no differences in circulating total leukocyte counts. Femoral artery ligation revealed a deficit in arteriogenesis, perfusion recovery, and accumulation of macrophages around the collateral arteries.[39] The above studies do not rule out a positive role for

lymphocytes, but clearly demonstrate the importance of functional monocytes on collateral artery growth.

To determine a role for lymphocytes, Stabile and colleagues examined $CD4^{-/-}$ mice which are deficient in $CD4^{+}$ T-lymphocytes but have a normal complement of $CD8^{+}$ T-lymphocytes and monocytes.[43] Perfusion recovery was impaired in knock-out mice concurrent with impaired macrophage accumulation in ischemic tissue.[43] Collateral lumen diameters were not measured; therefore effects on arteriogenesis cannot be conclusively determined. The impaired phenotype was completely rescued by infusion of exogenously purified $CD4^{+}$ lymphocytes.[43] In a follow-up study, the role of $CD8^{+}$ lymphocytes in arteriogenesis was tested in $CD8^{-/-}$ mice.[44] They found impaired perfusion recovery and reduced $CD4^{+}$ lymphocyte recruitment to the collateral artery. The phenotype was rescued by administration of exogenous $CD8^{+}$ lymphocytes or IL-16 at the time of ligation.[44] Together, the data suggests that early on in arteriogenesis, $CD8^{+}$ lymphocytes are first recruited to collaterals where they secrete cytokines, such as IL-16, to recruit $CD4^{+}$ lymphocytes that in turn recruit monocytes and that all cell types are necessary for proper collateral growth. While it has been shown that purified leukocytes can secrete a variety of growth factors and cytokines *in vitro*, it is not known what molecules are responsible for the promotion of arteriogenesis *in vivo*.

In addition to recruited cell types, resident cells are also critical for arteriogenesis. Endothelial cells respond to the shear stress stimulus and express adhesion molecules on their lumenal surface. While the shear stress

sensing mechanism and endothelial responses to shear have been extensively studied in vitro,[45, 46] it is not known if these same mechanisms act in vivo in collaterals. Proper growth and migration of vascular smooth muscle cells are also required to accommodate the increased forces exerted on the collateral wall following vessel occlusion. One of the most potent arteriogenic molecules, monocyte chemotactic protein-1 (MCP-1), is a smooth muscle cell mitogen in addition to its role in recruiting circulating monocytes. Other stimulators of smooth muscle growth and migration need to be elucidated to design optimal therapies to promote arteriogenesis. The collateral adventitia also extensively remodels during arteriogenesis and may provide a resident source of progenitor cells to contribute to the growth of collaterals.[9, 47, 48]

MOLECULAR MEDIATORS OF ARTERIOGENESIS

Most of the molecules examined have been growth factors or chemo/cytokines. To date, the most potent arteriogenic cytokine is MCP-1. Exogenous administration of MCP-1 at the time of ligation greatly enhances monocyte recruitment and collateral growth.[49-51] Similarly, in genetic mouse models of MCP-1 or CCR2 (MCP-1 receptor) deletion, arteriogenesis is inhibited.[52, 53] Interestingly, the actions of MCP-1 may, in part, be mediated by VEGF.[54, 55] Inflammatory molecules such as TNF-α, which increase the mobilization and recruitment of leukocytes, are also strong arteriogenic cytokines.[56] Toll-like receptor-4 has been shown to be important in outward remodeling of arteries[57]; however its role in arteriogenesis has not been

examined. Leukocytes, while important, are ineffective unless they can interact with the collateral endothelium and transmigrate through the vascular wall. Thus, as expected, adhesion molecule expression is critical in mediating collateral artery growth.[58, 59]

Tyrosine kinase receptors are essential for arteriogenesis. Systemic inhibition with a non-selective protein tyrosine phosphatase inhibitor enhanced collateral blood flow in a rat model of peripheral vascular disease.[60] In vitro, these investigations observed enhanced signaling from VEGFR-2 and Tie-2, receptors for VEGF and Ang-1 respectively.[60] Studies administering VEGF[61-75] or Ang-1[76] either singly or in combination[77, 78] have shown enhanced arteriogenesis and collateral perfusion. Placental growth factor (PlGF), which belongs to the VEGF family of proteins, has also been shown to be a positive modulator of arteriogenesis.[79, 80] Platelet derived growth factor-B (PDGF-B)[81, 82], basic fibroblast growth factor (bFGF)[81, 83-92], and hepatocyte growth factor (HGF)[93] are other growth factors demonstrated to augment collateral growth of tissue perfusion.

The extracellular matrix (ECM) is a complex structure surrounding collaterals. It provides support for the collateral artery and may also provide a reservoir of growth factors for the stimulation of arteriogenesis.[94] Many of the growth factors listed above have protein domains which confer ECM binding affinity. Components of the ECM also have angiostatic properties.[95] The role of these molecules has not been studied with respect to arteriogenesis. In order for the collateral to enlarge in length and diameter, it is necessary for the ECM to

remodel to accommodate the growing collateral. Matrix proteases such as MMP-2 have been shown to become activated in angiogenesis, but a role in arteriogenesis has yet to be determined.

VASCULAR ENDOTHELIAL CELL GROWTH FACTOR

Vascular endothelial growth factor (VEGF), or vascular permeability factor (VPF), was originally isolated as a secreted cytokine that increased permeability in tumor vessels.[96] It was subsequently identified as an endothelial specific mitogen.[97, 98] In endothelial cells, VEGF is also a potent chemotactic agent and pro-survival factor.[99] VEGF is also a potent vasodilator due to its downstream activation of endothelial nitric oxide synthase (eNOS) and subsequent nitric oxide production.[100-102] In addition to its actions on endothelial cells, VEGF also acts as a pro-migratory, anti-apoptotic, and survival factor in numerous other cell types, including skeletal muscle[103-106], vascular smooth muscle[107, 108], and monocytes[109, 110].

In mice, VEGF mRNA is alternatively transcribed into three major isoforms, VEGF120, 164, and 188. The high molecular weight isoforms, VEGF164 and VEGF188, contain heparin-binding motifs, which establish a distribution of the growth factor. In contrast, VEGF120 lacks this motif assigning it diffusible and circulating activities. The differential splicing mechanism regulating which isoforms are produced is not clear, however each isoform is biologically and functionally unique. Mice that lack the heparin binding isoforms have impaired vessel branching morphogenesis and larger caliber vessels.

Conversely, mice that express only VEGF188, contain an excess of endothelial cell filopodia and vessel branching.[111] Evidence from this and subsequent studies using targeted mice demonstrate that all three isoforms are critical to establish the proper VEGF gradient in tissues to allow correct development and patterning of the vasculature and related organs.

VEGF-A acts through two high-affinity receptor tyrosine kinases, VEGFR-1 (flt-1) and VEGFR-2 (flk-1/KDR). Numerous studies have demonstrated that the VEGF signaling cascade is primarily mediated by VEGFR-2. In embryonic development, VEGFR-1 is thought to act predominantly as a negative regulator of VEGF signaling through the alternate transcription of a soluble VEGFR-1 antagonist. Because of its weak kinase activity, it has been difficult to dissect any signaling pathway mediated by VEGFR-1.[112] However, recent experiments in adult mice using the VEGFR-1 specific agonist PlGF and targeted mice lacking the kinase domain of VEGFR-1, demonstrate a positive role for VEGFR-1 signaling.[109, 113] VEGFR-2 is primarily expressed on endothelial cells, but it also can be found on a sub-population of bone marrow derived cells termed endothelial progenitor cells (EPCs). VEGFR-1 is expressed on endothelial cells but also on vascular smooth muscle cells, skeletal muscle, and on T-lymphocytes and monocytes where it acts as a chemotactic receptor.[107, 110]

SOURCES AND ACTIONS OF VEGF-A IN THE COLLATERAL ZONE

The microenvironment surrounding collateral vessels contains numerous other cell types that may be influenced by actions of VEGF. In an in vitro co-

culture system, VEGF stimulated migration and proliferation of aortic smooth muscle migration in an endothelial cell-dependent manner.[108] Interestingly, VEGF also mediates MCP-1 induced smooth muscle migration and proliferation[55] and angiogenesis[54]. To date, MCP-1 is the most potent factor mediating collateral artery enlargement. In skeletal muscle, VEGF is a pro-survival molecule that also stimulates migration of skeletal myoblasts in culture.[103] In damaged muscle tissue, exogenously delivered VEGF promotes the growth and survival of myogenic fibers.[104] In a further adaptation to a hypoxic stimulus, VEGF stimulates the expression of myoglobin, thus greatly increasing oxygen carrying capacity in ischemic skeletal muscle.[105] VEGF also affects circulating cell types. It acts on receptors on cells from the hematopoetic lineage to increase proliferation and mobilization of leukocytes.[114] VEGF stimulates the expression of adhesion molecules such as, vascular adhesion molecule-1 (VCAM), intercellular adhesion molecule-1 (ICAM-1), and E-selectin in endothelial cells, which facilitates the recruitment and diapedesis of leukocytes, including monocytes, neutrophils, and T-lymphocytes.[115]

Collateral and peri-collateral cells are also potential sources of VEGF in addition to responding to VEGF. Efforts to quantitate tissue VEGF have been complicated by the relatively low abundance of endogenous VEGF in the adult and the complex milieu surrounding the collateral arteries. Current antibodies used for Western blot analysis are unable to distinguish between the various isoforms of VEGF. While qRT-PCR is used to distinguish the relative contribution of each isoform in a tissue, no such studies have been done in the

context of collateral artery growth (Chapter 2 and 3). It is likely that increases in VEGF production, if any, will be limited to the region immediately surrounding the collateral (Chapter 3). No studies have described the cellular expression pattern of VEGF or its receptors in the collateral and peri-collateral region. A study by Couffinhal and colleagues described the identity of VEGF producing cells in a mouse hindlimb ischemia model. In ischemic lower limb muscles, VEGF secretion was markedly upregulated in skeletal muscles up to day 14 post-op, with expression decreasing up to 35 days post-op. In addition, VEGF immuoreactivity was seen in macrophages and T-lymphocytes in the ischemic lower limb. The authors also identified VEGF mRNA and protein expressed in endothelial cells of the ischemic lower limb.[116] Whether or not these cell types also express VEGF in the collateral region has not been examined (Chapter 2 and 3).

Skeletal muscle is potentially the largest source of VEGF in the collateral microenvironment. Despite the lack of detectable hypoxia in this region[117], VEGF could be upregulated by mechanical stimulation of the muscle. Increases in mechanical load and capillary shear stress, which could occur in the thigh in models of PAD, lead to increases in skeletal muscle VEGF secretion and capillary density.[118] Ischemic lower limb muscles, which are far removed from collateral vessels, are potentially the largest source of circulating VEGF. Plasma VEGF may then stimulate proliferation and mobilization of leukocytes from bone marrow. Targeted deletion of skeletal muscle VEGF leads to muscle apoptosis

and a decrease in capillary density, stressing the importance of even basal skeletal muscle VEGF in the maintenance of the tissue.[119]

Vascular wall cells are an additional source of VEGF. Skeletal muscle capillary endothelial cells express increased amounts of VEGF in response to increased capillary shear stress, which could occur in the thigh in resonse to flow-mediated dilation upstream of collaterals after femoral ligation..[26] Shear stress and stretch have been shown to increase the expression and activation of VEGFR-2 in endothelial cells, thus providing a local autocrine positive feedback loop.[120, 121] Vascular smooth muscle cells are a third source of VEGF. In vitro, smooth muscle cells increase expression of Hif-1α and VEGF in response to cyclic mechanical stretch.[122]

Cells recruited to remodeling collateral vessels are also potential sources of VEGF production. In vitro studies demonstrate that bone marrow-derived cells can secrete large amounts of VEGF.[123] Local injection of these cells into the collateral zone shows co-localization of VEGF. However, whether this VEGF contributes to collateral growth has not been clearly defined. An important aim of this dissertation is to analyze the role of local paracrine VEGF signaling in collateral growth in ischemia.

VEGF-A IN ARTERIOGENESIS

Human trials and animal studies using exogenous VEGF to promote arteriogenesis have met with mixed results.[61-75, 117, 124-127] The complex biology of VEGF[128] (ie, multiple isoforms, receptors, and actions) and the complex nature

of the collateral microenvironment, make it difficult to assess the effect of exogenously delivered VEGF on collateral artery growth. Studies have shown that increases in VEGF expression correlates with increases in capillary density but not with increases in angiographic score.[129] The authors concluded therefore, that VEGF was not a mediator of arteriogenesis. However, the biological mechanism of angiogenesis and arteriogenesis are distinct. Therefore it is incorrect to assume that VEGF has similar temporal properties in these two processes. In addition, the method used by these investigators to detect VEGF does not distinguish between the isoforms. It is likely that the amount and localization of an isoform, rather than the total amount of all isoforms, is important in collateral growth. In a subsequent study, Hershey and colleagues delivered adenovirus encoding human VEGF165 to the thigh of rabbits and failed to show improvement of the angiographic score.[124] They were only able to elicit a transitory increase in VEGF expression lasting from 3 to 5 days depending on the viral dose. In addition, the timing of injection was 10 days post-ligation. The events that initiate collateral remodeling begin immediately ligation[21], thus it is possible that events already set into motion may not be susceptible to further augmentation by later administration of VEGF. Moreover, higher doses of adenovirus-VEGF165 induced edema in the upper hindlimb.[124] This edema, which suggests that the level of VEGF was too high, could have reduced flow in the collaterals and therefore prevented growth of the collaterals.

Developmentally, VEGF is regulated in a narrow window of expression such that overexpression or underexpression is embryonic lethal.[130-133] Likewise, it is

possible in the adult that VEGF has a narrow therapeutic window, such that too much leads to edema and no collateral remodeling and too little has no effect at all.

In contrast, several reports support a role for VEGF in arteriogenesis. Delivery of plasmid encoding VEGF165 intramuscularly or intraarterialy[67, 70] reportedly increases angiographic score, calf blood pressure ratio, and flow. Importantly, plasma VEGF and arterial pressure were not measured. If VEGF were released systemically from the transfected tissue, it could have caused systemic vasodilation, including in the ligated limb which would favor increased flow in collateral circulation and collateral growth. This effect would be secondary to VEGF's systemic actions and not a direct effect of VEGF on collateral vessels. This limitation holds true for all studies that deliver VEGF systemically. In addition, systemically released VEGF could further confound the interpretation of direct actions on the collateral vasculature by augmenting angiogenesis in the lower limb, distal to the ligation, and thus potentially alter the hemodynamics in a way favorable for collateral growth. Furthermore, systemic VEGF release could increase the mobilization of bone marrow derived cells, thus augmenting leukocyte chemotaxis to the collateral vessel and increasing their positive role in collateral growth.

The above makes clear that systemic VEGF delivery can lead to complications in the interpretation of VEGF's direct role in collateral growth. An alternative approach is to block the actions of endogenous VEGF and study its affect on collateral growth. To this end, three groups have demonstrated that

blockade of VEGF greatly impairs collateral growth in hindlimb[134, 135] and myocardial ischemia models[136]. However, like systemic delivery of VEGF, systemic blockade can also confound the interpretation of any direct role. Systemic inhibition has the potential to increase peripheral resistance by the removal of a potent vasodilator. This in turn could result in a decrease in flow and shear stress in the collaterals thus preventing a powerful stimulus of growth in the collateral arteries. Systemic inhibition might also block mobilization and recruitment of leukocytes, negating their role in collateral growth. Systemic blockade could also inhibit angiogenesis in the ischemic tissue, creating greater ischemia and tissue damage. This is especially important in the coronary collateral circulation, where the ischemic myocardium overlaps the collateral zone. Edema from ischemic tissue damage could block arteriogenesis irrespective of VEGF's actions. To elucidate VEGF's direct contribution to collateral growth, it is necessary to block VEGF's actions only in the collateral zone (Chapter 3). It is also important to develop strategies for local disruption of VEGF production in select cell types to determine each cell's contribution to arteriogenesis.

CHAPTER 2

Vascular Endothelial Growth Factor-A Specifies Formation of Native Collaterals and Regulates Collateral Growth in Ischemia

ABSTRACT

The density of native (pre-existing) collaterals and their capacity to enlarge into large conduit arteries in ischemia (arteriogenesis) are major determinants of the severity of tissue injury in occlusive disease. Mechanisms directing arteriogenesis remain unclear. Moreover, nothing is known about how native collaterals form in healthy tissue. Evidence suggests VEGF, which is important in embryonic vascular patterning and ischemic angiogenesis, may contribute to native collateral formation and arteriogenesis. Therefore, we examined mice heterozygous for VEGF receptor-1 (VEGFR-$1^{+/-}$), VEGF receptor-2 (VEGFR-$2^{+/-}$), and over-expressing and under-expressing VEGF-A ($VEGF^{hi/+}$ and $VEGF^{lo/+}$). Recovery from hindlimb ischemia was followed for 21 days after femoral artery ligation. All statements below are $p<0.05$. Compared to wild-type mice, VEGFR-$2^{+/-}$ showed no deficits in recovery of hindlimb perfusion, collateral enlargement or angiogenesis, and ischemic scores were comparable. In contrast, VEGFR-$1^{+/-}$ showed reduced recovery of perfusion, reduce collateral enlargement and worse ischemic scores, whereas angiogenesis was comparable. Compared to wild-

type mice, $VEGF^{lo/+}$ had 2-fold lower perfusion immediately after ligation (suggesting fewer native collaterals which was confirmed by angiography) and blunted recovery of perfusion. $VEGF^{hi/+}$ mice had 3-fold greater perfusion immediately after ligation, more native collaterals, and improved recovery of perfusion. These differences in collateral density were confirmed in the cerebral circulation; compared to wild-type mice, $VEGF^{lo/+}$ formed fewer and $VEGF^{hi/+}$ formed more collaterals during the perinatal period during which adult density was established. Our findings indicate VEGF and VEGFR-1 are determinants of arteriogenesis. Moreover, they identify VEGF as the first signaling molecule specifying formation of native collaterals in healthy tissues.

INTRODUCTION

Ischemic vascular disease is the leading cause of morbidity and mortality in the developed world.[25] The number and diameter of native (pre-existing) collaterals in a healthy tissue and their capacity to enlarge (remodel) are critical determinants of the severity of ischemic injury following arterial obstruction. While molecules regulating collateral remodeling in ischemia are beginning to be understood,[8] nothing is known regarding when native collaterals form or the responsible signaling mechanisms.[29]

Collateral enlargement in ischemia requires proliferation of endothelial cells, leukocyte recruitment, proliferation and migration of mural cells, and remodeling of the extracellular matrix. Evidence suggests VEGF participates in each of these processes.[137] However, trials testing whether exogenous VEGF

can augment collateral enlargement have met with mixed results.[25] The complexities of VEGF signaling (ie, multiple isoforms, receptors and intracellular pathways) plus the inaccessibility of the collateral circulation make it difficult to determine a direct effect on collateral remodeling. Most studies have administered a single VEGF isoform with limited control of concentration, leading to defective neovascularization, tissue edema and impaired vessel growth.[138] Recent studies have shown that multiple gradient-forming isoforms are expressed within narrow concentration windows that, if exceeded too greatly in either direction, lead to aberrant vessel formation, embryonic lethality and disturbed vessel maintenance and growth in the adult.[130-133, 139] Interestingly, administration of an engineered zinc-finger transcription factor that drives expression of multiple VEGF isoforms improved recovery of limb perfusion following femoral artery ligation.[140, 141]

While the above studies examined therapeutic VEGF, findings from several studies using VEGF antagonists given systemically suggest that endogenous VEGF may contribute to ischemic collateral remodeling.[134-136] However, no study has examined this question with a genetic approach or identified the responsible VEGF receptor type. VEGF acts primarily through two high-affinity receptor tyrosine kinases, VEGFR-1 (flt-1) and VEGFR-2 (flk-1/KDR). VEGF-induced angiogenesis is mediated predominantly by VEGFR-2. However, during embryonic development alternately transcribed soluble VEGFR-1 (sFlt-1) also participates as a negative regulator of VEGF signaling.[137, 142] Because of weak kinase activity, it has been difficult to clarify potential signaling

by membrane-anchored VEGFR-1. However, experiments in adult mice using the VEGFR-1 specific agonist, PIGF, and targeted mice lacking the kinase domain of VEGFR-1 demonstrate a positive role for VEGFR-1 signaling.[80, 113] In this study, we examined the effect of targeted over- and under-expression of VEGF, VEGFR-1 and VEGFR-2 on collateral remodeling in ischemia.

It is not known when the native collateral circulation develops or what signaling pathways specify formation of these rare vessels that interconnect adjacent arterial trees through artery-to-artery anastomoses. A previous study in our laboratory identified two inbred mouse strains —C57BL/6 and BALB/c— that differ in collateral density (BALB/c are markedly deficient), and identified an expression quantitative trait locus at or near *Vegfa* that positively correlated with low VEGF expression at the BALB/c allele.[23] Thus in the present study, we also tested the hypothesis that VEGF expression is a determinant of collateral formation in newborn and adult mice genetically targeted to over- and under-express VEGF.

MATERIALS AND METHODS

Unilateral hindlimb ischemia. Twelve-to-16 week-old VEGFR-$1^{+/-}$, VEGFR-$2^{+/-}$, $VEGF^{hi/+}$ and $VEGF^{lo/+}$ mice[131, 143-145] (kindly provided by J Rossant and A Nagy), maintained on the CD-1 background and genotyped by X-gal staining of tail biopsies, were compared to wild-type littermate controls. Animals were randomized and procedures and analyses were conducted blindly. Femoral artery ligation was performed as described.[23, 34] Briefly, mice were

anesthetized with 1.25% isoflurane/O_2 and the hindlimbs depilated. Temperature was maintained at 37.0±0.5ºC. The right femoral artery was exposed through a 2mm incision and ligated with two 7-0 ligatures placed distal to the origin of the lateral caudal femoral and superficial epigastric arteries (the latter was also ligated) and proximal to the genu artery. The artery was transected between the sutures and separated by 1-2 mm. The wound was irrigated with sterile saline and closed, and cefazolin (50mg/kg, im), furazolidone (topical) and pentazocine (10mg/kg, im) were administered. Procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Laser Doppler perfusion imaging. As detailed previously,[23, 34] under 1.125% isoflurane/O_2 anesthesia and 37±0.5ºC, non-invasive perfusion imaging of the adductor thigh region and plantar foot of both limbs was performed before, immediately after, and at 3, 7, 14, and 21 days after femoral ligation. Regions of interest were drawn to anatomical landmarks.

Assessment of ischemia. An "appearance score" after ligation was obtained, where 0=normal, 1=cyanosis or loss of nail(s), 2=partial or complete atrophy of digit(s), 3=partial atrophy of fore-foot.[23]

Leukocyte counts. Three days after femoral ligation blood was collected under anesthesia by retro-orbital puncture. Leukocyte counts were performed with a Heska's Animal Blood Counter.

***Histology*.** 21 days after ligation, animals were pressure-perfused transcardially (100mmHg) with phosphate-buffered saline (PBS, pH7.4) containing 20mmol/L adenosine, 10^{-4}mol/L papaverine and 10U/ml heparin,

followed by pressure-perfused fixation with 4% paraformaldehyde in 100mmol/L sodium phosphate (PFA; pH7.4) for 15min. The adductor below the femur was excised in a 5mm block extending ~1mm medial of the "mid-zone" of the gracilis muscle collaterals that interconnect the lateral caudal femoral artery and saphenous artery trees, to the lateral portion of the thigh. A 5mm block of the calf was excised beginning at the origin of the Achilles tendon. Blocks were paraffin-embedded and sectioned at 5μm. Lumen diameter of collaterals in the anterior and posterior gracilis muscles was determined from sections of their mid-zone stained with cyano-Massons trichrome. For all histochemical analyses, results reported for each animal were averages of values from two sections separated by 250μm. *Griffonia Simplicifolia* isolectin-1-B_4 (Vector Labs) was used to determine capillary density and capillary-to-muscle fiber number in two 40x fields from the posterior head of gastrocnemius muscle. Leukocyte density around gracilis collaterals was determined with CD45 (BD Pharmingen) immunohistochemistry.[23, 34]

Post-mortem arteriography. As detailed previously,[23] animals were maximally dilated and perfusion-fixed 7 days after ligation when the contralateral femoral artery was also ligated. A high-viscosity barium sulfate solution (85%w/v) with impaired capillary transit was infused after ligation of the ankles to minimize arterial-venous shunting, followed by X-ray angiography. A Rentrop-like line analysis was performed on digitized films by counting vessels intersecting a line drawn perpendicular to the femur beginning at a point distal to the femoral ligation that approximated the midpoint of the gracilis collaterals.

Cerebral pial arteriography was obtained in separate groups following maximal dilation and perfusion-fixation.[23] After removal of the dorsal calvarium, high-viscosity polyurethane[146] (60% resin in 2-butanone with blue dye) that is unable to cross capillaries was infused via the thoracic aorta during stereoscopic imaging, followed by topical and the whole-brain fixation in 2% PFA overnight at 4ºC. Brains were imaged and collaterals interconnecting the middle and anterior cerebral artery trees were counted.[23]

Quantitative RT-PCR. The hindlimbs were perfused 36h after unilateral femoral artery ligation with RNA-later (Ambion) via that abdominal aorta under deep anesthesia. 5mm thick sections of proximal calf and the adductor centered on the collateral mid-zone and perpendicular to the femur were excised and stored in RNA-later at -80ºC. Tissue was pulverized under liquid nitrogen. Total RNA was extracted (RNeasy Fibrous Tissue mini kit, Qiagen) according to the manufacturer. Genomic DNA was removed by on-column DNase treatment (Qiagen). Total RNA was reverse-transcribed (Verso cDNA kit, Abgene). Real-time PCR was performed with triplicate cDNA samples diluted 1:100 using SYBR Green chemistry (Sigma) on a Rotor-Gene 3000 (Corbett Life Science). Data were analyzed according to the Pfaffl method.[147]

Statistics. All data are reported as means ± SEM. Significance ($P<0.05$) was determined by two-way ANOVA followed by Dunn-Bonferonni Corrected *t*-tests. One-way ANOVA was used for comparisons between VEGF genotypes ($VEGF^{hi/+}$, $VEGF^{lo/+}$, wild-type), followed by paired or un-paired *t*-tests. Capillary

number-to-muscle fiber ratios and appearance scores were subjected to Mann Whitney U tests.

RESULTS

Reduced recovery after femoral artery ligation in VEGFR-1 heterozygous mice.

Although VEGFR-1$^{-/-}$ or VEGFR-2$^{-/-}$ mice are embryonic lethal, mice heterozygous for the receptors have defects in cardiac preconditioning but otherwise appear normal.[148, 149] We examined VEGFR-1$^{+/-}$ and VEGFR-2$^{+/-}$ mice for recovery from femoral ligation. Compared to wild-type, VEGFR-1$^{+/-}$ had larger paw appearance scores indicating greater ischemia[23] (**Fig. 2.1a**). In contrast, VEGFR-2$^{+/-}$ showed no differences (**Fig. 2.1b**). In agreement, recovery of plantar perfusion (**Fig. 2.1c**), which correlates with overall hindlimb blood flow[34], was attenuated in VEGFR-1$^{+/-}$ but not in VEGFR-2$^{+/-}$ mice (**Figs. 2.1d,e**).

To investigate the responsible mechanisms, we measured capillary density in gastrocnemius muscle. While capillary-to-muscle fiber number ratios at baseline were comparable among groups, ratios significantly increased at day-21 in VEGFR-2$^{+/-}$, and trended similarly in VEGFR-1$^{+/-}$ mice. (**Figs. 2.2a,d,g**). The increase in VEGFR-2$^{+/-}$ was associated with slightly greater capillary area density and muscle atrophy (**Suppl. Figs. 2.1b,f**). Capillary area density and muscle atrophy were not different in VEGFR-1$^{+/-}$ (**Suppl. Figs. 2.1a,e**). We next examined collateral diameters in the anterior and posterior gracilis muscles and perfusion in the superficial adductor region containing these collaterals. Lumen diameter was not different at baseline among groups. However, outward

remodeling at day-21 was less in VEGFR-$1^{+/-}$ (p=0.07, 2-tailed) (**Figs. 2.2b,e,h**). In addition, VEGFR-$1^{+/-}$ had reduced adductor perfusion (**Suppl. Fig. 2.2d**).[23, 34] Collateral remodeling and perfusion were not different in VEGFR-$2^{+/-}$ mice (**Fig. 2.2h, Suppl. Fig. 2.2e**). Leukocytes contribute importantly to collateral artery growth.[8, 25, 29] Peri-collateral $CD45^{+}$ leukocytes were not different in VEGFR-$2^{+/-}$, but were reduced in VEGFR-$1^{+/-}$ mice (**Figs. 2.2c,f,i**), consistent with their attenuated collateral remodeling. In addition, circulating leukocytes, lymphocytes, and monocytes were lower three days after ligation in VEGFR-$1^{+/-}$ (**Suppl. Table 2.1**). These findings indicate that reduced expression of VEGFR-1 results, after femoral artery occlusion, in fewer circulating and peri-collateral leukocytes, reduced collateral enlargement and flow, reduced recovery of hindlimb flow, and greater tissue ischemic injury. They suggest VEGFR-1 is important in collateral remodeling.

The VEGF hypermorphic allele augments —and hypomorphic allele attenuates— recovery after femoral artery ligation.

Studying the role of endogenous VEGF levels has been difficult because heterozygous and homozygous null mice are embryonic lethal. Thus, VEGF-A reporter mice have been constructed to generate hypermorphic or hypomorphic alleles.[130, 131, 145] Homozygosity of either allele results in embryonic lethality due to excess or insufficient VEGF. However, $VEGF^{hi/+}$ and $VEGF^{lo/+}$ are viable and appear normal despite expressing VEGF at intermediate levels to homozygous and wild-type counterparts.[130, 131] Since data are not available, we assessed

expression of the VEGF isoforms, VEGF120, VEGF164, and VEGF188, by qRT-PCR in calf and collateral zone adductor muscles.

Baseline VEGF expression in calf muscle from the non-ligated leg was 2-fold higher in $VEGF^{hi/+}$ and 25-75% lower in $VEGF^{lo/+}$ compared to wild-type (**Suppl. Fig. 2.3a**). Thirty-six hours after femoral ligation, expression in the calf increased in all three strains for VEGF120 (**Figs. 2.3a-c**). In contrast, expression in the adductor of the ligated leg, which does not experience detectable ischemia in this model,[117] showed selective upregulation of the high-molecular weight isoforms, VEGF-164 and -188, in wild-type and $VEGF^{hi/+}$ mice (**Figs. 2.3d,e**). Interestingly, all VEGF isoforms decreased in the $VEGF^{lo/+}$ mice (**Fig. 2.3f**). To determine involvement of HIF signaling in this unique pattern of upregulation in the adductor collateral zone, we performed qRT-PCR for Hif-1α, Hif-1β and Hif-2α. Whereas expression was similar in all strains in the non-ligated leg's adductor (**Suppl. Fig. 2.3b**), Hif-1α increased 2-fold while Hif-1β was unaffected in the ligated leg's adductor (**Figs. 2.3g-i**). Interestingly, Hif-2α decreased, suggesting an alternative role for this alpha subunit. These data suggest that ischemia promotes alternative splicing of VEGF favoring the soluble isoforms, whereas shear-induced collateral remodeling is associated with expression of the matrix-bound isoforms.

Compared to wild-type mice, $VEGF^{hi/+}$ exhibited less (**Fig. 2.4a**) whereas $VEGF^{lo/+}$ experienced greater (**Fig. 2.4b**) ischemic appearance scores after femoral artery ligation. Likewise, $VEGF^{hi/+}$ mice exhibited less reduction in perfusion immediately after ligation and better recovery thereafter (**Figs.**

2.4c,e,f), whereas VEGF$^{lo/+}$ experienced greater reduction and worse recovery (**Figs. 2.4d,e,f**). The milder ischemia in VEGF$^{hi/+}$ is consistent with their trend toward a smaller increase in VEGF120 in the calf, compared to wild-type (**Fig. 2.3b**). The slight reduction of VEGF120 in VEGF$^{lo/+}$, compared to wild-type, and the decrease of VEGF164 (**Fig. 2.3c**) may reflect the reduced stability of VEGF transcripts in the VEGF$^{lo/+}$ strain.[131]

Since vascular smooth muscle tone is absent immediately after femoral artery ligation,[150] leg perfusion measured at this time largely reflects anatomical differences in pre-existing collateral density and/or diameter, and to a lesser extent, capillary density. Thereafter, recovery of perfusion is primarily determined by collateral remodeling, with a smaller contribution of angiogenesis. To distinguish VEGF's potential effect on these determinants, we first examined capillary-to-muscle fiber ratio in gastrocnemius at baseline (**Figs. 2.5a,b**). There was no difference between wild-type and VEGF$^{hi/+}$ or VEGF$^{lo/+}$ mice. This suggests a difference in collateral number and/or diameter underlies the differences in perfusion immediately after ligation. Capillary-to-fiber ratio increased at 21 days in VEGF$^{hi/+}$. Interestingly, baseline fiber size was greater in VEGF$^{hi/+}$, consistent with VEGF's role in skeletal muscle development (**Suppl. Figs. 2.1c,g**).[106] Capillary-to-fiber ratio decreased at 21 days in VEGF$^{lo/+}$ mice. While capillary number-per-tissue-area of VEGF$^{lo/+}$ did not differ from wild-type, greater atrophy occurred (**Suppl. Figs. 2.1d,h**), resulting in reduced capillary-to-muscle fiber ratio. These differences in capillary density which are consistent with VEGF's role in ischemic angiogenesis, could contribute to the differences in

recovery of hindlimb perfusion and ischemic scores (**Fig. 2.4**), although collateral remodeling is the primary determinant of recovery of hindlimb perfusion.

Given the importance of collateral conductance following femoral artery ligation, we determined collateral diameter at baseline and 21 days after ligation. Lumen diameters in $VEGF^{hi/+}$ mice were significantly smaller than wild-type at both time-points; however percent remodeling and adductor perfusion in $VEGF^{hi/+}$ were not different (**Fig. 2.5c, Suppl. Fig. 2.2f**). In contrast, baseline diameters in $VEGF^{lo/+}$ mice did not differ from wild-type, however remodeling and adductor perfusion were significantly reduced (**Fig. 2.5d, Suppl. Fig. 2.2g**). The baseline diameter data suggest (since baseline capillarity do not differ) that differences in collateral density may underlie the differences in perfusion immediately after ligation, ie, greater number of smaller diameter collaterals in $VEGF^{hi/+}$, versus fewer in $VEGF^{lo/+}$. In addition, less remodeling in $VEGF^{lo/+}$ mice suggests VEGF contributes to collateral remodeling.

VEGF gene dosage determines the number of pre-existing collaterals.

To determine if VEGF expression influences native collateral density, as suggested by the above findings, we performed X-ray arteriography immediately and 7 days after ligation (the latter to improve detection of smaller collaterals after onset of remodeling). Analogous to Rentrop analysis, we counted vessels crossing a line drawn through the middle of the adductor collateral zone (**Fig. 2.6a**); this method agrees with other methods of quantifying pre-existing collateral capacity in the hindlimb.[23] An apparent VEGF gene dose-relationship

was detected, wherein VEGF$^{hi/+}$ had more and VEGF$^{lo/+}$ had fewer collaterals than wild-type mice (**Fig. 2.6b**).

We next sought to determine if collaterals are present at birth and if VEGF expression correlates with newborn and adult density in another tissue besides skeletal muscle. We recently reported that differences in collateral density among mouse strains exist across multiple tissues.[23] This includes collaterals of the cerebral cortical circulation, which are confined to the pial (leptomeningeal) membrane and thus readily quantified.[23] We therefore counted pial collaterals in VEGF$^{hi/+}$, VEGF$^{lo/+}$ and wild-type mice using a polyurethane casting agent (**Fig 2.6c**).[146] Interestingly, collaterals were present at birth (P1; **Fig 2.6d**). As in the hindlimb (**Figs 2.6b**) an apparent VEGF gene dose-relationship was detected at P1, P21 and 3-months of age, with VEGF$^{hi/+}$ mice having more and VEGF$^{lo/+}$ having fewer collaterals (**Figs. 2.6c,d**). Wild-type and VEGF$^{hi/+}$ mice were born with the same number of collaterals while VEGF$^{lo/+}$ were born with fewer, suggesting VEGF levels specify the density of nascent collaterals formed during embryogenesis. In VEGF$^{hi/+}$, pial collateral number remained constant throughout post-natal development. In contrast, wild-type mice experienced a decline in collateral density by P21 that remained unchanged in adults. VEGF$^{lo/+}$ mice also lost collaterals with age. These data suggest that collaterals form during the perinatal period, that VEGF is important in their formation, and that VEGF is a determinant of their post-natal maturation and establishment of the adult collateral density.

DISCUSSION

Our findings suggest that VEGFR-1 positively regulates arteriogenesis through mobilization and recruitment of leukocytes to the peri-collateral region. We also provide the first examination of when the collateral circulation becomes established, finding that pial collaterals achieve their adult density by the third postnatal week. In addition, we identify the first gene, VEGF-A, whose expression impacts native collateral formation.

The signaling pathways that direct remodeling of pre-existing collaterals in ischemia remain unclear.[25] Few studies have addressed whether endogenous VEGF mediates collateral growth. Supportive evidence exists in hindlimb[134, 135] and heart[136] ligation models, where systemic administration of sFlt1/sFlk1 adenovirus[134], VEGF receptor inhibitor[135] or neutralizing antibody[136] impaired recovery of perfusion and angiographic collateral growth. Our findings that VEGFR-$1^{+/-}$ mice show reduced recovery of plantar and adductor perfusion, greater ischemia, reduced peri-collateral leukocyte accumulation and reduced collateral enlargement —but normal angiogenesis— suggest that VEGF acting through VEGFR-1 mediates collateral remodeling. This is in contrast to VEGFR-1's role as a VEGF inhibitor during development.[137, 142] It is difficult to measure VEGFR-1 signaling due to its weak kinase activity.[113] However, studies using the VEGFR-1 specific agonists, PlGF and VEGF-B, and kinase-dead mutants find that VEGFR-1 possesses signaling functions in the adult.[137, 142] Our data add collateral growth to these functions. They also agree with evidence that mice lacking PlGF have impaired recovery from hindlimb ischemia.[80] In addition, our

findings of fewer leukocytes circulating and residing around remodeling collaterals, which suggest reduced leukocyte mobilization and transmigration, are consistent with their known role in arteriogenesis.[80] These results are congruent with VEGFR-1's expression on leukocytes[142] and its proposed role in the bone marrow niche[151], in homing[152] and in mediation of chemotaxis.[110] No deficits were evident in VEGFR-$2^{+/-}$ mice. However given VEGFR-2's strong kinase activity, a single allele may specify sufficient receptor density to mediate VEGFR-2-dependent ischemic angiogenesis as well as any potential contribution of this receptor to arteriogenesis. This could explain why angiogenesis in the ischemic gastrocnemius was not impaired in VEGFR-$2^{+/-}$. Conditional deletion of VEGFR-2 will be required to confirm that VEGFR-2 does not participate in collateral growth.

Collaterals are small in diameter and few in number. In addition, current antibodies cannot distinguish among VEGF isoforms and have limited resolution to detect secreted ligand. Thus, it is not known if VEGF isoforms are increased around remodeling collaterals to contribute, directly or indirectly along with other cytokines, to arteriogenesis. Deindl and colleagues[117] did not detect increased VEGF in rabbit collaterals or in whole quadriceps muscle 3 days after femoral ligation. In contrast, we found that mRNAs for high-molecular weight isoforms of VEGF increased in the adductor collateral zone of wild-type mice 36 hours after ligation. Since transcript levels of VEGF agree with protein expression[153] this discrepancy may be due to differences in duration of ligation, tissue analyzed or species studied. We assayed a 5mm wide section in the middle of the adductor

that contains hindlimb collaterals, including those in the gracilis muscles that we measured histologically. In contrast to the adductor, only VEGF120 was upregulated in the gastrocnemius. This differential expression in tissues where collaterals are remodeling versus where capillaries are sprouting, is consistent with the different physical properties and functions of VEGF isoforms. In the calf, free diffusion of VEGF120 would promote a wide area of angiogenesis. In addition, VEGF120 released into the circulation from ischemic tissue aids mobilization of leukocytes from bone marrow.[151] In contrast, collateral growth in the thigh is presumed to require temporal and spatial release of proteases, cytokines, and growth factors. Elaboration of the heparin-binding VEGF164 and VEGF188 isoforms around collaterals may establish VEGF gradients that stimulate diapedesis and proliferation of leukocytes, proliferation of endothelial cells, and migration of smooth muscle cells. It will be important in future studies to determine the cell source(s) and stimulus for VEGF release. Endothelium, smooth muscle, peri-collateral leukocytes and fibroblasts, and skeletal muscle are all potential sources of VEGF.

Deindl and colleagues[117] also did not detect increased Hif-1α in the adductor, although differences in species, time after ligation and tissue sampled could be important. In contrast, we found a ~two-fold increase in Hif-1α mRNA (and no increase in Hif-1β) in the adductor collateral zone. Since Hif-1α levels are regulated by tissue oxygen over a broad range,[154] it is possible that oxygen declines sufficiently, though not to ischemic levels, to increase transcription and stabilization of Hif-1α transcripts. In addition, it is possible that other factors in

the extracellular milieu surrounding remodeling collaterals that regulate Hif-1α, such as reactive oxygen species, nitric oxide and certain growth factors[155], may contribute to the increase in Hif-1.

Consistent with deficient recovery of flow after femoral ligation in VEGFR-$1^{+/-}$mice, a direct relationship was evident between VEGF gene dosage and recovery among $VEGF^{lo/+}$, wild-type and $VEGF^{hi/+}$ mice. Compared to wild-type mice, $VEGF^{lo/+}$ had worse ischemic appearance scores and blunted recovery of plantar perfusion that were similar to VEGFR-$1^{+/-}$. In contrast, $VEGF^{hi/+}$ mice showed enhanced recovery of perfusion and little ischemia. In addition, $VEGF^{lo/+}$ mice exhibited attenuated adductor perfusion and impaired collateral remodeling like that seen in the VEGFR-$1^{+/-}$ mice. On the other hand, collateral remodeling and adductor perfusion were not enhanced in $VEGF^{hi/+}$. Although this appears discordant, several considerations are important. Immediately after ligation, perfusion decreased more in $VEGF^{lo/+}$ and less in $VEGF^{hi/+}$ mice. The increase in shear stress in collaterals immediately after ligation favors inhibition of collateral smooth muscle tone. Moreover, ischemia and low pressure cause myogenic and metabolic inhibition of tone in the vasculature below the point of ligation.[150] These considerations suggest that the differences in perfusion immediately after ligation arise from anatomical differences in pre-existing collateral number, collateral lumen size and/or capillary density. Capillary density was not different among $VEGF^{hi/+}$, $VEGF^{lo/+}$ and controls, and collateral lumen diameter was comparable in the latter two groups while being *smaller* in $VEGF^{hi/+}$ mice. Collectively, these data suggest that density of native collaterals varies directly

with level of VEGF expression. In $VEGF^{hi/+}$ mice, a greater number of collaterals in parallel favors lower flow in individual collaterals and thus smaller baseline diameters, which is what we observed. Shear stress is the proximate stimulus of arteriogenesis.[8, 25] A greater number of collaterals favors less increase in shear in individual collaterals, which is consistent with our finding that remodeling was not greater in $VEGF^{hi/+}$ mice than in wild-type despite increased VEGF expression. In $VEGF^{lo/+}$ mice with fewer collaterals, the expected higher shear forces may not be able to overcome the deficit in VEGF expression. This could explain the reduced collateral remodeling and impaired perfusion that we observed.

We found that VEGF expression determines collateral density in a second tissue —the pial circulation— where like skeletal muscle, density was greater in $VEGF^{hi/+}$ and lower in $VEGF^{lo/+}$ mice. An additional intriguing finding was that $VEGF^{lo/+}$ mice were born with fewer pial collaterals compared to wild-type and $VEGF^{hi/+}$ mice. These data suggest that VEGF levels contribute to collateral formation in the embryo. We previously hypothesized that collaterals form during embryonic arterial tree formation and that reduced VEGF expression results in reduced collateral formation.[23] VEGF has been implicated in branching morphogenesis.[111, 156-158] Our data support the postulate that collateral density and vascular branching are intimately related. In addition, collateral density was reduced by post-natal day 21 in wild-type and $VEGF^{lo/+}$ mice, while density was maintained in $VEGF^{hi/+}$ mice. This is consistent with VEGF's role in stabilizing nascent blood vessels.[137, 138, 142]

We reported that native collateral density and VEGF expression differ strongly in two mouse strains.[23] Inducible VEGF expression and collateral density in hindlimb, cerebral cortex and intestine were high in C57BL/6 mice and low in BALB/c. This association led us to hypothesize that VEGF is a determinant of native collateral formation. Our present studies using mice with a single targeted genetic difference, ie altered VEGF expression,[130, 131, 145] provide strong support for this hypothesis. Furthermore, they suggest that genetic polymorphisms and environmental factors that reduce VEGF expression during embryonic or perinatal periods could reduce collateral formation, resulting in increased severity of stroke, myocardial infarction and peripheral artery disease. How VEGF affects collateral formation and stabilization are intriguing questions for future study. While no studies have described when and how collaterals form, our data suggests that collaterals form prior to birth and mature within a narrow three-week period in the mouse.

While these findings indicate VEGF specifies collateral density in normal tissue, other molecules in the VEGF signaling pathway may also contribute to collateral density. Resar and coworkers recently identified a HIF-1α polymorphism in exon 12 that associated with the presence of coronary collaterals in patients.[159] The C-to-T polymorphism results in a P582S amino-acid substitution that confers increased HIF-1 activity. However, patients with the T allele did not have visible collaterals, which is at variance with our findings. Among mechanisms proposed by the authors to explain the apparent discrepancy between higher HIF-1 activity and lack of visible collaterals,[159] we

propose an additional hypothesis. Because clinical angiography can only detect large vessels, individual collaterals in a person with a small native density would be more likely to experience larger increases in shear and thus to enlarge to detectable diameters during progression of coronary artery disease. In contrast, collaterals in a person with a high native density would be expected to experience smaller increases in shear, resulting in less outward remodeling during disease progression and absence of detection by angiography.

In addition to upstream activators of VEGF, downstream effectors may also influence native collateral density. Endothelial nitric oxide synthase (eNOS) is an important signaling molecule induced by VEGF that contributes significantly to arteriogenesis.[160] In a recent report, Mees and colleagues compared arteriogenesis in mice over-expressing eNOS (eNOS-Tg) versus mice lacking functional eNOS ($eNOS^{-/-}$).[160, 161] Of note, laser Doppler perfusion immediately after ligation decreased more in $eNOS^{-/-}$ mice and less in eNOS-Tg mice, which we suggest may indicate a smaller versus larger native collateral density, respectively. If correct, these results which parallel our present data would further support an important role for VEGF in establishing the density of native collaterals in tissues.

In conclusion, our results suggest that VEGFR-1 mediates collateral growth in ischemic disease by mobilizing leukocytes and recruiting them to the peri-collateral space. In addition we show that collaterals form prior to birth and stabilize at their adult density by the third postnatal week in mice. Lastly, we identify VEGF as the first molecule specifying pre-existing collateral density in

normal tissue. We propose a model whereby collaterals form in a VEGF-dependent manner during embryogenesis. Further, these nascent collaterals require adequate VEGF during a critical period after birth to stabilize and mature sufficiently to achieve their full adult density. Further studies will be required to define when and how VEGF contributes to collateral formation, as well as the downstream effectors that stabilize and maintain collaterals in the adult. It is known that coronary collateral flow in healthy patients varies over a wide range.[162] Moreover, several VEGF polymorphisms linked to altered expression have been described.[163] Thus, it will be important to examine whether polymorphisms affecting the expression of VEGF and other genes are associated with altered collateral density in humans. An understanding of the pathways that specify collateral formation in normal tissues may lead to therapies to induce formation of new collaterals in adults with ischemic disease.

Figure 2.1: Impaired recovery of perfusion in VEGFR-$1^{+/-}$ mice after femoral artery ligation. (**a,b**) VEGFR-$1^{+/-}$ mice have greater hindlimb ischemic appearance score. Scale: 0=normal, 1=cyanosis or loss of nail(s), 2=partial or complete atrophy of digit(s), 3=partial atrophy of fore-foot; n=6-20 per data-point. (**c**) Laser Doppler perfusion images of plantar foot with region of interest (ROI). (**d,e**) Quantification of plantar perfusion measured over ROI. Data are mean±SEM for this and other figures. Two-way ANOVA followed by Dunn-Bonferonni corrected *t*-Test; *$p<0.05$ vs. wild-type CD-1; n=6-16 mice per data-point.

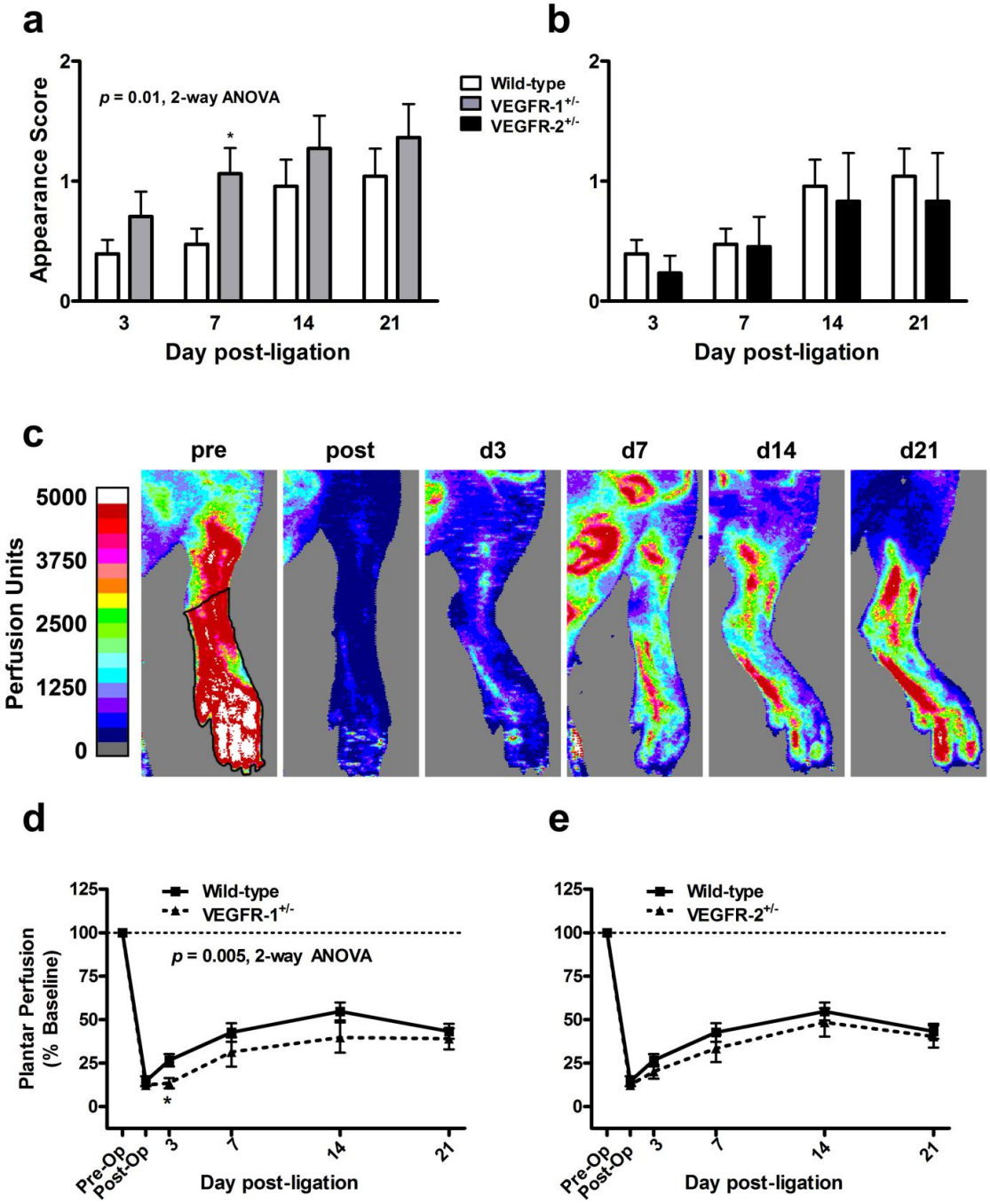

a
Appearance Score
p = 0.01, 2-way ANOVA
*
Day post-ligation
b
Wild-type
VEGFR-1+/-
VEGFR-2+/-
Day post-ligation
c
pre
post
d3
d7
d14
d21
Perfusion Units
5000
3750
2500
1250
0
d
Plantar Perfusion (% Baseline)
Wild-type
VEGFR-1+/-
p = 0.005, 2-way ANOVA
Pre-Op
Post-Op
Day post-ligation
e
Wild-type
VEGFR-2+/-
Day post-ligation

Figure 2.2: Reduced collateral remodeling (lumen expansion) and leukocyte recruitment in VEGFR-$1^{+/-}$ mice. (**a**) Lectin-stained capillaries in gastrocnemius muscle. (**d,g**) Capillary number-to-muscle fiber number ratios at baseline (before) and 21 days after femoral ligation. (**b**) Cyano-Massons-elastin-staining of collateral in gracilis muscle. (**e,h**) Collateral lumen diameter at baseline and 21 days after ligation. Numbers inside bars here and elsewhere are percent increase from baseline. (**c,f,i**) $CD45^{+}$ leukocytes in a 1-diameter area around anterior gracilis collaterals. Paired *t*-Test vs. baseline; Un-paired *t*-Test vs. wild-type; #$p<0.05$ vs. percentage change from wild-type; n=6-11 mice per data-point.

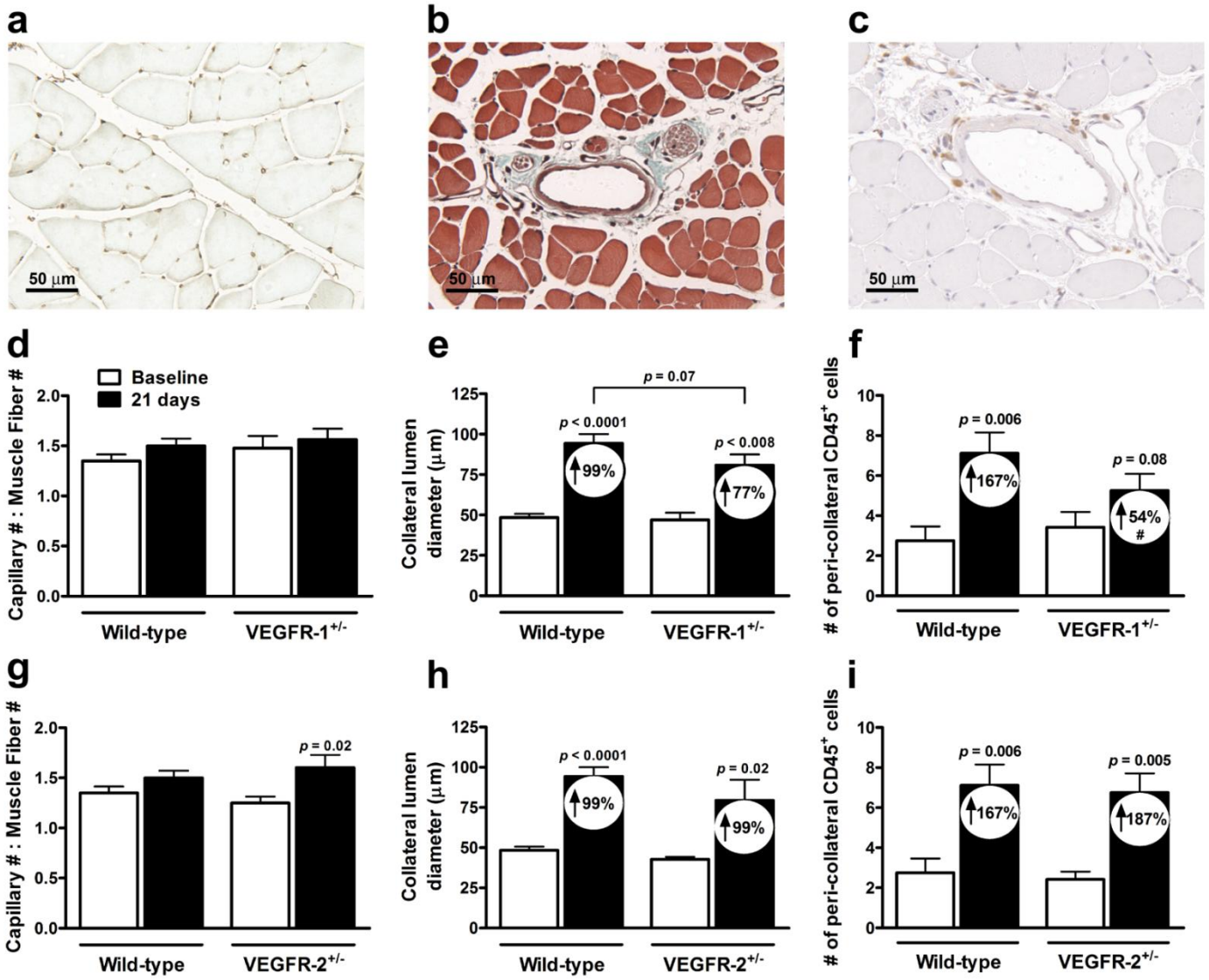

a
50 μm
b
50 μm
c
50 μm
d
Baseline
21 days
Capillary # : Muscle Fiber #
2.0
1.5
1.0
0.5
0.0
Wild-type
VEGFR-1+/-
e
p = 0.07
p < 0.0001
p < 0.008
99%
77%
Collateral lumen diameter (μm)
125
100
75
50
25
0
Wild-type
VEGFR-1+/-
f
p = 0.006
p = 0.08
167%
54%
#
# of peri-collateral CD45+ cells
10
8
6
4
2
0
Wild-type
VEGFR-1+/-
g
p = 0.02
Capillary # : Muscle Fiber #
2.0
1.5
1.0
0.5
0.0
Wild-type
VEGFR-2+/-
h
p < 0.0001
p = 0.02
99%
99%
Collateral lumen diameter (μm)
125
100
75
50
25
0
Wild-type
VEGFR-2+/-
i
p = 0.006
p = 0.005
167%
187%
# of peri-collateral CD45+ cells
10
8
6
4
2
0
Wild-type
VEGFR-2+/-

Figure 2.3: Quantitative RT-PCR of VEGF isoforms (**a-f**) and HIF subunits (**g-i**) in calf (**a-c**) and adductor muscle (**d-i**) of wild-type (**a,d,g**), $VEGF^{hi/+}$ (**b,e,h**), and $VEGF^{lo/+}$ (**c,f,i**) mice. Data for muscle taken from the ligated leg are relative to muscle from the non-ligated leg and normalized to 18S rRNA. Non-parametric *t*-Test vs. non-ligated; n=5-6 mice per data-point.

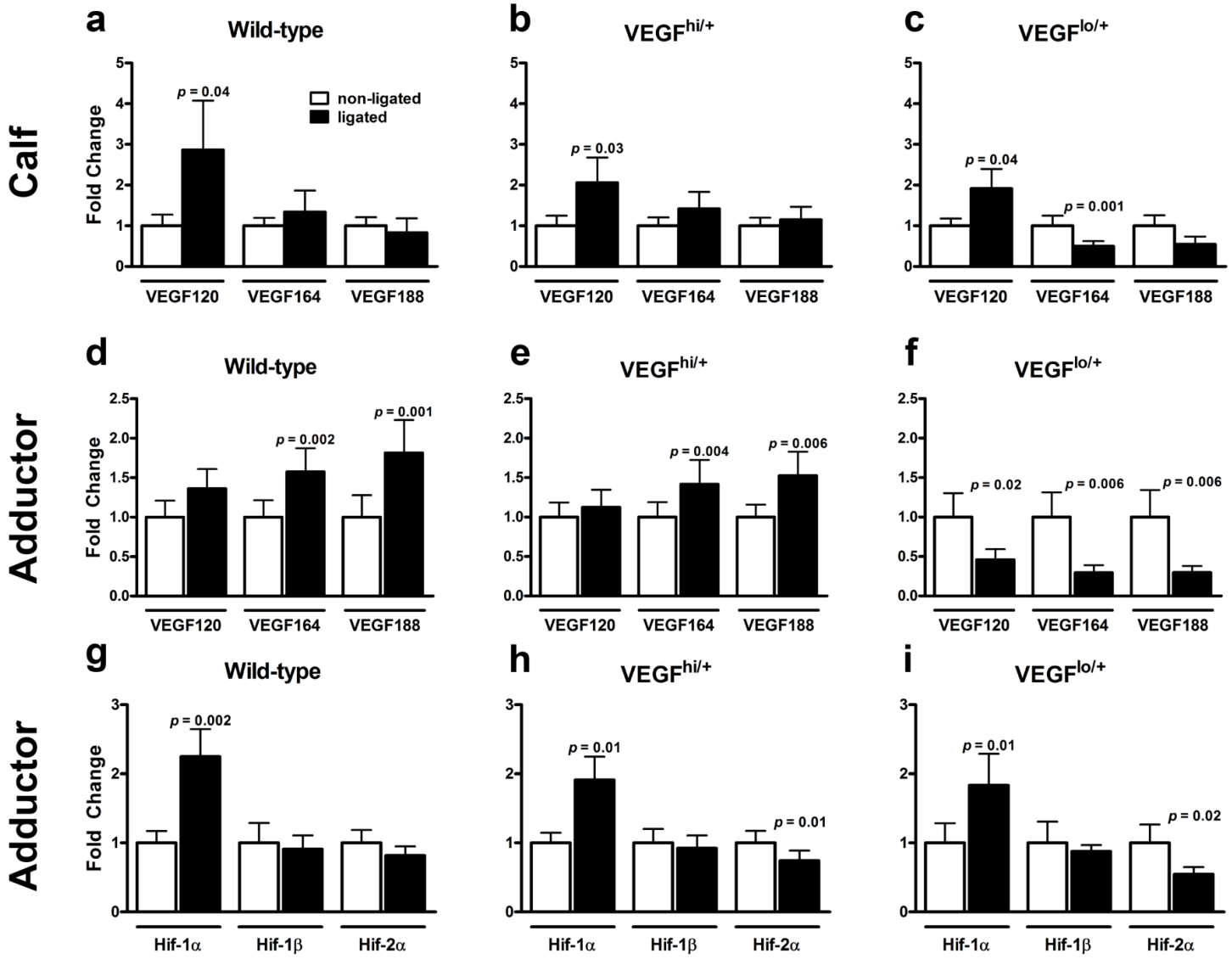

a
Wild-type
b
VEGF^hi/+
c
VEGF^lo/+
Calf
Fold Change
non-ligated
ligated
p = 0.04
p = 0.03
p = 0.04
p = 0.001
VEGF120
VEGF164
VEGF188
d
Wild-type
e
VEGF^hi/+
f
VEGF^lo/+
Adductor
Fold Change
p = 0.002
p = 0.001
p = 0.004
p = 0.006
p = 0.02
p = 0.006
p = 0.006
VEGF120
VEGF164
VEGF188
g
Wild-type
h
VEGF^hi/+
i
VEGF^lo/+
Adductor
Fold Change
p = 0.002
p = 0.01
p = 0.01
p = 0.01
p = 0.02
Hif-1α
Hif-1β
Hif-2α

Figure 2.4: $VEGF^{hi/+}$ mice have better and $VEGF^{lo/+}$ worse recovery after femoral ligation. (**a,b**) Lower ischemic appearance score in $VEGF^{hi/+}$ and more ischemia in $VEGF^{lo/+}$ (see Fig. 2.1 for scale); n=6-20 per data-point. (**c,d,e**) Quantification of perfusion measured over plantar foot ROI shows less reduction in perfusion immediately after ligation ("Post-Op") and better recovery in $VEGF^{hi/+}$, and opposite effects in $VEGF^{lo/+}$. (**f**) Representative Doppler images of Pre- and Post-Op time points. ANOVA followed by Dunn-Bonferonni corrected *t*-Test; **p*<0.05, ***p*<0.01, ****p*<0.001 vs. wild-type; n=6-16 per data-point.

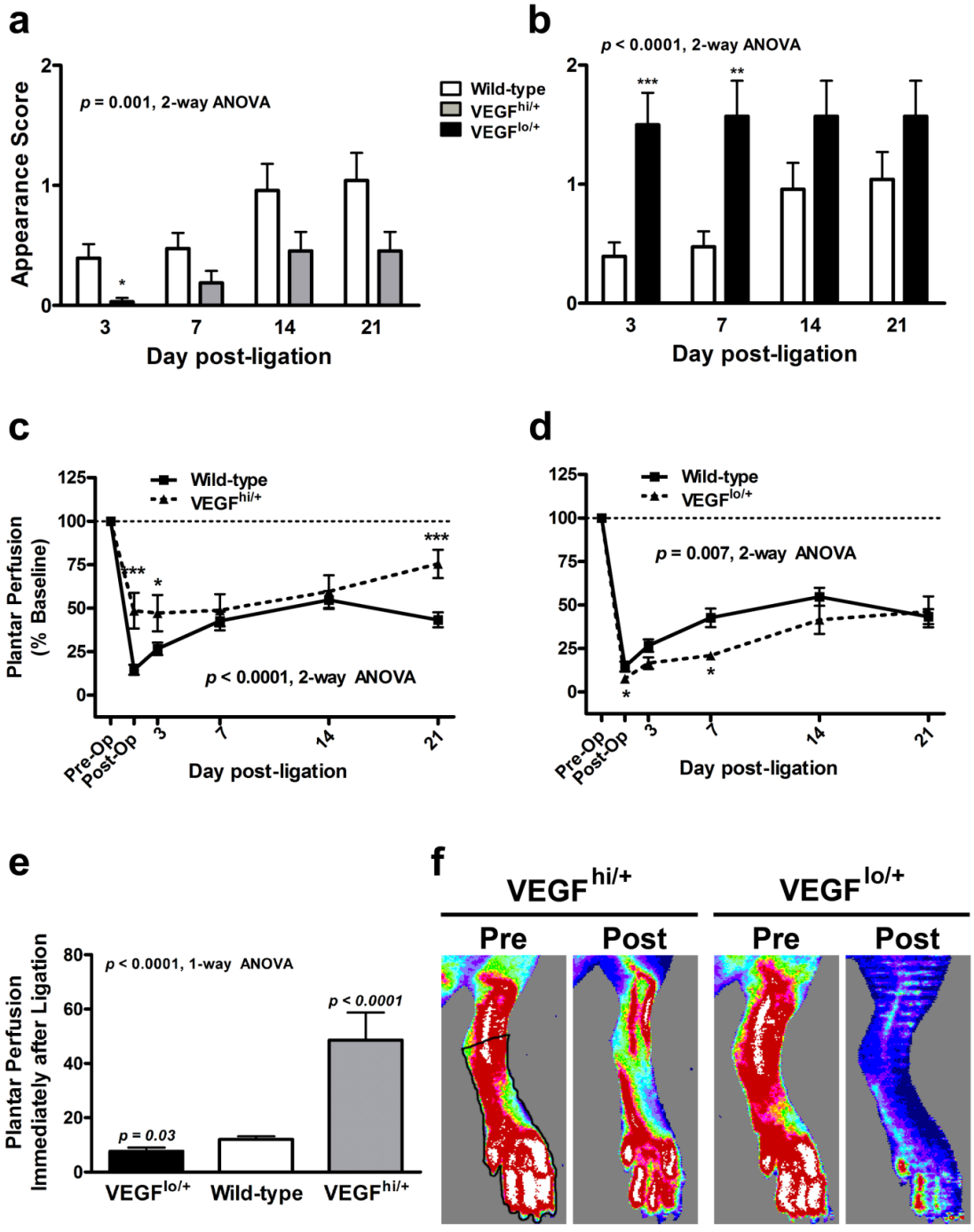

a
Appearance Score
p = 0.001, 2-way ANOVA
Wild-type
VEGFhi/+
VEGFlo/+
Day post-ligation
b
p < 0.0001, 2-way ANOVA
Day post-ligation
c
Plantar Perfusion (% Baseline)
Wild-type
VEGFhi/+
p < 0.0001, 2-way ANOVA
Pre-Op
Post-Op
Day post-ligation
d
Wild-type
VEGFlo/+
p = 0.007, 2-way ANOVA
Day post-ligation
e
Plantar Perfusion Immediately after Ligation
p < 0.0001, 1-way ANOVA
p = 0.03
p < 0.0001
VEGFlo/+
Wild-type
VEGFhi/+
f
VEGFhi/+
VEGFlo/+
Pre
Post
Pre
Post

Figure 2.5: Collateral remodeling and angiogenesis are attenuated in $VEGF^{lo/+}$ mice. Capillary number-to-muscle fiber number ratios (**a,b**) and collateral lumen diameters (**c,d**) before and 21 days after ligation. Paired *t*-Test vs. Baseline; Unpaired *t*-test vs. wild-type; #$p<0.05$ vs. percentage change from wild-type; n=7-11 per data-point

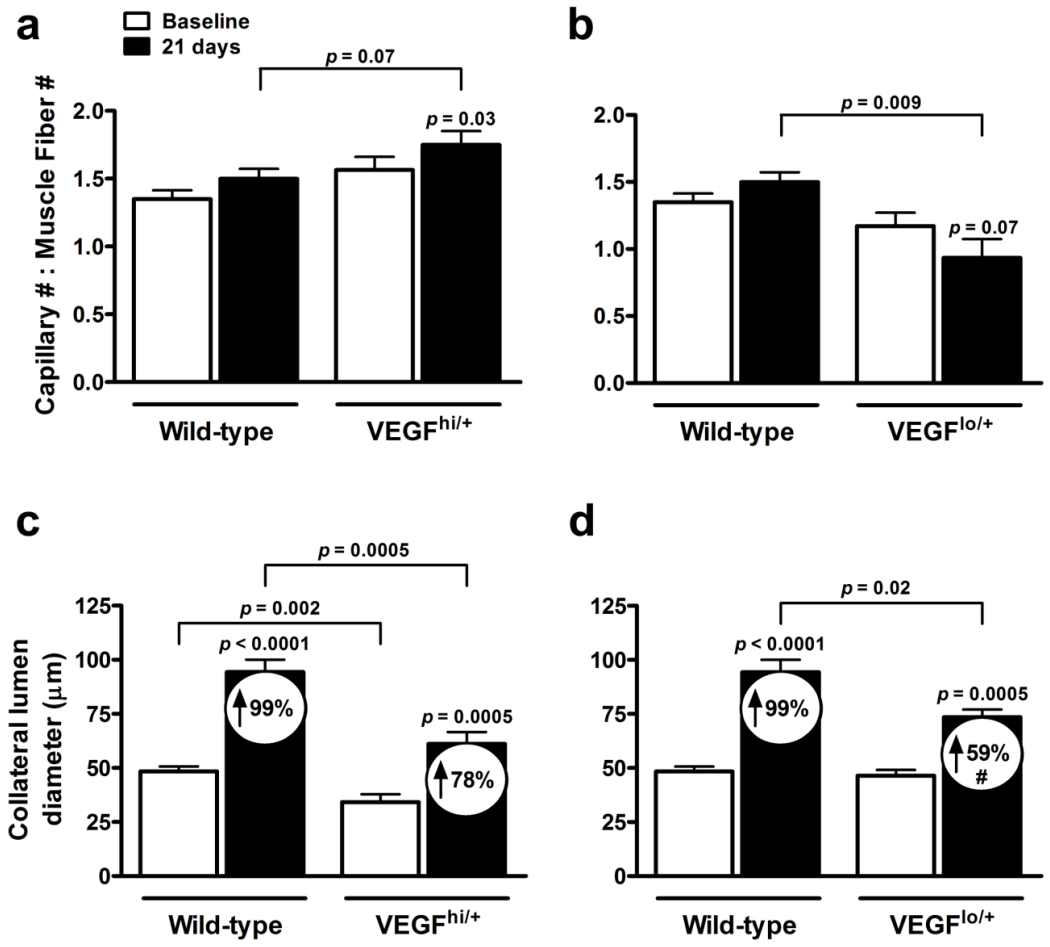
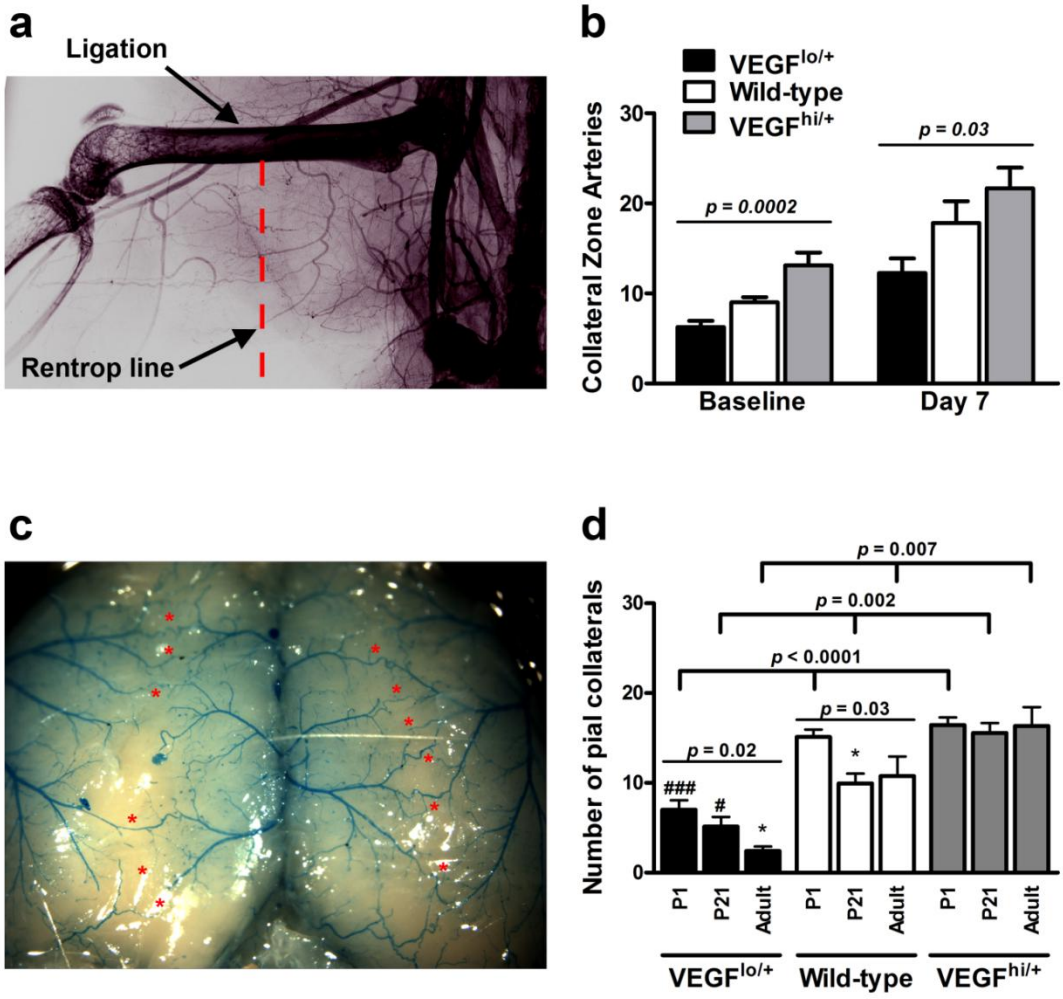

a
Baseline
21 days
p = 0.07
p = 0.03
Capillary # : Muscle Fiber #
2.0
1.5
1.0
0.5
0.0
Wild-type
VEGFhi/+
b
p = 0.009
p = 0.07
Wild-type
VEGFlo/+
c
p = 0.0005
p = 0.002
p < 0.0001
99%
p = 0.0005
78%
Collateral lumen diameter (μm)
125
100
75
50
25
0
Wild-type
VEGFhi/+
d
p = 0.02
p < 0.0001
99%
p = 0.0005
59%
#
Wild-type
VEGFlo/+

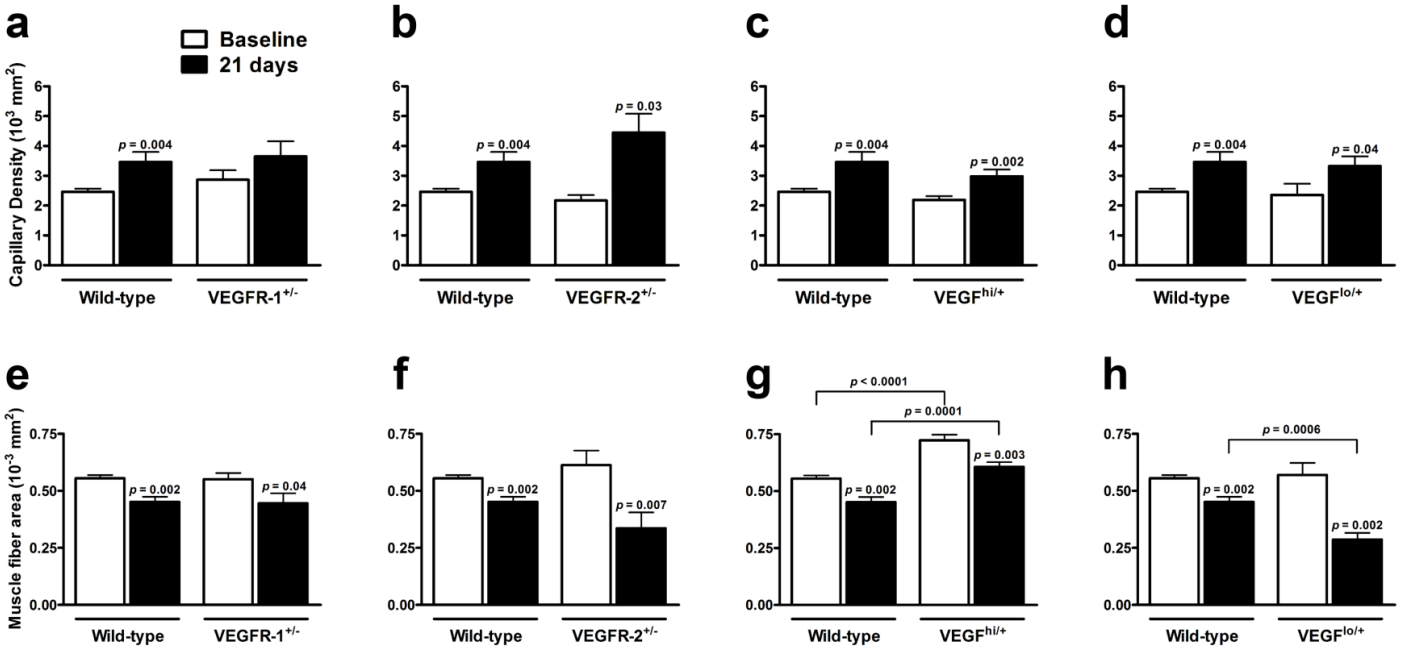
Figure 2.6: Collateral density in skeletal muscle and pial circulations correlates with VEGF gene dosage. (**a**) Post-mortem X-ray arteriogram of thigh. Vessels were counted (data given in (**b**)) that crossed a line drawn from the mid-point between the femoral ligations through the thigh collateral zone before and 7 days after ligation. (**c**) Post-mortem polyurethane arteriograph of pial circulation. Collaterals were counted (data given in (**d**)) that interconnected the middle and anterior cerebral artery trees at post-natal day-1, day-21 and 12 weeks. Brackets, 1-way ANOVA followed by Dunn-Bonferonni corrected *t*-Test; **p*<0.05 vs. P1; #*p*<0.05, ###*p*<0.001 vs. corresponding wild-type time-point; n=8-11 per data-point.


a
Ligation
Rentrop line
b
VEGF lo/+
Wild-type
VEGF hi/+
Collateral Zone Arteries
30
20
10
0
p = 0.0002
p = 0.03
Baseline
Day 7
c
d
p = 0.007
p = 0.002
p < 0.0001
p = 0.03
p = 0.02
Number of pial collaterals
30
20
10
0
###
#
*
P1
P21
Adult
VEGF lo/+
Wild-type
VEGF hi/+

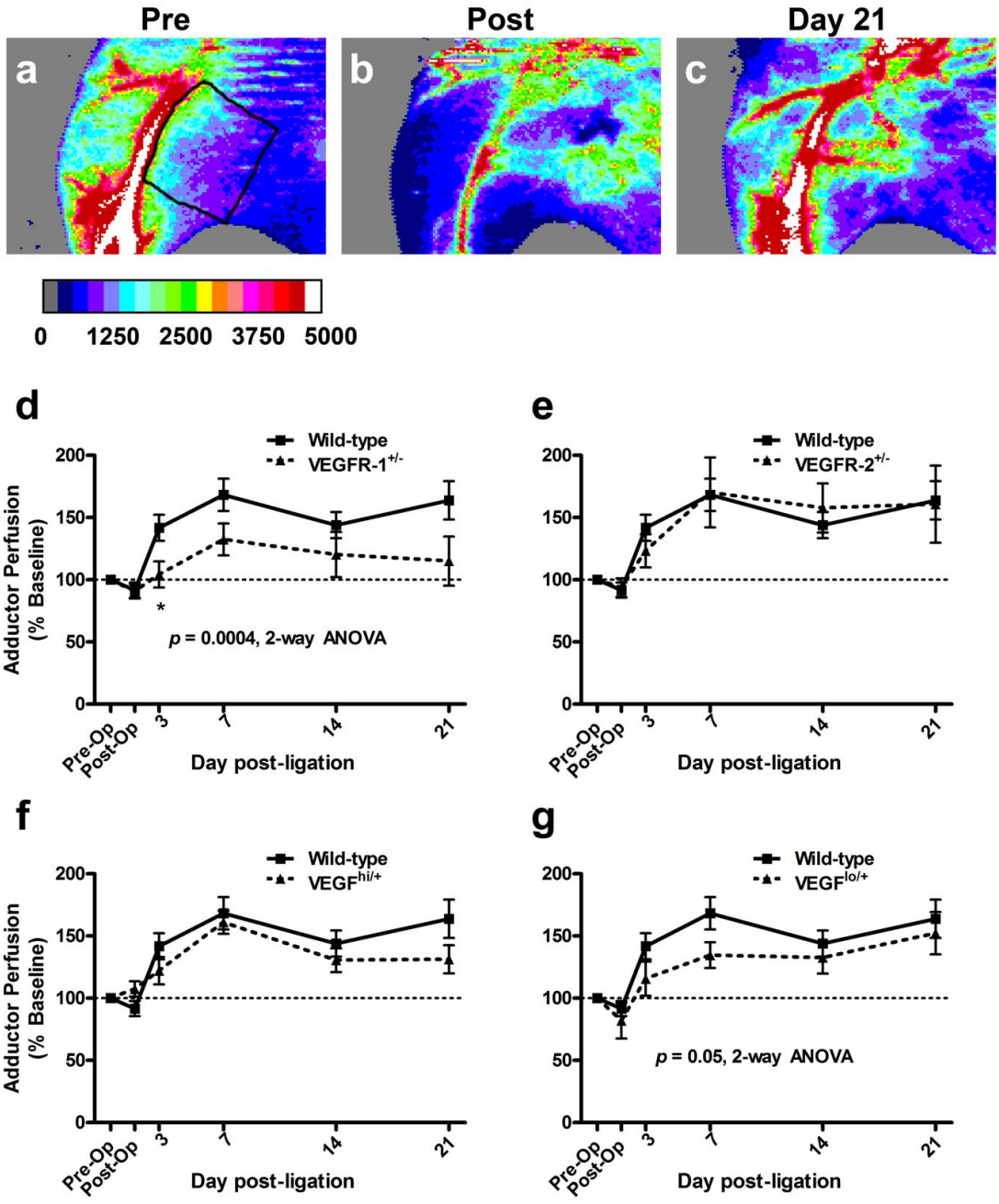
Supplemental Table 1: Whole blood leukocyte counts ($10^3/\mu l$) are reduced in VEGFR-$1^{+/-}$ but not VEGFR-$2^{+/-}$ mice.

	WBC	**LYMF**	**GRAN**	**MONO**
Wild-type	4.44 ± 0.27	2.92 ± 0.30	1.08 ± 0.08	0.44 ± 0.02
VEGFR-$1^{+/-}$	3.50 ± 0.19	2.18 ± 0.19	0.96 ± 0.04	0.36 ± 0.02
***p* value**	0.01	0.04	0.11	0.02
Wild-type	3.84 ± 1.13	2.52 ± 1.02	0.86 ± 0.14	0.46 ± 0.08
VEGFR-$2^{+/-}$	4.32 ± 0.99	2.72 ± 0.78	1.14 ± 0.26	0.46 ± 0.07
***p* value**	0.49	0.39	0.15	0.45

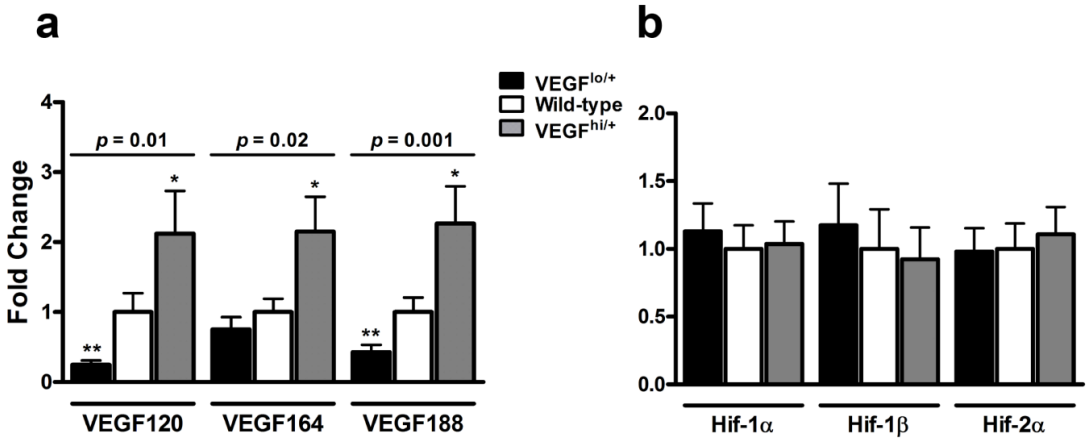
Supplemental Figure 2.1: Capillary area density (**a-d**) and muscle fiber area (**e-h**) for VEGFR-$1^{+/-}$ (**a,e**), VEGFR-$2^{+/-}$ (**b,f**), $VEGF^{hi/+}$ (**c,g**), and $VEGF^{lo/+}$ (**d,h**) mice before (open bars) and 21 days after femoral ligation (black bars). Capillary area density increased in wild-type mice, with a similar trend in VEGFR-$1^{+/-}$ mice (**a**). Muscle fiber area declined similarly in wild-type and $VEGFR^{+/-}$ mice (**e**). Together, the data suggest that VEGFR-1 haplo-insufficiency does not affect angiogenesis or muscle atrophy. Capillary area density tended to increase more (**b**) and muscle fiber area tended to decrease more (**f**) in VEGFR-$2^{+/-}$ mice. The higher capillary area density and smaller muscle fiber area resulted in an increase in capillary-to-muscle fiber number ratio (**Fig. 2f**), which suggests that heterozygosity does not impair angiogenesis. Capillary area density increases slightly less in $VEGF^{hi/+}$, consistent with a less ischemia in the hindlimb (**c**). Interestingly, $VEGF^{hi/+}$ muscle fiber area was greater at baseline and at 21 days (**g**). The difference in muscle fiber area may indicate altered skeletal muscle physiology from chronic VEGF overexpression. Capillary area density was not different in $VEGF^{lo/+}$ mice, which is incongruent with increased ischemia (**d**). However, there is greater atrophy of skeletal muscle, which is consistent with increased ischemia in $VEGF^{lo/+}$ mice (**h**). Together, these data explain the slight decrease in capillary-to-muscle fiber number ratio (**Fig. 5d**).


a
Baseline
21 days
Capillary Density (10^3 mm^2)
p = 0.004
Wild-type
VEGFR-1+/-
b
p = 0.004
p = 0.03
Wild-type
VEGFR-2+/-
c
p = 0.004
p = 0.002
Wild-type
VEGFhi/+
d
p = 0.004
p = 0.04
Wild-type
VEGFlo/+
e
Muscle fiber area (10^{-3} mm^2)
p = 0.002
p = 0.04
Wild-type
VEGFR-1+/-
f
p = 0.002
p = 0.007
Wild-type
VEGFR-2+/-
g
p < 0.0001
p = 0.0001
p = 0.002
p = 0.003
Wild-type
VEGFhi/+
h
p = 0.0006
p = 0.002
p = 0.002
Wild-type
VEGFlo/+

Supplemental Figure 2.2: VEGFR-$1^{+/-}$ and $VEGF^{lo/+}$ mice have attenuated, and VEGFR-$2^{+/-}$ and $VEGF^{hi/+}$ mice have normal perfusion over the adductor collateral zone. (**a-c**) Representative laser Doppler images of the ventral adductor with ROI outlined. Collateral perfusion is blunted in VEGFR-$1^{+/-}$ suggesting a positive role for VEGFR-1 in mediating arteriogenesis (**d**). Impaired collateral perfusion is likely due to a decrease in collateral remodeling (**Fig. 2e**) as a result of a decrease in leukocyte recruitment (**Fig. 2f**) and leukocyte mobilization (**Suppl. Table 1**). Adductor perfusion is unchanged in VEGFR-$2^{+/-}$ mice, suggesting that VEGFR-2 is not required for collateral remodeling or, despite heterozygosity, receptor density and/or signaling is sufficient to mediate normal arteriogenesis (**e**). In $VEGF^{hi/+}$ mice, collateral perfusion is not different from wild-type (**f**) while adductor perfusion is decreased in $VEGF^{lo/+}$ (**g**). Inhibition of adductor perfusion in $VEGF^{lo/+}$ is consistent with attenuation of perfusion in VEGFR-$1^{+/-}$ and suggests a model whereby sufficient VEGF levels are required to adequately recruit VEGFR-1-containing leukocytes to the growing collateral. However, collateral perfusion from $VEGF^{hi/+}$ would seem to contradict that model. This could reflect that VEGF expression exceeding wild-type levels may not be able to further augment collateral remodeling. Consistent with this, no further augmentation of collateral lumen diameter was observed in $VEGF^{hi/+}$ mice (**Fig. 5c**). Alternatively, the greater number of native collaterals in $VEGF^{hi/+}$ mice (**Fig. 6**) favors reduced flow and shear in any individual collateral. A decrease in the shear stimulus, combined with an increase in VEGF production, may result in a similar amount of remodeling compared to wild-type mice.


Pre
Post
Day 21
a
b
c
0
1250
2500
3750
5000
d
Wild-type
VEGFR-1+/-
Adductor Perfusion (% Baseline)
200
150
100
50
0
*
p = 0.0004, 2-way ANOVA
Pre-Op
Post-Op
3
7
14
21
Day post-ligation
e
Wild-type
VEGFR-2+/-
Pre-Op
Post-Op
3
7
14
21
Day post-ligation
f
Wild-type
VEGFhi/+
Adductor Perfusion (% Baseline)
Pre-Op
Post-Op
3
7
14
21
Day post-ligation
g
Wild-type
VEGFlo/+
p = 0.05, 2-way ANOVA
Pre-Op
Post-Op
3
7
14
21
Day post-ligation

Supplemental Figure 2.3: Quantitative RT-PCR of non-ischemic calf skeletal muscle confirms genotype of $VEGF^{hi/+}$ and $VEGF^{lo/+}$ mice (**a**). VEGF expression in normal skeletal muscle has not been previously reported in these adult mouse models. $VEGF^{hi/+}$ mice have 2-fold greater levels of the major VEGF isoforms while there is a 25-75% decrease in them in $VEGF^{lo/+}$ mice. Non-ischemic adductor HIF subunit expression is similar in all genotypes demonstrating no VEGF-dependent alteration in HIF expression. Data show expression in non-ischemic muscle of $VEGF^{hi/+}$ or $VEGF^{lo/+}$ relative to non-ischemic wild-type muscle. Data are normalized to 18S rRNA. Non-parametric *t*-Test vs. non-ligated; n=5 mice per data-point.


a
b
VEGF^lo/+
Wild-type
VEGF^hi/+
Fold Change
p = 0.01
p = 0.02
p = 0.001
VEGF120
VEGF164
VEGF188
Hif-1α
Hif-1β
Hif-2α

CHAPTER 3

Paracrine VEGF Signaling is Essential for Arteriogenesis

ABSTRACT

Arteriogenesis (collateral artery growth in ischemia) is a critical vascular adaptation to ischemic vascular disease. It is a complex process that requires the recruitment of leukocytes and elaboration of growth factors, cytokines, and proteases. The role of VEGF in arteriogenesis is controversial. Studies administering exogenous VEGF have shown both positive and negative results for improved arteriogenesis and perfusion recovery. Alternatively, systemic inhibition of VEGF has demonstrated a positive role for endogenous VEGF in arteriogenesis. However, both of these approaches can indirectly lead to augmentation or inhibition of arteriogenesis through secondary actions on hemodynamics and leukocyte mobilization. Currently, no studies have conclusively identified a direct role for VEGF in arteriogenesis. Therefore, we examined the role of local VEGF secretion and signaling in mediating collateral growth. We found selective VEGF expression in cells surrounding remodeling collaterals, but not quiescent collaterals or similarly sized arterioles. Deletion of VEGF in the adductor via electroporation of Cre recombinase in $VEGF^{loxP/loxP}$ mice led to increased ischemic appearance and limb use impairment scores, and attenuated recovery of plantar perfusion. These results suggest that tissue

resident cells are a source of arteriogenic VEGF. Electroporation of FltIgG (VEGF trap) similarly increased ischemic appearance and limb use scores. While recovery of plantar perfusion was unaffected, adductor perfusion was attenuated in FltIgG animals, suggesting impaired collateral remodeling. Together, our results suggest a role for local, endogenous VEGF signaling in arteriogenesis.

INTRODUCTION

The molecular mediators of arteriogenesis, ie, collateral artery growth in ischemia, remain incompletely defined. Studies have found that exogenous administration of certain growth factors and cytokines, including VEGF can both augment[61-66] and impair[124, 125] collateral growth. In the case of VEGF, disagreements could arise from differences in methods of delivery which have included plasmids, viruses, and protein, and variation in concentrations. It is clear from studies of therapeutic angiogenesis that a narrow window of VEGF expression is required, such that too much or too little, renders the molecule ineffective.[130-133] Not only is the concentration of VEGF important, but the cellular and tissue distribution of the isoforms can alter its function.[138]

Systemic administration of exogenous VEGF is problematic in that it is a pleiotropic molecule with both direct and indirect actions on each of the cell types involved in arteriogenesis. VEGF mobilizes leukocytes from the bone marrow, and increases their transmigration, which is required for arteriogenesis.[36-39, 59, 110, 114] VEGF, through activation of eNOS, is a potent vasodilator, which may

increase flow and shear in the collateral circulation and thus indirectly augment collateral remodeling.[100] Similarly, VEGF-induced angiogenesis in ischemic tissue favors reduction in resistance downstream of the occlusion and increased flow and shear in the collateral circulation.

The above difficulties make clear that strategies are needed to manipulate endogenous VEGF locally in the collateral region to understand the biological function of VEGF in arteriogenesis. Reports have shown that systemic inhibition of VEGF can attenuate collateral remodeling and perfusion recovery.[134-136] However, similar to systemically increasing VEGF expression, systemic blockade can indirectly cause inhibition of arteriogenesis through similar secondary effects. In the present study, we tested the hypothesis that local endogenous VEGF is necessary for arteriogenesis and recovery of perfusion in occlusive vascular disease. We used in vivo electroporation to deliver plasmids encoding Cre-recombinase into the adductor of $VEGF^{loxP/loxP}$ mice, the site of the main collateral circulation to hindlimb. We also delivered FltIgG, a VEGF-trap, into the adductor of C57BL/6 mice and assessed perfusion recovery and arteriogenesis.

MATERIALS AND METHODS

Immunofluorescence. 7 days after ligation, $VEGF^{hi/+}$ mice were pressure-perfused transcardially (100mmHg) with phosphate-buffered saline (PBS, pH7.4) containing 20mmol/L adenosine, 10^{-4}mol/L papaverine and 10U/ml heparin, followed by pressure-perfused fixation with 4% paraformaldehyde in 100mmol/L sodium phosphate (PFA; pH7.4) for 15min The adductor was

excised in a 5mm block extending ~1mm medial of the “mid-zone” of the gracilis collaterals that interconnect the lateral caudal femoral artery and saphenous artery trees, to the lateral portion of the thigh. Blocks were paraffin-embedded and sectioned at 5µm. β-galactosidase was detected with rabbit anti-β-galactosidase antibody (ab616, Abcam, 1:100) followed by Cy5-conjugated donkey anti-rabbit antibody (Jackson Immuno Research, 1:500). Leukocytes were detected with rat anti-CD45 antibody (clone 30-F11, BD Pharmingen, 1:100) followed by FITC-conjugated donkey anti-rat antibody (Jackson Immuno Research, 1:500).

Plasmid construction. The parental plasmid used in this study, pIRES (Clontech) contains a CMV promoter driving the transcription of the genes of interest. pIRES-LacZ: LacZ was PCR amplified (Phusion high-fidelity polymerase, NEB) from pBI-G (Clontech) with primers LacZ F-1 and LacZ R-1 (see Table 3-1 for list of oligos used in this study) and ligated into the XbaI / NotI site of pIRES. pCre-IRES-LacZ: Cre was amplified from pCMV5-Cre-NF (gift from R Sealock) with Cre F-1 and Cre-R1 and ligated into the NheI / EcoRI site of pIRES-LacZ.

pIRES3: pIRES was subjected to 3 rounds of mutagenesis. Round 1: pIRES was amplified with IRES F-1, IRES R-1, mutIRES2-F, and mutIRES2-R. The PCR fragment was digested with NheI and XbaI and ligated into the corresponding sites of pIRES to yield pIRES2. Round 2: pIRES2 was amplified with IRES F-2, IRES R-1, mutIRES3-F1, and mutIRES3-R1. PCR product was digested with PflMI and XbaI and ligated into the corresponding sites of pIRES2

to yield pIRES3x. Round 3: pIRES3x was amplified with IRES F-3, IRES R-2, mutIRES3-F2, and mutIRES3-R2. The PCR product was digested with NdeI and XhoI and ligated into the corresponding sites of pIRES3x to yield pIRES3.

pLuc-IRES3: Luciferase was amplified from pBI-GL (Clontech) with Luc-F1 and Luc-R1, digested with XbaI and SalI and ligated into NheI / XhoI of pIRES3. pLuc-IRES3-LacZ: LacZ was amplified from pBI-G with LacZ F-2 and LacZ R-2, digested with XbaI and SalI and ligated into XbaI / SalI of pLuc-IRES3. pLuc-IRES3-FltIgG: FltIgG was amplified from pAdEasy-FltIgG (kindly provided by N Ferrara) with FltIgG F-1 and FltIgG R-1, digested with XbaI and SalI and ligated in XbaI / SalI of pLuc-IRES3.

In vivo electroporation. Mice were anesthetized with 1.125% isoflurane/O_2. Dorsal and ventral hindlimbs were depilated. Using a 32g needle collared to confine penetration to ~2 mm and micro-manipulator control, 50μl of 0.8 U/μl hyaluronidase (Sigma) was injected into 3 sites in the adductor and 3 sites in the abductor, adjacent to the body wall in both right and left legs (**Fig. 3.2 a**). 2 hours after hyaluronidase treatment, 50μl plasmid, 1 μg/μl, was injected into the same sites in the adductor and abductor muscle groups of both legs. Electrode paste was applied to the thigh and 8 pulses of 1ms duration at 125 V/cm were applied to the thigh with 1cm caliper electrodes (BTX).

In a separate study, Ibuprofen (0.2 mg/ml, IBU) was added to the drinking water 7 days before electroporation and for the duration of the study, to assess the “inflammatory” hyperemic response to electroporation.

Unilateral hindlimb ischemia. 3 and/or 13 days after electroporation, mice received unilateral femoral artery ligation. 12 to 14 week-old mice were used in all studies. $VEGF^{loxP/loxP}$ mice were kindly provided by N Ferrara.[164] C57BL/6 mice were purchased from Jackson Laboratories. $VEGF^{hi/+}$ mice were a gift of A Nagy.[145] Animals were randomized and procedures and analyses were conducted blindly. Femoral artery ligation was performed as described.[23, 34] Briefly, mice were anesthetized with 1.25% isoflurane/O_2 and the hindlimbs depilated. Temperature was maintained at 37.0±0.5ºC. The right femoral artery was exposed through a 2mm incision and ligated with two 7-0 ligatures placed distal to the origin of the lateral caudal femoral and superficial epigastric arteries (the latter was also ligated) and proximal to the genu artery. The artery was transected between the sutures and separated by 1-2 mm. The wound was irrigated with sterile saline and closed, and cefazolin (50mg/kg, im), furazolidone (topical) and pentazocine (10mg/kg, im) were administered. Procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Laser Doppler perfusion imaging. As detailed previously,[23, 34] under 1.125% isoflurane/O_2 anesthesia and 37±0.5ºC, non-invasive perfusion imaging of the adductor thigh region and plantar foot of both limbs was performed before, immediately after, and at 3, 7, 14, and 21 days after femoral ligation. Regions of interest (ROI) were drawn to anatomical landmarks.

Assessment of ischemia. An “appearance score” after ligation was obtained, where 0=normal, 1=cyanosis or loss of nail(s), 2=partial or complete

atrophy of digit(s), 3=partial atrophy of fore-foot.[23] A "use score" was similarly obtained, where 0=normal, 1=no toe flexion or toe spreading, 2=ambulation with the foot but no plantar flexion, 3=mouse drags foot.[23]

Leukocyte counts. Three days after femoral ligation, blood was collected into EDTA tubes (Sarstedt) under anesthesia by sub-mandibular puncture (Medipoint). Leukocyte counts (CBC) were performed with a Heska's Animal Blood Counter.

Whole mount X-gal staining. 7 or 21 days after electroporation, the abdominal aorta was cannulated and animals were pressure-perfused (100mmHg) with phosphate-buffered saline (PBS, pH7.4) containing 20mmol/L adenosine, 10^{-4}mol/L papaverine and 10U/ml heparin, followed by pressure-perfused fixation with 4% paraformaldehyde in 100mmol/L sodium phosphate (PFA; pH7.4) for 15min. X-gal wash buffer (PBS, 2mM $MgCl_2$) was perfused followed by X-gal staining solution (X-gal wash buffer, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, X-gal at 1mg/ml). Hindlimbs were removed and incubated in X-gal staining solution at 37ºC overnight.

Xenogen luciferase imaging. Animals were injected IP with 30mg/kg luciferin (Caliper Life Science) and allowed to ambulate for 5 minutes. Mice were then anesthetized with 2% isoflurane/O_2 until recumbent and positioned in the light tight chamber of the IVIS100 under 1.5% isoflurane/O_2 . Photons were collected and integrated for 1 second intervals at 10 and 15 minutes following injection of luciferin for the ventral surface and 20 and 25 minutes for the dorsal

surface. Living Image software was used to automatically generate ROI's above 10% threshold. Data are reported as photons/sec.

Statistics. All data are reported as means ± SEM. Significance ($P<0.05$) was determined by two-way ANOVA followed by Dunn-Bonferonni Corrected *t*-tests. One-way ANOVA was used for comparisons of plasmid effect on CBC over time, followed by paired or un-paired *t*-tests.

RESULTS

VEGF is localized to remodeling collaterals and not to other arterioles.

While it is known that *in vitro*, cells surrounding the collateral and cells of the collateral wall are capable of expressing VEGF, it is not clear whether or not these cells express VEGF in the context of arteriogenesis. To assess this, we examined β-galactosidase (β-gal) expression in $VEGF^{hi/+}$ mice. $VEGF^{hi/+}$ mice have a targeted mutation that permits β-gal to be expressed, in a bi-cistronic manner, with VEGF.[145] β-gal is thus a surrogate for VEGF expression. Immunofluorescence of β-gal in gracilis collaterals reveals increased staining in skeletal muscle fibers surround the remodeling collateral (**Fig. 3.1a**) whereas very little staining is seen in non-ligated collaterals (**Fig. 3.1d**). To determine if this staining was collateral specific, we analyzed β-gal immunoreactivity in other arterioles in the adductor (identified as 25-50 μm diameter vessels with no associated vein or nerve). An arteriole found in the adductor magnus muscle group showed reduced staining similar to that seen in the non-ligated collateral (**Fig. 3.1g**). In addition to skeletal muscle expression of β-gal, we see co-

localization of β-gal with CD45 (**Fig. 3.1b**), a marker for leukocytes, suggesting that recruited leukocytes also express VEGF.

In vivo electroporation produces localized and sustained gene expression.

After optimization of plasmid concentrations and electrical parameters (data not shown), we wanted to examine the extent and duration of plasmid expression in electroporated thighs. First, we injected and electroporated pIRES-LacZ. Despite injecting in the medial portion of the thigh (**Fig. 3.2a**), we show high level of X-gal staining throughout the length of the muscle fiber. This expression turns on relatively early (day 7) and lasts for at least three weeks (**Fig. 3.2b,c**). In addition, plasmid electroporation results in tissue-restricted expression in that we see no evidence of LacZ expression in the gastrocnemius muscle (**Fig. 3.2d**).

A disadvantage of using LacZ as a reporter of plasmid expression, is that the extent of tissue transfection can only be assessed by sacrificing the animal. We therefore, constructed a plasmid containing a luciferase gene to allow non-invasive imaging of the same animal over an extended time-course, pLuc-IRES3-LacZ. Consistent with data from pIRES-LacZ, in vivo luciferase imaging of pLuc-IRES3-LacZ showed restricted expression in the thigh and long duration of expression, up to 21 days post-ligation (24 days post-electroporation) (**Fig. 3.5a-e**). Quantitation of luciferase expression is easily done and shows a high level of sustained expression (**Fig. 3.5e**). A disadvantage of luciferase imaging is that the resolution is not as precise as LacZ staining.

Cre-mediated VEGF deletion in the thigh results in impaired perfusion recovery.

We wanted to test the hypothesis that local VEGF expression is necessary for collateral growth. Three days after plasmid electroporation with pIRES-LacZ (control) or pCre-IRES-LacZ, $VEGF^{loxP/loxP}$ mice underwent unilateral femoral artery ligation. Consistent with this hypothesis, ischemic appearance score tended to be worse and limb use was more impaired in mice receiving Cre expressing plasmid (**Fig. 3.3a,b**). In addition, plantar perfusion recovery tended to be reduced in mice receiving Cre plasmid in the adductor (**Fig. 3.3c,e**). There was no difference in adductor perfusion (**Fig. 3.3d,f**) between the two groups.

The tendency for adductor perfusion to be *greater* in the group receiving Cre plasmid (**Fig. 3.3f**) was unexpected, and we were concerned that plasmid electroporation, alone, might be altering blood flow in the thigh. Analysis of the raw perfusion values revealed a "hyperemic" response in the control group that was resolved by 7 days post-ligation (10 days post-electroporation) (**Fig. 3.4a,b**). Interestingly, this response was attenuated in mice receiving Cre-plasmid (**Fig. 3.4b**), suggesting an involvement of VEGF. We then assessed whether this was an inflammatory mediated "hyperemic" response. We tested administration of ibuprofen (in drinking water) beginning 7 days prior to electroporation and throughout the experiment. Ibuprofen administration did not attenuate the local hyperemia (**Fig. 3.4c**).

Position of gene of interest in pIRES affects level of expression.

In a recent report it was shown that gene expression in pIRES was dependent on the position, multiple cloning site (MCS) -A or –B (upstream and downstream of IRES respectively), and the sequence of the IRES.[165] pIRES is an attenuated mutant designed to give equal expression at both MCS-A and MCS-B. The wild-type IRES gives approximately 100 fold greater expression of the gene inserted into MCS-B than when inserted into MCS-A. The relatively low expression of Cre-recombinase may explain our failure to reach significance in our experiments. We therefore redesigned our plasmids by replacing the attenuated IRES sequence with the wild-type IRES sequence to generate pIRES3. We then cloned our reporter gene in MCS-A and FltIgG into MCS-B to ensure a high level of expression.

Adductor expression of FltIgG, a VEGF sequestering molecule, worsens ischemic appearance and limb use and impairs adductor perfusion.

While local VEGF deletion defines a role for VEGF expression from resident cells (ie, collateral wall cells, tissue macrophages, skeletal muscle), it does not address the possibility that systemic VEGF sources (ie, from ischemic calf) can affect collateral growth. To address this possibility we electroporated a plasmid encoding FltIgG, a VEGF-Trap, into the adductor and analyzed recovery from hindlimb ischemia.

FltIgG is a chimeric molecule designed for increased stability and half-life.[164] It also contains residues that confer increased binding to extracellular

matrix.[166] It is not a true antagonist, in that it does not bind to the receptor. Instead, it binds VEGF with high affinity thus preventing its binding and activation of VEGF receptors, acting as a molecular VEGF sponge.

Expression of FltIgG in the adductor significantly increased the ischemic appearance scores and impaired limb use scores compared to LacZ control (**Fig. 3.6a,b**). Plantar perfusion was not different in the two groups consistent with the absence of VEGF inhibition in the circulation and in calf (**Fig. 3.6c**). Adductor perfusion was significantly attenuated in FltIgG animals consistent with a role for VEGF inhibition in collateral remodeling and perfusion.

Adductor FltIgG does not affect circulating white blood cell populations.

The distribution of white blood cell types was not different between FltIgG and LacZ control groups (**Fig 3.7**). This is consistent with the failure of FltIgG to escape from the adductor tissue and circulate systemically, inhibiting leukocytes mobilization. The white blood cell count revealed a distinct pattern during the course of ligation. Lymphocytes gradually decreased approximately 20% by day 7 of ligation (day 10 post electroporation) (**Fig. 3.7a**). Granulocytes showed an increase by day 7 of ligation, consistent with an acute inflammatory response (**Fig. 3.7b**). Monocytes did not change for the first 2 weeks, but increased at 21 days. This may suggest a transient inflammatory state.

DISCUSSION

Our findings reveal a positive role for paracrine VEGF signaling in arteriogenesis in the thigh. We have shown that deletion of VEGF and expression of FltIgG in the thigh impairs perfusion recovery in the plantar foot and adductor, respectively. In addition, we have successfully used in vivo electroporation to obtain long term, sustained, and tissue restricted expression of our gene of interest.

The role for VEGF in arteriogenesis is still controversial with studies showing both impairment and augmentation of collateral growth and tissue recovery with systemic delivery. [61-75, 117, 124-127] This conflict is largely due to the complex biology of VEGF and its pleiotropic actions on multiple cell types involved in arteriogenesis. The importance of endogenous VEGF has been demonstrated in studies blocking the actions of VEGF.[134-136] However, the caveats of systemic VEGF manipulation remain.

We have utilized in vivo electroporation as a tool to locally express inhibitors of VEGF only in the adductor, where collaterals are found. This technique permits VEGF to function in other tissues, such as in the calf to mediated angiogenesis and in the circulation to mediate vasodilation and leukocyte mobilization. For the first time, we have dissected a direct role for VEGF in arteriogenesis. In the thigh, the predominant cell type that is transfected is skeletal muscle.[167] Muscle is also potentially the largest source of secreted VEGF. Therefore, deletion of VEGF from this tissue was our first goal. Using conditional VEGF mice, $VEGF^{loxP/loxP}$,[164] and plasmid expressing Cre-

recombinase, we showed attenuation in plantar perfusion, which is consistent with impaired collateral remodeling. In a parallel experiment, we electroporated a plasmid carrying the FltIgG gene, a VEGF-trap molecule. We saw similar gross phenotypes, impaired limb use and increased ischemic appearance. We also observed attenuation in adductor perfusion, which is consistent with impaired collateral remodeling (See Chapter 2).[23]

Although we did not see significance in the plantar perfusion deficit in the Cre-recombinase treated animals, we have reason to believe that further optimization of the electroporation protocol will allow us to measure a significant change. These experiments were performed before the report demonstrating differences in gene expression in various IRES constructs was published. With the re-engineering of Cre into the high expressing plasmid pIRES3, repetition of the experiment will likely see positive results.

The addition of luciferase as a reporter gene allowed us to serially examine plasmid expression in the same mice over an extended time period. Future studies will allow the normalization of various parameters to luciferase expression, such that high and low expressers can be distinguished. While we were able to measure attenuation in adductor perfusion, we saw no difference in plantar recovery in mice receiving FltIgG or LacZ in the adductor. The mice used were C57BL/6 and it has been previously shown that this strain has a robust angiogenic response to femoral artery ligation.[21-23] It is possible that in the absence of FltIgG in the calf, that a robust angiogenic response occurred to

increase the Doppler perfusion signal. Histological studies will need to be performed on calf and adductor tissues to verify this possibility.

In conclusion, we have identified a positive role for paracrine VEGF in arteriogenesis. To understand the biology of collateral growth, it will be necessary to study the contributions of a host of endogenous molecules. With the increasing availability of conditional mouse strains and peptide/protein inhibitors coupled with in vivo electroporation, we can now temporally and spatially study collateral growth. Plasmid constructs used in this study use the CMV promoter to drive non-cell selective expression. Future studies are not restricted to non-selective expression. Cell-type specific promoters can be used to drive cell specific deletion or expression of molecules of choice. The field is no longer limited by mouse models that die embryonically or factors that exhibit multiple functions. We can now ask specific questions about a molecules direct role in arteriogenesis.

Table 3.1: List of primers used in this study

Primer name	Sequence	Features
LacZ F-1	CC**TCTAGA**GATCCACCATGGGGCC	XbaI
LacZ R-1	ATAAGAAT**GCGGCCGC**CCGGTTATTATT	NotI
Cre F-1	GAAT**GCTAGC**GTGGCCTCGAACACC	NheI
Cre R-1	TGCT**GAATTC**CTACTTGTCGTCATCATCC	EcoRI
IRES F-1	TAGGCGTGTACGGTGGGAGGTC	
IRES F-2	CTCTCCTCAAGCGTATTCAACAAG	
IRES F-3	GCCTTTTTACGGTTCCTG	
IRES R-1	GGTTACAAATAAAGCAATAGCATCAC	
IRES R-2	TTCCTTCACGACATTCAACAG	
mutIRES2-F	GAAAAACACGATGATAATATGGCCACA**TCTAGA**GTCGACCCG	XbaI
mutIRES2-R	CGGGTCGAC**TCTAGA**TGTGGCCATATTATCATCGTGTTTTTC	XbaI
mutIRES3-F1	CATGTGTTTAGTCGAGGTT**AAAAAA**CGTCTAGGCCCCCCGAACC	Single nucleotide deletion
mutIRES3-R1	GGTTCGGGGGGCCTAGACG**TTTTTT**AACCTCGACTAAACACATG	
mutIRES3-F2	CCGTCAGATCACTAG**AAGGTT**TATTGCGGTAGTTTA	Abolish HindIII site
mutIRES3-R2	TAAACTACCGCAATA**AACCTT**CTAGTGATCTGACGG	
Luc F-1	ATATCACG**TCTAGA**ATGGAAGACGCCAAAAACATAA	XbaI
Luc R-1	ATAAGAAT**GTCGAC**TTACAATTTGGACTTTCCGCCC	SalI
LacZ F-2	ATATCACG**TCTAGA**ATGGGGCCCAAGAAGAAACGCA	XbaI
LacZ R-2	ATAAGAAT**GTCGAC**TTATTATTATTTTTGACACC	SalI
FltIgG F-1	ATATCACG**TCTAGA**ATGGTCAGCTGCTGGGAC	XbaI
FltIgG R-1	ATAAGAAT**GTCGAC**TTATTTACCCGGAGACCT	SalI

Figure 3.1: Immunofluorescence of $VEGF^{hi/+}$ mice reveal increased β-galactosidase staining around remodeling collateral artery. β-galactosidase expression recapitulates VEGF expression.[145] Gracilis collateral at d7 post-ligation (**a-c**), and at d0 (**d-f**). Arteriole in adductor magnus muscle (**g-i**). Immunostaining for β-galactosidase (**a,d,g**), CD45 (**b,e,h**), and merged images (**c,f,i**).

β-gal
CD45
Merge
d7 collateral
d0 collateral
arteriole
a
b
c
d
e
f
g
h
i
50 µm

Figure 3.2: In vivo electroporation of plasmid encoding β-galactosidase yields long-term and restricted gene expression. (**a**) Diagram depicting the inner thigh (adductor). Site of hyaluronidase and plasmid injections are shown as asterisks. (**b-e**) Two separate mice injected and electroporated with pIRES-LacZ and stained with X-gal at d7 post-electroporation (**b**) and d21 post-electroporation (**c,d**). X-gal staining is excluded from the calf and is restricted to the thigh muscles (**d**).

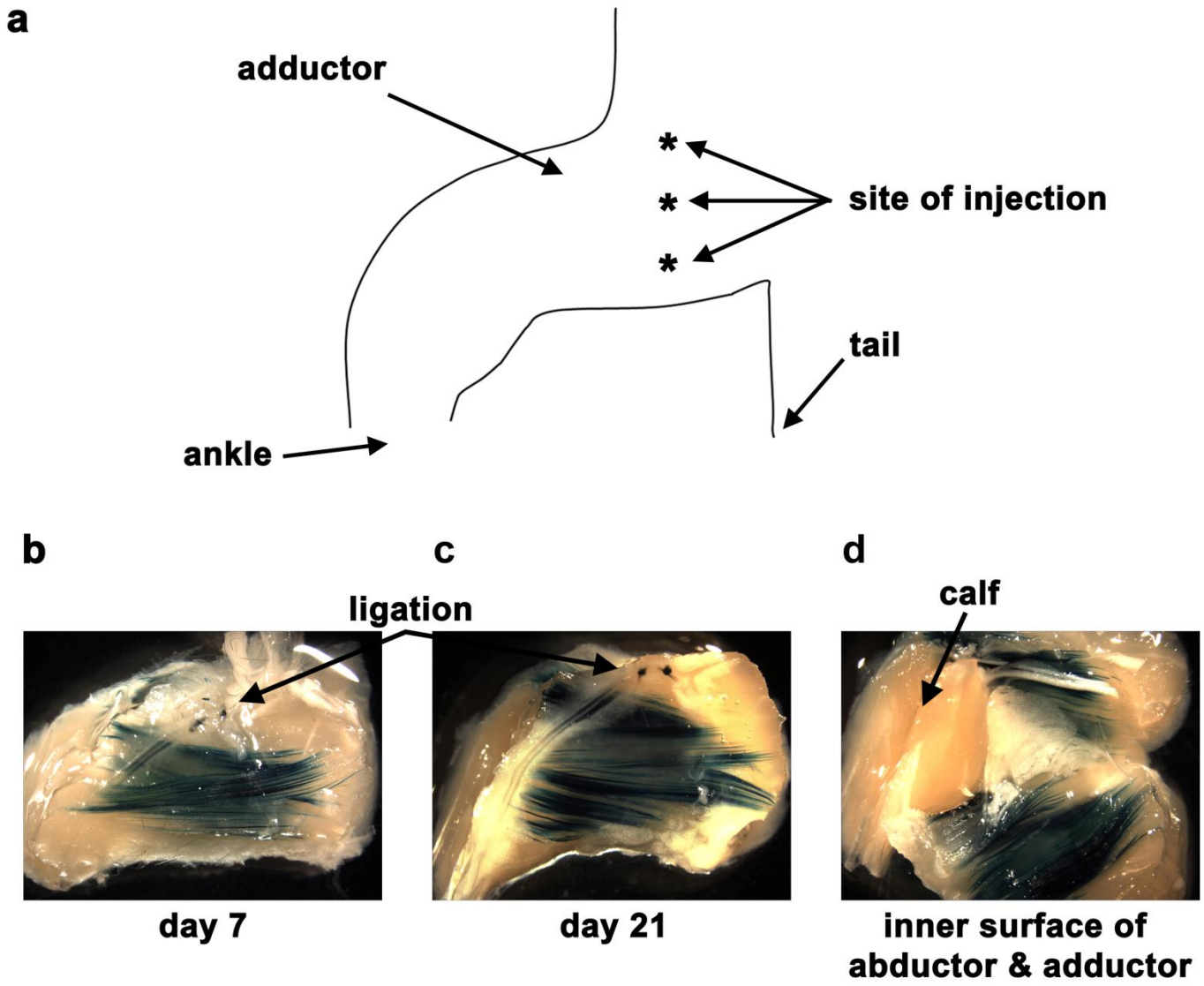

a
adductor
site of injection
tail
ankle
b
c
d
ligation
calf
day 7
day 21
inner surface of
abductor & adductor

Figure 3.3: $VEGF^{loxP/loxP}$ mice receiving electroporation of plasmid expressing Cre recombinase into the adductor show increased ischemic appearance, poor use, and impaired recovery of plantar perfusion. (**a**) Appearance score. Scale: 0=normal, 1=cyanosis or loss of nail(s), 2=partial or complete atrophy of digit(s), 3=partial atrophy of fore-foot; n=9 per data-point. (**b**) Use score. Scale: 0=normal, 1=no toe flexion/spreading, 2=no plantar flexion, 3=foot dragging; n=9 per data point. Laser Doppler perfusion images of plantar foot (**c**) and ventral adductor (**d**) with region of interest (ROI). Quantification of plantar perfusion (**e**) and adductor perfusion (**f**) measured over ROI. Data are mean±SEM for this and other figures. Two-way ANOVA followed by Dunn-Bonferonni corrected *t*-Test; n=9 mice per data-point.

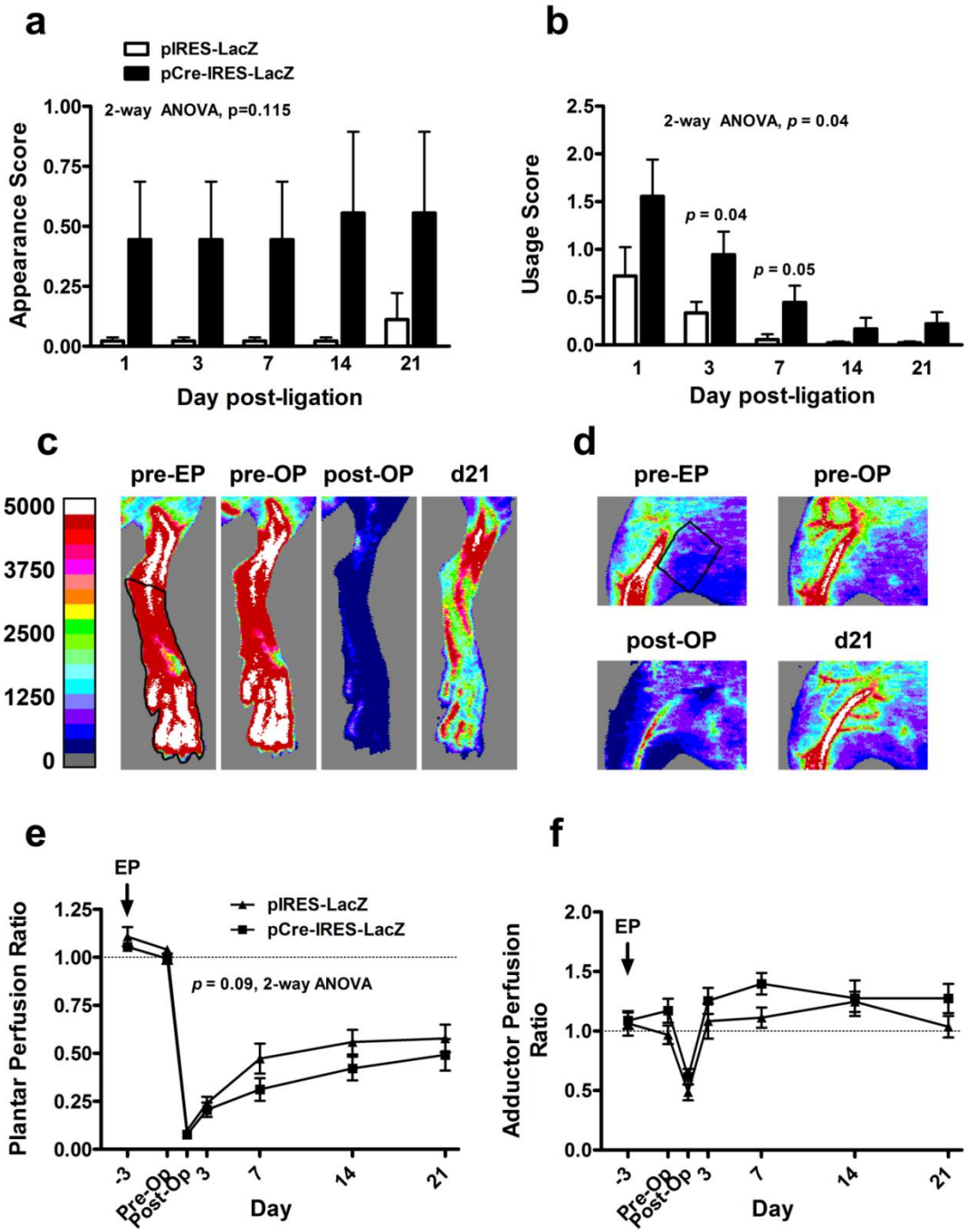

a
pIRES-LacZ
pCre-IRES-LacZ
2-way ANOVA, p=0.115
Appearance Score
1.00
0.75
0.50
0.25
0.00
1
3
7
14
21
Day post-ligation
b
2-way ANOVA, p = 0.04
p = 0.04
p = 0.05
Usage Score
2.5
2.0
1.5
1.0
0.5
0.0
1
3
7
14
21
Day post-ligation
c
pre-EP
pre-OP
post-OP
d21
5000
3750
2500
1250
0
d
pre-EP
pre-OP
post-OP
d21
e
EP
pIRES-LacZ
pCre-IRES-LacZ
p = 0.09, 2-way ANOVA
Plantar Perfusion Ratio
1.25
1.00
0.75
0.50
0.25
0.00
-3
Pre-Op
Post-Op
3
7
14
21
Day
f
EP
Adductor Perfusion Ratio
2.0
1.5
1.0
0.5
0.0
-3
Pre-Op
Post-Op
3
7
14
21
Day

Figure 3.4: Electroporation induces an “inflammatory” hyperemic response in the adductor that is not ameliorated by anti-inflammatory drugs. Raw perfusion values in the ligated adductor (**a**) and non-ligated adductor (**b**). Time of electroporation is depicted by arrow and EP. (**c**) Raw perfusion values following administration of 0.2 mg/ml Ibuprofen (IBU) in drinking water 7 days before electroporation and throughout the time course. Two-way ANOVA followed by Dunn-Bonferonni corrected *t*-Test, #$p<0.05$ pCre-IRES-LacZ vs. pIRES-LacZ; *$p<0.05$ pCre-IRES-LacZ vs. pIRES-LacZ + IBU; n=9 per data point.

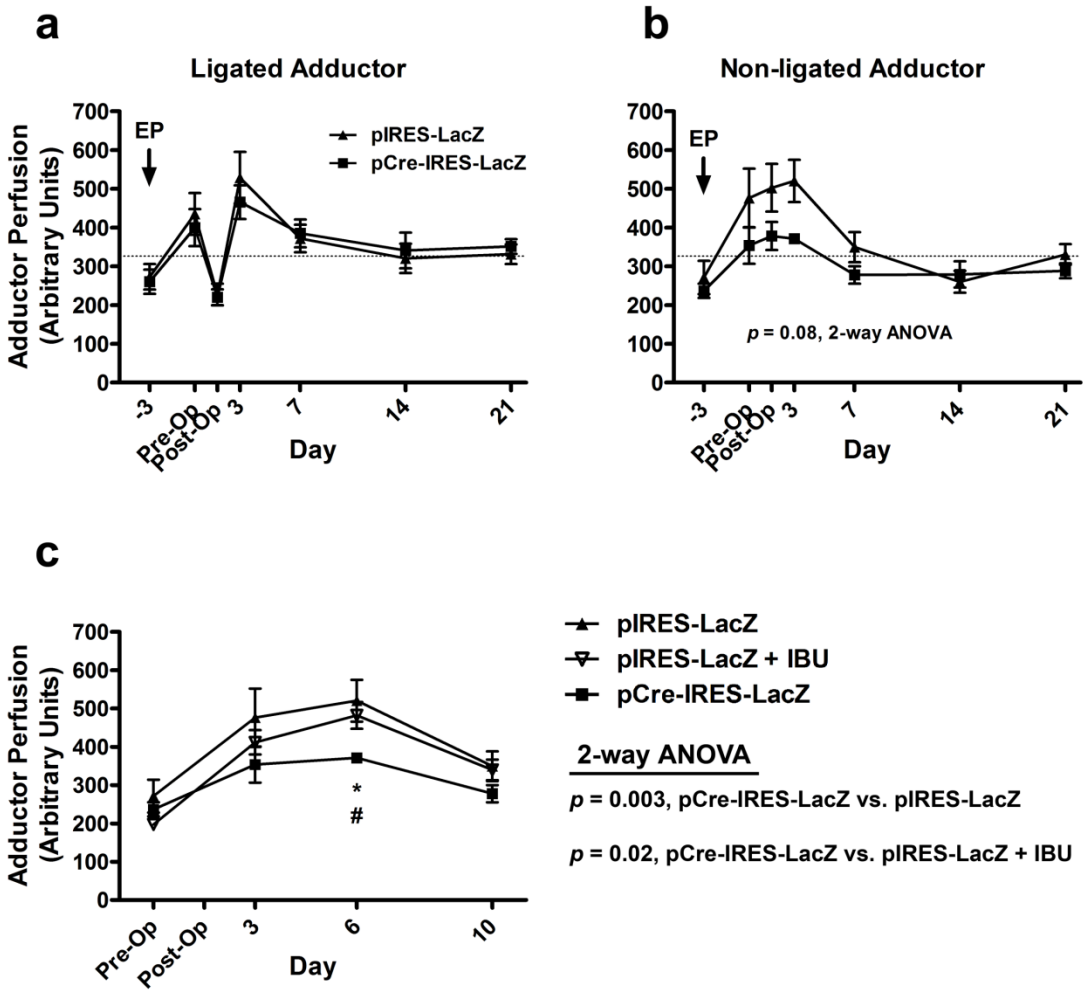

a
Ligated Adductor
Adductor Perfusion (Arbitrary Units)
EP
pIRES-LacZ
pCre-IRES-LacZ
Day
b
Non-ligated Adductor
p = 0.08, 2-way ANOVA
c
pIRES-LacZ
pIRES-LacZ + IBU
pCre-IRES-LacZ
2-way ANOVA
p = 0.003, pCre-IRES-LacZ vs. pIRES-LacZ
p = 0.02, pCre-IRES-LacZ vs. pIRES-LacZ + IBU

Figure 3.5: In vivo electroporation of a plasmid expressing luciferase allows for non-invasive imaging of plasmid expression over 21 days. (**a-d**) Pseudo-colored images of integrated photons at specified time points in the same mouse. (**e,f**) Quantitation of radiance, photons/s/cm^2/sr.

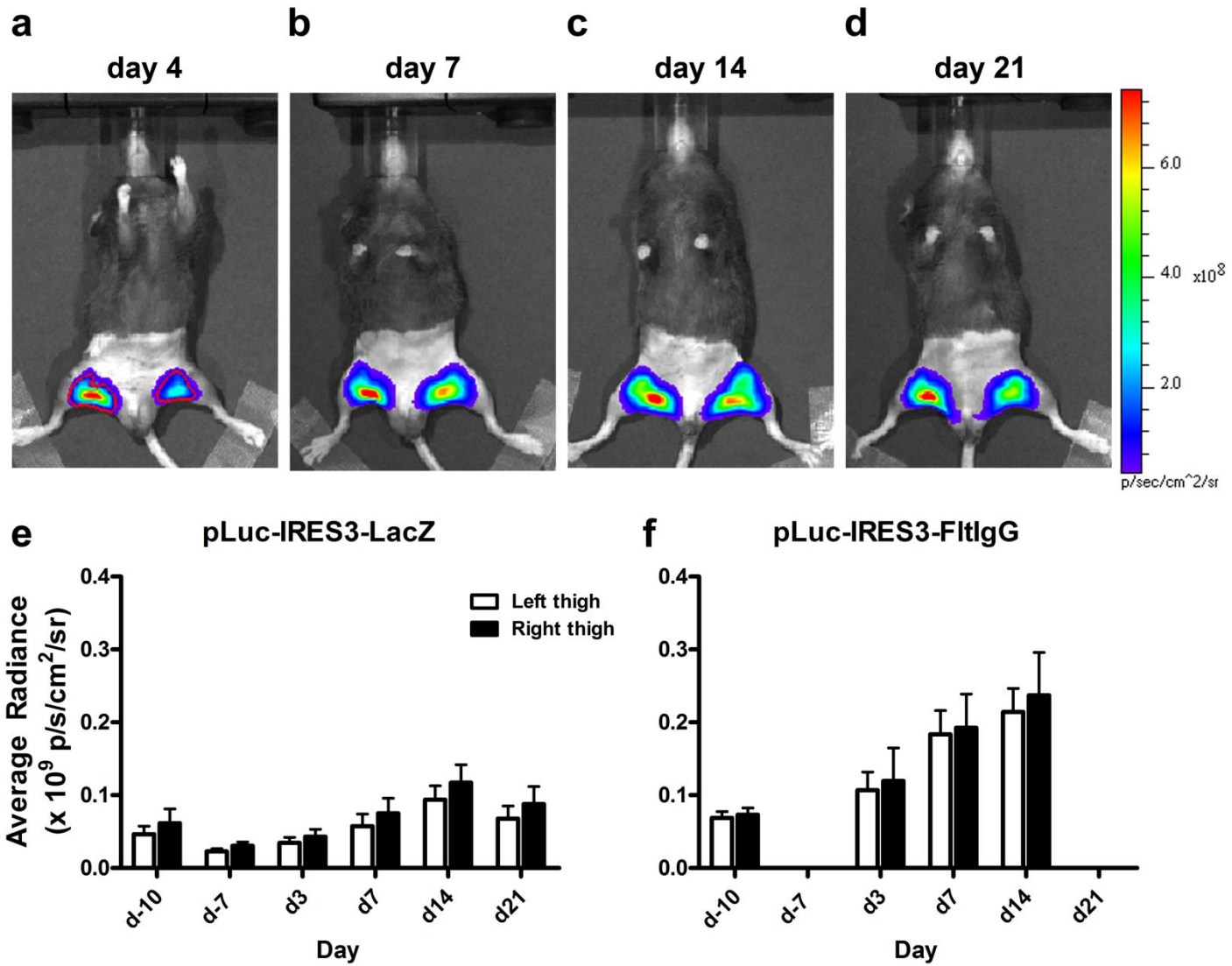

a
b
c
d
day 4
day 7
day 14
day 21
6.0
4.0
2.0
x10^8
p/sec/cm^2/sr
e
pLuc-IRES3-LacZ
Average Radiance (x 10^9 p/s/cm^2/sr)
Left thigh
Right thigh
0.4
0.3
0.2
0.1
0.0
d-10
d-7
d3
d7
d14
d21
Day
f
pLuc-IRES3-FltIgG
0.4
0.3
0.2
0.1
0.0
d-10
d-7
d3
d7
d14
d21
Day

Figure 3.6: Adductor expression of FltIgG leads to impaired limb use, increased ischemic appearance, attenuated adductor perfusion, but no changes in plantar perfusion. Appearance (**a**) and Use (**b**) scores of mice receiving pLuc-IRES3-LacZ (control) or pLuc-IRES3-FltIgG. See Fig. 3.6 for scales. Plantar perfusion ratio (**c**) and adductor perfusion ratio (**d**) with times of electroporation indicated by arrows. EP=electroporation. Two-way ANOVA followed by Dunn-Bonferonni corrected *t*-Test, **p*<0.05, ***p*<0.01; pLuc-IRES3-LacZ vs. pLuc-IRES3-FltIgG; n=9 per data point.

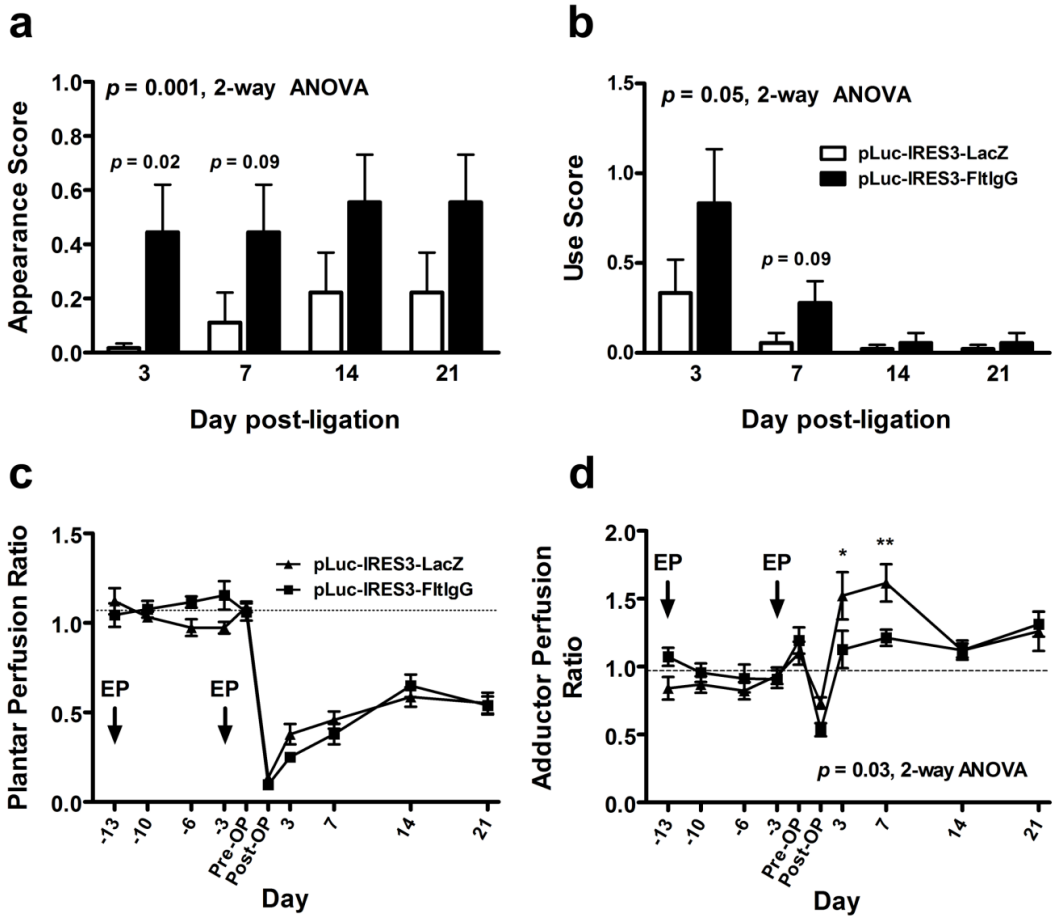
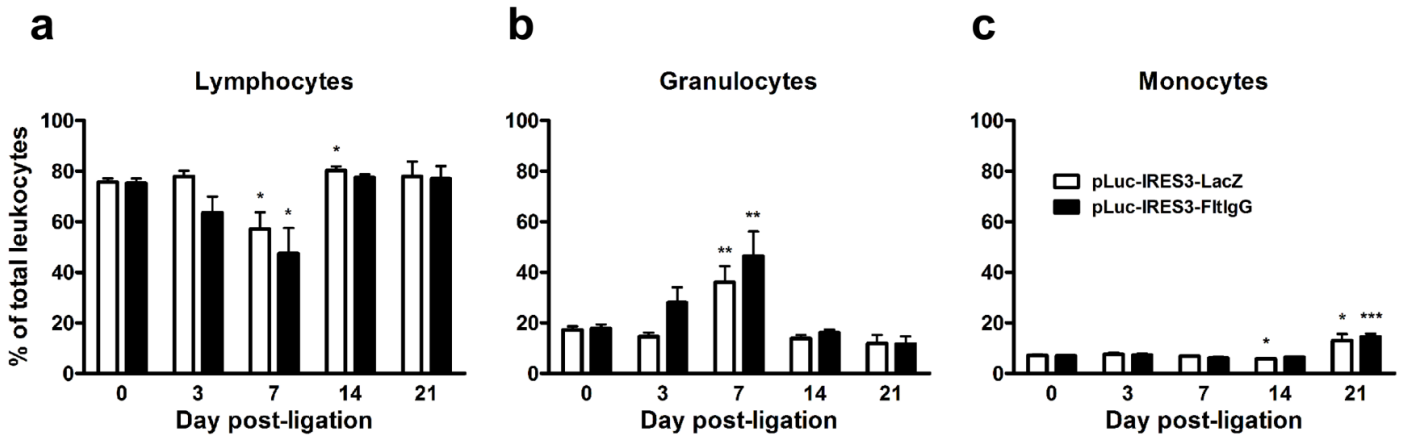

a
p = 0.001, 2-way ANOVA
p = 0.02
p = 0.09
Appearance Score
1.0
0.8
0.6
0.4
0.2
0.0
3
7
14
21
Day post-ligation
b
p = 0.05, 2-way ANOVA
pLuc-IRES3-LacZ
pLuc-IRES3-FltIgG
p = 0.09
Use Score
1.5
1.0
0.5
0.0
3
7
14
21
Day post-ligation
c
pLuc-IRES3-LacZ
pLuc-IRES3-FltIgG
EP
EP
Plantar Perfusion Ratio
1.5
1.0
0.5
0.0
-13
-10
-6
-3
Pre-OP
Post-OP
3
7
14
21
Day
d
EP
EP
*
**
Adductor Perfusion Ratio
2.0
1.5
1.0
0.5
0.0
p = 0.03, 2-way ANOVA
-13
-10
-6
-3
Pre-OP
Post-OP
3
7
14
21
Day

Figure 3.7: Whole blood leukocyte counts following electroporation and femoral artery ligation. Data are expressed as percentage of total white blood cell counts for lymphocytes (**a**), granulocytes (**b**), and monocytes (**c**).


a
Lymphocytes
% of total leukocytes
100
80
60
40
20
0
0
3
7
14
21
Day post-ligation
b
Granulocytes
Day post-ligation
c
Monocytes
pLuc-IRES3-LacZ
pLuc-IRES3-FltIgG
Day post-ligation

CHAPTER 4

SUMMARY AND FUTURE DIRECTIONS

VEGF EXPRESSION AND ARTERIOGENESIS

Early studies by Couffinhal and colleagues have shown that VEGF is upregulated in ischemic calf muscles in a hindlimb ischemia model.[116] In addition to skeletal muscle, VEGF expression was also seen in lymphocytes, monocytes and endothelial cells in the ischemic calf. Whether this expression pattern was recapitulated in the adductor (ie, surrounding growing collaterals) was not known. I have shown that VEGF is expressed selectively in skeletal myocytes surrounding a remodeling collateral artery. This expression was exclusive to remodeling collaterals in that there was little or no expression in non-remodeling collaterals and other arterioles. There was also evidence of peri-collateral $CD45^+$ leukocytes expressing VEGF. It was difficult to ascertain VEGF expression in other cell types. High resolution confocal microscopy will be necessary to determine what other cell types express VEGF in the context of a remodeling collateral.

Additionally, I have shown a unique expression pattern of VEGF isoforms in the adductor compared to the ischemic calf. There was selective upregulation of the soluble VEGF isoform, VEGF120, in the ischemic calf. This is consistent with properties of a soluble isoform in mediating extensive angiogenesis in the

calf and for mobilizing leukocytes in the bone marrow. In the adductor, the opposite pattern was observed. The high molecular isoforms, VEGF164 and VEGF188, were selectively upregulated in the adductor. The physical properties of these heparin binding isoforms allow for restricted and localized expression to allow for tighter control of VEGF signaling. The resolution of our qRT-PCR experiments does not allow us to define precise cellular localization, however it does demonstrate that different physiological processes are eliciting different VEGF expression patterns. In situ hybridization is a technique that can identify the cell types that express VEGF mRNA. The use of locked nucleic acid (LNA) probes may allow one to discriminate the specific isoforms that are expressed.

One intriguing question that remains to be answered is how different physiological processes affect mRNA splicing. The molecular mechanism of alternative splicing has been well studied, however mechanisms describing tissue specific splicing patterns or splicing patterns altered by different physiological processes is less well understood. A recent study has described hypoxia as a regulator of mRNA splicing by altering the expression of components of the splicing machinery.[168] It is likely that hypoxia in the ischemic calf muscle is driving an alternative splicing pattern of VEGF. MicroRNA's have recently been identified as modifiers of gene expression. Indeed, microRNA's have shown to promote tissue specific pre-mRNA splicing.[169] In vitro experiments with cells that express VEGF may be more amenable to study the mechanical and metabolic factors that regulate VEGF mRNA splicing.

GENETIC CONTRIBUTION TO ARTERIOGENESIS

Analysis of arteriogenesis in genetic mouse models requires the maturity of mice into adults. Unfortunately, many mutant mouse models of genes that regulate angiogenesis are embryonic lethal and thus cannot be studied for their role in arteriogenesis. However, the increasing incidence of conditionally targeted mutants together with tissue specific Cre lines, now allows one to study molecules in a tissue specific manner for their effects on collateral growth.

In a recent study, our laboratory has reported strain specific differences in arteriogenesis. C57BL/6 mice had increased collateral remodeling and perfusion recovery than BALB/c mice.[23] In addition, C57BL/6 mice had numerous collaterals in skeletal muscle, intestine, and pia while BALB/c mice had fewer.[23] Genetic dissection of factors that regulate arteriogenesis and collateral development can be facilitated by analyzing various crosses of these mice. Indeed, mRNA expression quantitative trait loci (eQTL) analysis of VEGF has shown that C57BL/6 has polymorphism on or near the *Vegfa* locus that is associated with increased VEGF expression, compared to BALB/c. Given this association of VEGF expression with arteriogenesis and collateral density, I wanted to test the role of genetic perturbations of VEGF and its receptors in collateral artery growth in ischemic disease and collateral artery development in normal tissue.

Homozygous deletion of VEGFR-1 and VEGFR-2 is embryonic lethal so it is difficult to study their roles in arteriogenesis. In Chapter 2, I showed that heterozygosity of VEGFR-1 impaired arteriogenesis while VEGFR-2

heterozygosity had no effect on recovery. Due to the high kinase activity of VEGFR-2, my studies do not rule out a positive role for VEGFR-2. It is possible that sufficient receptor density exists to allow proper signaling through VEGFR-2 and thus demonstrate no adverse effects of femoral artery ligation. Conditional mice have now been generated for both receptors.[139, 170] Breeding these mice to tissue specific Cre-recombinase driver strains will be important to determine the cells and the receptors that are required to mediate arteriogenesis.

VEGF heterozygosity leads to early embryonic lethality,[132, 133] thus the study of the genetic contribution of this gene is impossible in adults. However, mutant mouse strains have been created that confer hypermorphic or hypomorphic VEGF phenotypes.[130, 131, 145] Using these mice, I was able to show that VEGF hypomorphs, $VEGF^{lo/+}$, have impaired arteriogenesis and perfusion recovery. No increase in arteriogenesis was observed in VEGF hypermorphs, $VEGF^{hi/+}$. However, it is possible that a ceiling effect was reached, such that greater increases in VEGF could not further augment arteriogenesis as that seen in wild-type mice. In addition, we observed a VEGF genotype – collateral number relation, such that $VEGF^{hi/+}$ mice had more collaterals and $VEGF^{lo/+}$ mice had fewer. Together these observations support a genetic role of VEGF in mediating arteriogenesis and collateral development.

PARACRINE VEGF IS REQUIRED FOR ARTERIOGENESIS

Systemic VEGF administration or inhibition has provided evidence for a positive role in arteriogenesis. However, VEGF's actions systemically can

confound any direct action on collaterals. I thus designed experiments to locally restrict VEGF inhibition only to the thigh where arteriogenesis takes place. Using conditional VEGF mice[164], I electroporated a plasmid expressing Cre-recombinase to the mouse thigh. Thigh selective VEGF deletion resulted in impaired limb use and increased ischemic appearance over LacZ control electroporated mice. Adductor VEGF deletion also impaired plantar perfusion recovery. This data suggests that resident cells in the thigh, including skeletal muscle, collateral vessel wall cells, and resident leukocytes are important contributors of VEGF and mediate arteriogenesis and perfusion recovery.

In a parallel experiment, I electroporated a plasmid expressing the VEGF-trap molecule, FltIgG into the thigh of C57BL/6. This mouse strain was chosen for its characterization as a robust model of arteriogenesis.[21-23] Inhibition of arteriogenesis was most likely to be detected in this strain. Indeed, mice receiving FltIgG in the thigh experienced greater ischemic appearance and impaired limb use. Additionally, attenuated adductor perfusion was observed while plantar perfusion recovery was unaffected. A benefit of FltIgG expression in the adductor, is that it will bind to all VEGF produced in the thigh as well as VEGF that is delivered to the thigh from the circulation. It is possible that increased angiogenesis in the calf compensated for reduced collateral growth in the thigh to give no change in plantar perfusion measurements. Histological measurements are necessary to distinguish between reduced collateral growth and increased angiogenesis.

The technique of in vivo electroporation has allowed targeted inhibition, both spatially and temporally, of VEGF in order to examine its role in arteriogenesis. This technology does not need to be restricted to VEGF. In vivo electroporation opens up new possibilities to study the paracrine role of a host of growth factors, cytokines, and matrix proteins. Plasmid electroporation can be combined with conditional mouse mutants, tissue specific promoters and performed at various time points during collateral remodeling to more precisely pin-point the responsible molecules and the time at which they act. By understanding the precise biological mechanisms of arteriogenesis including cell types, molecules, and temporal progression, researchers can rationally design improved therapies to treat patients with ischemic vascular disease.

FUTURE DIRECTIONS

The elucidation of genetic factors regulating collateral growth in ischemia and collateral development in the embryo or adult is the next frontier in arteriogenesis research. The model system of the BALB/c and C57BL/6 provides strong tools to dissect these factors. eQTL analysis has the potential to identify novel pathways that determine collateral development. The current eQTL database for BALB/c and C57BL/6 crosses are limited. We have the ability to expand this database and probe additional factors with ischemic and non-ischemic calf muscle and ligated and non-ligated adductor muscles. In addition, genetic mapping experiments are underway to identify loci linked to collateral density and growth.

The addition of a stroke model of middle cerebral artery occlusion has enabled us to visualize collateral growth and hemodynamics that was not possible with the femoral artery ligation model. The stroke model is amenable to manipulation of molecular pathways identified in eQTL and QTL analysis of BALB/c and C57BL/6 crosses.

The identification of VEGF as the first molecule implicated in establishing collateral development is the first step in understanding the complex process of collateral growth and development. Undoubtedly, elucidation of novel pathways utilizing genetic crosses will further our knowledge and potentially enable development of new treatments of vascular occlusive disease in humans.

REFERENCES

1. Schaper W, Scholz D. Factors regulating arteriogenesis. *Arterioscler Thromb Vasc Biol*. 2003;23:1143-51.

2. Heil M, Schaper W. Arteriogenic growth factors, chemokines and proteases as a prerequisite for arteriogenesis. *Drug News Perspect*. 2005;18:317-22.

3. Luttun A, Carmeliet P. De novo vasculogenesis in the heart. *Cardiovasc Res*. 2003;58:378-89.

4. Carmeliet P. Angiogenesis in health and disease. *Nat Med*. 2003;9:653-60.

5. Risau W. Mechanisms of angiogenesis. *Nature*. 1997;386:671-4.

6. Burri PH, Tarek MR. A novel mechanism of capillary growth in the rat pulmonary microcirculation. *Anat Rec*. 1990;228:35-45.

7. Heil M, Schaper W. Insights into pathways of arteriogenesis. *Curr Pharm Biotechnol*. 2007;8:35-42.

8. Heil M, Eitenmuller I, Schmitz-Rixen T, Schaper W. Arteriogenesis versus angiogenesis: similarities and differences. *J Cell Mol Med*. 2006;10:45-55.

9. Deindl E, Schaper W. The art of arteriogenesis. *Cell Biochem Biophys*. 2005;43:1-15.

10. Helisch A, Schaper W. Arteriogenesis: the development and growth of collateral arteries. *Microcirculation*. 2003;10:83-97.

11. Scholz D, Cai WJ, Schaper W. Arteriogenesis, a new concept of vascular adaptation in occlusive disease. *Angiogenesis*. 2001;4:247-57.

12. Van Royen N, Piek JJ, Schaper W, Bode C, Buschmann I. Arteriogenesis: mechanisms and modulation of collateral artery development. *J Nucl Cardiol*. 2001;8:687-93.

13. Scholz D, Ito W, Fleming I, Deindl E, Sauer A, Wiesnet M, Busse R, Schaper J, Schaper W. Ultrastructure and molecular histology of rabbit hind-limb collateral artery growth (arteriogenesis). *Virchows Arch*. 2000;436:257-70.

14. Herzog S, Sager H, Khmelevski E, Deylig A, Ito WD. Collateral arteries grow from preexisting anastomoses in the rat hindlimb. *Am J Physiol Heart Circ Physiol*. 2002;283:H2012-20.

15. Baroldi G, Mantero O, Scomazzoni G. The collaterals of the coronary arteries in normal and pathologic hearts. *Circ Res*. 1956;4:223-9.

16. Kornowski R. Collateral formation and clinical variables in obstructive coronary artery disease: the influence of hypercholesterolemia and diabetes mellitus. *Coron Artery Dis*. 2003;14:61-4.

17. Tepper OM, Galiano RD, Capla JM, Kalka C, Gagne PJ, Jacobowitz GR, Levine JP, Gurtner GC. Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation*. 2002;106:2781-6.

18. Werner GS, Richartz BM, Heinke S, Ferrari M, Figulla HR. Impaired acute collateral recruitment as a possible mechanism for increased cardiac adverse events in patients with diabetes mellitus. *Eur Heart J*. 2003;24:1134-42.

19. Weihrauch D, Lohr NL, Mraovic B, Ludwig LM, Chilian WM, Pagel PS, Warltier DC, Kersten JR. Chronic hyperglycemia attenuates coronary collateral development and impairs proliferative properties of myocardial interstitial fluid by production of angiostatin. *Circulation*. 2004;109:2343-8.

20. Couffinhal T, Silver M, Kearney M, Sullivan A, Witzenbichler B, Magner M, Annex B, Peters K, Isner JM. Impaired collateral vessel development associated with reduced expression of vascular endothelial growth factor in ApoE-/- mice. *Circulation*. 1999;99:3188-98.

21. Scholz D, Ziegelhoeffer T, Helisch A, Wagner S, Friedrich C, Podzuweit T, Schaper W. Contribution of arteriogenesis and angiogenesis to postocclusive hindlimb perfusion in mice. *J Mol Cell Cardiol*. 2002;34:775-87.

22. Helisch A, Wagner S, Khan N, Drinane M, Wolfram S, Heil M, Ziegelhoeffer T, Brandt U, Pearlman JD, Swartz HM, Schaper W. Impact of mouse strain differences in innate hindlimb collateral vasculature. *Arterioscler Thromb Vasc Biol*. 2006;26:520-6.

23. Chalothorn D, Clayton JA, Zhang H, Pomp D, Faber JE. Collateral density, remodeling, and VEGF-A expression differ widely between mouse strains. *Physiol Genomics*. 2007;30:179-91.

24. Carmeliet P, Conway EM. Growing better blood vessels. *Nat Biotechnol*. 2001;19:1019-20.

25. Grundmann S, Piek JJ, Pasterkamp G, Hoefer IE. Arteriogenesis: basic mechanisms and therapeutic stimulation. *Eur J Clin Invest*. 2007;37:755-66.

26. Milkiewicz M, Brown MD, Egginton S, Hudlicka O. Association between shear stress, angiogenesis, and VEGF in skeletal muscles in vivo. *Microcirculation*. 2001;8:229-41.

27. Pipp F, Boehm S, Cai WJ, Adili F, Ziegler B, Karanovic G, Ritter R, Balzer J, Scheler C, Schaper W, Schmitz-Rixen T. Elevated fluid shear stress enhances postocclusive collateral artery growth and gene expression in the pig hind limb. *Arterioscler Thromb Vasc Biol*. 2004;24:1664-8.

28. Eitenmuller I, Volger O, Kluge A, Troidl K, Barancik M, Cai WJ, Heil M, Pipp F, Fischer S, Horrevoets AJ, Schmitz-Rixen T, Schaper W. The range of adaptation by collateral vessels after femoral artery occlusion. *Circ Res*. 2006;99:656-62.

29. Sherman JA, Hall A, Malenka DJ, De Muinck ED, Simons M. Humoral and cellular factors responsible for coronary collateral formation. *Am J Cardiol*. 2006;98:1194-7.

30. Heil M, Schaper W. Influence of mechanical, cellular, and molecular factors on collateral artery growth (arteriogenesis). *Circ Res*. 2004;95:449-58.

31. Kinnaird T, Stabile E, Burnett MS, Lee CW, Barr S, Fuchs S, Epstein SE. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circ Res*. 2004;94:678-85.

32. Scholz D, Schaper W. Enhanced arteriogenesis in mice overexpressing erythropoietin. *Cell Tissue Res*. 2006;324:395-401.

33. Vogel J, Kiessling I, Heinicke K, Stallmach T, Ossent P, Vogel O, Aulmann M, Frietsch T, Schmid-Schonbein H, Kuschinsky W, Gassmann M. Transgenic mice overexpressing erythropoietin adapt to excessive erythrocytosis by regulating blood viscosity. *Blood*. 2003;102:2278-84.

34. Chalothorn D, Zhang H, Clayton JA, Thomas SA, Faber JE. Catecholamines augment collateral vessel growth and angiogenesis in hindlimb ischemia. *Am J Physiol Heart Circ Physiol*. 2005;289:H947-59.

35. Schaper W. Tangential wall stress as a molding force in the development of collateral vessels in the canine heart. *Experientia*. 1967;23:595-6.

36. Schaper J, Konig R, Franz D, Schaper W. The endothelial surface of growing coronary collateral arteries. Intimal margination and diapedesis of monocytes. A combined SEM and TEM study. *Virchows Arch A Pathol Anat Histol*. 1976;370:193-205.

37. Hoefer IE, Grundmann S, van Royen N, Voskuil M, Schirmer SH, Ulusans S, Bode C, Buschmann IR, Piek JJ. Leukocyte subpopulations and arteriogenesis: specific role of monocytes, lymphocytes and granulocytes. *Atherosclerosis*. 2005;181:285-93.

38. Heil M, Ziegelhoeffer T, Pipp F, Kostin S, Martin S, Clauss M, Schaper W. Blood monocyte concentration is critical for enhancement of collateral artery growth. *Am J Physiol Heart Circ Physiol*. 2002;283:H2411-9.

39. Bergmann CE, Hoefer IE, Meder B, Roth H, van Royen N, Breit SM, Jost MM, Aharinejad S, Hartmann S, Buschmann IR. Arteriogenesis depends

on circulating monocytes and macrophage accumulation and is severely depressed in op/op mice. *J Leukoc Biol*. 2006;80:59-65.

40. Felix R, Cecchini MG, Hofstetter W, Elford PR, Stutzer A, Fleisch H. Impairment of macrophage colony-stimulating factor production and lack of resident bone marrow macrophages in the osteopetrotic op/op mouse. *J Bone Miner Res*. 1990;5:781-9.

41. Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H, Sudo T, Shultz LD. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature*. 1990;345:442-4.

42. Wiktor-Jedrzejczak W, Bartocci A, Ferrante AW, Jr., Ahmed-Ansari A, Sell KW, Pollard JW, Stanley ER. Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. *Proc Natl Acad Sci U S A*. 1990;87:4828-32.

43. Stabile E, Burnett MS, Watkins C, Kinnaird T, Bachis A, la Sala A, Miller JM, Shou M, Epstein SE, Fuchs S. Impaired arteriogenic response to acute hindlimb ischemia in CD4-knockout mice. *Circulation*. 2003;108:205-10.

44. Stabile E, Kinnaird T, la Sala A, Hanson SK, Watkins C, Campia U, Shou M, Zbinden S, Fuchs S, Kornfeld H, Epstein SE, Burnett MS. CD8+ T lymphocytes regulate the arteriogenic response to ischemia by infiltrating the site of collateral vessel development and recruiting CD4+ mononuclear cells through the expression of interleukin-16. *Circulation*. 2006;113:118-24.

45. Tzima E, Irani-Tehrani M, Kiosses WB, Dejana E, Schultz DA, Engelhardt B, Cao G, DeLisser H, Schwartz MA. A mechanosensory complex that mediates the endothelial cell response to fluid shear stress. *Nature*. 2005;437:426-31.

46. Barakat AI. A model for shear stress-induced deformation of a flow sensor on the surface of vascular endothelial cells. *J Theor Biol*. 2001;210:221-36.

47. Cai WJ, Koltai S, Kocsis E, Scholz D, Kostin S, Luo X, Schaper W, Schaper J. Remodeling of the adventitia during coronary arteriogenesis. *Am J Physiol Heart Circ Physiol*. 2003;284:H31-40.

48. Khmelewski E, Becker A, Meinertz T, Ito WD. Tissue resident cells play a dominant role in arteriogenesis and concomitant macrophage accumulation. *Circ Res*. 2004;95:E56-64.

49. Ito WD, Arras M, Winkler B, Scholz D, Schaper J, Schaper W. Monocyte chemotactic protein-1 increases collateral and peripheral conductance after femoral artery occlusion. *Circ Res*. 1997;80:829-37.

50. Schirmer SH, Buschmann IR, Jost MM, Hoefer IE, Grundmann S, Andert JP, Ulusans S, Bode C, Piek JJ, van Royen N. Differential effects of MCP-1 and leptin on collateral flow and arteriogenesis. *Cardiovasc Res*. 2004;64:356-64.

51. Voskuil M, van Royen N, Hoefer IE, Seidler R, Guth BD, Bode C, Schaper W, Piek JJ, Buschmann IR. Modulation of collateral artery growth in a porcine hindlimb ligation model using MCP-1. *Am J Physiol Heart Circ Physiol*. 2003;284:H1422-8.

52. Heil M, Ziegelhoeffer T, Wagner S, Fernandez B, Helisch A, Martin S, Tribulova S, Kuziel WA, Bachmann G, Schaper W. Collateral artery growth (arteriogenesis) after experimental arterial occlusion is impaired in mice lacking CC-chemokine receptor-2. *Circ Res*. 2004;94:671-7.

53. Voskuil M, Hoefer IE, van Royen N, Hua J, de Graaf S, Bode C, Buschmann IR, Piek JJ. Abnormal monocyte recruitment and collateral artery formation in monocyte chemoattractant protein-1 deficient mice. *Vasc Med*. 2004;9:287-92.

54. Hong KH, Ryu J, Han KH. Monocyte chemoattractant protein-1-induced angiogenesis is mediated by vascular endothelial growth factor-A. *Blood*. 2005;105:1405-7.

55. Parenti A, Bellik L, Brogelli L, Filippi S, Ledda F. Endogenous VEGF-A is responsible for mitogenic effects of MCP-1 on vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol*. 2004;286:H1978-84.

56. Hoefer IE, van Royen N, Rectenwald JE, Bray EJ, Abouhamze Z, Moldawer LL, Voskuil M, Piek JJ, Buschmann IR, Ozaki CK. Direct evidence for tumor necrosis factor-alpha signaling in arteriogenesis. *Circulation*. 2002;105:1639-41.

57. Hollestelle SC, De Vries MR, Van Keulen JK, Schoneveld AH, Vink A, Strijder CF, Van Middelaar BJ, Pasterkamp G, Quax PH, De Kleijn DP. Toll-like receptor 4 is involved in outward arterial remodeling. *Circulation*. 2004;109:393-8.

58. van Royen N, Voskuil M, Hoefer I, Jost M, de Graaf S, Hedwig F, Andert JP, Wormhoudt TA, Hua J, Hartmann S, Bode C, Buschmann I, Schaper W, van der Neut R, Piek JJ, Pals ST. CD44 regulates arteriogenesis in mice and is differentially expressed in patients with poor and good collateralization. *Circulation*. 2004;109:1647-52.

59. Hoefer IE, van Royen N, Rectenwald JE, Deindl E, Hua J, Jost M, Grundmann S, Voskuil M, Ozaki CK, Piek JJ, Buschmann IR. Arteriogenesis proceeds via ICAM-1/Mac-1- mediated mechanisms. *Circ Res*. 2004;94:1179-85.

60. Carr AN, Davis MG, Eby-Wilkens E, Howard BW, Towne BA, Dufresne TE, Peters KG. Tyrosine phosphatase inhibition augments collateral blood flow in a rat model of peripheral vascular disease. *Am J Physiol Heart Circ Physiol*. 2004;287:H268-76.

61. Banai S, Jaklitsch MT, Shou M, Lazarous DF, Scheinowitz M, Biro S, Epstein SE, Unger EF. Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs. *Circulation*. 1994;89:2183-9.

62. Takeshita S, Zheng LP, Brogi E, Kearney M, Pu LQ, Bunting S, Ferrara N, Symes JF, Isner JM. Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. *J Clin Invest*. 1994;93:662-70.

63. Takeshita S, Pu LQ, Stein LA, Sniderman AD, Bunting S, Ferrara N, Isner JM, Symes JF. Intramuscular administration of vascular endothelial growth factor induces dose-dependent collateral artery augmentation in a rabbit model of chronic limb ischemia. *Circulation*. 1994;90:II228-34.

64. Lopez JJ, Laham RJ, Stamler A, Pearlman JD, Bunting S, Kaplan A, Carrozza JP, Sellke FW, Simons M. VEGF administration in chronic myocardial ischemia in pigs. *Cardiovasc Res*. 1998;40:272-81.

65. Crottogini A, Meckert PC, Vera Janavel G, Lascano E, Negroni J, Del Valle H, Dulbecco E, Werba P, Cuniberti L, Martinez V, De Lorenzi A, Telayna J, Mele A, Fernandez JL, Marangunich L, Criscuolo M, Capogrossi MC, Laguens R. Arteriogenesis induced by intramyocardial vascular endothelial growth factor 165 gene transfer in chronically ischemic pigs. *Hum Gene Ther*. 2003;14:1307-18.

66. Tsurumi Y, Takeshita S, Chen D, Kearney M, Rossow ST, Passeri J, Horowitz JR, Symes JF, Isner JM. Direct intramuscular gene transfer of naked DNA encoding vascular endothelial growth factor augments collateral development and tissue perfusion. *Circulation*. 1996;94:3281-90.

67. Takeshita S, Weir L, Chen D, Zheng LP, Riessen R, Bauters C, Symes JF, Ferrara N, Isner JM. Therapeutic angiogenesis following arterial gene transfer of vascular endothelial growth factor in a rabbit model of hindlimb ischemia. *Biochem Biophys Res Commun*. 1996;227:628-35.

68. Becit N, Ceviz M, Kocak H, Yekeler I, Unlu Y, Celenk C, Akin Y. The effect of vascular endothelial growth factor on angiogenesis: an experimental study. *Eur J Vasc Endovasc Surg*. 2001;22:310-6.

69. Takeshita S, Tsurumi Y, Couffinahl T, Asahara T, Bauters C, Symes J, Ferrara N, Isner JM. Gene transfer of naked DNA encoding for three isoforms of vascular endothelial growth factor stimulates collateral development in vivo. *Lab Invest*. 1996;75:487-501.

70. Tsurumi Y, Kearney M, Chen D, Silver M, Takeshita S, Yang J, Symes JF, Isner JM. Treatment of acute limb ischemia by intramuscular injection of vascular endothelial growth factor gene. *Circulation*. 1997;96:II-382-8.

71. Babiak A, Schumm AM, Wangler C, Loukas M, Wu J, Dombrowski S, Matuschek C, Kotzerke J, Dehio C, Waltenberger J. Coordinated activation of VEGFR-1 and VEGFR-2 is a potent arteriogenic stimulus leading to enhancement of regional perfusion. *Cardiovasc Res*. 2004;61:789-95.

72. Asahara T, Tsurumi Y, Takeshita S, Isner JM. Naked cDNA encoding secreted proteins for intra-arterial and intramuscular gene transfer. *Semin Interv Cardiol*. 1996;1:225-32.

73. Bauters C, Asahara T, Zheng LP, Takeshita S, Bunting S, Ferrara N, Symes JF, Isner JM. Physiological assessment of augmented vascularity induced by VEGF in ischemic rabbit hindlimb. *Am J Physiol*. 1994;267:H1263-71.

74. Bauters C, Asahara T, Zheng LP, Takeshita S, Bunting S, Ferrara N, Symes JF, Isner JM. Site-specific therapeutic angiogenesis after systemic administration of vascular endothelial growth factor. *J Vasc Surg*. 1995;21:314-24; discussion 324-5.

75. Chang DS, Su H, Tang GL, Brevetti LS, Sarkar R, Wang R, Kan YW, Messina LM. Adeno-associated viral vector-mediated gene transfer of VEGF normalizes skeletal muscle oxygen tension and induces arteriogenesis in ischemic rat hindlimb. *Mol Ther*. 2003;7:44-51.

76. Shyu KG, Manor O, Magner M, Yancopoulos GD, Isner JM. Direct intramuscular injection of plasmid DNA encoding angiopoietin-1 but not angiopoietin-2 augments revascularization in the rabbit ischemic hindlimb. *Circulation*. 1998;98:2081-7.

77. Shyu KG, Chang H, Isner JM. Synergistic effect of angiopoietin-1 and vascular endothelial growth factor on neoangiogenesis in hypercholesterolemic rabbit model with acute hindlimb ischemia. *Life Sci*. 2003;73:563-79.

78. Siddiqui AJ, Blomberg P, Wardell E, Hellgren I, Eskandarpour M, Islam KB, Sylven C. Combination of angiopoietin-1 and vascular endothelial growth factor gene therapy enhances arteriogenesis in the ischemic myocardium. *Biochem Biophys Res Commun*. 2003;310:1002-9.

79. Scholz D, Elsaesser H, Sauer A, Friedrich C, Luttun A, Carmeliet P, Schaper W. Bone marrow transplantation abolishes inhibition of arteriogenesis in placenta growth factor (PlGF) -/- mice. *J Mol Cell Cardiol*. 2003;35:177-84.

80. Pipp F, Heil M, Issbrucker K, Ziegelhoeffer T, Martin S, van den Heuvel J, Weich H, Fernandez B, Golomb G, Carmeliet P, Schaper W, Clauss M.

VEGFR-1-selective VEGF homologue PlGF is arteriogenic: evidence for a monocyte-mediated mechanism. *Circ Res*. 2003;92:378-85.

81. Hao X, Mansson-Broberg A, Gustafsson T, Grinnemo KH, Blomberg P, Siddiqui AJ, Wardell E, Sylven C. Angiogenic effects of dual gene transfer of bFGF and PDGF-BB after myocardial infarction. *Biochem Biophys Res Commun*. 2004;315:1058-63.

82. Hao X, Mansson-Broberg A, Blomberg P, Dellgren G, Siddiqui AJ, Grinnemo KH, Wardell E, Sylven C. Angiogenic and cardiac functional effects of dual gene transfer of VEGF-A165 and PDGF-BB after myocardial infarction. *Biochem Biophys Res Commun*. 2004;322:292-6.

83. Baffour R, Berman J, Garb JL, Rhee SW, Kaufman J, Friedmann P. Enhanced angiogenesis and growth of collaterals by in vivo administration of recombinant basic fibroblast growth factor in a rabbit model of acute lower limb ischemia: dose-response effect of basic fibroblast growth factor. *J Vasc Surg*. 1992;16:181-91.

84. Lazarous DF, Scheinowitz M, Shou M, Hodge E, Rajanayagam S, Hunsberger S, Robison WG, Jr., Stiber JA, Correa R, Epstein SE, et al. Effects of chronic systemic administration of basic fibroblast growth factor on collateral development in the canine heart. *Circulation*. 1995;91:145-53.

85. Unger EF, Banai S, Shou M, Lazarous DF, Jaklitsch MT, Scheinowitz M, Correa R, Klingbeil C, Epstein SE. Basic fibroblast growth factor enhances myocardial collateral flow in a canine model. *Am J Physiol*. 1994;266:H1588-95.

86. Yang HT, Deschenes MR, Ogilvie RW, Terjung RL. Basic fibroblast growth factor increases collateral blood flow in rats with femoral arterial ligation. *Circ Res*. 1996;79:62-9.

87. Rajanayagam MA, Shou M, Thirumurti V, Lazarous DF, Quyyumi AA, Goncalves L, Stiber J, Epstein SE, Unger EF. Intracoronary basic fibroblast growth factor enhances myocardial collateral perfusion in dogs. *J Am Coll Cardiol*. 2000;35:519-26.

88. Lazarous DF, Unger EF, Epstein SE, Stine A, Arevalo JL, Chew EY, Quyyumi AA. Basic fibroblast growth factor in patients with intermittent claudication: results of a phase I trial. *J Am Coll Cardiol*. 2000;36:1239-44.

89. Lazarous DF, Shou M, Scheinowitz M, Hodge E, Thirumurti V, Kitsiou AN, Stiber JA, Lobo AD, Hunsberger S, Guetta E, Epstein SE, Unger EF. Comparative effects of basic fibroblast growth factor and vascular endothelial growth factor on coronary collateral development and the arterial response to injury. *Circulation*. 1996;94:1074-82.

90. Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, Wu Y, Bono F, Devy L, Beck H, Scholz D, Acker T, DiPalma T, Dewerchin M, Noel A, Stalmans I, Barra A, Blacher S, Vandendriessche T, Ponten A, Eriksson U, Plate KH, Foidart JM, Schaper W, Charnock-Jones DS, Hicklin DJ, Herbert JM, Collen D, Persico MG. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat Med*. 2001;7:575-83.

91. Asahara T, Bauters C, Zheng LP, Takeshita S, Bunting S, Ferrara N, Symes JF, Isner JM. Synergistic effect of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in vivo. *Circulation*. 1995;92:II365-71.

92. Kondoh K, Koyama H, Miyata T, Takato T, Hamada H, Shigematsu H. Conduction performance of collateral vessels induced by vascular endothelial growth factor or basic fibroblast growth factor. *Cardiovasc Res*. 2004;61:132-42.

93. Morishita R, Nakamura S, Hayashi S, Taniyama Y, Moriguchi A, Nagano T, Taiji M, Noguchi H, Takeshita S, Matsumoto K, Nakamura T, Higaki J, Ogihara T. Therapeutic angiogenesis induced by human recombinant hepatocyte growth factor in rabbit hind limb ischemia model as cytokine supplement therapy. *Hypertension*. 1999;33:1379-84.

94. Taipale J, Keski-Oja J. Growth factors in the extracellular matrix. *Faseb J*. 1997;11:51-9.

95. Distler O, Neidhart M, Gay RE, Gay S. The molecular control of angiogenesis. *Int Rev Immunol*. 2002;21:33-49.

96. Dvorak HF, Senger DR, Dvorak AM, Harvey VS, McDonagh J. Regulation of extravascular coagulation by microvascular permeability. *Science*. 1985;227:1059-61.

97. Connolly DT, Heuvelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, Siegel NR, Leimgruber RM, Feder J. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J Clin Invest*. 1989;84:1470-8.

98. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science*. 1989;246:1306-9.

99. Gupta K, Kshirsagar S, Li W, Gui L, Ramakrishnan S, Gupta P, Law PY, Hebbel RP. VEGF prevents apoptosis of human microvascular endothelial cells via opposing effects on MAPK/ERK and SAPK/JNK signaling. *Exp Cell Res*. 1999;247:495-504.

100. Gelinas DS, Bernatchez PN, Rollin S, Bazan NG, Sirois MG. Immediate and delayed VEGF-mediated NO synthesis in endothelial cells: role of PI3K, PKC and PLC pathways. *Br J Pharmacol*. 2002;137:1021-30.

101. Feliers D, Chen X, Akis N, Choudhury GG, Madaio M, Kasinath BS. VEGF regulation of endothelial nitric oxide synthase in glomerular endothelial cells. *Kidney Int*. 2005;68:1648-59.

102. Reihill JA, Ewart MA, Hardie DG, Salt IP. AMP-activated protein kinase mediates VEGF-stimulated endothelial NO production. *Biochem Biophys Res Commun*. 2007;354:1084-8.

103. Germani A, Di Carlo A, Mangoni A, Straino S, Giacinti C, Turrini P, Biglioli P, Capogrossi MC. Vascular endothelial growth factor modulates skeletal myoblast function. *Am J Pathol*. 2003;163:1417-28.

104. Arsic N, Zacchigna S, Zentilin L, Ramirez-Correa G, Pattarini L, Salvi A, Sinagra G, Giacca M. Vascular endothelial growth factor stimulates skeletal muscle regeneration in vivo. *Mol Ther*. 2004;10:844-54.

105. van Weel V, Deckers MM, Grimbergen JM, van Leuven KJ, Lardenoye JH, Schlingemann RO, van Nieuw Amerongen GP, van Bockel JH, van

Hinsbergh VW, Quax PH. Vascular endothelial growth factor overexpression in ischemic skeletal muscle enhances myoglobin expression in vivo. *Circ Res*. 2004;95:58-66.

106. Bryan BA, Walshe TE, Mitchell DC, Havumaki JS, Saint-Geniez M, Maharaj AS, Maldonado AE, D'Amore PA. Coordinated vascular endothelial growth factor expression and signaling during skeletal myogenic differentiation. *Mol Biol Cell*. 2008;19:994-1006.

107. Wang H, Keiser JA. Vascular endothelial growth factor upregulates the expression of matrix metalloproteinases in vascular smooth muscle cells: role of flt-1. *Circ Res*. 1998;83:832-40.

108. Cucina A, Borrelli V, Randone B, Coluccia P, Sapienza P, Cavallaro A. Vascular endothelial growth factor increases the migration and proliferation of smooth muscle cells through the mediation of growth factors released by endothelial cells. *J Surg Res*. 2003;109:16-23.

109. Niida S, Kondo T, Hiratsuka S, Hayashi S, Amizuka N, Noda T, Ikeda K, Shibuya M. VEGF receptor 1 signaling is essential for osteoclast development and bone marrow formation in colony-stimulating factor 1-deficient mice. *Proc Natl Acad Sci U S A*. 2005;102:14016-21.

110. Tchaikovski V, Fellbrich G, Waltenberger J. The molecular basis of VEGFR-1 signal transduction pathways in primary human monocytes. *Arterioscler Thromb Vasc Biol*. 2008;28:322-8.

111. Ruhrberg C, Gerhardt H, Golding M, Watson R, Ioannidou S, Fujisawa H, Betsholtz C, Shima DT. Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. *Genes Dev*. 2002;16:2684-98.

112. Shibuya M. Vascular endothelial growth factor receptor-1 (VEGFR-1/Flt-1): a dual regulator for angiogenesis. *Angiogenesis*. 2006;9:225-30; discussion 231.

113. Hiratsuka S, Maru Y, Okada A, Seiki M, Noda T, Shibuya M. Involvement of Flt-1 tyrosine kinase (vascular endothelial growth factor receptor-1) in pathological angiogenesis. *Cancer Res*. 2001;61:1207-13.

114. Heil M, Clauss M, Suzuki K, Buschmann IR, Willuweit A, Fischer S, Schaper W. Vascular endothelial growth factor (VEGF) stimulates monocyte migration through endothelial monolayers via increased integrin expression. *Eur J Cell Biol*. 2000;79:850-7.

115. Kim I, Moon SO, Kim SH, Kim HJ, Koh YS, Koh GY. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-kappa B activation in endothelial cells. *J Biol Chem*. 2001;276:7614-20.

116. Couffinhal T, Silver M, Zheng LP, Kearney M, Witzenbichler B, Isner JM. Mouse model of angiogenesis. *Am J Pathol*. 1998;152:1667-79.

117. Deindl E, Buschmann I, Hoefer IE, Podzuweit T, Boengler K, Vogel S, van Royen N, Fernandez B, Schaper W. Role of ischemia and of hypoxia-inducible genes in arteriogenesis after femoral artery occlusion in the rabbit. *Circ Res*. 2001;89:779-86.

118. Rivilis I, Milkiewicz M, Boyd P, Goldstein J, Brown MD, Egginton S, Hansen FM, Hudlicka O, Haas TL. Differential involvement of MMP-2 and VEGF during muscle stretch- versus shear stress-induced angiogenesis. *Am J Physiol Heart Circ Physiol*. 2002;283:H1430-8.

119. Tang K, Breen EC, Gerber HP, Ferrara NM, Wagner PD. Capillary regression in vascular endothelial growth factor-deficient skeletal muscle. *Physiol Genomics*. 2004;18:63-9.

120. Staalesen T, Risberg B, Mattsson E. The kinase insert domain-containing receptor (KDR) is regulated by shear stress. *Scand Cardiovasc J*. 2002;36:368-72.

121. Zheng W, Christensen LP, Tomanek RJ. Stretch induces upregulation of key tyrosine kinase receptors in microvascular endothelial cells. *Am J Physiol Heart Circ Physiol*. 2004;287:H2739-45.

122. Chang H, Shyu KG, Wang BW, Kuan P. Regulation of hypoxia-inducible factor-1alpha by cyclical mechanical stretch in rat vascular smooth muscle cells. *Clin Sci (Lond)*. 2003;105:447-56.

123. Kinnaird T, Stabile E, Burnett MS, Shou M, Lee CW, Barr S, Fuchs S, Epstein SE. Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. *Circulation*. 2004;109:1543-9.

124. Hershey JC, Baskin EP, Corcoran HA, Bett A, Dougherty NM, Gilberto DB, Mao X, Thomas KA, Cook JJ. Vascular endothelial growth factor stimulates angiogenesis without improving collateral blood flow following hindlimb ischemia in rabbits. *Heart Vessels*. 2003;18:142-9.

125. Masaki I, Yonemitsu Y, Yamashita A, Sata S, Tanii M, Komori K, Nakagawa K, Hou X, Nagai Y, Hasegawa M, Sugimachi K, Sueishi K. Angiogenic gene therapy for experimental critical limb ischemia: acceleration of limb loss by overexpression of vascular endothelial growth factor 165 but not of fibroblast growth factor-2. *Circ Res*. 2002;90:966-73.

126. Nikol S, Pelisek J, Engelmann MG, Shimizu M, Fuchs A, Golda A, Armeanu S, Mekkaoui C, Rolland PH. Vascular endothelial growth factor (VEGF165) and its influence on angiogenesis versus arteriogenesis in different vascular beds. *J Endovasc Ther*. 2002;9:842-54.

127. Pelisek J, Fuchs A, Engelmann MG, Shimizu M, Golda A, Mekkaoui C, Rolland PH, Nikol S. Vascular endothelial growth factor response in porcine coronary and peripheral arteries using nonsurgical occlusion model, local delivery, and liposome-mediated gene transfer. *Endothelium*. 2003;10:247-55.

128. Mukhopadhyay D, Zeng H, Bhattacharya R. Complexity in the vascular permeability factor/vascular endothelial growth factor (VPF/VEGF)-receptors signaling. *Mol Cell Biochem*. 2004;264:51-61.

129. Hershey JC, Baskin EP, Glass JD, Hartman HA, Gilberto DB, Rogers IT, Cook JJ. Revascularization in the rabbit hindlimb: dissociation between capillary sprouting and arteriogenesis. *Cardiovasc Res*. 2001;49:618-25.

130. Miquerol L, Langille BL, Nagy A. Embryonic development is disrupted by modest increases in vascular endothelial growth factor gene expression. *Development*. 2000;127:3941-6.

131. Damert A, Miquerol L, Gertsenstein M, Risau W, Nagy A. Insufficient VEGFA activity in yolk sac endoderm compromises haematopoietic and endothelial differentiation. *Development*. 2002;129:1881-92.

132. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature*. 1996;380:439-42.

133. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature*. 1996;380:435-9.

134. Jacobi J, Tam BY, Wu G, Hoffman J, Cooke JP, Kuo CJ. Adenoviral gene transfer with soluble vascular endothelial growth factor receptors impairs angiogenesis and perfusion in a murine model of hindlimb ischemia. *Circulation*. 2004;110:2424-9.

135. Lloyd PG, Prior BM, Li H, Yang HT, Terjung RL. VEGF receptor antagonism blocks arteriogenesis, but only partially inhibits angiogenesis, in skeletal muscle of exercise-trained rats. *Am J Physiol Heart Circ Physiol*. 2005;288:H759-68.

136. Toyota E, Warltier DC, Brock T, Ritman E, Kolz C, O'Malley P, Rocic P, Focardi M, Chilian WM. Vascular endothelial growth factor is required for coronary collateral growth in the rat. *Circulation*. 2005;112:2108-13.

137. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med*. 2003;9:669-76.

138. von Degenfeld G, Banfi A, Springer ML, Wagner RA, Jacobi J, Ozawa CR, Merchant MJ, Cooke JP, Blau HM. Microenvironmental VEGF distribution is critical for stable and functional vessel growth in ischemia. *Faseb J*. 2006;20:2657-9.

139. Haigh JJ, Morelli PI, Gerhardt H, Haigh K, Tsien J, Damert A, Miquerol L, Muhlner U, Klein R, Ferrara N, Wagner EF, Betsholtz C, Nagy A. Cortical and retinal defects caused by dosage-dependent reductions in VEGF-A paracrine signaling. *Dev Biol*. 2003;262:225-41.

140. Yu J, Lei L, Liang Y, Hinh L, Hickey RP, Huang Y, Liu D, Yeh JL, Rebar E, Case C, Spratt K, Sessa WC, Giordano FJ. An engineered VEGF-activating zinc finger protein transcription factor improves blood flow and limb salvage in advanced-age mice. *Faseb J*. 2006;20:479-81.

141. Xie D, Li Y, Reed EA, Odronic SI, Kontos CD, Annex BH. An engineered vascular endothelial growth factor-activating transcription factor induces therapeutic angiogenesis in ApoE knockout mice with hindlimb ischemia. *J Vasc Surg*. 2006;44:166-75.

142. Cross MJ, Dixelius J, Matsumoto T, Claesson-Welsh L. VEGF-receptor signal transduction. *Trends Biochem Sci*. 2003;28:488-94.

143. Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature*. 1995;376:66-70.

144. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature*. 1995;376:62-6.

145. Miquerol L, Gertsenstein M, Harpal K, Rossant J, Nagy A. Multiple developmental roles of VEGF suggested by a LacZ-tagged allele. *Dev Biol*. 1999;212:307-22.

146. Krucker T, Lang A, Meyer EP. New polyurethane-based material for vascular corrosion casting with improved physical and imaging characteristics. *Microsc Res Tech*. 2006;69:138-47.

147. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res*. 2002;30:e36.

148. Addya S, Shiroto K, Turoczi T, Zhan L, Kaga S, Fukuda S, Surrey S, Duan LJ, Fong GH, Yamamoto F, Maulik N. Ischemic preconditioning-mediated cardioprotection is disrupted in heterozygous Flt-1 (VEGFR-1) knockout mice. *J Mol Cell Cardiol*. 2005;38:345-51.

149. Thirunavukkarasu M, Addya S, Juhasz B, Pant R, Zhan L, Surrey S, Maulik G, Menon VP, Maulik N. Heterozygous Disruption Of Flk-1

Receptor Leads To Myocardial Ischemia Reperfusion Injury In Mice: Application Of Affymetrix Gene Chip Analysis. *J Cell Mol Med*. 2008.

150. Yang HT, Laughlin MH, Terjung RL. Prior exercise training increases collateral-dependent blood flow in rats after acute femoral artery occlusion. *Am J Physiol Heart Circ Physiol*. 2000;279:H1890-7.

151. Hattori K, Heissig B, Wu Y, Dias S, Tejada R, Ferris B, Hicklin DJ, Zhu Z, Bohlen P, Witte L, Hendrikx J, Hackett NR, Crystal RG, Moore MA, Werb Z, Lyden D, Rafii S. Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. *Nat Med*. 2002;8:841-9.

152. Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, MacDonald DD, Jin DK, Shido K, Kerns SA, Zhu Z, Hicklin D, Wu Y, Port JL, Altorki N, Port ER, Ruggero D, Shmelkov SV, Jensen KK, Rafii S, Lyden D. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature*. 2005;438:820-7.

153. Yuan A, Yu CJ, Chen WJ, Lin FY, Kuo SH, Luh KT, Yang PC. Correlation of total VEGF mRNA and protein expression with histologic type, tumor angiogenesis, patient survival and timing of relapse in non-small-cell lung cancer. *Int J Cancer*. 2000;89:475-83.

154. Jiang BH, Semenza GL, Bauer C, Marti HH. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. *Am J Physiol*. 1996;271:C1172-80.

155. Chun YS, Kim MS, Park JW. Oxygen-dependent and -independent regulation of HIF-1alpha. *J Korean Med Sci*. 2002;17:581-8.

156. Kearney JB, Kappas NC, Ellerstrom C, DiPaola FW, Bautch VL. The VEGF receptor flt-1 (VEGFR-1) is a positive modulator of vascular sprout formation and branching morphogenesis. *Blood*. 2004;103:4527-35.

157. Tomanek RJ, Ishii Y, Holifield JS, Sjogren CL, Hansen HK, Mikawa T. VEGF family members regulate myocardial tubulogenesis and coronary artery formation in the embryo. *Circ Res*. 2006;98:947-53.

158. Childs S, Chen JN, Garrity DM, Fishman MC. Patterning of angiogenesis in the zebrafish embryo. *Development*. 2002;129:973-82.

159. Resar JR, Roguin A, Voner J, Nasir K, Hennebry TA, Miller JM, Ingersoll R, Kasch LM, Semenza GL. Hypoxia-inducible factor 1alpha polymorphism and coronary collaterals in patients with ischemic heart disease. *Chest*. 2005;128:787-91.

160. Yu J, deMuinck ED, Zhuang Z, Drinane M, Kauser K, Rubanyi GM, Qian HS, Murata T, Escalante B, Sessa WC. Endothelial nitric oxide synthase is critical for ischemic remodeling, mural cell recruitment, and blood flow reserve. *Proc Natl Acad Sci U S A*. 2005;102:10999-1004.

161. Mees B, Wagner S, Ninci E, Tribulova S, Martin S, van Haperen R, Kostin S, Heil M, de Crom R, Schaper W. Endothelial nitric oxide synthase activity is essential for vasodilation during blood flow recovery but not for arteriogenesis. *Arterioscler Thromb Vasc Biol*. 2007;27:1926-33.

162. Wustmann K, Zbinden S, Windecker S, Meier B, Seiler C. Is there functional collateral flow during vascular occlusion in angiographically normal coronary arteries? *Circulation*. 2003;107:2213-20.

163. Balasubramanian SP, Brown NJ, Reed MW. Role of genetic polymorphisms in tumour angiogenesis. *Br J Cancer*. 2002;87:1057-65.

164. Gerber HP, Hillan KJ, Ryan AM, Kowalski J, Keller GA, Rangell L, Wright BD, Radtke F, Aguet M, Ferrara N. VEGF is required for growth and survival in neonatal mice. *Development*. 1999;126:1149-59.

165. Bochkov YA, Palmenberg AC. Translational efficiency of EMCV IRES in bicistronic vectors is dependent upon IRES sequence and gene location. *Biotechniques*. 2006;41:283-4, 286, 288 passim.

166. Holash J, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, Boland P, Leidich R, Hylton D, Burova E, Ioffe E, Huang T, Radziejewski C, Bailey K, Fandl JP, Daly T, Wiegand SJ, Yancopoulos GD, Rudge JS. VEGF-Trap: a VEGF blocker with potent antitumor effects. *Proc Natl Acad Sci U S A*. 2002;99:11393-8.

167. Miyazaki S, Miyazaki JI. In vivo DNA electrotransfer into muscle. *Dev Growth Differ*. 2008.

168. Schmidt-Kastner R, Yamamoto H, Hamasaki D, Parel JM, Schmitz C, Dorey CK, Blanks JC, Preising MN. Hypoxia-regulated components of the U4/U6.U5 tri-small nuclear riboprotein complex: possible role in autosomal dominant retinitis pigmentosa. *Mol Vis*. 2008;14:125-35.

169. Makeyev EV, Zhang J, Carrasco MA, Maniatis T. The MicroRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. *Mol Cell*. 2007;27:435-48.

170. Ambati BK, Nozaki M, Singh N, Takeda A, Jani PD, Suthar T, Albuquerque RJ, Richter E, Sakurai E, Newcomb MT, Kleinman ME, Caldwell RB, Lin Q, Ogura Y, Orecchia A, Samuelson DA, Agnew DW, St Leger J, Green WR, Mahasreshti PJ, Curiel DT, Kwan D, Marsh H, Ikeda S, Leiper LJ, Collinson JM, Bogdanovich S, Khurana TS, Shibuya M, Baldwin ME, Ferrara N, Gerber HP, De Falco S, Witta J, Baffi JZ, Raisler BJ, Ambati J. Corneal avascularity is due to soluble VEGF receptor-1. *Nature*. 2006;443:993-7.