Femoral Artery Regeneration in Ischemic Hind Limb by AAV9 Expressing Conditionally Silenced VEGF

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FEMORAL ARTERY REGENERATION IN ISCHEMIC HIND LIMB BY AAV9
EXPRESSING CONDITIONALLY SILENCED VEGF

By
Jeffrey P. Boden

A DISSERTATION

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of the University of Miami
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FEMORAL ARTERY REGENERATION IN ISCHEMIC HIND LIMB BY AAV9 EXPRESSING CONDITIONALLY SILENCED VEGF

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Peripheral artery disease (PAD) is caused by buildup of atherosclerotic plaque along the inside of arterial walls that supply oxygenated blood to the limbs. PAD affects 7% of all people over 60 years of age, and 23% of all people over the age of 80 (1). Over time, PAD progresses in severity, leading to critical limb ischemia (CLI), defined as an ankle-brachial index (a measurement of blood flow in the lower limb) of 0.40 or less. Surgical bypass and other endovascular techniques are capable of providing temporary relief from symptoms of CLI in some subjects. However, recurrence of the disease is seen in over 30% of patients following surgical stent or graft intervention (2). As of yet there are no effective pharmacological agents available to treat CLI. When surgical intervention fails or is not possible, a patient is left with only one option; limb amputation. The outlook is bleak for lower limb amputees: 30% require subsequent amputation of the contralateral limb within 1 year and 50% of all lower limb amputees die within 5 years (3). Since 1996 there have been 11 Phase I, 6 Phase II, and 1 Phase III clinical trials that have aimed to treat PAD with transgene delivery of pro-angiogenic growth factors (3).
For diseases such as PAD and other cardiovascular indications that are chronic and recurring, there is a need for a physiological responsive vector with long-term, regulated expression and the ability to switch on and off in response to cellular micro-environmental cues associated with the disease. As of now, most PAD remains untreatable by pharmacological or biotechnological approaches and there are many cases where vascular surgery is not an option. It is the opinion of this laboratory that the failure of biotechnology, in particular gene therapy, but also stem cell therapy, is related to the absence of appropriate regulation of the therapeutic agent(s). This aspect, as it relates to gene therapy, will be the subject of my thesis. In our studies of VEGF gene therapy for treatment of CLI, we have attempted to address some of the concerns that have plagued past clinical trials. We have created a hypoxia regulated DNA backbone expressing human VEGF165 that is encapsulated within a muscle-trophic AAV serotype 9 shuttle vector. This vector targets only ischemic tissue and mediates semi-permanent transgene expression with minimal immunogenicity and no apparent toxicity or adverse side effects.

The mouse hindlimb model has been widely used to assess the activities of pro-angiogenic mediators, but few studies have attempted to characterize the molecular pathways and their responses to treatments. Such studies may provide clues to the molecular and cellular events that determine success or failure of specific therapeutic approaches. Studies in this laboratory have defined a novel system using the mouse hindlimb model in which we can reproducibly promote recovery or failure of the vasculature following surgical ischemia. Using
the mouse hindlimb model we have established conditions that promote full and partial recovery of limb perfusion, or failure of the therapy (and attendant limb loss). Adenoviral delivery of VEGF promotes an early burst of angiogenesis but does not support limb salvage. Adeno-associated virus-mediated VEGF delivery by a constitutive promoter supports limb salvage but is inferior to AAV that employs a hypoxia-regulated VEGF gene. My hypothesis is that ectopically administered VEGF can stimulate successful stable revascularization of ischemic muscle, provided a pro-angiogenic signaling gradient (directing endothelial tip cell migration via the Notch signaling pathway) is established. Unregulated VEGF expression or premature withdrawal causes the process to fail. Progression through this linear process will be defective qualitatively or quantitatively at one or more points when the therapy is inadequate.

By using conditional silencing we have created a gene switch that responds to ischemia with greater that 100-fold increase in gene expression, more than an order of magnitude greater than any previous reports for in vivo regulation. Adenovirus VEGF expression is lost several weeks following injection into ischemic muscle and does not support permanent revascularization of the mouse hind limb. AAV gene expression persists for more than 6 months in vivo and supports limb salvage when delivered in a hypoxia regulated construct. Unregulated AAV-CMV-VEGF does not support limb salvage due to over-expression of VEGF. Use of the conditionally silenced vector was shown to support early reperfusion of the hind limb, tight auto-regulation by ischemia, permanent salvage of the hind limb and evidence for regeneration of a large
artery within the femoral tract at 1-year post-surgery. Unregulated vectors do not demonstrate large artery regeneration and result in limb auto-amputation.
Acknowledgement Page

I would like to thank the members of the Webster lab for their help with the work that went into completing the work described in this thesis. I would especially like to thank Hans Layman for teaching me how to perform hind limb ischemia surgery. Hans also completed the surgeries for limb salvage evaluations of the AAV9-HRE-VEGF, AAV9-PGK-VEGF, and Ad-VEGF vectors, in association with Grazia Spiga who completed the Doppler analyses. Grazia also completed the cloning work to create our early hypoxia regulated constructs and the AAV9-HRE-VEGF vector. Huilan Wang conducted the cloning work that went into the construction of the AAV9-TOAD-VEGF vector.
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Chapter 1: Ischemic Disease and Gene Therapy

Peripheral Artery Disease

Peripheral artery disease (PAD) is caused by buildup of atherosclerotic plaque along the inside of arterial walls that supply oxygenated blood to the limbs. PAD affects 7% of all people over 60 years of age, and 23% of all people over the age of 80 \(^1\). Over time, PAD progresses in severity, leading to critical limb ischemia (CLI), defined as an ankle-brachial index (a measurement of blood flow in the lower limb) of 0.40 or less. Surgical bypass and other endovascular techniques are capable of providing temporary relief from symptoms of CLI in some subjects. However, recurrence of the disease is seen in over 30% of patients following surgical stent or graft intervention \(^2\). As of yet there are no effective pharmacological agents available to treat CLI. When surgical intervention fails or is not possible, a patient is left with only one option; limb amputation. The outlook is bleak for lower limb amputees: 30% require subsequent amputation of the contralateral limb within 1 year and 50% of all lower limb amputees die within 5 years \(^3\). Since 1996 there have been 11 Phase I, 6 Phase II, and 2 Phase III clinical trials that have aimed to treat PAD with transgene delivery of pro-angiogenic growth factors \(^3\). Overall, the trial results have been disappointing, failing to reach established trial endpoints and in most cases, not impacting amputation frequency.
Origins of Gene Therapy

The earliest seeds of gene therapy were planted in the 1940’s when scientists began experimenting with pneumococci transformation techniques. Avery, McLeod and McCarty were the first to describe the induction of heritable characteristics through the transfer of deoxyribonucleic acid isolates of one pneumococcal type to another. By the 1960’s scientists had begun experimenting with viral vectors of gene transmission. This work was inspired by findings that SV40, found in monkeys and humans, was capable of integrating a portion of its genome into the host cell DNA and that this integrated DNA was stable and transmissible to daughter cells. These early experiments struggled to make headway in terms of gene therapeutics because recombinant DNA technology was not yet available. As a consequence progress was limited.

Rogers and Pfuderer (1968) first reported on the delivery of modified genetic material to eukaryotic cells using a viral vector. These authors infected plant cells with a tobacco mosaic virus containing a polyA repeat in the 3’ genomic DNA and demonstrated the expression of polylysine in the host cells. Seven years later the same team published the first gene therapy clinical trial that attempted to use a papilloma virus expressing high levels of the arginase gene to treat patients with arginase deficiency. The trial was a failure, possibly due to insufficient gene transfer and/or arginase expression. During the same period, findings from several other studies had supported the possibility that viral vectors were promising vehicles for the delivery of genetic information, and the
field gained momentum. On the heels of these new discoveries the first gene therapy focused meeting held by the National Institute of Health in 1971.

The advent of recombinant DNA technology ushered in a golden age of discovery for gene therapy (reviewed in 7, 8). Early success of bone marrow cell transfection with plasmids containing the thymidine kinase gene, led to the first human gene therapy clinical trial using recombinant DNA in 1980 9, 10, 11. The clinical trial attempted to treat thalassemia by delivering an intact globin gene into bone marrow cells ex-vivo, followed by reinfusion of the transfected cells. The trial, implemented without the permission of a university internal review board (IRB), failed and was highly criticized on ethical grounds 12, 13, 14. Lively debate raged throughout the scientific community regarding the ethics of human genetic research. This rapidly led to the creation of official public policies to govern human genetic trials.

The next major breakthrough in gene therapy research came with the discovery of retroviral vectors. Retroviral genomes integrate into host cell DNA, establishing stable gene transduction, a desirable yet potentially dangerous characteristic. The potential for harmful mutations resulting from insertion mutagenesis is a cause for concern when using these vectors for gene therapy. Gene transfer of HPRT to Lesch-Nyhan cells and adenosine deaminase to T-lymphocytes served as proof of concept for retroviral vectors in genetic deficiency models of disease 15, 16. Although unable to infect non-dividing cells, retroviral vectors are highly efficient gene delivery vehicles, capable of attaining nearly 100% infection of dividing cells. Retroviral vectors were the most highly
favored gene vector until retroviral-associated incidences of leukemia were reported in four of nine patients who were successfully treated for X-SCID\textsuperscript{17, 18}. Clinical trials using these vectors were allowed to continue under conditions of informed patient consent, a condition now required for all gene and stem cell trials.

Adenoviral vectors can transduce dividing and non-dividing tissues, thus broadening the range of therapeutic application. Most adenoviral vectors that are used for gene therapy are derived from adenovirus serotype 5. This virus does not significantly integrate into host chromosomes; therefore, the transferred genetic material exists episomally within the nucleus eliminating problems associated with insertion mutagenesis. However, this also means that gene delivery by adenoviral vectors is transient in nature, and long term therapy requires multiple dosing, a condition that is largely precluded due to immune rejection of adenoviral capsid proteins. A potentially lethal aspect of adenovirus immunogenicity was evidenced by the death of teenager Jesse Gelsinger in a study to treat ornithine transcarbamylase deficiency using adenoviral gene delivery, conducted by the University of Pennsylvania in 1999\textsuperscript{19}.

Non-viral methods of gene delivery have also been explored\textsuperscript{20}. Naked DNA injections in the form of plasmids, as well as plasmids encased in a cationic lipid shield or liposome, have been used for cardiovascular targets and in multiple clinical trials\textsuperscript{3, 21}. Transgenes delivered in this manner also do not integrate significantly into host cell DNA and their effects are transient in nature. Plasmid gene delivery is of low efficiency but may be safer than retrovirus or
adenovirus for gene therapy. Until recently plasmids have been the vector of choice for most gene therapy trials for treating critical limb ischemia. 22.

### Angiogenic Gene Therapy for PAD

The early work of Judah Folkman and others to identify and isolate angiogenic growth factors gave rise to the idea of therapeutic angiogenesis for the treatment of ischemic disease. 23, 24, 25, 26, 27. On the heels of these revelations, a number of studies demonstrated the potent angiogenic effects of growth factors *in vivo*. 28, 29, 30, 31, 32. The first pre-clinical use of therapeutic angiogenesis to treat critical limb ischemia was conducted in 1992. 33. Baffour et al. administered recombinant basic fibroblast growth factor (bFGF) protein to rabbit hind limb at a dose of 1 or 3 µg of total protein via daily intramuscular injection for two weeks following ischemia surgery. 33. bFGF treated animals displayed increased blood vessel network regeneration as shown by angiography, faster recovery of transcutaneous oximetry (TcPo2) in thigh and calf muscles, increased capillary density, and increased thigh muscle viability. The following year a similar study using acidic FGF, known now as FGF1, confirmed the proangiogenic potential of this family of growth factors in ischemic recovery. 34. Continued study of direct injection of FGF protein to ischemic hearts in dog and pig models demonstrated increased myocardial collateral flow and function. 35, 36.
By the end of the 1980’s experimentation with plasmid and virally delivered gene therapy constructs gained popularity among researchers searching for methods to increase gene transfer efficiency over direct protein injection. Lipofection and retroviral delivery of transgenes were found to increase transfection efficiencies by more than two orders of magnitude and could be delivered selectively to blood vessel walls \textit{in vivo} by balloon angioplasty \textsuperscript{37,38}. Adenovirus vectors could also be site specifically delivered to coronary endothelium in association with surgical angioplasty \textsuperscript{39}. Isner and colleagues first experimented with site-directed delivery of the vascular endothelial growth factor (VEGF) transgene to arterial walls using a direct deposition technique \textsuperscript{40}. Shortly thereafter the same group reported results of a pre-clinical study in which 400 $\mu$g of plasmid encoding VEGF$_{165}$ was delivered to the iliac artery of rabbits via balloon angioplasty \textsuperscript{41}. The therapeutic response was manifested in increased collateral vessel growth, capillary density, and increased blood pressure in the calf of the ischemic limb \textsuperscript{41}.

Following almost a decade of supportive preclinical studies, Isner et al. (1996) first reported the results of a case study using the human vascular endothelial growth factor gene (hVEGF$_{165}$) cloned into a plasmid vector for intra-arterial delivery. The 71 year old female patient suffered from severe peripheral artery disease as evidenced by gangrene of several toes on the right foot \textsuperscript{42}. Treatment involved a one time delivery of 2mg of plasmid DNA applied to the hydrogel polymer coating of an angioplasty balloon and administered directly to the distal wall of the popliteal artery \textsuperscript{42}. Angiography at 4-weeks follow-up
revealed increased collateral vessels at the knee, mid-tibial, and ankle, which persisted through 12-weeks and intra-arterial Doppler-flow measurements showed an 82% increase in resting blood flow and a 72% increase in maximal flow. However, the authors noted the development of spider angiomas and unilateral peripheral edema. These were determined to have been caused by the vascular permeability effects of plasmid delivered VEGF and the patent’s leg was amputated below the knee 5 months after gene therapy.

Isner’s pioneering work was followed by multiple gene therapy trials aimed at regenerating arterial systems and cardiac function following ischemic insult. The results reported for these studies have been largely disappointing. A failure to meet primary endpoints has prevented most studies from progressing to Phase II or Phase III clinical trials. For example, the results of the first Phase II trial using VEGF to treat PAD were published in 2003. The Regional Angiogenesis with Vascular Endothelial Growth Factor (RAVE) trial was the first double-blind, randomized, Phase II clinical trial using adenovirally delivered VEGF for the treatment of PAD. A pool of 105 patients were randomly assigned into one of two treatment groups to receive intramuscular injections of either high (4x10^{10} PU) or low dose (4x10^{9} PU) adenoviral VEGF. A third group of patients received placebo. Injections were administered over 20 sites in a single session. Change in peak walking time (PWT) was used as a primary endpoint at 12 weeks after gene therapy. After 12 weeks there was no difference between treatment groups and placebo for measurements of PWT. Patients receiving VEGF therapy also experienced
an increased incidence of peripheral edema, leading investigators to conclude that local delivery of VEGF121 by adenovirus was not a viable treatment strategy in patients with unilateral PAD\textsuperscript{45}.

Several years after the RAVE trial, another double-blind randomized placebo controlled trial aimed to determine the effect of intramuscular injection of plasmid VEGF\textsubscript{165} on CLI in 54 adult diabetic patients\textsuperscript{50}. This time, instead of increases in PWT, the researchers used amputation frequency as a primary endpoint, measured 100 days after plasmid injection. The authors found that plasmid treatments resulted in fewer amputations (3 versus 6, \( p = \text{NS} \)), improved circulation (7 versus 1, \( p = 0.05 \)), reduced skin ulcers (7 versus 0, \( p = 0.01 \)), decreased pain (5 versus 2, \( p = \text{NS} \)), and displayed an overall clinical improvement (14 versus 3, \( p = 0.003 \)) versus placebo. The conclusions of the study noted a failure to meet the primary objective of amputation reduction; however, there were marked improvements in plasmid treated patients without substantial side effects\textsuperscript{50}.

The results of a randomized, double-blind, placebo-controlled study involving 44 patients were published in 2010\textsuperscript{59}. The study aimed to treat CLI with IM injection of 4mg of HGF plasmid diluted in a total of 24ml of sterile saline and distributed over 8 injection locations in the calf and distal thigh muscles of the affected limb. 4 weeks after injection, patients received a second round of injections containing another 4mg of total plasmid, making for a total HGF plasmid therapy of 8mg. Evaluations were carried out over 12 weeks and primary endpoints included improvement of rest pain in patients without ulcers
and reduction of ulcer size in patients with ulcers. The researchers found significant improvement in ulcer size and healing in patients displaying ischemic ulcers but did not report a significant reduction in resting pain. There was no significant improvement in the secondary endpoint evaluation of ABI between HGF and placebo groups. Overall the researchers concluded that HGF plasmid was well tolerated and reduced the size of ischemic ulcers and improved overall quality of life \(^{59}\).

There is still no FDA approved gene or stem cell treatment for PAD. Most recently, the results of a placebo-controlled Phase III trial of FGF-1 for CLI were presented. The TAMARIS trial was the largest trial ever implemented for a gene therapy procedure and involved 525 patients with CLI \(^{60}\). Patients were given 8 IM injections of 0.5mg per site for a total of 4mg of FGF plasmid per treatment session. A total of four treatments were conducted for each patient over two weeks. Unfortunately no efficacy was show and there were even trends to worsening of symptoms in treatment compared with placebo groups. The conclusion of TAMARIS stated that FGF plasmid therapy was not effective in delaying time to amputation or death in patients with CLI \(^{60}\).

The reasons for the modest to negative outcomes of plasmid and adenoviral gene therapy clinical trials for cardiovascular indications, have been evaluated by many experts (for reviews see \(^{3,61,62}\)). Key factors include gene delivery method, dosage, and persistence of expression. Clearly insufficient delivery, level or duration of expression precludes therapy. Such may be the case for plasmid-mediated therapies because plasmids have low transduction
efficiencies that lead to low transgene expression and limited duration of expression. Intramuscular delivery is the preferential delivery method for the treatment of peripheral ischemia. Intramuscular delivery is more efficient than systemic delivery, vector wash-out is low, and the vector is delivered directly to the muscle cells without having to cross blood vessel walls. Procedures that inject vectors systemically have a greater risk for off-target delivery. This is an important safety concern for angiogenic growth factors. Intra-arterial gene delivery is also technically more difficult for skeletal muscle targets. In contrast intracoronary delivery tends to be the route of choice for the myocardial gene and stem cell transfers \(^{49, 51, 52}\).

A major challenge to using gene therapy with pro-angiogenic growth factors for the treatment of patients with PAD is the metering of a safe and effective dose. Preclinical studies have been used as gauges to establish the clinical dose, and the emphasis has been on maximizing DNA delivery and transgene expression, for example by promoter strength, from the vector. However an optimal clinical dose has not yet been determined (for review see \(^{62}\)). In most clinical trials that use plasmids, the vectors are injected at the maximal dose that the tissue or subject can tolerate without major side effects, and expression is directed by a powerful promoter, usually from cytomegalovirus (CMV). In this setting it has been generally assumed that more is better. For adenovirus this assumption was starkly challenged by the adenoviral/immune-response-associated death of Jesse Gelsinger in 1999 (referred to above). Gelsinger was an 18-year-old, otherwise healthy patient, being treated by
adenoviral gene therapy for ornithine transcarbamylase \textsuperscript{19}. He was given an infusion of type 5 adenovirus containing the ornithine transcarbamylase cDNA, in the right hepatic artery, at a dose of 6x10^11 vp/kg \textsuperscript{63}. Four days later Mr. Gelsinger died from a severe innate immune response to the virus. The tragedy dealt a blow to the progress of gene therapy in the clinic that retarded progress for over a decade. This unfortunate accident highlighted the risk of dose escalation, as well as uncertainty in patient immune response. Mr. Gelsinger received the same dose as a previous trial patient but his immune response was much more severe \textsuperscript{64}. It is my opinion that the mechanism and regulation of gene therapy dosing has not received sufficient attention. Duration of expression is also a major issue; adenoviral vectors transduce efficiently but expression is limited to a few weeks \textsuperscript{65}. Plasmids on the other hand transduce inefficiently but can express for several months, albeit weakly \textsuperscript{66}.

For diseases such as PAD and other cardiovascular indications that are chronic and recurring, there is a need for a physiological responsive vector with long-term, regulated expression and the ability to switch on and off in response to cellular micro-environmental cues associated with the disease. As of now, most PAD remains untreatable by pharmacological or biotechnological approaches and there are many cases where vascular surgery is not an option. It is the opinion of this laboratory that the failure of biotechnology, in particular gene therapy, but also stem cell therapy, is related to the absence of appropriate regulation of the therapeutic agent(s). This aspect, as it relates to gene therapy, will be the subject of my thesis. In our studies of VEGF gene therapy for
treatment of CLI, we have attempted to address some of the concerns that have plagued past clinical trials. We have created a hypoxia regulated DNA backbone expressing human VEGF_{165} that is encapsulated within a muscle-trophic AAV serotype 9 shuttle vector. This vector targets only ischemic tissue and mediates semi-permanent transgene expression with minimal immunogenicity and no apparent toxicity or adverse side effects.
Chapter 2: Hypoxia Driven Vasculogenesis and Arteriogenesis

VEGF Family of Proteins

The VEGF family of proteins consists of five known members; VEGFA, VEGFB, VEGFC, VEGFD and placental growth factor (PIGF). These ligands are dimeric glycoproteins with an approximate size of 40 kilodaltons. Each ligand binds to a transmembrane receptor tyrosine kinase (VEGFRs), which can form homo- or heterodimeric configurations each with its own proclivity to binding a specific VEGF ligand. The members of the VEGF family elicit specific responses that are temporally, spatially and developmentally required for maintenance of a normal vascular physiological state. Additional functional dimensions are bestowed upon each VEGF family ligand through pre- and post transcriptional modifications that result in variable sizes of the translated protein \(^67\). Figure 1 summarizes the binding characteristics of each VEGF ligand and receptor combination.

VEGFA

While the existence of VEGFA was first documented in the 1970’s \(^23\); the gene was not isolated and cloned until 1989 \(^68\). The most potent inducer of angiogenesis, VEGFA is essential for normal embryonic development to the point where the loss of one allele is lethal \(^69\). VEGFA binds to both VEGFR-1 and VEGFR-2 but has a much higher binding affinity to VEGFR-1 (Kd ~ 2-10pM) \(^70\).
However, most of the angiogenic effects of VEGFA are elicited through VEGFR-2 signaling as evidenced by experiments in VEGFR2-null mice and low tyrosine kinase activity of VEGFR-1. The interplay between high-affinity binding of the VEGFA/VEGFR-1 interaction and strong signaling via VEGFR-2/VEGFA, indicates that VEGFR-1 may act as a VEGFA sink, whereby VEGFR-1 or soluble VEGFR-1 is able to attenuate VEGFA/VEGFR-2 signal by sequestering free VEGFA. Supporting evidence for this comes from studies of VEGFR-1 in embryonic development showing that mice expressing VEGFR-1 that lack a tyrosine kinase domain develop normal vasculature, and that VEGFR-1 plays a role in the proper maintenance of angiogenic sprout formation.
VEGFA is also known as vascular permeability factor because it can cause blood vessels to leak, creating an edema; this was first described for vascular conduits within the tumor microenvironment \(^{78}\). The crystal structure of VEGFA revealed that the ligand exists naturally as a homodimer, consisting of monomeric subunits bound in an antiparallel fashion \(^{79}\). Receptor binding sites for VEGF molecules are located at the poles of each monomeric subunit \(^{79}\).

There are four major VEGFA isoforms of varying amino acid length that can be produced through alternative splicing of the VEGF transcript; VEGF\(_{121}\), VEGF\(_{165}\), VEGF\(_{189}\) and VEGF\(_{206}\). There are also 5 minor isoforms that can be expressed to a lesser degree. Each VEGF isoform contains binding sites for the co-receptor heparin sulfate proteoglycan (HSPG). The longer length of the translated protein corresponds with increased HSPG binding capability such that VEGF\(_{189}\) strongly binds HSPG in the ECM, whereas VEGF\(_{121}\) does not bind to the ECM and is freely diffusible. Isoforms of intermediate length have intermediate HSPG binding affinities. The function of having different VEGF isoforms is to regulate the spatial VEGF signal via a VEGF gradient along a cellular plane. While freely diffusing VEGF\(_{121}\) is capable of distant recruitment signaling to chemotactic cells, locally higher concentrations of VEGF\(_{189}\) affixed to HSPG, drive location-specific direction to recruited cells and acute cellular response within the region of more concentrated signals \(^{80}\). VEGFA binds primarily to VEGFR-2 in order to elicit a cellular response. VEGFA gene expression is primarily regulated by stabilized HIF-1\(\alpha\) binding to HREs in the VEGF promoter \(^{81}\). VEGF mRNA induction in skeletal myoblasts has been
shown to increase by 5-fold under anoxic culture conditions\textsuperscript{82}. Additional factors that induce VEGFA gene expression are low temperature, growth factors and steroid hormones\textsuperscript{83, 84, 85, 86}. VEGFA assists in the processes of wound healing, cancer disease progression and maintenance of normal endothelium\textsuperscript{87}. Deletion of the VEGFA allele within the endothelial cell lineage causes degradation of the vasculature\textsuperscript{87}. VEGFA is produced by most parenchymal cells.

**The Physiologic Hypoxic Angiogenic Response**

Both angiogenesis and vasculogenesis are required for tissue regeneration and/or repair of damage caused by ischemia. VEGFA is strongly induced by hypoxia, an obligate component of ischemia. In cooperation with the drosophila (and mammalian) morphogen, sonic hedgehog (SHH), VEGF has been shown to orchestrate embryonic arteriogenesis by acting at multiple levels to regulate the expression of downstream effectors\textsuperscript{88}. Recent studies have shown that a balance between proliferation and differentiation signals must be established in order to construct a stable vascular network\textsuperscript{89, 90}. In other words angiogenesis, vasculogenesis and arteriogenesis are tightly controlled and require precise organization of factors at both temporal and spatial levels. Hypoxia plays an important role in this regulation both by initiating the program through VEGF induction and modulating cellular responses. As a consequence of this requirement, widespread, unregulated expression of growth factors such as VEGF, as practiced by most gene therapy procedures, may not alone be able
to create the proper niche for neovascularization. Rather it must be precisely regulated both at the temporal and spatial levels.

Much of what we know of the processes of neovascularization comes from studies of the developing mouse retina (reviewed in 91). Neovascularization occurs in embryonic tissues by the processes of vasculogenesis and angiogenesis. Vasculogenesis is a de novo process that involves recruitment of progenitor stem cells, many of which originate in the bone marrow. When parenchymal tissues are avascular or become hypoxic, VEGFA gene expression is upregulated by the binding of Hif-1α to hypoxia response elements (HREs) upstream of the VEGF 5’ and 3’ ends of the coding sequence. Binding of Hif-1α recruits accessory factors including the adaptor protein p300 that confers its associated histone acetyl transferase (HAT) activity and upregulates gene expression. As a result, Vegf-a protein is secreted into the surrounding lumen, leading to the establishment of a local Vegf-a gradient. SDF-1, a potent inducer of hematopoietic stem cell migration, is also secreted by somatic cells within the hypoxic environment in direct response to Hif-1α. Progenitor cells follow this signaling gradient and congregate into what is known as an angioblast, to create a primordial vascular network. Primitive plexi are pruned through budding and branching angiogenic processes that lead to the formation of venous and arterial endothelium. Endothelial cells begin to phenotypically differentiate into arterial and venous endothelium through differential expression of cell surface proteins. Notch target genes HEY1 and HEY2 denote pro-arterial vascular phenotypes, whereas suppression of Notch signaling by COUP-TF2, denotes
venous endothelium. In adult tissues, the process of angiogenesis functions to revascularize damaged and/or ischemic tissues by creating collateral vessels that supplement the basic vasculature.

**Network Formation and the Role of Notch Signaling**

The formation of a stable bi-polar blood-conducting network from a primitive vascular plexus requires intricate coordination of endothelial cell behavior. Our assertion is that the production of stable vessels by gene therapy requires the same or similar degrees of physical and temporal signaling, including gradients of growth factors and cytokines that support development of the cardiovascular system during embryogenesis and normal adult tissue collateral generation. This is also strongly supported by the work of others. One of the principal initiating events in new vessel development is the specialization of endothelial cells into tip cells that lead the outgrowth of blood vessels. During collateral vessel formation, endothelial cells of viable tissue within or surrounding ischemic regions proliferate, migrate, and remodel into neovessels that grow into the ischemic tissue by following cytokine gradients.

Mature arteries are composed of tightly bound endothelial cells that maintain a closed luminal circumference through tight cell junctions and obligate autocrine VEGFA signaling. Downstream of VEGF signaling, Ephrin-B2 recruits pericytes and vascular smooth-muscle cells to the vessel exterior for added strength. In this state vascular luminal endothelial cells are quiescent.
and remain so until growth factor stimulus within the micro-environment trigger endothelial activation and migration. Protease degradation of basement membranes allows endothelial cells on the leading migratory edge to extend filopodia and creep along chemotactic gradients often caused by hypoxia \(^{93, 94, 99, 100}\).

Angiogenic stimulation of quiescent endothelial cells triggers a migratory response that can be either attractive or repulsive, depending upon specific microenvironmental signals \(^{99, 100}\). To maintain luminal integrity, endothelial cells signal to one another in a process known as 'lateral inhibition,' where the differentiation state of a cell is determined by competitive stimuli between neighboring cells. Lateral inhibition is mediated through Notch cell surface receptor activation \(^{88, 89, 101}\). Tip cells lead the migratory movement of an endothelial layer extension along these signaling gradients. Trailing stalk cells are less motile, maintain a connection to the existing vasculature, and serve as parental cells to the newly formed vascular lumen \(^{102, 103}\). Tip cells continue to migrate along angiogenic gradients until encountering another tip cell or vascular bed. At this moment, the little understood process of anastomosis, connects adjacent vessels. Macrophage chaperones and growth factors are thought to promote anastomosis and stabilize vessel walls \(^{94, 104, 105}\). In the presence of continuous growth factor signaling, this process recurs, followed by subsequent rounds of pruning of extraneous vessel lumen \(^{106}\).

In order for luminal integrity to be maintained during this migratory process, proper distributions of tip and stalk cells must be tightly controlled. If too
many tip cells are created, luminal structure can be compromised resulting in vessel leakiness, whereas, with a relative absence of tip cells tissues can become ischemic. Tip and stalk cell differentiation are governed by the Notch signaling pathway in coordination with VEGFA.

**Molecular mechanism of Notch regulation**

VEGFR2 binding of VEGFA at the plasma membrane of an endothelial cell, signals to the nucleus via tyrosine-kinase induced signaling cascades that include the MAPK and AKT pathways, to activate cell proliferation, migration, and survival signals. The VEGFR2/VEGFA interaction also leads to an up-regulation ofDll4 expression at the cell surface. Dll4 binding to Notch1 of an adjacent cell sends a bi-directional signal whereby the cell expressing Dll4 takes on tip cell characteristics and the Notch1 expressing cell becomes a stalk cell. The actual process of tip and stalk cell differentiation is complex; all endothelial cells express both Dll4 and Notch1 on their cell surfaces. A cell becomes a tip cell through up-regulation of Dll4 and binding to the Notch1 sites of a neighboring stalk cell. Following ligand-receptor binding, proteolytic cleavage of Notch1 separates the protein into an intracellular and extracellular domain. The Dll4-bound extracellular domain is trans-endocytosed by the ligand expressing cell, conferring a tip cell fate. The Notch intracellular domain translocates to the nucleus to activate Notch target genes. Activation of Notch transcriptional targets causes a down regulation of VEGFR2 transcription, which limits the
cellular response to VEGF, and confers a stalk cell phenotype. Additionally, the expression of Notch ligand Jag1 is restricted to stalk cells. Jag1 acts as a sink for excess Notch1 presentation on the surface of tip cells. Because, Notch1 signal can only be transmitted by binding to Dll4, a Notch1:Jag1 interaction does not activate the notch signaling pathway, maintaining the tip cell phenotype of the neighboring cell. Increased VEGF signaling concomitant with decreased Notch signaling via Dll4 can lead to an over-expansion of tip cells, erratic vessel branching and poor conductance. Recent studies have shown that the Dll4 ligand is essential for active, productive vascularization and upregulation of Ephrin-B2 expression. Haploinsufficiency of Dll4 has been shown to yield malformation of the vascular system and embryonic lethality. Another recent study implicated Dll1 signaling as a key regulator of postnatal arterial development.

Thus the process of active vascularization requires balanced signaling of VEGF and Notch pathways to produce organized sprouting and branching of endothelial tubes. This coordination is essential if activated ECs and branched vessels are to progress into mature blood conducting networks, a process that leads to the coordinated production of arteries and veins. The delicate balance of Notch signal inhibition/activation that translate to a coordinated cellular response mandate that exogenous delivery of stimuli to regenerate damaged vasculature mirror this coordinated cacophony of signaling directed by endogenous stress signals. A major part of my thesis involves analyses of the
downstream events that follow regulated VEGF gene therapy, including the coordinated production and regulation of Notch factors (see Chapter 8).
Chapter 3: Adeno-Associated Virus

Atchison et al. (1965) first reported the discovery of Adeno-Associated Virus (AAV). Originally thought to be a defective virus found in adenovirus cultures, AAVs are members of the Paroviridae family of viruses, subclassified in the genus Dependovirus due to their inability to replicate in the absence of replicative trans-acting proteins, supplied by adenovirus or herpes simplex virus (known as helper viruses). In the absence of helper virus, wild-type AAV maintains a latent infection where the viral genome persists inside the nuclei either integrated into the DNA at a preintegration site (human chromosome 19q13.3–qter) or episomally. AAV is a non-enveloped virus containing a linear, single stranded DNA genome with a maximal size of about 4.7kb. AAV DNA is housed within an icosahedral capsid and the virion has a diameter of 20-22 nm. Wild type AAV is non-pathogenic and highly prevalent in the human population, with approximately 80% of all serum samples testing positive for AAV serotype 2. AAV is currently the gene therapy vector of choice for many applications at least in part because it has shown efficacy for a range of indications and has an impeccable safety record. Other attractive characteristics of AAV include its ability to infect dividing and non-dividing cells, its ubiquitous nature of tissue infectivity, and its ability to sustain long-term transgene expression. Interestingly, cells infected with AAV do not activate a CTL response. While humoral response to AAV antigens have been reported
during *in vivo* administration, these do not appear to preclude the successful use of AAV for gene therapy.

**Creating a Recombinant AAV Gene Therapy Vector**

AAV2 has traditionally been used as the viral backbone for most recombinant viral constructs. The process of generating a suitable vector involves “neutering” the parental virus by removing all genes that act in the viral replication process. Then, the rAAV is made by simply inserting a promoter/gene construct of interest between the 145 nucleotide-long ITRs. Since the *Rep* and *Cap* genes have been eliminated from the rAAV genome, they must be supplied in *trans*. The serotype origin of the Rep and Cap proteins will confer serotypic cellular specificity to viral progeny based upon the capsid protein configuration and receptor/co-receptor interactions that result. To ensure that the resulting stock of viral particles are free of potentially harmful adenovirus or other helper virus, plasmid constructs that express helper genes are used in culture. Co-transfection of HEK 293 cells with helper plasmids and the pro-viral transgene construct leads to the packaging of AAV particles. Stocks of AAV viral particles are concentrated by cesium chloride gradient where AAV virions have a buoyant density of 1.39-1.41 g/cm$^3$. Chromatography can also be used to isolate AAV virions by exclusion based upon particle charge, binding affinity, or size. Final virus stock concentrations are generally denoted in terms of viral particle number (total number of DNA packaged virions per volume), infectious units (number of
particles capable of infecting host cells), or transducing units (number of particles capable of transferring their DNA to host cells).

**AAV Tropism**

The ability to tailor the tropism of AAV to specific tissues makes it an attractive gene therapy shuttle vector. By changing the configuration of the capsid proteins we have the ability to influence the binding affinity of virions to cell specific receptor complexes. Current strategies to maximize the targeting efficiency of AAV delivery to tissues revolve around the process of creating AAV pseudotypes. Generally, this involves combining the rep genes from AAV2 with the cap genes from another AAV serotype. For example, by creating an AAV2/9 pseudotype one can systemically deliver gene therapy vectors that have a high tropism for cardiac and skeletal muscle. The vast array of cellular specificities that can be targeted by recombinant AAV vectors is summarized in several review articles 113, 115, 116. Strategic manipulation of capsid confirmations has given researchers the ability to target therapies to almost every cellular phenotype and thus a great number of AAV capsid/therapeutic gene combinations are being tested to treat diseases stemming from protein deficiencies, allelic polymorphisms, autoimmune disease, and cancer, to name a few. Capsid manipulation is achieved through a process known as capsid shuffling. Changing the ratio of capsid proteins refines AAV tropism and generates new chimeric AAV serotypes. Researchers at the University of North
Carolina have developed an in vivo “panning” method to isolate chimeric AAVs that are most specific to target tissues. This technique has been used to isolate newly recombined AAV shuttle vectors comprised of capsid proteins from several serotypes that confer a higher level of tissue specificity. Antibody targeting methods have also been used to target AAV to non-permissive cells through the fusion of bispecific antibodies to the AAV capsid.

Membrane Penetration

Optimal infection efficiency for rAAV vectors of AAV2 origin occurs via its primary cell surface receptor, heparan sulfate proteoglycan. Co-receptors aid in the process of internalization and include αvβ5 integrin heterodimers, c-Met (hepatocyte growth factor receptor), and fibroblast growth factor receptor type 1. Some cell surface receptors for AAV are shared among the different serotypes while other receptors are exclusive. The configuration of capsid proteins determine the specificity. Serotypes that share a larger percentage of homology in capsid structure can infect a correspondingly greater array of tissues while those with fewer shared structures infect non-overlapping tissues. A summary of AAV serotypes and their cell surface receptors are presented in Table 1.
<table>
<thead>
<tr>
<th>Serotype</th>
<th>Receptor</th>
<th>Co-Receptors</th>
<th>Target Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV1</td>
<td>Not described</td>
<td>N-linked sialic acids</td>
<td>Skeletal muscle, liver, CNS neurons, ependymal and glial cells, retinal pigmented epithelium, pancreas</td>
</tr>
<tr>
<td>AAV2</td>
<td>HSPG 120</td>
<td>FGFR1 124, HGFR 123, AvB5 integrin 121, 37/67kd laminin receptor 126, A5B1 integrin 127</td>
<td>CNS cells, ubiquitous</td>
</tr>
<tr>
<td>AAV3</td>
<td>HSPG 128</td>
<td>37/67kd laminin receptor, FGFR1 128, HGFR 129</td>
<td>Megakaryocytes</td>
</tr>
<tr>
<td>AAV4</td>
<td>O-linked 2,3-sialic acid 130</td>
<td>Not described</td>
<td>Retinal pigmented epithelium, CNS neurons</td>
</tr>
<tr>
<td>AAV5</td>
<td>N-linked sialic acids 131</td>
<td>PDGFR 132</td>
<td>Lung alveolar cells, liver cells, CNS neurons, ependymal and glial cells, retinal pigmented epithelium, photoreceptors</td>
</tr>
<tr>
<td>AAV6</td>
<td>HSPG 133</td>
<td>N-linked sialic acids</td>
<td>Skeletal muscle, apical airway cells, liver cells</td>
</tr>
<tr>
<td>AAV7</td>
<td>Not described</td>
<td>Not described</td>
<td>Skeletal muscle, CNS neurons, retinal pigmented epithelium, photoreceptors</td>
</tr>
<tr>
<td>AAV8</td>
<td>Not described</td>
<td>37/67kd laminin receptor</td>
<td>Liver cells, skeletal and cardiac muscles, hepatocytes, CNS neurons, retinal pigmented epithelium, photoreceptors, pancreas</td>
</tr>
<tr>
<td>AAV9</td>
<td>Not described</td>
<td>37/67kd laminin receptor</td>
<td>Skeletal muscle, hepatocytes, apical airway cells, retinal pigmented epithelium, pancreas, brain</td>
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<td>Not described</td>
<td>Brain 134</td>
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<tr>
<td>AAV11</td>
<td>Not described</td>
<td>Not described</td>
<td>Not described</td>
</tr>
<tr>
<td>AAV12</td>
<td>Not described</td>
<td>Not described</td>
<td>Nasal epithelium 135</td>
</tr>
</tbody>
</table>

Membrane to Nuclear Entry

While much is known about the cellular receptors required for AAV infection, less is known about the steps that guide the viral particle to the nucleus. Studies on AAV internalization suggest that clathrin coated vesicles form following receptor binding 136, 137 and serve as the major mechanism for virion internalization. However, alternative routes of viral penetration may
Following endocytosis, AAVs escape endosomal degradation and are transported to the nucleus along microtubules through association with motor proteins. AAV has a diameter of approximately 22nm making it one of the smallest viruses and allowing it to cross the nuclear envelope directly through the nuclear pore complex (NPC). DNA is released inside the nucleus in the form of oligomers, linked head-to-tail, or as circular monomers. The entire process of infection, from endocytosis to nuclear entry ranges from 40 minutes to 24 hours.

**Single to Double Strand Conversion and Replication**

Following viral uncoating within the nucleus, single stranded AAV DNA must be converted to double stranded DNA in order to be transcribed. This is considered to be the rate limiting step in AAV infection. Single to double strand conversion is accomplished either by host-cell mediated second strand synthesis or complementary intermolecular base pairing with another rAAV single stranded genome. Only trace amounts of single stranded rAAV DNA templates are ever present in infected host nuclei indicating that the processes of conversion to dsDNA or ssDNA degradation occurs rapidly after viral uncoating. dsDNA conversion occurs by *de novo* second strand synthesis and also by complimentary binding of ssDNA genomes of opposite polarities, known as strand annealing, facilitating transgene expression. Following the generation of duplex AAV genomes, episomes and concatameres can form
through intra or intermolecular recombination, respectively, at the ITRs\textsuperscript{146}.

Transgene expression can be detected by 24 hours following AAV infection\textsuperscript{147}.

**Recombinant AAV Persistence and Safety**

Transgene persistence is an important attribute for most gene therapy applications. Proteins, plasmids, and adenovirus persist in the target tissues on the order of hours, days, and weeks, respectively. Because therapeutic angiogenesis may require sustained presence of growth factors to generate stable vessels, the strategies of protein/gene delivery are likely to require repeat dosing. The AAV vectors on the other hand can persist *in vivo* for several years, making them the first semi-permanent gene therapy option\textsuperscript{114}. A single-dose of appropriately targeted AAV-mediated gene transfer has the potential to offer a permanent solution to recurrent genetic disease or cardiovascular ailments such as myocardial and peripheral ischemia.

The exact mode by which AAV derives semi-permanent persistence is not fully understood. Long-term persistence of episomal rAAV genomes has been reported in studies of murine livers\textsuperscript{144,148,149} and episomal forms of rAAV DNA contribute an overwhelming proportion of the transgene signal in transduced cells\textsuperscript{149}. However, a small population of transduced cells may undergo stable chromosomal integration of the rAAV genome\textsuperscript{149,150,151}. Genome sequencing indicates that integration events occur in regions of the DNA that are transcriptionally active\textsuperscript{152} and rAAV insertion events occur more frequently in
DNA that has been cleaved by endonuclease activity or has suffered chemical or radiation induced damage. Therefore, it is believed that double stranded rAAV genomes insert within host chromosomal DNA at double stranded breaks, suggesting that rAAV delivered DNA is probably no more hazardous to the host genome than plasmid DNA. Overall, AAV, like plasmid integration events, are low and without any apparent toxicity in human or animal studies.
Chapter 4: Hypoxia Regulation of Transgene Expression to Treat Ischemic Disease

The most obvious strategy for gene regulation to treat peripheral ischemia is to target muscle and restrict expression to regions of ischemia by the use of hypoxia-response elements. AAV serotype 9 has demonstrated high tropism for skeletal and cardiac muscle, making it a good candidate as a shuttle vector to deliver a genetic payload to these tissues. Our group first reported that a vector containing hypoxia response enhancer elements (HREs) was activated in the myocardium by ischemia. Subsequently other groups reported similar results in different models of ischemia and different methods of gene regulation by hypoxia. These studies have demonstrated the functionality of Hif-1α and tissue specific promoters to regulate AAV and plasmid derived transgene expression in cardiac tissues. However, these studies have failed to restrain normoxic gene expression in target tissues that resulted in excessive leakiness of the vectors. Until now, the major problem associated with regulation by hypoxia/ischemia has been the weak induction by hypoxia and more importantly by ischemia in vivo, and presence of high basal expression in normoxic tissue. The limited level of regulation precludes the use of tissue-specific or strong basal promoters because of unacceptable levels of gene expression in non-ischemic tissues. Leaky angiogenic growth factors may activate dormant tumors, increase the growth of active tumors and exacerbate conditions such as diabetic retinopathy. Therefore for clinical applications, the growth factors must be
contained within the target tissue, expression must be limited to periods of ischemia, and must be without significant systemic leakage.

Based upon our current knowledge of gradient-based, endothelial and stem cell migratory behavior, we propose that hypoxia regulation of transgene expression is the most appropriate way to deliver therapeutic VEGF, or other pro-angiogenic factors, to ischemic muscle. The limb of a CLI patient is probably not ubiquitously ischemic, if it were there would be global necrosis. In the early stages of PAD, ischemia may be localized to small pockets of unperfused tissue downstream of occluded vessels or damaged capillary networks. As arterial blockages increase in size and number so does the severity of ischemia in downstream tissues. At the most injurious pathological state of CLI, regions of ischemic (hypoxic) and perfused (normoxic) tissue may be closely aligned throughout the musculature of the limb leading to areas of graded ischemia. Predictably, the most severe ischemia is commonly found at the extremities, usually the distal portions the limbs, especially toes because these tissues depend on perfusion through multiple upstream vessels that are subject to atherosclerotic narrowing (e.g. the popliteal and femoral arteries). Even within the most severely ischemic tissues there must exist micro-pockets of tissues that receive blood supply and are perhaps mildly hypoxic; if this were not the case, necrosis would spread much more rapidly than it appears to do in affected subjects.

As discussed above, vascular regeneration begins with endothelial tip and stalk cell activation within normoxic endothelium. Tip cells migrate towards
adjacent hypoxic regions along a chemotactic gradient, primarily driven by VEGF. The processes of endothelial tip and stalk cell activation and migration involves transendocytosis of Notch signaling ligands and receptors. These signals are translated into directional and morphological cellular behaviors. Tight and specific control over growth factor signaling is required for proper guidance of endothelial sprouts toward hypoxic tissues, whereby a single endothelial cell is selected, via lateral inhibition, to lead the endothelial migration. Based upon our knowledge of the role of VEGF in embryonic vascular development we believe that the establishment of a three-dimensional lattice of graded VEGF stimulus, which directly parallels the hypoxic gradient, predetermines endothelial migration, vasculogenesis, and angiogenesis; thus, obligating the need for VEGF transgene stimulus to be directed by a hypoxia regulated promoter.

**Conditional Silencing**

Gene silencers recruit HDAC to eukaryotic promoters and cause transcriptional silencing by chromatin condensation. Suppression of neuronal genes in non-neuronal tissues is achieved when the ubiquitous neuronal responsive silencer factors (NRSF) bind to neuronal responsive silencer elements (NRSE) in the promoters of neuronal genes\(^{162,163}\). The NRSF requires the sequence GCACCGCGG for DNA binding and silences gene expression by recruiting histone deacetylases (HDACs)\(^{163}\). Gene enhancers work in the opposite way by recruiting HAT, relaxing chromatin and activating transcription.
We reasoned that it would be possible to create conditional silencing by combining a constitutive silencer function with a strong inducible enhancer, such that the HAT activity of the enhancer would antagonize the silencer selectively in the induced state. This process is illustrated in Figure 2 (a-c).

Figure 2d describes conditional silencing of a constitutive promoter with high baseline expression levels under the direction of transcriptional hypoxia activation. To create a conditionally silenced vector we inserted tandem repeats (3x) of HREs at -86bp and a conditional silencing switch element (CSE) containing alternating HREs and silencer elements at -164. The phosphoglycerate kinase (PGK) gene promoter has endogenous HREs situated between -280 and -365 upstream of the transcription start site and expression is induced 5.1 ±0.3-fold in skeletal myocytes by hypoxia. Insertion of a CSE at position -495 resulted in >90% silencing of aerobic expression that was reversed by hypoxia. The fold induction of the CS-PGK promoter was 116± 32 (Fig 2d). Conditional silencing also conferred hypoxia-inducibility of >200-fold to the muscle creatine kinase (MCK) and myosin light chain 2 (MLC-2) promoters (not shown). To test for this, tandem alternating NRSEs and HREs were cloned upstream of a luciferase reporter in a truncated SV40 promoter as described in Methods. We found that expression of pCS-SV in C2C12 skeletal myocytes was low when the cells were cultured aerobically but was induced by more than 200-fold in hypoxia.
Figure 2. Conditional Silencing. (a) Gene enhancers (blue box) recruit histone acetyltransferase (HAT) by binding Hif-1α. The neuronal responsive silencer elements (NRSE) recruits histone deacetylase by binding ubiquitous neuronal responsive silencer factors. (b) Addition of multiple NRSEs upstream of a constitutive promoter causes suppression. Gene silencing is antagonized by the addition of gene enhancers. (c) Conditional silencing is achieved by combining a constitutive silencer function with a strong inducible enhancer such that the HAT activity of the enhancer would antagonize the silencer selectively in hypoxia. Silencers suppress gene signaling in the absence of Hif-1α. (d) C2C12 cardiac myocytes were co-transfected with PGK or PGK-CS plasmids and Renilla luciferase plasmids as a control. 24 hours after transfection the cultures were incubated under an aerobic or hypoxic atmosphere for an additional 24h and harvested for reporter expression. (e) C2C12 skeletal myocytes were transfected with pSV-CS (2μg) and pcDNA, pNRSF, or dnNRSF (2μg) and exposed to normoxia (21% O2) or hypoxia (0.5% O2). (f) C2C12 skeletal myocytes were transfected with pSV-CS and subjected to aerobic and hypoxic incubation as above, parallel plates were treated with Trichostatin A (250ng/ml) or vehicle.
Induction was attenuated by over-expression of wild type or dominant negative NRSF. Over-expression of wild type NRSF resulted in hypoxic silencing and over-expression of dnNRSF resulted in the loss of aerobic silencing (Figure 2e). The HDAC inhibitor trichostatin A mimicked the effect of dnNRSF, confirming the role of HDAC in conditional silencing (Figure 2f). Trichostatin A reduced the fold induction from 255 ±44 to 38 ±21, similar to cotransfection with NRSF plasmids. These results confirm an important role for NRSF binding and HDAC activity as mechanisms of conditional silencing.

We have demonstrated that in vitro gene expression can be reversibly silenced by the addition of NRSE sites in tandem with HREs upstream of the PGK promoter. The HAT activity of HREs increased gene induction over 100-fold in response to hypoxia while HDAC activity of NRSF binding silenced normoxic gene expression. Tight control over normoxic gene signal has not been reported in previous studies of angiogenic gene therapy. We believe that the strong induction of hypoxic transgene signal and tight normoxic restriction makes our hypoxia regulatory mechanism ideally suited to drive pro-angiogenic gene expression to treat PAD. We have demonstrated strong hypoxic induction of transgene expression, which we believe is necessary for the establishment of a hypoxia driven signaling gradient, while simultaneously limiting baseline expression and thus reducing the risk of VEGF induced angioma and edema.
**In Vitro and In Vivo Conditional Silencing**

To examine the use of conditional silencing for regulating therapeutic genes in vivo we created adeno-associated virus serotype 9 (AAV9) vectors expressing human VEGF and GFP under the direction of a phosphoglycerate kinase (PGK) promoter with or without conditional silencing elements as described in Methods. AAV9 has been shown to have muscle tropism. Expression of the shuttle vectors in C2C12 skeletal myocytes is shown in Figure 3a. As shown by fluorescence microscopy, myocytes transfected with the HRE vector did not express GFP until they were exposed to hypoxia, consistent with conditional silencing. Also consistent with this, VEGF secretion into the culture medium was enhanced 117-fold under hypoxia (Figure 3b). In data not shown we found that re-oxygenation extinguished gene expression and reactivation was possible by a second episode of hypoxia.

We used a mouse ischemic hindlimb model to determine whether conditional silencing was an effective method to regulate gene expression in vivo. Severe ischemia was induced in Balb/c mouse hind limbs by excision of the entire superficial femoral artery between its junctions with the external iliac and popliteal arteries as described in Methods. In the absence of treatment, limb degeneration is rapid with discoloration of digits apparent during the first 3 days, loss of toes and feet within the first week and auto-amputation of the lower limb within 2 weeks.
Figure 3. *In Vitro and In Vivo Characterization of Conditionally Silenced VEGF Expression*. a) AAV shuttle plasmids expressing GFP and VEGF directed by the PGK promoter or conditionally silenced PGK promoter (CS) were transfected into C2C12 skeletal myocytes and cultured for 48h under normoxia followed by an additional 24h hypoxia. GFP was visualized by fluorescence microscopy and VEGF by ELISA as described in Methods. b) Mouse hind limbs were made ischemic or subjected to sham operation as described in Methods. Mice were sacrificed at 4, 8, 24 and 48h. The thigh muscles removed and cut into 2 longitudinal pieces proximal (P) and distal (D) to the first femoral artery ligation. RNA was extracted and equal amounts separated by agarose gel and analyzed by Northern blots as described previously [40]. c) qRT-PCR analysis of human VEGF (hVEGF) gene expression in mouse hind limb tissue sampled at the indicated time points following limb ischemia surgery. Each limb was injected with 1×10^11vp of either AAV9-HRE-VEGF or AAV9-PGK-VEGF prior to surgery. Tissue sampled from PBS injected limbs at the corresponding time points were used as a control (n=3). d) Doppler ratios show recovery from ischemia in response to AAV9-HRE (red line) and AAV9-PGK (blue line) therapies. Mouse limbs injected with PBS (grey line) or AAV9-GFP (green line) did not recover (n=4).
To detect hypoxia after surgery we measured the expression of marker genes metallothionein (MT) and heme oxygenase (HO)\textsuperscript{167, 168} in anterior (A) and posterior (P) segments of the adductor magnus muscle within and downstream of the excised femoral artery. The transcript levels of both of these markers was induced within 8h of ischemia and remained elevated at 72h (Figure 3c). This result confirms that our model of hindlimb ischemia causes hypoxia in hindlimb musculature and that the stabilized P300/HIF-1\(\alpha\) complexes that bind to hypoxia response elements within nuclear DNA are functional. This is especially important as HIF-1\(\alpha\) complex binding to HREs is at least part of the mechanism that drives transgene expression in our hypoxia regulated gene therapy construct.

The Balb/C ischemic hind limb model was used to determine the contribution of additional HREs to gene expression directed by the PGK promoter. To do this, mice were divided into two experimental groups and the left limbs injected intramuscularly with 50ul PBS containing \(1\times10^{11}\) vp of either AAV9-HRE-VEGF or AAV9-PGK-VEGF (n=3 per time point). Control limbs received only PBS. Injections were administered five days prior to ischemia surgery to ensure that the viral transgene was fully transcriptionally active at the time of induction of ischemia. Three mice from each treatment group served as baseline controls of gene expression in normoxic tissue. Mice were sacrificed at 1-day, 3-days, 7-days, 14-days, 1-month, 2-months, and 6-months after surgery as described in Methods. Adductor magnus muscles of the ischemic limbs were dissected and processed for RNA extraction. Real-time PCR was performed to
evaluate transgene (human VEGF) expression of each vector. We found that baseline hVEGF levels in AAV9-PGK-VEGF showed higher amounts of basal (normoxic) hVEGF gene expression (359 +/- 10 gene copies) than hypoxia regulated AAV9-HRE-VEGF (291 +/- 44 gene copies). AAV9-HRE-VEGF gene expression was induced 181-fold by ischemia and returned to baseline by 1-week after ischemia surgery (Figure 3d). AAV9-PGK-VEGF gene expression was induced 35-fold by ischemia and remained elevated over 2-fold for at least two months following ischemia surgery (Figure 3d). The differences seen in maximal hypoxic induction of the HRE and PGK vectors demonstrates the in vivo functionality of HREs to enhance ischemic signaling in response to our model of CLI. To monitor the recovery of blood flow in response to VEGF gene therapy, Doppler images of ischemic and contralateral limbs were taken over eight weeks post-ischemia surgery as described in Methods. All animals in the AAV9-HRE-VEGF and AAV9-PGK-VEGF therapy groups (n=4 each) demonstrated blood flow recovery through 8 weeks (Figure 3e). Control animals that received PBS or AAV9-GFP vector did not experience blood flow restoration following ischemia surgery (Figure 3e).

Gene Expression Profile and Safety of Unregulated VEGF Gene Therapy

Having confirmed that hypoxia-inducible genes are activated in response to femoral artery ligation/excision surgery within the proximal region of the muscle, and that the addition of HRE/CSs upstream of the PGK promoter
increased hypoxic induction while decreasing baseline gene expression, I expanded my repertoire of vectors to include a completely unregulated AAV9-CMV-VEGF group. Balb/c mice again received 50ul intramuscular injections of AAV9-CMV-VEGF in PBS (1x10^{11} vp; n=3) five days prior to ischemia surgery and three additional mice were set aside to serve as baseline controls for gene expression in normoxic tissue. Mice were sacrificed at 1-day, 3-days, 7-days, 14-days, 1-month, 2-months, and 6-months after surgery. As expected, baseline hVEGF levels in AAV9-CMV-VEGF samples were high (17669 +/- 695 gene copies) compared to AAV9-HRE-VEGF (291 +/- 44 gene copies) (Figure 4). One day after surgery, CMV-VEGF was induced by 3.8 fold over baseline compared with 181-fold for AAV9-HRE-VEGF (Figure 4) consistent with minimal hypoxia-regulation of the CMV promoter. AAV9-CMV-VEGF gene expression remained 440-fold higher than AAV9-HRE-VEGF at six months after ischemia surgery and expression equivalent to 14,000 gene copies were present in the tissue for the 6-month duration of the experiment. Clearly, such high constitutive expression precludes the use of AAV-CMV-VEGF for clinical gene therapy because of side effects associated with spillover and the exposure of tissues to chronically elevated levels of VEGF. Adverse side effects include edema and a risk of enhanced vascularization and thence growth of tumors. Indeed most clinical protocols of angiogenic gene therapy require exclusion of patients with current or history of cancer.

To observe the physiological effect of prolonged exposure to high amounts of VEGF transgene expression, mice were given i.m. injections of
$1 \times 10^{11}$ vp of AAV9-CMV-VEGF or AAV9-HRE-VEGF. Mice were sacrificed two weeks after virus administration without undergoing surgery to induce ischemia. At the time of tissue harvest it was noted that in the CMV treatment group, all mice had pronounced tissue edema and restricted muscle flexibility. After sacrifice and dissection, a yellowish buildup overlying the hindlimb muscle was noted, possibly caused by plasma leakage due to extreme vascular permeability and/or inflammation (data not shown). Edema has been reported as the main side effect of VEGF gene therapy both in preclinical and clinical settings. No such side effects were seen in AAV9-HRE-VEGF treated limbs even in the presence of ischemia perhaps because of the transient high-level of expression of this vector and the rapid establishment of blood flow following treatment (see below). Adductor magnus muscles of the injected limbs were fixed in 10% formalin and embedded in paraffin for tissue sectioning as described in Methods. CD31 stain was performed on tissue cross-sections to evaluate capillary density presented as CD31+ vessels per muscle fiber. Following vessel quantification, CMV treated mice displayed significantly more capillaries than HRE-VEGF treated limbs and normal limb tissue (Figure 5). There was heterogeneity in vessel density within the tissues of AAV9-CMV treatment group, with some regions displaying extremely high capillary density (>20/fiber; see Figure 4a) while other regions were less dense. Higher density regions may be associated with peripheral edema and increased inflammation caused by high VEGF expression. There was no difference in capillary density between AAV9-HRE-VEGF and normal tissues. These results confirm the harmful effects of
prolonged exposure to elevated VEGF or other angiogenic growth factors caused by unregulated AAV gene therapy.

Figure 4. In Vivo Comparison of AAV-Conditionally Silenced and AAV-CMV-VEGF Gene Expression. a) qRT-PCR results for total gene copies of human VEGF (hVEGF) relative to mouse hindlimbs injected with PBS. RNA used for each measurement was isolated from mouse hindlimbs at the indicated times after hindlimb ischemia surgery. b) Fold induction of hVEGF production for each viral vector was calculated as compared to baseline measurements taken from mouse hindlimbs that were injected with viral gene therapy construct but did not have ischemia surgery. * p < 0.05.
Summary

Hypoxia-responsive vectors have been used to target therapeutic genes, including VEGF and heme oxygenase, to cardiac and skeletal muscles. The goal is to auto-regulate growth factor expression by ischemia, promote endothelial activation in adjacent tissues and support the growth of new vessels through regions of ischemia. A major goal is to restrict gene expression to the ischemic tissue, fully extinguish expression when the tissue is reperfused and prevent angiogenic activity of potentially opportunistic recipients such as latent tumors or the retina of diabetics. Our choice of VEGF is...
based on previous work showing that VEGF as sole therapy is capable of promoting a complete program of angiogenesis and possibly arteriogenesis by initiating and modulating a cascade of inter-dependent downstream events that include the activation of other growth factors, cytokines, and modulators of angiogenesis\textsuperscript{170, 171} (this will be expanded on below in our regulated gene therapy model, see Figures 17, 18, and 19). By using conditional silencing we have created a gene switch that responds to ischemia with approximately 180-fold increase in gene expression, more than an order of magnitude greater than any previous reports for \textit{in vivo} regulation\textsuperscript{158, 159, 160, 161}. It should be noted that conditional silencing does not produce a super-active promoter because much of the effect involves suppression of gene expression in normoxic tissue as opposed to an exaggerated response to hypoxia.

We chose to characterize transgene delivery and therapeutic efficacy in ischemic muscle of Balb/c mice based upon recent findings of differences in angiogenic potential among different strains of mice\textsuperscript{172}. Balb/c mice are deficient in their intrinsic angiogenic response following femoral artery ligation compared to other strains such as C57BL/6 mice. The source of this deficiency is described as a lack of response to hypoxia and in particular, defective induction of the VEGFA gene following femoral artery ligation\textsuperscript{172}. To confirm this finding, we measured VEGFA gene expression in Balb/c mice 24h, 72h, and 1-week after ischemia surgery by real-time PCR. The results were compared to normoxic Balb/c limb tissue. The results shown in Figure 6 confirm the results of Chalothorn et al. The blunted hypoxia/VEGF response makes Balb/c mice an
appropriate mouse model for testing gene therapy vectors to treat ischemic
disease because these mice are highly sensitive to ischemia surgery and
recovery poorly in the absence of therapeutic intervention. Patients with arterial
disease and/or diabetes also have defective responses to hypoxia and related
angiogenesis. These results also confirm that any response of these mice to
VEGF gene therapy can be attributed to the exogenous transgene because the
endogenous response is absent.

![Mouse VEGFA Gene Induction](image)

**Figure 6. Deficient Hypoxic VEGF Induction in Balb/c Mice.** In order to determine the level of VEGF-A
gene transcription that is induced by femoral artery ligation/excision surgery, RNA was
isolated from the adductor magnus of Balb/c mice at various times following ischemia
surgery (n= at least 3 per time point). Normal adductor magnus tissue was used as a
baseline = 1. Quantitative real-time PCR results show that Balb/c mice do not mount a
hypoxia induced VEGF-A response.

Our comparison of transgene expression profiles of AAV9-HRE-VEGF and
AAV9-CMV-VEGF (Figure 4) confirm the stringent regulation of the conditionally
silenced vector by hypoxia/ischemia. The levels of hVEGF contained in the hind
limb muscle after intramuscular vector injection, indicate efficient gene transfer by this technique. The rapid responses to ischemia, one day after surgery is also consistent with rapid transcriptional activation of the transgene and high sensitivity to physiological hypoxia. Baseline levels of hypoxia-regulated hVEGF transcription remained low and there appears to be no superfluous capillary expansion observed with the regulated vector. This is a testament to the power of the NRSE normoxic silencing component of the AAV9-HRE-VEGF vector. At $1 \times 10^{11}$ vp, a dose that I estimate to be 500 times higher than necessary for therapeutic efficacy in mouse hindlimb, no adverse effects of the therapy were observed, even after 1 year (see later chapters below). On the other hand, gene therapy with AAV9-CMV-VEGF is harmful to the target tissue and potentially to other organs by systemic transmission. In my studies, this was evidenced by massive vascular proliferation even in the absence of ischemia. These results confirm that gene therapy with AAV-CMV-VEGF and possibly other vectors including plasmids that use the CMV promoter to direct VEGF expression may be unsafe. Clinical studies also suggest that they are lack efficacy in human patients (TAMARIS) $^{60}$. 
Chapter 6: Therapeutic Angiogenesis By Conditionally Silenced VEGF

AAV9-HRE-VEGF vs Adenovirus-VEGF

Previous reports have shown that adenoviral delivery of the VEGF gene was without therapeutic efficacy in patients with PAD (RAVE trial; \(^45\)). Therefore we compared the efficacy of our conditionally silenced AAV9 vector to an adenoviral vector in which a CMV promoter drives hVEGF expression. Mice were separated into three groups (n=5) and intramuscular injections of either 5x10^8vp AAV9-HRE-VEGF, 5x10^8vp Ad-VEGF, or 5x10^9 Ad-VEGF were administered at the time of hind limb ischemia surgery. Laser Doppler perfusion imaging (LDPI) was performed weekly to quantify the recovery rates of each experimental group. As shown in Figure 7a, mice given either high or low dose Ad-VEGF therapy experienced recovery of limb perfusion for approximately eight weeks following surgery. However, perfusion declined precipitously after 12 weeks and by six months after surgery, all limbs auto-amputated as a result of vascular insufficiency and ischemia-induced necrosis. AAV9-HRE-VEGF treated mice displayed similar recovery trends to those of the Ad-VEGF treatment groups during the first eight weeks; however perfusion recovery of the HRE-VEGF treatment group remained stable through 12 weeks. Isometric limbs in this group had recovered approximately 70% perfusion compared to contralateral limb at six months post surgery (Figure 7).
Figure 7. VEGF Mediated Ischemic Recovery: Ad-VEGF vs AAV9-HRE-VEGF. a) Laser Doppler perfusion imaging of ischemic hindlimb. Limbs injected with AAV9-HRE-VEGF maintain stable perfusion 6 months after limb ischemia surgery. Adenovirus treated limbs lose perfusion around 8-weeks and limbs autoamputate by 6-month follow-up. b) RT-PCR results showing total hVEGF expression in ischemic limbs injected with Ad-VEGF at the indicated time points. hVEGF gene expression steadily decreased over 12 weeks. The loss of adenovirus hVEGF expression correlates with a loss of limb perfusion. c) Representative Dil confocal images of 10x fields of view in Ad- and AAV9-HRE-VEGF treated hind limb muscle, 12 weeks after ischemia surgery. Velocity statistical evaluation of Dil images revealed 2.4 times higher vascular density in the AAV9-HRE-VEGF therapy group versus Ad-VEGF therapy (n=4).
The successful outcome of AAV9-HRE-VEGF gene therapy on limb salvage compared to Ad-VEGF can be partially attributed to the non-pathogenic, non-immunogenic properties of the AAV vector. Because of immune rejection, gene expression from adenoviral vectors is transient. This phenomenon is manifested in our experiments by a loss of perfusion after eight weeks. Loss of perfusion correlates with a parallel loss of adenoviral-directed gene expression that approached zero between 8-12 weeks after delivery (Figure 7b).

To further explore the differences between angiogenic response to AAV9-HRE-VEGF and Ad-VEGF therapies, DiI perfusion imaging was conducted on mice (n=4) at time points of 12-weeks or later after treatments (see Methods). DiI images of AAV9-HRE limbs and Ad-VEGF limbs revealed 2.4 times higher vascular density (p<0.05) in AAV9 limbs as measured by Volocity statistical software (Figure 7c and see Methods). This is consistent with our observation that Ad-VEGF limbs experience decreased perfusion at 12-weeks compared to AAV9-HRE-VEGF treated limbs. In a previous report from this laboratory we tested adenoviral gene therapy in a rabbit model. In this study we found that an initial burst of angiogenesis and capillary production following Ad-hVEGF-medicated gene therapy was followed by apoptosis and degradation of de novo vessels \(^{174}\). This is also consistent with our findings in the Balb/c model described here.

In summary, gene expression profiles demonstrate that AAV9 vectors are competent for strong gene expression for at least six months after delivery to ischemic muscle and the presence of conditional silencing elements in these
vectors confers tight regulation by ischemia (Figure 4a). The loss of VEGF expression by adenoviral gene therapy and the sustained expression by AAV9-HRE-VEGF contributes to vascular collapse in the former and may be required for stable reperfusion in the latter. The consequence is auto-amputation or permanent limb salvage, respectively. These results are consistent with our previous report using a rabbit model and highlight the high therapeutic potential of hypoxia-regulated AAV as a gene therapy vector.

Hypoxia Regulated AAV9-HRE-VEGF is Superior to Unregulated AAV9-CMV-VEGF

(i) Dynamics of reperfusion and limb salvage by AAV9-CMV-VEGF

To investigate the relative therapeutic efficacies of conditionally silenced versus unregulated AAV vectors directing hVEGF gene expression I compared therapy by low-dose AAV9-HRE-VEGF and AAV9-CMV-VEGF in the Balb/c ischemia hind limb model using Doppler score and limb salvage as outcomes. These experiments were designed to specifically test the effects of regulation in the same semi-permanent AAV vector system. Adductor magnus muscles were injected with $5 \times 10^8$ vp of either AAV9-HRE-VEGF or AAV9-CMV-VEGF diluted in 50ul PBS and a third group with PBS as placebo/vehicle control, as described in Methods. Doppler images were taken weekly for 4 weeks to monitor reperfusion and paws were photographed to document necrosis (see Methods). Limbs treated with conditionally silenced AAV9-HRE-VEGF experienced only
minor necrosis evidenced by discoloration of digits and toenails after the one-week and perfusion was significantly recovered at two weeks (Figure 8a and b). In mice treated with AAV9-CMV-VEGF there was early evidence of severe necrosis of toes and paws within one-week of surgery and 50% of the limbs auto-amputated within 2 weeks. Indeed the AAV9-CMV-VEGF treatment group suffered the same degree of tissue degeneration and limb loss as the control (PBS) group (Figure 8a and b). Composite Doppler scores (Figure 8a) confirmed absence of reperfusion in AAV9-CMV-VEGF and PBS groups and progressive recovery of the AAV9-HRE-VEGF group.

Figure 8. Dynamics of Reperfusion and Limb Salvage: AAV9-CMV-VEGF vs AAV9-HRE-VEGF. Intramuscular injections of either AAV9-HRE-VEGF (5x10^8 vp), AAV9-CMV-VEGF (5x10^8 vp), or 50ul PBS were administered in Balb/C hindlimb at the time of limb ischemia surgery (n=4). a) Doppler ratio measured by laser doppler perfusion imaging shows recovery of blood flow with hypoxia regulated gene therapy. Unregulated gene therapy and PBS treated limbs do not recover. b) Light images showing representative limbs for each treatment 1-week after surgery (top). Doppler images of mouse hindquarters show similar levels of blood flow in normal and HRE treated mice. PBS and CMV mice are missing their right hind limb due to necrotic auto-amputation by 2 weeks after femoral artery ligation/excision.
(ii) Histology and immunostaining for capillary density

Muscles were harvested and sections immunostained with anti-CD31 antibodies as described in Methods. As shown in Figure 9, quantification of CD31 positive vessels in the hindlimb cross-sections revealed significantly higher levels of vascular proliferation in limbs treated with AAV9-CMV-VEGF relative to controls at one and two weeks after ischemia surgery (Figure 9a and b). The capillary densities of AAV9-HRE-VEGF treated limbs were intermediate between the CMV-VEGF and control groups (Figure 9a). Notably, there were significantly more CD31+ vessels in the AAV-HRE-VEGF and AAV-CMV-VEGF treatment groups compared with the PBS. This indicates that vessel degeneration apparent in the PBS group does not occur in the VEGF treatment groups, presumably because the tissue is protected by VEGF expression in the latter conditions. One month after surgery the vessel densities in muscles from, both AAV9-CMV-VEGF and AAV9-HRE-VEGF treatments were similar to normal limb muscle (Figure 9c). These results were confirmed by imaging a fluorescent Isolectin B4 dye (Figure 9d).
These results highlight a unique feature and perhaps a paradox of VEGF gene delivery that underlies a central theme of our approach to gene therapy, namely that capillary density does not necessarily correlate with recovery from ischemia, limb salvage or protection from necrosis. Mice treated with unregulated (CMV) VEGF maintained higher densities of CD31 and isolectin-B4 positive vessels compared with conditionally silenced or control groups, yet this group sustained severe necrosis and ultimately auto-amputation of the lower limb. The results indicate two scenarios of muscle insufficiency, one caused by a capillary density that is too low to sustain muscle integrity (PBS), the other caused by high, but possibly disorganized, capillary density that leads to the same outcome of insufficient oxygen and nutrient transport to the muscle. In
contrast, the use of a conditionally silenced vector confers protection against necrosis of the toes, allows limb salvage and promotes rapid tissue reperfusion. We hypothesize that this is because hypoxia-regulated gene therapy reproduces the natural course of a healthy response to ischemia where VEGF expression is restricted to hypoxic tissue and directs an organized program of directional vessel growth and regeneration.

DiI Perfusion Imaging Reveals Morphological Differences Conferred by Hypoxia Regulation; Evidence for Arteriogenesis

(i) Essential roles for hypoxia-regulated angiogenesis

Neovascularization in embryonic as well as adult tissues occurs by the processes of angiogenesis and vasculogenesis. During angiogenesis, endothelial cells of viable tissue within or surrounding ischemic regions proliferate, migrate, and remodel into neovessels that grow into the ischemic tissue. Vasculogenesis is a de novo process that involves recruitment of progenitor stem cells, many of which originate in the bone marrow. Both angiogenesis and vasculogenesis are required for tissue regeneration and/or repair of damage caused by ischemia. VEGF is strongly induced by hypoxia, an obligate component of ischemia. In cooperation with the drosophila (and mammalian) morphogen, sonic hedgehog (SHH) VEGF has been shown to orchestrate embryonic arteriogenesis by acting at multiple levels to regulate the expression of downstream effectors. Recent studies have shown that a
balance between proliferation and differentiation signals must be established in order to construct a stable vascular network \cite{89,90}. However, widespread, unregulated expression of growth factors such as VEGF may not alone be able to create the proper niche for neovascularization. In fact, my results described above, demonstrate that unregulated expression of VEGF in ischemic mouse hind limb produces erratic vessel growth that does not appear to support conduction to the limb extremities.

(ii) Adaptation of Dil imaging for skeletal muscle

Although originally developed for visualization of the capillary networks of the eye, I used Dil perfusion imaging as an essential tool to demonstrate the regenerative capability of hypoxia regulated AAV9 gene therapy. As described in Methods, this technique has enabled me to visualize vessels ranging in sizes from arteries to capillaries in whole mount tissue samples to a depth of approximately 500 microns from the tissue surface (Ms submitted to *Biotechniques*, 2012). The use of a motorized stage and preset imaging fields allows me to visualize the entire sagittal plane of a mouse hind limb between the iliac/superficial femoral artery junction and the popliteal/saphenous arteries in as little as 10 minutes. Composite images measuring approximately 11mm by 4mm are created through the use of photo stitching software or can also be done by hand, using simple photo editing programs (see Methods).
Balb/c mice were injected with $5 \times 10^{10}$ vp of either AAV9-HRE-VEGF (n=4) or AAV9-CMV-VEGF (n=4), five days prior to surgery. Following femoral artery ligation and excision the mice were housed for eight months to allow imaging and characterization of regenerated vessels. At that time the mice were sacrificed for DiI perfusion imaging analysis. All animals in the AAV9-HRE-VEGF treatment group recovered limb perfusion and retained paws and toes with minimal evidence of tissue necrosis. In contrast all mice in the AAV9-CMV-VEGF treatment group lost paws and most of the lower limb to necrosis. DiI imaging revealed that large blood vessels regenerated across the ischemic zone along the path of the femoral nerve, only in the AAV9-HRE-VEGF treatment group. In contrast, limbs of mice in the AAV9-CMV-VEGF treatment group displayed erratic disorganized vessel growth in the same region. Representative images are shown below in Figure 10a.

(iii) Quantification of directional vessel regeneration

Direct visualization of Dil-stained muscle sections suggested to me that vessel regeneration by AAV9-HRE-VEGF was directional in character, with vessels growing in a longitudinal manner similar to the longitudinal orientation of the femoral nerve. This did not appear to be the case for vessel growth in response to AAV9-CMV-VEGF. To test for this possibility, I implemented a quantitative analysis of vessel directionality, as indicated by divergence from longitudinal growth, using the DiI images and Volocity software (see Methods section for details).
In this analysis, the path of the femoral nerve was used as a standardized path (divergence = 0) across the ischemic zone. Each vessel was measured for “directionality” with respect to the path of the original femoral artery. The results of this analysis show that regenerated vessels in limbs treated with AAV9-HRE-VEGF grow in a direction that adheres significantly more closely to the original femoral artery (less divergence) than do those treated with AAV9-CMV-VEGF.
Significantly longer vessels were also regenerated in response to AAV9-HRE-VEGF than to unregulated VEGF (Figure 10c).

**Artery Regeneration is Observed over the Course of 1 Year, Post-Ischemia Surgery**

I used Dil composite imaging to observe the pattern of vessel regeneration in Balb/c mice following gene therapy with AAV9-HRE-VEGF over the course of one year following ischemia surgery. To do this, mice were sacrificed at multiple time points and the region between proximal and distal sutures was imaged (Figure 11). The results of this time-course clearly display the progressive and coordinated nature of arterial regeneration resulting from hypoxia regulated /CS-VEGF gene therapy. The beginnings of large vessels can be seen originating from the distal and most hypoxic region of the hypoxic zone, where ischemia is more severe and the VEGF concentrated highest. This is evidenced by angiogenic sprouts seen as early as 1-week after surgery (Figure 11, top). By 12-weeks these sprouts begin to coalesce into larger arterioles growing longitudinally and parallel with the femoral nerve, the path along which the formerly excised femoral artery and vein once lay. By one year after surgery, arteries of comparable size and length to the intact femoral artery have regenerated. These results demonstrate for the first time that hypoxia-regulated VEGF production in a gene therapy setting supports directional vessel growth that culminates with arteriogenesis. The results also suggest that regulation by hypoxia provides the cues that support such growth. By extension, unregulated
VEGF production does not provide such cues, and the ensuing gene therapy promotes disorganized vessels that may even be detrimental to reperfusion by diverting blood flow.

To confirm these results I implemented live animal MRI angiography followed by immunohistochemical analyses of tissue sections through the region of the femoral tract. Representative results from a 43 week AAV9-HRE-VEGF treatment group are shown in Figure 12. Light microscope images of the hypoxic zone reveal a large vessel that closely followed the path of the femoral nerve (Figure 12a) and could also be detected by MRI angiography (Figure 12c). H&E stain and fluorescent images of cross-sections of the same limb show arteries with thick layers of smooth muscle actin lining the vessel walls (Figure 12b). These results suggest that the regenerated vessel is an artery with structure and dimensions comparable with the original femoral artery. The mode of arterial growth was unique to the AAV9-HRE-VEGF group.
Figure 11. One Year Time-Course Reveals AAV9-HRE-VEGF Mediated Arteriogenesis. Composite Dil images of ischemic mouse hind limb treated with AAV9-HRE-VEGF. Angiogenic sprouts can be seen 1 week after ischemia surgery originating at the distal suture location (right side of the image). Progressive regeneration of the femoral artery along the femoral tract continues over the course of 1 year. Regenerated vessels are of similar size and length as a normal femoral artery (bottom image). Time-points are indicated at the left side of each image. X represents the location of sutures used in ischemia surgery. Yellow arrows highlight the path of the femoral nerve.
Conditionally Silenced AAV9-HRE-VEGF is Superior to Partial Hypoxia-Regulated AAV9-PGK-VEGF in the Generation of Large Arteries

My results show that there are marked morphological differences between vessels regenerated by regulated (AAV9-HRE-VEGF) versus unregulated (AAV9-CMV-VEGF) gene therapy. To further explore the possible requirements for stringent hypoxia regulation in this respect I, conducted additional experiments to compare tight hypoxia regulation by conditional silencing with conventional hypoxia-regulation by a natural HIF-1α promoter. For the former I used AAV9-HRE-VEGF, for the latter I used the phosphoglycerate kinase promoter, one of the most strongly hypoxia-regulated gene promoters. This comparison is especially relevant because AAV9-HRE-VEGF contains the PGK
promoter with additional conditional silencing elements to tighten the regulation. The only difference between AAV9-PGK-VEGF and AAV9-HRE-VEGF is the presence or absence of these CS elements.

Morphological comparisons of blood vessels present within the ischemic region were conducted following ischemia surgery in mice given intramuscular injections of $5 \times 10^{10}$ vp of either AAV9-HRE-VEGF or AAV9-PGK-VEGF ($n=3$). After 12 weeks mice were sacrificed and hind limb muscle fixed and stained and quantified by Volocity software as described in Methods. Fluorescence imaging of smooth muscle actin revealed vessels of significantly larger cross-sectional area in tissue sections from mice injected with AAV9-HRE-VEGF gene therapy compared with those treated with AAV9-PGK-VEGF (Figure 13a and b). Dil imaging of the ischemic zone also revealed that the arterial regenerative capability of AAV9-PGK is inferior to that of AAV9-HRE (Figure 13c). Comparisons of multiple Dil images ranging in recovery times from four months to over one-year post-ischemia surgery, revealed significantly more directional growth (adherence to the femoral tract) in AAV9-HRE-VEGF treated limbs compared with AAV9-PGK-VEGF ($n=6$ per treatment) (Figure 13d).
Figure 13. Arteriogenesis: AAV9-PGK-VEGF vs AAV9-HRE-VEGF. a) Representative images of smooth muscle actin stained blood vessel cross-sections. b) AAV9-HRE-VEGF gene therapy yields vessels of significantly larger cross-sectional area 12 weeks after limb ischemia surgery than AAV9-PGK-VEGF therapy (n=3). c) Representative composite Dil images of artery regeneration one year after AAV9-HRE-VEGF therapy (top) and AAV9-PGK-VEGF therapy (bottom). d) Quantification of vessel directionality with respect to the femoral nerve reveals significantly more deviation in vessels that respond to AAV9-PGK-VEGF gene therapy than those that respond to AAV9-HRE-VEGF. (n=6)
Summary

Use of a conditionally silenced vector was shown to support early reperfusion of the hind limb, tight auto-regulation by ischemia, permanent salvage of the hind limb and evidence for regeneration of a large artery within or close to the femoral tract at 6-months to more than one-year post-surgery. Large arteries of comparable diameter to the excised femoral artery were only regenerated by VEGF that was expressed under stringent regulation by hypoxia. Mature regenerated vessels stained positively for smooth-muscle actin, confirming that the procedure promotes regenerating arteries and that regenerated arteries grow longitudinally parallel to the femoral groove, the region where the formerly excised femoral artery and vein once lay. Vessel regeneration and remodeling appears to continue over at least 6 months following ischemia surgery and gene therapy. This may mean that a semi-permanent source of pro-angiogenic signal (from AAV) is essential to guide arteriogenic maturation. However, despite being a semi-permanent form of VEGF gene therapy, full artery regeneration did not appear to be associated with the less stringently regulated AAV9-PGK-VEGF vector or unregulated AAV9-CMV-VEGF. In the latter case I hypothesize that leaky expression in normoxic tissue is responsible for the difference. The results indicate that vector persistence within target tissues is not the main factor for directional arteriogenesis. Because artery regeneration only occurred in response to AAV9-HRE-VEGF gene therapy we believe that unique VEGF signaling conferred by conditional silencing, which responds to the hypoxic gradient of the ischemic
microenvironment by generating a parallel gradient of VEGF, is the key regulator of arteriogenesis.

Thus far, the results of my analyses suggest that hypoxia-regulation is the driving force for directional arteriogenesis in this model whereas dose and persistence play more minor roles. Conditionally silenced gene therapy promotes longitudinal vessel growth down the localized gradients of ischemia and this is superimposed on the global thigh to toe gradient that is created by the femoral excision. Longitudinal vessel growth within the ischemic region is predicted to allow contacts with vessels upstream of the ischemia region as well as downstream of the region of femoral artery excision, which is the most severely ischemic. Such directional vessel growth may facilitate contacts between the regenerating vessel(s) and the iliac artery upstream that would allow continuity of blood flow from upstream circulation. Such communications and consequentially enhanced blood flow may generate the increased shear stress that is required for arteriogenesis. In contrast the toxic effects of AAV9-CMV-VEGF gene therapy indicate that sustained VEGF in the absence of regulation by hypoxia is not sufficient to resolve ischemia and may be detrimental. Therefore, my results show for the first time that stringent regulation by hypoxia, and baseline silencing conferred by the addition of tandem HREs and NRSEs to the PGK promoter create a gene therapy vector that has superior properties and may be able to regenerate the entire femoral artery.
Chapter 7: Optimization of AAV9-HRE-VEGF; Addition of Non-Tissue Specific Silencer Function

Construction of AAV9-TOAD-VEGF

Even low basal, normoxic levels of expression of a pro-angiogenic gene is a concern when using vectors such as AAV that confer semi-permanent gene delivery. To address this concern we sought to minimize transcriptional leakiness by enhancing the silencer function of the AAV9-HRE-VEGF vector. To do this we identified two silencer elements contained in the B-cell specific B29 promoter that were described 15 years ago and named FROG and TOAD\textsuperscript{176}. FROG/TOAD are cis-acting DNA regulatory elements that down-regulate transcription of the downstream linked genes. Like the NRSE element, FROG/TOAD bind specific nuclear DNA-binding protein complexes and function in both a position and orientation independent manner. The two motifs do not share any significant sequence homology\textsuperscript{176}. The combination of a single FROG and TOAD silencing element has been shown to maximally silence basal levels of gene transcription and the addition of multiple copies of these two unique sequences did not confer additional silencing to the B29 promoter\textsuperscript{176}. We created the FROG/TOAD plasmid by inserting a double alternating FROG-TOAD sequence downstream of the HRE/NRSE conditional switch motif present in the HRE-VEGF construct. Thus, the sequence of the inserted DNA fragment is shown below:
The new, more stringently regulated vector is referred to as AAV9-TOAD-VEGF.

**In Vivo Gene Expression Profile**

To evaluate the effects of adding the TOAD silencing motif to the HRE construct, mice were given intramuscular injections of $1 \times 10^{11}$ vp one week before ischemia surgery and divided into groups of three for sacrifice at day-1, day-3, day-7, day-14, 1-month, 2-months, and 6-months after surgery. Following harvest of the adductor magnus muscle of the ischemic hind limb, real-time PCR was performed to evaluate human VEGF expression of each vector. As expected, hVEGF transcription from AAV9-TOAD-VEGF produced lower numbers of gene copies than AAV9-HRE-VEGF when maximally induced at Day-1 after surgery (Figure 14), however baseline levels of hVEGF gene expression, as measured from non-surgery control mice, were not significantly different ($p=0.1$). Maximal gene induction over baseline in response to hypoxia was muted in AAV9-TOAD-VEGF compared to AAV9-HRE-VEGF; 12-fold vs. 181-
fold increase at Day-1 (Figure 14b). These results confirm a high level of silencing by addition of the TOAD silencer suggesting that for this vector there is insufficient HIF-1α to compete with the silencer proteins even under severe ischemia in the Balb C muscles.

Figure 14. Comparison of AAV9-HRE and AAV9-TOAD-VEGF Gene Expression. a) qRTPCR analysis of human VEGF (hVEGF) gene expression in mouse hind limb tissue sampled at the indicated time points following limb ischemia surgery. Each limb was injected with 1x10^11 vp of either AAV9-HRE-VEGF or AAV9-TOAD-VEGF prior to surgery. Tissue sampled from PBS injected limbs at the corresponding time points were used as a control. b) Fold induction of hVEGF production for each viral vector was calculated compared to baseline measurements taken from mouse hindlimbs that were injected with viral gene therapy construct but did not have ischemia surgery. *p<0.05, n=3.
Therapeutic Benefit: TOAD vs. HRE

To evaluate the regenerative capabilities of AAV9-TOAD-VEGF, mice were administered intramuscular injections of $5 \times 10^8$ vp and hindlimb ischemia surgery was performed to occlude blood flow to the limb ($n=4$). Doppler images were captured over a period of eight weeks and the results were compared to those of AAV9-HRE-VEGF therapy. Doppler flow results demonstrated a similar trend in re-establishment of blood flow in both treatments over the course of eight weeks (Figure 15a and b), however, visual inspection of the limbs, paws and digits of the TOAD-treatment group revealed significantly less necrosis compared with the HRE-treatment group (Figure 15c). Therefore enhanced silencing conferred by the addition of TOAD silencers to the prototype HRE construct may allow more efficient and rapid reperfusion during the early response to severe ischemia in this model.

![Graph showing Doppler flow results](image1)

![Representative doppler images showing blood flow recovery](image2)

![Images showing limb necrosis](image3)
DiI Perfusion Imaging Reveals a Similar Patterns of Blood Vessel Recovery Among Hypoxia Regulated Therapies

Mice received 5x10^{8}vp of either AAV9-HRE-VEGF or AAV9-TOAD-VEGF i.m., and evaluations of the regenerated vasculature were conducted six months after ischemia surgery (n=4). Volocity software was used to trace continuous vessel paths within the area between the proximal and distal sutures that marked the positions of the occluded the femoral artery. The results of these analyses revealed that both vectors conferred similar degrees of directional vessel growth and equivalent reperfusion (Figure 16b). To highlight the benefit of hypoxia regulated VEGF therapy this evaluation was expanded to include all groups of vectors, including AAV9-CMV-VEGF (n=3), AAV9-PGK-VEGF (n=6), AAV9-HRE-VEGF (n=14) and AAV9-TOAD-VEGF (n=4). Quantitative data was obtained from mice with recovery times of at least four months and included vector doses between 5x10^{8} and 5x10^{10}vp. There was no apparent difference between dose (not shown); the results show that regenerated vessels in the limbs of AAV9-CMV or PGK-VEGF mice deviated significantly from the original path of the femoral nerve as compared to those of either of the conditionally silenced vectors (Figure 16c). Images of normal hindlimb vasculature were used as controls (n=4).
Summary

In this chapter I have shown that the addition of a non tissue-specific silencer element to the parent AAV9-HRE-VEGF in the form of the TOAD silencer reduced baseline leakiness in normoxic tissue. RNA analysis of hind limbs treated with AAV9-TOAD-VEGF reveal the lowest absolute and baseline values of VEGF transgene expression following limb ischemia surgery of any of our AAV9-VEGF constructs. Although the added silencer muted the hypoxic
inducibility of AAV9-TOAD-VEGF, this did not negatively impact reperfusion rates following induction of ischemia. In fact I observed an increase in the rate of reperfusion with less necrosis of digits compared to AAV9-HRE-VEGF therapy. Maintaining tight control over baseline normoxic transgene expression is a clinical goal. Therefore I believe that this can be achieved with AAV9-TOAD-VEGF without sacrificing therapeutic benefit.
Chapter 8: Notch Signaling within Expanding Endothelium is Required for Successful Recovery from Ischemia Surgery and Regeneration of the Femoral Artery

Angiogenic homeostasis must be maintained to prevent pathological tissue disorders. For patients with severe PAD, major arteries become occluded by atherosclerotic plaque on the inside of vessel walls. An insufficient growth factor response to the ensuing ischemia limits angiogenesis and prevents adequate collateral vessel generation that in turn precludes alleviation of ischemia, promotes oxygen and nutrient insufficiency and progressive necrosis. Gene therapy clinical trials using plasmid or adenoviral vectors have attempted to compensate for tissue insufficiency of patients by delivering exogenous growth factors. However these have so far been ineffective to provide long-term limb salvage, the most important endpoint of therapy. It seems possible that this is due to an overly simplistic approach to the problem. We believe that for successful gene therapy the production of stable vessels requires at the minimum the establishment of signaling gradients of the exogenously supplied growth factors. My results presented above support this and suggest that these gradients can be achieved by delivering hypoxia-regulated gene therapy vectors. Ample evidence from other work also supports this conclusion.

In a scenario of limb ischemia, when a quiescent endothelial cell layer encounters a VEGF gradient endothelial cells initiate an organized migratory response that is dependent upon a strict balance of cell migration, transformation
and replication processes. Migratory endothelial cells follow the signaling gradient traveling from regions of low VEGF to high VEGF signal. These cells, known as ‘tip cells’, guide trailing endothelial cells, known as ‘stalk cells’, to the chemo-attractant source. In order for luminal integrity to be maintained during this migratory process, proper distribution of tip and stalk cells must be maintained. My hypothesis is that ectopically administered VEGF stimulates successful stable revascularization of ischemic muscle, by orchestrating the reversible activation of multiple other genes that are required for functional vessel regeneration. Particularly important in this respect is the ratio of VEGF:Notch pathway genes. To test for this I measured a battery of hypoxia marker genes, growth factors and angiogenic modulators during ischemia and following gene therapy with conditional silencing.

Activation of Notch Pathway Genes and Regulation by VEGF and Ischemia

qRT-PCR measurements of marker genes involved in hypoxia and vessel regeneration were analyzed at 1-day, 3-days, 7-days and 28-days following ischemia surgery as described in Methods. To reconfirm the presence of hypoxia in the muscle adjacent to the femoral nerve following femoral artery excision I first quantified the expression of two hypoxia-regulated genes. Heme oxygenase (HO) is strongly induced by hypoxia at the transcription level; HIF-1α has been shown to be regulated both transcriptionally and post translationally by
hypoxia (reviewed in 178). As shown in Figure 17, HO and HIF-1α gene transcription were both strongly induced during the first week after femoral artery excision; HO peaked at day 4 and declined close to basal level after 28 days in all experimental groups suggesting that the most severe hypoxia occurs within the first 3 days after surgery as expected. HIF-1α levels displayed similar regulation although it remained elevated in the control (PBS) group perhaps suggesting that ischemia is not well resolved in the thigh muscles of this group even after 4 weeks, a time when the lower limb auto-amputated. Ischemia appears to be resolved in each of the AAV treatment groups including AAV9-CMV-VEGF despite lower limb auto-amputation. These results confirm a period of intense hypoxia within the muscles surrounding the excised femoral artery for at least 1-week following surgery. The results are consistent with those described in Figure 3. It is noteworthy that expression of the hVEGF transgene from conditionally silenced promoters delivered by AAV9 (HRE and TOAD) appears to be activated sooner and is turned off more rapidly than the endogenous genes. The reason for this is not clear, but may reflect competition of HREs for available HIF-1α and/or the epigenetic, non-integrated condition of the transgene that presumably lacks chromatin and may be subject to different regulation compared with endogenous genes. Animals treated with AAV9-CMV-VEGF maintained significantly higher levels of HO and HIF-1α (p < 0.01) gene expression at Day 28 (Figure 4 and Figure 17) compared to AAV9-CS therapeutic groups.
Notch ligand DLL4 gene promoters are induced by hypoxia through 5’ HREs. In the PBS group, DLL4 levels remained elevated approximately 5-fold over baseline at all time points with little change over time suggesting an absence of dynamic regulation of DLL4 effectors in these muscles. It is also possible that persistent hypoxia contributes to the constitutively elevated expression. Both factors are consistent with the absence of reperfusion or resolution of ischemia in this group. Similarly DLL4 expression increased by >7-fold in the AAV9-CMV-VEGF group and remained elevated for the duration of the experiment. The enhanced, constitutive upregulation of DLL4 in this group is likely due to the combination of constitutive VEGF expression from the CMV promoter and by sustained hypoxia. Dll4 is known to be indirectly up-regulated by VEGF signaling through VEGFR2. These trends are in contrast to those seen for the AAV9-CS-VEGF groups. In the latter groups, there are clear trends for dynamic regulation with peak expression during the first week that is sharply extinguished after 28 days. The trend is consistent with high-level angiogenesis and vessel regeneration during the first week after FA excision followed by decrease angiogenic activity as functional vessels are formed.
reperfusion occurs and hypoxia is resolved. Similar trends were seen for the expression of HEY1 and HEY2; both PBS and AAV9-CMV-VEGF groups displayed increased expression of these genes that did not shut down. In contrast there was tight regulation of both genes in the CS groups. HEY1 and HEY2 are transcriptionally upregulated by activated Notch signaling and may be involved in artery vs vein specification, an event that needs to occur early in the process of arteriogenesis.

DLL1 is essential for post-natal arteriogenesis and active DLL1 expression is an indication of a dynamic vascular environment. I found that DLL1 gene expression was again tightly regulated in the CS gene therapy groups but not so in the PBS or AAV9-CMV-VEGF groups. DLL1 remained significantly higher in PBS and AAV9-CMV-VEGF groups than either AAV9-CS-VEGF group at day 28 (Figure 19a; p<0.05). Interestingly, whereas HO and VEGF expression peaked
early within 3 days, that of DLL4 and 1, and both Hey genes peaked later at 1 week, consistent with secondary responses to VEGF induction. Ephrin-B2 expression, which functions in a supportive fashion to add tensile strength to arterial walls by pericyte recruitment, demonstrated tight regulation in the CS groups but remained significantly elevated at day 28 in PBS and CMV treatment groups (Figure 19b).

To investigate the potential role of endogenous pro-angiogenic gene induction in ischemia/reperfusion recovery, I measured the levels of gene expression for fibroblast growth factor-2 (FGF2), hepatocyte growth factor (HGF), and stromal derived factor-1 (SDF-1) (Figure 20). FGF2 and HGF have been previously tested in gene therapy trials as single-agent inducers of angiogenic remodeling to treat peripheral limb ischemia. SDF-1 is hypoxia-regulated through HIF-1α and a potent inducer of stem cell migration. I observed similar trends of growth factor gene induction in all VEGF treatment groups. PBS treatment resulted in weak induction of FGF2, HGF, and SDF-1 at days 1, 3, and 7. Gene expression at day 28 was significantly higher (p<0.05) in PBS than
all other treatment groups, possibly due to continuous induction by hypoxia. The induction of other growth factors in the VEGF treatment groups is consistent with the position of VEGF at the top of an angiogenic hierarchy that begins with hypoxia and HIF-1α. Such a hierarchy can perhaps explain why VEGF but perhaps not other angiogenic growth factors is capable of single factor therapy when appropriately regulated in response to hypoxia. At day 28, HGF gene expression was significantly higher (p<0.05) in CMV-VEGF treated limbs versus CS-VEGF therapy. Also, at this time I observed silenced SDF-1 gene expression in AAV9-TOAD-VEGF limbs (p<0.0001), consistent with a wind down and loss of hypoxia regulation caused by reperfusion in these groups. There was no difference in FGF2 signal at day 28 between the VEGF treatment groups.

Figure 20. Growth Factor Gene Expression. qRT-PCR analysis of (a) FGF2, (b) HGF, and (c) SDF-1 gene expression (n=3). RNA was extracted from the hind limb adductor magnus at the indicated time-points following femoral artery ligation/excision surgery.
Summary

This evaluation of gene expression in the mouse hind limb represents the first ever documentation of multiple angiogenic genes responding to ischemia and recovery mediated by hypoxia-regulated gene therapy in a model of PAD. The study reveals a marked activation and dynamic regulation of Notch signaling only in response to conditionally silenced gene therapy. Notch signaling was activated by ischemia in the AAV-CMV-VEGF and PBS groups but there was no evidence of regulation, an observation that is consistent with absence of therapy or resolution of ischemia in the latter treatment groups. The results suggest that gene therapy with conditionally silenced VEGF mediates a wave of enhanced angiogenic activity that includes the coordinated activity of multiple pathways and results in the production of blood conducting vessels that support permanent reperfusion of the limb and perhaps regeneration of the femoral artery, an effect not seen for gene therapy with unregulated AAV. Limb reperfusion only in the CS treatment group was followed by the coordinated shut down of the genetic program activated by hypoxia and VEGF.

The “ability” of regulated therapies to silence Notch signal activity is most likely attributed to early reperfusion following ischemia surgery. The elimination of hypoxia induced genes seen in Figure 17 suggests restoration of blood flow in limbs treated with CS-VEGF. Lingering ischemia associated with unregulated VEGF and PBS treatments affects downstream activation of endogenous hypoxia inducible growth factor signaling resulting in a dynamic constitutively active endothelium. An endothelial environment that is in a dynamic phase of
morphological cell transition and migration is incapable of forming mature arteries. Elevated DLL1 expression one month after ischemia surgery is an indication of a dynamic vascular environment\textsuperscript{112}, as seen in CMV-VEGF and PBS treated limbs. Furthermore, steady state arteriogenesis is dependent upon the establishment of a quiescent vascular phenotype, where Dll1, Ephrin-B2, and Notch transcriptional target activity are low\textsuperscript{112}. The endogenous angiogenic response to conditionally silenced VEGF gene therapies was quiescent within one month after ischemia surgery as evidenced by baseline levels of Notch signaling. Taken together, my results suggest that early reestablishment of blood flow in response to hypoxia regulated VEGF gene therapy, eliminates hypoxia and hypoxia induced growth factor signaling, and facilitates arteriogenic maturation of collateral vessels.
Chapter 9: Discussion

In this dissertation work I have tested the preclinical efficacy and safety of four gene therapy AAV vectors in a Balb/c mouse model of critical limb ischemia. In addition I have compared the efficacy of optimized AAV vectors with adenoviral and plasmid vectors. The latter comparisons are particularly relevant to the current state-of-the-art for human gene therapy because plasmids have been the vectors of choice and have been extensively tested clinically. However their efficacy has recently been questioned because of the recent failure of the TAMARIS trial, a Phase III multicenter, placebo controlled trial involving 525 patients, the largest trial ever implemented for a gene therapy procedure. The TAMARIS trial not only showed absence of efficacy, but also indicated trends for worsening symptoms in the treatment group that may preclude further clinical use of plasmids for this indication.

The main conclusions of my study are:

1. The addition of HRE/CSs upstream of the PGK promoter increases \textit{in vivo} hypoxic induction approximately 180-fold while decreasing baseline gene expression.

2. Gene therapy with AAV-CMV-VEGF is unsafe and not a candidate for the treatment of PAD.
3. Conditionally silenced AAV-VEGF supports early reperfusion of the hind limb, tight auto-regulation by ischemia, permanent salvage of the hind limb and regeneration of large arteries within or close to the femoral tract.

4. The addition of a non tissue-specific silencer element to the prototype AAV9-HRE-VEGF in the form of FROG/TOAD silencer elements reduces baseline leakiness in normoxic tissue and confers optimal therapy.

5. Hypoxia-regulated VEGF transgenic expression transactivates a cascade of proangiogenic factors that contribute to successful regeneration of the vasculature and lead to arteriogenesis.

6. Hypoxia regulation of VEGF gene expression rather than dose or persistence of expression is the critical property responsible for limb salvage following ischemia surgery, more so than VEGF dose and persistence.

7. Tight regulation of gene expression by ischemia though conditional silencing confers auto-regulation of gene therapy and provides a safety switch that may provide for a vigilant state that can be reactivated by recurrent ischemia.
Taken together my results suggest that regulation of VEGF transgene expression by hypoxia is required for successful gene therapy for PAD/CLI. Typically, patients with CLI experience rest pain in the feet or calves, ischemic lesions, and gangrene beginning at the toes and distal portions of the limb \(^{184}\). However, patients in the early stages of PAD are often asymptomatic or experience intermittent claudication \(^{184}\). Thus, as arterial blockages increase in size, so does the severity of ischemic disease presentation. As such, CLI does not create global ischemia in the affected limb but rather graded ischemia, the highest level of which is most distal to the arterial blockage.

In order for the vasculature to regenerate, endothelial tip and stalk cells must migrate from perfused regions of quiescent luminal endothelial cell layers toward hypoxic tissues. The process of angiogenic sprouting is governed by endothelial cell migration along a VEGF gradient in association with morphogenic Notch signaling \(^{177}\). Tight and specific control over VEGF signaling is required for proper guidance of endothelial sprouts toward hypoxic tissues. Hypoxia directed VEGF gradients guide embryonic arterial development \(^{177}\) and it is my belief that recapitulation of these developmental patterns is required for post-embryonic (adult) artery regeneration.
A Molecular Argument for Hypoxia Regulated Vectors to Treat Ischemic Disease

VEGF, the most potent inducer of endothelial cell migration, acts upstream of the Notch pathway, and influences the expression patterns of other pro-angiogenic genes to govern almost all morphological angiogenic events (Reviewed in 91, 94, 177, 185). Based upon this knowledge we believe that VEGF is the only gene that can singularly treat CLI. We created a hypoxia regulated DNA backbone expressing human VEGF$_{165}$ that is encapsulated within a muscle-trophic AAV serotype 9 shuttle vector. This vector targets only ischemic tissue and mediates semi-permanent transgene expression with minimal immunogenicity and no apparent toxicity or adverse side effects. We believe that the strategic design of our vector addresses the issues that have plagued CLI gene therapy trials in the past, namely, insufficient duration of expression, inappropriate/unregulated gene expression, and inadequate targeting of gene therapy vectors to ischemic tissue.

As previously discussed, the hypoxic VEGF response in Balb/c mice is deficient and mice that do not receive AAV9-CS-VEGF gene therapy lose their limb following ischemia surgery due to severe necrosis. By presenting the tissue with more HREs, AAV9-CS-VEGF competes effectively for the available HIF-1$\alpha$ relative to endogenous binding sites. VEGF transgene is activated in the presence of stabilized HIF-1$\alpha$/P300 and the amount of hypoxia induced VEGF
signal is proportional to the level of localized hypoxia, as it is in embryonic development \(^{177}\). Studies of the developing retina have shown that VEGF gradients drive endothelial expansion and maturation along the hypoxic front \(^{186}\) and reviewed in \(^{187}\) and \(^{188}\) and that VEGF expression is localized to the avascular periphery \(^{177}\). Differences in local VEGF concentrations lead to the designation of venous and arterial phenotypes, whereby increased VEGF expression is highly associated with venous endothelium due to lower oxygen tension in the surrounding tissues \(^{189}\). In quiescent adult endothelium, constant low level expression of VEGF is required for cell survival and vessel maintenance \(^{87}\). Indeed, VEGF functions at all levels of growth and development to establish and maintain the cardiovascular system.

In mouse hind limb following ischemia surgery, tissue ischemia is most severe at the foot and less severe towards the location of arterial occlusion (thigh) resulting in a gradient of ischemia across the entire limb (Figure 21a). AAV9-CS-VEGF responds to the hypoxic gradient, establishing a VEGF gradient that is most concentrated at locations that are distal to arterial occlusion (Figure 21b). Endothelial tip cells migrate along a VEGF gradient, from low signal to high signal, bringing with them a trailing luminal cavity containing a fresh source of oxygenated blood \(^{177}\) (Figure 21a). Anastomosis of collateral vessels creates conducting junctions to deliver oxygenated blood to hypoxic tissues from the arterial network, the source of which is proximal to the original arterial occlusion. The physical forces exerted upon the vessel walls by shear stress increase vessel diameter and conductance \(^{190}\). Shear stress also activates
endothelial recruitment of monocytes to the vessel walls. Monocyte derived cytokine signaling controls endothelial proliferation and arterial maturation.

Figure 21. Hypoxia Regulated VEGF Expression and Vascular Response. a) Illustration of VEGF expression in ischemic tissue (red=highest concentration). In the case of hypoxia regulated expression of VEGF (top), gene expression follows along a hypoxic gradient and therefore VEGF signal is most highly concentrated at the distal portion of the limb. Endothelial sprout cells (blue rectangles) migrate along VEGF gradients from regions of low VEGF to high VEGF. Unregulated VEGF gene expression (a, lower panel) is ubiquitous throughout ischemic tissue and therefore endothelial sprouts do not migrate towards the most hypoxic tissues. b) Following limb ischemia the foot becomes purple in color due to lack of oxygenated blood. In the case of hypoxia regulated gene therapy a gradient of VEGF signal (green dots) is most highly concentrated near the foot resulting in regeneration of large arteries that span the hypoxic zone and limb salvage. Vessels grow erratically in response to unregulated gene therapy, resulting in loss of the ischemic limb.
I believe that hypoxia regulated coordination of intercellular transgene expression establishes a VEGF gradient and is responsible for the therapeutic effects of CS-VEGF gene therapy described in this dissertation. Recreation of developmental, ischemia driven VEGF gradients, facilitates arteriogenesis and limb salvage in our model of critical limb ischemia. My results have demonstrated that the application of AAV9-CS-VEGF gene therapy to ischemic hind limb muscle enhances the induction of endogenous growth factors and Notch signals (Figures 18-20), reestablishes blood flow, and promotes arterial development (Figure 12).

Conditionally silenced, hypoxia regulated gene therapy is unique and we believe it to be essential for successful arteriogenic gene therapy. The addition of HIF-1α binding motifs upstream of a promoter region enhances gene transcription in a Balb/c hind limb model of CLI. Strict normoxic silencing is not attainable by HRE alone 157, 158, 159, 160. The juxtaposition of gene enhancers (HREs) with silencer elements (FROG/TOAD and NRSE) mediates transgene induction that responds optimally to cellular hypoxia and silences normoxic signaling. The addition of the FROG/TOAD silencing element to the HRE/NRSE construct sacrificed maximal hypoxic VEGF induction for a higher degree of normoxic silencing. However, this did not have a negative effect upon ischemia/reperfusion outcome. In fact I observed an increased rate of blood flow reestablishment and decreased toe necrosis associated with strict normoxic silencing of AAV9-TOAD-VEGF therapy compared to AAV9-HRE-VEGF (Figure 15).
I have observed that high dose therapy of AAV9-CMV-VEGF is associated with vascular leakiness and inflammation. Ubiquitous, unregulated VEGF signaling, independent of dose, may be a biotechnological artifact that rarely if ever occurs in nature; it precludes directional cues for endothelial cell migration because there is no gradient of VEGF signal. *Vegf* activation of *Vegfr2* upregulates DLL4 gene expression, causing increased membrane bound *Dll4* presentation to neighboring cells. Competitive signaling through *Dll4/Notch* interaction is proposed as the mode of tip cell selection whereby activation of *Notch* leads to downregulation of VEGFR-2 and stalk cell fate. Unregulated VEGF delivery is not suited to treat vascular disease given that competitive endothelial signaling cannot be accomplished in the absence of a *Vegf* gradient. Unregulated VEGF expression produces non-directional angiogenic growth, as we have seen in our experiments with AAV9-CMV-VEGF. Disruption of the Notch signaling pathway also causes erratic vessel branching and poor conductance in hypoxic tumor environments and retinal development. Taken together, it is clear that a gradient of VEGF transgene signal is required to ensure that there is a proper balance of migratory VEGF and Notch signaling to guide endothelial cell migration and promote artery/vein maturation (Figure 21).

Our CS-VEGF construct is unique among current strategies to treat CLI because it responds acutely to cellular hypoxia to create an ischemia induced VEGF gradient. VEGF signal is terminated when a sufficient supply of oxygenated blood has been reestablished. The ineffectiveness of unregulated protein, plasmid, and adenovirally delivered angiogenic gene therapy is well
In my study of VEGF gene therapy I have observed that naked plasmid DNA of the Toad-VEGF construct was capable of promoting vascular reperfusion (data not shown). However, I believe that our optimized AAV9-CS-VEGF vector is the most clinically appropriate tool currently available to treat CLI. AAV9 is muscle-trophic and can be injected directly into ischemic muscle. The semi-permanent nature of AAV eliminates the need for multi-dosing strategies that have been implemented in protein or plasmid trials. Silenced normoxic VEGF expression protects against the development of peripheral edema and reduces the risk of complications associated with prolonged VEGF exposure. In our model of CLI, hypoxia driven VEGF expression from AAV9-CS-VEGF guides endothelial sprouts to ischemic tissues, enhances endogenous growth factor signaling, and produces large arteries.
Methods and Materials

Cell Culture, Transfections, and Hypoxia
Our methods for aerobic and hypoxic culture have been described previously \(^{193}\).

The Lipofectin reagent (New England Biolabs) was used for all transfections.

Luciferase or \(\beta\)-gal was measured in cell lysates using a dual-luciferase reporter assay kit (Promega) or enzyme enhanced x-gal assay kit (Cal Biochem).

Vector Constructs
Oligonucleotides containing the sequences 5' CTTCAGCACCAGCG GACAGT 3' (NRSE) and 5' GCCTGTCACGTGCACGACT 3' (HRE) were inserted as multimers (3X) or tandem repeats of NRSE-HRE (also 3X) into pGL3-PV (Promega). Sequences of the TOAD and FROG silencer oligonucleotides were 5-CTCTTCCAGAGCA AGGCAACCACAGGAGACC-3 (TOAD) and 5-GGTGTGCATTTAGCTAAATTCCCCA-3 (FROG). To create vectors with \(\alpha\)-MHC, MCK, and PGK promoters, the SV40 promoter was excised from pGL3 and its progeny and replaced respectively with \(\alpha\)-MHC\(_{164}\) or PGK\(_{495}\). To insert 3XHRE into CS-\(\alpha\)-MHC164 the 3XHRE oligonucleotide was first inserted 5' of \(\alpha\)-MHC86 and a second oligonucleotide containing the sequence -86 to -164 inserted after the HRE. Mutations in the regulatory elements were made by replacing the core CCGCG of the NRSE element with AATCG, or the core ACGTC of the HRE with ATAGC. Plasmids containing the \(\alpha\)-MHC promoter were gifts from Bruce Markham, (Pfizer Global Research and Development, Ann Arbor, Michigan), the muscle creatine kinase enhancer-promoter (-1256) was a gift from Stephen
Hauschka, (University of Washington, Seattle) and the mouse myosin light chain promoter was a gift from Yi Tang, (University of Florida, Gainsville, FL); vectors expressing wild type and dominant negative forms of NRSF (REST) were gifts from Gail Mandel, NYU, Stony Brook, N.Y. The PGK promoter to 5’ -493 was generated by PCR using primers designed from sequence NCBI accession number AF335420. All clones were verified by sequencing.

**Generation of rAAV9 and in vitro expression**
Two recombinant AAV vector plasmids were constructed using the pAAV-IRES-hrGFP plasmid (Stratagene) as backbone. A 1.2kb Mlu/Sall containing the cytomegalovirus (CMV) promoter was replaced with the PGK or CS-PGK promoter upstream of the human VEGF165 cDNA. After transfection into C2C12 myocytes cultures were exposed to an aerobic or hypoxic atmosphere; GFP expression was monitored by fluorescence microscopy and culture supernatants were measured by ELISA for secreted hVEGF165 (RayBiotech).

**AAV9 virus production**
AAVs were generated in HEK 293 cells without using contaminating adenovirus using the pHelper plasmid and replication and packaging functions provided by pRC, generously provided by Dr James Wilson, (University of Pennsylvania). Cell lysates were fractionated using an iodixanol density gradient (Optiprep, Sigma). Virus containing fraction was concentrated using an Amicon Ultra 100k column
(Millipore). After obtaining genomic DNA, virus titers were obtained by RT-PCR using primers directed to the IRES region.

Ischemic hindlimb model
Balb/C mice (Charles River Laboratories; Boston, MA) of age 6 - 8 weeks, weighing approximately 25g, were cared for and operated following IUCAC guidelines at the University of Miami, Miller School of Medicine. Mice were anesthetized an intraperitoneal (IP) injection of ketamine (100 mg/ml) dose 40 mg/kg and xylazine (20 mg/ml) dose 8 mg/kg. The anesthetized mouse was placed on a surgical board covered with an absorbent bench pad and the mouse’s feet were fixed with adhesive tape onto the pad. Hair was removed from the right lower abdominal quadrant and the right leg by briefly (one minute) applying Nair hair removal cream. Excess cream and dissolved hair were then wiped from the surgical area. A longitudinal 15 mm incision was made beginning at the saphenous branches along the femoral vessels visible through the skin using surgical scissors and microsurgery forceps (ASSI.JFAL-3). The incision continued until the branch point of the epigastrica and Profunda femoris branches could be visualized. Connective tissue was removed beginning just proximal to the saphenous branches, by gripping the connective tissue with forceps and using a second set of forceps to remove the layer of connective tissue that covers the femoral artery, vein and nerve. This procedure was continued along the path of the femoral tract until the Profunda femoris was exposed. The femoral nerve was carefully separated from the artery and vein.
The femoral nerve was brushed away from the artery and vein using the natural flexing motion of the forceps. Gripping the femoral artery and vein gently with one pair of forceps, the entire length of the vasculature was separated from the underlying muscle. The tip of a pair of microsurgical forceps was slipped underneath the artery and nerve at a location immediately proximal to the branch location of the epigastrica and Profunda femoris. A length of 7-0 silk (Ethicon) was placed in the grasp of the forceps and pulled underneath the femoral artery and vein. Measures were taken to ensure that the femoral nerve was not occluded. The proximal femoral artery and vein was occluded using double surgical knots. The silk ends were cut using dissecting scissors and knots tied tightly by pulling silk ends. The occlusion procedure was repeated at the location proximal to the saphenous branches where the initial incision was made. To remove the femoral artery and vein forceps were used to hold the proximal end of the ligated vessels. Using dissecting scissors the femoral artery and vein were cut just distal to the proximal suture. The femoral artery and vein were pulled back along their original path and any branches were severed until the distal suture was reached. The vessels were cut at a point just distal to the suture. Any bleeding was absorbed with a cotton swab. The incision was closed using a simple running stitch and 6-0 silk (Ethicon).

Mice were divided into 6 groups: PBS, AAV9-CMV-VEGF, AAV9-HRE-VEGF, AAV9-TOAD-VEGF, AAV9-PGK-VEGF and Adenoviral-VEGF (Ad-VEGF with a CMV promoter). Injections were delivered in 50µL PBS into the quadriceps muscle (3 injection sites). For the purposes of viral transgene
evaluation, mouse hind limbs were injected with equal doses of the indicated AAV construct 5 days before limb ischemia surgery to ensure gene integration into the host cell and minimize lag in the hypoxic response.

**DNA, RNA, PCR**
Viral delivery and spread were determined by harvesting tissues 1-week after injections, purifying genomic DNA and subjecting the DNA to PCR using IRES primers common to all AAVs (n=3). RNA was extracted from skeletal muscle at indicated time points and purified using Tri Reagent (Sigma) followed by the RNeasy kit (Qiagen) with RNase-free DNase. Purity and RNA concentration were determined using a NanoDrop spectrophotometer. RNA was reverse transcribed using a cDNA High Capacity Kit (Applied Biosystems) and VEGF expression analyzed using the human VEGF165-specific primers: 5’AGGAGGAGGCCAGAATCATCAC and 3’ GGACTCCAGTGGGCACCACA). Endpoint amplification with Taq Red was evaluated after 30 cycles.

**Confirmation of Virus-free RNA Stock**
The purity of RNA samples was confirmed by PCR amplification of hypoxia response element-specific primers: 5’AACTCCATCAGAGGGTTC and 3’GCTGGAGGCTCTACTTGAAG. Viral stocks were mixed in 20mg/mL proteinase K and 0.5% SDS solution and incubated at 37°C for 15 minutes to release viral DNA for positive controls. Viral DNA was then precipitated in 100% ethanol and collected in RNeasy spin columns.
Quantitative Real-Time Polymerase Chain Reaction
RNA was extracted from skeletal muscle at indicated time points. Hind limb
musculature between proximal and distal sutures was dissected and placed in 1
mL of TRI Reagent. Tissues were homogenized using a 2000 Geno/Grinder and
centrifuged at 12000g for 10 minutes at 4 °C to remove the insoluble material.
RNA was extracted as recommended by the TRI Reagent manufacturer (Sigma).
Purity and RNA concentration were determined using a NanoDrop
spectrophotometer (Thermo Scientific). RNA was reverse transcribed using a
cDNA High Capacity Kit (Applied Biosystems). Gene expression was quantified
using TaqMan assays (Applied Biosystems) for each gene of interest. Individual
genes and their associated assay numbers are listed below:

Mm00456503_m1 - Angiopoietin 1
Mm00433287_m1 - FGF2
Mm01215897_m1 - Ephrin B2
Hs_00900054_m1 – Human VEGFa
Mm00468875_m1 – HIF-1α
Mm00437304_m1 – Mouse VEGFa
Mm00435245_m1 – Notch1
Mm00496902_m1 – Jag1
Mm00444619_m1 – Dll4
Mm01279269_m1 – Dll1
Mm00469280_m1 – Hey2
Mm00445553_m1 – Cxcl12
Mm01292123_m1 – Cxcr4
Mm01242584_m1 – Pecam1
Mm00516005_m1 – Hmox1
Mm99999915_g1 - GAPDH

Real-time PCR analysis was performed using a StepOnePlus Real-Time PCR System and a 7900HT Fast Real-Time PCR System (Applied Biosystems) with Taqman Fast Universal PCR Mastermix (4352042). Significance was evaluated using t-test and fold differences calculated using the formula $2^{\Delta Ct}$.

**Doppler Analyses**

Hindlimb perfusion was assessed using Periscan PIM II laser Doppler perfusion imaging (LDPI; Perimed Medical Systems, New York, NY), and data were acquired as previously reported \(^{195}\). Briefly, color images were obtained and hindlimb perfusion was determined by comparing the intensity of a murine hindlimb before surgery or to contralateral limb to a specific time point: post-surgery. Doppler frequency shift due to light reflecting off the moving red blood
cells produces more intense LDPI signal. Mice were sedated and secured on a monochromatic surface then scanned from their lower abdomen to the ends of the toes. An ischemic Doppler ratio was established for all groups and compared.

**Magnetic Resonance Imaging**
MRI was performed on 4.7-Tesla (200MHz) 40-cm bore magnet interfaced with a Bruker Avance™ console employing a gradient set with an inner diameter of 70-mm and a maximum gradient strength of 1000 mT/m.

**Immunohistology**
**CD31 Imaging**
For CD31 imaging, limbs were removed, skinned, placed in 10% formalin and paraffin embedded. Sections were deparaffinized using standard techniques and antigens retrieved by immersion in 95°C Tris-EDTA buffer. CD31 positive vessels were stained with ImmunoCruz™ goat ABC Staining System (sc-2023) and anti-Pecam-1 primary antibody (SC-1506, Santa Cruz). 40x fields of view were imaged for counting of CD31 positive stain (3 mice, 10 fields of view per time point) using an Olympus BX51 microscope mounted with an Olympus DP71 camera. CD31 positive stain was counted and expressed as a ratio of CD31 positive vessels to muscle fibers in any given field of view.
**Isolectin B4 Imaging**

Biotinylated lectin I isolectin B4 was purchased from Vector Laboratories (Burlingame, CA). Following deparaffinization antigens were retrieved by soaking in 95°C sodium citrate buffer for 20 minutes. After rinsing biotinylated lectin was applied to tissue sections at a concentration of 10µg/mL for 30 minutes at room temperature then washed with PBS + 0.05% Tween 20. Streptavidin alexa fluor 488 from Invitrogen was then applied at a concentration of 15µg/mL at room temperature for another 30 minutes. Finally the slides were washed in PBS and mounted. Fluorescence imaging was done on an Olympus BX51 microscope.

**Smooth Muscle Actin Imaging**

Tissue sections were prepared as described above and antigens retrieved in sodium citrate buffer. Sections were then washed for 10 minutes in TBS + 0.1% Triton X-100. Slides were blocked in 10% serum and TBS for 2 hours at room temperature. Primary anti-SMA (Sigma) was diluted in TBS and 1% BSA (1:200), applied to sections and incubated overnight at 4°C. After rinsing in TBST secondary Alexa Fluor 568 goat anti-mouse IgM (Invitrogen) diluted in TBS and 1% BSA (1:300) was applied to tissue sections for 1 hour at room temperature. Sections were rinsed for 20 minutes in TBS then mounted with ProLong® Gold antifade reagent with DAPI (Invitrogen). Staining was visualized using a Zeiss Axiovert 200 fluorescent microscope. Vessel area was measured.
using Zeiss Axiovert software from at least 20 fields and statistical significance
determined by t-test.

**DiI Imaging**
1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)

perfusion staining was used to directly visualize vessel architecture, collateral
vessel growth and organization. Mice were sacrificed by CO₂ overdose and the
heart rapidly exposed by thoracotomy. A butterfly needle was inserted into the
left ventricle and the right atrium was punctured to allow outflow of blood and
excess perfusate. Two mL of PBS was injected into the LV, followed by 5-10 mL
of Dil solution (42364, Sigma-Aldrich), and finally 5-10 mL of 4%
paraformaldehyde solution. The entire limb was severed and placed on a 35mm
glass bottom dish (MatTek Corporation) for confocal microscopy. The area
between sutures was then imaged at 10x magnification on an LSM510 or
LSM710 scanning confocal microscope set to image at an individual step size of
10um. Vessels within approximately 500 microns from the tissue surface were
imaged. Z-series were stacked and compressed into individual 2D images that
were merged using Zen software to create a composite image of blood vessels in
the ischemic region (between the sutures).

**Vessel Directionality**
To measure vessel directional growth, full size composite Dil images were
imported into Volocity software (Improvision; PerkinElmer) and a directional
reference was determined manually by tracing along the path of the femoral nerve in each image. Unbranched vessels were traced using Volocity and the values for bearing on an XY-axis were obtained and normalized to a 180-degree scale. The bearing of the reference was then subtracted from the bearing of each individual measured vessel in an image (minimum of 10 per limb) and the absolute value of each bearing was averaged to determine an overall measurement of angular deviation in collateral vessel growth for each individual hind limb. Student’s t-test was used to determine statistical significance between therapeutic groupings.

**Vessel volume**
Z-series obtained in confocal imaging were analyzed using Volocity high performance imaging software. Vessel volume data was generated using a uniform region of interest for each field of view. 10 fields of view from 3 separate mice were used to evaluate mean vessel density (volume of total Dil stained vessels) for each treatment.
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