Intradermal Delivery of Plasmids Encoding Angiogenic Growth Factors by Electroporation Promotes Wound Healing and Neovascularization

Bernadette Ferraro
University of South Florida

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Intradermal Delivery of Plasmids Encoding Angiogenic Growth Factors by Electroporation Promotes Wound Healing and Neovascularization

by

Bernadette Ferraro

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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College of Medicine
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Keywords: Gene therapy, vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), angiogenesis, wound healing, peripheral artery disease, ischemia

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<th>Definition</th>
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<tbody>
<tr>
<td>4PE</td>
<td>Four Plate Electrode</td>
</tr>
<tr>
<td>ABI</td>
<td>Ankle brachial index</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>CLI</td>
<td>Critical limb ischemia</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EP</td>
<td>Electroporation</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FGFs</td>
<td>Fibroblast growth factors</td>
</tr>
<tr>
<td>FGF-1</td>
<td>Fibroblast growth factor-1</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Fibroblast growth factor-2</td>
</tr>
<tr>
<td>FGFR1</td>
<td>FGF receptor 1</td>
</tr>
<tr>
<td>FGFR2</td>
<td>FGF receptor 2</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HPF</td>
<td>High power field (400X magnification)</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>IL-12</td>
<td>Interleukin-12</td>
</tr>
<tr>
<td>I/NI</td>
<td>In the hindlimb ischemia model, the ratio of blood flow in the ischemic limb to the blood flood in the non-ischemic limb</td>
</tr>
<tr>
<td>IC</td>
<td>Intermittent claudation</td>
</tr>
<tr>
<td>KGF-1</td>
<td>Keratinocyte growth factor-1</td>
</tr>
<tr>
<td>KPO₄</td>
<td>Potassium phosphate</td>
</tr>
<tr>
<td>LDPI</td>
<td>Laser Doppler Perfusion Imaging</td>
</tr>
<tr>
<td>MEA</td>
<td>MultiElectrode Array</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen activated protein- Erk kinase</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Magnesium sulfate</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonyl phenoxyphethoxylethanol</td>
</tr>
<tr>
<td>PAD</td>
<td>Peripheral artery disease</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>P-E-</td>
<td>No treatment</td>
</tr>
<tr>
<td>pFGF</td>
<td>Plasmid encoding human FGF-2</td>
</tr>
<tr>
<td>pFGFE-</td>
<td>Intradermal injection of pFGF without EP</td>
</tr>
<tr>
<td>pFGFE+</td>
<td>Intradermal injection of pFGF with EP</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>pLuc</td>
<td>Plasmid encoding the luciferase reporter gene</td>
</tr>
<tr>
<td>pLucE-</td>
<td>Intradermal injection of pLuc without EP</td>
</tr>
<tr>
<td>pLucE+</td>
<td>Intradermal injection of pLuc with EP</td>
</tr>
<tr>
<td>POD</td>
<td>Postoperative day</td>
</tr>
<tr>
<td>pVAXE+</td>
<td>Intradermal injection of the pVAX plasmid with EP</td>
</tr>
<tr>
<td>pVEGF</td>
<td>Plasmid encoding human VEGF&lt;sub&gt;165&lt;/sub&gt;</td>
</tr>
<tr>
<td>pVEGFE-</td>
<td>Intradermal injection of pVEGF without EP</td>
</tr>
<tr>
<td>pVEGFE+</td>
<td>Intradermal injection of pVEGF with EP</td>
</tr>
<tr>
<td>RSF</td>
<td>Random skin flap</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
</tbody>
</table>
INTRADERMAL DELIVERY OF PLASMIDS ENCODING ANGIGENIC GROWTH FACTORS BY ELECTROPORATION PROMOTES WOUND HEALING AND NEOVASCULARIZATION

Bernadette Ferraro

ABSTRACT

Gene therapy techniques delivering exogenous angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF-2), are currently being investigated as potential treatments for ischemia resulting from a variety of conditions, such as peripheral artery disease (PAD) and chronic wounds. Despite these intense efforts, a viable clinical option to promote therapeutic neovascularization remains elusive. Electroporation is a simple in vivo method to deliver normally impermeable molecules, such as plasmid DNA, to a variety of tissues including skin and muscle. This study investigated intradermal injection of plasmids encoding angiogenic growth factors with electroporation as a novel therapeutic approach to increase perfusion in areas of ischemia. Two common animal models of ischemia were employed: a skin flap model, used to study wound healing, and a hindlimb ischemia model, used to investigate potential therapies for PAD. In the skin flap model, delivery of plasmid VEGF with electroporation significantly increased VEGF expression for 5 days after delivery compared to injection of the plasmid alone. While the increase in VEGF expression was short-term, it significantly increased expression of the downstream angiogenic growth factor endothelial nitric oxide synthase, as well as perfusion and
healing in the distal area of the skin flap. To facilitate the translation of electroporation to the clinic, a novel electrode configuration was previously designed for cutaneous delivery of plasmids to a large surface area. The design of the Multielectrode Array allows for delivery to a large surface area without the need to increase the applied voltage. Conditions for plasmid delivery with this electrode were optimized and it was then utilized to deliver plasmid FGF-2 (pFGF) to the hindlimb ischemia model. FGF-2 expression, perfusion, and angiogenesis were assessed. FGF-2 expression was significantly higher for 10 days after treatment with pFGF with electroporation compared to injection of pFGF alone. This increase in FGF-2 expression induced a significant increase in perfusion and angiogenesis in the ischemic limb. The research presented here suggests intradermal injection of plasmids encoding angiogenic factors by electroporation is a novel potential therapeutic approach to increase perfusion to areas of ischemia and promote wound healing.
GENERAL INTRODUCTION

Neovascularization

Neovascularization plays a major role in both health and disease. The formation of new blood vessels occurs during normal physiological processes, such as embryonic development and wound healing, but excessive neovascularization contributes to the pathology of several diseases, notably cancer as well as ocular and inflammatory disorders. Aberrant neovascularization is also associated with many other diseases including diabetes mellitus, asthma, psoriasis and arthritis. In contrast, insufficient neovascularization is characteristic of other diseases including ischemic heart and limb diseases, neurodegeneration and osteoporosis [1]. New vessel growth occurs by three main mechanisms: angiogenesis, arteriogenesis and vasculogenesis. In embryonic development, vasculogenesis refers to the formation of the capillaries by the mobilization of bone marrow-derived endothelial stem cells that will eventually form the vasculature of the adult [2]. In the adult, vasculogenesis is simply a term for angiogenesis characterized by intussusception of progenitor cells in and around the new vascular structures [3]. Angiogenesis refers to the sprouting of new capillary-like structures from existing vasculature initiated in response to tissue hypoxia [4]. In contrast, arteriogenesis occurs in response to chronic or acute occlusion of a major artery, such as the coronary or
femoral artery. In arteriogenesis, preexisting arteriolar connections are recruited to bypass the site of occlusion in response to an increase in fluid shear stress [5].

It is currently not well understood how the stimulus of increased shear stress is transmitted from the endothelial cell (EC) membrane to the nucleus, but increases in fluid shear stress result in the production of the vasodilator nitric oxide (NO) and the activation of ECs and transcription factors, including early growth response factor-1 (Egr-1) and activator protein-1 (AP-1) [5]. Once activated, transcription factors increase the expression of chemokines, notably MCP-1, and adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1) [2, 6]. The second stage of arteriogenesis is mediated by an inflammatory response. The presence of MCP-1 and other chemokines on the cell surface of the activated ECs results in a chemotactic gradient that recruits circulating monocytes. Adherence of monocytes to the vascular endothelium is enhanced by the presence of selectins, intracellular adhesion molecules and vascular adhesion molecules. Monocytes, or after maturation macrophages, then migrate into the deeper areas of the collateral vessel wall where they induce EC and smooth muscle cell (SMC) proliferation. This process results in the release of growth factors and cytokines that promote vascular wall remodeling [3, 5]. In several months the new collateral artery is almost indistinguishable from a normal artery, except for a slightly higher collagen content between the smooth muscle layers [7].

The initiation and progression of angiogenesis is mediated through the temporal and spatial production of multiple growth factors and cytokines. Consistent with hypoxia being the driving force of angiogenesis, the transcription of multiple genes that play a role in angiogenesis is induced by hypoxia, primarily through the action of hypoxia-
inducible transcription factors HIF-1β, HIF-1α and HIF-2α. Angiogenesis initiates with a hypoxia-mediated increase in nitric oxide synthases leading to increased NO production and subsequently vasodilation [8, 9]. Following vasodilation, vascular permeability increases in response to VEGF signaling allowing for the extravasation of plasma proteins that lay down a provisional scaffold for migrating ECs. The destabilization of mature vessels by matrix metalloproteinases (MMPs) and other proteases then liberates ECs that migrate to the distal site of neovascularization. The ECs then proliferate to form an immature vessel lumen which is stabilized by a covering of pericytes and vascular SMCs recruited to the new vessels by the chemotactic action of PDGF-ββ [9].

**VEGF regulation of neovascularization**

VEGF is a highly specific mitogen for vascular ECs and exerts its effects mainly during angiogenesis, not arteriogenesis. VEGF does not naturally associate with or induce arteriogenesis in vivo because it occurs in a normoxic environment [10]. VEGF165 (referred to as simply VEGF throughout this manuscript) is the predominant isoform of 5 isoforms (VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206) generated by alternative splicing of the VEGF-A gene. These isoforms differ not only in their molecular mass but also in their biological properties [11, 12]. VEGF expression levels are increased in response to hypoxia by both transcriptional mechanisms, through the actions of HIF-1, and by stabilization of and increased translation of VEGF mRNA [13-15]. Several angiogenic growth factors, including epidermal growth factor, PDGF and FGFs, and inflammatory cytokines, such as interleukin-2, also upregulate VEGF mRNA [16]. In vitro and in vivo studies have demonstrated that the level of VEGF expression
must be finely regulated to induce successful angiogenesis [17-20]. High levels of constitutive VEGF expression can result in edema [21], formation of hemangiomas and unstable immature vessels [17-20, 22]. In contrast, low levels or premature cessation of VEGF expression results in regression of the newly formed vessels [23].

The downstream effects of VEGF are mediated by two tyrosine kinase receptors, VEGF Receptor 1 (VEGFR1) and VEGF Receptor 2 (VEGFR2). VEGFR1 has a high affinity for VEGF, however its tyrosine kinase activity is approximately 10-fold weaker than VEGFR2. VEGF binding to VEGFR1 weakly induces EC proliferation, simulates the migration of monocytes and induces MMPs and proteases that degrade the ECM resulting in the release of angiogenic growth factors [16, 24]. It is generally accepted that VEGFR2 is major mediator of the mitogenic, angiogenic and vessel permeability enhancing effects of VEGF. VEGF binding of VEGFR2 also induces the phosphorylation of PI3K to promote EC survival and activates the Raf-MEK-ERK pathway to increase EC proliferation [16]. VEGF-mediated increases in vascular permeability are largely due to an upregulation of endothelial nitric oxide synthase (eNOS) that stimulates the production and release of NO [25-27].

**FGF regulation of neovascularization**

In comparison to the VEGF family, the FGF family is much more complex containing 23 known isoforms that share approximately 30–70% homology in their amino-acid sequences. FGF-1 and FGF-2 differ from other members of the FGF family in that they lack a signal sequence for extracellular transport, but are instead exported by an alternative pathway during active angiogenesis [28]. FGF-1 (also known as acidic
FGF), FGF-2 (also known as basic FGF) and FGFs 4, 5 and 8 have been shown to play a role in neovascularization, but much of the *in vivo* and *in vitro* research centers on the actions of FGFs 1 and 2.

After FGF-1 and FGF-2 are exported from ECs they bind FGF receptor 1 (FGFR1) or FGF receptor 2 (FGFR2) to promote both angiogenesis and arteriogenesis by increasing vascular EC proliferation, migration and degradation of the ECM. Activation of FGFR1 and FGFR2 by the binding of FGF-1 or FGF-2 increases EC proliferation through activation of MAPK signaling pathway [29]. In addition to activation of the MAPK signaling cascade sustained activation of PKC is required for FGF-2 to exert its full mitogenic effect in ECs [30]. Activation of both the MAPK pathway and PKC are also required for FGF-1 and FGF-2 mediated cell migration [31]. During arteriogenesis, the release of FGF-2 from macrophages enhances both EC and SMC proliferation in the vascular wall [32]. FGF-1 and FGF-2 promote ECM degradation by upregulating the expression of uPA and the expression of the uPA receptor on the surface of ECs. uPA converts plasminogen to the serine protease plasmin. Plasmin degrades fibrin, as well as other ECM proteins, and activates MMPs. Expression of the uPA receptor on ECs localizes proteolytic degradation to leading edge of the front of EC migration. While FGF-1 and FGF-2 initially promote ECM degradation, during the later steps of neovascularization they promote the formation of the ECM to stabilize the newly formed vessels [31]. FGF-2 also regulates EC morphogenesis, the reorganization and assembly of ECs into three-dimensional tubes, by signaling through VEGFR1 [33]. There is a considerable degree of cross-talk between FGF-2 and VEGF/VEGFRs. Several studies have shown that FGF-2 may require the activation of VEGF/VEGFRs to promote
angiogenesis, and, under some conditions VEGF may also require FGF-2 to exert its angiogenic effects [34, 35].

Methods of therapeutic gene delivery

Therapeutic delivery of exogenous angiogenic growth factors and cytokines to increase neovascularization, or therapeutic angiogenesis, has been investigated as a potential treatment for ischemia-related diseases and to promote wound healing. The main obstacle for widespread use of gene therapy approaches for the treatment of diseases in general is the lack of a safe and efficient system for delivery of transgenes.

The simplest gene transfer approach is direct injection of naked plasmid DNA. This method has had some success, but is limited by low expression levels due to inefficient uptake of the plasmid by cells [36]. Thus, methods to increase the expression of plasmids encoding genes of therapeutic interest for the treatment of a particular disease are actively being explored. In vivo gene therapy approaches can broadly be divided into two categories, viral and non-viral. Viral approaches utilize a variety of genetically engineered viral vectors for gene delivery while non-viral approaches focus on the use of chemical or physical methods to increase the uptake and expression of plasmid DNA. Direct injection to the target tissue or intravenous administration of recombinant proteins has also been investigated as a therapeutic approach. Recombinant proteins can also be topically applied to promote wound healing. The efficacy of recombinant protein delivery is often hampered by the short-half life, and subsequently low bioavailability, of many proteins of therapeutic interest [37-41].
Viral vectors have been employed to deliver genes to provide either transient or permanent transgene expression. Viral vectors that do not integrate into the host genome, such as adenoviral and vaccinia vectors, confer transient expression that is relatively short-term (1 to 2 weeks) compared to expression levels achieved with viral vectors that do integrate into the host gene. Vectors that do integrate into the host genome, such as retroviral and adeno-associated viral vectors, confer high levels of permanent transgene expression [42]. Through 2007, viral vectors have been used in approximately 67% of gene therapy clinical trials worldwide, with adenoviral (25%) and retroviral (23%) vectors being the most common [43]. The safety of viral vectors has been questioned as a result of adverse events in human clinical trials. In clinical trials, adenoviral vectors have caused severe inflammatory responses, formation of antibodies to the adenoviruses, transient fever, hepatotoxicity and in rare cases, death [44-46]. The use of retroviral vectors was questioned after patients developed leukemia in a clinical trial treating adenosine deaminase deficiency, a fatal autosomal recessive form of severe combined immunodeficiency (SCID) [47]. The preference of retroviral vectors to integrate into the genome at transcriptional start sites and introns is the most probable cause of the adverse events reported in the first SCID trial [48], but the frequency of integration was also likely to be higher than normal due to a number of secondary risk factors associated with SCID [49]. In contrast to earlier reports, a more recent SCID gene therapy clinical trial reported positive results and no adverse effects in patients transfused with autologous bone marrow cells transduced with a retroviral vector containing the adenosine deaminase gene [50]. This recent success may lead to renewed interest and more investigations of therapeutic gene delivery using retroviral vectors.
As mentioned above, non-viral approaches for therapeutic gene delivery encompass chemical or physical methods to increase transfection of plasmid DNA. Chemical methods for non-viral gene delivery are largely based on the use of positively-charged cationic liposome complexes. Combining the cationic liposomes with negatively-charged plasmid DNA facilitates the uptake of the plasmid by the cell membrane, and subsequently, plasmid internalization. Cationic liposome-mediated gene transfer is technically simple and can be used for the delivery of a large amount of DNA by direct injection or intravenous administration, but this approach generally does not confer high levels of transgene expression [51, 52]. Interestingly, the transfection of plasmid DNA with cationic liposomes was not enhanced in mouse skin with the addition of in vivo EP (discussed below) indicating other physical methods of delivery may not be efficacious for enhancing the effects of these reagents [53].

A variety of physical methods have been explored to increase plasmid gene transfer including ultrasound, laser, particle bombardment, magnetic fields, hydrodynamic pressure and in vivo EP. The use of ultrasound, focused lasers, particle bombardment (also known as the gene gun) and magnetic fields share the common advantage of being non-invasive approaches, but generally do not confer a substantial increase in transgene expression compared to direct injection of plasmid DNA alone [54]. Hydrodynamic pressure, rapidly injecting a large volume of plasmid DNA into the tail vein of rodent models, has been successfully used to transflect hepatocytes throughout the majority of the liver [55, 56]. This approach has also been successful for gene delivery to the kidney [57]. While useful for studies in rodent models, however, it is currently not directly applicable to humans.
In vivo electroporation for gene delivery

In vivo EP involves the application of a controlled pulsed external electric field that slightly surpasses the capacitance of the cell membrane. Application of the electric pulse transiently increases cell membrane permeability allowing for an increase in the uptake of plasmid DNA. The first significant demonstrations of successful delivery of plasmid DNA with in vivo EP were conducted in rat liver [58], murine brain tumors [59] and murine skeletal muscle [60]. Since these initial experiments, in vivo EP has also been used for plasmid delivery to a wide variety of tissues including skin [61-77], brain [78-81], kidney [82] and lung [83-86].

EP delivery of plasmids encoding genes with therapeutic potential to the skin could potentially be used to treat a variety of diseases. The skin is an attractive target for delivery of plasmid DNA because it is easily accessible, which allows for enhanced control over expression levels. If higher expression levels are needed, the area treated or number of treatments can be increased. Further, plasmid DNA has been successfully delivered to the skin with in vivo EP for both systemic and tissue-specific expression [61-63, 67, 69]. This approach has shown promise in several fields including vaccines [69-73] and wound healing [74-77]. A recent publication by Daud et al. [87] reported the results of the first human trial utilizing in vivo EP for plasmid delivery. The trial investigated intra-tumoral delivery of a plasmid encoding interleukin-12 (IL-12) with in vivo EP as a possible therapeutic option for the treatment of metastatic melanoma. The results of this Phase I trial indicated the potential treatment to be promising as well as safe, effective, and reproducible. Importantly, intra-tumoral delivery of plasmid IL-12 by in vivo EP allowed for control of IL-12 expression levels.
Previous work in the Heller laboratory led to the development of a novel electrode configuration, the Four Plate Electrode (4PE), that was optimized for cutaneous plasmid delivery with EP with minimal tissue damage [68]. As the name suggests, the 4PE is composed of 4 metal plates that grip the injection site and a nonconductive ‘stopper’ placed in the center of the 4 plates (Figure 1). The use of an electric field to increase plasmid uptake and expression is more efficient when the electric field is applied in more than one direction [88-90]. This approach permeabilizes a greater area of the cell surface and a larger number of cells in the target tissue [91]. The design of the 4PE allows for the application of 2 sets of electric pulses, with the second set of pulses rotating 90° relative to the first set of pulses, without removing the electrode from the delivery site. Prior to the development of the 4PE, electrically mediated delivery of plasmids to skin was typically facilitated with a 2-plate caliper electrode. One drawback to this approach was the requirement of the electrode plates to be manipulated around the treatment site to administer 2 sets of pulses. Removal and replacement of the caliper electrode from the delivery site resulted in variation in the distance between the electrode plates and thus variation in the applied voltage. The nonconductive ‘stopper’ that is placed in the center of the 4 plates of the 4PE ensures a constant distance between the plates and thus constant voltage across the delivery site.
Wound Healing

Wound healing is frequently defined as the repair or reconstitution of a defect in an organ or tissue, commonly the skin. Cutaneous wounds are classified as acute, having occurred within the past 3 to 4 weeks, and chronic or delayed healing, persisting 6 to 8 weeks or longer [92]. Surgical intervention to facilitate wound healing can be described as healing by primary intention, delayed primary closure or secondary intention. Primary intention and delayed primary closure are achieved by suturing or stapling to join the wound edges, but the latter refers to wounds that are not immediately closed due to infection or other complications. Secondary intention refers to allowing the wound to close through the normal healing process.

The process of wound healing occurs in three general overlapping phases: (1) an inflammatory phase which encompasses coagulation and the initial migration of inflammatory cells to the wound site (2) a proliferative phase (also known as the migration phase) involving the migration and proliferation of keratinocytes, fibroblasts and endothelial cells, leading to re-epithelialization and granulation tissue formation and (3) an extended remodeling phase which results in a reduction of both cell content and blood flow in the scar tissue [92, 93]. There is a significant temporal overlap in the progression through these phases and the later phases of wound repair are critically dependent on the initial events of the healing process. A deficiency in one or more essential growth factors or cytokines may result in asynchronous progression through the phases of wound healing leading to the development of a chronic wound. Several systemic conditions or diseases, such as malnutrition, immunodeficiency or diabetes
mellitus, are frequently the underlying cause of the cytokine or growth factor deficiency and are often associated with impaired healing [94-96].

The inflammatory phase of wound healing begins immediately following injury with the formation of a fibrin clot to maintain tissue homeostasis and prevent infection. The fibrin clot consists of platelets embedded in a mesh composed largely of polymerized fibrin and smaller amounts of fibronectin, vitronectin and thrombospondin [92]. In addition to serving as a temporary cover of the wound, the fibrin clot also functions as a scaffold for inflammatory cells. Inflammatory cells are recruited by cytokines and growth factors released by platelet degranulation and by peptides cleaved from bacterial proteins [97, 98]. For the first 48 hours following injury, neutrophils are the predominant inflammatory cell type in the wound. Neutrophils serve several functions in the wound bed including the removal of dead tissue, prevention of infection and degradation of the ECM to prepare the wound for the later phases of healing. Additionally, neutrophils are also a source of proinflammatory cytokines that may provide the earliest signals to activate keratinocytes, the major constituent of the epidermis, and fibroblasts, which synthesize the ECM [99]. Between 48 to 72 hours post-injury monocytes / macrophages replace neutrophils as the predominant inflammatory cell type in the wound area. Circulating monocytes are recruited to the wounded through the chemotactic effects of growth factors and cytokines, primarily MCP-1 [100]. Monocytes / macrophages are essential for effective wound healing. If macrophage infiltration is prevented, wound healing will be severely impaired [101].

The hypoxic environment of the wound also plays a key role in mediating the early stages of healing. While prolonged ischemia is deleterious to the healing process
[96], hypoxia serves a positive role during the early stages of injury. Hypoxia increases the proliferation of fibroblasts, migration of keratinocytes for re-epithelialization and promotes early angiogenesis. Moreover, hypoxia induces the transcription of growth factors, such as VEGF, that are integral to the wound healing process [95, 102].

Due to the temporal overlap of the phases of wound healing, there is some discrepancy in the time, post-injury, that the proliferative phase begins. In general, the onset is thought to occur within 2 to 4 days after injury and persist through days 14 to 21 after injury [92, 93]. The proliferative phase is marked by the formation of granulation tissue and ECM, entrance of macrophages into the wound, angiogenesis and completion of re-epithelialization. Approximately 4 days after injury, the provisional fibrin clot is gradually replaced by granulation tissue, which provides a framework for angiogenesis and cell migration. Granulation tissue is composed of three cell types: ECs, macrophages and fibroblasts, which drive the production of the ECM. Macrophages in the granulation tissue continue to secrete growth factors and cytokines to induce fibroblast proliferation, migration and ECM deposition. Release of angiogenic growth factors, such as VEGF and FGF-2, by macrophages also facilitates EC morphogenesis and new vessel formation. Angiogenesis is integral to the wound healing process. If angiogenesis is inhibited, the healing of a wound will be severely impaired [92, 103]. Another key event that begins during the proliferative phase is wound contraction. Wound contraction occurs if a wound was not surgically closed and functions to decrease the wound area. This process is mediated by fibroblasts that have acquired a contractile phenotype and transformed into myofibroblasts. Wound contraction begins approximately 5 days after injury and continues into the onset of the remodeling phase of healing [96].
The remodeling phase is the longest phase in the wound healing process. It begins approximately 14 to 21 days after injury and persists for as long as a year, depending on the size and location of the wound. Currently, remodeling is the least characterized phase of healing and its regulation is poorly understood. At the onset of the remodeling phase, new blood vessels that are no longer required to supply nutrients to the injured tissue regress and re-epithelialization is completed. During the remodeling phase, the type III collagen that was synthesized by fibroblasts in the granulation tissue is slowly degraded by the action of MMPs and replaced by type I collagen. The replacement of type III collagen by the stronger type I collagen, coupled with an increase in collagen cross-linking, slowly improves the tensile strength of the wound [92, 93, 96]. At the beginning of the remodeling phase, the strength of the wound is approximately 20% of intact skin, but it continues to increase during the phase to reach a maximum of 80% at 1 year following injury [104].

Skin flaps

Skin flaps are frequently used in plastic and reconstructive operations to repair large skin defects and deep wounds formed from injuries, operations, ulcerations or congenital defects. In skin flap surgery, a piece of full thickness skin is transferred to the affected area while remaining attached to the donor site by a vascular pedicle. Because skin flaps receive their blood supply from the donor site they are preferable over skin grafts to cover recipient beds of poor vascularity, such as areas of exposed bone and tendons [105]. Moreover, skin flaps often provide better cosmesis when repairing
delicate areas, such as facial structures, and are less prone to wound contraction than skin grafts [106, 107].

Skin flaps are grouped into two general categories, axial and random. The pedicle of axial pattern skin flaps is based on a defined vascular territory and the blood is supplied through the vascular pedicle by a direct subcutaneous artery. In contrast, the pedicle of random skin flaps (RSFs) is based on random, non-dominant vessels and the vascular pedicle contains only small musculocutaneous or septocutaneous perforators [108]. In both axial and RSFs, insufficient arterial supply and inadequate venous drainage can lead to ischemia and necrosis of the distal portion of the skin flap. RSFs are considered more at risk for necrosis than axial skin flaps, due to the low level of blood supplied to the flap by the small vessels in the pedicle, and the instability of RSFs further increases when the length to pedicle width ratio exceeds 2.5 to 1 [109]. The tendency of the distal region of skin flaps to suffer severe ischemia and necrosis remains a significant issue in the clinic, but has also provides researchers with a valuable tool to study wound healing in animal models in the laboratory [110].

Currently, the use of the technique of surgical delay is the standard method used clinically to increase skin flap survival. Surgical delay consists of partially elevating and undermining the skin flap 7 to 14 days prior to full elevation and inset. Investigations of the molecular mechanisms by which the delay procedure increases skin flap viability have found that the procedure increases the expression of both FGF-2 and VEGF, but not TGF-β or PDGF [111-113]. Gene therapy techniques delivering exogenous growth factors, such as VEGF and FGF-2, may therefore mimic the endogenous response to
surgical delay and are currently being investigated to increase skin flap perfusion and healing.

Therapeutic angiogenesis to promote wound healing

Preclinical studies have shown that therapeutic angiogenesis is a promising option for increasing wound healing, but as of 2007 there were only a small number of clinical trials investigating gene transfer approaches targeting wound healing [41, 51]. A multitude of growth factors have been investigated to promote all phases of wound healing in both skin flap and incisional wound models [51, 98]. For example, therapeutic delivery of members of the FGF family has shown promise for increasing wound healing. In vivo EP delivery of a plasmid encoding KGF-1, (also known as FGF-7) increased healing in both a diabetic mouse [76] and septic rat model [77]. Also, liposome-mediated delivery of plasmid KGF-1, in combination with a plasmid encoding IGF-1, accelerated re-epithelialization and increased expression of VEGF in an acute wound model [114]. Further, transfer of FGF-2 by recombinant protein injection [115] or plasmid with in vivo EP increased skin flap survival [116].

PDGF and VEGF have been the most intensely studied growth factors for promoting therapeutic angiogenesis to increase wound healing. In preclinical studies, delivery of exogenous PDGF by viral-mediated transfer [117-119] and by direct injection [120] or liposome-mediated [121] delivery of a plasmid encoding PDGF successfully increased wound healing. In addition, a topical gel containing recombinant PDGF, Regranex (Ortho-McNeil), is currently prescribed for the treatment of diabetic neuropathic foot ulcers [39]. In several multicenter trials, daily application of Regranex
only modestly increased ulcer closure. Specifically, ulcer closure ranged from 25% to 36% with the placebo gel and from 36% to 50% with Regranex [122]. Topical application of recombinant VEGF protein has also been investigated as potential treatment to increase healing of diabetic neuropathic foot ulcers. In a Phase I clinical trial, application of a cream containing recombinant VEGF protein every 2 days for 6 weeks showed a positive, but not statistically significant, trend in ulcer healing [123]. In contrast, preclinical studies have shown direct injection or systemic delivery of recombinant VEGF protein improves skin flap survival [124-127]. One study demonstrated that intravenous delivery of VEGF protein was more effective than localized injection [124], but this approach raises the concern for pathological angiogenesis that may result from systemic VEGF expression [17-20]. Also in preclinical models, viral-mediated [128-131] transfer of VEGF and direct injection [132-134], liposome-mediated [120] or transfer in a fibrin matrix [135, 136] of plasmid VEGF have all increased skin flap survival. Nonetheless, non-protein methods for gene transfer of PDGF or VEGF have yet to be explored as a method to increase healing in clinical trials.

Peripheral Artery Disease

Peripheral artery disease (PAD), commonly resulting from atherosclerosis, is one of the leading causes of morbidity and mortality in the western world [137, 138]. The primary pathophysiology of PAD is a reduction in blood flow to the lower extremities caused by stenosis or occlusion of a collateral artery. The reduction in blood flow generally results in one of two clinical presentations: intermittent claudication (IC) or critical limb ischemia (CLI). IC is the most common presentation of PAD and is
characterized by muscular leg discomfort induced by exercise that is relieved by rest. Patients with IC are limited in walking speed and distance, and in other measures of quality-of-life [139]. CLI, a less common but more severe presentation of PAD, results from insufficient blood flow to the affected limb even at rest. The decrease in blood flow to the limb with CLI is so severe that limb loss is likely without therapeutic revascularization. There are a myriad of symptoms associated with CLI, including: rest pain in the extremity, metatarsalgia (pain and inflammation of the metatarsal region of the foot), ulcers or wounds that fail to heal, and gangrene [140].

Current treatment options for PAD include risk factor reduction, physical therapy and training, and pharmacological treatment of the underlying atherosclerosis. Also, in patients with severe IC or CLI, interventional (catheter based) or open surgical procedures may be used to revascularize the ischemic limb. A pharmacotherapy to increase perfusion to the effected limb is not available, and treatments targeting the underlying atherosclerosis are largely ineffective. Cilostazol, a reversible phosphodiesterase-3 inhibitor, has shown some clinical benefit for treatment of IC, but cannot be used in patients with any degree of heart failure [141]. Pentoxifylline, a pharmacological treatment that decreases blood viscosity and thereby increases circulation in the limb, is widely prescribed in the United States for the treatment of IC but has little or no clinical benefit [141, 142]. As mentioned above, revascularization of the lower extremity for patients with severe IC or CLI can be accomplished by interventional catheter-based techniques as well as open surgical procedures. However, direct revascularization is often impossible due to the anatomic extent of PAD and is also limited by associated co-morbidities, such as diabetes, coronary artery disease or stroke.
requiring a majority of patients to undergo limb amputation [138, 143-145]. The lack of an effective pharmacological treatment and the limits of interventional and surgical procedures have spurred an intense investigation of alternative approaches, such as therapeutic angiogenesis, for the treatment of PAD.

*Preclinical models of limb ischemia*

Animal models of hindlimb ischemia are the standard preclinical model for evaluating potential therapies for limb ischemia. The first characterization of an animal model of hindlimb ischemia was in the 1950s with studies in the rabbit [146]. Since this initial work, experimental hindlimb ischemia models have been developed in both large and small animals. In general, large animal models, such as swine and canine, are advantageous because the limb vessels and associated branches are easily identifiable while small animal models, such as mouse and rat, are advantageous because of their low cost and the availability of transgenic lines.

The design of preclinical limb ischemia models varies, and the level of ischemia achieved by each design depends greatly on the species and even the strain of animal [147]. The most common approaches to induce hindlimb ischemia are the tourniquet technique, simple ligation of the common femoral or common iliac arteries, and ligation with excision of these arteries and their distal branches. The tourniquet technique is a non-invasive approach that has been used in both large and small animal models to interrupt all arterial and venous blood flow to the limb, including blood flow from potential collateral vessels [148]. This approach results in a rapid onset of severe muscle necrosis and is more appropriate for studying acute ischemic injury than the chronic
occlusive disease characteristic of PAD. The level and duration of ischemia achieved by ligation of the common femoral or common iliac artery depends greatly on the level of collateral blood flow in the species. In general, this technique fails to produce chronic ischemia at rest, as seen in patients with CLI, but in some species and transgenic models it does result in a reduction in blood flow with exercise, characteristic of patients with IC [147, 149, 150]. In animals with an extensive network of collateral vessels, such as the rat [151], ligation of the common femoral [152] or common iliac artery [153] only transiently decreases blood flow to the limb. However, a study by Angersbach et al. [154] suggested that prolonged ligation of the femoral artery in the rat will eventually result in similar symptoms to IC. In comparison to simple ligation, ligation and excision of the most distal point of the external iliac or common femoral and associated distal branches reduces blood flow to the limb at rest, even in model systems with extensive collateral networks such as the rat [147, 155-157]. Thus, the ligation and excision method is considered an appropriate model to study potential therapies for CLI [147, 155-157].

Although there are differences in the methodology and resulting ischemia in the above mentioned preclinical models, all of these models are associated with some degree of perfusion recovery even without therapeutic intervention. Thus, the timing of delivery of potential therapeutics and assessment of limb perfusion is critical. Interventions delivered at the time of surgery, or shortly thereafter, will effectively measure the ability of the intervention to improve limb perfusion. If the intervention is delivered at later time points, usually no later than postoperative day (POD) 7, the ability to detect the effectiveness of the intervention will be hampered because perfusion in the ischemic limb
will be naturally nearing that found in the non-ischemic limb, which is often used as a control [147]. There are multiple methods to determine the effect of a potential treatment on limb blood flow in preclinical models. Histological assessment of capillary density alone is not an accurate measure of perfusion and should be supplemented by a method that detects limb blood flow directly such as calf blood pressure ratio, laser Doppler perfusion imaging (LDPI) or magnetic resonance imaging.

*Therapeutic angiogenesis for the treatment of limb ischemia*

Gene therapy techniques delivering angiogenic growth factors are emerging as promising options for the treatment of IC and CLI in patients when direct revascularization is not possible. A large number of angiogenic growth factors and cytokines have been explored as therapeutic candidates in both preclinical models and clinical trials [158]. Of these, FGF-1, FGF-2 and members of the VEGF family have been the most actively investigated, but several other growth factors have successfully promoted therapeutic angiogenesis in preclinical models including HIF-1α [159, 160], MCP-1 [161, 162] and eNOS [163, 164]. Hepatocyte growth factor (HGF) has also shown promise for therapeutic angiogenesis in both preclinical models [165, 166] and Phase I clinical trial as a treatment for CLI [167]. In clinical trials, several end points are measured to determine the efficacy of the treatment. In patients with IC, leg pain induced by exercise is the main clinical manifestation. Thus, quality-of-life questionnaires, peak walking time, and measurements of limb blood flow, such as ankle brachial index (ABI) or LDPI, are considered appropriate clinical trial end points. Because the clinical manifestations and prognosis of limb ischemia are more severe with CLI, trials
investigating therapeutic angiogenesis for the treatment of CLI often include additional end points, such as the size and healing of ulcers and, when appropriate, loss of tissue [140].

There have been multiple Phase I and II clinical trials investigating VEGF as a therapeutic agent for the treatment of PAD. Phase I clinical trials investigating intramuscular injection [168-170] and intra-arterial infusion [171] of plasmid VEGF\textsubscript{165} showed the treatment increased angiographic score, ABI, and ulcer healing and decreased the incidence of limb loss in patients with CLI. In a Phase II trial conducted in patients with both CLI and diabetes mellitus, intramuscular injection of plasmid VEGF\textsubscript{165} increased ABI and ulcer healing [172]. Also, in a Phase II trial intra-arterial administration of adenoviral-VEGF\textsubscript{165} in combination with plasmid VEGF\textsubscript{165} increased angiographic score in patients with CLI [173]. Clinical trials have also been conducted with adenoviral VEGF\textsubscript{121}. In Phase I trials, intramuscular injection of adenoviral VEGF\textsubscript{121} increased peak walking time [174], but when moved into Phase II trials there was not a significant improvement in the end points measured (quality-of-life and peak walking time) [175].

Of the FGF family members FGF-1 and FGF-2 have been the most intensely studied and most frequently utilized in emerging therapeutic angiogenesis approaches. In a Phase II clinical trial intramuscular injection of plasmid FGF-1 was well tolerated and decreased the incidence of limb amputation in patients with CLI [176]. Patients for a Phase III trial are currently being recruited to continue the study of intramuscular injection of plasmid FGF-1 for the treatment of PAD [177]. There have been several clinical trials investigating the use of recombinant FGF-2 protein to promote therapeutic
angiogenesis in patients with PAD. In a Phase I trial, intra-arterial delivery of recombinant FGF-2 significantly increased calf blood flow [178]. In another Phase I / II trial, recombinant FGF-2 suspended in a biodegradable gelatin hydrogel to promote sustained release was injected into the gastrocnemius muscle in patients with CLI. In comparison to baseline, the patients in this study showed significant improvements in peak walking time, limb perfusion and healing of foot ulcers [179]. In the Phase II Therapeutic Angiogenesis with FGF-2 (TRAFFIC) trial, intra-arterial injection of recombinant FGF-2 protein significantly improved peak walking time and ABI, but no differences were seen in claudication onset time or quality-of-life measurements [180]. Non-protein methods delivering FGF-2 to promote therapeutic angiogenesis have yet to be explored in clinical trials. Given the success intramuscular injection of plasmid FGF-1 in CLI patients, it would be of interest to explore other gene therapy approaches to deliver FGF-2 in clinical trials.
CENTRAL HYPOTHESIS

The end goal of therapeutic angiogenesis is to increase the amount of nutrients supplied to and the rate of waste product removal from ischemic tissue by increasing vasodilation, vascular permeability, neovascularization and, subsequently, blood flow. As a whole, this research explored the hypothesis that intradermal injection of plasmids encoding angiogenic growth factors followed by non-invasive in vivo EP is a feasible approach for promoting therapeutic angiogenesis in ischemic tissue to increase perfusion.
INTRADERMAL DELIVERY OF PLASMID VEGF_{165} BY ELECTROPORATION PROMOTES WOUND HEALING

Introduction

In addition to direct injection of plasmid DNA, the most prevalent methods of therapeutic delivery of VEGF to skin flaps documented in the literature are adenoviral-mediated gene transfer and recombinant protein injection. These methods have shown promise for increasing skin flap healing [124-134, 181] but have drawbacks (see General Introduction, Methods of therapeutic gene delivery). Moreover, in comparison to non-viral approaches, transgene expression with adenoviral-mediated delivery persists longer increasing the potential for the occurrence of the adverse side effects associated with continuous unregulated VEGF expression [17-20]. Thus, the development of a gene transfer approach that allows for tight control over expression level and duration would be advantageous for VEGF-mediated therapeutic angiogenesis. The research presented

Figure 2. The random skin flap model

*Left* Approximate placement of the RSF. *Center* Location of the distal area monitored for perfusion and healing. *Right* approximate location of the treatment sites
in this Chapter investigated intradermal injection of pVEGF followed by *in vivo* EP, using the 4PE, as potential therapy to increase wound healing. This strategy was explored using a modified McFarlane [182, 183] 8 cm x 3 cm RSF model (Figure 2). Specifically, pVEGF+ or control treatments were delivered at 4 sites to the RSF on POD 2. VEGF expression kinetics and the ability of the treatment to increase an endogenous angiogenic response, perfusion and healing were evaluated.

**Results**

*In vitro plasmid VEGF expression*

The ability of pVEGF to increase VEGF expression was tested *in vitro* prior to use *in vivo*. The promoter and coding region of VEGF was removed from pBLAST-VEGF and cloned into the backbone of pVAX1 to allow for more efficient preparation of large quantities of the plasmid. The level of VEGF expression with pVEGF was equivalent to that with pBLAST-VEGF in both the cell lysate and cell culture supernatant 48 hours after lipid-mediated transfection (Figure 3).
VEGF expression kinetics in the gastrocnemius muscle

Plasmids encoding a wide variety of genes with therapeutic potential, including VEGF, have been successfully delivered to skeletal muscle by intramuscular injection and in vivo EP [54, 184] resulting in increased transgene expression compared to injection of the plasmid alone. After determining pVEGF increases VEGF expression in vitro, in vivo expression was confirmed by delivering pVEGF+ and pVEGF- to the rat gastrocnemius muscle. On days 2, 5 and 7 after pVEGF delivery, muscle was excised

Figure 3. In vitro plasmid VEGF expression

Average VEGF expression ± SD was determined by ELISA 48 hours after lipid-mediated transfection of B16F10 cells with pVEGF or pBLAST-VEGF. For the mock transfection, cells were treated with the transfection reagent without plasmid DNA. n=3 for all groups.
from the delivery sites and assayed for VEGF expression using an ELISA that specifically detects human VEGF\textsubscript{165}. Compared to pVEGFE-, VEGF expression was significantly greater with pVEGFE+ for 5 days after delivery (Figure 4).

![Figure 4. pVEGFE+ increases VEGF expression in the gastrocnemius muscle](image)

**Figure 4. pVEGFE+ increases VEGF expression in the gastrocnemius muscle**

pVEGFE+ increased VEGF expression in gastrocnemius muscle compared to pVEGFE-. Day 2 and day 7: n=6 per group, Day 5: pVEGFE- n=5; pVEGFE+ n=4. * p < 0.05.

*VEGF expression kinetics in the random skin flap*

Because pVEGFE+ successfully increased VEGF expression in gastrocnemius muscle it was then investigated whether the same increase could be achieved with pVEGFE+ in the skin of the RSF model. Skin flaps were treated on POD 2. On PODs 4, 7, 10 and 14, skin samples from the treatment sites were excised and assayed for VEGF
expression using an ELISA that specifically detects human VEGF\textsubscript{165}. Necrosis prevented some sites from being harvested, especially in the pVEGFE- group at later time points. On both PODs 4 and 7, VEGF expression was significantly higher in the pVEGFE+ treatment group compared to the pVEGFE- treatment group (p< 0.05, both time points) (Figure 5). VEGF expression was determined for each treatment group in the proximal area, closest to the RSF pedicle, and distal area, farthest from the RSF pedicle. Interestingly, on POD 4, VEGF expression was significantly higher in the distal area of the RSF, compared to the proximal area, for the pVEGFE+ treatment group (p<0.05). There was a 2-fold increase in VEGF expression in the distal area, compared to the proximal area, with pVEGFE-, but this increase was not statistical significant (p= 0.157). The level of VEGF expression achieved with pVEGFE+ in the distal area of the RSF was similar to the level and duration observed in the gastrocnemius (Figures 4 and 5). Compared to POD 4, there was an overall significant decrease in VEGF expression on POD 7 (p< 0.05, both treatment groups in both the proximal and distal areas) but levels were still significantly higher with pVEGFE+ compared to pVEGFE- in each area (p< 0.05). At POD 10, there was not a significant increase in VEGF expression with pVEGFE+ in either the proximal or distal area of the RSF, and by POD 14 only background levels of VEGF were detected for both pVEGFE+ and pVEGFE-. VEGF expression was not detected when the vector backbone lacking the VEGF cDNA insert was delivered to the RSF with EP (pVAXE+) or in the skin of untreated controls (P-E-). Also, VEGF expression was not detected in serum samples at any time point during the study (data not shown) indicating pVEGFE+ only increases VEGF expression locally, not systemically.
Figure 5. VEGF expression kinetics in the random skin flap

On PODs 4 and 7 VEGF expression was significantly greater with pVEGFE+ than pVEGFE- in the RSF. VEGF expression was further increased in the distal region of the RSF. pVEGFE-: POD 4 proximal n=8, distal n=6; POD 7 proximal n=3, distal n=4; POD 10 proximal n=3, distal n=2; and POD 14 proximal and distal n=2. pVEGFE+: POD 4 proximal n=4, distal n=7; POD 7 proximal n=7, distal n=6; POD 10 proximal n=3, distal n=4; and POD 14 both proximal and distal n=3. For groups with n≤3 the mean ± SD is reported. For groups with n >3 the mean ± SEM is reported. * p< 0.05.
Delivery of plasmid VEGF with electroporation increases eNOS expression

Both in vitro and in vivo studies [26, 185] have shown increases in VEGF can increase levels of eNOS. To determine if pVEGFE+ and pVEGFE- increased eNOS in the RSF, skin samples from the distal area of the RSF were assayed for eNOS by ELISA at both PODs 4 and 7. On POD 4 eNOS was significantly higher following pVEGFE+ treatment compared to pVEGFE- treatment (p < 0.05), but by POD 7 eNOS levels were similar in the pVEGFE+ and pVEGFE- treatment groups (Figure 6). The application of an electric field to tissues alone can transiently induce expression of some genes [186-188] but pVAXE+ did not increase eNOS in the RSF model (p < 0.01).

![Graph showing eNOS expression](image)

**Figure 6. pVEGFE+ increases eNOS expression**

pVEGFE+ significantly increased eNOS expression in the distal area of the RSF on POD 4. n=7 per group per time point. * p< 0.05 compared to pVEGFE-. ‡ p< 0.01 compared to pVAXE+. 
Effect of plasmid VEGF with electroporation on distal skin flap blood flow

Because RSF survival shows little change after POD 15 [182] skin flap perfusion and healing were monitored through POD 14. pVEGFE+ resulted in the highest level of VEGF expression in the distal area of RSFs, which is more at risk for ischemia and necrosis since it is farthest from the vascular pedicle. Thus, it was determined if pVEGFE+ treatment increased perfusion more than control treatments in the distal area of the RSF, corresponding to approximately 25% of the total RSF area. LDPI was used to measure perfusion preoperatively (baseline), immediately postoperatively, and on PODs 2, 4, 7, 10, and 14. A sharp decrease in distal perfusion was observed immediately postoperatively through POD 4 indicating the RSF was effectively rendered ischemic. Perfusion for each treatment group is reported as the percent recovery of baseline (Figure 7a) and postoperative perfusion (Figure 7b). At PODs 10 and 14 perfusion was significantly different between all treatment groups for both recovery of baseline and postoperative perfusion (p< 0.01). Perfusion in the pVEGFE+ treatment group was significantly greater than all controls on both PODs 10 and 14 for both recovery of baseline (p< 0.01) and postoperative (p< 0.05) perfusion.
Figure 7. pVEGFE+ increases distal skin flap blood flow

Perfusion was measured in the distal region of the RSF by LDPI at the indicated time points. pVEGFE+ significantly increased recovery of baseline (a) and postoperative (b) perfusion on PODs 10 and 14 compared to all controls. n=7 per group, per time point. ** p< 0.01, *p < 0.05 compared to all controls.
Plasmid VEGF with electroporation promotes distal skin flap healing

Visual assessment of RSF viability over the time course of the study showed a clear decrease in distal necrosis in the pVEGFE+ treatment group compared to control groups (Figure 8, top panel). The surviving region of the distal area of the RSFs was quantitated on PODs 7 and 14 (Figure 8, bottom panel). To quantitate the percent survival of the distal area, the area of skin with normal texture and color was expressed as a percent of the total distal area. Non-surviving areas were defined as dark, rigid necrotic skin or scabbed necrotic lesions and surviving areas were defined as skin normal in texture and color. The pVEGFE+ treatment increased the percent of distal RSF survival on POD 7 (89.4 ± 4.3) (p < 0.05) compared to pVEGFE- (56.6 ± 10.6), pVAXE+ (59.3 ± 9.8) and P-E- (60.2 ± 9.6). The surviving area in all treatment groups increased approximately 5% to 10% on POD 14, but healing was still greater and more consistent in the pVEGFE+ group (95.2 ± 2.2) (p < 0.01) compared to pVEGFE- (65.6 ± 9.3), pVAXE+ (70.6 ± 7.4) and P-E- (73.5 ± 4.1). Adverse side-effects associated with increased VEGF expression levels, such as edema, [17-20] were not observed by gross visual assessment in any of the treatment groups. The mean percent survival of the pVEGFE- group on POD 7 in this study is similar to previously reported values delivering a plasmid encoding VEGF to a similar RSF model [120, 134]. On POD 14, skin samples were excised 2-cm from the distal end of the RSFs for histological analysis by Hematoxylin-Eosin (H&E) staining (Figure 9). If the skin was necrotic at 2-cm from the distal end, samples were excised near the edge of the surviving border. Skin from the pVEGFE+ treatment group appeared healthy (Figure 9, a-b) while skin from the
pVEGFE- treatment group showed evidence of acute inflammation, necrosis and myonecrosis (Figure 9, c-f).

**Figure 8.** pVEGFE+ increases distal skin flap healing

*(Top)* Representative images of RSFs from each treatment group over the time course of the study. *(Bottom)* Quantification of distal survival on PODs 7 and 14. n=7 per group, per time point. **p< 0.01, * p< 0.05 compared to all controls.*
Figure 9. pVEGFE+ decreases inflammation and myonecrosis

Representative images of skin excised 2-cm from the distal end of RSFs on POD 14 and stained with H&E from the pVEGFE+ (panels a and b) and pVEGFE− (panels c through f) treatment groups. Open arrows indicate areas of epidermal lifting characteristic of necrosis with pVEGFE− (panels c and d) compared to a normal epidermis with pVEGFE+ (panels a and b). Panel f, solid arrow indicates an example of an area of myonecrosis of the panniculus carnosus muscle observed with pVEGFE−. Open circles (panels d, e and f) indicate areas of inflammation characterized by the dark staining of numerous inflammatory cells. Inflammatory infiltrate was not observed with pVEGFE+ (panels a and b).
Preliminary studies were completed to explore if alternative treatment time points would increase RSF healing more than pVEGFE+ treatment on POD 2. It was first confirmed that pVEGFE+ and pVEGFE- increased VEGF expression when delivered to the RSF on POD 0 (Figure 10a). While immediate postoperative delivery of pVEGFE+ increased VEGF expression on POD 2, compared to pVEGFE-, the increase was not as robust as on POD 4 when treating on POD 2 (Figures 5 and 10a). It is possible the lower level of VEGF expression when treating on POD 0 was due to the need for the tissue to recover prior to treatment. Also, because VEGF primarily mediates the proliferative phase of wound healing [189], high levels of VEGF expression during the inflammatory phase may have been recognized as deleterious to the endogenous wound healing process. Thus, treatment on POD 0 may have resulted in the exogenous VEGF mRNA or protein being targeted for degradation. Because of the exploratory nature of these experiments, the use of experimental animals was minimized. Thus, the small number of treatment sites assayed resulted in a large variation in VEGF expression (Figure 10a).

RSF healing was also evaluated for the alternative treatment time points (Figure 10b). RSFs treated with pVEGFE- on both PODs 0 and 2 showed the largest increase in healing of the alternative treatment time points, but healing with pVEGFE+ treatment on POD 2 was still greater. Interestingly, RSF healing was much lower with pVEGFE+ treatment on both PODs 0 and 2 compared to treatment on POD 2 alone. This may be because excessively high levels of VEGF expression can have negative effects on the process of angiogenesis [17-22].
Figure 10. pVEGFE+ treatment on POD 2 increases healing more than treatment at POD 0 or multiple time points.

(a) pVEGFE+ treatment on POD 0 increases VEGF expression on POD 2. n=2 per group for both proximal and distal areas. Mean ± SD is reported (b) Quantification of distal RSF survival on PODs 7 and 14 when treating on POD 0, POD 2 or both POD 0 and 2. Treatment on POD 0 and POD 0 and 2 n=2 per group. Treatment on POD 2 n= 7 per group.
Discussion

The data presented in this chapter demonstrate a novel therapeutic approach to increase VEGF expression, induce an endogenous angiogenic response, and increase perfusion and healing of the distal area of RSFs. pVEGFE+ is an attractive alternative to viral and recombinant protein gene transfer approaches to promote healing because it allows for the control of localized expression levels and duration. This approach also circumvents the adverse side-effects and practicality issues associated with viral-mediated and recombinant protein approaches. Previous gene therapy studies delivered VEGF to skin flaps either preoperatively or intraoperatively [120, 124-133, 135, 136]. This study was the first to deliver pVEGF on the second POD. VEGF modulates the proliferative phase of wound healing that begins 2 to 4 days after the initial injury [189]. By administering pVEGFE+ on POD 2, VEGF expression was timed to peak during the beginning of the proliferative phase of healing. Preliminary studies treating the RSFs on POD 0, and on both POD 0 and 2 suggested that pVEGFE+ treatment on POD 2 increases healing more than treatment at these alternative time points (Figure 10b). Also, by administering treatment on POD 2, when the distal area of the skin flap is most hypoxic [190], it was determined that electrically-mediated delivery of plasmid DNA to a large area of ischemic skin is in fact possible.

These results demonstrate that pVEGFE+ significantly increased VEGF expression, compared to pVEGFE-, and that VEGF expression further increased with distance from the vascular pedicle. Also, in the distal area of the RSF, pVEGFE+ resulted in similar levels of VEGF expression as delivery to the gastrocnemius muscle
(Figures 4 and 5). Higher VEGF expression in the distal area of the RSF, compared to proximal region, could be due to hypoxia-induced stabilization of the VEGF mRNA or increased translation [191]. The current known mechanisms to increase VEGF mRNA stability and translation involve genetic regions not contained within the plasmid used in this study. It is possible other unknown mechanisms could enhance VEGF levels and may be responsible for the increase in VEGF expression in the distal area, compared to the less hypoxic proximal region. Importantly, VEGF expression was transient and not detected systemically, indicating that the increase was localized to the delivery area. Moreover, pVEGFE+ confers a short-term localized increase in VEGF expression thereby decreasing the possibility of the occurrence of the adverse side-effects that are associated with long-term high levels of VEGF expression [17-22].

The increase in eNOS expression after the pVEGFE+ and pVEGFE- treatments indicates that the observed increase in exogenous VEGF successfully stimulated an endogenous angiogenic response. pVEGFE+ induces an earlier and more robust increase in eNOS expression compared to pVEGFE- (Figure 6). These results are consistent with reports that 

*in vitro* VEGF-induced upregulation of eNOS is time and dose dependent [192] and with a study by Huang et al. [193] that demonstrated adenoviral delivery of VEGF165 to RSFs increased eNOS. Relative to pVEGFE-, there was a 2.2-fold increase in eNOS protein with pVEGFE+ in the distal area of the RSF on the fourth POD that was mediated by a 6.6-fold increase in VEGF protein (Figures 5 and 6). Significant progress has been made elucidating the mechanisms underlying VEGF-mediated increases in eNOS, but many aspects of the complete mechanism remain unclear despite the well established role of this pathway in wound healing and angiogenesis [163, 193, 194].
vitro [26, 192] studies utilizing the optimal VEGF dosage, and in vivo [193] studies, have shown that VEGF-mediated increases in eNOS protein levels are typically not greater than 2- to 5-fold over background levels. In the work presented here, eNOS levels with pVEGFE+ remain similar from POD 4 to POD 7, but increase with pVEGFE- over this time period, suggesting that maximal level of eNOS has been reached in the pVEGFE+ group on POD 4. The small increase in eNOS levels, relative to VEGF levels, may be due to inhibition of eNOS production by high levels of NO [195]. Also, the persistence of eNOS expression in the pVEGFE+ and pVEGFE- treatment groups at POD 7, despite a decrease in VEGF expression, may be the result of a VEGF-mediated increase in eNOS mRNA stability [26]. eNOS mRNA has a normal half-life of 48 hours [196] and further extending the half-life would allow for the increase in eNOS protein to persist despite a decrease in VEGF levels.

The early increase in eNOS observed with pVEGFE+ could contribute to the increased perfusion and healing observed at the later time points. eNOS production of NO is important in the early stages of skin flap healing [197] and contributes to maintaining peripheral skin flap circulation [198]. Another possible mechanism for the increased perfusion and healing of the RSF after treatment with pVEGFE+ is increased neovascularization. There was not a significant increase in vessel density (data not shown) in skin samples harvested 2-cm from the distal end of the RSFs on POD 14. Other studies delivering exogenous VEGF to wound healing models have reported the same phenomenon of increased healing without an associated increase in vessel density [127, 133] while other studies do not discuss changes in capillary density. To prevent ischemic necrosis there must be adequate perfusion pressure to the distal region of the
skin flap [199]. In this study, increased eNOS, and the subsequent increase in vasodilation, could be the primary mechanism of the pVEGFE+-mediated increase in perfusion and healing, but determination of the exact mechanism will require further studies.

In summary, the pVEGFE+ treatment significantly increased VEGF expression for 5 days after treatment (through POD 7) and this short-term increase was sufficient to significantly increase eNOS, compared to pVEGFE- treatment, 2 days after treatment. Further, pVEGFE+ significantly increased recovery of baseline and postoperative perfusion and as well as healing compared to all control treatment groups. Thus, electrically-mediated intradermal delivery of pVEGF is a potential non-invasive therapeutic approach to increase perfusion and wound healing.

**Future directions**

To move this approach forward into a clinical setting, the efficacy of pVEGFE+ to increase perfusion and healing of RSFs should next be examined in a large animal model. Porcine skin physiologically resembles that of human skin. Also, direct injection of plasmid DNA in porcine skin results in a similar expression pattern as in human skin, suggesting the swine as an appropriate large animal model for future work [200]. Also, because wound healing is impaired in patients with diabetes mellitus [92, 95], it would be interesting to explore if pVEGFE+ increases the healing of RSFs in a diabetic model. Diabetes has been successfully induced in both the rat [201] and swine [202] with streptozotocin. Also, transgenic rat models of diabetes are available that could be used for these future experiments [203, 204].
Experiments investigating the mechanism by which pVEGFE+ significantly increases VEGF expression in the distal area, compared to the proximal area, of the RSF could elucidate a novel mechanism of VEGF mRNA stability. The ELISA used in these studies specifically detects human VEGF\textsubscript{165} and because only background levels of VEGF were detected with P-E- and pVAXE+, it is unlikely that the increase in VEGF in the distal area of the RSF is due to non-specific detection of endogenous VEGF. The expression of endogenous VEGF is increased and attenuated during hypoxia by both transcriptional regulation and mRNA stabilization. HIF-1 increases VEGF transcription through binding the hypoxia response element in the endogenous VEGF promoter and VEGF mRNA is stabilized in 5’ and 3’ untranslated regions of the endogenous mRNA [13-15]. Neither of these genetic regions is present in pVEGF suggesting the increase in VEGF expression occurs through an unknown mechanism. Several genes, such as c-fos, c-myc and β-tubulin, contain RNA regulatory elements within their coding regions [205] and it is possible the increase in VEGF expression in the distal area of the RSF occurs through a similar mechanism. Endogenous VEGF is increased during hypoxia by transcriptional mechanisms, but it has been shown that the increase in VEGF protein occurs mainly through an increase in mRNA stability [13]. In vitro experiments could be used to determine if the increase in VEGF with pVEGFE+ in the distal area of the RSF results from increased mRNA stabilization through a regulatory region in the coding sequence. To evaluate this hypothesis, assays similar to those reported by Shima, et al. [13] could be used. Briefly, pVEGF would be transfected into a non-human cell line, in order to distinguish endogenous and recombinant VEGF, and then cultured under both hypoxic and normoxic conditions. After 15 hours, the culture media would be replaced
with new media containing actinomycin D to inhibit new RNA synthesis and cells would then be cultured under normal conditions. VEGF mRNA levels would then be determined by northern blot, using a human VEGF<sub>165</sub> specific probe, at time points up to 8 hours after the addition of actinomycin D. Changes in endogenous mRNA could be used as a positive control. If VEGF<sub>165</sub> mRNA levels are increased in cells cultured under hypoxic conditions it would indicate that there is a putative hypoxia-regulatory element within VEGF mRNA coding region. Further experiments could then be carried out to determine the location of the regulatory region and the RNA binding proteins that bind it.

It would also be of interest to determine if increased healing with pVEGFE+ is primarily mediated through an early increase in eNOS expression. In a rat model of hindlimb ischemia Namba, et al. [163] demonstrated increased perfusion with intramuscular injection of a plasmid encoding eNOS was mediated through VEGF. In their study they inhibit eNOS activity by administering N<sup>(G)</sup>-nitro-L- arginine methyl ester (L-NAME), a general inhibitor of nitric oxide synthase activity, through the animals drinking water. Employing the same strategy, eNOS could be inhibited and RSF perfusion and survival could be assessed. Effective inhibition of the VEGF-mediated increase in eNOS should first be confirmed on the fourth POD, when the increase is first seen, by ELISA. Although L-NAME is a general inhibitor of nitric oxide synthase activity, and not eNOS specifically, these experiments would elucidate if inhibiting the VEGF-mediated increase eNOS effects RSF healing.
OPTIMIZATION OF DELIVERY CONDITIONS FOR THE MULTIELECTRODE ARRAY

Introduction

The design of the MultiElectrode Array (MEA) (Figure 11) expands on the configuration of the 4PE. The MEA is composed of an array of 16 small electrodes, in a 4 by 4 configuration, with a 2-mm gap width between electrodes. Similar to the 4PE, the design of the MEA allows for multiple sets of electrical pulses to be applied in 2 electric field orientations at a 90° angle without removing the electrode from the delivery site. One drawback to current electrode designs is that delivery of plasmid to a large surface area requires the gap width between the electrode pairs to be increased thereby the applied voltage must also be increased for cell permeabilization to be effective. The design of the MEA allows for its dimensions to be expanded by incorporating a larger number of electrode pairs thus enabling a larger surface area to be treated without the need to increase the distance between electrodes. Further, the small gap width between the electrode pairs decreases the depth of the electric field penetration and thus should decrease the amount of nerve stimulation and pain.

Figure 11. The MultiElectrode Array
The goal of the work presented in this chapter was to further evaluate and optimize delivery parameters for the MEA. This work was completed using a plasmid encoding the luciferase reporter gene (pLuc) delivered by intradermal injection in a guinea pig model followed by in vivo EP with the MEA. Specifically, expression kinetics and the effects of varying field strength, pulse duration, and plasmid dose were evaluated.

Results

Effect of field strength and pulse width on transgene expression

For in vivo EP, the voltage applied and pulse width are considered the main determinants of the level of transgene expression, while other pulse parameters, such as pulse number, have a lesser impact [65, 206]. Previous work determined that a field strength of 300 V/cm and 150 ms pulse width applied with the MEA resulted in similar luciferase expression levels achieved with the 4PE at 200 V/cm and 20 ms [Heller, R et al., manuscript in preparation]. To further evaluate the effects of field strength and pulse width for plasmid delivery with the MEA, 3 field strengths (300 V/cm, 250 V/cm and 225 V/cm) were examined with varying pulse widths, ranging from 350 ms to 50 ms (Figure 12). Two days after plasmid delivery, the delivery sites were excised and luciferase activity determined by a standard in vitro luciferase assay. For all 3 field strengths and pulse widths, except for 250 V/cm, 50 ms pulses, there was a significant increase in luciferase expression with pLucE+ compared to pLucE- (p< 0.001). In general, a pulse duration of longer than 150 ms resulted in slightly higher levels of luciferase expression, but 150 ms provided the highest level of expression with the least variation and minimal visual damage to the delivery area.
**Figure 12. Effect of field strength and pulse width luciferase expression**

Luciferase expression was determined 48 hours after delivery using an *in vitro* luciferase assay for the indicated field strengths (V/cm) and pulse widths (ms). * p<0.001 compared to pLucE-. 
Transgene expression kinetics

Because a pulse width of 150 ms for each of the field strengths examined in Figure 12 (300 V/cm, 250 V/cm and 225 V/cm) resulted in the highest level of transgene expression with the least variation and visible tissue damage, the duration of transgene expression was then evaluated using all 3 field strengths with 150 ms pulses. There was a significant increase in luciferase expression with all 3 field strengths beginning 24 hours after delivery. For 300 V/cm and 250 V/cm, luciferase expression remained significantly higher than pLucE- through day 17 after delivery (Figure 13) (p< 0.001). With all field strengths luciferase expression decreased to background levels 21 days after delivery (data not shown). For 225 V/cm, luciferase expression remained significantly higher with pLucE+ through day 10 after delivery (p< 0.001). Fourteen days after delivery there was no significant increase in luciferase expression with 225 V/cm compared to pLucE- (p=0.103). However, on day 17 luciferase expression with 225 V/cm increased slightly and expression was significantly higher compared to pLucE- at that time point (p< 0.001). Although there was a slight increase in expression on day 17 with 225 V/cm (3.4 x 10^5 photons / s) compared to day 14 (1.6 x 10^5 photons / s), the significant difference on day 17 between pLucE+ and pLucE- for 225 V/cm is most likely due to the decrease in luciferase expression with pLucE- on day 17 (3.8 x 10^4 photons / s) compared to day 14 (1.2 x 10^5 photons / s). Luciferase expression for all field strengths did not significantly decrease from the initial peak expression at 24 hours until day 10 after delivery (p< 0.001). In contrast, luciferase expression with pLucE- significantly decreased 96 hours after delivery (p< 0.001).
Figure 13. Luciferase expression kinetics

Luciferase expression was determined at the indicated time points by *in vivo* luciferase assay for the indicated field strengths (V/cm) with 150 ms pulse widths. pLucE- and 225 V/cm n=16 per group per time point. 250 V/cm n=15 per group per time point. 300 V/cm n=14 per group per time point. * p< 0.001 compared to pLucE- for that time point.
Control of transgene expression by plasmid dose

Control over transgene expression levels is desirable for delivery of plasmids encoding genes of therapeutic potential. As such, it was next determined whether a dose response relationship existed between the amount of plasmid delivered and the resulting level of transgene expression. A range of pLuc doses (20 µg, 50 µg, 100 µg, 150 µg and 200 µg) was delivered at 250 V/cm and 150 ms pulses. Two days after delivery, luciferase expression was determined by in vivo luciferase assay (Figure 14). For pLucE+, compared to 100 µg of pLuc (the dose delivered in Figures 12 and 13), a significant decrease in luciferase expression was seen when delivering 20 µg of pLuc (p<0.01) and a significant increase was seen when delivering 200 µg of pLuc (p<0.01). While altering the pLuc dose required altering the intradermal injection volume, varying injection volume did not significantly affect transgene expression with pLucE+ (Figure 15).
Figure 14. Control of luciferase expression by plasmid dose

Luciferase expression was determined 48 hours after pLuc delivery by *in vivo* luciferase assay. pLucE+ = 250 V/cm 150 ms. For 20 µg, 150 µg and 200 µg, n=6 per group for both pLucE- and pLucE+; For 100 µg n=8 per group for both pLucE- and pLucE+; For 50 µg pLucE- n=6 and pLucE+ n=5. * p< 0.05 for that plasmid dose, ‡ p< 0.05 compared to expression with pLucE+ delivering 100 µg.
Figure 15. Increasing plasmid injection volume does not affect luciferase expression

Luciferase expression was determined using an in vivo luciferase assay 48 hours after delivery of 100 µg pLuc in the indicated injection volume. pLucE+ = 250 V/cm, 150 ms. n=8 per group. * p< 0.001 compared to pLucE- for that injection volume.
Discussion

As a whole, the data presented in this chapter demonstrate that the level and duration of transgene expression achieved for pLucE+ delivered with the MEA can be adjusted by altering either electroporation parameters or plasmid dose. The duration and magnitude of the voltage applied has a significant impact on the level of electropermeabilization of the cell membrane in the target tissue [206]. Delivery of pLuc at 300 V/cm, compared to 250 V/cm and 225 V/cm, resulted in slightly higher luciferase expression at all pulse widths examined, but there was frequently visible tissue damage after delivery. It is possible that damage to the tissue is the reason for increased variability with 300 V/cm. Compared to 225 V/cm, luciferase expression was higher with 250 V/cm at all pulse widths. The lack of a significant increase in luciferase expression with 50 ms pulses delivered at 250 V/cm (Figure 12) indicates that 100 ms may be the minimum pulse duration to reach the necessary level of cell membrane permeabilization to increase plasmid uptake at a field strength of 250 V/cm or less.

The results presented in Figure 12 utilized in vitro luciferase assays to evaluate transgene expression. In vivo bioluminescence imaging allows for the use of fewer experimental animals and was used to determine luciferase expression in Figures 13, 14 and 15. Prior to using in vivo luciferase assays, it was determined that the results obtained by the in vivo assay were highly correlated with the results obtained by the in vitro assay ($r^2 = 0.94$, n=8).

It was previously reported [200] transgene expression decreases 3 days after plasmid DNA delivery by intradermal injection [200]. Consistent with these findings, in this study luciferase expression with pLucE- significantly decreased between days 3 and
4 after delivery compared to the initial peak expression at 24 hours (Figure 12). For all pLucE+ conditions, there was a significant decrease in luciferase expression 10 days after delivery, compared to the initial peak at 24 hours, and expression with 300 V/cm and 250 V/cm remained greater than pLucE- for 17 days. Luciferase expression with 225 V/cm was not significantly greater than pLucE- 14 days after delivery, but on day 17 expression with 225 V/cm slightly increased and was significantly higher than expression with pLucE+. The significant difference in expression with 225 V/cm, compared to pLucE-, at day 17 is most likely due to the decrease in luciferase expression with pLucE-.

Also, 225 V/cm resulted in an overall lower level of luciferase expression than 300 V/cm and 250 V/cm beginning at day 10 after delivery. Preliminary data not reported here determined that a field strength of 200 V/cm and 100-150 ms pulses increases luciferase expression approximately 2-fold compared to pLucE- 2 days after delivery. Overall, these results indicate that if a lower level of expression is desired, it could be achieved by decreasing the field strength.

Adjusting the level of transgene expression can also be accomplished by increasing or decreasing the dose of plasmid delivered. Compared to delivery of 100 µg pLuc (7.3 x 10^8 photons / s), delivery of 200 µg (4.5 x 10^9 photons / s) resulted in a 6-fold increase and delivery of 20 µg a 3.4-fold decrease (2.1 x 10^8 photons / s) in luciferase expression. Luciferase expression continued to increase up to 200 µg of pLuc and there was a linear correlation between the amount of plasmid delivered and luciferase expression (r^2 = 0.88) indicating the maximum expression level was not reached. Further experiments will be needed to determine the dose required to achieve the maximum level of expression.
In summary, for pLucE+, the level of luciferase expression increased with the magnitude of the voltage applied. For each of the field strengths examined, a pulse duration of 150 ms achieved the highest level of expression with the least variation and visible tissue damage. Also, for all 3 field strengths, luciferase expression did not decrease from initial peak expression at 24 hours until 10 days after delivery and with 300 V/cm and 250 V/cm remained significantly higher than pLucE- for at least 17 days. In addition to adjusting transgene expression levels by altering fielding strength, levels can also be controlled by adjusting the plasmid dose.

**Future directions**

While the MEA was designed for plasmid delivery to the skin, it may also be a useful non-penetrating electrode design for the delivery of plasmid to other tissues. Plasmid DNA can be delivered to internal organs using both penetrating (needle arrays) and non-penetrating electrodes. Generally, if a non-penetrating electrode design is selected to deliver plasmid DNA to internal organs, such as the liver, *in vivo* EP would be carried out with a paddle electrode. The concerns with this approach are similar to those when delivering plasmid to the skin with the caliper electrode. In order to apply 2 sets of pulses, the electrode plates need to be manipulated around the treatment site, and removal and replacement of the electrode plates results in variation in the distance between the plates and thus a variation in the applied voltage. The design of the MEA would eliminate this variation. In addition to being effective for plasmid delivery to the skin, the MEA has already been shown to be effective for *in vivo* EP plasmid delivery to
cardiac muscle [Marshall, WG et al., submitted for publication] and could easily be explored for delivery to other organs, such as the liver.

Experiments optimizing delivery conditions for the MEA were initially planned to be carried out in a hairless guinea pig model because the structural characteristics and vascularity of hairless guinea pig skin closely resemble human skin [207, 208]. Unfortunately, hairless guinea pigs were not available at the time these experiments were completed or for the foreseeable future. Confirmation of the data presented here, generated in Hartley guinea pigs, in the hairless guinea pig, if available, would be helpful when moving this technology into clinical trials. If hairless guinea pigs are not available then confirmation of this data in a porcine model, which also have skin similar to human skin [200], would be an alternative option.

The MEA was designed to allow for its dimensions to be expanded by incorporating a larger number of electrode pairs to treat a larger surface area without the need to increase the distance between electrodes. While the data presented here confirms the principle of the MEA design, experiments employing a larger electrode (composed of more electrode pairs) than the one utilized in these experiments have not yet been completed. A further understanding of the expression levels and kinetics that can be achieved with a larger MEA would be useful for future studies and for translation into clinical applications.
INTRADERMAL DELIVERY OF PLASMID FGF-2 BY ELECTROPORATION INCREASES PERFUSION AND ANGIOGENESIS IN AN ISCHEMIC HINDLIMB

Introduction

Gene therapy approaches delivering plasmid FGF-2 by intramuscular injection of plasmid FGF-2 alone [209] or followed by intramuscular in vivo EP [210, 211] have shown promise as a treatment for PAD in preclinical models. Also, delivery of recombinant FGF-2 has been shown to be beneficial in patients with PAD in clinical trials [178-180]. Currently, a simple non-invasive technique to deliver plasmid FGF-2 is not available. The primary focus of the research presented in this chapter was to determine if delivery of a plasmid encoding human FGF-2 (pFGF) to the skin with the MEA would increase FGF-2 expression, blood flow, and angiogenesis in a rat model of hindlimb ischemia. In addition, this was the first study utilizing the MEA to deliver a plasmid encoding a gene with therapeutic potential and expression levels were compared to those achieved with the 4PE.

To create the rat model of hindlimb ischemia, the most distal end of the external iliac artery and the femoral artery proximal and distal to the bifurcation of the superficial epigastric artery and vein were ligated. The femoral artery was then cut between the ligation of the external iliac, adjacent to the inguinal ligament, and dissected free to the bifurcation of the superficial artery and vein (Materials and Methods for complete...
description). This method of inducing hindlimb ischemia, the ligation and excision method, effectively decreased blood flow to the limb at rest for the duration of the study (14 days) and is considered an appropriate model to study potential therapies for CLI [147, 155-157]. After postoperative perfusion assessment with LDPI, pFGF was delivered to the ischemic hindlimb at 2 sites on the medial aspect of the ischemic limb by intradermal injection followed by EP with the MEA.

Results

In vitro plasmid FGF-2 expression

The ability of pFGF to increase FGF-2 expression was tested in vitro prior to use in vivo. The promoter and coding region of FGF-2 was removed from pBLAST-bFGF and cloned into the backbone of the pVAX1 to allow for more efficient preparation of large quantities of the plasmid. The level of FGF expression with pFGF was slightly lower compared to pBLAST-bFGF, but pFGF still effectively increased FGF-2 expression. As expected, FGF-2 was detected in the cell lysate and not the cell culture supernatant because FGF-2 is only secreted from cells during active angiogenesis [212] (Figure 16).
Optimization of electroporation conditions for plasmid FGF-2 delivery

The work described in the previous chapter utilized a reporter plasmid (pLuc) in a guinea pig model to evaluate delivery conditions for the MEA. It was expected that delivery of a plasmid encoding a gene of therapeutic potential (pFGF) in a different animal model would result in different expression kinetics. Thus, it was next evaluated whether pFGFE+ delivered with the MEA increased FGF-2 expression in the skin of the rat flank and if the level of expression achieved was similar to that achieved when

Figure 16. *In vitro* pFGF expression

Average FGF-2 expression ± SD was determined by ELISA 48 hours after lipid-mediated transfection of HaCat cells with pFGF, pBLAST-bFGF or the pVAX1 vector backbone without a cDNA insert. pFGF n=3 pBLAST-FGF-2 n=1, pVAX1 n=1.
delivered with the 4PE. Two days after pFGFE- or pFGFE+, skin samples from the delivery sites were excised and assayed for FGF-2 expression by ELISA (Figure 17). The level of FGF-2 expression was approximately equal for pFGFE+ delivered with the 4PE at 200 V/cm, 20 ms (4,763 ± 1,105 pg / sample) and the MEA at 250 V/cm, 150 ms (4,257 ± 815 pg / sample). FGF-2 expression was slightly greater when pFGF was delivered with the MEA at 300 V/cm, 150 ms (5,482 ± 901 pg / sample).

**Figure 17. Comparison of FGF-2 expression after pFGF delivery with the 4PE or MEA**

FGF-2 expression was determined by ELISA 48 hours after delivery of pFGF to the rat flank with either the 4PE or MEA at the indicated field strengths and pulse widths. n=4 per group. Average expression ± S.D. is reported. * p< 0.02 compared to pFGFE-. 
FGF-2 expression kinetics

The duration of FGF-2 expression was next determined for pFGFE+ delivered at 300 V/cm, 150 ms with the MEA. At the time points indicated in Figure 18, skin from the treated areas were excised and assayed for FGF-2 expression by ELISA. For 10 days after plasmid delivery pFGFE+ significantly increased FGF-2 protein levels compared to pFGFE- (p< 0.05) before decreasing to background levels at days 14 and 17 after delivery. The application of an electric field to tissues alone can transiently induce expression of some genes, including angiogenic growth factors [186-188], however, pVAXE+ resulted in similar levels of FGF-2 expression as P-E- (n=4, 1416 ± 326 total pg / sample).

Plasmid FGF-2 with electroporation increases blood flow in the ischemic hindlimb

It was next evaluated whether the pFGFE+ treatment could increase blood flow in a rat model of hindlimb ischemia. Immediately postoperatively, pFGFE+ or control treatments were administered at 2 sites on the medial aspect of the ischemic limb. LDPI was used to measure blood flow in the distal area of both the ischemic and non-ischemic limbs preoperatively (baseline), immediately postoperatively and on PODs 1, 3, 7, and 14 (Figure 19a). Immediately postoperatively, perfusion decreased to approximately 40% of baseline indicating the hindlimb was effectively rendered ischemic. In Figure 19b blood flow is reported for each treatment group as the ratio of blood flow in the ischemic hindlimb to the non-ischemic hindlimb (I/NI) (Figure 19b, top panel) and as a percent of the perfusion recorded at baseline (Figure 19b, bottom panel). There was significant
difference in limb blood flow beginning on the third POD between all treatment groups for I/NI (p< 0.02) and as a percentage of baseline blood flow (p< 0.001). Also on POD 3, blood flow in the pFGFE+ treatment group was significantly greater than all of the control groups for I/NI and percentage of baseline blood flow (p< 0.001). Perfusion in the ischemic limb continued to be higher in the pFGFE+ treatment group compared to the control treatment groups for all subsequent time points in the study (p< 0.05, all time points for I/NI and percent of baseline blood flow). The pFGFE- treatment group showed

Figure 18. FGF-2 expression kinetics

At the indicated time points, skin samples were harvested from the delivery sites and assayed for FGF-2 protein expression by ELISA. To determine FGF-2 expression resulting from pFGFE+ and pFGFE- the average FGF-2 expression in untreated skin (n=4, 1416 ± 326 total pg / sample) was subtracted from the total pg/sample determined for each treatment site. Day 2 n=10, days 4, 7, and 10 n=6 and days 14 and 17 n=8 per group per time point. pFGFE+= 300 V/cm, 150 ms. ** p< 0.01, * p< 0.05.
a slight, but not significant, increase in perfusion compared to the pVAXE+ and P-E-
treatment groups on PODs 7 and 14.

Figure 19a. pFGFE+ increases hindlimb blood flow

Representative LDPI images of the ischemic limb from the pFGFE+ and pFGFE-
treatment groups at baseline and on PODs 0, 3, 7 and 14. As indicated in the color
legend on the right, areas red or white in color represent higher perfusion and areas in
blue lower perfusion. The white box indicates the approximate area where perfusion was
assessed. The black arrows indicate the absence of perfusion in the area of the femoral
artery after the operation to induce hindlimb ischemia.
Figure 19b. pFGFE+ increases hindlimb blood flow

Perfusion was determined by LDPI. (Top) Ratio of perfusion in the ischemic limb to the non-ischemic limb (I/NI) for all treatment groups. (Bottom) Postoperative perfusion at the indicated time points relative to baseline perfusion for all treatment groups. Percent of baseline perfusion was calculated using the I/NI determined during the baseline perfusion measurements. n=6 per group per time point. * p< 0.05 compared to all controls.
Plasmid FGF-2 with electroporation increases angiogenesis in the ischemic hindlimb

Next, it was determined whether the increase in limb perfusion in the pFGFE+ treatment group resulted from an increase in angiogenesis. In rodent models of hindlimb ischemia, as well as in patients with PAD, angiogenesis typically occurs in the gastrocnemius muscle, or distal to the arterial occlusion, while arteriogenesis occurs higher in the limb near the site of the occlusion [147, 213]. Thus, on POD 14 samples were harvested from the gastrocnemius muscle of the ischemic limb and capillary density was visualized by immunohistochemical staining for the endothelial cell marker factor-VIII associated antigen. Representative fields (400X) from each of the 4 treatment groups are shown in Figure 20. Also in Figure 20, the average number of vessels in 5 randomly selected high power fields (HPFs) (400 X) for 5 animals in each treatment group is reported. There was approximately a 2-fold increase in capillary number in the pFGFE+ treatment group compared to all control groups (p< 0.001) indicating the increase in perfusion to the ischemic limb with pFGFE+ was in fact due to an increase in angiogenesis.
Figure 20. pFGFE+ increases angiogenesis in the ischemic hindlimb

(Left) Representative cross-sections (400X) of Factor VIII immunohistological staining of samples excised from the gastrocnemius on POD 14. (Right) Quantification of capillary density. The average number of capillaries in 5 fields, for 5 animals in each treatment group. * p< 0.01 compared to all controls.
Discussion

In addition to direct injection of naked plasmid DNA, the most prevalent methods of FGF-2 delivery documented in the literature are viral-mediated gene transfer and recombinant protein injection. These methods are promising for treatment of PAD but have drawbacks (See General introduction, Methods of therapeutic gene delivery). Further, the unregulated expression and long term expression conferred by some viral vectors, relative to plasmid DNA vectors, increases the possibility of the occurrence of the adverse side-effects associated with high levels of FGF-2 expression, such as potential toxicity and proteinuria [140, 214]. In both animal models [21, 215, 216] and clinical trials [178-180], intramuscular injection or intra-arterial administration of recombinant FGF-2 protein improved perfusion to the ischemic limb or PAD related symptoms, respectively. Similar to VEGF, the use of recombinant FGF-2 in a clinical setting is not practical due to its short half-life and poor bioavailability. Consequently frequent administration of the recombinant protein is often required to sustain lasting effects [37, 38, 140].

The strategy presented here is a simple, non-invasive approach that effectively increases FGF-2 protein expression, ischemic limb blood flow and angiogenesis. FGF-2 expression was similar for pFGFE+ delivered with the 4PE and MEA (Figure 17) and was significantly higher with pFGFE+ than pFGFE- for at least 10 days after delivery (Figure 18). Beginning on POD 3, there was a significant difference in blood flow in the ischemic hindlimb among all treatment groups, and blood flow in the pFGFE+ treatment group was significantly greater than all control groups (p< 0.05). The increase in
hindlimb perfusion with pFGFE+ treatment persisted from POD 3 through POD 14, the end point of the study (Figure 19b). There was also a significant increase in the number of capillaries in the gastrocnemius muscle with pFGFE+ treatment indicating the increase in limb blood flow is, at least in part, the result of an increase angiogenesis (Figure 20) (p< 0.001).

The increases in perfusion and angiogenesis in the pFGFE+ treatment group may be occurring by several known in vivo mechanisms. FGF-2 is normally confined to the cell compartment but during active angiogenesis it is exported to the extracellular space to exert downstream effects [28]. Therefore, although pFGFE+ treatment is administered to the skin, increased levels of FGF-2 protein after treatment and subsequent export could activate downstream angiogenic pathways. For example, a recent study by Fujii, et al. [209] demonstrated that in a model of hindlimb ischemia intramuscular injection of a plasmid encoding FGF-2 increased the expression of placental growth factor via the upregulation of VEGF expression. Placental growth factor alone is known to increase angiogenesis in models of hindlimb ischemia, and it is possible a similar mechanism may be occurring with pFGFE+ [217, 218]. Another possible mechanism for the increase in angiogenesis and hindlimb blood flow with pFGFE+ treatment is the upregulation of HGF. FGF-2 stimulates HGF expression, which itself has also been successful in increasing perfusion in preclinical models [165, 166] and in a clinical trial [167]. Determination of the exact mechanism of pFGFE+ mediated angiogenesis will require further studies.

In summary, electrically-mediated intradermal delivery of pFGF is a potential non-invasive non-viral therapeutic approach to increase perfusion and angiogenesis in a
hindlimb ischemia model and warrants further investigation as a possible treatment for PAD.

**Future Directions**

Unlike humans, the rat has an abundant network of collateral vessels in the lower limbs due to the physiological absence of the profunda femoris artery [151]. Prior to investigating this approach as a potential therapy for limb ischemia in the clinic, it should be further tested in a larger animal model with a vascular anatomy that more closely resembles that of humans, such as the rabbit or swine [147]. If pFGFE+ is explored as a potential treatment for PAD in a larger animal model, the field strength and pulse width should first be optimized for that animal model. In this study, a lower field strength was used for pFGF delivery to the hindlimb (250 V/cm, 150 ms) to achieve FGF-2 expression levels that were comparable to the levels observed after pFGF delivery to the flank at a higher field strength (300 V/cm, 150 ms). Relative to the skin of rat flank, the skin of the hindlimb is markedly thinner. Therefore, it is expected that a higher field strength would be required to achieve equivalent expression in a larger animal model, such as the swine, with thicker skin than that of the rat hindlimb.

Optimally, both angiogenesis and arteriogenesis should be stimulated to increase perfusion to the lower limb [144, 219]. At the time these experiments were carried out, the technology used to assess collateral vessel formation in a small animal model was not available. When moving this approach to a larger animal model, it would also be beneficial to determine if pFGFE+ increases arteriogenesis in addition to angiogenesis. Gross observation of the skeletal muscle in the ischemic limbs on POD 14 of this study
suggested there may be an increase in arteriogenesis with pFGFE+ as indicated by the healthier color of the muscle and the presence of superficial immature collaterals identified by an increase in calibres and their typical corkscrew pattern [5]. The presence of immature collaterals would be expected as a result of inducing ischemia in the hindlimb alone, and therefore, is not necessarily indicative of pFGFE+ increasing collateral vessel growth.

In this study, pFGFE+ increased blood flow in the ischemic hindlimb, but the mechanism was not defined. One possible pathway for FGF-2 to increase blood flow and angiogenesis is through the upregulation of VEGF. Other studies have shown in models of hindlimb ischemia that delivery of exogenous FGF-2 increases VEGF expression and that blocking VEGF abrogates the therapeutic effects of FGF-2 [21, 209]. Future studies could be conducted using a VEGF neutralizing antibody to block endogenous VEGF expression, similar to those conducted by Masaki et al. [21], to determine if pFGFE+ mediates its therapeutic effects through VEGF. Briefly, a VEGF neutralizing antibody would be administered the same day as pFGFE+ treatment, hindlimb perfusion would be monitored through POD 14 and capillary density would be determined in gastrocnemius muscle samples excised at the end point of the study. Successful neutralization of endogenous VEGF expression, and the duration of the neutralizing effects of the antibody, should first be determined in tissue samples from the gastrocnemius muscle using a VEGF ELISA. If pFGFE+ exerts its angiogenic effects by upregulating VEGF, perfusion and capillary density should be similar in the pFGFE+ group receiving the VEGF neutralizing antibody and the pVAXE+ and P-E- control groups.
The skin is an attractive target for transgene delivery with *in vivo* EP because it is easily accessible and thus frequency and/or number of treatments can be easily increased. For delivery to the rat hindlimb (250 V/cm, 150 ms) and the flank (300 V/cm, 150 ms), pFGFE+ increased FGF-2 expression approximately 2-fold compared pFGFE-. This increase was sufficient to induce a biological effect, but it would be of interest to evaluate if treatment at more than 2 sites on the ischemic hindlimb or if increasing the plasmid dose would further increase limb perfusion and neovascularization. Of note, the observed increase in FGF-2 with pFGFE+ may have been diminished by the presence of high levels of endogenous FGF-2 in the rat skin (1416 ± 326 total pg/sample). The skin of the medial aspect of the hindlimb is exceptionally thin and increasing the field strength above 250 V/cm resulted in visual tissue damage. Thus, increasing the field strength is not a viable option to increase FGF-2 expression with pFGFE+ treatment in the rat model of hindlimb ischemia and increasing the dose of plasmid and number of treatments should be further explored. As mentioned above, increasing the field strength will likely be necessary to increase the level of FGF-2 expression achieved with pFGFE+ in a larger animal model. Also, in a larger animal model with thicker skin, a larger range of EP parameters could be examined and it is possible that increasing the field strength or pulse width would result in a larger fold-increase in FGF-2 expression with pFGFE+, compared to pFGFE-.

The establishment of new functional vascular networks is a complex process orchestrated by a myriad of growth factors and cytokines. Transfer of a single angiogenic growth factor or cytokine has shown promise in preclinical and clinical studies, but the efficacy of these therapeutic approaches may be improved by delivering a
combination of angiogenic growth factors and / or cytokines. An elegant study by Cao, et al. determined that delivery of both FGF-2 and PGDF-ββ to a rat and rabbit model of hindlimb ischemia synergistically increases neovascularization [215]. Synergistic increases in neovascularization have also been observed with combined delivery of FGF-2 and VEGF in rabbit [220] and mouse[211] models of hindlimb ischemia. The combination of FGF-2 with HGF has also been shown to synergistically increase neovascularization more than either growth factor alone in a mouse model of hindlimb ischemia [221]. pVEGFE+ and pVEGFE- did not increase VEGF expression above background levels in this or a more severe hindlimb ischemia model (data not shown).

Despite the decrease in blood flow to the muscles of the ischemic limb, the skin most likely remained normoxic and did not allow for VEGF mRNA stabilization and expression. If the combination of FGF-2 and VEGF were to be explored, VEGF could be delivered to the gastrocnemius muscle while still delivering pFGFE+ to the skin of the ischemia limb, but the potential treatment would no longer be non-invasive. Future experiments exploring if pFGFE+ in combination with a plasmid encoding PDGF-ββ or HGF increases limb blood flow more than either treatment alone would be useful for moving this approach toward clinical trials, especially if the experiments were conducted in a larger animal model as suggested above.
MATERIALS AND METHODS

Plasmids

The CMV promoter was removed from the pVAX1 plasmid (Invitrogen) to create a promoterless pVAX1. The human VEGF165 plasmid was cloned by sub-cloning the hEF1-HTLV-hVEGF165 sequence from pBLAST49-hVEGF (Invivogen) into the promoterless backbone of pVAX1. The human FGF-2 plasmid (pFGF) was cloned by sub-cloning the hEF1-IF4g-hbFGF sequence from pBLAST45-hbFGF2 (Invivogen) into the promoterless backbone of pVAX1. The luciferase plasmid used in this research was gWiz-Luc (Aldevron). For in vivo use, all plasmids were commercially prepared (Aldevron).

Cell culture and transfection

Experiments confirming in vitro expression of pVEGF were performed using the B16F10 mouse melanoma cell line maintained in McCoy's Media 5A with 10% FBS and 0.2% Gentamycin. In vitro expression of pFGF was confirmed in the immortalized keratinocyte HaCaT cell line maintained in DMEM media with 10% FBS and 0.2% Gentamycin. Twenty-four hours prior to transfection, 2 x 10^5 cells were plated per well in 6-well cell culture dishes. Cells were transfected with 2.5 μg of plasmid per well using the TransIT-LT1 Reagent (Mirus) according to manufacturer’s instructions. Forty-eight
hours after transfection cells were harvested and lysed in 100 μl of lysis buffer (150 mM NaCl, 20 mM sodium phosphate pH 7.4, 10% glycerol, 1% NP-40 and protease inhibitors (Roche)) for 30 min on ice. Lysates were then centrifuged at 4°C for 10 minutes at 10,000 rpm and protein expression determined by ELISA.

**Animals and anesthesia**

All procedures were approved by the Animal Use and Care Committee of the University of South Florida College of Medicine. Male Sprague Dawley rats (250-300 g) (Harlan) were used for both the RSF model and hindlimb ischemia model. Female Hartley guinea pigs (250-300 g) were purchased from Charles River. Prior to operations and all procedures, animals were placed in an induction chamber and anesthetized with 5% isoflurane in oxygen. After the animals were anesthetized they were fitted with a standard rodent mask and maintained under anesthesia with 3.0% isoflurane in oxygen. All operations were performed using standard aseptic technique. At indicated time points animals were humanely euthanized by exposure to CO₂.

**The random skin flap model**

To create the rostral-based single pedicle RSF on the left lateral flank, the rat was shaved and an 8-cm by 3-cm template was traced with a surgical marker. Full thickness incisions were made along the traced template lines. The skin and subcutaneous tissue were then elevated and sutured back to its bed using simple interrupted and running stitches with 4-0 non-absorbable sutures.
The hindlimb ischemia model

Hindlimb ischemia was induced in right hindlimb of the Sprague Dawley rats. Through an approximately 2-cm long incision parallel to the inguinal ligament the saphenous artery was ligated distally of the bifurcation of the femoral artery into the saphenous and popliteal arteries. The most distal end of the external iliac was ligated immediately adjacent to the inguinal ligament and the femoral artery was ligated proximal and distal to the bifurcation of the superficial epigastric artery and vein. The superficial epigastric artery and vein were also ligated at 2 sites adjacent to the femoral bifurcation and cut between these sites. The femoral artery was then cut between the ligations adjacent to the inguinal ligament and distally of the superficial epigastric artery and vein. The femoral artery was then dissected free from the point of the distal ligation of the saphenous artery to the ligation of the femoral placed distally to the bifurcation of the superficial artery and vein.

In vivo electroporation

For all in vivo experiments, all plasmids were at a concentration of 2 µg/µL in sterile injectable saline and all injections were 50 µL in volume unless otherwise noted. All injections were performed using a 25 gauge, 5/8-inch length needle.

Plasmid delivery to muscle

The rat gastrocnemius muscle was exposed using a 1-cm incision. pVEGF was then injected into the center of the muscle and the injection site marked with a single stitch using a 4-0 non-absorbable suture to later identify the delivery site to be excised for
protein extraction. For pVEGFE+, a 4-needle electrode array, with a 6-mm gap width between needles, was then placed around the delivery site and 8 20 ms pulses were delivered at 200 V/cm.

**Plasmid delivery to skin**

For delivery to the RSF with the 4PE: Injections were centered along the midline of the RSF at 1, 3, 5, and 7-cm from the pedicle. Injection sites were marked with a surgical marker if they were to be excised later for protein extraction. Where noted, proximal delivery sites were placed at 1-cm and 3-cm from the RSF pedicle and distal delivery sites were placed at 5-cm and 7-cm from the RSF pedicle. After injection of plasmid DNA, the 4PE [68] was used to deliver 8 20 ms electric pulses at 200 V/cm to pVAXE+ and pVEGFE+ treatment groups.

For delivery of pLuc to guinea pig skin with the MEA: Plasmid was intradermally injected at sites on the guinea pig flank. The MEA was then placed over the injection bubble and an electric field applied at the indicated field strengths and pulse widths. Delivery sites were marked with a surgical marker for later identification for quantification of luciferase expression.

For delivery of pFGF with the MEA: pFGF was delivered to 2 sites on the medial aspect of the ischemic hindlimb immediately after assessment of postoperative perfusion. After injection of plasmid DNA, the MEA was placed over the injection bubble and 150 ms pulses were applied at 300 V/cm (delivery to skin of rat flank for initial expression kinetics) and 250 V/cm (delivery to hindlimb). FGF-2 expression levels from pFGF+ and pFGF- were equivalent at 300 V/cm for delivery to the rat flank and 250 V/cm for delivery to the hindlimb.
hindlimb. FGF-2 expression with pFGF+ and pFGF- did not differ between the ischemic and non-ischemic hindlimb.

**In vitro luciferase assays**

At the indicated time points animals were humanely euthanized and the delivery sites were excised and snap frozen on dry ice. Tissue samples were homogenized in 1 to 2 mL of homogenization buffer (25 mM Tris, pH 7.8, 2 mM EDTA, 1 mM DTT and 10% glycerol). Homogenates were then centrifuged at 4°C for 2 minutes and the supernatants were removed and assayed for luciferase activity in Luciferase Assay Buffer (25 mM glycyglycine, pH 7.8, 15 mM KPO$_4$, pH 7.8, 15 mM MgSO$_4$, 4 mM EGTA, 2 mM ATP, 1 mM DTT and 100 µM Luciferin). Luciferase activity was quantitated using a MLX microtiter plate luminometer (Dynex Technologies). Using a standard preparation of recombinant luciferase (Promega) relative light unit values were converted to pg of luciferase and luciferase activity is reported as total pg luciferase per tissue sample.

**In vivo luciferase assays**

**In vivo** bioluminescence imaging was completed using the IVIS 100 system (Xenogen Corp.) in conjunction with the Living Image acquisition and analysis software (Xenogen Corp.). Luciferin (Xenogen Corp.) was dissolved to 7.5 mg/ml in PBS, filter-sterilized, and stored in single use aliquots at -20°C. At the indicated time points the guinea pigs were anesthetized and received a 50 µL intradermal injection of Luciferin solution at the delivery site. Images were acquired 2 minutes after Luciferin injection.
Quantitation of luciferase activity was based on the total flux (photons / sec) of emitted light from the delivery site.

**Determination of protein expression**

At the indicated time points the delivery sites were excised and snap frozen on dry ice. Tissue samples were homogenized in 1 to 2 mLs of lysis buffer (150 mM NaCl, 20 mM sodium phosphate pH 7.4, 10% glycerol, 1% NP-40 and protease inhibitors (Roche)). Homogenates were then centrifuged at 4°C for 10 minutes at 10,000 rpm. The supernatants were removed and assayed for protein expression using commercially available ELISAs for human VEGF165 (R&D Systems, DY293B), eNOS (R&D Systems, DEN00) and FGF-2 (R&D Systems, DY233) according to the manufacturer’s instructions.

**Laser Doppler Perfusion Imaging**

A Laser Doppler Perfusion Imager (Moor Instruments Ltd.) was used to record perfusion at the indicated time points. A low intensity (2 mW) laser light beam (λ = 632.8 nm) scanned the surface of the skin without contact at a standardized working distance of 28 cm. Scan modus was set at 10 ms/pixel and resolution at 256 x 256 pixels. To control for variability that may result from fluctuations in temperature and ambient light, animals were placed on a heating pad and measurements were completed in a low lit room.
Random skin flap model

Three scans were completed per time point for each animal and average perfusion in arbitrary units (flux) for the distal region of the skin flap (5-cm$^2$ area) was determined for each time point. Baseline perfusion was assessed immediately preoperatively and postoperative perfusion was assessed immediately after the operation to create the RSF. Perfusion results are reported as the percentage of perfusion units relative to baseline or postoperative perfusion (normalized to 100%).

Hindlimb ischemia model

Three scans were completed per time point for each animal for both the ischemic and non-ischemic limbs and average perfusion in arbitrary units (flux) was determined separately for each limb. Perfusion in the ischemic limb was normalized to the contralateral non-ischemic limb to minimize variation due to ambient light and temperature. Baseline perfusion was assessed immediately preoperatively and postoperative perfusion assessed immediately after completion of the operation to induce hindlimb ischemia. The average I/NI was used to calculate percentage of baseline perfusion at the postoperative time points.

Assessment of skin flap healing

The percent survival of the distal 5-cm$^2$ area of the RSF was quantitated on PODs 7 and 14. Areas dark in color and / or covered in scabs were defined as necrotic / non-surviving areas. Areas with normal skin texture and color were defined as surviving
areas. The surviving areas were calculated using the Moor imaging software (Moor Instruments Ltd.) and images were recorded using a digital camera (Nikon).

**Histology**

On POD 14 animals were humanely euthanized. Tissue samples were excised 2-cm from the distal end in the center of the RSF, fixed in 10% neutral buffered formalin for at least 12 hours at room temperature, embedded in paraffin and sectioned (4-μm). If the tissue was necrotic at 2-cm from the distal end, samples were excised at the border of the necrotic and surviving tissue. Sections were then stained with H&E using a standard protocol. Stained sections were analyzed using a standard light microscope (Olympus BX51) and representative fields photographed with a Spot Insight 2 digital camera (Pathtech).

**Immunohistochemistry**

On POD 14 animals were humanely euthanized and tissue samples from the gastrocnemius muscle were excised and fixed in 10% neutral buffered formalin for at least 12 hours at room temperature before embedding in paraffin and sectioning (4-μm). Capillaries in the cross-sections were visualized using the Blood Vessel Staining Kit (Chemicon) according to manufactures instructions for Factor VIII with Hematoxylin counterstaining. The number of Factor VIII positive vessels was manually counted in a blinded fashion in 5 randomly selected fields (400X) for 5 animals from each treatment group. Stained sections were analyzed using a standard light microscope (Olympus BX51). The average capillary density per HPF (400X) for each treatment group is
reported. Representative fields from each treatment group were photographed at 400X with a digital camera (Spot Insight 2).

**Statistical Analysis**

All values are reported as the mean ± SEM unless otherwise noted. Statistical significance was assumed at p≤ 0.05. All statistical analysis was completed using the Statistical Package for the Social Sciences (SPSS).

Analysis of VEGF expression was completed using a 2-tailed Student’s t-test. Analysis of eNOS expression and distal RSF perfusion was completed using ANOVA with a post-hoc Dunnett’s test to adjust for multiple comparisons to a single control group (pVEGFE+). Analysis of distal RSF survival was completed using the Mann-Whitney-Wilcoxon test with a post-hoc Bonferroni-Dunn correction to adjust for multiple comparisons. Analysis of luciferase activity was completed using a 2-tailed Student’s t-test when comparing 2 groups. Analysis of 3 or more groups was completed by ANOVA with a post-hoc Fisher’s Least Significant Difference adjustment for multiple comparisons. Analysis of FGF-2 expression and capillary density in the ischemic hindlimb was completed using a 2-tailed Student’s t-test. A post-hoc Bonferroni correction was used to adjust for multiple comparisons to pFGFE+ for analysis of capillary density. Analysis of hindlimb perfusion was completed by ANOVA with post-hoc Fisher’s Least Significant Difference adjustment for multiple comparisons.
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122. www.regranex.com, *U.S. Prescribing Information for REGRANEX (becaplermin) Gel 0.01%*.


ABOUT THE AUTHOR

Bernadette Ferraro grew up in the Hudson Valley area of New York State. She began her undergraduate studies at the University of South Florida in 1996 and graduated with a Bachelor of Science in Microbiology in May 2000. She returned to USF in the fall semester of 2003 to pursue a Master of Public Health in Epidemiology while working the laboratory of Eric Haura, M.D. Upon deciding to advance to a career in basic science research, Bernadette began graduate work in the USF Department of Biochemistry and Molecular Biology in 2004, and later enrolling in the PhD Plus program to complete her MPH and PhD concurrently. While in the program, Bernadette received several awards to present her work at both national and international conferences, and she filed a provisional patent as a co-inventor with her co-major professor Richard Heller, Ph.D.. Bernadette plans to pursue a career as an independent investigator.