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Tissue Engineering Strategies for the Treatment of Peripheral Vascular Diseases

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TISSUE ENGINEERING STRATEGIES FOR THE TREATMENT OF PERIPHERAL VASCULAR DISEASES

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TISSUE ENGINEERING STRATEGIES FOR THE TREATMENT OF PERIPHERAL VASCULAR DISEASES

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Peripheral vascular diseases such as peripheral artery disease (PAD) and critical limb ischemia (CLI) are growing at an ever-increasing rate in the Western world due to an aging population and the incidence of type II diabetes. A growing economic burden continues because these diseases are common indicators of future heart attack or stroke. Common therapies are generally limited to pharmacologic agents or endovascular therapies which have had mixed results still ending in necrosis or limb loss. Therapeutic angiogenic strategies have become welcome options for patients suffering from PAD due to the restoration of blood flow in the extremities. Capillary sprouting and a return to normoxic tissue states are also demonstrated by the use of angiogenic cytokines in conjunction with bone marrow cell populations. To this point, it has been determined that spatial and temporal controlled release of growth factors from vehicles provides a greater therapeutic and angiogenic effect than growth factors delivered intramuscularly, intravenously, or intraarterialy due to rapid metabolization of the cytokine, and non-targeted release. Furthermore, bone marrow cells have been implicated to enhance angiogenesis in numerous ischemic diseases due to their ability to secrete angiogenic cytokines and their numerous cell fractions present which are implicated to promote mature vessel formation. Use of angiogenic peptides, in conjunction with bone marrow

cells, has been hypothesized in EPC mobilization from the periphery and marrow tissues to facilitate neovessel formation.

For this purpose, controlled release of angiogenic peptides basic fibroblast growth factor (FGF-2) and granulocyte-colony stimulating factor (G-CSF) was performed using tunable ionic gelatin hydrogels or fibrin scaffolds with ionic albumin microspheres. The proliferation of endothelial cell culture was determined to have an enhanced effect based on altering concentrations of growth factors and method of release: co-delivery versus sequential. Scaffolds with these angiogenic peptides were implanted in young balb/c mice that underwent unilateral hindlimb ischemia by ligation and excision of the femoral artery. Endpoints for hindlimb reperfusion and angiogenesis were determined by Laser Doppler Perfusion Imaging and immunohistochemical staining for capillaries (CD-31) and smooth muscle cells (α-SMA). In addition to controlled release of angiogenic peptides, further studies combined the use of a fibrin co-delivery scaffold with FGF-2 and G-CSF with bone marrow stem cell transplantation to enhance vessel formation following CLI. Endpoints also included lipophilic vascular painting to evaluate the extent of angiogenesis and arteriogenesis in an ischemic hindlimb. Tissue engineering strategies utilizing bone marrow cells and angiogenic peptides demonstrate improved hindlimb blood flow compared to BM cells or cytokines alone, as well as enhanced angiogenesis based on immunohistochemical staining and vessel densities.

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Chapter 1. Introduction and Background

1.1 Peripheral Artery Disease and Critical Limb Ischemia

Peripheral artery disease (PAD) has grown exponentially in the Western world due to an increase in an aging population as well as incidence of type II diabetes (Aranow, 2005; Aranow, 2007; Hackam et al, 2005). Summarized by Kuhlmann, it is estimated that 20% of Americans over age 65 suffer from PAD, in Germany 20% of men and 17% of women over age 65, and in France 11% of people aged 40-80 show signs of PAD (Kuhlmann et al, 2007). Although PAD is not as severe as heart attack, or stroke it is generally an excellent indicator of future cerebral vascular and cardiovascular traumatic events. This disease is generally caused by a reduction of normal blood flow to the extremities of the body (i.e. toes, feet) because of atherosclerotic narrowing of vital arteries to these areas. Over time the narrowing may cause ambulatory pain, ulcers, or gangrene and could eventually result in amputation at points from the knee or below depending on the severity.

In order to determine if a patient is at risk for PAD, a widely accepted method of measuring blood pressure in the ankle and directly comparing it to the blood pressure along the brachial artery, also known as the ankle-brachial index (ABI) is used, and calculated (Nicolai et al, 2008; Obermayer et al, 2006). A normal reading is generally between the range of 0.9 to 1.3, whereas candidates at values less than 0.9 may have signs of early PAD. Between 0.4 to 0.9, signs of moderate PAD are present and if below 0.4, severe PAD, or critical limb ischemia (CLI) is diagnosed. If the ABI is at lower levels amputation of the limb may be required.

Treatments for PAD are generally limited to risk factor modification such as smoking cessation, light exercise, or diet change when non-severe levels of PAD are present (Comerota, 2001). However, these methods are often insufficient to achieve clinical significance due to patients requiring a high degree of discipline and motivation during old age and may not be a possible method of treatment in severe cases of PAD. Revascularization procedures using balloon angioplasty or stenting have become preferred options. These methods exploit the stenosed section of the artery and expand it to a working proper size to allow normal flow to ameliorate ischemic tissues. As demonstrated in numerous patients and previous clinical trials (Comerota, 2001; Welten et al, 2008), the use of stenting generally leads to restenosis of the artery and further angioplasty or stenting may be required leading to loss of vessel integrity. Lastly, vein grafting has become an alternative method for patients with severe PAD or CLI because this method does not need deployment of stents and utilizes the patient's own vessels from other regions in the body (Smith and Clagett, 2008). Primarily, veins are used to bypass the stenotic arterial segment in order to augment flow in the tissues. However, due to the low elasticity of veins, in comparison to arteries, the pulsatile flow commonly seen in the iliac or common femoral arteries is severely affected, and may result in vessel regression.

1.2 Angiogenesis

The endogenous response to ischemic events such as PAD and CLI include two major processes, arteriogenesis and angiogenesis, ultimately leading to vessel formation (Carmeliet, 2005). Angiogenesis refers to the formation of new blood vessels from pre-existing vasculature induced by the proliferation and migration or pre-existing fully

differentiated endothelial cells resident within parent vessels in response to various stimuli such as hypoxia, ischemia, mechanical stretch, and inflammation (Yancopoulos et al, 2000). Many studies are currently ongoing to delineate differences in the various pathways of angiogenesis as there are many processes involved. Figure 1.1, adapted from Yancopoulos et al, demonstrates these processes. First, vasculogenesis is initiated in early development where endothelial cells differentiate and proliferate within a previously avascular site and these cells coalesce to form primitive tubular structures. Second, angiogenic remodeling occurs where the initial tubular structure is modified to form interconnection branch patters characteristic of mature vasculature. Lastly, angiogenic sprouting occurs and involves the sprouting from pre-existing vessels into a previously avascular tissue. This process is responsible for vascularizing certain structures during normal development, such as the neural tube or the retina, as well as a majority of the new vessels formed in an adult.

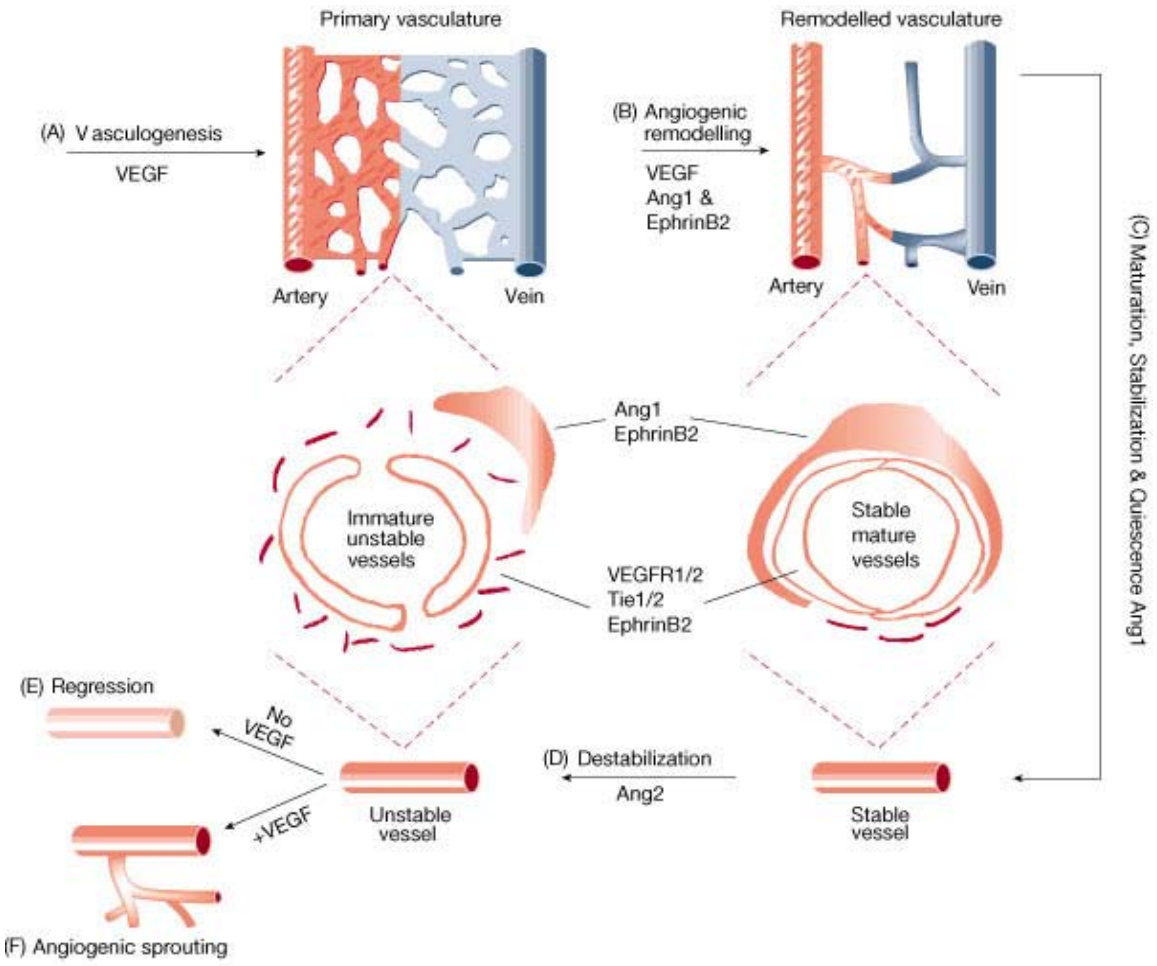


Figure 1.1. Schematic representation of the roles of growth factors associated with vessel formation including vasculogenesis (stage A), angiogeneic remodeling (stage B), stabilization and maturation (stage C), destabilization (D), regression (E), and sprouting (F). Adapted from Yancopoulos et al, 2000, *Nature*.

The process of angiogenesis is highly complex and involves several angiogenic cytokines and stem cells in order to induce vessel formation. The generally accepted process of angiogenesis involves the regulation of hypoxia-inducible factor-1α (HIF-1α) which is considered the master switch in cellular release of vascular endothelial growth factor (VEGF) (Rivard et al, 1999; Germani et al, 2009). As VEGF is released, the recruitment and proliferation of endothelial cells at the ischemic site occurs and the basement membrane of the extracellular matrix is degraded by basic fibroblast growth

factor (FGF-2) which acts to align endothelial cells that have migrated to the ischemic tissue. FGF-2 is also chemoattractant for mesenchymal stem cells, bone marrow stem cells, and peripheral blood cells to further the angiogenic cascade (Goto et al, 1993; Przybylski, 2009; Hall, 2007). Beyond this time point, it has not clearly been defined as to what master switches occur to prune and trim vasculature to mature it, but there are numerous growth factors that are generally accepted as maturation factors to recruit endothelial progenitor cells, hematopoietic stem cells, and other stem cell lineages: platelet derived growth factor (PDGF), granulocyte-colony stimulating factor (G-CSF), and angiopoietins 1 and 2 (Richardson et al, 2001; Jeon et al, 2006b; Peters et al, 2004; Fukahara et al, 2010).

Of more specific interest, G-CSF has been studied extensively as a potent mobilizer for bone marrow cells, hematopoietic stem cells, and endothelial progenitor cells to the systemic circulation (Bussolino et al, 1991; Minamino et al, 2005; Okhi et al, 2005). Upon mobilization, these cells are typically homed to the site of ischemia and assist in the remodeling phase of angiogenesis. As these cells proliferate and differentiate at the ischemic site, a plethora of additional endogenous cytokines are released to facilitate additional endothelial cell maturation by the formation of the lumen, smooth muscle cells, and pericytes surrounding the endothelial cells. Moreover, G-CSF alone is not enough to sustain angiogenesis in ischemic regions and depends on an environment of multiple factors and cells to play a role in mature vessel formation.

1.3 Therapeutic Angiogenesis

In order to develop mature vessel formation in ischemic tissues, studies have utilized therapeutic angiogenic strategies to abate the deleterious effects observed with

traditional revascularization methods. Pioneering studies by Folkman and Isner have led the way in exploiting endogenous angiogenic growth factors FGF-2 and VEGF for revascularization (Ingber and Folkman, 1989; Asahara et al, 1995; Takeshita et al, 1994). Primarily, these growth factors are administered as a bolus injection in order to rapidly vascularize ischemic tissue. In 2002, the TRAFFIC study was the first large-scale clinical trial involving angiogenic growth factor administration of FGF-2 (Lederman et al, 2002). This study resulted in decreases in ambulatory pain and increased walking distance but did not investigate important angiogenic endpoints such as mature vessel formation and bloodflow recovery. Administration of growth factors in a bolus fashion leads to problems due to the resident half-life of many growth factors, systemic toxicity, and non-site specific delivery. These pitfalls of growth factors can easily be overcome when incorporated with a vehicle generally as a genetic vector for DNA delivery encoding for the angiogenic protein of interest (Yockman et al, 2008), or by encapsulation in a polymeric, biocompatible scaffold (Young et al, 2005; Layman et al, 2007).

Genetic vectors are constructed by transfer of genetic material into somatic cells of an organism with the aim of achieving high levels of gene expression. When transferred into vascular cells at the ischemic site, local production of the transgene product will translate into meaningful biological effects and ultimately a therapeutic benefit. Transfer vectors facilitate cellular penetration and intracellular trafficking of the transgene, and local delivery systems deliver the vector to the vicinity of the target cells. Genetic vectors are commonly composed of viral vectors and are transfected via adeno or retro-viral association. Many studies have investigated the beneficial effect of delivering

adeno-associated VEGF however, the recovery is only over a transient period, ultimately resulting in vessel regression (Gounis et al, 2005; Rivard et al, 1999). Additionally, the efficiency of loading angiogenic growth factor into the virus is very low and if the expression of the growth factor is low to begin with, this will ultimately lead to improper dosing at the site of ischemia (Tong et al, 2009).

In order to prolong dosing of growth factors, numerous studies have employed the use of polymeric scaffolds to delivery angiogenic cytokines (Ikada and Tabata, 1998; Wood and Sakayima-Elbert, 2008). Synthetic and natural materials have been used extensively for the delivery of polypeptides at controlled rates. Material properties such as crosslinking density, surface modifications, or spatial and temporal variables, can be easily altered to tailor the rates at which growth factors are released. In terms of therapeutic angiogenesis, materials that have established the greatest compatibility are poly-lactide-co-glycolide (PLGA), fibrin, gelatin, and alginate. A landmark study by Richardson et al in 2001 utilized delivery of VEGF and PDGF from a PLGA scaffold which resulted in enhanced vessel ingrowth, maturation, and hindlimb bloodflow in a critical limb ischemia model (Richardson et al, 2001). We have also demonstrated that the co-delivery of FGF-2 and G-CSF results in enhanced hindlimb perfusion and angiogenesis by the controlled, tunable delivery from ionic-gelatin scaffolds, and fibrin hydrogels (Layman et al, 2009). Numerous reports by Lee et al have shown that alginate hydrogels doped with VEGF can have an enhanced angiogenic effect as the rate of release is sustained over a long period (Lee and Lee, 2009; Lee et al, 2010). Although these studies have initiated an angiogenic response, they have demonstrated that multiple cues are needed in order to revascularize ischemic tissues.

The co- or sequential delivery of growth factors has had the most promising therapeutic effect toward peripheral vascular disease and ischemic tissue treatment. The importance of these systems is the initial burst from the bulk of the scaffold followed by a prolonged, sustained release of growth factor from the second phase. The release kinetics can easily be altered by enzymatic degradation of the scaffold and, depending on the dose needed at specific time points, the growth factors can be released at these rates (Lee, 1985). Lastly, the interplay of multiple growth factors leads to stable, functional, mature vessel formation that is characterized by smooth muscle cell and pericyte formation around the endothelial cell layer. An example of the two-phase delivery system with VEGF and PDGF is depicted in Figure 1.2(Folkman, 2007; Carmeliet, 2000). In this case with VEGF and PDGF, VEGF is released first to stimulate endothelial cell formation, and PDGF is released at a later time to induce mature vessel formation by smooth muscle cell recruitment, and pericyte capping of the endothelial cells which are generally leaky and highly permeable. It is currently unclear as to which growth factor and to what sequence is necessary to provide the greatest angiogenic effect, but it is known that multiple cytokines, signals, and cells are needed to enhance therapeutic angiogenesis.

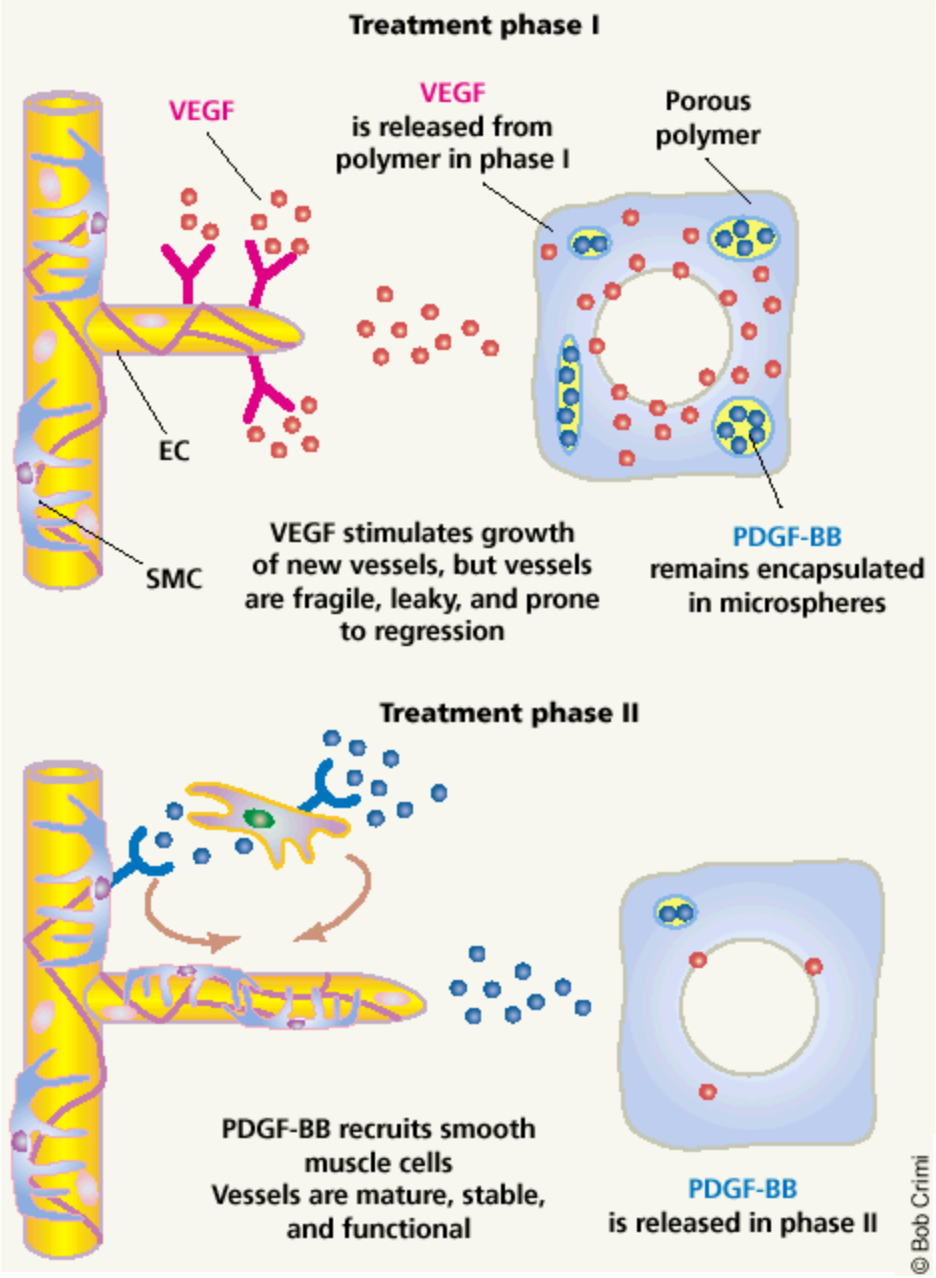


Figure 1.2. Interplay of angiogenic cytokines VEGF and PDGF-BB released from PLGA scaffolds in a sequential fashion to induce mature, functional, and stable neovasculature. Adapted from Carmeliet and Conway, *Nat Biotechnol*, 2001.

1.4 Therapeutic Stem and Progenitor Cell Transplantation

A landmark study by Asahara and Isner in 1997 identified endothelial progenitor cells and their potential use in multiple applications including angiogenesis (Asahara et al, 1997). Specifically these EPCs are can home to foci of angiogenesis suggesting their

potential use as autologous treatment because these cells are already circulating within. Since this study, numerous reports have been published to determine the most appropriate mode of isolation and to further explore their potential use in therapeutic angiogenesis (Kobayashi et al, 2008; Hung et al, 2009; Yu et al, 2009). One major pitfall associated with isolation of EPCs is their low frequency, even during ischemic injury (Powell et al, 2005). For example, in patients with coronary artery disease, the percentage of EPC phenotype cell present, shown by surface markers CD34+/CD133+ or CD133+/VEGFR-2+, is generally less than 0.02%. For this reason, large numbers of cells must be harvested to isolate a significant amount of EPCs. In order to overcome this, bone marrow cells have been harvested which are rich in stem cells such as mesenchymal and hematopoietic stem cells which have the capability to differentiate toward EPCs and mature endothelial cells (Rafii and Lyden, 2003). Table 1.1 highlights the various sources and origins of vascular cells from bone marrow toward endothelial precursors as well as mesenchymal cells and their potential role in angiogenesis. Several studies have already demonstrated the potential use of bone-marrow derived cells toward angiogenesis during limb ischemia but the method of incorporation and as to how they remodel blood vessels remains under investigation (Lawall et al, 2010). One of the advantages of using bone marrow cells for this purpose is that they contain fraction of cells is rich in EPCs, HSCs, MSCs, and numerous other cell lineages that participate in angiogenesis. Bone marrow cells have also been implicated in their ability to secrete numerous angiogenic cytokines (Gazitt, 2000) which assist in cellular proliferation, differentiation, and recruitment to ischemic tissues.

Table 1.1. Common sources of vascular and progenitor cells, their source of origin and potential and determined end targets toward angiogenesis.

Cell Type	Source/Origin	End Target/Outcome
Endothelial Progenitor Cell (EPC)	Bone marrow, vascular parenchyma, organ-specific EPC, cord blood	Mature endothelial cells (Asahara et al, 1999a)
Circulating endothelial progenitor (CEP)	Peripheral blood, cytokine-mobilized peripheral blood	Arterial or venous endothelial cell (Rafii and Lyden, 2003)
Mesenchymal stem cells (MSC)	Bone marrow	Smooth muscle cell and pericyte formation (Carmeliet and Luttun, 2001)
Circulating Endothelial Cells (CEC)	Vessel wall mature endothelium	Lining of blood vessels (Lawall et al, 2010)
Hematopoietic Stem Cell (HSC)	Bone marrow	Arterial endothelium (Carmeliet and Luttun, 2001)
Hematopoietic Progenitor Cell (HPC)	Bone marrow	Lymphatic endothelium (Carmeliet and Luttun, 2001)

Upon vessel injury, soluble angiogenic factors such as VEGF-A and PlGF are released to the plasma which activate MMP-9 (Figure 1.3). MMP-9 activates the bioavailability of dormant stem cells from the bone marrow which enhance the proliferation and circulation of hematopoietic and vascular progenitors (Rafii and Lyden, 2003). These cells are then homed to the ischemic organ upon release into the circulation lining new blood and lymphatic vessels. As these cells line new vessels in the ischemic tissue, a plethora of additional endogenous cytokines is released such as IGF, PDGF,

VEGF, G-CSF, and others (Gazitt, 2000). These additional cytokines present a feed-back loop for the circulating cells as the endothelial cells are pruned and the mature vasculature is formed. They may also provide chemoattract properties for the cells to form pericytes and smooth muscle cells as FGF-2 and VEGF have shown to cause leakiness of the vessels (Cao et al, 2004).

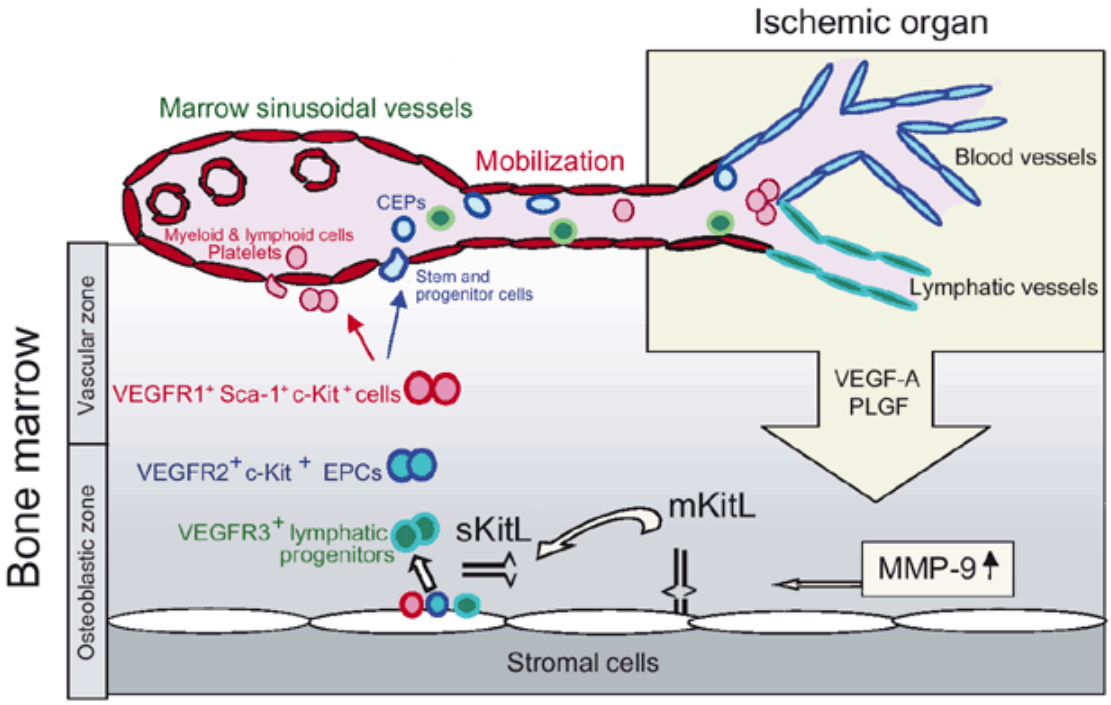


Figure 1.3. Molecular switches involved in the mobilization and recruitment of endothelial, lymphatic and hematopoietic stem and progenitor cells. Adapted from Rafii and Lyden, *Nat Med*, 2003.

Although success of bone marrow derived cell transplantation has been demonstrated in several animal models, the efficaciousness has not been duplicated in clinical trials for peripheral artery disease. Endpoints such as ABI, transcutaneous oxygen pressure, rest pain, and pain free walking all improved over 24 weeks (Lawall et al, 2010) however important endpoints such as limb perfusion, vessel densities, and incorporation of the cells was not investigated. Most of the clinical studies using stem

cells therapies have not had the greatest success due to patient drop-out or unwillingness to harvest their own cells. Furthermore the concentration of cells needed is currently undefined. In the TACT study, approximately $1.6x10^9$ mononuclear cells were harvested, resulting in, at best estimates, only 35,000 EPCs present possibly resulting in the need for multiple treatment. Higher doses have been explored in the Higashi study (Higashi et al, 2004), however, no additional therapeutic effects were observed at higher doses with patients.

1.5 Tissue Engineering Strategies

Engineering of functional tissues depends on the development of suitable scaffolds to support cellular growth. Furthermore, scaffolds must be able to biologically interact with adhering and invading cells and effectively guide cell growth and development by releasing bioactive proteins like growth factors and cytokines (Tessmar and Gopferich, 2007). Although much work has been done to understand the release of bioactive agents from scaffolds (Katz and Burdick, 2009; Isihara et al, 2007) as well as compatibility of cells within scaffolds (Young et al, 2005), it is still unclear as to how polymeric scaffolds can direct cell fate in the microenvironment. The timing of release and the dose released are equally responsible in directing cell fate at a mirco-level (Sands and Mooney, 2007) because angiogenesis depends on specific timing of cytokines and cells which has not been clearly defined. Specifically, the extracellular matrix is continually processed, degraded, and synthesized anew, altering the scaffolds presentation, composition, and elasticity. Meanwhile, soluble bioactive factors are secreted and destroyed as cells migrate, differentiate, proliferate, and undergo apoptosis.

Many bioactive factors have been investigated toward cell growth, however it was not fully understood that factors have short half lives and must be compartmentalized in order to sustain bioactivity (Gombotz and Pettitt, 1995). Angiogenic cytokines such as VEGF and FGF-2 have half lives of less than one hour (Stein et al, 1995; Nakamura et al, 2009), and in order to sustain bioactivity these factors must be delivered at high doses. Significant progress has been made in the area of sustaining bioactivity of growth factors by encapsulation within polymeric matricies by the Hubbell and Mooney labs (Zisch et al, 2003; Sands and Mooney, 2007). For example, Schense investigated the enzymatic activity of factor XIIIa to covalently incorporate exogenous bioactive peptides within fibrin during coagulation (Schense et al, 2000). From this study, they determined that the amount of nerve regeneration was significantly greater when using controlled release vehicles than the administration of nerve growth factor alone. Alternatively, Mooney's lab has extensively investigated a gas foaming reaction to form polymer scaffolds containing poly-L-lactic acid (PLLA) in order to abate undesirable by-products from material synthesis. They have demonstrated from their studies that a burst of VEGF (about 80%) can be released within 2-3 days and the release is then sustained over 80 days. Many forms of PLLA exist, and by altering material properties no burst effect can be utilized and release of 62% of VEGF can be achieved over 80 days (Sheridan et al, 1999). By being able to tailor release rates based on material properties or enzymatic incorporation, engineered tissue constructs will be more sustainable due to the tight control of cell and growth factor interaction at appropriate times.

Numerous biopolymeric controlled delivery matricies currently exist that have been used toward angiogenic growth factors. Biomaterial schemes for clinical growth

factor administration must begin to take better account of biological principles of growth factor function as well as the cell biological basis necessary to produce functional engineered tissues, namely blood vessels (Cao and Mooney, 2007). Synthetic materials include polyethylene glycol (PEG), poly-L-lactide, poly-co-lactide-glycolic acid (PLGA), and poly-L-glycolic acid (PGA). Many problems exist with synthetic materials due to their breakdown of products, release of undesirable by-products from synthesis, or pathogenicity (Zisch et al, 2003). Breakthroughs have occurred with PEG because PEG-based gels normally suppress the adhesion of all cells (Tessmar and Gopferich, 2007). PEG can be chemically modified by the addition of acrylic acid derivatives with or without a PEG spacer (Wain et al, 2001) which enhances cellular attachment for potential ischemic revascularization. Furthermore, West and Mann used PEG and incorporated it into the polymer backbone of bioactive sites that permit direct molecular interaction between matrix and cells for applications of adhesion and peptide cleavage (West and Mann, 2002). This could be potentially useful for appropriate cellular signaling for organ revascularization. The use of PEG has become widespread due to the inertness of the material, however the chemical modifications that must be performed for proper cellular or molecular interaction may result in undesirable chemicals being leached to the surrounding environment in vivo.

Alternatively, natural materials provide a more hospitable environment with no such undesirable by-products from chemical synthesis, unless modifications are necessary. Common natural materials include alginate, chitosan, gelatin, and fibrin (DeFail et al, 2006; Niu et al, 2008; Lee et al, 2010; Hall, 2007). Of specific interest, fibrin is a critical blood component and is responsible for wound healing and is formed

by cleaving of peptide fragments of soluble fibrinogen by thrombin to yield a fibrin mesh, this mesh becomes a clot by transglutaminase activity through Factor XIII (Ahmed et al, 2008). Synthesis of fibrin occurs by the coagulation cascade (Figure 1.4) which has two distinct pathways: extrinisic and intrinsic. Although fibrin has disadvantages such as low mechanical stiffness and rapid degeneration (Mol et al, 2005; Hall, 2007), these disadvantages can be easily prevented by combination with other scaffolds (i.e. microspheres, nanospheres), or incorporation of a fixative agent (Jockenhovel et al, 2001). Fibrin has been used in numerous tissue engineering applications with excellent results including therapeutic angiogenesis toward vascular tissue lineages (Dietrich and Lelkes, 2006; Liu et al, 2007)

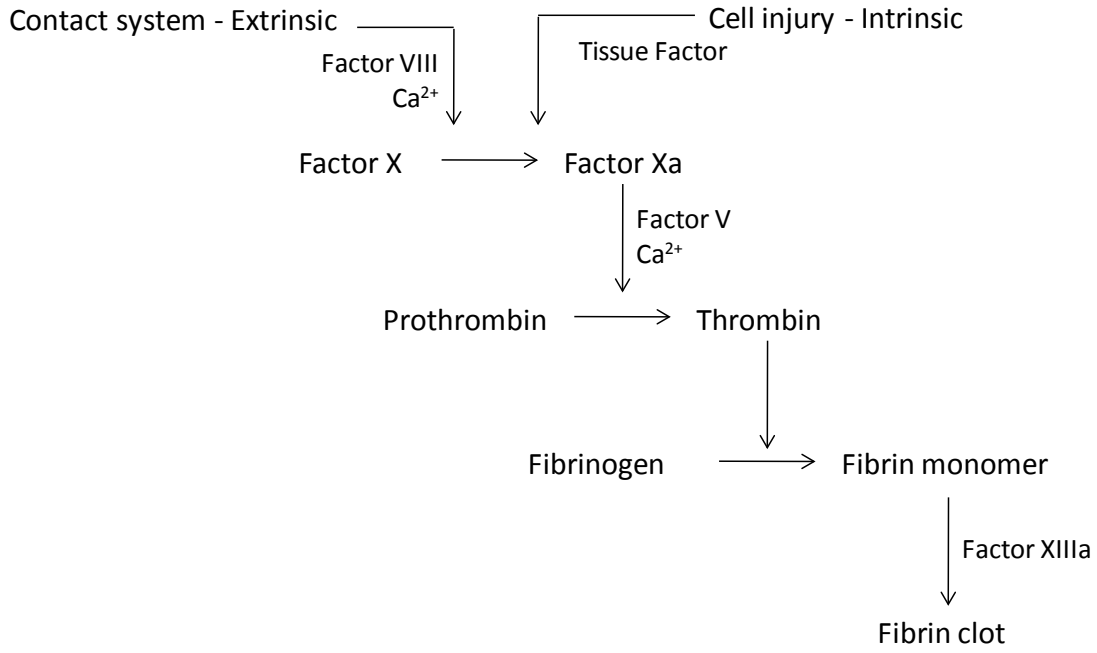


Figure 1.4. Formation of crosslinked fibrin clot by extrinsic and intrinsic pathways, ultimately leading to the common pathway, for wound healing purposes.

In order to reperfuse ischemic tissues, studies have explored the potential use of tissue-engineering vascular networks where a host of angiogenic cytokines and cell

fractions have been investigated (Zhang and Suggs, 2007). There are several potential targets for therapeutic manipulation which include, but may not be limited to: (1) stimulation induced, transient angiogenic growth factor upregulation; (2) upregulated growth factor administration to activate nearby endothelial cells and recruit stem cells; (3) cell-cell and cell-matrix interaction to guide capillary formation; (4) stabilization of vessel by recruited pericytes and smooth muscle cells. Growth factors are not limited to FGF-2, G-CSF, and VEGF for facilitation of angiogenesis. Platelet-derived growth factor (PDGF) is a pluirpotent angiogenic growth factor which is responsible for inflammatory cell chemotaxis and recruiting stem cells from bone marrow (Alvarez et al, 2006). It has been utilized in conjunction with VEGF and FGF-2 and promoted vascular maturation of newly formed blood vessels (Richardson et al, 2001; Cao et al, 2003).

Angiopoietins (e.g. ang-1 and ang-2) function through the Tie-2 receptor whose signaling controls survival and migroation of endothelial cells and regulates vascular remodeling (Morisada et al, 2006). Ang-1 is the best characterized factor of the subclass and is secreted by pericytes and through interactions with its Tie-2 receptor on endothelial cells, stabilizes vessels by recruiting additional perictyes to the vessel wall and mediating interactions between pericytes and endothelial cells. The status of the Tie-2 receptor determines the state of the endothelium. Activation of Tie-2 via ang-1 leads to the quiescent resting state of the endothelium. Alternatively, ang-2 has an antagonistic effect toward the endothelium as it destabilizes it and the endothelium is then susceptible to exogenous stimuli from nascent angiogenic cytokines (Yancopoulos et al, 2000; Kobayashi and Lin, 2006). Additional growth factors such as TGF-β, HGF, and IGF have been implicated for capillary formation toward angiogenesis, specifically toward

endothelial cell function but their methods of action toward angiogenesis has not yet been clearly defined (Zhang and Suggs, 2007).

In terms of cellular therapies endothelial progenitor cells are the gold standard for treatment of ischemic diseases. These cells have been isolated from human umbilical cord blood, peripheral blood, bone-marrow derived mononuclear cells, and hematopoietic stem cells. These cells have the capability to incorporate into growing vasculature as demonstrated in several ischemic models (Waksman et al, 2009; Qian et al, 2007; Liu et al, 2010), and have been shown to differentiate toward endothelial cells with and without angiogenic cytokines present (Gehling et al, 2000). Rafii and Lyden have demonstrated that EPCs mobilized from bone marrow into the blood stream can be actively recruited and incorporated into sites of active neovascularization in ischemic tissues (Rafii and Lyden, 2003). Numerous growth factors currently exist that are chemoattractive for EPCs, but of keen interest are G-CSF and SDF-1α. These factors will bind chemokine receptors on EPCs and if these factors are present in the ischemic tissue, the uptake of EPCs at the site will facilitate neovessel formation via endothelial cell maturation.

Few studies have yet encompassed the realm of growth factor administration in combination with bone marrow cells. Jeon et al have demonstrated the potential therapeutic effect of administration of bone marrow mononuclear cells in conjunction with G-CSF in a murine critical limb ischemic model. In this study, the transplantation of EPCs in conjunction with G-CSF resulted in increased density of microvessels, CD-31 and SMA+, as well as localized concentrations of VEGF and FGF-2 (Jeon et al, 2007). Treatments involving cells alone or cytokines alone did not have as significant an effect as demonstrated by the tissue engineered therapy. Figure 1.5 provides a method for EPC

isolation because a fraction of isolated bone marrow derived cells result in endothelial precursor cells leading to endothelial cells that play a pivotal role in lining newly formed blood vessels. By providing a large population of bone marrow cells, the probability of the cells becoming lineage positive toward EPCs is much greater, and when injected into an environment with G-CSF, the capability of these cells to differentiate toward EPCs is further enhanced. This study did elucidate the potential of cells and cytokines together; however, it did not address the idea of the controlled release of growth factors from a matrix to avoid complications associated with bolus administration of cytokines, and multiple treatments.

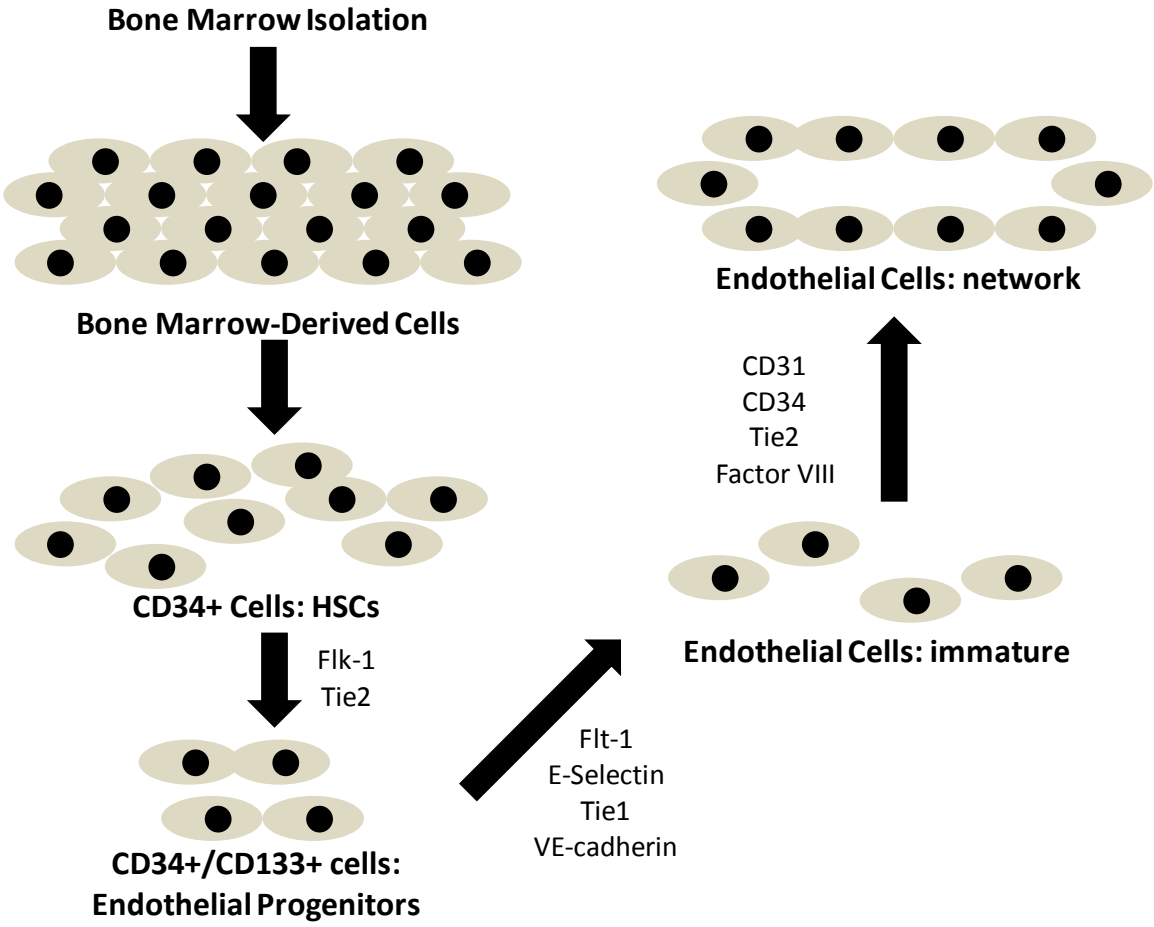


Figure 1.5. Flow diagram of characterization of EPC lineage cells toward endothelial cells.

Lastly, a study by Jay et al has investigated the use of controlled delivery of bioactive VEGF from alginate microspheres with endothelial cell transplantation. This study has documented that limb perfusion increases, and capillary density increases after 28 days of treatment (Jay et al, 2008). Although the endpoints were deemed positive, toward the end of the study, the hindlimb perfusion scores and vessel densities were decreasing suggesting another dose may be needed due to possible vessel regression. In order to facilitate vessel maturation, additional factors or a stem cell population would be of therapeutic benefit (Jain, 2003; Jain et al, 2005). These studies suggest that endothelial cells, perivascular cell precursors, or myoblasts be transplanted in conjunction with a cocktail of angoigenic cytokines to grow tissue engineered vasculature. The major challenge that still remains for this field is to deliver the minimal number of molecules in an optimal fashion in a coordinated manner. Without this caveat complete, tissue engineered constructs for ischemic diseases will be difficult to utilize in clinical applications.

1.6 Specific Aims

The specific goal of this work is to develop bioengineered-based therapeutic angiogenic modalities to treat peripheral artery disease and critical limb ischemia. This could be achieved by delivery of exogenous growth factors in a controlled manner in combination with cellular therapy. The rate, duration, and sequence of peptide release will depend on cell-mediated biodegradation, scaffold design characteristics (e.g. biodegradable sites, crosslinking density, swellability), method of encapsulation within the scaffold, and the loading capacity of the peptide. The central hypothesis of this investigation is that blood flow in ischemic tissues (i.e. hindlimb) could be restored by

implantation of a cytokine-loaded hydrogel scaffold that is programmed to deliver sustained angiogenic and stem cell mobilization signals to the ischemic tissue in conjunction with cellular therapies (i.e. bone marrow cells). We expect to test this hypothesis by pursuing the following specific aims:

(1) Synthesize an ionic-gelatin based scaffold for the localized and sustained delivery of angiogenic peptides FGF-2 and G-CSF. FGF-2 and G-CSF were selected as angiogenic cytokines because of their capability in remodeling the extracellular matrix, line endothelial cells, and recruit endothelial progenitor and stem cell populations to ischemic tissues. Ionic-gelatin based materials were selected as a carrier of the angiogenic peptides because of their biocompatibility and the ability to prolong the release of the growth factors.

(2) Develop a sequential delivery system using a fibrin hydrogel containing ionic albumin microspheres to deliver FGF-2 and G-CSF at different sustained rates in critical limb ischemia. Synthesis of materials will be achieved by the following methods; for fibrin: mixing thrombin and fibrinogen at various ratios; for albumin: microsphere synthesis by oil-in-water emulsion followed by crosslinking via water-soluble carbodiimide reaction. The effect of method of release will be determined in a murine critical limb ischemia model.

(3) Assessment of the synergistic effects of FGF-2, G-CSF, and bone marrow derived cells toward critical limb ischemia. The sustained delivery of FGF-2 and/or G-CSF will be achieved from fibrin matricies and complimented with administration of bone marrow cell transplantation to assess neo-vessel formation in a critical limb ischemia model.

Chapter 2. Co-Delivery of FGF-2 and G-CSF From Gelatin-Based Hydrogels as Angiogenic Therapy in a Murine Critical Limb Ischemic Model

2.1 Co-delivery approaches toward peripheral artery disease and critical limb ischemia

Peripheral artery disease (PAD) afflicts more people today because of the prevalence of diabetes mellitus and the aging population (Allaqaband et al, 2006). Endarterectomy is currently the most available treatment option for patients; however, this method is highly invasive (Comerota, 2001). Therapeutic angiogenesis induced by growth factor administration is a promising non-invasive treatment option for PAD because it allows for capillary outgrowth (Sugano et al, 2004), re-establishes perfusion (Tsui and Dashwood, 2005) and restores tissues to normoxic conditions (Lee et al, 2005). The delivery of exogenous growth factors, such as basic fibroblast growth factor (FGF-2) and granulocyte colony-stimulating factor (G-CSF), has shown a marked capability in restoring regional blood flow and recovering ischemic tissues (Sugano et al, 2004; Tsui and Dashwood, 2005; Lee et al, 2005). FGF-2 is a single-chain polypeptide that is mitogenic and chemotactic for both fibroblasts and endothelial cells, which play significant roles during angiogenesis (Cote et al, 2004). Additionally, FGF-2 stimulates wound healing and augments collateral artery development to relieve peripheral ischemia (Nugent and Iozzo, 2000). G-CSF facilitates survival, proliferation and differentiation of all cells within the neutrophil lineage and assists in the amelioration of infections. G-CSF is a potent mobilizer of hematopoietic stem cells from the bone marrow, has a neuroprotective effect, activates endothelial proliferation and stimulates angiogenesis via mobilizing bone marrow stem cells (BMSCs) (in particular, endothelial progenitor cells, EPCs) to the ischemic site (Lee et al, 2005; Okhi et al, 2005; Roberts, 2005; Hubel and

Engert, 2003). Although administration of single growth factors may induce angiogenesis, multiple triggers may be necessary to form mature vasculature. Recent studies have suggested that by using two or more growth factors in combination may in fact promote mature vessel formation (Cao et al, 2003; Richardson et al, 2001). Growth factors administered via bolus injections can be rapidly metabolized, promote immature vasculature formation or cause systemic toxicity due to improper dosing.

An approach to circumvent problems associated with bolus delivery is the localized and controlled delivery of growth factors at the site of injury. Controlled delivery vehicles based on synthetic (Sharifi et al, 2006; Willerth and Sakiyama-Elbert, 2007; Goldberg et al, 2007) and natural biomaterials (George and Abraham, 2007; Chitkara et al, 2006) have been designed for the sustained delivery of growth factors, or plasmid DNA (Otani et al, 1996; Ikada and Tabata, 1998; Zisch et al, 2003; Agarwal et al, 2005; Kasper et al, 2005). Polymer-based controlled delivery vehicles are excellent candidates for growth factor or gene delivery because they can provide spatial and temporal control of presenting the therapeutic agent at the site of interest, are non-immunogenic and can be prepared with tunable biodegradability (Zisch et al, 2003; Agarwal et al, 2005; Chen et al, 2007; Davis et al, 2004). In addition to polymer-based delivery vehicles, the use of plasmids or viral vectors for the delivery of angiogenic cytokine encoding genes has been employed in a number of peripheral artery disease/critical limb ischemic models with notable success (Gounis et al, 2005; Rajagopalan et al, 2007; Madeddu, 2005; Yla-Herttuala and Alitalo, 2003). Although viral vectors can successfully deliver angiogenic genes at the ischemic site with acceptable transfection rates, issues with patient safety, controlling the dosage of the

growth factor being expressed and developing a switch-off mechanism upon repair have so far prevented the clinical use of gene therapy in therapeutic angiogenesis.

Herein, we report on the co-delivery of FGF-2 and G-CSF from ionic gelatin-based hydrogels and their synergistic effect in establishing reperfusion and mature vessel formation in a murine critical limb ischemic model. We envision that the timely release of FGF-2 and G-CSF could facilitate angiogenesis, with FGF-2 directing endothelial migration and proliferation, and G-CSF inducing endothelial progenitor cell migration to the wound site (Kong et al, 2004). Gelatin was selected as the primary component of the delivery vehicle because of its known cytocompatibility and its ability to stabilize proteins via poly-ion complexation and assist in endothelial cell matrix remodeling (Tokounaga et al, 2004).

2.2 Materials and Methods

2.2.1 Materials

Gelatin B from bovine skin (pI = 9.0; ~225 bloom), poly-L-glutamic acid (PLG; MW = 50,000 – 100,000 kDa), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), and N-hydroxysuccinimide (NHS) were purchased from Sigma Aldrich (St Louis, MO) at their highest purity. Human recombinant basic fibroblast growth factor (FGF-2; MW = 16,000 kDa; pI = 9.6), and granulocyte-colony stimulating factor (G-CSF; MW = 18,800 kDa; pI = 6.2) were purchased from Peprotech (Rocky Hill, NJ). Human umbilical vein endothelial cells (HUVEC) were purchased from ATCC (Manassas, VA). All other chemicals were purchased from Sigma Aldrich at their highest grade purity unless otherwise noted.

2.2.2 Hydrogel Synthesis

Ionic, gelatin-based hydrogels were prepared from the polymerization of gelatin and PLG via amide bond formation in the presence of the EDC/NHS activation catalyst (Otani et al, 1996; Layman et al, 2007) as demonstrated in Figure 2.1. Briefly, hydrogels were synthesized by combining 10, and 15 wt-vol% buffered solutions (pH = 7.4) of gelatin with 1 wt-vol% PLG. PLG was previously solubilized and activated in an aqueous buffered solution (pH = 7.4) containing 1.3 mg/ml EDC and 0.44 mg/ml N-hydroxysuccinimide. Equal volume solutions of gelatin (150 µL) and PLG/EDC/NHS (150 µl) were merged and crosslinked for 10 minutes at 4 °C.

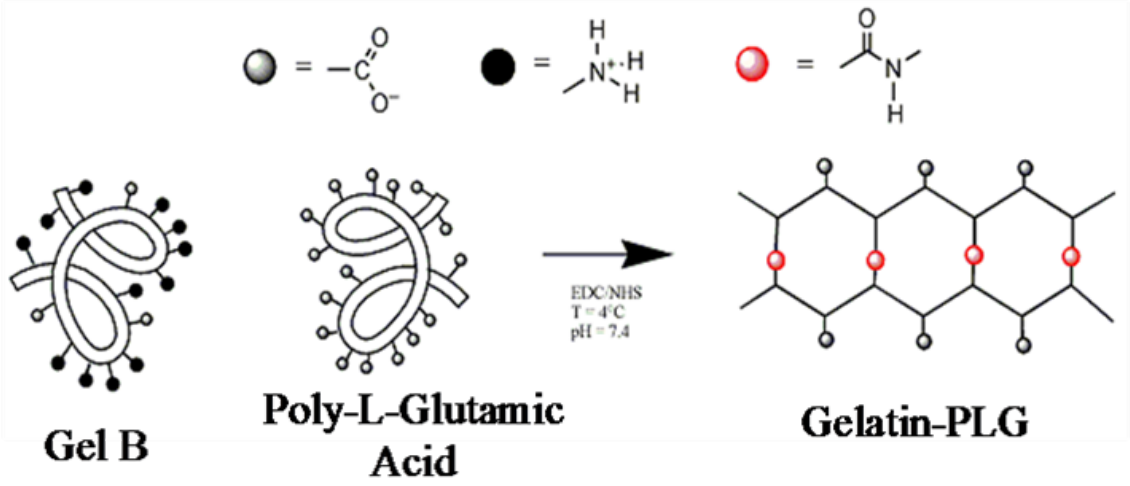


Figure 2.1. Reaction scheme for the synthesis of ionic, gelatin-based hydrogels. Gelatin B was co-crosslinked with PLG (anionic gels) via an EDC/NHS coupling reaction.

2.2.3 Controlled Release of G-CSF and FGF-2

The release kinetics of G-CSF and FGF-2 were determined using a method described previously (Layman et al, 2007). Briefly, G-CSF and FGF-2 were reconstituted in double-distilled water or Tris-HCl (pH = 8.0) respectively to a concentration of 0.1 mg/ml. Plastic cylinders (h = 4.0 mm, D = 6.5 mm, V = 135.0 mm^3)

were sealed to the bottom of a 6-well culture plate using silicon glue (Dow Corning). 150 μL of 10, and 15 wt-vol % gelatin B solution was combined with the growth factors to make a final concentration of FGF-2 or G-CSF at 1.66 μg/ml, and cross-linked with 150 μL of 2.6 mg/ml EDC, 0.88 mg/ml NHS, and 2 wt-vol% PLG solution at 4 °C for 10 minutes. Release experiments were conducted on a MaxQ4000 orbital shaker (Barnstead International; Dubuque, IA) at 100 rpm, and 37 °C. The hydrogels were immersed in 10 mL of PBS (pH = 7.4) and samples were collected at random time points over 14 days and stored at -20 °C prior to quantification in triplicate. The amount of G-CSF (R&D Systems; Minneapolis, MN) and FGF-2 (EMD Biosciences; San Diego, CA) released was determined using an ELISA. The optical density was determined at 490 nm with a reference wavelength at 650 nm for G-CSF and at 450 nm with reference at 570 nm for FGF-2.

2.2.4 Activity of FGF-2 and G-CSF on Endothelial Cell Culture

2.2.4.1 Proliferation

HUVECs were cultured to confluence (passage six) in T-75 flasks at 37 °C and 5% CO_2, trypsinized, and transferred to 24-well plates (10,000 cells/well). Fresh Endothelial Cell Basal Medium (EBM; Lonza; Walkersville, MD), supplemented with 2% fetal bovine serum (FBS; Lonza; Walkersville, MD), gentamicin, hEGF, bovine brain extract, and hydrocortisone was added to the cells followed by FGF-2 and/or G-CSF at final concentrations of 0.1, 1, 10, and 100 ng/ml. After 4 days of incubation without medium replacement, the cells were trypsinized and counted using a Coulter Particle Counter (Beckman Coulter; Miami, FL) and assayed in triplicate experiments.

Human recombinant vascular endothelial growth factor (VEGF; Sigma Aldrich; St Louis, MO) was used as a positive control at a concentration of 10 ng/ml.

2.2.4.2 Migration

A modified Boyden chamber assay was performed using HTS FluoroBlok™ inserts with 8.0 μm pore size (BD Biosciences; Minneapolis, MN) and 24-well plates. Briefly, HUVECs were seeded in the upper chamber (100,000 cells/well) while the lower chamber was supplemented with EBM containing 2% FBS, and growth factors at 10, 100, and 2000 ng/ml in the following groups: 1) negative control (no growth factor), 2) FGF-2 alone, 3) G-CSF alone, 4) FGF-2 and G-CSF. After incubation for 24 hours at 37 °C and 5% CO_2 the inserts were removed, the cells were trypsinized and counted using a Beckman Coulter Particle Counter (triplicates). The cells that had migrated to the lower chamber were counted as positively migrated cells.

2.2.4.3 In Vitro Tube Formation Assay

HUVEC tube formation was assessed in a Matrigel (100 mg/cm^2; BD Biosciences). HUVECs (20,000 cells/well) were seeded on 24-well plates coated with Matrigel in EBM and 2% FBS. Cells were treated with 10 or 100 ng/ml of: 1) G-CSF alone, 2) FGF-2 alone, 3) both G-CSF and FGF-2. After 24 hours, HUVEC tube formation was quantified using light microscopy at 10x, and branch points were counted using NIH Image J 1.73 software. At least five fields were examined per well in triplicate.

2.2.4.4 Activity of Released G-CSF and FGF-2

The activity of the released G-CSF and/or FGF-2 was determined on the proliferation of HUVECs. Cells (10,000 cells/well) were seeded in 24-well culture plates

in EBM supplemented with 2% FBS and then incubated for 12 hours at 37 °C and 5% CO_2. Five treatment groups were investigated: 1) No scaffold, 2) empty scaffold, 3) G-CSF releasing gelatin-PLG scaffolds, 4) FGF-2 releasing gelatin-PLG scaffolds, and 5) FGF-2 and G-CSF releasing gelatin-PLG scaffolds. Prior to seeding, the scaffolds (300 μl) were cross-linked as described previously in transwell baskets (Dow Corning) at 10 wt-vol% and loaded with various concentrations of G-CSF and FGF-2 (10, 100, or 2000 ng/ml) for 10 min. at 4 °C. After cross-linking, the baskets were placed in the wells for two and four days without medium replacement. The activity of the HUVECs was assessed by cell count using a Coulter Particle Counter as previously described (Defail et al, 2006). All assays were performed in triplicate experiments.

2.2.5 Animal surgery

Balb/C mice (Charles River Laboratories; Boston, MA) of age 6 to 7 weeks were cared for and operated on under IACUC guidelines at the Miller School of Medicine at the University of Miami. Prior to surgery, mice were anesthetized using a ketamine/xylazine/acepromazine cocktail at 40 mg/kg, 8 mg/kg, and 10 mg/kg body weight respectively. The surgery was performed according to standard methods (Layman et al, 2007; Rivard et al, 1999). Briefly, an incision was made from the patella to the lower abdomen exposing the femoral and external iliac arteries. A 7-0 grade silk suture was used to ligate the lower superficial femoral artery (above the saphenous branches), and at the junction of the external iliac, deep femoral, and profunda femoris. The artery was then excised and removed. The surgery is pictorially described in Figure 2.2.

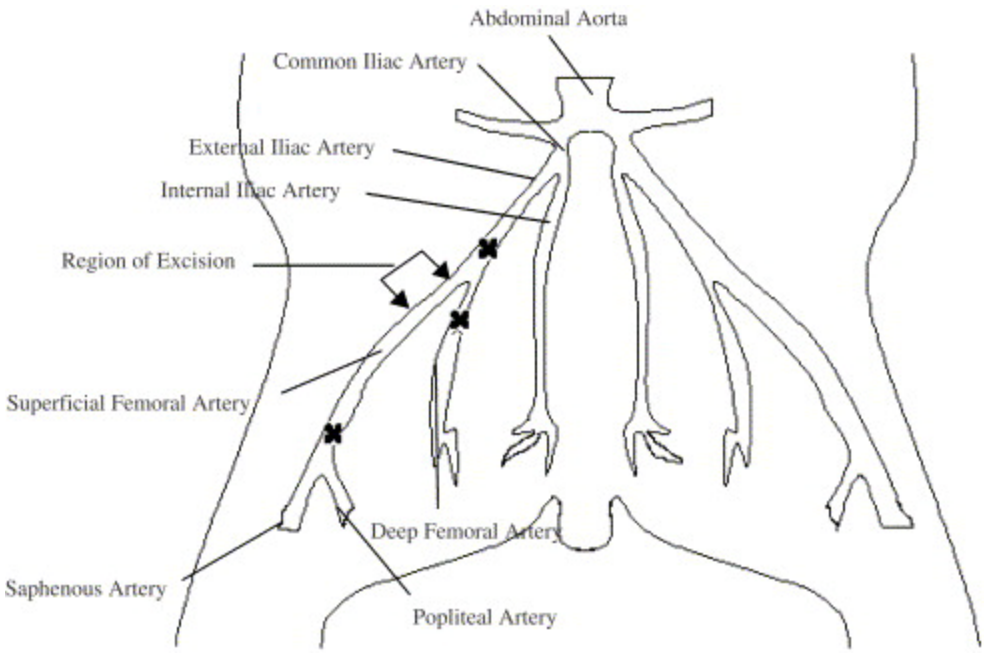


Figure 2.2. Schematic representation for the induction of critical hindlimb ischemia in a murine model.

After surgery, mice were randomized for treatment in two categories (*N*=5 per group per time point). First, bolus administration (300 μl) of growth factors were administered as follows: 1) No treatment (PBS); 2) 100 ng/ml FGF-2; 3) 100 ng/ml G-CSF; 4) 100 ng/ml of G-CSF and FGF-2. Second, gelatin-PLG scaffolds (10 wt-vol% gelatin; 1 wt-vol% PLG; 300 μl) were implanted at two growth factor concentrations (100 ng/ml and 1000 ng/ml) of FGF-2, G-CSF, or both.

2.2.6 Laser Doppler Perfusion Imaging

Hindlimb perfusion was assessed using Laser Doppler Perfusion Imaging (LDPI; Perimed Medical Systems; New York, NY), and data acquisition was obtained as previously reported (Rivard et al, 1999). Briefly, color photographs were obtained and hindlimb perfusion was determined by comparing the intensity of a murine hindlimb prior to surgery to a specific time point: post-surgery, one, two, three, four, and eight weeks. An ischemic Doppler ratio was established for all groups and compared.

2.2.7 Immunohistochemistry

The mice were sacrificed at predetermined time points (2, 4 and 8 weeks) with an overdose of halothane inhalation. Quadriceps muscle tissue was removed and immediately placed in 10% formalin in PBS overnight. After fixation, tissue was embedded in paraffin and 5 μm tissue sections were cut and mounted on positively charged slides. The tissue's antigenicity was recovered using a warmed proteinase K solution for CD31+ staining, and 10 mM citrate buffer (pH 6.0) for actin staining. Samples were then treated with diluted primary antibody (1:25), goat PECAM-1 monoclonal antibody overnight (Santa Cruz Biotechnology; Santa Cruz, CA) or α-actin (0.N.5) diluted at 1:500 (Santa Cruz Biotechnology; Santa Cruz, CA) for 2 h. Samples were then rinsed, and incubated in secondary biotinylated antibody (1:200), rabbit anti-goat polyclonal antibody (Santa Cruz Biotechnology). Samples were then treated with ABC (Vector Labs; Burlingame, CA) and DAB chromogen reagent (DAKO; San Diego, CA). Slides were then viewed using light microscopy at 60x using a LEICA Microsystems AF6000LX brightfield/fluorescence microscope (Bannockburn, IL), and images were captured using MetaMorph 5.1 Software. Positively stained CD31 capillaries were manually counted in minimum five separate fields and expressed as capillaries per millimeter squared. The number of vessels associated with a-SMA positively stained cells was also quantified in a similar fashion.

2.3 Results and Discussion

2.3.1 Controlled Release of FGF-2 and G-CSF

To assess the effect of gelatin concentration on the release kinetics of FGF-2 and G-CSF, two different gelatin concentrations (10, and 15 wt-vol%) were used to prepare gelatin-PLG hydrogels that contained the releasing growth factors (0.83 μg/ml). The hydrogels were swollen within the cylinder and the growth factors were released one-dimensionally into the surrounding medium. In the case of G-CSF, changes in the gelatin concentration significantly affected the growth factor's release profile in the presence or absence of the other growth factor, FGF-2. Specifically, as the gelatin concentration increased from 10 to 15 wt-vol%, the total amount of G-CSF released from the gelatin-PLG hydrogels over 14 days was decreased by almost 55% (92.2% ± 2.0%, and 40.1% ± 5.1% of G-CSF respectively, Figure 2.3A). However, in the case of FGF-2, its release from the gelatin-PLG hydrogels was not significantly affected by the changes in gelatin concentration (Figure 2.3B). Furthermore, the overall amount of FGF-2 released over 14 days was about 60% of its initial loading concentration and about 25% lower than the total amount of G-CSF been released from similar hydrogels. This is likely attributed to the cationic nature of FGF-2 at the pH (7.4) of the incubation medium and the ionic interactions between FGF-2 and the gelatin-PLG matrix that prolonged the release of FGF-2. There is also a significant ($p < 0.01$) difference between the release profiles of FGF-2 when G-CSF is also encapsulated within the gelatin-PLG hydrogels during the first 6 days of release. Specifically, there is a 25% drop at the rate of FGF-2 release when G-CSF is also immobilized within the hydrogel matrix suggesting that there might be some interactions (e.g. ionic complexation) between the two growth factors that alters the

diffusion kinetics (Figure 2.3B). Our data along with other studies suggest that by altering the chemical composition (i.e. reactant ratios, crosslinker concentrations), ionic nature, or crosslinking density of the delivery vehicle, the release profiles of FGF-2 or G-CSF may be controlled to meet the requirements of natural angiogenic progression in a CLI model (Ikada and Tabata, 1998; Otani et al, 1998, Haraguchi et al, 2007; Thyagarajapuram et al, 2007; Pieper et al, 2002; Park et al, 2002).

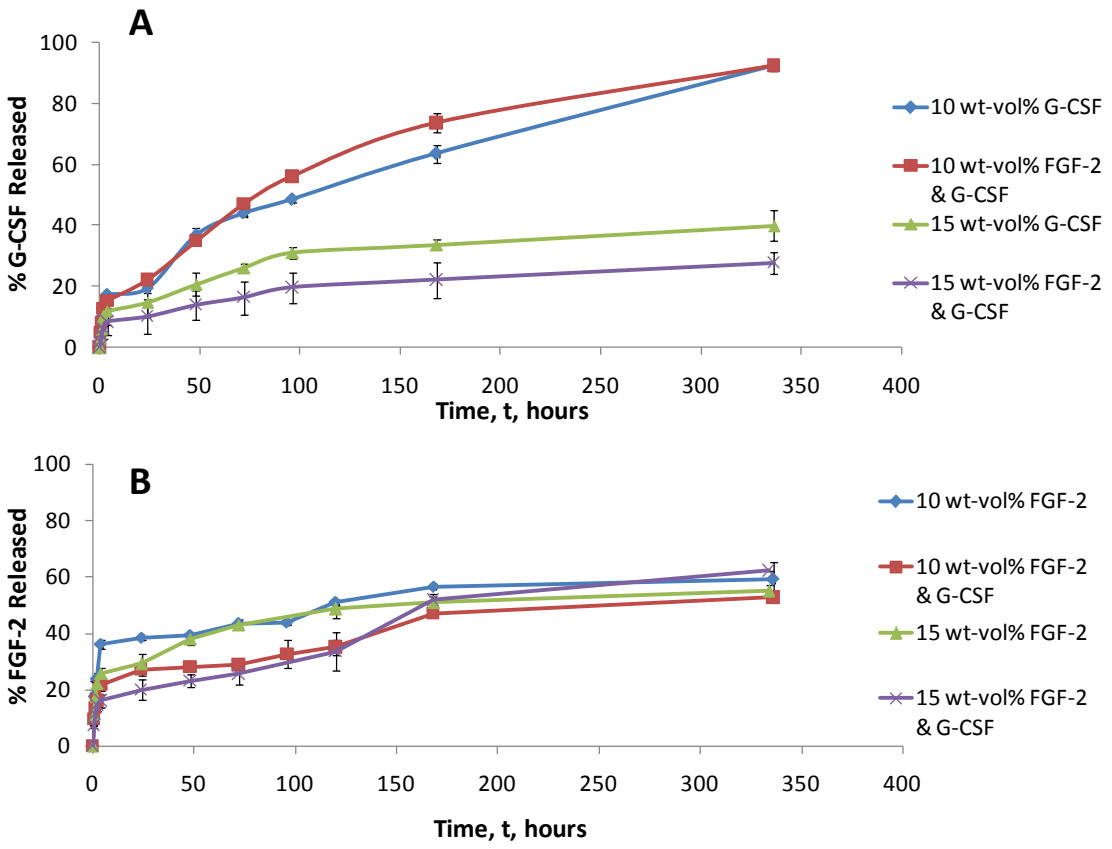


Figure 2.3. (A) Percent release of G-CSF over 14 days from gelatin–PLG hydrogels prepared with gelatin concentrations of 10 or 15 wt.-vol.% ($N = 3$ per time point per group). G-CSF release is shown in the presence or in the absence of FGF-2. (B) Percent release of FGF-2 over 14 days from gelatin–PLG hydrogels prepared with gelatin concentrations of 10 or 15 wt.-vol.%. FGF-2 release is shown in the presence or in the absence of G-CSF.

2.3.2 Effect of FGF-2 and G-CSF on HUVEC culture

A number of studies have explored the effect of FGF-2 or G-CSF on endothelial cell proliferation, migration, and tube formation however to our knowledge their combinatory effect has yet to be evaluated in a HUVEC culture (Pieper et al, 2002; Park et al, 2002; Bussolino et al, 1993). In accordance with other studies, FGF-2 had a positive effect on endothelial proliferation in a concentration dependent manner (Figure 2.4A) (Kong et al, 2004). Specifically, as the concentration of FGF-2 increased from 0.1 ng/ml to 100 ng/ml, the total cell population increased by 20% (29,640 cells ± 772 cells to 37,080 cells ± 1,116 cells). On the other hand, G-CSF had no significant proliferative effect on HUVECs at the tested concentrations (0 to 100 ng/ml). When FGF-2 and G-CSF were used in combination, the results mirrored those of FGF-2, suggesting that FGF-2 plays a more prominent role in accelerating endothelial cell proliferation compared to G-CSF (Figure 2.4A). The combinatory effect of FGF-2 and G-CSF on endothelial cells was similar to the effect of VEGF at 10 ng/ml (34,983 cells ± 786 cells) (Figure 2.4A).

A modified Boyden chamber was used for the migration assay. Cell migration is an integral facet of the angiogenic response as endothelial cells move through the protease-degraded basement membrane across a concentration gradient of growth factors. The HUVECs that had migrated to the lower chamber were counted as positively migrated cells after trypsinization. Neither G-CSF nor FGF-2 had a significant effect on migration at 10 ng/ml, however when used in combination, there was a significantly greater effect on migration than either growth factor acting alone ($p < 0.05$). As the concentration of growth factor was increased to 2 μg/ml, G-CSF induced a 76% ± 4.2% greater migratory response on HUVECs, and likewise, combinatory delivery of growth

factors induced a 77.4% ± 3.9% greater migration compared to control. On the other hand, FGF-2 had a potent migratory capability at the lower concentrations, but reached a maximum response at 100 ng/ml (Figure 2.4B).

A tube formation assay was conducted in a 24-well plate coated in Matrigel and the branch points were counted to assess neovascularization. Figure 2.4C shows that the number of branch points was dependent on the concentration of growth factor administered. Although the effect of FGF-2 and G-CSF at 100 ng/ml was not significantly different compared to each other, when used in combination, the number of branch points formed were 21% higher ($p < 0.05$) compared to the other treatment groups.

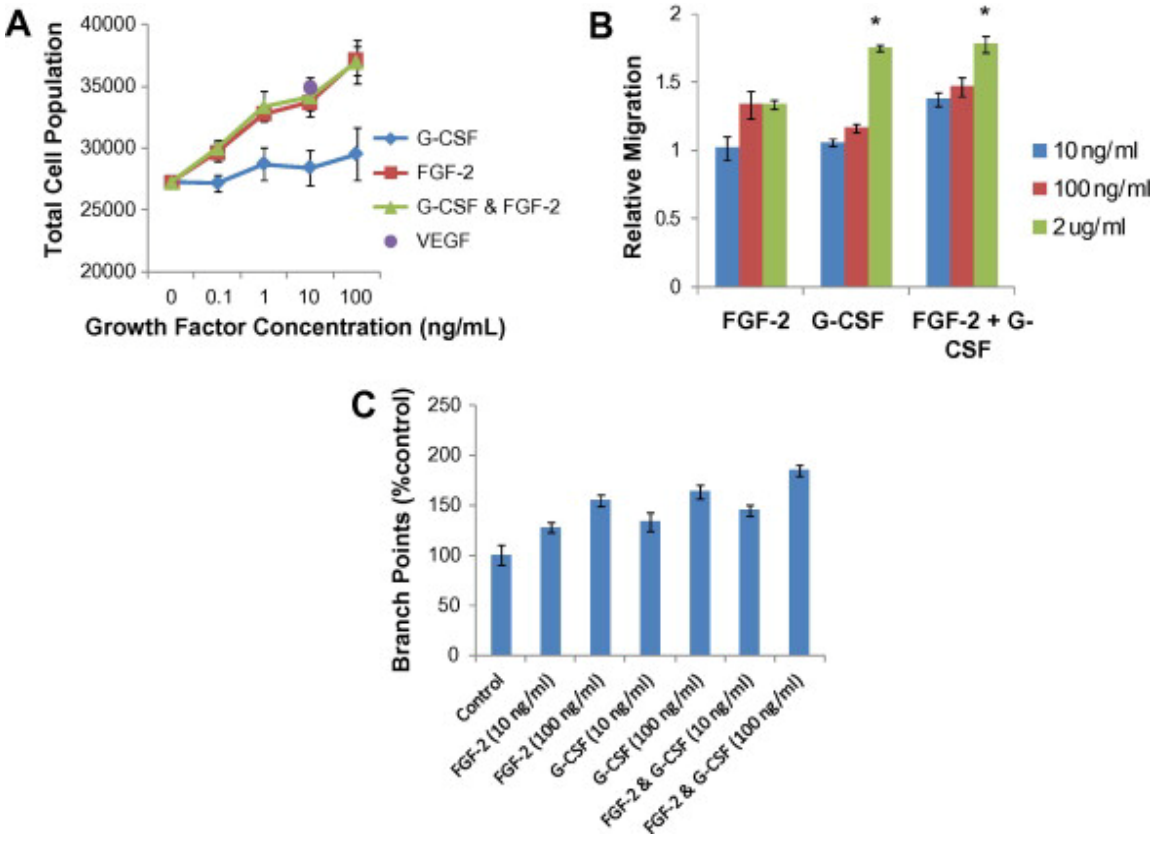


Figure 2.4. FGF-2 and G-CSF in vitro activity on the proliferation and tube length formation of HUVECs. (A) Effect of varying concentrations of FGF-2, G-CSF or both on HUVEC proliferation over 4 days cultured at 37 °C and 5% CO_2. (B) Relative migration

of HUVECs at various growth factor concentrations using a Boyden chamber assay. Cells that had migrated to the bottom chamber were counted. (C) Effect of various growth factor concentrations on tube formation of HUVECs in Matrigel (100 mg cm^{-2}) estimated at 10× after 24 h. *Statistical significance between the two groups with $p < 0.05$.

The activity of the released G-CSF and FGF-2 was assessed on cell proliferation. Figure 2.5 depicts the effect of the released FGF-2 and G-CSF from the gelatin-PLG hydrogels on the proliferation of HUVECs cultured in EBM supplemented with 2% FBS. The released FGF-2 induced cellular proliferation after 2 and 4 days of incubation with the most pronounced effect at the higher loading concentrations (2000 ng/ml vs. 100 ng/ml) in contrary to the released G-CSF which had a minimum effect (Figure 2.5A,B). The co-delivery of G-CSF and FGF-2 had similar proliferative effect to the release of FGF-2 alone over the same incubation period (Figure 2.5A,B). The proliferative effect of the releasing growth factors was in agreement with the effect that the bolus administration of the growth factors had (FGF-2 in particular) assuming that only about a small fraction of the loading concentration of the FGF-2 was released over 4 days.

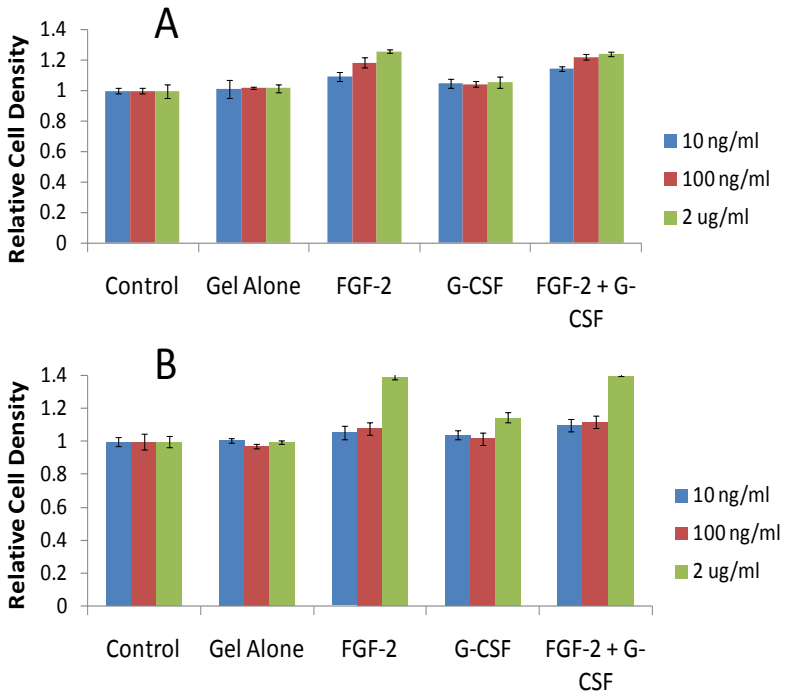


Figure 2.5. Endothelial cell activity in the presence of a controlled release vehicle (10 wt.-vol.% gelatin, 1 wt.-vol.% PLG) loaded with 10, 100 or 2000 ng ml^{-1} of FGF-2, G-CSF or both over 2 days (A) and 4 days (B) (N = 3 per time point per group).

These data show that FGF-2 has potent mitogenic activity toward proliferation and migration of endothelial cells in a dose dependent manner suggesting that FGF-2 dominates the early stages of endothelial cell cycle progression as well as mobilizing adjacent endothelial cells to migrate (Cote et al, 2004). Conversely, G-CSF has a minimum proliferative effect conferring previous results (Bussolino et al, 1993). However, G-CSF facilitates endothelial tube formation, suggesting its involvement in the development of an endothelial cell network (Lee et al, 2005; Kondoh et al, 2004).

2.3.3. Effect of dual growth factor delivery and dose response in a critical limb ischemic model

To assess the effect of the controlled co-delivery of G-CSF and FGF-2 on reperfusion, capillary sprouting and vessel formation, we utilized a well-accepted murine

critical limb ischemic model. Bolus injections of growth factor solutions (no vehicle) were administered immediately following surgery (300 μl) and followed-up on 1, 2, 3, 4 and 8 weeks with LDPI, and at 2, 4 and 8 weeks with immunohistochemistry. LDPI revealed that maximal reperfusion occurred at 3 weeks for all treatment groups (Fig. 2.6A). When G-CSF and FGF-2 were administered in combination, the LDPI ratio was 10% greater (0.59 ± 0.03, $p < 0.05$) than all other treatments. In addition, there was at least a 75% increase in capillary density in mice treated with G-CSF and FGF-2 compared to non-treated ischemic limbs at all time points (Fig. 2.6B). After 8 weeks, the combinatory bolus treatment was statistically greater than all other treatments for capillary outgrowth (1183 ± 36 vessels mm^{-2}; 0.54 ± 0.04), which also corresponded to a greater hindlimb perfusion shown in Fig. 2.6B. Conversely, after 3 weeks there was a significant decrease in the hindlimb perfusion in FGF-2 treated mice up to 8 weeks ($p < 0.05$). This decrease could be attributed to growth factor clearance, or immature vasculature formation resulting in apoptosis of endothelial cells (Richardson et al, 2001; Jeon et al, 2006a). In order to assess mature vessel formation, immunostaining for α-actin was performed. For the FGF-2 treated group, the number of positively stained SMA vessels decreased significantly (from 225 ± 14 to 175 ± 12 positive-stained SMA cells mm^{-2}; $p < 0.05$) after 4 weeks of treatment (Fig. 2.6C). On the other hand, the group that was treated with G-CSF and FGF-2 demonstrated a significant increase in the amount of mature vessels formed from 4 to 8 weeks (from 244 ± 25 to 312 ± 18 positive-stained SMA vessels mm^{-2}; $p < 0.05$).

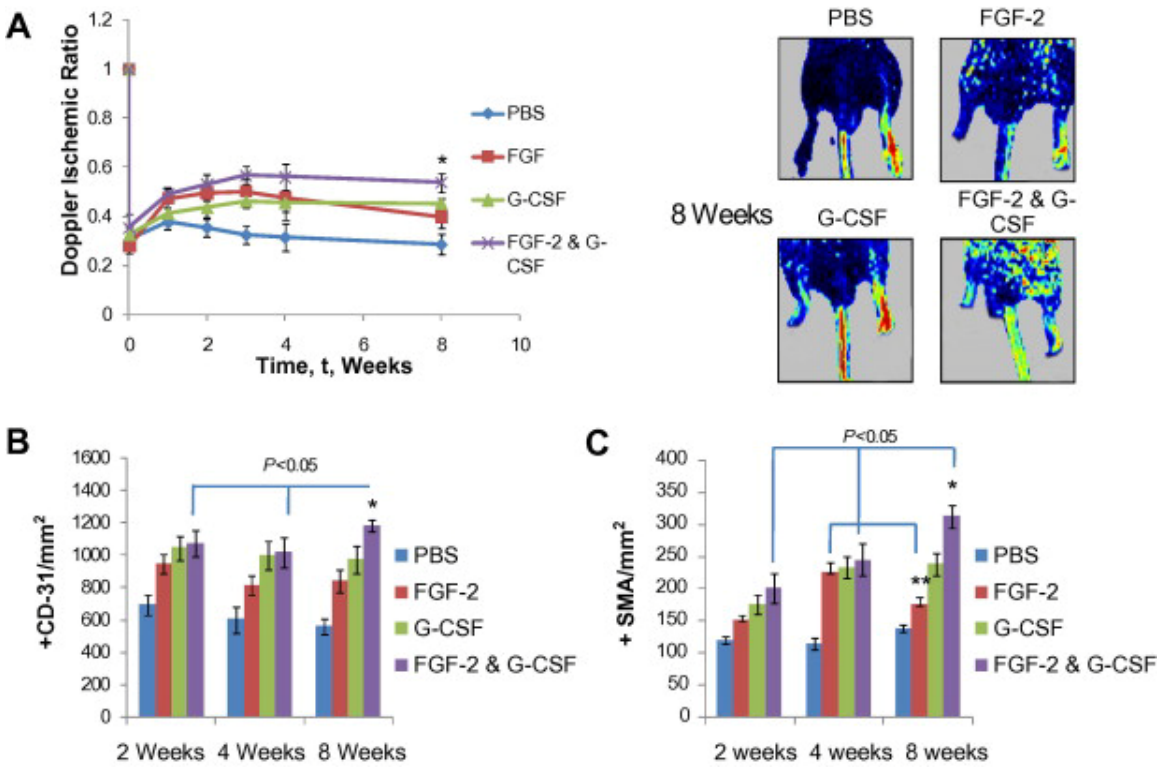


Figure 2.6. The effect of bolus administration (no vehicle) of FGF-2 and G-CSF on reperfusion, capillary density and positively stained α-SMA vessels. Growth factor solutions of FGF-2 (300 µl, 100 ng ml^{-1}), G-CSF (300 µl, 100 ng ml^{-1}) or both were injected in the muscle adjacent the excision site ($N = 5$ mice per time point per group). (A) Doppler perfusion ratio of the ischemic limb compared to the non-ischemic limb prior to surgery for the murine model up to 8 weeks after surgery. (B) Capillary density of quadriceps muscle in a CLI mouse 2, 4 and 8 weeks post-ligation/excision. Laser Doppler perfusion images of ischemic limbs for all treatments using bolus injections at 8 weeks post-ligation/excision. (C) Total number of capillary vessels per unit area as confirmed by positive α-actin staining of quadriceps muscle; *,**Groups that are statistically different ($p < 0.05$) from the marked groups.

Since the dual administration of FGF-2 and G-CSF in the absence of a vehicle produced only a 60% return to the pre-ischemic perfusion levels (by LDPI), we then assessed the effectiveness of a delivery vehicle (gelatin–PLG hydrogels) to protect the growth factors from degradation and rapid clearance while at the same time providing a localized, sustained release profile of the polypeptides at the ischemic site (Richardson et al, 2001; Zisch et al, 2003; Chen et al, 2007). Fig. 2.7A shows that the controlled delivery of 100 ng ml^{-1} of G-CSF alone increased hindlimb perfusion by an additional 13%

(0.67 ± 0.04) in comparison to bolus administration of both factors over an 8 week treatment ($p < 0.05$). Compared to bolus administration, after 8 weeks from the induction of ischemia, there was only a 9% increase in capillary density for controlled release G-CSF treated limbs at 100 ng ml^{-1} ($p = 0.21$), suggesting that a higher concentration of G-CSF may be needed to induce further capillary sprouting (Fig. 2.7B). The LDPI score and capillary density decreased significantly for the controlled release of 100 ng ml^{-1} of FGF-2 between 4 and 8 weeks; specifically, there was a 14% decrease in blood flow perfusion and a 10% decrease in capillary sprouting. This decrease might be due to insufficient initial FGF-2 loading concentration, growth factor degradation or the requirement of multiple triggers (Richardson et al, 2001; Jeon et al, 2006a; Jain, 2003). As hypothesized, the co-delivery of G-CSF and FGF-2 (at 100 ng ml^{-1}) from the gelatin–PLG hydrogels also had a superior effect on capillary density and hindlimb reperfusion after 8 weeks (1278 ± 92 vessels mm^{-2}; 0.80 ± 0.04) compared to all controlled release groups ($p < 0.05$) and bolus treatments ($p < 0.05$). Vessel maturation as quantified by the α-SMA immunostaining assay was also highly dependent on the co-delivery of FGF-2 and G-CSF (Fig. 2.7C). The controlled delivery of 100 ng ml^{-1} of FGF-2 from the gelatin–PLG hydrogels resulted in a significant decrease in the amount of positively stained SMA vessels between 4 and 8 weeks (from 272 ± 19 to 222 ± 34 positive-SMA vessels mm^{-2}; $p < 0.05$). Conversely, the highest increase in positively stained SMA vessels (317 ± 24 SMA vessels mm^{-2}) was seen for the controlled co-delivery of FGF-2 and G-CSF treatment group after 8 weeks of treatment (Fig. 2.7C, Gel+FGF-2 & G-CSF group).

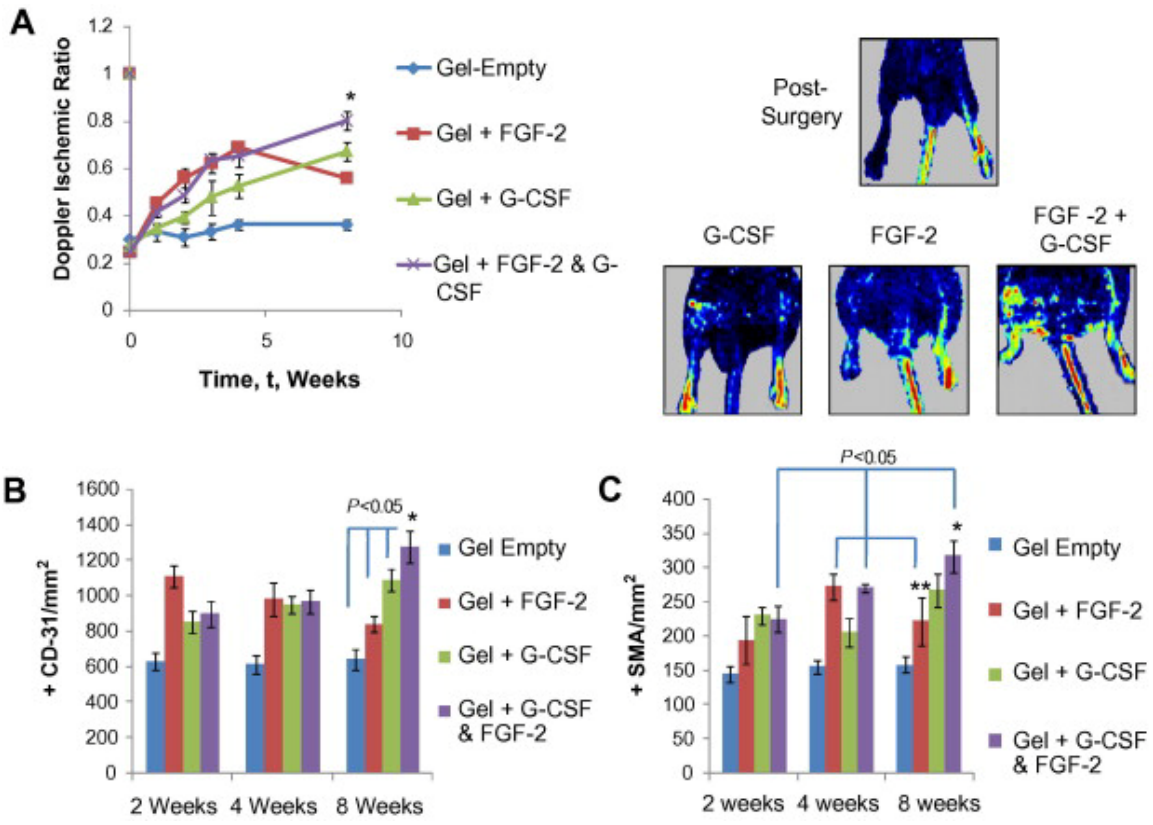


Figure 2.7. The effect of the controlled delivery of FGF-2 (100 ng ml^{-1}), G-CSF (100 ng ml^{-1}) or both from gelatin–PLG hydrogels on reperfusion, capillary density and positively stained α-SMA vessels ($N = 5$ mice per time point per group). The growth factor-containing hydrogels were implanted adjacent to the excision site. (A) Doppler perfusion ratio of the ischemic limb compared to the non-ischemic limb prior to surgery for 8 weeks post-surgery. (B) Capillary density of quadriceps muscle in a PAD mouse 2, 4 and 8 weeks post-ligation/excision. Laser Doppler perfusion images of ischemic limbs for all treatments using controlled delivery vehicles at 8 weeks post-ligation/excision. Micrographs at 60× of CD31^{+} stained capillaries at 8 weeks post-ligation and excision for indicated groups. (C) Total number of capillary vessels per unit area as confirmed by positive α-actin staining of quadriceps muscle.

In order to assess the effect of growth factor concentration on vessel formation and maturation, the amount of the growth factors loaded within the gelatin–PLG hydrogels was increased 10-fold. By increasing the growth factor loading concentration from 100 to 1000 ng ml^{-1} there was a significant increase in blood flow perfusion at all time points ($p < 0.05$) compared to gel-empty, as shown in Fig. 2.8A. The peak blood flow for hydrogels loaded with 1000 ng ml^{-1} of G-CSF and FGF-2 was 0.867 ± 0.03,

with minor variations up to 8 weeks. For the FGF-2-releasing hydrogels there was a 26% decrease in regional blood flow (LDPI) and a noticeable decrease on cell density and positively stained SMA vessels from 4 to 8 weeks, suggesting the inability of a single growth to induce a mature and stable vasculature (Fig. 2.8A-C) (Jain et al, 2003). Additionally, after 8 weeks, the capillary density was found to be insignificant between lower and higher concentrations of growth factor loading for FGF-2+G-CSF and G-CSF loaded hydrogels (Fig. 2.8B). This suggests that G-CSF may play a larger role in reperfusion through arteriogenesis because of the significant increase in hindlimb perfusion rather than capillary density (Minamino et al, 2005).

Although the bolus administration of FGF-2 and G-CSF led to an increase in capillary density after 8 weeks and the perfusion was significant compared to all other groups, only 60% normal perfusion levels were seen. Alternatively, the co-delivery of FGF-2 and G-CSF from the gelatin–PLG scaffolds led to the highest level of reperfusion (87% of normal vessels), capillary density and number of positively stained SMA vessels in comparison to all other groups after 8 weeks of treatment. This suggests that G-CSF complements the angiogenic effect of FGF-2 by acting upon other phases of angiogenesis, such as EPC migration, arteriogenesis or mature vessel stabilization, as shown in previous studies (Minamino et al, 2005; Jeon et al, 2006b; Hematti et al, 2003). Studies to delineate G-CSF's mechanism of action on angiogenesis in a critical ischemic model are currently underway.

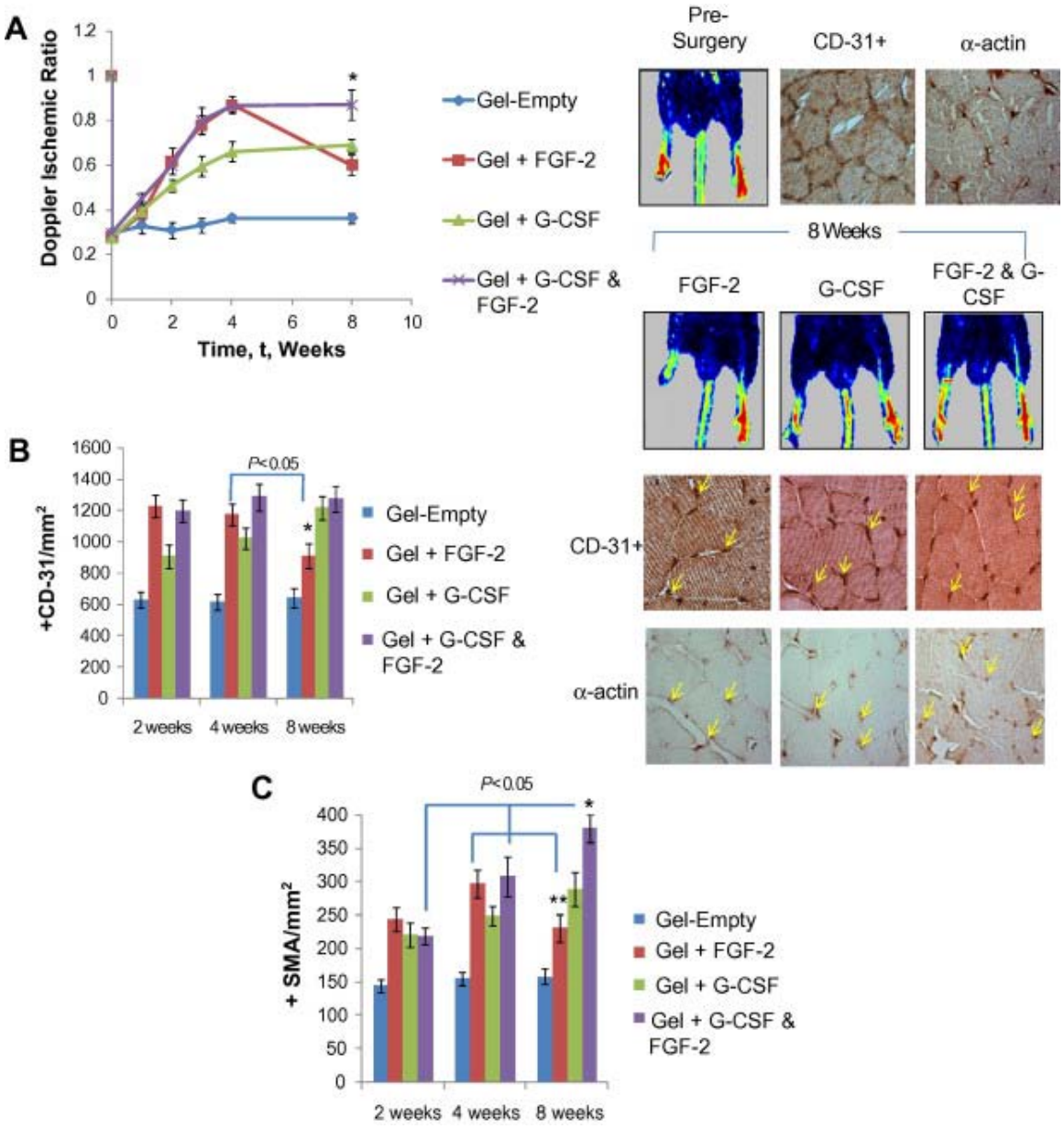


Figure 2.8. Assessment of in vivo reperfusion, capillary density and mature vessel formation of critical limb ischemic mice followed by the implantation of a gelatin–PLG hydrogels loaded with 1000 ng ml^{-1} of FGF-2, G-CSF or both (N = 3 mice per time point per group). (A) Doppler perfusion ratio of the ischemic limb compared to non-ischemic limb prior to surgery for 8 weeks post-surgery following administration of 1000 ng ml^{-1} of growth factor. (B) Capillary density of quadriceps muscle in a CLI mouse 2, 4 and 8 weeks post-ligation. Laser Doppler perfusion images are given for 8 weeks after surgery and treatment. Immunohistochemistry micrographs are presented at 60× for capillaries at 8 weeks following treatment with the growth factor-releasing gelatin–PLG hydrogels. (C) Total number of capillary vessels per unit area as confirmed by positive α-actin staining of quadriceps muscle.

2.4 Conclusions from Co-delivery of FGF-2 and G-CSF from Gelatin-Based Hydrogels as Angiogenic Therapy in a Murine Critical Limb Ischemic Model

Tunable gelatin-based ionic hydrogels were synthesized via a water soluble carbodiimide reaction for the delivery of multiple growth factors in an acute model of critical limb ischemia. G-CSF and FGF-2 were encapsulated in anionic gelatin hydrogels because of their angiogenic capability and their release kinetics were determined to be a function of the material properties. The effects of the G-CSF and FGF-2 were evaluated in endothelial cell culture for proliferative, migratory, and tubular formation capability at various concentrations. Additionally, these growth factors were tested in a murine model for blood flow reperfusion and capillary ingrowth. Studies are currently underway to elucidate the pathways of angiogenesis associated with action of these growth factors as well as spatially compartmentalized materials or sequential release of growth factors offering additional alternatives for an enhanced therapeutic effect *in vivo* for treatment of CLI.

Chapter 3. Sequential Delivery of FGF-2 and G-CSF from fibrin hydrogels in Critical Limb Ischemia

3.1 Sequential Delivery Strategies for the Treatment of Ischemic Tissues

Current therapies to regenerate ischemic tissues (e.g., muscle, heart, brain) in the body rely on the bolus administration of a single growth factor. However, success of pre-clinical trials administering angiogenic cytokines has been limited. Namely, studies have shown that administration of vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (FGF-2) alone stimulates vessel growth for a transient period but may result in fragile and leaky vessels prone to regression (Carmeliet and Conway, 2001; Richardson et al, 2001; Layman et al, 2007). The limitations of such treatment may be related to the mode of cytokine delivery as well as the multiple cues needed to initiate mature blood vessel formation. In most studies, proteins are delivered as a bolus injection to the ischemic tissue or administered to the blood stream (Lederman et al, 2002; Mifune et al, 2008; Powell et al, 2005). This strategy has received mixed results as the exact dosage of growth factors required to reperfuse ischemic tissues is unknown, growth factors can be rapidly cleared from the tissue, and non-specific localization of the drug at distant sites (Cao and Mooney, 2007). In addition to angiogenic factors, recent studies have shown that stem or endothelial precursor cell transplantation (Nishimura and Asahara, 2005; Rafii and Lyden, 2003; Asahara et al, 1999b), and gene delivery (Gounis et al, 2005; Rivard et al, 1999) could be useful for therapeutic angiogenesis. The molecular mechanisms of angiogenesis, as well as timing, are still being delineated and it is clear that numerous growth factors and cell types play a pivotal role at different stages of angiogenesis. For example, FGF-2 is mitogenic and chemotactic for fibroblasts and

endothelial cells, driving the alignment of the basement membrane of newly formed capillaries, and recruiting bone marrow stem cells. However, FGF-2 alone is not adequate to restore and sustain the tissue perfusion at the pre-ischemic level, as demonstrated previously (Layman et al, 2007; Cao et al, 2003). Granulocyte-colony stimulating factor (G-CSF) recruits endothelial progenitors from the periphery and bone marrow to ischemic tissues, and these progenitor cells have the capability to differentiate to cardiomyocytes, endothelial cells, and vascular smooth muscle cells, as well as secrete numerous angiogenic peptides to facilitate angiogenesis (Minamino et al, 2008).

One approach to bypass limitations associated with bolus administration drug delivery is the localized, sustained release of growth factors at the site of ischemia from a biodegradable natural polymer scaffold. For example, hydrogels and micro- or nano-particles have been utilized extensively and shown remarkable success in protecting peptides from enzymatic degradation (Gombotz and Pettit, 1995). A variety of natural and synthetic polymers have been used to form hydrogels in vivo (Richardson et al, 2001; Lee et al, 2010; Patel et al, 2008b; Schense et al, 2000). Of particular interest, fibrin has found a niche in wound healing and tissue regeneration as it is biocompatible, and has widespread use in tissue engineering applications (Schense et al, 2000; Hall, 2007; Rowe et al, 2007; Wong et al, 2003). Conversely, very few studies have utilized albumin as a carrier matrix for angiogenic peptides (Okoroukwu et al, 2010). Albumin's physiochemical properties can be modified to deliver bioactive agents at therapeutic rates for various end uses (Okoroukwu et al, 2010).

The release kinetics of growth factors from hydrogels or microspheres have been thoroughly investigated but the rate of release of growth factors from these systems in

combination has not yet clearly been defined in vivo. The sequence, timing, and concentration of growth factor from a delivery system needs to be defined in order to direct therapeutic angiogenesis (Cao and Mooney, 2007; Zhang and Suggs, 2007; Tessmar and Gopferich, 2007). Generally, hydrogels rapidly release proteins, and polymeric micro- and nano-particles are susceptible to macrophage digestion in vivo. We have previously demonstrated that G-CSF and FGF-2 can be co-delivered and is completely released from fibrin hydrogels in a period of five days (unpublished data). Furhtermore, we have demonstrated that the rate of release can be prolonged by incorporating growth factors in scaffolds that carry a charge opposite their ionic charge at neutral pH (Layman et al, 2009). Herein, we hypothesized that we could sequentially release FGF-2 and G-CSF by using a fibrin hydrogel embedded with anionic and cationic microspheres for the treatment of critical limb ischemia in a mouse model.

Herein, we report on the sequential delivery of FGF-2 and G-CSF from a fibrin scaffold containing ionic albumin microspheres in a murine critical limb ischemia model to promote neo-vessel formation and increased hindlimb perfusion. Sequential delivery was achieved by the incorporation of anionic and cationic nature of albumin microspheres in a fibrin matrix, and the release kinetics were determined as a function of the degree of swelling and crosslinking time. The activity of the releasing growth factors was assessed in HUVEC culture as well as in a murine critical limb ischemia model. Scaffolds were implanted and the in vivo efficacy was determined using Laser Doppler Perfusion Imaging to estimate hindlimb reperfusion, and the extent of angiogenesis was determined by the amount of capillaries and mature vessels present, using

immunohistochemical staining for CD-31 and α-smooth muscle actin (SMA), respectively.

3.2 Materials and Methods

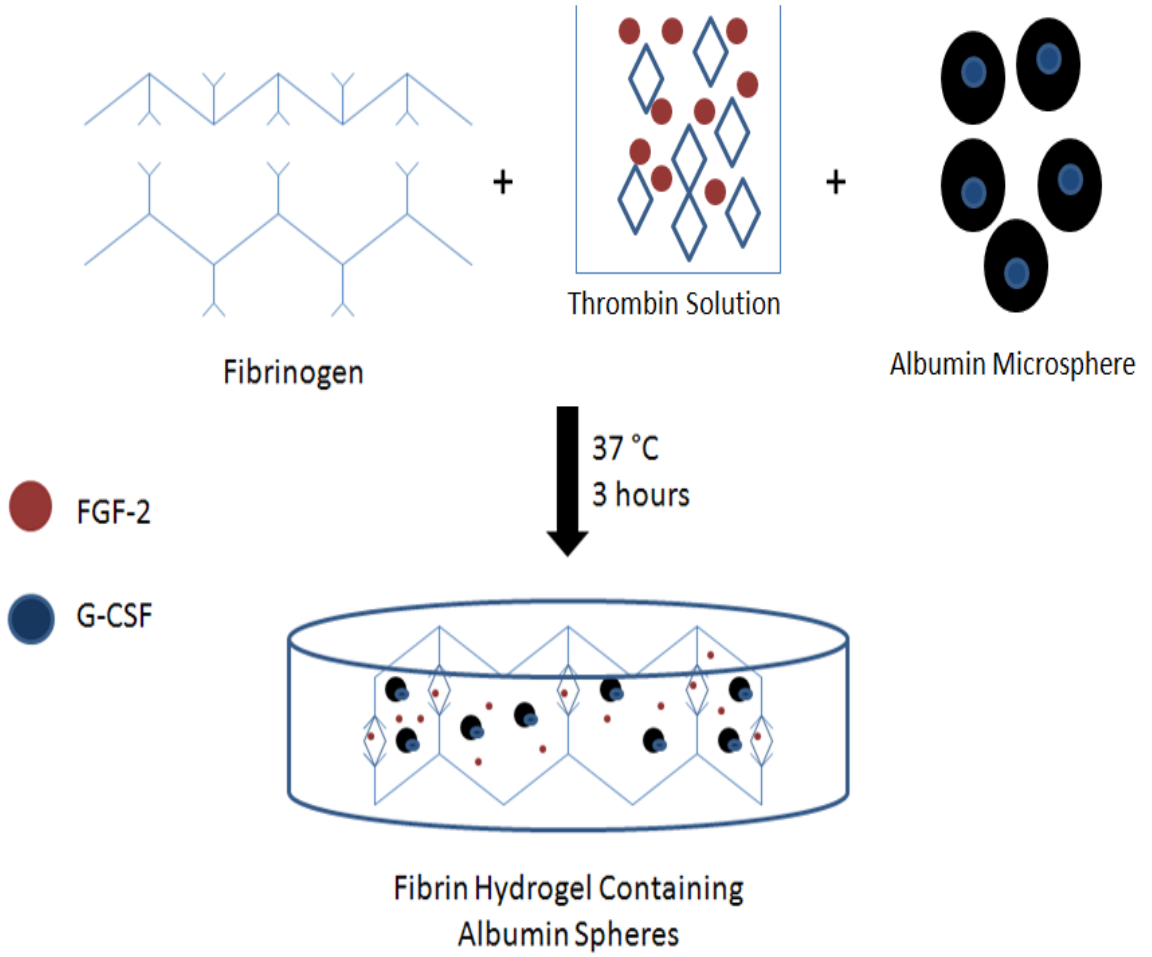
3.2.1 Materials

Fibrinogen (fraction I, type I; from human plasma), bovine serum albumin (BSA; purified fraction V), thrombin (from bovine plasma), poly-L-lysine hydrobromide (PLL; MW = 90000 – 140000 Da), poly-L-glutamic acid sodium salt (PLG; MW = 75000 – 120000 Da), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDC), and calcium chloride were obtained from Sigma Aldrich (St. Louis, MO) and were used as specified by the manufacturer. Human recombinant basic fibroblast growth factor (FGF-2; MW = 16000 Da; IEP = 9.6) and human recombinant granulocyte-colony stimulating factor (G-CSF; MW = 18800 Da; IEP = 6.2) were obtained from Peprotech (Rocky Hill, NJ). All other materials were obtained at their highest purity from Sigma Aldrich unless otherwise noted.

3.2.2 Scaffold preparation

Fibrin scaffolds were synthesized according to a previously established method with minor modifications (Ehrbar et al, 2008; Scheme 3.1). Briefly, fibrinogen (4 mg/ml) was dissolved in a 0.9% sodium chloride solution at 37 °C for 2 hours. Separately, a 10 mM solution of calcium chloride was combined with thrombin at various concentrations. At the time of crosslinking, ionic-albumin microspheres were dispersed throughout the fibrin scaffold and were synthesized in conjunction with previous methods (Patel et al, 2008a; Adhirajan et al, 2007). Precisely, 5 g of BSA and PLL or PLG at a 10:1 weight-

to-weight ratio was dissolved in 50 ml of ddH_2O and warmed to 40 °C. This solution was added dropwise to 50 ml of heated vegetable oil (60 °C) while stirring at 400 rpm for 10 minutes. The oil-in-water emulsion was then chilled to 4 °C for 30 minutes and continued stirring at 400 rpm. 15 ml of acetone was added to the emulsion to precipitate albumin spheres while being stirred and chilled at the previous conditions for 1 hr. Ionic albumin spheres were collected via centrifugation at 2500 rpm and rinsed in acetone three times to remove oil residues. The collected spheres were placed into a 100 mM solution of EDC and stirred at 400 rpm for 12 hr to facilitate crosslinking. The ionic-albumin microspheres were collected over a 0.2 μm filter and washed in acetone followed by sieving of the microspheres to obtain spheres less than 75 micron and then freeze-dried for 24 hours.



Scheme 3.1. Formation of a fibrin scaffold embedded with albumin microspheres for the sequential delivery of FGF-2 and G-CSF. A fibrinogen solution is combined with a thrombin solution with FGF-2 and allowed to crosslink for 3 hours with the addition of ionic albumin microspheres containing G-CSF.

10 µl of FGF-2 or G-CSF was absorbed into 10 mg of albumin microspheres for 12 hrs at 4 °C at final concentrations of 1 or 10 µg/ml. Alternatively, FGF-2 and G-CSF were directly added to the fibrinogen/thrombin solution prior to crosslinking for final concentrations of 100 or 1000 ng/ml. Microspheres were dispersed within the fibrinogen solution and these two solutions were combined in a 35 mm x 10 mm style polystyrene culture dish (Dow Corning; Corning, NY) at a 5:1 ratio (fibrinogen : thrombin/$CaCl_2$)

and crosslinked for various times at 37 °C. Scaffolds were removed using a punch biopsy with a total volume of 400 μl.

3.2.3 Scaffold properties

Following material synthesis, microspheres and gels were equilibrated in 40 ml of phosphate buffered saline (PBS; pH = 7.4; Gibco; Carlsbad, CA) and the crosslinking density of microspheres and fibrin gels was determined by the degree of swelling ratio. As described previously (Layman et al, 2007), the wet weight of material was determined following equilibration and the sample was allowed to freeze dry for 24 hours and the dry weight was measured in triplicate. Microspheres were further characterized by scanning electron microscopy (XL 30, Philips, Eindhoven, The Netherlands) to determine the range of diameters present. Briefly, microspheres were mounted on aluminum stubs using double-sided sticky tape, sputter-coated with gold for 60 seconds and viewed using SEM. At least 15 images were obtained per sample to measure the range of microsphere diameter.

3.2.4 Degradation of ionic albumin microspheres

The rate of degradation of albumin microspheres in vitro was determined by a previously established method with modification (Adhirajan et al, 2007). Briefly, 50 mg of crosslinked ionic albumin microspheres was immersed in 10 ml of PBS containing a physiological concentration, 373 ng/ml, of collagenase (Calbiochem, San Diego, CA). At predetermined time points over eight weeks, samples were centrifuged and the supernatant removed. The microspheres were freeze-dried overnight and the dry weight taken and compared to the initial mass.

3.2.5 Activity of releasing FGF-2 and G-CSF sequentially in endothelial cell culture

The activity of released FGF-2 and G-CSF was determined on the proliferation of human umbilical vein endothelial cells (HUVEC; ATCC; Manassas, VA). Cells (10,000 cells/well) were seeded in 24-well culture plates in endothelial cell basal media (EBM; Lonza, Walkersville, MD) supplemented with 2% fetal bovine serum. The following groups were evaluated: 1) growth factor delivery from fibrin scaffolds; 2) growth factor delivery from ionic albumin microspheres; 3) sequential delivery of GF from fibrin gels containing albumin microspheres; 4) sequential delivery of GF from fibrin gels containing ionic microspheres at a low (40 ng) and high (400 ng) concentrations of FGF-2 and G-CSF. Scaffolds were synthesized in transwell baskets with 8.0 μm pore size (Corning Incorporated; Corning, NY) and then placed in the wells containing HUVECs for 2 and 4 days without medium replacement. The activity of HUVECs was determined using a coulter particle counter (Beckman Coulter, Miami, FL) in triplicate. A representative table of all groups is shown below (Table 3.1).

Table 3.1. Treatment groups for sequential delivery studies.

Group	Treatment
A	Fibrin gel releasing FGF-2
B	Fibrin gel releasing G-CSF
C	Co-delivery of FGF-2 and G-CSF from fibrin gel
D	Albumin microsphere releasing FGF-2
E	Albumin microsphere releasing G-CSF
F	Cationic albumin-PLL microsphere releasing G-CSF
G	Anionic albumin-PLG microsphere releasing FGF-2
H	Fibrin gels releasing FGF-2 embedded with albumin microspheres releasing G-CSF
I	Fibrin gels releasing FGF-2 embedded with cationic albumin-PLL microspheres releasing G-CSF
J	Fibrin gels releasing G-CSF embedded with albumin microspheres releasing FGF-2
K	Fibrin gels releasing G-CSF embedded with cationic albumin-PLL microspheres releasing FGF-2

3.2.6 Sequential release kinetics of FGF-2 and G-CSF

The controlled, sequential delivery of FGF-2 and G-CSF were determined as previously reported (Layman et al, 2007). Briefly, FGF-2 and G-CSF were reconstituted according to manufacturer's specifications at a concentration of 0.1 mg/ml. Plastic cylinders (h = 4.0 mm, D = 6.5 mm, V = 135.0 mm^3) were sealed to the bottom of a 6-well culture plate using silicon glue (Dow Corning). Next, scaffolds were synthesized in the plastic cylinders combining 250 μl fibrinogen with 50 μl thrombin/$CaCl_2$ with 10 mg of ionic microspheres containing 100 ng of FGF-2 and/or G-CSF. Release experiments were conducted on a MaxQ4000 orbital shaker (Barnstead International; Dubuque, IA) at 100 rpm and 37 °C. The samples were immersed in 10 ml of PBS and samples were collected at specific time points over 7 days and stored at -20 °C prior to quantification in triplicate experiments. The amounts of G-CSF (R&D Systems; Minneapolis, MN) and FGF-2 (EMD Biosciences; San Diego, CA) released were determined using an enzyme-

linked immunosorbent assay. The optical density was determined at 450 nm with wavelength correction to 570 nm using a microplate reader (BioRad; Philadelphia, PA) for FGF-2 and G-CSF.

In addition to release in phosphate buffered saline, the release kinetics associated with fibrin gels and ionic albumin microspheres was determined in the presence of collagenase to determine if there is an enzymatic release effect associated with growth factor release. The release of FGF-2 and G-CSF was performed in the identical manner as previously described with the addition of 373 ng/ml of collagenase added to PBS solution (pH = 7.4). The medium was replaced with fresh buffer daily containing collagenase for 5 days for fibrin gels and 7 days for albumin microspheres.

3.2.7 Mathematical Modeling of Release Kinetics

The mathematical model describing the growth factors release profile as a result of one-dimensional diffusion, and release from the scaffold can be described as a power function of time (1):

$$M/M_{\infty} = kt^{n} \qquad \textbf{(1; adapted from Lee, 1985)}$$

which is derived from a second-order partial differential equation, where k is a constant characteristic of the system and n is an exponent characteristic of the mode of transport.

For n = 0.5, the solvent diffusion or drug release follows Fickian diffusion mechanisms. For n > 0.5, non-Fickian diffusion behavior is observed. The mathematical parameters can be determined from comparing the amount of growth factor released ($\boldsymbol{M}$) at time t and compared to the amount of growth factor released at infinity (maximal loading). These parameters were calculated from release experiments without collagenase releasing FGF-2 and G-CSF from co- or sequential delivery scaffolds with n

and k calculated for each group (N = 3 per group). Linear-regression analysis was performed using Microsoft Excel 2007 to determine these parameters for the release of factors.

3.2.8 Critical Limb Ischemia Model

Balb/c mice (Charles River Laboratories; Boston, MA), aged 6–7 weeks, were cared for and operated on under IACUC guidelines at the Miller School of Medicine at the University of Miami. Prior to surgery, the animals were anesthetized using a ketamine/xylazine/acepromazine cocktail at 40, 8 and 10 mg/kg body weight, respectively. The surgery was performed according to standard methods (Limbourg et al, 2009). Briefly, an incision was made from the patella to the lower abdomen, exposing the femoral and external iliac arteries. A 7-0 grade silk suture was used to ligate the lower superficial femoral artery (above the saphenous branches), and at the junction of the external iliac, deep femoral and profunda femoris. The artery was then excised and removed. Following surgery, mice were randomized for treatment (N = 5 per time point per group) in three groups. The first group received treatment with co-delivery of FGF-2 and/or G-CSF from fibrin scaffolds; second, FGF-2 or G-CSF was released from ionic albumin microspheres; lastly, sequential delivery of FGF-2 and G-CSF from fibrin scaffolds containing ionic albumin microspheres. All treatments were loaded with 40 or 400 ng of growth factor contained in a sequential delivery scaffold of 400 μl in total volume removed by punch biopsy from the polystyrene dish and applied atop the region of excision.

3.2.9 Monitoring of Hindlimb Bloodflow

Hindlimb perfusion was assessed using Laser Doppler Perfusion Imaging (LDPI; Perimed Medical Systems; New York, NY), and data acquisition was obtained as previously reported (Layman et al, 2007). Briefly, color photographs were obtained and hindlimb perfusion was determined by comparing the intensity of a murine hindlimb prior to surgery to a specific time point: post-surgery, and at 1, 2, 3, 4 and 8 weeks. An ischemic Doppler ratio was established for all groups and compared.

3.2.10 Immunohistochemistry

The mice were sacrificed at 2, 4, and 8 weeks post-surgery with an overdose of halothane inhalation. Quadriceps muscle tissue was removed and immediately placed in 10% formalin in PBS overnight. After fixation, tissue was embedded in paraffin and 5 μm tissue sections were cut and mounted on positively charged slides. The tissue's antigenicity was recovered using a warmed proteinase K solution for CD31+ staining, and 10 mM citrate buffer (pH 6.0) for α-actin staining. Samples were then treated with diluted primary antibody (1:25), goat PECAM-1 monoclonal antibody overnight (Vector Labs; Burlingame, CA) or α-actin (0.N.5) diluted at 1:100 (Vector Labs; Burlingame, CA) for 2 h. Samples were then rinsed, and incubated in secondary biotinylated antibody (1:200), rabbit anti-goat polyclonal antibody (Vector Labs). Samples were then treated with ABC (Vector Labs; Burlingame, CA) and DAB chromogen reagent (DAKO; San Diego, CA) for CD31+ stained cells. For SMA+ stained cells, HRP substrate was added for 10 minutes until a light brown stain was visible. Slides were then viewed using light microscopy at 20× using a LEICA Microsystems AF6000LX brightfield/fluorescence microscope (Bannockburn, IL), and images were captured using MetaMorph 5.1

Software. Positively stained CD31 capillaries were manually counted in minimum five separate fields and expressed as capillaries per millimeter squared. The number of vessels associated with α-SMA positively stained cells was also quantified in a similar fashion.

3.3 Results

3.3.1 Scaffold Properties

In the present study, fibrin hydrogels were synthesized by the addition of soluble fibrinogen to thrombin to form a crosslinked fibrin mesh (Figure 3.1). The crosslinking density, measured by the degree of swelling, was evaluated at three thrombin concentrations with the addition of calcium chloride (10 mM) at various time lengths of crosslinking (Figure 3.1A). It is shown that the amount of thrombin in relation to a fixed amount of fibrinogen (4 mg/ml) has a significant effect ($p < 0.01$) on the extent on the degree of swelling at all crosslinking time points. For example, after three hours of crosslinking with thrombin at 0.02 units/mg fibrinogen, fibrin gels absorbed 143.8 ± 5.8 times their weight in water, whereas using the middle concentration (0.2 units/mg fibrinogen) and crosslinking for 3 hours, the gels absorbed 55.2 ± 7.6 times their weight, and at the high concentration (2 units/mg fibrinogen) the degree of swelling observed was 18.5 ± 1.7, significantly less ($p < 0.05$) than all other groups. Calcium chloride also plays a critical role in fibrin clot formation and the degree of swelling in the presence or absence of $CaCl_2$ was determined at various fibrinogen:thrombin (F:T; weight-to-weight) ratios (Figure 3.1B). When $CaCl_2$ is present in the crosslinking reaction, no significant effect in the degree of swelling is observed with a degree of swelling of 19.6 ± 3.1 at a 5:1 F:T ratio compared to a degree of swelling of 21.4 ± 2.1 at a 20:1 F:T ratio. Without

the presence of $CaCl_2$ there is a significant decrease in the degree of welling between 20:1 and 5:1 F:T ratio groups ($p < 0.01$). At a 20:1 ratio the degree of swelling is 49.1 ± 8.9, whereas the degree of swelling at 5:1 ratio is 28.2 ± 3.8. Based on the assessment of degree of swelling, the fibrin gels crosslinked for 3 hours, at a ratio of 5:1 (fibrinogen:thrombin), and 2 U T/mg F were determined to have the best hydrogel properties (mechanical strength, swellability, crosslinking time) for use in our following studies.

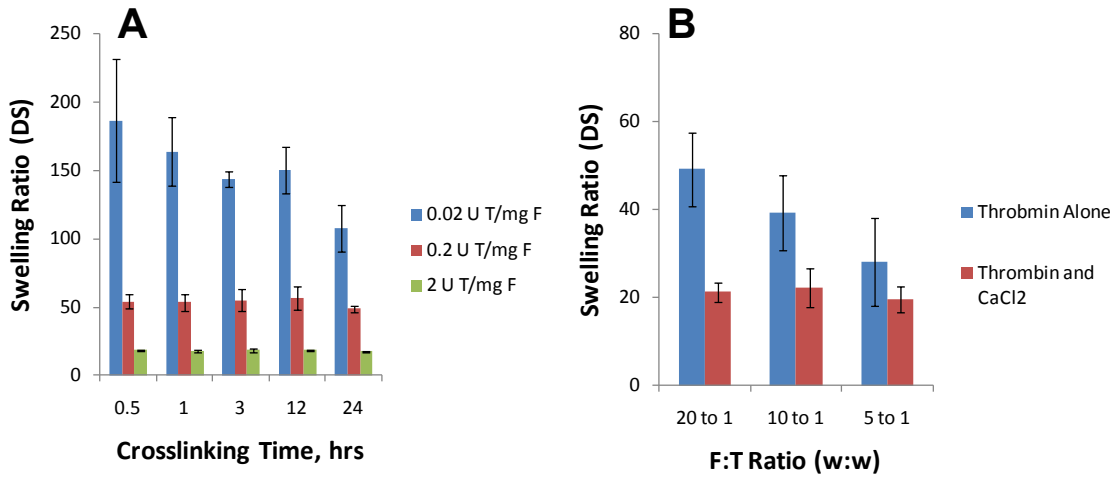


Figure 3.1 Extent of fibrin crosslinking density as measured by the degree of swelling. The degree of swelling of fibrin hydrogels was measured as a function of crosslinking time and thrombin concentration (Fig 3.1A) where the concentration of fibrinogen was maintained at 4 mg/ml. The degree of swelling was measured as the hydrogels wet weight in comparison to its dry weight. Alternatively, the crosslinking density was measured as a function of fibrinogen to thrombin present and in the presence of calcium chloride at 10 mM after crosslinking for 3 hours (Fig 3.1 B).

Additionally, ionic albumin microspheres were characterized by degree of swelling measurements and the range of diameters present. The degree of swelling was significantly different between all groups ($p < 0.05$) where the degree of swelling was 1.3 ± 0.1, 3.5 ± 0.4, and 2.2 ± 0.2 for albumin, albumin-PLL, and albumin-PLG microspheres respectively. The percent distribution of spheres at a range of diameters is shown in

Figure 3.2A and demonstrates that ionic spheres have larger diameters (35-45 micron), than albumin spheres (25-35 micron). 47% of albumin-PLG and 42.9% of albumin-PLL spheres were present at diameter 35-45 micron, and 30.6% of albumin spheres were present at a diameter between 25-35 micron as shown by SEM (Figure 3.2 C-E).

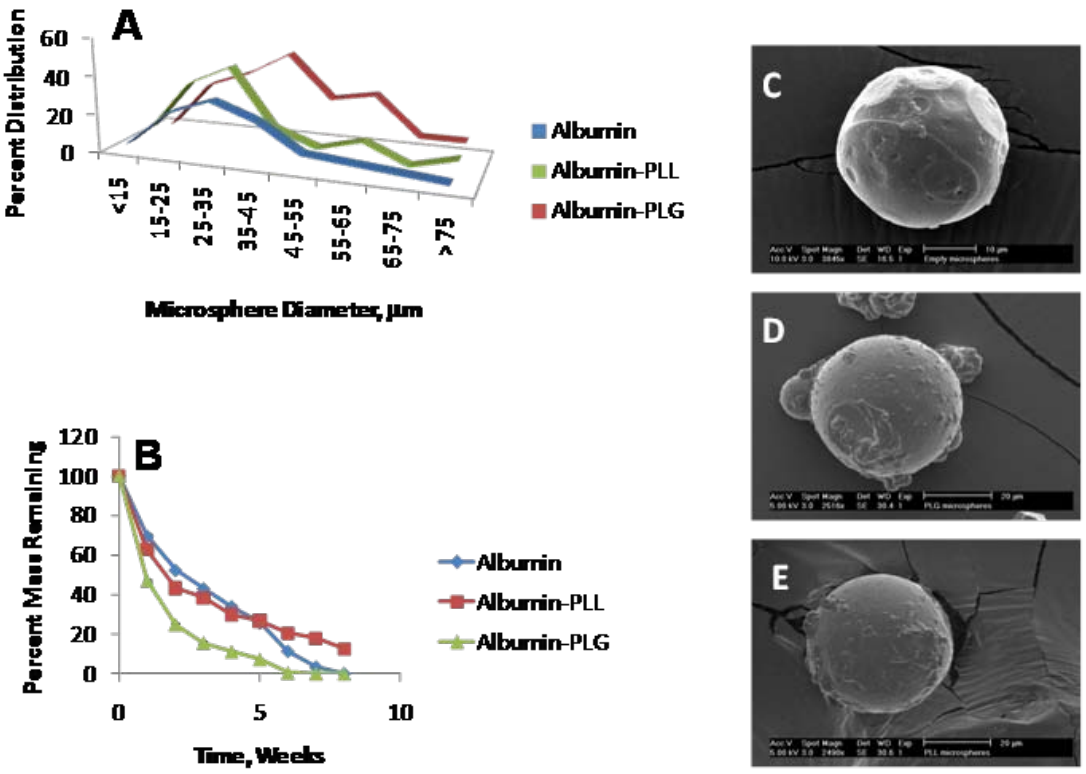


Figure 3.2. Characterization of ionic-albumin microspheres. The average microparticle size of normal, cationic, and anionic microspheres was determined after sieving particles with diameter greater than 70 µm by scanning electron microscopy (Fig 3.2 A). The degradation of the microspheres was performed in a physiological concentration of collagenase (373 ng/ml) over eight weeks and the dry weight of the microspheres was determined at each time point (Fig 3.2 B). Representative SEM images of albumin (3.2C), albumin-PLL (3.2D), and albumin-PLG (3.2E) microspheres are shown.

3.3.2 In vitro degradation of ionic-albumin microspheres

The degradation rate of ionic albumin microspheres was employed based on enzymatic degradation and was determined in a physiological based (373 ng/ml)

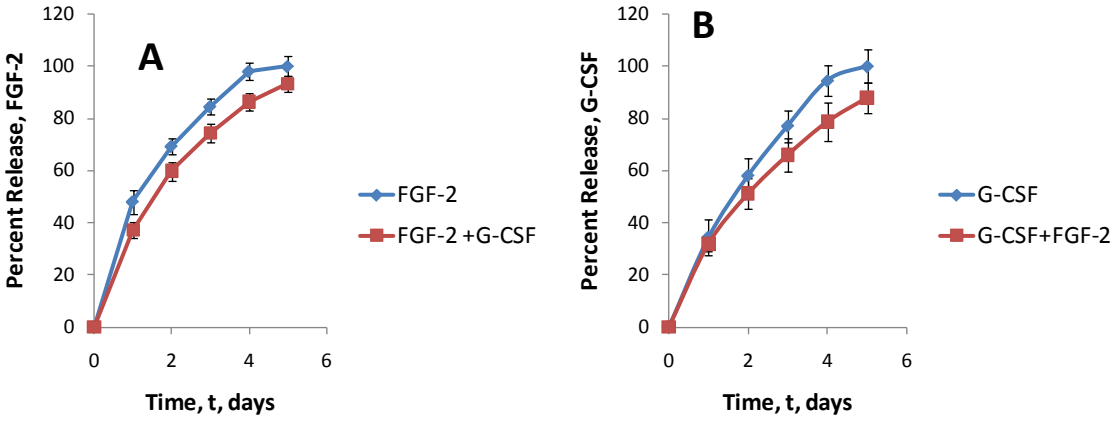
concentration (Patel et al, 2008b) of collagenase to assess if there were any differences in rates of degradation between ionic and non-ionic albumin microspheres. After one week, the rate of anionic sphere degradation was significantly greater ($p < 0.01$) than cationic spheres (Figure 3.2B) where 53.1 ± 2.0% of albumin-PLG spheres degraded after one week in comparison to 37.2 ± 1.4% albumin-PLL spheres, and 30.5 ± 3.3% albumin spheres at the one week time point. Within six weeks all of the anionic microspheres had been degraded, and after eight weeks all of the non-ionic spheres had degraded. At eigth weeks 12 % of the cationic spheres were still present in collagenase solution.

3.3.3 Sequential release of FGF-2 and G-CSF in vitro

In the present study, the effect of sequentially delivering FGF-2 and G-CSF from fibrin matricies and ionic-albumin microspheres was determined in a temperature controlled environment. Fibrin matricies and albumin spheres were swollen within the cylinder and released to the surrounding medium one-dimensionally. Within 7 days, 100% of FGF-2 was released from albumin and albumin-PLL spheres whereas 70.8 ± 2.7% of the growth factor was released over the same time span from albumin-PLG spheres (Figure 3.3A). Alternatively, 92.9 ± 1.7%, 82.3 ± 2.2%, and 45.4 ± 3.6% of G-CSF was released over seven days from albumin-PLG, albumin, and albumin-PLL microspheres, respectively (Figure 3.3B).

The sequential delivery of FGF-2 and G-CSF from ionic albumin microspheres dispersed in fibrin hydrogels was quantified in a similar fashion (Figure 3.3 C-F). The release of growth factor from ionic albumin spheres distributed within the fibrin hydrogel exhibit decelerated release kinetics compared to non-ionic albumin spheres. When releasing GF from fibrin hydrogels, there is no significant difference between releasing

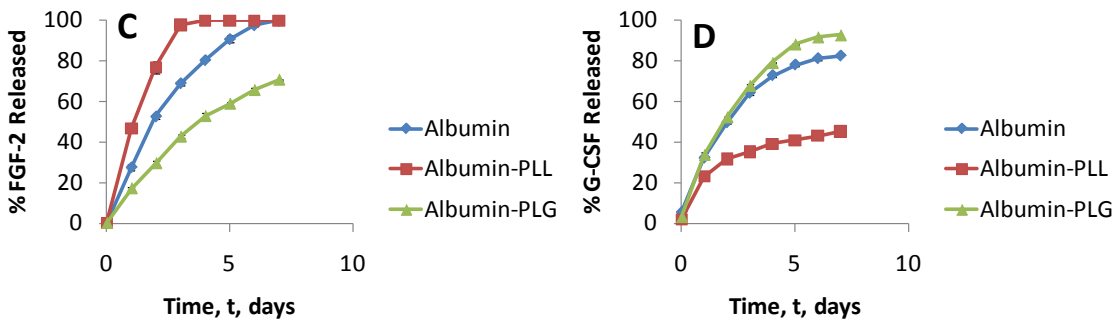
one growth factor versus co-delivery (Figure 3.3A&B). Release of FGF-2, G-CSF, or co-delivery from fibrin hydrogels results in at least 90% of all growth factor being released in five days.



Experimental Group	Initial Growth Factor Loading Location	Growth Factor	k	n	R^2
A	Fibrin	FGF-2	0.3282	0.2606	0.9691
B	Fibrin	G-CSF	0.3694	0.3232	0.9711
C	Fibrin	FGF-2	0.3825	0.2373	0.9862
		G-CSF	0.4192	0.1087	0.9973

Figure 3.3 A&B. Release of 100 ng FGF-2, G-CSF, or both from fibrin hydrogels over five days. Release kinetics were determined based on Equation 3.1 to determine release coefficient *k*, and transport coefficient *n*.

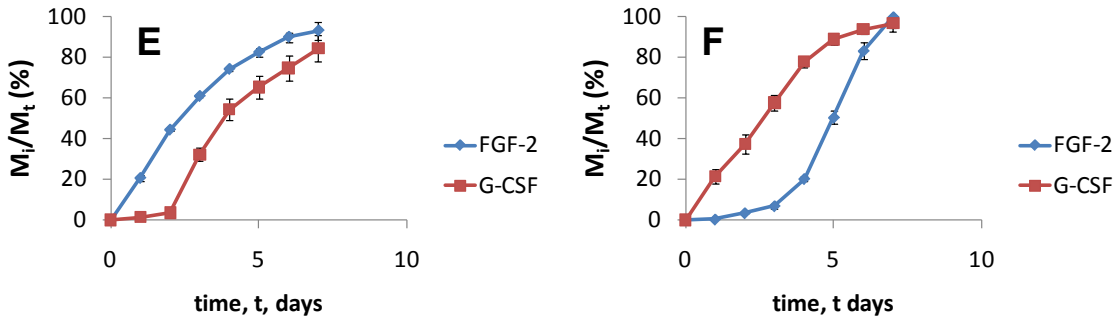
When G-CSF is released from albumin spheres or albumin-PLL spheres (Fig. 3.3D) the amount of growth factor released over seven days is significantly greater when not ionically complexed ($p < 0.01$) where 82 ± 3.2% was released, whereas 45.4 ± 2.4% of G-CSF was released from albumin-PLL spheres. The release of FGF-2 from albumin microspheres and albumin-PLG microspheres (Fig. 3.3C) also demonstrated decelerated release kinetics as shown with release of G-CSF. For example, albumin microspheres released 100 ± 2.7% whereas albumin-PLG microspheres released 70.8 ± 1.4% of loaded FGF-2 after seven days.



Experimental Group	Initial Growth Factor Loading Location	Growth Factor	k	n	R^2
D	Albumin	FGF-2	0.5949	0.085	0.9965
---	Albumin-PLL	FGF-2	0.5459	0.1712	0.9307
E	Albumin-PLG	FGF-2	0.7257	0.4483	0.9731
F	Albumin	G-CSF	0.4052	0.1309	0.9865
G	Albumin-PLL	G-CSF	0.2871	0.0714	0.9897
---	Albumin-PLG	G-CSF	0.5122	0.1091	0.9778

Figure 3.3 C&D. Release of 100 ng FGF-2, G-CSF, or both non-ionic and ionic albumin microspheres. Release kinetics were determined based on Equation 3.1 to determine release coefficient *k*, and transport coefficient *n*.

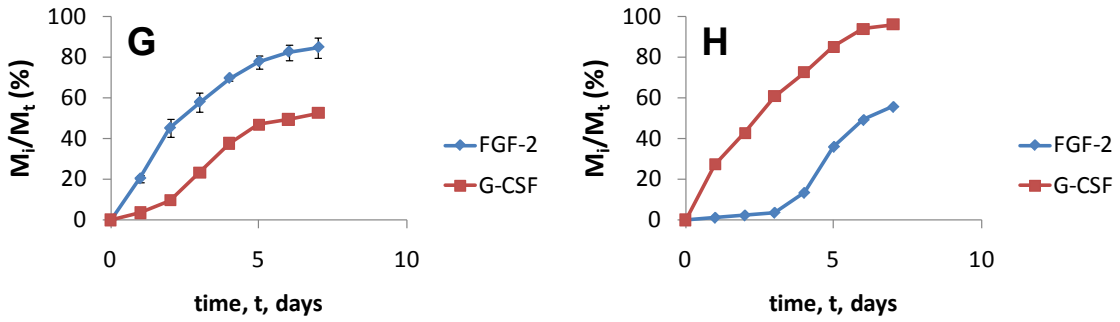
The release of FGF-2 and G-CSF was determined in our sequential delivery scaffold using non-ionic albumin microspheres embedded within the fibrin gel (Figure 3.3 E&F). The scaffold demonstrates that the growth factor complexed with the microspheres can be delayed up to two days where 3.4% of the FGF-2 was released to the surrounding medium after 2 days (Fig. 3.3F) and 3.5% of the G-CSF (Fig 3.3E) had been released over the same time period. After seven days, there is no difference in the amount of growth factor released whether released from the spheres or the fibrin gel.



Experimental Group	Initial Growth Factor Loading Location	Growth Factor	k	n	R^2
H	Fibrin	FGF-2	0.6424	0.0499	0.9675
	Albumin	G-CSF	0.7643	0.0143	0.7993
I	Fibrin	G-CSF	0.8245	0.023	0.9157
	Albumin	FGF-2	0.746	0.409	0.5857

Figure 3.3 E&F. Release of 100 ng FGF-2, G-CSF, or both from a fibrin scaffold containing one growth factor and the non-ionic albumin spheres containing the other. Release kinetics were determined based on Equation 3.1 to determine release coefficient *k*, and transport coefficient *n*.

Furthermore, the release kinetics of FGF-2 and G-CSF were determined from a fibrin scaffold containing ionic albumin microspheres (Figure 3.3 G&H). This demonstrates that the release of growth factor from the spheres within the hydrogel are still delayed (at least two days) and the release is sustained further. After seven days the amount of FGF-2 released from albumin-PLG microspheres dispersed in the fibrin scaffold was 55.7 ± 2.7%, and the amount of G-CSF released from albumin-PLL spheres dispersed within the fibrin hydrogel was 52.7 ± 3.9% over the same time period.

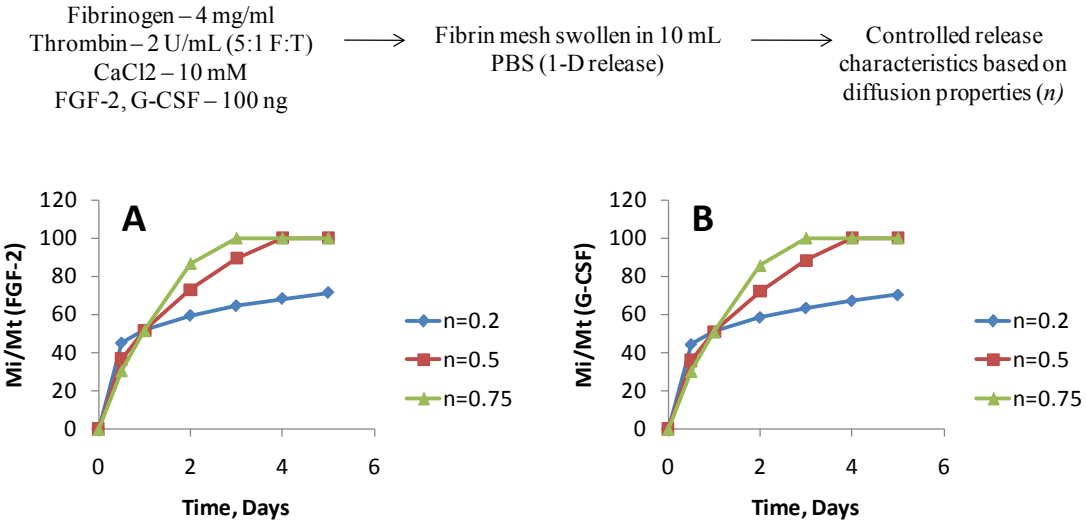


Experimental Group	Initial Growth Factor Loading Location	Growth Factor	k	n	R^2
J	Fibrin	FGF-2	0.6204	0.0504	0.9903
	Albumin-PLL	G-CSF	0.4186	0.1937	0.89
K	Fibrin	G-CSF	0.6573	0.0517	0.9689
	Albumin-PLG	FGF-2	0.7974	0.4451	0.6368

Figure 3.3 G&H. Release of 100 ng FGF-2, G-CSF, or both from a fibrin scaffold containing one growth factor and ionic albumin spheres containing the other. Release kinetics were determined based on Equation 3.1 to determine release coefficient *k*, and transport coefficient *n*.

Variables that can affect the mode of transport (*n*) include, but are not limited to, crosslinking density of the hydrogel, crosslinking density of the microspheres, interaction between carrier and growth factor, degradation characteristics, and the nature of interaction of hydrogel with microspheres. These parameters can be assessed by varying the mode of transport variable from Equation 3.1. By altering this variable, the concentration released from the hydrogels or microspheres is modified and shown in Figure 3.4 and 3.5. The input to the system is the material and its specific characteristics, the output, which is the material synthesized with growth factor embedded in the material, and the calculation is the release kinetics based on changing *n* and determining the initial burst by adjusting concentration. Assuming Fickian release ($n = 0.5$), only 36% of the growth factor (FGF-2 or G-CSF) will be released within the first 12 hours at our given conditions. The amount of growth factor released over this period can be

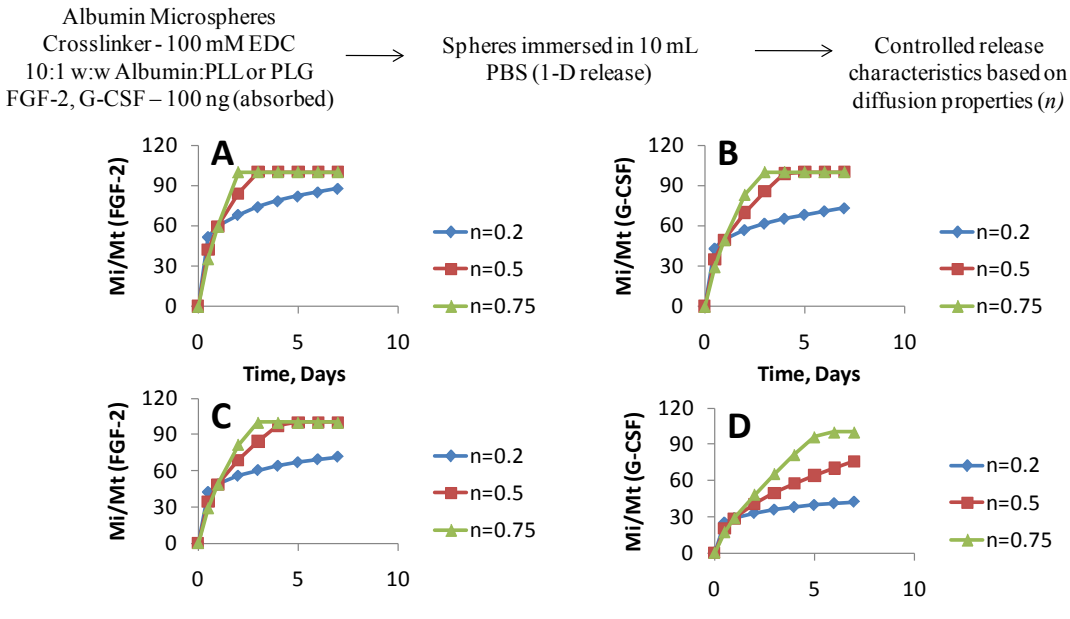
modfied based on adjustment of crosslinking concentrations, crosslinking time, or fibrinogen concentration.



Initial Rate of Release (12 hrs)			
	n=0.2	n=0.5	n=0.75
FGF-2 (A)	44.9	36.5	30.7
G-CSF (B)	44.3	36	30.3

Figure 3.4. Modeling of release kinetics of FGF-2 (A) and G-CSF (B) from fibrin hydrogels by variation of the mode of transport (*n*). The rate constant (*k*) was held constant for fibrin gels and the release of FGF-2 or G-CSF as determined previously. The amount of growth factor released was then determined by Equation 3.1 assuming constant *k* and varying *n*.

The release of growth factor from microspheres depends on the ionic charge associated with the sphere (cations/anions) and the amount of growth factor released can change depending on the amount of growth factor loaded, significantly ($p < 0.05$). Figure 3.5 demonstrates the initial burst of growth factor released from non-ionic and ionic microspheres. Again, assuming ideal Fickian conditions with *n* equal to 0.5, the amount of growth factor released when ionically complexed is significantly reduced ($p < 0.05$) compared to non-ionic conditions in the first 12 hours.



Initial Rate of Release (12 hrs)

	n=0.2	n=0.5	n=0.75
Albumin:FGF-2 (A)	51.7	42	35.3
Albumin:G-CSF (B)	43	34.9	29.4
Albumin-PLG:FGF-2 (C)	42.3	34.4	28.9
Albumin-PLL:G-CSF (D)	25	20.3	17.1

Figure 3.5. Modeling of release kinetics of FGF release from albumin (A) and albumin-PLG microspheres (C), and G-CSF release from albumin (B) and albumin-PLL microspheres (D) at different concentrations of growth factor loading and initial burst of growth factor released in 12 hours. The rate constant (k) was held constant for fibrin gels and the release of FGF-2 or G-CSF as determined previously. The amount of growth factor released was then determined by Equation 3.1 assuming constant k and varying n.

Next, the release kinetics of fibrin hydrogels and albumin microspheres was determined in the presence of a physiological concentration of collagenase in vitro (373 ng/ml). Figure 3.6 demonstrates the effect that collagenase has toward the release of FGF-2 and G-CSF from fibrin gels and albumin microspheres. Specifically, there is no significant effect in the amount of FGF-2 or G-CSF released from fibrin hydrogels when delivered on its own or in a co-delivery fashion. For example, when releasing FGF-2 alone 100 ± 1.5% of the growth factor is released over five days, whereas 89.9 ± 3.1% is released over five days when co-delivered (Fig 3.6A). This demonstrates that the release

of growth factor from fibrin hydrogels is not significantly affected by the presence of collagenase therefore the release depends on diffusion and not enzymatic degradation. Additionally, the release of growth factors from albumin microspheres is diffusion limited and not dependent on enzymatic degradation, as the microspheres take eight weeks to degrade. For example, albumin spheres degrade over eight weeks, whereas a 100% of FGF-2 is released within five days.

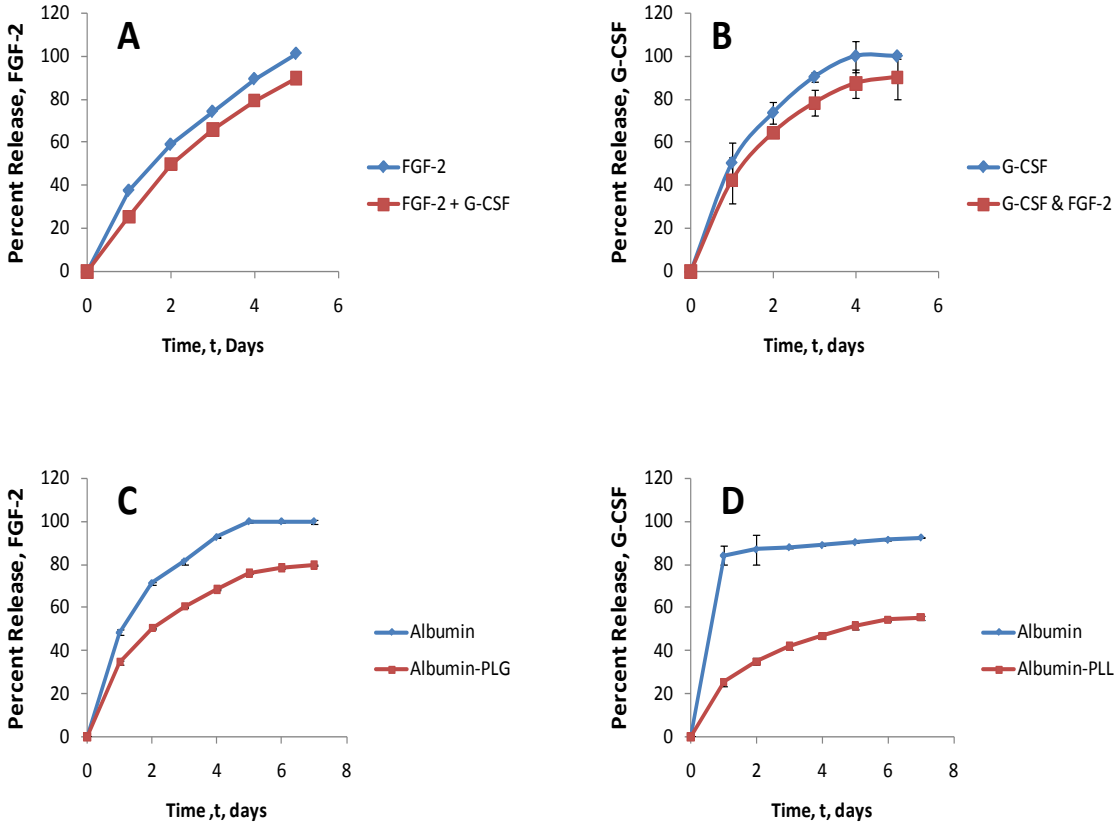


Figure 3.6. Release kinetics of FGF-2 and G-CSF from fibrin hydrogels (A&B) as well as ionic-albumin microspheres (C&D) in the presence of a physiological concentration of collagenase (373 ng/ml). Fibrin hydrogels were loaded with 100 ng/ml of FGF-2 (A), G-CSF (B), or both and released into medium containing collagenase. Albumin microspheres were loaded with 100 ng of FGF-2 (C) or G-CSF (D) and released into medium containing collagenase. The amount of growth factor released is expressed as total mass percent released over time.

3.3.4 Activity of released FGF-2 and G-CSF on Endothelial Cell Culture

The activity of released FGF-2 and G-CSF was assessed on cell proliferation. Tables 3.2-3.5 demonstrate the effects of delivery strategies and growth factor concentration on endothelial cell growth. G-CSF had a minimal effect on cellular growth over two days of incubation regardless of the mode of delivery supporting previous findings that G-CSF has no proliferative effect on endothelial cells (Bussolino et al, 1991). However, at high concentration (400 ng), G-CSF had a noticeable proliferative effect on HUVEC cells after 2 days compared to control ($p < 0.05$). After four days, no observable difference is seen in endothelial cell culture activity between control and vehicles releasing G-CSF (Table 3.3). Conversely, release of FGF-2 induces cellular proliferation of endothelial cells. The release of active FGF-2 after two days induces a 74.5 ± 10.8% increase in endothelial cell populations from albumin microspheres when loaded with high dose of FGF-2 and after four days the increase in endothelial cell population was 84.9 ± 8.6% which was significantly greater than any other treatment group ($p < 0.01$).

Co-delivery of FGF-2 and G-CSF resulted in significant endothelial cell growth compared to controls at both concentrations and time points (Tables 3.2, 3.3; $p < 0.05$). Alternatively, sequential delivery of growth factors from fibrin matricies and ionic albumin microspheres (Tables 3.4, 3.5) had no significant changes in cellular proliferation between treatment groups. Increasing concentration of growth factor delivered to endothelial cells results in a significant increase ($p < 0.05$) in cell proliferation when releasing FGF-2 from fibrin gels or from non-ionic microspheres. Specifically, in the sequential delivery system releasing FGF-2 from fibrin and G-CSF

from microspheres there was a 40% increase in viable cells, and using a fibrin scaffold releasing G-CSF with microspheres containing FGF-2 there was a 36.7% increase in viable cells compared to the lower concentration at day 2 (Table 3.4). However, after four days, only a 22.7% increase ($p < 0.05$) in the amount of viable endothelial cells at 400 ng compared to 40 ng when fibrin releasing G-CSF with albumin microspheres containing FGF-2 was administered. All other groups did not have a significant effect on endothelial cell proliferation with respect to concentration or the method of release.

Table 3.2. Proliferation of HUVEC at low concentration of growth factor loading (40 ng) using fibrin gels or albumin microspheres. * denotes significant increase compared to control at day 2 ($p < 0.05$), ** denotes significant increase compared to control at day 4 ($p < 0.05$). Control was determined as normal HUVEC growth in 24-well plates over two and four days in medium supplemented with 2% FBS.

Low Concentration GF		
	Relative Proliferation ±SD	
Group	Day 2	Day 4
Fibrin	99.1 ± 3.4%	101.6 ± 4.4%
Spheres	96.5 ± 5.4%	97.8 ± 3.9%
A	135.4 ± 9.2%, *	142.3 ± 6.6%, **
B	110 ± 9.9%	99.2 ± 5.5%
C	136 ± 12.4%, *	139.6 ± 9.7%, **
D	171.9 ± 5.7%, *	158.7 ± 3.6%, **
E	120 ± 10.2%, *	109.4 ± 4.9%
F	108.8 ± 9.9%	106.7 ± 3.8%
G	128.2 ± 4.3%, *	121 ± 7.1%, **

Table 3.3. Proliferation of HUVEC at high concentration of growth factor loading (400 ng) using fibrin gels or albumin microspheres. * denotes significant increase compared to control at day 2 ($p < 0.05$), ** denotes significant increase compared to control at day 4 ($p < 0.05$), *** denotes significant increase compared to low concentration. Control was determined as normal HUVEC growth in 24-well plates over two and four days in medium supplemented with 2% FBS.

High Concentration GF		
	Relative Proliferation ±SD	
Group	Day 2	Day 4
Fibrin	99.1 ± 3.4%	101.6 ± 4.4%
Spheres	96.5 ± 5.4%	97.8 ± 3.9%
A	162.6 ± 10.4%, *, ***	160.5 ± 6.5%, **, ***
B	116.5 ± 2.1%	106.3 ± 4.3%
C	177.5 ± 16.6%, *, ***	160.6 ± 8.7%, ***
D	174.5 ± 11.7%	185 ± 8%, ***
E	129.4 ± 7.2%, *	109 ± 4.2%
F	134.8 ± 8.4%, *, ***	108.4 ± 5.1%
G	161 ± 6.4%, *, ***	128 ± 6.6%

Table 3.4. Sequential delivery of FGF-2 and G-CSF on endothelial cell culture at low concentration of growth factor loading (40 ng). * denotes significant increase compared to control at day 2 ($p < 0.05$), ** denotes significant increase from day 2 to day 4 ($p < 0.05$). Control was determined as normal HUVEC growth in 24-well plates over two and four days in medium supplemented with 2% FBS.

Low Concentration GF		
	Relative Proliferation ±SD	
Group	Day 2	Day 4
H	139.7 ± 8.7%, *	132.7 ± 6.1%
I	129.8 ± 2.5%, *	129.9 ± 3.6%
J	132 ± 4.2%, *	127.6 ± 1.2%
K	130.4 ± 6.5%, *	158.8 ± 7.7%, **

Table 3.5. Sequential delivery of FGF-2 and G-CSF on endothelial cell culture at high concentration of growth factor loading (400 ng). * denotes statistical increase compared to control at day 2 ($p < 0.05$), ** denotes statistical increase from day 2 to day 4 ($p < 0.05$), *** denotes significant increase in cell proliferation dependent on concentration ($p < 0.05$). Control was determined as normal HUVEC growth in 24-well plates over two and four days in medium supplemented with 2% FBS.

High Concentration GF		
	Relative Proliferation ±SD	
Group	Day 2	Day 4
H	149.8 ± 7.2%, *	139.8 ± 8.2%
I	132 ± 4.6%, *	124.8 ± 2.4%
J	144.7 ± 5.5%, *, ***	150 ± 6.6%, ***
K	170.3 ± 6.2%, *, ***	168.8 ± 4.7%

The mode of delivery of FGF-2 and G-CSF had no significant effect on endothelial cell proliferation over two and four days of incubation. However, proliferative activity is dependent on the presence of FGF-2 and the concentration of growth factor administered. G-CSF had minimum effect on endothelial cell activity and only at high concentration (400 ng) was any effect observed.

3.3.5 Hindlimb blood flow restoration

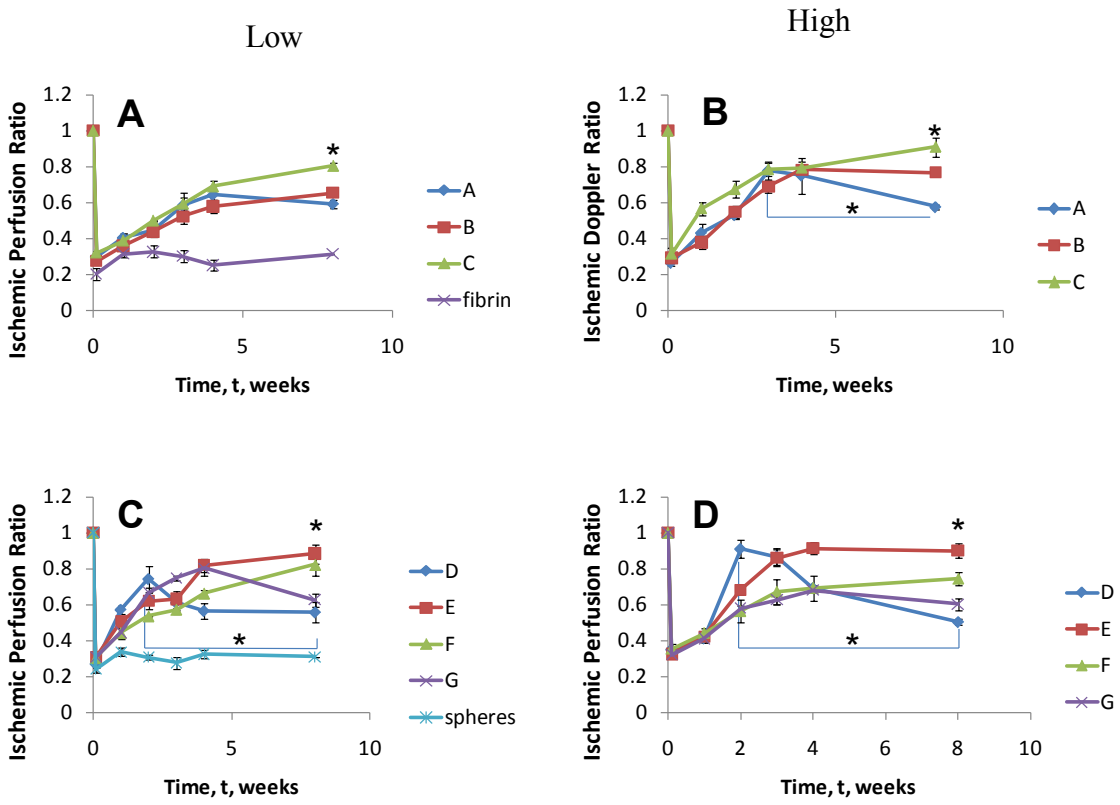
The effects of co- and sequential delivery of FGF-2 and G-CSF were evaluated in vivo with laser Doppler perfusion imaging. Following induction of critical limb ischemia, fibrin hydrogels with or without albumin microspheres were administered with FGF-2 and G-CSF at concentrations of 40 or 400 ng per formulation and followed to eight weeks. An ischemic Doppler ratio was estimated based on pre-surgery levels to each time point with treatment administered. Release of G-CSF from fibrin hydrogels (Fig. 3.7A&B; group B) or albumin microspheres (Fig. 3.7 C&D; group E) had a significant reperfusion effect ($p < 0.05$) in the amount of blood flow restored after eight

weeks at both concentrations. Specifically, after eight weeks, delivery of 400 ng G-CSF from fibrin hydrogels recovered 76.7 ± 2.9% of the original blood flow, and albumin microspheres recovered 90 ± 1.8% of the original blood flow. The recovery of hindlimb blood flow using G-CSF was significantly greater ($p < 0.05$) than treatments utilizing ionically-complexed G-CSF or any FGF-2 treatment at eight weeks at the low or high concentrations due to the amount of growth factor released to the ischemic tissue from the albumin microsphere. Release of 400 ng FGF-2 from albumin microspheres (Fig. 3.7D; group D) resulted in the greatest extent of reperfusion after two weeks where 90.9 ± 5.0% of hindlimb blood flow recovery was observed and afterwards resulted in a significant decrease ($p < 0.01$) to eight weeks with only 50.7 ± 1.8% of blood flow to the hindlimb remaining. Contrastingly, release of FGF-2 from fibrin gels resulted in a 9.6% and a 17.5% decrease ($p < 0.01$) in perfusion levels between four to eight weeks at low and high concentrations respectively.

The co-delivery of FGF-2 and G-CSF (Fig. 3.7A&B; group C) from fibrin hydrogels significantly restored blood flow to ischemic limbs out to eight weeks compared to delivery of a single growth factor. A significant increase ($p < 0.05$) in hindlimb bloodflow was observed where 80.4 ± 3.3% and 91.0 ± 5.2% blood flow recovery was found for treatment at 40 and 400 ng of FGF-2 and G-CSF from fibrin hydrogels respectively suggesting a concentration dependence on blood flow recovery.

Lastly, the extent of hindlimb blood flow recovery was determined based after administration of our sequential delivery groups (Fig. 3.7 E&F). It was determined that the sequence of release is not significant in the extent of blood flow recovery at either concentration at eight weeks. For example, release of FGF-2 from albumin-PLG

microspheres and G-CSF from fibrin hydrogels compared to release of G-CSF from albumin-PLL microspheres and FGF-2 from fibrin hydrogels had no significant difference in hindlimb blood flow where perfusion ratios were 93.0 ± 3.2% and 88.7 ± 4.1% respectively at a concentration of 40 ng. Alternatively, there is no significant difference between any sequential delivery scaffold at a higher concentration at eight weeks where 92.0 ± 2.1% blood flow recovery was observed for release of G-CSF from albumin-PLL microspheres and FGF-2 from fibrin hydrogels.



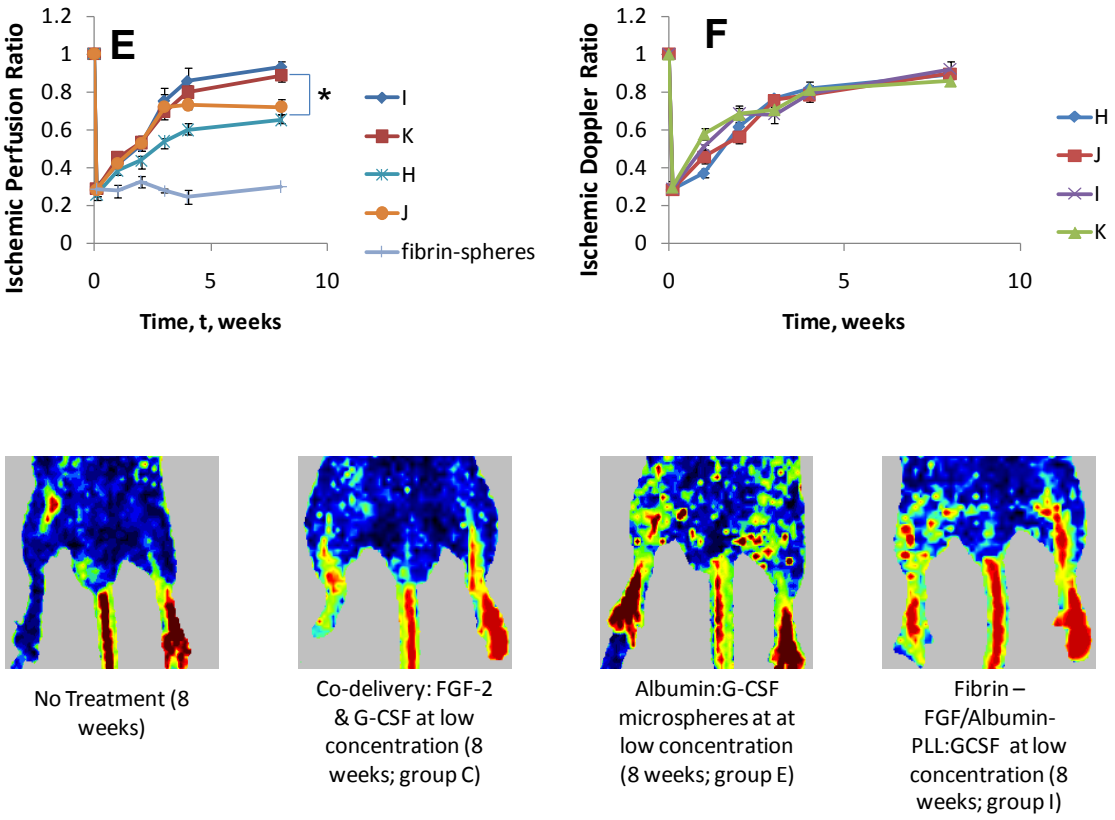
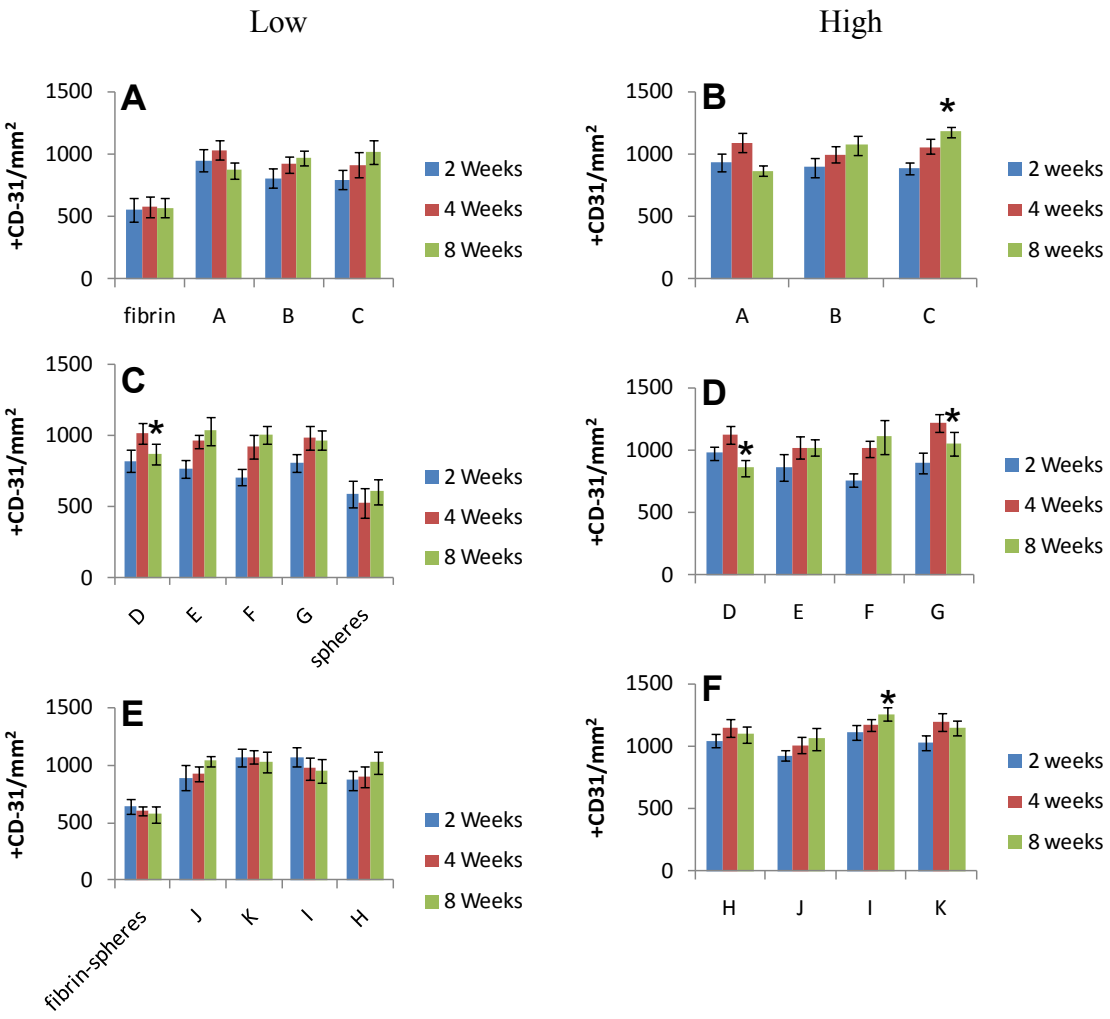


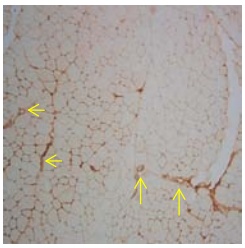
Figure 3.7. Extent of hindlimb reperfusion using sequential delivery scaffolds. Fibrin scaffolds (fibrin, treatments: A – FGF-2; B – G-CSF; C – co-delivery), and albumin microspheres (spheres, treatments: D – Albumin-FGF-2; E – Albumin-G-CSF; F – PLL-G-CSF, G – PLG-FGF-2), as well as sequential delivery vehicles were loaded with 40 ng (Fig 3.6 A,C,E; low concentration) or 400 ng (Fig 3.6 B,D,F; high concentration) of growth factor respectively. Ischemic perfusion ratios were determined by direct comparison of treatment to pre-ischemic levels in the same hindlimb. Representative Doppler images were captured at eight weeks post-surgery. Statistical significance was determined at a value of at least $p < 0.05$ and denoted by *.

3.3.6 Immunohistochemistry

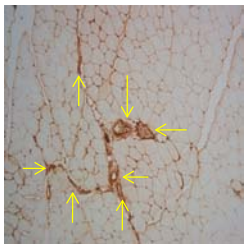
To quantify the extent of angiogenesis following ischemic insult, capillary density and mature vessel formation was determined utilizing immunohistochemical staining for CD-31+ and α-SMA+ antibodies on paraffin embedded ischemic quadriceps muscle. CD-31+ staining (Figure 3.8) demonstrates that there is a significant increase ($p < 0.05$) in the capillary density when multiple growth factors are administered compared to administration of a single growth factor from fibrin or albumin vehicles. Precisely, using

co-delivery (Fig. 3.8B; group C) of high concentration (400 ng), 1181 ± 64 CD-31+ cells/mm^2 were present after 8 weeks, and the sequential delivery scaffold using anionic spheres with G-CSF dispersed in fibrin containing FGF-2 (Fig. 3.8F; group I) resulted in a capillary density of 1258 ± 77 CD-31+ cells/mm^2. Alternatively, administration of fibrin gels releasing G-CSF (Fig. 3.8B; group B) resulted in 1075 ± 88 CD-31+ cells/mm^2, and albumin spheres releasing G-CSF (Fig. 3.8D; group F) had 1025 ± 48 CD-31+ cells/mm^2 at the high dose. Additionally, a significant decrease ($p < 0.05$) in CD-31+ cells was found in treatments using FGF-2 between four to eight weeks, except at the low concentration (40 ng) using cationic microspheres. For example, in mice treated with fibrin hydrogels releasing FGF-2 (Fig 3.8B; group A) (high dose) there was a 20.7% decrease in total CD-31 cell populations from four weeks (1094 ± 81 cells/mm^2) to eight weeks (869 ± 39 cell/mm^2).

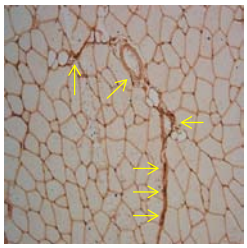




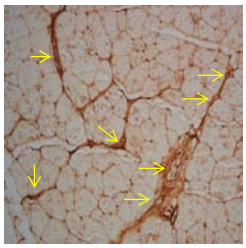
Empty fibrin and empty albumin spheres (8 weeks; group fibrin-spheres)



Co-Delivery: FGF-2 and G-CSF at high concentration (8 weeks; group C)



Albumin-PLL:G-CSF microspheres at high concentration (8 weeks group F)

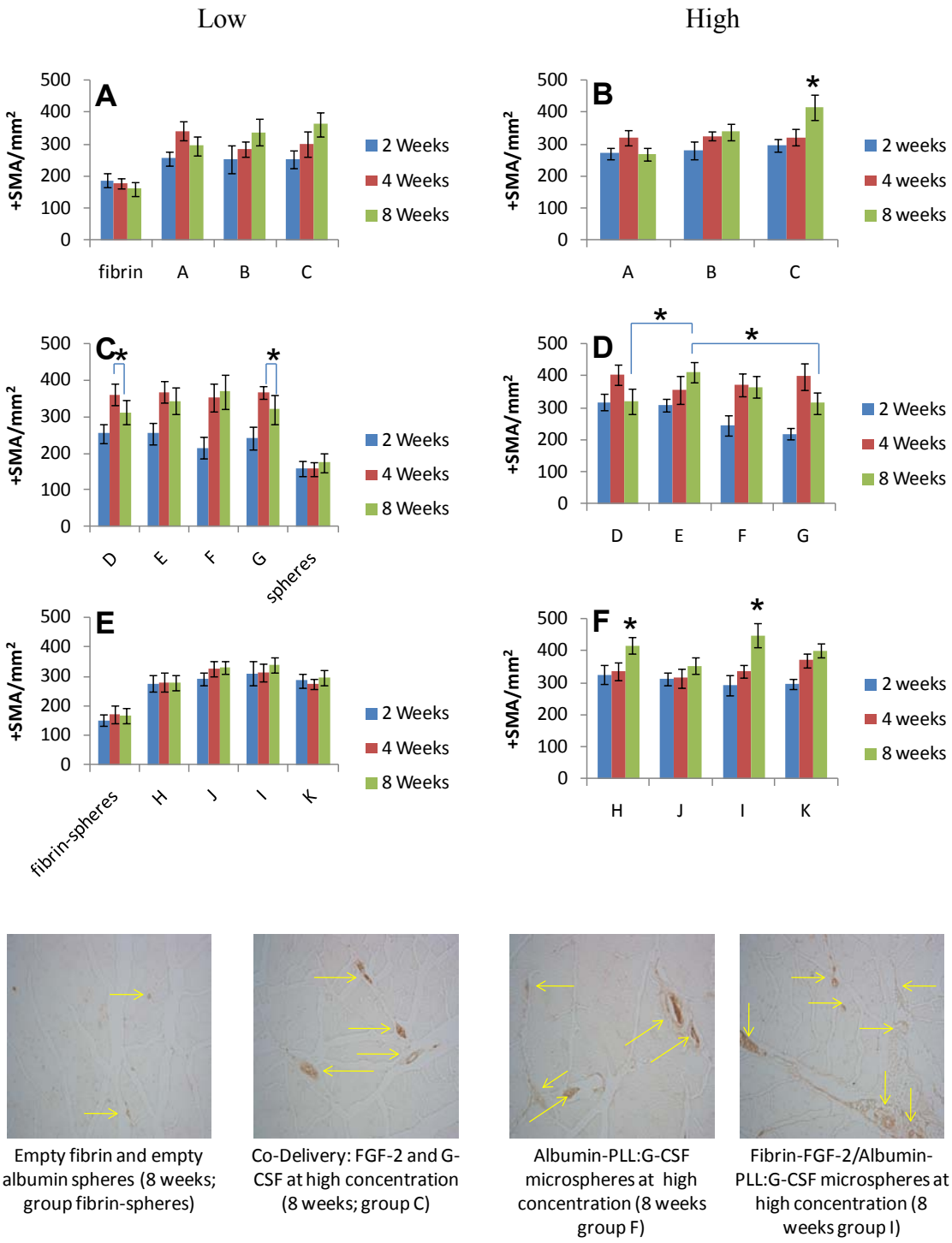


Fibrin-FGF-2/Albumin-PLL:G-CSF microspheres at high concentration (8 weeks group I)

Figure 3.8. Effect of sequential delivery of FGF-2 and G-CSF toward capillary formation as determined by CD-31 staining of ischemic quadriceps muscle at 2, 4, and 8 weeks. Fibrin scaffolds (fibrin, treatments: A – FGF-2; B – G-CSF; C – co-delivery), and albumin microspheres (spheres, treatments: D – Albumin-FGF-2; E – Albumin-G-CSF; F – PLL-G-CSF, G – PLG-FGF-2), as well as sequential delivery vehicles were loaded with 40 ng (Fig 3.7 A,C,E; low concentration) or 400 ng (Fig 3.7 B,D,F; high

concentration) of growth factor respectively. Representative micrographs were captured at 20x magnification. Statistical significance was determined at a value of at least $p < 0.05$ and denoted by *.

Angiogenesis was also estimated by mature vessels present by staining for smooth muscle actin (Figure 3.9). The increased concentration of growth factor resulted in significant increases ($p < 0.05$) in mature vessels present at all time points and treatment groups, except when using microsphere treatments. At eight weeks, when using co-delivery (Fig. 3.9A & 3.9B; group D) there was a 14% increase in SMA+ stained cells from the low concentration (362 ± 29 cells/mm^2) to the high concentration (415 cells ± 31 cells/mm^2), and using a sequential delivery with anionic spheres releasing G-CSF dispersed in fibrin containing FGF-2 (Fig. 3.9E & 3.9F; group I) resulted in a 33% increase in mature vessels with 338 ± 22 cell/mm^2 at 40 ng to 448 ± 61 cells/mm^2 at 400 ng. As shown previously with CD-31 staining, there are significant decreases ($p < 0.05$) in the SMA cell populations from four to eight weeks in treatments using only FGF-2. For example, the administration of cationic microspheres releasing FGF-2 resulted in a 20.7% decrease (397 ± 42 cells/mm^2 to 315 ± 49 cells/mm^2) in SMA cell populations as shown in Figure 3.9D (group G).



Empty fibrin and empty albumin spheres (8 weeks; group fibrin-spheres)

Co-Delivery: FGF-2 and G-CSF at high concentration (8 weeks; group C)

Albumin-PLL:G-CSF microspheres at high concentration (8 weeks group F)

Fibrin-FGF-2/Albumin-PLL:G-CSF microspheres at high concentration (8 weeks group I)

Figure 3.9. Assessment of mature vessel formation in critical limb ischemic mice determined by SMA staining of ischemic quadriceps muscle at 2, 4, and 8 weeks. Fibrin scaffolds (fibrin, treatments: A – FGF-2; B – G-CSF; C – co-delivery), and albumin microspheres (spheres, treatments: D – Albumin-FGF-2; E – Albumin-G-CSF; F – PLL-G-CSF, G – PLG-FGF-2), as well as sequential delivery vehicles were loaded with 40 ng (Fig 3.7 A,C,E; low concentration) or 400 ng (Fig 3.7 B,D,F; high concentration) of

growth factor respectively. Representative micrographs were captured at 20x magnification. Statistical significance was determined at a value of at least $p < 0.05$ and denoted by *.

Lastly, the sequence of administration of cytokine has no significant effect ($p = 0.23$) on the extent of angiogenesis. For example, when using PLL-G-CSF microspheres dispersed in a fibrin hydrogel releasing FGF-2, CD-31 cell populations were 952 ± 87 cells/mm^2 and SMA cell populations were 338 ± 31 cells/mm^2, whereas when using PLG-FGF-2 microspheres dispersed in the hydrogel releasing G-CSF cell populations were 1024 ± 82 cells/mm^2 and 294 ± 34 cells/mm^2 for CD-31 and SMA cells respectively at 8 weeks with low concentration.

3.4 Discussion

Therapeutic angiogenesis has the specific goal to utilize and augment the natural processes of ischemic revascularization through the delivery of bioactive agents with the ultimate goal restoring tissue function through blood vessel regeneration. Systemic delivery of angiogenic growth factors such as VEGF, FGF-2, IGF, and PDGF have been studied extensively, however adverse side-effects such as hypotension or tissue edema may develop from improper dosing (Vale et al, 2001). In order to overcome such limitations, sustained, localized delivery of growth factors from polymeric scaffolds has been utilized to facilitate formation of blood vessels and induce their maturation leading to tissue regeneration (Ricahrdson et al, 2001; Ehrbar et al, 2008). Many studies have recommended the use of hydrogel implants or microsphere systems for the controlled delivery of angiogenic cytokines in vivo as well as bone marrow stem cell or peripheral blood transplantation (Asahara et al, 1997; Iba et al, 2002) for the treatment of ischemic

diseases, but few studies have been reported on the in vivo efficacy of sequential delivery growth factor systems. Additionally, the remodeling of the ECM and mature vessel formation is tightly controlled by multiple angiogenic triggers (Folkman, 2007) and sequential dosing may optimize such parameters. This study aims to delineate the effect of multiple growth factor delivery and examine the sequence of release toward the formation of mature, stable, functional vasculature. These aims are demonstrated through tuning of the rate of release of growth factor through interactions between the vehicle and the releasing cytokine. In respect to our data, angiogenesis does not depend on the sequence of release of FGF-2 and G-CSF at our prescribed concentrations over eight weeks from our resorbable polymeric scaffold, but depends on the type of growth factors and delivery kinetics.

Fibrin hydrogels have been used extensively as a polymeric scaffold for delivery of angiogenic factors for treatment of ischemic events (Ehrbar et al, 2008; Ahmed et al, 2008), however we have previously demonstrated that the release of angiogenic factors from these scaffolds is within a matter of days (unpublished data). For this reason, a delivery system composed of both microspheres and hydrogel were fabricated by dispersing ionic albumin microspheres into a fibrin hydrogel to prolong and control the sequence of growth factors released. This combination was beneficial in controlling release behaviors of angiogenic growth factors based on their isoelectric points. Growth factors formed ion-complexes to their opposite charge (i.e. FGF's pI is 9.6 and PLG's pI is 4.5) and decelerated the release of the growth factor significantly compared to albumin spheres with no ionic modification. Although the sequence of release had no significant effect on the endothelial cell growth, the angiogenic factors, together, had an important

effect on endothelial cell growth because FGF-2 has been implicated to be highly mitogenic toward endothelial cells (Vale et al, 2001) whereas G-CSF is more important for the migration of endothelial cells (Bussolino et al, 1991).

The delayed release of growth factors from ionic microspheres dispersed in fibrin hydrogels initiated a therapeutic angiogenic effect as determined by hindlimb blood flow, capillary ingrowth, and mature vessel formation. These findings were not observed in treatments with single growth factor administration from microspheres, hydrogels, or by co-delivery at low concentrations. It has been implicated that angiogenesis depends on timing and sequence of growth factors present, and delivery vehicles must be modified to adapt to these conditions (Carmeliet and Conway, 2001; Richardson et al, 2001). For example, FGF-2 degrades the basement membrane of the ECM and recruits endothelial cells to line neo-vessel formation early in angiogenesis and at a later time point G-CSF homes endothelial progenitor cells to ischemic regions to form stable vasculature around endothelial cells (i.e. pericytes, smooth muscle cells), decreasing the likelihood of leaky vessels (Rafii and Lyden, 2003). At our administered concentrations, the sequence of growth factor release did not play an important role in the angiogenesis of the ischemic limb at 8 weeks, as determined by laser Doppler perfusion imaging and immunohistochemical staining. At higher concentrations, the findings at lower concentrations were amplified as blood flow, capillary ingrowth and mature vessel formation were significantly affected.

Most importantly, between four and eight weeks the release of FGF-2 from fibrin matricies or microspheres resulted in vessel regression and decreased hindlimb blood flow, which was amplified at higher concentrations. Previous studies have demonstrated

growth factors that are specific for endothelial cell proliferation, such as FGFs and VEGFs, induce angiogenesis that consists of immature, and leaky vessels (Carmeliet and Conway, 2001; Layman et al, 2007; Greenberg et al, 2008). Additional triggers are needed to provide a maturation function, as demonstrated by the use of G-CSF. Other studies have utilized PDGF (Richardson et al, 2001) or angiopoietin-1 (Baffert et al, 2006) to facilitate mural cell and pericyte recruitment to form stable, functional vessels. Furthermore, the decelerated release from microspheres dispersed in the fibrin scaffold significantly increased hindlimb blood flow. This could be due to directional migration of endothelial and smooth muscle cells, as well as vessel branching as growth factors may be administered in correspondence with physiological demands from our scaffold (Cao and Mooney, 2007; Sands and Mooney, 2007). The localized, sequential delivery of factors employed in this study allows for the control over the doses required for therapeutic angiogenesis and demonstrates that low doses can be equally effective. Also, the system developed for this study demonstrates that controlling the dose of growth factor delivered can result in an increase in the formation and maturation of the new vessels as shown by immunohistochemical staining. Previous studies using bolus injections of growth factors have had minimal success in sustaining mature vessel density and blood flow in ischemic limbs because of the short half-life of common angiogenic cytokines (Layman et al, 2009; Jeon et al, 2006b; Kuhlmann et al, 2007). The mature vessels induced by sequential delivery did not appear to regress, as indicated by the sustained number of vessels (CD-31+ and SMA+) out to eight weeks.

3.5 Conclusions on sequentially delivery of FGF-2 and G-CSF from fibrin hydrogels in critical limb ischemia

Our findings demonstrate that sequential delivery of FGF-2 and G-CSF from ionic albumin microspheres dispersed within a fibrin hydrogel is capable of restoring regional blood flow and forming neovasculature in a critical limb ischemic model. The properties of the material can be easily modified by crosslinking constituents used (i.e. thrombin, $CaCl_2$, transglutaminse activity) or the crosslinking density to tailor release rates to ischemic tissues. While optimal angiogenic growth factor concentrations and doses are currently under investigation, FGF-2 and G-CSF demonstrate a synergistic effect in sustaining hindlimb reperfusion, sprouting capillaries, and increasing mature vessel densities than either growth factor alone. Finally, this study demonstrates that the number of administrations of treatment can be significantly reduced. By combining stem cell populations with FGF-2 and G-CSF, the extent of angiogenesis may be significantly enhanced further as a promising approach to treat ischemic diseases.

On a dose basis, sequential delivery provides a better platform for neo-vessel formation than co-delivery of FGF-2 and G-CSF. At lower concentrations, hindlimb blood flow recovery is shown at 93%, whereas co-delivery shows 80% reperfusion. CD-31+ and SMA+ cell populations were also in agreement with reperfusion increases at these lower concentrations demonstrating that the timing of growth factor presentation may be important in facilitating angiogenesis.

Chapter 4. Synergistic Angiogenic Effect of Co-Delivering FGF-2 and G-CSF from Fibrin Scaffolds and Bone Marrow Transplantation in Critical Limb Ischemia

4.1 Delivery of Angiogenic Cytokines and Bone Marrow Cells for Ischemic Treatment

The incidence of peripheral artery disease (PAD) and critical limb ischemia (CLI) is growing in an alarming pace due to an aging population and the prevalence of diabetes mellitus (Comerota, 2001). PAD is caused by a reduction of normal blood flow due to atherosclerotic narrowing of vessel diameter leading to hypoxic tissues, ambulatory pain, and possible limb amputation. Therapeutic angiogenesis strategies to improve limb perfusion include, but are not limited to, cytokine administration (i.e. VEGF, PDGF-BB, FGF-2) (Kuhlmann et al, 2007; Belting et al, 2004), stem cell therapies (Asahara et al, 1997; Rafii and Lyden, 2003; Asahara et al, 1999a; Majka et al, 2003), gene therapy (Gounis et al, 2005; Rivard et al, 1999; Rivard et al, 2000), and tissue engineering strategies that combine cytokine therapy and stem cell administration (Jeon et al, 2006a; Mifune et al, 2008; Jeon et al, 2007; Layman et al, 2009; Jain, 2003; Layman et al, 2007).

Cytokines play a critical role in the development of capillaries as their localized administration has been shown to augment collateral circulation in animal models and clinical trials (Jeon et al, 2006a; Mifune et al, 2008; Jeon et al, 2007; Layman et al, 2009; Takahashi et al, 1999; Lederman et al, 2002). Various growth factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF-2), platelet-derived growth factor (PDGF), and angiopoietin-1 among others have been indentified to play critical roles in angiogenesis (Jain, 2003). Administration of exogenous VEGF or FGF-2 can improve regional blood flow in underperfused regions of the heart or leg.

Their effectiveness has been demonstrated by intra-arterial injection or administration directly to the ischemic region, and also with sustained release from adenovirus or polymeric vehicles (Gounis et al, 2005; Rivard et al, 1999; Rivard et al, 2000; Layman et al, 2009). Unfortunately, the exact dosage of growth factor required to induce mature vessel formation without the occurrence of adverse systemic effects is not yet certain. Furthermore, treatment involving single administration of a growth factor has produced mixed results. Specifically, therapeutic angiogenic studies using VEGF have had minimal success due to leaky vasculature formation and suboptimal dosing (Carmeliet, 2000). Although delivery of a single cytokine may induce angiogenesis, multiple triggers are needed to continue the angiogenic cascade (Schense et al, 2000; Rowe et al, 2007; Wong et al, 2003). A number of studies have suggested that by using two or more growth factors in combination may promote mature vessel formation (Layman et al, 2009; Richardson et al, 2001; Jeon et al, 2006b). Recently we have demonstrated that the localized co-delivery of FGF-2 and G-CSF from gelatin based hydrogels had a synergistic angiogenic effect by significantly restoring hindlimb blood flow and promoting mature vessel formation in a murine critical limb ischemic model (Layman et al, 2009).

Therapeutic angiogenesis strategies also include the transplantation or the mobilization of stem cell populations to the ischemic region. Recent studies suggest that the transplantation of bone marrow or vascular progenitor cells promotes tissue neovascularization and regeneration (Rafii and Lyden, 2003; Majka et al, 2003). Autologous bone marrow transplantation has been shown to increase blood vessel densities, increase tissue concentrations of VEGF and FGF-2, and increase blood flow to

the ischemic limb of a diabetic mouse (Sica et al, 2006). In a pilot clinical trial, autologous BM cell transplantation in patients with limb ischemia resulted in improved ankle-brachial index, transcutaneous oxygen pressure, and rest pain compared to saline treatment at four and 24 weeks (Lawall et al, 2010). Furthermore, Jeon et al., demonstrated that the transplantation of BM cells in ischemic mouse limbs in addition to the mobilization of endogenous CD34+/AC133+ endothelial progenitor cells resulted in marked increase in capillary density and limb perfusion (Jeon et al, 2007).

Stem cell mobilization and homing to the ischemic area have also been exploited as a potential angiogenic therapy. Functional stem cells can be recruited to the ischemic region to participate in angiogenesis by inflammatory markers released from, or exogenous growth factors injected to, the site of injury (Rafii and Lyden, 2003; Cao and Mooney, 2007; Powell et al, 2005). The corecruitment of hematopoietic cells that secrete specific angiogenic factors facilitates the incorporation of EPCs into newly sprouting blood vessels (Rafii and Lyden, 2003). A pre-clinical study by Powell et al., demonstrated that the administration of G-CSF to patients with coronary artery disease resulted in an increase in the amount of circulating hematopoietic progenitor cells and a transient increase in the amount of EPC colony forming units at the ischemic site (Powell et al, 2005). EPC mobilization has also been demonstrated with similar results using stromal-derived factor-1 (SDF-1) (Tan et al, 2009). Finally, strategies to overcome the limitations (i.e. immature vasculature, vessel regression) of current angiogenic therapies are focused on the delivery of angiogenic cytokines in combination with stem cell transplantation (Jeon et al, 2006a). Asahara et al., demonstrated that VEGF can mobilize EPCs from bone marrow resulting in an enhanced neovascularization effect in a corneal

angiogenesis study in mice (Asahara et al, 1999b). Numerous growth factors, notably G-CSF, play a pivotal role in recruitment and differentiation of bone marrow stem cells to ischemic sites. It has been shown that the local administration of G-CSF and BM cells to an ischemic site improves wound healing and angiogenesis. Jeon et al demonstrated that the controlled delivery of G-CSF in conjunction with BM cells resulted in more extensive neovascularization than growth factor or stem cells alone in a murine model (Jeon et al, 2007). The choice of FGF-2 and G-CSF was based on the ability of FGF-2 to direct endothelial cell migration and proliferation, and G-CSF to induce endothelial progenitor cell homing for mature vessel formation. Specifically, FGF-2 is highly mitogenic and chemotactic toward fibroblasts and endothelial cells. FGF-2 also facilitates the alignment of smooth muscle cells in the basement membrane of newly formed capillaries (Layman et al, 2009). Alternatively, G-CSF is a potent mobilizer of hematopoietic stem cells from the bone marrow, has a neuroprotective effect, activates endothelial proliferation and stimulates angiogenesis via mobilizing bone marrow stem cells (BMSCs) (in particular, endothelial progenitor cells, EPCs) to the ischemic site (Rafii and Lyden, 2003).

In this study we explore the angiogenic potential of co-delivering FGF-2 and G-CSF from a fibrin matrix in combination to the localized bolus administration of BM cells in a murine critical limb ischemia. End points include the blood flow in ischemic limb by Laser Doppler Perfusion Imaging, immunohistochemical staining for neovascular indicators CD31 and α-smooth muscle actin (SMA), which are marker of immature (capillary) and mature vessels, respectively, and a lipophilic tracer carbocyanine DiI, which stains endothelial cells that can be imaged using confocal laser scanning microscopy to determine the extent of mature and functional vessel formation.

4.2 Materials and Methods

4.2.1 Materials

Fibrinogen (fraction I, type I; from human plasma), thrombin (from bovine plasma), and calcium chloride were purchased at their highest purity from Sigma Aldrich (St Louis, MO). Human recombinant basic fibroblast growth factor (FGF-2; MW= 16,000 Da; pI= 9.6) and granulocyte colony-stimulating factor (G-CSF; MW= 18,800 Da; pI= 6.2) were purchased from Peprotech (Rocky Hill, NJ). All other chemicals and reagents were purchased from Sigma Aldrich unless otherwise noted.

4.2.2 Scaffold Fabrication

Fibrin scaffolds were synthesized according to a previously established method (Schense et al, 2000) with minor modifications. Briefly, fibrinogen (4 mg/ml) was dissolved in a 0.9% sodium chloride solution at 37 °C for 2 hours. Separately, a 10 mM solution of calcium chloride was combined with thrombin at a concentration of 0.2 NIH U/ml. 10 μl of phosphate buffered saline (PBS; pH = 7.4; Gibco; Carlsbad, CA) containing 100 ng of FGF-2, G-CSF or both were added to the thrombin/$CaCl_2$ solution prior to crosslinking. These two solutions were combined in a 35 mm x 10 mm polystyrene culture dish (Dow Corning; Corning, NY) at a 5:1 ratio (fibrinogen : thrombin/$CaCl_2$) and crosslinked for 3 hours at 37 °C. Fibrin hydrogels (400 μl) were removed using a punch biopsy with a growth factor concentration of 100 ng/ml.

4.2.3 Release Kinetics of FGF-2 and G-CSF

The release kinetics of G-CSF and FGF-2 were determined using a previously described method (Layman et al, 2009). Briefly, G-CSF and FGF-2 were reconstituted in double-distilled water or Tris–HCl (pH 8.0) respectively, to a concentration of 100 μg/ml.

Plastic cylinders (h = 4.0 mm, D = 6.5 mm, V = 135.0 mm^3) were sealed to the bottom of a 6-well culture plate using silicon glue (Dow Corning). Next, 250 µl of fibrinogen (4 mg/ml) was combined with the growth factors, thrombin, and calcium chloride at a 5:1 ratio, using previously described concentrations, to make a final concentration of FGF-2 or G-CSF at 100 ng/ml, and cross-linked at 37 °C for 3 hours. Release experiments were conducted on a MaxQ4000 orbital shaker (Barnstead International; Dubuque, IA) at 100 rpm and 37 °C. The hydrogels were immersed in 10 ml of PBS and samples were collected daily over five days and stored at -20 °C prior to quantification in triplicate experiments. The amounts of G-CSF (R&D Systems; Minneapolis, MN) and FGF-2 (EMD Biosciences; San Diego, CA) released were determined using an enzyme-linked immunoabsorbent assay. The optical density was determined at 490 nm with a reference wavelength at 650 nm for G-CSF and at 450 nm with reference at 570 nm for FGF-2.

4.2.4 Isolation of Bone Marrow Cells

Bone marrow cells were obtained utilizing a well-established method with modifications (Asahara et al, 1999a). Tibias and femurs of age-matched (6-7 weeks), donor male Balb/C mice were flushed with PBS using an 18-gauge needle and collecting them over a 70-micron filter. The homogenized BM cell collection was centrifuged, aspirated, and resuspended in PBS twice and then placed into culture medium containing DMEM (Invitrogen; Carlsbad, CA) supplemented with 10% fetal bovine serum, 1% sodium pyruvate, L-glutamate, and penicillin-streptomycin (Invitrogen). Cells were incubated overnight at 37 °C and 5% CO_2 and the unattached cell portion was isolated at 1 x 10^6 cells.

4.2.5 Critical Limb Ischemia Model

Balb/C mice (Charles River Laboratories; Boston, MA), aged 6–7 weeks, were treated under IACUC guidelines of the Miller School of Medicine at the University of Miami. Prior to surgery, the animals were anesthetized using a ketamine/xylazine/acepromazine cocktail at 40, 8 and 10 mg/kg body weight, respectively. The surgery was performed according to previously described methods (Layman et al, 2009). Briefly, an incision was made from the patella to the lower abdomen, exposing the femoral and external iliac arteries. A 7-0 grade silk suture was used to ligate the lower superficial femoral artery (above the saphenous branches), and at the junction of the external iliac, deep femoral and profunda femoris. The artery was then excised and removed. Following surgery, mice were randomized for treatment ($N = 5$ per time point per group) in two groups. In group one, fibrin hydrogels containing 100 ng/ml of FGF-2, G-CSF, or both were implanted adjacent to the ischemic site. In group two, in addition to the growth factor containing fibrin hydrogels, BM cells were administered (1×10^6) in four locations in the quadriceps muscle of the ischemic site.

4.2.6 Monitoring of Hindlimb Blood Flow

Hindlimb perfusion was assessed using Laser Doppler Perfusion Imaging (LDPI; Perimed Medical Systems; New York, NY), and data acquisition was obtained as previously reported (Layman et al, 2009). Briefly, color photographs were obtained and hindlimb perfusion was determined by comparing the intensity of a murine hindlimb prior to surgery to a specific time point: post-surgery, and at 1, 2, 3, 4 and 8 weeks. An ischemic Doppler ratio was established for all groups and compared.

4.2.7 Immunohistochemistry

The mice were sacrificed at 2, 4, and 8 weeks post-surgery with an overdose of halothane inhalation. Quadriceps muscle tissues were removed and immediately placed in 10% formalin in PBS overnight. After fixation, tissue was embedded in paraffin and 5 μm tissue sections were cut and mounted on positively charged slides. The tissue's antigenicity was recovered using a warmed proteinase K solution for CD31+ staining, and 10 mM citrate buffer (pH 6.0) for α-actin staining. Samples were then treated with diluted primary antibody (1:25), goat PECAM-1 monoclonal antibody overnight (Santa Cruz Biotechnology; Santa Cruz, CA) or α-actin (0.N.5) diluted at 1:500 (Santa Cruz Biotechnology; Santa Cruz, CA) for 2 h. Samples were then rinsed, and incubated in secondary biotinylated antibody (1:200), rabbit anti-goat polyclonal antibody (Santa Cruz Biotechnology). Samples were then treated with ABC (Vector Labs; Burlingame, CA) and DAB chromogen reagent (DAKO; San Diego, CA) for CD31+ stained cells. For SMA+ stained cells, HRP substrate was added for 10 minutes until a light brown stain was visible. Slides were then viewed using light microscopy at 60× using a LEICA Microsystems AF6000LX brightfield/fluorescence microscope (Bannockburn, IL), and images were captured using MetaMorph 5.1 Software. Positively stained CD31 capillaries were manually counted in minimum five separate fields and expressed as capillaries per millimeter squared. The number of vessels associated with α-SMA positively stained cells was also quantified in a similar fashion.

4.2.8 Fluorescent Vessel Painting

A direct labeling procedure was employed to visualize arteries and capillaries in the murine hindlimb according to a well-established procedure (Li et al, 2008). Briefly,

1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (100 mg, DiI; Invitrogen) was dissolved in 16.7 ml of 100% ethanol overnight with gentle agitation and covered from light. 200 μl of the DiI solution was added to a diluent solution containing PBS and a 5% glucose solution at a 1:4 ratio respectively. Following solution work-up, mice were sacrificed using an overdose of carbon dioxide inhalation and underwent cardiac perfusion. First, 3-4 ml of PBS was perfused through the left ventricle at a rate of 1 ml/min to flush the vascular system through the right atrium/vena cava. Second, 5 ml of the DiI work-up solution was administered through the left ventricle at a rate of 1-2 ml/min or until a light pink discoloration was observed at the extremities. Lastly, the mouse was fixed via perfusion with 5 ml of 4% paraformaldehyde through the left ventricle at a rate of 1-2 ml/min. The hindlimb was removed and stored in ddH_2O until viewing using a confocal microscope with a rhodamine filter. A stack of images was obtained ranging from 200-400 μm at 100x magnification with 0.3 NA and reconstructed to map the vasculature of the hindlimb using Zeiss Axiovision 4.7 software and the extent of angiogenic sprouting with respect to treatment was quantified by the relative color signal intensity (RGB density) above and below the proximal point of ligation in at least five fields per animal and compared to an animal with no surgery performed. Additionally, the approximate range of diameter, based on previous estimates (Helisch et al, 2006), was determined at the proximal ligation point in at least eight fields per animal.

4.3 Results and Discussion

4.3.1 Growth Factor Release Kinetics

The rate of release of FGF-2 and G-CSF from our fibrin networks was determined using an immunoabsorbent assay. Fibrin, a critical blood component of the coagulations

cascade, is a versatile biopolymer used in a number of tissue engineering applications, including bone, vascular, cartilage and skin regeneration (Wong et al, 2003; Ahmed et al, 2008; Zhang and Suggs, 2007). Fibrin matrices are biodegradable, non-toxic, can serve as provisional extracellular matrix to stabilize and control the delivery of growth factors and can be easily fabricated to desired specifications under mild conditions (e.g. controlling thrombin/fibrinogen/$CaCI_2$/transglutaminase ratios) (Rowe et al, 2007; Jeon et al, 2005). Because of these attributes we've chosen fibrin as the delivery vehicle of FGF-2 and G-CSF. The growth factors were released via Fickian diffusion, one-dimensionally from the top of the plastic cylinders and their rates of release were measured with or without the other growth factor. All of the FGF-2 was released from the fibrin gels within 5 days (Figure 4.1 A), while in the presence of G-CSF, the amount of FGF-2 been released from the scaffolds was lower at 93.4 ± 2.9% ($p < 0.05$). This could be attributed to possible binding interactions between the two growth factors that led to the deceleration of the rate of FGF-2 release when the other growth factor was present. At pH of 7.4 at which the release experiment was performed, the overall charge of FGF-2 is slightly cationic (pI=9.6) while G-CSF's is anionic (pI=6.2). Similarly, all of the G-CSF was released (100%) within five days in the absence of FGF-2, while in the presence of FGF-2, 87.9 ± 2.6% ($p < 0.05$) of G-CSF was released during the same time (Figure 4.1 B). Similar observations on the release rates of FGF-2 and G-CSF were made when these factors were released from ionic gelatin hydrogels (Layman et al, 2007).

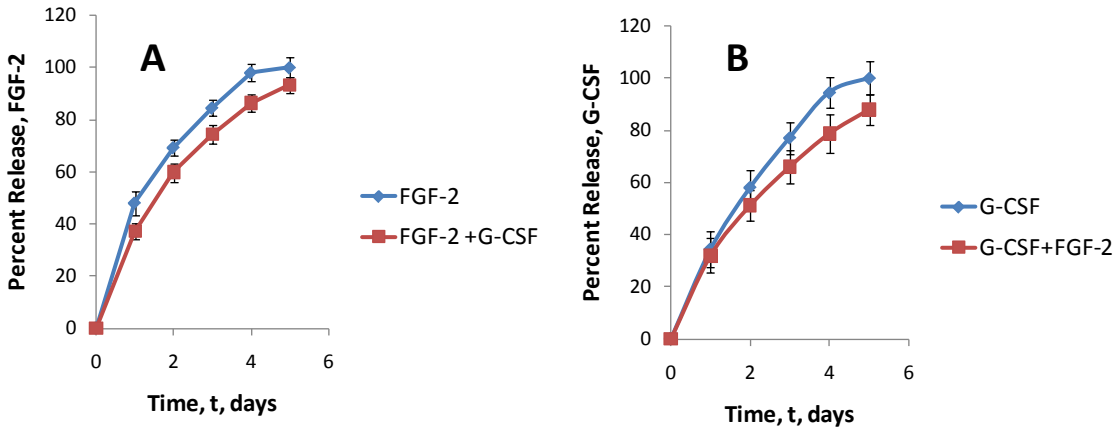


Figure 4.1. Release kinetics of cytokines from fibrin matrices. **(A)** Percent release of FGF-2 (100 ng/ml) in the presence or absence of G-CSF over five days from fibrin scaffolds ($N = 3$ per time point per group). **(B)** Percent release of G-CSF (100 ng/ml) in the presence or in the absence of FGF-2 over five days from fibrin scaffolds ($N = 3$ per time point per group).

4.3.2 Assessment of Angiogenesis in CLI model

4.3.2.1 Hindlimb Perfusion Mapping

A murine critical limb ischemia model was used to assess the synergistic angiogenic effect of co-delivering FGF-2 and G-CSF and administrating BM cells at the ischemic region. Mice were scanned post-surgery, and at 1, 2, 3, 4, and 8 weeks after surgery. LDPI revealed a significant increase in the reperfusion of the ischemic hindlimb when FGF-2 and G-CSF were co-delivered to the ischemic site (Figure 4.2 A). At 8 weeks after injury, the perfusion values in the ischemic hindlimb of in mice treated with either FGF-2 or G-CSF alone are 59± 3% and 65 ± 1% of the pre-injury values, respectively (Fig 2A). Interestingly, between four to eight weeks there is a significant decrease (by 9.5%; $p < 0.05$) in the reperfusion values in mice treated with FGF-2 alone. This may suggest that FGF-2 is prone to leaky vessel formation or vessel regression due to non-sustained dosing (Cao et al, 2004). By increasing the dose, prolonging the release,

multiple treatments of FGF-2, or administration of additional cytokines the problems associated with vessel regression may be amended. Alternatively, the co-delivery of FGF-2 and G-CSF revealed a significant increase in the reperfusion of the hindlimb compared to release FGF-2 or G-CSF at eight weeks ($p < 0.05$); Figure 4.2 A). The co-delivery of FGF-2 and G-CSF in the absence of bone marrow cell transplantation improved the LDPI ratios by 15% and 21% in comparison to the delivery of only G-CSF or FGF-2 respectively ($p < 0.05$ for both). However, by only recovering 80% of normal perfusion levels by LDPI after 8 weeks, additional angiogenic triggers might be necessary to achieve vessel regeneration and blood flow recovery (Kuhlmann et al, 2007; Rafii and Lyden, 2003).

Several preclinical studies have demonstrated that the transplantation of BM cells in ischemic regions improves collateralization and tissue reperfusion (Nishimura and Asahara, 2005; Shi et al, 2008). BM cell transplantation can promote angiogenesis by the direct differentiation of the transplanted cells to endothelial cells and vascular smooth muscle cells, by supplying endothelial progenitor cells (EPC) that can differentiate into endothelial cells, or by supplying a number of angiogenic growth factors. The transplantation of bone marrow cells had a significant increase of hindlimb perfusion at four and eight weeks post-surgery when used in conjunction with G-CSF or co-delivery. At four weeks there was a 58 ± 4% recovery in blood flow (by LDPI) for G-CSF treated mice and 67 ± 6% for the mice treated with G-CSF and BM cells (Figure 4.2B, $p < 0.05$).

The hindlimb recovery was significantly greater in the case of co-delivering FGF-2 and G-CSF in conjunction to bone marrow transplantation in comparison to all other experimental groups. Specifically, the co-delivery of FGF-2 and G-CSF and bone

marrow transplantation induced an 87 ± 4% of blood flow recovery in the mouse hindlimb significantly higher ($p < 0.01$) than the co-delivery of FGF-2 and G-CSF without BM cells (80 ± 2%) at eight weeks. Similar to our previous findings the co-delivery of FGF-2 and G-CSF is superior in improving blood flow in comparison to single growth factor delivery (Layman et al, 2007). Furthermore, BM cells alone induced only a marginal recovery in blood flow (56 ± 7%) in the ischemic limb. In summary a combination of growth factor delivery and bone marrow transplantation has a syngenistic effect on angiogenesis in the a CLI model, and the best results is achieved when bone marrow and the co-delivery of FGF-2 and G-CSF are used together.

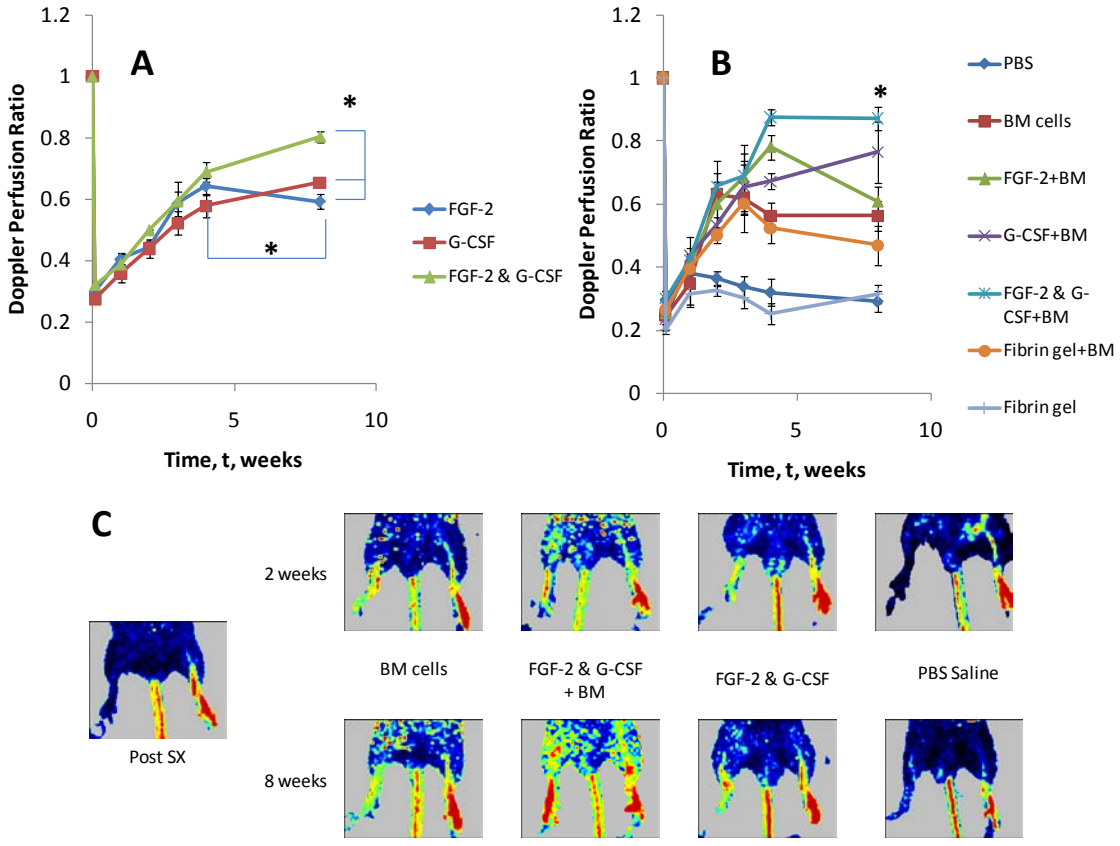


Figure 4.2. Hindlimb perfusion using Laser Doppler Perfusion Imaging (N = 5 per time point per group). **(A)** LDPI ratios of mice treated with fibrin gels releasing FGF-2 (100 ng/ml) and/or G-CSF (100 ng/ml). Doppler perfusion ratio, established as a ratio of pre- and post-surgery perfusion intensity. **(B)** LDPI ratios of mice treated with fibrin gels

releasing FGF-2 (100 ng/ml) and/or G-CSF (100 ng/ml) in addition to bone marrow transplantation (300 μL bolus inenjection of one million cells at four locations in the quadriceps muscle) **(C)** Representative Doppler scans at two and eight weeks following surgery compared to a non-perfused limb following surgery. Statistical significance was determined when $p < 0.05$ and denoted by * between treatments and time points.

4.3.2.2 Capillary and Mature Vessel Density

Blood vessels were identified by staining for CD-31 (capillary density) and smooth muscle α-actin (SMA) in quadriceps muscles of the ischemic hindlimbs at 2, 4, and 8 weeks post-surgery. The effect of FGF-2/G-CSF co-delivery (100 ng/ml) on capillary density at eight weeks post surgery was significantly greater (1017 ± 44 cells/mm^2; $p < 0.05$) than treatment with either FGF-2 (874 ± 89 cells/mm^2) or G-CSF alone (905 ± 58 cells/mm^2; Figure 4.3A). In line with previous reports a significant decrease, 16%, in the capillary density by LDPI and immunohistochemistry (CD31 and α-SMA) from 4 to 8 weeks was observed in mice treated with FGF-2 releasing scaffolds (Figures 4.3,4.4), suggesting that FGF-2 may stimulate neo-vessel formation for a transient period, which ultimately lead to fragile and leaky vessels that are prone to regression (Layman et al, 2007; Layman et al, 2009; Cao et al, 2004). At 8 weeks after injury, co-delivery of FGF-2 and G-CSF resulted in an increase in positively stained SMA vessels by 12% (not statistically significant; $p = 0.09$) and 19% ($p < 0.05$) when compared to treatment with either G-CSF or FGF-2 alone, respectively. A statistically significant increase on vessels stained with SMA was observed for the co-delivery group from 2 (253 ± 40 cells/mm^2) to 8 (362 ± 34 cells/mm^2) weeks post treatment ($p < 0.05$).

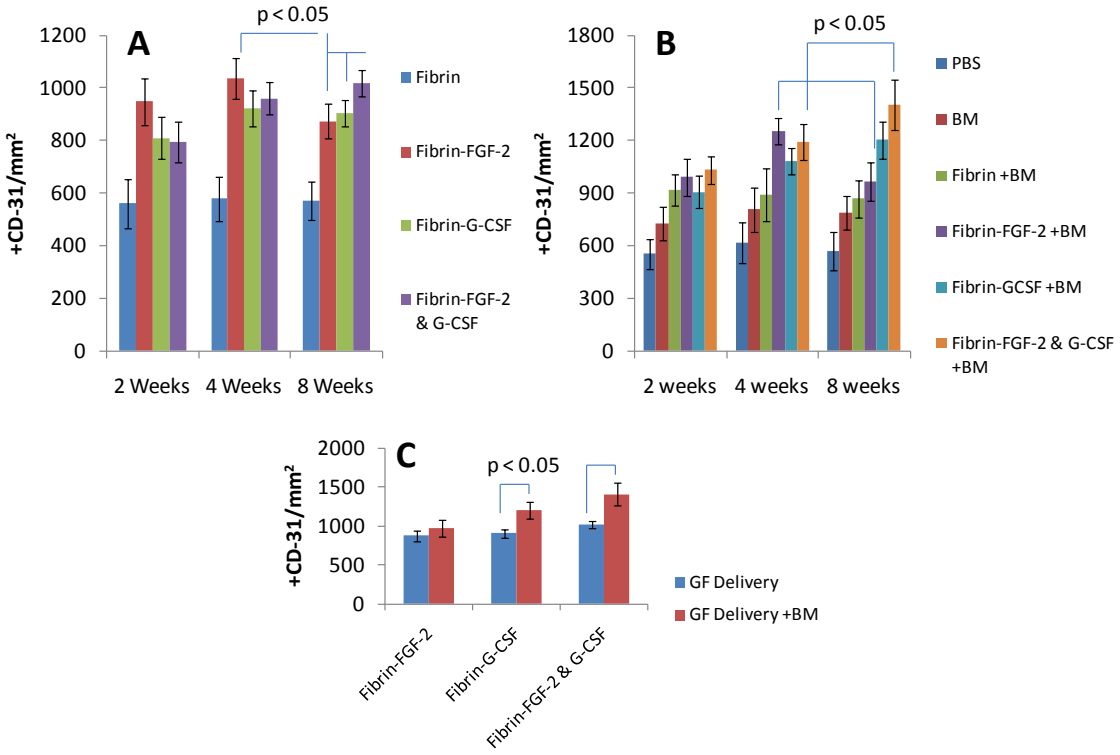


Figure 4.3. Immunohistochemical analysis for CD-31 positively stained cells (estimated capillary density) in the ischemic quadriceps muscle of the mouse (N = 3 per time point per group). Representative images were captured at 60 X. **(A)** Capillary density at 2, 4, and 8 weeks post-surgery in mice treated with fibrin gels releasing FGF-2 (100 ng/ml) and/or G-CSF (100 ng/ml). **(B)** Capillary density at 2, 4, and 8 weeks post-surgery in mice treated with fibrin gels releasing FGF-2 (100 ng/ml) and/or G-CSF (100 ng/ml) and bone marrow cell transplantation (1 million). **(C)** Capillary density at 8 weeks to compare statistical difference with treatment using growth factor (GF) delivery compared to GF delivery with BM transplantation. Statistical significance was determined when $p < 0.05$ and denoted by * between treatments and time points.

The transplantation of bone marrow cells (1.0 x 10^6 per mouse) in conjunction with growth factor delivery exhibited a significant increase in CD-31+ and SMA+ cells at all time points compared to controlled delivery of growth factors (Figures 4.3B and 4.4B). Specifically, the addition of bone marrow stem cells in comparison to only FGF-2/G-CSF delivery elicited a 38% increase (1405 cells/mm^2 ± 98 cells/mm^2 versus 1017 ± 44 cells/mm^2; $p < 0.05$) in capillary sprouting based on CD-31+ stained cells and a 65% increase in positively stained smooth muscle cells (599 cells/mm^2 ± 24 cells/mm^2 versus

362 ± 34 cells/mm^2; $p < 0.05$). Comparatively, the administration of BM cells with FGF-2 or G-CSF released from fibrin hydrogels demonstrated a significantly smaller ($p < 0.05$) density of CD-31+ and SMA+ cells in ischemic quadriceps muscles. For example, the release of G-CSF from fibrin hydrogels with BM cell transplantation resulted in 1202 ± 98 cells/mm^2 and 509 ± 22 cells/mm^2 for CD-31+ and SMA+ cells respectively, demonstrating the need for additional angiogenic triggers to facilitate mature vessel formation and capillary ingrowth. Figures 3C and 4C demonstrate the significant differences ($p < 0.05$) in capillary density and smooth muscle cell formation when BM cells are transplanted in combination with fibrin scaffolds releasing G-CSF, or FGF-2 and G-CSF at eight weeks. For example, when treating with FGF-2, G-CSF and BM cells, there is are 38% and 40% increases in CD-31+ and SMA+ cell populations, respectively, in ischemic tissues compared to treatment with co-delivery at eight weeks. Interestingly, there is no significant difference in CD-31+ (874 ± 66 cells/mm^2 versus 967 ± 108 cells/mm^2 $p = 0.14$) and SMA+ (295 ± 29 cells/mm^2 versus 350 ± 30 cells/mm^2 $p = 0.09$) cells at eight weeks when treated with FGF-2 versus FGF-2 with BM cell transplantation. This suggests a potential interaction between G-CSF and BM cell fractions to facilitate neo-vessel formation. Previous studies by Jeon et al (2006, 2007) have extensively investigated the sustained delivery of FGF-2 or G-CSF in conjunction with transplantation of bone marrow cells to four weeks showing elevated capillary density in a mouse critical limb ischemia model, with FGF-2 being superior out to four weeks. However, our study demonstrates that the administration of bone marrow cells in combination with the sustained release of FGF-2 and G-CSF are highly advantageous toward angiogenesis by increasing capillary density and smooth muscle cell formation.

Bone marrow cell transplantation allows for the delivery of undifferentiated and differentiated cells, which in turn promote endothelial and vascular smooth muscle cells proliferation and migration allowing for robust new vessel formation (Asahara et al, 1997; Rafii and Lyden, 2003; Asahara et al, 1999a). BM cells secrete numerous angiogenic growth factors such as VEGF, PDGF, IGF, and GM-CSF that can mobilize EPC from the bone marrow and peripheral blood, and promote endothelial cell migration to ischemic tissue (Gazitt, 2000). The delivery of FGF-2 promotes endothelial cell proliferation and migration whereas G-CSF is been shown to mobilize EPC from the bone marrow to the ischemic tissue (Jeon et al, 2007). Earlier studies have suggested that multiple cytokines are needed to facilitate two important steps in angiogenesis: (1) endothelial cell recruitment and alignment in the ECM, and (2) pericytes and smooth muscle cell formation around endothelial cells to form stable, mature vasculature (Layman et al, 2009; Richardson et al, 2001; Jeon et al, 2006a). This is generally achieved by one factor that is specific for endothelial cells and ECM reorganization (i.e. FGF-2, VEGF) and another factor specific for maturation to recruit mural cells, pericytes, smooth muscle cells, or endothelial progenitor cells (i.e. PDGF, G-CSF, SDF-1α, etc.). Other studies have documented the potential use of one growth factor in combination with cell fractions, specifically bone marrow cell fractions, toward angiogenesis (Jeon et al, 2006; Jeon et al, 2007; Asahara et al, 1999b). Specifically, the release of FGF-2 and the transplantation of bone marrow cell fractions demonstrated enhanced angiogenesis in a mouse model compared to either treatment alone (Jeon et al, 2006b). However, no studies have yet investigated the interaction of bone marrow cell transplantation in combination with multiple growth factor delivery. Our study demonstrates that the

controlled release of G-CSF, as well as FGF-2, potentiates the angiogenic efficacy of a single therapy in a critical limb ischemic model by recruitment of endothelial progenitor cells, and the migration and proliferation of endothelial cells at a therapeutic rate. With the combinatory therapy, transplanted BM cells, EPCs, and migrating endothelial cells could proliferate and differentiate under the control of the released FGF-2 and G-CSF (Rafii and Lyden, 2003). The interaction of FGF-2, G-CSF, and BM cells leads to functional, stable, mature vasculature.

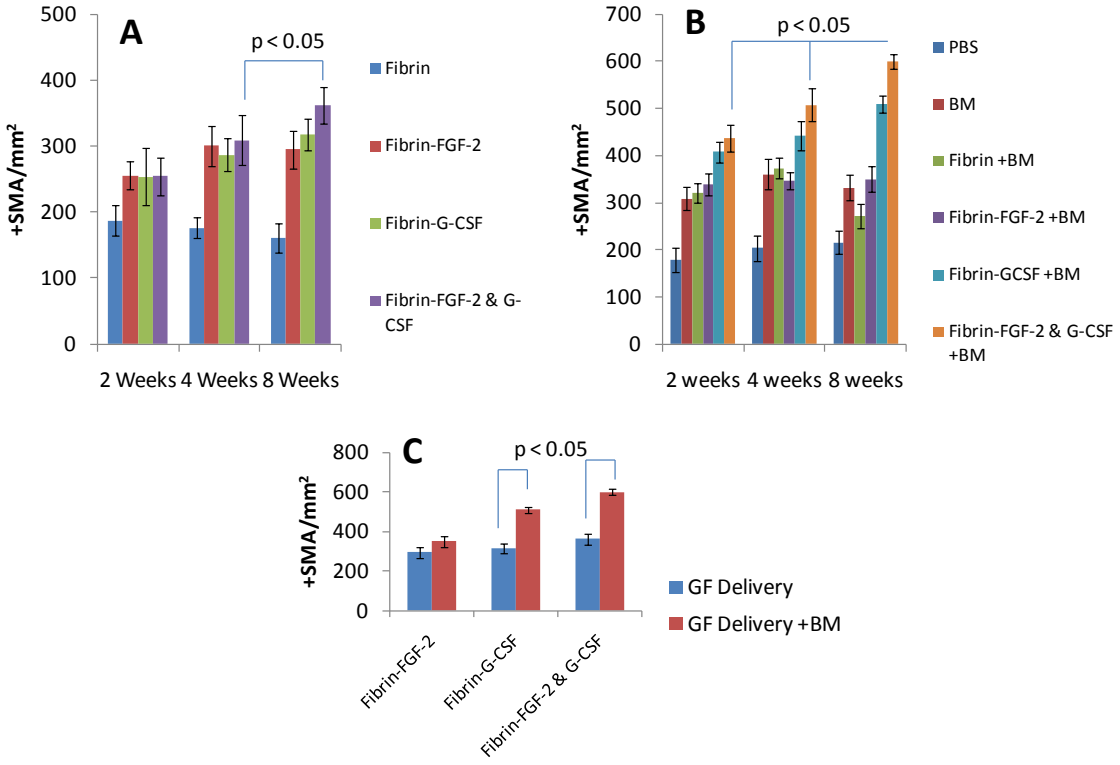
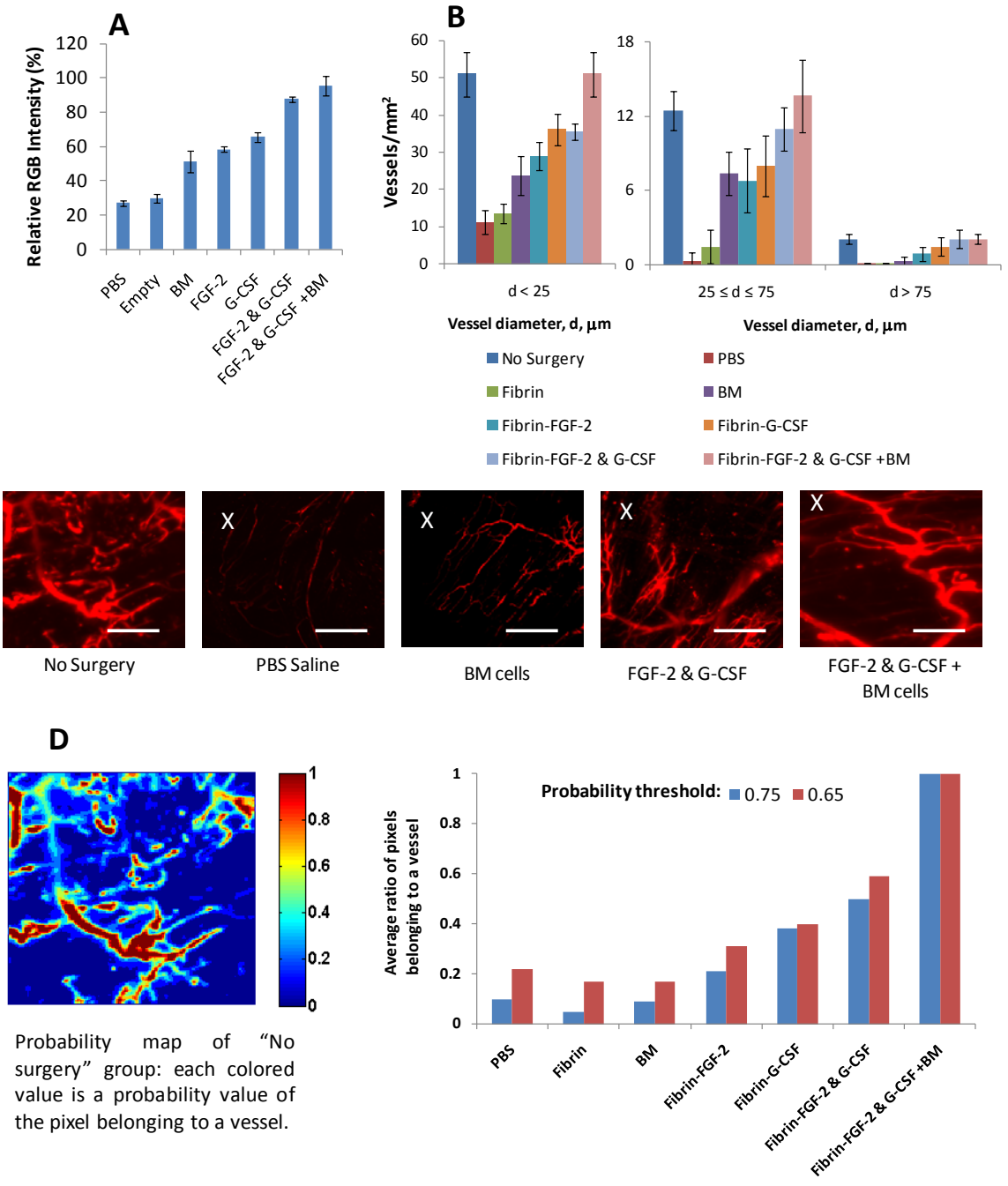


Figure 4.4. Immunohistochemical analysis for α-smooth muscle actin positively stained cells (mature vessels) in the ischemic quadriceps muscle of the mouse (N = 3 per time point per group, 60X magnification). **(A)** SMA positively stained cells at 2, 4, and 8 weeks post-surgery in mice treated with fibrin gels releasing FGF-2 (100 ng/ml) and/or G-CSF (100 ng/ml). **(B)** SMA positively stained cells at 2, 4, and 8 weeks post-surgery in mice treated with fibrin gels releasing FGF-2 (100 ng/ml) and/or G-CSF (100 ng/ml) and bone marrow cell transplantation (1 million). **(C)** Smooth muscle cell population at 8 weeks to compare statistical difference with treatment using growth factor (GF) delivery compared to GF delivery with BM transplantation. Statistical significance was determined when $p < 0.05$ and denoted by * between treatments and time points.

4.3.2.3 Direct Vessel Labeling

To more precisely identify the extent of neovascularization and angiogenesis in the murine hindlimb ischemic model, we used direct labeling of the vasculature using a lipophilic carbocyanine dye, DiI, through cardiac perfusion. Mice were sacrificed and labeled eight weeks post-surgery and the extent of neovascularization was determined at the proximal point of ligation. The extent of neovascularization was determined by two methods. First, RGB signal intensity, calculated by direct comparison of painted vessels at the proximal point of ligation for treated hindlimbs versus normal vasculature. This was expressed as a relative percentage to control animals where no surgery was performed. Second, the approximate diameter of vessels present at the proximal site of ligation at eight weeks post surgery was estimated. Co-delivery of FGF-2 and G-CSF exhibited greater vessel signal density than either growth factor used alone ($p < 0.05$) where there was a 29.2% increase compared to FGF-2 and a 22.0% increase compared to G-CSF (Figure 4.5A). Additionally, the administration of bone marrow cells in combination with co-delivery of FGF-2 and G-CSF results in a 7.9% increase in the RGB signal density ($p < 0.05$) compared to co-delivery of growth factors without administration of BM cells.



Probability map of "No surgery" group: each colored value is a probability value of the pixel belonging to a vessel.

Figure 4.5. Lipophilic tracer DiI perfused ischemic hindlimb at eight weeks post ligation. **(A)** The Red-Green-Blue (RGB) color density was determined by comparing panels above and below the point of ligation using Image J 1.73 software and compared, on a percent basis, directly to hindlimbs with no surgery performed. **(B)** The range of vessel diameter in at least eight fields per treatment group at eight weeks post-ligation. Ranges shown are for capillaries (d<25 micron), arterioles (25 micron<d<75 micron), and arteries for (d>75 micron) sizes respectively. Images were captured in a 2-D plane and rendered

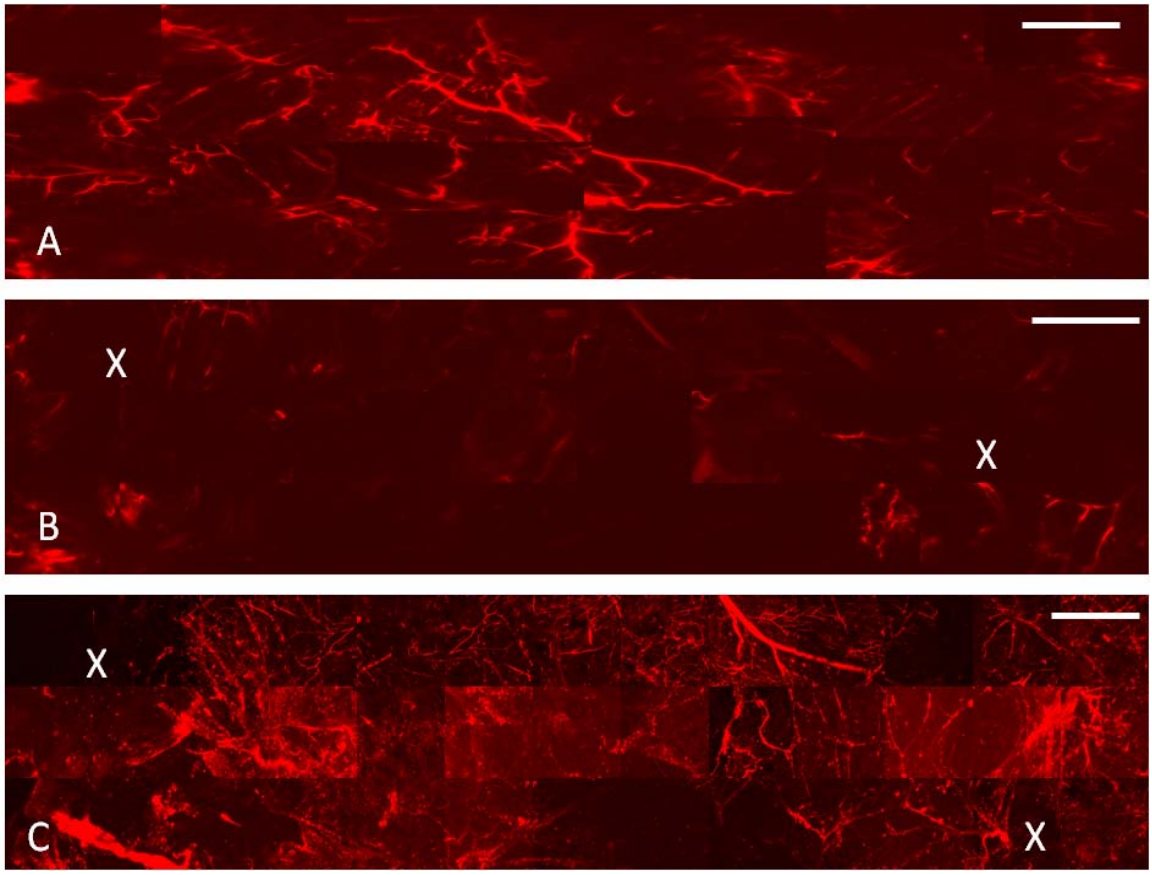
three-dimensionally with a stack of confocal images at 100 X total magnification with a 0.3 NA ranging in size 200-400 micron set on a rhodamine filter with excitation wavelength of 562 nm using a confocal laser scanning microscope, scale bar represents 35 microns. **(C)** Probability map: each colored value is a probability value (based on average percentages over several sample images) of the pixel belonging to a vessel. For illustration purposes the plots are normalized using the maximum value. In blue: the plot estimated after thresholding the probability map with the value 0.65. In red: distribution estimated using the value 0.75 for thresholding the probability map. (Note: the higher the threshold, the fewer pixels are considered to belong to vessels.

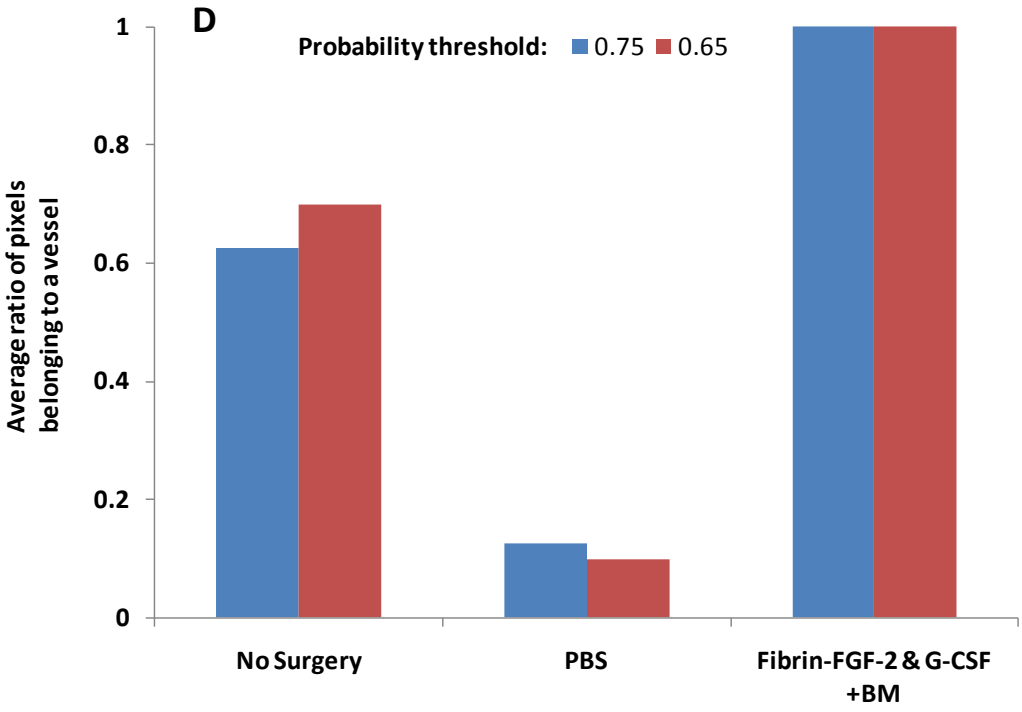
Comparatively, Figure 4.5B shows the range of vessel diameters (less than 25 micron, 25-75 micron, and greater than 75 micron) present in the mouse hindlimb for each treatment based on previous estimates (Helisch, et al, 2006). For vessels that are less than 25 microns, particularly the capillaries, total recovery was observed in mice treated with FGF-2/G-CSF and BM cells with 51 ± 6 vessels/mm^2, which was equivalent to the amount of small vessels found in a mouse with no surgery performed. This treatment had a significantly greater impact than all other treatment groups ($p < 0.05$) at eight weeks post-surgery. For vessels with diameter size between 25-75 microns (arterioles), there is a significant increase ($p < 0.05$) over using one growth factor, FGF-2 (7 ± 3 vessels/mm^2), G-CSF (8 ± 2 vessels/mm^2), or BM cells (7 ± 2 vessels/mm^2), compared to the use of both growth factors, FGF-2 and G-CSF, which had 11 ± 1 vessels/mm^2. There was not a significant difference found between the amount of vessels per square area between using co-delivery, or by using co-delivery with BM cells (14 ± 3 vessels/mm^2; $p = 0.38$). Lastly, vessels with size greater than 75 microns were only found in mice treated with G-CSF and the combination of growth factor co-delivery and bone marrow transplantation. However, every treatment was significantly less than control animals (no surgery, $p < 0.05$) where the amount of vessels greater than 75 microns was 2.07 ± 0.4 vessels/mm^2. The synergistic angiogenic effect of growth factor

co-delivery and bone marrow transplantation was also demonstrated by mapping the whole ischemic region between the two ligation points using Dil vessel painting and confocal microscopy (Figure 4.6). Clearly, the localized co-delivery of FGF-2/G-CSF from fibrin gels and BM cell transplantation induced new vessel formation over the entire ischemic region salvaging the animal's hindlimb (Figure 4.6C). The overall recovery of the hindlimb, pictorially, can be demonstrated in Figure 4.7. This figure demonstrates that our tissue engineered therapy can improve limb salvage over no treatment used or by the controlled release of growth factors alone, FGF-2 and G-CSF.

To extract the vessel regions and to calculate the image statistics, we also used a region classification method, namely the Collaborative Conditional Random Field (CoCRF), whose main advantage is the appearance-driven smoothness in the obtained vessel regions (Tsechpenakis et al, 2009) (Figure 4.5C). Region classification includes a machine learning-based family of methods for labeling regions in an image, based on their appearance, namely their intensity or, more generally, their texture. Methods of this kind assign class labels to regions, in a probabilistic manner, i.e., assign probabilities to pixels/regions of belonging to pre-defined classes, given their appearance. In our application, there are two class labels of interest: "vessel" and "background" (non vessel region); the vessel regions appear in higher intensity than their surroundings (in grayscale), and a classification approach would assign higher probability (in the interval [0,1]) to regions with higher intensities. Classification tackles noise, due to its probabilistic nature, and yields more robust results. After obtaining the class probabilities, one thresholds the resulting probabilities (probability maps/images) to obtain the binary vessel maps. In our experiments, we used two probability thresholds

(0.65 and 0.75). Under both probability thresholds the co-delivery of FGF-2 and G-CSF in addition to BM cell transplantation led to an enhanced vessel formation superior to all other treatment groups (Figure 4.5C and 4.6D).

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Probability threshold: 0.75 0.65
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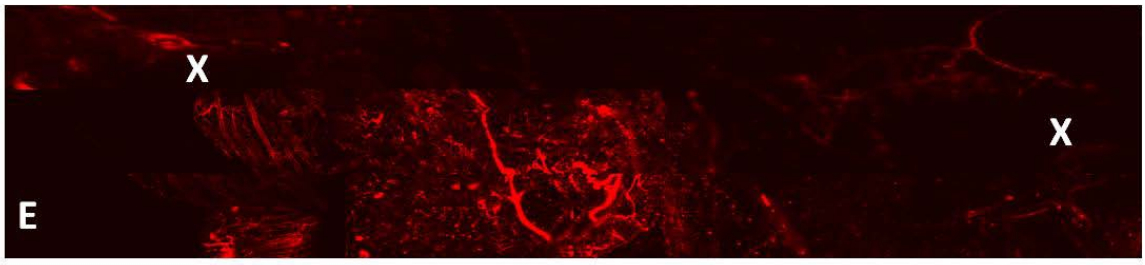
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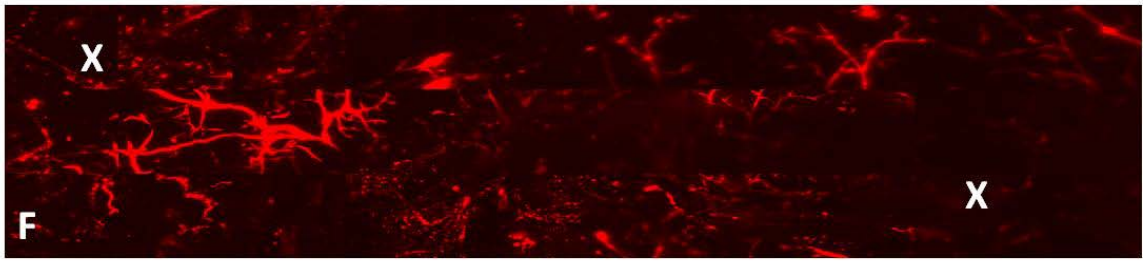
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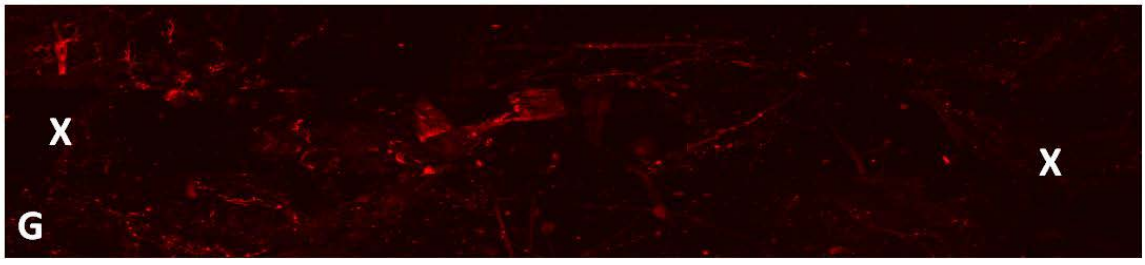
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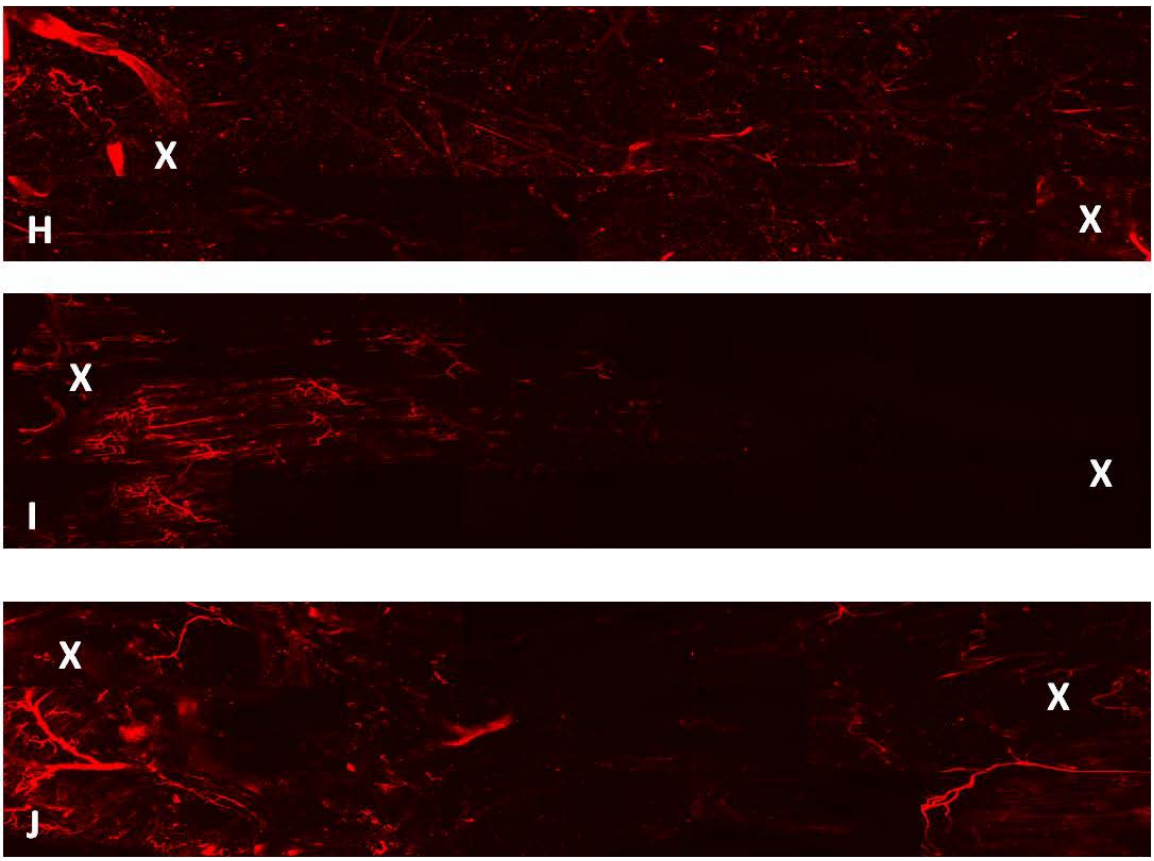

Figure 4.6. Reconstructed map of the vasculature of the murine hindlimb using a lipophilic tracer DiI at eight weeks post ligation using confocal laser microscopy. **A),** reconstructed image of normal leg, **B),** ischemic limb treated with 300 μL saline intramuscular injection and **C),** limb treated with bone marrow transplantation (one million) and fibrin gels releasing FGF-2 (100 ng/ml) and/or G-CSF (100 ng/ml). Images were captured using a confocal laser microscope at 100X total magnification with a 0.3 NA in a 2D-plane and reconstructed in a 3D-plane with a stack of images ranging in size 200-400 microns. The filter of the microscope was set on rhodamine with an excitation wavelength of 562 nm. Scale bar represents 900 microns. **D),** Percentages of pixels belonging to vessels, w.r.t the total number of the pixels of the "mosaic" images (A), (B) and (C). In blue: the plot estimated after thresholding the probability map with the value 0.65. In red: distribution estimated using the value 0.75 for thresholding the probability map. **E)**, administration of 100 ng/ml of G-CSF released from fibrin hydrogel, **F)**, co-delivery of FGF-2 and G-CSF (100 ng/ml) from fibrin hydrogels, **G)**, administration of bone marrow cells alone (one million cells injected intramuscularly), **H)**, 100 ng/ml FGF-2 released from fibrin hydrogels, **I)**, administration of 100 ng/ml FGF-2 released from fibrin in conjunction with one million bone marrow-derived cells, **J)**, administration of 100 ng/ml G-CSF released from fibrin in conjunction with one million bone marrow-derived cells.

A therapeutic angiogenic approach utilizing BM cell transplantation in conjunction with FGF-2 and G-CSF is shown to have an enhanced angiogenic effect by restoring blood flow in an ischemic leg. An increase in capillary density and mature vessel formation was observed in animals that were treated with BM cell transplantation and FGF-2/G-CSF delivery. It is hypothesized that the controlled release of G-CSF and FGF-2 allows for mobilization of endothelial progenitor cells, recruitment and proliferation of endothelial cells and fibroblasts, and alignment of cells in the newly formed blood vessels (Jain, 2003). The transplantation of BM cells at the ischemic site augments angiogenesis by supplying numerous fractions of differentiated and undifferentiated cells at the site of ischemia that have the capacity to become vascular cells or release critical angiogenic growth factors like VEGF, PDGF, and IGF, which in turn induce collateral vessel formation, increase collateral blood flow, upregulate eNOS expression, and increase subsequent nitric oxide release (Jeon et al, 2007; Shi et al, 2008). Conversely, if these cells are administered systemically, a minimal amount of the cell population will be delivered to the site of ischemia (Nishimura and Asahara, 2005). The localized delivery of FGF-2 and G-CSF in combination with bone marrow cell transplantation established a stable and functional vasculature within eight weeks. New vasculature is formed at all vessel levels except arteries (d>75 micron) which may need prolonged dose of growth factor, additional growth factors, or a higher concentration of stem cell populations to fully regenerate the excised artery and recover the ischemic tissue.

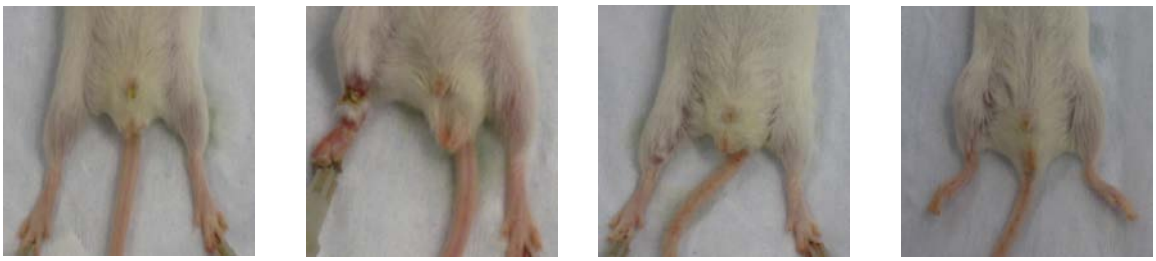

Figure 4.7. Visual recovery of critical ischemic limbs utilizing tissue engineered strategies, PBS saline (no treatment), or the co-delivery of low concentration of FGF-2 and G-CSF.

4.4 Conclusions for Synergistic Angiogenic Effect of Co-delivering FGF-2 and G-CSF from Fibrin Scaffolds and Bone Marrow Transplantation in Critical Limb Ischemia

The present study demonstrates that a therapeutic angiogenesis approach that combines cell therapy with multiple growth factors is essential in establishing a stable and mature vasculature in regions of ischemia. Herein, the co-delivery of FGF-2 and G-CSF in combination with unfractionated BM cell transplantation promoted mature vessel formation that is superior to each approach alone. Studies are currently underway to optimize growth factor and cell concentrations in order to prevent vessel regression over longer periods of time (6 months to a year). Furthermore, we are investigating the angiogenic potential of specific BM subpopulations (i.e MSC, EPC) as alternative cell transplantation sources.

Chapter 5. Conclusion and Future Directions

5.1 Conclusions

Our studies demonstrate the need for tissue engineered therapies including, but not limited to, peripheral vascular diseases and critical limb ischemia. We are able to synthesize hydrogel scaffolds that can release growth factors at various rates depending on crosslinking density, swellability, and ionic interactions. The release of these peptides increase therapeutic effects depending on the specific application of the scaffold, and in this study it is toward PAD and CLI models. We can also sequentially deliver multiple growth factors in a delayed fashion from albumin microspheres dispersed within a fibrin hydrogel. By releasing these angiogenic cytokines in endothelial cell culture, we have shown that FGF-2 and G-CSF have potent mobilization and proliferation capabilities when delivered in a controlled manner.

We have also modeled the release of the peptides from our synthesized hydrogels and microspheres to determine what effect fibrin, gelatin and albumin have on transport properties. By adjusting the varying the transport variable, n, the diffusion patterns are dependent upon loaded growth factor concentration, and as n increases, the amount of growth factor released over time increases significantly. By knowing these parameters, we can tailor our release based on concentration using identical material properties. Obviously, the in vivo environment is dramatically different in comparison to in vitro conditions and these adjustments must be considered when tailoring a material to deliver at pre-determined rates based on these calculations.

Furthermore, we have further demonstrated that bolus delivery of growth factors, regardless of concentration, will have no sustained therapeutic effect unless delivered in a

sustained manner or by use of multiple administrations. We have also demonstrated that the release of multiple growth factors induce the formation of and sustains functional and mature vessels in a CLI murine model. By altering concentrations, the extent of hindlimb perfusion can be elevated to at least 75% of normal levels within two weeks. As thought by previous experiments, the sequence of growth factor administration did not have a significant effect on the extent of hindlimb perfusion or neovascularization at our concentrations and time points demonstrating that sequence and timing do not play a pivotal role in stable neo-vessel formation. With the addition of bone marrow cells to co-delivery of angiogenic cytokines, hindlimb bloodflow recovery, and vessel densities (i.e. CD31+ and SMA+) were significantly greater, suggesting that the extent of neo-vessel formation depends on a unique combination of cell populations and cocktail of angiogenic growth factors.

Based on the studies performed, the use of cytokines and cells play an important role in angiogenesis. Growth factor administration with cells will have the capability to allow cellular proliferation and differentiation of cells transplanted and induces an endogenous response. The release of G-CSF and FGF-2 from fibrin hydrogels with BM cell transplantation leads to the greatest extent of mature vessels and capillaries formed in ischemic quadriceps muscle and restores nearly 90% of the blood flow to the limb, whereas other treatments at higher concentrations result in the same amount of blood flow but fewer capillaries and mature vessels. Also, because angiogenic peptides are rather expensive, using higher concentrations to obtain similar results in terms of perfusion is not practical. There are other numerous cell fractions and growth factors that have been extensively investigated for use toward therapeutic angiogenesis, CLI, and

PAD such as iPS cells, endothelial cells, as well as VEGF and PDGF, however these studies have not demonstrated stability, synergy and recovery to the extent that our study has with bone marrow cells that are highly differentiable, secrete growth factors in combination with our fibrin hydrogel co-delivering FGF-2 and G-CSF.

Prior to this study, no groups had investigated the use of cell fractions with multiple growth factors. Jeon et al (2007) was the first study to use a maturation factor, G-CSF, with bone marrow cell fractions in a mouse model. The demonstrated that there was an increase in the amount of capillaries and smooth muscle cells present, but did not investigate parameters such as time course of treatment, or perfusion levels. They administered a bolus of G-CSF along with BM cells and showed limb salvage, recovery, and increased capillary densities but only out to one week. Our study encompasses a whole new facet as we have delivered, in a sustained manner, multiple growth factors, and transplanted bone marrow cells and demonstrated limb recovery, increased capillary and mature vessel densities, and limb perfusion out to eight weeks.

Interestingly, as previously demonstrated by earlier studies, the extent of angiogenesis does not depend on the sequence of growth factor presentation, at least in terms of FGF-2 and G-CSF (Jain, 2003; Yancopoulos et al, 2000; Rafii and Lyden, 2003). The previous ideology suggested that priming growth factors such as FGF-2 or VEGF promoted endothelial cell growth and recruitment to the ischemic site first, whereas, second, maturation factors like PDGF, G-CSF, ang-1, ang-2, and SDF-1α have all been implicated to promote mature vessel formation (i.e. arteriogenesis, angiogenesis) and decrease permeability and leakiness. Our study shows that this in fact is not the case at our concentrations and time points where FGF-2 and G-CSF have synergistic roles in

maintaining blood flow, endothelial cell growth, as well as smooth muscle cell and pericyte capping of endothelial cells to maintain mature vessel integrity. These previous studies generally investigate hypoxia as the main determinant and this pathway triggers HIF-1α which generally signals the downstream target VEGF. While our model is somewhat hypoxic, it is mostly an ischemic model where the wound is severe and may need alternate treatment, such as our prescribed sequential delivery scaffold.

5.2 Future Experiments and Directions

In this dissertation many facets of tissue engineered therapies toward peripheral vascular diseases were investigated. It was determined that the optimization of growth factor delivery is crucial to the success of amelioration of ischemic tissues. Precisely, the temporal and spatial gradients of growth factors within the hydrogel or microsphere are currently unknown. By understanding the spatial and temporal gradients of the materials with FGF-2 and G-CSF loaded within will further enhance the understanding of this study so the time of presentation of growth factor can be delineated. Knowing how the material degrades and the timing of the presentation of growth factors will be essential for optimization of healing parameters. Furthermore, better understanding of the action of FGF-2 and G-CSF toward the bone marrow derived cells is needed. From this particular study, and other studies, the action mechanisms toward bone marrow derived cells with FGF-2 and G-CSF has not been defined.

Comparatively, many cellular functions participate toward functional, mature vessel growth and smooth muscle cells play an important role in this mechanism. To date, no one has elucidated the synergistic pathway of FGF-2 and G-CSF toward smooth muscle cells. It is interesting to note that the administration of G-CSF initiated a large

growth of smooth muscle cells in vivo and it has not been determined in vitro if G-CSF would induce proliferation more so than FGF-2. A pilot study by culturing aortic smooth muscle cells in the presence of FGF-2 and G-CSF and assessing the tubule formation, proliferation, and migration would be important to understand the functional relationship between smooth muscle cells and the growth factors. An ideal experiment would be a co-culturing experiment with endothelial cells and smooth muscle cells and using FGF-2 and G-CSF at differing concentrations to assess proliferation, migration, and tubule formation as this would be the primary condition similar to in vivo as multiple cells and cytokines play pivotal roles toward angiogenesis.

The delivery of either growth factor, or both, may have induced bone marrow derived stem cells or progenitor cells to differentiate into endothelial and vascular smooth muscle cells to facilitate angiogenesis. Without knowing how FGF-2 and G-CSF effect bone marrow derived cells, it will be unclear as to how these two angiogenic factors facilitate endothelial cell migration and proliferation as well as recruiting progenitor and stem cell fractions. This last study could be accomplished by utilizing cell sorting techniques to see what lineages of cells are present after certain concentrations of growth factor are applied over time.

In addition to further in vitro testing, studies to delineate mechanisms in vivo are needed. Two specific studies are needed to further understand the release of factors and how these factors recruit bone marrow derived cells. First, we need to clearly define the release of growth factor in vivo. This can be accomplished by radiolabeling FGF-2 or G-CSF and releasing it into the ischemic muscle. By removing the quadriceps muscle and determining the half-life of growth factor present in the muscle, we can get an idea of the

in vivo degradation of the hydrogel scaffold as well as the release kinetics in vivo. Upon understanding the release kinetics in vivo, we can further tailor our hydrogels to deliver FGF-2 and G-CSF in a tunable and/or sustained manner. We must further explore the potential use of bone marrow cells in vivo, particular the homing ability of G-CSF toward these cells during ischemic insult. In order to identify this, we could implant bone marrow from GFP mice into the ischemic muscle of wild-type mice and track them to see if they incorporate into new blood vessels or if they are lost to the periphery. Upon understanding this, we can begin to explore the mechanisms of incorporation of bone marrow cells for therapeutic angiogenesis and tissue engineered strategies for a host of ischemic diseases.

Lastly, a clinical based assessment of the recovery should be addressed. Generally, a score of one to five is assigned to a limb that would be necrotic (1) to a limb that has no scarring and has no signs of limping after treatment (5). By assigning a score based on treatment, we will be able to delineate, clinically, what treatment provides the best option qualitatively. Of course we can readily demonstrate mature vessels, capillaries, reperfusion, but what does this really mean in terms of visual evidence (shown in Figure 4.7). By assessing a score we can readily show that our treatment may return a limb to normal walking characteristics with no distress or limping.

By further clarifying the mechanisms of cell-cell interaction, cell-matrix interaction, as well as cytokine-cell interaction in terms of the treatment utilizing fibrin hydrogels containing ionic-albumin microspheres in conjunction with bone marrow derived cells we can begin to finely tune necessary parameters to provide the optimal strategy for therapeutic angiogenesis. For example, by knowing specific concentrations

at certain times of FGF-2 and G-CSF in vivo in conjunction of knowing when to supply a certain amount of bone marrow derived cells would be, unequivocally, a landmark finding. However, right now, these parameters are still being defined and will classify the next set of experiments for this study.

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