Engineering Vascular Networks

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ENGINEERING VASCULAR NETWORKS

by

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The cure for boredom is curiosity. There is no cure for curiosity.

Dorothy Parker
DEDICATION

To all my family,

in particular to my father.
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Less than five years ago I crossed the Atlantic towards the USA in order to start this journey. Now it is time to express my gratitude to all those individuals who have helped make this thesis possible. I am today incommensurable more scientifically and personally gifted than I was when I started, and all of that I owe you all.

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To everyone else who believed in me but is not individually named (you know who you are) I salute you!

Eduardo A. Silva

June 2007
ABSTRACT

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by

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Neovascularization driven by therapeutic agents represents a powerful strategy to treat ischemic diseases and is also critical to virtually all tissue-engineering approaches. Specifically, spatiotemporal control over the delivery of angiogenic agents may be crucial to guide the formation of new vascular networks. The hypothesis underlying this thesis is that the formation of neovascularization in an ischemic hindlimb can be governed by the regulated delivery of endothelial progenitor cells and growth factors from polymeric materials.

The initial studies of this thesis examined the role of different vascular endothelial growth factor (VEGF) isoforms in endothelial cell proliferation and differentiation. Further, a biodegradable alginate hydrogel system was designed to provide temporal control and appropriate spatial biodistribution of VEGF in ischemic hindlimbs. The controlled delivery of VEGF resulted in significantly improved angiogenesis, as compared to bolus delivery, and led to the return of tissue perfusion to normal levels by day 28. Normal levels of perfusion were never achieved with
bolus delivery. This system represents an attractive new generation of therapeutic delivery vehicles for treatment of cardiovascular diseases, as it combines long term in vivo therapeutic benefit with minimally invasive delivery.

This thesis also examined a novel cell delivery approach in which a material system is utilized to create a depot of cells in vivo that exit over time to repopulate damaged tissue. Microenvironmental conditions sufficient to maintain the viability and outward migration of human progenitor cells were delineated and validated in vitro. A material incorporating these signals dramatically improved engraftment of transplanted cells in ischemic murine hindlimb musculature, resulting in the complete prevention of necrosis. In contrast, direct cell injection was minimally effective, while untreated limbs underwent autoamputation within 3 days. These results demonstrate that progenitor cell utility is highly dependent on the mode of delivery, and control over cell fate after transplantation.

Overall, this work is leading to a new generation of therapeutic strategies for treating ischemic vascular diseases, and will also have a great impact on current efforts to engineer and regenerate a wide variety of tissues via cell transplantation.
RESUMO

ENGINEERING VASCULAR NETWORKS

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A neovascularização induzida por agentes terapêuticos representa uma potente estratégia para tratar doenças isquêmicas, sendo também um elemento fundamental a praticamente todas as estratégias de engenharia de tecidos. Em concreto, a obtenção de um controlo espaço-temporal da libertação de agentes angiogénicos poderá ser crucial para guiar a formação de novas redes vasculares. A hipótese subjacente a esta tese é que a formação de novos vasos sanguíneos, em regiões isquêmicas de membros inferiores de ratinhos, pode ser orquestrada através da libertação controlada de células progenitoras endoteliais e factores de crescimento por materiais poliméricos.

Nos estudos iniciais desta tese, examinou-se a função de diferentes isoformas do factor de crescimento vascular endotelial (VEGF) na proliferação e diferenciação de células endoteliais. No seguimento desses estudos, um hidrogel biodegradável alginato foi especificamente concebido para proporcionar um controlo temporal e biodistribuição espacial adequados do VEGF em regiões isquêmicas de membros
inferiores. A libertação controlada do VEGF provocou um aumento significativo de angiogênese, quando comparada com a simples injeção do VEGF em solução aquosa, resultando no retorno da circulação sanguínea para valores normais após 28 dias. Em contraste, não foram observados níveis normais de circulação sanguínea em animais tratados com solução aquosa contendo VEGF. Este sistema polimérico à base de alginato representa uma nova geração de veículos atractivos para libertação terapêutica visando o tratamento de doenças cardiovasculares, visto associar um benefício terapêutico *in vivo* a longo prazo com uma libertação minimamente invasiva.

Na presente tese examinou-se também uma nova estratégia para o transplante de células, na qual um sistema polimérico é utilizado para criar, *in vivo*, um depósito de células localizado, responsável por uma libertação faseada no tempo e dessa forma originando uma repopulação do tecido isquémico. Foram delineadas e validadas *in vitro* as condições microambientais suficientes para assegurar a viabilidade e migração de células progenitoras humanas para o exterior. O sistema polímero-células utilizado promoveu a integração das células transplantadas na musculatura do tecido isquémico, resultando na completa prevenção de necrose dos membros inferiores de ratinhos. Em contraste, a injeção directa das células progenitoras apresentou uma eficácia reduzida foi minimamente efectiva, enquanto que animais não tratados com células progenitoras exibiram auto-amputação dos membros inferiores em menos de três dias após cirurgia. Estes resultados demonstram que a utilidade biológica de células progenitoras é extremamente dependente do modo de libertação e do controlo obtido sobre o destino celular após transplantação.

Globalmente, este trabalho conduz a uma nova geração de estratégias terapêuticas direccionadas para o tratamento de doenças vasculares isquémicas,
podendo também vir a ter um impacto elevado nos actuais esforços para regenerar uma grande variedade de tecidos por intermédio de transplantação células.
RÉSUMÉ

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La néo-vascularisation induite par agents thérapeutiques constitue une stratégie puissante pour le traitement des maladies ischémiques, ainsi qu’un élément fondamental de pratiquement toutes les approches d’ingénierie des tissus. Plus spécifiquement, le contrôle spatio-temporel de la libération des agents angiogéniques peut être crucial pour guider la formation de nouveaux réseaux vasculaires. L’hypothèse sous-jacente à cette thèse est que la formation de nouveaux vaisseaux sanguins dans un membre inférieur ischémique peut être gouvernée par la libération de cellules endothéliales progénitrices et de facteurs de croissance régulée par matériaux polymères.

Les premières études de cette thèse ont examiné le rôle de différentes isoformes de facteur de croissance de l’endothélium vasculaire (VEGF) dans le processus de prolifération et de différentiation des cellules endothéliales. À la suite de ces études, un hydrogel alginate biodégradable a été spécifiquement développé afin de
permettre un contrôle temporel et une distribution spatiale adaptée de la libération de VEGF dans les membres inférieurs ischémiques. Cette libération contrôlée de VEGF a résulté en une augmentation significative de l’angiogenèse en comparaison d’une simple injection de VEGF en solution aqueuse, et a permis un retour des tissus à un niveau normal de perfusion 28 jours après induction d’une ischémie sévère dans les tissus des membres inférieurs chez la souris. Un retour au niveau normal de perfusion n’a jamais été observé chez les animaux traités par VEGF injecté en solution aqueuse. Ce système polymère constitue une attrayante nouvelle génération de véhicules de libération thérapeutique pour le traitement des maladies cardio-vasculaires, puisqu’il combine un effet thérapeutique in vivo à long terme avec une libération ministalement invasive.

La présente thèse a également examiné une nouvelle approche de greffe de cellules, dans laquelle un système polymère a été utilisé pour créer un dépôt localisé de cellules in vivo, permettant une libération progressive pour repeupler le tissu endommagé. Les conditions micro-environnementales suffisantes au maintien de la viabilité et à la migration vers l’extérieur de cellules progénitrices humaines ont été définies et validées in vitro. Un matériel polymère répondant à ces critères a considérablement amélioré la greffe des cellules transplantées dans la musculature ischémique de membres inférieurs de souris. Par contraste, l’injection directe des cellules progénitrices a eu un effet minimal, tandis que les membres non traités ont présenté une auto-amputation en l’espace de trois jours. Ces résultats démontrent que l’utilité des cellules progénitrices dépend fortement du mode de libération, ainsi que du contrôle possible sur le devenir de la cellule après transplantation.

En conclusion ce travail ouvre la voie à une nouvelle génération de stratégies thérapeutiques pour le traitement des maladies vasculaires ischémiques, mais il a
également un impact important sur les efforts actuels en matière d’ingénierie des tissus et de régénération d’une grande variété de tissus par transplantation cellulaire.
# TABLE OF CONTENTS

Dedication .................................................................................................................. IV

Acknowledgements ...................................................................................................... V

Abstract ..................................................................................................................... VIII-XIII

  English .................................................................................................................... VIII

  Portuguese ............................................................................................................... X

  French ..................................................................................................................... XIII

List of Figures ............................................................................................................ XIX

List of Tables ............................................................................................................ XXV

List of Appendices .................................................................................................... XXVI

## CHAPTER 1

Introduction .................................................................................................................. 1

  1.1 Problem Statement ............................................................................................. 1

  1.2 Hypothesis and Specific Aims ......................................................................... 7

  1.3 Significance ....................................................................................................... 7

  1.4 Outline of Thesis .............................................................................................. 9

  1.5 References ....................................................................................................... 9

## CHAPTER 2

Mechanisms of neovascularization, synthetic extracellular matrix approaches for therapeutic angiogenesis ............................................................................................................. 11
2.1 Introduction ............................................................. 11
2.2 Mechanisms of Neovascularization ............................... 11
2.3 Growth Factors ....................................................... 22
2.4 Therapeutic Angiogenesis ........................................... 27
2.5 Synthetic Extracellular Matrices for Therapeutic Angiogenesis... 28
2.6 Summary .................................................................. 34
2.7 References .............................................................. 35

CHAPTER 3
The role of distinct VEGF isoforms on endothelial cell behavior and the kinetics of endothelial cell sprouting in vitro ................................................................. 49

3.1 Introduction ............................................................. 49
3.2 Materials and Methods .............................................. 54
3.3 Results .................................................................. 57
3.4 Discussion .............................................................. 68
3.5 References .............................................................. 71

CHAPTER 4
Spatiotemporal control of VEGF isoform delivery enhances in vivo neovascularization ................................................................. 76

4.1 Introduction ............................................................. 76
4.2 Materials and Methods .............................................. 79
4.3 Results .................................................................. 86
4.4 Discussion .............................................................. 114
4.5 References .............................................................. 120
CHAPTER 5
Alginate as synthetic extracellular matrix for progenitor cell delivery ............ 124

5.1 Introduction ............................................................................. 124
5.2 Materials and Methods .......................................................... 130
5.3 Results ................................................................................... 138
5.4 Discussion ............................................................................. 166
5.5 References ............................................................................. 173

CHAPTER 6
Conclusions, implications, and future directions ............................................ 179

6.1 Conclusions ........................................................................... 179
6.2 Implications ........................................................................... 182
6.3 Future directions ..................................................................... 185
6.4 References ............................................................................. 189

APPENDICES .................................................................................. 191
Figure D1 Definition of nascent sprout, mature sprout and lumen ............... 196
Figure F1 Schematic illustration of alginate formulation and processing .... 198
Figure II Image of lower ventral view from a female SCID mice and identification of different regions ................................................................. 202
LIST OF FIGURES

CHAPTER 1

Figure 1.1 Main causes of mortality worldwide .................................................. 2
Figure 1.2 Bioactive factor delivery strategy for therapeutic angiogenesis .......... 4
Figure 1.3 Schematic representation of two tissue engineering approaches using synthetic ECMs .......................................................... 6

CHAPTER 2

Figure 2.1 Schematic representation of blood vessel hierarchy and systematic organization .......................................................... 13
Figure 2.2 Mechanisms of neovascularization: vasculogenesis in embryo and adult .......................................................... 15
Figure 2.3 Mechanisms of neovascularization: angiogenesis .......................... 18
Figure 2.4 Mechanisms of neovascularization: arteriogenesis ...................... 21
Figure 2.5 Cross talk between cells mediated by growth factors .................... 24
Figure 2.6 The different isoforms of human VEGF ....................................... 25
Figure 2.7 Two different forms of synthetic ECMs ...................................... 31

CHAPTER 3

Figure 3.1 DIC photomicrograph of human microvascular endothelial cells in culture .......................................................... 52
Figure 3.2 Photomicrograph of microcarriers coated with HMVEC-d .......... 53
Figure 3.3 Quantification of endothelial cell proliferation under different VEGF_{165} concentrations .......................................................... 58
Figure 3.4 Quantification of endothelial cell proliferation under different VEGF_{121} concentrations.......................................................... 60

Figure 3.5 In vitro VEGF bioactivity degradation by endothelial cells.............. 61

Figure 3.6 Quantification of different human cells VEGF secretion under different oxygen tensions........................................................................... 62

Figure 3.7 Photomicrograph of sprouting cells from a microcarrier............... 64

Figure 3.8 Quantification of number of sprouts and length formed by HMVEC-d in response to VEGF stimulation......................................................... 65

Figure 3.9 Quantification of number of sprouts and length formed by HMVEC-d in response to different temporal exposure to VEGF................................. 67

CHAPTER 4

Figure 4.1 Alginate structure and gelation mechanism........................................... 78

Figure 4.2 VEGF_{165} release from alginate gels formulated with different solvent solutions and polymer molecular weight distributions................................. 88

Figure 4.3 Chemical structure of partially oxidized alginate................................... 89

Figure 4.4 Dry weight loss of different alginate gels............................................... 90

Figure 4.5 Rheological properties of pre-gelled alginate solutions......................... 92

Figure 4.6 Release of different VEGF isoforms from alginate gels......................... 93

Figure 4.7 Effect of partially oxidation on VEGF_{165} release from different alginate gels............................................................................................................. 94

Figure 4.8 Bioactivity of VEGF_{165} released from alginate gels......................... 95

Figure 4.9 Endothelial cell sprouting in response to VEGF isoforms delivered from alginate gels.............................................................................................. 96
Figure 4.10 Quantification of phosphorylated ERK1/2 in VEGF_{165} stimulated endothelial cells ................................................................. 98

Figure 4.11 Gross photograph of ischemic hindlimb tissue model displaying tissue regions utilized for analysis .................................................. 99

Figure 4.12 VEGF localization on injection site 24 hours after delivery .......... 100

Figure 4.13 Relative VEGF spatial biodistribution in hindlimb tissue 12 hours after delivery ................................................................. 102

Figure 4.14 Temporal distribution of VEGF_{165} delivered in bolus form into ischemic hindlimb tissue ....................................................... 103

Figure 4.15 Temporal distribution of VEGF_{121} delivered from gels in ischemic hindlimb tissue ............................................................. 104

Figure 4.16 Temporal distribution of VEGF_{165} delivered from gels in ischemic hindlimb tissue ............................................................. 105

Figure 4.17 VEGF levels present in peripheral serum 24 hours after delivery ................................................................. 106

Figure 4.18 VEGF_{165} dose response in ApoE^{-/-} ischemic hindlimb model..... 108

Figure 4.19 Photomicrographs of tissue sections from hindlimbs of ApoE^{-/-} mice at postoperative day 42, immunostained for the endothelial marker CD31..... 109

Figure 4.20 Quantification of blood vessel densities of hindlimb ApoE^{-/-} muscle .............................................................................. 110

Figure 4.21 Representative color-coded LDPI images at various time points, and gross photographs of ApoE^{-/-} mice at postoperative day 42................................. 112

Figure 4.22 Regional perfusion profile from ApoE^{-/-} mice hindlimbs at various time points ....................................................................... 113

Figure 4.23 Distribution of hindlimb ischemia severity during 6 weeks after
CHAPTER 5

Figure 5.1 RGD-modified alginate structure .......................................................... 128

Figure 5.2 Schematic illustration of proposed cell delivery vehicle .................. 129

Figure 5.3 DIC photomicrographs of EPCs and OECs isolated from human umbilical cord blood ................................................................. 140

Figure 5.4 Immunohistochemical photomicrographs of EPCs and OECs isolated from human umbilical cord blood ........................................ 141

Figure 5.5 Telomerase activities of different human endothelial cell populations .......................................................... 142

Figure 5.6 Analysis of angiogenic protein secretion by EPCs, OECs and ECs ........................................................................................................ 143

Figure 5.7 Proliferation of OECs and ECs in response to different VEGF isoforms .......................................................... 144

Figure 5.8 Representative photomicrographs and quantification of OECs that migrated out from alginate scaffolds ......................... 146

Figure 5.9 Quantification of OECs that populated tissue mimics after scaffold migration .......................................................... 147

Figure 5.10 Visualization and quantification of OECs cell viability after migration out from scaffolds .......................................................... 148

Figure 5.11 Proliferation of control OECs and OECs that migrated out from scaffolds ................................................................................ 149

Figure 5.12 In vitro analysis of the angiogenic effect of EPCs and OECs utilizing a sprouting assay .......................................................... 151
Figure 5.13 Representative gross photographs of hindlimb SCID mice at postoperative day 3 and day 15 ................................................................. 152

Figure 5.14 Distribution of hindlimb ischemia severity on SCID mice during 2 weeks after surgery ................................................................. 154

Figure 5.15 Representative photomicrographs tissue sections of hindlimb SCID mice at postoperative day 15, immunostained for the mouse endothelial marker CD31 ................................................................. 155

Figure 5.16 Quantification of blood vessel densities of hindlimb SCID mice at 15 days postoperative ................................................................. 156

Figure 5.17 Photomicrographs of tissue sections from hindlimbs of SCID mice at postoperative day 15, immunostained for human endothelial marker CD31 ................................................................. 157

Figure 5.18 Gross photographs of ischemic hindlimbs as a function of time post-surgery ................................................................. 159

Figure 5.19 Distribution of hindlimb ischemia severity during 6 weeks after surgery ................................................................. 160

Figure 5.20 Regional blood perfusion profile from SCID mice hindlimbs at various time points ................................................................. 161

Figure 5.21 Gross photographs and perfusion images of ischemic hindlimbs as a function of time post-surgery ................................................................. 162

Figure 5.22 Photomicrographs of tissue sections from hindlimb SCID mice at 42 days postoperative, stained with haematoxylin and eosin ................................................................. 163

Figure 5.23 Photomicrographs of tissue sections from hindlimb SCID mice at 42 days postoperative, immunostained for the mouse endothelial marker CD31 ................................................................. 164
Figure 5.24 Quantification of blood vessel densities of hindlimb SCID mice at 42 days postoperative...
LIST OF TABLES

CHAPTER 2

Table 2.1 Description of positive and negative factors involved in blood vessel formation........................................................................................................19
LIST OF APPENDICES

APPENDIX .......................................................................................................................... 191

A  VEGF bioactivity degradation in vitro ............................................................. 191
B  Cell culture under hypoxic conditions ............................................................. 193
C  3D in vitro sprouting assay .............................................................................. 194
D  Schematic representation of sprout definition ................................................. 196
E  Alginate oxidation ............................................................................................. 197
F  Alginate gel processing ....................................................................................... 198
G  VEGF$_{121}$ iodination ........................................................................................ 199
H  Immunological detection of phosphorylation .................................................. 200
I  Mouse regions of body – lower ventral view ..................................................... 202
J  Animal model of severe hindlimb ischemia ....................................................... 203
L  In vivo protein quantification ........................................................................... 205
M  Mouse CD31 immunostaining for mouse tissue .............................................. 206
N  Laser Doppler perfusion imaging (LDPI) ........................................................ 208
O  Ischemic grade .................................................................................................... 209
P  Isolation and culture of endothelial progenitors cells from
human cord blood .................................................................................................... 210
Q  Peptide modification of alginate ...................................................................... 213
R  Fluorescent cell labeling in sprouting assay ..................................................... 215
S  Human CD31 immunostaining for mouse tissue .............................................. 216
CHAPTER 1

Introduction

1.1 Problem Statement

The establishment of a vascular network represents an essential step in the all tissue development, and dysfunctional and unregulated formation of vascular networks are intrinsically associated with many pathologically disorders (e.g., ischemic diseases, cancer). There exists a profound need to control blood vessel formation in ischemic diseases, in particular, as they remain the dominant cause of mortality worldwide (Figure 1.1).

More specifically, one appealing clinical target for neovascularization therapies is peripheral vascular disease (PVD). PDV is typically caused by atherosclerotic occlusion of the vessels to the limbs. It is associated with a substantial risk of illness and death, resulting in a marked reduction in ambulatory capacity and quality of life. Not only do a large number of patients suffer from other pathologies predisposing them to develop PVD (e.g. diabetes, hypertension), but also the severity of peripheral arterial disease is closely related with the risk of myocardial infarction,
Figure 1.1 – Main causes of mortality worldwide.

1. Source
ischemic stroke, and death from vascular causes. In the United States alone PVD affects more than 8 million people, and it is predicted to become increasingly prevalent as life expectancy increases. Current therapies for PVD rely on pharmacologic treatments or surgical procedures. However, a considerable number of patients are not candidates for these treatments. Instead, the large majority of these pharmacologic treatments are specifically directed either to induce a risk-factor modification (e.g., use of antiplatelet drugs) or to treat the symptoms, rather than targeting neovascularization mechanisms. Despite the great advances in medicine in the last decades, PVD is still considered as an undertreated disease, motivating the development of alternative therapies. The induction of new blood vessels throughout branching from pre-existing vessels could be a very useful strategy to treat these patients (Figure 1.2).

Beyond prospects for PVDs treatment, it is also necessary to develop alternative therapies to treat patients who generally suffer from the loss or failure of organs and tissues. The numbers of individuals willing or able to donate their organs and tissues versus those that require transplantation therapies represents a major discrepancy. For example, the annual number of putative candidates for heart transplantation is approximately 18,000, but only 2,192 patients received heart transplants in the United States in 2006 due to the lack of donor organs. In fact, every 16 minutes a new patient requires organ transplantation. The fields of tissue engineering and regenerative medicine represent sophisticated attempts to address this significant number of patients who need a new or improved organ or tissue. In these fields, a variety of life sciences and engineering disciplines are integrated with the goal of promoting and controlling new tissue formation or regeneration. The ultimate goal is to develop synthetic constructs that restore and enhance the functions of
Figure 1.2 – Bioactive factor delivery strategy for therapeutic angiogenesis.

Cells located more than hundred micrometers (oxygen diffusion limit) can likely experience progressive hypoxia (cells in magenta) as compared with cells in nearby vessels (cells in yellow). New blood vessel formation directly stimulated via the delivery of specific bioactive factors (blue gradient) can rescue these hypoxic cells.
healthy tissues (Figure 1.3). Development biology is one of the main sources of inspiration for tissue engineering strategies. The developmental mechanisms and events serve as templates to design new synthetic extracellular matrices (ECMs). A long and extensive list of tissues are currently being develop using tissue engineering approaches, but their success will rely on the creation of a functional vascular network capable of providing the metabolic needs of the engineered tissue and facilitating integration with native tissue. Thus there exists a critical need for neovascularization in all tissue engineering strategies.

The molecular and genetic mechanisms involved in neovascularization have been extensively studied in the past decades, and several factors have been identified as important regulators of neovascularization, including vascular endothelial growth factor (VEGF). In particular, VEGF has been tested in several pre-clinical and clinical studies with a therapeutic objective. However, the predominant delivery strategies used in these studies were very rudimentary, ignoring several critical aspects needed for neovascularization, including appropriate targeting, low systemic exposure and extended time of exposure.

An alternative strategy to induce neovascularization is the delivery of specialized cell populations, and several cell populations have recently been reported to directly contribute to new blood vessel formation, including mature endothelial cells, bone marrow derived cells and stem cell populations. Surprisingly, the limitations of the strategies utilized for growth factor delivery had not been addressed in these more recent cell based therapies, as basic biologic requirements necessary to obtain a therapeutic benefit are likely being disregarded. A significant enhancement in therapeutic benefit can potentially be achieved with the development of effective carriers capable of providing angiogenic factors in a spatiotemporal fashion matching
Figure 1.3 – Schematic representation of two tissue engineering approaches using synthetic ECMs.

Bioactive factors (cyan spheres) can be delivered from synthetic ECMs (blue) to activate target quiescent cell populations (orange cells) and instruct them to migrate towards diseased site (red area) and direct tissue regeneration (A). Cell transplantation strategies utilize the delivery of specific cell population (green cells) to the defect site in order to orchestrate new tissue formation in association with native cells (B).
biological needs, and formulating of cell delivery vehicles that presents specific cues to transplanted cells, using localized factor delivery, may allow one to regulate and govern neovascularization.

1.2 Hypothesis and Specific Aims

The hypothesis guiding this dissertation research is that a functional and mature vascular network may be asserted in an ischemic inferior limb by the appropriate delivery of endothelial progenitor cells and angiogenic growth factors using biodegradable polymer carriers.

This hypothesis will be addressed with the following specific aims:

Aim 1: Determine the kinetic effects of growth factor stimuli on endothelial cell sprouting in a three-dimensional in vitro sprouting assay.

Aim 2: Develop an injectable biodegradable polymer carrier capable of minimally invasive delivery of angiogenic factors in desirable spatiotemporal profile in an in vivo model of ischemia.

Aim 3: Design a biodegradable polymer scaffold embedded with a bioactive architecture that governs localized progenitor cell delivery in an in vivo model of ischemia.

1.3 Significance
The neovascularization strategies developed in this thesis may have a direct impact on current efforts to reestablished perfusion of ischemic inferior limbs. Neovascularization events are highly regulated by the local presentation of specific microenvironmental cues, and this work describes how host tissues respond to a local presentation of angiogenic factors. The systems developed in this thesis may greatly improve the utility of both the growth factors and progenitors cells currently being scrutinized for use in therapeutic angiogenesis. The delivery systems formulated in this thesis will likely be quite versatile, and could easily be replicated for applications perhaps more complex than those proposed in this thesis, including the treatment of ischemic heart diseases\(^7\). Regardless of recent advances in understanding the molecular and cellular mechanisms involving neovascularization, several aspects of these processes are still unclear and these material systems may provide useful models to decipher some of these signaling pathways. From a different perspective, the mechanisms related to the destabilization and regression of blood vessels can also likely be investigated using these systems, by taking advantage of the delivery principles delineated in this thesis to provide alternative approaches for anti-angiogenic therapies (e.g. tumor regression). More broadly, these polymer systems provide novel models to study and manipulate a multiplicity of physiologic events that rely on the signaling of multiple growth factors. The multifaceted ability of scaffold architecture to instruct cell survival and migration postulated in this thesis may also be replicated to obtain a greater control over other cell populations targeting distinct tissues (e.g. diabetes). Furthermore, it may be particularly important from a clinically perspective, to translate some of the principles developed in this thesis to obtain control over the mechanisms of stem cell differentiation.
1.4 Outline of Thesis Contents

Chapter 2 provides a review of the background necessary to understand the motivations and challenges addressed by this thesis. Chapter 3, which addresses Aim 1, evaluates the mechanisms that govern in vitro endothelial cell sprouting kinetics. This chapter also provides important information relating growth factors and modulation of endothelial cell behavior in vitro. Chapter 4, which addresses Aim 2, describes efforts to design a biodegradable delivery vehicle that can be delivered using minimally invasive techniques and are capable of releasing growth factors in a spatiotemporal fashion. This chapter also describes how appropriate presentation of angiogenic factors can direct functional angiogenesis in vivo. Chapter 5, which focuses on Aim 3, describes an alternative approach for cell delivery, in which a material system is used to create microenvironment containing cues sufficient to extend the viability and outward migration of human vascular progenitor cells following their transplantation. The results of this chapter describe how this type of system can dramatically improve the efficacy of these cells in salvaging ischemic murine limbs and returning perfusion to normal levels, and also investigate the specific role of distinct vascular progenitor cells in the stimulation of angiogenesis. Finally, Chapter 6 presents a summary of principal findings, their implications for therapeutic angiogenesis and regenerative medicine, and possible future research directions.

1.5 References


CHAPTER 2

Mechanisms of neovascularization, synthetic extracellular matrix approaches for therapeutic angiogenesis

2.1 Introduction

This chapter provides a comprehensive framework to better understand the motivations and hypothesis supporting this thesis. First this section describes the biologic mechanisms associated with blood vessel formation in both development and in the adult, including normal and pathologic formation. In particular, the importance of certain key biologic players in the regulation of neovascularization will be reviewed. Subsequently, different and alternative strategies utilized to mimic the natural neovascularization events will be addressed. Finally, the utilization of synthetic extracellular matrices for the delivery of growth factors and cells capable of orchestrating neovascularization events is reviewed.

2.2 Mechanisms of Neovascularization
Vascular system

All tissues of mammalian organisms are derived from three different embryonic germ layers: mesoderm, endoderm and ectoderm. Mesoderm originates bone, muscle and mesenchymal connective tissue. Endoderm gives rise to digestive organs and the epithelium of the lungs. Finally, the ectoderm layers produce the nervous system and the epithelium of the skin and its derivatives. The development of the vascular system, germinated from the mesodermic germ layer, is one of the earliest systems to be established during organogenesis.

The vascular system is responsible for blood transport to and from all tissues, and it can be decomposed into two distinct parts: the systemic circulation and the pulmonary circulation. The heart is the central organ of the vascular system, coordinating the blood circulation to all tissues of body. Blood is responsible for delivering nutrients and other essential substances (such as oxygen) and removing wastes and regulating the body temperature. Blood leaves the heart via the aorta and this vessel subsequently divides into smaller arteries and arterioles, throughout the body, terminating in a multibranching system (capillary beds) that match up to venules and ultimately return blood to the heart via the vena cava (Figure 2.1). Although there exists different divisions (arteries, veins) and subdivisions (venules, arterioles), all blood vessels are tubular channels formed from a single layer of endothelial cells, which could or could not be coated with mural cells (smooth muscle cells or pericytes). For example, capillaries (≈1 mm long and 5-10 μm in diameter) lack the mural cell coat, while the aorta (≈ 2.5 cm in diameter) displays multiple layers of smooth muscle cells coating the single layer of endothelial cells. The densities of vessels in a particular tissue rely on the metabolic tissue needs, ranging
Figure 2.1 – Schematic representation of blood vessel hierarchy and systematic organization.

Blood circulating in the arteries reaches all body tissues via the arterioles. The capillary bed is the meeting point between the arterioles and the venules. At this intersection, blood is transferred to venules and re-directed towards the heart. The majority of nutrient and waste removal occurs in these small capillaries.
from dense in human myocardium to sparse on the cornea of the eye. The nutrient transport and waste removal mainly take place across the small capillaries, and this mechanism is predominantly regulated by diffusion, resulting in substances with low permeability (large molecules, lipid insoluble molecules) diffusing slowly, while substances that can go through cell-cell connections (small molecules) diffusing rapidly (for example O₂ and CO₂). Overall, vascular networks are tightly regulated in vivo, resulting in a high level of spatial organization critical to preserve a tissue’s viability and function. The remodeling or formation of new vascular networks, neovascularization, can be pursued by three different mechanisms: vaculogenesis, angiogenesis and arteriogenesis.

Vasculogenesis

Vasculogenesis is a term used to describe blood vessel formation directly orchestrated by endothelial precursor cells. Until very recently vasculogenesis was exclusively associated with the concept of blood vessel formation during embryogenesis, however recent data documented the presence of endothelial progenitors cells in the adult ², leading to speculation that vasculogenesis can also occur in adults ³⁵ (Figure 2.2).

In the embryo, vasculogenesis arises very early after the initiation of gastrulation, and the starting step is intrinsically associated with mesoderm formation. A specific population of cells described as fibroblasts-like cells migrate out from ectoderm and endoderm forming the early mesoderm. These subsets of migratory cells expressing vascular endothelial growth factor receptor-2 (VEGFR-2 / Flk-1 – fetal liver kinase / KDR - kinase-insert domain receptor) are recognized as the
Figure 2.2 – Mechanisms of neovascularization: vasculogenesis in embryo and adult.

Vasculogenesis can occur during embryonic development or in the adult. In the embryo, clusters of angioblasts (precursor of endothelial cells) surround agglomerates of hematopoietic cells. These cells fuse with each other to form a capillary network (primary capillary plexus). Vasculogenesis, in the adult, involves the recruitment of endothelial precursor cells to a specific location where they will mediate new blood vessel formation.
angioblast precursor cells \(^6\text{-}^9\). Under the influence of specific molecular and microenvironmental cues \(^{10}\), these cells are instructed to self-organize and assemble into small clusters that are called blood islands \(^{11}\). The peripheral cells lining the blood islands are the precursors of endothelial cells – angioblasts; and the cells localized inside will give rise to the hematopoietic stem cells \(^7\text{-}^{13}\). Then a process of remodeling and adaptation will drive these blood islands to fuse together, resulting in the formation of a lumen structure, and finally, the creation of a primitive vascular plexus \(^{11}\). The exact molecular mechanisms that govern vasculogenesis are still uncertain, however several genes and proteins have been reported to regulate the process of vasculogenesis during embryonic stages, including \textit{Hex} \(^{14}\), basic helix-loop-helix (bHLH) \(^{15}\) and \textit{Vegf} \(^{13}\). The proteins responsible for regulating this process include basic fibroblast growth factor (bFGF) \(^{11,13,16}\), bone morphogenic protein 4 (BMP4) \(^{7,12,15}\) and substantially \textit{VEGF} \(^{7,11,13,15-20}\).

The identification of endothelial progenitor cells circulating in adults suggests that not only can these cells migrate from vessels in the embryo, but they can also be recruited to form capillaries in the adult \(^{21}\) leading to the idea that vasculogenesis and angiogenesis may occur simultaneously in the adult. The isolation of circulating progenitors cells in peripheral blood followed by their differentiation by local signals may allow one to direct vasculogenesis in a therapeutic sense to treat tissue ischemia. However, opposite to vessel formation in the embryo, where the activity and proliferation of endothelial cell precursors is high, in adults these cells remain in an extraordinary latent phase and they circulate in the blood stream in very small numbers \(^{22}\). Studies estimate that the interval for ECs entrance to the circulation is less than 1000 days \(^{23}\). Granulocyte monocyte-colony stimulation factor (GM-CSF) \(^{24,25}\) and \textit{VEGF} \(^{15,25,26}\) have been reported to play critical roles in the control of
vasculogenesis in adults, however very little is known about the direct mechanism of interactions between the microenvironment cues and soluble factors.

**Angiogenesis**

The designation “angiogenesis” was introduced in 1935 to describe the formation of new blood vessels in the placenta 27. Nowadays the term is more specific, and describes the sprouting and stabilization of new blood vessels from pre-existing vessels 15 (Figure 2.3). This process involves a cascade of events, including endothelial cell activation, recruitment and proliferation followed by interactions with mural cells for the stabilization of the initially immature new vasculature. A large number of angiogenic and anti-angiogenic signals (Table 2.1) and microenvironment cues have been identified, and these factors are presented locally in a well-regulated way to control angiogenesis. In a simple view, angiogenesis may be described as a process first involving the vasodilation of existing vessels, an event that is mainly mediated by VEGF, accompanied by an increase of the vascular permeability. Associated with these processes is degradation of the extracellular matrix (ECM), which is necessary for the subsequent endothelial cell migration. These processes are regulated by angiopoietin-1 (Ang1), and its antagonist angiopoietin-2 (Ang2) 15. Degradation of extracellular matrix is mainly orchestrated by matrix metalloproteinases (MMPs) secreted by the ECs. The assembly of immature vessels networks follows endothelial cell proliferation and migration. Recently it was reported that monocyte chemotactic protein (MCP)-1 can also elicit endothelial cell proliferation 28. The immature endothelial cell assemblies are susceptible to regression due to endothelial cell apoptosis. VEGF, Ang1 and Ang2 are key players in
Figure 2.3 – Mechanisms of neovascularization: angiogenesis.

Angiogenesis, the sprouting of new blood vessels from pre-existing vessels, requires several essential events: protease induction followed by degradation of ECM; local recruitment of endothelial cells; outward migration of endothelial cells in tandem to form a capillary sprout; proliferation within the sprout; and the formation of a lumen structure. This process is largely regulated by VEGF. Thereafter, the vessel sprout is stabilized by the recruitment of mural cells (pericytes or smooth muscle cells).
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<tr>
<th>Pro angiogenic factors</th>
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<td>Angiopoietin</td>
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<td>Acidic and basic Fibroblast Growth Factor (aFGF and bFGF)</td>
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<td>Epidermal Growth Factor (EGF)</td>
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<td>Hepatocyte Growth Factor (HGF)</td>
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<td>Insulin-like Growth Factor (IGF-1 and IGF-2)</td>
<td>Pigment Epithelium-Derived Factor (PEDF)</td>
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<td>Granulocyte Colony-stimulating Factor (G-CSF)</td>
<td>Interferon (IFN-α and IFN-β)</td>
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<td>Placental Growth Factor (PIGF)</td>
<td>Thrombospondin-1 (TSP-1)</td>
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<td>Transfroming Growth Factor (TGF-α and TGF-β)</td>
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Table 2.1 – Description of positive and negative factors involved in blood vessel formation.
all steps of these processes. Finally, the vessels mature to form stable vascular networks via the recruitment of smooth muscle cells (SMC), and this process is regulated by platelet derived growth factor (PDGF).

Angiogenesis is not only critical for organ growth and repair, but is also associated with several physiologic events such as menstruation and wound healing and repair. On the other hand, misgoverned angiogenesis is also linked with more than 70 pathologic disorders, including cancer, diabetes and multiple sclerosis. The predominant mechanism of neovascularization in adults is angiogenesis, and an advanced control and manipulation of the angiogenic regulators will likely boost certain therapies in the medical field.

Arteriogenesis

Arteriogenesis describes the compensatory growth of blood vessels after major occlusion of blood flow (Figure 2.4). In spite of the fact that angiogenesis and arteriogenesis share some common characteristics, these two neovascularization mechanisms present distinct triggers and molecular mechanisms. For example, angiogenesis is mainly induced by hypoxia and results in new capillaries, while arteriogenesis is triggered by physical forces resulting in pre-existing vessel enlargement. Specifically blood flow shear stresses induce the compensatory process of adaptation of the existing collaterals, resulting in profound effects on the functional expression of the endothelial and mural cells. The exact molecular mechanisms required to adapt to changes in physiologic demands of blood are unknown, however distinct genes and growth factors have been described to be controlled by shear stress including GM-CSF, transforming growth factor-β (TGF-β),
Figure 2.4 – Mechanisms of neovascularization: arteriogenesis.

Arteriogenesis results in the growth of pre-existing collateral vessels after occlusion of a supply vessel. Blood shear stresses directly induce the remodeling and compensatory process of enlargement of collaterals vessels.
and monocyte chemoattractant protein-1 (MCP-1) \(^{35,38}\). Subsequent to the enlargement of the pre-existing collateral vessels, a process of maturation is necessary to maintain appropriate blood circulation. In this process, monocytes play a critical role not only by releasing several bioactive factors including proteases, chemokines and growth factors, but also by mediating cell migration \(^{36}\).

### 2.3 Growth Factors

Growth factors are soluble secreted signaling proteins capable of instructing specific cellular responses \(^{39}\). The final result of cellular cross talk intermediated by a growth factor results in a very wide range of cellular instructions, including cell survival, and control over migration, differentiation or proliferation of a specific subset of cells. This frequent intercellular transmission mechanism initiates with growth factor secretion by the producer cell directed to reach the target cell populations. The growth factor subsequently and specifically binds to the target via transmembrane cell receptors (Figure 2.5). After the binding of the ligand, an intricate signal transduction pathway is activated, involving protein phosphorylation, ion fluxes, changes in metabolism, gene expression, protein synthesis and ultimately an integrated biological response \(^{40}\). The ability of a signaling molecule to deliver a particular message to a distinct subpopulation of cells is not only determined by the soluble factor, but also by its receptor and the intracellular signal transduction. Growth factors can be multifunctional, where the same growth factor can convey different instructions depending on the receptor type to which it binds on or in the target cell. Moreover, the same receptor can translate different messages depending on the intracellular transduction pathways, which can differ from one cell type to
Figure 2.5 – Cross talk between cells mediated by growth factors.

The producer cell secretes soluble growth factors that bind to target cell receptors. The instructions are translated into the cell through complex signal transduction networks resulting in a specific biological cellular response.
another. The ultimate response of a target cell to a particular soluble growth factor can also be governed by external factors, including the ability of the factors to bind to ECM, ECM degradation, and growth factor concentration and cell target location \cite{41}. Control over this overall growth factor regulatory system is critical to instruct specific cellular decisions.

**Vascular Endothelial Growth Factor**

VEGF-A (or vascular permeability factor – VPF) is a key regulator of new blood formation during vasculogenesis and physiological and pathological angiogenesis that was isolated and purified in 1989 \cite{42-44}. VEGF-A is a member of the VEGF family, and this family includes 6 other members: VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and finally placental growth factor (PIGF). Humans do not produce VEGF-E and VEGF-F, while VEGF-C and VEGF-D are primarily lymphoangiogenic factors. On the other hand the exact role of PIGF in new blood vessel formation is not yet well established \cite{45}. VEGF-A displays a dimeric cysteine-knot structure related to platelet-derived growth factors \cite{46,47} (all further references in this thesis to VEGF refer to VEGF-A member). The human VEGF gene is located at chromosome 6p21.3 and encodes 8 exons and 7 introns \cite{26,48} (Figure 2.6). Alternative mRNA splicing generates six VEGF isoforms, and these differ by the presence or absence of exons 6 and 7 \cite{49}, meaning that each monomer has 121, 145, 165, 189, or 206 amino acids, respectively \cite{45}. The splice variants of VEGF with 121 and 165 amino acids are the most common and abundantly secreted. The exon 7 contains the heparin binding domain, with the result that VEGF_{121} and VEGF_{165} differ by the presence of the binding sites for heparan sulphate and extracellular matrix \cite{26,45,48,50,51}.  

24
Figure 2.6 – The different isoforms of human VEGF.

The human *VEGF* gene encodes 8 exons and 7 introns, and by alternative splicing, 6 different isoforms are produced. VEGF<sub>121</sub> and VEGF<sub>165</sub> are the most abundant isoforms. The presence of exon 7 on VEGF<sub>165</sub> isoform provides ECM binding competence. VEGF<sub>121</sub>, however, lacks this exon and its absence makes this isoform a very diffusible factor.
Due to the lack of the heparin binding domain VEGF_{121} is a very diffusible protein, in contrast to VEGF_{165}, which moderately binds to extracellular matrix. In contrast, VEGF_{189} and VEGF_{206} are secreted, but strongly bond to heparin-containing proteoglycans on the cell surface or basement membrane, and consequently remain cell associated \(^{45}\).

All VEGF isoforms bind with high affinity to two receptors: VEGF receptor 1 (VEGFR-1 / Flt-1 – fms-like tyrosine kinase) \(^{52}\) and VEGFR-2 \(^{12,53}\). These receptors express seven extracellular immunoglobulin (Ig) homology domains, followed by a transmembrane region and a conserved intracellular tyrosine kinase \(^{45,50}\). The second and the third Ig domains have been reported as the high-affinity ligand binding areas for VEGFR-1 and VEGFR-2 respectively \(^{49}\). Despite the higher binding affinity of VEGF to VEGFR-1 than to VEGFR-2, VEGFR-2 predominantly translates VEGF signals in endothelial cells \(^{54}\). However, VEGFR-1 can heterodimerize with VEGFR-2 resulting in a synergistic effect on the signaling properties \(^{55}\). High levels of VEGF are generally linked with hypoxia (and ischemia) essentially because the VEGF gene promoter is upregulated by the activation of hypoxia inducible factor -1 (HIF-1) \(^{15}\). Expression of VEGF receptors is not exclusive of endothelial cells \(^{50}\), as VEGFR-1 is also expressed in monocytes \(^{56-58}\), smooth muscle cells \(^{59}\), megakaryocytic precursors \(^{58}\), and renal mesangial cells \(^{60}\). VEGFR-2 is present in hemapoietic cells \(^{61-64}\), testicular tissue \(^{65}\), retinal progenitor cells \(^{66}\) and in pancreatic duct cells \(^{67}\). Other cell surface membranes had been reported to act as VEGF co-receptors, including \(\alpha_v\beta_3\) integrins \(^{19}\) and cadherins \(^{50}\). Neuropilin 1 (Nrp-1) can also serve as a VEGF co-receptor enhancing VEGF_{165} - VEGFR-2 interactions \(^{68,69}\).

VEGF primarily targets endothelial cells, and is responsible for the proliferation and migration of these cells. However, VEGF also acts as a potent
survival factor for several other cell types \(^{70,71}\), and has been reported to directly influence osteoblasts \(^{72}\), monocytes \(^{57,73}\), macrophages \(^{73,74}\), neurons \(^{75-78}\) and specific hematopoietic populations \(^{26,79,80}\). In the presence of very low levels of oxygen, cells are instructed to produce and secrete VEGF signals. Despite the fact that endothelial cells are the main target of VEGF, these cells are not the main VEGF suppliers in physiological environment \(^{70,81}\). Interestingly, large pools of VEGF have been found in all malignant tumors types \(^{32}\), emphasizing the concept that VEGF promotes tumor progression and metastasis \(^{32,34,82}\). Because the importance of VEGF as a key regulator in new blood vessel formation is undisputed, this protein has been widely utilized in numerous therapeutic applications including clinical trials in patient with coronary or limb ischemia \(^{83,84}\).

### 2.4 Therapeutic Angiogenesis

Therapeutic angiogenesis describes the delivery of angiogenic factors to drive new blood vessel formation in an ischemic tissue. The therapeutic implications of angiogenic growth factors were idealized more than 35 years ago, when Judah Folkman documented that tumor growth is dependent on neovascularization, suggesting that this might imply local regulation of specific angiogenic factors \(^{82}\). The concept that tumors are angiogenesis-dependent inspired considerable attention and efforts orientated not only to better understand the mechanisms that govern blood vessel formation, but also on the discovery and purification of the key factors responsible for the physiological and pathological angiogenesis. In parallel to the studies aimed to inhibit blood vessels formation, several researchers began to investigate the induction of blood vessel formation in ischemic areas, as stimulated by
angiogenic factors. Subsequently, the idea that the delivery of angiogenic factors may be utilized to develop therapeutic approaches for ischemic diseases was tested by an intracoronary injection of FGF in a canine model subjected to coronary obstruction, resulting in an improvement of cardiac systolic function and reduction of the infarcted area. Currently, the number of growth factors tested in therapeutic angiogenesis is considerable, including VEGF and PDGF. Therapeutic angiogenesis can be achieved by using either recombinant proteins or by the delivery of genes encoding these proteins.

Recently these strategies for angiogenesis were translated to several clinical trials. VEGF and b-FGF have been extensively used in phase I/II clinical trials, but no results for phase III trials have been reported to date. Phase I trials typically have reported promising results. However the results obtained in the larger phase II trials have not shown the expected benefit to patients. Nevertheless, this disappointing clinical result with angiogenic factors represents a challenge for the development of alternative strategies capable of inducing a sustainable physiological neovascularization.

2.5 Synthetic Extracellular Matrices for Therapeutic Angiogenesis

The failure experienced with the phase II clinical trials of the therapeutic angiogenesis may be attributable to several causes, including the formulation of the growth factor used, the dose utilized, the route of administration, and/or inappropriate clinical trial design (e.g. selection of patients). One potential limitation to current approaches in therapeutic angiogenesis is the mode of delivery. Typically, this involves the introduction of large doses of potent angiogenic molecules, formulated in
solution form, directly into the body. This administration of supraphysiolog-
cal concentrations of growth factors may not allow sufficient levels of the factors to be
sensed by target tissue for the necessary time frame, and may lead to severe side
effects. Degradation of growth factors in vivo can occur via several and distinct
pathways including denaturation, oxidation or proteolysis. In particular, VEGF
presents a biological half-life of less than 30 minutes when infused intravenously,
resulting in the need for massive doses and multiple injections. However, the use of
large quantities of VEGF should be avoided because it can lead to catastrophic
pathological vessel formation at non-target sites (e.g. tumor formation). The currently
delivery strategies intrinsically lack targeting of specific cell populations, and result in
a transient and inadequate biological response.

Synthetic ECMs may be utilized to bypass these limitations by providing
angiogenic factors in a localized and sustainable manner to desired cell populations.
The bioactive factors are encapsulated into the polymer, preventing their denaturation,
and their release can be controlled by the degradation rate of the polymer or by their
diffusion through the polymer. An alternative approach to direct protein delivery is
to instead use cell therapy strategies to provide locally specific cell populations
capable to orchestrate directly or indirectly new tissue formation.

Protein Delivery

Synthetic ECMs provide an approach to factor delivery that more closely
mimics the normal processes for presentation of the many factors involved in tissue
development and regeneration. This approach allows one to deliver physiologically
relevant concentrations of the factors in a sustainable and localized manner.
Polymeric materials are attractive candidates for this approach, as the protein can be slowly and controllably released from the construct while the polymer protects the incorporated protein from denaturation.

Polymeric delivery vehicles may be either implantable or injectable, and can be fabricated from a variety of synthetic and natural polymers (Figure 2.7). Implantable materials require invasive procedures at the desired site, while injectable materials may be delivered using minimally invasive technologies. A variety of polymers, including poly(glycolide) (PGA), poly(L-lactide) (PLA) and their copolymers (poly(lactide-co-glycolide) (PLG)) have been widely used for protein delivery. These materials are generally considered to be biocompatible and have been used in biomedical applications for over 30 years. These polymers can be formed into a variety of physical structures relevant to drug delivery using several processes, but a key issue is maintenance of the bioactivity of the encapsulated growth factors. A high-pressure gas foaming process was recently developed to allow factor incorporation without the use of organic solvents or high temperatures (both likely lead to protein denaturation), and this system can be used for the delivery of growth factors in vivo in a bioactive form, resulting in sustainable release that can lead to new tissue formation. Polymeric materials can also be useful to investigate the role of combinations of growth factors in angiogenesis, since a synergy between different growth factors has been demonstrated in angiogenesis. For example, a 3-D PLG scaffold has been developed that allows the release of sequences of growth factors. Release of VEGF followed by PDGF led to both a significant increase in the local blood vessel density, and to maturation of the newly formed vessels. Simultaneous delivery of the two factors led to little to no revascularization.


Both synthetic and naturally-derived hydrogel forming materials are also used in growth factor delivery essentially due to their ability to be injected. Synthetic materials (e.g. Poly(ethyleneoxide) (PEO), poly(vinyl alcohol) (PVA)) are advantageous in that their chemistry and physical properties can be readily controlled \(^{108,109}\). On the other hand, naturally-derived gel forming polymers (e.g. alginate, chitosan) typically exhibit excellent biocompatibility. Some physical properties, such as the degradation rate, can be difficult to control with naturally-derived materials. However, covalent modifications can be used to allow control over features such as degradation \(^{109}\). Hydrogels may be particular useful for clinical applications, as they can supply bioactive factors using minimally invasive procedures, and several angiogenic molecules, including PDGF, VEGF, b-FGF have been incorporated into hydrogels \(^{110,111}\). Factor release is typically controlled by diffusion and/or polymer degradation. However, the mechanical environment of the local site may also dramatically influence the release rate \(^{112}\). This effect could be exploited to deliver bioactive molecules in locations under mechanical stress (e.g., heart). VEGF bioactivity is maintained in these systems, and in some cases it seems to be enhanced \(^{113}\).

*Cell Transplantation*

Limitations associated with growth factor strategies (e.g. unpredictable and uncontrolled cell type recruitment, time gap between matrix delivery and tissue development) could be circumvented by directly transplanting the desired cell population to the target site. Cell populations may be delivered without the use a synthetic ECM \(^{114}\), but the use of a synthetic ECM provides an opportunity to both
regulate the gene expression of the transplanted cells and to control the structure and function of the tissues formed either in vitro or in vivo.

The identification of the specific types and sources of cells to be transplanted is critical to the success of this approach. Most often, differentiated cell types related to the target tissue have been used (e.g., dermal fibroblasts are used to engineer dermal tissues) in past work, as this provides more certainty regarding the functionality of the transplanted cell population and the possibility that they may have the genetic pre-programming to reassemble appropriately into the desired tissue or organ structure \(^{115-118}\). The cells may potentially be obtained from autologous, allogeneic or xenogeneic sources. Autologous tissues are the most direct source of cells and reduce problems related with compatibility and immune reactions to the transplanted cells. Allogeneic cells are typically used when there exists a limited supply of autologous cells and/or banking and use of a constant cell source is desirable. Xenogeneic cells are attractive when the autologous and allogeneic sources are insufficient to achieve the necessary cell mass. Recent advances in stem cell research provide encouragement that either embryonic stem cells or adult tissue-derived multipotent cells could be used for tissue repair or formation \(^{119,120}\). Stem cell populations offer the possibility of significant expansion in culture from a small starting tissue mass, and the ability to form multiple cell types from a single starting source.

Synthetic ECMs are frequently utilized to deliver cells to the desired tissue site, to provide the space for tissue development, and to evoke specific cellular responses to modulate tissue architecture and function \(^{121,122}\). The materials utilized include synthetic macroporous scaffolds fabricated from collagen and PLG \(^{123-125}\), and hydrogels formed from synthetic or naturally-derived polymers (e.g., alginate) \(^{126}\).
Due to their structural similarity to the ECM native to many developing tissues (e.g., highly hydrated, space-filling gel), hydrogels are appealing for cell transplantation approaches. Alginate and PGA have been used extensively for new cartilage regeneration via the transplantation of autologous chondrocyte. Combining growth factor delivery approaches with cell transplantation may allow one to modulate the fate of transplanted cells, and this approach is being pursued to regulate more precisely new tissue development and remodeling.

The recently developed ability to isolate specific stem cells populations has generated much enthusiasm in the medical community and led to several clinical trials, but the result of these trials are consistently failing or demonstrating minimal benefit. Rather than a lack of intrinsic bioactivity of the cells, success in this field may instead be limited by our ability to deliver and control the engraftment of these cell populations. In that perspective, the development of sophisticated synthetic extracellular matrices capable of nursing cells during transplantation, and also directly instructing them in vivo will likely bypass some of these pitfalls and eventually boost the therapeutic effect of these cells.

2.6 Summary

There is a tremendous therapeutic need for achieving a regulated control of new blood vessel formation. Therapeutic angiogenesis aims to delivery angiogenic factors capable of reverting tissue ischemia. The biological multitasking properties of VEGF in its induction of angiogenesis have made it an appealing and promising factor for therapeutic applications. Meanwhile, the recent advances in stem cell biology provide the possibility of using these specialized cell populations for clinical
use. However, the standard delivery strategies for both VEGF and cells lack control over location and bioactivity of both after introduction into the body, limiting their therapeutic effect. The biological effects of these factors may be augmented if these suboptimal delivery strategies can be avoided in the future. The use of synthetic extracellular matrices that biologically cooperate with angiogenic factors may represent an attractive new generation of therapeutic delivery vehicles for the treatment of ischemic diseases.

2.7 References


CHAPTER 3

The role of distinct VEGF isoforms on endothelial cell behavior and the kinetics of endothelial cell sprouting \textit{in vitro}

3.1 Introduction

Neovascularization can occur via three different mechanisms: vasculogenesis, angiogenesis and arteriogenesis, and in each process endothelial cells play a critical role, since they constitute the central cellular organizational unit of vascular networks. As reviewed in Chapter 2, endothelial cells can work as a integrators, transducers and effectors of local environmental signals and the recruitment, proliferation, migration and survival of endothelial cells are concisely regulated by microenvironmental cues. The aim of this chapter is to investigate how endothelial cell behavior can be modulated under the influence of different biological signals, such as VEGF isoforms and hypoxia. Further, it evaluates the signals that govern \textit{in vitro} endothelial cell sprouting in a 3-D environment. Finally, the chapter's information on the signals governing endothelial cell behavior will help design alternative approaches for \textit{in vivo} delivery of angiogenic agents.
Endothelial cells (ECs) assemble and remodel vascular networks in a direct response to spatially and temporally environmental cues. These signals vary from soluble cues, such as growth factors, or insoluble cues associated with the local matrix signals. One of most investigated angiogenic factors involved in the regulation of EC fate during neovascularization is VEGF, and many lines of evidence highlight the influence of VEGF over migration, proliferation and survival of ECs. The effects of VEGF on endothelial cell behavior can range from seconds to days: for example, while VEGF will trigger VEGFR-1 and VEGFR-2 phosphorylation and downstream signaling within seconds; survival, proliferation, protection against senescence and reprogramming of gene expression will occur within hours or days. From mRNA alternative splicing of a single gene, several VEGF isoforms are generated, although VEGF$_{121}$ and VEGF$_{165}$ are the most commonly expressed. Cellular responses to VEGF$_{165}$ have been intensely studied, but very little is known about the specific biological responses that VEGF$_{121}$ might induce in ECs. In fact the studies regarding the potency of these isoforms as EC mitogens have generated some contradictory results, and investigating the role of action of these two isoforms may provide useful knowledge to better pattern EC response.

Despite the emphasis on growth factor signaling in mediating EC fate, the insoluble matrix cues presented by ECM are also critical to govern neovascularization events. ECM can regulate the spatial presentation of growth factors by controlling the extent of binding of these factors to the matrix. ECM is also critical in mediating EC migration and maintaining ECs in a quiescent state, by the local presentation of physical and structural links allowing the anchorage of the cellular motility machinery (e.g. actin stress fibers, integrins). For example, several studies have demonstrated
that integrins, a large family of cell surface receptors, are active in regulating angiogenic signaling and EC behavior in physiologic and pathologic events\textsuperscript{16-18}.

\textit{In vitro} models are remarkably useful tools for the study of neovascularization events, since they allow one to screen the effects of specific conditions (e.g. only one or two growth factors) on EC behavior in a tightly controlled environment. Nowadays, it is possible to and obtain isolate human microvascular endothelial cells with a consistent phenotype and genotype from a commercial source, providing a useful cell source for mimicking angiogenic events \textit{in vitro}. It is also important to select the source of the endothelial cells used for culture (e.g. microvascular cells vs umbilical vein cells), since responses of distinct cells may vary considerably\textsuperscript{19}. In this thesis all \textit{in vitro} studies used endothelial cells, refer to human microvascular endothelial cells from a dermal source (HMVEC-d) (Figure 3.1). Several two-dimensional (2-D) \textit{in vitro} models have been frequently used to examine the effect of different angiogenic factors on EC sprout formation, such as collagen gel assays\textsuperscript{20}. However, some of these 2-D assays do not reproduce \textit{in situ} conditions, and typically effects observed in 2-D are not reproduced in 3-D assays\textsuperscript{21-23}. Recently a versatile three-dimensional (3-D) \textit{in vitro} sprouting assay was developed, providing a suitable model to investigate EC behavior under different stimuli. In this assay, ECs attach to gelatin-coated microcarriers (30 cells/microcarrier) (Figure 3.2), as a monolayer, and subsequently are gently immobilized in fibrin gel, resulting in a depot of cells prone to form capillary structures in a 3-D environment. The specific goals of this chapter are to examine the mitogenic potential of different VEGF isoforms on human microvascular endothelial cells. In addition, the effects of distinct oxygen tensions in stimulating HMVEC-d proliferation were also investigated. This chapter investigates
Figure 3.1 DIC photomicrograph of human microvascular endothelial cells in culture.

HMVEC-d, in monolayer culture, display a cobblestone-like morphology. These cells have a central nucleus and a 15-30 μm diameter, and when seeded in appropriate cell density (5 000 cells/cm²) typically will reach confluency within 3-4 days. (100x magnification)
Figure 3.2 Photomicrograph of microcarriers coated with HMVEC-d.

DIC image of microcarriers confluent with HMVEC-d (A). The microcarriers were incorporated within fibrin gels only when cells were confluent on the beads. LIVE/DEAD image display the high level of cell viability within the microcarriers (B). (100x magnification)
the role of different VEGF isoforms in sprouting formation, and also addresses some of mechanisms that govern the kinetics of EC sprouting

3.2 Materials and Methods

Proliferation and Viability Assay

HMVEC-d (Cambrex, NJ, USA) (passage 5) were seeded into 24 well plates (5,000 cells/cm² cell seeding density) overnight with EGM-2MV (Cambrex, USA). Endothelial cells were then washed twice with PBS and cultured with EGM-2MV without growth factors, EGM-2MV without growth factors supplemented with VEGF₁₆₅ (kindly provided by the Biological Resources Branch of National Cancer Institute) or VEGF₁₂₃ (R&D systems, USA) at different concentrations, including 3, 5, 10, 20, 30, 50, 100 ng/ml. The cells were fed everyday and after 3 days of culture, the endothelial cells were detached via trypsinization and counted in a Coulter Counter (Beckman Corp.). The proliferation rate was normalized by comparing the cell number of cells treated with VEGF to cell counts of no growth factor media. The viability of the cells was quantified by trypan blue exclusion with a Viacell Counter (Beckman Corp., USA).

Endothelial cell VEGF secretion

HMVEC-d (passage 6) were seeded into 24 well plates (5,000 cells/cm² cell seeding density) overnight with EGM-2MV. Endothelial cells were then washed twice with PBS and cultured with EGM-2MV without growth factors for 3 days.
Human bone marrow stromal cells (kindly gifted by Darnell Kaigler) were isolated as described previously \(^{24}\) and cultured with \(\alpha\)-MEM (Gibco, USA) media containing 10% FBS (Gibco, USA) and 1% P/S (Gibco, USA). Human cardiomyocytes HL-1 (kind gift of William Claycomb) were isolated and cultured as previously described \(^{25}\). The HL-1 cells were feed with Claycomb medium (JRH Biosciences, USA) and supplemented with 10 %FBS (JRH Biosciences, USA), 1% penicillin-streptomycin (Gibco, USA), 1% L-glutamine (10 mM) (Gibco, USA) and 1% of norepinephrine (0.1 mM) (Sigma, USA) The conditioned media was collected and stored at \(-80^\circ\text{C}\) before use. Total recombinant human VEGF levels present in samples were measured via quantitative human ELISA (Quantikine, R&D Systems, MN).

*Endothelial Cell VEGF Degradation*

HMVEC-d (passage 6) were seeded into 12 well plates (5,000 cells/cm\(^2\) cell seeding density) overnight with EGM-2MV (Cambrex, USA) (see Appendix A for detailed protocol). Endothelial cells were cultured until they reached 80-90% confluency, washed twice with PBS and cultured with EGM-2MV without growth factors supplemented with VEGF\(_{165}\) or VEGF\(_{121}\) (50 ng/ml). At different experimental time points the media was collected from the wells and centrifuged. Subsequently, the media was reconstituted with EGM-2MV without growth factors and used to culture HMVEC-d (50% confluent) present in 12 well plates. Cells were cultured for 48 hrs, and then cells detached via trypsinization and counted in a Coulter Counter.

*Hypoxic Experiments*
All experiments under hypoxic conditions (95% N₂, 5% CO₂) were performed by incubating cells inside a modular incubator chamber (Billups-Rothenberg, USA), and following manufacture instructions (see Appendix B for detailed protocol). This chamber was subsequently introduced in a conventional continuous flow CO₂ incubator. The cells were treated (e.g. seeding, feeding, media changes) in exactly the same conditions as cells under normoxia conditions.

Sprouting Assay

Cytodex 3 microcarriers (Amersham Biosciences, Piscataway, NJ, USA) were hydrated in PBS at room temperature (0.2 ml/mg of dry Cytodex 3), and after 3 hours the supernatant was decanted and replaced with fresh PBS, followed by sterilization with autoclaving (see Appendix C for detailed protocol). HMVEC-d (passage 4) were cultured in EGM-2M with 50 mg of microcarriers (combined in a 8:1 (cell: microcarrier) ratio) in a spinner vessel (Bellco Glass Inc., Vineland, NJ, USA). After 3 hours, microcarriers with adherent cells were incubated for 20 hrs with continuous stirring, and subsequently transferred to tissue culture flasks for an additional 1-2 days of culture. To perform the sprouting assay, the beads in suspension (57 ul) were combined with 170.5 ul of fibrinogen (Sigma, USA) solution (4 mg/ml) and 22.7 ul of aprotinin (Sigma, USA) (500 ug/ml). This solution was then added to 200 ul of thrombin (Sigma, USA) (22.72 units/ml), and incubated at 37°C for 20 min to allow gel formation. Cultures were fed every day with 0.8 ml of EGM-2MV without growth factors, or EGM-2MV with control VEGF (50 ng/ml). After 5 days, gels were washed twice with PBS and subsequently incubated with 4% formaldehyde overnight at 4°C. The formaldehyde solution was then aspirated, and
gels were washed twice with PBS (for certain conditions cells were labeled LIVE/DEAD prior fixation and then stained with DAPI (Molecular Probes – Invitrogen, USA) after overnight fixation) (see Appendix C and R for detailed protocol). Sprouts per bead were analyzed and visualized at 100x and 200x with an Olympus-IX81 light microscope (Japan) connected to an Olympus DP70 digital image capture system (Japan) (average of 100 beads analyzed per condition). Only the participation of two or more endothelial cells extending from the microcarriers was validated and counted as a sprout (see Appendix D for detailed definition of sprouting).

Statistical Analysis

All statistical comparisons were performed using the Students t-test (two-tail comparisons), and analyzed using InStat 2.01 (Graphpad, USA) software. Differences between conditions were considered significant if \( p<0.05 \).

3.3 Results

VEGF_{121} and VEGF_{165} Dose Response in HMVEC-d Proliferation

To investigate the mitogenic potential of VEGF_{121} and VEGF_{165} on EC proliferation, HMVEC-d were cultured with discrete doses for 3 days, and the cell counts were normalized to no growth factor controls. Increasing the VEGF_{165} dose induced a gradual increase in EC proliferation (Figure 3.3). Doses ranging from 5 – 20 ng/ml of VEGF_{165} resulted in the same level of cell proliferation, approximately
Figure 3.3 Quantification of endothelial cell proliferation under different VEGF_{165} concentrations.

HMVEC-d were counted after 3 days of culture under different VEGF_{165} concentrations, including 3 ng/ml (□), 5 ng/ml (■), 10 ng/ml (▲), 20 ng/ml (●), 30 ng/ml (▲), 50 ng/ml (■) and 100 ng/ml (▲). Values represent mean and standard deviations (n=6). * indicates statistically significant difference (p<0.05) between conditions and N.S. indicates no statistically significant difference (p>0.05) between conditions.
25% more proliferation than the control. Interestingly, these results demonstrated that the cell proliferation rate reached a plateau at 50 ng/ml, since no further increase was observed with a dose of 100 ng/ml, and for both conditions cells proliferated approximately 60% more than compared to no VEGF_{165} treated cells. A similar proliferation profile was observed when cells were stimulated with VEGF_{121} (Figure 3.4). No statistically significant differences in proliferation were observed between the two isoforms. Interestingly, doses of VEGF_{121} ranging from 3 – 20 ng/ml stimulated the same level of proliferation. As noted with VEGF_{165} stimuli, no further differences in proliferation were observed as the concentration was increased from 50 ng/ml to 100 ng/ml VEGF_{121}.

*In vitro VEGF degradation by HMVEC-d*

To investigate if the endothelial cells degrade distinct VEGF isoforms in a different fashion, HMVEC-d were stimulated with VEGF isoforms that were previously exposed to cells, and monitored over time (Figure 3.5). No differences in kinetics were observed between the bioactivity of the two isoforms, as they were degraded by cell exposure in similar manner.

*HMVEC-d VEGF Secretion under Distinct Oxygen Tensions*

Next the levels of VEGF secretion by ECs under normoxia and hypoxia conditions were evaluated (Figure 3.6). Two different cell types were utilized as controls: human bone marrow stromal cells and human cardiomyocytes. Interestingly, human cardiomyocytes were incapable of producing physiologically
Figure 3.4 Quantification of endothelial cell proliferation under different VEGF<sub>121</sub> concentrations.

HMVEC-d were counted after 3 days of culture under different VEGF<sub>121</sub> doses, including 3 ng/ml (□), 5 ng/ml (■), 10 ng/ml (■), 20 ng/ml (■), 30 ng/ml (■), 50 ng/ml (■) and 100 ng/ml (■). Mean values are presented with standard deviations (n=6), * indicates p<0.05 between conditions and N.S. indicates p>0.05 between conditions.
Figure 3.5 *In vitro* VEGF bioactivity degradation by endothelial cells.

The bioactivity of VEGF$_{121}$ (○) and VEGF$_{165}$ (●) was degraded by endothelial cells in a similar fashion over time. Mean values are presented with standard deviations (n=4)
Figure 3.6 Quantification of different human cells VEGF secretion under different oxygen tensions.

Human cells were submitted to normoxia (■) and hypoxia (■) conditions, and the VEGF secretion was normalized to cell number and secretion time. High levels of VEGF were secreted by endothelial cells as compared with human bone marrow stromal cells (HBMSCs) and human cardiomyocytes (HL-1). Cardiomyocytes secreted physiologically insignificant levels of VEGF. Mean values are presented with standard deviations (n=5).
relevant levels of VEGF either in normoxia or in hypoxia conditions. In contrast, HMVEC-d and stromal cells exhibited the same higher level of VEGF secretion under normoxia conditions. HMVEC-d were more sensitive to hypoxia as this resulted in one order of magnitude in VEGF secretion, as compared with normoxia conditions. Under hypoxic conditions, stromal cells exhibited an approximately 2-fold increase in VEGF secretion, as compared with normoxia.

*Effect of VEGF isoforms on HMVEC-d Sprouting*

The effects of different VEGF isoforms on eliciting HMVEC-d to create capillaries was next analyzed in a 3-D sprouting assay, a standard in vitro model that mimics some early events of angiogenesis \(^{26-28}\). A sprout was defined as an elongated structure extending from the bead with the participation of two or more endothelial cells (Figure 3.7). The occurrence of lumens in the sprouts was very low. The ability of HMVEC-d to forms sprouts was correlated with the passage number, as increasing passage number resulted in unorganized cell migration off the beads and in a decrease in sprouting. All studies used HMVEC-d with a low passage number (passage 4) from the same lot (lot number 5F1362).

To examine if different VEGF isoforms induce endothelial cell sprouting formation differently, fibrin gels containing microcarriers coated with HMVEC-d were feed with VEGF\(_{121}\), VEGF\(_{165}\) or VEGF\(_{121}\) and VEGF\(_{165}\) (1:1) for 5 days (Figure 3.8A). Media without growth factors was used as the experimental control. As expected, VEGF stimulated the formation of sprouts as compared with no VEGF controls. VEGF\(_{121}\) and VEGF\(_{165}\) induced the same level of sprouts formation at day 1 and day 5, but interestingly the combination of VEGF\(_{121}\) and VEGF\(_{165}\) did not result in
Figure 3.7 Photomicrograph of sprouting cells from a microcarrier.

DIC image of HMVEC-d sprouting formation with more than two cells (arrows indicate different cells) (A). DAPI stains were very useful to identify the individual cell contribution for sprout formation (B). (Scale bar in equals to 100 μm)
Figure 3.8 Quantification of number of sprouts and length formed by HMVEC-d in response to VEGF stimulation.

The ability of HMVEC-d to form sprouts was examined (≈ 90 beads/sample) under media containing VEGF₁₂₁ (50 ng/ml) (■), VEGF₁₆₅ (50 ng/ml) (□), VEGF₁₂₁ and VEGF₁₆₅ (total dose 50 ng/ml, 1:1) (■) and control media (no growth factor) (□) (A). The length of sprouts formed was also quantified (≈ 90 beads/sample) under the same conditions (B). Mean values are presented with standard deviations (n=4), * indicates p<0.05 between no growth factor control.
a higher level of sprout formation as compared with each alone. Further, the lengths of the sprouts formatted in these conditions were examined, and again no difference was observed between experimental conditions. Sprouts typically extended around 200 μm in length (Figure 3.8B). Interestingly, the average of sprout length at day 1 was the same observed at day 5.

Effect of VEGF on HMVEC-d Sprouting Kinetics

The effect of VEGF dose distribution on HMVEC-d sprouting was also examined (Figure 3.9). HMVEC-d were submitted to the same total VEGF$_{165}$ exposure (200 ng of VEGF) for 5 days, however four different dose distributions were used, including a constant dose distribution (50 ng/ml everyday), an decreased dose distribution (100 ng/ml, 50 ng/ml, 50 ng/ml, 25 ng/ml, 25 ng/ml; throughout 1-5 days respectively), a delayed dose distribution (100 ng/ml, 100 ng/ml, 20 ng/ml, 20 ng/ml, 10 ng/ml; throughout 1-5 days respectively) and an increased dose distribution (25 ng/ml, 25 ng/ml, 50 ng/ml, 50 ng/ml, 100 ng/ml; 1-5 days respectively). After 5 days the number and length of sprouts formed under these conditions was quantified. Media containing no VEGF was used as the experimental control. Cells exposed to higher doses of VEGF$_{165}$ in the early days exhibited higher number of sprouts as compared to a constant dose distribution or to an increasing dose with time (Figure 3.9A). Interestingly, no differences were observed in terms of sprouts formed, between a constant dose distribution and an increased dose distribution. However, the sprouts length was constant between distinct dose distributions of VEGF$_{165}$ (Figure 3.9B).
Figure 3.9 Quantification of number of sprouts and length formed by HMVEC-d in response to different temporal exposure to VEGF.

The number of sprouts formed by HMVEC-d when submitted to four different VEGF_{165} dose distributions: a constant dose distribution (50 ng/ml everyday) (■), decreased dose distribution (100 ng/ml, 50 ng/ml, 50 ng/ml, 25 ng/ml, 25 ng/ml; throughout 1-5 days respectively) ( ), a delayed dose distribution (100 ng/ml, 100 ng/ml, 100 ng/ml, 200 ng/ml, 200 ng/ml, 10 ng/ml; throughout 1-5 days respectively) ( ) and a increased dose distribution (25 ng/ml, 25 ng/ml, 50 ng/ml, 50 ng/ml, 100 ng/ml, 100 ng/ml; 1-5 days respectively) ( ); was analyzed. Media with no VEGF was utilized as a control ( □ ). The length of sprouts formed was also examined (B). Mean values are presented with standard deviations (n=4), * indicates statistically significant differences (p<0.05) and N.S. indicates no statistically significant difference between conditions (p>0.05).
3.4 Discussion

This chapter demonstrates that VEGF$_{121}$ and VEGF$_{165}$ are capable of governing *in vitro* EC behavior in similar fashion, and highlighted the ability of VEGF$_{121}$ and VEGF$_{165}$ to control angiogenesis via dose-dependent mechanisms. Due to the distinct ECM binding capabilities presented by these two isoforms, the findings in this chapter represent an important piece of information for delineating alternative strategies to control neovascularization *in vivo*. Furthermore, the 3-D model introduced in this chapter was utilized to screen the role of dose distribution in the kinetics of capillary formation.

Neovascularization is a complex process that is governed by an array of cytokines and growth factors, of which VEGF plays a critical role $^3$. Although there exist several VEGF isoforms arising from alternative mRNA splicing $^5$, VEGF$_{121}$ and VEGF$_{165}$ are the predominant in normal tissue $^{29}$. However, little is known regarding differences in the *in vitro* activities of the distinct isoforms. The results of this chapter demonstrate that both isoforms present similar mitogenic potency to endothelial cells (Figure 3.3 and 3.4). The mitogenic potential of different VEGF isoforms has generated some contradictory results in past studies. In certain studies, VEGF$_{121}$ was described as a 100-fold less potent mitogen than VEGF$_{165}$ $^{12}$. However, these results have been challenged by other studies revealing no differences between the two isoforms $^{6,30}$. The dose proliferation studies conducted in this chapter revealed that the mitogenic potential of both VEGF$_{121}$ and VEGF$_{165}$ saturated at 50 ng/ml, suggesting that this concentration is likely to be the optimal dose of VEGF for *in vitro* studies. Furthermore, the results of this chapter demonstrated that endothelial cells *in*
*in vitro* consumed VEGF<sub>121</sub> and VEGF<sub>165</sub> in similar fashion (Figure 3.5). Interestingly, some studies have reported that VEGF<sub>165</sub> stimulated a 2-fold higher ERK1/2 phosphorylation, as compared with VEGF<sub>121</sub> <sup>8</sup>, however this study utilized a smaller VEGF dose, and the endothelial cell type utilized in these studies was different the ECs used in chapter.

Oxygen tension plays a critical role in controlling angiogenesis by regulating the expression of a variety of genes, including VEGF <sup>2</sup>. The results of this chapter demonstrated that VEGF secretion by endothelial cells is highly stimulated by hypoxic conditions (Figure 3.6), and these results are in agreement with other studies <sup>31</sup>. Interestingly, endothelial cells were the main producers of VEGF, as compared with the other cell populations studied. Under hypoxia conditions cardiomyocytes (that represents approximately 90% of total cells present in heart <sup>32</sup>) did not produce relevant levels of VEGF, and this finding suggests that the severity observed in ischemic heart diseases, is likely related to the VEGF shortage founded in mammals hearts. Several lines of evidence have described that cardiomyocyte plasticity to revert injuries tissues in other lower vertebrates species, such as zebrafish, is directly linked with cardiomyocyte secretion of angiogenic factors, including VEGF, PDGF and MMPs <sup>33</sup>. However, mammalian hearts has been considered as an organ that is unable to regenerate basically due to the irreversibly exit the cell cycle shortly after development <sup>34</sup>. VEGF acting both as survival factor and angiogenic initiator has been described to restore cardiac function improving neovascularization and myocardial tissue viability, when delivered in some cardiac animals models <sup>35,36</sup>.

Many models for angiogenesis have been described <sup>37,38</sup>, however they often fail to mimic several in *vivo* characteristics (e.g., 3-D environment). Recently, an attractive fibrin gel-based model has been described and modified to investigate the
mechanisms associated with endothelial cell sprouting, elongation and lumenization. This system was used to test the effects of VEGF_{121} and VEGF_{165} on capillary sprouting. No differences in terms of number of sprouts formed and length of sprouts were observed between VEGF_{121} and VEGF_{165} (Figure 3.8). These results are in agreement with a recent study, suggesting that these two isoforms are both capable of stimulating angiogenesis in the same magnitude. Interestingly, no synergistic effect was observed with the combination of both angiogenic signals. In physiological conditions VEGF signals are constantly regulated, and the timing of VEGF presentation can elicit the temporal extension of angiogenic response. The effect of distinct timing of VEGF availability on EC sprouting formation was tested by using different VEGF_{165} dose distributions (Figure 3.9). High levels of VEGF in early time points resulted in an increase of sprouting, but no differences were observed regarding the sprouts length, which likely suggests that other factors promote further maturation and elongation of the capillaries. As described in several studies, these support the importance of VEGF in regulating the early steps of angiogenesis.

Overall, this chapter indicates that VEGF_{121} and VEGF_{165} are both potent angiogenic factors, and the ability of differentiated ECM binding from VEGF_{121} might be particularly attractive to evoke an angiogenic response in an alternative fashion than obtained with VEGF_{165} alone. The use of this two isoforms can be very useful for in vivo applications, essentially because ECM only display a control, regarding the local tissue presentation, over VEGF_{165} resulting in distribution associated with hypoxic areas. However, in some cases there is a need to activate cells distant of ischemic regions, because some of cells present in hypoxic could be less sensitive to VEGF signaling. The use of VEGF_{121} will be very useful to activate and induce...
migration of cell populations that are not present in ischemic regions. In addition, the results of this chapter regarding the effects of temporal presentation of VEGF in capillary formation, will be very useful for the design of new delivery systems, as showed that the VEGF doses needs to be relatively higher in the early time points to induce greater angiogenic response. Finally, some of the study models and results described in this chapter will be utilized and further investigated in the next chapters of this thesis.

3.5 References


CHAPTER 4

Spatiotemporal control of VEGF isoform delivery enhances in vivo neovascularization

4.1 Introduction

Therapeutic angiogenesis involves new blood vessel formation by delivery of specific mediators\(^1\), and the delivery of recombinant angiogenic growth factors to ischemic tissues has been commonly pursued in pre-clinical and clinical studies\(^2\text{-}^5\). However, as reviewed in chapter 2, no significant improvement in phase II trials has been achieved\(^2\text{-}^7\) to date. It is likely that these disappointing clinical results are due to the transient growth factor exposure in the ischemic site, as the factors likely need to be present for relatively long time periods to prevent regression of the newly formed vessels\(^8\). As noted in chapter 2, polymeric systems can deliver bioactive molecules in a localized and controlled fashion, and the aim of this chapter was to create an injectable polymeric delivery system capable of presenting bioactive growth factors in a spatiotemporal controlled manner. Furthermore, this chapter describes the in vivo application of some of the growth factors examined in vitro in chapter 3, and
translates some of the findings generated in the previous chapter to better design polymeric delivery vehicles.

Atherosclerosis is the main cause of PVD, and typically results in the obstruction of the blood supply to the upper and lower limbs. Despite recent advances, there still remains a major need for new approaches to successfully treat this disease. One of the most widely investigated factors to treat PVD is VEGF_{165}, but the typical approach to administering VEGF_{165} or other angiogenic factors involves either bolus injections into the ischemic site or systemic administration of recombinant protein solutions. Recent analysis of the pharmacokinetics of VEGF_{165} delivery in phase II clinical trials revealed that the bioactive VEGF_{165} levels fell below critical levels in less than 8 hours after treatment. In addition, although many studies have been devoted to understand the role of VEGF_{165} in angiogenesis, very little is known about the specific biological responses that other VEGF forms, particularly VEGF_{121}, might induce.

Polymeric delivery systems that allow localized and sustained presentation of therapeutic agents may allow one to bypass the limitations of current VEGF delivery. In particular, hydrogels may be an especially appealing class of delivery vehicle, as they can be introduced into the body with minimally invasive procedures, and are often highly biocompatible due to their high water content. Hydrogels have been tested for their ability to provide localized VEGF delivery, and alginate is an attractive material for this application. Alginate is a naturally occurring (linear unbranched) polysaccharide comprised of a α-L-guluronic and β-D-mannuronic acid sugar residues (Figure 4.1). Sodium salts of alginate are water soluble, but in the presence of divalent cations (such as calcium) alginate chains form ionic cross-links, leading to gelation. Due to the gentle nature of this gelation process, calcium cross-
Figure 4.1 Alginate structure and gelation mechanism.

The chemical structure of alginate, a naturally-derived copolymer consisting of monomers of mannuronic acid (M) and guluronic acid (G). The chemical properties of alginate are intrinsically related with the ratio and sequence of the two monomers (A). One appealing characteristic of alginate polymer chains (green and red, representing G and M blocks respectively) rely on the polyelectrolyte properties of G blocks, resulting in a remarkable affinity for divalent cations (e.g. Ca²⁺) (yellow spheres), and the presence of these cations results in the formation of ionic cross-links forming gels between the G blocks of the different polymer chains (B).
linked alginate hydrogels have been widely used in several medical applications (e.g., dental impressions, drug delivery devices and as immobilization matrices for cells) \(^{16,20}\). Despite its biocompatibility, however, these gels present a slow and unpredictable degradation in vivo that limits their utility. Previous studies have reported that control over alginate gel degradation can be achieved by partially oxidizing the polymer chains with sodium periodate \(^{21}\), and low levels of oxidation maintain the gel biocompatibility \(^{22}\). An alternative strategy to control degradation relies on controlling the polymer molecular weight distribution, and the use of a binary molecular weight formulation allows the incorporation of low molecular weight chains, which more readily disassociate from the gel and can be subsequently excreted from the body \(^{21}\). In contrast with non-degradable alginate hydrogels, where diffusion is the predominant mechanism of growth factor release, factor release from degradable gels could be tuned by controlling both diffusion and degradation.

This chapter describes the development of an injectable alginate hydrogel system that can provide a sustained delivery and spatial control of the presentation of bioactive VEGF isoforms in ischemic hindlimbs. The role of alginate gel biodegradation in controlling the rate of growth factor delivery was first analyzed in vitro, and the utility of this system was subsequently evaluated in a mouse model of PVD (ApoE\(^{-/-}\)) \(^{25}\). This system was utilized to investigate how spatiotemporal control over growth factor delivery can directly stimulate neovascularization \textit{in vivo}, and return limb perfusion to normal levels to prevent limb necrosis.

4.2 Materials and Methods

\textit{Binary Molecular Weight Alginate Gel Formulation and Modifications}
Ultrapure alginates were purchased from ProNova Biomedical (Norway). MVG alginate, a high G containing alginate (M/G ratio of 40/60 as specified by the manufacturer) was used as the high molecular weight (MW=250,000 Dalton) component to prepare gels. Low molecular weight alginate (MW=50,000 Dalton), was obtained by gamma (γ)-irradiating high molecular weight alginate with a cobalt-60 source for 4 h at a γ-dose of 5.0 Mrad (Phoenix Lab, University of Michigan, Ann Arbor, USA) \(^2\). The alginate used to form gels was a combination of the two different molecular weight polymers at a ratio of 7.5:2.5. Both alginate polymers were diluted to 1% w/v in ddH\(_2\)O, and 1% of the sugar residues in the polymer chains were oxidized with sodium periodate (Aldrich, St. Louis, MO, USA) by maintaining solutions in the dark for 17 h at room temperature, as previously described \(^2\) (see Appendix E for detailed protocol). An equimolar amount of ethylene glycol (Fisher, USA) was added to stop the reaction, and the solution was subsequently dialyzed (MWCO 1000, Spectra/Por\textsuperscript{®}) over 3 days. The solution was sterile filtered, frozen (-20°C overnight), lyophilized and stored at -20°C. To prepare gels, modified alginates were reconstituted in EBM-2 (Cambrex) to obtain 2% w/v solution (75% LMW; 25% MVG used in all experiments) prior to gelation. The 2% w/v alginate solutions were cross-linked with aqueous slurries of a calcium sulfate solution (0.21 g CaSO\(_4\)/ml d H\(_2\)O) in a ratio of 25:1 (40 μl of CaSO\(_4\) per 1 ml of 2% w/v alginate solution) using a 1 ml syringe. Reconstituted alginate was stored at 4°C.

*Growth Factor Incorporation and Release Kinetics*
Alginates were first mixed with recombinant human VEGF\textsubscript{165} protein (kindly provided by the Biological Resources Branch of National Cancer Institute) or VEGF\textsubscript{121} (R&D systems, USA) by using two syringes coupled by a syringe connector, and the calcium slurry (Sigma, USA) was then mixed with the resulting alginate/growth factor solution using two syringes coupled by a syringe connector to facilitate the mixing process and prevent entrapment of air bubbles during mixing (see schematic representation in Appendix F). The mixture was allowed to gel for 30 minutes and was then maintained at 4°C prior to animal injections. $^{125}$I-VEGF\textsubscript{165} was purchased from PerkinElmer Life Sciences (USA). VEGF\textsubscript{121} was iodinated via IODO-GEN\textsuperscript{®} pre-coated iodination tubes (PIERCE, USA) using Iodine-125 radionuclide (PerkinElmer, USA) (see Appendix G for detailed protocol). Alginates were mixed with radiolabeled growth factor as described above in certain experiments to quantify VEGF release in vitro. The resulting mixtures were cast between two glass plates separated with 1 mm spacers and allowed to gel for 30 minutes. The gels were divided into four samples and subsequently incubated in 3 ml of PBS buffer solution (PBS - Invitrogen with 0.1g/l of MgCl\textsubscript{2}*6H\textsubscript{2}O and 0.132g/l of CaCl\textsubscript{2}*2H\textsubscript{2}O Sigma) at 37 °C. At each experimental time point, the radiolabeled growth factor present in the buffer solution was measured using a gamma counter (1470 WIZARD (PerkinElmer, USA)) and compared with the initial total radiolabeled factor incorporated into the sample.

**Rheologic Properties of Pre-gelled Hydrogel Solutions**

The viscosities of pre-gelled alginate solutions were investigated by using a controlled-stress rheometer (CS-50, Bohlin Instrument) at 25 °C. Preceding the
measurement, all samples were pre-sheared at a high shear rate followed by 5 minutes rest. While increasing the shear stress from 0.008 to 10 Pa, the resulting strains were measured, and the corresponding viscosity (η) was calculated.

**Bioactivity Assay**

Recombinant human VEGF protein was incorporated into alginate solutions before gelling (250ng/ml), and gels were cast as previously described for analysis of release kinetics. After gels polymerized, they were transferred to 24 well plates, and 3 ml of EGM-2MV media without growth factors (Cambrex Corporation, NJ, USA) was added to each well. At certain time points media was collected and added to cultured endothelial cells, and fresh media was introduced to the alginate gels. Human microvascular dermal endothelial cells (HMVEC) (Cambrex Corporation, NJ, USA) (passage 6) were seeded into 24 well plates (5,000 cells/cm² cell seeding density) overnight with EGM-2MV. Endothelial cells were then washed twice with PBS and cultured with EGM-2MV without growth factors, EGM-2MV with control VEGF, and EGM-2MV with VEGF released from alginate gels (in both of the latter cases the VEGF concentration in the medium was 25 ng/ml at day 1, and 4 ng/ml from day 2 to 4). After 24 hours and 4 days, the endothelial cells were detached via trypsinization and counted in a Coulter Counter (Beckman Corp.). The biological activity of VEGF released from the gels was determined by comparison to the stimulatory effect observed in the culture wells containing control VEGF.

60 x 15 mm tissue culture dishes of HMVEC (passage 5) were cultured in EGM2-MV to subconfluency. The media was then removed and cells were washed twice in PBS. Subsequently, starvation media was added and cells cultured for 18 hrs
(starvation media was comprised of EBM). The starvation media was then removed and cells were submitted to starvation media supplemented with control VEGF$_{165}$ or VEGF$_{165}$ released from alginate gels (for both conditions the VEGF concentration was 40 ng/ml). After 5, 15 and 45 min, medium was removed from dishes and cells were washed twice with ice-cold PBS. Levels of phosphorylated intracellular ERK1/ERK2 in cell lysates were then quantified via a two-site sandwich ELISA (R & D Systems, MN) (see Appendix H for detailed protocol).

_Sprouting Assay_

Cytodex 3 microcarriers (Amersham Biosciences, Piscataway, NJ, USA) were hydrated in PBS at room temperature (0.2 ml/mg of dry Cytodex 3), and after 3 hours the supernatant was decanted and replaced with fresh PBS, followed by sterilization with autoclaving. HMVEC-d (passage 4) in EGM-2MV were then combined with 50 mg of sterile microcarriers in a 7:1 (cell:microcarrier) ratio in a spinner vessel (Bellco Glass Inc., Vineland, NJ, USA). After 3 hours, microcarriers with cells were incubated for 20 hrs with continuous stirring. The microcarriers with cells were subsequently transferred to tissue culture flasks, and cultured for 1-2 days. To perform the sprouting assay, the beads in suspension (57 ul) were combined with 170.5 ul of fibrinogen (Sigma) solution (4 mg/ml) and 22.7 ul of aprotinin (Sigma) (500 ug/ml). This solution was then added to 200 ul of thrombin (Sigma) (22.72 units/ml), and incubated at 37°C for 20 min. allowing gel formation. Cultures were fed every day with 0.8 ml of EGM-2MV without growth factors, EGM-2MV with control VEGF, and EGM-2MV with VEGF released from alginate gels (in both of the latter cases the VEGF concentration in the medium was 50 ng/ml at day 1, and 10 ng/ml from day 2.
to 3). After 3 days, gels were washed twice with PBS and incubated with 4% formaldehyde overnight at 4°C. The formaldehyde solution was then aspirated, and gels were washed twice with PBS, and sprouts per bead were quantified from microscopic images (average of 100 beads analyzed per condition).

**Animals and Surgical Procedures**

All animal work was performed in compliance with NIH and institutional guidelines. Female ApoE−/− mice aged 6 weeks, and 6-7 week old female C57BL/6J (Jackson Laboratories, Bar Harbour, ME) were used for these studies. ApoE−/− mice were fed a high fat diet (21% fat, 0.15% cholesterol, Harlan Teklad) for at least 6 weeks prior to enrollment in the study. Mice were anesthetized with an intraperitoneal injection of a mixture of ketamine 80 mg/kg and xylazine 5 mg/kg prior to all surgical procedures. Hindlimb ischemia was induced by unilateral external iliac and femoral artery and vein ligation as previously described 25 (see Appendix J for detailed protocol). After the vessel ligation, mice were injected with a total volume of 50 μl of alginate gel with or without growth factor. For some experiments the dose of growth factor ranged from 3-10 μg, for the dose dependent experiments. Unless specified, in all experiments gels contained 3 μg of growth factor total. The bolus controls were made of phosphate buffered saline (PBS) solution also containing 3 μg of growth factor. Injections were performed, using a 25G needle (Becton Dickinson, USA) directly into the muscle tissue where the vessels were ligated. The incisions were subsequently surgically closed and animals monitored over time.

**VEGF Localization In Vivo**
At various time points, three distinct hindlimb muscle regions were dissected from the ischemic hindlimbs (+/- 5 mm apart from each other), weighed and digested with T-Per Reagent (Pierce, USA). Tissue samples were subsequently sonicated for 5 seconds and centrifuged at 13,000 rpm for 5 min. (4°C). Supernatant was collected and stored at -20°C for future VEGF quantification. Blood samples were collected retro-orbitally using hematocrit heparinized capillaries (Fisher Scientific) and maintained at room temperature for 30 minutes to allow coagulation. After coagulation, blood samples were centrifuged at 13,000 rpm for 10 min. (4°C) (see Appendix L for detailed protocol). Blood serum was collected and stored at –20°C. Total recombinant human VEGF levels present in samples were measured via quantitative human ELISA (Quantikine, R&D Systems, MN).

*Immunohistochemistry and Blood Vessel Quantification*

Hindlimb muscle tissues (n=6/timepoint/experimental condition) were retrieved, fixed, paraffin embedded, and stained for mouse CD31 (BD Biosciences Pharmingen, San Diego CA) (see Appendix M for detailed protocol). For measurement of capillary densities, 30 randomly chosen high-power fields of the tissue were analyzed. The number of positively stained blood vessels were manually counted and normalized to the tissue area. Sections from each sample were visualized at 200x and 400x with an Olympus-IX81 light microscope (Japan) connected to an Olympus DP70 digital image capture system (Japan) and analyzed using IPLab 3.7 software (Scanalytics, Rockville, MD).
Laser Doppler Perfusion Imaging (LDPI) and Limb Integrity

Before surgery and 0, 1, 3 and 7 days, and 2, 4, and 6 weeks post-surgery measurements of the ischemic/normal limb blood flow ratio were performed on anesthetized animals (n=6/timepoint/experimental condition) using a Periscan system blood perfusion monitor laser Doppler equipment (Perimed, Sweden). Perfusion measurements were obtained from the right (ischemic) and left (non-ischemic) limb. To minimize variability due to ambient light and temperature, the index was expressed as a ratio of ischemic to non-ischemic limb blood flow (see Appendix N for detailed protocol).

Ischemic hindlimbs were visually observed at 1, 2, 4 and 6 weeks postoperative in order to grade limb integrity, and categorized according to degree of necrosis: normal compared to non-surgical limb, one necrotic toe, multiple necrotic toes, and necrotic foot (see Appendix O for detailed grading of limb integrity).

Statistical Analysis

All statistical comparisons were performed using the Students t-test (two-tail comparisons), and analyzed using InStat 2.01 (Graphpad, USA) software. Differences between conditions were considered significant if p<0.05.

4.3 Results

Optimization of Alginate Gel Formulation
Growth factor release from alginate hydrogels can possible be controlled by a wide variety of parameters, including the polymer molecular weight distribution and the solution utilized for alginate reconstitution. The effect of using different alginate solvent solutions was first tested on VEGF₁₆₅ release. No significant effects on VEGF₁₆₅ release were observed when PBS, water or EBM2 media were utilized as the alginate solvent (Figure 4.2A). VEGF₁₆₅ released from distinct polymer molecular weights distributions was also investigated. Increasing the amount of low molecular weight polymer in the gel resulted in increased the rate of VEGF release (Figure 4.2B). Based on these results, the binary molecular weight distribution gels formed with a polymer ratio of 7.5:2.5 (low:high) using EBM2 media solution was used in subsequent experiments.

*In vitro* Validation of Injectable Polymeric System

Alginate hydrogels with well controlled degradation rates can be generated by combining control over molecular weight distribution and partial oxidation to make the polymer chains susceptible to hydrolysis (Figure 4.3), and the ability of these gels to serve as sustained VEGF release vehicles was first assessed in vitro. The degradation of hydrogels formed from a combination of high and low molecular weight polymers that were partially oxidized (1%), and control gels formed from both high molecular alginate and a mixture of high and low molecular weight polymer that were not oxidized was evaluated by dry weight loss as a function of degradation time (Figure 4.4). As expected, the degradation rate of the gels formed from partially oxidized alginate was relatively rapid, with significant mass loss within 7 days, and almost complete mass loss by 40 days. In contrast, neither of the control gels
Figure 4.2 VEGF$_{165}$ release from alginate gels formulated with different solvent solutions and polymer molecular weight distributions.

VEGF$_{165}$ release from gels formulated with EBM2 media (■), PBS (□) and water (○) was examined as a function of time following incubation in PBS (A). VEGF$_{165}$ release from gels formed from distinct polymer molecular weight distributions, including a ratio of 0:10 (low:high molecular weight polymers) alginate (▽), 5:5 (▼), 6:4 (▲), 7:5:2.5 (●) and 8:2 (△) binary molecular weight partially oxidized alginate (■) was analyzed as a function of time following incubation in PBS (B). Values represent mean and standard deviation for A and B (n=5).
Figure 4.3 Chemical structure of partially oxidized alginate.

The partial oxidation of alginate chains with periodate creates an open chain adduct liable to hydrolytic scission (arrow indicating the open chain) in the presence of water.
Figure 4.4 Dry weight loss of different alginate gels.

The dry masses of gels formed from high molecular weight non-oxidized alginate (△), binary molecular weight non-oxidized alginate (□), and binary molecular weight partially oxidized alginate (■) were analyzed as a function of time following incubation in PBS. Values represent mean and standard deviation (n=5).
degraded significantly over this time frame. The capabilities of alginate hydrogels to be used as an injectable delivery system were evaluated by characterizing the rheological properties of pre-gelled solutions (Figure 4.5). The combination of low and high molecular weight alginates exhibited an intermediate low-shear viscosity (η), as compared to the high or low molecular weight polymers alone.

To investigate the relation between gel degradation rate and growth factor release, $^{125}$I-labelled VEGF isoforms were incorporated into the gels, and their release following incubation in medium monitored over time (Figure 4.6). After an initial burst, VEGF$_{165}$ was released in a sustained manner over time, and within 7 days approximately 60% of the total VEGF$_{165}$ loaded was released. The release of VEGF$_{121}$ displayed a similar burst, but the release was accelerated over time when compared with release of VEGF$_{165}$. After one week of in vitro incubation approximately 80% of total loaded VEGF$_{121}$ was released. In contrast, VEGF$_{165}$ delivered from non-oxidized alginates illustrated a slower release, and after 30 days only approximately 40% was released (Figure 4.7). VEGF$_{165}$ is susceptible to rapid degradation in vitro and in vivo, and VEGF$_{165}$ incorporated and released from the hydrogels was examined to determine if it maintained its bioactivity. This was first monitored by quantifying its capability to stimulate endothelial cell proliferation in vitro. The VEGF released for the first 4 days from the gels was not only bioactive, but even more potent than the same VEGF concentration added directly to the culture media (Figure 4.8). It was also investigated whether VEGF isoforms released from the gels were able to promote endothelial cell sprouting in vitro. After 3 days, VEGF isoforms released from alginate gels led to approximately 2.5 fold increase in the number of sprouts per bead, as compared to the control VEGF isoforms (Figure 4.9). Finally, it was investigated
Figure 4.5 Rheological properties of pre-gelled alginate solutions.

The viscosity of solutions of high (□), low (■) and binary molecular (●) weight partially oxidized alginate solutions. Values represent mean and standard deviation (n=5).
Figure 4.6 Release of different VEGF isoforms from alginate gels.

The release kinetics of $^{125}$I – VEGF$_{165}$ (■) and $^{125}$I – VEGF$_{121}$ (○) from gels formed from binary molecular weight partially oxidized alginate was monitored over time. Values represent mean and standard deviation (n=5).
Figure 4.7 Effect of partially oxidation on VEGF\textsubscript{165} release from different alginate gels.

The release kinetics of \textsuperscript{125}I – VEGF\textsubscript{165} from gels formed from binary molecular weight alginate either partially oxidized (■) or non-oxidized (□) was monitored over time (C). Values represent mean and standard deviation (n=5).
Figure 4.8 Bioactivity of VEGF$_{165}$ released from alginate gels.

A higher endothelial cell proliferation was observed when cells were cultured with VEGF released from binary molecular weight partially oxidized alginate gels ( ), as compared to media without VEGF (■) and control VEGF added to media (□), as measured from the cell number counts from day 0 to day 4. Values represent mean and standard deviation (n=6) and * indicate statistically significant difference (p<0.05).
Figure 4.9 Endothelial cell sprouting in response to VEGF isoforms delivered from alginate gels.

VEGF isoforms delivered from gels induced a greater number of endothelial cell sprouts, as compared with control VEGF isoforms, or no VEGF added to culture medium. Values represent mean and standard deviation (n=4) and * indicate statistically significant difference (p<0.05).
if VEGF released from gels can alters signal transduction in endothelial cells. VEGF\textsubscript{165} released from gels was studied for its effect on activation of the ERK1/ERK2 intracellular pathway, in terms of magnitude and duration of activation. The VEGF\textsubscript{165} released from alginate gels was able to activate and maintain ERK1/ERK2 intracellular signal in a distinct manner as control VEGF\textsubscript{165} (Figure 4.10). As expected, phosphorylation induced by the VEGF\textsubscript{165} control peaked in the early time points and decreased over time, but in contrast VEGF\textsubscript{165} released from the gels induced a constant level of ERK1/ERK2 phosphorylation over this time course.

\textit{In vivo Localization of VEGF isoforms Delivered from Gels}

Next it was examined whether degradable hydrogels had the potential to serve as an injectable system to deliver VEGF isoforms in a sustained and localized manner in vivo. C57BL/6J mice were submitted to ischemic hindlimb surgery and immediately injected with either VEGF\textsubscript{121} or VEGF\textsubscript{165} in PBS (bolus delivery) or hydrogels containing VEGF isoforms. The total VEGF dose and volume of injection were the same for both conditions. At different time points, the levels of exogenous VEGF in the blood and in discrete tissue regions of the hindlimb relative to the injection site (Figure 4.11) were assessed via ELISA. During the first 24 hours after injection, higher concentrations of VEGF\textsubscript{165} in the injection region were found when VEGF\textsubscript{165} was delivered from the alginate, as compared with bolus delivery (Figure 4.12). VEGF\textsubscript{121} delivered from gels was present in lower levels, as compared with VEGF\textsubscript{165} released from gels. At 12 hours after injection, the spatial distribution of VEGF isoforms across the different hindlimb tissue regions was analyzed. Approximately 95% of the total VEGF\textsubscript{165} dose in the gels was still localized in the
Figure 4.10 Quantification of phosphorylated ERK1/2 in VEGF$_{165}$ stimulated endothelial cells.

Control VEGF$_{165}$ (■) and VEGF$_{165}$ released from gels (□) activated ERK1/2 signaling in a different fashion over time, as opposed to control medium containing no VEGF (■) (F). Values represent mean and standard deviation (n=4) and * indicate statistically significant difference (p<0.05).
Figure 4.11 Gross photograph of ischemic hindlimb tissue model displaying tissue regions utilized for analysis.

A, B and C represents regions of the mice hindlimb at an increasing distance away from the injection site. The area of VEGF injection was labeled as region A. The region B was ± 5 mm distant from the injection site. Region C was ± 9 mm distant from the region A. At different time points tissue was retrieved from these regions and exogenous VEGF levels were detected via ELISA.
Figure 4.12 VEGF localization on injection site 24 hours after delivery.

Higher concentrations of bioactive VEGF$_{165}$ were present in the injected region (region A) when VEGF$_{165}$ were released from binary alginate gels (□), as compared to VEGF$_{121}$ released from gels (△) and to VEGF$_{165}$ bolus delivery (■). Mean values are presented with standard deviations (n=5).
region (A) closest to the injection site, while VEGF\textsubscript{165} delivered via bolus injection was much more widely dispersed (43% of VEGF located in regions B and C) (Figure 4.13). The VEGF\textsubscript{121} released from gels displayed broader spatial biodistribution across the different hindlimb regions, as compared with VEGF\textsubscript{165} delivered from alginate (5.4% of VEGF\textsubscript{121} was located in region C, while practically no VEGF\textsubscript{165} was detected in that region). The bolus VEGF\textsubscript{165} injection led to small amounts of bioactive VEGF\textsubscript{165} at the injection site at 24 hours, and complete VEGF\textsubscript{165} deprivation was observed 72 hours after injection (Figure 4.14). The levels of VEGF\textsubscript{121} delivered from gels were also physiologically insignificant at ischemic regions 72 hours after injection (Figure 4.15). In contrast, physiological levels of VEGF\textsubscript{165} were still present in the ischemic hindlimbs 15 days after delivery with the alginate injectable system (Figure 4.16). Bolus delivery did lead to very high VEGF\textsubscript{165} concentrations in peripheral serum within hours after injection, while the VEGF\textsubscript{165} levels in serum were low in the first 12 hours when gels were used for VEGF\textsubscript{165} delivery, and physiological insignificant or zero levels were observed after 12 hours (Figure 4.17). As expected, very high levels of VEGF\textsubscript{121} were detected in peripheral serum.

**VEGF Dose Response in Ischemic Hindlimb Model**

The effects of delivering different doses of VEGF from hydrogels on vascularization and perfusion of ischemic tissues was next analyzed in ApoE\textsuperscript{-/-} mice that were subjected to femoral artery and vein ligation, a standard model of peripheral ischemia that mimics some aspects of human atherosclerosis \textsuperscript{25}. The angiogenic response to delivery of three different doses (3, 5 and 10 \textmu g) of VEGF\textsubscript{165} from alginate gels was analyzed in terms of returning blood perfusion to ischemic regions.
Figure 4.13 Relative VEGF spatial biodistribution in hindlimb tissue 12 hours after delivery.

The relative VEGF (121 and 165 isoforms) distribution in distinct hindlimb regions, 12 hrs post injection for bolus delivery and delivery from alginate hydrogels. Mean values are presented (n=5).
Figure 4.14 Temporal distribution of VEGF\textsubscript{165} delivered in bolus form into ischemic hindlimb tissue.

The ischemic hindlimb tissues were analyzed after 12 hrs (●), 1 day (○) and 3 days (○). After 3 days of injection no bioactive VEGF\textsubscript{165} level was detected in ischemic hindlimbs. Mean values are presented with standard deviations (n=5).
Figure 4.15 Temporal distribution of VEGF$_{121}$ delivered from gels in ischemic hindlimb tissue.

The ischemic hindlimb tissues were analyzed after 12 hrs (●), 1 day (○) and 3 days (□). VEGF$_{121}$ was not detected in ischemic hindlimb tissues after 3 days of injection. Mean values are presented with standard deviations (n=5).
Figure 4.16 Temporal distribution of VEGF_{165} delivered from gels in ischemic hindlimb tissue.

The ischemic hindlimb tissues were analyzed after 12 hrs (●), 1 day (○), 3 days (○), 7 days (●) and 15 days (■). Physiological levels of bioactive VEGF_{165} were present in ischemic hindlimb tissues after 15 days of injection. Mean values are presented with standard deviations (n=5).
Figure 4.17 VEGF levels present in peripheral serum 24 hours after delivery.

The levels of VEGF₁₂₁ present in peripheral serum when released from binary alginate gels (Δ) were very high, likely due to the fact that VEGF₁₂₁ is a very diffusible protein as compared to VEGF₁₆₅. Low levels of VEGF₁₆₅ were observed in the peripheral serum when delivered from gels (□), as compared to bolus delivery (◇). Mean values are presented with standard deviations (n=5).
The LDPI technique was utilized to directly quantify blood perfusion in the entire hindlimbs. Animals treated with blank gels were incapable of recovering the normal levels of hindlimb blood perfusion after surgery. In contrast, animals treated with alginate releasing VEGF_{165} displayed a constant recovery of regional blood flow over time. However, no significant differences were observed between the three different doses delivered (Figure 4.18), indicating that 3 µg was an appropriate VEGF dose.

*Hydrogel VEGF isoform Delivery Promotes Angiogenesis, Alleviates Ischemia, and Prevents Necrosis*

The effects of delivering different VEGF isoforms from hydrogels on vascularization and perfusion of ischemic tissues were also examined in the ApoE-/- ischemic hindlimb model. These experiments had three experimental conditions: alginate gels releasing VEGF_{121}, VEGF_{165} and VEGF_{121} plus VEGF_{165} (ratio 1:1). Three different control conditions were also used: no treatment (neither gel or VEGF), gel with no VEGF, and bolus injection of VEGF_{165}. Retrieval of muscle tissue 42 days postoperative, and immunohistochemical analysis revealed that VEGF_{165} delivering gels increased blood vessel densities, compared with bolus delivery of VEGF_{165} or injection of a gel with no VEGF (Figure 4.19). The delivery of VEGF_{121} either alone or associated with VEGF_{165} also resulted in an increase in blood vessel densities, as compared with controls. Quantification of this data revealed that bolus delivery of VEGF_{165} had no significant effect on vascularization, as expected, and delivery of blank gels resulted in a small increase in blood vessel densities (Figure 4.20). Single VEGF_{121} and VEGF_{165} delivery from the gels resulted in an approximately 2-fold and 2.5-fold increase (respectively) in vessel density, as
Figure 4.18 VEGF$_{165}$ dose response in ApoE$^{-/-}$ ischemic hindlimb model.

LDPI blood perfusion profile of hindlimbs with injection of blank control gels (◇), VEGF delivered from alginate hydrogels (total load of 3 µg VEGF$_{165}$) (●), VEGF delivered from alginate hydrogels (total load of 5 µg VEGF$_{165}$) (○), and VEGF delivered from alginate hydrogels (total load of 10 µg VEGF$_{165}$) (□), in ApoE$^{-/-}$ mice. Mean values are presented with standard deviations (n=6) and * indicates statistically significant differences (p<0.05), as compared to blank control gels.
Figure 4.19 Photomicrographs of tissue sections from hindlimbs of ApoE-/- mice at postoperative day 42, immunostained for the endothelial marker CD31.

Representative images from CD31 immunostained sections of hindlimb muscle tissues. VEGF isoforms released from alginate hydrogels induced higher vessels densities as compared to experimental controls.
Figure 4.20 Quantification of blood vessel densities of hindlimb ApoE^−/− muscle.

Quantification of blood vessel densities after 6 weeks with: no injection of gel or VEGF (---); bolus delivery of VEGF_{165} (+++); injection of a gel with no VEGF (+-+); delivery of VEGF_{165} from a gel (++-); delivery of VEGF_{121} from gel (+++); or delivery of VEGF_{121}, and VEGF_{165} from a gel (++-) in ApoE^−/− mice revealed a statistically significant enhancement with VEGF delivering alginate gels. No synergistic effect was observed with the dual delivery of VEGF_{121} and VEGF_{165} as compared with single factor delivery. Mean values are presented with standard deviations and * indicates statistically significant differences (p<0.05), as compared to blank control gels.
compared to the condition in which there was no intervention. Interestingly, VEGF$_{121}$ delivery from gels resulted in a statistically significant increase in blood vessel density as compared to alginate releasing VEGF$_{165}$, but no synergistic effect was observed with VEGF$_{121}$ and VEGF$_{165}$ dual delivery from gels.

The effects of VEGF delivery from these gels on perfusion and amelioration of limb ischemia were also analyzed. LDPI was used to quantify perfusion in the entire hindlimbs, and tissue necrosis was quantified with visual observation. Regional blood flow was abruptly reduced after surgery in all conditions, as expected (Figure 4.21). No treatment led to little increase in perfusion over time, and the ischemic limbs became necrotic. Bolus VEGF delivery resulted in little difference from the no treatment control. In contrast, sustained and localized VEGF (either single or dual) isoform delivery from the gels led to an increase in tissue perfusion over time, and largely spared the limbs from necrosis. Quantification confirmed the beneficial effect of gel-based VEGF$_{165}$ delivery on return of perfusion, as compared to no treatment, bolus VEGF$_{165}$ delivery, or delivery of gel without VEGF (Figure 4.22). VEGF$_{121}$ delivery from alginate also induced a gradual increase in regional blood flow over time, as compared with experimental controls. However, a synergistic effect with the combination of simultaneous delivery of VEGF$_{121}$ and VEGF$_{165}$ was not observed in terms of blood perfusion. After a small increase in perfusion of the ischemic hindlimbs, the level of perfusion was almost constant over time for bolus VEGF$_{165}$ delivery as well as for the other control conditions. In contrast, animals treated, for example, with alginate gels delivering VEGF$_{165}$ showed a gradual and marked increase in blood flow over time (45% by day 3 and 83% of the normal level by day 42). The surgically induced hindlimb ischemia led to severe toe or foot gangrene, and animals were observed and categorized by the level of limb integrity. Necrosis of
Figure 4.21 Representative color-coded LDPI images at various time points, and gross photographs of ApoE<sup>−/−</sup> mice at postoperative day 42.

No treatment (no gel nor VEGF injection) (top, left panel), bolus VEGF<sub>165</sub> injection (top, center panel), blank gel (top, right panel), alginate gels releasing VEGF<sub>121</sub> (bottom, left panel), alginate gels releasing VEGF<sub>165</sub> (bottom, central panel) and alginate gels releasing VEGF<sub>121</sub> and VEGF<sub>165</sub> (bottom, right panel) conditions were analyzed using LDPI technique. Hindlimb normal baseline (before) perfusion is also provided. Immediately after unilateral femoral artery ligation (after) animals were scanned, and then again at different time points indicated in the images.
Figure 4.22 Regional perfusion profile from ApoE⁻/⁻ mice hindlimbs at various time points.

LDPI blood perfusion profiles of hindlimbs with no injection of gel or VEGF (□), injection of blank control gels (Δ), bolus VEGF₁₆₅ delivery (■), VEGF₁₆₅ delivered from alginate hydrogels (■), VEGF₁₂₁ delivered from alginate hydrogels (○), and VEGF₁₆₅ delivered from alginate hydrogels (○) in ApoE⁻/⁻ mice. * indicates statistically significant difference (p<0.05), as compared to controls (blank alginate, bolus, and no gel or VEGF) and mean values are presented with standard deviations (n=6).
toes or foot loss was prevented by gel-based VEGF<sub>165</sub> delivery in 4 of the animals in that cohort by week 6 (Figure 4.23). Animals treated with alginate gels delivering VEGF<sub>121</sub> displayed a similar distribution of ischemia severity as compared with gel-based VEGF<sub>165</sub> delivery. In contrast, bolus VEGF<sub>165</sub> delivery was incapable of preventing toe necrosis and foot loss, and the other control conditions led to similar results.

4.4 Discussion

The results of this chapter indicate that VEGF delivered from injectable alginate hydrogels promotes angiogenesis, alleviates ischemia, and prevents necrosis. Alginate gels can be manipulated and designed to exhibit favorable degradation kinetics and rheological properties, allowing their use as injectable delivery vehicles capable of displaying proteins in a spatially and temporally regulated order. This injectable system revealed a sustained and localized release of VEGF<sub>165</sub> in ischemic hindlimbs, resulting in a high retention of the VEGF<sub>165</sub> at the site of interest at physiologically relevant concentrations. The VEGF<sub>165</sub> delivered from this injectable system was biologically active and available for long temporal periods, and led to significant increases in blood vessel formation and subsequent increases in tissue perfusion. Although VEGF<sub>121</sub> and VEGF<sub>165</sub> presented distinct tissue biodistribution, there were few differences between these two factors in promoting neovascularization in vivo in these studies. Moreover, no synergistic effect on neovascularization was obtained with the dual delivery of these two growth factors from the gels. These findings indicate that VEGF<sub>121</sub> and VEGF<sub>165</sub> were both capable of promoting
Figure 4.23 Distribution of hindlimb ischemia severity during 6 weeks after surgery.

The probability of developing severe necrosis was dramatically reduced when animals were treated with either VEGF₁₂₁ or VEGF₁₆₅ delivered by alginate gels. In contrast, control animals demonstrated a gradual progression of ischemia severity over time.
neovascularization events but the results also suggest that they may have different roles in angiogenesis or could simply be due to saturation of effect (Figure 4.18).

Alginates are multifaceted polymers that may be formulated for specific applications by simply altering formulation parameters, including molecular weight distribution and solvent solution. The release kinetics of bioactive factors from alginates can be controlled by polymer formulation conditions. The results of this chapter demonstrated that different solvents might be utilized to formulate alginate gels with little or no effect on growth factor delivery rates (Figure 4.2A). In contrast, the molecular weight distribution may be utilized to directly control factor delivery from alginate gels (Figure 4.2B). Incremental increases in the low molecular weight component resulted in faster growth factor release. Furthermore, alginate gels can be assigned specific degradation rates by combining partial oxidation and a controlled molecular weight distribution, resulting in an injectable delivery vehicle capable of sustained release of bioactive factors (Figures 4.4-4.8). The results of this chapter indicate that protein release was controlled by the alginate gel degradation rate, as faster degrading gels led to more rapid release kinetics (Figure 4.4 and 4.7). Alginate gels present a highly porous (5nm-200nm) nanostructure, favorable for high diffusion rates for macromolecules. However, diffusion is also regulated by protein-polymer interactions, and alginate has been reported to reversibly bind heparin-binding growth factors such as VEGF$_{165}$, which slows the release and increases its dependency on gel degradation. For that reason, the absence of heparin-binding domain on VEGF$_{121}$ is likely responsible for the faster release observed with this factor (Figure 4.6). The control over gel degradation in this study was consistent with previous reports, where alginate gel degradation was demonstrated to be controllable by combining partial oxidation of the polymer chains and the utilization of a binary molecular weight
distribution in gel formation. Further, this injectable delivery vehicle was capable of not only maintaining, but also increasing the biological activity of VEGF incorporated into gels (Figure 4.8). This result was consistent with previous studies suggesting that VEGF bioactivity can be enhanced through interactions with alginate, perhaps due to a shielding of the VEGF from environmental conditions and protection from premature denaturation. This increase of the biological activity of VEGF released from gels correlated with an increased endothelial cell sprouting in vitro, likely resulting from activation of ERK1/2 (Figure 4.9 and 4.10). Interestingly, the levels of ERK1/2 activation induced by VEGF released from gels remained constant over different times of induction, in contrast with the VEGF control. These results may indicate that the cellular response to VEGF signal could be internalized in an extended temporal fashion.

The data presented in this chapter indicates that a sustainable and localized release of VEGF from alginate gels in ischemic hindlimbs preservers VEGF presentation at the hypoxic regions to a higher level than does bolus injection (Figure 4.12). The specific biophysical properties of these alginate gels led to VEGF delivery in vivo in a regulated temporal manner (Figure 4.16). The control over the factor spatial distribution also resulted in marginal levels of VEGF in the peripheral serum (Figure 4.17). Not surprisingly, the opposite behavior was observed with VEGF, which is again likely linked with the absence of heparin-binding domain on this VEGF isoform. This factor was transiently available in ischemic regions (Figure 4.15) resulting in relatively high levels being detected in the peripheral serum (Figure 4.17) after injection.

Notwithstanding the evidence that in certain physiologic and pathologic events VEGF has been found to be the most abundant isoform, the utilization of this
isoform in pre-clinical and clinical studies has been almost neglected. The results of this chapter reinforce the diffusible capabilities of this factor, but some control over its delivery was obtained by using alginate gels as delivery vehicles. In contrast, many lines of evidence indicate that VEGF_{165} is an attractive molecule to revascularize ischemic regions \textsuperscript{27,30,31}, however VEGF_{165} delivery has failed in the large clinical trial performed to date \textsuperscript{10}. Due to the short VEGF half-life in vivo, massive doses of VEGF_{165} were typically delivered into the circulation \textsuperscript{5} to stimulate a therapeutic effect in past trials, likely resulting in an absence of spatial resolution (ineffective microenvironmental localization) and a lack of appropriate temporal dynamics of VEGF_{165} presentation (local environment exposed to marginal VEGF_{165} doses). Because the timing of availability of angiogenic factors is believed to be crucial to obtain a physiological adequate angiogenic response in vivo \textsuperscript{31}, the use of polymeric constructs to deliver these types of therapeutic agents is very appealing \textsuperscript{15,19}. The specific hydrogel system developed in these studies was capable of maintaining bioactive VEGF_{165} in the ischemic tissue over significant time periods (7-15 days) with little biodistribution outside of the ischemic regions, and this contrasts with results of bolus delivery.

The results of this chapter document that therapeutic angiogenesis would not only likely benefit from sustained and localized VEGF_{165} delivery from the injectable and degradable alginate gels, but also that the VEGF_{121} therapeutic potential had been undervalued. The sustained VEGF_{165} tissue exposure made possible with this injectable system led to significant increases in blood vessel formation in the ischemic muscle tissue (Figures 4.19 and 4.20). Strikingly, VEGF_{121} led to higher blood vessel densities after 42 days of treatment (Figure 4.20) as compared to VEGF_{165}, despite the fact that VEGF_{121} exposure is more transient in ischemic tissues. These findings
suggest distinct roles of the different VEGF isoforms. One may conjecture that VEGF\textsubscript{165} is responsible for the short-range signaling providing guiding cues for local cells, while the freely diffusing and chaotic distribution characteristic of VEGF\textsubscript{121} may be critical for long-range cell activation. Furthermore, the controlled and localized delivery of both VEGF isoforms led to significant increases in tissue perfusion (Figure 4.21 and 4.22), alleviating ischemia and preventing necrosis associated with ischemia (Figure 4.21 and 4.23).

Altogether, these data indicate that VEGF delivered from alginate gels regulates the formation of functional new blood vessels in ischemic hindlimbs. Previous studies indicate that lipid disorders can directly impair or retard angiogenesis\textsuperscript{25}, and the results of this chapter indicate that the low endogenous angiogenic potential of ApoE\textsuperscript{-/-} mice can be bypassed with a sustainable VEGF delivery. However, while it was possible to rescue and reverse limb necrosis, it was not possible to achieve normal tissue perfusion nor were synergistic effects observed with dual delivery of VEGF isoforms. These findings suggest that VEGF delivery alone may not be sufficient, and that future studies may involve combining VEGF delivery with other angiogenic factors to augment and mature the angiogenic response, as previously demonstrated with polymer systems that require surgical implantation\textsuperscript{15}.

In summary, an injectable biodegradable alginate hydrogel allowing a sustained and localized release of VEGF has been developed. This system is particularly appealing for therapeutic use, since it allows for minimally invasive factor delivery. These results demonstrated that this injectable gel system provides VEGF\textsubscript{165} at a desirable concentration for extended time periods. The spatiotemporal factor bioavailability provided by this system led to a significant angiogenic response in ischemic hindlimbs, and this system may also be broadly useful to manipulate or
exploit the presentation of a wide variety of other growth factors and an examination of their roles in this and other biological processes.

4.5 References


CHAPTER 5

Alginate as synthetic extracellular matrix for progenitor cell delivery

5.1 Introduction

As described in Chapter 4, a spatiotemporal control over VEGF delivery from alginate gels regulates the formation of functional new blood vessels in ischemic sites, but an alternative approach to promoting angiogenesis is to recapitulate the process of vasculogenesis, and deliver appropriate endothelial progenitor cells to the desired site. As reviewed in Chapter 2, distinct cell populations have been utilized in several clinical trials, but the results of these trials are consistently failing or demonstrating marginal benefit\(^1^3\). A likely cause of these disappointing results is that the delivery methods for the cells in the clinical trials lack control of cell fate after transplantation. The objective of this chapter is to develop a material delivery system capable of creating a localized depot of cells \textit{in vivo} that exit over time to repopulate the damaged tissue and participate in its regeneration. The polymer described in Chapter 4 was manipulated to present cell specific microenvironmental cues and fabricated to serve as an implantable macroporous delivery system. This chapter describes the
utility of this material system to induce neovascularization in a hindlimb ischemic model, and also investigates the role of transplanted progenitor cells in tissue regeneration.

Cell therapies hold tremendous potential to treat a wide array of diseases ranging from diabetes to neurologic disorders, and have been the focus of considerable research and clinical efforts over the past ten years \(^2\text{-}^4\). The potential of circulating progenitor cells for the treatment of ischemic diseases has been a particularly prominent focus recently, with over 80 clinical trials initiated over the past five years. Such trials have involved harvesting a patient’s own cells, multiplying these cells in vitro, and reinfused them to the target tissue (e.g., ischemic muscle). While initial animal studies yielded promising results, recent clinical trials have not been nearly as successful \(^1\text{-}^5\). These trials, while supporting the feasibility and safety of utilizing such cells therapeutically, indicate that simple infusions of cell suspensions may have significant limitations \(^6\). More specifically, work with many cell types indicates that the vast majority (typically > 90%) of cells transplanted in this manner will fail to engraft in the target tissue and rapidly die.\(^7\) Thus the control over these cells’ fate is lost once they are placed in the body. In addition, the actual mechanism(s) by which endothelial progenitor populations function to enhance ischemic tissue vascularization and promote tissue regeneration has been reported to vary from direct participation in rebuilding of the tissue to a support role \(^8\), and this also complicates efforts to design an appropriate strategy for their deployment to damaged tissues. These same issues are also likely to plague other ongoing clinical trials using various cell populations and disease targets (e.g., neuroprogenitors for Parkinson’s disease).
Some of these limitations may be directly associated with the delivery approaches which appear incapable of providing new vascular networks that can support the physiologic needs of the tissue and integrate the new vessels with the surrounding host tissue. One may bypass certain of these limitations by delivering the cells on sophisticated material carriers that promote tissue formation by the cells, using the material as a template. However, this approach does little to address defects in the host tissue, and integration of the new tissue mass and the host tissue (e.g., vascularization, innervation, mechanical continuity) is often problematic. The natural ECM represents an appealing source of inspiration to design new materials systems capable of bypassing some of these problems.

The ECM plays a number of critical roles in regulating tissue regeneration, including the binding of cells via specific receptors, conveyance of mechanical signals, and presentation of growth factors and cytokines\textsuperscript{9}, and these signals vary in a temporally dynamic manner due to the constant remodeling of the ECM. One particularly important function of the ECM is providing a foundation for cell attachment, as cell anchorage plays an important role in the regulation of cell growth, differentiation, and apoptosis. The adhesion of cells to native ECM is mediated by specific cell-surface receptors, such as integrins, that interact with short amino acid sequences presented in the ECM molecules\textsuperscript{10}. This signaling is frequently mimicked in material system architecture by the presentation of cell binding motifs from the polymers used to fabricate the synthetic ECMs. The amino acid sequence arginine-glycine-aspartic acid (RGD), a ubiquitous cell-binding domain derived from fibronectin and laminin, is the peptide used most frequently to promote cellular attachment to synthetic ECMs\textsuperscript{11-13}, and the density of these ligands presented to cells
from the material has been shown to regulate the cellular response in vitro and in vivo.

Cell adhesion peptides can be linked to materials via different techniques including, physical immobilization and covalent coupling. Covalent couplings in an appealing approach due to the presence of terminal amine group on the adhesion peptide chains, and carboadiimide chemistry has been utilized to couple RGD sequences to different materials including alginate. The RGD coupling to alginate (Figure 5.1) confers specific cellular interactive properties to the otherwise non-adhesive structure, and the RGD-ligand density and distribution can be manipulated to provide control over cell adhesion, proliferation and cell fate after transplantation.

This chapter describes the development of a sophisticated material system capable of regulating the sustained egress of progenitor cells into ischemic hindlimbs. The alginate system developed in this chapter accommodates a variety of cell specific microenvironmental cues, including physical properties, and soluble and insoluble biological signals (Figure 5.2). The biological and physical architecture designed for this system was first examined in vitro by examining whether endothelial progenitor cells would egress in a viable and sustainable fashion. Next, this cell delivery vehicle was utilized to investigate how microenvironmental conditions designed to maintain the viability and outward migration of human vascular progenitor cells would effect the efficacy of these cells in salvaging ischemic murine limbs and returning perfusion to normal levels. Finally, these systems demonstrated great utility in directly investigating the specific role of different endothelial progenitor populations in neovascularization.
Figure 5.1 RGD-modified alginate structure.

Carbodiimide chemistry mediates the formation of an amide bond between the carboxyl group of alginate and the N-terminal amine of the RGD peptide sequence.
Figure 5.2 Schematic illustration of proposed cell delivery vehicle.

Presentation of cell adhesion ligands (RGD containing peptides) (red dots) and local morphogens (VEGF) (yellow dots) from the material to maintain cell viability, and to activate and induce cell migration out of scaffold (A). After implantation, cells within scaffolds will proliferate, while others will follow VEGF release and orchestrate tissue regeneration outside scaffolds (B).
5.2 Materials and Methods

**Human Umbilical Cord Blood Mononuclear Cell Preparation and Culture**

Human cord bloods (50-80 ml) were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA, USA). Human umbilical cord blood mononuclear cells (CBMNCs) were obtained as previously described with some modifications \(^{25,26}\) (see Appendix P for detailed protocol). Specifically, blood was diluted 1:1 with Hanks balanced salt solution (HBSS; Sigma, St. Louis, MO, USA) and layered over an equivalent volume of Histopaque 1077 (Sigma, St. Louis, MO, USA). Cells were centrifuged for 30 minutes at room temperature at 400 g for the early endothelial progenitor cells (EPCs) preparation. To obtain EPCs, the isolated CBMNCs were resuspended in EGM-2 MV medium (Cambrex, Walkersville, MD) consisting of EMB2, 5% fetal bovine serum, hEGF, VEGF, hFGF-B, IGF-1, ascorbic acid and antibiotics, and 1 x 10^6 CBMNCs per cm^2 were seeded on fibronectin-coated (Sigma, St. Louis, MO, USA) tissue culture plates and incubated in 5% CO\(_2\) incubation at 37°C. At \(\approx\)5 days after plating, non-adherent cells were aspirated and adherent cells were gently washed twice with culture media. Adherent cells EPCs were detached with 0.25% trypsin-EDTA (Mediatech, Herndon, VA, USA), washed and used in experiments.

To obtain outgrowth endothelial cells (OECs), blood diluted 1:1 with HBSS was centrifuged at 740 g, and the resultant CBMNCs were cultured on type I collagen coated tissue culture plates (BD Biosciences, Bedford, MA, USA) with EGM2-MV medium supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbed, CA,
USA). After 36 hours of culture, non-adherent cells were removed and adherent cells were washed once with medium. Medium was changed daily for 7 days and then every other day until the first passage. Colonies of OECs appeared between 7-14 days of culture. OECs derived from the colony were released from the original tissue culture plate with 0.05% trypsin-EDTA (Invitrogen, Carlsbed, CA, USA), resuspended in EGM2-MV medium and plated onto 25 cm² tissue culture flasks. OECs at second passage were used for in vivo experiments, and at passage 3 for in vitro experiments. EPCs and OECs were isolated from human cord blood 7 times, and similar results were obtained from each donor.

To characterize cells with immunohistochemistry markers, 1x10⁴ OECs and EPCs were cultured on fibronectin – coated chamber slides (Nalgen Nunc, Rochester, NY, USA) for 12 hours. Cells were fixed with an ice cold acetone/methanol solution (1:1) for 10 minutes, and incubated at room temperature for 30 minutes with primary antibodies, followed by 10 minute incubations with LSAB2 link-biotin and streptavidin-HRP (DAKO, Carpenteria, CA, USA), and then developed with DAB solution (DAKO) for 5 minutes. The following primary antibodies were utilized: anti-CD14, anti-CD31, anti-CD34, anti-CD144 (all from DAKO), anti-VEGFR-2 (KDR) (Sigma, USA), and VWF (BD-Biosciences, USA). Slides were analyzed by visual inspection under 100x magnification.

Macroporous Alginate Scaffolds

Alginate molecules rich in guluronic acid blocks (LF 20/40, FMC Biopolymer, NJ, USA) were first oxidized using sodium periodate (NaIO₄), as previously described ²⁷ (see Appendix E for detailed protocol), to generate
hydrolytically labile polymers. In brief, NaIO₄ was added to 1% (w/w) aqueous solutions of alginate. The molar ratio between NaIO₄ and uronic acids was kept constant at 0.01:1. After 10 hr, ethylene glycol was added to stop the reaction, and the oxidized alginates were dialyzed against deionized water for two days (molecular weight cut-off, 3,500) followed by lyophilization. Then, oxidized alginates were coupled with oligopeptides containing the Arg-Gly-Asp cell adhesion sequence (Commonwealth Biotech Inc., USA) following aqueous carbodiimide chemistry as previously described ²⁰ (see Appendix Q for detailed protocol). Briefly, N-hydroxysulfosuccinimide (sulfo-NHS, Pierce, USA), 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC, Sigma, USA), and oligopeptides (GGGGRGDS) were sequentially added to alginates dissolved in 2-(N-morpholino) ethanesulfonic acid (MES, Sigma) buffer at pH 6.5. The molar ratio between oligopeptides and uronic acids was kept constant at 1:1000, yielding two oligopeptides per alginate molecule. After reacting for 24 hours, alginate molecules modified with RGD peptides were purified with dialysis against deionized water for four days (molecular weight cut-off, 3,500) followed by sterilization through a 0.22 µm filter, and purification through membrane-based dialysis. Following lyophilization, the samples were reconstituted with EBM cell culture medium (Cambrex, MD, USA) at 2% (w/w).

Hydrogels were prepared by mixing the alginate solution with calcium sulfate slurry, and the molar ratio between calcium and uronic acids was kept constant at 0.40. The mixture was injected between glass plates with a spacer of 1 mm. After curing for 25 min, gel disks with diameter of 10 mm were punched out. These gel disks were frozen and stored at -20°C, and after 24 hr, gel disks were lyophilized to yield
macroporous materials. Gel disks were soaked with a cell suspension to infiltrate cells into pores.

To incorporate VEGF (1 - 3 μg total per scaffold, for in vitro and in vivo experiments respectively) alginate solutions were mixed with recombinant human VEGF_{121} (R & D Systems, MN, USA) or VEGF_{165} protein (Biological Resources Branch of National Cancer Institute, USA) before formation of gels and macroporous scaffolds. The alginate/VEGF solutions were then fabricated into macroporous scaffolds as described above.

_In Vitro Cell Assays_

Human microvascular dermal endothelial cells (HMVEC-d) (Cambrex Corporation, NJ, USA) (passage 6) and OECs (passage 2) were seeded into 24 well plates (5,000 cells/cm² cell seeding density) overnight with EGM-2MV. The cells were then washed twice with PBS and cultured with EGM-2MV without growth factors, EGM-2MV without growth factors but supplemented with VEGF_{121} (50 ng/ml), and EGM-2MV without growth factors supplemented with VEGF_{165} (50 ng/ml). After 3 days, the endothelial cells were detached via trypsinization and counted in a Coulter Counter (Beckman Corp.).

The ability of cells to migrate outwards from macroporous alginate scaffolds with no VEGF, or from scaffolds containign VEGF_{121} or VEGF_{165} (1 μg total incorporated per scaffold, respectively), was analyzed by seeding 5 x 10⁵ of OECs (passage 3) into the scaffolds, and then placing the scaffolds in contact with a collagen gel (3.0 mg/ml) (PureCol). The combined scaffold and collagen gel was then cultured with EBM2-MV without growth factors (Cambrex, NJ, USA) media at
37°C. At different experimental time points, the scaffold was removed and the cells that had populated the collagen gel were obtained by washing the collagen gels, and dissolving the gels, and counting cells in a Coulter Counter (Beckman Corp., USA). The total number of cells that migrated out of the scaffold was normalized to the total number of cells seeded initially into scaffolds. The viability of the cells populating the collagen gel was quantified by trypan blue exclusion with a Viacell Counter (Beckman Corp., USA).

The ability of the EPCs and OECs to modulate angiogenesis was analyzed in vitro using an endothelial cell sprouting assay \(^9\) (see Appendix C for detailed protocol). Cytodex 3 microcarriers (Amersham Biosciences, Piscataway, NJ, USA) were hydrated in PBS at room temperature (0.2 ml/mg of dry Cytodex 3), and after 3 hours the supernatant was decanted and replaced with fresh PBS, followed by sterilization with autoclaving. EPCs, OECs (passage 3) and HMVEC-d (Cambrex, MD, USA) (ECs) (passage 5), alone, selectively co-cultured (ECs/OECs - 1:1 ratio), or seeded all together (1:1:1 ratio) were cultured in EGM-2M (50 mg of microcarriers was combined in a 8:1 (cell:microcarrier) ratio in a spinner vessel (Bellco Glass Inc., Vineland, NJ, USA)). After 3 hours, microcarriers with adherent cells were incubated for 20 hrs with continuous stirring, and subsequently transferred to tissue culture flasks for an additional 1-2 days of culture. To perform the sprouting assay, the beads in suspension (57 ul) were combined with 170.5 ul of fibrinogen (Sigma, USA) solution (4 mg/ml) and 22.7 ul of aprotinin (Sigma, USA) (500 ug/ml). This solution was then added to 200 ul of thrombin (Sigma, USA) (22.72 units/ml), and incubated at 37°C for 20 min to allow gel formation. Cultures were fed every day with 0.8 ml of EGM-2MV without growth factors, or EGM-2MV with control VEGF\(_{165}\) (50 ng/ml). After 5 days, gels were washed twice with PBS and subsequently incubated with 4%
formaldehyde overnight at 4°C. The formaldehyde solution was then aspirated, and gels were washed twice with PBS (for certain conditions cells were labeled LIVE/DEAD prior fixation and then stained with DAPI (Molecular Probes – Invitrogen, USA) after overnight fixation) (see Appendix R for detailed protocol). Sprouts per bead were analyzed and visualized at 100x and 200x with an Olympus-IX81 light microscope (Japan) connected to an Olympus DP70 digital image capture system (Japan) (average of 100 beads analyzed per condition).

*Ischemic Hindlimb model in SCID Mouse*

All procedures were approved by the Experimental Animal Committee of Harvard University. For evaluation of *in vivo* angiogenesis, surgery to induce hindlimb ischemia was performed as described ³ (see Appendix J for detailed protocol). In brief, female SCID mice (Taconic, Cambridge, MA, USA) 8 to 9 weeks old, and 17 to 20 g in weight, were anesthetized with xylazine (20mg/kg body weight) and ketamine (100mg/kg body weight). The cell-loaded alginate scaffolds (5x10⁶ cells/scaffold) were implanted on the medial side of thigh muscle or 5x10⁶ cells in 50 μL serum-free EBM medium were injected into the hindlimb intramuscularly. The groups (n=6 per condition) were as follows: (1) blank scaffold, (2) bolus (containing 3 μg of VEGF₁₂₁) intramuscular injection of OEC (5x10⁶ cells), (3) OEC-loaded scaffolds (without VEGF₁₂₁) (5x10⁶ cells) and (4) OEC-loaded scaffolds (with VEGF₁₂₁ – 3 μg per scaffold) (5x10⁶ cells). These animals were humanely euthanized two weeks after surgery. A different group of animals were subjected to hindlimb ischemia surgery (as described above) and euthanized six weeks post-operative. After the vessel ligation, mice were injected intramuscularly with a total volume of 50 μl of
a solution (containing 3 μg of VEGF₁₂₃) of EPCs and OECs (5x10⁶ cells total in a 1:1 ratio), EPC-loaded scaffolds (5x10⁶ cells), OEC-loaded scaffolds (5x10⁶ cells), and EPC and OEC-loaded scaffolds (5x10⁶ cells total/scaffold in a 1:1 ratio). All scaffolds also contained 3 μg total VEGF₁₂₃ in this experiment. Before surgery and 0, 1, 3 and 7 days, and 2, 4, and 6 weeks post-surgery measurements of the ischemic/normal limb blood flow ratio were performed on anesthetized animals (n=6/timepoint/experimental condition) using a Periscan system blood perfusion monitor laser Doppler equipment (Perimed, Sweden) (see Appendix N for detailed protocol). Perfusion measurements were obtained from the right (ischemic) and left (non-ischemic) limb. To minimize variability due to ambient light and temperature, the index was expressed as a ratio of ischemic to non-ischemic limb blood flow 3⁰. Ischemic hindlimbs were also visually observed at 1, 2, 4 and 6 weeks postoperative in order to grade limb integrity, and categorized according to degree of necrosis: normal compared to non-surgical limb, one necrotic toe, multiple necrotic toes, and necrotic foot.

After euthanization, hindlimb muscle tissues (n=6/timepoint/experimental condition) were retrieved, fixed, paraffin embedded, and immunostained for mouse CD31 (BD Biosciences Pharmingen, San Diego CA) (see Appendix M for detailed protocol), or human CD31 (Dako, USA) (see Appendix S for detailed protocol). For measurement of capillary densities, 30 randomly chosen high-power fields of the sections were analyzed. The number of positively stained blood vessels were manually counted and normalized to the tissue area. Sections from each sample were visualized at 200x and 400x with an Olympus-IX81 light microscope (Japan) connected to an Olympus DP70 digital image capture system (Japan) and analyzed
using IPLab 3.7 software (Scanalytics, Rockville, MD). Vessel quantification was determined using ImageJ (NIH, USA) software.

Detection of Telomerase Activity

The telomerase activity in ECs, EPCs and OECs was measured with a commercially available quantitative detection kit (XpressBio, Frederick, MD, USA). Samples were prepared according to the manufacturer’s instructions. Telomerase activities were determined from cycle threshold $s(C_T)$ after 40 PCR cycles with SYBR Green I using the MJ Opticon real time PCR machine (MJ Research, Reno, NV, USA).

Viability and Proliferation of OECs that Migrated out from Scaffolds

OECs that had migrated into collagen gels were labeled with a LIVE/DEAD kit (Molecular Probes - Invitrogen, USA) according to the manufacturer’s instructions and incubated at room temperature for 30 min. Images were obtained at different time points using an Olympus-IX81 light microscope (Japan) connected to an Olympus DP70 digital image capture system (Japan). To measure the proliferation ability of these cells, OECs were detached from collagen gels via trypsinization and subsequently seeded into 24 well plates (5,000 cells/cm$^2$ cell seeding density) overnight with EGM-2MV. Control OECs (cells never placed in scaffolds) at the same passage number (p3) were seeded in the same conditions. OECs (both control and migrated from scaffolds) were then washed twice with PBS and cultured with EGM-2MV without growth factors, or EGM-2MV with control VEGF$_{165}$ (50 ng/ml).
Media was changed every day and at day 3 cells were detached via trypsinization and counted in a Coulter Counter.

**Angiogenesis antibody arrays**

Conditioned media from ECs, EPC and OEC cultures were obtained by maintaining cells (1x10⁶ cells) for 2 days in 2 ml with culture EBM-2MV medium. Expression of angiogenic factors in the supernatants was compared using an angiogenesis antibody array kit (Panomics, Fremont, CA, USA). All procedures were based the manufacturer’s instructions.

**Statistical Analysis**

All statistical analysis was performed using Students t-test (two-tail comparisons), and analyzed using InStat 2.01 (Graphpad, USA) software. Differences between conditions were considered significant if p < 0.05.

Note: Some of the experiments described on this chapter were done in collaboration with Eun-Suk Kim and Hyun Joon Kong

**5.3 Results**

*Morphologic and phenotypic features of EPCs and OECs*

Human umbilical cord blood can provide a useful source of progenitors cells for therapeutic applications, including different subpopulations of endothelial cells."
Both endothelial progenitor cells (EPCs), and blood outgrowth endothelial like cells (OECs) were isolated from human umbilical cord blood to analyze their potential utility in relieving ischemia and contributing to angiogenesis. In culture, the EPCs consisted of round cells forming colonies, and spindle shaped cells at the periphery of the colonies forming cord-like structures, as noted in previous studies\textsuperscript{31,32} (Figure 5.3A). Distinctively, OECs exhibited a cobblestone-like morphology similar to human microvascular endothelial cells (ECs), and multiple population doublings without senescence, again in agreement with past studies\textsuperscript{8,31} (Figure 5.3B). Immunohistochemistry confirmed that EPCs were monocyte/macrophage lineage cells and OECs were vascular endothelial lineage cells (Figure 5.4). Specifically, OECs expressed vascular endothelial cell surface antigens, including CD31, CD144, vWF, KDR and CD34. EPCs also expressed CD31, CD144, KDR and CD34, but revealed weak expression of vWF, since it was observed only in a small number of EPCs. CD14, the monocyte/macrophage cell surface antigen, was expressed only in EPCs. Further, the telomerase activity of these cells was also investigated (Figure 5.5). As expected, OECs revealed approximately 2-fold more telomerase activity, as compared with mature endothelial cells. In contrast, EPCs displayed very low levels of telomerase activity, as compared with ECs and OECs. The profile of protein secretion by these different endothelial cells populations was also investigated using an angiogenesis antibody array (Figure 5.6). EPCs, OECs and ECs exhibited different profiles of secretion. Leptin was exclusively secreted at high levels by EPCs and only OECs expressed relatively high levels of PIGF secretion. Finally, the proliferation rate of OECs under VEGF influence was examined (Figure 5.7). In contrast with ECs, OECs displayed distinct proliferation rates when treated with different VEGF isoforms, the VEGF\textsubscript{121} isoform specifically induced a higher proliferation rate of
Figure 5.3 DIC photomicrographs of EPCs and OECs isolated from human umbilical cord blood.

EPCs were organized in colony forming units, emerging 5-6 days after cord blood isolation. EPCs displayed central clusters of rounded cells surrounded by spinal cells (A). OECs display a cobblestone-like morphology typical of mature microvascular endothelial cells. This cell population emerged 20 days after cell isolation and culture (B). (100x magnification for A and B)
Figure 5.4 Immunohistochemical photomicrographs of EPCs and OECs isolated from human umbilical cord blood.

Immunohistochemical staining for a variety of cell surface antigens demonstrated positive staining of EPCs for CD14, CD31, CD34, CD144, KDR, and negative staining for vWF. In contrast, the OECs demonstrated positive staining for CD31, CD144, KDR, and vWF. (100x magnification)
Figure 5.5 Telomerase activities of different human endothelial cell populations.

OECs (■) displayed high levels of telomerase activity as compared with EPCs (□) and microvascular endothelial cells (□). Values represent mean and standard deviation (n=7) and * indicates statistically significant difference (p<0.05).
Figure 5.6 Analysis of angiogenic protein secretion by EPCs, OECs and ECs.

Each cell type had a distinct profile of secretion, but it was particularly notable that EPCs exclusively secreted high levels of leptin (red boxes) and OECs alone exhibited relatively high levels of PIGF secretion (blue boxes) (A). The code for the array is also provided (B).
Figure 5.7 Proliferation of OECs and ECs in response to different VEGF isoforms.

After 3 days of culture, OECs exhibited high levels of proliferation as compared with ECs. Interestingly, VEGF<sub>121</sub> (■) induced a higher rate of proliferation in OECs, as compared with VEGF<sub>165</sub> (■). As described in Chapter 3, VEGF<sub>121</sub> (▪) and VEGF<sub>165</sub> (▪) induced the same level of proliferation in ECs. Values represent mean and standard deviation (n=6) and * indicates statistically significant difference (p<0.05).
OECs, as compared to VEGF_{165}. As expected, OECs revealed a higher disposition to proliferate, as compared to ECs.

*In vitro validation of implantable alginate scaffolds as cell delivery vehicle*

Implantable alginate scaffolds were fabricated containing RGD signals and VEGF signals capable of both mediating scaffold cell adhesion and of maintaining cell viability, proliferation and migration from the scaffolds. To investigate the effect of the VEGF isoforms in mediating cell migration, OECs were seeded into scaffolds and outward migration was observed over time (Figure 5.8). Three days after cell seeding, scaffolds presenting VEGF_{121} mediated higher outwards OEC migration as compared with scaffolds presenting VEGF_{165}. Quantification of cells that repopulated the tissue mimics (collagen matrix) revealed that the presence of VEGF in scaffolds increased migration 2-fold, as compared with blank scaffolds (Figure 5.9). Inclusion of no VEGF led to very few cells migrating out of the scaffolds, while VEGF_{165} led to approximately 47% of the initial cell number migrating into the surrounding collagen by 3 days. VEGF_{121} was capable of inducing higher cell migration out of scaffolds (62%) as compared to VEGF_{165}. In addition, the viability of these migratory cells was also analyzed (Figure 5.10). Approximately 60% of the cells that migrated out from scaffolds presenting VEGF_{121} were viable, in contrast to only 28% of cells remaining viable when migrating from blank scaffolds. Subsequently, the proliferation capacities of the cells that migrated out of scaffolds were tested, and compared with cells that never were in contact with scaffolds (Figure 5.11). Both OECs populations displayed the same rate of proliferation when stimulated with VEGF_{165}.
Figure 5.8 Representative photomicrographs and quantification of OECs that migrated out from alginate scaffolds.

Quantification of OECs migrating out of scaffolds presenting VEGF and RGD cues (■), scaffolds with RGD and no VEGF (□) and scaffolds without RGD or VEGF (○) after 24 hrs (A). Phase-contrast photomicrographs of OECs that have migrated out from scaffolds that contain no VEGF (blank), VEGF₁₂₁, or VEGF₁₆₅ and populated the surrounding tissue mimic (collagen gel) after 72 hrs (B). Values represent mean and standard deviation (n=6) and * indicates statistically significant difference (p<0.05). (200x magnification in all images)
Figure 5.9 Quantification of OECs that populated tissue mimics after scaffold migration.

Quantification of OECs populating the collagen matrix when VEGF₁₂₁ was incorporated into scaffolds (■), as compared with the presentation of VEGF₁₆₅ (■), or no VEGF (□). Values were normalized to the initial cell number placed in scaffolds. Values represent mean and standard deviation (n=6) and * indicates statistically significant difference (p<0.05).
Figure 5.10 Visualization and quantification of OECs cell viability after migration out from scaffolds.

Phase-contrast photomicrographs of cells labeled with a LIVE/DEAD kit indicating live cells (green) viability and few dead cells (red) when migrating from scaffolds presenting VEGF$_{121}$ after 24 hrs, 36 hrs, and 72 hrs (A). 72 hours after cell seeding, the viability of the cells that migrated out of scaffolds with VEGF$_{121}$ (■) was higher, as compared with scaffolds releasing VEGF$_{165}$ (■) or blank (no VEGF) scaffolds (□) (B). Values represent mean and standard deviation (n=6) and * indicates statistically significant difference (p<0.05). (200 x magnification in all photomicrographs)
Figure 5.11 Proliferation of control OECs and OECs that migrated out from scaffolds.

Quantification of the proliferative ability of OECs that had migrated out of scaffolds (OEC from scaffolds) ( ), and OECs of the same passage that were not placed in scaffolds (control OEC) (■) following VEGF stimulation in vitro. OECs that were not exposed to VEGF served as a negative control (control) (□). Values represent mean and standard deviation (n=6) and N.S. indicates no statistically significant difference between conditions.
Role of EPCs and OECs in neovascularization - in vitro evaluation

The sprouting assay, described in Chapter 3, was utilized as a model to address the role of EPCs and OECs on neovascularization events (Figure 5.12). OECs demonstrated significant migration off beads, and typically no sprouts were observed with these cells (Figure 5.12A). However, co-culture of ECs and OECs resulted in a significantly increased sprout formation, as compared to ECs alone, and also increased the disposition for lumen formation in the sprouts. Interestingly, co-culture of EPCs, OECs and ECs resulted in massive sprouting, as compared with co-culture of OECs and ECs, and contrasted with the absence of sprouts when EPCs alone (Figure 5.12B). Interestingly, co-culture of EPCs and OECs on top of the fibrin gel induced EC migration towards the EPCs and OECs.

Controlled cell delivery enhances angiogenesis in ischemic hindlimbs

The utility of this approach to enhance the efficacy of transplanted OECs in alleving tissue ischemia was next assessed in SCID mice that were subjected to femoral artery and vein ligation. This evaluation was first addressed by using VEGF121 presenting scaffolds loaded with OECs. Three different control conditions were also used: blank scaffolds (neither cells or VEGF), infusion of a solution of OECs, and scaffolds loaded with OECs but with no incorporated VEGF. Animals treated with blank scaffolds suffered dramatic tissue necrosis, exhibiting auto-amputation within three days after scaffold implantation (Figure 5.13). An infusion containing OECs and VEGF121 (same quantities as placed in scaffolds) resulted in
ECs alone form sprouts (arrows) when immobilized on microcarrier beads that are placed into fibrin gels (top, left panel). OECs alone, in contrast, exhibit significant migration off beads (arrows), and typically do not form sprout structures (Top, right panel). Combining ECs and OECs significantly increased sprout formation, as compared to ECs alone, and increased lumen formation (areas delineated by yellow dashed lines) in the sprouts (bottom, left panel) (A). Culture of EPCs alone led to no sprouting, but co-culture of EPCs, OECs and ECs led to significant sprouting, and sprouts exhibited lumens (top, center and right panel). Cells were labeled with DAPI and LIVE/DEAD stains to more clearly demonstrate sprout formation (top, right panel). Culture of EPCs and OECs on top of gels containing carrier beads with adherent ECs led to significant migration of the ECs towards the EPCs and OECs (bottom, left panel). The inset demonstrates cells on the top of the gel, with the bead on a different focal plane (indicating cells in main image were not OECs or EPCs on top of gels) (B). (200x magnification in all images)
Figure 5.13 Representative gross photographs of hindlimb SCID mice at postoperative day 3 and day 15.

Implantation of blank scaffolds resulted in severe limb necrosis and auto-amputation after 3 days (top, left panel). Bolus injection of OECs and VEGF$_{121}$ (same quantities placed in scaffolds) prevented limb auto-amputation (top, right panel), as did also transplantation of OECs on scaffolds lacking VEGF$_{121}$ (bottom, left panel), but significant necrosis in the foot and toes was still noted in these conditions. Transplantation of OECs on scaffolds presenting VEGF$_{121}$ prevented auto-amputation, and decreased foot and toe necrosis (bottom, right panel).
limb auto-amputation. Transplantation of OECs on scaffolds presenting VEGF_{121} prevented auto-amputation, and decreased the extent of foot and toe necrosis (Figure 5.14). Hindlimb tissue retrieval at 15 days postoperative on these animals, and immunohistochemical analysis revealed that OECs transplanted with scaffolds presenting VEGF_{121} induced higher blood vessels densities as compared with the controls (Figure 5.15). Interestingly, capillary size and distribution induced by OECs delivered by scaffolds presenting VEGF_{121} were very similar to those observed in healthy animal hindlimbs. In contrast to this vessel distribution and organization, the hindlimbs of the animals treated with OEC delivered via bolus injection or with a scaffold lacking VEGF_{121} demonstrated disorganized vessel presentation. Subsequent quantification of this set of tissue sections demonstrated that transplantation of OECs from scaffolds presenting VEGF_{121} resulted in a 2-fold increase in vessel density, as compared with scaffolds lacking VEGF_{121} (Figure 5.16). Further, the fate of transplanted cells in terms of engraftment and incorporation into the host vascular network were analyzed (Figure 5.17). In the absence of scaffolds, no engraftment of cells was noted, while scaffolds lacking VEGF_{121} but loaded with OECs revealed low levels of cell engraftment. Finally, scaffolds loaded with OECs and VEGF_{121} displayed not only significant engraftment of cells, but also the presence of functional human-murine chimeric blood vessels.

Co-transplantation of EPC and OEC enhance neovascularization in vivo

The effects of controlled EPC and OEC delivery on hindlimb necrosis and regional perfusion were further evaluated using the same peripheral hindlimb model over a 42 day time period, using an LDPI system to monitor the functionality of new
Figure 5.14 Distribution of hindlimb ischemia severity on SCID mice during 2 weeks after surgery.

Hindlimbs subjected to surgery were also visually examined, and grouped as normal (displaying no discrepancy in color or limb integrity from non-ischemic hindlimbs of the same animal), or presenting one necrotic toe, multiple necrotic toes, or a complete necrotic foot. Animals treated with scaffolds delivering OECs and VEGF_{121} presented reduced distribution of ischemia as compared with control conditions.
Figure 5.15 Representative photomicrographs tissue sections of hindlimb SCID mice at postoperative day 15, immunostained for the mouse endothelial marker CD31.

Transplantation of OECs on scaffolds presenting VEGF_{121} (bottom, left) induced higher vessel densities as compared with experimental controls. Interestingly, the organization and distribution of blood vessels observed on scaffolds delivering OECs and VEGF_{121} was similar to healthy hindlimb tissue sections (bottom, right panel). Scale bar represents 100 μm.
Figure 5.16 Quantification of blood vessel densities of hindlimb SCID mice at 15 days postoperative.

Quantification of the total blood vessel densities in hindlimb muscle tissue after 2 weeks with bolus injection of VEGF$_{121}$ and OECs (+-+), scaffold delivery (no VEGF$_{121}$) of OECs (++-) or scaffold delivering OECs with VEGF$_{121}$ (+++) in SCID mice. Mean values are presented with standard deviations and * indicates p<0.05 between conditions.
Figure 5.17 Photomicrographs of tissue sections from hindlimbs of SCID mice at postoperative day 15, immunostained for human endothelial marker CD31.

Bolus cell/VEGF<sub>121</sub> (top, left panel) injection resulted in no human cells engrafting in the mouse hindlimbs. OECs delivered from scaffolds (top, right panel) resulted in the presence of human cells in the murine muscle, however the presence of VEGF<sub>121</sub> in the scaffolds (bottom, left panel) led to the formation of functional chimeric human-murine blood vessels.
vascular networks. Co-transplantation of EPCs with OECs was examined to directly investigate the role of these two cell populations to orchestrate in vivo neovascularization. A bolus injection of EPCs and OECs was used as a control condition for these studies. Animals treated with an EPC and OEC infusion developed necrosis, but did not progress to autoamputation as did limbs of animals treated with cells (blank) (Figure 5.18 and 5.19). Also animals treated with scaffolds presenting VEGF_{121} and either delivering EPCs or OECs presented some levels of ischemia. Co-transplantation of EPCs and OECs via alginate scaffold delivery completely prevented ischemia for 50% of the animals, and 30% of mice displayed normal limbs 6 weeks after surgery. The levels of regional blood perfusion did correlate with the distribution of ischemia described above (Figure 5.20 and 5.21). Animals that were subjected to co-transplantation of EPCs and OECs via scaffolds recovered nearly 100% of the initial blood perfusion. Interestingly, animals that received EPCs and OECs individually did not present the same level of recovery of blood perfusion, and also there were significant differences in recovery between conditions. Bolus injection of EPCs and OECs did not induce a significant increase in blood perfusion. Finally, immunohistochemical analysis and subsequent quantification of blood vessel densities was also performed (Figure 5.22, 5.23 and 5.24). Interestingly, animals transplanted with scaffolds delivering EPCs displayed significant high levels of fat tissue in hindlimbs (Figure 5.22), while animals treated with co-delivery of EPCs and OECs using implantable scaffolds revealed normal tissue organization and capillary density (Figure 5.23). Quantification of vessel densities revealed that the capillary density increased over 2.5 fold when cells were delivered from the scaffolds, compared to EPC and OEC infusions (Figure 5.24).
Figure 5.18 Gross photographs of ischemic hindlimbs as a function of time postsurgery.

Limbs with no treatment (blank scaffold), demonstrated a dramatic and rapid limb necrosis, resulting in auto-amputation. Animals treated with bolus injection of EPCs and OECs had progressive limb necrosis over time. Transplantation of either EPC or OEC alone in scaffolds decreased the progression towards limb necrosis. Finally, transplantation of scaffolds loaded with the EPCs and OECs resulted in a reversion of limb necrosis and ischemia.
Figure 5.19 Distribution of hindlimb ischemia severity during 6 weeks after surgery.

Animals that were treated with blank scaffolds experienced accelerated severe ischemia, resulting in hindlimb amputation. In contrast, animals that were treated with scaffolds delivering EPCs, OECs and VEGF displayed a gradual recovery of initial ischemia over time.
Figure 5.20 Regional blood perfusion profile from SCID mice hindlimbs at various time points.

Quantification of hindlimb perfusion for conditions including bolus injection of EPC and OEC (▼), EPC transplantation with scaffolds (□), OEC transplantation with scaffolds (△), and EPC and OEC combined transplantation on scaffolds (●) in SCID mice. Mean values are presented with standard deviations (n=6), and * indicates statistically significant difference (p<0.05), as compared to control (bolus injection of EPC and OEC), and # represents statistically significant difference (p<0.05) between conditions.
Figure 5.21 Gross photographs and perfusion images of ischemic hindlimbs as a function of time post-surgery.

Limbs with no treatment (blank scaffold), demonstrated precocious and rapid limb necrosis, and no perfusion images were obtained. For other conditions, hindlimbs were maintained over time, and perfusion images could be obtained. The normal baseline (before) perfusion was immediately reduced after unilateral femoral artery ligation (after), and subsequent recovery tracked as a function of time post-surgery.
Figure 5.22 Photomicrographs of tissue sections from hindlimb SCID mice at 42 days postoperative, stained with haematoxylin and eosin.

All animals receiving EPCs demonstrated adipose tissue in the transplanted limb, with high amounts when EPCs alone were transplanted. The size bars in photomicrographs represents 50 μm.
Figure 5.23 Photomicrographs of tissue sections from hindlimb SCID mice at 42 days postoperative, immunostained for the mouse endothelial marker CD31.

Transplanting EPCs or OECs alone from scaffolds resulted in a significant increase in capillary density in hindlimb muscle, as compared with bolus injection, and co-transplantation led to the highest densities of capillaries. The size bars in photomicrographs represents 50 μm.
Figure 5.24 Quantification of blood vessel densities of hindlimb SCID mice at 42 days postoperative.

Quantification of the total blood vessel densities in hindlimb muscle tissue after 6 weeks with bolus injection of EPCs and OECs (++--), scaffold delivery (VEGF<sub>121</sub>) of EPCs (+--), scaffold delivery (VEGF<sub>121</sub>) of OECs (+++) or scaffold co-delivery (VEGF<sub>121</sub>) of EPCs and OECs (+++) in SCID mice. Mean values are presented with standard deviations (n=6) and * indicates p<0.05 between conditions.
5.4 Discussion

As described in the previous chapter therapeutic angiogenesis can be achieved by the controlled released of recombinant growth factors, however, it was not possible to reach normal tissue perfusion. One may argue that other angiogenic factors are needed to further augment and mature the angiogenic response initiated by VEGF delivery. Another possibility can be that native endothelial cells lack responsiveness to growth factor cues. One alternative approach to promoting angiogenesis can arise from the delivery of endothelial progenitor cells. Cell transplantation might complement protein delivery therapies, because transplanted cells can integrate with host tissues and direct regeneration, and can also provide a regulated source of secreted growth factors and cytokines 33,34.

This chapter describes a radically different approach to transplant potentially therapeutic vascular progenitor cell populations, in which cells are delivered on a bioactive material carrier that provides a microenvironment enhancing cell survival, and the sustained release and repopulation of the surrounding tissue by outwardly migrating cells (Figure 5.2). The feasibility of this approach was examined using endothelial progenitors isolated from human cord blood to treat ischemic muscle tissue.

Two potentially clinical useful cell populations were isolated from cord blood, both a rapidly adhering population commonly referred to EPCs 32, and a population that arises later in the culture of cord blood cells, designated OECs 8,31. The isolated EPCs formed colonies with surrounding spindle-shaped cells (Figure 5.3), and expressed CD14, CD31, CD34 and CD144 cell surface antigens (Figure 5.4), in
agreement with past descriptions of this cell population. Interestingly, EPCs expressed CD14, a cell surface antigen frequently expressed by macrophages and monocytes, suggesting that this cell population may be derived from those lineages. This finding clearly supports others recent studies that argue that the cells commonly designed as “EPCs” are likely to be monocytes. However, these cells are still an appealing population for therapeutic applications, because they have been implicated in mechanisms of neovascularization via different biologic pathways, by providing a supply of regulatory cytokines and also working as a supporting cells during vascular repair. In contrast, OECs demonstrated the cobblestone morphology typical of endothelial cells (Figure 5.3), and labeled positively for the surface antigens CD31, CD144, and VWF (Figure 5.4), again in harmony with previous reports. The EPCs had low telomerase expression (Figure 5.5), consistent with the low growth potential of these cells observed in these studies. In contrast, the OECs maintained a high telomerase expression level, relative to mature human endothelial cells, consistent with the high level of proliferation that these cells demonstrated, compared to ECs (Figure 5.7). Strikingly, OECs demonstrated a higher proliferation response to VEGF than to VEGF.

A macroporous polymer scaffold was synthesized to provide the cell delivery vehicle, and the inclusion of cell adhesion anchors and morphogens was explored to create a microenvironment to maintain the viability of resident cells and increase their outward migration. The polysaccharide used to form the scaffolds, alginate, does not mediate cell adhesion itself, and in accordance with many previous reports of the importance of cell adhesion in migration, very few OECs (< 3% of the cells seeded into the scaffold) migrated out of devices formed from the native polymer to populate surrounding tissue-mimics in vitro (Figure 5.8A). However,
coupling of an appropriate density of adhesion ligands (GGGGRGDSP) to the polymer chains prior to scaffold formation increased OEC outward migration by approximately 5-fold (15% of seeded cell number; Figure 5.8A). Two isoforms of vascular endothelial growth factor (VEGF_{121} and VEGF_{165}), potent angiogenic factors, were then tested for their ability to further enhance cell activation into a migratory state and their subsequent ability to populate tissue mimics adjacent to the delivery vehicle. VEGF_{165} modestly increased cell activation and outward migration, but VEGF_{121} led to an order of magnitude increase in outward migration of the progenitor cells from the scaffold (62% of the initial cell number) (Figure 5.8B and 5.9). Endothelial cells are known to be both activated by VEGF_{165} and to migrate up the gradient of this factor that is established as a result of its binding to the ECM\(^\text{43}\), and the high concentration of VEGF_{165} in the scaffold in this system likely traps a high percentage of the activated cells. VEGF_{121} appears to be more useful to activate and drive cells out of a material, likely due to its lack of ECM binding\(^\text{34}\) which in turn causes a more even spatial distribution. In support of the favorable influence of the adhesion peptides and VEGF on cell migration, the viability of cells remaining in the scaffolds was maintained at ~30% with presentation of adhesion ligands alone, and at greater than 60% with presentation of both adhesion ligands and VEGF (Figure 5.10). Moreover, the cells migrating outward from the scaffolds presenting soluble and insoluble cues maintained a high viability and proliferative potential (Figure 5.10A and 5.11). We next investigated the role of OECs in angiogenesis, via an in vitro cell sprouting assay (a widely used system to model early steps in angiogenesis)\(^\text{29}\). Human microvascular endothelial cells exhibit significant sprouting from carrier beads into surrounding fibrin gel, with formation of capillary-like extensions consisting of interconnected cells with central lumen (Figure 5.12A). In contrast, the
OECs demonstrated relatively low ability to participate in sprout formation in this assay, revealing instead a highly migratory behavior. However, co-culture of ECs with OECs on beads led to a significant increase in sprout formation. The role of the EPCs in this process was further examined using the in vitro endothelial cell sprouting assay. EPCs did not participate in sprout formation when cultured alone on beads (Figure 5.12B), but addition of EPCs to OECs and ECs led to the formation of organized and lumen-containing sprouts. Culture of the EPC and OEC on top of the gels, instead of in contact with the ECs on the beads, induced high levels of EC migration towards the EPCs and OECs. Altogether, these in vitro studies indicate that one can create a 3-D niche for progenitor cell populations that activates the cells and directs their outward migration over time, and the combination of the cell adhesion ligand RGD and the morphogen VEGF_{121} appears to be particularly useful for the OECs. Further, these cells, in vitro, support angiogenic processes of endothelial cells, which is in agreement with recent reports 39.

To investigate the utility of this system to deliver progenitors to ischemic muscle tissue in vivo, scaffolds (with and without VEGF_{121}) were loaded with human OECs and transplanted into SCID mice with surgically-induced peripheral ischemia, and the colonization of the muscle tissue was analyzed 2 weeks post-operative. Control conditions in this experiment included placement of a scaffold with no cells and bolus injection of a quantity of suspended cells and VEGF equal to that loaded into scaffolds (mimicking current clinical protocols for the delivery of these cell populations). Placement of the scaffolds without cells had little to no benefit, as auto-amputation of the ischemic limbs was noted within three days (Figure 5.13). This precluded the use of this control condition in any further analysis. Examination of tissue sections immunostained to identify blood vessels derived from host vascular
cells suggested that bolus injection of the cells led to a modest density of capillaries in the muscle tissue. The low enhancement of capillary density in this condition likely results from VEGF delivery. In contrast, delivery of cells in the scaffold system (containing VEGF) led to a significant increase in the muscle tissue capillary densities (Figure 5.15). Strikingly, cell delivery with a scaffold system lacking VEGF led to a low and comparable density of capillaries as found with bolus injection, and both control conditions resulted in the formation of large disorganized capillaries with an erratic distribution, as compared to animal healthy limb. On the other hand, cells delivered from scaffolds presenting VEGF induced formation of capillaries with a size and spatial distribution that mimics normal limbs. Quantification of mouse vessel densities confirmed that the capillary density increased over two-fold when cells were delivered from the scaffolds instead of bolus injection (Figure 5.16). To investigate the engraftment of OECs and their incorporation into the vascular network, tissue sections were also immunostained with human-specific antibodies. Bolus injection showed little engraftment, while OECs transplanted on VEGF loaded scaffolds displayed significant engraftment at this time point, resulting in the formation of functional human-murine chimeric blood vessels (Figure 5.17). In contrast, OECs transplanted in scaffolds without VEGF revealed limited engraftment and only contributed to the formation of small vessels. The formation of these chimeric structures has been described in certain studies, but usually they are more frequent in early time points and tend to become transient over time due to the natural remodeling processes. Finally, OEC delivery on VEGF containing scaffolds led to a significant decrease in tissue necrosis, while bolus OEC delivery was ineffective in preventing toe necrosis and foot loss (Figure 5.14).
The ability of this approach to cell transplantation to promote recovery from ischemia was then tested in the same peripheral ischemia model over a 6-week time period, and co-transplantation of EPCs with OECs was investigated to determine if combinations of the two cell types would enhance the therapeutic effect. Bolus injection of the two cell types together salvaged the limbs, as did sustained delivery of each cell population alone and in combination (Figure 5.18). Analysis of vessel densities using quantitative histomorphometry revealed that the capillary density increased over 2.5 fold when cells were delivered from the scaffolds in place of bolus injection (Figure 5.24). These results confirm that transplantation of EPCs and OECs increases neovascularization of ischemic muscle tissue over long periods of time, consistent with results at 2 weeks and previous reports. However, these results also demonstrate a critical role for sustained delivery of appropriately activated progenitor cells in building new vasculature, in place of simply bolus injection. The effects of cell delivery on recovery of blood perfusion and hind limb necrosis were also evaluated using a Laser Doppler Perfusion (LDPI) system, and by visual examination, respectively. Femoral artery and vein ligation led to a rapid loss in perfusion to the ligated limb, as expected (Figure 5.20 and 5.21). Animals treated with blank scaffolds (no cells) again rapidly suffered from extreme necrosis and loss of the ischemic hindlimb, resulting in complete auto-amputation of the limb within a week after surgery, and were not further analyzed. Animals treated with a bolus injection of both cell types demonstrated a marginal recovery of regional blood flow over time (Figure 5.18 and 5.19). In contrast, animals treated with scaffolds delivering cells showed a gradual and marked increase in blood flow over time. Transplanting a combination of EPCs and OECs on the scaffolds led to a markedly superior perfusion recovery, as compared with transplanting either EPCs or OECs alone, resulting in a
return to normal perfusion levels by 4 weeks. Necrosis of toes or foot loss was also prevented in 50% of the animals at week 6 by EPC and OEC co-delivery from scaffolds, while bolus EPC and OEC delivery was ineffective in preventing toe necrosis and foot loss. Notably, EPC and OEC deployment into ischemic muscle utilizing this material system led to significant functional recovery, as also monitored by limb locomotion abilities. Mice treated with EPCs/OECs from the scaffold were undistinguished in their locomotion from normal (non-operated) mice (support video).

The level of control exhibited by the material system over the vascular progenitors (OECs and EPCs) used in the current study made possible therapeutic angiogenesis, reversal of ischemia, and prevention of necrosis and auto-amputation. While past reports have indicated similar cell functions \(^{31,32,39}\), the current results indicate that the clinical utility of these cell populations can be dramatically improved by delivering the cells in a sustained and viable fashion over time, in a manner that guides the function of the exogenous cells and their integration with native cells to together orchestrate tissue regeneration. EPCs and OECs each provided benefit when delivered individually, but together they provided a greater advantage. This result is likely due to their distinct participation in this process, with EPCs mainly contributing to cytokine production, while OECs directly interact with native EC, supporting new blood vessel formation \(^{39}\). Interestingly, OECs and EPCs both secrete high levels of FGF-α, IL-12 and IP-10, which all play a role in angiogenesis. Strikingly, EPCs alone exhibited high levels of leptin secretion (Figure 5.6), which may underlie the influence of these cells on revascularization and formation of fat tissue in hindlimbs when EPCs were delivered alone (Figure 5.22). Leptin coordinates the levels of fat tissue \(^{45,46}\), but has also been reported to directly stimulate in vivo angiogenic activity \(^{46}\) by both increasing EC proliferation and survival and synergistically increasing
VEGF stimulation \(^4^5\). OECs alone were also noted to express PIGF (Figure 5.6), which is a potent angiogenic factor \(^4^7\), and this finding may underlie certain of its effects in this study.

The cell delivery approach described in this report may be broadly useful to solve some of the fundamental problems associated with current cell-based therapies – the rapid loss of cell viability, low engraftment efficiency, and absence of control over cell fate after introduction into the body. Some of the failures and pitfalls recently experienced in clinical trials of cell transplantation approaches \(^1^,^2\) may arise directly from the manner of administration of the cells, rather than a lack in their intrinsic bioactivity, as suggested by the results with the OECs and EPCs in the current study. The findings of this chapter clearly support the potential of progenitors cells, and stem and differentiated cell populations, in tissue regeneration if their delivery and in vivo fate is appropriately regulated with microenvironmental cues provided by the delivery vehicle. While the specific cues will likely be distinct for different cell populations, the importance of controlling this therapeutic effect by locally regulating cell activation, migration and tissue engraftment is anticipated to remain constant.

5.5 References


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CHAPTER 6

Conclusions, implications, and future directions

6.1 Conclusions

This thesis describes novel strategies to enhance angiogenesis \textit{in vivo}. Material systems were developed and used to deliver angiogenic factors capable to orchestrate neovascularization. The angiogenic factors examined in this thesis ranged from potent recombinant human growth factors to a combination of growth factors and progenitor cell populations. The material used relies on a polymer system that was biochemically manipulated and physically processed to serve either as an injectable system or as an implantable scaffold. The polymeric delivery vehicles developed in this thesis were capable to enhance and extend the biological action of this angiogenic factors, resulting in reconstitution of blood flow perfusion in ischemic hindlimbs sufficient to restore tissue viability and preventing severe necrosis in mice. In addition, the polymers systems developed proved to be a useful model system revealing some of the molecular and cellular mechanisms involved on neovascularization.
VEGF is well recognized as a key regulator of neovascularization both *in vitro* and *in vivo*, but typically studies are focused on only one VEGF splice variant, ignoring the presence of others isoforms in several physiological and pathological conditions. The thesis further expands the actual knowledge regarding the role of VEGF in angiogenesis and represents also an effort to clarify the role of different VEGF isoforms in neovascularization. *In vitro* studies revealed that two of the most common VEGF isoforms – VEGF_{121} and VEGF_{165}, evoked the same magnitude of mitogenic potential in endothelial cells (Chapter 3). This mitogenic potential did correlate with the ability to stimulate sprout formation. VEGF_{121} and VEGF_{165} demonstrated the same power/ability to induce endothelial cell sprouting, but no synergistic effect was observed with simultaneous stimulation of these VEGF isoforms. The results of this thesis emphasized the critical presence of VEGF in early steps of sprout formation to orchestrate angiogenic events.

Recent phase II clinical trials testing the safety and efficacy of VEGF delivery revealed no significant improvements on patients, and the findings exhibited in this thesis linked these disappointing phase II results with the transient VEGF exposure obtained by delivering VEGF in a bolus manner (Chapter 4). Alginate was successfully manipulated to allow control over degradation *in vivo*, and subsequently validated to serve as a controlled and sustained VEGF delivery vehicle. The alginate gels developed in this thesis coupled the advantage of minimally invasive properties with the aptness to extend significantly the VEGF bioactivity both *in vitro* and *in vivo*. The injectable alginate hydrogel system provided a pronounced spatiotemporal control over VEGF_{121} and VEGF_{165} delivery, and this control was critical to induce neovascularization in an ischemic mouse model of PVD. The alginate gel led to complete return of regional tissue blood perfusion to normal levels by day 28,
whereas normal levels were not observed with bolus VEGF delivery. Both VEGF\textsubscript{121} and VEGF\textsubscript{165} led to a significant angiogenic response in ischemic hindlimbs, but again no synergistic effect was observed in this work.

As described for VEGF delivery, clinical trials involving simple infusions of progenitor cells failed to show significant improvement, and the results presented in this thesis indicated that these failures likely emerge from the manner of delivering cells (Chapter 5). This thesis demonstrates that EPCs and OECs delivered in a bolus formulation were capable to prevent complete amputation, but ineffective in preventing toe necrosis and foot loss. The results of this thesis demonstrated that polymeric systems can also be manipulated and designed to accommodate cells in a 3-D environment providing a niche to support cell growth and outward migration. In this thesis, alginate hydrogels were formulated to serve as implantable scaffolds and designed to present three different cell specific cues: bioactive cell anchorage sites that mediated cellular recognition and adhesion (RGD); an interconnected porous structure aimed to facilitate cell mobility; and a morphogen factor that enhanced cell survival, mitogenic potential and induces cell migration (VEGF). The freely diffusing properties of VEGF\textsubscript{121} observed in Chapter 4 were favorably used to regulate the egress of human endothelial progenitor cells into ischemic hindlimb tissues. Transplanting a combination of EPCs and OECs on the alginate scaffolds prevented necrosis of toes or foot loss in 50% of the animals, and 30% of animals exhibited normal healthy limbs. The results of this thesis provided new evidences regarding the specific role of these cell populations in neovascularization, in which EPCs mainly acted as cytokines producers and OECs participated directly in new blood vessel formation.
6.2 Implications

This thesis demonstrated that the local presentation of angiogenic factors governs the formation of new vascular networks, and the information generated in this study will have a specific implication and application on current strategies to reestablished perfusion of ischemic inferior limbs. The spatiotemporal control of angiogenic factors capable to regulate blood vessel formation described in this thesis may have implications more broadly, since angiogenesis has been directly involved in at least 70 disorders, with a potential market of 500 million patients worldwide. This thesis also represents an effort to understand some of the reasons behind the recent failures of clinical trials designed to treat cardiovascular diseases. An effective clinical trial program for therapeutic angiogenesis will likely include, on the one hand, the appropriate choice of biological agent for the specific application, and, on the other hand, an effective delivery modality. This work provides valuable information that could help delineate a new generation of therapeutic angiogenesis trials. Typically, the strategies of these studies was to use factor(s) tested in previous clinical trials, but to supplement these with specifically designed delivery approaches/systems which help increase the control over factor fate and ultimately allow a synchronization between biological presentation of factors and the ischemic tissue needs.

A spatiotemporal control over VEGF presentation proved to be critical when creating a functional vasculature. The results presented in this thesis indicated the importance of regulating VEGF local presentation for extended time periods to govern neovascularization. Some reports had previously suggested that VEGF delivery via bolus formulations were possibly responsible for insufficient local
exposition and ineffective temporal presentation. However, the thesis reveals a direct correlation between inadequate new blood vessel formation and biologically inappropriate VEGF delivery, and these findings should be taken into consideration for the design of new therapeutic applications. The results of this thesis also documented that VEGF121 exhibit potential for future therapeutic applications. Although VEGF121 exposure in ischemic tissues was very transient, the level of neovascularization obtained was similar to VEGF165 and these findings suggest different roles. VEGF121 can be used as an angiogenic factor for long-range cell activation, being particularly useful, for example, for the mobilization of circulating progenitors cells from a therapeutic perspective.

Control over local presentation of VEGF signals is critical to elicit a regulated vascularization response, however non-regulated VEGF hyper-expression is typically associated with solid tumor development. Several strategies speculated that anti-angiogenic factors should destroy tumors by blocking local vascularization, while others postulated that normalizing the abnormal and unregulated tumor vascular networks would improve the delivery of therapeutics targeted to attack tumors. The materials systems developed in this thesis were designed to accommodate pro-angiogenic factors, however the formulation parameters and delivery principles delineated can be easily re-adapted to accommodate anti-angiogenic factors and ultimately used to control tumor growth.

Cell therapies have great potential to treat a wide range of disorders, however the current delivery strategies have displayed absence of cell fate control after transplantation. The materials systems designed in this thesis represented an effort to obtain control over cell fate during and after transplantation, and these systems could become a potentially powerful new strategy with wide therapeutic implications.
These material matrices accommodates a higher and novel level of biological sophistication that relies upon two levels of control: the material structure and composition temporally controls egress of cells and the presence of bioactive factors spatially or directionally governs cell migration to populate injured tissues. This specific control over cell fate obtained through these systems is critical because transplanted cells are not just tolerated in the existing environment but are further nurtured by the presence of specific growth factors released by the material matrices. Specifically, this thesis focused on human endothelial progenitor cells that can be isolated from umbilical cord blood. The results described in this thesis provided new knowledge regarding the specific roles of the different endothelial progenitor cells, which in turn provides great utility in the delineation of new cell transplantation protocols. In addition, the results showed that VEGF_{121} is an appropriated factor to drive cell viability and migration outward scaffolds, and these findings revealed an interesting new utility for the use of this factor. In this work alginate scaffolds were specifically designed to present a bioactive microenvironment for transplanting endothelial progenitor cells at ischemic hindlimbs, but these systems can be further optimized and designed for any cell phenotype.

The new material systems developed and described across this thesis represents a detailed set of tools to quantitatively determine the dose, timing, and spatial presentation of angiogenic factors needed to orchestrate the formation of new vascular networks. The tissue engineering strategies developed throughout this thesis emphasize the concept that passive materials are not sufficient to complete new tissue regeneration. Instead, materials need to be primed with specific bioactive cues to drive new tissue formation. In addition these materials systems provide an appealing
platform to manipulate and to test the *in vivo* functions of a multiplicity of factors that govern tissue regeneration.

### 6.3 Future directions

This thesis work illustrates the utility of material systems to obtain control over angiogenic factors delivery, and the importance that spatiotemporal control over these factors have in governing neovascularization. Nonetheless, this thesis also stimulated further questions that will be particularly interest to address in the near future.

In the short term, it will be interesting to investigate the precise timing of *in vivo* degradation displayed by the materials systems developed in this thesis. In aim 2 of this thesis alginate was modified to allow degradation. Degradation was monitored and quantified *in vitro*, and the *in vivo* experiments demonstrated that 6 weeks after injection no alginate was visually observed. However, no precise information regarding the *in vivo* degradation kinetics of alginate gels was generated throughout this thesis. One way to monitor alginate degradation *in vivo* may be pursued by labeling alginate chains with radioactive or fluorescent markers via covalent coupling, allowing for the tracking of the polymer chains after tissue injection. Similarly, no quantitative information regarding the degradation of the implantable scaffolds was obtained *in vivo*. The 6-weeks experiments exhibited complete degradation, but some scaffold fragments were visually observed during the 2-weeks sample retrieval. The precise knowledge about material degradation assumes fundamental importance, because a close control over material degradation has been reported to be critical to
achieve optimal tissue regeneration by directly improving the quantity and quality of new tissue formation.

The long-term studies will involve investigating the specific mechanisms that govern capillary formation. The Chapter 3 of this thesis described a new 3-D *in vitro* sprouting assay. This model can be extended to investigate how important cellular cross-talk and organization is during sprout formation. A particularly interesting set of questions regards the machinery behind the initiation and maintenance of sprouts. Typically endothelial cells coated in microcarriers exhibited organized sprouts under influence of angiogenic factors, however very little is known about the characteristics of cells that lead the sprouts. One interesting question regarding the presence of a leading cell could be examined by testing if there is one leading cell or if the leading cell alters and change position over time. One possible way to test this question could be the simple observation of several time laps of the sprouting process. Another interesting experiment would examine if the cell “decision” of participating or not in sprout formation relies on a purely stochastic phenomenon or if it is instead associated with the presence of some specific phenotypic characteristics.

The effects of VEGF\textsubscript{121} on governing neovascularization was extensively investigated across different studies described in this thesis, however due to the little information regarding the specific role of this isoform in angiogenesis, further studies will be necessary. As described in Chapter 4, VEGF\textsubscript{121} exhibited a freely diffusible characteristic, which may indicated that this form could be capable to stimulate cell population in a long range. VEGF has been reported as responsible for the recruitment and mobilization of bone marrow cell populations \(^6\), and one may speculate whether VEGF\textsubscript{121} presents the appropriate binding properties for that specific assignment. Alginate gels releasing VEGF\textsubscript{121} may be applied to investigate
the recruitment and mobilization of vascular precursor cells \textit{in vivo} by using animals that undergone whole bone marrow cell transplantation. Basically, the cells present in bone marrow of these animals exclusively express an fluorescent label (e.g. GFP cells) that allow easy cell tracking. As described in this thesis, no synergistic effect was observed with the \textit{in vivo} delivery of VEGF$_{121}$ and VEGF$_{165}$, however these two factors were delivered at the same time. One interesting experiment could be a delivery of these isoforms but with a temporal delay between them. For example, speculating that VEGF$_{121}$ is responsible for activation of cell populations distant from ischemic regions, a synergistic effect could be observed in case of initial delivery of VEGF$_{121}$, followed by a VEGF$_{165}$ delivery 1-2 weeks after initial injection.

As studies in aim 2, an injectable alginate system was developed for the controlled delivery of angiogenic factors \textit{in vivo}, and VEGF was consistently used throughout this thesis as a factor capable to evoke neovascularization. However as reviewed in Chapter 2, several others angiogenic factors are critical in this process. For example, dual delivery of VEGF and PDGF has been reported to enhance new blood vessel formation and subsequently maturation\textsuperscript{10}. The injectable system developed in this thesis can be utilized to investigate the role of combinatory delivery of distinct angiogenic factors (e.g. Ang2, PDGF) in areas of difficult and critical access such as the heart\textsuperscript{11}. In addition, the spatial biodistribution that the injectable system developed can also be utilized to create a gradient of across ischemic areas. This gradient can be obtained by multiple injections of growth factors at distinct regions of tissue.

Very little is known about the effects that delivering exogenous factors can evoke on the presentation and secretion of the endogenous levels. In particular it
could be interesting to use some of the tools and protocols described in this thesis to address this question.

Chapter 5 of this thesis described the development of an implantable material system capable to control progenitor cell fate after transplantation. Although the best results were observed with co-delivery of EPCs and OECs from the scaffolds in 1:1 combination, no studies were performed to investigate if the results could be tailored when a different cell ratio is utilized. Further studies aimed to examine the optimal cell ratio and concentration of morphogen will be very useful and interesting to pursue. In addition, the timing of cell delivery can also be critical to orchestrate neovascularization. Therefore, a re-design of the material system aimed to allow distinct temporal waves of cell releases would be valuable. One particularly appealing challenging in the future will be to translate the bioactive environmental cues coupled with the implantable systems into the design of injectable systems capable of cell delivery. Neovascularization therapies via non-invasive techniques will represent a tremendous advantage and utility for clinical applications. Additionally and in a broad perspective, the flexibility exhibited by these implantable systems can also be useful in eliciting an antigen-specific immune response, ultimately working as delivery vehicles for cancer vaccines. The implantable system can be re-designed to attract immune cells inside scaffold and subsequently program these cells to attack pathologic disorders (e.g., solid tumors).

The results of this thesis generated a new contribution in some of recent studies of tissue engineering 12-14, and here were some questions that will be interesting to follow up in the near future. Others questions will also undoubtedly arise from the continuous advances being made in biology, medicine, chemistry,
engineering and materials sciences, contributing to a bright and rapidly evolving future for the tissue engineering field.

6.4 References


APPENDIX

SELECTED DETAILED PROTOCOLS AND PRELIMINARY STUDIES FOR FUTURE WORK

APPENDIX A

VEGF bioactivity degradation \textit{in vitro}

Materials:

- Amicon centrifuge tubes (5 000 MWCO) Millipore # UFC900
- Recombinant Human VEGF$_{165}$ 010 R&D Systems # 293-VE-
- Recombinant Human VEGF$_{121}$ 005 R&D Systems # 298-VE-
- PBS Gibco # 21600-010
- 12 well plate culture dish BD # 353043
- HMVEC-d cell Cambrex # CC 2543
- EBM - 2MV media Cambrex # CC 3202

Protocol

1. Seed two set of HMVEC-d in 12 well plates. One set will be cultured with EBM-2MV until they reach 80-90% confluency and used on point 2. The other set of cells will be also feed with EBM-2MV until they reach 50-60% confluency and used on point 7.
2. Aspirate media and wash cells twice with PBS.
3. Feed cells with EMB-2MV without growth factors supplemented with VEGF (50 ng/ml).
4. At different time points (e.g. 0 min; 30 min; 60 min; 240 min...) collect media (1 ml).
5. Add collected media to the Amicon centrifuge tubes and centrifuge for 15 min. at 4 000 g (this step will concentrate 10x your sample).
6. Reconstitute media collected after centrifugation and dilute 1:10 (obtaining 1 ml) with EBM-2MV without growth factors.
7. Aspirate media and wash cells (see point 1) twice with PBS.
8 Introduce the media diluted 1:10 to feed cells (use as controls media supplemented with VEGF (50 ng/ml), EMB-2MV without growth factors).
9 Culture cells in that conditions for two days.
10 Perform cell counting.

Notes:
A) store on 4°C the media with the VEGF degraded by cells, and just add all at the same time.
B) Do not forget to sterilefilter the media after centrifuge, since the Amicon tubes are not sterile.
C) The 0 min condition will be used as the “100%” bioactive condition. The % of bioactivity will be calculated by dividing the cell number of a specific time point over the 0 min cell count number.
APPENDIX B

Cell culture under Hypoxic conditions

Materials:

- Hypoxic chamber
- Flow meter
- Gas tanks (95% N₂, 5% CO₂)

- Billups-Rothenberg # MIC-101
- Billups-Rothenberg # DFM3002
- Igos

PROTOCOL

1. Clean chamber with dd H₂O and then with 70% ethanol.
2. Introduce a Petri dish with 10-20 ml of sterile dd H₂O (to prevent excessive evaporation of cultures).
3. Introduce tissue culture plates in the chamber.
4. Grasp chamber and ring clamp, make sure clamp is centered.
5. Open both inlet and outlet ports and attach inlet port tubing to a gas mixture.
6. Using the flow meter, set a rate of 20 liters/min and allow chamber to purge for 5 min.
7. Disconnect gas source and seal chamber (by closing both inlet and outlet ports).
8. Put chamber inside conventional incubator (37°C).
APPENDIX C

3D in vitro sprouting assay

Materials:
Cytodex 3 0485-01
Sigmacoat
Spinflar flask
Formaldehyde
Fibrinogen
Aprotinin
Thrombin
Syringe filter

GE Healthcare # 17-
Sigma # SL2
Belco # 1967-00050
EMS # FX0410-13
Sigma # F3879
Sigma # A4529
Sigma # T6884
Pall Life # PN 4612

Solutions:

_Fibrinogen solution:_

<table>
<thead>
<tr>
<th>NaCl solution</th>
<th>NaCl 0.9 g</th>
<th>dd H2O 100 ml</th>
</tr>
</thead>
</table>

NaCl solution 12.5 ml
Fibrinogen 50 mg

Rest fibrinogen solution for 10 min. at 37°C, to ensure complete fibrinogen dissolution. Sterile fibrinogen solution using 0.22 μm syringe filters. Avoid vacuum filtration. Store at 4°C, and its good for no more than week.

_Thrombin working solution:_
Dissolve thrombin in of sterile PBS and aliquot (25 u/ml). Store aliquots at -20°C. Stable for at least 6 months.

_Aprotinin working solution:_
Dissolve aprotinin in of sterile PBS and aliquot (500 μg/ml). Store aliquots at -20°C. Stable for at least 6 months.

_PROTOCOL_

Day zero - Preparation of bioreactor
1 Add some Sigmacoat (1.5-2 ml) in the flask and soak inside of the flask.
2 Drain the Sigmacoat and dry it in the hood
3 Wash by DDH20 thoroughly.
4 Autoclaving
Do not need to do this process every time. Once a month.

Preparation of microcarriers

1 Weigh 50 mg of dry microcarriers (MC) and allow to swell by adding 10 ml of PBS. Rest for at least 3h at room temperature with occasional agitation.
2 Remove the supernatant gently and replace new PBS (5 ml), and the MC are sterilized by autoclaving (no more than 120°C).
3 Prior to use, the supernatant was discarded and the MC are briefly rinsed in warm culture medium (5 ml of EGM-2MV), and transfer to the spinnar’s flask.
4 Add worm EGM-2MVmedium (15 ml) to the Spinnar’s flask containing the MC and incubated at 37°C in the incubator for 5 minutes, with continuous stir.
6 Trypsin, centrifuge and reconstitute the pellet cells with 5 ml of EGM-2MV (1X10^6 cells/ml)
5 Transfer cell suspension into spinnar’s flask, and start to stirring (2 mins-stirring and 28 mins no stirring). Perform the intermittent stirring for 3 hrs., at about 60 rpm.
6 3 hrs later, incubate the spinnar’s flask with continuous stirring for 20 hrs.
7 Take the entire medium out of the flask and transfer for a 50 ml tube. Allow mc to pellet, aspirate supernatant and add 15 ml of EGM-2MV.
8 Transfer the 15 ml mc suspension to 3 T25 tissue culture flasks.
9 Using microscope, observe cell incorporation to beads. Feed T25 flasks daily with 5 ml of EGM-2MV.
10 MC are ready to use when are confluent. Cells typically take 2-3 days to cover the mc surface.

Fibrin gel preparation

1 Take 1 ml of beads in medium from T25 flask to a 15 ml tube, and wait for 2-3 min. to pellet. Aspirate supernatant and introduce 1ml EGM-2MV.
2 Prepare “solution A”: fibrinogen (3ml) + aprotinin (0.4ml) + beads solution (1ml); and “solution B”: PBS (3.3 ml) + thrombin (0.3ml).
3 Mix A and B solution in a 5:4 ratio. Typically, introduce 0.25 ml of solution A to the well (24 well plate) (pipette up and down 5 times before transfer to well). Then introduce 0.20 ml the solution B (mixing inside well 7 times, but without making air bubbles); total 0.45 ml per well in case of a 24 well plate.
4 Incubate for at least 25 min. at 37°C, and then add media of interest (0.8 ml per well in case of a 24 well plate). Feed gels every day.
5 To terminate experiment, aspirate media and wash wells twice with 0.8 ml of sterile PBS.
6 Add 0.5 ml of 4% formaldehyde to fix cells and incubate overnight at 4°C.
7 Aspirate formaldehyde solution and wells twice with 0.8 ml of sterile PBS.
8 Add 0.5 ml of PBS and store at – 4°C. Gels are stable for 1-2 months.
APPENDIX D

Schematic representation of sprout definition

SPROUTING ASSAY

**Nascent Sprout** - a unique cell still connected to bead but extending into gel

**Mature Sprout** - extension of 2 or more cells connected to each other with at least one cell connected to bead

**Lumen** - The inner open space between sprouting cells

Figure D1 Definition of nascent sprout, mature sprout and lumen.
APPENDIX E

Alginate oxidation
(adapted from Hyun Joon Kong)

Materials:
Sodium Periodate (NaIO₄, MW= 213.89 g/mol) Sigma # 311448
Ethylene glycol (HOCH₂CH₂OH, MW= 62.07 g/mol) EM Sciences # EX0565
Dialysis membranes (MWCO 3500) Spectrumlabs #S632724
Sterile filter top (0.22 μm) Millipore # SCGPT05RE
Sterile bottle unit (0.22 μm) Nalgene # 125-0020

PROTOCOL

1 Weigh 2 g of alginate (low and high molecular weight), reconstitute alginate powder with 198 ml of dd H₂O (1% alginate solution (w/w)), and allow to stir overnight.

2 Prepare aqueous sodium periodate solution. For 1% oxidation, weigh 21 mg of sodium periodate and add 10 ml of dd H₂O, briefly vortex to allow complete dissolution (see table for different percentages of oxidation).

3 Add aqueous solution of sodium peroxide, drop by drop, to the alginate solution while maintain solution stirring. (use aluminum foil to cover beaker, because reaction is light sensitive)

4 Stir reaction at room temperature for 17 hours (keep protected from light).

5 Add an equimolar amount of ethylene glycol, 6 μl in case of 1% oxidation (see table for different percentages of oxidation), and stir for 30 minutes at room temperature.

6 Transfer alginate solutions to dialysis membranes (approximately 25 cm per 100 ml of alginate solution) by using clamps to entrap solutions inside the membranes.

7 Transfer dialysis membranes to a 4 L bucket with dd H₂O. Place a stir bar in bucket and let stir on stir plate. Change water for three days, twice on the first day and once for another two days.

8 Remove alginate solutions from membranes and sterile filter into a sterile, autoclaved glass bottle using a sterile filter top (0.22 μm)

9 Unscrew filter top of sterile bottle unit. Pour alginate solution into a sterile bottle unit, and screw in filter top.

10 Freeze overnight at –20°C.

11 Lyophilize for approximately 8-10 days until alginate is completely dry.

12 Reconstitute alginate in appropriate concentration and solution.

<table>
<thead>
<tr>
<th>Oxidation (%)</th>
<th>Alginate (g)</th>
<th>Sodium periodate (mg)</th>
<th>Ethylene glycol (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>2</td>
<td>5.401</td>
<td>1.5</td>
</tr>
<tr>
<td>0.5</td>
<td>2</td>
<td>10.803</td>
<td>3.1</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>21.605</td>
<td>6.2</td>
</tr>
<tr>
<td>2.5</td>
<td>2</td>
<td>54.013</td>
<td>15.6</td>
</tr>
</tbody>
</table>
APPENDIX F

Alginate gel processing

Figure F1 Schematic illustration of alginate formulation and processing.
APPENDIX G

VEGF$_{121}$ Iodination

Materials:
- IODO-GEN tubes
- PBS
- Recombinant Human VEGF$_{121}$
- 005
- Iodine-125 radionuclide
- Pierce #28601
- Gibco # 21600-010
- R&D Systems # 298-VS-005
- PerkimElmer# NEZ033H

PROTOCOL

1. Dissolve the VEGF$_{121}$ (5 μg) in PBS (0.025 μg/μl)
2. Add the VEGF$_{121}$ solution (200 μl) to a pre-coated tube.
3. Add carrier-free $^{125}$I radionuclide to the solution in the pre-coated tube (52.281 μCi per 1 μg of protein to label).
4. Agitate gently the vessel and wait for 15 minutes in order to achieve the reaction.
5. Extract the sample from the tube (you should use your sample as soon as possible in order to avoid decay).
6. OPTIONAL: You can use NaI or KI, in the step 3 or just add in the end. The carrier Iodide (NaI or KI) is used to enhance safety (when added in the end – final concentration 1 mM to final mixture) or to drive the reaction (in the step 3) using 0.25 mM-1 mM to 0.5 μCi-1.0 μCi.
7. Confirm the efficiency of the reaction (by measuring the amount of protein (simple protein assay) that you have, and the amount of radioactivity you have on that protein solution)
APPENDIX H

Immunological detection of phosphorylation

Materials:

- Alginate gels
- Recombinant Human VEGF₁₆₅
- PBS
- 60 x 15 tissue culture dish
- HMVEC-d cell
- EBM – 2MV media
- Phospho – ERK1/ERK2 Immunoassay Kit

R&D Systems # 293-VE-
Gibco # 21600-010
BD # 353002
Cambrex # CC 2543
Cambrex # CC 3202
R&D System # SUV1018

PROTOCOL

Alginate gels

1. Make a 2% alginate solution (1% oxidized) of low and High MW (reconstituted overnight with Zero media – EBM2).
2. Make gel using 75/25 low/high ratio- 3.1 ml gel (make a gel with VEGF ([VEGF₁₆₅]=1.075 ng/ul of gel and without VEGF)
3. After gel mixing, transfer 500 ul to a 15 ml centrifuge tube and incubate at room temperature for 30 min (allowing gelation)
4. To gels containing VEGF add 4.5 ml of starvation media to each 15 ml centrifuge tube. For blank gels, add the starvation media supplemented with VEGF in concentration equivalent to which will be released.
5. Incubate at 37°C for 24 hrs;
6. After 24 hrs, gently extract 4.5 ml of media and add new media (starvation).
7. Sample 0.6 ml to an appendorf (to run an VEGF ELISA to double check the amount of VEGF released), and store the rest (-20°C) for feeding cells after starvation.

Preparation of cell extracts

1. Seed HMVEC-d (p4-p7) cells (5000 cells/cm² cell density) in 60x15 tissue culture, and culture them in EBM-2MV (2 ml everyday) until they reach confluency.
2. Remove culture media, add 2ml of starvation media to each well plate, and culture a further 18 hr. (Make sure that the wells remain flat and all the medium covers the entire well. Serum starvation makes the cells quiescent, which can be achieved under these conditions within 14 to 24 hr. Starvation for too long or any change in temperature or pH may be stressful to the cells and may induce activation of one or more signaling pathways.)
3. Remove starvation media, add starvation, VEGF media and VEGF released from gels in media to cells. Return well plates to the incubators for 5, 15, and 45 min. (first to the wells with the longest incubation. It is useful to make and use a time
chart to ensure that stimuli will be given at the appropriate times and the cells harvested within a short period of time (5 to 10 min.))

4 After 5, 15 and 45 min, remove medium from wells. Rinse well twice with 5 ml of ice-cold PBS.

5 Remove ice-cold PBS and add 0.5 ml ice-cold 1X cell lysis Buffer (provided by KIT).

6 Scrape cells off the well and transfer to a labeled eppendorf. Keep on ice!

7 Sonicate on ice.

8 Microcentrifuge (10 000 rpm) for 10 min at 4°C and transfer the supernatant to a new eppendorf. The supernatant is the cell lysate. Store at -80°C if not used right away.

9 Run the protocol included on the kit according manufacture directions.
APPENDIX I

Mouse regions of body – lower ventral view

I - V Digitus primus, secundus, tertius, quartus, quintus

A - Regio pubica  
B - Regio inguinalis  
C - Regio femoris  
D - Regio cruris  
E - Regio tarsi  
F - Regio pedis

Figure 11 Image of lower ventral view from a female SCID mice and identification of different regions (source: Hedrich, H.J. & Bullock, G.R. The laboratory mouse, (Elsevier Academic Press, Amsterdam; Boston, 2004)).
APPENDIX J

Animal model of severe hindlimb ischemia

Materials:

Surgical tools
   Surgical scissors
   Needle driver
   Fine-tipped forceps
Sterilization Wraps
Sterile gauze pads (10.2 cm x 10.2 cm)
5-0 Ethilon sutures
Alcohol swab
Liquid adhesive
Antiseptic iodine solution
Clips
6062
Ketamine
Xylazine
Isoflurane
Roboz
   # RS5910, RS5859
   # RS7800
   # RS8208, RS5153, RS5155
VWR
   # 58752-964
   # 82030-642
Johnson & Johnson
   # 661G
BD
   # 326895
Ferndale Lab.
   # 0523
Medi-Flex
   # 260261
Harvard Apparatus
   # BS472-
Henry and Schein
   # 9952949
   # 4015809
   # 2099589

Before surgery

Autoclave surgical tools wrapped in sterilization wraps.

PROTOCOL

1 Anesthetize the animal with an intraperitoneal injection of a 7:1 mixture of ketamine:xylazine. Inject = 35 μl of the anesthetic on the opposite site of the animal that will be surgically altered. Wait between 4-7 min, allowing anesthesia action.

2 Completely shave left and right regio: pubica, inguinalis, femoris, crusis, tarsi and pedis (see Appendix I for orientation). Wipe lower ventral regions (regio pubica, inguinalis and femoris), first with alcohol swab and then with antiseptic iodine solution.

3 Using a fine-tipped forceps lift animal skin in the border between regio inguinalis and femoris and make a transversal incision (± 7 mm long) using surgical scissors.

4 Separate skin and lift the layer of fat tissue (pars inguinalis) and make an incision to exposure the underlying hindlimb muscle. The femoral artery will be easily visible.

5 The iliac artery and vein and the hypogastric artery (the vessel branching from the external iliac artery and vein) are easy to identify, since they run on the hindlimb muscle surface.

6 Holding the needle attached to the suture with needle driver, slip the needle directly underneath the external iliac artery and vein in a superficial fashion without grabbing surrounding muscle tissue.
7 Gently pull the suture through, and perform a securely tie a suture knot around the external iliac artery and vein.
8 Repeat to ligate the femoral artery and vein.
9 Make an incision on the femoral artery and vein between the two knots. Expect a small amount of bleeding and blot using the sterile gauze pads.
10 Place the polymeric system (in case of injectable system, inject alginate superficially between the two knots. In case of implantable system just directly place the scaffold between the two knots).
11 Cover skin incision with a continuous suturing technique or using suture clips.
APPENDIX L

In vivo protein quantification

Materials:

T-Per reagent
Hematocrit tubes
Recombinant Human VEGF$_{121}$
005
Iodine-125 radionuclide

Pierce #78510
Fisher # 22-362-566
R&D Systems # 298-VS-

PerkimElmer# NEZ033H

PROTOCOL

Blood samples

1 Anesthetize animals, preferably using isoflurane.
2 Retro-orbitally collect 5 full hematocrit tubes per mouse to a 1.5 ml eppendorf.
3 Allow the blood to clot by standing tubes vertically at room temperature for 45-60 minutes.
4 Centrifuge 13 000 rcf for 10 minutes at 4°C.
5 Transfer supernatant (serum) to a new eppendorf.
6 Perform growth factor quantification (e.g., ELISA) or store at -80°C.

Tissue samples

1 Euthanize animal.
2 Retrieve tissue sample from animal.
3 Transfer tissue samples to eppendorf (weight and label each eppendorf before use).
4 Weight tissue samples.
5 Use a ratio of ~1 g of tissue to 20 ml T-Per Reagent (typically add 1 ml to 150-350 mg of tissue sample).
6 Sonicate for 10 sec all samples (to allow homogenization).
7 Centrifuge sample at 13,000 rcf for 5 minutes at 4°C to pellet cell/tissues.
8 Collect supernatant and be ready to run some protein quantification (e.g., ELISA). Samples can be stored at -20°C.
APPENDIX M

Mouse CD31 immunostaining for mouse tissue

Materials:

- Rat anti-mouse CD31 antibody: Pharmingen # 557355
- Anti rat secondary antibody: Vector Laboratories # BA-4001
- TSA Biotin System kit: PerkinElmer # NEL 700A
- DAB Chromagen: Dako # K3466
- Dako Pen: Dako # S2002
- Proteinase K: Sigma # P2308
- Tween 20: Sigma # P1379
- Sodium chloride: EMD # SX0420-3
- Tris-HCl: Calbiochem # 648317
- Hydrogen peroxide (30%): VWR # VW3742-1
- Rapid mounting media solution: EMD # 65037-71

Solutions:

**Proteinase K:**

Proteinase K Solution (20 ug/ml in TE Buffer, pH 8.0):

\[
\text{TE Buffer (50mM Tris Base, 1mM EDTA, pH 8.0):}
\begin{align*}
\text{Tris Base} & : 6.10 \text{ g} \\
\text{EDTA} & : 0.37 \text{ g} \\
\text{Distilled water} & : 1000 \text{ ml}
\end{align*}
\]

Mix to dissolve. Adjust to pH 8.0 using concentrated HCl (10N HCl). Store at room temperature.

Proteinase K Stock Solution (20x, 400 ug/ml):

\[
\begin{align*}
\text{Proteinase K} & : 0.004 \text{ g (4 mg)} \\
\text{TE Buffer, pH8.0} & : 10 \text{ ml}
\end{align*}
\]

Mix well, aliquot and store at -20°C.

Working Solution (1x, 20 ug/ml):

\[
\begin{align*}
\text{Proteinase K Stock Solution (20x)} & : 1 \text{ ml} \\
\text{TE Buffer, pH8.0} & : 19 \text{ ml}
\end{align*}
\]

Mix well. This solution is stable for 1 month at 4°C. For longer storage, aliquot and store at -20°C.

**TNB Blocking solution:**

For 100 ml TNB:

- 1.57 g Tris-HCl
- 0.87 g of NaCl
- 0.5 g Blocking agent (provided on TSA Biotin System kit)

(Store at 4°C, is good for 2 months)
TNT washing solution

0.1M Tris-HCl, 0.15 M NaCl, 0.05% Tween 20.

- Tris-HCl: 15.76 g
- NaCl: 8.77 g
- dd H2O: 1 l
- Tween 20: 0.5 ml

(Adjust pH=7.5)

Peroxidase block

- 2 ml of H2O2 30%
- 18 ml of PBS

(make fresh everytime)

PROTOCOL

1 Heat slides at 65°C for 30 min.
2 Xylene I and xylene II bath (10 min each); followed by ethanol bath gradient (100%-90%-70%) 3 min each; and then place for 5 min in dd H2O.
3 Peroxidase block for 30 min.
4 TNT wash for 5 min.
5 Proteinase K -10 min at 37°C and 5 min room temperature.
6 TNT wash for 5 min.
7 TNB blocking buffer 30 min.
8 Tip excess.
9 CD31 Primary Antibody (1:250 diluted with TNB buffer) incubate overnight at 4°C.
10 TNT wash for 5 min.
11 Secondary antibody (1:200 diluted with TNB buffer) incubation for 30 min.
12 TNT wash for 5 min.
13 TSA strepavidin (1:100 diluted with TNB buffer) incubation for 30 min.
14 TNT wash for 5 min.
15 TSA biotinyl tyramide amplification working solution (1:50 diluted with amplification diluent) for 7 min.
16 TNT wash for 5 min.
17 TSA strepavidin incubation for 30 min.
18 DAB Chromagen +/- 5 seconds (use white paper as background to assess color change).
19 Tap H2O rinse and let stay for 3 min
20 Counterstain slides in Harris Hematoxylin for 4 seconds. Rinse with tap water until runs clear.
21 Dehydrate slides using graduated ethanol, 2 changes for 2 minutes each.
(70%, 90%, 100% ethanol)
22 Clear with xylenes, 2 changes for 2 minutes each.
23 Coverslip using a permanent mounting media
APPENDIX N

Laser Doppler perfusion imaging (LDPI)

Materials:

LDPI model: PeriScan PIM II Perimed # 44-00079-07

PROTOCOL

Before use:

1. Turn on LDPI system (e.g., scan head, computer) and leave it on for at least 1 hour (warm up the device before use for best results).
2. Verify settings on LDPIwin software: 40x40 sampling points (area of scanning), single mode, high resolution, threshold 5.4 V
3. Confirm if the scanner head is aligned and parallel to the table and verify the distance between laser head and scan area is greater than 12 cm (36 cm was the distance used).
4. Run a control scan to confirm the alignment of the outgoing laser beam.

Scanning procedure:

1. Induce animals with anesthesia (Isoflurane).
2. Shave left and right regio: pubica, inguinalis, femoris, crusis, tarsi and pedis (see Appendix I for orientation).
3. Center laser beam on regio pubica area. Extend both hindlimbs and verify if they are horizontal aligned to each other. The foot should be flat on the scanning pad. Use a dark fabric (non-reflective) to cover the tail.
4. Run the option “mark the area” to confirm that the entire hindlimb is included in the scan area. The key regions to scan are the regio: inguinalis, femoris, crusis, tarsi and pedis.
5. Turn off all lights (critical) and start scan. Save the scan file.

Note: Animals need to be shaved, since the hairs clearly affect the quality of scan.

Analysis:

1. Open saved file in LDPIwin.
2. Use the marker tools and draw starting on border between regio crusis and tarsi down to the digitus region. Draw an area for both hindlimbs (left and right).
3. Automatically a dialog window will display several parameters for two drew regions.
4. Take notes of the “mean perfusion” values and the number of “sites”.
5. To plot results, multiply “mean perfusion” with “sites”, and express it as a percentage of the non-ligated normal limb to ligated limb.
APPENDIX O

Ischemic grade
(modified from Ruth Cheng)

Animals are placed in a ventral position and the limb integrity is visually analyzed. Animals are graded in respect to following:

0 - Normal limbs: animals maintain intact all regions and parts of limb;
1 - Tips of toes black: the digitus are intact, but the nails or the digitus extremity (one or several) are slightly black
2 - One necrotic toe: one of digitus present a necrotic region.
3 - Multiple necrotic toes: several digitus are missing or necrotic.
4 - Necrotic foot: complete loss of the all digitus and regio cruris and tarsi necrotic.
5 - Amputation: complete loss of the limb until the regio femoris.
APPENDIX P

Isolation and culture of endothelial progenitors cells from human cord blood
(adapted from Eun-Suk Kim)

Materials:

Hanks’ balanced salts - HBSS
Sigma # H4891
Sodium bicarbonate
Sigma # S5761
Histopaque 1077
Sigma # 10771
RBC lysis buffer
eBioscience # 00-4333-57
0.25% Trypsin
CellGro # 25-050-C1
EGM-2MV media
Cambrex # CC-3202
Fibronectin
Sigma # F2006
Biocoat well plates
BD # 354400
100 mm culture dish
BD # 353003
10 ml syringe
BD # 309604
18G ½” needle
BD # 305196

Solutions:

Hanks’ balanced salts:

HBSS package
Sodium bicarbonate 0.35 g
Distilled water 1000 ml
Mix to dissolve. Adjust to pH 7.4. Store at room 4°C.

Fibronectin coating solution:

Reconstitute 5 mg of fibronectin with 5 ml of sterile dd H₂O.
Incubate for 15-30 with occasional mixing to allow complete fibronectin reconstitution.
Make aliquots of 500 µl and store at -20°C. Solution is good for 6 months.

Culture media:

For “endothelial progenitor cells – EPCs”:
EGM-2 MV without hydrocortisone supplemented with FBS
(total FBS – 10%)

For “outgrowth endothelial cells – OECs”:
EGM-2 MV supplemented with FBS (total FBS – 10%) (media utilized until first passage, after the 1:1 passage use commercial EGM-2MV media)

OECs freezing solution:
95% FBS, 5% DMSO

PROTOCOL
Immediately after cord blood arrival, dilute 1:1 with HBSS (20 ml of cord blood to 20 ml of HBSS in a 50 ml centrifuge tube).

2. Gently lay 20 ml of diluted blood on 20 ml of Histopaque 1077.

3. Centrifuge at 400 or 740 rcf (EPC and OEC respectively) for 30 min. at 25°C (Note: it’s important to control the temperature). Centrifugation details: acceleration 6 out of 9 and brake 0 out of 9. (See point 15 in EPC protocol for detail)

4. Discharge top layer (yellow layer - serum) and collect mononuclear cell layer (translucent layer localized right bellow top yellow layer - serum) using a 10 ml syringe attached with an 18G½ needle (typically collects 7-10 ml per centrifuge tube). Transfer each 20 ml of mononuclear cells to a new 50 ml tube.

5. Add 20 ml of HBSS to each 20 ml of mononuclear cells.


7. Aspirate supernatant and gently disperse cell pellet.

8. Resuspend and add 7 ml of RBC lysis buffer.

9. Stand for 10 min. with very occasional gentle mixing.

10. Add 30 ml of HBSS (to re-establish 40 ml in each 50 ml tube).


12. Aspirate supernatant and resuspend with 30 ml of EGM-2MV media.


For EPC

14. During #3 procedure, coat 100 mm culture dish with fibronectin coating solution (100 µg/ml) and then allow air-dry (3 dish / 80 ml of cord blood).

15. From #13, resuspend cells with 30 ml of EPC culture media and transfer 10 ml to each 100 mm tissue culture dish.

16. Culture for 4 day (no change media).

17. After 5 days, remove media gently and wash the dishes gently with HBSS (twice).

18. Add 1.5 ml of 0.25% trypsin and incubate for 4 min. at 37°C.

19. Add 10 ml culture media (in case cells not detached completely, use the cell scraper) and transfer cell solution to 15 ml tube.


21. Wash cells with serum free media (EBM-2MV).

22. Centrifuge at 300 rcf for 7 min. at 25°C. Centrifugation details: acceleration 9 out of 9 and brake 6 out of 9.

23. Count cell number and use for experiment.

For OEC

14. From #13, resuspend cells with 24 ml of OEC culture media and transfer 4 ml to each well of the Biocoat well plate (3 well plates / 80 ml of cord blood).

15. Culture for 36 hrs without media change.

16. Aspirate media and wash the well once with OEC culture media.

18. Add 2 ml of OEC culture media and change media everyday for 7 days.
19 After 7 days, observe all wells carefully everyday and change media, until endothelial cell colony is observed.

20 When the colony become large enough to occupy the whole visual field of low magnification (4X objective) of a phase contrast microscope, means that is ready to passage.

21 Detach cells by regular trypsinization and passage cells into regular tissue culture 6 well plate (1 well to 1 well passage).

22 Culture cells with EBM-2MV, changing media everyday.

23 When cells reaches 80-90% of confluency, either use cells or freeze them.
APPENDIX Q

Peptide modification of alginate
(adapted from Susan Hsiong)

Materials:

- EDC (FW 191.7)
- Peptide (GGGGCRGDSPC-OH; MW=1189 g/mol)
- Sulfo-NHS (FW 217.13)
- Dialysis membranes (MWCO 3500)
- MES
- Hydroxylamine hydrochloride
- Sterile filter top (0.22 µm)
- Sterile bottle unit (0.22 µm)
- Alginate MVG (LF20/40)

Sigma # E1769
Commonwealth Biotec.
Fisher # PI-24510
Spectrumlabs # S632724
Sigma # M8250
Sigma # H9867
Millipore # SCGPT05RE
Nalgene # 125-0020
FMC Biopolymer

Solutions:

MES solution:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>MES</td>
<td>9.76 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.77 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>500 ml</td>
</tr>
</tbody>
</table>

Mix to dissolve. Adjust to pH 6.5 with high concentration NaOH. Store at room temperature.

NaCl dialyze

NaCl per 4l of dd H₂O: 30 g - 25 g - 20 g - 15 g - 10 g - 5 g - 0 g - 0 g - 0 g - 0 g

Calculations

For a degree of substitution (DS) 2 (meaning 2 peptides per alginate chain)
(calculations are based on alginate MW and peptide sequence MW utilized. Then amount of peptide to couple dictates the amount of EDC and sulfo-NHS to add.)

EDC:sulfo-NHS (2:1)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGD</td>
<td>15 mg</td>
</tr>
<tr>
<td>Sulfo-NHS</td>
<td>27.40 mg</td>
</tr>
<tr>
<td>EDC</td>
<td>48.42 mg</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>18 mg</td>
</tr>
</tbody>
</table>

PROTOCOL

1 Reconstitute, for example 1 g, alginate powder (after oxidation) with 99 ml of MES solution (1% alginate solution (w/w)), and allow to stir overnight.

2 Mixing quickly, add in order sulfo-NHS, EDC and peptide (to assure more uniform distribution can dissolve in 10 ml of MES before adding to alginate).
3 Allow reaction to proceed for 20 hrs.
4 Quench solution with hydroxylamine and allow to stir for 30 min.
5 Transfer alginate solutions to dialysis membranes (approximately 25 cm per 100 ml of alginate solution) by using clamps to entrap solutions inside the membranes.
6 Transfer dialysis membranes to a 4 L bucket with dd H₂O. Dialyze with decreasing salt solutions of dd H₂O. Place a stir bar in bucket and let stir on stir plate. Change the solution 2-3 times per day.
7 Remove alginate solutions from membranes and sterile filter into a sterile, autoclaved glass bottle using a sterile filter top (0.22 um)
8 Unscrew filter top of sterile bottle unit. Pour alginate solution into a sterile bottle unit, and screw in filter top.
9 Freeze overnight at – 20°C.
10 Lyophilize for approximately 8-10 days until alginate is completely dry.
11 Reconstitute alginate in appropriate concentration and solution.
APPENDIX R

Fluorescent cell labeling in sprouting assay

Materials:

- DAPI
- Live/Dead kit
- Formaldehyde

Solutions:

LIVE/DEAD staining solution:

- Ethidium homodimer-1 solution (provided in LIVED/DEAD kit) 20 µl
- sterile PBS 10 ml
- Calcein AM solution (provided in LIVED/DEAD kit) 5 µl

Add 10 ml of sterile PBS to 20 µl of ethidium homodimer-1 solution. Mix the solution very well. Then add 5 µl of calcein AM solution (provided in LIVE/DEAD kit). Protect the resultant solution from light for the rest of the steps. Store at 4°C.

PROTOCOL

1. Aspirate media and wash wells twice with 0.8 ml of sterile PBS.
2. Add 0.3 ml of LIVE/DEAD solution.
3. Protect from light and incubate for 30 min. at room temperature.
4. Aspirate LIVE/DEAD solution and wash wells twice with 0.8 ml of sterile PBS.
5. Add 0.5 ml of 4% formaldehyde to fix cells and incubate overnight at 4°C.
6. Aspirate formaldehyde solution and wells twice with 0.8 ml of sterile PBS.
7. Add 1 ml of DAPI (300 ng/ml) and incubate for 30 min. at room temperature.
8. Aspirate DAPI solution and wells twice with 0.8 ml of sterile PBS.
9. Add 0.5 ml of PBS and store at – 4°C. Protect the well plate from light.
APPENDIX S

Human CD31 immunostaining for mouse tissue

Materials:

Monoclonal Mouse Anti-Human CD31 Dako # M0823
ARK (Animal Research Kit) Peroxidase Dako # K3954
Protein Block Serum-free Dako # X0909
DAB Chromagen Dako # K3466
Proteinase K Sigma # P2308
Dako Pen Dako # S2002
Tween 20 Sigma # P1379
Sodium chloride EMD # SX0420-3
Tris-HCl Calbiochem # 648317
Rapid mounting media solution EMD # 65037-71

Solutions:

Proteinase K:

Proteinase K Solution (20 ug/ml in TE Buffer, pH 8.0):

TE Buffer (50mM Tris Base, 1mM EDTA, pH 8.0):
- Tris Base 6.10 g
- EDTA 0.37 g
- Distilled water 1000 ml
Mix to dissolve. Adjust to pH 8.0 using concentrated HCl (10N HCl). Store at room temperature.

Proteinase K Stock Solution (20x, 400 ug/ml):
- Proteinase K 0.004 g (4 mg)
- TE Buffer, pH8.0 10 ml
Mix well, aliquot and store at –20°C.

Working Solution (1x, 20 ug/ml):
- Proteinase K Stock Solution (20x) 1 ml
- TE Buffer, pH8.0 19 ml
Mix well. This solution is stable for 1 month at 4 °C. For longer storage, aliquot and store at –20 °C.

TNT washing solution

0.1M Tris-HCl, 0.15 M NaCl, 0.05% Tween 20.
- Tris-HCl 15.76 g
- NaCl 8.77 g
- dd H2O 11
- Tween 20 0.5 ml
(Adjust pH=7.5)
PROTOCOL

1 Heat slides at 65°C for 30 min.
2 Xylene I and xylene II bath (10 min each); followed by ethanol bath gradient (100%-90%-70%) 3 min each; and then place for 5 min in dd H2O.
3 Peroxidase block (present in ARK kit) for 30 min.
4 TNT wash for 5 min.
5 Proteinase K -10 min at 37°C and 5 min room temperature.
6 TNT wash for 5 min.
7 Protein Block Serum-free for 30 min..
8 Tip excess.
9 Dilute anti-Human CD31 (1:50 diluted as directed in ARK protocol) and incubate slides overnight at 4°C.
10 TNT wash for 5 min.
11 Incubate with Streptavidin-Peroxidase (present in ARK kit) for 30 min.
12 TNT wash for 5 min.
13 DAB Chromagen for 5-8 min (use white paper as background to assess color change).
14 Tap H2O rinse and let stay for 3 min.
15 Counterstain slides in Harris Hematoxylin for 4 seconds. Rinse with tap water until runs clear.
16 Dehydrate slides using graduated ethanol, 2 changes for 2 minutes each. (70%, 90%, 100% ethanol)
17 Clear with xylenes, 2 changes for 2 minutes each.
18 Coverslip using a permanent mounting media