Biomaterial-based Strategies to Build Vascularized Modular Tissue Engineered Constructs

by

Ema Cristina Ciucurel

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Chemical Engineering and Applied Chemistry
Institute of Biomaterials and Biomedical Engineering
University of Toronto

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Doctor of Philosophy
Graduate Department of Chemical Engineering and Applied Chemistry
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2013

Abstract
Survival of engineered tissues in vivo requires the presence of an internal vascular network and immediate connection to the host vasculature. Modular tissue engineering approaches the vascularization ‘design’ requirement through fabrication of submillimeter-sized collagen microtissues (‘modules’) with endothelial cells (EC) seeded on the surface of the modules and functional or vascular support cells inside the modules. Several modules are then packed together to build a larger tissue. In this work, we explored biomaterial-based strategies to build vascularized modular tissue engineered constructs. A photocrosslinkable poloxamine-polylysine acrylate biomaterial was first synthesized to improve the mechanical limitations of collagen modules under flow, while still supporting EC attachment. An extracellular matrix (ECM)-based strategy was then explored to enhance the vascularization of the modules in vivo. Manipulation of the ECM was accomplished through lentiviral transduction of EC to overexpress Developmental endothelial locus-1 (Del-1), a pro-angiogenic ECM molecule. Supporting the hypothesis that Del-1 overexpression ‘tilts’ the balance in EC from a quiescent to a pro-
angiogenic phenotype, human umbilical vein endothelial cells transduced to overexpress Del-1 (Del-1 HUVEC) formed more sprouts and had a distinct expression profile of angiogenic genes in vitro, relative to control eGFP HUVEC. While very few blood vessels formed upon subcutaneous injection of empty collagen modules coated with Del-1 or eGFP HUVEC in a SCID/Bg mouse model, embedding adipose derived mesenchymal stem cells (adMSC) inside the modules increased blood vessel formation. Moreover, Del-1 HUVEC and adMSC modules consistently had more blood vessels (donor-derived and total number of vessels) compared to eGFP HUVEC and adMSC, over the 21 day duration of the study, with the greatest difference observed at day 7 post-transplantation. In addition, more α-smooth muscle actin (SMA+) staining was observed in Del-1 implants compared to eGFP, suggestive of increased vessel maturation through recruitment of SMA+ pericytes and smooth muscle cells. Perfusion studies showed that the implant vasculature was connected to the host vascular network as early as day 7, and throughout the 21 day duration of the study, for both Del-1 and eGFP implants. Nevertheless, further normalization of the vasculature is likely required to improve perfusion at early time points after transplantation.
Acknowledgements

I thank Dr. Michael Sefton, my supervisor, for his mentorship during my graduate training. It has been a privilege to learn from him about the important research questions in tissue engineering and regenerative medicine, and to pursue research ideas under his guidance. His continual encouragement and understanding throughout the completion of this project were very much appreciated. I also thank my thesis committee members, Dr. Molly Shoichet and Dr. Jeffrey Medin, for their advice and support.

I thank Chuen Lo for all his help with animal surgeries and perfusion experiments, and his support in making sure research materials were always ordered and delivered on time. I am very grateful for his willingness to help and his encouragements over the years. I also thank Alex Vlahos for his help while working together on the Del-1 project over the last two summers. His hard work and enthusiasm while conducting the sprouting assays and the blood vessel and cell counts were very much appreciated. I thank all other members of the Sefton lab (Mark, Dean, Brendan, Omar, Lindsay, Tom, Derek, Rohini, Sasha, Gabi, Laura) for their support.

I also thank all the collaborators who made this project possible: Orlay Lopez-Perez and Bryan Au (Dr. Medin lab, University Health Network, Toronto) for the lentiviruses used in this study, Dr. Quertermous (Stanford University) for supplying the Del-1 cDNA, the Toronto General Hospital Pathology Research lab (Carmelita, Art, Melanie, Yi) for all the immunohistochemistry services, Lisa Yu (Dr. Henkelman’s lab, Mouse Imaging Centre, Toronto Centre for Phenogenomics) for performing the microCT imaging, and Melissa Yin (Dr. Foster’s lab, Sunnybrook Research Institute, Toronto) for performing the ultrasound imaging.

I acknowledge scholarship funding from the Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT) and the Natural Sciences and Engineering Research Council of Canada (NSERC). The research project was funded through grants from the Canadian Institutes of Health Research (CIHR) and the US National Institutes of Health (NIH).

I thank my grandparents, my parents and my sister for never doubting me and giving me strength and confidence in myself, and I thank Dan for always pushing me to succeed and for his patience.
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<td>adMSC</td>
<td>adipose derived mesenchymal stem cells</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
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<tr>
<td>Ang 1</td>
<td>angiopoietin 1</td>
</tr>
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<td>Ang 2</td>
<td>angiopoietin 2</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<td>AVL</td>
<td>arteriovenous loop</td>
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<td>BCP</td>
<td>biphasic calcium phosphate</td>
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<td>bFGF</td>
<td>basic fibroblast growth factor (also called FGF2)</td>
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<td>BMA</td>
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<td>BMA-MAA</td>
<td>poly(butyl methacrylate –co– methacrylic acid)</td>
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<td>BMEL</td>
<td>bipotential mouse embryonic liver cells</td>
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<td>bmMSC</td>
<td>bone marrow derived mesenchymal stromal cells</td>
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<td>BMP-2</td>
<td>bone morphogenetic protein-2</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CAM</td>
<td>chick chorioallantoic membrane</td>
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<td>CCl₄</td>
<td>carbon tetrachloride</td>
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<tr>
<td>CHN</td>
<td>carbon hydrogen nitrogen</td>
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<td>CHO</td>
<td>chinese hamster ovary cells</td>
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<td>COC</td>
<td>cumulus granulosa-oocyte complexes</td>
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<td>COUP-TFII</td>
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<td>DLL-4</td>
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<td>DMF</td>
<td>(N,N)-dimethylformamide</td>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>E</td>
<td>mean amplicon efficiency</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>EGDMA</td>
<td>ethylene glycol dimethacrylate</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>eGFP</td>
<td>enhanced green fluorescent protein</td>
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<td>EPC</td>
<td>endothelial progenitor cells</td>
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<tr>
<td>ERK</td>
<td>extracellular signal related kinase</td>
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<td>EthD-1</td>
<td>ethidium homodimer-1</td>
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<td>focal adhesion kinase</td>
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<tr>
<td>FBS</td>
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<td>FGF2</td>
<td>fibroblast growth factor 2 (also called bFGF)</td>
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<td>FNIII</td>
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<td>G-CSF</td>
<td>granulocyte colony-stimulating factor</td>
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<td>GF</td>
<td>growth factor(s)</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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<td>HAF</td>
<td>human artery-derived fibroblasts</td>
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<td>HBPA</td>
<td>heparin-binding peptide amphiphiles</td>
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<td>hESdC</td>
<td>human embryonic stem cell-derived cells</td>
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<td>HGF</td>
<td>hepatocyte growth factor</td>
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<td>hypoxia-inducible factor-1</td>
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<td>HMEC</td>
<td>human microvascular endothelial cells</td>
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<td>HoxD3</td>
<td>homeobox D3</td>
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<tr>
<td>HS</td>
<td>heparan sulfate</td>
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<td>HSGAG</td>
<td>heparan sulfate-like glycosaminoglycans</td>
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<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
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<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule-1</td>
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<tr>
<td>ICP-AES</td>
<td>inductively coupled plasma atomic emission spectrometry</td>
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<td>IEM</td>
<td>2-isocyanatoethyl methacrylate</td>
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<td>IFN-γ</td>
<td>interferon gamma</td>
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<td>IGF-1</td>
<td>insulin-like growth factor-1</td>
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<td>IL-1α</td>
<td>interleukin-1 alpha</td>
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<td>Krueppel-like factor 2</td>
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<td>lactate dehydrogenase</td>
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<td>lipopolysaccharide</td>
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<td>LSD</td>
<td>least significant difference</td>
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<td>MAA</td>
<td>poly(methacrylic acid –co– methyl methacrylate)</td>
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<tr>
<td>MAETAC</td>
<td>(2-(methacryloyloxy) ethyl)-trimethylammonium chloride</td>
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<td>MEMS</td>
<td>microelectromechanical systems</td>
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<td>MHC</td>
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<td>microCT</td>
<td>microcomputed tomography</td>
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<td>MMP-2</td>
<td>matrix metalloproteinase-2</td>
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<td>matrix metalloproteinase-14</td>
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<td>MOI</td>
<td>multiplicity of infection</td>
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<td>MPC</td>
<td>mesenchymal progenitor cells</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MSC</td>
<td>mesenchymal stromal cells</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cut off</td>
</tr>
<tr>
<td>NHF</td>
<td>normal human fibroblasts</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
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<td>NOV</td>
<td>nephroblastoma-overexpresed protein</td>
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<tr>
<td>NRQ</td>
<td>normalized relative quantity</td>
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<td>OEC</td>
<td>outgrowth endothelial cells</td>
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<td>OPN</td>
<td>osteopontin</td>
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<td>OTS</td>
<td>octadecyltrichlorosilane</td>
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<td>PA</td>
<td>peptide amphiphiles</td>
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<td>PAI-1</td>
<td>plasminogen activator inhibitor 1</td>
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<td>PBAE</td>
<td>poly(β-amino esters)</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PDGF-B</td>
<td>platelet-derived growth factor B</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>PDGFR-β</td>
<td>platelet-derived growth factor receptor beta</td>
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<td>PDMS</td>
<td>poly(dimethyl siloxane)</td>
</tr>
<tr>
<td>PE</td>
<td>peak enhancement</td>
</tr>
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<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
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<td>PEI</td>
<td>poly(ethyleneimine)</td>
</tr>
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<td>PFDC</td>
<td>perfluorodecalin</td>
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<td>polyethylene glycol diacrylate</td>
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<td>quantitative real-time polymerase chain reaction</td>
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<td>ROI</td>
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<td>SCF</td>
<td>stem cell factor</td>
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<td>SCID/Bg</td>
<td>severe combined immunodeficient/beige</td>
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<tr>
<td>SDF-1</td>
<td>stromal derived factor -1 (also called CXCL12)</td>
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<tr>
<td>SDS</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<td>Shh</td>
<td>sonic hedgehog</td>
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<td>SMA</td>
<td>smooth muscle actin</td>
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<td>SMC</td>
<td>smooth muscle cells</td>
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<tr>
<td>SPARC</td>
<td>secreted protein, acidic and rich in cysteine</td>
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<td>SPCL</td>
<td>starch – poly(caprolactone)</td>
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<td>tissue culture treated polystyrene</td>
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<td>Acronym</td>
<td>Full Form</td>
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Chapter 1
Introduction

1 Research Motivation

Tissue engineering promises to provide artificial tissues to replace or restore function to damaged tissues and organs. A blood supply is needed to meet oxygen, nutrient and waste transport demands in engineered tissues and thus maintain cell viability within the tissue. Without an internal vasculature, and due to diffusion limitations, only tissue constructs of a few hundred microns or less in thickness can be made, thus limiting the clinical therapeutic value of these constructs [1]. In the Sefton lab, we use a modular approach to generate vascularized tissues [2, 3]. In the modular approach, functional cells or vascular support cells are embedded in submillimeter-sized gel cylinders (‘modules’) and endothelial cells (EC) are seeded on the surface of the modules. Several modules are randomly packed together to obtain a larger tissue. The modular approach has several advantages. First, the tissue constructs already include a vascular component by design, with the EC seeded on the surface of the modules. Second, the modules have uniform cell distribution. This method of tissue fabrication is also scalable. Finally, the modular approach is minimally invasive as the modules can simply be injected using a syringe and needle.

Collagen type I alone or a mixture of collagen and poloxamine have been used in our lab to fabricate the modules. Collagen modules support cell attachment, but have limited resistance to compression under flow in vitro. Poloxamine is a synthetic four arm block copolymer of poly(ethylene oxide) and poly(propylene oxide). Poloxamine is biocompatible, but it does not allow cell attachment. Modules made of a mixture of collagen and poloxamine are stiff and support cell attachment to a greater extent than poloxamine alone [4, 5]. However, EC attachment on these surfaces is patchy. The first part of this thesis project focused on chemically synthesizing a suitable biomaterial substitute to solve some of the mechanical or cell attachment challenges encountered with collagen or poloxamine methacrylate, respectively.

Ultimately, the success of the modular tissue engineering approach is dependent on the ability of the transplanted EC to form a functional vascular network in vivo upon transplantation, and to
rapidly connect to the host vasculature. A rapidly formed, functional vascular network would enable the survival of the transplanted functional cells, and a successful therapeutic outcome. Therefore, controlling the EC phenotype with a view to enabling the formation of a vascular network in vivo has emerged as a critical design goal in our lab. While much effort has been directed in the field of tissue engineering towards delivering growth factors in vivo with the goal of stimulating angiogenesis, more limited attention has been directed toward controlling and exploiting the interactions between the scaffold biomaterial and the cells to promote vascularization. We presumed that it would be possible to engineer the extracellular matrix (ECM) composition of the modules to ‘tilt’ the angiogenic balance in the EC coating the modules from quiescent to pro-angiogenic, which would then result in enhanced blood vessel formation in vivo. The second (and major) part of the thesis focused on examining the in vivo vascularization benefit of altering the ECM composition of the modules by using lentiviral transduction of the EC to overexpress a pro-angiogenic ECM molecule. The use of lentiviral constructs has several advantages. Lentiviral constructs have relatively large packaging capacity compared to other viral systems, reduced immunogenicity, and high transduction efficiency, they can transduce both dividing and non-dividing cells, and when they integrate into the host genome, they induce stable long-term gene expression [6]. Hence, we presumed that transduction of EC with a lentiviral construct to overexpress a pro-angiogenic ECM protein would ensure stable expression of this protein in vivo, and the EC would thus act as both drivers of tissue remodeling and vascularization, and as delivery vehicles. Both pre-clinical and clinical studies with lentiviral systems have shown that their use as gene delivery systems was safe and did not lead to tumorigenesis following viral insertion into the host genome [7-9]. In fact, several mutations are believed to be necessary to eventually lead to oncogenesis, which suggests that the risk of occurrence of disease due to insertional mutagenesis in several ‘wrong spots’ exists, but it is low [10].

2 Hypothesis and Objectives

The overall goal of this thesis was to develop biomaterial-based strategies to build vascularized modular tissue engineered constructs. We used two approaches: (1) chemical modification of synthetic scaffolds; (2) manipulation of the ECM through lentiviral transduction of EC.
2.1 Chemical Modification of Synthetic Scaffolds

The goal of this first part of the project was to produce a synthetic biomaterial substitute to overcome the mechanical limitations of collagen, and the low cell attachment properties of poloxamine, the materials typically used in our group to fabricate the modules. We hypothesized that a poloxamine-polylysine acrylate polymer would be suitable for this purpose. We reasoned that adding polylysine peptides to the poloxamine backbone would support EC attachment to the hydrogels. In addition, chemically adding acrylate groups would enable photocrosslinking of aqueous solutions of the polymer, and mechanically strong hydrogels (modules) would be obtained. Moreover, we presumed that polylysine would also be a good model molecule for future polymer synthesis aimed at introducing other desired functionalities (through other peptides) into the scaffold biomaterial. The objectives of the first part of the project were to: (1) synthesize the poloxamine-polylysine acrylate polymer; (2) test the ability of the synthesized polymer to support EC attachment and viability in vitro.

2.2 Manipulation of the Extracellular Matrix through Lentiviral Transduction of Endothelial Cells

The goal of the second part of the project was to enhance the vascularization of modular tissue engineered constructs upon implantation in vivo. The hypothesis was that we could use lentiviral transduction of EC to overexpress Developmental endothelial locus-1 (Del-1, an ECM protein shown by others to have angiogenic properties in vivo) as a means of tipping the angiogenic balance from a quiescent to a pro-angiogenic phenotype in EC incorporated in modular tissue engineered constructs, and as a means of enhancing the vascularization of modular tissue engineered constructs coated with these EC, upon their implantation in vivo. Specifically, the objectives were to (1) produce EC that overexpress Del-1 through lentiviral transduction; (2) characterize in vitro the behavior of the EC overexpressing Del-1 (gene expression, sprouting assay etc.); (3) characterize in vivo the remodeling and the vascular network formed upon implantation of collagen modules coated with Del-1 EC in a severe combined immunodeficient/beige (SCID/Bg) subcutaneous implant model; (4) characterize in vivo the remodeling and the vascular network formed upon implantation of collagen modules coated with Del-1 EC and also containing embedded adipose derived mesenchymal stem cells (adMSC), as vascular support cells.
Figure 1-1. Del-1 effect on EC. Our view was that transduction with Del-1 would ‘tilt’ the angiogenic balance in EC.

3 Thesis Content

The current chapter (chapter 1) is an introduction to the thesis. The research motivation, overall goal, hypothesis and objectives of the thesis are introduced. Chapters 2, 3 and 4 are a literature review highlighting the existing knowledge and advancements in scaffold vascularization and tissue engineering (chapter 2), with a particular emphasis on modular tissue engineering (chapter 3). Del-1 is introduced in chapter 4, as well as other pro-angiogenic matricellular proteins. In chapters 5, 6 and 7 two biomaterial-based strategies to build vascularized modular tissue engineered constructs are described. First, in chapter 5, the use of a synthetic polymer, poloxamine-polylysine acrylate, is examined as a mechanically stronger, cell adhesive hydrogel scaffold substitute to collagen and poloxamine, the materials typically used to fabricate the modules in our group. Next, in chapters 6 and 7, the manipulation of the ECM through lentiviral transduction of EC to overexpress Del-1 is examined as a means of enhancing the vascularization of modular tissue engineered constructs in vivo. Both the in vitro characterization of EC transduced to overexpress Del-1, as well the in vivo remodeling process and fate of transplanted cells are discussed, with an emphasis on donor-derived blood vessel formation (chapter 6). I also explore whether the combination of EC overexpressing Del-1 and adipose-derived stem cells (adMSC) in the context of modular tissue engineering enhances the vascularization of the tissue constructs compared to co-transplantation of control EC and adMSC, in terms of blood vessel density, as well as blood vessel maturation and functionality (chapter 7). Finally, chapter 8
highlights some research questions related to modular tissue engineering and the Del-1 component of this project in particular, that would perhaps be interesting to explore further in the future.

4 References


Chapter 2

Literature Review: Angiogenesis and Scaffold Vascularization in Tissue Engineering

1 Introduction

Evolution of multi-cellular organisms required the development of a circulatory system capable of distributing gases, nutrients and signaling molecules. Due to the poor diffusional distance of oxygen and nutrients, all cells within the human body are within 100 – 200 µm of a blood vessel with few exceptions (e.g. cells in the cornea, cartilage, ligaments) [1]. Like the evolution of larger organisms, engineered tissues of clinically relevant sizes require the incorporation of a vascular network capable of supplying seeded or recruited cells deep within the scaffold a means of exchanging gases, nutrients, signaling molecules and waste. This chapter focuses on the methods currently being developed to vascularize biomaterial scaffolds.

1.1 The Native Vascular Network

The vascular network in mammals is a hierarchical network of vessels that circulate the blood to and from the heart through the tissues. For most vessels, there are three layers (Figure 2-1). The intima is the innermost layer and is a monolayer of endothelial cells (EC) supported by a basement membrane. EC interface with blood and have a number of functions, which include the regulation of capillary permeability, hemostasis, and leukocyte recruitment and translocation [2, 3]. The middle layer is the media, which is composed of layers of contractile vascular smooth muscle cells (SMC) and elastin. The adventitia is the outermost layer and contains fibroblasts, mast cells, nerve endings and, in large vessels such as the aorta, small capillaries [4].

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Figure 2-1. Structure of nascent and mature blood vessels. (a) EC alone assembled into tube-like structures (nascent blood vessel). (b) Capillaries are the smallest type of blood vessels (only 5 - 10 µm in diameter), and are composed of a single layer of EC supported by a basement membrane of matrix proteins, and an incomplete covering of pericytes that stabilize the vessel. (c) Compared to capillaries, arterioles and venules are invested with a higher number of mural cells. Arterioles have a thinner media layer than arteries, and regulate the blood flow between the arteries and the capillaries. Venules (and veins) have thin walls and fewer SMC, relative to arteries. (d) Larger blood vessels have three specialized layers: intima (containing EC), media (containing SMC) and adventitia (containing fibroblasts). Arteries have a thick media composed mainly of SMC, which provide mechanical stability to the vessel wall, and allow the vessels to vasodilate and vasoconstrict in order to regulate blood flow and pressure. [Reprinted by permission from Macmillan Publishers Ltd.: Nat Med [4], copyright 2003]

In the embryo, the formation of the vascular network starts early on during development, to match the oxygen, nutrients and waste removal requirements of the growing organism. During vasculogenesis, endothelial cells differentiate from mesodermal precursor cells, with vascular endothelial growth factor receptor 2 (VEGFR2) being one of the earliest markers of the endothelial cell lineage. The cells proliferate, differentiate, aggregate and form blood islands. The blood islands then fuse together and ultimately form the primary vascular plexus, a honeycomb-like structure of primitive blood vessels [5-7]. Further remodeling of this primitive vascular structure occurs through either sprouting angiogenesis or intussusception. The arterial-venous differentiation is genetically predetermined, and contrary to what was initially assumed,
it does not rely on the start of blood flow. The bidirectional signaling between ephrinB2 (expressed by arteries) and its receptor EphB4 (expressed by veins) plays an important role in defining the boundaries between the arterial and venous sides. A number of genes upstream of ephrinB2/EphB4 are involved in the signaling that ultimately results in the specification of arteries and veins. Sonic hedgehog (Shh), vascular endothelial growth factor (VEGF), neuropilin-1, notch-1 and its ligand delta-like-4 (DLL-4), are among the genes upstream of ephrinB2 and are known to be involved in establishing the arterial cell fate, while neuropilin-2 and COUP-TFII (chicken ovalbumin upstream promoter-transcription factor II) are upstream of EphB4 and are involved in determining the venous identity [8, 9].

1.2 Angiogenesis: the Formation of New Blood Vessels

In adults, new blood vessels are typically formed through a process called angiogenesis, in which new microvessels sprout from pre-existing vessels. While angiogenesis is not the only mechanism of vascularization, it is the most extensively studied. Two other mechanisms have been described: post-natal vasculogenesis, in which vessels are formed de novo by recruited bone marrow-derived progenitor cells, and intussusception, in which the lumen of an existing blood vessel divides to form two separate vessels [10].

The process of forming new blood vessels follows the same general pattern in all tissue types. However, the final density of the vascular network depends on the specific requirements of the tissue undergoing vascularization. Tissues with high metabolic requirements, such as muscle, require a rich vascular network to provide sufficient amounts of oxygen, nutrients and waste removal to match the metabolic demand of the active muscle tissue. In addition, the structure of the blood vessels also reflects the function of the organ or tissue that these vessels supply. While most tissues are supplied by continuous, non-fenestrated capillaries, continuous fenestrated capillaries are present in organs and tissues involved in filtration or secretion, such as the endocrine glands, the gastrointestinal mucosa, or the glomerulus [11, 12]. Fenestrations are transcellular pores that extend through endothelial cells and facilitate the transport of water and small molecules across the endothelium. Other organs, such as the liver, contain discontinuous capillaries, which have even larger fenestrations (100–200 nm) [11, 12].

Numerous conditions can induce angiogenesis including tissue hypoxia, low pH, hypoglycemia and inflammation [13]. The angiogenic process is divided into three phases: 1) vasodilation and
increased vessel permeability, 2) EC proliferation and migration, 3) vessel maturation. The first stage of angiogenesis is marked by nitric oxide (NO) -mediated vasodilation, and increased permeability of the blood vessels, which are stimulated by increased levels of VEGF [14]. The increased permeability allows plasma proteins to extravasate into the surrounding tissue to form a provisional matrix for EC migration. This process is tightly regulated by the angiopoietin (Ang)/Tie system. Ang-1 and Ang-2 both bind the Tie2 receptor, however Ang-1 acts to stabilize blood vessels, while Ang-2 antagonizes Ang-1 and is associated with vessel destabilization and either vessel growth (in the presence of VEGF) or vessel regression [15-17]. Prior to migration, EC loosen their inter-endothelial contacts and release from the supporting mural cells, further destabilizing the blood vessel [18]. Proteinases, such as matrix metalloproteinases (MMPs) and plasminogen activators, degrade the extracellular matrix (ECM) allowing EC migration, and liberate matrix-bound pro-angiogenic growth factors (GF) including VEGF and basic fibroblast growth factor (bFGF) [18].

To prevent en masse migration of EC toward an angiogenic signal, and potential dissolution of a pre-existing vessel, EC compete to lead the migrating sprout along the gradient of angiogenic factors[19]. This leading cell, called a tip cell, is selected through a spatial cell patterning mechanism involving VEGF, and notch pathway ligand DLL-4 (Figure 2-2). Adjacent to the tip cell are stalk cells, which proliferate and elongate the stalk. An in depth look at tip and stalk cell selection and function is beyond the scope of this chapter, however it is reviewed in detail elsewhere [20-22]. As the tip cell migrates and the stalk elongates, the stalk cells form a lumen and stabilize the sprouting vessel by reestablishing tight inter-endothelial junctions, laying down a basement membrane and recruiting pericytes (mediated by platelet-derived growth factor B (PDGF-B), Ang-1, transforming growth factor beta (TGF-β) and other factors) [19, 21]. To become a functional, perfused vessel, the tip cell of one sprout must fuse with a neighboring sprout, a process called anastomosis, that is thought to be facilitated by macrophages [23]. As the new vessel matures, the EC resume their quiescent phalanx phenotype [21].

Angiogenesis is tightly regulated in vivo by maintaining a balance between pro-angiogenic and angiostatic (also called anti-angiogenic) stimuli [18]. Angiogenesis inhibitors (e.g. angiostatin, endostatin, anti-thrombin III, interferon-β, leukemia inhibitory factor and platelet factor 4, thrombospondin-1) can suppress EC proliferation and migration, or lumen formation [18, 24].
Other molecules (TGF-β1, tumor necrosis factor alpha (TNF-α)) can both stimulate or inhibit angiogenesis [18]. In order to form and maintain mature, functional vessels, a balance must exist between factors that induce and inhibit angiogenesis. For example, in cancer the balance is typically shifted too far in favour of angiogenesis, which results in the formation of tortuous, leaky, immature, vessels [10, 21, 25] (Figure 2-2). Often times, the vascular network formed within tissue engineered constructs is also leaky and lacks functionality, due to the absence of an adequate balance or spatio-temporal coordination between signals initiating angiogenesis and signals promoting vessel maturation in the implant area.

An alternative mechanism for forming new blood vessels is vasculogenesis. Previously, it was thought that vasculogenesis occurred only during development, however recent studies have demonstrated the role of vasculogenesis in adult vascularization (although the extent of contribution is unclear) [13, 26-28]. During vasculogenesis, blood vessels are formed *de novo* through the recruitment and differentiation of a heterogeneous (and controversial) family of bone marrow-derived cells called endothelial progenitor cells (EPC). Once recruited to a tissue, EPC proliferate, differentiate and incorporate themselves into immature vessels or form new vessels [13, 29].
Figure 2-2. Molecular basis of vessel branching, and normal vs. abnormal vessel formation. (a) Vessel branching is tightly regulated by a variety of key factors. The principal molecular signals are depicted for each of the consecutive steps of a branching process (b) In normal angiogenesis, nascent tube-like structures become invested with mural cells and form a mature, organized and stable vascular network. (c) In abnormal angiogenesis seen in established tumours, the newly formed blood vessels are generally abnormal in structure and function. Note: Blood vessel networks formed in tissue engineered constructs are often times leaky, tortuous and unstable. [Reprinted by permission from Macmillan Publishers Ltd.: Nature, copyright 2011 and Nat Rev Drug Discov, copyright 2011 [21, 30]]
2 Vascularization of Biomaterial Scaffolds

Vascularization of biomaterial scaffolds is currently a major barrier to creating viable, complex engineered tissues, such as liver, heart, bone and adipose, at a clinically relevant scale. There are a few key challenges in creating a vascularized tissue. The first is creating the vascular network within the scaffold. The second is connecting (anastomosing) the scaffold vasculature with the host vasculature so that the scaffold is perfused \textit{in vivo}. Finally, the newly formed vascular network has to mature, be functional, and persist over time. Researchers are currently using a variety of approaches to achieve scaffold vascularization (Figure 2-3). GF delivery (section 2.1) from biomaterial scaffolds is one of the first methods used to induce vessel formation \textit{in vivo}. Single or multiple GF can be released from the scaffold in a controlled and sustained manner to encourage the ingrowth of host vessels within the scaffold.

Another approach is vascular cell transplantation (section 2.2), where EC alone or in combination with support cells are delivered in a bulk scaffold or in small microtissues (modular tissue engineering). Gene delivery to overexpress angiogenic factors in transplanted cells has also been used. Cells can be cultured and transplanted using scaffold-free approaches or, conversely, scaffolds alone can be used to mobilize and recruit endogenous cells to vascularize the scaffold \textit{in situ} (section 2.3).

A common issue with both GF and cell transplantation approaches is the lack of immediate connection to the host vasculature and perfusion. Scaffold prevascularization (section 2.4) and decellularization strategies (section 2.5) aim to provide a solution to this problem. In some cases, biomaterials themselves (without the addition of GF or cells) were also shown to induce angiogenesis (section 2.6) and microfabrication methods can be used to create pre-defined, hierarchical vascular networks \textit{in vitro} (section 2.7).
Figure 2-3. Scaffold vascularization strategies. (a) Delivery of single or multiple angiogenic GF to stimulate the ingrowth of host vasculature into the scaffold. (b) Transplantation of EC (alone or in combination with support cells). The EC are expected to re-assemble in vivo, participate in blood vessel formation, and connect to the host vasculature. Support cells stabilize the nascent capillary-like structures. (c) Bio-functionalized materials are used to mobilize and capture endogenous cells at the implant site and stimulate de novo blood vessel formation. (d) In vivo prevascularization strategies use the host body as a bioreactor to vascularize the scaffold, prior to relocating the construct to its target implant site. In the case of arteriovenous loops, the prevascularization step takes place in a chamber enclosing an arteriovenous graft. (e) Decellularized scaffolds provide a natural template to recreate the architecture of the vasculature network. After decellularization, organs are reseeded with EC to repopulate the vascular tree and with functional cells to create a functional tissue engineered construct. (f) Upon implantation,
some biomaterials induce angiogenesis by themselves, without the addition of any cells or GF. (g) Using microfabrication techniques, predefined, hierarchical pseudo-vasculatures are created (although mostly for in vitro applications thus far).

2.1 Growth Factor Delivery

2.1.1 Single Growth Factor Delivery

One of the primary approaches to vascularize tissue engineering scaffolds is delivering angiogenic GF, such as VEGF, bFGF, and PDGF, to stimulate the ingrowth of host vasculature into the scaffold. While initial attempts to induce scaffold vascularization used single GF delivery systems, recent efforts have focused on dual or multiple GF delivery to form and maintain a vascular network.

GF have narrow therapeutic windows and short half-times in vivo, and in order to induce and maintain vascularization, the supply of exogenous GF must be sustained over several weeks to prevent vessel regression and to allow vessel maturation [31]. Bolus injection of soluble GF into the systemic circulation or directly into an ischemic zone have generally failed to achieve the prolonged, therapeutically relevant concentrations necessary to produce a lasting vascular network [32, 33]. In addition, systemic exposure may lead to adverse side effects such as hemorrhage, hypotension and the vascularization of undesired sites [33, 34]. Consequently, to ensure patient safety and efficacy of treatment, the dose, location and duration of exogenously administered GF must be tightly controlled. The incorporation of GF into slow-releasing biomaterial scaffolds provides the ability to have sustained local delivery of GF to the site of interest. The pharmacokinetics of protein release can be controlled through the polymer properties (e.g. polymer composition, porosity, degradation kinetics) and the method by which the GF is incorporated into the scaffold (e.g. absorbed, encapsulated or tethered).

One of the simplest methods to incorporate a GF into a polymer scaffold is to impregnate the scaffold with an aqueous solution containing the desired protein. Gelatin hydrogels loaded with bFGF were used to promote vascularization of the thigh muscle following the excision of the femoral artery in a rabbit model of hind limb ischemia [35]. Angiographic assessment with Laser Doppler Perfusion Imaging (LDPI) demonstrated that single intramuscular injection of the
bFGF-loaded hydrogel was sufficient to improve tissue blood flow 4 weeks post surgery, suggesting the therapeutic potential of the bFGF-containing gelatin scaffold.

To prolong the release of a GF of interest, the protein can be encapsulated into microspheres that are then embedded into the bulk of the scaffold, and this method has been used to deliver a variety of GF [36, 37]. For example, Ennet and colleagues used this method to deliver VEGF by pre-encapsulating it in poly(lactide-co-glycolide) (PLG) microspheres prior to incorporation into PLG scaffolds [36]. The encapsulation method resulted in a smaller burst and a slower, prolonged protein release, and the release kinetics were further adjusted by varying the polymer composition and microsphere size. The concentration of VEGF within the tissue infiltrating and surrounding the VEGF-releasing scaffold was present at physiologically relevant concentrations (more than 10 ng/mL) for up to 21 days, with negligible release into the systemic circulation [36]. Similarly, when implanted in vivo, composite alginate scaffolds containing encapsulated bFGF increased the von Willebrand factor (vWF)-positive microvessel density, compared to control scaffolds[37].

Alternatively, GF can be immobilized onto the scaffold to promote desired cell-material interactions [38-40]. GF can be modified to facilitate covalent binding to the scaffold, thereby prolonging their biological activity in the tissue. Ehrbar and collaborators created a variant of VEGF (TG-VEGF_{121}) containing a transglutaminase (TG) substrate sequence that allowed it to be directly bound to a fibrin scaffold by spontaneously cross-linking with fibrinogen during factor XIII-mediated fibrin polymerization [31, 38]. The TG-VEGF_{121} was released with the gradual degradation of the fibrin gel by enzymes (e.g. plasmin or MMPs) secreted by local cells; providing a sustained, local, low-level release of VEGF. In comparison, the passive release of freely diffusible wild-type VEGF from fibrin resulted in an initial burst release and a shorter sustained delivery (the fibrin gel was almost completely depleted 2 weeks post-implant). The active release of TG-VEGF_{121} from the fibrin gels induced the formation of blood vessels that were stabilized by SMC at 3 weeks post-implant. However, the newly formed vessels had regressed by 6 weeks despite the presence of the SMC at 3 weeks [38]. The regression was attributed to the absence of a physiological demand, although it could also indicate that administration of a single GF was insufficient for stable vessel development. This is a common issue when exogenously promoting vessel formation.
Other ways to immobilize GF include using carbodiimide chemistry, which covalently links the protein to the scaffold through an amide bond [40], or physical entrapment of the GF within the crosslinked scaffold [41]. Regardless of the method, immobilization is expected to provide persistent guidance cues for cell behaviour and migration [41], similar to what is observed in the ECM during angiogenesis. However, it also prevents the internalization of the GF upon binding to its receptor, which can eventually lead to the desensitization of the cell to the GF signal [40]. Moreover, certain GF/receptor complex signaling cascades are dependent on internalization [42] and consequently are not suitable for this method of delivery.

More complex scaffold designs exploit synthetic components to direct cell invasion, matrix degradation and controlled GF release to induce vasculature growth in vivo. Phelps et al. created a polyethylene glycol (PEG) hydrogel using photo-crosslinkable PEG diacrylate (PEGDA) monomers conjugated to MMP-sensitive peptides, integrin binding peptides (arginine-glycine-aspartic acid; RGD) and VEGF [32]. This elegant synthetic system encouraged vascularized tissue invasion by allowing cells to bind and remodel the scaffold, while presenting pro-angiogenic signals throughout the matrix. There were higher microvessel densities at 2 and 4 weeks, compared to hydrogels that did not contain all three components (i.e. MMP-cleavage sites, RGD and VEGF), by microCT (indicating also that vessels were connected to the host). Furthermore, in a mouse hind-limb ischemia model, injection of the hydrogel in ischemic muscle improved the perfusion of the leg and foot of the ischemic limb 7 days after vessel ligation, compared to the negative control [32].

### 2.1.2 Multiple Growth Factor Delivery

While single factor delivery typically results in the formation of a branched network of vessels, the vessels often fail to mature and stabilize. A reason for this outcome is that a single factor is likely incapable of inducing the full cascade of events that occur in angiogenesis from sprouting to maturation of the newly formed vessel. Hence, a strategy for promoting mature vessels is to initiate angiogenesis with a pro-angiogenic factor such as VEGF, then promote vessel stabilization by PDGF-mediated SMC and pericyte recruitment [43-45].

In one study, VEGF and PDGF were included in a calcium phosphate-crosslinked alginate gel to induce mature vessel formation. The alginate scaffold released PDGF more slowly than VEGF, likely due to the difference in affinity of the two GF to the alginate. VEGF had a high release
rate during the first 12 days after which it diminished, and PDGF had a slower but sustained release up to 30 days [44]. In vivo, the dual delivery system did not increase the vessel density in a myocardial infarct model, compared to VEGF alone. However, it did increase the density of vessels staining positive for smooth muscle actin (SMA; a marker for SMC and pericytes), indicative of greater vessel remodeling and maturation. Cardiac function was improved compared to single GF therapy [44].

A more controlled, spatially compartmentalized system for sequential VEGF and PDGF delivery was developed in the Mooney laboratory to pattern blood vessel formation and maturation [43]. A bilayered, porous PLG scaffold was designed to deliver the GF with a spatio-temporal gradient. The outer layer (first to be degraded) contained only VEGF, while the second, inner layer contained both VEGF (at a lower concentration) and microencapsulated PDGF. The VEGF was released quickly and created a concentration gradient in the surrounding tissue and within the scaffold that induced the infiltration of sprouting vessels. Encapsulation of the PDGF within PLG microspheres in the inner layer delayed its release, and created a localized gradient of PDGF within the scaffold, concentrated mainly within the inner layer. The porous structure of the scaffold allowed for tissue invasion in vivo. When implanted into mouse ischemic hind limbs, the bilayered, dual delivery system yielded a slightly lower blood vessel density, but a significantly more mature vascular network, characterized by large vessels and association of SMA-positive SMC (as compared to single GF systems or empty scaffolds) [43].

One caveat that is relevant to these studies is that there is an antagonistic relationship between VEGF and PDGF; when both VEGF and PDGF are used VEGF activation of VEGFR2 suppresses PDGF-β signaling in SMC, disrupting SMC/pericyte function during neovascularization [46]. How these observations relate to the vascularization results reported above is not clear.

2.2 Cell Delivery and Transplantation

2.2.1 Scaffolds Seeded with Cells

Transplantation of vascular cells (EC alone or in combination with support cells) to facilitate the vascularization of the scaffold is another common approach. Typically, EC are seeded onto the scaffold, which can be either a natural or synthetic biomaterial (or a decellularized scaffold, see
section 2.5), and these endothelialized constructs are then used for transplantation. The EC are expected to re-assemble in vivo, participate in blood vessel formation (i.e. vasculogenesis), and connect to the host vasculature. Other cell types can be co-cultured and transplanted along with EC to support EC survival and/or to stabilize the nascent capillary-like structures.

A quiescent EC layer is needed to create a non-thrombogenic surface [47, 48]. EC produce antithrombotic factors such as thrombomodulin, heparan sulphate, NO and prostacyclin[47]. Moreover, EC express tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor 1 (PAI-1), and play a key role in balancing fibrinolytic versus coagulation events. An intact, non-activated endothelium also provides a non-adherent surface for the platelets and leukocytes present in the blood, whereas activated EC up-regulate the expression of adhesion molecules such as E-selectin, inter-cellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), and facilitate the binding of circulating leukocytes to the endothelialized surface [47].

Initial implant studies revealed that EC rapidly undergo apoptosis when transplanted without a supportive scaffold. In a study by Kraehenbuehl and collaborators, only 3% of the EC injected directly into the site of ischemia engrafted, due to extensive cell death [49]. Seeding the EC onto a biomaterial substrate (or embedding the EC within the biomaterial scaffold) can help prevent anoikis (programmed cell death induced by lack of adhesion sites), but the choice of the biomaterial is a factor. For example, seeding human umbilical vein EC (HUVEC) on collagen eliminated anoikis, but the cells still had limited survival in vivo after subcutaneous implantation in an immunodeficient mouse model. On the other hand, when the EC were seeded onto fibronectin (another ECM protein)-coated collagen instead of collagen alone, HUVEC survival was significantly improved (30 – 45% over the first 3 weeks) and the number of HUVEC-lined blood vessels increased by 100% at day 7 and 14 post-implantation [50].

As an alternative to the use of ECM-based substrates, synthetic polymers have also been designed to promote EC adhesion, survival, migration and organization into tube-like structures. MMP-responsive PEG hydrogels with thymosin β4 (a small bioactive peptide with pro-angiogenic properties) and RGD sequences, created a 3D environment conducive for HUVEC attachment and survival, and induced vascular-like network formation in vitro, although the potential of this system to promote EC survival and increased vascularization remains to be
demonstrated in vivo [49]. Apoptosis in the absence of supporting cells is an issue, regardless of the scaffold.

Angiogenesis does not only involve EC, but is the result of a well-coordinated sequence of interactions among EC, supporting cells, and the surrounding ECM. Consequently, co-culture systems with one or multiple support cell types in addition to EC are a closer mimic of the in vivo environment. Several groups reported the development of a functional microcirculation in vivo when using this approach, as reviewed elsewhere [51, 52]. Seminal work by the Jain group demonstrated the formation of blood vessels and their persistence over at least 1 year in vivo when HUVEC and 10T1/2 cells were embedded together in a fibronectin-collagen gel and implanted in a transparent window, immune-compromised mouse model. The constructs prepared with HUVEC alone showed minimal perfusion and no cells were present beyond 60 days. In contrast, when HUVEC and mesenchymal precursor cells were implanted together, the 10T1/2 cells provided an abundant source of mural cells to stabilize the HUVEC-lined blood vessels that were connected to the host vasculature [53].

The West group also exploited the cellular interactions between EC and 10T1/2 cells in a synthetic PEG hydrogel containing cell attachment (RGD) and protease (MMP)-sensitive substrates. The co-cultured cells actively remodeled the hydrogel, secreted their own ECM proteins, and formed tube-like structures that were stable for at least 28 days in vitro [54].

Levenberg and collaborators used a tri-culture system to create a vascularized muscle tissue, with EC, myoblasts and embryonic fibroblasts seeded on porous biodegradable polymer scaffolds composed of 50% poly(L-lactic acid) (PLLA) and 50% polylactic-glycolic acid (PLGA) [55]. In vitro, this tri-culture system spontaneously formed tubular structures within the scaffold, with some of the embryonic fibroblasts becoming SMA-positive over time (suggestive of their differentiation into SMC), and co-localizing with the EC to stabilize the newly formed vascular structures. The skeletal muscle constructs were cultured in vitro for 2 weeks and then implanted either subcutaneously or intramuscularly in immunocompromised mouse and rat animal models. Evaluation of the implants 2 weeks after surgery showed the formation of blood vessels within the construct, with 41% of the blood vessels of human origin being perfused with lectin following lectin injection through the tail vein of the animals, thus confirming their connection to the host vasculature. Control injections with EC alone did not result in vessel formation, again
presumably due to apoptosis. Furthermore, the implanted muscle construct continued to differentiate and mature over time, with implanted myotubes elongating, becoming multinucleated and expressing myogenin, a muscle-specific marker [55].

### 2.2.2 Modular Tissue Engineering

Modular tissue engineering was pioneered by the Sefton group, and provides an alternative platform to creating endothelialized bulk scaffolds. Rather than seeding EC within one large scaffold, modular tissue engineering uses small, EC-coated constructs (modules, ~ 1 mm long and 0.5 mm in diameter, typically made out of collagen) that randomly pack together within a larger chamber to form a three-dimensional construct (Figure 2-4) [56]. Vascular support cells or therapeutic cells of interest (e.g. hepatocytes) can be embedded during fabrication, prior to EC seeding [57]. The network of EC-lined void spaces formed in between the modules is amenable to perfusion, forming a pseudo-vasculature *in vitro* [58]. Furthermore, the small dimension of the modules (less than 400 µm following contraction by EC) is within the diffusional distance of oxygen and consequently, in individual modules even high densities of embedded cells did not experience the hypoxia that is often observed in thick cell constructs [59]. The three-dimensional tissues assembled using this method have an intrinsic vasculature, are scalable, have uniform cell density throughout the construct, and allow for the mixing of different cell types within the modules or by using modules with different types of embedded cells.
Figure 2-4. Modular tissue engineering. Sub-millimeter sized collagen gels containing therapeutic cells are coated with EC and packed together into a larger structure. The network of void spaces formed in between the endothelialized modules is amenable to perfusion.

When collagen modules coated with rat aortic EC (RAEC) were transplanted into the omental pouch of allogeneic rats with immunosuppressive drug treatment, the RAEC started to form blood vessels within the first 7 days [60]. Over time, the blood vessels matured, were supported by endogenous SMC, and anastomosed with the host vasculature as shown by the accumulation of erythrocytes. Although these vessels persisted for at least 60 days, some of them were leaky, as shown by microCT perfusion studies. Moreover, a robust inflammatory response was noted around the implant [60]. When bone marrow-derived MSC were added as support cells to the endothelialized modules, the number of blood vessels did not change, but resulting blood vessels were more stable (i.e. less leaky) compared to vessels formed by RAEC alone [61]. Encapsulated MSC became SMA positive and lined the EC layer suggesting their differentiation into pericyte-like cells. In addition, the presence of MSC decreased the total number of macrophages and shifted macrophages to what was perhaps a more tissue repair phenotype, which further improved RAEC survival and consequently, vascularization [61].
Modular tissue engineering was also tested in islet transplantation and in cardiac tissue engineering [62, 63]. In the case of islet transplantation, vessel density in the implant area was significantly increased, and a trend toward increased islet viability was observed for islets implanted in modules coated with EC as compared to islets implanted in collagen modules without EC at 21 days. However, the endothelialization of the modules did not bring any significant benefit in terms of islet function [62]. In the case of the modular cardiac constructs, RAEC-lined collagen modules supplemented with Matrigel™ were embedded with a neonatal rat heart cell population enriched in cardiomyocytes, and the modules were gathered to form a sheet-like, porous structure. The constructs were electrically responsive and presented cardiac markers at inter-module junctions, suggesting the potential of this approach for cardiac tissue engineering [63].

Materials other than collagen have been used to fabricate the modules. For example, the surface composition of the modules was altered by coating them with other ECM proteins, such as fibronectin [50], or the modules were made mechanically stronger by replacing collagen with a photo-crosslinkable, cell adhesive synthetic polymer, such as poloxamine-polylysine acrylate [64]. In addition, other methods such as micromolding were developed to produce similar modular constructs, demonstrating the ease of module manufacturing and the potential of this technology for scale-up and construction of larger 3D tissues [65].

Although fabrication of modular tissues from different cell types is easily feasible, fabricating modular tissues with specific micro-architectures preserved into larger tissue constructs remains a challenge. The Khademhosseini group introduced a sequential assembly method that allows for better control over the relative spatial arrangement of the different building blocks [66]. The group used PEG micro-gels fabricated by sequential photo-crosslinking through two overlaying masks to generate a concentric design that emulated the EC and SMA vascular layers, with HUVEC encapsulated in the internal layer and SMC encapsulated in the external layer of the micro-gels. Several micro-gels were then reassembled into a tube-like structure upon immersion in mineral oil [66]. However, the cells were immobilized in a non-degradable PEG gel within which cells cannot proliferate or migrate. To make this approach applicable to tissue engineering, other scaffolds with chemical and mechanical properties that better mimic the natural ECM, need to be considered. The remodeling of the initial tissue architecture in vivo also needs to be explored for this system.
2.2.3 Genetically Modified Growth Factor-Producing Cells

Transplanted cells (EC or other) can be genetically modified to overexpress angiogenic factors. This approach obviates some limitations associated with the classical scaffold-GF delivery strategy, such as the short half-life of GF \textit{in vivo} and GF degradation during scaffold manufacturing, while, ideally, the genetically modified cells can continuously produce angiogenic factors at constant rates. However, obtaining pharmacologically useful rates is problematic [67, 68].

The Laurencin group combined EC transplantation with \textit{ex vivo} gene transfer for bone tissue engineering applications [69]. Human adipose-derived mesenchymal stromal cells (adMSC) were isolated and transduced with an adenovirus to express VEGF. The co-transplantation of EC and adMSC overexpressing VEGF resulted in significant vascularization of the three-dimensional PLGA sintered microsphere scaffolds implanted subcutaneously in severe combined immunodeficient (SCID) mice. The scaffolds seeded with VEGF-transduced adMSC and EC resulted in the highest number of blood vessels 2 and 3 weeks post implantation, compared to the blank scaffolds, as well as the scaffolds seeded with either EC or adMSC alone, or with co-cultured EC and non-transduced adMSC. The group speculated that VEGF-transduced adMSC increased migration of endogenous EC into the scaffold and proliferation of transplanted EC, and enhanced the differentiation of EC progenitors present within the adMSC population [69].

Soker and colleagues suspended VEGF-transfected myoblasts (using Lipofectamine) in collagen and injected them subcutaneously into nude mice [70]. While VEGF expression was noted at 1 week but was absent at 3 weeks, increased microvessel density (by vWF staining) was observed. The resulting muscle tissue progressively increased in volume up to 4 weeks post-implant, a volume that was preserved for the duration of the study (8 weeks). This tissue expressed typical muscle markers (desmin and sarcomeric tropomyosin) and lacked connective tissue formation [70].

Yang and colleagues used a biodegradable polymer-DNA nanoparticle system based on poly(β-amino esters) (PBAE) to deliver VEGF cDNA into human MSC and hESC-derived cells (hESdC) [71]. Scaffolds seeded with VEGF-transfected MSC and implanted subcutaneously into athymic mice led to 2 - 4 fold increase in blood vessel density at 2 and 3 weeks after the implant compared to controls (acellular scaffolds, scaffolds seeded with luciferase transfected cells, and
scaffolds seeded with cells transfected with VEGF using Lipofectamine as transfection reagent). A similar trend was noted for the transfected hESdC group. The same study reported enhanced angiogenesis 4 weeks after intramuscular injection of the VEGF-transfected MSC in a mouse ischemic hind limb model, suggesting that transient, non-viral gene delivery of angiogenic factors has great potential for therapeutic angiogenesis applications (at least in hind limb muscle), while providing a safer alternative to viral gene delivery[71].

2.2.4 Scaffold-Free Cell Delivery Approaches

Scaffold-based tissue engineering strategies involve seeding of cells onto scaffolds designed to act as ECM substitutes. An alternative strategy uses the cells' ability to create their own ECM and implant the intact cell-ECM construct [72]. The cell-generated ECM may represent the optimal scaffold, because it recapitulates the native instructive signals to promote tissue growth and maturation. The Okano group cultured cells on temperature-responsive surfaces (cell culture dishes grafted with poly(N-isopropylacrylamide), a temperature-responsive polymer) that allowed cell attachment and proliferation at 37°C, then promoted spontaneous cell detachment without enzymatic digestion when the temperature was lowered below 32°C [73]. Once confluent, the cell sheets were detached from the culture surface and stacked together to build a three-dimensional multilayered tissue. When a sparse layer of HUVEC was inserted in between two myoblast cell sheets, EC sprouted and formed capillary-like structures (Figure 2-5). In a five-sheet construct, EC capillary-like structures connected through all five layers of the construct within 4 days. The five-layer myoblast sheets, with or without HUVEC, were then placed on a fibrin gel and implanted subcutaneously in nude rats. Histological examination showed that HUVEC-free myoblast sheets had no vessels visible within the graft after 7 days. In contrast, the presence of HUVEC resulted in the formation of blood vessels containing endogenous erythrocytes, suggesting these blood vessels were connected to the host vasculature. One can envision that more complex tissues could be made by incorporating micro-fabrication techniques to integrate patterning and micro-texture, as discussed in section 2.7.
Figure 2-5. Cell sheet stacking method. (a) Cells (myoblasts) are cultured on a temperature-responsive culture dish. Once confluent, they are harvested as a cell sheet using a hydrogel-coated plunger and overlaid on top of a second cell sheet. After allowing the two cell sheets to attach, the plunger is lifted and the double-layered construct is placed on top of a third cell sheet. The procedure is repeated several times until a multi-layered construct is formed. (b) To create a vascularized construct, layers of sparsely seeded EC are inserted in between the myoblast cell sheets, using a similar stacking method. [Reprinted from Biomaterials [73], copyright 2010, with permission from Elsevier]

Another scaffold-free approach was introduced by Kelm and collaborators [74]. They used a microtissue self-assembly method to create small diameter tissue-engineered blood vessels. First, a hanging drop method resulted in microtissues (spheroids) of human artery-derived fibroblasts (HAF), with 10,000 cells/drop seeded in an inverted 60-well plate. After 2 days, HUVEC were added to coat the spheroids and the final microtissues were harvested after five additional days of culture. The microtissues were collected in a bioreactor and cultured under either static or dynamic conditions (under pulsatile flow and circumferential mechanical stimulation). Accumulation of capillary-like structures occurred within 14 days. Under dynamic conditions, although a layered tissue structure was observed after 14 days of culture, it was not characteristic
of native vasculature since EC were present inside the wall while SMA-positive fibroblasts lined the lumen [74].

2.3 In Situ Vascularization with Endogenous Cells

Instead of seeding scaffolds with EC prior to implantation, bio-functionalized materials have been used to mobilize endogenous cells such as EPC in situ. This approach differs from the use of GF-containing scaffolds in that it aims to stimulate de novo blood vessel formation through mobilization of vessel forming cells, instead of promoting GF-induced sprouting from existing blood vessels. This alternative in situ strategy exploits the patient's own cells, eliminating any immune response issues.

However, the definition of what constitutes an EPC cell is controversial, since there is no specific marker that is unique to this cell population, and different methods are currently used to isolate, identify and culture these cells. As reviewed by Yoder and collaborators, there are currently several approaches to isolate EPC, which typically involve some form of in vitro cell culture of peripheral monocytes and the distinguishing of particular phenotypes as say early outgrowth or late outgrowth EPC [75, 76]. Various cell marker combinations have been used (CD34⁺AC133⁺KDR⁺ is a common one) [77], but none can specifically and uniquely identify EPC. In one study, two types of EPC were suspended in a mixture of collagen type I and fibronectin, and the gelled constructs were implanted in the flank of NOD-SCID mice. CD31 staining showed donor (human) derived blood vessels were only seen with EPC that had produced colonies of cobblestone shaped cells after in vitro culture. While both EPC expressed EC markers (such as CD31, VWF, UEA-1 and others), the EPC that did not form vessels also expressed monocyte/macrophage markers (CD14, CD115), as well as the hematopoietic marker CD45. The abundance of the two cell populations was also different, with only ~ 0.02 vessel forming EPC colonies/10⁶ MNC, instead of ~ 4 colonies/10⁶ cells of the non-functioning EPC [78]. In a different study, these functioning EPC cells were used to create a construct for bone tissue engineering applications using a porous biphasic calcium phosphate (BCP)/bone morphogenetic protein-2 (BMP-2) scaffold. Vasculogenesis by the EPC was shown 4 weeks after the implant, with donor (human) derived blood vessels containing host red blood cells [27].

During ischemic injury, bone marrow derived ("hematopoietic") EPC have the ability to home to the site of injury and re-endothelialize the injured blood vessels. Designing a system that can
mobilize and capture EPC is an attractive concept, but in addition to further clarifying what cells are actually being mobilized, several key issues must be addressed before its successful implementation. In order to increase the number of circulating EPC, a strategy will need to be devised to mobilize the EPC from the bone marrow, since only very few EPC are thought to be circulating in peripheral blood. Several factors are known to increase the number of circulating EPC, including SDF-1, IL-8, Ang-1, and granulocyte colony-stimulating factor (G-CSF) [79-83]. The main challenge lies in creating a signal that is strong enough to attract EPC and that is above the “background noise” associated with inflammation and tissue injury associated with biomaterial implantation. Moreover, since the implanted biomaterial will not be lined with EC, strategies to inhibit thrombosis and prevent undesirable protein adsorption and cell adhesion will be required.

Alobaid and collaborators evaluated the potential of a nanocomposite biomaterial based on polyhedral oligomeric silsesquioxane nanocages with incorporated RGD sequences to promote capture of circulating EPC in vitro. When peripheral blood mononuclear cells containing 1-2% CD34+ cells were plated onto nanocomposite sheets incorporating the RGD sequence, or on control nanocomposite sheets without the RGD sequence, the RGD-nanocomposite biomaterial showed a significant increase in the number of attached EPC colonies, thus showing the potential of this approach for endothelialization with EPC [84]. However, in order for this method to be applicable in vivo, bioactive sequences other than RGD will need to be considered, since RGD is a universal cell adhesion peptide, and cells more abundant than EPC, such as monocytes, would most likely dominate the surface attachment sites.

SDF-1, a potent chemotactic cytokine, promotes angiogenesis (directly or by modulating the production of GF such as VEGF), and vasculogenesis by modulating cell homing to areas of neovascularization. In a study performed by Simock et al. using a nude rat animal model, a vascularized tissue was first created by placing an arteriovenous loop (AVL) inside a polycarbonate chamber in the groin area, leading to the formation of a highly vascular fibrous tissue (see section 2.4) [85]. SDF-1 was administered postoperatively into the chamber via a catheter connected to a pump, for a total of 1 mg of SDF-1 over 1 week. Human CD34+ cells (collected by leukapheresis from patients after up to 3 days stimulation with G-CSF, sorted by magnetic affinity for CD34, and stored frozen until used) were injected intracardially 6 days after creating the AVL chamber and the tissue was explanted and studied 2 and 7 days following cell
injection. This approach was shown to be successful in delivering cells to the implant site, as the number of fluorescently pre-labeled CD34+ cells was 8 times higher for groups receiving SDF-1 treatment compared to the untreated group. However, these cells did not line the blood vessels at the AVL implant site, and there was no significant difference in the extent of vessel density or vascular maturity in the control versus the SDF-1 treated group. An increase in the number of leukocytes, including neutrophils and macrophages, was also observed when SDF-1 was administered. The concomitant influx of leukocytes and CD34+ cells raised the concern over the non-specificity of EPC homing signals. Moreover, the increased number of leukocytes in the treated group also presents a challenge for further studies to decouple the effects due to the homing of CD34+ cells from the effects potentially caused by the presence of other infiltrating cells[85].

2.4 Scaffold Prevascularization

A relevant concern of scaffold vascularization schemes via GF and/or cell delivery is that the length of time it takes to form a functional vasculature (> 1 week) is often too long maintain viability of cells co-implanted within the scaffold. Consequently, a vasculature may eventually form, but the therapeutic cells within the engineered construct will have already died from lack of oxygen and nutrients in the interim. To address this issue, scaffolds have been prevascularized prior to implantation at the target site in vivo, so the scaffold vessels need only anastomose with the host vasculature. The prevascularization can occur in vitro [55, 86] or in vivo [87, 88]. However, the co-culture of multiple cell types in vitro prior to implantation is not trivial, since different cell types typically have different culture requirements. Different cell culture media, as well as different cell ratios between the co-cultured cells can significantly impact the angiogenic activity and the maintenance of cell phenotypes [47, 55].

Hiscox and colleagues investigated the potential of a prevascularized collagen construct to enhance the survival of transplanted islets [86, 89]. Islets are highly vascularized microtissues. However, the isolation process destroys much of the islet microvasculature, and prompt revascularization and perfusion of transplanted islets is essential to islet survival and function [90]. Freshly isolated microvessel fragments from rat adipose tissue were cultured within collagen gels and were shown to form a vascular network in vitro and rapidly integrated with the host vasculature in vivo [91]. Isolated islets were embedded within a thin collagen gel, which
was then sandwiched between two layers of this prevascularized collagen gel. This construct was implanted subcutaneously into SCID mice, and was shown to improve islet survival and insulin production over the 28 days of the study, compared to islets implanted without the preformed vasculature, which had no viable islets or insulin production [86]. Furthermore, insulin-producing cells were detected and were co-localized with intra-islet EC illustrating an association between islet viability and the presence of intra-islet vasculature [86]. While further in vivo work is required to demonstrate the therapeutic potential of this approach, this prevascularization strategy significantly improved the viability of the implanted cells, and could be used to create prevascularized constructs for other target tissues.

In vitro prevascularization strategies were used for bone tissue engineering by long-term co-culture of EC and osteoblasts seeded on a variety of scaffolds [92, 93]. Co-culture of primary human osteoblasts and human outgrowth EC (OEC) on silk fibroin scaffolds resulted in highly organized prevascular structures and mineralized matrix after 4 weeks in culture. Individual phenotype and functionality of both OEC and osteoblasts needs to be further assessed in this co-culture system since qPCR analysis indicated a decrease in some EC and osteogenic markers [93]. OEC, a subset of mononuclear cells of peripheral blood, can potentially serve as an easily accessible autologous EC source due to their minimally invasive isolation, high expansion potential and differentiated EC phenotype similar to such cells as HUVEC [94]. When implanted subcutaneously, starch – poly(caprolactone) fibre meshes (SPCL) prevascularized for 7 days with OEC-osteoblast cocultures had CD31-positive vessels in the scaffold within 48 hours of implant[95]. The presence of erythrocytes within the donor-cell lined vessels suggested rapid anastomoses of the prevascular structure with the host vasculature. The vessels persisted within the scaffold for the duration of the 14-day study.

In vivo prevascularization strategies use the host as a bioreactor to vascularize the scaffold, prior to implanting the construct at its target site. One strategy uses an AVL and vascular pedicles to generate a vascularized matrix within an enclosing chamber. In this approach, an AVL or a pedicle, which contains both an artery and a vein, is enclosed within a polymer chamber that contains a matrix (such as collagen or Matrigel) or can be left empty [96]. Within the chamber, a microvascular bed is formed within the enclosed matrix, or in the case of empty chambers, new vascularized granulation tissue is formed. Functional cells are generally incorporated once the prevascularized tissue is formed [87, 97]. The newly vascularized tissue is then relocated to the
desired target site or left in place [98]. This technique has been used to create a variety of vascularized tissues including adipose [98, 99], skeletal muscle [100], pancreatic (islet) [87, 97], and cardiac tissues [101, 102]. An example of this strategy utilized prevascularized chambers to support the survival and function of transplanted islets [87, 97]. A chamber containing GF-reduced Matrigel™ and supplemental bFGF was placed around the epigastric pedicle in the groin of a diabetic mouse and formed a highly vascularized adipose tissue [87]. After 21 days, isolated islets were transplanted into the prevascularized chamber and the implant was left in place. After another 3 weeks, there was a significant reduction in blood glucose levels and improved glycemic control as measured by a glucose tolerance test [87]. The improvement in blood glucose regulation was due to the transplanted islets, as the mice returned to the initial hyperglycemic state after the chambers were removed [87, 97].

The highly vascularized omentum is a natural “bioreactor” that has been used to vascularize tissue engineering scaffolds, such as prevascularized cardiac patches for the repair of the myocardial tissue following an infarct [88]. A mixture of neonatal cardiac cells with GF-reduced Matrigel™ and pro-survival and angiogenic factors (insulin-like growth factor 1 (IGF-1), SDF-1, and VEGF) were seeded into an alginate scaffold containing alginate-sulfate groups to enhance binding and sustained release of the GF. After 48 hour in vitro, the patch was transplanted into the omentum and was allowed to mature and remodel. After 7 days in vivo, the patch contained cardiac muscle and a network of perfused blood vessels with associated perivascular cells. The prevascularized cardiac patches were then transplanted onto infarcted rat hearts 7 days after induction of myocardial infarction. The evaluation at 28 days post implant showed that prevascularized cardiac patches were structurally and electrically integrated into the host myocardium. Moreover, the presence of the vascularized cardiac patch induced thicker tissue formation, prevented further dilation of the chamber and ventricular dysfunction. This study provided evidence that prevascularization of the cardiac patch resulted in better grafting of the patch and led to improved cardiac function after myocardial infarction.

2.5 Decellularized Scaffolds

While still having elements of cell transplantation (see section 2.2), decellularized scaffolds take advantage of the preexisting architecture of a tissue that has been rendered acellular. The scaffolds are produced by perfusing an organ with detergents and buffers that effectively remove
all the immunogenic cellular components from the tissue while preserving the tissue architecture and matrix, including the vascular basement membrane. The advantage of using an organ is that there is a vascular inlet and outlet. This allows the organ to be connected to a perfusion system that can distribute the decellularizing agents throughout the entire organ. As the vascular architecture has been preserved, it is hypothesized that the entire vascular tree of the organ, including the capillaries, is re-endothelialized by perfusing the decellularized organ with EC. The scaffold is then seeded with functional cells, such as hepatocytes or cardiomyocytes, to create a vascularized tissue engineering construct.

In 2007, the Mertsching group engineered liver-like tissue using a decellularized porcine jejunal segment [103]. After removing the original cells, the preserved acellular vasculature was seeded with porcine microvascular EC, which were allowed to attach and grow for 1 week. The luminal surface of the decellularized intestine was then seeded with primary porcine hepatocytes suspended in a collagen gel, and the cells grew in multiple layers around the endothelialized capillaries. The three-dimensional liver tissue construct was maintained for 3 weeks in vitro, and the hepatocytes retained the capacity to perform liver specific functions, as measured by urea synthesis [103]. In other studies, decellularized arterial [104] and ureter [105] scaffolds have been used to engineer vascular grafts.

Taylor and colleagues presented a seminal study in which they engineered a bioartificial heart using an intact, decellularized cadaveric heart [106]. The cadaveric heart was decellularized by perfusing a sodium dodecyl sulfate (SDS) - based detergent through the heart chambers for 12 hours. The optimized decellularization scheme fully removed all cellular components from the matrix, while maintaining the micro and macro structures within the heart, including, presumably, a perfusable vasculature. The heart tissue was repopulated with neonatal cardiac cells through intramural injections and was maintained in a specialized bioreactor that simulated the physiological perfusion of the heart and electrical stimulation. The construct was allowed to mature under simulated physiological conditions and after 8 days, developed into a contractile tissue capable of approximately 2 % of the pump action of an adult heart [106]. The group also demonstrated that re-endothelialization of the decellularized whole-heart could be achieved by perfusion of EC into the vascular conduits. After 7 days, EC formed single layers in both larger and smaller vessels and within ventricular cavities. In a similar study, Petersen et al. decellularized adult rat lungs, then repopulated the airways with pulmonary epithelial cells and
the vasculature with EC [107]. The lungs were successfully transplanted, perfused, ventilated for 2 hours, and were shown to be functional and effective at gas exchange [107].

2.6 Angiogenic Biomaterials

In some cases, biomaterials themselves may induce angiogenesis without the addition of bioactive components such as GF or cells. By comparison to other vascularization strategies, using biomaterials as agonists of angiogenesis may be associated with lower cost, ease of storage, and increased versatility (the same biomaterial can be used to induce vascularization in various circumstances). The Sefton group has demonstrated the angiogenic effect of two synthetic methacrylic acid-based copolymers: poly(methacrylic acid –co– methyl methacrylate) (MAA) beads and poly(butyl methacrylate –co– methacrylic acid) (BMA-MAA) scaffolds. The MAA beads (150 – 200 µm diameter; 45 mol% methacrylic acid, 64 mol% methyl methacrylate and 1% ethylene glycol dimethacrylate (EGDMA)) are non-biodegradable, and have a negative surface charge and rough surface topography [108]. In vivo, MAA beads significantly improved the vascularization of skin grafts in rats [108], and diabetic wounds in mice [109], compared to control poly(methyl methacrylate) (PMMA, 100% methyl methacrylate, 150 - 200 µm diameter) beads or no biomaterial. For tissue engineering applications, a porous BMA-MAA scaffold (45 mol% methacrylic acid, 54 mol% n-butyl methacrylate, 1% EGDMA) was developed [110]. BMA-MAA scaffolds were implanted subcutaneously in mice and promoted aggressive tissue penetration. The tissue invading the BMA-MAA scaffolds had a higher microvessel density compared to control poly(butyl methacrylate) (BMA) scaffolds at 21 and 30 days.

The mechanisms of action of MAA beads and BMA-MAA scaffold are unclear. The pro-angiogenic effect of the MAA beads was attributed to the methacrylic acid content, as no increased angiogenesis was observed in response to PMMA beads (same diameter, no methacrylic acid), and in early studies MAA beads and gels with low methacrylic acid content had a reduced effect of angiogenesis. It had been presumed that the anionic charge of MAA beads and BMA-MAA scaffold might bind endogenous cationic GF then release them slowly, extending the duration of the pro-angiogenic signaling. Also, in the context of beads, the surface topography may also play a role; MAA beads are rough while PMMA beads are smooth.
To clarify the biological mechanism of MAA-induced angiogenesis, the group has used quantitative real-time PCR to identify the effect of MAA beads on HUVEC and macrophage-like cells (dTHP-1) [111]. The MAA beads did not modify the gene expression of typical angiogenic genes (such as VEGF), but did modulate the expression of cytokines important in wound healing [111]. Gene expression analysis in MAA-treated cutaneous wounds in diabetic mice showed a significant increase in the amount of Shh (sonic hedgehog; pro-angiogenic morphogen) mRNA at day 4 post-wound, which was followed by increased microvessel density at day 7 [112]. Despite these insights, the mechanism remains unclear.

Based on earlier work by Brauker and collaborators [113], the Ratner group has used spherical, interconnected pores to enhance vascularization in poly(2-hydroxyethyl methacrylate) (pHEMA) scaffolds [114]. They extended the use of this scaffold microtemplating strategy for cardiac tissue engineering by adding parallel channels to encourage the organization of aligned cardiomyocyte bundles in poly(2-hydroxyethyl methacrylate-co-methacrylic acid) (pHEMA-co-MAA) scaffolds [115]. Histological examination demonstrated that the scaffold pores and the surrounding interface were filled with vascular granulation tissue, and that an optimal pore size of 30 – 40 \( \mu m \) maximized neovascularization while minimizing fibrous encapsulation. Other groups have developed self-assembling, peptide-based nanofiber scaffolds (reviewed by [116]) that enhance EC survival and angiogenesis, despite using peptide sequences that are not naturally occurring and are presumed to be biological inactive [116-120]. Narmonova and colleagues demonstrated regulation of EC activation and angiogenesis \textit{in vivo} using injectable RAD16-II (AcN-RARADADARADADA-CNH\textsuperscript{2}) peptide nanofibers [117, 121]. In a diabetic wound healing mouse model, wound treatment with RAD16-II nanofibers significantly enhanced angiogenesis and improved healing at day 7 post-wounding [117]. The presence of nanofibers resulted in significant increases of VEGF protein levels in the wound tissue. Biotinylated lectin injections demonstrated that most of the vessels formed within the granulation tissue were anastomosed with the host vasculature by day 7. Addition of \( \beta1 \) and \( \beta3 \) integrin inhibitors completely abrogated both \textit{in vitro} and \textit{in vivo} effects caused by the nanofibers suggesting that nanofiber-induced angiogenesis was, at least in part, mediated by integrins despite the low-affinity binding kinetics of the integrin-nanofiber interactions [117].

Nanofiber gels formed by self-assembled peptide amphiphiles (PA) have also demonstrated angiogenic activity \textit{in vivo} [118-120]. PA consist of a hydrophobic alkyl tail and a hydrophilic
peptide head, which contains a sequence to promote self-assembly and bioactive domains that can be customized for specific applications [118-120]. Heparin-binding PA (HBPA) were engineered to self-assemble in the presence of heparan sulfate or heparan sulfate-like glycosaminoglycans (HSGAG). These HBPA-HS nanostructures were originally designed to stimulate extensive vascularization by binding minute amounts of VEGF and bFGF [119]. In a serendipitous discovery during an in vivo biocompatibility study, the control nanofibers (without the addition of any GF) were found to promote angiogenesis and the formation of vascularized tissue in both murine subcutaneous implant and dorsal skinfold chamber models, in the absence of a fibrotic response [118]. However, by day 60 macrophages had completely degraded the nanofiber gel and the neovasculature had regressed. The observed vascularization at earlier time points (day 10 and 30) was not noted in nanofiber gels prepared without heparan sulfate or for only heparan sulfate treatment, suggesting that the angiogenic effect was attributed specifically to the presence of heparan sulfate within the nanofiber gel. It is possible that the heparan sulfate presented on the surface of HBPA-HS binds, stabilizes and gradually releases GF secreted by infiltrating endogenous cells, thereby enhancing angiogenesis [118].

Another example of an angiogenic biomaterial is a PA nanostructure designed to mimic the activity of VEGF [120]. The PA incorporated a synthetic oligopeptide that was designed to mimic the α-helical receptor-binding domain of native VEGF, and was capable of binding and inducing the phosphorylation of the VEGF receptor [120]. During in vitro culture, the VEGF-mimetic PA (VEGF-PA) enhanced EC proliferation, survival and migration compared to untreated control and to a higher degree than recombinant VEGF. In a chicken chorioallantoic membrane assay, VEGF-PA nanogels elicited a strong angiogenic response, with a > 2x increase in microvessel density at 3 days post implantation, compared to the initial blood vessel density (day 0). To evaluate the therapeutic efficacy of the VEGF-mimetic nanofibers, they were injected into the ischemic muscle 3 days following the induction of critical hind-limb ischemia. The nanofibers decreased tissue necrosis, and improved tissue perfusion, functional recovery, and limb salvage at day 21 and 28 compared to controls (VEGF peptide, mutant PA and saline), and the nanofibers performed as well or better than the administration of recombinant VEGF protein. The most significantly affected measure was the increased CD31-positive capillary density that VEGF-PA induced in the ischemic muscle, compared to a 20-μg bolus injection of VEGF_{165} and a saline control [120].
2.7 Microfabrication Methods

Many of the strategies for creating vascularized scaffolds rely on manipulating the biology of vessel formation, using angiogenic factors, transplanted cells or bioreactors to induce vessel formation within the scaffold. Another vascularization paradigm focuses on generating predefined, hierarchical pseudo-vasculatures in vitro, with a high degree of spatial control using technologies such as MicroElectroMechanical Systems (MEMS) and cell/protein printing. These approaches allow the vascular network to be designed, ensuring optimal blood flow and mass transport characteristics [122, 123].

The Vacanti group pioneered the concept of engineering a vasculature in vitro using micromachining technologies to create a blueprint for a microvascular network within the scaffold [124]. While early experiments aimed at growing spatially patterned cell sheets that were layered to form a three-dimensional construct [124], the group eventually focused on forming polymer films from non-degradable poly(dimethyl siloxane) (PDMS) [125, 126] and degradable poly(glycerol sebacate) (PGS)[122]. These contained branching, vessel-like channels throughout the construct that were partially lined with EC to form a pseudo-vasculature in vitro. Master molds of the vascular blueprint were created using MEMS, then used to cast the PDMS or PGS films [125]. The patterned films were then bonded to flat films, to create an enclosed, perfusable microfluidics network [122] (Figure 2-6). The constructs were either seeded under static conditions with EC directly, or were coated with cell adhesive molecules prior to seeding. Once attached, the cells were exposed to flow for 2 to 4 weeks and formed confluent endothelial monolayers that covered some sections of the microchannels [122, 126].

Zheng et al. used a microfabrication technique to create a scaffold with a well-defined microchannel network capable of modulating scaffold vascularization in vivo [127]. A PDMS micromold with patterned microstructures (cylindrical pillars with 100 to 400 µm diameters and connected slots with 100 µm width and 600 µm length) was assembled within a larger PDMS macromold and used to cast 2% collagen microstructured tissue templates. The microstructured collagen templates were implanted subcutaneously and the void spaces within the collagen gels were rapidly infiltrated with endogenous cells (compared to the 2% collagen volume). After 14 days, vascularized host tissue had invaded the patterned pores, with vessels running along the length of the pores. The large vessels (20 – 30 µm diameter) surrounded by SMA-positive cells.
and containing erythrocytes were found along the axis of the pores, while smaller capillaries were found penetrating the deepest regions of the pores and the lateral branches of invaded tissue [127]. While over time, the collagen scaffold would likely undergo remodeling and degradation, at early time points it demonstrated the potential to guide the initial cellular invasion and vascularization.

**Figure 2-6.** Microfabrication of capillary networks. (a) A silicon micromold coated with sucrose was first used to transfer a capillary network pattern to a PGS layer. (b) The patterned PGS layer was then placed on top of a flat PGS layer and the two were bonded together to close the opened capillary channels in the patterned PGS. (c) The PGS microchannels were seeded with HUVEC and a confluent layer of EC was observed in some sections of the capillaries within the first 14 days of culture [Reprinted from Tissue Eng Part A [122], copyright 2005, with permission from Mary Ann Liebert, Inc.].

Micropatterning techniques can also be used to pattern surfaces with cell instructive ligands, to spatially direct a desired cell response. The West group used this approach to regulate the formation of capillary-like structures [128]. The surface of nonadhesive PEGDA hydrogels was patterned with a cell adhesive ligand, Arg-Gly-Asp-Ser (RGDS), in stripes of varying width and ligand concentration. Both stripe width and ligand concentration (µg/cm²) modulated the endothelial morphogenesis, with the EC forming cord-like structures on 50 µm-wide stripes with a ligand concentration of 20µg/cm². This response was inhibited on wider stripes, and stripes containing a higher concentration of ligand, suggesting that both geometrical and biochemical cues were controlling EC morphogenesis [128]. A micropatterning technique was also used to direct the fate of progenitor cells *in vitro* [129]. Angiogenic progenitors obtained from
differentiating embryoid bodies were seeded on 100 μm-wide lanes micropatterned with either collagen alone or collagen displaying immobilized VEGF. Endothelial progenitors attached to the VEGF-collagen surface differentiated into mainly EC, while cells grown on collagen surfaces differentiated mainly into SMC, resulting in the formation of EC stripes lined with SMC, roughly mimicking a blood vessel [129].

While the technology is still in its infancy, three-dimensional bioprinting may offer an interesting means to creating thick tissue engineered constructs with pre-defined architecture. Nakamura and colleagues used an inkjet-based platform capable of printing cells and biomolecules, layer by layer to form three-dimensional constructs (reviewed by [130]). However, to date no efforts have been made to attempt to print the complex three-dimensional architecture that would be required for a vascular network.

3 Limitations and the Road Ahead

Much effort has been devoted in the last decade to develop functional, vascularized tissue engineered constructs. Despite significant progress, especially in terms of expanding our current understanding of the design requirements for creating a functional vasculature within the construct, there is still much more required. The vascularization process is typically too slow to maintain the viability of the therapeutic cells of interest, and only tissues with low vascularization requirements, such as thin skin tissue or cartilage, are currently available clinically.

Most of the current vascularization approaches have proven successful in inducing blood vessel formation in vivo, but many of these blood vessels are not fully functional (leaky or not connected to the host vasculature), and many regress over time. Where some of these approaches have resulted in a functional, persistent blood vessel network, the time required to achieve such functional vascularization is still too long to be clinically relevant (several weeks for a millimeter sized implant) [131]. Without an immediate vascular supply, the majority of the therapeutic cells (ex. liver cells, islets) within the construct will not survive. Going forward, much effort will need to be directed towards accelerating the formation of a perfused (i.e. anastomosed) vascular network within the construct and ensuring the new vasculature matures and persists.
Another key lesson learned from previous attempts to generate vascularized tissues is that any vascular structures pre-formed *in vitro* prior to implantation will most likely be remodelled *in vivo*. For example, when EC-coated modular constructs were implanted in mouse or rat animal models, the EC migrated off the surface of the modules and formed blood vessels in the channels between the modules instead of staying on the modules and providing non-thrombogenic surface to the channels randomly formed between the modules, as was originally expected [56]. The implanted cells were presumed to be the drivers of the remodelling process, with endogenous cells invading and remodelling the construct [60-62, 132]. Finding ways to predict and guide the remodelling process *in vivo* will likely emerge as a key feature for the next generation of vascularized scaffolds, and the focus of vascularization research will likely switch from building pre-vascularised structures to controlling how the vascular structures will be remodelled and integrated with the host vasculature *in vivo*.

# 4 Summary

The tissue engineered constructs that are being developed currently have limited clinical usefulness due to the lack of inherent vasculature, which critically constrains the construct size. A vascular supply is needed to maintain the viability of a clinically relevant number of therapeutic cells (in clinically relevant tissue densities) in order to achieve a functional benefit upon transplantation. A broad range of vascularization approaches are being explored to promote blood vessel formation within the construct. Initial studies using single GF delivery from tissue engineering scaffolds were successful in inducing blood vessel formation at early time points, but failed to produce a lasting vascular network. Hence, it was reasoned that the use of multiple GF, with different functions in angiogenesis, would be more suitable to recapitulate all stages of vessel formation. Multiple GF delivery improved vessel maturation and functionality, although issues still remain in terms of delivering physiological doses of GF, in a spatio-temporal controlled manner. Scaffolds are also used to deliver (transplant) cells to a target site, or to mobilize a specific cell type *in situ*. Transplanted or mobilized vascular cells contribute to the formation of a vascular network both directly, by re-assembling into vessel-like structures, as well as indirectly, by secreting various GF and driving the construct remodelling process and host response. However, the time frame required to develop a perfusable, functional blood vessel network within the construct is on the order of weeks, which significantly hinders the clinical
benefit of this approach since most functional cells will not be able to survive without a vascular supply for such an extended period of time.

To circumvent this last issue, scaffold prevascularization approaches have been developed. The scaffold can be seeded with vascular cells and cultured in vitro to create tube-like structures within the scaffold prior to implantation (in vitro prevascularization). Alternatively, avascular scaffolds can be implanted in a highly vascularized site in vivo (such as the omentum) to facilitate construct vascularization, followed by relocation and connection to the host vasculature at the desired target site once the construct has been fully vascularized. Decellularized scaffolds have been used to provide a natural template for recreating the vascular tree architecture. Tissues or whole organs are first decellularized (while preserving the tissue architecture), and then repopulated with new cells (EC to repopulate the vascular tree and the desired functional cell populations). Another approach to induce vascularization is the use of biomaterials that act as angiogenic agonists. The use of angiogenic biomaterials provides a simple, cost effective and versatile method of inducing angiogenesis, as the same biomaterial can potentially be used to induce vascularization of various tissues with minimal modification. Finally, microfabrication techniques are currently being explored to create predefined, hierarchically organised vascular structures in vitro. The remodelling that occurs upon implantation of these structures in vivo remains to be further clarified.

Overall, significant progress has been made in expanding our understanding of the design criteria for fabricating vascularized tissue engineered constructs. However, there is still a limited number of clinically relevant tissue engineered constructs available, and further work is required in resolving the issue of poor tissue construct vascularity. Moving forward, vascularization strategies need focus on accelerating the vascularization process and on improving the functionality of the vascular network formed.

5 References


Chapter 3
Literature Review: Modular Approach in Tissue Engineering

1 Introduction

Modular tissue engineering was first introduced by the Sefton lab as a means of building intrinsically vascularized tissue constructs [1, 2]. Instead of using the conventional method of seeding a “large” porous scaffold with cells, the group proposed seeding smaller constructs, which they named ‘modules’, and packing these modules together to obtain a larger tissue construct. With this method of assembly, empty channels form among the modules upon packing of several modules, and these channels are interconnected to provide the porosity that is otherwise generated by starting with a conventional scaffold. Since these modules were covered with endothelial cells (EC) prior to assembly, the channels were lined with EC and amenable to perfusion (with blood), similarly to a vascular network: hence the intrinsically vascularized nature of the modular approach. Vascular supporting cells (such as smooth muscle cells, SMC [3], or mesenchymal stromal cells, MSC [4]) or therapeutic cells of interest (cardiomyocytes [5], islets [6], or others.) can be embedded inside the modules. Modules containing different supporting or therapeutic cells can also be mixed together in a desirable ratio to build more complex functional tissue structures (Figure 3-1).

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Figure 3-1. Schematic of modular tissue engineering with endothelialized building blocks. Different modular units that contain different cell types can be made separately and then mixed together in various ratios to form more complex structures. These complex structures can then be used for *in vitro* modeling of tissues (under static or flow conditions) or implanted. There are two basic types of modular units. Functional modules contain the therapeutic cells or the cells of interest for study. Support modules contain cell types (such as MSC) that support the function of the therapeutic cells or improve the vascularity of the implanted construct.

The modular approach, when first proposed by the Sefton group, aimed to address the lack of an internal vascular network, a key issue in tissue engineering. A vascular supply is required to supply nutrients to the cells, to remove waste products, for gas exchange, and for circulation of signaling molecules. Due to diffusion limitations within tissues, all cells must be within 100 – 200 µm of a blood vessel [7]. Therefore, from a tissue engineering perspective, an immediate vascular supply is required in order to preserve cell viability and tissue function at clinically relevant sizes. Compared to the conventional approach of seeding cells on top of scaffolds and relying mostly on host cell infiltration to remodel and vascularize the scaffold (slow process), the modular approach has the advantage of a ‘built-in’ vascular network, with vascular channels pre-formed by design between the modules. The small size of the modules (~ 2 mm long and 0.6 mm in diameter starting size which is typically further contracted by embedded functional cells and EC) also ensures that the cells embedded in any individual module will not experience hypoxia [8]. Hypoxia is typical in thick tissue constructs fabricated using the conventional approach.
Other advantages of modular tissue engineering are that the modular design allows for uniform cell seeding within the construct (by controlling the cell density within each of the individual modular building blocks), as well as controlled mixing of different cell populations (by mixing together modules encapsulating different functional cells, in different ratios), and this approach is scalable (increasingly larger tissue constructs can be made by assembling increasing numbers of modular building blocks). The modules can also be easing their utility in some situations. Moreover, from a biomimetic standpoint, the modular approach recapitulates the “design” of native tissues and organs, which are often composed of repeating functional units. For example pancreatic islets, hepatic lobules, muscle fibers etc. each consist of repeating functional units to form larger structures. Many research groups have expanded the use of modular tissue engineering from what was initially envisioned as a method of building intrinsically vascularized and scalable constructs, to a method of controlling tissue architecture and building larger tissue constructs from the ‘bottom-up’ [9-14], thereby expanding the definition of a “module” (Figure 3-2).
Figure 3-2. Approaches to modular tissue engineering. A) Types of modular units (schematic cross-section) that are currently being used in tissue engineering. Advantages and disadvantages of each method are listed. B) The classes of materials that are being used for scaffolds in modular tissue engineering with advantages and disadvantages.
This chapter focuses on the different variations of the modular tissue engineering approach that are currently being explored. Figure 3-2 illustrates some of these current trends. Modules containing encapsulated cells and fabricated using different natural or synthetic biomaterials (section 2) are used to either create intrinsically vascularised tissue constructs (section 3), or as building blocks for generating larger tissue constructs in vitro, sometimes through controlled assembly of the modules with the goal of controlling the architecture of the final assembled construct (section 4). Modules integrated in microfluidics systems are being developed as in vitro tissue models for studying cell interactions, for drug testing, and for performing different bioassays in a controlled, three-dimensional, perfusable cell culture environment, with multiple cell types co-cultured inside the device (section 5).

2 Materials Used to Fabricate the Modular Building Blocks

Both natural extracellular matrix (ECM) components and synthetic polymers are being used to make modular tissue engineered constructs. Collagen (an ECM component) and poly(ethylene glycol) (PEG)-based synthetic polymers are the most widely used.

2.1 ECM-based Materials

The Sefton group fabricated cylindrical collagen modules by gelling a neutralised collagen type I solution (with or without embedded functional cells) at 37°C inside a polyethylene tubing and then cutting the tubing into small pieces using an automatic custom-made tube cutter (Figure 3-3A). The resulting cylindrical pieces of collagen were separated from the tubing by vortexing and coated with endothelial cells through a combination of static and dynamic seeding. These endothelialized modules were then randomly packed together to obtain larger, intrinsically vascularized modular tissue constructs [1, 2, 15]. In a newer fabrication method, the collagen liquid was sheared into liquid modules upon contact with a perpendicular stream of pressurized air, resulting in individual collagen modules separated by air spaces inside the polyethylene tubing. After gelation of the collagen inside the tubing, the modules were simply expelled using a syringe, thus eliminating the need for cutting of the tubing, which is time consuming and therefore less practical for larger batches [16] (Figure 3-3B). A combination of ECM materials was also used, with fibronectin coating of collagen modules shown to improve the survival of EC
upon subcutaneous implantation in a severe combined immunodeficient/beige (SCID/Bg) animal model [17].

A) Automated cutter method of making modules

Collagen with or without embedded cells is gelled in a length of poly(ethylene) tubing. The tubing is then cut into small (~2 mm in length) segments and collected in a 50 ml tube containing medium. After 1 hr of incubation at 37°C the tube is vortexed separating the tubing and the modules. The modules are collected and coated with EC.

B) Air plug method of making modules

Collagen with or without embedded cells are generated and loaded in the poly(ethylene) tubing. The collagen is gelled at 37°C and the modules are then forced out of the tubing using a syringe in a tube containing medium. The modules are then coated with EC.

**Figure 3-3.** Methods of making endothelialized modules. A) Automated cutter method. Collagen with or without embedded cells is gelled in a length of poly(ethylene) tubing. The tubing is then cut into small (~2 mm in length) segments and collected in a 50 ml tube containing medium. After 1 hr of incubation at 37°C the tube is vortexed separating the tubing and the modules. The modules are collected and coated with EC. B) Air plug method. Using a T junction, droplets of the collagen with or without embedded cells are generated and loaded in the poly(ethylene) tubing. The collagen is gelled at 37°C and the modules are then forced out of the tubing using a syringe in a tube containing medium. The modules are then coated with EC.
Other groups use collagen to fabricate modules. Using a soft-lithography method, the Whitesides group fabricated collagen microgels inside a polydimethylsiloxane (PDMS) mold. The microgels had various shapes (circular, square, cross) depending on the shape of the mold, and contained encapsulated NIH/3T3 fibroblasts, HepG2 liver cells, or primary rat cardiomyocytes. Matrigel™ and agarose modules were also fabricated using the same method [18]. The Demirci group used a droplet-based system, with SMC suspended in collagen and deposited in droplets under precise spatial control [19].

Gelatin, a denatured form of collagen, has also been used to build modular constructs, either in the form of gelatin microcarriers, or as photocrosslinkable gelatin derivatives. In one study, gelatin microcarriers seeded with fibroblasts were assembled into a three-dimensional dermal tissue [20]. In a different study, human amniotic mesenchymal stem cells were seeded onto gelatin microcarriers, underwent osteogenic differentiation, and assembled into a bone construct in a perfusion chamber [21]. Gelatin hydrogels were fabricated by synthesizing a gelatin methacrylate derivative and exposing the synthesized polymer solution to UV light (photocrosslinking) to form a gel [22]. Similarly, gelatin methacrylate mechanically reinforced with carbon nanotubes, or mixed with pullulan methacrylate (a fungal polysaccharide), have been photocrosslinked into microgel building blocks [23, 24].

Another natural material, hyaluronic acid, has also been used to fabricate modules. Cells (NIH/3T3 fibroblasts or murine embryonic stem cells R1 strain) were mixed with a methacrylate derivative of hyaluronic acid (MeHA) and deposited in a PDMS mold [25]. After exposure to UV light and gelling of the polymer solution, hydrogel modules of different shapes and sizes (as defined by the micropatterns of the PDMS mold) were collected from the mold. Although not done here, these hydrogels could presumably be assembled together to build a larger modular tissue. However, as with all systems that rely on photocrosslinking for formation of the gels, the exposure time to UV light, as well as the concentration of the photoinitiator required to initiate photocrosslinking need to be minimized in order to preserve cell viability. This is often not a trivial requirement, since short UV exposure times and low photoinitiator concentrations also make gelling of the hydrogels difficult, leading to soft hydrogels that are difficult to manipulate, let alone assemble into a larger porous structure without collapsing.
2.2 PEG-based Materials

Modular tissue engineering is also amenable to the use of purely synthetic materials that can form hydrogels. PEG is a synthetic polymer commonly used in tissue engineering to make hydrogel scaffolds. Specifically in modular tissue engineering, PEG has been used extensively to fabricate hydrogel building blocks of different sizes and shapes (circles, squares, lock-and-key structures, saw-shaped microgels etc.), that can then be assembled into larger structures with controlled architecture [26-30]. PEG-based hydrogels are typically obtained through placement under a photomask of a solution of PEG polymer functionalized with methacrylate or acrylate groups, and exposure to UV light for photocrosslinking. By varying the size and shape of the photomask and the thickness of the PEG layer, hydrogels of various sizes and shapes are obtained. These hydrogel building blocks are then assembled in a random or controlled manner using various techniques, such as acoustic assembly, thermodynamically-driven assembly in multiphase liquid systems etc. (see section 4 of this chapter) [29-31]. The assembled final structures can be further stabilized through a secondary UV crosslinking step [29, 30].

PEG-based hydrogel systems offer tremendous opportunities in terms of controlling the initial architecture of the building blocks. However, including cells in these PEG-based gels can be difficult due to the toxicity of the photoinitiator at the concentration and UV exposure time required to form mechanically strong gels through photocrosslinking. Unlike PEG, ECM materials such as collagen do not require such chemical crosslinking to form gels; the process of making collagen gels is entirely cell compatible, as collagen gels are formed by simply incubating a neutralised collagen solution at 37°C, the standard cell culture temperature. Moreover, PEG based systems generally lack the biological properties required to build functional tissues (at least without further modification), starting with their low cell binding and non-degradable properties. They are therefore generally limited to proof-of-concept studies, with a focus on controlling the initial architecture, but not the long-term functionality of the assembled tissue construct; most of these studies only investigated the short-term (i.e. typically in the order of a few hours to a few days) survival of cells. Mixing the PEG-based polymer solution with cell compatible ECM components, such as collagen, can be useful for maintaining cell viability in these photocrosslinkable hydrogels. There are, of course, many reports in the tissue engineering and biomaterials literature (although not necessarily in the context of modular tissue engineering) describing PEG-based scaffolds that have been chemically or physically
modified to include cell-responsive components (cell attachment, enzymatic degradation etc.). For example, in one study reporting the fabrication of 3D hepatic tissues from building blocks, primary hepatocytes were successfully cultured on PEG-based hydrogels functionalised with RGD sequences, and assembled into a 3D construct. The hepatocytes maintained liver-specific functions over 12 days in culture *in vitro* under perfusion [32].

Poloxamine (a four-arm block co-polymer of poly(propylene oxide) and poly(ethylene oxide) with properties similar to PEG) has also been used in modular tissue engineering. Methacrylate groups were chemically added to poloxamine and the synthesized polymer was gelled inside a polyethylene tubing by exposure to UV light [33, 34]. To improve cell attachment and viability of cells embedded within or seeded onto poloxamine modules, poloxamine was mixed with collagen [33, 34], chemically modified to introduce positively charged groups into the scaffold biomaterial [35, 36], chemically linked to polylysine peptide chains [37], or poloxamine modules were coated with laminin, another ECM protein [38]. Since the ability of the cells to remodel the tissue engineered construct is also an important design factor, remodelable poloxamine-based modules were also fabricated by synthesizing a lactoyl-poloxamine methacrylate derivative [39]. In a related approach, PEG methacrylate was mixed with gelatin methacrylate and photocrosslinked together to obtain microgels that combined the favorable mechanical properties of PEG and the cell attachment and degradable properties of gelatin [40].

### 3 Intrinsically Vascularized Tissue Constructs

When first presented by McGuigan and Sefton, modular tissue engineering was envisioned as a method to create intrinsically vascularized tissue constructs, with a quiescent layer of EC lining the module surface and creating nonthrombogenic vessel-like channels between the modules. Modules coated with human umbilical vein endothelial cells (HUVEC) were indeed nonthrombogenic *in vitro*, which is an important requirement for implantable vascular structures [41, 42]. When exposed to whole blood, the endothelialized modules showed reduced platelet activation (reduced microparticle formation) and platelet-leukocyte associations (platelet positive events in a leukocyte gated sample by flow cytometry) compared to the non EC coated collagen modules [41]. The EC coated modules also delayed clotting when exposed to whole blood on a rocking platform, and there was limited fibrin and platelet deposition on the surface of the modules under static conditions [41]. The endothelialized constructs also enabled whole blood
perfusion, with limited platelet loss/deposition in the case of the endothelialized modules [41]. HUVEC on collagen modules also expressed low levels of tissue factor (TF) and high levels of thrombomodulin (TM), suggestive again of their nonthrombogenic phenotype [42]. While nonthrombogenicity was confirmed in vitro, in vivo studies showed that endothelial cells did not remain attached to the collagen gel and in a quiescent state. Rather they drove a remodeling process which resulted in a chimeric vasculature that was different than what was originally envisaged (see section 3.1).

3.1 In Vivo Fate of Endothelialized Modules

In vivo, host response and remodelling of the modular construct become decisive factors for the outcome of the implant. Like all tissue constructs, biofabrication of implantable modular tissues should include in vivo remodelling as a fundamental design parameter. The number of in vivo reports using the modular tissue engineering approach is however still limited, at least by comparison to the larger number of in vitro studies.

In one of the first in vivo modular tissue engineering studies by Gupta et al. [43], collagen modules (without embedded cells) coated with HUVEC were implanted in the omental pouch of nude rats, with temporary depletion of macrophages (using clodronate liposomes injections) to improve EC survival. Histology images showed that the modules randomly assembled and the channels formed between the modules persisted for at least 14 days. However, the HUVEC did not remain on the surface of the modules, as initially predicted, but instead migrated off the modules and within 7 days after implantation, started to form primitive blood vessels in the area between the modules. There were signs of incipient vascularization of the tissue, with host rat EC, SMC and red blood cells investing these primitive blood vessels over the 14 day time course of the experiment. However, the EC survival and blood vessel formation were still limited, presumably due to the inflammatory and immune host response directed against the implanted human cells in this only partially immune compromised animal model; clodronate had only a temporary depleting effect on invading macrophages.

Overall, this first in vivo modular tissue engineering study brought attention to the importance of properly modulating the remodeling and host response if the end goal is to obtain a vascularized tissue construct. More attention to modulating the remodeling process in vivo is required and perhaps less on the initial shape and architecture of the constructs pre-fabricated in vitro, which
will most likely eventually be remodeled *in vivo*. For example, the degree of confluence or quiescence of the attached EC appears to be less important than it was originally deemed. Creating a construct that will drive a favorable remodeling response *in vivo* is perhaps the key, at least for applications where the end goal is not an *in vitro* tissue model.

In a subsequent study, collagen modules coated with rat aortic EC (RAEC) were implanted in the omental pouch of immunosuppressed (allogeneic) Sprague-Dawley rats [44]. No T cells (TCR αβ+) were observed in the implant area, and the inflammatory cell numbers (CD68+) were reduced in the drug immunosuppressed animals compared to the untreated control animals. Similarly to the previous study, the authors reported the remodeling of the modular implants. Initially (day 3 after the implant), the RAEC were still seen on the surface of the modules. At later time points (day 7), the RAEC had migrated off the surface of the modules and had started forming primitive blood vessels. At even later time points (day 14, 21 and 60), the blood vessels became invested with SMC and erythrocytes (suggestive of connection to the host vasculature). Both host and donor derived EC formed the endothelial cell lining of these blood vessels. Figure 3-4A shows a representative picture of the implant 21 days after surgery.

MicroComputed Tomography (microCT) studies performed on the whole omental pouch at 21 days (Figure 3-4B) after the surgery proved that the vessels were perfusable and connected to the host vasculature, although the vessels were somewhat leaky (in this case without added bone marrow derived MSC, bmMSC). The authors attributed the formation and (partial) stabilization of the blood vessels formed to the ability of the transplanted endothelial cells to elicit a beneficial host response that involved macrophages, fibroblasts, SMC and host EC, and which eventually led to the remodeling of the implant and the formation of a perfusable vasculature, anastomosed to the host vascular system. In a later study using the same animal model, the authors embedded bmMSC inside the RAEC-coated collagen modules, and succeeded in creating a less leaky, more mature vasculature, with the hematoma almost fully resolved by 21 days [4] (Figure 3-4C).

They reported significant remodeling of the pre-fabricated modular constructs in this case as well. At later time points (day 14 and day 21) a high percentage of the implanted bmMSC were associated with the blood vessels formed in the implant area, forming the smooth muscle layer of these blood vessels. This study also showed that the bmMSC had a significant effect on the host macrophage response, with a decrease in the total number of macrophages (CD68+) apparent at
day 14 and at day 21, and most importantly, with an increase in the number of “pro-angiogenic” macrophages (CD163+) inside the implant area for modules embedded with bmMSC and coated with EC compared to modules without bmMSC. Thus, the addition of the bmMSC seemed to have a beneficial effect in terms of modulating the host response to the implanted modular tissue and creating a less leaky vasculature.

In a SCID/Bg mouse subcutaneous model with HUVEC coated modules, modules were fabricated either using collagen alone, or coating the collagen modules with fibronectin before seeding with HUVEC [17]. Fibronectin coated modules decreased apoptosis of implanted HUVEC by nearly 40% (TUNEL staining) at day 3 after the surgery. This resulted in a nearly 2 fold increase in donor derived (UEA-1+) blood vessel formation at day 7 and day 14 post surgery, although these differences did not persist at later time points (day 21).

HUVEC readily undergo apoptosis upon implantation even in SCID/Bg mice [45] and this needs to be minimized (e.g. by using MSC or by genetic manipulation, [45, 46]) to drive vascular remodeling.

Adipose derived MSC (adMSC) were embedded inside collagen modules, coated with human microvascular endothelial cells (HMEC), and implanted subcutaneously in a SCID/Bg mouse model [47]. In the absence of adMSC, there was limited HMEC survival past 14 days, whereas with the embedded adMSC, HMEC derived primitive vessels formed as early as day 3, matured over time, connected to the host vasculature, and were still present in the implant area 90 days after surgery.

In turn, this early vascularization of the tissue was beneficial for fat development over time, with the implanted adMSC surviving (in the vascularized environment) and presumably differentiating over time into mature adipocytes. Approximately 60% of the implant showed fat accumulation (Oil Red O+) by 90 days (the duration of the study) when HMEC were included (Figure 3-4D). With modules embedded with adMSC but not coated with HMEC, there was limited fat accumulation over time, presumably due to the lack of early tissue vascularization and consequently, early adMSC death. This study shows the advantages of this modular approach; while the adMSC initially contributed to HMEC survival and blood vessel formation (presumably due to adMSC secreted pro-angiogenic and pro-survival factors), this early tissue
vascularization proved to be beneficial at later time points for adMSC survival and differentiation into what appears to be fat.

The creation of functional tissues using the modular tissue engineering approach has also been explored in vivo. An in vivo study investigated islet transplantation in the context of modular tissue engineering, using both syngeneic and allogeneic immunosuppressed diabetic rat omental pouch models [6]. In this case, the coating of the modules with EC resulted in increased blood vessel formation compared to transplantation of free islets or non endothelialized islet modules. However, this increase in blood vessel density did not result in improved islet function (as determined through blood glucose and insulin measurements), presumably due to delayed blood vessel maturation and lack of initial functionality.

In an in vivo cardiac tissue engineering study, collagen modules (with or without Matrigel™) containing a rat neonatal cardiomyocyte-enriched cell population and coated with rat cardiac EC (both isolated from GFP transgenic animals) were implanted in syngeneic Lewis rats [48]. The modules were injected in the peri-infarct region of the heart 7 days after inducing myocardial infarction, and explanted and analysed by histology 3 weeks after implantation. Donor-derived cardiomyocyte bundles (GFP+/myosin heavy chain (MHC) +) were observed at the implant site throughout the collagen modules, for both EC coated and non EC coated modules. EC coating of the collagen modules (without Matrigel™) was beneficial in terms of increasing total blood vessel density (donor and host derived, CD31+) in the implant area compared to the cardiomyocyte-only implants (without EC coating). Some of these blood vessels contained erythrocytes, suggestive of connection to the host vasculature. Moreover, most of the blood vessels formed following implantation of the EC coated modules contained donor-derived EC (GFP+). However, this increase in vascularization for the EC coated modules did not lead to improved cardiomyocyte survival compared to the cardiomyocyte only implants, at least not 3 weeks post implantation (time point included in this study).

In contrast to the collagen modules, modules made of a mixture of collagen and Matrigel™ performed less well in this animal model. The Matrigel™ containing implants elicited a much stronger macrophage (CD68+) and T-cell (T-cell receptor+) infiltration in the implant area, which presumably resulted in the loss of donor cardiomyocytes, emphasizing again the importance of modulating host response. No GFP+/MHC+ cardiomyocytes were detectable 3
weeks after implantation in the case of cardiomyocyte, EC coated modules with Matrigel™. Some GFP+/MHC+ cardiomyocytes were still found in cardiomyocyte only implants, but mostly on the perimeter of the modules.

3.2 Cell Sheet Technology

Cell sheet stacking, as introduced by the Okano group, is another biofabrication technology that was used to build pre-vascularised modular tissue constructs for transplantation, with each cell sheet acting as a modular building block [49-51]. As has been demonstrated by others [52], cells may be cultured on thermoresponsive poly(N-isopropylacrylamide) (PNIPAAm) based hydrogels at 37°C, then the temperature is decreased below the lower critical solution temperature, and the cells can be detached as a consequence of hydrogel swelling without enzymatic digestion. Using a hydrogel-coated plunger to harvest the cell sheet at the lower temperature, the first cell sheet is placed on top of a second cell sheet, the two cell sheets are allowed to attach, and the plunger is then lifted along with the double-layered cell sheet construct. The procedure is repeated to overlay multiple cell sheets and create thicker, 3D constructs (Figure 3-4E).
Figure 3-4. *In vivo* modular tissue engineering. A) Trichrome image of RAEC coated modules (without embedded cells) implanted into a rat omental pouch for 21 days. Blood vessels (arrows) formed around and near the modules (outlined by dashed red line). B) MicroCT image of the vascular system formed from the RAEC modules (of A) implanted into a rat omental pouch after 21 days. There was a large leaky core on the microCT images showing that these vessels were immature and leaky. [Reprinted from Tissue Eng Part A [44], copyright 2011, with permission from Mary Ann Liebert, Inc.] C) MicroCT image of the vascular system formed from RAEC coated bmMSC embedded modules implanted into a rat omental pouch after 21 days. The addition of the MSC decreased the size of the leaky core consistent with the improved maturation of the blood vessels. [Reprinted from Tissue Eng Part A [4], copyright 2012, with
permission from Mary Ann Liebert, Inc.] D) Fat development (Oil red O staining) within modules containing human adMSC with or without HMEC in a SCID/Bg mouse after 90 days (subcutaneous injection). Without the presence of the HMEC the adMSC did not develop into a fat pad (bottom panel) suggesting that the vascularization is a prerequisite for the formation of the fat pad. [Reprinted from Tissue Eng Part A [47], copyright 2012, with permission from Mary Ann Liebert, Inc.] E) Schematic illustration of the fabrication of a cell sheet construct containing myoblasts and HUVEC (top panel). Bottom panels: cross-sectional images of the construct showing vascularisation (white arrowheads). The sheet is stained with anti-human CD31 antibody (green) or UEA-I (red) and counterstained with Hoechst 33342 (blue). [Reprinted by permission from Macmillan Publishers Ltd: Nature Protocols [51], copyright 2012; originally modified from Biomaterials [49], copyright 2010, with permission from Elsevier.]

In one study aiming to create a pre-vascularised construct using the cell sheet technology, HUVEC sheets were sandwiched between myoblast cell sheets to obtain a five-layered construct [49]. The HUVEC formed tubular structures inside the construct after 4 days of culture in vitro. When these pre-vascularised multi-layered constructs were implanted subcutaneously in nude rats, some HUVEC-derived blood vessels (human CD31+ and UEA-1+) were visible 7 days after implantation. Some transplanted myoblasts (desmin+) were also present in the implant area at day 7 post implantation.

In a different study using a similar approach, EC were co-cultured with neonatal rat cardiomyocytes to create a pre-vascularized multi-layered cardiac tissue [50]. In this study, each individual cell sheet contained both EC and cardiomyocytes, and three cell sheets were stacked together to build a larger construct. These three-layered constructs were grafted onto the infarcted myocardium of athymic rats, two weeks after inducing myocardial infarction. Implants containing co-cultured EC and cardiomyocytes showed increased blood vessel formation (isolectin B4 staining) compared to implants with cardiomyocytes alone (without EC co-culture), at 4 weeks after implantation. The co-culture implants also reduced fibrosis compared to the cardiomyocyte only implants. Cardiac function, as assessed by fractional shortening, was improved compared to sham, although only when higher EC densities (≥4x10^5 EC/cell sheet) were implanted, but not for lower EC densities or cardiomyocyte only implants; the end diastolic anterior wall thickness was increased compared to sham transplantation at the 4 week time point for all implants (with or without EC). The cell sheet technology was used in several other studies
to improve cardiac function upon transplantation, using various other cell sources (skeletal myoblasts, mesenchymal stem cells), as reviewed elsewhere [53].

### 3.3 *In Vitro* Modular Tissue Engineering

Cardiac and liver tissues with an intrinsically vascularized structure are examples of tissue constructs built *in vitro* using a modular approach to include the presence of a vascular component as a biofabrication design requirement. In a cardiac modular tissue engineering study, a neonatal cardiomyocyte-enriched cell population was embedded in submillimeter sized modules made of collagen and Matrigel™, and RAEC were seeded on the surface of the modules [5]. These modular units were then assembled into a macroporous sheet-like structure by gelling alginate on top of a single layer of modules in order to partially immobilize the modules together. The premise was that multiple sheets could ultimately be stacked together to create a 3D multi-sheet endothelialized cardiac tissue. Cardiac troponin I+ and connexin-43+ muscle bundles were observed in these modular structures, and the single module sheets were able to contract upon external field stimulation, although the presence of the EC coating on the modules interfered with the responsiveness of the constructs.

A modular approach has also been used to form vascularized liver tissue *in vitro* [54]. Primary rat hepatocytes were first cultured on a rotary shaker to form spheroids, then coated with collagen, and finally seeded with HUVEC. The endothelialized hepatocyte spheroids were then packed together inside hollow fibers (otherwise used for plasma separation). The hepatocytes retained liver specific function (measured through albumin secretion) and endothelial cells were present inside the construct (vWF+) for the duration of the study (9 days *in vitro*).

Another *in vitro* study investigated the interaction of SMC and EC in the context of modular tissue engineering [3]. Depending on the presence or absence of serum in the culture medium, the authors found that the SMC phenotype changed and directly affected the phenotype of the co-cultured EC. SMC pre-conditioning in serum-free medium prior to embedding inside the modules improved EC adherent junction formation on the surface of the modules (VE-cadherin expression, used as indicator for EC quiescence) compared to SMC cultured in medium containing fetal bovine serum; however, it also increased HUVEC proliferation, thus emphasising once more the importance of modulating the phenotype of each of the co-cultured cell types for a successful outcome. The goal had been to use SMC-EC co-culture as a means of
maintaining an intact, quiescent, nonthrombogenic layer of EC on the surface of the modules. However this goal has now been achieved using SMC as described above.

4 Building Tissue Constructs by Assembly of Building Blocks

Several groups used the modular approach as a bottom-up approach to build larger tissues from smaller building blocks \textit{in vitro}, but without EC coating of the modules for vascularization. The final assembly of the modular units into a larger structure was done either in a random (section 4.1), or in a controlled manner, if the objective was to control the architecture of the final construct (section 4.2).

4.1 Self-assembly of Modules

Microtissues are sub-millimetre sized cell aggregates (generally spheroids) that form by cellular self-assembly when cells are cultured under conditions that prevent their attachment to the culture dish surface and favor aggregation of adhesion-dependent cells. Microtissues are fabricated using different methods, such as culture on non-adherent surfaces, culture in hanging drops, culture in spinner flasks, centrifugation based methods etc. [55, 56]. They can serve as building blocks to form larger structures, and can be assembled into defined shapes (Figure 3-5A).
A) Self-assembly of modules

Cells, Spheroids or Cells on microcarriers

Mold of shape (rod, toroid, honeycomb, etc.)

Culture constructs until units fuse

B) Directed assembly of modules

1) Simple Molding

Theca cell honeycomb
Granulosa spheroids

2) Phase Assembly

Gel

3) Oligonucleotide Hybridization / Peptide crosslinker

Complementary Linkers

4) Gel Etching

PEG gel
Alginate gel
Remove Ca which dissolves alginate gels

5) Dielectrophoresis

Gels in unpolymerized bulk solution

ITO

6) Acoustic Excitation

Apply voltage to align gels on ITO pattern

Excite the units with sound to effect assembly.

7) Multilayer Additive Photopatterning

Add 2nd layer and crosslink
Add 3rd layer and crosslink

8) Thermoresponsive Mold

Mold

1st Gel

24°C to 37°C expands mold

2nd Gel
**Figure 3-5.** Methods of assembly of modules.  

**A)** Self-assembly of modules. The self-assembly of modular units (cells, spheroids, microcarries, etc.) is driven by the aggregation of the individual units into a higher order shape (rod, toroid, honeycomb, etc.) within a mold.  

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**B)** Different methods of directed assembly of modular units.  
1) Simple molding. A honeycomb pattern was formed from Theca cells (an ovary stromal cell) and spheroids of granulosa cells were placed inside the voids in the honeycomb pattern. This formed an ovary-like structure.  

[Reprinted from J Assist Reprod Genet [57], copyright 2010, with permission from Springer]  
2) Phase assembly uses two or more phases to thermodynamically drive the aggregation of the modular units together. After the units have aggregated they are crosslinked again to hold the structure together.  

[Reprinted from Biotechnol Bioeng [26], copyright 2011, with permission from John Wiley & Sons]  
3) Oligonucleotide hybridization and peptide crosslinkers. Individual units are formed and surface decorated with complementary linkers. These linkers can be oligonucleotides or peptides with free thiol groups. The units are aggregated and conditions are changed to react the linkers together.  
4) Gel etching uses units made from alginate and another polymer, such as PEG, to build the 3D architecture of the construct. After crosslinking the units together, the construct is washed with buffers to remove calcium from the gels. This causes the alginate gels to depolymerize allowing channels and voids to be formed in the 3D construct.  
5) Dielectrophoretic patterning uses a pattern laid out using a dielectric mask on indium tin oxide (ITO) to pattern gel microcarriers. The microcarriers are loaded into the device in an unpolymerized bulk solution of a polymer, such as PEG. A voltage is applied to the system which causes the microcarriers to migrate to the ITO surface forming the pattern. The bulk phase polymer is then polymerized to lock the pattern of microcarriers in place.  
6) Acoustic excitation uses sound waves to move differently shaped units to form complex repeating patterns.  
7) Multilayer additive photopatterning uses a UV crosslinkable polymer as a mask to form a shape. The mask is then moved and a new layer is added on top of the old layer. This process can be repeated several times.  
8) Thermoresponsive mold. A mold is made from a substance that changes shape due to a temperature change such as PNIPAAm. A gel is formed in this mold at low temperature. The temperature is then increased to expand the mold. This causes a space to form around the first gel which allows a second gel to be formed around the first.
The Morgan group investigated the *in vitro* parameters controlling cell aggregation within individual microtissues, as well as the self-assembly of multiple microtissues [58-64]. They showed that the duration of the pre-culture step before mixing the modular blocks together (hence the maturity of the microtissue building blocks) plays a particularly important role in the outcome of the self-assembly process [62]. When normal human fibroblasts (NHF) spheroids were pre-cultured for 1, 4 or 7 days and then assembled inside the recesses of through-shaped agarose gels, the spheroid modular blocks fused and assembled into a rod shape within 24 hours, regardless of the duration of the pre-culture step. However, the remodeling and self-assembly process was considerably slower for modules pre-cultured for longer periods of time. On the other hand, the initial size of the module building blocks surprisingly had no effect on the kinetics of the assembly process, or the length of the final rod structure, with similar results for both small and large modular building blocks. When mixing modules containing two different types of cells (NHF and H35 rat hepatoma cells), the position of each cell type within the final structure could be modulated by varying the pre-culture time of the individual building blocks. The structures obtained ranged from a single NHF core coated with H35 cells, or several NHF spherical cores, but completely coated with H35 cells and fused together, or inside-out structures etc. [62].

Furthermore the assembled modules could be reassembled or remodeled by subsequent culture steps. When HUVEC spheroids pre-cultured for 7 days were mixed with NHF cells, the HUVEC spheroids were able to re-assemble and form microtissues with a completely distinct structure, with the NHF cells on the inside and coated with HUVEC on the outside [63]. The kinetics of cellular self-assembly also varied from one cell type to the other [59, 64]. While H35 cells formed and maintained relatively stable rod, tori or honeycomb structures inside the recesses of micromolded agarose gels, NHF cells were found to quickly re-assemble the initial rod structures to a final spheroid structure, and the NHF cells also formed fewer stable tori and honeycomb structures as well [64].

In another study, multiple H35 toroid structures were randomly assembled together, with some of the toroids fused along their outer rim and giving rise to multi-lumen structures, while others were fused along their top and bottom surfaces, elongating the tubes. The authors suggested that the assembly of toroid modules might be useful in the context of tissue engineering for creating 3D porous structures with high cell density. Nevertheless, remodelling of the individual toroid
structures over time and the eventual loss of the lumen due to remodelling still remains a concern. It was observed that the inner diameter of individual toroids significantly decreased over time (up to 85% decrease within 10 days, although it did not completely close, presumably due to the slower rate of self-assembly of H35 cells) [61]. Overall, these studies emphasize the importance of understanding self-assembly and tissue remodelling processes and perhaps finding ways to control or at least modulate these processes toward a successful outcome.

Exploiting another approach, self-assembly of fibroblast coated gelatin microcarriers was used to build a dermal tissue equivalent [20]. Primary bovine fibroblasts were seeded onto gelatin microcarriers and cultured in a spinner flask, and these microtissues were then transferred to an assembly chamber. Histology and qRT-PCR analysis showed the presence of a newly synthesized layer of type I collagen (one of the main components of dermal tissue) inside and around these microtissues; the diameter of the beads also visibly increased over time, due to the synthesis and deposition of new ECM by the fibroblasts. After 1 week of culture and maturation in the assembly chamber, compact and homogenous disc shaped dermal tissue equivalents ~ 1 cm diameter and 1 mm thick were obtained. The study showed that with this modular tissue engineering approach, fibroblasts were able to maintain their natural functions, and quickly generated a dermal substitute that is rich in collagen type I and easy to handle without breaking.

In another study using gelatin microcarriers, human amniotic MSC were seeded onto gelatin microcarriers and induced to differentiate towards the osteogenic lineage within 28 days in spinner flask culture, with the goal of creating a bone substitute. The microtissues had increased mineral deposition over time (Alizarin red S staining), and also increased alkaline phosphatase (ALP) activity and increased mRNA expression of collagen type I and osteocalcin, indicative of osteogenic differentiation. These bone microtissues were then transferred to a perfusion chamber and cultured for 7 days under pulsatile flow with osteogenic culture medium, resulting in a larger bone tissue construct (~ 2 cm diameter x 1 cm long) [21].

4.2 Directed Assembly of Modules

Much effort has also been devoted towards devising methods to control the assembly of the modules (Figure 3-5B). The premise is that by directing the assembly of the modular building blocks (instead of randomly packing multiple modules together), it will be possible to recreate
native tissue architecture, as well as the native cell-cell interactions between the multiple cell types present in a tissue, with the overall goal of building better mimics of native tissues. Nevertheless, remodeling of these pre-formed \textit{in vitro} structures upon implantation \textit{in vivo} is a question still requiring further investigation, as these pre-formed modular assemblies have been generally characterized only \textit{in vitro} so far.

4.2.1 Manual Assembly of Functional Tissue Components

The functional benefit of modular assembly was eloquently demonstrated in an \textit{in vitro} study aiming to fabricate a human ovary mimic [57]. The ‘human artificial ovary’ was assembled from constituent modular blocks containing the three functional cell types that are naturally found in an ovarian follicle, namely the theca cells, the granulosa cells and the oocytes. Theca cells were first placed in an agarose mold containing honeycomb-shaped wells. Once formed (within 1 day), the honeycomb-shaped theca cell microtissues were then removed from the agarose mold and placed in a petri dish. Cumulus granulosa-oocyte complexes (COC) were then transferred inside the openings of the honeycombs formed by the theca cells. Within a few hours, the theca cells contracted and immobilized the COC inside the construct. One of three oocytes cultured using this method and extracted from the construct at 45 hours demonstrated polar body extrusion, which indicated maturation of the oocyte. Within a few days, the microtissue remodelled to a spheroid shape containing all three cell types. The authors concluded that the suggested assembly and co-culture of the three types of cells were beneficial for oocyte maturation, demonstrating the potential of a 3D modular assembly and the importance of cell interactions between the three functional cell types in order to create a successful \textit{in vitro} maturation culture system or an \textit{in vitro} toxicology model for the ovarian follicle.

4.2.2 Thermodynamically-driven Assembly in Multiphase Systems

The Khademhosseini group developed several methods of directed module assembly based on the thermodynamic tendency of multiphase systems to minimize the contact surface area between phases [26-30, 65]. These methods rely on the tendency of hydrogels (with hydrophilic properties) to pack together in the presence of a hydrophobic phase in order to minimize the surface of contact with the hydrophobic phase. By varying the different fabrication parameters, such as the initial size and shape of the hydrogels etc., it is possible to build different modular structures, with lock-and-key shapes being particularly well suited for controlling the assembly
of the building blocks. In one study, PEG methacrylate hydrogels were fabricated by photolithography, than placed in a petri dish containing mineral oil. Using a pipette tip, the modules were manually agitated inside the petri dish, which led to module aggregation. In order to preserve the structure of the modular assembly, the modules were re-exposed to UV light prior to removal from the oil phase (secondary UV crosslinking step). Most of the NIH/3T3 fibroblasts encapsulated inside the hydrogels survived the assembly process (Live/Dead® assay immediately after each biofabrication step), although both the agitation and the UV photocrosslinking steps lowered the cell viability somewhat [30].

Using the same method, double-layered, concentric tubular constructs that mimic the structure of a blood vessel were assembled [26]. EC were encapsulated in the internal ring and SMC were encapsulated in the external ring of each PEG hydrogel module (two-step photolithography), then several modules were assembled together in oil to form a double-layered tubular structure. This proof-of-concept study showed that the layered tubular structure of a blood vessel can be re-created using this assembly approach. However, since PEG has low cell attachment properties, and it is not degradable, it is expected that the long term survival of the cells within this matrix will be limited and the use of a more bioactive hydrogel material is likely necessary for future long term studies.

Using a similar principle, hydrogels floating on the surface of high density hydrophobic liquids such as perfluorodecalin (PFDC) or carbon tetrachloride (CCl₄), were driven by the surface tension on the liquid-air interface to move towards each other and aggregate [27]. This method was used to assemble together modules containing two different cell types (HepG2 and NIH/3T3), such that each HepG2 module was surrounded by six NIH/3T3 modules. Gelatin methacrylate was used instead of PEG to fabricate these hydrogels to allow cell attachment and migration. As an alternative to module assembly in hydrophobic liquids, hydrogel modules were also placed on glass surfaces patterned with hydrophobic and hydrophilic regions. The glass slides were patterned with hydrophobic regions using PDMS stamps coated with octadecyltrichlorosilane (OTS). The modules had a tendency to assemble onto the hydrophilic regions of the glass slides, driven by surface tension [28]. The assembled modular sheets were then stabilized through a secondary UV photocrosslinking step.
4.2.3 Assembly through Chemical Approaches

Gartner and Bertozzi suggested a method to control the connectivity between cells; their method involved functionalization of the cells with short DNA sequences, followed by formation of controlled cell-cell contacts through hybridization of complementary DNA sequences, and purification of the desired cellular structures from the by-products or unreacted components by fluorescence-activated cell sorting [66]. This method of assembly controls the architecture of the microtissues at the cellular level, with each individual cell becoming a building block. The authors used this assembly method as a proof-of-concept to build a paracrine signalling network in a 3D environment in vitro. CHO cells engineered to express interleukin-3 (IL-3) and a hematopoietic progenitor cell line (FL5.12) which undergoes apoptosis in the absence of IL-3 were functionalized with complementary oligonucleotides and assembled together. The assembled microtissues were then embedded in an agarose gel. The study showed that FL5.12 cells that had formed oligonucleotide mediated cell-cell contacts with the CHO cells expressing IL-3 were indeed able to survive and proliferate, but not FL5.12 that were not part of a CHO/FL5.12 module, thus confirming the formation of a signalling network between CHO and FL5.12 cells connected through complementary oligonucleotides.

Using a related directed assembly approach based on hybridization of complementary DNA sequences, the Bhatia group functionalized PEG diacrylate photocrosslinked hydrogels with DNA sequences [67]. The PEG microgels were first decorated with streptavidin moieties, which were then linked to biotinylated DNA sequences. The assembly of the microgels was directed through hybridization of the DNA sequences present on the surface of the microgels to the complementary DNA sequences spotted in a controlled fashion onto a DNA array template. It was shown that this microgel directed assembly method is applicable to multiple cell types (J2-3T3 rat fibroblasts, TK6 human lymphoblasts, A549 human lung adenocarcinoma cells were encapsulated inside the hydrogels). However, only immediate survival (3 hours post-encapsulation) was demonstrated, and the long term survival and functionality of the microtissues assembled using this method remain to be investigated in future studies.

Another method of assembly of PEG hydrogels relies on using a peptide crosslinker with free thiol groups to bridge the PEG diacrylate hydrogels together (Michael-type addition reaction) [68]. PEG hydrogels of various shapes (squares, circles, stars) were fabricated and assembled
using this method, with star-shaped hydrogel assemblies having the highest porosity and pore interconnectivity. NIH/3T3 fibroblasts embedded inside star-shaped hydrogels and assembled using this method were able to survive for at least 3 days under perfusion culture in vitro. Long term survival studies, as well as functional assays, potentially with therapeutic cells of interest, remain to be investigated using this biofabrication method.

4.2.4 Porous Assembly Using Sacrificial Building Blocks

Porous 3D modular constructs were also fabricated by stacking several layers of PEG-based modules together and using alginate microgels as sacrificial building blocks to create pores within the construct. Modules were first fabricated by photocrosslinking a PEG diacrylate solution inside a PDMS mold. The hydrogels were then retrieved and assembled in a monolayer structure, further stabilized by a secondary UV crosslinking step. Multiple layers were then stacked together to create a larger 3D construct and stabilized through further photocrosslinking [69]. While NIH 3T3 cells remained ~80% viable in all four layers of the multilayered construct, HepG2 cells were only ~70% viable in the top and bottom layer 24 hours after fabrication, potentially due to the toxicity of photocrosslinking and the lack of bioactive components in PEG. Most importantly, the majority of HepG2 in the middle layer of the three layered construct did not survive (less than 15-20% survival after 24 hour in culture). In some cases, alginate microgels were mixed with the PEG microgels, and dissolved after assembly to create pores within the final structure. Live/Dead® fluorescence images showed that the initial survival (24 hours) of HepG2 in the middle layer was improved when porous structures were created using sacrificial alginate microgels, presumably due to better oxygen and nutrient accessibility through the pores. Future studies are needed to evaluate the survival of the cells at later time points and functional assays are required to test the suitability of the construct for tissue engineering.

4.2.5 Assembly through Microfluidic Approaches

Another method of directed assembly of modular building blocks makes use of dielectrophoretic (DEP) patterning [70]. Hydrogel microtissues with encapsulated cells are mixed with a bulk-phase polymer solution of low conductivity and low viscosity (amenable to DEP patterning) and placed inside a DEP patterning chamber. Micropatterning of a dielectric layer on the lower glass slide of the DEP chamber allows for directed positioning of the microgels in the areas of the DEP chamber with higher electric field strength. Once the microgels are assembled in the desired
regions of the chamber, the bulk-phase polymer is gelled either through exposure to UV light or a change of temperature, to preserve the microgel assembly. The whole construct can then be transferred to a cell culture dish. This directed assembly biofabrication method has been used with bipotential mouse embryonic liver (BMEL) cells encapsulated in alginate microgels and then mixed with an agarose solution. After positioning the microgels inside the agarose solution, the agarose was gelled by brief immersion in ice-water to stabilize the microgel assembly. Most BMEL cells survived the biofabrication process (Live/Dead® assay performed 1 hour after polymerization), although long-term viability and functional assays remain to be performed in future studies to confirm the biocompatibility of this method.

Acoustic excitation was also used as a method to assemble microgel modules of different sizes and shapes (cubes, tetris shapes, saw-like shapes, lock-and-key shapes) [31]. PEG-based microgels were fabricated using common photolithography methods and then assembled by exposure to acoustic excitation inside a Petri dish assembly chamber. The assembly process was complete within seconds. Several layers were also assembled using this method by first photocrosslinking the first layer of assembled modules, followed by step-by-step assembly and photocrosslinking of each subsequent layer. Cells encapsulated in the microgels were viable immediately after the assembly process (Live/Dead® assay performed on NIH/3T3 fibroblasts immediately after acoustic assembly). However, the long term and functional performance of these constructs remains to be evaluated.

A different technique, multilayer additive photopatterning, has also been used to build modular tissues with controlled architecture [32]. The individual layers (which served as modular building blocks in this case) were made through placement under a photomask (of the desired shape) and UV photocrosslinking of a PEG-based polymer solution functionalised with RGD cell attachment sequences. The polymer solution that was not crosslinked during the first step of photopatterning was then washed away and a second layer of PEG-based polymer was added using a thicker spacer and photocrosslinked using a new photomask. The procedure was repeated to obtain the third layer. Primary hepatocytes were encapsulated in each of the layers of the construct to obtain a 3D multilayered hepatic tissue. The design of the photomasks ensured that none of the regions of the cell polymer mixture was exposed to UV light more than once, thus enhancing cell viability. By assembling the three layers, a hexagonal branching structure was obtained. An MTT assay performed 24 hours after cell encapsulation within a single layer
construct (hexagon shape with photopatterned features of 500 µm) showed that hepatocyte short-term survival was improved in photopatterned hydrogels compared to unpatterned bulk hydrogels that exhibited a necrotic core. The necrotic core was presumably due to poor oxygen and nutrient transport at the center of the unpatterned hydrogel discs. Three layered hexagonal branched constructs cultured for 12 days \textit{in vitro} under perfusion produced more albumin and urea compared to unpatterned hydrogels, indicators of improved functionality of the engineered liver tissue when fabricated using the multilayer modular approach.

Two-layered, sequentially patterned modules were also formed using thermoresponsive PNIPAAm micromolds [71]. Using the swelling or shrinking properties of the PNIPAAm polymer below or above its lower critical solution temperature (~32°C), the shape of the micromold was dynamically adjusted by changing the temperature, and two separate agarose layers (in some cases with encapsulated cells) were sequentially gelled inside the mold. The second agarose gel and cell population surrounded the original hydrogel layer and formed a two-layered construct with cylindrical, square or stripe shapes, depending on the shape of the micromold.

Microfluidics was also combined with modular tissue engineering with the purpose of fabricating large numbers of modules in a continuous and rapid manner. Using stop-flow lithography, microgels were formed by flowing a PEG diacylate polymer solution through a microchannel and photocrosslinking the polymer solution by passing pulses of UV light through a photomask [72]. Large numbers of microgels of different shapes were fabricated using different photomasks. However, the viability of NIH 3T3 fibroblast cells encapsulated in the hydrogels fabricated using SFL was only 68% at 1 hour post fabrication, and further improvements to this method are likely necessary to make it more widely applicable.

5 Modular Tissue Engineering Combined with Microfluidics

Beyond what has been described in the previous section, microfluidic systems have been used by a few groups in the context of modular tissue engineering as a means of creating controlled 3D co-culture systems and \textit{in vitro} tissue models. These systems combine the high-throughput and controlled microenvironment advantages of microfluidic devices with the advantages of modular
tissue engineering, such as 3D cell culture and potential to culture multiple cell types together by mixing modules encapsulating different cell populations.

In a proof-of-concept study, collagen modules with embedded NIH/3T3 fibroblasts or HepG2 cells were fabricated inside a PDMS mold, then loaded inside a microfluidics chamber, and cultured under perfusion for 24 hours [73]. The pores formed between the modules allowed for culture medium to circulate in the spaces between the modules and reach the encapsulated cells. The cells maintained high viability over 24 hours in perfusion culture (~99%). Narrowing the channels within the microfluidic chip allowed for ordered packing of the modules within the device. Sequentially loading three different batches of modules, each batch containing a differently labeled population of NIH/3T3 fibroblasts, resulted in an organized string of distinct modules. This technique is thus presumably amenable to the formation and culture of ordered modular tissue structures within the microfluidic chamber. Nevertheless, a material that is more amenable to perfusion culture (better mechanical properties) than collagen would presumably need to be used, as the authors noticed that the weak collagen modules deformed under flow, which limited the flowrate, as well as the maximum length of the construct that could be perfused to only a few millimeters.

In a different study combining modular tissue engineering and microfluidics, a microfluidic chamber loaded with EC-coated collagen modules was used to characterize the remodelling of the modular construct and the changes in EC phenotype under flow conditions [74]. The study found that EC proliferation was significantly reduced for flow compared to static conditions after 24 hours (BrdU uptake assay), but similarly low proliferation levels were observed for both cases after 48 hours. KLF2 (a transcription factor upregulated with laminar shear stress and which upregulates anti-thrombotic factors) was upregulated at 24 hours under flow. However, expression of VE-cadherin (used as an indicator of EC quiescence) was downregulated and discontinuous under flow conditions, presumably due to EC reorientation and remodeling of the construct under flow. No statistically significant differences in VCAM-1 or ICAM-1 expression (indicators of EC activation) were observed between the static and flow conditions over 24 hours. Overall, the study suggested that perfusion of the modular constructs did not significantly increase EC activation; however, remodeling of the constructs under flow occurred, with discontinues VE-cadherin expression, and with the void spaces (flow channels) formed in between the modules decreasing in size over time due to compaction, even at low flow rates.
When bmMSC were embedded inside the collagen modules prior to EC seeding, and using the same microfluidic remodelling chamber experimental setting, it was noted that flow conditioning of the modular constructs lead to increased expression of smooth muscle cell markers (SMA+, desmin+ staining) by the MSC and increased migration from the inside of the modules towards the module surface compared to the static control, as well as compared to the MSC only (no EC coating) control, over the 21 day time course of the study [75]. All modules also remodeled and contracted significantly over time, with the least amount of contraction observed for the MSC only modules under static conditions. A change in the extracellular matrix composition was also observed for MSC containing modules under flow conditions, with or without the EC coating, with more proteoglycan deposition occurring in these cases (Alcian Blue staining). Overall, this study demonstrated the potential usefulness of these flow-conditioned modular tissues as model systems to study the remodelling of tissue engineered constructs composed of multiple cell types over time in vitro, and as a tool to better understand and predict the remodelling that would occur upon implantation in vivo.

Other studies combine microfluidics with a modular approach to create in vitro 3D liver tissue models for drug metabolism and toxicology assays [76, 77]. Several precision-cut rat liver slices were embedded in Matrigel™, placed inside a PDMS microfluidic device and cultured under perfusion using an optimized culture medium formulation [77]. Each of the precision-cut liver slices (modular building blocks) maintains the native liver architecture and cellular components, making the system a closer mimic to the native tissue. Embedding the liver slices in Matrigel™ and using an optimized culture medium composition maintained high viability of the liver slices for at least 72 hours in culture (~90% by lactate dehydrogenase (LDH) activity assay), and preserved the liver metabolic activity (determined through measurement of metabolite formation upon addition of substrate), although only partially. Encouragingly, phase II metabolism was similar between fresh slices and slices embedded in Matrigel™ and cultured inside the microfluidic device for 72 hours; however, the phase I metabolism decreased significantly, with only 5% of the initial value after 72 hours.

6 Conclusion

Biofabrication of tissue constructs using a modular approach offers tremendous opportunities in terms of creating tissue constructs that recapitulate the complexity of native tissues. This in turn
translates into more realistic *in vitro* models for drug testing or other biological assays, compared to conventional 2D systems or bulk scaffolds seeded with cells. For transplantation purposes, it also brings us a step closer towards creating tissue or organ replacements that are better mimics of their native counterparts.

Using the modular approach, multiple cell types can be mixed together, either within the same modular unit or in separate modules, and the relative abundance of each cell type can be individually controlled by simply changing the number of modules containing that particular cell population. Furthermore, the modular approach is 3D in nature and it is versatile in terms of the materials that can be used to fabricate the modules, thus offering the opportunity to recreate the cells’ natural ECM and microenvironment. Moreover, the porous structures created upon assembly of several modules are amenable to perfusion, enabling researchers to conduct biological assays under physiologically relevant flow conditions. The modular approach is also scalable, paving the way to fabricating tissues and organs of clinically relevant size. Increasingly larger structures, with cells evenly distributed throughout the construct, can be obtained by simply increasing the number of modular building blocks mixed together, and controlling the cell density within each individual building block.

Moving forward, the question of remodelling of these pre-formed modular structures, particularly *in vivo*, needs to be further investigated. Host response and remodelling play a critical role in determining the success or failure of the implanted tissue construct. Therefore, the *in vivo* remodelling of the engineered tissue needs to be integrated as a design parameter for the fabrication of these structures *in vitro*. Finding ways to modulate the remodelling of the tissue constructs after implantation, is perhaps more important than achieving perfect control over the tissue microarchitecture prior to implantation. Distinct sets of design criteria may in fact be necessary depending on whether the envisioned application is fabrication of tissue or organ replacements for transplantation, in which case host response and *in vivo* remodelling are probably the key design parameters to consider, or whether the envisioned application is fabrication of *in vitro* tissue models, in which case faithful imitation of the native tissue structure may be of highest importance.
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Chapter 4

Literature Review: Del-1 and Other Pro-Angiogenic Matricellular Proteins

1 Del-1

Del-1 (Developmental endothelial locus-1) is an extracellular matrix (ECM) protein containing three N-terminal EGF-like repeats, and two discoidin I-like domains at the C-terminus [1] (Figure 4-1). An RGD motif is located at the end of a long protruding loop in the second EGF domain, mediating cell attachment [2]. The discoidin domains direct deposition of Del-1 in the ECM [3]. The major Del-1 transcript contains 480 amino acids (~52kDa), with a ten amino acid sequence sometime absent between the first and second EGF repeats. A minor transcript also exists (less frequent), and it only contains the three EGF repeats and a portion of the first discoidin-I-like domain [1]. Only the major transcript has angiogenic properties [4].

![Figure 4-1. Schematic representation of Del-1 protein domains.](image)

Initial reports focused on Del-1 expression by endothelial cells (EC) during vascular development [1], and its re-expression in adult tissues only under ischemic conditions [4]. Its expression was also shown to be increased after electroconvulsive seizure treatment, and it was associated with an increase in vascular density in some regions of the hippocampus [5]. More recent reports show a broader physiological role for Del-1 in the adult endothelium, such as its anti-inflammatory properties [6, 7], or its role in microparticle clearance [8, 9]. In addition to the endothelium, Del-1 is also secreted by some macrophages [10], and it was also reported in chondrocytes [11]. Del-1 is also expressed in some tumor tissues and tumor cell lines [12, 13].

In vitro, Del-1 was shown to support the attachment of EC, with phosphorylation of signaling proteins including p125FAK, MAPK, and Shc, and formation of focal contacts, with recruitment
of talin and vinculin [14]. Del-1 also stimulates migration of EC [14]. Del-1 interaction with EC first involves binding of Del-1 to integrin αβ, on the resting endothelium. The interaction of Del-1 with the αβ integrin triggers the expression of the transcription factor HoxD3, which then induces the expression of a number of factors involved in angiogenesis, such as integrin αβ and urokinase-type plasminogen activator (uPA) [15, 16]. Del-1 was also shown to protect EC from undergoing apoptosis upon exposure to inducers of both the intrinsic and extrinsic apoptosis pathways, when EC were plated on Del-1 coated surfaces. The anti-apoptotic effect was mediated via binding of the αβ integrin, and involved FAK/ERK and Akt signaling. The same study showed that combining Del-1 with VEGF or bFGF had an additive anti-apoptotic effect [17]. Expression of Del-1 mRNA by EC was only shown to be induced by exposure to IFNγ [17], while others found that VEGF, IL-1α, and TNF-α also stimulate Del-1 expression [18]. On the other hand, yolk sac cells transfected with Del-1 were inhibited from forming tubes in a Matrigel™ assay [1]. Del-1 also supports the adhesion, the formation of focal contacts enriched in vinculin, the migration, and the proliferation of smooth muscle cells (SMC), effects mediated through interaction with the same αβ integrin [19].

In vivo, Del-1 was shown to initiate angiogenesis in the absence of exogenous growth factors in hind-limb, cerebral and cardiac ischemia animal models [4, 15, 20, 21]. Del-1 plasmid delivery in a mouse hind limb ischemia model stimulated vascularization of the ischemic muscle, and restored muscle function, evaluated as run time to exhaustion, four weeks after treatment [15]. In a different study, Del-1 daily protein injection for three weeks also increased blood vessel density and perfusion using a similar mouse hind limb ischemia model [4]. Similarly, Del-1 plasmid injection in a rabbit hind-limb ischemia model improved vascularization of the tissue, as shown by angiography imaging one month after treatment [15]. Safety studies in mice and rabbits showed that intravenous or intramuscular administration of Del-1 plasmid did not have significant adverse effects on the health of the animals [22]. Adeno-associated viral delivery of Del-1 in a cerebral ischemia mouse model [20], or adenoviral delivery in a cardiac ischemia model also resulted in improved vascularization of the ischemic tissues [21]. Del-1 was also shown to induce significant vascular remodelling in a chick chorioallantoic membrane (CAM) assay [1, 14].

Nevertheless, clinical trials investigating the use of Del-1 as a treatment option for patients suffering from intermittent claudication caused by peripheral arterial disease were less successful
Del-1 plasmid was injected intramuscularly using a poloxamer formulation, or the poloxamer formulation was injected by itself as control. No adverse health effects due to treatment were recorded, and significant improvements in peak walking time and quality of life were observed after treatment, but no significant difference in outcome between Del-1 and placebo groups.

Consistent with the role of Del-1 in enhancing vascularization, tumor cells transfected to overexpress Del-1 and injected into mice gave rise to larger tumors in vivo compared to control-transfected tumor cells, as a consequence of increased vascularization of the tumors [12]. Another study found that Del-1 was expressed in approximately half of the samples from hepatocellular carcinoma patients, and Del-1 expression correlated with poor survival outcome for these patients [25]. Moreover, injection of Del-1-shRNA plasmid to reduce Del-1 expression in tumors generated by implantation of human colon cancer cells into mice, effectively reduced tumor growth, emphasizing an important role for Del-1 in stimulating vascularization and thus supporting tumor tissue growth [26].

Recent studies on Del-1 focused on the role of Del-1 as an endogenous inhibitor of inflammation [6, 7, 27-29]. Lymphocyte function associated molecule-1 (LFA-1 or αLβ2) is expressed by leukocytes and plays an important role in inflammation through its involvement in leukocyte-endothelial cell binding and transmigration via the interaction with its endothelial cell ligand, ICAM-1 [30]. Del-1 was shown to antagonize LFA-1 dependent adhesion [7], and thus interfere with ICAM-1/LFA-1 interactions. In vitro experiments showed that unlike ICAM-1, which allowed firm neutrophil adhesion under both static and flow conditions, Del-1 only bound neutrophils under static conditions, weakly under low shear flow conditions, and neutrophil attachment to Del-1 surfaces was completely eliminated at high shear. Therefore, not surprisingly, increasing Del-1 density on surfaces with co-immobilized Del-1 and ICAM-1 effectively reduced neutrophil adhesion to ICAM-1 under flow [7]. In vivo, Del-1 intravenous administration decreased the number of neutrophils after stimulation with thioglycollate into the peritoneum of wild-type mice. Also, Del-1 deficient mice (Del-1−/− mice) had increased accumulation of neutrophils in the bronchoalveolar lavage fluid after lipopolysaccharide (LPS) induced lung inflammation. Dell1−/− mice also had more leukocytes adherent to post-capillary venules, both under basal conditions, and after stimulation with TNF-α, and the leukocytes had slower rolling velocity [7]. In addition, in a periodontitis inflammatory disease model, it was
shown that in both aging-associated periodontitis, as well as in Del-1⁻⁻ young mice having developed periodontitis, the higher number of neutrophils at the site of inflammation was associated with lower (or lack of) Del-1 expression and higher IL-17 expression. Conversely, local administration of Del-1 inhibited neutrophil recruitment and decreased local IL-17 expression [6].

Del-1 was also shown to bind to phosphatidylserine on apoptotic cells via its discoidin I-like domains, and it is believed to act as a bridge between apoptotic cells and phagocytic cells; a subset of macrophages, including fetal thymus and fetal liver macrophages, the macrophage cell lines BAM3 and J774A.1, and bone-marrow derived macrophages were shown to express Del-1 [10]. Binding of Del-1 to phosphatidylserine was also shown to be involved in the clearance of phosphatidylserine-expressing microparticles by EC, both in vitro and in vivo [8].

Other properties attributed to Del-1 in the literature include stimulation of hair regrowth [31], no significant effect on wound healing [31], enhancement of efficiency of non-viral gene delivery both in vitro [32] and in vivo [33] (effect mediated via the third EGF domain by increasing endocytosis efficiency), very weak apoptosis-inducing effect on COS-7 cells (effect mediated through the third EGF domain as well) [34], and a role in the proper forebrain development in the Xenopus embryo [35].

2 Other Pro-Angiogenic Matricellular Proteins

Del-1 shares some of its properties with some of the ECM proteins named collectively ‘matricellular’ proteins. In fact, Del-1 is considered a matricellular protein itself [17]. Matricellular proteins were defined as ECM proteins whose primary role is not to provide structural support to the tissue, but to modulate cell behavior by interacting with and coordinating the functions of several biological factors, including cells, cytokines, growth factors, proteases, and other ECM components [36, 37]. Some matricellular proteins including some members of the CCN family (CCN1, CCN2 and CCN3), tenascin-C (TN-C) and osteopontin (OPN) were shown to be pro-angiogenic, while others such as thrombospondin-1 (TSP-1), thrombospondin-2 (TSP-2), and secreted protein, acidic and rich in cysteine (SPARC) are generally considered as inhibitors of angiogenesis, although SPARC can also be pro-angiogenic, depending on the biological context [37, 38]. Matricellular proteins modulate processes such as cell adhesion, migration, survival, proliferation, and ECM deposition. They are
mostly expressed during development, or in response to injury. Expression of these proteins is also often detected in tumor tissues [39]. As a general rule, matricellular proteins are present at sites of remodelling, or at sites of chronic inflammation. Perhaps not surprisingly, their functions are contextual, depending on the combination of biological factors that predominate in the tissue and that interact with the matricellular protein in that particular biological context [37]. The phenotypes of mice deficient in these matricellular proteins are generally mild, given the lack of a structural function for these ECM proteins [36].

The CCN family of extracellular matrix proteins consists of six members, with CCN1, CCN2 and CCN3 shown to have angiogenic properties. It is believed that these proteins do not stimulate angiogenesis simply by manipulating the phenotype of a particular type of cells, but rather by coordinating the actions of multiple biological factors in the tissue microenvironment towards a vascularization outcome. The modular structure of these proteins, with four conserved functional domains, including the insulin-like growth factor binding protein, von Willebrand factor type C repeat, thrombospondin type 1 repeat and the carboxyl-terminal module, allows them to interact with a variety of cell receptors, proteoglycans, growth factors and other ECM proteins [40-44]. The first member of the CCN family is CCN1 (or Cyr61, named after its cysteine rich structure). CCN1 was reported to stimulate attachment and migration of EC [45]. The \( \alpha_6\beta_1 \) and \( \alpha_\gamma\beta_3 \) integrins were shown to be involved in the interaction of CCN1 with EC [46], and the \( \alpha_6\beta_1 \) integrin and heparan sulfate proteoglycan in supporting the attachment and chemotaxis of smooth muscle cells [47]. In addition, CCN1 was shown to liberate bFGF bound to the ECM and thus enhance bFGF induced proliferation of EC [48]. CD34+ progenitor cells were also shown to attach and migrate in response to CCN1 [49]. In vivo, CCN1 delivery was shown to stimulate vascularization in a rat corneal pocket angiogenesis assay [45] and in a rabbit hindlimb ischemia model as well [50]. It was also reported to enhance tumor growth by stimulating vascularization [45]. The second member of the CCN family is CCN2 or CTGF, for connective tissue growth factor. CCN2 interaction with EC is believed to occur similarly to CCN1, and the enhanced survival and migration of EC in the presence of CCN2 were shown to be mediated via the \( \alpha_\gamma\beta_3 \) integrin [51]. On the contrary, CCN2 was also shown to bind VEGF and thereby reduce VEGF-induced tube formation \textit{in vitro} and angiogenesis \textit{in vivo} in a Matrigel™ injection assay [52]. Experiments in CCN2-deficient mice showed that CCN2 also plays an important role in retention of pericytes around EC lined blood vessels, by influencing
PDGF signaling and expression of basement membrane components [53]. The third pro-angiogenic member is CCN3 or NOV, for nephroblastoma-overexpressed protein. Similar to the other CCN family members, $\alpha_6\beta_1$, $\alpha_V\beta_3$ and $\alpha_5\beta_1$ integrins, and heparan sulfate proteoglycans were all shown to mediate the pro-angiogenic effects of CCN3, and CCN3 was shown to induce vascularization \textit{in vivo} in a rat corneal pocket angiogenesis assay [54]. Less is known about the pro-angiogenic effects for CCN4, CCCN5, and CCN6, and in fact there were reports suggesting that these ECM proteins had anti-angiogenic properties [40].

Tenascin-C is an extracellular matrix glycoprotein, upregulated upon tissue injury, and minimally expressed in healthy tissues. It has a modular structure, including an assembly domain, several EGF-like repeats, several fibronectin type III-like repeats (FNIII) and a fibrinogen-like globe at the C-terminus. Due to its modular structure, it can interact with a variety of biological factors and has many functions during tissue repair or remodelling, including angiogenesis [55]. Tenascin-C deficiency in mice significantly decreased blood vessel formation in a mouse corneal injury model [56]. Evidence for tenascin-C overexpression surrounding tumor blood vessels compared to normal blood vessels, and at sites of vascular repair after injury, suggests a role for tenascin-C in angiogenic remodelling as well [55, 57, 58]. \textit{In vitro}, tenascin was expressed by sprouting EC, but not by quiescent EC, and sprouting was reduced upon exposure of EC to antibodies to tenascin [59]. The angiogenic properties of tenascin-C are mediated via integrin $\alpha_V\beta_3$, and through regulation of VEGF expression, among other factors [58].

Osteopontin is a glyco-phosphoprotein that is upregulated during tissue remodelling and is involved in processes including vascularization of ischemic tissues, inflammation and tumor progression [60]. It supports EC migration, proliferation and tube formation on Matrigel\textsuperscript{TM}, and protects EC from apoptosis induced by serum-withdrawal [61, 62]. These effects on EC are mediated via interaction with the $\alpha_V\beta_3$ integrin, and involve upregulation of VEGF and activation of PI3K/Akt and ERK1/2 in EC [62]. Osteopontin itself is upregulated by VEGF [61], and osteopontin upregulates VEGF, thus suggesting a potential positive feedback loop between VEGF and osteopontin [63]. FGF-2, another angiogenic growth factor, was also shown to upregulate osteopontin [64]. Osteopontin delivery \textit{in vivo} enhanced vascularization in a CAM assay, in a rabbit corneal micropocket assay, and in the tumor tissue formed by transplantation of tumor cells transfected with osteopontin, or with osteopontin treatment, in a mammary fat pad
mouse model; the osteopontin-induced vascularization effect was reduced following treatment with anti-OPN or anti-VEGF antibodies [62, 63]. In a different study, osteopontin pro-angiogenic activity in a CAM assay was shown to be independent of the αvβ3 integrin, but was accompanied by increased macrophage recruitment, presumably having pro-angiogenic phenotype [64]. Interaction with CD44 was shown to also play an important role in the biological activity of osteopontin, particularly in host-inflammatory responses and tumor growth [60].

3 References


Chapter 5
A Poloxamine-Polylysine Acrylate Scaffold for Modular Tissue Engineering

Abstract

A new polymer, poloxamine-polylysine acrylate (PPA) was synthesized for tissue engineering applications. Polylysine was used to confer endothelial cell attachment properties to the poloxamine-based polymer, while the acrylate groups made it photocrosslinkable. The PPA polymer was synthesized in three reaction steps. (1) Polylysine was acrylated using N-hydroxysuccinimide acrylic acid ester and the reaction product was characterised by $^1$H-NMR. (2) The hydroxyl groups on poloxamine were activated by tresylation with tresyl chloride and the reaction product (tresylated poloxamine) was characterised by ICP-AES for sulphur content. (3) The acrylated polylysine was reacted with the activated poloxamine to obtain the final product, poloxamine-polylysine acrylate. The final product was characterised by CHN elemental analysis. Aqueous solutions containing a mixture of PPA and poloxamine methacrylate were photocrosslinked by exposure to a 365nm UV light source in the presence of a photoinitiator to obtain hydrogels. The synthesized PPA polymer enhanced endothelial cell adhesion on poloxamine based, photocrosslinked hydrogel scaffolds. The same synthesis methods can be used in the future to introduce other desired functions (through other peptides) into the scaffold biomaterial.

Keywords: modular tissue engineering; poloxamine; polylysine; hydrogels; photo-cross-linkable scaffolds

1 Introduction

Tissue engineering promises to provide artificial tissues to replace or restore function to damaged tissues and organs. However, due to the lack of a vascular network and to diffusion limitations, only tissue constructs of a few hundred microns or less in thickness can be made [1]. Our group uses a modular approach to generate vascularized tissues: the functional cells of interest are embedded in submillimetre sized gel cylinders (modules) and endothelial cells (EC) are seeded on the surface of the modules. When several modules are randomly packed together, the spaces between them create a network of interconnected channels lined with EC, mimicking a vascular network [2, 3].

Collagen type I alone or a mixture of collagen and poloxamine methacrylate have been used to fabricate the modules. Collagen modules support cell attachment, but have limited resistance to compression under flow. Poloxamine is a synthetic four arm block copolymer of polyethylene oxide and polypropylene oxide. Methacrylate groups are added to each of the 4 poloxamine arms to allow photocrosslinking and the formation of hydrogels. Poloxamine is biocompatible, but it does not allow cell attachment [4, 5]. Poloxamine has biological properties (low protein adsorption and low cell binding) similar to poly(ethylene glycol) (PEG), and compared to PEG, it also has the advantage of a higher functionality (four arms). The higher functionality makes poloxamine amenable to more extensive chemical modification. Moreover, a higher degree of crosslinking compared to PEG can be achieved for the same polymer molar concentration. However, the low protein adsorption and low cell binding properties of poloxamine make it an unsuitable material for scaffolds in tissue engineering applications without further modification.

Different approaches have been pursued to increase the cell compatibility and cell attachment properties of poloxamine. Positive charges have been introduced into the scaffold biomaterial by mixing poloxamine methacrylate with a quaternary ammonium methacrylate ([2-(methacryloyloxy) ethyl]-trimethylammonium chloride - MAETAC) prior to photocrosslinking [6]. Another approach has been to synthesize a positively charged poloxamine by methylating the tertiary amine groups on poloxamine and then adding methacrylate groups to this material, to make it photocrosslinkable [7]. Hydrogel scaffolds containing a mixture of poloxamine methacrylate and collagen have also been tested, although the collagen does not appear to be uniformly mixed with the poloxamine, limiting cell attachment [4, 5]. Similarly, different
strategies have been used to modify PEG and make it more cell adhesive. Peptides such as RGD [8, 9], or YIGSR [10] for instance have been chemically linked to PEG to improve its cell attachment properties.

The present work explores the possibility of chemically linking a polylysine peptide chain to each of the four poloxamine arms. We hypothesized that by chemically adding polylysine peptides to the poloxamine backbone, a poloxamine-based polymer that supports cell adhesion would be obtained. Consistent with the low protein adsorption properties of poloxamine, previous work in our lab suggested that mixtures of poloxamine and collagen were not homogenous, with poloxamine and collagen phases separating in the mixture. Therefore, we expected that chemically linking polylysine chains to each of the four poloxamine arms would eliminate the need for physical mixing of the two components and solve the phase separation problem. We also acrylated the polylysine chains in order to allow photocrosslinking of the polymer and the formation of hydrogel scaffolds upon photocrosslinking.

The ε-amino group of the lysines in polylysine is positively charged at physiological pH (pKa = 9.3-9.5; [11]). The positive charges on polylysine are likely to enable the adsorption of serum proteins that support cell attachment on the hydrogel surface, as well as to mediate electrostatic interactions with the cell membrane. Moreover, the presence of reactive amino groups on the polylysine side chains also make polylysine a good model molecule for future polymer synthesis aimed at introducing other desired functions into the scaffold biomaterial.

2 Materials and Methods

2.1 Synthesis

2.1.1 Poloxamine Methacrylate

Poloxamine methacrylate was synthesized as before [4]. Briefly, dried poloxamine (10.1 g of Tetronic 1107, a gift from BASF, New Jersey, NJ) was reacted with 2-isocyanatoethyl methacrylate (420 µL IEM, molar ratio IEM: poloxamine = 4.4 : 1; Sigma, St. Louis, MO ), 2-ethyl-hexanoate (0.4g, Sigma) and hydroquinone (0.01 g, Sigma) in dimethyl sulfoxide (DMSO, EMD, Germany) at 70-72°C under dry nitrogen atmosphere and magnetic stirring for 3 hours. The reaction product was dissolved in chloroform (JT Baker inc, Phillipsburg, NJ), filtered and precipitated twice in 800 mL petroleum ether (Fisher Scientific, Fair Lawn, NJ). After drying,
the degree of acrylation was quantified by $^1$H-NMR with a Mercury 400MHz NMR spectrometer. The spectra were obtained using 15% wt CDCl$_3$ (Sigma) solutions at room temperature.

2.1.2 Polysine Acrylate

Adapting the method of Hubbell et al [9], poly(L-lysine) hydrobromide (MW = 1000 – 5000, average MW = 4200, Sigma) in 50 mM bicarbonate buffer solution (1-10 mg/mL, pH 8.5) was added to a reaction flask containing the succinimidyl ester of acrylic acid (Sigma, mole ratio 10:1 relative to polysine) and allowed to react for 2 hours at room temperature, under magnetic stirring (Figure 5-1). The product was dialysed against deionized water for up to 7 days (500 – 1000 MWCO Biotech Cellulose Ester Dialysis Membrane, Spectrum Laboratories, Rancho Dominguez, CA) and lyophilized. The extent of functionalization was quantified by $^1$H-NMR with a Mercury 400MHz NMR spectrometer using 25 mg/mL solutions in deuterated water (D2O, Sigma) at room temperature.

**Figure 5-1.** Reaction step 1: acrylation of polysine.

2.1.3 Poloxamine Tresylate

Adapting a method of Griffith et al [12], dried poloxamine (2g Tetronic 1107, a gift from BASF) in tetrahydrofuran (THF, Sigma) was reacted with tresyl chloride (88 µL, Sigma) and triethylamine (TEA, 112 µL, Aldrich, Milwaukee, WI) for 3 hours on ice (Figure 5-2). The product was precipitated in petroleum ether and dried. The degree of poloxamine activation was determined by inductively coupled plasma atomic emission spectrometry (ICP-AES) for sulphur using a Perkin Elmer Model Optima 3000DV ICP AEOS equipment and by thin-layer chromatography (TLC) using general-purpose, silica gel on glass 20 cm x 20 cm TLC plates (Sigma).
2.1.4 Poloxamine – Polylysine Acrylate (PPA)

The tresylated poloxamine (4mM in N,N-Dimethylformamide, DMF, Sigma) was reacted overnight with a 2 fold molar excess of acrylated polylysine in the presence of triethylamine (Aldrich), at room temperature (Figure 5-3). The reaction product was dialysed (Standard Regenerated Cellulose Dialysis Tubing MWCO 6000-8000Da, Spectrum Laboratories) and analysed by SDS-PAGE (4-16.5% gels, Tris-Tricine buffers) with Coomassie staining and by elemental analysis using a Perkin Elmer Model 2400II CHN analyzer with Perkin Elmer AD-6 autobalance. Calibration against thermal standard acetanilide (C:71.09, H:6.71, N:10.36) was performed before and after elemental analysis of the sample.
2.2 Hydrogels

Aqueous solutions containing a mixture of PPA and poloxamine methacrylate, or poloxamine methacrylate alone were photocrosslinked to obtain hydrogels in a 96 well-plate, following the methods previously described [4]. The solutions were exposed to UV light (Spectroline EN-180 lamp, one 8-W 365 nm tube, 1.850 mW/cm², 15 cm distance) for at least 10 min, in the presence of a photoinitiator (Irgacure 2959, Ciba, NY). Poloxamine methacrylate/collagen gels were made by mixing the poloxamine methacrylate solution with a type I bovine dermal collagen solution (PureCol, Inamed Biomaterials, CA) and neutralising the collagen solution with sodium bicarbonate, followed by 30 min incubation at 37°C, and exposure to UV light. Collagen only gels were prepared by neutralising the collagen solution with sodium bicarbonate, and incubating the solution for 45 min at 37°C [2-5]. The hydrogels were typically kept in culture medium for at least 24 hours prior to cell seeding.

2.3 Cells

Human Microvascular Endothelial Cells (HMEC-1, a cell line) were maintained in MCDB-131 medium (VEC Technologies Inc, Rensselaer, NY) with 10% foetal bovine serum (FBS, Sigma) at 37°C in a 5% CO₂ humidified air atmosphere. Culture medium was changed every 2-3 days. Calcein AM and ethidium homodimer-1 (EthD-1) staining (Live/Dead™ kit, Molecular Probes, Eugene, OR) was used to visualize live (green fluorescence) and dead (red fluorescence) endothelial cells seeded on top of the gels after 1 and 7 days in culture. Staining was visualized with a Zeiss Axiovert fluorescence microscope.

Endothelial cells seeded on top of different hydrogels were incubated in fresh medium with or without 50 ng/mL tumor necrosis factor α (TNF-α, Invitrogen, OR) or 50 µg/mL lipopolysaccharides (LPS, Sigma) for 22 hours, washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 min at 37°C. After washing (x3) with PBS, the cells were incubated with 100% FBS for 30 minutes, washed again (x3) with PBS and incubated with the primary antibodies against vascular cell adhesion molecule 1 (VCAM-1, goat IgG, 1:50 dilution in PBS, Santa Cruz Biotechnology, Santa Cruz, CA) and intercellular adhesion molecule 1 (ICAM-1, mouse IgG, 1:50 dilution in PBS, Santa Cruz Biotechnology) for 30 minutes. After another wash with PBS (x3), samples were incubated with the secondary antibodies (1:100 dilution in PBS of Alexa Fluor® 568 rabbit anti-mouse or Alexa Fluor® 488 rabbit anti-goat,
Invitrogen) and Hoechst (bisBenzimide H 33342 trihydrochloride, Sigma-Aldrich) nuclear stain in the dark for 30 minutes. After washing with PBS (x3), the samples were visualised with a Zeiss Axiovert fluorescence microscope.

3 Results

3.1 Synthesis

A poloxamine-polylysine acrylate polymer was synthesized using a three step chemical synthesis strategy. Typical coupling efficiencies are summarised in Table 5-1.

Table 5-1. Synthesis of poloxamine-polylysine acrylate: typical coupling efficiencies

<table>
<thead>
<tr>
<th>Reaction step</th>
<th>Coupling efficiency (analysis method)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: Acrylation of polylysine</td>
<td>3-4 acrylate groups/polylysine molecule (1H-NMR)</td>
</tr>
<tr>
<td>2: Tresylation of poloxamine</td>
<td>90% or higher (ICP-AES for sulphur and TLC)</td>
</tr>
<tr>
<td>3: Poloxamine-polylysine acrylate synthesis</td>
<td>Up to 70% (CHN elemental analysis and SDS-PAGE)</td>
</tr>
</tbody>
</table>

Methacrylate groups were added to poloxamine to enable subsequent photocrosslinking and the formation of hydrogels. The degree of functionalization was determined by 1H-NMR, as previously described [4, 5]. Typically, the reaction proceeded with a high yield (around 70%) and degree of substitution (close to completion), consistent with the literature [4, 5]. The hydroxyl groups on poloxamine were separately activated with tresyl chloride. The yield of the reaction was typically around 80% and the degree of tresylation was typically found to be 90-100%, by ICP-AES for sulphur; 100% tresylation corresponded to 41.1ppm S for a 5 mg/mL poloxamine tresylate aqueous solution.

The N-hydroxy succinimidyl ester of acrylic acid was used to add acrylate groups to polylysine and the number of acrylate groups per polylysine was estimated by 1H-NMR (Figure 5-4). The values of the integrals for the vinyl protons (5.6-6.4 ppm, 3 protons per acrylate group added) and for the beta, gamma and sigma protons on the polylysine chain (1-2 ppm, 6 protons per lysine residue) were used to calculate the degree of substitution. Depending on the reagent ratio and reaction time, as few as 1 and as many as 20 acrylate groups per polylysine molecule were added. With a typical reagent ratio of 1:10 and a reaction time of 2 hours, 3-4 acrylate groups per polylysine molecule were added.
Figure 5-4. $^1$H-NMR spectrum of polylysine acrylate. 3.7 acrylate groups/polylysine molecule. Recorded on a Mercury 400 MHz NMR spectrometer. Reaction step 1, conditions used: room temperature, pH 8.5, 2 h, polylysine hydrobromide/N-hydroxysuccinimide acrylate = 1:10.

The acrylated polylysine was reacted with tresylated poloxamine to obtain the final product, poloxamine-polylysine acrylate (PPA). The coupling efficiency was estimated to be up to 70% by elemental analysis (example of elemental analysis results: C: 55.1% ± 0.3%, H: 9.1% ± 0.3%, N 7.0% ± 0.3%) and the SDS-PAGE and Coomassie staining confirmed that any unreacted polylysine acrylate left was successfully removed by dialysis. The yield of the reaction was approximately 50%, suggesting that some of the reaction product was lost during dialysis, presumably through binding to the dialysis membrane. Using centrifugal filter devices (Centriplus YM-10, MWCO 10 000Da, Millipore, Bedford, MA) as an alternative to the dialysis tubing also resulted in significant product loss.
3.2 Cell Behaviour

Photocrosslinked hydrogel scaffolds containing the synthesized PPA polymer were able to support HMEC-1 cell survival and attachment onto the hydrogel surface for at least 7 days after cell seeding, as shown by the Live/Dead™ images in Figure 5-5. At both time points studied here (day 1 and day 7), there were visibly more HMEC-1 cells on PPA scaffolds than on scaffolds made from poloxamine methacrylate alone. The latter are known not to support cell attachment, but cell attachment is greater when poloxamine methacrylate is mixed with collagen [4, 5]. Cell coverage on PPA hydrogels was similar to hydrogels containing such mixtures at day 1 (although not as good as on collagen only hydrogels). Moreover, it appeared that while HMEC-1 cells are stably adhered and proliferating on the PPA material between day 1 and day 7, the mixture of poloxamine methacrylate and collagen showed a decrease in cell number over time. This confirms the potential benefit of using PPA as a scaffold material.

![Figure 5-5. Live/Dead™ assay of HMEC-1 (200 000 cells/gel) seeded on top of different hydrogels (200 μL hydrogels in a 96 well-plate) assayed on day 1 (A-D) and day 7 (E-H) after](image)

In this study we were solely interested in evaluating the long term (i.e. over 7 days) ability of endothelial cells to attach and survive on hydrogels containing PPA, with the overall goal of determining the suitability of these hydrogels to be used for future in vitro experiments requiring cell culture on the surface of the hydrogels for several days. Nevertheless, for further characterization of the cell adhesion properties of the PPA polymer, it would be useful to also quantify the kinetics and extent of initial cell adhesion (i.e. within the first few hours after seeding the cells on the hydrogel surface). [This note was added for clarification purposes in this Ph.D. thesis after publication of the chapter in J Biomater. Sci. Polym. Ed. 2010; 22:2515-2528.]
seeding. (A,E) 5 wt% poloxamine methacrylate mixed with 6 wt% PPA, (B,F) 5 wt% poloxamine methacrylate, (C,G) 0.3 wt% collagen, (D,H) 5 wt% poloxamine methacrylate mixed with 0.2 wt% collagen. No or few dead cells are seen. A high number of HMEC-1 attach and survive for at least 7 days on photo-cross-linked hydrogel scaffolds containing PPA. The number of cells that attach and survive on PPA scaffolds was higher than on scaffolds made out of poloxamine methacrylate alone, both at day 1 and day 7, although not as high as on collagen only scaffolds. Scale bar = 100 μm.

The responsiveness of endothelial cells seeded on various hydrogel biomaterials and exposed to activation factors (TNF-α or LPS) was assessed through VCAM-1 and ICAM-1 staining and confocal microscopy (Figure 5-6). Under basal conditions, HMEC-1 express some ICAM-1, as expected, for cells on PPA. There was more ICAM-1 expression on hydrogels made of mixtures of poloxamine methacrylate and collagen. Upon exposure to inflammatory factors, ICAM-1 expression increased and some VCAM-1 expression also became apparent.

Figure 5-6. HMEC-1 (200 000 cells/gel) seeded on different hydrogels (200 μL hydrogels in a 96 well-plate). (A-D) Level of activation under basal conditions and responsiveness to inflammatory factors (E-H) TNF-α and (I-L) LPS, as measured by ICAM-1 (red) and VCAM-1 (green; see arrows) expression. Hoechst stain (blue) for the nucleus. (A,E,I) 5 wt% poloxamine methacrylate mixed with 6 wt% PPA, (B,F,J) 5 wt% poloxamine methacrylate, (C,G,K) 0.3 wt% collagen, (D,H,L) 5 wt% poloxamine methacrylate mixed with 0.2 wt% collagen. Scale bar = 100 μm.
4 Discussion

Poloxamine has been used by our group to overcome the mechanical limitations associated with collagen in modular tissue engineering applications. Poloxamine methacrylate hydrogels, as well as hydrogels made out of a mixture of collagen and poloxamine methacrylate, are mechanically stronger than collagen hydrogels. However, poloxamine methacrylate based hydrogels do not have the same cell attachment properties as collagen hydrogels. The present work focused on the chemical modification of poloxamine with the goal of creating a synthetic, cell adhesive, poloxamine-based material that would overcome these limitations.

A three step chemical synthesis method was developed to synthesize the poloxamine-polylysine acrylate polymer. First, acrylate groups were added to the polylysine molecule to allow further photocrosslinking. Then, the four hydroxyl end-groups on poloxamine were activated using tresyl chloride. Finally, the activated poloxamine was reacted with the acrylated polylysine to obtain the final product, poloxamine-polylysine acrylate. The final product was photocrosslinkable, cell compatible, and cell adhesive.

Poloxamine was activated with tresyl chloride in THF, and the tresylated poloxamine was recovered by precipitation in petroleum ether. Alternatively, we used dichloromethane and the dried product was washed several times at -20°C with methanol, similarly to a published methodology by Sperinde et al [13]. Using this alternative method, we obtained a high coupling efficiency (90-100% by ICP-AES for sulphur and thin layer chromatography), but the reaction yield was lower (around 50%), since much of the product was lost during the washing steps.

For the reaction of tresylated poloxamine with acrylated polylysine, we used DMF as a solvent and an organic base, TEA. We chose an organic solvent because the tresylated poloxamine is susceptible to hydrolysis, and therefore performing the reaction in an aqueous environment is typically less efficient, as the coupling reaction of poloxamine tresylate with polylysine competes with the hydrolysis of the tresylate groups. The denaturation of the polylysine molecule was not considered to be a relevant issue for this study, and the use of an organic solvent was appropriate in this case. However, if other peptides or proteins are to be linked to poloxamine in the future and depending on the nature of the study involved, it may be necessary to perform the reaction in an aqueous solvent in order to prevent protein denaturation.
Using similar synthesis methods, others have modified PEG to make it more cell adhesive. PEG attachment to proteins or peptides is most commonly accomplished through the side chain amino groups present in proteins and peptides. The lysine residues found in proteins or peptides are particularly useful for chemical modification, since they contain the ε-amino group which is a reasonably good nucleophile above pH 8. The carboxyl and thiol groups on proteins can also be used for PEGylation, as well as enzymatic methods, but they have been less commonly used compared to the methods making use of the amino groups [11, 14-17]. The side chain amino groups have been PEGylated using different activated PEG molecules reactive towards amino groups, such as PEG succinimidyl succinate, PEG succinimidyl carbonate, PEG p-nitrophenyl carbonate, PEG benzotriazol carbonate, PEG trichlorophenyl carbonate, PEG carbonylimidazole, PEG dichlorotriazine, or PEG tresylate [11]. We have used the tresylated form of poloxamine to react with the amino groups on the polylsine side chains. Tresylation is a relatively straightforward method to activate hydroxyl groups. Tresyl chloride is a very reactive reagent and the tresylation reaction has a high efficiency. For example, this method was used to activate PEG and link it to glutaminamide [18].

Instead of first activating PEG and then reacting it with the peptide or protein, others have added acrylate groups to both PEG and the peptide or protein, and subsequently photocrosslinked the PEG acrylate and peptide acrylate together by exposure to UV light in the presence of a photoinitiator [8, 9, 19]. Similarly, we added acrylate groups on each of the polylsine chains to enable photocrosslinking and hydrogel formation, but we chose to link the acrylated polylsine to poloxamine by reacting the amino groups on polylsine with the tresylated form of poloxamine. We chose this strategy in an attempt to maximize the number of polylsine chains linked to each poloxamine molecule, thus enhancing the cell attachment properties of the material. Simply mixing acrylated poloxamine and acrylated polylsine together would have presumably resulted in a less homogenous mixture, containing non-cell adhesive, poloxamine rich regions in with acrylated poloxamine molecules are linked together instead of being linked to polylsine molecules.

Polylysine was used here to confer cell attachment properties to the poloxamine-based polymer, while the acrylate groups make it photocrosslinkable. Moreover, the reactive amino groups on the polylsine side chains also make the chemical methods described here a useful platform for
future polymer synthesis aimed at introducing other peptides or proteins into the poloxamine based scaffold biomaterial.

Polylysine-coated tissue-culture surfaces (T-flasks, well plates) are commercially available and they are used to enhance the adhesion and growth of a variety of cell types, particularly neurons, glial cells, and numerous cell lines. Different groups have also used polylysine as a coating for various biomaterials in order to enhance the cell attachment properties of these biomaterials. Boura et al have shown that human umbilical vein endothelial cells (HUVEC) seeded onto multilayered polyelectrolyte (poly(L-glutamic acid)/poly(D-lysine)) films were able to attach to poly(D-lysine) surfaces, they maintained a normal morphology, and cell growth on these films was similar to that on tissue-culture polystyrene (TCPS). These polyelectrolyte multilayer films did not induce any cytotoxic effects and did not alter the phenotype of the endothelial cells. HUVEC seeded onto these surfaces were also able to resist shear stress under flow conditions better than HUVEC seeded on glass surfaces or on fibronectin [20, 21]. Another study showed similar results, with HUVEC being able to attach, spread, and become elongated on multilayered surfaces terminated with a poly(L-lysine) layer [22]. Budd et al showed that coating of polytetrafluoroethylene (PTFE) vascular grafts with poly(L-lysine) increased HUVEC attachment to the vascular grafts [23]. Poly(glycolic acid) scaffolds coated with poly(L-lysine) for cardiovascular tissue-engineering applications were shown to enhance cell attachment of myofibroblasts cells [24]. Others have chemically immobilized poly(D-lysine) to chitosan and showed that poly(D-lysine) improved neuron cell survival and adhesion [25].

Polylysine is also used for gene-delivery applications. However, various studies have shown that high-molecular-weight polylysine solutions, as well as polylysine-DNA complexes can be cytotoxic. A study performed using L929 mouse fibroblasts showed that the magnitude of the cytotoxic effects of polycations (polylysine and others) in solution was dependent on the molecular weight of the polymer, with higher molecular weights causing more cell damage due to the stronger electrostatic interactions with the cell membrane [26]. Another study showed that solutions of high-molecular-weight polylysine (average MW higher than 40 000) had cytotoxic effects on HUVEC and that the poly(D-lysine) configuration caused more cell damage than the poly(L-lysine) configuration [27]. The same authors showed in another study using HUVEC that the cytotoxic potency of poly(L-lysine) solutions increased over the 40 000 – 120 000 molecular weight range. However, unlike the high-molecular-weight polylysine solutions, cell-culture well
plates coated with high molecular weight poly(L-lysine) had no cytotoxic effect. Also, solutions of low-molecular-weight poly(L-lysine) (MW less than 25 000) were shown not to have a cytotoxic effect [28].

We used a poly(L-lysine hydrobromide) with an average molecular weight of 4200 Da (MW = 1000 – 5000 Da) for the synthesis of the polymer. We hypothesised that the number of lysine units and amino groups of the 4200 Da polylysine (20 lysine units on average) was adequate to allow both chemical modification and cell attachment, while not causing any cell damage. Moreover, the polylysine molecules are surface-bound in this case instead of being in soluble form, which was also expected to greatly reduce any potential cytotoxic effects. However, in future studies it may be useful to also test higher-molecular-weight polylysines for the synthesis of the polymer.

Using the synthesized PPA material, we made photocrosslinked hydrogel scaffolds by exposure to UV light, in the presence of a photoinitiator. HMEC-1 attached and survived on PPA hydrogels for at least 7 days after cell seeding. On the other hand, cell attachment and survival on hydrogels made exclusively of poloxamine methacrylate was very limited. This suggests that the polylysine chains attached to the poloxamine backbone were indeed able to confer cell attachment properties to the scaffold, as intended. Cell coverage on hydrogels made exclusively of collagen was nevertheless better than on PPA hydrogels. Thus, the synthesized PPA polymer appears to enhance endothelial cell adhesion on poloxamine based hydrogel surfaces, but cell attachment is not quite as good as on collagen only hydrogels.

Finally, we were interested in the responsiveness to inflammatory factors of HMEC-1 cells seeded on PPA scaffolds. Upon stimulation with activation factors, the endothelium undergoes changes which allow it to participate in the inflammatory response. LPS and inflammatory cytokines such as TNF-α stimulate the expression of endothelial cell surface adhesion molecules. These adhesion molecules are involved in the interaction between endothelial cells and circulating leucocytes. The upregulation of adhesion molecules on the endothelial cell surface following tissue injury or infection slows down the circulating leucocytes and facilitates their adhesion to endothelial cells, as well as their transmigration into the injured tissue.

ICAM-1 (CD54) is a 80-115 kDa single-chain membrane glycoprotein that is expressed constitutively by endothelial cells and whose expression is upregulated on the activated
endothelium [29]. ICAM-1 expression contributes to the inflammatory response by supporting the adhesion and diapedesis of various leucocytes. ICAM-1 is the receptor for leukocyte function-associated antigen-1 (LFA-1), expressed on neutrophils, monocytes, lymphocytes, and natural killer cells [30, 31]. VCAM-1 (CD106) is another adhesion molecule expressed by endothelial cells. It is a transmembrane glycoprotein of 110 kDa, expressed only on cytokine-activated endothelium [29]. VCAM-1 interacts with the very late antigen-4 (VLA-4), expressed on lymphocytes and monocytes [30, 31].

HMEC-1, an endothelial cell line, retained the typical endothelial cell expression pattern for ICAM-1 and VCAM-1, with constitutive expression of ICAM-1, but not VCAM-1. Upon activation by inflammatory factors, ICAM-1 expression increases and VCAM-1 expression is also observed [32]. We wanted to determine if the same pattern of expression of VCAM-1 and ICAM-1 was maintained when HMEC-1 were cultured on PPA hydrogels. As expected, HMEC-1 seeded on PPA hydrogels expressed some ICAM-1 under basal conditions, and upon exposure to inflammatory factors, there was increased ICAM-1 expression and some VCAM-1 expression was observed. This suggests that PPA hydrogels, similarly to the other hydrogels tested here, are able to maintain normal endothelial cell responsiveness to inflammatory factors.

5 Conclusions

A poloxamine-polylysine acrylate (PPA) polymer was synthesized using a three step chemical synthesis strategy. The synthesized PPA polymer enhanced endothelial cell adhesion on poloxamine based, photocrosslinked hydrogel scaffolds.

These chemical methods can be used in future studies to introduce other peptides or proteins into the scaffold. Of particular interest in the context of modular tissue engineering is the vascularization of the modular constructs in vivo. Since the endothelial cell phenotype is presumably strongly influenced by the choice of scaffold properties, the interactions between endothelial cells and the scaffold could potentially be controlled and exploited with the view of enhancing the vascularization process in vivo.
6 Acknowledgements

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Scaffold precoating with human autologous extracellular matrix for improved cell attachment in

al. Polylysine-functionalised thermoresponsive chitosan hydrogel for neural tissue engineering.


Chapter 6
Transduction with the Extracellular Matrix Protein Del-1 as a Means of Tipping the Angiogenic Balance in Endothelial Cells Incorporated in Modular Tissue Engineered Constructs

Abstract

Modular tissue engineering is a method of building vascularized tissue engineered constructs. Submillimeter-sized microtissues (‘modules’) coated with a layer of endothelial cells (EC; vascular component), and with embedded functional cells, are self-assembled into a larger, three-dimensional tissue. In this study we examined the use of Developmental endothelial locus-1 (Del-1), an extracellular matrix (ECM) protein with pro-angiogenic properties, as a means of tipping the angiogenic balance in EC incorporated in modular tissue engineered constructs. The motivation was to enhance the vascularization of these constructs upon transplantation in vivo. EC were transduced using a lentiviral construct to overexpress Del-1. The Del-1 EC formed more sprouts in a fibrin gel sprouting assay in vitro compared to eGFP (control) transduced EC, consistent with the expectation that Del-1 would enhance the angiogenic state of the EC. Del-1 EC also had a distinct profile of gene expression (upregulation of matrix metalloproteinase-9 (MMP-9), urokinase-type plasminogen activator (uPA), vascular endothelial growth factor (VEGF-A) and intercellular adhesion molecule-1 (ICAM-1); downregulation of angiopoietin-2 (Ang2)), also supporting the notion of ‘tipping the angiogenic balance’. Del-1 had no effect on EC proliferation or protection from apoptosis in vitro. On the other hand, contrary to our expectations, when Del-1 EC coated modules were implanted subcutaneously in a SCID/Bg

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animal model, the pro-angiogenic effect of Del-1 was less remarkable. We observed only a small increase in the number of blood vessels formed in Del-1 implants compared to the eGFP implants, and only few blood vessels formed at the implant site in both cases. This was due to limited EC survival after transplantation. We speculate that if we could improve EC survival in our study (for example by adding other pro-survival factors or supporting cells), we would see a greater Del-1 induced angiogenic benefit in vivo as a consequence of increased Del-1 secretion by a higher number of surviving cells.

1 Introduction

The lack of rapid vascularization of tissue engineered constructs upon transplantation remains a critical issue in tissue engineering. A vascular supply is required for circulation of nutrients, signaling molecules, gas exchange, and waste removal. In the absence of an internal vascular network that can rapidly connect to the host vasculature, the majority of the transplanted therapeutic cells do not remain viable. Here, we report a potential means of enhancing the vascularization of tissue engineered constructs. Our hypothesis was that we could use transduction with Del-1, an ECM protein shown to have angiogenic properties in vivo, as a means of tipping the angiogenic balance from a quiescent to a pro-angiogenic phenotype in EC incorporated in tissue engineered constructs.

Del-1 is a matricellular protein containing three EGF-like repeats, and two discoidin I-like domains [1]. Del-1 was shown to initiate angiogenesis in vivo in the absence of exogenous growth factors in hind-limb, cerebral and cardiac ischemia animal models [2-5]. In vitro, Del-1 was shown to support the attachment and migration of EC and to trigger the expression of the transcription factor HoxD3, which then induces the expression of a number of factors involved in angiogenesis, such as integrin αVβ3 and uPA [2]. Del-1 was also shown to protect EC from undergoing apoptosis upon exposure to inducers of both the intrinsic and extrinsic apoptosis pathways [6]. Del-1 also supports the adhesion, migration and proliferation of smooth muscle cells (SMC) [7]. Del-1 was also shown to interfere with lymphocyte function associated molecule-1 (LFA-1)-dependent leukocyte-EC adhesion [8] and thus act as an endogenous inhibitor of inflammation [8-11].
To fabricate the tissue engineered constructs in our study, we used a modular approach. Modular tissue engineering was first introduced by the Sefton group as a method to build larger tissue engineered constructs with an intrinsically built-in vascular component [12]. Sub-millimeter sized tissue units (modules) are typically made of collagen type I and EC are seeded on the outer surface of the modules. Vascular supporting cells or therapeutic cells of interest can be embedded inside the modules. Larger tissue structures are formed by mixing together several modules [13]. Using this modular approach, our group previously showed that when empty collagen modules coated with human umbilical vein endothelial cells (HUVEC) were implanted in the omental pouch of nude rats, HUVEC assembled into vascular tube-structures in the area between the modules, although the extent of vascularization was still limited, due to limited EC survival. A similar study in severe combined immunodeficient/beige (SCID/Bg) mice and with subcutaneous implants showed that fibronectin coating of the collagen modules prior to seeding with HUVEC significantly reduced the extent of HUVEC apoptosis in vivo, as well as in vitro, and contributed to a significant increase in tissue vascularization in vivo. Still in a SCID/Bg animal model, but using human microvascular endothelial cells (HMEC) to coat the modules, and with embedded human adipose derived mesenchymal stromal cells (adMSC), our group showed there was extensive tissue vascularization by day 21. However, in the absence of adMSC, there was limited EC survival in vivo and limited vascularization. In a Sprague-Dawley rat, drug-immunosuppressed, omental pouch model, allogeneic rat aortic endothelial cells (RAEC)-coated modules were able to give rise to a rich vascular network. The maturation and functionality of these blood vessels was improved through the addition of bone marrow derived rat MSC (bmMSC).

The results reported in this paper are an extension of these previous studies with modular tissue engineered constructs. The goal was to explore using Del-1 transduction as an alternative to MSC incorporation. Specifically, this study aimed to: (i) produce EC that overexpress Del-1; (ii) characterize in vitro the behaviour of the EC overexpressing Del-1 (gene expression, sprouting assay etc.); (iii) characterize in vivo the remodeling and the vascular network formed upon implantation of collagen modules coated with Del-1 EC in a SCID/Bg subcutaneous implant model, in the absence of any vascular supporting cells.
2 Materials and Methods

2.1 Cells

Primary HUVEC (Lonza) were maintained in EGM-2 cell culture medium (Lonza) at 37°C in a 5% CO₂ humidified air atmosphere, with cell culture medium changed every 2–3 days. HUVEC were stably transduced with HIV-1-based recombinant lentivirus encoding for either eGFP alone (eGFP HUVEC), or Del-1-IRES-eGFP (Del-1 HUVEC), with mouse Del-1 major cDNA, a kind gift from Dr. T. Quertermous lab, Stanford University. After transduction (MOI of 5, 24 hrs incubation with the lentivirus in EGM-2 medium supplemented with 8μg/mL protamine sulfate (Sigma)), transduced HUVEC were maintained in culture and used for experiments up to passage 6. The transduction efficiency was measured by flow cytometry for both eGFP and Del-1 HUVEC, and ~ 100% of cells were consistently eGFP⁺ throughout the cell culture period (Beckman Coulter Epics XL cytometer with Expo32 ADC version 1.1C software).

The lentivirus constructs were designed and prepared by Dr. J. Medin’s lab, University Health Network, Toronto. Briefly, the transfer vector (pDY.Del-1.IRES-eGFP or pDY.eGFP.WS; see Figure 6-1), the packaging plasmid (pCMVdeltaR8.91), and the envelope plasmid (pMDG), were first mixed together with polyethylenimine (PEI; Sigma-Aldrich). Next, 293T cells (cultured in DMEM medium (Sigma) supplemented with 10% FBS, 2mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma)) were incubated with the plasmid mixture for 16-18 hrs at 37°C and 5% CO₂ atmosphere. At the end of the incubation period, the medium was replaced with fresh cell culture medium. The supernatant was collected 24 and 48 hrs after the medium change, filtered (0.45μm filter), concentrated by ultracentrifugation, resuspended in DMEM medium, and stored at -80°C. The viral titer was determined by transduction of 293T cells.

2.2 Sprouting Assay

As adapted from [14, 15], Cytodex3™ beads (gelatin coated microcarriers, GE Healthcare) were coated with EC (0.6x10⁶ HUVEC/ 10³ beads in EGM-2 complete medium) by gentle shaking every 20 min for 4h at 37°C, followed by overnight incubation. The beads were resuspended in a fibrin gel the next day. To prepare the fibrin gel, a fibrinogen solution (Sigma; 2 mg/mL dissolved in saline solution) was first mixed with aprotinin (Sigma; 0.15 Units/mL). Next, the beads coated with HUVEC were resuspended in the fibrinogen solution (~400 beads/mL
fibrinogen solution). The beads and fibrinogen were then mixed with thrombin (Sigma; 0.625 Units/mL) in the wells of a 24 well plate and allowed to form a clot at 37°C for 20 min. EC culture medium was added to the wells after the clot was formed and changed periodically, as per standard cell culture protocols. Sprout formation was monitored over 1 week (day 1, day 4, and day 7). Pictures of the beads were taken using a Zeiss Axiovert light microscope with a 5x objective lens and equipped with a CCD camera (N=3; 10 beads/condition at each time point). The sprouts were counted and measured manually using ImageJ software (ImageJ 1.45, NIH).

2.3 qRT-PCR

HUVEC (Del-1 or eGFP) were plated on thin collagen gels (type-I bovine dermal collagen solution; PureCol, Inamed Biomaterials; 3.1 mg/mL) or on tissue culture treated polystyrene (TCPS) plates (5x10³ cells/cm²). The collagen gels were prepared by adding a thin layer of neutralized collagen solution to a non tissue culture treated cell culture dish (30µL/cm²) and allowing the collagen to gel for one hour at 37°C prior to seeding the cells. After 7 days in culture, the cells were lysed and the RNA was isolated using the Qiagen RNeasy® Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer’s instructions. The concentration and purity of the RNA were measured using a NanoDrop Spectrophotometer (ND1000, Thermo Scientific). The 260/280 ratio was greater than 2.0 for all samples. cDNA synthesis from RNA was performed using the SuperScript™ III First-Strand Synthesis kit (Invitrogen) according to the manufacturer’s instructions, with both random and oligo(dT) primers. The Applied Biosystems 7900HT Real-Time PCR system with the SDS 2.3 software (Applied Biosystems) was used for fluorescence detection during PCR (SYBR® Green chemistry). Gene-specific primers (Table 6-1) were designed using either Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) or PrimerBank (http://pga.mgh.harvard.edu/primerbank/) and synthesized by Sigma Genosys. The data analysis was performed as described elsewhere [16]. To perform the analysis, the individual threshold Cycle values (Ct) and the mean amplicon efficiencies (E) were estimated using the SDS 2.3 software and the LinRegPCR software (LinRegPCR version 12, Heart Failure Research Center, Netherlands), respectively. GAPDH was used as reference gene for the relative quantification. The normalized relative quantity (NRQ) of each gene of interest relative to the endogenous reference gene (GAPDH) was first calculated for each sample using the following formula:
Normalized Relative Quantity (NRQ) = \frac{E_{\text{GAPDH}}^{Ct}}{E_{\text{gene of interest}}^{Ct}}

The relative expression ratio for each gene of interest was then calculated as the ratio between the mean NRQ for Del-1 samples and the mean NRQ for eGFP samples for that gene of interest:

\text{Ratio (R)} = \frac{\text{NRQ}_{\text{Del-1}}}{\text{NRQ}_{\text{eGFP}}}

Results were presented as the relative expression ratio ± standard error of the mean (SEM), N=3, for each gene of interest. A t-test (paired, one tailed) statistical analysis was performed on the log transformed NRQ to determine if there are any statistical differences between paired Del-1 and eGFP samples. Differences were considered significant when 0.67 ≤ R ≥ 1.5 and p < 0.05 (*) or p < 0.10 (**).
Table 6-1. Primer sequences for qRT-PCR.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Forward (F) and Reverse (R) Primers</th>
<th>GenBank Accession</th>
</tr>
</thead>
</table>
| GAPDH     | F: AAATTGAGCCGCCAGCAGCTCCC  
R: TGAACCGACGCCACATCAGAC | NM_002046.3       |
| Del-1     | F: GGGTTAATGCTGTTCCTGAGGCG  
R: CAACCAAAAGGCTGCTGCTACC | AF031524.1       |
| Ang2      | F: CAAAACAGCTAAAGGCACCCCATGTG  
R: CGCTGCCATCCTCAGTCGCA | NM_001147.2       |
| bFGF      | F: ATCAAAAGGAGTGTGCTAACC  
R: ACTGCCAGCTGTTTCAGTG | NM_002006         |
| CXCL12    | F: ATGCCCATGCGATTCTTCG  
R: GCCGAGGTAGATCAGG | NM_000609         |
| PDGF-B    | F: TCTCTGCTGCTACCCTGCTG  
R: CAAGGGCTAGGATGCTGGGCAGCTG | NM_002608.2       |
| VEGF-A    | F: CAACATCACCATGCAAGATTATGC  
R: GCTTTTCGTGCTCCTTCCTTC | NM_003376         |
| HoxD3     | F: AGAGCTCGACAAGCACTCCAAG  
R: GCGTTCCGTGAGATTCAGC | NM_006898         |
| Integrin β3 | F: ATACCCTGCGACCTGCTTGTG  
R: AGGCACACGTGCTGACTACAACCTG | NM_00212.2       |
| ICAM-1    | F: TTGAACCCACAGTACACCTAT  
R: CCTCTGGCTGCTCAGAATCA | NM_000201         |
| uPA       | F: AGCGACTCCAAAGGCAGGAAATGAG  
R: TTCTTTGGGACTGCTGACGAC | NM_002658.3       |
| MMP2      | F: GGAAAGGCAGGATCCATTCTT  
R: ATGCCAGCTTAACTTGGA | NM_004530         |
| MMP9      | F: AGACGGGTATCCCTCCGACG  
R: AAACCGAGTTGGAAACCACGAC | NM_004994         |
| MMP14     | F: GAAGCCTTGCTACAGCAATATG  
R: TGCAAGCCCGTAAAACCTTCGC | NM_004995         |

2.4 Implants

Sub-millimeter sized collagen cylinders (modules, initial size ~ 2 mm long and 0.6 mm in diameter) were prepared by neutralising and gelling a type-I bovine dermal collagen solution (PureCol, Inamed Biomaterials; 3.1 mg/mL) inside polyethylene tubing (Intramedic™ PE60, Becton Dickson), followed by cutting of the tubing into small pieces with an automatic tube cutter (FCS Technology Inc.), as described elsewhere [13]. The collagen modules were seeded with either Del-1 HUVEC or eGFP HUVEC overnight (~4x10⁶ HUVEC/mL of settled modules). The next day, approximately 200 modules in 0.1 ml PBS were injected subcutaneously, through an 18 gauge needle, in the dorsum of SCID/Bg mice (6–7 weeks of age, male, Charles River Laboratories; N=4), similar to [17]. After surgery, mice were individually housed in sterile cages.
and provided free access to sterilized food and water under the approval of the University of Toronto animal care committee.

2.5 Immunohistochemistry

2.5.1 *In Vivo* Samples

The modules were explanted 7, 14 and 21 days after the implant, fixed in 10% neutral buffered formalin and processed for immunohistochemistry. Paraffin embedded, 4 µm thick sections were analysed using the following stains and antibodies: Hematoxylin and Eosin (H&E), Masson Trichrome, anti-GFP (Abcam #AB6556, 1:1000, rabbit polyclonal antibody, detects (e)GFP expressing cells), Biotinylated Ulex Europaeus Agglutinin I (UEA I) (Vector Laboratories #B1065, 1:400, binds to human endothelial cells), anti-CD31 (Santa Cruz Biotechnology Inc. #SC-1506-R, 1:2000, rabbit polyclonal antibody, detects both mouse and human CD31), and anti-α Smooth Muscle Actin (SMA) (Sigma #A5228, 1:200, mouse monoclonal antibody, detects both mouse and human αSMA).

*In vivo* Blood Vessel Counts: The number of donor-derived (GFP+) blood vessels (with defined lumen), as well as the total number of blood vessels (donor+host, CD31+) present at the implant site were counted manually using digitized histology slides (ScanScope XT brightfield scanner, Aperio Technologies; 20X objective lens) and the Aperio ImageScope viewing software (Aperio Technologies; version 11). The number of vessels in the implant area was normalized to the area occupied by the implant on the whole histological section to obtain the vessel density at the implant site (N=4). The diameter of the GFP+ and CD31+ vessels was manually measured using the Aperio ImageScope viewing software. Vessels were binned based on their size, with capillaries < 9 µm, small arterioles or venules 9-15 µm, large arterioles or venules 15-75 µm, and other (abnormal) ≥ 75 µm, similarly to [18]. Digitized SMA-stained histology slides were analysed using the Positive Pixel Count Algorithm available with the Aperio ImageScope software to determine the SMA density at the implant site (N=4).

2.5.2 *In Vitro* Samples

Some modules were collected immediately after coating with HUVEC (day 0 *in vitro* samples, N=4) or were cultured *in vitro* for an additional 7 days under standard cell culture conditions (day 7 *in vitro* samples, N=4). Samples were collected and fixed in 4% paraformaldehyde
(Electron Microscopy Sciences), embedded in a 2% agarose block (Roche Diagnostics), processed for histology (paraffin sections) and stained with an anti-Ki67 antibody (Novus Biologicals #NB110-90592, 1:1000, rabbit polyclonal antibody) to detect proliferating cells. Some modules were also kept and cultured in vitro for 7 days in complete medium, then cultured for another 7 days in serum-free medium to induce apoptosis (apoptosis in vitro samples). Modules were then collected and fixed in 4% paraformaldehyde, embedded in a 2% agarose block, processed for histology (paraffin sections) and stained with an anti-cleaved caspase 3 antibody (Cell Signaling Technology #9661, 1:600, rabbit polyclonal antibody) to detect apoptotic cells.

**Cell Counts for In Vitro Samples:** Proliferating cells (Ki67+) and apoptotic cells (cleaved caspase 3+) were counted on histology slides prepared from in vitro samples. An Olympus BX61 light microscope with a 20X objective lens and equipped with a Olympus DP70 camera was used to take up to 5 pictures (hot spots) per sample (N=4, n ≤ 5). ImageJ software (ImageJ 1.45, NIH) was used to analyze the images (manual counting) and the average of the counts/sample was used for statistical analysis.

### 2.6 Statistical Analysis

A one-way analysis of variance (ANOVA) with LSD post hoc was used to compare means between multiple groups, unless otherwise noted. Differences between means were considered statistically significant at p < 0.05. Statistical analysis was performed with the SPSS Statistics software (IBM Corp.; version 20).

### 3 Results

#### 3.1 Delivery of Del-1 through Lentiviral Transduction

The maps of the transfer vectors included in the lentiviral constructs are shown in Figure 6-1. The transfer vectors contained either the Del-1-IRES-eGFP sequence (Figure 6-1A), or eGFP alone, as control (Figure 6-1B). The eGFP sequence was included in both Del-1, and control eGFP vectors to enable tracking of implanted HUVEC in vivo.
Figure 6-1. Maps of transfer vectors included in lentiviral constructs: (A) vector containing Del1-IRES-eGFP sequence; (B) vector containing eGFP alone sequence, used as control for experiments.

Modules coated with transduced HUVEC were analyzed by immunohistochemistry immediately after fabrication to confirm eGFP expression by both Del-1 and eGFP HUVEC and also to confirm good coverage of the modules with the HUVEC prior to transplantation (Figure 6-2). Qualitatively, the histology images indicated that all the EC (UEA-1+, CD31+) also expressed eGFP (GFP+). Additionally, good cell coverage was observed for both Del-1 and eGFP HUVEC. It was important to verify that similarly good cell coverage was obtained for both Del-1 and eGFP HUVEC to ensure that equally high numbers of cells were transplanted in both cases.
Figure 6-2. Representative histology images of modules \textit{in vitro} immediately after fabrication. The samples were serially cut; the same modules are seen with the different histology stains. All of the EC (UEA-1+, CD31+) appeared to express eGFP (GFP+). Good EC coverage of the modules was observed for both Del-1 and eGFP samples. Scale bar is 100μm for lower magnification images and 50μm for higher magnification (black squares indicate areas that are shown in higher magnification images). Red circle highlights one individual module. Each oval structure in the high magnification images also represents one module.

3.2 Sprouting Assay

Sprouting of Del-1 HUVEC was compared to eGFP HUVEC, from the surface of gelatin coated microcarriers (Cytodex3™ beads) in a fibrin gel (Figure 6-3). Del-1 HUVEC formed significantly more sprouts compared to eGFP HUVEC. The differences in number of sprouts were significant (p < 0.05) as early as day 1 after suspending the HUVEC coated beads in the fibrin gel, and the differences remained significant at day 4 and day 7 as well (Figure 6-3B). The sprouts formed by the Del-1 cells were also significantly (p < 0.05) longer than eGFP for all time points (Figure 6-3C). For both Del-1 and eGFP cells, the sprouts elongated from day 1 to day 4 (with less pronounced elongation observed for eGFP cells), while simultaneously decreasing in number. The initial elongation from day 1 to day 4 was followed by regression of the sprouts.
between day 4 and day 7 (decrease in both number and length of sprouts) for both Del-1 and eGFP cells. This is expected for EC, especially in the absence of supporting cells (smooth muscle cells, fibroblasts, mesenchymal stem cells etc.) that were shown to delay sprout disintegration \textit{in vitro} [14, 19].
Figure 6-3. Cytodex3™ bead fibrin gel sprouting assay. (A) Representative images of sprouts. Scale bar = 100μm. (B) Del-1 HUVEC formed more sprouts compared to eGFP as early as day 1, and more sprouts were apparent for Del-1 for at least 7 days in culture. (C) The length of the sprouts was also greater for Del-1 compared to eGFP for all time points. Sprouts were measured for 10 beads per condition. Graphs show average ± SEM; N=3 (n=10); ANOVA with LSD post hoc and p < 0.05 (*) considered significant.

3.3 qRT-PCR

qRT-PCR analysis was performed to identify differences in gene expression (mostly genes involved in angiogenesis) for Del-1 HUVEC compared to eGFP HUVEC. Specifically, we compared the expression of the ECM molecule Del-1, the expression of some growth factors (Ang2, basic fibroblast growth factor (bFGF), stromal cell-derived factor 1 (SDF-1 or CXCL12), platelet-derived growth factor (PDGF-B), VEGF-A), the expression of the transcription factor HoxD3, of integrin β3, of ICAM-1 and of some proteases (uPA and matrix metalloproteinases MMP-2, MMP-9, MMP-14). The gene expression results are shown in Figure 6-4.

qRT-PCR data confirmed Del-1 expression by cells transduced with the integrating Del-1 lentivirus. Del-1 HUVEC expressed 20 and 18 times more Del-1 on average than eGFP HUVEC (p < 0.05) when cultured on TCPS and on collagen gels, respectively (Figure 6-4A).
qRT-PCR data also showed that Del-1 HUVEC had a distinct expression profile of genes involved in angiogenesis compared to eGFP, particularly when cells were cultured on TCPS. Differences in gene expression were small, but statistically significant. Specifically, when cultured on TCPS for 7 days, Del-1 HUVEC differentially expressed Ang2, VEGF-A, ICAM-1, uPA, and MMP-9 compared to eGFP (Figure 6-4B). When cultured on collagen, MMP-9 was upregulated in Del-1 HUVEC compared to eGFP (Figure 6-4C). For the rest of the genes analyzed, there was no significant difference.
Figure 6-4. Expression of genes involved in angiogenesis in Del-1 vs. eGFP HUVEC. (A) Del-1 HUVEC expressed much higher levels of Del-1 mRNA compared to eGFP. (B) When cultured on TCPS for 7 days, Del-1 HUVEC differentially expressed Ang2, VEGF-A, ICAM-1, uPA, and MMP-9 compared to eGFP. Differences were small, but statistically significant. (C) When cultured on collagen, MMP-9 was upregulated in Del-1 HUVEC compared to eGFP. Data is
presented as ratios of relative gene expression (Del-1 vs. eGFP, relative to GAPDH). Graphs show average of ratios ± SEM; N=3; paired t-test one tailed with p < 0.05 (*) or p < 0.10 (**) and 0.67 ≤ Ratio ≥ 1.5 highlighted.

3.4 *In Vitro* Proliferation

We did not observe any statistically significant difference in cell proliferation (Ki67+ cells) for Del-1 HUVEC vs. eGFP, both initially after seeding the cells onto the collagen modules (Day 0), as well as after culturing the EC coated modules for 7 days *in vitro* (Day 7) (Figure 6-5). We observed a higher number of proliferating cells at Day 0 compared to Day 7 (5-10 times more proliferating cells) for both Del-1 and eGFP HUVEC, although the number of proliferating cells was still low at both Day 0 and Day 7.

**Figure 6-5.** Proliferation of HUVEC *in vitro*. (A) Quantitative analysis of proliferating HUVEC *in vitro*. Significantly more proliferating HUVEC were observed immediately after seeding the modules with HUVEC (‘Day 0’) compared to after keeping the modules for 7 days in culture *in vitro*. No significant differences were observed between the proliferative state of Del-1 HUVEC vs. eGFP, both at day 0 and after 7 days in culture. Proliferating cells were counted in up to 5 hot-spots (20x objective lens) per sample. Graph shows average ± SEM; N=4 (n≤5); ANOVA with LSD post hoc and p<0.05 (*) considered significant. (B) Representative histology images of
proliferating HUVEC at day 0 and after 7 days in culture in vitro. Each oval structure represents one module. Scale bar = 50µm.

3.5 In Vitro Apoptosis

We did not observe any statistically significant difference in the number of apoptotic cells between Del-1 and eGFP HUVEC, when HUVEC were cultured under serum-free conditions on collagen modules, although there were slightly more (1.2X) apoptotic cells for the Del-1 samples (Figure 6-6).

![Cleaved Caspase-3+ Cell Counts](image)

Figure 6-6. HUVEC apoptosis in vitro. (A) Quantitative analysis of apoptotic HUVEC in vitro. No significant differences were observed between Del-1 HUVEC vs. eGFP, when cultured under serum-free conditions for 7 days to induce apoptosis. Apoptotic cells were counted in up to 5 hot-spots (20x objective lens) per sample. Graph shows average ± SEM; N=4 (n≤5); ANOVA with LSD post hoc and p<0.05 (*) considered significant. (B) Representative histology images of apoptotic HUVEC in in vitro culture. Each oval structure represents one module. Scale bar = 50µm.
3.6 In Vivo Vascularization

Representative images of the visual appearance of the tissues at explant are shown in Figure 6-7, with some blood vessels visible (red color) inside the implant area. Figure 6-8 shows various histological stains of the implants at day 7, 14 and 21. Implanted HUVEC migrated off the surface of the modules and formed blood vessels in the area between the modules. Trichrome and H&E histology images show the presence of red blood cells in some of these blood vessels as early as day 7, suggestive of perfusion (Figure 6-8). The average density of both donor-derived (GFP+) and total number of blood vessels (CD31+) was slightly higher for Del-1 implants compared to eGFP at both day 7 and day 14 (Figure 6-9A and 6-9B), although these differences were small. Both the number of donor-derived, as well as the total number of blood vessels remained relatively constant over time, with a slight decrease at day 14, followed by recovery at day 21 (Figure 6-9A and 6-9B). Some SMA staining was present in the implant area (Figure 6-9C), and some of the blood vessels were invested with a layer of SMA+ cells. The average size of the blood vessels at the implant site did not change over time and was similar between Del-1 and eGFP implants (Figure 6-10). The binning of these vessels based on size is shown in Table 6-2.

![Figure 6-7](image.png)

**Figure 6-7.** Representative photographs of tissues at explant. Some blood vessels are visible (red color) within the implant area. Arrows indicate the location of implants.
Figure 6-8. Representative histology images of explants at (A) Day 7; (B) Day 14; (C) Day 21 after implantation (serially-cut tissue samples were treated with the different stains and antibodies). Implanted HUVEC migrated off the surface of the modules and formed blood vessels in the area between the modules (GFP+ for donor-derived vessels; CD31+ for donor- OR host-derived EC-lined blood vessels). Examples of donor- or host-derived blood vessels are indicated with arrows. The majority of blood vessels at the implant site were donor-derived; examples of predominantly host-derived blood vessel are indicated with (*). Some of these blood
vessels (both donor- and host-derived) were invested with smooth muscle cells (SMA+; examples indicated with arrows), perhaps suggestive of incipient vessel maturation. Some of the blood vessels (both donor- and host-derived) also contained erythrocytes (examples indicated with arrows), suggestive of connection to the host vasculature. Scale bar is 100μm for lower magnification images and 50μm for higher magnification. Black squares indicate areas that are shown in higher magnification images. Red circle highlights one individual module.

![Graph showing total number of GFP+ tube-like structures](image-url)

**A)**

**Total Number of GFP+ Tube-like Structures**

(no of vessels/mm²)

<table>
<thead>
<tr>
<th>Day</th>
<th>Del-1 HUVEC</th>
<th>eGFP HUVEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 7</td>
<td>15 ± 2</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>Day 14</td>
<td>10 ± 1</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>Day 21</td>
<td>15 ± 2</td>
<td>12 ± 1</td>
</tr>
</tbody>
</table>
Figure 6-9. Density of blood vessels and SMA staining at the implant site. Transplantation of Del-1 HUVEC (black bars), led to a slightly higher number of donor-derived blood vessels (GFP+; Fig. 6-9A), as well as total number of blood vessels (donor+host, CD31+; Fig. 6-9B), and SMA staining (Fig. 6-9C) compared to eGFP HUVEC (patterned bars). The number of
blood vessels observed at day 7 remained relatively constant over time, with a slight decrease at day 14, followed by recovery at day 21, although these differences were small. All vessels (with defined lumen) were counted and normalized to the area occupied by the implant on the whole histological section. The Aperio ImageScope Positive Pixel Count Algorithm was used for SMA staining. Graphs show average ± SEM; N=4; ANOVA with LSD post hoc and p < 0.05 (*) considered significant.
Figure 6-10. Average size of blood vessels at the implant site. All donor-derived (GFP+) or donor+host (CD31+) EC-lined vascular tubes within each implant were manually measured using digitized whole slides and the Aperio ImageScope viewing software. The average GFP+ or CD31+ blood vessel size per implant was calculated. No significant change in average vessel
size was observed over time, or between Del-1 and eGFP implants. Graphs show average ± SEM; N=4; ANOVA with LSD post hoc and p < 0.05 (*) considered significant.

Table 6-2. Blood vessel size distribution for HUVEC implants.

<table>
<thead>
<tr>
<th>Size Distribution of GFP+ Vessels</th>
<th>Size Range</th>
<th>Day 7 Del-1</th>
<th>eGFP</th>
<th>Day 14 Del-1</th>
<th>eGFP</th>
<th>Day 21 Del-1</th>
<th>eGFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillaries</td>
<td>&lt; 9 µm</td>
<td>34%</td>
<td>35%</td>
<td>36%</td>
<td>39%</td>
<td>39%</td>
<td>43%</td>
</tr>
<tr>
<td>Small Arterioles or Venules</td>
<td>9-15 µm</td>
<td>43%</td>
<td>50%</td>
<td>56%</td>
<td>40%</td>
<td>46%</td>
<td>46%</td>
</tr>
<tr>
<td>Large Arterioles or Venules</td>
<td>15-75 µm</td>
<td>23%</td>
<td>15%</td>
<td>9%</td>
<td>21%</td>
<td>15%</td>
<td>11%</td>
</tr>
<tr>
<td>Other (Abnormal)</td>
<td>≥ 75 µm</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
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</table>

<table>
<thead>
<tr>
<th>Size Distribution of CD31+ Vessels</th>
<th>Size Range</th>
<th>Day 7 Del-1</th>
<th>eGFP</th>
<th>Day 14 Del-1</th>
<th>eGFP</th>
<th>Day 21 Del-1</th>
<th>eGFP</th>
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<tbody>
<tr>
<td>Capillaries</td>
<td>&lt; 9 µm</td>
<td>24%</td>
<td>30%</td>
<td>34%</td>
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<td>24%</td>
<td>24%</td>
<td>26%</td>
<td>24%</td>
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<tr>
<td>Other (Abnormal)</td>
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<td>0%</td>
<td>0%</td>
<td>1%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Note: The percentages represent average value for N=4.

4 Discussion

4.1 Increased Sprouting and Differential Angiogenic Gene Expression for Del-1 HUVEC

Some in vitro results (sprouting, mRNA) were consistent with the expectation that Del-1 can enhance the state of transduced HUVEC. The sprouting assay was used here as an in vitro measure of the angiogenic state of Del-1 HUVEC compared to eGFP HUVEC. We observed an increase in the number and length of sprouts for Del-1 HUVEC compared to eGFP. We speculate that the increased sprouting for Del-1 HUVEC may be, at least in part, a consequence of increased protease activity (MMPs, uPA etc.). One of the steps involved in the sprouting of EC from the surface of the beads is the degradation of the surrounding ECM by the cells in order to
form vascular guidance channels in the matrix, and several literature reports showed the involvement of both MMPs and uPA in sprout formation in fibrin gels [19-21]. Our qRT-PCR clearly showed that Del-1 HUVEC expressed more MMP-9 and uPA compared to eGFP, although measured on collagen gels and TCPS, not on fibrin. The upregulation of these proteases in Del-1 HUVEC is consistent with a more invasive and pro-angiogenic state for Del-1 HUVEC compared to eGFP.

From an *in vivo* angiogenesis perspective, proteases such as MMP-9 and uPA, which we found upregulated in Del-1 HUVEC, are necessary to break down and remodel the ECM in a localized manner, and allow the EC to form vascular channels and migrate into these spaces to eventually re-assemble into tube-like structures and form blood vessels [19-24]. Moreover, other than tissue remodelling in itself, ECM proteolysis also has consequences in terms of exposing pro-angiogenic cryptic sites within ECM proteins, thus facilitating cell attachment to these sites and further contributing to tissue vascularization. In addition, proteases also play an important role in releasing various growth factors otherwise sequestered in the ECM, thus further stimulating blood vessel formation. For example, release of matrix sequestrated VEGF [25] and exposure of a pro-angiogenic cryptic site in collagen IV are among the known *in vivo* functions of MMP-9 [26], while uPA has been shown to proteolytically activate HGF (hepatocyte growth factor, another growth factor involved in angiogenesis) [27].

Other than the increased expression of MMP-9 and uPA mRNA, we observed a distinct pattern of gene expression for Del-1 vs. eGFP HUVEC for other factors involved in angiogenesis as well. VEGF-A was upregulated in Del-1 HUVEC compared to eGFP when cells were cultured on TCPS. As reviewed by others [28-30], VEGF is a potent mitogen and pro-survival factor for EC, it stimulates sprout formation in *in vitro* models, and it was shown to stimulate angiogenesis *in vivo*. The measured upregulation of VEGF by Del-1 EC may thus play an important role in autocrine regulation of Del-1 EC behavior and is consistent with the reported pro-angiogenic properties of Del-1.

We also observed a tendency for Ang2 downregulation in Del-1 HUVEC compared to eGFP when cells were cultured on TCPS. Under physiological conditions, Ang2 expression is low. Ang2 is upregulated by hypoxia, at sites of vascular remodelling, or under pathological conditions (for example tumor growth), and it is involved in both vascularization and vessel
regression, depending on the context. In a rat glioma model, it was shown that overexpression of Ang2 in tumor cells led to impaired SMC coverage of the tumor blood vessels and impaired vascularization. On the contrary, in combination with growth factors such as VEGF, Ang2 enhances VEGF-induced angiogenesis [as reviewed by [31]].

ICAM-1 was also upregulated in Del-1 HUVEC compared to eGFP when cells were cultured on TCPS. Physiologically, ICAM-1 is expressed at low basal levels on the surface of EC and it is upregulated on the activated endothelium, such as during the inflammation process associated with angiogenesis. The ICAM-1 upregulation may therefore confirm the enhanced angiogenic state of Del-1 EC relative to eGFP. The upregulation of ICAM-1 perhaps also suggests a compensatory mechanism triggered in Del-1 transduced cells, as Del-1 was shown to inhibit leukocyte-endothelial cell binding via its antagonizing interaction with the lymphocyte function associated molecule (LFA-1) [8], the main leukocyte ligand of ICAM-1 [32].

We recognize that the fold changes for the genes differentially regulated in Del-1 versus eGFP HUVEC were not very high (less than 2X), and in combination with the inherent sample variability, it also led to marginally statistically significant differential mRNA expression in some cases (p<0.05[*] criterion was used for mRNA expression analysis in some cases, but p<0.10 [**] in others). This is however not surprising given the multitude of factors other than Del-1 (such as other ECM, growth factors in the cell culture medium) that the HUVEC were exposed to, and likely also the higher total amount of these other factors compared to Del-1 in this in vitro 2D culture system. It is therefore encouraging that Del-1 was still able to trigger a distinct, although subtle, increase in pro-angiogenic gene expression in the presence of a multitude of other pro-angiogenic molecules.

In contrast with the above data in support of “tilting the balance”, there was other data that did not support this notion. In our hands, Del-1 transduced EC did not show upregulation of the pro-angiogenic transcription factor HoxD3 or the integrin αβ3 compared to eGFP HUVEC, contrary to literature reports [2, 33]. We speculate that the differences with the literature might be due to the intrinsic experimental differences between the means of exposure of cells to Del-1, specifically a continuous/chronic mode of exposure in our case versus an acute mode of exposure in the literature reports, perhaps with a higher amount of Del-1 present as well in the latter case (although we could not measure the amount of Del-1 present in our study to confirm). RT-PCR
literature reports were based on characterizing EC plated on basement membrane ECM and with Del-1 protein added to the culture medium (10μg/mL Del-1 protein) for 24 hrs [2]. On the other hand, we transduced EC to stably express Del-1 and characterized these cells after 7 days in culture. In our experimental model, the cells therefore continuously produced and were exposed to Del-1 during the 7 days in culture. Others have shown that chronic exposure of microvascular endothelial cells to TNF-α through stable retroviral transduction leads to a distinct pattern of gene expression compared to acute exposure through adding TNF-α to the cell culture medium, with only 36% of the changes in gene expression relative to non-activated cells being similar between the acute and chronic modes of exposure (microarray analysis) [34]. In addition, we also cannot exclude the possibility that the Del-1 induced pro-angiogenic effects that we observed in our study were in fact due to intracellular Del-1-mediated pro-angiogenic signaling (if Del-1 was not secreted by the EC), rather than due to signaling triggered by integrin binding to Del-1 after Del-1 deposition in the ECM, as established in all previous literature reports. If this was the case, it is conceivable that molecules other than HoxD3 and αβ might be involved in the intracellular Del-1-mediated signaling, although the identity of these other molecules is currently unknown. However, to our knowledge there are no reports in the literature indicating any biological effects of intracellular Del-1; this topic certainly warrants further investigation.

4.2 No Difference in Proliferation or Anti-Apoptotic Effect In Vitro

Our in vitro proliferation results are consistent with literature reports showing that Del-1 did not affect proliferation of EC [6]. The decrease in proliferation over time was also expected, as HUVEC on collagen modules form a confluent, quiescent cell layer on the surface of the collagen modules after a few days in culture in vitro [12, 35, 36].

On the other hand, our apoptosis results were contradictory to a literature report comparing HUVEC plated on Del-1 coated plates versus HUVEC plated on bovine serum albumin (BSA) coated plates, and exposed to inducers of apoptosis (TNFα+IFNγ, or etoposide). The Del-1 coated surfaces protected HUVEC from apoptosis by comparison to BSA surfaces, an effect mediated by integrin binding and adhesion to Del-1 (2-10 fold decrease in apoptosis, depending on the assay) [6]. We suspect that in contrast to Del-1, the BSA surfaces did not provide strong attachment of HUVEC, as BSA does not have integrin binding sites and does not support focal adhesion formation, and thereby BSA surfaces facilitated apoptosis. On the contrary, in our case,
both Del-1 and eGFP HUVEC were seeded onto collagen modules, thus having integrin binding in both cases (although perhaps via a different combination of integrins); therefore, integrin binding to Del-1 did not elicit any significant additional anti-apoptotic benefit in our case.

4.3 *In Vivo Vascularization*

HUVEC are susceptible to undergo apoptosis upon transplantation even in immuno-compromised animal models such as SCID/Bg mice [37, 38], presumably triggered by the hostile inflammatory response, as well as by the hypoxic, nutrient-poor environment. Others have improved the outcome of the transplantation by using genetic manipulation with anti-apoptotic genes (transduction of EC with Bcl-2 in [38, 39]), by changing the composition of the ECM to improve HUVEC survival (added fibronectin in [37]), or by co-transplantation with supporting cells (bmMSC or adMSC in [17, 18]). In this first *in vivo* study using Del-1 transduced EC, we did not use any supporting cells or any additional pro-survival factors other than Del-1, relying on the literature reported properties of Del-1 to compensate for the absence of MSCs.

Some blood vessels formed in the implant area, and the presence of red blood cells inside the lumen of some of these blood vessels suggests they were perfused, although potentially only transiently, since these blood vessels also had limited SMA+ coverage, and it is likely that they failed to fully mature. Although the *in vitro* results showed a clear Del-1 induced angiogenic effect, the *in vivo* angiogenic benefits of Del-1 in our system were less dramatic, as we observed only a small increase in the number of blood vessels formed in Del-1 implants compared to the eGFP implants. We suspect that a limited number of HUVEC survived after transplantation, and as a consequence, a limited amount of Del-1 was also produced at the implant site by the remaining surviving HUVEC. Others in our group also showed that when EC were transplanted alone in collagen modules, limited or even no blood vessel formation occurred (depending on the type of EC used). In a study examining human microvascular EC (HMEC) transplantation subcutaneously in the same SCID/Bg mouse model, zero vessels were found at day 3, 7, and 14 post-transplantation. In fact, by 14 days, no HMEC were found at the implant site, presumably due to extensive cell death. Unlike HMEC and similarly to what we found in our study, some HUVEC were still present at the implant site at day 14, but they formed only few blood vessels around the modules (qualitative visual observation) [17]. In another study using the same animal model, coating of the collagen modules with fibronectin prior to seeding with HUVEC
significantly decreased the number of apoptotic cells \textit{in vivo} (by 40%; TUNEL staining) over the first three days after transplantation, which resulted in a 2X increase (microvascular density hot-spot counts) in the number of HUVEC-derived blood vessels at 7 and 14 days post-transplantation [37].

We speculate that if we could improve HUVEC survival in our study (for example by adding other pro-survival factors or supporting cells), we would see a greater Del-1 induced angiogenic benefit \textit{in vivo} as a consequence of increased Del-1 secretion by a higher number of surviving cells.

\subsection*{4.4 Platform for Future Manipulations}

The modular approach, along with lentiviral transduction of EC with pro-angiogenic molecules, can serve as a uniquely versatile platform for future studies. First of all, transduction of EC with various molecules of interest is a highly adaptable \textit{in vivo} delivery method, where cells act not only as drivers of \textit{in vivo} tissue reconstruction, but also as delivery vehicles. Secondly, the modular approach is intrinsically versatile, as one can envision that modules coated with EC transduced to overexpress various molecules of interest can be mixed together in controlled ratios to elicit a desired outcome \textit{in vivo}. Alternatively, the same strategy can be used as a screening tool to better understand \textit{in vivo} responses to various combinations of stimuli and better inform the design of tissue engineered constructs. Moreover, the lentiviral delivery system could be further manipulated to generate transient instead of permanent expression of certain molecules by using non-integrating instead of integrating lentiviruses.

We propose that our system can be further improved \textit{in vivo} by using a combination of pro-angiogenic and pro-survival signals rather than using Del-1 alone, and these signals may be a combination of ECM factors and growth factors. The cross-talk and synergy between integrins and growth factor receptors in angiogenesis is well recognized and is the subject of several reviews [40-42]. For example, [43] described cross-activation between the $\beta_3$ subunit of integrin $\alpha_v\beta_3$ and vascular endothelial growth factor receptor 2 (VEGFR2) in EC. Careful selection of combinations of ECM molecules (to bind specific integrins) and growth factors (to bind specific growth factor receptors) may lead to increased vascularization of tissue engineered constructs by comparison to delivery of growth factors alone or ECM molecules alone.
5 Conclusion

Here, we used transduction with the pro-angiogenic ECM protein Del-1 as a means of tipping the angiogenic balance in endothelial cells incorporated in modular tissue engineered constructs. At any given time, the EC receive a combination of pro- and anti-angiogenic signals from their environment, and the ratio of these signals determines the EC state (i.e. angiogenic vs. quiescent). We aimed to tilt this balance through Del-1 delivery and we showed that EC transduced to overexpress Del-1 have increased sprouting activity and a distinct angiogenic gene expression profile compared to eGFP HUVEC in vitro, although the pro-angiogenic effect of Del-1 in vivo was less remarkable.

6 Acknowledgements

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7 References


Chapter 7

Del-1 Overexpression in Endothelial Cells Increases Vascular Density in Tissue Engineered Implants Containing Endothelial Cells and Adipose-Derived Stem Cells

Abstract

We used a combination of strategies to stimulate the vascularization of tissue engineered constructs in vivo. We used a modular approach to build larger tissues from individual building blocks (‘modules’) mixed together. Each building block included vascular cells by design: modules were submillimeter-sized collagen gels with an outer layer of endothelial cells (EC), and with embedded adipose-derived mesenchymal stem cells (adMSC) to support EC survival and blood vessel maturation in vivo. We transduced the EC coating the modules with a lentiviral construct to overexpress the angiogenic extracellular matrix (ECM) protein Developmental endothelial locus-1 (Del-1). Del-1 was used here as a means of tipping the angiogenic balance in the transplanted EC from quiescent to pro-angiogenic. Upon injection of modules in a subcutaneous SCID/Bg mouse model, we observed an increase in the number of blood vessels (both donor-derived and total number of blood vessels) for implants with EC transduced to overexpress Del-1 compared to control implants (with eGFP transduced EC) over the 21 day duration of the study. The greatest difference between Del-1 and eGFP implants and the highest number of blood vessels was observed seven days after transplantation. The day 7 Del-1 implants also had increased SMA+ staining compared to control, suggesting increased blood vessel maturation through recruitment of SMA+ smooth muscle cells or pericytes to stabilize the newly formed blood vessels. Perfusion studies (microCT, ultrasound imaging, and systemic injection of fluorescent UEA-1 or dextran) showed that some of the newly formed blood vessels

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(both donor-derived and host-derived, in both Del-1 and eGFP implants) were perfused and connected to the host vasculature as early as seven days after transplantation, and at later time points as well. Nevertheless, perfusion of the implants did not appear to be very effective in some cases, suggesting further improvements are necessary to normalize the vasculature at the implant site.

1 Introduction

One of the key issues in tissue engineering is the lack of an internal vascular network and rapid connection to the host vasculature upon transplantation of tissue engineered constructs. Here, we approach this issue using a combination of strategies: (1) use a modular strategy to build endothelialized tissue constructs [1, 2]; (2) use Del-1, an angiogenic ECM protein, as a means of tipping the angiogenic balance in the transplanted EC from quiescent to pro-angiogenic (see chapter 6 of thesis); (3) use adMSC as vascular support cells to aid the survival of EC upon transplantation and to stabilize the newly formed blood vessels by adopting a pericyte role [3, 4].

The modular approach consists in fabricating small tissue constructs (‘modules’), and packing the modules together to form a larger tissue [1, 2]. Each module is typically made of collagen, with the outer surface of each module seeded with EC, and with either functional cells (such as cardiomyocytes [5], islets [6] etc.) or vascular support cells (such as MSC [3, 7]) embedded inside the modules. The modular approach has several advantages. First, the tissue constructs already include a vascular component by design, with the EC seeded on the surface of the modules. Second, the modules have uniform cell distribution. This method of tissue fabrication is also scalable. Finally, the modular approach is minimally invasive as the modules are simply injected using a syringe and needle.

In a previous study, we transduced EC to overexpress Del-1 using a lentiviral system, and we showed that Del-1 delivery successfully ‘tilted’ the angiogenic balance in EC towards a more pro-angiogenic phenotype (see chapter 6 of thesis). The EC overexpressing Del-1 formed more sprouts \textit{in vitro}, and differentially expressed a number of genes known to be involved in angiogenesis. However, the number of blood vessels formed \textit{in vivo} was still limited, in a severe combined immunodeficient/beige (SCID/Bg), subcutaneous implant model, due to extensive loss of EC (presumably through apoptosis) after implantation. In a different study using the same
animal model and the modular approach, adMSC embedded inside the modules prevented EC apoptosis [3]. Donor-derived vessels formed at the implant site, some of these vessels persisted for at least 90 days, and their connection to the host vasculature was confirmed by microCT 21 days after implantation.

Here, the goal was to combine these strategies to further improve the tissue vascularization outcome in vivo. Specifically, this study aimed to: (i) explore whether the combination of EC overexpressing Del-1 and adMSC in the context of modular tissue engineering enhances the vascularization of the tissue constructs compared to co-transplantation of control EC and adMSC, in terms of blood vessel density, as well as blood vessel maturation and function (i.e. connection to host vasculature); (ii) expand our understanding of the remodelling process and fate of transplanted cells.

2 Materials and Methods

2.1 Cells

Primary Human Umbilical Vein Endothelial Cells (HUVEC, Lonza) were transduced and cultured as described previously (see chapter 6 of thesis), with HIV-1-based recombinant lentivirus encoding for either Del-1-IRES-eGFP (Del-1 HUVEC), or eGFP alone (eGFP HUVEC, as control). The lentiviruses were prepared and designed by Dr. J. Medin’s lab, University Health Network, Toronto. Mouse Del-1 major cDNA was a kind gift from Dr. T. Quertermous lab, Stanford University. Human adMSC (Lonza) were maintained in DMEM medium (Sigma) with 10% foetal bovine serum (FBS, Sigma) and 1% pencillin/streptomycin (Life Sciences Corporation), with medium changes every 3-4 days. The adMSC were used for implants at passage 3.

2.2 Implants

The adMSC were embedded in sub-millimeter sized collagen cylinders (modules, initial size ~ 2 mm long and 0.6 mm in diameter). The modules were prepared by neutralising and gelling the collagen solution with suspended adMSC (PureCol, Inamed Biomaterials; 3.1 mg/mL type-I bovine dermal collagen; 1x10^6 adMSC/mL of collagen solution) inside polyethylene tubing (Intramedic™ PE60, Becton Dickson), followed by cutting of the tubing into small pieces with
an automatic tube cutter (FCS Technology Inc.), as described elsewhere [2, 3]. The adMSC modules were then seeded with either Del-1 HUVEC or eGFP HUVEC overnight (~4x10^6 HUVEC/mL of settled modules). The next day, approximately 200 modules in 0.1 ml PBS were injected subcutaneously, through an 18G needle, in the dorsum of SCID/Bg mice (6–7 weeks of age, male, Charles River Laboratories). After surgery, mice were individually housed in sterile cages and provided free access to sterilized food and water under the approval of the University of Toronto animal care committee. Some mice were transferred to a different location on day 6 post-surgery to perform a longitudinal ultrasound imaging study (day 7, day 14, and day 21 ultrasound analysis). The relocated mice were individually housed in sterile cages and provided free access to sterilized food and water for the duration of the ultrasound study, under the approval of Sunnybrook Research Institute animal care committee. The transferred mice were returned to the initial location on day 22 post-surgery for UEA-1 and dextran perfusion studies on the same day.

For all experiments, animals were separated in two groups: group 1 = Del-1; group 2 = eGFP. For immunohistochemistry analysis, we used N=5 animals/group for day 3 analysis (with all implant surgeries for day 3 analysis done in one single batch); and N=5 animals/group/time point for day 7, day 14 and day 21 analysis (total 30 animals for 2 groups and 3 time points; implant surgeries were done in 2-3 separate batches, with similar numbers of Del-1 and eGFP animals in each batch).

Separate surgeries were done for imaging. For microCT analysis, we used N=3 animals/group for analysis at day 7 post-surgery; implant surgeries were done in two separate batches. For dextran injection experiments, we used N=3 animals/group for analysis at day 7 post-surgery (with all day 7 implant surgeries done in one single batch). For UEA-1 lectin injection experiments, we used N=6 animals/group for analysis at day 7 post-surgery (with day 7 implant surgeries done in two separate batches, with n=3 animals/group/batch). For ultrasound imaging experiments, we used N=5-6 animals/group/time point at day 7, 14 and 21 post-surgery; the same animals that were analysed at day 7 were re-analysed at day 14 (minus one animal that died before day 14), and again at day 21 (minus one animal due to insufficient contrast agent left); all implant surgeries for ultrasound analysis were done in one single batch. At the end of the ultrasound analysis study, the same animals were used for either day 22 dextran perfusion studies (N=3 animals/group) or day 22 UEA-1 lectin perfusion studies (N=2-3 animals/group).
2.3 Immunohistochemistry

2.3.1 In Vivo Samples

The modules were explanted 3, 7, 14, and 21 days after the implant, fixed in 10% neutral buffered formalin and processed for immunohistochemistry. Paraffin embedded, 4 μm thick sections were analysed using the following stains and antibodies: Hematoxylin and Eosin (H&E), Masson Trichrome, anti-GFP (Abcam #AB6556, 1:1000, rabbit polyclonal antibody, detects (e)GFP expressing cells), Biotinylated Ulex Europaeus Agglutinin I (UEA I) (Vector Laboratories #B1065, 1:400, binds to human endothelial cells), anti-CD31 (Santa Cruz Biotechnology Inc. #SC-1506-R, 1:2000, rabbit polyclonal antibody, detects both mouse and human CD31), anti-α Smooth Muscle Actin (SMA) (Sigma #A5228, 1:200, mouse monoclonal antibody, detects both mouse and human αSMA), anti-Ki67 (Novus Biologicals #NB110-90592, 1:1000, rabbit polyclonal antibody, detects human Ki67), anti-cleaved caspase 3 (Cell Signaling Technology #9661, 1:600, rabbit polyclonal antibody, detects both mouse and human cleaved caspase 3), anti-CD45 (BD Pharmingen™ # 550539, 1:100, rat monoclonal antibody, detects mouse CD45).

**In vivo Blood Vessel Counts:** The number of donor-derived (GFP+) blood vessels (with defined lumen), as well as the total number of blood vessels (donor+host, CD31+) present at the implant site were manually counted using digitized histology slides (ScanScope XT brightfield scanner, Aperio Technologies; 20X objective lens) and the Aperio ImageScope viewing software (Aperio Technologies; version 11). The total number of vessels in the implant area was normalized to the area occupied by the implant on the whole histological section to obtain the vessel density at the implant site (N=5). The diameter of the GFP+ and CD31+ vessels was manually measured using the Aperio ImageScope viewing software and vessels were binned based on their size (with capillaries < 9 μm, small arterioles or venules 9-15 μm, large arterioles or venules 15-75 μm, and other (abnormal) ≥ 75 μm, as used by others [7]). Digitized SMA-stained histology slides were analysed using the Positive Pixel Count Algorithm available with the Aperio ImageScope software to determine the SMA density at the implant site (N=5).

**In vivo Cell Counts:** Proliferating cells (Ki67+), apoptotic cells (caspase-3 cleaved+) and leukocytes (CD45+) present at the implant site were manually counted in 5 hot-spots within each implant. A Zeiss Axiovert light microscope with a 20x objective lens and equipped with a CCD
camera was used to take pictures of the implants and ImageJ software (ImageJ 1.45, NIH) was used to manually count the cells. The average of the counts per sample was used for statistical analysis (N=5; n=5).

2.3.2 *In Vitro* Samples

Some modules were collected immediately after coating with HUVEC (day 0 *in vitro* samples, N=4) or were cultured *in vitro* for an additional 7 days under standard cell culture conditions using a 50:50 mix of EGM-2 medium and DMEM medium with serum (day 7 *in vitro* samples, N=4). Samples were collected and fixed in 4% paraformaldehyde (Electron Microscopy Sciences), embedded in a 2% agarose block (Roche Diagnostics) and processed for histology (paraffin sections). The following antibodies were used to analyse the *in vitro* samples: anti-Ki67, anti-GFP, UEA I, anti-CD31, anti-αSMA (all antibodies same as above), anti-desmin (Dako #M0760, 1:200, mouse monoclonal antibody), anti-PDGFRβ (Abcam # ab32570, 1:400, rabbit monoclonal antibody), anti-collagen IV (Dako #M0785, 1:100, mouse monoclonal antibody), anti-laminin (Sigma #L9393, 1:100, rabbit polyclonal antibody), and anti-fibronectin (BD Transduction Laboratories #610078, 1:2000, mouse monoclonal antibody). The histology slides were imaged using an Olympus BX61 light microscope with a 20X objective lens and equipped with an Olympus DP70 camera, or digitized using the ScanScope XT brightfield scanner with a 20X objective lens. Ki67+ cells were manually counted on up to 5 representative images per sample at 20X magnification using the ImageJ software and the average of the counts per sample was used for statistical analysis (N=4, n≤5).

2.4 Microfil® Injection and MicroComputed Tomography *(microCT)* Imaging

Mice were heparinized (100 units heparin, LEO Pharma Inc.; subcutaneous injection 5 minutes before the surgery), then anaesthetized with isofluorane. A small incision was made below the rib cage, the diaphragm was cut open and a 24G catheter was inserted into the left ventricle of the heart. Mice were then perfused with ~100 mL of PBS with 5U/mL heparin, warmed to 37°C, at a rate of 15 mL/min using a peristaltic pump. PBS perfusion was followed by perfusion with ~30mL of Microfil® Silicone Rubber solution (MV-122, Flow-tech), obtained by mixing the Microfil compound, the diluent and the curing agent in a 8/20/2 ratio by volume. The Microfil® solution was then allowed to polymerize for 2 hrs at room temperature. The tissue of interest was
explanted and fixed in 10% formalin for 24-48 hrs, then stored in PBS until analyzed (N=3; day 7 analysis). Before analysis, tissue samples were carefully dissected to remove extra skin and hair, embedded in 1% agar, then scanned at 14 µm resolution for 2 hours with a microCT scanner (GE eXplore Locus SP, GE Healthcare) at the Mouse Imaging Centre, Toronto Centre for Phenogenomics. A total of 720 views were acquired through 360° rotation with the x-ray source at 80 kVp and 80 µA. Three-dimensional microCT data was reconstructed at 14 µm resolution using the Feldkamp algorithm.

2.5 Injection of Rhodamine-labeled UEA-1 Lectin or Texas-Red®-conjugated Dextran

Mice received tail vein injections of Rhodamine conjugated UEA-1 (Vector Laboratories #RL-1062; 50 µg Rhodamine labeled UEA-1 diluted in 100 µL saline with 1mM CaCl2 per mouse) 10 minutes before sacrifice [similar to [8]]. Other mice received tail vein injections of Texas Red® conjugated dextran, 70 000 MW, lysine fixable (Life Technologies Inc. #D1864, 250 µL of a 25mg/mL solution per mouse) 30 minutes before sacrifice [adapted from [9]]. The tissues of interest were explanted and fixed in 10% formalin overnight at 4°C, then kept for another overnight in 30% sucrose solution at 4°C (UEA-1 study), or fixed in 4% paraformaldehyde overnight at 4°C (dextran study). The fixed tissues were embedded in Tissue-Tek® OCT compound (Sakura Finetek) and snap frozen in liquid nitrogen. Thick frozen sections (10-12µm) were then cut and imaged using an Olympus BX50 upright fluorescence microscope with automated whole slide tiling capabilities, 10x objective lens, CoolSNAP HQ2 (Photometrics) CCD camera, and the MetaMorph® (Molecular Devices) image analysis software (N=6 for UEA-1 lectin day 7; N=2-3 for UEA-1 lectin day 22; N=3 for dextran day 7; N=3 for dextran day 22).

2.6 High-Frequency Ultrasound Imaging

High-Frequency Ultrasound Imaging was performed using the Vevo®2100 Imaging System with a solid-state array transducer (MS-250) (VisualSonics Inc.) at a center frequency of 18MHz. Dual-mode presentation of a grayscale image side-by-side with a contrast-enhanced image, facilitated the selection of the region of interest (ROI). The following settings were kept consistent throughout the study: 4% power, wide beam width, 38 dB contrast gain and 35 dB dynamic range. In preparation for the imaging, mice were anaesthetized with 2% isoflurane
(Abbott Laboratories), shaved, and placed prone on an imaging stage. For contrast mode imaging, each mouse received a bolus of 50 µL (2x10⁹ microbubbles/mL) of the Vevo MicroMarker® Contrast Agent (VisualSonics Inc.) via tail vein injection, at a rate of 600 µL/min, using a syringe pump. Two dimensional (2D) image series were collected to measure ultrasound signal at the implant site before, during, and after microbubble injection. After imaging, mice were individually housed in sterile cages and provided free access to sterilized food and water under the approval of Sunnybrook Research Institute animal care committee. The same mice were used for imaging at day 7, 14 and 21 after implantation of the modules (N=5-6). The collected data was analysed using the Vevo2100 software (version 1.4.1.4510 with VevoCQ version 1.3.8.0). The implant region (ROI) was manually selected on the acquired ultrasound images, the imaging clip was corrected for movement during imaging using the built-in software function, and the change in ultrasound signal in the implant area following microbubble injection was quantified using the VevoCQ software algorithm. The Peak Enhancement (PE) and Wash-in Rate (WiR) results were used for comparison purposes between Del-1 and eGFP implants. PE in contrast mode represents the increase in nonlinear ultrasound signal (produced by microbubbles) after microbubble injection, with a high PE value corresponding to a high level of perfusion. Parametric images showing PE value distribution within the implant (heat maps) were also generated using the VevoCQ software. Wash-in Rate is a measure of the rate of ‘filling’ of the implant with microbubbles after microbubble injection, with high WiR values suggesting higher rate of perfusion.

2.7 Statistical Analysis

A one-way analysis of variance (ANOVA) with LSD post hoc was used to compare means between multiple groups. Differences between means were considered statistically significant at p < 0.05. All statistical analysis was performed with the SPSS Statistics software (IBM Corp.; version 20).

3 Results

3.1 Module Characterization Before Implantation

Modules with embedded adMSC and coated with transduced HUVEC were analysed by immunohistochemistry to confirm eGFP expression before implantation (Figure 7-1).
Qualitatively, the histology images indicated that all HUVEC (UEA-1+, CD31+) also expressed eGFP (GFP+), in both Del-1 and eGFP modules. Additionally, the modules were evenly coated with HUVEC in both cases. The embedded adMSC did not express SMA (a marker of pericyte differentiation) prior to implantation.
Figure 7-1. Histology images of modules *in vitro* (day 0 post-fabrication). The samples were serially cut; the same modules are seen with the different histology stains. All the EC (UEA-1+, CD31+) appeared to express eGFP (GFP+). Good EC coverage of the modules was observed for both Del-1 and eGFP modules. The black squares in the low magnification images in (A) indicate the areas shown in the high magnification images in (B). The red circles indicate one individual module. Scale bar = 100 µm for low magnification images, and 50µm for high magnification images.
3.2 *In Vivo* Vascular Density

Co-transplantation of Del-1 HUVEC with adMSC led to significant improvement in vascularization compared to co-transplantation of eGFP HUVEC with adMSC, with a higher number of blood vessels formed for Del-1 implants compared to eGFP. Figure 7-2 shows representative images of the visual appearance of the tissues at explant. Blood vessels were visible (red color) within all Del-1 and eGFP explants, with Del-1 day 7 explants having a relatively more vascularized appearance, consistent with the significantly higher vessel density results at day 7.

Implanted HUVEC migrated off the surface of the modules and started to form blood vessels in the area between the modules as early as day 3 (the earliest time point included in this study), in both Del-1 and eGFP implants. Both donor (GFP+/UEA-1+/CD31+) and host derived (CD31+/UEA-1-/GFP-) blood vessels were present at the implant site, with the majority of the donor-derived blood vessels formed in the area between the modules, and the majority of host-derived blood vessels formed in the fibrous tissue surrounding the modules, presumably supplying the donor-derived vasculature at the implant site (Figure 7-3). Red blood cells were visible inside the lumen of many of these donor-derived blood vessels, as early as day 3 and even more prominently at later time points, suggesting they were connected to the host vasculature and perfused (Figure 7-3).

The blood vessel density was consistently higher in Del-1 compared to eGFP implants, with the greatest overall difference (number of vessels formed and presence of SMA staining) observed 7 days after implantation. For Del-1 implants, the density of blood vessels increased significantly from day 3 to day 7, then decreased and remained relatively constant from day 14 to day 21. The highest blood vessel density for Del-1 implants, observed at day 7, was ~68 donor-derived vessels/mm$^2$ (GFP+), and ~84 total vessels/mm$^2$ (donor+host,CD31+) (Figures 7-4A and 7-4B). The blood vessel density at day 7 for eGFP implants was ~22 donor-derived vessels/mm$^2$ (GFP+), and ~44 total vessels/mm$^2$ (donor+host,CD31+), significantly lower than for Del-1 (Figures 7-4A and 7-4B). Many of the EC-lined vessels in the implant area were also surrounded by an SMA+ layer (presumably pericytes or smooth muscle cells wrapping around the blood vessels as part of the process of vessel maturation) as early as day 3, but most prominently at day 7 and later time points (as seen in Figure 7-4). The level of SMA+ staining
was higher for Del-1 implants relative to eGFP at day 7 and day 14, and similar at day 21. The average size of the blood vessels (both donor-derived and total) increased from day 3 to day 7 for both Del-1 and eGFP, and remained relatively unchanged from day 7 to day 21. The binning of the vessels based on size is shown in Table 7-1, with the majority of the blood vessels having the size characteristics of capillaries, small arterioles and small venules, as expected. Slightly larger donor-derived blood vessels (average size, Figure 7-5A) were observed for Del-1 compared to eGFP implants at day 7, with more donor-derived vessels in the 9-15 µm range, and fewer vessels smaller than 9 µm.

**Figure 7-2.** Photographs of representative tissues at explant. Blood vessels were visible (red color) within all Del-1 and eGFP explants, with Del-1 day 7 explants having a relatively more vascularized appearance. Arrows indicate the location of implants.
<table>
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</table>

Host-derived (CD31+/GFP-/UEA-1-) blood vessels at the edge of the implant supply the donor-derived vasculature (CD31+/GFP+/UEA-1+).
Figure 7-3. Histology sections of explants at (A) Day 3; (B) Day 7; (C) Day 14; (D) Day 21 after implantation (serially-cut tissue samples were treated with the different stains and antibodies). Implanted HUVEC migrated off the surface of the modules and formed many blood vessels in the area between the modules (GFP+ and UEA-1+ for donor-derived vessels; CD31+ for donor- OR host- derived vessels), in both Del-1 and eGFP implants. Examples of donor- or
host-derived blood vessels are indicated with arrows. In general, the majority of blood vessels at the implant site were donor-derived, for both Del-1 and eGFP implants. Host-derived blood vessels were mostly located next to the edge of the implant (for both Del-1 and eGFP implants), presumably supplying the donor-derived implant vasculature. Examples of host-derived blood vessels are indicated with (*); the extra row of images in Fig. 3B (bottom row) was added to show an example of host-derived blood vessels at day 7. Many of these blood vessels (both donor- and host-derived; in both Del-1 and eGFP implants) were invested with a smooth muscle cell layer (SMA+; examples indicated with arrows), suggestive of vessel maturation. Erythrocytes were visible (examples indicated with arrows) in the lumen of many of these blood vessels (both donor- and host-derived; in both Del-1 and eGFP implants) as well, suggestive of connection to the host vasculature. Scale bar is 100µm for lower magnification images and 50µm for higher magnification. Squares indicate the areas that are shown in the higher magnification images. Red circle shows an individual module.
Figure 7-4. Density of blood vessels and SMA staining at the implant site. Co-transplantation of Del-1 HUVEC and adMSC (black bars) led to an increase in the number of donor-derived blood vessels (GFP+; Fig. 7-4A), as well as total number of blood vessels (donor+host, CD31+; Fig. 7-4B), and increased SMA staining (Fig. 7-4C) compared to eGFP (patterned bars). These
differences were most noticeable at day 7. All vessels (with defined lumen) were counted and normalized to the area occupied by the implant on the whole histological section. The Aperio ImageScope Positive Pixel Count Algorithm was used for SMA staining. Graphs show average ± SEM; N=5; ANOVA with LSD post hoc and p<0.05 (*) considered significant.
**Figure 7-5.** Average size of blood vessels at the implant site. All donor-derived (GFP+) or donor+host (CD31+) blood vessels within each implant were manually measured using digitized whole slides and the Aperio ImageScope viewing software. The average GFP+ or CD31+ blood vessel size per implant was calculated. The average size of the blood vessels (both donor-derived and total) increased from day 3 to day 7 for both Del-1 and eGFP, and remained relatively unchanged from day 7 to day 21, but with Del-1 donor-derived blood vessels slightly larger than eGFP at day 7. Graphs show average ± SEM; N=5; ANOVA with LSD post hoc and \( p < 0.05 \) (*) considered significant.

**Table 7-1.** Blood vessel size distribution for HUVEC + adMSC implants

<table>
<thead>
<tr>
<th>Size Distribution of GFP+ Vessels</th>
<th>Day 3 Del-1</th>
<th>Day 7 Del-1</th>
<th>Day 14 Del-1</th>
<th>Day 21 Del-1</th>
<th>Day 3 eGFP</th>
<th>Day 7 eGFP</th>
<th>Day 14 eGFP</th>
<th>Day 21 eGFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillaries (&lt; 9 µm)</td>
<td>90%</td>
<td>91%</td>
<td>43%</td>
<td>59%</td>
<td>48%</td>
<td>46%</td>
<td>41%</td>
<td>38%</td>
</tr>
<tr>
<td>Small Arterioles or Venules (9-15 µm)</td>
<td>9%</td>
<td>7%</td>
<td>42%</td>
<td>29%</td>
<td>41%</td>
<td>41%</td>
<td>44%</td>
<td>47%</td>
</tr>
<tr>
<td>Large Arterioles or Venules (15-75 µm)</td>
<td>1%</td>
<td>2%</td>
<td>15%</td>
<td>11%</td>
<td>11%</td>
<td>13%</td>
<td>15%</td>
<td>15%</td>
</tr>
<tr>
<td>Other (abnormal, ≥ 75 µm)</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
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<table>
<thead>
<tr>
<th>Size Distribution of CD31+ Vessels</th>
<th>Day 3 Del-1</th>
<th>Day 7 Del-1</th>
<th>Day 14 Del-1</th>
<th>Day 21 Del-1</th>
<th>Day 3 eGFP</th>
<th>Day 7 eGFP</th>
<th>Day 14 eGFP</th>
<th>Day 21 eGFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillaries (&lt; 9 µm)</td>
<td>80%</td>
<td>73%</td>
<td>41%</td>
<td>48%</td>
<td>45%</td>
<td>52%</td>
<td>40%</td>
<td>33%</td>
</tr>
<tr>
<td>Small Arterioles or Venules (9-15 µm)</td>
<td>14%</td>
<td>18%</td>
<td>41%</td>
<td>34%</td>
<td>36%</td>
<td>31%</td>
<td>41%</td>
<td>47%</td>
</tr>
<tr>
<td>Large Arterioles or Venules (15-75 µm)</td>
<td>6%</td>
<td>9%</td>
<td>18%</td>
<td>18%</td>
<td>19%</td>
<td>17%</td>
<td>19%</td>
<td>20%</td>
</tr>
<tr>
<td>Other (abnormal, ≥ 75 µm)</td>
<td>0%</td>
<td>0%</td>
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3.3 MicroCT Imaging of Perfused Vessels

Connection of the implant blood vessels to the host vasculature was evaluated seven days after implantation using microCT imaging. The images in Figure 7-6 show a view of the entire implant region. We found many large blood vessels that were perfused with the Microfil® contrast agent seven days post-transplantation. Qualitatively, it appeared there were more blood vessels that were perfused and not leaky within Del-1 implants compared to eGFP. Large pools of contrast agent accumulated at the implant site in the eGFP case, but not Del-1, suggesting the eGFP implant blood vessels were leakier.

Figure 7-6. Microfil® perfusion 7 days after implantation. MicroCT images of (A) Del-1 and (B) eGFP subcutaneous implants show that by day 7 after implantation, many of the larger blood vessels formed in the implant area were perfused and connected to the host vasculature. It appears that more vessels were perfused and not leaky in Del-1 implants compared to eGFP ones. Images show a microCT view of the entire implant region. Scale bar is 1 mm; the arrow indicates a perfused blood vessel with a diameter of ~100µm.

3.4 Perfusion with Texas Red®-labeled Dextran

We observed that at both day 7 (Figure 7-7A) and day 22 (Figure 7-7B), and in both Del-1 and eGFP cases, some of the blood vessels at the implant site (donor-derived and host-derived) were perfused with the fluorescent dextran. The dextran filled the lumen of some of the donor-derived (GFP+) blood vessels and it did not leak into the surrounding tissue, suggesting that the vessels that were perfused had functional cell-cell junctions, restricting the leakage of their content into the surrounding tissue. Nevertheless, we also observed many donor-derived blood vessels that
were not perfused with the dextran at day 7, as well as day 22 (the low magnification images in Figure 7-7 show the whole implant region on the histology slide). In general, it appeared that the larger donor-derived blood vessels were preferentially perfused relative to the smaller vessels. Some host-derived blood vessels formed at the implant site were also filled with dextran (red, dextran-filled circular structures that are not GFP+ in Figure 7-7; some examples of host-derived blood vessels are shown with arrows). In general, the perfused host-derived blood vessels were seen at the edge of the implants, in both Del-1 and eGFP cases, and at both day 7 and day 22.

Figure 7-7. Identification of perfused blood vessels (both donor-derived and host-derived) at the implant site via tail vein injection of Texas Red®-labelled dextran. Both at day 7 (A), as well as day 22 (B), we observed that some of the donor derived blood vessels (GFP+, green) were filled...
with fluorescent dextran (red), and the dextran was contained within the lumen of these blood vessels, and not leaking into the surrounding tissue, for both Del-1 and eGFP implants. Some host-derived blood vessels at the implant site were also filled with dextran (red, dextran-filled circular structures that are not GFP+; some examples of host-derived blood vessels are shown with arrows). Scale bar is 200μm for lower magnification images and 100μm for higher magnification. The low magnification images show the whole implant region on the histology slide. Squares indicate the areas that are shown in the higher magnification images.

3.5 Perfusion with Rhodamine-labeled UEA-1 Lectin

We also used tail-vein injection with fluorescently labeled UEA-1 lectin to identify perfused donor-derived blood vessels, 7 and 22 days after transplantation. For both Del-1 and eGFP implants, we found that many of the blood vessels of donor origin (GFP+) present at the implant site were connected to the host vasculature by day 22, since many of these vessels were also labeled with the fluorescent UEA-1 lectin injected systemically (Figure 7-8C and 7-8D; the low magnification images show the whole implant region on the histology slide). On the contrary, only very few (if any) UEA-1 positive vessels were visible at the implant site at day 7 (Figure 7-8A and 7-8B).
Figure 7-8. Identification of perfused blood vessels of donor origin via tail vein injection of Rhodamine-labelled UEA-1 lectin. (A) At day 7, only very few of the donor-derived blood vessels (GFP+, green) were also stained with UEA-1 lectin (red), in both Del-1 and eGFP cases. (B) By day 22, many of the donor-derived blood vessels were perfused and labeled with the UEA-1 lectin, in both Del-1 and eGFP cases. Scale bar is 200μm for lower magnification images and 100μm for higher magnification. The low magnification images show the whole implant region on the histology slide. Squares indicate the areas that are shown in the higher magnification images.
3.6 High-Frequency Ultrasound Imaging of Perfused Vessels

Ultrasound imaging with microbubble contrast agent injection showed that the microbubbles reached the implant site after systemic injection, as the ultrasound nonlinear signal at the implant site (generated by the microbubbles) increased soon after injecting the microbubbles into the tail vein. This suggests that the implant blood vessels were indeed connected to the host vasculature. Heat maps of Peak Enhancement of ultrasound signal after microbubble injection show a high content of microbubble at the implant site (red areas), particularly at day 7, in both Del-1 and eGFP implants. The heat maps presented as example in Figure 7-9A show the same Del-1 and eGFP implants, at day 7, 14 and 21 (longitudinal study on the same animals). By day 21, the microbubble content of the implant (and the associated ultrasound signal) decreased, and more extended areas with low microbubble content (blue) are seen within the implants compared to day 7. Quantification of PE using the VevoCQ image analysis algorithms showed high microbubble content for both eGFP and Del-1 at day 7 and day 14, and a decrease in microbubble content at day 21. eGFP implants had more microbubble contrast agent than Del-1 at day 7 and day 14, although these differences were not statistically significant (Figure 7-9B). Quantification of the wash-in rate of microbubbles into the implant using the VevoCQ image analysis algorithms showed a small decrease over time from day 7 to day 21 for both Del-1 and eGFP implants, and similar values for Del-1 and eGFP implants (Figure 7-9C).
Figure 7-9. Peak Enhancement of nonlinear ultrasound signal and wash-in rate of microbubbles into the implant. (A) Heat maps show examples of PE value distribution within the implants after microbubble injection, with red areas indicating regions with high signal, and blue areas indicating regions of low signal. The regions of interest for heat maps were drawn to include the
whole implant region as seen on the 2D ultrasound images; the implants were visible as a ‘bump’ immediately under the skin. The images are from the same Del-1, and eGFP implants, respectively, monitored over time at day 7, 14, and 21. The heat maps show higher signal at day 7, and a decrease over time. (B) Quantification of ultrasound signal showed that PE was high for both eGFP and Del-1 implants at day 7 and day 14, and lower at day 21. We also observed an increase in PE signal for eGFP compared to Del-1 implants at day 7 and day 14, although this increase was not statistically significant. (C) Quantification of the wash-in rate of microbubbles into the implant showed a small decrease over time from day 7 to day 21 for both Del-1 and eGFP implants, and similar values for Del-1 and eGFP implants. Graphs show average ± SEM; N=5-6; ANOVA with LSD post hoc and p<0.05 (*) considered significant.

3.7 *In Vivo* Proliferation of Implanted Donor Cells

Quantitative analysis of proliferating donor-derived vascular cells *in vivo* showed that the number of proliferating cells was more than 2 fold higher for Del-1 relative to eGFP implants at day 7 (p < 0.05), and was increased by 35% at day 3 (Figure 7-10A). Additionally, the number of proliferating cells was increased at day 7 relative to day 3 for both Del-1 and eGFP. Proliferating cells observed in the implant area had migrated off the modules and were mostly located in the area between the modules, either as individual cells, or less frequently, associated with blood vessels presumably undergoing angiogenic remodelling. The proliferating cells were likely of both EC and adMSC origin.
Figure 7-10. Proliferating donor-derived cells in vivo. (A) Quantitative analysis of proliferating donor-derived cells in vivo. Significantly more proliferating cells of donor origin were observed for Del-1 compared to eGFP implants at day 7. Additionally, an increase in proliferating cells from day 3 to day 7 was observed for both Del-1 and eGFP implants. Proliferating cells were counted in 5 hot-spots (20x objective lens) within each implant. Graph shows average ± SEM; N=5 (n=5); ANOVA with LSD post hoc and p<0.05 (*) considered significant. (B) Histology images at day 7. Visual analysis of serially cut tissue sections shows that in general, Ki67+ cells were found in areas where both EC (GFP+) and pericytes (SMA+) were colocalized. For this same reason, the identity of Ki67+ cells (whether EC or of adMSC origin) was challenging to distinguish on serially cut sections. Nevertheless, it is likely that some of the Ki67+ proliferating
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cells were of EC origin (solid arrows), others were of adMSC origin and were expressing SMA+ pericyte markers (dashed arrows), while others were likely of adMC origin, but not expressing SMA(*). The red circle highlights one individual module. A blood vessel containing proliferating cells is also shown (b.v.). Scale bar is 50µm.

3.8  *In Vivo* Apoptosis of Cells at the Implant Site

We observed fewer apoptotic cells for Del-1 implants: the number of apoptotic cells in Del-1 relative to eGFP decreased by ~35% at both day 3 and day 7 (Figure 7-11A). Also, the number of apoptotic cells decreased by ~60% at day 7 relative to day 3 in both eGFP and Del-1 implants, and with the level of apoptosis in Del-1 implants already lower than eGFP at day 3 (Figure 7-11A). Some of the apoptotic cells were EC, and some were part of regressing blood vessels.

![Cleaved Caspase-3+ Cell Counts](image-url)
Figure 7-11. Apoptotic cells at the implant site. (A) Quantitative analysis of apoptosis *in vivo*. Fewer apoptotic cells were observed for Del-1 implants compared to eGFP at both time points (day 3 and day 7), although these differences were not statistically significant. Apoptosis decreased significantly in both eGFP and Del-1 implants from day 3 to day 7, with the level of apoptosis in Del-1 implants already lower than eGFP at day 3. Apoptotic cells were counted in 5 hot-spots (20x objective lens) within each implant. Graph shows average ± SEM; N=5 (n=5); ANOVA with LSD post hoc and p<0.05 (*) considered significant. (B) Histology images at day 3. Visual analysis of serially cut tissue sections showed that some of the apoptotic cells were EC (GFP+), as indicated by the arrows. Also, some of the apoptotic cells were part of tubular structures that likely were regressing primitive blood vessels (indicated as b.v. in Figure). Very few SMA+ cells were also found at the implant site at day 3, and their co-localization with the EC makes it challenging to distinguish on serially cut sections whether some of these SMA+ cells also underwent apoptosis. The red circle highlights one individual module. Scale bar is 50µm.

3.9 *In Vivo* Leukocyte Infiltration at the Implant Site

We quantified the leukocytes (CD45+) present in two regions of the implants: (1) between the modules (Figure 7-12A), and (2) in the thin fibrous tissue formed around the implant (Figure 7-12B). In the area between the modules, we observed no difference at day 3, and ~ 20% fewer leukocytes in Del-1 implants relative to eGFP at day 7 (although this difference at day 7 was not statistically significant) (Figure 7-12A). In general, we observed a decrease in the number of leukocytes from day 3 to day 7. In the fibrous tissue layer around the implant, we found there was a significant increase in leukocytes by ~50% for Del-1 relative to eGFP three days after
implantation (Figure 7-12B). By day 7, the number of leukocytes surrounding Del-1 implants decreased and it became ~ 20% lower relative to eGFP (although this difference at day 7 was not statistically significant) (Figure 7-12B).
**Figure 7-12.** Quantitative analysis of leukocyte infiltration *in vivo.* (A) Similar number of leukocytes were observed in the area between the modules for Del-1 and eGFP implants at both day 3 and day 7, with significantly more leukocytes present at day 3 compared to day 7. (B) Higher number of leukocytes (CD45+) were present in the fibrous tissue formed around the implant for Del-1 compared to eGFP implants at day 3, while by day 7 the number of leukocytes in the Del-1 implants decreased and was similar and slightly lower than eGFP. Leukocytes (CD45+) were counted in 5 hot-spots (20x objective lens) within each implant. Graph shows average ± SEM; N=5 (n=5); ANOVA with LSD post hoc and p<0.05 (*) considered significant. (C) Representative histology images of leukocyte infiltration in the implant area, at day 3 and day 7. Black squares and red squares in the low magnification images indicate the areas between the modules, and in the fibrous tissue surrounding the implant, respectively, which are shown at higher magnification in the row immediately below. The red circles highlight individual modules. Scale bar is 500µm in high magnification images, and 50µm in low magnification images.

### 3.10 *In Vitro* Proliferation of EC and adMSC co-Cultured in Modules

The number of proliferating cells was relatively low for both Del-1 HUVEC + adMSC modules and eGFP at day 0, and increased for both Del-1 and eGFP from day 0 to day 7 (Figure 7-13A). The proliferating cells were of both EC and adMSC origin. Based on these similar results for Del-1 vs. eGFP modules, we conclude that the effect of Del-1 on proliferation of either HUVEC or adMSC is limited in this *in vitro* culture system.
Figure 7-13. Proliferation of EC and adMSC co-cultured in modules in vitro. (A) Quantitative analysis of proliferating cells in vitro in collagen modules with embedded adMSC and coated with HUVEC. Significantly more proliferating cells were observed after 7 days in culture in vitro (day 7) compared to the initial time point immediately after fabricating the modules (day 0). No statistically significant differences were observed between the proliferative state of cells in Del-1 modules vs. eGFP, both at day 0 and day 7, although a small increase in proliferation for the Del-1 modules compared to eGFP was observed at day 0. Proliferating cells were counted in up to 5 hot-spots (20x objective lens) per sample. Graph shows average ± SEM; N=4 (n≤5); ANOVA with LSD post hoc and p < 0.05 (*) considered significant. (B) Identity of proliferating cells at day 7. Images show serially cut tissue sections. Some of the proliferating cells (Ki67+) were EC (GFP+), indicated with the arrows. Many proliferating cells were not GFP+, and therefore must be of adMSC origin (indicated with the stars). Also, many of the EC were not proliferating, as they were not Ki67+ (indicated with the dashed capped lines). The black squares indicate the areas shown at higher magnification below. Scale bar is 100µm in low magnification images, and 50µm in high magnification images. The red circle highlights one individual module within the larger microtissue; the larger microtissue formed after contraction and self-assembly of several modules after culture in vitro.
3.11 *In Vitro* Expression of Pericyte Markers by adMSC co-Cultured with EC in Modules

Some adMSC expressed PDGFRβ, but only very few expressed αSMA and desmin after seven days in *in vitro* co-culture with HUVEC in modules (Figure 7-14). We did not observe any significant difference between the expressions of these pericyte markers in Del-1 compared to eGFP modules in this *in vitro* system. The adMSC expressing pericyte markers appeared to be located in areas with EC present (GFP+ staining identifies EC in Figure 7-14).

**Figure 7-14.** Differentiation of adMSC into pericytes after seven days *in vitro*. PDGFRβ was expressed by some cells, but only very few cells expressed αSMA, and even fewer (if any) desmin. The arrows indicate adMSC expressing pericyte markers (PDGFRβ+ and αSMA+) and co-localized with EC (GFP+). The black squares indicate the areas shown at higher magnification below. Scale bar is 100µm in low magnification images, and 50µm in high magnification images. The red circle highlights one individual module within the larger microtissue; the larger microtissue formed after contraction and self-assembly of several modules after culture *in vitro*. 
3.12 *In Vitro* ECM Deposition by EC and adMSC co-Cultured in Modules

We observed deposition of basement membrane ECM components in both Del-1 and eGFP modules cultured for seven days *in vitro*, with no measurable difference between the two. Each individual structure in low magnification images in Figure 7-15 represents several modules contracted and fused together after *in vitro* culture, and forming a larger microtissue. Collagen IV was deposited in small amounts throughout each microtissue. A thick layer of laminin was deposited on the outer surface of each microtissue (darker stain on the outer surface; unspecific (lighter brown color) antibody staining of the interior of the modules). A thin layer of fibronectin was also observed as the outermost layer of the microtissues (darker stain on the outer surface; unspecific (lighter brown color) antibody staining of the interior of the modules).

**Figure 7-15.** ECM deposition after culturing the modules for seven days *in vitro*. Modules contracted and self-assembled into larger microtissues. Both Del-1 and eGFP modules deposited some collagen IV within the microtissues, a thick layer of laminin on the outer surface of the microtissues, and a thin layer of fibronectin, again on the outer surface of the microtissues. The arrows indicate examples of areas containing the ECM synthesized by the cells. The black squares indicate the areas shown at higher magnification in the row below. Scale bar is 500µm in low magnification images, and 50µm in high magnification images. The red circle highlights one individual module within the larger microtissue.
4 Discussion

4.1 Del-1 Significantly Increased the Density of Blood Vessels Formed at the Implant Site

In a previous study, when we delivered Del-1 \textit{in vivo} through transduction of HUVEC and implantation of HUVEC-covered collagen modules, but without embedded adMSC, we observed a very limited increase in vascularization for Del-1 implants compared to eGFP implants (see chapter 6 of thesis). In that previous study, we reasoned that the absence of a strong Del-1 induced pro-angiogenic effect was due to the limited survival of HUVEC upon transplantation, and therefore limited production of Del-1 from the remaining surviving HUVEC. In the current study, we co-transplanted adMSC and HUVEC (either Del-1 HUVEC or eGFP HUVEC) instead of HUVEC alone. In this case, we observed indeed a Del-1 induced angiogenic benefit. Both the number of donor-derived, as well as the total number of blood vessels at the implant site was consistently higher for Del-1 implants compared to eGFP implants over the 21 day duration of the study, with the greatest difference observed seven days after transplantation. We speculate this was due to improved HUVEC survival in the presence of pro-survival factors secreted by adMSC (VEGF, bFGF, HGF etc.) [10-12], which allowed for Del-1 to be produced by the surviving cells. The secreted Del-1 then ‘tipped’ the pro-angiogenic balance in HUVEC further and favored blood vessel formation. At day 7 post-transplantation, Del-1 HUVEC and adMSC implants had 68 donor-derived blood vessels/mm$^2$, and 84 total (donor and host) blood vessels/mm$^2$. By comparison, Del-1 HUVEC only implants (without adMSC) in our previous study (see chapter 6 of thesis) only had 14 donor blood vessels/mm$^2$, and 21 total blood vessels/mm$^2$ at day 7. Similarly, eGFP HUVEC and adMSC implants had more blood vessels than eGFP HUVEC implants, but less than the Del-1 and adMSC implants. eGFP HUVEC and adMSC implants had 22 donor vessels/mm$^2$, and 44 total vessels/mm$^2$ at day 7. By comparison, eGFP HUVEC only implants (no adMSC) only had 12 donor blood vessels/mm$^2$, and 16 total blood vessels/mm$^2$.

In addition, we observed an increase in SMA$^+$ staining at the implant site in Del-1 HUVEC and adMSC implants compared to eGFP HUVEC and adMSC implants. This likely also contributed to the increased number of blood vessels in Del-1 HUVEC and adMSC implants, as recruitment of SMA$^+$ cells (pericytes and smooth muscle cells) is an important step in the process of
maturation of newly formed blood vessels and it is required to prevent blood vessel regression. Presumably at least some of these SMA+ cells were of adMSC origin, which were shown by others to act as pericytes [4, 13-17]. In addition and supporting the hypothesis that at least some of the SMA+ cells were of adMSC origin, we found much more SMA+ staining in implants containing adMSC and HUVEC by comparison to implants with HUVEC alone that we had investigated in a previous study (see chapter 6 of thesis). For example, at day 7 post-transplantation we found 0.54 SMA+pixels/mm² in Del-1 HUVEC and adMSC implants by comparison to only 0.12 SMA+pixels/mm² in Del-1 HUVEC (without adMSC) implants, and 0.24 SMA+pixels/mm² in eGFP HUVEC and adMSC implants by comparison to only 0.11 SMA+pixels/mm² in eGFP HUVEC (without adMSC) implants. Moreover, literature reports showed that Del-1 increased smooth muscle cell proliferation and migration [18], and it is possible that it had a similar effect on the SMA+ cells present at the implant site, thus contributing to the increase in the number of SMA+ cells that we observed in vivo for Del-1 HUVEC and adMSC implants.

In addition, the interaction between adMSC/pericytes and EC in vivo may have also contributed to the proper assembly of Del-1 in the ECM. It is known that EC and pericytes are both required to properly deposit and assemble the basement membrane during blood vessel formation, and poor ECM assembly occurs if pericytes are not present along with the EC [19-22]. It is therefore tempting to speculate that improved ECM assembly (with incorporated Del-1) is another mechanism explaining the observed synergistic effect between Del-1 and adMSC co-transplantation with HUVEC in vivo. On the other hand, our in vitro results were less conclusive. We did not see differences in the expression of pericyte markers by the adMSC co-cultured in modules with either Del-1 or eGFP HUVEC, nor in the number of proliferating cells. In fact, while some of the adMSC expressed PDGFRβ, only very few expressed SMA or desmin after seven days in culture. We also did not see differences in the amount or type of ECM synthesized. Perhaps the cell culture medium that we chose with a view to facilitate co-culture of the two cell types (50/50 mixture of the medium typically used for EC and adMSC culture), as well as the static culture conditions (lack of flow) were not favorable for promoting pericyte differentiation and pericyte/EC interactions for basement membrane deposition [23, 24]. Others in our group found that culture of adMSC in EGM-2 cell culture medium (medium typically used for HUVEC culture) promoted a proliferative phenotype of adMSC (unpublished data).
Finally, despite the strong improvement in vascularization with Del-1 implants compared to eGFP, we cannot ignore the decrease in the number of blood vessels over time, down to a level statistically not different from eGFP, although still higher for Del-1, at day 21, the latest time point included in the study. Nevertheless, by comparison to implants with HUVEC alone (both Del-1 and eGFP) that we explored in a previous study (see chapter 6 of thesis), the number of blood vessels (both donor-derived and total (host and donor)) was still higher in implants with adMSC at 21 day post-transplantation, presumably due to the increased HUVEC survival in the presence of adMSC. For example, while Del-1 HUVEC and adMSC implants had 37 donor vessels/mm² and 41 total vessels/mm², Del-1 HUVEC alone implants only had 13 donor vessels/mm² and 17 total vessels/mm². We propose that the decrease in blood vessel density in Del-1 HUVEC and adMSC implants was not caused by a loss of Del-1 effect overtime, but rather physiologically justified. Under normal physiological conditions, the blood vessels that are no longer needed are pruned over time [25]. In our case, there was no additional functional, metabolically active tissue load within the implant (other than the vascular network itself), and therefore the existence of a rich network of blood vessels was not physiologically required. Still in the context of modular tissue engineering and co-transplantation of adMSC and EC subcutaneously in SCID/Bg mice, but using human microvascular endothelial cells (HMEC) instead of HUVEC, and without Del-1 transduction, our group has previously shown that the blood vessel density decreased over the initial time frame, but then it was accompanied by an increase in blood vessel size in that case [3]. We did not observe a similar increase in blood vessel size in the current study, perhaps due to the difference in the source of cells (HUVEC vs. HMEC; adMSC commercially available vs. isolated in house). The blood vessel size distribution in our implanted tissues was however physiologically consistent, with the majority of vessels falling under the capillary or small arteriole and venule category.

4.2 Blood Vessels Formed at the Implant Site Were Connected to the Host Vasculature

For all time points included in this study, and as early as day 3, red blood cells were visible inside the lumen of many of the blood vessels present at the implant site (both donor-derived and host-derived), suggesting these vessels were perfused and connected to the host vascular supply. We used several perfusion methods to evaluate the quality of the vasculature formed, and to compare between Del-1 and eGFP implants. All perfusion contrast agents were able to reach the
newly formed vessels, but to a different extent, depending on the perfusion method and time point studied. Overall, the perfusion results were encouraging in that they showed that both Del-1 and eGFP implants were connected to the host vasculature as early as day 7. On the other hand, the effectiveness of perfusion in all of these constructs was significantly impaired compared to normal tissues, and suggests improvements are still required in order to build a functional vascular tissue. The different perfusion studies offered complementary and sometime apparently conflicting information, and generated a more comprehensive image of the quality of perfusion. These perfusion results are discussed in further detail below.

MicroCT imaging of the implant vasculature showed that delivery of Del-1 improved the quality of the blood vessels formed, as early as seven days after transplantation. The perfused blood vessels in Del-1 implants were visibly less leaky compared to eGFP, indicative of enhanced blood vessel maturation. These microCT observations are consistent with the increased SMA+ staining in Del-1 implants compared to eGFP, as increased SMA+ staining suggests vessel maturation and stabilization through recruitment of pericytes or smooth muscle cells.

We also used systemic delivery of fluorescent-labeled UEA-1 lectin (tail-vein injection) to identify perfused blood vessels of donor-origin (UEA-1 lectin binds specifically to the human endothelium, and not the mouse endothelium). We found that day 22 implants had a highly functional vasculature, with the majority of the donor-derived blood vessels (GFP+) also labeled with the UEA-1 lectin. On the other hand, the day 7 implants showed very few UEA-1 lectin+ vessels. The day 7 results were unexpected given the evidently successful perfusion of these vessels with the Microfil® compound at day 7, at least in the case of Del-1 implants. This suggests that although the blood vessels in Del-1 implants were connected to the host vasculature at day 7 and not leaky, the perfusion of these vessels was still not very good. The microCT protocol involved perfusing the animal through the left ventricle of the heart with 30 mL of contrast agent at a rate of 15 mL/min using a peristaltic pump. On the other hand, the UEA-1 protocol only involved manual injection of 100µL, through the tail vein, hence using the physiological blood flow and pressure. At the early time points after implantation, the vasculature at the implant site is highly tortuous and still undergoing remodeling to achieve optimal branching for perfusion. Therefore, blood (or perfusion fluid) encounters more resistance to flow through the implanted tissue compared to the host vasculature, and preferentially takes the path of least resistance, i.e. through the normal tissue. Frequent bypassing of the implant
renders the circulation through the implanted tissue less effective compared to normal tissue, similarly to what is now recognized about the tumor vasculature [26]. Therefore, one might expect that more circulating volume, concentration of contrast agent, pressure, or time would be required in order to accumulate perfusion fluid/contrast agent at the implant site, and this explains the discrepancies between the microCT day 7 and UEA-1 lectin day 7 perfusion results, with the microCT study using both higher volume and pressure.

We also evaluated the quality of the vasculature at the implant site through systemic delivery of fluorescent dextran (tail-vein injection). The injected dextran was expected to fill all of the host vascular system, as well as implant vessels (both donor-derived and host-derived). One of the functions of the EC lining the blood vessels is to form a functional barrier that regulates the passage of molecules from the intraluminal space into the surrounding tissue and vice-versa. Typically, proteins such as albumin (66kDa) are retained, while smaller molecules can pass through. We injected a 70kDa fluorescent dextran (similar to [9]) and expected it to be retained inside the blood vessels. We found donor-derived (GFP+) vessels filled with dextran and not leaky as early as day 7, and at day 22 as well, for both Del-1 and eGFP implants. Nevertheless, mostly the larger diameter donor-derived vessels were filled with dextran, and not the smaller vessels. As discussed in reference to the UEA-1 perfusion results, we presume this is due to the less effective circulation of fluid through the implant by comparison to the rest of the host vasculature.

Finally, ultrasound imaging showed that the microbubble contrast agent injected systemically using a syringe pump accumulated at the implant site in both Del-1 and eGFP implants. In fact, higher ultrasound signal (PE) was measured at day 7 and day 14 compared to day 21 in both Del-1 and eGFP implants. In addition, we measured higher ultrasound signal in eGFP implants compared to Del-1 at day 7 and day 14, although these differences were not statistically significant. The Peak Enhancement in non linear ultrasound signal is proportional to the amount of microbubbles present in the region of interest, and it does not distinguish between microbubbles within the blood vessels, and microbubbles leaking out of the vessels and into the surrounding tissue (microbubbles are only 2-3 µm in size). Therefore, considering that our microCT images at day 7 showed large accumulation of contrast agent outside of leaky vessels for the eGFP implants, and that blood vessel counts were lower in eGFP compared to Del-1 (histology data), it is likely that the higher ultrasound signal in eGFP vs. Del-1 at day 7 is due to
microbubble accumulation in the tissue facilitated by leaky vessels, rather than by an increase in the number of functional vessels. Similarly, the leakiness of the blood vessels at early time points is likely also an important contributor to the increased ultrasound signal that we observed at day 7 and day 14 compared to day 21. In this latter case, the decrease in number of vessels by day 21 also justifies the lower ultrasound signal.

We also quantified the wash-in rate, a measure of the rate of filling of the implant with the microbubbles (perfusion rate). A high WiR value indicates high rate of perfusion, due to a higher number of blood vessels, or to the presence of larger blood vessels. We found slightly higher wash-in rates at day 7 and a decrease over 21 days in both Del-1 and eGFP implants (and similar values for Del-1 compared to eGFP), although this decrease over time was small (not statistically significant). We speculate that the higher rates of perfusion at day 7 compared to day 21 for both Del-1 and eGFP implants were due to the higher number of blood vessels (donor + host) at day 7 compared to day 21 (as shown by our histology blood vessel counts data), and potentially also due to the accumulation of microbubbles in the tissue from leaky vessels, particularly in the eGFP case. Our histology data showed minimal change in blood vessel size over time, so we speculate that blood vessel size did not play a role in the higher WiR at day 7.

4.3 Increased Vascular Cell Proliferation In Vivo for Del-1 Implants

We analysed the number of proliferating cells as an indication of the angiogenic state of the implanted cells. We used Ki67, a well-established proliferation marker expressed during all active phases of the cell cycle, but absent during the resting phase [27], to identify both proliferating HUVEC, as well as proliferating adMSC (both of human origin; the antibody did not stain host mouse cells), at day 3 and day 7. The higher number of proliferating cells for Del-1 compared to eGFP implants is consistent with the higher number of blood vessels formed within the Del-1 HUVEC+adMSC implants. Most of these proliferating cells were not associated with any blood vessels, as expected, since the cell layers forming blood vessels are normally quiescent. A fraction of the proliferating cells were however associated with blood vessels , indicative of the active angiogenic remodelling process that was occurring at the implant site. The proliferating cells were of both EC and adMSC origin, in both Del-1 and eGFP implants.
4.4 Decreased Apoptosis In Vivo for Del-1 Implants

We also evaluated the effect of Del-1 on apoptosis in vivo. We used the activated (cleaved) form of caspase-3 as indicator of cellular apoptosis. Caspase-3 is one of the effector caspases and it is a common effector of both the extrinsic and intrinsic apoptotic pathways. We found fewer apoptotic cells for Del-1 vs. eGFP HUVEC with adMSC implants at both day 3 and day 7; suggesting Del-1 had some anti-apoptotic protective effect on the cells in the implant area.

Consistent with our data in this study, literature reports showed that Del-1 had a protective anti-apoptotic effect on HUVEC, mediated by binding and signaling through the αvβ3 integrin [28]. However, in our previous study investigating transplantation of HUVEC alone (either Del-1 HUVEC or eGFP HUVEC), only a limited number of cells survived after transplantation and consequently formed a limited number of blood vessels in both Del-1 and eGFP cases (see chapter 6 of thesis). We suspect that the limited HUVEC survival that we observed in our previous study was a consequence of extensive HUVEC apoptosis (although we did not stain with anti-apoptotic markers to confirm the cause of limited survival), and Del-1 did not have a significant anti-apoptotic effect in that system. In our current system, adMSC were also included in both Del-1 and eGFP implants. The adMSC secrete a number of pro-survival factors themselves, such as VEGF, bFGF, HGF etc. [10-12], and literature reports showed that the anti-apoptotic effect of Del-1 on HUVEC was in fact additive to the anti-apoptotic effect of VEGF and bFGF [28]. Therefore, as expected, we found that many more cells survived and formed blood vessels after transplantation for both Del-1 and eGFP HUVEC co-transplanted with adMSC, compared to our previous study involving transplantation of HUVEC alone (presumably due to the pro-survival factors secreted by the adMSC), and we also found that apoptosis was further decreased in Del-1 HUVEC + adMSC implants compared to eGFP HUVEC + adMSC implants (presumably due to the anti-apoptotic effects of Del-1).

The higher level of apoptosis observed early after transplantation at day 3 compared to day 7 for both Del-1 and eGFP HUVEC with adMSC implants is likely a consequence of both the inflammatory response and of the hypoxic, nutrient-poor environment within the implanted tissue early after implantation. Most apoptotic cells were seen in the area between the modules. We also noticed that some of the apoptotic cells lined the surface of what were likely regressing blood vessels. Apoptosis is part of the process of eliminating non-functional blood vessels.
(absence of blood flow) or vessels that fail to mature (lack of recruitment of supporting pericytes or SMC), as well as part of the normal pruning of blood vessels that are no longer needed if there is no physiological demand from the functional tissue that the blood vessels supply [29].

4.5 Inflammatory Response for Del-1 Implants

We used CD45 (also called leukocyte common antigen) as a pan-leukocyte marker to characterize the leukocyte infiltration in the implant area. While leukocytes can be detrimental to the survival of implanted cells, they can also secrete a great number of pro-angiogenic factors, creating an environment that is favorable for vascularization, with both innate and adaptive immune cells responsible for these effects [30, 31]. Although we used an immune-compromised animal model (SCID/Bg), which is T-cell and B-cell deficient and with impaired natural killer cells, all other components of the immune system are still active.

We found that Del-1 induced a changed inflammatory response, and this may have contributed, in part, to the increased vascularization we observed by day 7 post-transplantation. The higher number of leukocytes for Del-1 versus eGFP in the fibrous tissue surrounding the modules at day 3 suggests that the Del-1 transduced endothelial cells played a role in either recruiting or retaining these cells at the implant site. Of course, the higher number of apoptotic cells at day 3 compared to day 7 (for both Del-1 and eGFP implants) also suggests that other than their role in stimulating vascularization, the inflammatory cells also played a role in inducing and clearance of the apoptotic cells from the implant site.

By day 7, the number of leukocytes decreased compared to day 3 in both Del-1 and eGFP constructs, with even lower numbers in Del-1 implants. Literature reports showed that Del-1 interfered with lymphocyte function associated molecule-1 (LFA-1) mediated leukocyte-endothelial cell interactions and acted as an endogenous inhibitor of inflammation [32-36]. The lower number of leukocytes for Del-1 versus eGFP implants at day 7 may therefore be a consequence of the anti-inflammatory properties of the secreted Del-1. Our data suggests that Del-1 has a bimodal effect on inflammatory cell recruitment, depending on the biological context. On one hand, Del-1 played a role in recruiting or retaining more inflammatory cells to the implant site at day 3, presumably facilitating the clearance of apoptotic cells and the initiation of tissue vascularization at this early stage after cell transplantation. On the other hand, at day 7,
presumably in a different biological context, after the initial tissue repair after implantation has occurred, Del-1 decreased the number of inflammatory cells.

5 Conclusion

Manipulation of the extracellular matrix, here through lentiviral transduction of EC with Del-1, is a fruitful method to accelerate tissue construct vascularization. Co-transplantation of Del-1 HUVEC and adMSC in a SCID/Bg animal model increased the number of blood vessels in the implant area compared to co-transplantation of eGFP HUVEC and adMSC, suggesting a Del-1-induced angiogenic effect. Most importantly, perfusion studies showed that many of these blood vessels were indeed connected to the host vasculature as early as seven days after transplantation. However, the blood flow through the implant vasculature was still less effective compared to the native host vasculature. Developing new strategies to normalize the implant vasculature and improve perfusion effectiveness is a key next step.

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7 References


Chapter 8
Conclusions and Recommendations for Future Work

1 Conclusions

Modular tissue engineering was first introduced in the Sefton lab as a novel approach to build vascularized tissue engineered constructs [1, 2]. The focus of this thesis was on developing biomaterial-based strategies to be used in modular tissue engineering. While the first part of the project focused on developing a synthetic scaffold biomaterial, the second part of the project used lentiviral transduction of endothelial cells (EC) as a delivery method of pro-angiogenic extracellular matrix (ECM) proteins.

We synthesized a poloxamine-polylysine acrylate biomaterial to overcome the mechanical limitations of collagen, and the lack of cell attachment properties of poloxamine. We used a three step chemical synthesis strategy. We first added photocrosslinkable acrylate groups to the polylysine peptides. Then we activated the hydroxyl end groups of poloxamine by tresylation. Finally, the activated poloxamine was reacted with the polylysine peptides to obtain the final product. As hypothesized, the synthesized poloxamine-polylysine acrylate polymer formed mechanically strong hydrogels in aqueous mixtures with methacrylated poloxamine, and it supported the attachment of EC on the surface of the hydrogels for at least seven days in vitro.

We then transduced EC with a lentiviral construct to overexpress Developmental endothelial locus-1 (Del-1), expecting that Del-1 will ‘tilt’ the angiogenic balance in these EC from quiescent to pro-angiogenic. In support of our hypothesis, an in vitro sprouting assay with EC coated beads in a fibrin gel showed the Del-1 HUVEC (human umbilical vein endothelial cells) formed more sprouts compared to enhanced green fluorescent protein (eGFP, control) transduced HUVEC, over seven days in culture. In addition, the Del-1 HUVEC also had a distinct pro-angiogenic gene expression profile compared to eGFP HUVEC in vitro. However, contrary to our expectations, the pro-angiogenic effect of Del-1 in vivo was less remarkable. Similarly low numbers of blood vessels formed after implantation of Del-1 HUVEC coated modules or eGFP HUVEC (control) coated modules in a severe combined immunodeficient/beige (SCID/Bg)
mouse subcutaneous implant animal model. We suspected that the limited number of blood vessels in vivo was due to limited HUVEC survival after transplantation, and therefore, limited production of Del-1 by the surviving cells.

Next, we incorporated adipose-derived mesenchymal stem cells (adMSC) in the modules coated with either Del-1 or eGFP HUVEC. The adMSC are known to secrete a number of pro-survival factors [3, 4] and we expected that these pro-survival factors will support HUVEC survival in vivo. In turn, we expected that increased HUVEC survival would result in increased Del-1 production, and we would then see a Del-1 induced pro-angiogenic effect. Indeed, we observed increased cell survival and blood vessel formation compared to transplantation of HUVEC alone without adMSC in both Del-1 and eGFP with adMSC implants. Del-1 implants with adMSC had decreased apoptosis in vivo at both day 3 and day 7 compared to eGFP implants with adMSC, as well as increased proliferation of donor-derived cells at day 7, suggesting that Del-1 had a protective anti-apoptotic and proliferative effect. Most importantly, we consistently found more blood vessels (both donor-derived and total number of blood vessels) at the implant site in Del-1 implants with adMSC compared to eGFP implants with adMSC over the 21 day duration of the study. The differences in blood vessel counts were most prominent 7 days post-transplantation. We also observed an increase in α-smooth muscle actin (SMA+) staining in Del-1 compared to eGFP implants. This likely also contributed to the increased number of blood vessels in Del-1 implants, as recruitment of SMA+ pericytes and smooth muscle cells (SMC) is an important step in the process of maturation of newly formed blood vessels and it is required to prevent blood vessel regression. These findings suggest that Del-1 supported blood vessel assembly and maturation in vivo.

Finally, we investigated whether the blood vessels that we found at the implant site (donor or host-derived) were connected to the host vascular network and perfused. We used several perfusion methods including nonlinear contrast agent ultrasound imaging, microcomputed tomography (microCT), and systemic injection of fluorescent UEA-1 (Ulex Europaeus Agglutinin I) and dextran. For both Del-1 and eGFP implants, we found that the perfusion agent reached the implant site as early as day 7 post-transplantation, and the vessels continued to be connected to the host vasculature and perfused over the 21-22 day duration of the study. Nevertheless, with some perfusion methods, we found that the number of blood vessels that were perfused at day 7 was very limited, and we suspect this was due to non-optimal branching
structure, tortuosity and leakiness of the implant vasculature compared to the host vasculature. Future efforts should be directed towards normalizing the implant vasculature to facilitate perfusion.

We conclude that our experimental data is in support of our initial hypothesis. Lentiviral transduction of EC to overexpress Del-1 was successful in tipping the angiogenic balance towards a pro-angiogenic phenotype in EC incorporated in modular tissue engineered constructs, and it enhanced the vascularization of modular tissue engineered constructs coated with these EC upon their implantation in vivo, albeit only when adMSC were also embedded inside the modules as vascular support cells.

2 Recommendations for Future Work

This section discusses some key research questions related to modular tissue engineering in general and to the Del-1 component of this project in particular, that may be explored further in the future.

2.1 Study the Effect of the Local Environment on the Vascularization Outcome In Vivo

2.1.1 Implant Location

We used a subcutaneous implant model for the Del-1 project. Intuitively, the local environment is expected to play an important role in determining the outcome of the transplantation therapy. First, the vascularization needs of different tissues are not identical, with the skin for example representing a less well vascularized environment than muscle. Since the final vascular density within the implanted tissue is expected to eventually reach a physiological equilibrium with the surrounding tissue, one would also expect that a subcutaneous implant would also become less densely vascularized. Therefore, a subcutaneous model is likely more hostile in terms of providing the rich vascular supply needed for the survival of transplanted cells. On the other hand, the subcutaneous model also provides a good ‘worst case scenario’ model, potentially enabling to distinguish some subtle pro-angiogenic effects that would otherwise be missed in a strongly pro-angiogenic local environment.
Other than the difference in vascular density itself, differences in recruitment of host cells to the implant site have also been reported among different implantation locations. One study showed that while only fibroblasts were recruited in subdermal implants in rabbits, implants in the adipose tissue recruited host fibroblasts, EC, and SMC in some cases. Implants in this study were elastin tubes (prepared from porcine carotid arteries) filled with either stromal derived factor -1α (SDF-1α) or basic fibroblast growth factor (bFGF)-loaded agarose gels. The study also found that while in the adipose implant model bFGF recruited fibroblasts, SMC, and EC, SDF-1α only recruited fibroblasts and EC, but not SMC. Also, fibroblasts in the bFGF subdermal model were SMA+, while in the SDF-1α subdermal model they were not [5].

Literature reports showing that Del-1 delivery enabled revascularization of ischemic tissues in vivo mostly focused on hind limb ischemia models, with some reports also investigating cardiac and cerebral ischemia models as well. These studies only involved Del-1 delivery, without cell transplantation. It would be interesting to investigate the effect of the implantation site on the Del-1 implant vascularization outcome. From a tissue engineering perspective, although in most cases the ultimate goal would be to transplant the functional tissue to its proper anatomical location, one can envision that a vascularized tissue can be first built in a strategically selected location, then surgically removed and re-transplanted to its proper location. Remodeling of the pre-formed vascular network upon transplantation to a new location, and the time lag before reconnection to the host vasculature at the new location are the key issues with this approach.

Others showed that upon implantation of Matrigel™ hydrogels containing endothelial progenitor cells (EPC) and mesenchymal progenitor cells (MPC) in a subcutaneous SCID mouse model, a few of the donor-derived blood vessels were perfused and connected to the host vascular supply five days after transplantation, and the number of perfused blood vessels further increased at later time points [6]. Upon harvest of the implanted vascularized tissue and re-transplantation in a different animal, a few of the donor-derived blood vessels were re-connected to the host vascular network three days after transplantation. These results show that pre-vascularization of the implant before re-transplantation to a different site preserved some of the pre-formed vascular network and decreased the lag time before connection to the host vascular supply by two days in this study [6].
To further accelerate re-connection to the host vasculature upon relocation to a different implant site, one can envision strategically delivering factors that are involved in (1) sprouting angiogenesis (for example vascular endothelial growth factor, VEGF) to initiate host vessel sprouting and direct the elongation of the host-derived sprouts towards the implant area, (2) in recruiting bone-marrow derived progenitor cells to the implant site (for example SDF-1) to participate in the formation of new blood vessels connecting the implant vasculature with the host vasculature, as well as (3) in anastomosis between host blood vessel sprouts and donor-derived sprouts (possible receptor-ligand candidates that facilitate the interaction between tip EC and macrophages (macrophages act as a bridge facilitating connection) include Tie2-Angiopoietin 2, CXCR4-SDF-1, Notch-Delta-like 4) [7, 8].

The omentum is likely a promising pre-vascularization site, given its highly vascularized nature [9-12]. In one study, a cardiac patch was first fabricated by seeding neonatal rat cardiac cells onto an alginate gel mixed with Matrigel™ and with added pro-survival and pro-angiogenic factors. The cardiac patch was then transplanted onto the omentum and pre-vascularized at this location for seven days. After the seven days, blood vessels, as well as bundles of cardiac muscle were present throughout the cardiac patch. The cardiac patch was then re-located onto infarcted rat hearts. Analysis 28 days after re-transplantation showed that the cardiac patch remained vascularized, it was electrically integrated with the host myocardium, and it had a positive therapeutic effect on the infarcted heart [9].

Previous studies from our group also used the omentum for transplantation purposes. EC transplanted in an omental pouch were able to survive and form a dense network of blood vessels in the absence of any vascular support cells [13]. The addition of MSC as vascular support cells further improved the maturation of the newly formed blood vessels [14]. When EC were transplanted into the omental pouch along with pancreatic islets, as therapeutic cells, and in the absence of MSC, donor-derived blood vessels formed around the transplanted islets. However, further improvement in blood vessel maturation was presumed to be necessary in this case in order to improve islet engraftment, perhaps by adding MSC [15]. It may be interesting to investigate whether the addition of Del-1 in this omentum implant model would lead to further improvements in vascularization. In addition, another extension to these studies is to investigate the use of the omentum solely as a pre-vascularization site (for example for cardiac tissue...
engineering applications), then relocate the construct to the target site (the heart) at the end of this short pre-vascularization period.

Another *in vivo* pre-vascularization strategy that could be adapted for modular tissue engineering consists in placing the implanted tissue around an arteriovenous loop (AVL) *in vivo*. Modules would first be placed inside an implantable chamber, and the chamber would then be surgically inserted around a surgically created AVL. With this experimental setting, blood vessel formation is expected to occur rapidly within the chamber, with sprouts elongating from the AVL and connecting to donor-derived vessels formed by assembly of the transplanted EC, delivered on modules. After a short pre-vascularization step, the pre-vascularized tissue could then be surgically removed and relocated to the target site. From a practical point of view, some of the advantages of the AVL system are that the implanted chamber can be easily localized and surgically removed when desired; also, timed delivery of therapeutic cells or additional growth factors is possible, as the tissue of interest is spatially contained within the chamber walls. Hence, modules with therapeutic cells could potentially be delivered a few days after the initial surgical placement of the chamber, to enable initiation of the vascular network assembly prior to addition of therapeutic cells, which would otherwise be more susceptible to undergo apoptosis in the initial hypoxic environment. Also, while factors promoting vessel sprouting or bone marrow progenitor cell recruitment could be delivered inside the chamber during the initial surgery step, additional growth factors involved in blood vessel maturation could be delivered at a later time point. Literature reports showed successful use of the AVL strategy for various tissue engineering applications, including vascularized pancreatic islets, adipose, cardiac and skeletal muscle tissues [16-20].

### 2.1.2 Functional Cells

In the context of modular tissue engineering, functional cells, or therapeutic cells, are defined as the cells that are responsible for performing the main physiological function of the organ or tissue that they are part of. For example, hepatocytes are the functional cells of the liver, while cardiomyocytes are the functional cells of the heart. Since this thesis project focused on the formation of a vascular network *in vivo* and on exploring ways to build vascularized tissues in general (i.e. we did not focus on building cardiac tissue, or liver tissue or any other specific
tissue applications), we only included vascular cells in the modular tissue engineered constructs (EC, with or without adMSC as vascular support cells), but no ‘functional’ cells.

Nevertheless, the presence or absence of these functional cells within the implanted tissue is also expected to influence the vascularization outcome. The functional cells will increase the metabolic load of the implant, and likely also the physiological steady state vascular density threshold compared to implants containing only vascular cells, such as those used for this thesis project. In fact, in contrast to other cell types, EC are particularly well suited to survive in a hypoxic environment and in general have relatively low oxygen consumption rates [21]. Under resting conditions in quiescent blood vessels, EC are exposed to an abundance of oxygen and use aerobic metabolism. On the other hand, migratory EC (i.e. during sprouting angiogenesis) are exposed to a hypoxic environment in the avascular tissue that they invade to vascularize, and during this sprouting phase EC mostly derive their energy through anaerobic glycolysis, thus relying to a lesser extent on oxygen availability [21]. The fact that EC are well equipped with a metabolic mechanism that enables them to survive and proliferate in a hypoxic environment, while other cells are not, reinforces the idea that adding functional cells will increase the oxygen demand within the tissue engineered implant, thus providing a physiological stimulus for maintaining a rich network of blood vessels.

The higher number of metabolically active cells will create a hypoxic environment at the implant site. In turns, hypoxia is one of the main physiologic drivers of angiogenesis. In a hypoxic environment, there is an increase in the amount of HIF-1 present (hypoxia-inducible factor 1, a heterodimeric transcription factor, and one of the master regulators of angiogenesis), with the HIF-1α subunit of HIF-1 no longer targeted for degradation in the absence of oxygen. HIF-1 then increases the expression of a number of pro-angiogenic factors stimulating sprouting of blood vessels, including VEGF, or recruitment of bone marrow derived progenitor cells, including SDF-1α [22, 23]. Overall, the biological factors secreted by the functional cells, including angiogenic growth factors, will tilt the angiogenic balance between pro- and anti-angiogenic factors at the implant site towards stimulating vascularization. Once a physiological balance between supply and demand of oxygen (and nutrients) has been reached at the implant site, the number of blood vessels will likely stabilize to enable oxygen homeostasis [21, 22, 24]. The presence of blood flow, the recruitment of pericytes and the synthesis of new ECM stabilize the newly formed vessels [25]. In addition, since the hypoxic stimulus is removed upon formation of
a network of blood vessels that delivers sufficient oxygen, the expression of molecules stimulating sprouting also decreases, contributing to the stability and quiescence of the blood vessel network formed [25].

Further analyzing the number of blood vessels formed in Del-1 implants, we notice that the highest density of blood vessels was 84 vessels/mm² (donor+host vessels) at day 7 post-surgery, and that the density of blood vessels decreased over time, down to 41 vessels/mm² at day 21. We hypothesize that if functional cells were present, the number of blood vessels that we observed at day 7 (or perhaps an even higher number of blood vessels) would have been maintained over time to match the oxygen and nutrients demand from the functional cells and thus to continue to support the viability of the implanted tissue. Wound healing is an example of physiological process characterized by an initial peak in blood vessel formation to support the high number of cells recruited at the injury site to contribute to tissue repair; followed by vessel regression during the final stages of tissue remodelling, when the number of cells involved in tissue repair at the injury site also decreases, thus matching the blood vessel supply with the metabolic load of the tissue [26, 27].

Solely considering the diffusion limit of oxygen (~0.1-0.2 mm [28]), while ignoring the specific metabolic requirements of the implanted tissue, it is expected than a minimal blood vessel density of 25-100 vessels/mm² (also assuming uniform distribution of blood vessels within the implant) is required to supply oxygen within tissues with dimensions greater than the diffusion limit of oxygen. Therefore, the blood vessel density that we observed at day 7 (84 vessels/mm²) fulfills this minimum requirement. However, tissues with high metabolic activity such as cardiac tissue for example have a much denser network of blood vessels (> 4000 vessels/mm² in the native mouse heart [29]; i.e. one order of magnitude higher than the minimum requirement). Nevertheless, the cell density in tissue engineered constructs is typically also much lower than normal tissue (i.e. $10^6$-$10^7$ cells/cm³ instead of $10^9$ cells/cm³), and therefore the minimum blood vessel density as defined by the oxygen diffusion limit is likely sufficient to maintain the viability of tissue engineered constructs.

In conclusion, we propose that the addition of functional cells to the tissue engineered constructs would maintain a relatively high number of blood vessels over time. However, one caveat to this approach is that the higher density of implanted cells will perhaps increase hypoxia immediately
after transplantation and prior to the assembly of a vascular network up to a level that might actually jeopardize the initial survival of the cells. Perhaps adding the functional cells after the initial assembly of the primitive blood vessel network, but before the onset of vessel regression (i.e. combining a pre-vascularization strategy, see section 2.1.1 of thesis) may provide a successful alternative.

2.1.3 Host Inflammatory Response and Animal Model

For the purpose of this thesis, we only briefly investigated the host inflammatory response to the implanted tissue, by investigating the number of CD45+ cells present at the implant site. Our data suggested that Del-1 had a bimodal effect on inflammatory cell recruitment, depending on the biological context. On one hand, Del-1 played a role in recruiting or retaining more inflammatory cells to the implant site at day 3, presumably facilitating the clearance of apoptotic cells and the initiation of tissue vascularization at this early stage after cell transplantation. On the other hand, at day 7, presumably in a different biological context, after the initial tissue repair after implantation has occurred, Del-1 decreased the number of inflammatory cells.

Literature reports showed that Del-1 binds to phosphatidylserine on apoptotic cells via its discoidin I-like domains, and it is believed to act as a bridge between apoptotic cells and phagocytic cells [30]. On the other hand, other literature reports showed that Del-1 acts as an endogenous anti-inflammatory factor, by antagonizing lymphocyte function associated molecule-1 (LFA-1) dependent adhesion and thus interfering with intercellular adhesion molecule-1 (ICAM-1)/LFA-1 interactions between endothelial cells and leukocytes [31, 32]. These multiple functions of Del-1 are consistent with our data suggesting a bimodal effect on inflammatory cells.

It would be interesting to further characterize the inflammatory response in our system, perhaps including specific markers for neutrophils and different subsets of macrophages, to first determine the identity, as well as phenotype of the CD45+ cells (CD45 is a pan-leukocyte marker), and evaluate whether there is also a difference in the identity or phenotype of these inflammatory cells depending on the implant type and time point. We could also analyze the expression levels of chemokines known to be involved in recruitment of bone-marrow derived progenitor cells (such as SDF-1) both within the implant and in the serum. We would expect to detect upregulation of these cytokines for Del-1 versus eGFP implants, and at day 3 compared to
day 7, if Del-1 played an indirect role in stimulating recruitment of bone-marrow derived progenitor cells via its interactions with the cells at the implant site. In a simplified *in vitro* system, we could also investigate the conditions under which Del-1 overexpression in endothelial cells promotes or inhibits inflammatory cell retention. In a system mimicking blood flow through an endothelial lined tube, we could examine whether in the context of either hypoxic endothelial cells or endothelial cells just starting the apoptosis process, Del-1 expression by the endothelial cells indeed favors enhanced adhesion of neutrophils or macrophages flown through the system compared to a similar system, but with EC not transduced to overexpress Del-1. Next, in the context of healthy, but activated (i.e. with upregulated expression of ICAM-1) EC and under normal oxygen culture conditions, we would expect to find the opposite effect, i.e. we would expect Del-1 overexpression by EC to decrease the ability of the inflammatory cells to adhere to ICAM-1 expressed on the activated EC. Still *in vitro*, we could also analyze the expression of various pro-angiogenic factors by the inflammatory cells that are retained to the EC layer, and perhaps also compare to the expression profile of the inflammatory cells that are not retained. Overall for our purposes, it would be interesting to further investigate and relate the (anti)inflammatory and pro-angiogenic properties of Del-1 in the context of tissue engineering of vascularized constructs.

Another aspect deserving further attention is the animal model used. For this thesis, we used an immunocompromised animal model (SCID/Bg mouse), which is T-cell and B-cell deficient and with impaired natural killer cells, while having all other components of the immune system still functional. Other animal models may provide additional insight into the mechanism and functions of Del-1. Perhaps using an immunocompetent animal model with syngeneic tissue transplantation would provide additional information. Del-1 literature studies used immunocompetent mice, and the anti-inflammatory properties of Del-1 were mainly reported in different treatment-induced or age-induced inflammatory contexts, and by comparison to Del-1 deficient mice (Del-1−/− mice) [31, 32].

### 2.2 Develop Additional Strategies to Normalize the Implant Vasculature

In our study, Del-1 overexpression increased the number of blood vessels formed *in vivo* and some of these vessels were connected to the host vasculature and perfusable as early as day 7.
However, the vasculature at the implant site at day 7 was tortuous, and the branching structure was presumably still not optimal for perfusion, since depending on the perfusion method used, the perfusion agent did not reach some of these blood vessels in some cases. Therefore, Del-1 overexpression is probably not sufficient by itself and much effort is likely still required to build a truly functional network of blood vessels.

The modular approach, along with lentiviral transduction of EC with pro-angiogenic molecules, can serve as a uniquely versatile platform and screening tool for future studies aimed at establishing the critical factors required for the formation of a functional vasculature, including, but not limited to Del-1. EC transduction with various molecules of interest can be used as an in vivo delivery method, where cells act not only as drivers of in vivo tissue reconstruction, but also as delivery vehicles. Modules coated with EC transduced to overexpress different pro-survival or pro-angiogenic factors can be mixed together in controlled ratios to elicit a desired outcome in vivo.

Moreover, by using non-integrating lentiviruses instead of integrating lentiviruses, we could also control the time frame of expression of these factors, with either transient or permanent expression. An example of combination of factors that could be tested in vivo are stem cell factor (SCF), interleukin 3 (IL-3), and SDF-1α. The Davis group recently showed that this unique combination of three factors was able to support tube formation under defined serum-free conditions in vitro, in a collagen type I gel, with EC alone or co-cultured with pericytes [33]. By comparison to this combination of three factors, VEGF-A was not able to induce tube formation in this serum-free system. However, pre-treatment with VEGF-A and FGF-2 upregulated the EC receptors for the three cytokines and thus primed the EC to respond to the combination of the three cytokines and form tubes. One can envision that a similar strategy could be tested in vivo in the context of modular tissue engineering, with modules coated with EC transduced to transiently express VEGF-A and FGF-2 mixed together with modules coated with EC transduced to stably express SCF, IL-3 and SDF-1α. Nevertheless, even with this combination of factors, the key question that remains to be answered is whether the blood vessels that presumably will form at the implant site will also have adequate function and normal structure. Perhaps adapting the principles of vessel normalization currently under investigation in cancer research or other angiogenic diseases [34, 35] would help induce vessel normalization at the implant site, which
would in turn improve delivery of oxygen and nutrients and the overall implant survival and function.

2.3 Further Characterize the Quality of the Implant Vasculature

*In Vivo*

For this thesis project, we used several methods to investigate whether the implant vasculature was connected to the host vascular network and to evaluate the extent of perfusion. These methods included nonlinear contrast agent ultrasound imaging, microCT, and systemic injection of fluorescent UEA-1 and dextran. While we now know that the implanted constructs were somewhat perfused at day 7, we do not know whether this level of perfusion would be sufficient to maintain the survival of functional implanted cells, and whether there is a functional difference between Del-1 and eGFP implants. Photoacoustic imaging is a technology enabling the quantification of oxygen saturation of blood via detection of the concentration of oxygen bound hemoglobin (oxyhemoglobin) in the blood [36]. It relies on the different light absorption properties of oxyhemoglobin versus hemoglobin not bound to oxygen (deoxyhemoglobin). Using photoacoustic imaging, we would be able to obtain a visual map of the hypoxic versus normoxic areas within the implant, which would then allow us to make a more informed functional assessment of the vascular network formed, based on the level of oxygenation of the implant. This technique has been used by others in studies including investigation of hypoxia in tumor vasculature [37], or examination of the brain vasculature [38].

3 References


