Tissue Engineering Blood Vessels

Macrovasculature, Microvasculature and Molecular Mechanisms

Doctoral Thesis

Mia Marie-Elisabeth Hauge Rambøl

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Faculty of Medicine

Institute of Basic Medical Sciences, Department of Molecular Medicine

University of Oslo

and

Norwegian Center for Stem Cell Research

Department of Immunology, Oslo University Hospital, Rikshospitalet

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List of papers included

Paper I: Rambøl MH, Hisdal J, Sundhagen JO, Brinchmann JE, Rosales A. **Recellularization of Decellularized Venous Grafts Using Peripheral Blood: A Critical Evaluation.** *EBioMedicine*. 2018;32:215–222. doi:10.1016/j.ebiom.2018.05.012

Paper II: Rambøl MH, Han E, Niklason L. **Microvessel network formation and interactions with pancreatic islets in 3D chip cultures.** Submitted manuscript.

Paper III: Rambøl MH, Sundaram A, Brinchmann JE. **Mapping of crosstalk between mesenchymal stem cells and endothelial cells in a 2D co-culture model of neovascularization.** Manuscript.

Abbreviations

AT	adipose tissue
DLL4	delta like canonical notch ligand 4
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
EC	endothelial cell
ECM	extracellular matrix
EPC	endothelial progenitor cells
ESC	embryonic stem cell
FBS	fetal bovine serum
FLT1	fms-related tyrosine kinase 1 (VEGFR-1)
FPKM	Fragments Per Kilobase of transcript per Million fragments mapped.
GF	growth factor
GO	gene ontology
GSEA	gene set enrichment analysis
H&E	hematoxylin and eosin stain
HGF	hepatocyte growth factor
HUVEC	human umbilical vein endothelial cell
IHC	immunohistochemistry
iPSC	induced pluripotent stem cell
iPSC-ECFC	iPSC derived endothelial colony forming cell
mRNA	messenger RNA
MSC	mesenchymal stem (or stromal) cell
OCT	optimal cutting temperature compound
PCL	polycaprolactone
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PDGFR	platelet derived growth factor receptor
PDMS	polydimethylsiloxane
PGA	polyglycolic acid
PLA	polylactic acid
RNA	ribonucleic acid
RNA-seq	RNA sequencing
ROBO4	roundabout guidance receptor 4
RT-qPCR	quantitative reverse transcription PCR
scRNA-seq	single cell RNA sequencing
SEM	scanning electron microscopy
SMC	smooth muscle cell
TGFB	transforming growth factor beta
UNC5B	unc-5 netrin receptor B
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
vSMC	vascular smooth muscle cell
vWF	von Willebrand factor

Introduction

The focus of this thesis is tissue engineering of the vasculature. In this introduction, I will first give a brief introduction to the general field of tissue engineering and regenerative medicine. This includes defining the concept, introducing cells and materials commonly used for tissue engineering, and briefly reviewing the current state of the field. For studies on vascular tissue engineering, an understanding of vascular anatomy and vascular development is important. These topics will be given some attention, with special emphasis on the process of angiogenesis. Tissue engineering of the vasculature includes both generation of macrovascular structures as well as development of microvascular beds, both of which will be covered. For tissue engineered grafts, manufacturing methods and scaffold materials will be described, as well as current advancements and applications. For microvascular engineering, various approaches for generation of microvascular networks will be presented.

Tissue engineering and regenerative medicine

The concept of tissue engineering and regenerative medicine

Tissue engineering and regenerative medicine are interdisciplinary fields that apply the principles of engineering and life sciences toward development of biological substitutes to restore, repair, maintain, replace or improve the function of damaged or missing tissues, or entire organs[1,2]. A major goal is to mitigate the critical shortage of organ donors via fabrication of biological structures, but the concept also applies to other uses, including development of specialized extracorporeal life support systems such as the bioartificial liver and kidney[3,4]. Moreover, tissue engineering can be used to generate platforms for drug testing or basic studies on tissue development and morphogenesis[5-7].

The terms tissue engineering and regenerative medicine are often used interchangeably, although tissue engineering was originally considered a subfield within the broader and more generalized category of regenerative medicine[8]. While tissue engineering often makes use of a combination of cells, biomaterials and growth factors (GF) to encourage growth of

cells and tissues, regenerative medicine includes this but may also utilize other strategies including cell based therapies, immunomodulation, gene therapy and nanomedicine[2]. This can involve transplantation of genetically engineered cells without the use of scaffolds, or pharmaceutical targeting of stem cell developmental pathways as a means of therapy[9]. However, because of similar objectives, namely to replace diseased organs with newly functioning ones, there is an increasing tendency treat the fields as a single research pursuit, which have originated the combined concept of Tissue Engineering and Regenerative Medicine; "TERM"[2,8,10].

Design approaches for tissue engineering

There are three basic tissue engineering design approaches for the creation of new tissues for use in patients. These include the use of cells only, where freshly isolated or cultured cells are implanted into the patient, the use of engineered tissues assembled *in vitro* from cells and scaffolds, or the use of scaffolds only, relying on *in situ* tissue regeneration[11]. For cellular implantation, cells or cell aggregates can be injected into the damaged tissue directly, or be combined with a degradable scaffold *in vitro* prior to implantation. Engineered autologous cartilage tissues have been used for nasal reconstruction[12], and engineered scaffold-free cartilage spheroids have been used for treatment of cartilage defects in the knee[13]. Where whole engineered tissues are used, a complete three-dimensional tissue is grown in vitro using cells and a suitable scaffold, and implanted once it has matured into a functioning organ structure. The first successful use of this method was tissue engineering of bladders, where urothelial and muscle cells derived from the patients were expanded in culture, seeded on a bladder-shaped scaffold, and implanted into the patients seven weeks later[14]. For *in situ* regeneration, a scaffold is directly implanted into the body, where it integrates and is "made alive" by cellular migration and recruitment from the host. An example of this is bioengineered human acellular vessels that have been shown to recellularize and evolve into living blood vessels after implantation into humans[15]. The choice of method is dependent on the structure and function of the tissue to be repaired or replaced.

Cells for tissue engineering

Identifying the most suitable cell source is important for design and development of engineered tissues and organs. To be able to recapitulate the characteristics of the native tissue in vitro, cells of tissue specific phenotypes must be available in sufficient numbers[16]. Cells like endothelial cells (EC), smooth muscle cells (SMC) and beta cells of the pancreas all perform tasks that might be integral to the function of the tissue. In addition, the cells' production of tissue specific extracellular matrix (ECM) influences tissue function[17]. For instance, the function of articular cartilage found in the knee is dependent on the composition of its ECM[18].

The cells used for tissue engineering can be derived from the patient's own cells (autograft), from another individual (allograft), or from a non-human animal species (xenograft), and can be fully differentiated cells or cells with stem cells properties[6].

Differentiated cells

Many tissue engineering applications have relied on the use of differentiated somatic cells taken from the patient. These cells are representative with regards to the functional aspects of a tissue, and can be isolated from patient tissue biopsies[16]. Moreover, patient derived cells are immunologically compatible, and will not be rejected by the body[19]. However, tissue engineering may require a large number of cells, and as adult cells are often fully differentiated and post-mitotic with a limited life span, they may have a limited proliferation potential[16]. In addition, the potentially invasive nature of cell harvest as well as complex cell isolation procedures, and the potential of these cells to be in a diseased state, confer some limitations on the use of fully differentiated cells[17,20].

For these reasons, attention has become more focused on stem cells. Stem cells are undifferentiated cells capable of self-renewal and differentiation into one or more cell types, and can be isolated from several sources, such as embryos, fetuses, umbilical cords and adult tissues, where the cell source determines the differentiation capability of the cell[16]. Several types of stem cells have been considered for tissue engineering applications, including embryonic stem cells (ESC), mesenchymal stem cells (MSC) and induced pluripotent stem cells (iPSC)[17].

Stem cells for tissue engineering

Embryonic stem cells.

ESCs are pluripotent, having ability to differentiate into to all of the cell types found in the body[21]. These cells can be maintained in culture for long periods of time, thereby potentially providing large amounts of cells that cannot be derived directly from a tissue source. Cultured ESCs have a demonstrated potential to differentiate into a multiple tissue types, and could in theory allow for production of type matched tissues for each patient through stem cell banking or by the use of therapeutic cloning[17,22]. However, the use of ESC is surrounded by political and ethical controversy. Moreover, ESCs are allogeneic in nature, and may be subject to immune reactions *in vivo*[23]. ESCs are also prone to teratoma formation *in vivo*, which complicates their clinical use. Therefore, good methods to control and ensure terminal differentiation are required[24].

Induced pluripotent stem cells.

iPSCs are pluripotent cells derived from somatic cells by overexpression of key transcription factors[25,26]. Generation of iPSCs provides the means to surpass the allogenicity issue by using the patient's own cells. As ESCs, these self-renewing cells can be differentiated into virtually any cell type. Techniques also exist for trans-differentiation of some differentiated cell types into other specialized cells, without reverting to the stem cell state[27-29]. Still, human iPSC could potentially offer a virtually unlimited supply of cells for tissue engineering, therapeutic discovery, and disease modelling[20]. The persistence of iPSC in differentiated cell populations still pose a risk for teratoma formation, and efficient purging of stem cells from tissue engineered grafts is required for their clinical use[30].

Mesenchymal stem cells

MSCs have emerged as a promising and frequently used cell source for tissue engineering[31]. A defining feature of MSCs is their trilineage differentiation potential: MSCs can readily differentiate into osteogenic, chondrogenic and adipogenic lineages[32]. Moreover, MSCs have also been shown to hold the potential to differentiate into myogenic, and possibly neurogenic, lineages[33-36,37]. The secretome from MSCs has also been shown to have

therapeutic potential, with demonstrated abilities to modulate the local immune microenvironment, reduce injury, promote epithelial repair and promote angiogenesis[38-40].

MSCs can be isolated from several tissue sources, including bone marrow, adipose tissue (AT), lung, liver, muscle, cord blood, amniotic fluid, umbilical cord and dental pulp[33,41-45], and can easily be expanded in culture to obtain clinically relevant numbers. MSCs thus represent a readily available autologous cell type with potential for use in tissue engineering[40].

Scaffolds for tissue engineering

Where injection or implantation of cells or cell-aggregates alone is not an option, such as in cases where the structure and shape of the implanted tissue is important for its function, combining cells with scaffolds is a well-known approach. Scaffolds for tissue engineering can be made of a variety of materials utilizing a large number of manufacturing techniques[46]. These scaffolds are intended to mimic the cells' natural three dimensional environment, and must be appropriate for the desired local environment within the human body, as well as for the tissue specific cell types[6,47]. The scaffolds do not only provide temporary structural integrity, but are involved in interaction with cells and biomolecules, cell attachment and growth, and in the tissue development process[48].

Design criteria for scaffolds for tissue engineering

Tissue engineered scaffolds serve various purposes, depending on which cells and tissues it should support. Scaffolds can provide mechanical support for regenerating tissue, can be used as vehicles for signaling molecules, aid in directing cell growth and tissue remodeling, as well as contribute to establishment of a suitable microenvironment in which tissue repair and regeneration can take place[49]. Some key considerations are important when designing or determining the suitability of a scaffold. One important design criteria is that of *biocompatibility*[46]. Cells must be able to adhere to and migrate through the surface, and proliferate and function normally in the scaffold. What is more, the scaffold material must not elicit any immune reactions and inflammatory responses that will prevent healing or cause graft rejection. Second, for many applications the scaffold should be *biodegradable*, allowing the body's own cells to take over and produce their own ECM[50]. The by-products of the degradation process should also be non-toxic, and there should not be any fibrous

encapsulation or residues remaining in the body[51,52]. Third, the scaffold should exhibit *mechanical properties* consistent with the task it is to perform in the body, and be strong enough for handling during implantation surgery. Depending on the tissue and anatomical site, the required mechanical properties such as toughness, rigidity and elasticity will vary[53]. Especially for cardiovascular and orthopedic applications, this can be a challenge. For engineering of bone or cartilage, producing scaffolds with adequate mechanical properties can be demanding, as the implanted scaffolds should have sufficient strength and integrity to function from the time of implantation[54]. Lastly, the *scaffold architecture* is of critical importance, and should provide for *structural support for cells*. Scaffolds should have interconnected pores and be porous enough for cell penetration and migration, as well as for diffusion of nutrients. In addition, cellular waste products and degradation products from the scaffold should readily escape the scaffold. Pore size has also been shown to impact on cell attachment, and thus affects cell viability in the construct[53,55]. Most scaffold materials aim to recapitulate important features of the cells' local tissue environment, the ECM.

Extracellular matrix

The ECM is the other major component of human tissue, in addition to the cells themselves. The ECM is a three-dimensional network of extracellular macromolecules that provides structural and biochemical support for the cells. The amount of ECM varies between tissues[49]. Connective tissues such as articular cartilage and bone are largely comprised of ECM, with only 2% and 10% of the tissue volume made up of cells[56,57]. Conversely, epithelial tissues are composed largely of cells and contain relatively little ECM[58].

The ECM is generally composed of molecules from three categories: fibrous proteins such as collagen, elastin, fibrillin and fibulin, adhesive glycoproteins such as laminin, fibronectin, tenasin, thrombospondin, and glycosaminoglycans[49,59]. The cells of the tissue are constantly synthesizing, secreting, modifying and orienting these components, which are directly participating in promoting cell adhesion, migration, growth and differentiation through interactions with cell surface receptors[60].

The diversity in morphology and composition of ECM in the body contributes significantly to the specific properties and function of organs and tissues[59]. In the body, the ECM provide

structural support in addition to biological functions during tissue regeneration and maintenance. The ECM is involved in a number of other processes important for tissue morphogenesis and organ development that rely on close interactions with the cells. These include establishing and maintaining the cellular microenvironment, providing structural information and biochemical cues to surrounding cells, regulating signaling molecule activity as well as affecting cell behavior, including morphology, survival, proliferation, migration and differentiation through complex and dynamic cell-ECM interactions[49,59,61]. These interactions are also important for pattern formation, morphogenesis and phenotype acquisition during development[60]. Moreover, the ECM is also essential for wound healing by providing structural integrity, regulation of cellular functions, and mediating interactions between cells, as well as serving as a reservoir and modulator of GFs and cytokines[62].

Cells bind to ECM through integrins and focal adhesions. These adhesions provide a means through which cells can sense and respond to the extracellular environment. Integrin mediated cell-ECM adhesion may have profound effects on cellular behavior, including their morphology, proliferation, differentiation, migration and survival[63]. Similarly, changes within a cell can alter the cells' surface receptors and modify their interaction and binding to ECM components. The cellular response to ECM signaling can also alter the state of the ECM itself, and the ECM is constantly modified in response to the metabolic activities of the cells residing in the tissue, the mechanical demands of the tissue, and the microenvironmental niche conditions[64]. For instance, cells may release matrix metalloproteases to break down dense ECM to allow for cell migration or proliferation[60]. A number of ECM proteins or proteins regulating cell-ECM contact has also been shown to be indispensable for embryonic development or normal organ function[65].

The interactions between the ECM and the resident cell population rely on "dynamic reciprocity"[66]. This gives the use of decellularized ECM or ECM-derived components for tissue engineering an advantage compared to synthetic materials. This also emphasizes the significance of aiming to maintain or mimic the native composition and ultrastructure of the ECM during scaffold preparation[64]. Scaffolds used for tissue engineering should ideally provide the same level of support and functions as native ECM, and should accurately meet the needs of the tissue[67,68].

Types of scaffolds for tissue engineering – natural, synthetic or composite

Scaffolds for tissue engineering are generally classified as natural or synthetic. Natural scaffolds include scaffolds produced from isolated ECM components or native ECM structures derived from decellularized tissues of organs. Synthetic scaffolds can be derived from a variety of synthetic materials with the aim of mimicking ECM properties. Hybrid scaffolds, containing both natural and synthetic components, are also being used. Scaffolds can be fabricated by a variety of techniques, including, but not limited to, freeze drying, solvent casting, gas foaming, molding, textile techniques such as electrospinning, weaving or knitting, CAD-aided design such as 3D-printing or solid-freeform fabrication, lithography techniques and decellularization, depending on the chosen material[60,68-70].

Natural scaffolds from ECM components

Fabricating scaffolds from natural ECM components could potentially provide features similar to those of native ECM, thus allowing the cells to interact with the scaffold in a natural manner[49,67]. Receptors on the cells can bind to natural ECM components, promoting attachment, proliferation and differentiation, in a similar manner to what is naturally occurring in the body. However, different tissues have different ECM composition, and which components should be included depends on the properties of the tissue as well as cell type[69]. The native ECM is a complex structure, and developing an ECM analogue for use in tissue engineering is challenging[49,51]. Several different ECM proteins have been used for generation of scaffolds for tissue engineering applications.

Collagen is the most abundant protein in the human body, providing structural stability and mechanical rigidity for tissues and organs[71]. These properties can be modified, depending on bundle thickness and directional order of the fibers[72,73]. More than 20 forms of collagen have been identified, and collagen type I is often used for scaffold production[69,74]. Interactions with collagen has been shown to influence cell growth and differentiation, determined by the cells' ability to penetrate the fibrillar collagen ECM[75]. However, for cells not naturally residing in collagen type I rich ECM, being seeded into a scaffold largely composed of this polymer might elicit unwanted responses. For example, articular chondrocytes, which normally exist in hyaline cartilage consisting of a collagen type II

network, are induced to form fibrocartilage by collagen type I[69]. This underlines the importance of tailoring ECM to specific applications.

Elastin is a self-assembling, highly elastic macromolecule, synthesized by several different cell types including fibroblasts, ECs, SMCs, chondrocytes and keratinocytes[76]. Elastin is critical for the elasticity, compliance and resilience for a range of tissues, including the vasculature, skin and lung[77]. In addition, elastin provides mechanical integrity and is involved in regulation of cell behavior[78]. Elastin is often used to reinforce the mechanical properties of collagen based scaffolds[79,80]. Incorporation of other ECM molecules, such as laminin, fibulin, fibrillin, fibronectin and tenacin, or peptide derivatives of these, is also being investigated for use in tissue engineering scaffolds[60,74].

Some limitations to the use of natural ECM materials for production of tissue engineered scaffolds include poor mechanical strength, batch variations of engineered products, and unpredictable or varying degradation rates[71]. Moreover, potential antigenicity and immune responses, limited availability of raw material, and labor intensive processes for isolation and preparation could potentially make natural scaffolds less desirable. An additional risk could be transmission or animal and prion-based diseases, however some of these issues would be addressed by the use of recombinant protein technology[69].

Natural scaffolds from decellularized tissue

One way to produce a scaffold that is (near) identical in ECM composition to that of the native tissue, is by means of decellularization. This process aims at completely removing antigenic cellular material from the tissues, while the overall composition, mechanical properties and biological activity should remain intact[81]. This method has the potential to alleviate some of the limitations and drawbacks described for the use of isolated ECM components, including insufficient mechanical integrity and inflammatory reactions, however, rapid degradation rates might still be an issue. Decellularized native ECM has been shown to induce a positive host response promoting cell infiltration, rapid scaffold degradation, increased host production of new matrix and tissue remodeling with a reduced amount of scarring[81].

Entire tissues and organs can be decellularized, but one can also take advantage of the inherent ability of the cells to produce ECM. By seeding ECM-secreting cells on pre-designed scaffolds, it is possible to produce scaffolds of specified dimensions, tailored to certain applications. Heart valves is one example of a tissue structure made using this approach, produced from a tube of decellularized cell-produced matrix and mounted on a frame[82]. The engineered valve demonstrated strength and organization comparable to native leaflets, and animal studies, where the engineered scaffolds were implanted as aortic valve replacements into sheep, showed that the valves were functional for at least 24 weeks.

In another study, vascular SMCs were seeded onto degradable tubular scaffolds in a bioreactor delivering cyclic radial strain, yielding mechanically robust vessel structures composed of cell-produced ECM and cells. Decellularization of this structure yielded acellular vessels for use in tissue engineering, and could allow for production of several scaffolds of desired dimensions from one cell donor[15,83]. This method could also open up for off-the-shelf production of tissue constructs for clinical use.

Decellularized cell-produced ECM could eventually help meet the demands for new tissues and organs, by circumventing restrictions on tissue production that are due to limited availability of donor tissue. Using decellularized tissues from animals that have an organ anatomy resembling that of the human would further expand the available donor pool[84]. Decellularized xenografts of porcine origin has been tested for clinical use, including ureters and heart valves. Decellularized heart valves of porcine origin mounted in a rigid stent have in fact been very clinically successful[85]. The porcine valves were treated with glutaraldehyde, believed to reduce antigenicity of xenogenic collagen[86].

Decellularized ECM has been successfully used to recreate several tissues and organs, some of which have demonstrated clinical success. These include tissue engineered blood vessels[15,87-92], urinary bladder[93] and trachea[94]. Tissue engineered heart valves, mentioned above, have demonstrated promising results in animal studies[82]. Additional decellularized tissues and organs are not yet ready for clinical use, but researchers have nonetheless managed to recreate important aspects of the native organ. These include the heart[95,96], lung[97,98], liver[99-102], kidney[103,104], cornea[105,106] and esophagus[107]. For the most complex organ

structures, decellularization of native organs is currently the only way to obtain a scaffold that accurately recapitulates the detailed architecture of the organ[108].

The process of decellularization

In the decellularization process, cells and genetic material are removed from ECM through a combination of mechanical, chemical or enzymatic steps[109]. By using decellularization techniques suited to specific organs or tissues, the native composition, ultrastructure and 3D architecture of the tissue can be largely preserved[110,111]. Which methods and decellularization agents are most suitable largely depends on the composition of the tissue in terms of cellularity, density, lipid content and thickness[112]. A number of decellularization agents have been explored including various chemical agents such as acids and bases, hypotonic and hypertonic solutions, detergents such as Triton X-100, sodium dodecyl sulfate (SDS) or 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), as well as alcohols. Other solvents like acetone or tributyl phosphate (TBP) have also been used. Use of enzymes such as nucleases, trypsin, collagenase, lipase, dispase, thermolysin, and α-galactosidase have also been reported[112,113]. Specifically, α-galactosidase is used for treatment of xenogeneic tissues, for removal the immunogenic cell surface antigen galactose-α-(1,3)-galactose (Gal epitope)[114,115]. When utilizing solutions, perfusion of whole organs, or alternatively immersion and agitation are commonly used[64]. Other approaches include induction of a pressure gradient or supercritical gas decellularization[112,113]. In addition, physical treatments for decellularization, such as freeze-thaw processing, or application of force and pressure, have been used for removal of cellular materials[112].

Requirements for successful decellularization

One of the most critical requirements for decellularization is to reduce the scaffold's immunogenicity[113]. A number of criteria have been suggested to assess successful decellularization, including < 50 ng/mg dsDNA in dry weight decellularized ECM, a remaining DNA fragment length of < 200 bp, and no visible DAPI-staining[112]. DNA components remaining in the scaffold could potentially cause inflammatory reactions, and enzymes such as DNase and RNase are used to break down fragments of nucleic acids[113,116,117]. Native antigens could also induce immune responses, and should be completely removed[113]. For xenogeneic scaffolds, this also includes reducing the xeno-antigenicity of the material. If the

issue of antigenicity is not sufficiently addressed, the result could be a graft-specific host rejection response, putting both the patient and the survival of the graft at risk[118,119].

Protein content in the decellularized ECM should also be evaluated, with emphasis on structural proteins, and decellularized scaffolds should display mechanical properties matching the original tissue[113]. The decellularization process leaves an acellular matrix scaffold composed mainly of collagen and elastin. For tissue engineering of scaffolds, the patients' own cells can then be used to repopulate the decellularized structures.

Synthetic scaffolds

A number of scaffolds mimicking ECM properties have been developed using synthetic materials, for use in tissue engineering and reconstruction[120]. Synthetic materials can offer some advantages, including the technical possibility of optimizing chemical and physical properties for particular applications[121]. Synthetic materials may provide considerable mechanical strength, and are readily available. Moreover, many synthetic materials are bioresorbable with a known degradation rate, thus degradation time should not vary much between hosts[60]. However, accumulation of degradation products from synthetic scaffolds could possibly cause unwanted reactions, and may elicit chronic diseases associated inflammatory responses[122]. Surface and structural characteristics of the synthetic scaffold can be controlled, but their synthetic nature means that they are lacking the biological components of the native ECM[71,123]. Tissue engineering scaffolds composed of purely synthetic polymers lack the critical binding moieties that is required for interactions between the cells and the scaffolds[69].

The primary focus of synthetic materials for tissue engineering has been biodegradable polymers, mostly of the polyester family[60], such as PGA (polyglycolic acid), PLA (polylactic acid), PLGA (polylactide-co-glycolide), PCL (polycaprolactone), PDO (polydioxanone), PEG (polyethylene glycol) and PEO (polyethylene oxide)[124]. These polymers are thermoplastics, making them easy to form into 3D scaffolds with the desired shape and microstructure. Synthetic polymers have been investigated for a number of tissue engineering applications, including liver, cartilage, bone, ligament, tendon, vessels, nerve, bladder, and skin[60].

Scaffolds promoting tissue development

In addition to serving as an ECM analogue providing structure and cell interactions, scaffolds can be designed with additional properties promoting development of the tissue. Most notably, cell-stimulating molecules can be incorporated into the scaffolds. Scaffolds can be designed to release GFs that induce cellular differentiation and promote cell growth *in vitro*, or cell migration i*n vivo*[11]. Scaffolds engineered for staged release of PDGF and VEGF, mimicking the physiological production of these GFs, have been shown to promote neovascularization[125]. Scaffolds releasing Nerve growth factor have been shown to improve viability of fetal neural cells transplanted into the rat brain[126], and local sequential scaffold release of VEGF and BMP-2 has similarly been shown to possibly enhance bone regeneration[127]. Signaling molecules can be incorporated both in natural and synthetic scaffolds, and can include a large number of cytokines and chemokines to promotes cell growth, guide the differentiation process, and promote migration[48].

Levels of complexity in tissue engineering

Four levels of complexity can be defined for categorizing fabrications of tissues and organs. Flat structures, such as skin, followed by tubular organs such as blood vessels are the two least complex structures. Next comes nontubular hollow organ structures like the bladder, whereas the most complex category encompasses solid organs such as the heart, lungs, kidney and liver.[6]

For tissues of less complex structures, such as skin and cartilage, tissue engineering applications have reached the clinic. Skin tissue has been created using dermal fibroblasts and epidermis derived from normal immortal keratinocytes (NIKS-cells)[128,129], used to facilitate healing of traumatic cutaneous wounds. Autologous chondrocyte implantation has been used to treat cartilage effects over the past years, and cartilage for implantation has also been created in the lab[130].

Fabricated tubular organs like blood vessels and trachea have also been used in patients. Recently, a five-year follow up was reported on the implantation of a tissue engineered trachea in a pediatric patient, demonstrating long time viability for this technique. The successful graft was created by decellularization of a trachea from a deceased donor, and

then repopulation of the scaffold with the patient's own epithelium and MSCs[94]. Tissue engineered vascular grafts, both autologous and allogeneic, have been transplanted into patients, mostly for hemodialysis access. Various methods were used to produce these grafts, including sheet based techniques, dehydration or decellularization of tubular fibroblast or vascular smooth muscle cell (vSMC) -produced ECM structures[15,83,89,90,131]. Moreover, in 2011 five boys aged 10-14 with urethral defects received autologous tissue engineered urethras. The engineered urethras were similar to native urethras in terms of histological and functional characteristics, and all the boys remain continent[132].

Tissue engineered autologous vaginas have been implanted into patients with vaginal aplasia, and the engineered organs have been demonstrating normal functions for years after implantation[133]. Autologous bladders have also been fabricated in vitro and been shown to function normally after implantation[14,134].

Compared with solid specialized organs, the above mentioned structures are less challenging from a bioengineering perspective. Hollow structures and thin walls reflect their mechanostructural functions, and their architecture can relatively easily be recapitulated using biomaterial scaffolds, such as tubes or sheets. Also, void of a parenchymal core, they can rely on diffusion of oxygen and nutrients from adjacent tissues while developing new vasculature, if required. More complex organs are composed of multiple cell types and may have intricate microarchitectural structures, such as the nephron in the kidney[135].

Whereas both flat, tubular and hollow organs have been tissue-engineered for clinical use, solid organs still have a way to go. Functional penises have been tissue engineered in a rabbit model[136], but human solid organs for clinical use have not been constructed this far[6]. In addition to structural complexity and scaffold preparation, as well as the need for several different and specialized cell types, a major hurdle for fabricating solid organs is proper vascularization[137,138]. The ability to vascularize tissues is one of the most significant challenges in the field of tissue engineering. All cells require a sufficient supply of nutrients and oxygen, as well as the ability to remove waste[139-143]. A functional microvasculature is necessary to ensure proper function and survival of engineered biological tissues that are too large to be maintained by diffusion[6,144,145]. Several different techniques have been

investigated to achieve organ vascularization. Seeding cells directly into decellularized scaffolds, taking advantage of the structures remaining from the pre-existing vascular network, has been one promising approach[141]. Tissue engineering of the vasculature will be described in more detail later. This process not only requires an understanding of the process of vascular development, but also knowledge on the structure of the vascular system, which will be reviewed next.

The vascular system

Two major circulatory systems exist in the human body: the blood and lymphatic systems. The blood vascular system of the human body is comprised of an extensive network of blood vessels. This is a circulatory transport system, where the heart pumps blood through the vasculature as a means to deliver and remove substances. The circulation provides all the cells in the body with the oxygen and nutrients required for normal function, and removes waste products of cell metabolism, thereby maintaining cellular homeostasis[146,147].

Broadly, the blood vasculature contains three main types of blood vessels. *Arteries* carry oxygen-rich blood away from the heart, to all of the body's tissues. Through a branching network of progressively smaller arteries and arterioles, the blood reaches the *capillary network*. The thin walls of the capillaries allow oxygen and nutrients to pass into the cells, whereas carbon dioxide and waste products can be transported out of the cells. Capillaries connect the arteries and *veins*. The venous system collects blood from the capillaries, leading it back to the heart through progressively larger venules and veins[146] When blood circulates through the capillary system, fluids and proteins leak out into the surrounding tissue. The task of the lymphatic system is to collect this fluid, and transfer it back to the venous circulation through lymphatic vessels[148]. The lymphatic system will not be further discussed in this thesis.

Cells of the blood vessels

The blood vessels are composed of two interacting cell types. The inner lining of all vessels is composed of a layer of ECs, the endothelium or *tunica intima*. The endothelium has critical roles in the control of vascular function[149]. Interacting with circulating cells in the blood, as well as cells present in the vascular wall, ECs are the main regulators of vascular homeostasis. Being the interphase between blood and tissue, they are responsive to changes in blood composition and blood flow.

The endothelium is supported by perivascular cells. These are vSMCs and pericytes, collectively known as mural cells[150,151]. These associate with the abluminal side of the vessel and express characteristics specific to their location[152,153]. Pericytes are mural cells of the microcirculation, and play key roles in regulation of microvascular morphogenesis and

stability. Capillaries are partially covered with pericytes, although their coverage vary between vessel of different organ systems[150,152,153]. Pericytes have been shown to play important roles in regulation of EC proliferation and differentiation, contractility and tone, as well as microvessel stabilization and permeability[154-158]. The pericytes serve to stabilize capillary vessels, but also communicate with ECs through direct physical contact and paracrine signaling pathways[150,152].

Blood vessel structure

Larger blood vessels have a tri-layered structure to fulfil their physical requirements, where each layer is responsible for an independent and essential function. The innermost layer, the *tunica intima*, is an endothelial monolayer which is in direct contact with the blood stream, and is responsible for preventing thrombosis via active and passive mechanisms[159]. Active coagulant mediators, including prostacyclins, nitric oxide and tissue plasminogen activator are secreted by the ECs[160]. Moreover, the endothelial glycocalyx, which is a negatively charged mesh of proteoglycans, glycosaminoglycans, glycoproteins and glycolipids, shields the blood from the thrombogenic basement membrane, and plays an important role in vessel wall homeostasis[161].

The tunica intima is completely surrounded by single or multiple layers of vSMC, the *tunica media*, which is responsible for the mechanical strength of the vessel. The outermost layer, the *tunica adventitia*, consists of collagens and elastic fibers, which imparts vascularization and autonomic control[162]. This layered structure confer strength and stability to larger vessels, while at the same time enabling them to dynamically respond to changes in wall stress or metabolic demands. By contracting or relaxing in response to signals from the surrounding tissue, thus regulating the caliber of the vessels, vSMCs are responsible for redistributing blood according to the needs of the body. Arteries and veins have different composition reflecting their different tasks in the circular system, where arteries contain more smooth muscle and thicker vessel walls (Figure 1). The mural cells associated with intermediate vessels, arterioles and venules, have properties somewhere in between the typical vSMC and the pericyte[152]. It has been suggested that pericytes and vSMCs are phenotypic variants along a continuum of mural cell phenotypes, where the different

properties reflect different sub-specifications dependent on their location in the vascular system[163].

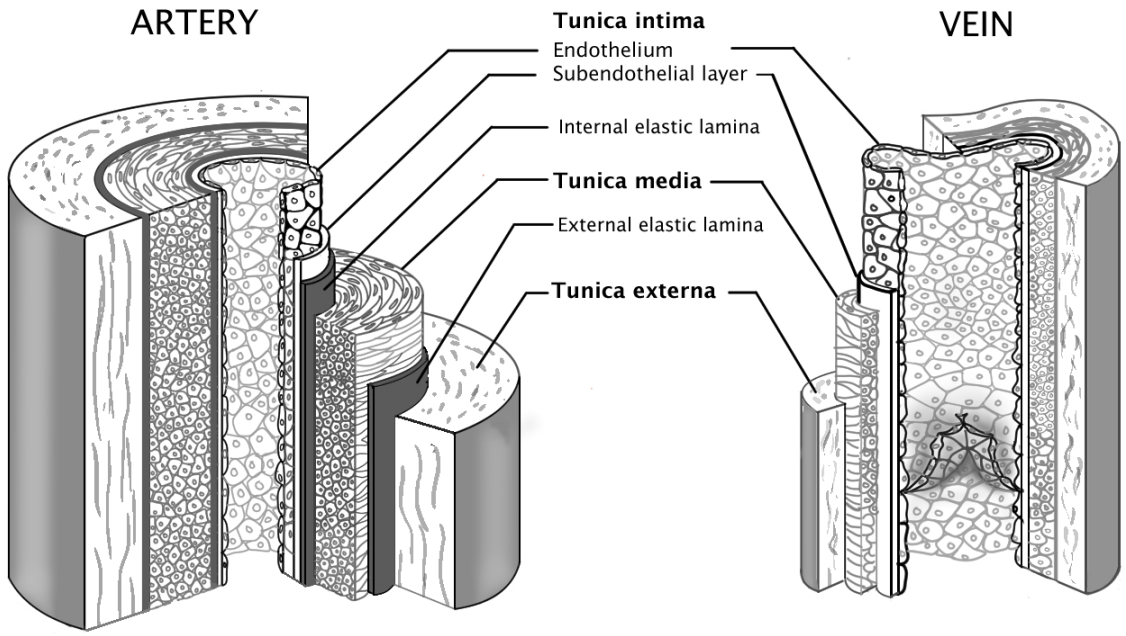

Figure 1. Anatomy of arteries vs veins.

Blood vessel development

Development of any new tissue requires formation of new blood vessels, both throughout embryo development and growth, and for *in vitro* tissue engineering. Without sufficient perfusion, tissues are unable to grow beyond the size that can be sustained by diffusion of necessary nutrients and oxygen, approximately 200 µm[137]. To engineer vascularized tissues, an understanding of the processes involved in vascular development is of great importance[141].

Blood vessel development is an intricate biological process. This process requires careful coordination and integration of orchestrated signaling cues, involving ECs, perivascular cells, as well as the surrounding tissue, leading to the formation and further remodeling of the vasculature into mature vessels[164-167]. New blood vessels can be formed by different mechanisms, including *de novo* assembly by endothelial precursor cells (vasculogenesis) and endothelial outgrowth from the vasculature through sprouting, proliferation and remodeling (angiogenesis; sprouting angiogenesis)[167,168], as shown in Figure 2. In addition, splitting of

existing vessels (intussusception; splitting angiogenesis) is another mechanism to expand and remodel an existing vascular network[168,169].

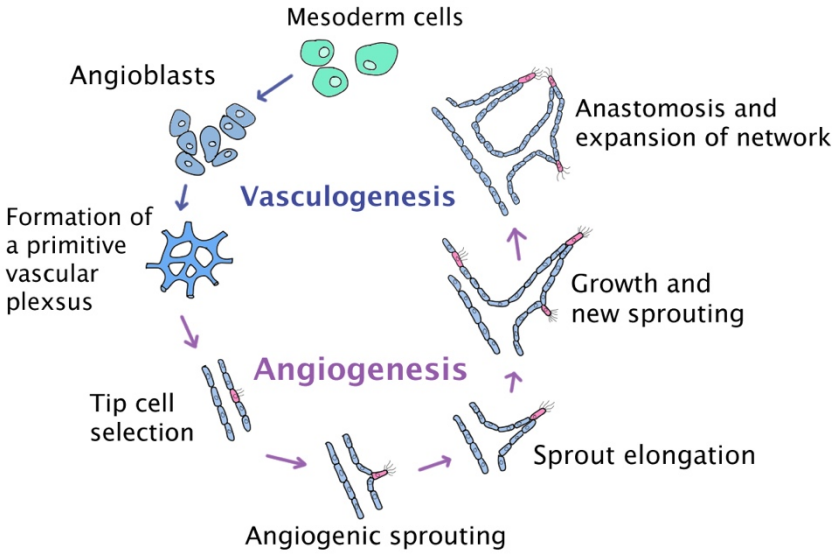

Figure 2. Vasculogenesis and angiogenesis. Formation of new blood vessels can occur via the processes of vasculogenesis and angiogenesis. Vasculogenesis refers to blood vessel formation through *de novo* assembly of endothelial progenitor cells, wheras angiogenesis refers to formation of new blood vessels through sprouting and outgrowth from pre-existing vessels.

Vasculogenesis

Vasculogenesis is the process where new vessels form *de novo* by specification and assembly of endothelial precursor cells. These cells, angioblasts, have the potential to differentiate into ECs, but have not yet acquired all characteristic EC markers. Angioblasts are derived from hemangioblasts, which together with hematopoietic stem cells are derived from mesodermal stem cells[170-172]. Angioblasts then differentiate, coalesce into clusters, and form new blood vessels *in situ*. Vasculogenesis is largely confined to the formation of the first primitive vascular plexus and the large axial vessels in the early embryo[173,174]. Following formation of the primitive capillary networks during vasculogenesis, ECs are specified into either arterial or venous fate, partly in response to hemodynamic forces[175]. Further development of the circulatory system requires angiogenesis[176].

Angiogenesis

Angiogenesis is the formation of neovessels from existing vessels, via extension and remodeling of the existing vascular plexus. This can occur by two different mechanisms known as sprouting angiogenesis and intussusceptive growth[173,177]. Sprouting angiogenesis is a multi-step process. The basic steps include degradation of the basement membrane, EC specification and proliferation, directed migration of ECs, tube formation, pericyte recruitment and anastomosis[177-183]. This process will be reviewed in more detail below. Sprouting angiogenesis underlies the formation of many structures during development, including vessels of the retina, limbs, heart and the central nervous system[170,184,185]. Sprouting angiogenesis is also seen in the adult organism, mainly in the female reproductive system during the menstrual cycle and in pregnancy, but also in response to hypoxia and in would healing[151,152]. Pathological angiogenesis is also involved in several diseases, such as in tumor growth, diabetic retinopathy, retinal vein occlusion, rheumatoid arthritis, endometriosis and many more[152].

Intussusceptive angiogenesis, or splitting angiogenesis is also referring to the development of vascular structures from pre-existing ones, but by means of a different mechanism. This process is capable of significantly modifying the structure of the microcirculation, and may be involved in the remodeling process of vascular development. Intussusception occurs by the formation of an endothelial-lined tissue pillar, a small structure spanning the lumen, extending into the middle of a (small) vessel. This mechanism can serve as a mechanism for pruning redundant or inefficient vessels, as a way to duplicate vessels, or to modify the branch angle of bifurcating vessels[186,187].

Overview of the angiogenic process

Initiation

Sprouting angiogenesis can be initiated as a response to local hypoxia, where cells of poorly perfused tissues up-regulate their expression of pro-angiogenic signaling molecules, or other triggering events[188,189]. These signals reach receptors expressed by the endothelium, leading to changes in phenotype for a subset of ECs, which then becomes motile, invasive, and initiate the sprouting process from the outer surface of the vessel[190,191].

The change from endothelial quiescence to the angiogenic phenotype is accompanied by enzymatic degradation of the vascular basement membrane, pericyte detachment as well as loosening of EC-EC junctions[192,193].

A key molecule involved in angiogenesis is Vascular Endothelial Growth Factor A (VEGFA), a GF which is the major regulator of angiogenic sprouting[194]. Together with Notch-signaling, VEGFA is known to coordinate EC behavior and regulate new vessel growth in a range of sites during the process of embryo development[181,191]. There are several members of the VEGF family, and multiple isoforms of the VEGFA protein, that can have different effects on vascular development[195,196]. VEGFA signals primarily through VEGF-receptors (transmembrane receptor tyrosine kinases), and have been shown to have diverse roles in endothelial differentiation, survival, regulation of vessel diameter, barrier integrity and chemotaxis[197-200]. In addition, VEGFA mediates sprout formation and vessel outgrowth[179,201].

Tip cells and stalk cells

High extracellular concentrations of VEGFA induces specification of endothelial tip cells, which are the cells located at the distal end of the outgrowing sprout[201,202]. These cells extend long dynamic filopodia expressing VEGFR2 and other receptors, probing the surrounding environment for cues[190]. The ECs immediately following the tip cell acquire a stalk cell phenotype, and will proliferate and make up the body of the growing sprout. Whereas tip cells respond to *gradients* of VEGFA by guided migration, the stalk cells proliferate in response to overall VEGFA concentration[201]. Stalk cells also form the lumen of the developing sprout, and establish junctions to maintain sprout integrity[203,204]. Tip and stalk cells also show differences in their gene expression profile, where tip cells express PDGFB, DLL4, UNC5B, VEGFR2 and FLT1 at higher levels than the cells of the stalk, which in turn express more of FLT1, ROBO4 and JAG1[190,201,205-207].

Tip cell selection

The tip and stalk phenotypes are not fixed once selected, but are dynamic states upheld through a mechanism of lateral inhibition involving Notch-signaling[194,208]. The identity of tip and stalk cells can switch throughout angiogenic development, with cells rearranging their positions dynamically competing for the tip[181,209,210]. Only a fraction of the ECs in the parent

vessel obtain a tip cell phenotype, and decision is regulated by Notch-signaling[211]. In response to VEGFA, the prospective tip cells upregulate their expression of the Notch ligand DLL4. As a result, Notch is up-regulated in the neighboring cells, causing down-regulation of VEGFR2 and reducing their responsiveness to VEGFA[180,211]. Tip cells, on the other hand, react stronger to VEGFA and acquire a motile, invasive and sprouting phenotype[211]. This regulation of tip versus stalk cell fate through Notch regulation of VEGF-receptor expression allows for precise coordination of response to the VEGFA signal[212]. In addition, down-regulation of VEGFR2 also indirectly inhibits DLL4 expression levels, and thereby reinforces the dominance of the current tip-cell[180,213-216].

Sprouting/outgrowth and anastomosis

Once the tip/stalk phenotypes are established, directed vessel outgrowth can begin. As mentioned, tip cells migrate toward increasing concentrations of VEGFA[201]. This gradient is further enhanced by stalk cell expression of VEGFR1 (FLT1), which binds and inactivates VEGFA on each side of the sprout, acting as a "VEGFA sink"[217,218]. The vessel sprout is also guided by longer range cues that affect sprout stability, and there are recognized similarities between endothelial tip cells and axonal growth cones[191,217]. Four classes of axon guidance cues have been implicated in regulation of blood vessel patterning: Ephrin-Eph, Slit-Robo, Netrin-UNC, and Semaphorin–Plexin–Neuropilin[191]. Experiments have shown that it is not the proliferation of the stalk cells that push the sprout forward; rather, the tip cell interactions with the surroundings serve to pull the stalk along in the direction of growth[190,219].

Lumen formation

In addition to proliferation, the stalk cells have a second important role in establishing the lumen of the sprout, in a process known as tubulogenesis[220]. Several models of lumen formation have been described[166]. One model proposed that lumens are formed by fusion of intracellular vacuoles, which fuse with vacuoles of neighboring ECs to hollow the sprout[221]. However, it was subsequently demonstrated that cell-cell junctions were present over the entire length of the vessels, and it was suggested that lumens form extracellularly, between the cells by exocytosis of vacuoles[222,223]. Alternatively, lumens have also been proposed to form via luminal repulsion, where VE-cadherin relocalizes CD34 sialomucins to the contact

sites between the ECs of the sprouts, leading to electrostatic repulsion and formation of a gap between the cells[224].

Anastomosis

Most vascular sprouts do not extend beyond 100 µm before they form connections with other vessels, and to expand the vasculature over larger distances, the steps of sprouting and tubulogenesis must be repeated. This also reinforces the transient tip-cell phenotype, as they too are incorporated into new vessels[190,225]. The process in which growing sprouts meet each other and connect is known as anastomosis, a process which is not completely understood[166]. VE-cadherin present at the endothelial tip cell sprouts may be involved in the early establishment of new cell-cell junctions[166,222]. It has also been suggested that macrophages can act as "bridge cells" that may facilitate contact and help stabilize the new connections[226-229].

Maturation and mural cell recruitment.

Significant remodeling of the network follows the establishment of new connections, where some branches are stabilized, whereas others regress. This is influenced by the oxygen levels of the surrounding tissue[230]. Formation of a stable microvasculature requires support from other structures, including perivascular cells and the ECM[231]. Capillary-associated pericytes have an elongated and flattened shape, and are oriented along the vessels with several long finger-like processes embracing the abluminal vessel wall[153]. Pericytes do not only serve as scaffolding, but communicate with the endothelium by direct physical contact and through paracrine signaling[153,155,232]. Even though pericyte recruitment has been shown to play an important role, the mere presence of pericytes is on its own not sufficient for vascular stabilization[232]. During angiogenesis, EC-released PDGFB bind PDGF-receptor beta on pericytes, stimulating their proliferation and recruitment along the capillary sprouts[233]. Pericytes can additionally be recruited to the vasculature by PDGFB/PDGFBR independent mechanisms, and PDGFB/PDGFBR-signaling is not required for pericyte recruitment in the liver vasculature[194]. However, the nature of PDGF-B independent mechanism of pericyte recruitment is not yet known[194]. PDGFB/PDGFRB knock-out mice invariably die at birth, suffering from generalized hemorrhage and edema[233], underlining the fundamental role of pericytes for vascular maturation. Whereas PDFG-B is a chemoattractant for mural cells,

signaling by angiopoietins and their receptors, and TGFB signaling, stabilize the interactions between ECs and smooth muscle[233-236].

The ECM also provides critical functions for angiogenesis, both through providing structural support, and by molecular signaling involved in all steps of blood vessel formation[192]. The ECM binds cytokines important for angiogenesis, and the dynamic remodeling and complex cell-ECM interactions suggest that that the ECM possible exerts significant control over several aspects of angiogenesis and blood vessel maturation[193].

Tissue engineering of the macrovasculature

Vascular grafts as treatment of cardiovascular disease

Ischemic diseases, such as atherosclerotic cardiovascular disease, remain one of the leading causes of death globally[237-239]. A common treatment option for advanced vascular disease is the use of vascular grafts to replace or bypass damaged or obstructed vessels, which have resulted in a high demand for vascular conduits[84,240]. In addition, clinical situations such as hemodialysis also make use of vascular conduits[241]. The autologous vessel graft remains the gold standard for vessel grafting, however not all patients have sufficient or healthy autologous veins[242]. Shortage of autologous or allogeneic grafts, complications related to harvest and immunological rejection of large animal derived vessels led to the introduction of synthetic grafts in the 1950's[84].

Autografts is the preferred alternative for surgical treatment of diseased small diameter vessels[243], with the saphenous vein being the most commonly used[244]. Internal mammary arteries and radial arteries have also been used[245-247], but the relative ease of access and harvest of the saphenous vein makes this extensively used for coronary artery bypass grafting surgery[248]. Saphenous veins are not ideal, however. Mechanical mismatch with the host vessel may cause dilation, intimal hyperplasia and accelerated atherosclerosis[249-252], and surgical intervention is often needed within 10 years of the initial surgery[253]. Synthetic alternatives, composed polytetrafluorethylene (PTFE) or woven polyethylene terephthalate fibers (also known as Dacron), are commercially available and have successfully been used for large diameter (> 6mm) applications[254,255]. However, for smaller diameter applications, these artificial grafts suffer from poor patency and unacceptably high failure rates, largely due to stenosis, myointimal hyperplasia, calcium deposition, infection and thromboembolization.[83,256-259]. However, even for larger diameter applications, artificial grafts are outperformed by autologous grafts, and autologous saphenous vein grafts have demonstrated superior long-term patency rates compared with synthetic alternatives in coronary artery bypass grafting[109]. Mechanical properties of the prosthetic materials are influencing the patency of synthetic vessel grafts. For instance, mismatches in compliance and caliber between native vessel and graft will contribute to unnatural wall shear stress, which is linked to intimal hyperplasia and poor long term patency[260,261]. Also, for more

complex cases such as reconstructive vein surgery requiring not only a blood conduit but intact valves, the available synthetic alternatives are not optimal[262]. Synthetic grafts are also not optimally suited for pediatric cases due to lack of growth potential of the graft, making repeated interventions necessary and thereby increasing the susceptibility for infections[84]. Despite advances in the field of synthetic graft fabrication, the overall mortality and morbidity has not been significantly decreased[263,264]. The scarcity of autologous vessels combined with the shortcomings of synthetic vessels have encouraged a quest to develop a functioning tissue engineered vessel graft, and recent advances in tissue engineering can now provide promising alternatives[265].

Design requirements for tissue engineered vessel grafts

Tissue engineered blood vessels should perform like native vessels, and for the vessel graft to meet this obligation, there are some design criteria that should be met[162,266,267]. First, as its main purpose is to serve as a conduit for blood, the graft should display mechanical properties compatible with local hemodynamic forces, and should not rupture or experience permanent deformation[268]. To prevent stresses on the graft anastomosis, the scaffold should possess suitable compliance, and the scaffold should have a geometry that support favorable flow characteristics[261,269-271]. Importantly, the mechanical properties of the graft should be similar to those of the native tissue, as mismatch may contribute to thrombogenicity and intimal hyperplasia[272]. Second, the tissue engineered vessel should be biocompatible, non-immunogenic, and demonstrate low risk of inducing thrombosis[265]. The luminal surface of the graft must be non-thrombogenic and anticoagulative, and neither promote platelet adhesion or activation, nor induce intrinsic or extrinsic clotting pathways[266]. Moreover, the surface should support the formation and maintenance of an endothelial lining, which on its own also prevents blood clotting[162]. Third, the size of the graft should match the native vasculature, in terms of inner diameter and length. A properly sized vessel graft is important for conduction of blood without any turbulence or resistance[162]. Lastly, the graft should integrate with the native host vessels, and be able to grow, remodel and self-repair *in vivo*[109,265]. Remodeling events include migration of ECs, and establishment of cell-cell contacts between host and graft ECs, migration and invasion of SMCs to smoothen the host-graft interphase, and establishment of mechanical homeostasis[84].

Manufacturing tissue engineered vessel grafts

There are several approaches for tissue engineering of vessel grafts, employing a variety of manufacturing techniques, including scaffolding based methods, decellularization techniques, and tissue self-assembly processes. In addition, several different scaffold materials are being explored, including natural and synthetic alternatives.[84,109,162,243,265,268] In the following, various approaches for generation of tissue engineered vessels will be discussed.

Synthetic biodegradable scaffolds

The most widely used degradable polymer scaffolds used for tissue engineering of vascular grafts are PCL, PGA, PLA and derived co-polymers, typically processed into a porous 3D tubular structure that is seeded with cells[109,162]. Shin'oka et al[273] used this approach to develop a vascular graft for treatment of cardiovascular disorders in children, where the graft should have the potential to grow, remodel and repair after transplantation into a growing child. The grafts were produced by culturing autologous bone marrow-derived mononuclear cells on a synthetic composite mesh scaffold *in vitro* prior to implantation. The grafts have remained patent for up to seven years, and has not suffered from rupture, aneurysms, infection or calcification, although angioplasty has been required in a small number of cases[274,275]. Examination of the explants revealed the formation of vascular tissue with SMCs and endothelialization of the lumen, as well as complete degradation of the scaffold material. It must be noted that these grafts were of a large internal diameter (12-24 mm) and were implanted in a high-flow low-pressure system, and that the encouraging results might not translate easily to smaller diameter applications with high-pressure arterial flow. Their approach has been adapted to develop a graft better suited for such applications, showing promising results in a mouse model so far[276].

Niklason et al. also used a similar approach to develop tissue engineered vascular grafts for dialysis access[83,277]. To make the graft, a PGA scaffold was seeded with human SMC and cultured in a bioreactor under pulsatile conditions for eight weeks. The gradual degradation of the PGA scaffold, and a gradual increase of the mechanical stress on the cells, stimulated ECM synthesis and resulted in vessels with sufficient mechanical strength. The engineered grafts may be decellularized, allowing for allogenous transplantation, and tests in animal

models were encouraging in terms of function and patency of the graft.[83,278]. Clinical trials of this human acellular vessel have demonstrated the safety of the graft, and the vessels have been shown to recellularize and evolve into living vessels after human transplantation[15,279]. These tissue engineered structures have no branches that must be ligated to create a continuous conduit, and their dimensions and diameter can be precisely controlled. This method could allow for off-the-shelf production and banking of tissue engineered vessel constructs[83].

A number of other studies based on synthetic polymer scaffolds for the creation of a tissue engineered vessel grafts have been reported, showing great variation in scaffold manufacturing methods, cell seeding and choice of polymers. Successful results have been reported for ovine, murine, canine, porcine and baboon animal models[83,278,280-284].

Natural scaffolds

Since the pioneering work of Weinberg and Bell, developing the first tissue engineered blood vessel construct from collagen and cells, there has been a significant focus on the use of natural biopolymers as scaffolds[285,286]. In their landmark paper from 1986[285], a multilayered tube was constructed to resemble a blood vessel, with distinct layers mimicking the intima, media, and adventitia of native arteries. A Dacron mesh sleeve had to be incorporated in the design to provide structural support, but the mechanical properties were poor compared to native vessels, with a vessel burst strength of only 120-180 mmHg, and as low as 10 mmHg without incorporation of the supporting mesh. In comparison, the physiological burst strength of human saphenous vein is between 2000 mmHg and 3000 mmHg[287]. However, well differentiated SMCs and a functional endothelium made this model attractive, sparking further research into tissue engineering of blood vessels.

Tissue engineered grafts based on purified forms of ECM proteins may to some extent mimic the native vessel, where vSMCs are embedded in the ECM. Cell entrapment during fibrillogenesis (gelation) means that the cellularity of the constructs will not only rely on cell invasion[288-290]. Moreover, the cells induce gel compaction around the center of the mold, leading to circumferential alignment of the medial layer and an increased burst strength[291]. The embedded cells can recognize matrix stimuli provided by the ECM scaffolds, promoting

binding and remodeling of the scaffold, as well as secretion of more ECM[162,292]. The choice of biopolymer influences cell behavior, and it has been shown that cells cultured within fibrin gels synthesize significantly more collagen and elastin than cells cultured in collagen gels[293].

Several groups have used fibrin gel as the basis for development of a tissue engineered vessel graft[294-297]. However, the mechanical properties of these grafts are generally poor[243,285], and strategies to reinforce the gels with "sleeves", replace collagen with fibrinogen, cross-linking of the matrices or mechanical conditioning strategies have been tested[243,289,298,299]. Application of mechanical stimulation has seemingly been the most successful strategy, demonstrated by Syedain et al.[298] Culturing the construct in a perfusion bioreactor, applying cyclic strain as well as luminal, abluminal and transmural flow, resulted in a tissue engineered vessel graft with a burst pressure comparable to that of active vasculature. Using this method applying ovine dermal fibroblasts, followed by an added decellularization step before implantation in the femoral artery of an ovine model, these grafts remained patent for up to 24 weeks[300].

Studies from other groups have also tested fibrin based constructs with SMCs in an ovine animal model, demonstrating patency for up to 15 weeks as a vein interposition graft[301,302]. The grafts integrated with the native vessels, but only reached 25% of the strength of the ovine aorta after complete remodeling of the graft. The use of bone marrow derived smooth muscle progenitor cells as well as increasing fibrinogen concentrations were subsequently shown to produce stronger vessels *in vitro*[296,302].

Other natural biopolymers that are being investigated for vascular tissue engineering applications include silk fibroin, collagen, elastin, chitosan and gelatin. In addition to conventional gelling, production methods for these can include weaving, knitting, electrospinning, gel spinning, crosslinking and freeze drying[303-310].

Hybrid scaffolds

Hybrids between natural and synthetic polymers are also being explored. Coating of synthetic polymers with natural polymers have been tested in an effort to improve biocompatibility and cell adhesion[311,312], and synthetic polymers have also been used in

order to mechanically reinforce natural polymer scaffolds[313]. A wide range of different material combinations have been investigated, where several of the hybrid grafts were tested in animal models[312-318]. The rationale for generating hybrid scaffold is to exploit both the strength, tunability and manufacturing control of synthetic materials, as well as the biocompatibility and biochemical cues derived from natural components[109].

Decellularized blood vessels

The use of decellularized natural matrices in tissue engineering exploits the structure and mechanical performance of natural tissue ECM, while potentially steering clear of adverse immunological reactions[109]. As described above, the process of decellularization aims at completely removing cellular material from tissues, while the overall composition, mechanical properties and biological activity should remain intact[81]. As decellularization largely preserves the native architecture of the ECM, and may be more favorable starting point for vessel remodeling, this may present an advantage over reconstituted soluble ECM or synthetic materials[84].

The first decellularized vascular grafts were developed in the 1960s using animal tissue[319], and since then several of these grafts have been made commercially available[320-325]. These have been used for vascular bypass surgeries and as access conduits, but their large-scale adaptation has not been seen. Several trials concluded that these xenografts offered no clear advantage compared with synthetic prosthesis, and that bovine artery grafts are more commonly associated with infection and thrombosis, as well as having a shorter patency period. Bovine grafts also demonstrated lower mechanical strength and were more difficult to repair as compared with synthetic grafts[326-328]. Decellularized xenografts are also considerably more expensive than synthetic alternatives[109].

Another limitation of decellularized xenografts is limited cell migration into the scaffold, possibly because of tight matrix organization combined with glutaraldehyde fixation effects[329]. Selective removal of collagen and elastin to increase the porosity of the scaffold did not enhance cell infiltration[330], underlining the importance of scaffold cell migratory signals, as well as a structure that promote cell recruitment into the scaffolds[162].

Potential rejection of xenografts transplanted into humans is another important consideration. In one case, where porcine decellularized conduits were implanted into children, the grafts failed catastrophically with three of the children dying[119]. The failure was explained by severe inflammation and immune rejection, attributed to incomplete removal of antigenic cellular components during the decellularization procedure[162].

Decellularized human donor veins have also been commercialized for use an arterio-venous fistula, under the brand name of SynerGraft. However, studies found no improved patency compared with established solutions, and even though the vessels appeared more resistant to infection than synthetic alternatives, they were more prone to aneurysms[331,332]. In addition, availability of human donor vessel may be unpredictable, and commercialization of such tissues are associated with regulatory and ethical issues. Decellularized small-diameter vascular allografts have also been developed in various animal models, and decellularized human umbilical veins have been shown to retain mechanical properties and demonstrate cellular integration after implantation into nude rats[333]. However, the nude rat is very permissive, and the implantation period was only 8 weeks.

Self-assembly of tissue engineered vessel grafts

Tissue engineering by self-assembly diverts from the classical paradigm that an exogenous scaffold is a prerequisite to develop a tissue engineered vessel graft[334]. The method of cell sheet rolling pioneered this scaffold free approach, which now also includes cell printing or microtissue aggregation[109,335]. The rolling technique/cell sheet strategy relies on the cells themselves to produce the scaffold, resulting in a completely biological tissue engineered vessel graft[162]. Sheet based tissue engineering to produce a tissue engineered vessel graft was first attempted by L'Heureux and colleagues[334,336]. The process involved production of ECM rich cell sheets by growing autologous MSCs and fibroblasts on a flat surface under conditions stimulating collagen synthesis. The cell sheets were then wrapped around a mandrel, with an acellular layer first, followed by SMC layers, and a fibroblast sheet forming the outermost layer, creating the tubular and layered structure of the vessel graft. After weeks in culture, allowing the layers to fuse and the construct to mature, the tubular support was removed, and ECs were seeded into the lumen. The burst strength of these

grafts were found to exceed those of native veins. Testing in animal models as well as clinical trials have demonstrated promising patency rates[89-91].

Tissue engineering of small versus large vessels

For smaller caliber applications, less than 5-6 mm in diameter, synthetic grafts have unacceptably high failure rates, with peripheral graft patency of less than 25% at 3 years as compared to over 70% patency for autologous veins[83]. Virtually no non-autologous conduits are useful in the coronary circulation, and synthetic arterio-venous grafts used for dialysis access have shown very high failure rates. There is clearly a significant need for suitable small diameter grafts for a range of clinical applications, and tissue engineering is a promising approach[142]. For larger caliber vessels (<6 mm), synthetic alternatives have been relatively successful for aortic, iliac and common femoral artery repairs[337]. However, as synthetic grafts lack the ability to grow and remodel, their use in pediatric recipients is not optimal. For patients with congenital cardiovascular defects, an engineered blood vessel that could grow with the patients would be ideal[142]. An engineered graft with growth potential was reported by Shin'oka in 2001[338]. For subsequent application of this approach, the cell source was changed from peripheral vein-derived cells to bone marrow-derived cells, and the cell culture time on the graft was reduced from 1 week to 2-4 hours[339]. Late-term follow up of 25 pediatric patients demonstrated very promising results, with 19 fully patent grafts, 6 grafts demonstrating some narrowing that were successfully treated with angioplasty in 4 of the cases[275].

Strategies to improve the patency of tissue engineered vascular grafts.

The clinical performance of existing grafts for small diameter applications is poor compared to the success of tissue engineered vessel grafts for larger diameter applications[340]. The main cause for engineered vessel graft failure is believed to be thrombosis. This can be caused by platelet activation upon contact with the exposed collagens of the vascular wall or the wall of a synthetic graft[341]. In healthy native vessels, the endothelium is preventing platelet adhesion to collagen, and much effort have been made to find methods to mitigate the thrombogenicity of the tissue engineered graft, including biological, biochemical and chemical approaches[162].

The biological strategy involves seeding the graft with ECs, growing a confluent endothelium prior to implantation[342]. Several seeding strategies and EC sources have been tested[83,343], and some studies have found that autologous EC lining improves the patency of collagen grafts[314], as well as synthetic small diameter vascular grafts[344,345]. However, other studies have suggested that although EC seeding of synthetic grafts may partially reduce early post-transplant thrombosis, the mid- or long-term prevention is rather small, with only marginal improvements over non-endothelialized grafts[346]. In the latter study, the grafts did remain endothelialized following implantation, thus insufficient seeding was not the likely cause. Another possible explanation could be the static culture conditions of ECs prior to seeding of the graft, as it has been demonstrated that static culture cause down-regulation of important anti-thrombogenic molecules, and up-regulation of procoagulant and proinflammatory molecules[347].

The chemical approach relies on coating of the lumen with substances that could attract circulating endothelial progenitors, inhibit platelet activation and adhesion, or reduce the electrostatic interactions at the graft-blood interphase[348]. A common approach has been addition of heparin by different methods, which is readily available and widely used as a clinical-grade anticoagulant[340,349-351]. The clinical success of heparin-coated synthetic vascular grafts has been limited[352,353], and alternative coating materials have also been explored[354-356]. Treatment of the graft surface to enhance endothelial recruitment has included conjugation of CD34 or SDF-1(CXCL12) to the lumen of the vessel graft, as well as plasma treatment of the lumen. These strategies have been suggested to facilitate neo-endothelialization of vascular grafts[357].

Whereas the resilient surface of synthetic grafts allows for harsh chemical treatment and controlled chemical coating, decellularized grafts are more susceptible to damage. ECM collagen fibers are denatured by solvents, destroying the mechanical properties of the graft[162]. Chemical coating of natural scaffold surfaces necessitates a more gentle approach, which may result in low binding of anti-thrombogenic molecules and exposed collagen[358]. One method to address this issue involved amplification of heparin binding by first attaching anchors, which are able to bind up to ten of the anti-thrombogenic molecules at once[340].

Tissue engineering of the microvasculature

Microvasculature for tissue engineering applications

Tissue engineering of the vasculature does not only pertain to the production of vessel constructs for transplantation, but also to the development of microvascular structures. Ischemic diseases can also arise at the microvasculature level, and simply replacing upstream arteries would not address the needs of downstream tissues[359]. Microvascularization and perfusion is also known to be important during regeneration and wound healing, and a delay of this process, as seen in e.g. diabetes, can have detrimental effects[360,361]. Capillaries, arterioles and venules have sub-millimeter diameters, and the fabrication methods used for larger vessels are not applicable for microvessels. It is not regeneration of individual capillary tubes that is the endpoint; rather, it is the regeneration of a network of microvessels, a capillary bed[84].

In addition to development of microvasculature as a treatment option for ischemic disease, generation of microvascular networks have other applications in tissue engineering. As noted above, the current inability to develop vascularized systems is one of the greatest hurdles in the field of tissue engineering, and the successful production of organs for transplantation[137-143,362]. Nearly all native tissues are supplied with oxygen and nutrients by the highly branched blood vessel system, which also have a crucial function in waste removal.[139-143]. A functional microvasculature is absolutely crucial to ensure proper function and survival of most engineered biological tissues that are too large to be maintained by diffusion[6,144]. For most tissues, the maximum distance between capillaries does not extend beyond 200 µm, which correlates with most tissues' diffusion limit for oxygen[363,364]. In some tissues, however, including skin, cartilage and cornea, nutrients and oxygen can be delivered to the cells via diffusion from blood vessel systems further away, which can explain the relative success of tissue engineered skin and cartilage[137].

Generation of stable and patent microvascular networks *in vitro* will be an important development towards successful engineering of organs and tissues[137,365], as well as for reliable modelling of microvasculature and organ systems[366]. Organ-on-a-chip models mimicking the characteristics of various organ systems have been established, however, the

clinical relevance of many of these may be improved by integrating a three dimensional microvasculature[367,368].

To generate a network of microvessels, hydrogels or other scaffold materials are used to provide a structure in which the ECs should reside, and possible also develop microvascular networks, depending on the approach. The scaffold can also provide regenerative cues that support formation and maintenance of the vascular bed, depending on the nature of the material[84]. As described in the general section about tissue engineering, natural biomaterials including fibrin and collagen are commonly used for microvessel engineering. Especially fibrin, which is one of the main components of the provisional matrix in wound healing, and is known to have naturally angiogenic properties, is widely used[369].

Two main strategies are utilized for generation of microvascular networks for tissue vascularization. The first strategy, known as the bottom-up approach, relies on the inherent ability of ECs to form new vessels. Here, the cells should recapitulate the physiological mechanisms for angiogenic or vasculogenic vessel formation that occurs during development and tissue regeneration events. The other approach focuses on generation of the tubular structures. In this approach, also known as the top-down approach, the geometry and architecture of the vascular bed are pre-designed and pre-made before introducing ECs to line the structure[84,137]. Each of the strategies encompasses several different methods to achieve vascularization.

Bottom up-approaches approaches for tissue engineering microvasculature

The bottom up approach to model a 3D vasculature relies on the ability of ECs to self-assemble into networks[370]. Recent advances has allowed for development of 3D perfusable microvascular networks by utilizing the morphogenic properties of ECs, thus alleviating the need for pre-made architecture to support the developing structures[144]. Suspending cells in a hydrogel such as fibrin or collagen and letting them spontaneously develop and remodel vascular network structures might more closely mimic vessel formation in vivo[368].

In an *in vitro* approach to prevascularization, tissue scaffolds are seeded with ECs and possibly other cells, followed by *in vitro* culture to allow for the formation of three-

dimensional prevascularized structures. When the prevascularized scaffold is transferred to the host, the preformed vessel network should anastomose with the recipient tissue, ideally speeding up the rate of integration compared to non-vascularized tissue[371]. In one study, tissue engineered "endothelialized reconstructed skin" was created, in which a network of human capillary-like structures formed in the dermal part upon *in vitro* culture. The prevascularized tissue required only four days to form a connection with the host vasculature following implantation. In comparison, non-endothelialized skin grafts acquired the same level of connection after 14 days[372].

The effect of prevascularization on anastomosis time have also been demonstrated by others. Chen et al.[373] demonstrated that human umbilical vein EC (HUVEC) and fibroblast-seeded fibrin constructs that were allowed to develop microvascular networks *in vitro* prior to implantation anastomosed with the host vasculature within 4-5 days, as opposed to a construct containing newly suspended cells, which were not perfused until 8-14 days.

In vitro prevascularization could potentially facilitate integration of the construct upon implantation by reducing the time needed to connect with the host vasculature. Although there is often no perfusion of the preformed vasculature until anastomosis with the host, and thus potential hypoxic conditions in the core, this approach may speed up the process, and increase survival and function of the graft[137].

In vivo prevascularization relies on *de novo* vascularization of a non-vascularized tissue construct via preliminary implantation in the host body. After a vascularization period at the initial transplant site, a microvasculature is formed in the engineered construct. The construct is then harvested and reimplanted at the defect site[374]. This methods has been used for studies on vascularization of larger bone constructs[375], but involves several surgeries and is not widely employed.

Strategies to enhance vascularization.

A number of strategies to enhance the ability of ECs to vascularize tissue have been tested. The use of angiogenic GFs to induce neovascularization is a well-known approach for engineered tissues. GFs can be delivered to cells both for *in vitro* and *in vivo* vascularization

approaches. However, soluble GFs tend to be instable with a high degradation rate, thus potentially disappearing quickly from the scaffold, and strategies for sustained delivery are necessary[376,377]. A great variety of natural[378-380], synthetic[381] and hybrid materials[382,383] have been adapted for use as delivery matrices for angiogenic GFs, and can be tailored to release the growth factors at suitable rates.

In addition to adding soluble GFs to the growth medium, ECs can also be co-cultured with other cell types that promote angiogenesis through secretion of angiogenic GFs. Co-culturing of ECs with MSCs or fibroblasts have been shown to induce the formation of microvascular networks[384-389]. As well as stimulation of angiogenesis in a paracrine manner, studies have shown that MSCs differentiate toward a smooth muscle like phenotype upon contact with ECs, and contribute to stabilization of the network[390,391].

Top down approaches for tissue engineering microvasculature

In the top-down approach, the dimensions and architecture of the vascular bed are pre-designed and pre-fabricated before cells are introduced[84]. This approach often relies on the presence of a scaffold, which is then coated with ECs to generate the microvascular structures. A number of methods for micropatterning of scaffolds for generation of vascular structures have been developed, some of which will briefly be described in the following.

3D printing

One quite successful 3D printing strategy for microvascular applications involves generation of hollow structures for cell seeding, in contrast to printing of the vascular cells themselves. This approach relies on the use of a “sacrificial” material, which can be printed with fine resolution and embedded in a hydrogel. The printed structures can then be dissolved, leaving hollow channels for cell seeding. The method of sacrificial printing for vascular fabrication was fist demonstrated by Miller et al.[392] Using a water-soluble sugar ink, a 3D vascular architecture was first printed, and then embedded in a soluble ECM that crosslinked around the print. The printed network structure was then dissolved, leaving hollow microfluidic channels that could be seeded with ECs, creating a vascular bed with channel diameters starting at 150 µm. Other printing materials have also been employed to print networks in a similar fashion[393,394]. The method of sacrificial 3D printing has been used to

generate large (cm scale) fibrin constructs loaded with human MSCs and fibroblasts, containing interconnected vascular network structures[395]. Upon perfusion of GFs through the HUVEC-lined vascular channels of the construct, MSCs were found to differentiate toward an osteogenic lineage. This methodology could hold the potential to vascularize engineered tissue for tissue replacement.

Some attempts have also been made of 3D printing the actual cell-containing vascular structures. Here, a suspension of living cells in a viscous prepolymer can be used for printing of pre-defined microvascular patterns[396,397]. However, 3D printing of the actual vasculature has proven difficult because of high resolution and stress on the cells, suggesting that the approach may be better suited for creation of tissues of more homogenous cell populations[398,399].

Laser degradation

Another method for fabricating channels within a hydrogel is to use laser technology to selectively degrade regions in a pre-defined geometry[400]. The hydrogel can be empty, or contain cells. Laser degradation was first used for vascular patterning by Heinz et al.[401], who fabricated channels in a pattern derived from scanned cerebral cortex vasculature. Channels as small as 3 µm could be made, however, lining these narrow channels with ECs without clogging was not attainable. The smallest microchannels which they could successfully perfuse and endothelialize were 50 µm.

Other micropatterning methods

Microvascular channels for cell seeding can also be fabricated by other methods. In the layer-by-layer method, the 3D structures is generated additively, by creating individual layers through lithography. The individual components are then joined together mechanically or chemically, and the resulting scaffold will contain hollow channels that can be seeded with cells[402-404]. Another method involves the use of microneedles to generate the hollow channels[405]. Yet another approach has been named “viscous finger patterning”, which takes advantage of basic fluidic principles[406,407]. This method applies passive pumping of culture media through an unpolymerized hydrogel, which generates a lumen in the hydrogel, which is then subsequently polymerized.

Applications

In vitro perfusable vessel analogues using micropatterned hydrogel channels or polydimethylsiloxane (PDMS) devices have been used to study several aspects of EC biology including migration, vascular barrier functions, inflammatory response, thrombosis, interactions of vasculature with tumor cells, as well as response to biomolecular and mechanical stimuli[403,405,408-411]. However, since these microvascular networks are generated by attachment of EC monolayers to channels in a pre-defined pattern, these models may be somewhat limited in their ability to replicate in vivo development of microvascular networks[366]. Moreover, depending on the scaffold material, these models are varying in the ability to reconstitute distinctive attributes and dynamics of EC responses in vivo. For instance, models in which ECs are seeded in tubular channels in a PDMS scaffold might be useful for studies on the effect of vessel geometry and hydrodynamic stresses on EC response[402,411], but does not take into account the interaction with ECM or with other cell types. Hydrogel scaffolds, that can also contain cells, can allow for endothelial sprouting and outgrowth, and thus may represent a more dynamic model of the microvasculature, with more immediate tissue engineering applications.

Aims of this work

The aim of this work was to obtain a better understanding of several aspects related to tissue engineering of the vasculature, including development of larger vessels as well as microvascular networks, and exploring molecular mechanisms involved in microvascular development.

Our first paper focused on tissue engineering of larger blood vessels. The aim of this study was to critically evaluate a published method for tissue engineering of blood vessels, in order to determine the feasibility of their use in a clinical setting at Oslo University Hospital. Specifically, we asked the following questions:

- Did the described recellularization method of using only a small volume of whole blood (peripheral blood) result in the formation of a continuous endothelial layer in the lumen of the vessel?
- Did the decellularization protocol fulfil the established criteria for successful decellularization?

Our second paper focused on microvascular structures, and the development of an *in vitro* model to study vascular development and vascularization of micro-organs. Our aims for this study were:

- To identify suitable cell types and culture conditions for modeling microvascular development in vitro.
- To establish an interconnected and perfusable microvasculature in a hydrogel chip.
- Establish a model to study real-time interactions of microvasculature with micro-organs, and particularly the interactions with isolated pancreatic islets to assess the potential for *in vitro* revascularization.

The third paper investigated the molecular mechanisms involved in microvascular network formation observed in a 2D co-culture model of microvascular development, in a fully humanized system without added GFs. The first part of this study involved assay optimization, including:

- Establishing a fully humanized and GF free version of the 2D co-culture model of vascular development, using clinically relevant cell sources.
- Optimizing methods for cell separation of co-cultured cells to yield pure cell populations for downstream analysis.

Following RNA sequencing (RNA-seq) of mono-cultured or co-cultured and adipose-derived MSCs and ECs, we further asked the following questions:

- How do the gene expression profiles of MSCs and ECs change in co-culture?
- What is nature of the MSC-EC connectome, and how do the interactions between MSCs and ECs change in co-culture?
- Can we identify cell-specific signaling molecules that might be involved in vascular development, and possibly identify new potential players?

Summary of results

Paper 1

In this paper, we were evaluating a published protocol for creating tissue engineered blood vessels for clinical use[412]. The premise of the re-evaluated paper was a claim that it was possible to completely recellularize a decellularized vessel graft by circulating only a small volume of peripheral blood through the vessel scaffold. The recellularization and decellularization processes were performed as in the original paper by the company NovaHep (today: Verigraft AB). We obtained samples from newly harvested cadaver veins, decellularized veins and veins that were attempted recellularized, in order to verify the method and their previous findings. The veins were examined by a number of different imaging techniques, including Scanning Electron Microscopy (SEM) and *en face* microscopy of the luminal surface, and immunostaining of vessel cross sections. Hoechst staining demonstrated that no entire nuclei were remaining in the vessel following the decellularization process, however, diffuse staining throughout the vessel wall indicated that there may be some residual DNA present. Moreover, quantification of DNA in the decellularized vessel revealed that all of the examined vessels still contained a higher amount of DNA than the established criteria of 50 ng/mg dsDNA. SEM confirmed the *en face* observation of no endothelialization, although CD31 and vWF staining were positive. Our suggested explanations for this were: a) vWF binds collagen upon cell lysis and was not removed in the decellularization process, and b) CD31 staining in the attempted recellularized samples was likely derived from platelets from the circulating recellularization blood solution, which was supported by positive CD41 staining of the recellularized grafts only. In conclusion, we found that not only did the method of whole blood recellularization fail to work as previously described, the decellularization procedure also failed to generate properly decellularized vessels judged by established criteria.

Paper 2

In this paper, we were focusing on developing a functional microvasculature in a microfluidics chip device, in order to create a platform to study revascularization of isolated rat pancreatic islets. To determine which cells types were best suited to development of a

microvascular network, and which culture medium performed best at inducing network formation, we initially tested a range of fluorochrome-tagged ECs co-cultured with either fibroblasts or adipose-derived MSCs in four different culture mediums, in a 2D assay of vascular network formation. The combination of adipose derived MSCs and HUVECs cultured in EGM-2MV medium demonstrated the most extensive and robust network formation. Combining these cell types in fibrin hydrogel, we could observe the formation of an interconnected vasculature. Addition of fluorescent microbeads to the chip confirmed that the vasculature was perfusable, and confocal microscopy confirmed the presence of luminal structures. Further adding isolated pancreatic islets to the chip culture allowed us to study the interaction of the developing microvasculature with the islets in real time. Our results demonstrated that the islets are recruiting vascular structures to their immediate vicinity, but we did not observe any invasion over the course of our study. Our established platform is useful for studies of microvasculature-islet interactions in real time, and can also be used for studies on vascularization or vascular interactions in other organ systems, as well as studies on tumor biology and drug design.

Paper 3

In this paper, we were investigating the crosstalk between AT-derived MSCs and ECs in a 2D model of vascular network formation. Using *in vitro* expanded AT-derived ECs and MSCs from the same donor, in a fully humanized system not containing exogenous GFs or Fetal Bovine Serum (FBS), we demonstrated that these cells could present a promising autologous cell source for vascular therapy and tissue engineering applications. Co-culture induced a morphological shift in the ECs, which evolved into elongated and interconnected structures, resembling microvascular networks. After 5 days of co-culture, we harvested the cells and separated the two cell populations by CD31-conjugated magnetic beads. Pure populations were confirmed by flow cytometry and RT-qPCR. Monocultured and separated co-cultured cells were then processed for RNA-seq. Comparing the expression levels of genes between mono- and co-culture demonstrated considerable changes in gene expression for both cell types following co-culture, and gene set enrichment analysis (GSEA) showed that these changes were related to vascular development and angiogenesis for both cell types. In addition, gene ontology (GO)-terms related to SMC activity were upregulated in co-cultured MSCs. Several SMC markers were upregulated in co-cultured MSCs, suggesting that MSCs

may be differentiating toward a mural cell phenotype upon contact with EC. Further exploring our results, we mapped our expression data to the ligand-receptor connectome provided by the RIKEN consortium to identify changes in communication between the cell types. Comparing expression data of ligands then let us identify the nature of the signaling initially contributed to the culture from each of the cell types, and which signaling molecules are up- or down-regulated in co-culture. Our results demonstrated that MSCs contribute several angiogenic genes into the co-culture, including VEGFA and HGF. However, co-culture expression levels indicated that several angiogenesis-inducing genes were down-regulated, and that a number of angiostatic genes were up-regulated. Taken together, this demonstrates that EC-co-culture induce a possible shift in MSC paracrine activity, from an angiogenesis-inducing to take on a more network stabilizing function.

Methodological considerations

In this section, some of the methodological approaches will be discussed, highlighting some of the benefits and challenges of our applied methods. Very standardized methods will not be discussed. Experimental issues or problems encountered over the course of the experiments will be addressed, and strategies to overcome these problems will be described. Where the chosen methods have clear limitations, alternative approaches will be mentioned. This section is not exhaustive. Detailed descriptions of all methods are found in the materials and methods section or the original papers.

Paper I

Decellularization technique

The aim of the decellularization process is to remove all cellular material from a tissue, leaving only the associated ECM. This generally involves treating the tissue with a combination of physical, chemical and/or enzymatical methods to remove all cellular material, including DNA, from the tissue[64,240]. This process should be stringent enough for complete removal of all cellular material, while at the same time aiming to preserve the composition, biological activity and mechanical integrity of the remaining ECM[413]. Three minimal criteria have been established to evaluate decellularization of ECM[112], suggesting that:

a) the decellularized ECM should contain < 50 ng/mg dsDNA in ECM dry weight

b) eventual DNA remnants in the ECM should be of < 200 bp fragment length

c) DAPI staining should demonstrate complete lack of visible cellular material in tissue sections.

For this study, the actual decellularization and recellularization was performed by NovaHep (today known as Verigraft), by the method patented by the company and described in their 2014 publication[412]. This method involved cyclic incubation with 1% Triton X-100, 1% tri-n-butyl phosphate (TBP) and 4mg/L deoxyribonuclease I, with extensive washing between

each cycle. Upon completion of the decellularization process, samples were sent to Oslo University Hospital for testing.

Our evaluation of criteria a) and c) revealed that the decellularization was incomplete, even though satisfactory results were claimed in the original publication. However, images from that paper revealed that recellularized vessels displayed DAPI staining of the tunica media, which could not have originated from the recellularization procedure. Moreover, it is not clear whether the DNA quantification in the original publication was performed on dry weight of tissue, which could explain the lower DNA content found in both native and decellularized vessels as compared with our findings.

It is possible that a higher number of cycles could be run to ensure complete decellularization. Alternatively, a number of other protocols for decellularization of vessels have been published, demonstrating complete removal of cellular materials.[413]

Recellularization technique

In the original paper, recellularization was performed using only a small volume of blood. The principle underlying this method is that blood contains circulating Endothelial Progenitor Cells (EPC)[414,415], which could differentiate into ECs and coat the luminal surface. However, as the number of circulating EPCs have been estimated to a maximum of 4 cells per mL of blood[416-418], 25 mL of blood would only yield 100 cells. Microscopy and immunohistochemistry (IHC) approaches showed that no reendothelialization had occurred following perfusion of whole blood through the graft, and only platelets and a fibrin meshwork were lining the luminal side.

The feasibility of blood-derived EPC recellularization of vascular grafts has been demonstrated by Tillman et al.[419] However, they were using a CD133+ antibody capture system to harvest ovine EPC from 1800 mL of peripheral blood, and the captured cells were subsequently expanded in culture to yield several million cells for recellularization.

There are also other potential cell sources for endothelialization of a vessel graft. The saphenous vein can be used as a source for ECs, and can be obtained after coronary artery

bypass grafting[420-422]. Saphenous vein ECs have been used for successful endothelialization of decellularized porcine aortic segments, as well as synthetic grafts[342,423]. However, unless harvest of the saphenous vein is required for immediate bypass surgery, using the saphenous vein as an EC source is invasive and unnecessary. Arterial-derived ECs have also been used[313]. However, this source is also unpractical in terms of invasiveness and autologous availability. Derivation of ECs from adipose derived MSCs have also been investigated for cell seeding purposes[424]. HUVECs, an available and widely used source for research on ECs, have also been used for studies of endothelialization of vessel grafts[425]. However, as HUVECs are generally not available as autologous cells, their suitability for tissue engineering purposes may be limited.

The ideal EC cell source for recellularization should be autologous, available in sufficient quantities, and their harvest should be possible without causing donor site morbidity[426]. In addition to blood derived EPC, and possibly stem cell derived ECs, AT-derived ECs also fulfil these criteria[427]. AT is an easily available source of large number of autologous cells for therapeutic application, and AT-derived ECs may hold great potential for tissue engineering applications.

Another question that needs an answer is whether endothelialization of vessel grafts is necessary. For smaller vessels, early endothelialization of the vessel lumen of the tissue engineered vascular graft is assumed to be a critical step. For decellularized vessels, this is particularly important, as they are mostly comprised of collagen[342,428-430]. However, for larger caliber vessels, recent clinical trials have demonstrated promising results of acellular constructs for dialysis access grafts[15,279].

Scanning Electron Microscopy

To obtain SEM images with good ultrastructural preservation, the samples must be prepared carefully by appropriate methods. In general, the sample preparation for SEM involves stabilization of the structures (fixation), dehydration, and coating with a conductive material. Our protocol involved sample fixation in 2.5% glutaraldehyde for 24 hours, followed by dehydration in increasing concentrations of ethanol before the final step of platinum coating. We tested several dehydration methods to identity the method that would best

preserve the structure of the endothelium. Critical point drying is the most widely used method to dry samples for examination with SEM[431], and is often included as a step in processing of endothelium[313,432-435]. Critical point drying prevents direct air drying, which can result in distortion of specimen shape due to effects of surface tension forces. Replacing high surface tension water with a transitional solvent, often CO_2, with lower surface tension may alleviate this problem[431]. However, critical point drying caused the endothelium of our samples to collapse and flatten, and we left this approach. Another method involves freeze drying of the samples[436], which removes water from the sample by the process of sublimation[431]. However, the endothelium of our samples were destroyed and distorted during this process. We also tested post fixation of our samples in 1% osmium tetroxide, which is included in some published protocols for SEM of the endothelium[433,437]. However, osmium tetroxide treatment did not improve our results. In addition, we tested low vacuum environmental SEM to look at unfixed and un-coated vessel samples, but this method did not yield clear images. In the end, conventional SEM with one-step glutaraldehyde fixation and direct air drying following ethanol dehydration produced the best results in our hands.

Immunohistochemistry and histology

IHC performed on tissue sections is a way to visualize proteins, and gives a spatial readout that is used to determine the tissue distribution of an antigen of interest. In our case, we used it to evaluate recellularization and decellularization of vessels. Initially, we started with paraffin embedded sections, which produced strong and consistent staining for vWF, but only faint and partial staining for CD31. Paraformaldehyde fixation of the tissue prior to paraffin embedding can have a negative effect on antigen detection caused by crosslinking and masking of the epitope. This masking can be reversed by antigen retrieval, which refers to the process of restoring the immunoreactivity of an epitope. We tested several protocols for antigen retrieval including tris/EDTA buffer, Proteinase K-buffer and citrate buffer, where the latter showed the best effect. However, we also tested cryosectioning of unfixed tissues, which yielded good staining for all tested antibodies, including CD31. The cryosectioning method may also involve a fixation step, which is done by immersion of the slide in ice-cold ethanol immediately post sectioning. As alcohol fixation does not induce crosslinking, this has no effect on the epitope. As the name implies, cryosectioning involves freezing of the tissue. The freezing process may cause artifacts, and paraffin sectioning perform better in

terms of preserving the structure of the tissue. Consequentially, cryosectioning was used for IHC, whereas paraffin sections were processed for H&E.

Paper II

This work was performed at Yale University (CT, USA), in the research group of Professor Laura E. Niklason.

Choice of cells

As ECs are unable to form stable microvascular networks on their own, we tested two potential supporting cell types. MSCs were found to better support microvascular network formation in the 2D model of microvascular network formation as well as in the 3D fibrin gel model. To identify the most suitable EC type, we tested three different EC sources, HUVECs, iPSC-derived endothelial colony forming cells (iPSC-ECFCs) and microvascular lung ECs. Of these both the HUVECs and iPSC-ECFCs were tagged with a fluorochrome, which made for easy visualization. HUVECs are widely used and easy to obtain, however, unless cells are banked at birth, these are not autologous. iPSC-ECFC can be a source of autologous cells, but these cell would still require a lot of investigations to enhance their angiogenic capacity as well as ensuring the safety of their use.[30] As neither HUVECs nor iPSC-ECFC were found to invade islets, we asked whether a microvascular source could be advantageous. We thus tested a microvascular EC source available in the lab, namely rat lung microvascular ECs. These cells did not perform very well, and the results were not included in the paper, partly because we did not have enough cells to run a sufficient number of replicates, and partly because these were rat cells, and thus not clinically relevant. A potential source for autologous ECs could be adipose derived microvascular ECs. These cells are readily available, and would not require additional surgical procedures, as they could be obtained from AT collected for isolation of MSCs. In addition, as shown by studies performed on these cells in the Brinchmann lab as part of paper III, these cells will readily develop into capillary-like structures in co-culture with MSC. These cells were also tested in a 3D fibrin bead angiogenesis assay, and were found to sprout and form microvascular structures. Unfortunately, these cells were not available in the Niklason lab in the period that I was there.

Choice of hydrogel system

Collagen, as well as a mix of collagen and fibrin, had previously been tested for this assay in the lab. Fibrin was chosen because it was found better in supporting network formation. Fibrin has also been shown to support angiogenesis and islet survival, as discussed in more detail in the paper, and is known to be a suitable material for microvascular tissue engineering. An alternative that was discussed was to use reconstituted pancreas hydrogel. This hydrogel would contain all the ECM composition of the pancreas, which might mimic more closely the natural environment of the islets. However, timing issues prevented this from being realized.

Cryosectioning of islets

Rat pancreatic islets are small, with a diameter ranging from 50 to 300 µm. This makes them very challenging to section and stain, as the sample is barely visible to the naked eye. With larger tissues, the samples are usually oriented and embedded in Optimal Cutting Temperature compound (OCT), frozen, and sectioned. With miniscule samples, this method would pose the risk of losing the islets in even the smallest mold, unless the sample supply is unlimited, which is rarely the case. Not knowing where in the cryoblock the sample is found would require examination of every slide under the microscope, with the risk of OCT melting and sample degradation, and even then it was hard to distinguish the tiny sectioned islets from the OCT material. To overcome this, we first embedded a minimum of 30 islets in a smaller volume of OCT dyed with food coloring, which does not affect subsequent IHC staining. This colored sample-containing OCT was then embedded in regular OCT in a regular cryomold. This way we could keep track of our sample, by looking for the stained area of the OCT block. After cutting, it was also important to mark the position of the section on the slide, as the islets were near invisible after OCT removal.

Paper III

Cell culture and supplements

AT was obtained from patients undergoing elective liposuction procedures. All patients gave informed consent prior to tissue donation. Isolation of AT-derived MSCs and ECs was performed using established protocols previously described by our group. For monolayer

culture, standard culture medium supplemented with antibiotics were used. Most cell types also need additional supplements, such as FBS, in order to survive and proliferate. Serum is a source of nutrients and GFs, which support cell proliferation and survival. In addition, serum generally contains ECM components which facilitate cell adhesion to tissue culture plastic. Our lab has moved from using FBS to human sources of culture supplements. A fully humanized system avoids issues related to xenoreactivity. In addition, variability between different batches of FBS has shown significant differences in its ability to support MSC proliferation[438]. For an animal-free system, we initially started out with human platelet lysate-rich plasma, however[439], the requirement of heparin addition to prevent clotting interfered with experiments involving fibrin gels. To avoid this problem, we then moved to using human AB-serum. For culture and expansion, we added 5 ng/mL bFGF to the culture medium to promote proliferation. For co-culture experiments, this addition was not made. The only GFs present in the co-culture system are thus the ones produced by the cells themselves, and GFs potentially present in the AB-serum. Addition of GF like VEGF or FGF could potentially lead to the formation of denser microvascular networks, however, being interested in the isolated effects the co-cultured cells have on each other, we chose to work with a more minimal system.

AT-ECs have previously been characterized by our lab[427], and represent a promising cell source for therapeutic applications. In contrast to the more widely used HUVECs, AT-EC can be derived from autologous sources. AT-EC have been shown to have angiogenic potential, and can easily be isolated and cultured to reach clinically relevant numbers. AT is also a source for MSCs, which is known to secrete a variety of angiogenic factors, promoting vascularization and tissue formation[440]. Bone marrow-derived MSCs are also extensively used in our lab, but as AT yields two cell populations suitable for studies on vascular tissue engineering, AT-derived cells were an natural starting point. In addition to being clinically relevant cell sources, they also performed well in all of our assays. A recent study comparing the ability of MSCs derived from bone marrow or AT to induce vascular network formation of ECs also demonstrated that AT-MSC were superior in that regard[441].

Separation of co-cultured cells

Co-cultured cells were separated with CD31-conjugated Dynabeads. Initially, we tested purity by RT-qPCR, as bead-bound cells are unsuitable for flow cytometry. To verify proper separation, we investigated genes previously shown in our lab to be expressed in only one of the cell types in a mono-culture setting. One pitfall here is that co-culture influences gene expression, and might up-regulate genes lowly expressed in mono-culture to a detectable level in co-culture, which then may suggest the presence of a contaminating cell type. However, surface expression of CD31 on ECs and CD90 on MSCs, were not changing in co-culture, and we modified our procedure to be able to run flow cytometry on our samples. This involved replacing the conventional Dynabeads with CELLection Dynabeads, where the magnetic beads are coupled to the antibody by a DNA linker, which can then be removed by DNAse after sorting, releasing the cells. Our initial flow results, as well as our RT-qPCR results, demonstrated that simply following the manufacturers protocol did not yield pure populations, as both isolations showed traces of the other cell type. Especially the less numerous EC isolation contained a significant amount of MSCs. To resolve this, we included five extra washing steps of the CELLection CD31 positive selection, which then removed most MSCs. In addition, we performed a second negative selection step of the remaining MSC-fraction using conventional CD31-conjugated Dynabeads after the first round removing the bulk of the ECs. This second step was to ensure complete purging of ECs from the remaining MSC population. These modifications yielded EC and MSC populations that were >99% pure, as assessed by flow cytometry.

RNA sequencing

RNA-seq is used to examine the cellular transcriptome of a biological sample at a given moment[442]. In addition to mRNA sequencing, which we have performed, other RNA populations such as total RNA, miRNA, tRNA can be investigated[443,444]. For our study, we were running RNA-seq on mono- and co-cultured cells to investigate changes in gene expression occurring as a result of co-culture. Our results revealed significant changes in gene expression for both cell types, but choice of sequencing protocol also has some limitations, which will be described here.

Running bulk RNA-seq on our two cell types, it is likely that we are missing information about cellular heterogeneity in the process of vascular network formation. It has been shown that MSCs in close proximity with ECs in a similar system will have a higher expression of SMC and pericyte markers compared to cells more distant from the EC cords[390]. Thus, averaging gene expression over the whole MSC population could mask strong effects in some cell types. This is also true for ECs, where tip- and stalk cell phenotypes are expressing discrete levels of some genes. Large alterations in only a subset of the cells would not be reflected by conventional RNA-seq. Immunostaining can help reveal some cellular heterogeneity[390], however, the usefulness of this assay critically depends on the localization of the protein. A more comprehensive way to resolve cellular heterogeneity on the gene level could be running single cell RNA sequencing (scRNA-seq) on the cell populations. This method allows for quantitative measurements of transcriptional diversity occurring within the same cell type[194]. The RNA-seq results for MSCs show that some genes known to be involved in promoting angiogenesis are down-regulated upon co-culture, whereas a number genes known to be involved in vascular stabilization are up-regulated. However, angiogenesis-inducing VEGFA is also up-regulated in co-cultured MSCs. scRNA-seq could help elucidate whether changes in gene expression are occurring in all MSCs, or only within some subsets of cells. For instance, direct EC contact might influence the gene expression pattern of the MSCs, and change their angiogenic activity, or direct differentiation toward a pericyte-like phenotype. It has also been suggested that pericytes and vSMCs are phenotypic variants along a continuum of mural cell phenotypes[163], and the steps and variation in MSC differentiation toward these phenotypes could also be elucidated by scRNA-seq. Running scRNA-seq on the co-cultured EC population would also likely reveal clustering of ECs into several subgroups. Our co-cultured samples did show enrichment of tip-cell markers, likely indicating that there are subtypes of specified ECs in the network.

Time points

Another limitation of our study is the fact that we have only included two time points – mono-culture and late co-culture, as we chose to harvest the cultures when the networks were well established. This allowed us to get information about changes that had happened as a result of co-culture, but not on the nature and timing of these changes. Thus, in

hindsight, it would have been useful to choose at least one additional time point, as we might miss early signals involved in the induction of microvascular network formation.

It is possible and likely that the observed changes are happening at different time points, with some gene expression changes occurring early on in the co-culture, while some are occurring toward the later stages. Some genes could also theoretically demonstrate an early increase/decrease, and then revert to the initial expression level after some time in co-culture. It would be interesting to follow certain genes through the entire process, from induction of the angiogenic process, through elongation to stabilization and maturation. In our current system, we have a picture of the signals involved in maintaining the mature network, displaying which changes have already happened. This is still useful information, as network stabilization is relevant for vascularization in relation to tissue engineering. Engineered networks should stabilize and seize further (aberrant) angiogenic activity once it has reached a certain level of vascularization. However, including more time points from early co-culture would be interesting to reveal the dynamics of gene expression for individual genes over the course of the co-culture.

RNA sequencing and genome mapping

Mapping our RNA-seq results against the hg38 genome, we are obtaining not only information about protein coding genes, but on non-coding genes and pseudogenes. Even though for instance some ncRNAs are shown to have an effect on angiogenesis and other cellular processes, we chose to limit our studies to only protein coding genes in this first analysis. Non-coding genes have been increasingly studied, and have been shown to play important roles in regulating gene expression. A number of long non coding RNAs have for instance shown to regulate the expression of VEGFA, which is one of the main genes promoting angiogenesis. We could also have chosen to look at miRNAs, a number of which have been shown to be involved in angiogenesis.[445]

Identification of expressed genes

In analyzing RNA-seq data, it has been common to apply an arbitrary expression threshold of 0.3-1 FPKM. Lowly expressed transcripts could be the result of noise and background rather than active transcipts[446]. In their publication “Finding the active genes in deep RNA-seq gene

expression studies", Hart et al.[446] showed that the human cell transcriptome can be divided into active genes, and other genes that are most likely the by-products of biological or experimental noise. They developed a method, "The zFPKM normalization method", which with high accuracy can predict which genes are the biologically relevant genes in the cell, those associated with active promoters, by integrating gene expression data with chromatin data from the ENCODE project. We thus chose to apply this metric to filter our genes, yielding a FPKM cutoff value of 0.16. This might include some background or noise, but we are less likely to lose relevant genes with low expression.

Lowly expressed genes may still be relevant and hold important functions. However, looking at changes in gene expression between the two conditions, significantly up-regulated genes involved in the co-culture induced changes will likely be expressed at higher levels than zFPKM cutoff threshold. Our study provide a snapshot of MSC-EC crosstalk, and to draw any conclusions on the role of particular genes in our system, further validations and experiments would be necessary.

General discussion

The overarching theme for this thesis is tissue engineering of the vasculature. Each of the papers is addressing one aspect of this broad and diverse field, from tissue engineering of macrovasculature (Paper I), tissue engineering of microvasculature (Paper II), to investigating molecular mechanisms involved in *in vitro* formation of microvascular structures by a bioinformatics approach (Paper III). Because the different papers cover separate, but related topics, the aims and methodologies vary significantly between the three studies. In the following section, each of the papers will be briefly discussed, including rationale, important results, and potential implications.

Paper I

The 2014 paper by Olausson et al.[412], "In Vivo Application of Tissue-Engineered Veins Using Autologous Peripheral Whole Blood: A Proof of Concept Study", received massive attention after it was published. Vascular disease is a major cause of death worldwide, and autologous grafts or synthetic alternatives cannot meet the current need for vessel grafts. As discussed above, vascular tissue engineering has emerged as a compelling strategy for generation of blood vessels for reconstructive or replacement surgeries.

The novel and exciting claim of the paper, that is was possible to use only 25 mL of blood from a simple blood draw to completely recellularize a tissue engineered vein, would represent a very promising new approach. This method would eliminate the need for invasive cell harvest and expansion in culture, thus limiting the complexity, cost and time of the procedure. The method was patented, and NovaHep AB, a biotech spinoff from the Karolinska Institute that was previously established by some of the authors, was aiming to commercialize the product. When talks began about a potential collaboration with Oslo University Hospital, including a REK-approved clinical phase 1 trial, measures were taken to control the accuracy of the scientific claims behind the research. This involved new and thorough *ex vivo* studies on the vessels, performed by our lab. As described in Paper I, several techniques were used to investigate the veins at different stages. Veins were examined immediately after harvest, after decellularization, and finally after completed

recellularization, aiming to assert whether the methods were sound and the product would be safe to use.

Paper I reports the findings of these investigations. The results clearly demonstrate that the recellularization method does not live up to its expectations. The premise of the blood recellularization technique is that EPCs are present in peripheral blood,[414,415] and will differentiate into ECs, proliferate and coat the luminal surface. As discussed in the Methodological considerations-section, the number of circulating EPCs in peripheral blood has previously been estimated to be as low as 4 cells/mL[416-418], which would require a massive expansive potential of these cells to fully endothelialize the vessel lumen in only a few days. In a previous publication, the authors refer to a VEGFR2+ cell population in peripheral blood, which have the potential to differentiate into ECs after culture, and could contribute to recellularization of balloon-injured femoral arteries of nude mice[447]. However, in that same publication, they show that this VEGFR2+ cell population constitute only 0.8% ± 0.5% of the total population of mononuclear cells in peripheral blood. These cells did not proliferate *in vitro*, but did so *in vivo*. For the recellularization process, they used $5 * 10^5$ cells that were incubated in the injured vessel area for 15-20 minutes. After 4 weeks of culture, 77±6% of the lesion was covered by VEGFR2+ cells. The larger amount of cells, longer culture time and smaller area for recellularization may explain this relative success. Using less cells, shorter culture time and having a larger luminal area to repopulate, it is not surprising that we did not see any recellularization. The authors do not comment on the amount of EPCs present in the blood sample used for recellularization, or speculate about proliferation rate or number of ECs needed for complete recellularization.

In addition to the logical flaw of speedy recellularization with the limited amount of EPCs present in blood, some of the images supporting the original publication were a bit puzzling. For the SEM images, where they claim to see ECs, the indicated structures do not look like endothelium. Our SEM images of native vessels demonstrate a clear endothelial monolayer, which is clearly not present in neither the decellularized or recellularized vessels. On the contrary, other structures, including platelets and fibrin mesh, are found lining the recellularized vessels.

Moreover, images of recellularized vessels show plentiful DAPI-staining in the tunica media, as well as presence of CD31+ cells in the tunica media in the original paper. This may indicate insufficient decellularization, which is confirmed by our studies. What is more, the presumed presence of CD31 positive cells in the tunica media might indicate overexposure of the images, as there should not be any ECs in the media. We did obtain clear VEGFR2-staining of native vessels but, unlike the original publication, saw no traces in the recellularized veins.

Surprisingly, given our SEM and *en face* results showing no traces of an endothelium, we observed some CD31 staining on the luminal surface of recellularized vessels. This lead us to ask whether this was due to platelet adhesion to the luminal surface, as was indicated by our SEM results, as platelets are known to express CD31. Staining for the platelet marker CD41 confirmed this, yielding a feasible explanation for the positive CD31 staining.

In this paper, we questioned the proof of concept about recellularization of decellularized veins with only a small volume of blood. Replication studies are essential for building confidence in medical research. Scandals such as the Macchiarini case may put patients severely at risk, and destroy the credibility and public trust in medical research. Aside from the scientific shortcomings, this research has been tainted by other issues. Two of the authors, Sumitran-Holgersson and Olausson, were found guilty to misconduct by their employer, Gothenburg University in 2017, after falsely claiming to have obtained ethics approval for surgeries carried out on three children, both in the re-evaluated paper [412], and one that was published earlier[448]. Moreover, the group has also been under investigation for image fraud, and retraction of 8 papers were recommended by the Central Ethical Review Board at Gothenburg University. Until our paper was published, no publications have questioned the biological message conveyed by papers from this group. Our publication demonstrate the importance of careful and comprehensive studies prior to clinical application of new methods, demonstrating how wrong interpretation of image data and hasty conclusions can give misleading results.

Paper II

This work was performed at Yale University, in the lab of Laura Niklason, as a part of a Fulbright funded research stay. This second paper of the thesis addresses tissue engineering

of microvasculature in a general sense, but also more specifically, in that we were attempting to vascularize isolated pancreatic islets.

The first part of this study involved identifying the most suitable cells for generation of microvascular networks *in vitro*. As has already been discussed, one of the most significant challenges facing the field of tissue engineering is the ability to vascularize tissues[137,139-141,374]. The AIM Biotech chip assay had already been established in the lab, with fibroblasts and HUVECs co-cultured in fibrin. However, after testing other cell types, we found AT-MSCs to perform better in inducing and supporting microvascular network formation. The potent angiogenic ability of MSCs was not surprising, given our previous experience with this cell type, which is also widely supported by the literature[39,389,449-463]. By adjusting the cell seeding density, the microvascular morphology could be adjusted, including the microvessel diameter and network density. This shows the potential to generate microvascular networks tailored to specific applications, such as organ specific microvascular beds. There are significant differences between the microvasculature structure and microvascular ECs of different organs, and every organ demonstrate specific vascular characteristics and functions[464,465]. Thus, being able to design microvascular networks with the desired parameters is important for vascularization for tissue engineering applications, and for organ-on-a-chip applications incorporating a vascular component.

In the second part of Paper II, we focused on applications of the microvascularized chip, more specifically by integrating pancreatic islets into the chips. This allowed for real time studies on islet interactions with the microvasculature, where the aim was endothelial invasion and vascularization of the islets. We did observe vasculature recruitment to the islets, but no invasion of the islets. As discussed above, there are several potential explanations for this, including the use of non-human islets and lack of possible key physiological conditions which might affect vascularization. Islet isolation severs the connections with the vasculature, and intra-islet ECs disappear after only a few days of culture[466]. Rapid vascularization is thought to be an important factor for islet graft survival and function, and could possibly mitigate the massive islet death post transplantation[467-469]. To speed up vascularization of transplanted islets, efforts have been made to vascularize islets *in vitro*. The prevascularized islets could then make faster connections with the host

vasculature, establishing perfusion through the islets[141]. One approach relied on coating of islets with ECs and MSCs, which induced EC ingrowth into the islets[463]. Yet another approach has been self-condensation culture. Here, several islets were cultured together in one non-stick well along with ECs and MSCs. The islets and the cells aggregated and condensed, and formed one larger islet structure. *In vivo* experiments demonstrated that this facilitated vascularization post-transplantation, and also improved tissue viability and function[469]. Islet reaggregation has also been investigated as a means to improve islet survival. This method involved dissolving the islets using accutase, and reaggregating them to spheroids of defined sizes[470-474]. Incorporation of other cell types into the islet spheroids has also been tested, as a means to promote islet function and vascularization[472,475]. We performed some experiments on islet spheroid generation, including generation of composite spheroids incorporating HUVECs. Preliminary testing indicated that spheroids and islets secreted insulin at similar rates, and that spheroid generation did not affect cell survival (unpublished results). However, we did not observe any invasion upon spheroid incorporation into the chips, and a fibrin angiogenesis assay demonstrated only occasional sprouting from the EC-islet spheroids. Generating smaller islet spheroids makes sense from several perspectives. Smaller sized islet have been shown to secrete more insulin per islet equivalent than larger islets[476]. Moreover, smaller islets/spheroids would possibly be less prone to death by hypoxia, as they would be easier to sustain by diffusion before a vasculature is established[477,478].

One shortcoming of our study is the lack of functional testing of the islets upon integration in the chips. Previous studies in the lab had demonstrated that islet tolerance for fibrin embedding depended largely on the concentration of the fibrin gel. Higher concentration gels, 10 and 5 mg/mL, yielded more dead islet cells than lower concentrations, where most cells were alive even after several days in culture. Our chips contained a final fibrin concentration of 2 mg/mL. We did not test whether chip co-culture with ECs and MSCs affected the survival or function of islets, which might be likely, given that several studies have suggested that co-culture with MSCs improves islet survival and function both *in vivo* and *in vitro*[479-486]. In addition to survival, functional testing of insulin and VEGFA secretion from the islets could be investigated as a part of characterizing our system.

Although our initial efforts of islet revascularization were rather disappointing, the organoid/microvasculature platform could still be useful for other applications. Organ-on-a-chip has gained increased attention over the recent year. Incorporating a functional microvasculature could improve the biological relevance of such systems by replicating the various physiological role of the endothelium in organ systems, including barrier functions and nutrient delivery[368].

Paper III

For this study, we were approaching the topic of vascularization from a more molecular angle, looking at the crosstalk between the cells in the process of vascular network assembly, and paracrine interactions in particular.

As described earlier, incorporation of a functional vasculature is a major hurdle for tissue engineering of larger constructs. I addition, the ability to generate self-assembling microvascular networks from cells can be useful for treatment of ischemic diseases. This paper relied on a previously published 2D *in vitro* model of vascular network formation, where ECs co-cultured with MSCs spontaneously change their morphology and form interconnected network structures. Not all cells have the ability to induce spontaneous network formation of ECs, and upon co-culture with human lung fibroblasts (demonstrated in paper II) or vascular SMCs, the ECs retained their cobblestone morphology.

The co-culture model is simple and easy to set up, and allows for easy assessment and quantification of vascular structure formation. Although the model is 2D in nature, studies have shown that the developing cord structures hold the potential to form 3D-structures and lumen containing vessels[487]. The model thus recapitulates several steps of *in vivo* angiogenic development, were the steps of sprouting, branching, anastomosis, lumen formation and mural cell recruitment all are observed. The model is useful for assessing factors and conditions that might impact vascular structure development, and for studies on communications between the participating cells.

In particular, we were interested in the signals derived from the MSCs that regulate induction of microvascular network formation, and the dynamics of this communication in

the co-culture setting. As our model does not contain any added GFs, aside from what might be present in the AB-serum, the changes in cell behavior in co-culture is a result of the stimuli the cells exerted on each other. To explore the interactions between the cells in our system, we mapped our expressed protein-coding gene data against the FANTOM5 database of ligand-receptor pairs[488]. Lots of potential ligand-receptor pairs were identified, including both autocrine and paracrine signaling.

Mapping ligands expressed by one cell type and receptors expressed by the other cell type does not necessarily give a clear picture of paracrine signaling. Many of the molecules were present on both cell types, but expressed at different levels. As an example, both cells expressed VEGFA, and both cell types showed an up-regulation of VEGFA in co-culture. However, even though the increase measured in fold change was greater in ECs (log2 fold change of 4.6 in ECs, vs log2 fold change of 1.2 in MCSs), the absolute level of MSC-expressed VEGFA was initially more than a hundred-fold higher in MSCs (FPKM 140 vs FPKM 1). Thus, we reasoned that the bulk VEGFA contribution was derived from MSCs, and we sought to identify receptors predominantly expressed by ECs to unravel what part of the signaling were specifically or primarily occurring between the two cell types.

Upregulated genes may help say something about which processes are initiated within the cell upon co-culture, however, it may not be a good indicator for assessment of which signaling molecules are important for the induced changes. From the cells' perspective, the major change occurs when moving from a mono-culture setting to co-culture setting, where the ECs are suddenly exposed to MSC-derived signaling molecules, such as VEGFA and HFG. The up- or down-regulation of these signaling molecules then tell us something about the feedback the MCSs receive from the ECs in co-culture, and about the dynamic role of the MSCs.

Our results show that pro-angiogenic molecules are expressed by MSCs at high levels by default. Some of these increase their expression level upon co-culture with ECs, whereas other decrease. Moreover, we find that a number of angiostatic molecules are up-regulated, indicating a potential change in the angiogenic potential of the MSCs. It seems like MSCs are taking on a more stabilizing role, also indicated by increased expression of mural cell

markers, suggesting a differentiation of MSCs toward a mural cell-like phenotype. As we performed bulk sequencing, we do not know whether all cells differentiate to the same extent, but it is probably likely that direct contact with ECs may help induce SMC differentiation. Whether these cells are also responsible for the increased angiostatic signaling, or if that is a global response of all MSCs in the co-culture, also remains to be elucidated.

As discussed above, scRNA-seq could potentially provide information about different subsets of phenotypes present in the co-culture setting, and potential differences in gene expression dynamics between these. Also, including more time points could be beneficial, perhaps especially for the early phases of the co-culture. In the later stages, it is likely that several processes are going on at once, as the network is expanding, where maturation steps and sprouting steps are occurring simultaneously at different locations in the culture. We could perhaps also benefit from including a negative control, such as the SMC-EC co-culture in which networks were not developing. This could help elucidate which signals were directly responsible for the induction of EC network formation.

Conclusion and future perspectives

In this section the major conclusions of our studies are presented, along with a brief discussion on future studies that could be pursued.

1. **Circulation of whole blood is not a viable strategy for successful endothelialization of decellularized vessel grafts**

If successful, endothelialization of a vein graft with only a small volume of blood would constitute a major advantage over more time-consuming recellularization processes involving invasive cell harvest, isolation and expansion. However, as this method failed, likely due to too few ECs present in the blood sample, alternative approaches for endothelialization should be applied. Cells could still be sourced from whole blood, however, isolation of cells from a larger volume and expansion in culture would likely still need to be a part of the process. An alternative cell source could be AT, which contain large numbers of ECs. Moreover, we have optimized the differentiation protocol to generate SMCs from AT-derived MSCs, which could possibly be used for recellularization of the media. It is also possible that for larger diameter venous grafts, endothelialization may not be necessary, and that a coating strategy to attract ECs upon implantation to prevent thrombosis could be an effective strategy. *In vivo* studies, preferably in large animal models, or clinical studies, would be necessary to establish this.

2. **Protocols for decellularization should be carefully optimized prior to clinical use. Alternatively, a different approach to generate decellularized scaffolds may be used.**

One rationale for using decellularized native veins were the presence of intact venous valves. Harvest and decellularization of cadaver veins is a labor intensive and time consuming process, yielding few veins per donor. At a minimum, the decellularization process should be optimized to ensure that the criteria for successful decellularization are fulfilled. Alternatively, pursuing an off-the-shelf approach where donor availability and quality of the harvested vein would not be limiting factors could be an option. The recent advances in cell-produced ECM scaffolds for vessel grafting, where the ECM-rich scaffolds

are decellularized and can be stored awaiting transplantation, opens up for a similar approach for generation of vein scaffolds. Valve-containing vessels have a more complex structure than simple tubes, but as leaflets have successfully been made from synthetic or biological materials[489], this challenge could likely be overcome. Generating tubular structures containing leaflets for cell seeding would initially require effort and optimization, but the subsequent testing procedures of the vessels, including valve patency, would be the same as for decellularized cadaver veins. This method would also require more advanced equipment, including bioreactors delivering cyclic radial strain, but could allow for up-scaling of the production.

3. **In both 2D and 3D models, human AT-derived MSC performed better than human lung fibroblasts for induction of vascular network formation of ECs, and HUVECs were superior to iPSC-ECFC in terms of their ability to form stable networks. However, the search for the optimal cell source has not been concluded.**

Fibroblasts are widely used in co-culture models with ECs. Fibroblasts, like MSCs, promote angiogenesis through the production of ECM, GFs and proteases[490-493], and have been shown to support 3D network formation by HUVECs[144,494]. However, we and others found that AT-MSCs demonstrated a better capability to induce vascular networks[495]. Our lack of success with fibroblasts, especially in 2D, could possibly be explained by fibroblast source, or by the fact that different fibroblast populations derived from the same source have shown different angiogenic abilities[496]. In addition to fibroblast heterogeneity[496,497], donor genotype or culture conditions (2D vs 3D) affecting fibroblast morphology and gene expression could possibly explain these results[498-500].

With regards to EC source, we have demonstrated that EC from various sources differ in their ability to be induced to form vascular networks. Even though HUVECs performed better than IPSC-ECFC in our setup, other EC sources may be more suitable. IPSC-ECFC can be derived from autologous sources, but performed sub-optimally. For clinical applications, HUVECs are not the obvious choice, and other more relevant EC sources should be tested. For future studies, it would be interesting to test human AT derived microvascular ECs in our platform. These cells have demonstrated angiogenic abilities and we already known them to

develop into microvascular network structures in co-culture with adipose derived MSCs. Moreover, in contrast to HUVECs, these are readily available from autologous sources, which would increase the clinical relevance of the study. Although widely used, HUVECs may not be representative of the actual *in vivo* conditions, as they are derived from immune-privileged fetal tissue and is known do differ in several aspects from adult vascular endothelium[501].

4. Microvascular networks are recruited to isolated rat islets, but are not invading and revascularizing the islets.

Native islet of the pancreas are highly vascularized, but the intra-islet ECs disappear after only few days in culture[466]. Speedy vascularization is thought to be an important factor in preventing massive islet death post transplantation[467-469]. Our islets were shown to recruit and possibly interact with the developing vasculature in our chip assay, however, we did not observe any invasion. There could be several explanations for this, and several things could be attempted in order to enhance vascularization.

One possible modification would be using human islets instead of rat islets, which also would make the platform more clinically relevant. Islets are known to secrete angiogenic factors like VEGF, which is also likely necessary for revascularization of islets *in vivo*[502]. We did not test whether rat VEGFA stimulates angiogenesis of human ECs as efficiently as human VEGFA, which could influence vasculature recruitment. Moreover, interactions between rat islets and human cells might not replicate the interactions of a fully humanized setup.

In addition, our preliminary setup does not incorporate some key physiological parameters that might influence the outcome of islet vascularization in the chips. Physical stimuli are known to affect blood vessel morphology and behavior[503], and incorporating flow could possibly alter the behavior of the developing vasculature in our setup[504]. Moreover, as hypoxic stimuli may enhance the angiogenic response, setting up this experiment under hypoxic conditions might have an effect of vascularization of the islets in our hydrogel chip system.

Finally, it is also possible that our EC source, HUVECs, was sub-optimal in terms of angiogenic invasion. As already mentioned, HUVECs are not microvascular, and testing other sources, including AT-ECs, may provide an advantage.

5. Successful application of a commercially available microfluidics device to establish a platform for studies of vasculature interactions with organoids.

We applied the described platform for studies on islet interactions with the vasculature. Although islet vascularization could be further explored in this setup, this platform could also be useful for other applications. Organ-on-a-chip has gained increased attention over the recent year, and incorporating a functional microvasculature could improve the biological relevance of such systems[368]. Several research topics would benefit from 3D platforms containing a perfusable microvasculature, including studies on tumor cell progression and vascularization, where a 2D culture might not accurately mimic the interplay between tumor cells and their environment[505,506]. In addition, variations in the phenotype of tumor cells grown in 2D vs 3D have been observed, and some aspects of tumor characteristics would not be recapitulated in monolayer cultures[507,508]. Chip setups such as ours can recapitulate tissue architecture and key characteristics of the mechanical and biochemical microenvironment[509-511]. These platforms have the potential to realize organ-level cell functions that cannot be recapitulated with conventional *in vitro* methods[512-514], and can be used for drug development and modeling of human physiology and disease[515]. For studies on organ-level cell functions, recapitulation of the vascular interphase of the organs is of great significance. An engineered perfusable microvasculature can deliver nutrients and cells to 3D tissue constructs, and has great potential to recapitulate cellular microenvironments and functions, and for studies of tissue interactions with the vasculature[368,515].

6. AT-derived ECs and MSCs readily form microvascular networks in a 2D co-culture assay with a fully humanized setup without addition of exogenous GFs.

The 2D co-culture assay is used for studies on MSC induced network formation of ECs[388]. The network-inducing potential of the supporting cells is dependent on direct EC interaction, and the cells have reciprocal effects on each other[390]. Both fibroblasts, SMCs and BM-MSCs have

demonstrated some potential to induce network formation of EC in this assay, however, AT-MSCs have been shown to promote vascular network formation more strongly than other cell types[388,441]. ECs of different origins have also been tested for their ability to organize into cord-like structures in this assay, including cord blood-derived ECs, human microvascular EC (HmVEC), human retinal ECs, iPSC-ECFC and HUVECs[388-390,441]. It has also been demonstrated that the heterogenous stromal vascular fraction from AT can develop into vascular networks[487]. However the resulting networks were rather sparse, and only half of the cultures developed into networks in one study[389]. This can likely be attributed to a low proportion of ECs in freshly isolated SVF. In our study, we isolated and expanded this EC population, and demonstrated a consistent ability of AT-EC to rearrange into cord networks in co-culture with AT-MSC. Importantly, no exogenous GFs were added, and the system was fully humanized, increasing its clinical relevance. This system could be used for testing of the effect of various culture additives, such as GFs or chemicals on vascular network formation. In addition, over-expression or knock-out of individual genes could help elucidate their potential role in vascular network formation.

7. RNA sequencing reveals major co-culture induced changes in gene expression for both cell types, where the role of MSCs seem shift from a predominantly angiogenic to a more network-stabilizing role.

RNA-seq revealed that co-culture induced major changes in gene expression levels for both ECs and MSCs, a significant part of which were related to the ligand-receptor connectome. This highlights the significance of intercellular communication in vascular development, where both signals derived from the other cell type is important, as well as communication within the same cell type to coordinate the process. Whereas transcriptional alterations in ECs seemed to be mostly related to enhanced angiogenic development, which was expected, given the observed morphological changes in co-culture, the changes in MSC expression pattern were more complex. While at the same time demonstrating an increase in VEGFA following co-culture, a number of genes known to be involved in vascular stabilization and inhibition of angiogenesis were induced. Also, angiogenesis-inducing genes were down-regulated. Our results demonstrate that MSCs clearly do have a role in promoting network formation of ECs, and our RNA-seq results suggest that this ability might be inherent to the

MSCs. Co-culture might enhance the ability of MSCs to induce microvascular networks, as indicated by the upregulation of VEGFA, but MSCs also may take a role in stabilizing the process, preventing excessive network formation.

As discussed above, our experimental setup has some limitations, including the possibility of bulk RNA-seq masking cellular heterogeneity, as well as investigating few time points. To address these shortcomings, and thus be able to further explore the nature of the genetic changes, a combination of more time points and scRNA-seq would be a powerful method to gain more detailed information about the expression dynamics of the genes involved in the process. Distinct genes are likely involved in the various stages of vascular network development, where different subsets of the cells also demonstrate varying expression levels.

Another possible improvement would be to use 3D co-culture instead of 2D-culture. In the body, cells are naturally existing in a 3D environment, and a 3D culture would more closely mimic the cells' natural conditions. In addition, by using a system similar to the AIM Biotech chip used in Paper II, eventual incorporation of flow could further increase the physiological relevance of the system.

The next logical step after RNA-seq would be to identify potential novel target genes that might play a role in vascular development. Over-expression studies or gene silencing could help assess the effect of genes or pathways on vascular network formation, and this platform allows for testing of multiple genes or substances in a relatively simple setup.

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Research Paper

Recellularization of Decellularized Venous Grafts Using Peripheral Blood: A Critical Evaluation

Mia H. Rambøl [a,b,*], Jonny Hisdal [b], Jon O. Sundhagen [b], Jan E. Brinchmann [a,c], Antonio Rosales [b]

[a] Norwegian center for stem cell research, Department of immunology, Oslo university hospital, Oslo, Norway
[b] Oslo vascular center, Department of vascular surgery, Oslo university hospital, Oslo, Norway
[c] Department of molecular medicine, University of Oslo, Oslo, Norway

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ABSTRACT

Vascular disease is a major cause of death worldwide, and the growing need for replacement vessels is not fully met by autologous grafts or completely synthetic alternatives. Tissue engineering has emerged as a compelling strategy for the creation of blood vessels for reconstructive surgeries.

One promising method to obtain a suitable vessel scaffold is decellularization of donor vascular tissue followed by recellularization with autologous cells. To prevent thrombosis of vascular grafts, a confluent and functional autologous endothelium is required, and researchers are still looking for the optimal cell source and recellularization procedure.

Recellularization of a decellularized scaffold with only a small volume of whole blood was recently put forward as a feasible option. Here we show that, in contrast to the published results, this method fails to re-endothelialize decellularized veins. Only occasional nucleated cells were seen on the luminal surface of the scaffolds. Instead, we saw fibrin threads, platelets and scattered erythrocytes. Molecular remnants of the endothelial cells were still attached to the scaffold, which explains in part why earlier results were misinterpreted.

Decellularized vascular tissues may still be the best scaffolds available for vascular tissue engineering. However, for the establishment of an adequate autologous endothelial lining, methods other than exposure to autologous whole blood need to be developed.

1. Introduction

Cardiovascular disease is the leading cause of death globally [1,2]. A common treatment for patients with advanced vascular disease is the use of vascular grafts to replace or bypass damaged or obstructed vessels [3]. Autologous vessel grafts remain the gold standard, but not all patients have sufficient or healthy autologous veins for vascular grafting [4]. Synthetic alternatives like Dacron and polytetrafluorethylene are being used with relative success for some applications needing large diameter (>6 mm) grafts, but for smaller diameter applications, synthetic grafts tend to suffer unacceptably high failure rates [5–7]. In addition, for cases requiring more than just a new conduit, like reconstructive vein surgery where valve function is essential, these alternatives are not optimal [8,9].

One technique utilized to make a vascular scaffold is to decellularize allogeneic vascular tissues. Decellularization refers to the removal of antigenic cellular material from tissue [10]. The starting point can be native tissue, or extracellular matrix (ECM) produced from cells ex vivo [11]. By using decellularized natural matrices, one can take advantage of the intrinsic properties of the tissue, including ECM composition, biocompatibility, shape and mechanical properties [12,13]. The decellularization process may involve a variety of chemical agents, solvents and enzymes, and must balance the task of removing all cellular material with the aim of preserving composition, biological activity and mechanical integrity of the remaining ECM [12]. Inadequate decellularization could potentially cause immune reactions and graft failure, while an aggressive decellularization process may remove essential ECM components, thus altering the mechanical properties of the tissue [14,15].

One challenge of using decellularized vessels might be limited recellularization in vivo, caused by the dense ECM of the vessel wall or chemical damage to the ECM in the decellularization process [16,17]. The limited success of current commercially available decellularized grafts has been, in part, explained with their lack of cellularity on implantation [18,19]. A viable endothelium is important to suppress thrombosis of smaller caliber vessel grafts, especially crucial for decellularized grafts with their exposed collagen luminal wall surface [20]. This makes a successful recellularization step essential, and a number of different cell sources and strategies have been employed [13,21]. The ideal cell source would be one that is readily available in sufficient

* Corresponding author at: Norwegian center for stem cell research, Department of immunology, Oslo university hospital, Oslo, Norway.
E-mail address: m.h.rambol@medisin.uio.no (M.H. Rambøl).

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amounts, can be obtained by a minimally invasive procedure, and that would willingly settle in the graft.

In 2014, Olausson et al. [22] reported two pediatric cases involving clinical transplantation of tissue engineered decellularized allogeneic veins. The veins were reported to be recellularized following exposure to 25 mL of autologous peripheral whole blood. It is known that normal human adult peripheral blood contains a small number of circulating endothelial cells (ECs), and cell cultures from blood have demonstrated endothelial outgrowth [23]. Using cells obtained from a simple blood sample for recellularization, this whole blood procedure would sidestep the time-consuming processes of harvest, isolation and expansion, and would present a promising and available approach.

The number of circulating ECs from peripheral blood has previously been estimated to four cells per mL of blood, or even less [24–26]. If the estimated 100 ECs from 25 mL blood could fully endothelialize a graft luminal surface of at least 10 cm^2 in <10 days, that would indicate a great expansive potential of these cells. This would contradict published evidence, and would suggest that there may be other cells present in blood which acquire an endothelial phenotype and contribute to the formation of neointima of a vessel graft.

Since the theoretical background on the prevalence of blood ECs does not fully integrate with the positive results on blood re-endothelialization published in the Olausson et al. study [22], we decided to re-evaluate the procedure with respect to both the quantity and the quality of the re-endothelializing cells. Of note, although these are new experiments, the procedure and the laboratory performing the de-cellularization and the re-endothelialization were the same as in the re-evaluated study.

2. Methods

2.1. Harvest of Vein Segments

The Norwegian Institutional Review Boards and Ethics Committees approved all research protocols. All methods were performed in accordance with relevant guidelines and regulations.

All veins used for decellularization and recellularization were harvested at Oslo University Hospital (OUH), Norway. Informed consent had been obtained. Donors were free from infectious disease. Segments from the femoral vein ($N = 9$), about 5–8 cm in length, were harvested from two adult human cadavers by using vascular surgical techniques, with careful ligation of all side branches. The segments were flushed thoroughly with saline to remove all blood. Vein samples were stored in individual labelled containers filled with PBS supplemented with antibiotics (0.5% penicillin, 0.5% streptomycin, and 0.5% amphotericin B), and kept at 2–8 °C for all transportation events. Shipping time between Oslo and Gothenburg never exceeded 24 h. Samples from two cadaver veins were kept at OUH for analysis.

Native control veins were obtained from remaining saphenous vein segments after bypass operations with the informed consent of patients. Vein samples were stored in PBS supplemented with antibiotics (0.5% penicillin, 0.5% streptomycin, and 0.5% amphotericin B) at 4 °C for maximum 24 h awaiting further processing.

2.2. Decellularization

The decellularization process was carried out by NovaHep, as described in Olausson et al. [22] Minor changes were introduced in a 2015 publication by the same group [9], considered improvements to the procedure. These are mentioned specifically in the following text. The improvements were used also in the current study. The segments were washed for 72 h in distilled water, and decellularized by 9–-14 cycles of sequential incubation in three different decellularization solutions (7 cycles in Olausson et al. [22]). The solutions used were 1% Triton, 1% tri-*n*-butyl phosphate (TnBP), and 4 mg/L DNAse.

All steps were performed at 37 °C with agitation and continuous perfusion with decellularization solution. The segments were immersed in each solution for 4 h per cycle (3 h in Olausson et al. [22]), and rinsed in distilled water between each chemical incubation. All solutions used for decellularization contained 0.5% penicillin, 0.5% streptomycin, and 0.5% amphotericin B.

After completion of the decellularization process, the segments were washed in PBS for 48 h, sterilized in EtOH and frozen at −80 °C. The complete decellularization process lasted 10 days. Samples for characterization were shipped to OUH after the sterilization step.

2.3. Recellularization

The recellularization was conducted by NovaHep, using a patented procedure developed in the lab of Sumitran-Holgersson at Sahlgrenska University Hospital [22]. Peripheral venous blood for recellularization was collected from three healthy donors aged 48, 52 and 63 in sterile heparin-coated Vacutainer tubes and transported to the laboratory within 2 h. The entire recellularization process was carried out under sterile conditions at 37 °C. Before recellularization, the veins were perfused with heparin at a concentration of 50 IU/mL in phosphate buffered saline (PBS) for 2 h. The heparin was drained off, and whole blood was immediately perfused for 48 h at 2 mL/min speed. 25 mL blood was used for recellularization of each sample.

After 48 h the blood was drained off, and the vein was rinsed with PBS containing 1% penicillin-streptomycin-amphotericin until all traces of blood were completely removed. The vein was subsequently perfused for 4 days with EC medium, and the vein segments were immersed in EC medium throughout the incubation period. Complete endothelial medium was prepared with MCDB131 (Life Technologies, Stockholm, Sweden) basal medium supplemented with 10% heat-inactivated human AB serum (Life Technologies), 1% glutamine, 1% penicillin-streptomycin-amphotericin, and EGM-2 SingleQuot kit (CC4176; Lonza). The recellularization method originally included an additional 4 day incubation step with perfusion of the graft with smooth muscle cell medium. This step was later abandoned, as Olausson et al. did not find that it significantly affected either the endothelialization or the proliferation of ECs in the graft [22]. In line with this, the vessels were not perfused with smooth muscle cell medium for the present study.

After recellularization, the segments were washed in PBS and shipped to OUH for further analysis. Upon arrival in Oslo, all segments were immediately rinsed in PBS and processed for downstream analysis.

2.4. DNA Quantification

Vein samples were freeze dried and DNA was extracted using DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions.

2.5. Histology

Vein samples were fixed in 4% paraformaldehyde overnight. The samples were dehydrated in gradients of ethanol and xylene, and embedded in paraffin according to standard protocol. Further, blocks were cut and mounted on SuperFrost Plus Adhesion Slides. 6 μm sections were deparaffinized and stained with hematoxylin and eosin (H&E) using standard laboratory technique. Sections were imaged in an Olympus BX51 microscope.

2.6. Immunohistochemistry

Vein samples were embedded in Frozen Section Medium (Richard-Allan Scientific Neg 50, Thermo Scientific) and frozen in dry ice-cooled isopenthane. Frozen tissue blocks were stored at —80C. The samples

were cut in 8 um thick sections on a CryoStar™ NX70 Cryostat (Thermo Scientific™), mounted on SuperFrost Plus Adhesion slides and immediately post-fixed for 30 s in cold 95% ethanol. Slides were stored at —20C awaiting immunostaining.

Sections were immunostained for the presence of CD31 (1:50; Abcam #ab9498), von Willebrand factor (1:500; Abcam #ab6994), VEGFR-2 (1:200; Acris Antibodies TA337222), alfa smooth muscle actin (1:500, Abcam #ab7817), CD41 (1:1000; Abcam #ab11024) and fibrinogen (1:250; Abcam #ab118488). Antibodies were diluted in blocking buffer (5% goat serum/3% BSA in PBS), and slides were incubated at 4 °C overnight. Negative controls were made omitting the primary antibody. The secondary antibodies used were goat anti-rabbit IgG conjugated to Alexa 488 and goat anti-mouse IgG conjugated to Alexa 594 (Life Technologies), used at 1:500. Each immunostaining procedure was performed in triplicate and repeated at least twice per vein sample.

The stained sections were mounted with prolong gold antifade (Invitrogen), containing DAPI for nuclear staining. Imaging was done using an upright Nikon Eclipse E600 microscope equipped with an Olympus ColorView III camera.

2.7. Scanning Electron Microscopy

Vein samples were fixed in 2.5% glutaraldehyde for 24 h and dehydrated in increasing concentrations of ethanol. Samples were mounted on aluminum stubs, coated with platinum in a vacuum sputter coater and examined in a Philips XL30 ESEM LaB6 Electron Scanning Microscope. SEM imaging was performed in quadruplicate per sample.

2.8. En Face Microscopy

Vein samples were fixed in 4% PFA for 24 h, washed in PBS and cut open longitudinally. Triplicate samples were stained with 2 μg/mL Hoechst 34,580 (Invitrogen) for 5 min, rinsed in PBS and placed on glass slides endothelial side down. The samples were viewed with a Zeiss Axio Vert 1A inverted microscope.

2.9. Data Availability

The data generated during the current study are available from the corresponding author upon reasonable request.

3. Results

3.1. Generation and Characterization of de- and Recellularized Vessel Scaffolds

Treatment with 1% Triton and 1% tri-*n*-butyl phosphate through 14 decellularization cycles resulted in generation of pale and translucent veins. The vein segments did not shrink significantly, and maintained their tubular appearance. H&E staining of the control vessels showed layers of pink ECM with purple nuclei surrounded by darker pink cellular material dispersed throughout the vessel wall and along the lumen (Fig. 1a). DAPI staining showed rounded nuclei near the luminal surface, with more elongated nuclei lying parallel to the luminal surface within the outer layers. There was weak DAPI background staining between the nuclei (Fig. 1d). Staining of the cadaver veins prior to the decellularization process showed that cells were still present, and that the ECM had not been severely degraded during the five post mortem days before harvest (Fig. S1a). Decellularized veins contained multiple layers of ECM within the vessel wall, with no visible obviously intact nuclei (Fig. 1b), indicating that the cells and their nuclei had been disrupted in the decellulariazation process. DAPI staining of decellularized scaffolds showed some diffuse blue staining and perhaps scattered nuclei throughout the vessel wall, and some very rare blue condensations along the luminal surface which may represent nucleated cells (Fig. 1e). We did not observe the emergence of increased number of nuclei after the recellularization procedure (Figs. 1c; f). In

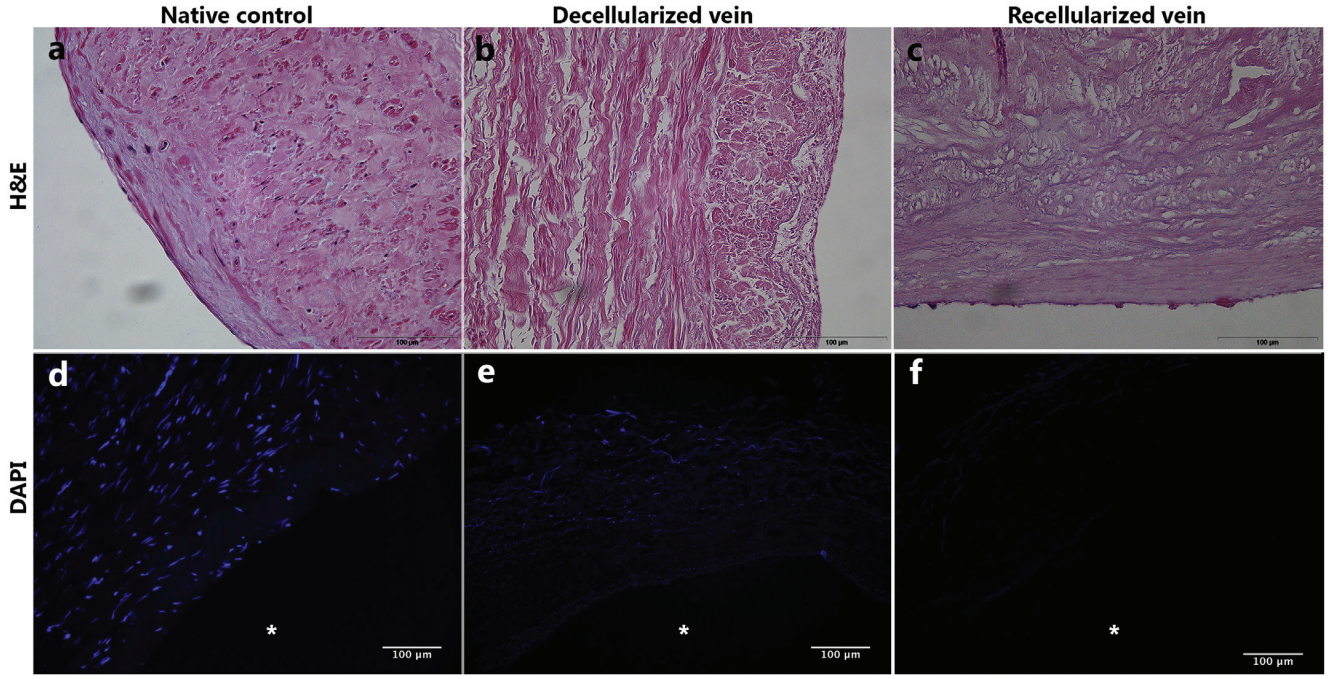

Fig. 1. Decellularization removes all nuclei throughout the vein, but no signs of successfull re-endothelialization. (a-c) Representative images of H&E stained paraffin cross sections. a) Native control vein displays purple nuclei along the endothelial surface and throughout the tissue. b) Complete removal of nuclear components from tissue post decellularization. c) There are no visible nuclei along the luminal surface after recellularization. (d-f) DAPI staining of cryo cross sections. d) Nuclei are evident along the endothelial surface and thoughout the tissue in the native vein. e) All intact nuclei are removed in the decellularization procedure. f) No visible nuclei along the luminal surface after the recellularization process.

particular, there was no evidence of increased numbers of nuclei along the luminal surface. Some background staining of the tunica adventitia was observed, however, there was no visible staining of the luminal surfaces. To quantify the removal of DNA from the vein wall, DNA was measured in three decellularized samples and found to be 105, 97 and 177 ng/mg tissue. For comparison, native saphenous veins contained 1110 ng/mg tissue, and cadaveric veins 723 ng/mg tissue.

3.2. Examination of the Luminal Surface

To evaluate the outcome of the recellularization process, we examined the luminal surface of the veins by en face microscopy. Hoechst staining demonstrated that native control vessels contained abundant round endothelial nuclei covering the intima, as well as the more elongated nuclei of the smooth muscle cells of the media (Fig. 2a). Decellularized vessels did not contain any nuclear structures, suggesting that intact cells were no longer present (Fig. 2b). However, there was some diffuse staining throughout the vessel wall, which may represent remaining DNA following disruption of the nuclei. The recellularized tissues also displayed some diffuse staining throughout the vessel wall, and with rare condensations of staining which may conceivably represent cell nuclei, but markedly different from the native vessels in both shape and amount (Fig. 2c).

For a more comprehensive analysis of the inner structure and morphology of the veins, we conducted Scanning Electron Microscopy (SEM) studies on all samples. All control vessels displayed a densely packed surface with elongated, smooth endothelial cells lining up in the direction of the blood flow (Figs. 2d; 2 g). The decellularized samples showed a flattened surface void of cell-like structures, but with an abundance of exposed ECM fibers (Figs. 2e; 2 h). Consistent with the en face findings, our SEM images did not indicate the presence of endothelial cell layers in any of the recellularized vein samples, but showed rare structures which may represent individual cells attached to the vessel wall. (Figs. 2f; i). However, a fibrous mesh was found to cover large areas of the luminal surface.

This meshwork was also evident in H&E cross sections at very high magnification covering areas of the lumen (Fig. 3a-c). Not infrequently, red blood cells trapped in the meshwork could be seen. SEM revealed more details of the luminal surface. Instead of an endothelium established after recellularization, we found exposed ECM fibers or red

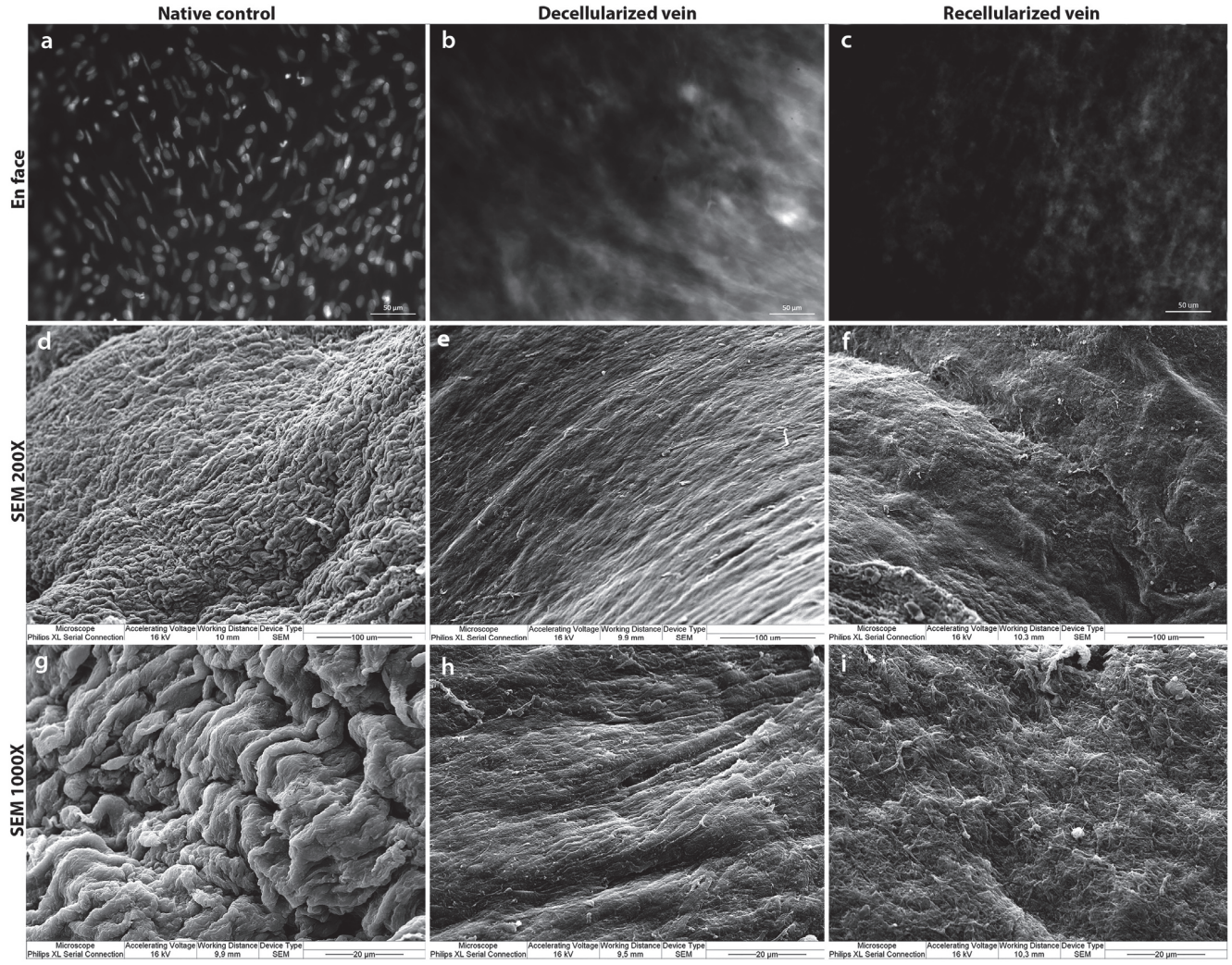

Fig. 2. A closer look at the luminal surface shows no evidence of recellularization. (a-c) En face view of luminal surface. a) Native control vein displays a confluent endothelial cell layer, evident by round nuclear staining. Smooth muscle cells of the media are visible in the form of elongated nuclei. b) En face microscopy of decellularized vein shows complete removal of intact nuclei. c) En face view of recellularized vein sample reveals that a new endothelial cell layer has not emerged. Small black dots are thought to be red blood cells. (d-i) Representative images of Scanning Electron Microscopy of luminal surface, 200× and 1000× magnifications. d and g) Native control veins display a confluent endothelial layer. e and h) Decellularized vein shows the complete removal of endothelial cells. The underlying ECM is exposed. f and i) The luminal side of recellularized vein does not contain anything resembling a confluent endothelial cell layer. The lumen appears to be covered by a fibrous meshwork.

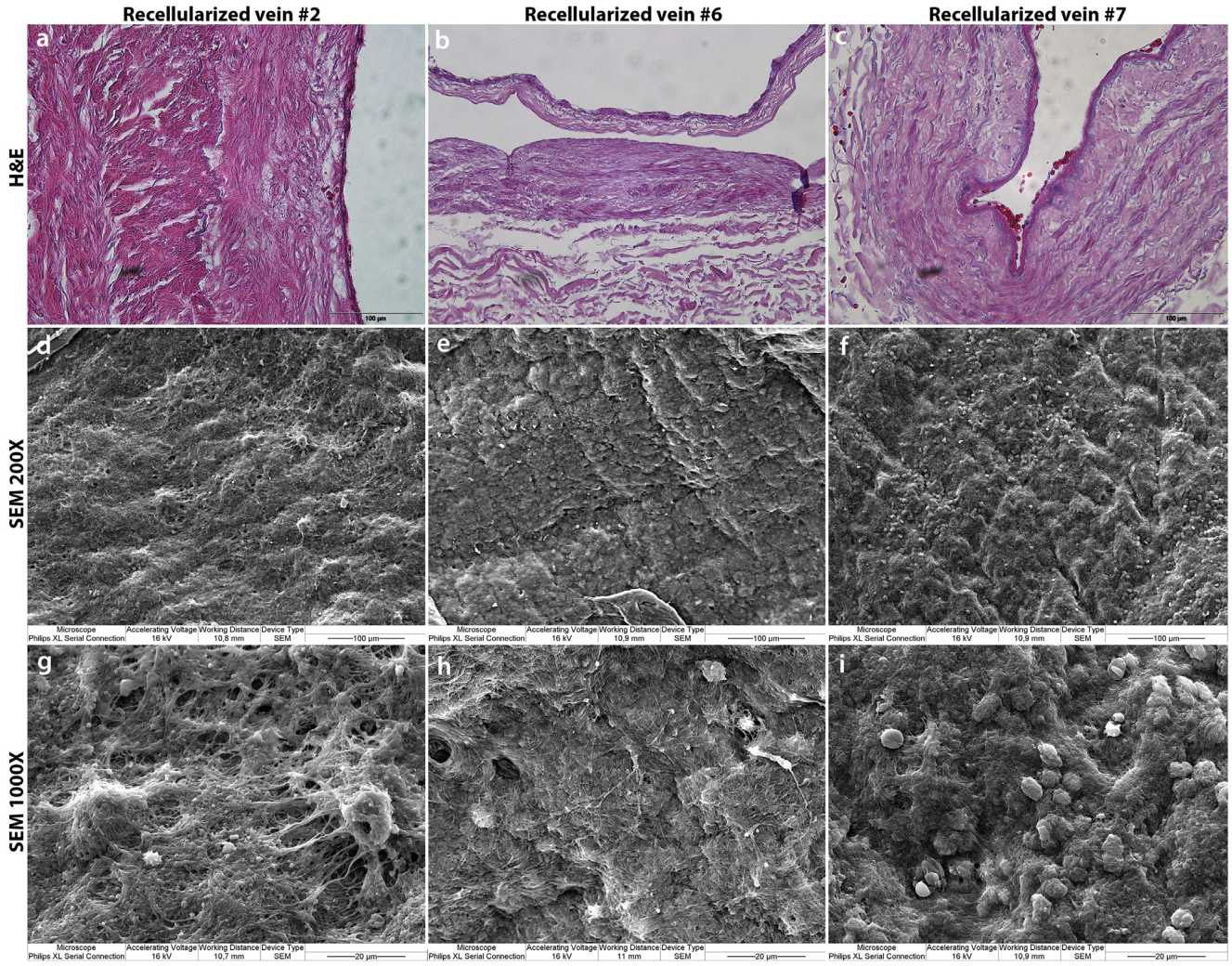

Fig. 3. Deposition of fibrin and platelets on the surface of recellularized veins. (a-c) H&E stainings show the formation of darker pink layers, possibly fibrin, along the luminal side of the vessel. Red blood cells trapped within the fibrin clot are noticeable several places. No new nuclei are seen along the luminal surface following the recellularization process. (d-f) SEM at 200× show a fibrous mesh covering the luminal surface, in addition to some round structures, especially evident in sample #7. No apparent re-endothelialization. (g-i) SEM at 1000× reveal details of fibrous mesh. Red blood cells and platelets are trapped in a fibrin network covering the entire surface of the vein scaffold. No traces of endothelial cells can be seen.

blood cells, platelet-like structures and long fibers covering parts of the vessel lumens (Fig. 3c-i). The larger magnifications reveal structures very similar to platelets in different stages of activation (Fig. 3g-i).

3.3. Immunohistochemistry

Two commonly used markers to demonstrate successful re-endothelialization are CD31 and von Willebrand factor (vWF) [22,27]. Remarkably, despite ECs not being present on the re-endothelialized surfaces, our IHC results showed that the recellularized veins stained positive for CD31 along the luminal surface (Fig. 4c). As expected, the native control veins also stained positive for CD31 (Fig. 4a), while the decellularized veins were negative for this marker (Fig. 4b). Also surprising, the staining for vWF was positive in all our preparations: the native veins, decellularized veins and the recellularized specimens (Figs. 4d-f).

Anti-VEGFR-2 was used in the original study to demonstrate successful repopulation of the graft with endothelial progenitor cells. As native saphenous veins, used as control here, do not express VEGFR-2 [28], our positive control for VEGFR-2 was a sample of the cadaver vein before decellularization. Staining of both the decellularized and recellularized veins with anti-VEGFR-2 were negative, in line with our results showing no re-endothelialization of the vessels (Figs. 4g-i).

As our SEM and H&E results indicated a meshwork of fibrin and platelets forming along the luminal surface, we stained for the specific platelet marker CD41 and for fibrin. IHC results confirmed the presence of platelets and fibrin along the lumen of the recellularized vessels (Figs. 5c; f). Native and decellularized veins were all negative for these markers, demonstrating that platelets and fibrin had been deposited on the luminal surface during the recellularization process (Figs. 5a; b; d; e).

4. Discussion

In contrast to the proof of principle publication [22], we could not find evidence for adequate recellularization of the decellularized scaffold, and did not observe the formation of a new endothelial layer. Cross-sectional histology staining did not show nuclei along the vessel luminal surface, confirmed by en face images covering larger areas of the luminal surface. SEM images similarly made it very clear that there was no settlement of new cells along the luminal surface post recellularization. Negative VEGFR-2 staining also confirms this. However, we may have found the explanation for why some of the

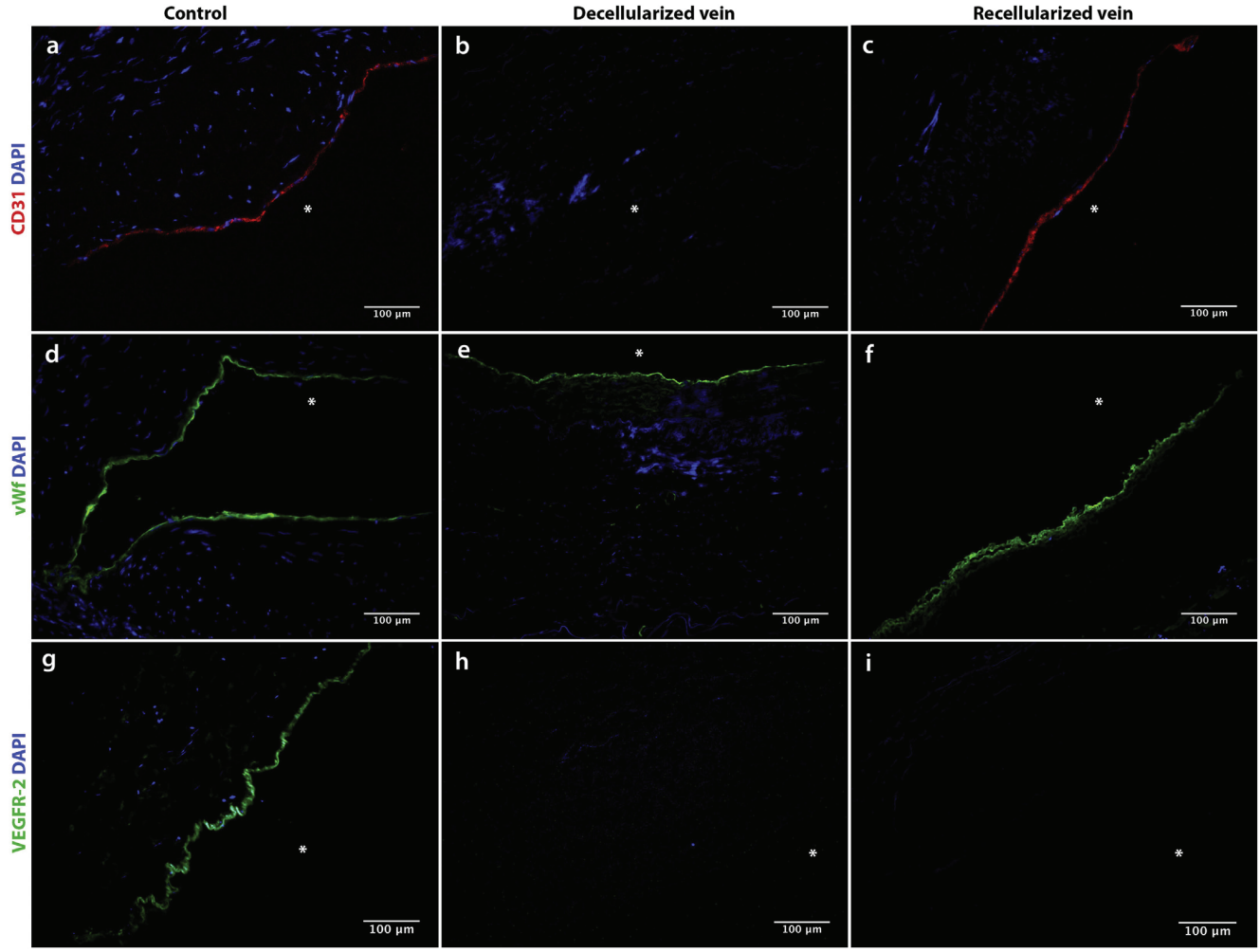

Fig. 4. CD31, von Willebrand Factor and VEGFR-2 immunostaining of native, decellularized and recellularized veins. a-c) CD31 immunostaining of cryosections. Asterisk indicates the lumen. a) Native control vein contains a confluent layer of CD31-staining cells. b) CD31-staining cell layer has been fully removed in the decellularization process. c) A CD31-positive layer has emerged on the luminal surface after recellularization. d-f) Von Willebrand factor immunostaining of cryosections. Asterisk indicates the lumen. d) Continuous vWF staining in the native control vein. e) Continuous vWf staining of decellularized vein. f) Continuous vWF staining of recellularized vein. g-i) VEGFR-2 factor immunostaining of cryosections. Asterisk indicates the lumen. g) Continuous VEGFR-2 staining in the cadaver control vein. h) VEGFR-2-staining layer has been removed in the decellularization process. i) No traces of VEGFR-2-positive cells in the recellularized vein.

immunohistochemical staining procedures may have given the false impression that the scaffolds were re-endothelialized.

Interestingly, antibodies specific for both vWF and CD31 stained positively in the recellularized specimens, even though no new endothelium had been formed. Both of these markers were used to verify the presence of ECs in the Olausson et al. paper, though the vWf staining results were not shown. VWF is a collagen binding protein [29], and upon rupture of the endothelial cells in the decellularization process, we believe that the vWF released from the cells binds to the collagen component of the luminal surface of the matrix. This will give similar staining with anti-vWF antibody both in the native specimens, where the vWF is inside the endothelial cells, and in the decellularized and recellularized specimens, where the vWF is bound to the ECM making up the luminal surface of the vessel scaffold. Consequently, as long as the vWF is not removed from the tissue in the decellularization process, this marker cannot be used as evidence for recellularization.

CD31, also known as PECAM-1 (Platelet Endothelial Cell Adhesion Molecule 1), is not exclusively expressed by endothelial cells, but also on platelets and other hematopoietic cells [30]. Based on our SEM images, and the fact that the decellularized vessels were negative for CD31, we made the hypothesis that platelets from the circulating blood had attached to the luminal surface of the recellularized vessels. This was tested and confirmed by staining with antibodies specific for the platelet-specific surface marker CD41 [31]. It is also possible that soluble CD31, present in circulating blood, could have attached to structures on the luminal surface of the vessel scaffold and contributed to the staining by the anti-CD31 antibody [32]. Thus, recellularization with whole blood does not endothelialize the vessel scaffold, but leads to positive staining with anti-CD31 antibodies and a false impression that the vessel scaffold is covered with endothelial cells. When whole blood is used for recellularization, CD31 cannot be used as a definite proof for the presence of endothelial cells.

The importance of a functional endothelium in preventing thrombosis has been well documented in the literature. It is not unlikely that the naked vessel wall, combined with retained vWF, may contribute to the formation of blood clots [33]. When collagen of the vessel wall is exposed, usually caused by damage to the endothelium, platelets will bind directly to the vessel wall and eventually initiate coagulation. The interaction between platelets and collagen is strengthened by vWF, which is known to be a mediator of the initiation and progress of

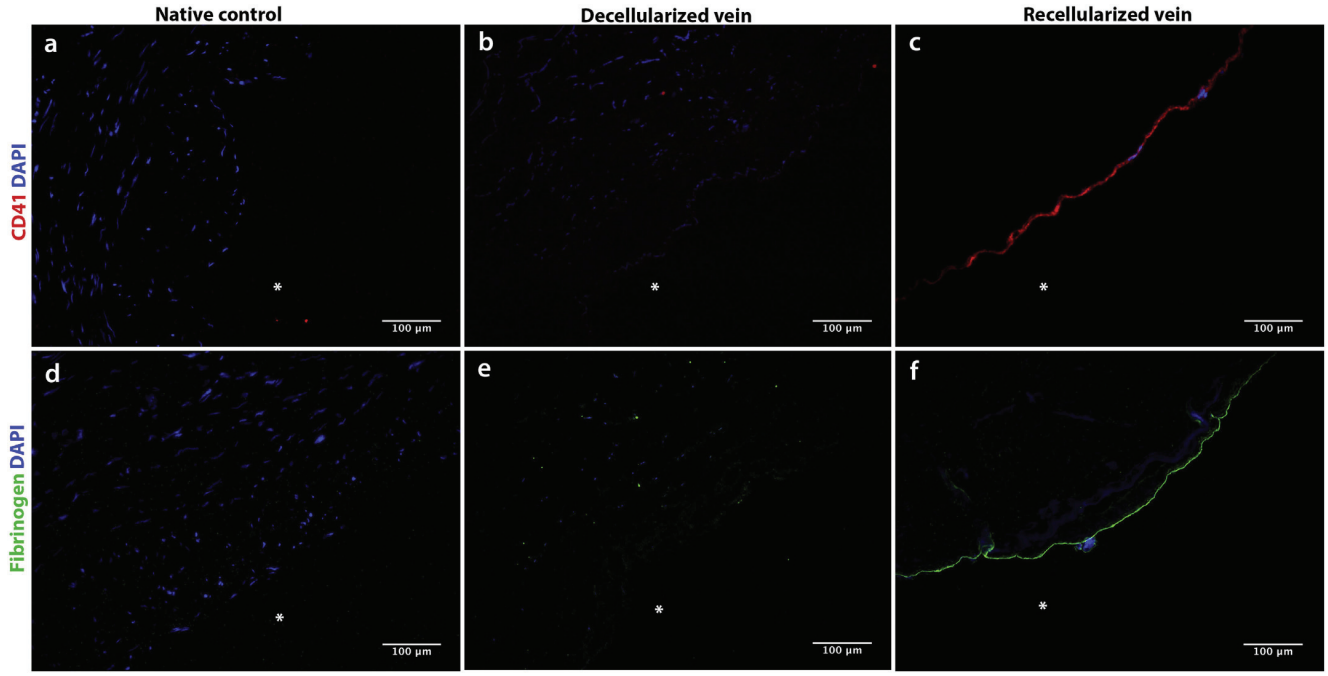

Fig. 5. CD41 and fibrin immunostaining of native, decellularized and recellularized veins. a-c) CD41 immunostaining of cryosections. Asterisk indicates the lumen. Native and decellularized veins stain negative (a and b), whereas c) shows continuous CD41 staining of luminal side after recellularization. d-f) Fibrin immunostaining of cryosections. Asterisk indicates the lumen. Native and decellularized veins stain negative (d and e), whereas f) shows continuous staining for fibrin along the luminal surface following recellularization.

thrombus formation by several means, including tethering of platelets, platelet adhesion and factor VIII binding [29,33].

In addition, these samples also stained positive for fibrin. Fibrin is formed from fibrinogen, a component of normal blood plasma. Fibrin, together with platelets, are known to be an essential ingredients for the formation of blood clots [34,35]. The presence of vWF, platelets and fibrin along the luminal surface may not only be a curious side effect of the use of blood for re-endothelialization, but may predispose to thrombosis in the treated veins.

The notion of using peripheral blood to regenerate a decellularized vessel would potentially make tissue engineered blood vessels available to a large number of patients. The peripheral blood re-endothelialization method was already used[22] for implantation of tissue engineered veins in extrahepatic by-pass procedures in two pediatric patients. However, two of the three grafts used in the two patients failed post implantation, at least one of them due to occlusion. Our results may offer explanations for these failures.

Successful re-endothelialization by peripheral blood derived EPCs has in fact been achieved, by Tillman et al. [36] Using a CD133+ antibody capture system to harvest ovine EPCs from 1800 mL of peripheral blood, they further expanded the cells in the laboratory for several weeks, obtaining millions of cells for recellularization. In addition to histological studies, convincing SEM images demonstrate that they get a complete re-endothelialization of the vessel graft. This shows that blood does contain cells suitable for recellularization purposes. However, our results show that whole blood preparations are not suitable for re-endothelialization. The amount of endothelial cells in whole blood is too limited, in addition to the potentially thrombogenic effects of the deposition of fibrin, platelets and vWF along the vessel wall. Thus, if peripheral blood is to be used as a source of cells for re-endothelialization, these cells need to be isolated from the blood and most likely expanded ex vivo. Alternatively, one may find autologous endothelial cells or their precursors in adipose tissue and the bone marrow [37,38]. As a complete, continuous and functional layer of ECs along the vessel wall is essential for the safe use of decellularized scaffolds in clinical applications, the work to identify the best cell source and the optimal recellularization process continues.

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Author Contributions

Conception and design: MHR, JH, JOS, JEB, AR.
Analysis and interpretation: MHR, JEB, AR.
Data collection: MHR, AR.
Writing the article: MHR, JEB,
Critical revision of the article: MHR, JH, JOS, JEB, AR.
Final approval of the article: MHR, JH, JOS, JEB, AR.
Obtained funding: JEB.

Competing Financial Interests

The authors declare no competing financial interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.05.012.

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II

Microvessel network formation and interactions with pancreatic islets in 3D chip cultures

Mia H. Rambøl[1*], Edward Han[2], Laura E. Niklason[2].

1 Department of Molecular Medicine, Faculty of Medicine, University of Oslo, 0316 Oslo, Norway.

2 Department of Biomedical Engineering, Yale University, New Haven, CT 06519, USA; Department of Anesthesiology, Yale University, New Haven, CT 06519, USA.

* Corresponding author

Author information

Mia H. Rambøl, M.Sc.

Phone: +47 22840480; E-mail: m.h.rambol@medisin.uio.no

Edward Han, M.Sc.

Phone: (973) 818-1108; E-mail: edward.han@yale.edu

Laura E. Niklason, Professor of Professor of Anesthesiology & Biomedical Engineering

Phone: (203) 737-1422; E-mail: laura.niklason@yale.edu

The work was performed at the Department of Biomedical Engineering, Yale University, New Haven, CT 06519

Abstract

The pancreatic islet is a highly vascularized micro-organ, and rapid revascularization post islet transplantation is important for islet survival and function. However, the various mechanisms involved in islet revascularization are not fully understood, and we currently lack good *in vitro* platforms to explore this. Our aim for this study was to generate perfusable microvascular networks in a microfluidic chip device in which islets could be easily integrated, to establish an *in vitro* platform for investigations on islet-microvasculature interactions. We compared the ability of MSCs and fibroblasts to support microvascular network formation by HUVECs and human iPSC-ECFC in 2D and 3D models of angiogenesis, and tested the effect of different culture media on microvessel formation. HUVECs that were supported by MSCs formed patent and perfusable networks in a fibrin gel, whereas networks supported by fibroblasts rapidly regressed. Network morphology could be controlled by adjusting relative cell numbers and densities. Incorporation of isolated rat islets demonstrated that islets recruit local microvasculature in vitro, but that the microvessels did not invade islets, at least during the course of these studies. This in vitro microvascularization platform can provide a useful tool to study how various parameters affect islet integration with microvascular networks, and could also be utilized for studies of vascularization of other organ systems.

Statement of Impact

To improve pancreatic islet graft survival and function post transplantation, rapid and adequate revascularization is critical. Efforts to improve islet revascularization are demanding due to an insufficient understanding of the mechanisms involved in the process. We have applied a microfluidics platform to generate microvascular networks, and by incorporating pancreatic islets we were able to study microvasculature-islet interactions in real time. This platform can provide a useful tool to study islet integration with microvascular networks, and could be utilized for studies of vascularization of other organ systems. Moreover, this work may be adapted towards developing a pre-vascularized islet construct for transplantation.

Introduction

One of the most significant challenges facing the field of tissue engineering is the ability to vascularize tissues[1,2]. All cells require a sufficient supply of nutrients and oxygen, as well as the ability to remove waste[3,4], and a functional microvasculature is needed to ensure proper function and survival of engineered biological tissues that are too large to be maintained by diffusion[5,6]. Generating perfusable microvascular networks *in vitro* will be an important development toward successful engineering of organs and tissues[7,8], as well as for reliable modeling of microvasculature and organ systems[9]. Currently, multiple *in vitro* systems mimicking characteristics of various organs have been established[10]. However, many current systems do not fully integrate a 3D microvasculature, and a vascularized model could consequently greatly improve their physiological relevance[11].

Several approaches have emerged for engineering *in vitro* microvessels. One method involves endothelialization of micropatterned hydrogel channels or polydimethylsiloxane (PDMS) devices, to make *in vitro* perfusable vessel analogues[9,11]. These models have been used to study several aspects of endothelial cell (EC) biology, including EC migration, vascular barrier function, inflammatory response, thrombosis, tumor cell interactions, and response to biomolecular and mechanical stimuli[12-17]. However, since these tubules are formed by the attachment of EC monolayers to channels in a pre-defined pattern, these models have only limited ability to replicate *in vivo* development microvascular networks[9]. Moreover, these models cannot fully reconstitute the distinctive attributes and dynamics of endothelial responses *in vivo*.

Another approach to model 3D microvasculature relies on the ability of ECs to self-assemble into networks[18]. Great advances have been made over recent years in developing 3D perfusable microvascular networks, where utilizing the morphogenic properties of EC eliminates the need for pre-made architecture to support the newly-formed blood vessels[5,11]. Suspending cells in hydrogels like fibrin or collagen more closely mimics vessel formation *in vivo*[11]. The inherent angiogenic ability of ECs, supported by cells secreting angiogenic growth factors, extracellular matrix (ECM) proteins and proteases, allows for the dynamic development and remodeling of microvascular networks[19].

EC tube formation is regulated by coordinated crosstalk between ECs and other cell types[20], and stromal cell-derived factors are important for EC sprouting and lumen formation[21]. A number of cell types from the stromal compartment, such as fibroblasts, vascular smooth muscle cells and mesenchymal stem cells (MSCs), have been shown to support self-assembly of microvascular networks in hydrogels[22-24]. Even though the specific dimensions in self-assembled microvascular networks cannot be strictly controlled, microvascular network morphology can be modified by adjusting parameters like cell seeding density, hydrogel composition and by the addition of growth factors[5].

Microvascular network morphology and attributes are regulated by the surrounding environment. There are significant differences between the microvasculature structure and microvascular ECs of different organs[25,26], since every organ demonstrates specific vascular characteristics and functions[27]. For instance, pancreatic islet vasculature shows considerable differences from that of the exocrine pancreas. Islet capillaries are highly fenestrated, are wider than those in the exocrine pancreas, and are present at a considerably higher vascular

density than in the exocrine organ[28]. Islets make up only 1% of the pancreas, but they receive up to 15% of the pancreatic blood supply[29]. The higher degree of vascularization is important for the islet's ability to quickly secrete insulin in response to fluctuating blood glucose levels[29]. Even though the outcomes of pancreatic islet transplantation have improved over the recent years[30-32], many challenges remain. Most islet transplants are done by intraportal vein injection[29], wherein infused islets are lodged in the liver vasculature. This site is suboptimal since it evokes early imflammatory reactions[33], and exposes cells to a relatively hypoxic environment[34], resulting in substantial islet loss post transplantation[35]. Up to 60% of the transplanted islet mass is estimated to be destroyed following the infusion into the portal vein[36].

Pancreatic islet isolation disrupts the connections between the islet vasculature and surrounding tissue, and intra-islet endothelial cell content decreases rapidly in culture, partially as a consequence of hypoxia and lack of nutrients in the devascularized islets[37]. After islet transplantation, the reestablishment of blood flow to transplanted islets requires at least several days[29]. Therefore, ischemia and inadequate blood supply are likely contributors to the significant islet death occurring in the immediate posttransplant period[37]. Hence, rapid and adequate revascularization is important for islet graft survival and function, and could improve the outcomes of islet transplantation[38-40]. Various methods to improve engraftment of transplanted islets have been suggested, including co-transplantation with other cell types[41-44], alternative sources for insulin producing cells[45], gene therapy[46], and the addition of ECM proteins and growth factors[47]. Moreover, alternative transplant sites are being explored. The subcutaneous site, with its accessibility, large size and ease of monitoring may be an option[45]. However, the subcutaneous option

suffers from inadequate vascularization and poor oxygen tension, and islet transplantation into an unmodified subcutaneous site has never reversed diabetes in humans or animals[48,49]. To improve the subcutaneous microenvironment several approaches have been tried, including the use of encapsulation devices[50], polymers[51] and meshes[52], co-transplantation with growth factors[52], MSCs[53] or fibroblasts[54], as well transient priming by temporary placement of a catheter[49]. Beyond these approaches, the *prevascularization* of islets *in vitro* could possibly improve islet survival *in vivo,* by speeding inosculation with the host after implantation. Takahashi et al.[40] showed that *in vitro* self-condensation of islets with ECs improves islet function in culture, as well as post-transplant reperfusion and engraftment.

The objective of our study was to establish a platform for the study of microvascular interaction with pancreatic islets. Despite recent advances, efforts to improve revascularization of transplanted islets have been hampered due to an insufficient understanding of the mechanisms involved in the process, including the responsible cells, ligands and receptors[38]. For this, good *in vitro* models are useful. The platform should support the development of patent and perfusable microvessels in a hydrogel system in which pancreatic islets could be easily integrated, and should allow for real time monitoring to closely follow islet-network interactions over time. Finally, the platform should also provide flexibility to investigate parameters that could potentially affect microvascular network development and islet integration, and should be easy to reproduce.

We have integrated microvascular networks engineered into a commercially available microfluidic chip, along with pancreatic islets, to produce a platform that will improve our understanding of islet revascularization. This setup could be adapted for the study of

microvascularization of other organ systems and tissues. Ultimately, this work may be adapted toward developing a prevascularized islet construct for transplantation.

Material and methods

Cells and culture

GFP- and RFP-tagged human umbilical vein endothelial cells (HUVECs) were purchased from Angio-proteomie (Boston, MA). Cells were seeded onto rat tail collagen type I (Corning) coated (50 ug/mL) tissue culture flasks and cultured in EBM-2 with complete EGM-2 MV Microvascular Endothelial Cell Growth Medium SingleQuot supplements (Lonza). Cells were passaged at 80% confluence, and passage 3-4 cells were used for all experiments.

Human adipose-derived MSCs were isolated from adipose obtained from Yale New Haven Hospital . Cells were maintained in DMEM supplemented with 1% Penicillin-Streptomycin (Gibco) and 10% FBS (HyClone). Cells were passaged at 80% confluence. Passage 2-4 cells were used for all experiments.

Human lung fibroblasts were received as a generous gift from Will Chang at the Department of Medicine and Section of Nephrology, Yale University. Cells were maintained in M199 supplemented with 1% Penicillin-Streptomycin and 10% FBS. Cells were passaged at 80% confluence. Passage 3-5 cells were used for all experiments.

tdTomato-tagged human induced Pluripotent Stem Cell-derived Endothelial Colony Forming Cells (iPSC-ECFCs) were a generous gift from Mervin Yoder, Department of Pediatrics,

Indiana University School of Medicine. Cells were maintained in VascuLife VEGF Endothelial Medium supplemented with complete Vasculife VEGF LifeFactors Kit (LifeLine Technology, Walkersville) on collagen-coated tissue culture flasks. Cells were passaged at 80% confluence. Cells from passage 11 were used for this study.

Rat islet isolation

This study was approved by the Yale University Institutional Animal Care and Use Committee. All animal care complied with the Guide for the Care and Use of Laboratory Animals. Human tissues and human cell populations that produced the human MSC were obtained using protocols approved by the Yale University Human Investigation Committee, and were discarded tissues, not categorized as human subjects research.

Sprague Dawley rats were euthanized by intraperitoneal injection of ketamine/xylazine at 75mg/kg Ketamine and 5mg/kg Xylazine. Pancreata were excised and digested in 1.5mg/mL collagenase P (Sigma-Aldrich) for 20 minutes in a shaking 37°C water bath. Collagenase digestion was stopped with a solution composed of HBSS (ThermoFisher) containing 10% FBS and 1% Pen-Strep. The digest was further homogenized by passage through a 14 G syringe needle followed by filtration through a 500 µm nylon mesh. The digested pancreas was washed three times with HBSS solution, and islets were purified using Histopaque -1077 (Sigma-Aldrich) density gradient centrifugation. After additional washes to eliminate residual exocrine tissue and histopaque solution, the islet pellet was resuspended in 10 mL RPMI 1640 medium (Gibco) supplemented with 10% FBS and 1% Pen-Strep, and cultured on uncoated dishes in a humidified 37°C CO_2 incubator. Medium was changed on day 1, and then every 3 days.

2D angiogenesis assay

For stromal cell-EC co-culture, adipose-derived human MSCs or human fibroblasts, and HUVECs or iPSC-ECFCs were mixed at a ratio of 4:1 and plated at $1x10^5$ cells/cm^2 on tissue culture plastic. Fluorescently labelled endothelial cells allowed the monitoring of cell behavior in real time over the course of the experiments.

To determine an optimal medium for 2D angiogenesis assay, we tested three different cell culture media. These were: EBM-2 with added EGM-2 MV Microvascular Endothelial Cell Growth Medium SingleQuots supplements) (Lonza); VascuLife VEGF Endothelial Medium supplemented with complete Vasculife VEGF LifeFactors Kit (LifeLine Technology, Walkersville); and MCDB131 (Gibco) supplemented with 10% FBS, 1%Pen-Strep and FGF2 vial from EGM2 bullet kit (Lonza). Medium were changed every day. Images were taken at day 3 and 6 with a Leica DM6000 fluorescent microscope (Leica Microsystems).

Microvascular network formation in fibrin

HUVECs or iPSC-ECFC, combined with MSC or fibroblasts, were resuspended in EGM basal medium with thrombin and aprotinin, and mixed with fibrinogen to yield a final fibrin concentration of 2 mg/mL. Final concentrations of thrombin and aprotinin were 1U/mL and 25 ug/mL, respectively. The ratio of endothelial cells (either HUVEC or iPSC-ECFC) to MSC/fibroblast cells was 5:1 for all experiments. For experiments comparing cell types, total cell number was 2.4 million/mL of fibrin gel. For investigations on the effects of cell number on network and lumen formation, the cell numbers were 3, 6 and 9 million/mL of fibrin gel. When looking at microvasculature recruitment to islets, 2.4 million cells/mL were used. Islets were included in the cell mixture before resuspension in basal medium for islet studies, and

then mixed with fibrinogen to form the fibrin gel encapsulating the islets and vascular cells. The cell/fibrinogen mixture was quickly injected into the middle gel channel of the DAX 1 3D microfluidic Cell Culture chip (AIM Biotech, Singapore). The chips were incubated at 37°C for 30 minutes before adding EGM-2MV medium to both medium channels. For perfusion assays, medium channels were coated with fibronectin (Sigma) at 10ug/mL for one hour after the gel had solidified, and HUVECs (2M/mL) were added and left for a minimum of four hours to allow cell attachment. Medium was then changed to remove unattached cells, and thereafter changed twice daily. Confocal images were taken with a Leica SP5 microscope.

Microvascular network analysis

To determine the effect of cell number on microvascular network formation, network density was analyzed using FIJI/ImageJ software. Images of cell networks (whole AIM Biotech chips, tiled images, n = 5) were converted to 8-bit images and an RFP fluorescence threshold was applied to render the images binary. Area coverage (vessel area) was determined as fluorescent area divided by total area of the gel. For further analysis, the FIJI plugin Angiogenesis Analyzer developed by Gilles Carpentier was used. Average vessel diameter was calculated as total length of vessels divided by size of vessel area in mm^2. Quantification of vascular density was calculated as total vessel length / total gel area (mm^2).

To study microvessel interaction with islets, microvascular networks on and immediately around the islets were analyzed. The region analyzed contained the islet and the area extending 100 µm around the islet. In a single experiment, the same islets were analyzed at day 1 and day 4. Average network coverage of total chips (n = 6) were also analyzed at day 1

and 4, as a benchmark of capillary density in the absence of intervening islets. Area coverage vas calculated as described above.

Immunostaining of Aimbiotech chips

Fibrin gels were processed for staining after 5 days of culture. Gels were fixed with 4% PFA for 15 minutes and blocked for 2 hours at room temperature in PBS containing 0.75% glycine (OmniPur) and 5% BSA (Gemini Bio-products). Primary antibody against ACTA2 (M0851, Dako) was diluted 1:100 in blocking buffer, and gels were incubated overnight at 4°C. After washing the gels for 3 x 5 minutes in PBS, fluorescence-conjugated secondary antibody (goat anti-mouse Alexa Fluor 555, Invitrogen A21424) was diluted 1:400 in blocking buffer and incubated with the gels for 1 hour at RT. Gels were then washed repeatedly in PBS and imaged by confocal microscopy (Leica SP5).

Microparticle perfusion

30 μL medium were aspirated from both ports of one medium channel to create a pressure drop across the gel. 10 μL 1 mg/mL green fluorescent microparticles were added to one port of the opposing medium channel, allowing the microparticles to flow into the medium channel and into the microvascular network. Images were taken with a Leica DM6000 microscope.

Statistics

Data were analyzed by a two-way Student's *T*-test, and expressed as group mean ± SEM. A *p* value of <0.05 was considered statistically significant. The R package ggplot2[55] was used for plotting.

Results

Effect of culture medium on 2D microvascular network formation

To evaluate the effect of culture medium type on microvascular network formation, HUVECs and MSCs were co-cultured on tissue culture plastic in three different culture media. EGM-2MV and Vasculife VEGF Endothelial Medium both contain undisclosed concentrations of the growth factors FGF-ß, VEGF-A, IGF-1 and EGF, in addition to hydrocortisone and ascorbic acid. We also tested MCDB131 that was supplemented with only FGF-ß to provide a "minimal medium" comparator. By day 3, HUVECs in all three culture conditions had changed morphology from cobblestone to elongated cells, and were starting to form interconnected networks (Figure 1A, upper panels). These networks were notably denser in the growth factor supplemented media, indicating a faster proliferation rate and/or an impact on EC phenotype. By day 6, the networks cultured in either Vasculife or EGM-2MV had developed and stabilized, forming fully interconnected networks covering the entire culture area (Figure 1A, lower panels). By contrast, networks cultured in minimal medium had regressed. These results indicate that growth factor-supplemented medium is required for microvascular networks formation by HUVECs in a 2D co-culture model with MSCs. Since EGM-2MV gave smoother and denser networks in this assay, as assessed by microscopy, we chose this medium for subsequent experiments.

Effect of cell types on microvascular network formation

As fibroblasts are widely used in co-culture models with endothelial cells, we investigated two different isolations of human lung fibroblasts in our 2D model, to assess their potential to support microvascular network formation by HUVECs. Cultured in EGM-2MV, the HUVECs

did not acquire an tube-forming phenotype at either day 3 or day 6 (Figure 1A) as assessed by elongated morphology, but instead remained clustered in cobblestone colonies. We obtained similar results from both fibroblast isolations, demonstrating that human lung fibroblasts cannot induce an angiogenic phenotype in HUVECs in these culture conditions.

Assessing the angiogenic potential of iPSC-ECFC, we co-cultured iPSC-ECFC with MSCs in EGM-2MV. This resulted in mostly cobblestone-shaped endothelial cells clustering together, with some elongated cells extending from the clusters (Figure 1A, rightmost panels). By day 6, these elongated cells had increased in number, and formed larger areas of interconnected networks between the cell clusters. Our results indicate that cells with some *in vitro* angiogenic capacity can be derived from iPSC, however, HUVECs were found to be superior in terms of network forming ability in this assay.

Effect of cell type on microvascular network formation in 3D.

To investigate the ability of cells to form stable microvascular networks in a fibrin gel, HUVECs were cultured alone, with MSCs, or with fibroblasts in a 2% fibrin gel in the AIM Biotech DAX 1 3D microfluidic Cell Culture chip in EGM2-MV medium (Figure 1B). Chips with iPSC-ECFC and MSC were also cultured. HUVECs alone failed to form stable networks in the fibrin gel. The cells were found to elongate and form tentative branches with poor interconnection, and by day 5 most branches had regressed. HUVECs cultured with both fibroblasts and MSCs did form networks, both of which were still present to various extents at day 5. However, HUVECs cultured with MSCs displayed a more consistent elongated morphology and formed smoother branches than did HUVECs cultured with fibroblasts. In the fibroblast-supported networks, not all parts of the cell culture were interconnected, and

the networks started to regress by day 4. iPSC-ECFC cultured with MSCs formed stable and mostly interconnected networks, although a proportion of the cells did not join in the network formation (Figure 1B, rightmost panels. These results demonstrate that HUVECs require a supporting cell type to form a stable microvascular network in this assay, and that MSCs performed better than fibroblasts in this regard. iPSC-ECFCs did also form networks in co-culture with MSCs, however, the HUVEC-MSC combination generated the most stable and interconnected networks.

To determine whether MSCs acquired a more contractile phenotype during co-culture with endothelium, we probed the HUVEC-MSC networks for ACTA2 expression. Immunofluorescent staining revealed consistent co-localization of ACTA2-positive MSCs with the HUVECs throughout the networks. (Supplementary figure S1). The co-localization of MSCs and ECs demonstrates a likely interaction between the cell types, suggesting that MSCs might have a role in stabilizing the newly formed microvascular network.

Effect of cell density on microvascular network formation

To assess the effect of cell density on formation of microvascular networks, fibrin gels were seeded with 3, 6 and 9 million total cells/mL, at a HUVEC:MSC ratio of 5:1. By day 5, all visible HUVECs were participating in stable interconnected networks (Figure 2A and B). Area coverage, as calculated as the area of the chip with RFP fluorescent cells, significantly increased with increasing cell number (3M vs 6M cells/mL: $p < 0.001$; 6M vs 9M cells/mL: $p < 0.01$, $n = 5$) (Figure 2C). The average vessel diameter also significantly increased, from 22.8 ± 1.0 µm with 3M cells/mL, to 28.2 ± 0.9 µm with 6M cells and 38.7 ± 2.6 µm for 9M cells/mL (3M vs 6M cells/mL: $p < 0.01$; 6M vs 9M cells/mL: $p < 0.01$, $n = 5$) (Figure 2D). The network

density (Figure 2E), calculated as tubule length/mm^2 showed a significant change from 3M to 6M cells ($p < 0.01$, $n = 5$), whereas further increasing cell number to 9M did not significantly change the length of the network. These results demonstrate that cell density can impact the morphology of microvascular networks grown in a fibrin gel, where higher cell densities yield networks with larger average vessel diameter.

Lumen formation in microvascular networks

Confocal imaging of 9M/mL vascular networks revealed consistent lumen formation throughout the network (Figure 3A; For projections, see Supplementary videos S2 A-B). Orthogonal views revealed multiple clear circular shapes, verifying that the networks had intact lumens. Furthermore, addition of microparticles to the medium channel of the chip resulted in perfusion into the network (Figure 3B).

Recruitment of microvasculature by rat islets in vitro

To generate an *in vitro* platform for the study of pancreatic islet interaction with microvasculature, isolated rat pancreatic islets were combined with our microvascular networks in the microfluidics device. Immediately after seeding, cells were evenly distributed in the gels. After days in culture, cells gradually formed interconnected networks throughout the gel and around the islets (Figure 4A and B; for projections of B, see Supplementary videos S3 A-B). Analyzing area coverage of the microvascular network immediately surrounding the islets at day 1 and day 4, the average coverage increased significantly (*P*-value < 0.0001, $n = 37$ islets), from 12.3 ± 0.5% to 20.0 ± 0.9% (Figure 4C). In comparison, the average microvascular area coverage for whole chips (the entire gels, including the area surrounding the islets) were 10.9 ± 0.35% and 12.7 ± 1.06% at day 1 and

day 4, and the change was not significant (p = 0.066, n = 6 gels). As the network densities increased significantly around the islets, but not for the entire gel, our results indicate that islets are attracting microvasculature to their immediate surroundings.

Discussion

In the present study, we successfully developed a functional microvascular network in a microfluidic device, where HUVECs that were supported by adipose derived MSCs formed interconnected microvessels. The generated microvasculature was patent and perfusable, thereby potentially allowing for delivery of nutrients and oxygen via the luminal space. By adjusting the cell seeding density, we can control microvascular morphology, including the microvessel diameter and network density, demonstrating the potential to generate networks that are tailored to specific applications.

In the absence of MSCs, we observed only impaired network formation and stability, confirming the stimulatory effect of MSCs on angiogenesis[56]. We also observed consistent co-localization of ACTA-2 positive MSCs with endothelial cells of the microvascular networks, supporting previous findings that MSCs can act in a pericyte-like manner under these types of co-culture conditions[57-60], and provide stabilizing effects on microvascular networks *in vitro* and *in vivo*[61,62]. Microvascular networks also formed in a co-culture of MSCs and iPSC-ECFCs, although these networks did not appear to be fully interconnected. In addition, iPSC-ECFC were only partially induced to form networks in a 2D assay, possibly reflecting a lack of angiogenic potential or a need for further stimuli than were provided in this set of studies.

Fibroblasts, like MSCs, promote angiogenesis through the production of ECM, growth factors and proteases[63-66], and have been shown to support 3D network formation by HUVECs[5,67]. We were therefore surprised to observe a rapid regression of HUVEC-fibroblasts networks, preventing the networks from reaching a fully interconnected and stable state. Moreover, human lung fibroblasts were unable to support endothelial network formation in 2D co-culture assays in an angiogenic cell culture medium. Evensen et al.[68] observed a similar difference between human dermal fibroblasts and other stromal cell types, whereas other investigators have found that human dermal fibroblasts do induce network formation of HUVECs in the same 2D model[69]. Newman et al.[21] compared human lung fibroblast populations having different angiogenic abilities, and found that a number of ECM proteins are more abundant in fibroblasts that readily induce endothelial sprouting in a fibrin model of angiogenesis. Shortage of secreted ECM proteins from fibroblasts as compared with MSCs, and thus insufficient structural support for the ECs to form networks, could possible explain why networks failed to form in the 2D model. As the fibrin gel itself provides some structural support, this could explain the difference in angiogenic potential of fibroblasts between these two platforms. Fibroblast heterogeneity[21,70] or donor genotype, as well as 2D vs 3D culture conditions affecting fibroblast morphology and gene expression[71-73] could also explain these contrasting results.

Combining islets with our engineered microvascular networks, we show that microvasculature is actively recruited to pancreatic islets *in vitro*. Islets are known to secrete angiogenic factors like VEGF, which is also likely necessary for revascularization of islets *in vivo*[74]. Rapid revascularization of islets post transplantation is considered important for islet graft survival and function, and could improve the outcomes of islet transplantation[37-39].

Moreover, secretion of VEGF and proteases by MSCs supports vascularization, and could promote EC migration into the islets[56,75]. MSCs have been shown to improve islet vascularization after islet transplantation[75-79], and MSCs from various sources have been reported to improve islet survival and function both *in vivo* and *in vitro*[80-86].

Providing islets with a readily available microvascular networks supported by MSCs may speed up the revascularization process, as well as improve islet survival until revascularization is complete. By demonstrating that this process can be started *in vitro* by attraction of vascular cells to the islets, our model could represent a first step toward creating a vascularized islet construct for transplantation. Prevascularization could also facilitate integration of the construct upon implantation. Allowing fibrin constructs to develop microvascular networks *in vitro* prior to implantation, as opposed to a construct containing newly suspended cells, has been shown to significantly improve anastomosis with host vasculature[87].

The fibrin base of the construct could also have a positive effect on islet outcome. In addition to promoting angiogenesis, fibrin has been shown to enhance islet function and survival[88,89]. Fibrin is biocompatible and biodegradable[90,91], and is also the body's natural structural scaffold for wound healing.

Even though we observed consistent microvasculature recruitment to islets, we only rarely observed invasion of endothelial cells into the islets themselves. Lack of invasion and penetrating revascularization of islets *in vitro* could have several possible explanations. Our model may benefit from incorporation of flow, since mechanic stimulation has been shown

to impact vasculature remodeling and development[92]. In addition, our *in vitro* model does not incorporate all of the physiological signals and signaling molecules found *in vivo* that influence islet revascularization.

For future investigations, alternative sources for endothelial cells should also be considered. HUVECs are widely used for *in vitro* studies and have a demonstrated ability to form neovasculature and microvascular networks, but differ from microvascular endothelial cell in some important aspects, including differences in cell surface receptors, cytoskeletal and secreted proteins[93-95]. In addition, for potential clinical applications, an autologous cell source is desirable. One possible source for autologous microvascular endothelial cells is iPSCs. These cells have an extensive capacity for self-renewal, and can be used to derive tissue-specific cells, including different EC subtypes[96]. iPSC-derived vascular endothelium has been shown to be highly plastic[97], and can be directed toward specific EC subtypes in response to biomechanical cues[98]. Although we and others[99] found iPSC-ECFCs to be outperformed by HUVECs in terms of angiogenic ability, iPSC-derived ECs have previously shown more plasticity in modulating their phenotype in response to flow, as compared with HUVECs[17]. The use of IPSC-EC would warrant further studies, and may present a viable alternative in the future of islet transplantation.

To conclude, we applied a commercially available microfluidics platform to generate perfusable microvascular networks, and by incorporating pancreatic islets we were able to study microvasculature-islet interactions in real time. This platform can provide a useful tool to study how various parameters affect islet integration with microvascular networks, and could also be utilized for studies of vascularization of other organ systems. Moreover, this

work may be adapted towards developing a prevascularized islet construct for transplantation.

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Author Disclosure Statement

L.E.N. is a founder and shareholder in Humacyte, Inc., which is a regenerative medicine company. Humacyte produces engineered blood vessels from allogeneic smooth muscle cells for vascular surgery. L.E.N.'s spouse has equity in Humacyte, and L.E.N. serves on Humacyte's Board of Directors. L.E.N. is an inventor on patents that are licensed to Humacyte and that produce royalties for L.E.N. L.E.N. has received an unrestricted research gift to support research in her laboratory at Yale. Humacyte did not influence the conduct, description, or interpretation of the findings in this report. The other authors report no conflicts.

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Image captions

Figure 1. Effect of cell types and culture medium on microvascular network formation in 2D and 3D. (A) Representative images of vascular networks formed by HUVECs and iPSC-ECFC cocultured with MSCs or human lung fibroblasts in different culture media. Images were taken after 3 and 6 days of culture. Scale bars, 500 µm. (B) Representative images of vascular networks formed by HUVECs alone, HUVECs + human lung fibroblasts, HUVECs + adipose derived MSCs, and iPSC-ECFC + adipose derived MSCs in a fibrin gel. Images were taken after 5 days of culture. Scale bars, 200 µm

Figure 2. Effect of cell number and cell type ratio on microvascular network. (A, B) Representative images of microvascular networks formed in a fibrin gel by increasing number of cells. Ratio of HUVEC to MSC is 5:1. Scale bars, 250 µm. (C) Quantification of vessel area of the fibrin gel shown in percentage of RFP-HUVEC signal of the total area. All values are means from five gels plus ± SEM. 3M vs 6M cells/mL: $p < 0.001$; 6M vs 9M cells/mL: $p < 0.01$ (D) Average vessel diameter calculated as total length of vasculature to area coverage. 3M vs 6M cells/mL: $p < 0.01$; 6M vs 9M cells/mL: $p < 0.01$ (E) Quantification of vascular density calculated as vessel length / mm^2. Significant increase from 3M to 6M cells, $p < 0.01$.

Figure 3. Microvascular networks form perfusable lumens. (A) Orthogonal projections of z-stack images of RFP-HUVECs cultured with AT-MSCs in a fibrin gel for 5 days. Images were composed from 0.9 um serial confocal images (72 slices) through the z-plane of the cells. Left panel shows stacked xy projection, and the side and bottom panels show yz and xz projections. Yellow crosshairs indicate intersection of yz and yx planes. Rightmost panel shows single slice from stack at z-depth as indicated by the x and y axis crosshairs within the projections. Scale bars, 100 µm. For lumen projections, see Supplementary animations S2A and S2B. (B) Fluorescent microparticles added to the medium channel enter the microvascular network, demonstrating that the engineered networks are perfusable. Scale bars, 25 µm

Figure 4. Microvascular network recruitment to islets. (A) Progression of microvascular network formation around a rat islet day by day. Cells were evenly dispersed in the gel immediately after seeding, and gradually formed a mostly interconnected network by day 5, assembling around the islets. 2 M RFP-HUVECs were co-cultured with AT-MSCs at a 5:1 ratio in a fibrin gel with integrated GFP rat islets. Scale bars, 200 µm. (B) Confocal image of microvascular network surrounding an islet after five days of culture. For projections, see Supplementary animations S3A and S3B. Scale bar, 100 µm. (C) Distribution of vascular network coverage immediately around islets at day 1 and day 4. For analysis of network area coverage around islets, the regions that were analyzed contained the islet and the area extending 100 µm from the islet boundary. FIJI was used to automatically threshold and analyze the images. The vascular density around the islets increased significantly from day 1 (12.3 ± 0.5%) to day 4 (20.0 ± 0.9%) (*P*-value < 0.00001, N = 37 islets). Red horizontal lines indicate vascular area coverage throughout the entire fibrin gel, this change was not significant. (10.9 ± 0.35% and 12.7 ± 1.06% at day 1 and day 4, respectively. *P*-value < 0.065699, *n* = 6 gels).

Supplementary figure S1. Colocalization of ACTA2-stained AT-MSCs with HUVECs of the microvascular network after five days of culture. Scale bar, 50 µm.

Supplementary video S2. Microvascular network form lumens.
(A) Run though of z-stack and (B) projection of a microvasculature region.

Supplementary video S3. Microvasculature around an islet.
(A) Run through of z-stack and (B) projection of microvascular network surrounding an islet

Figure 1. Effect of cell types and culture medium on microvascular network formation in 2D and 3D.

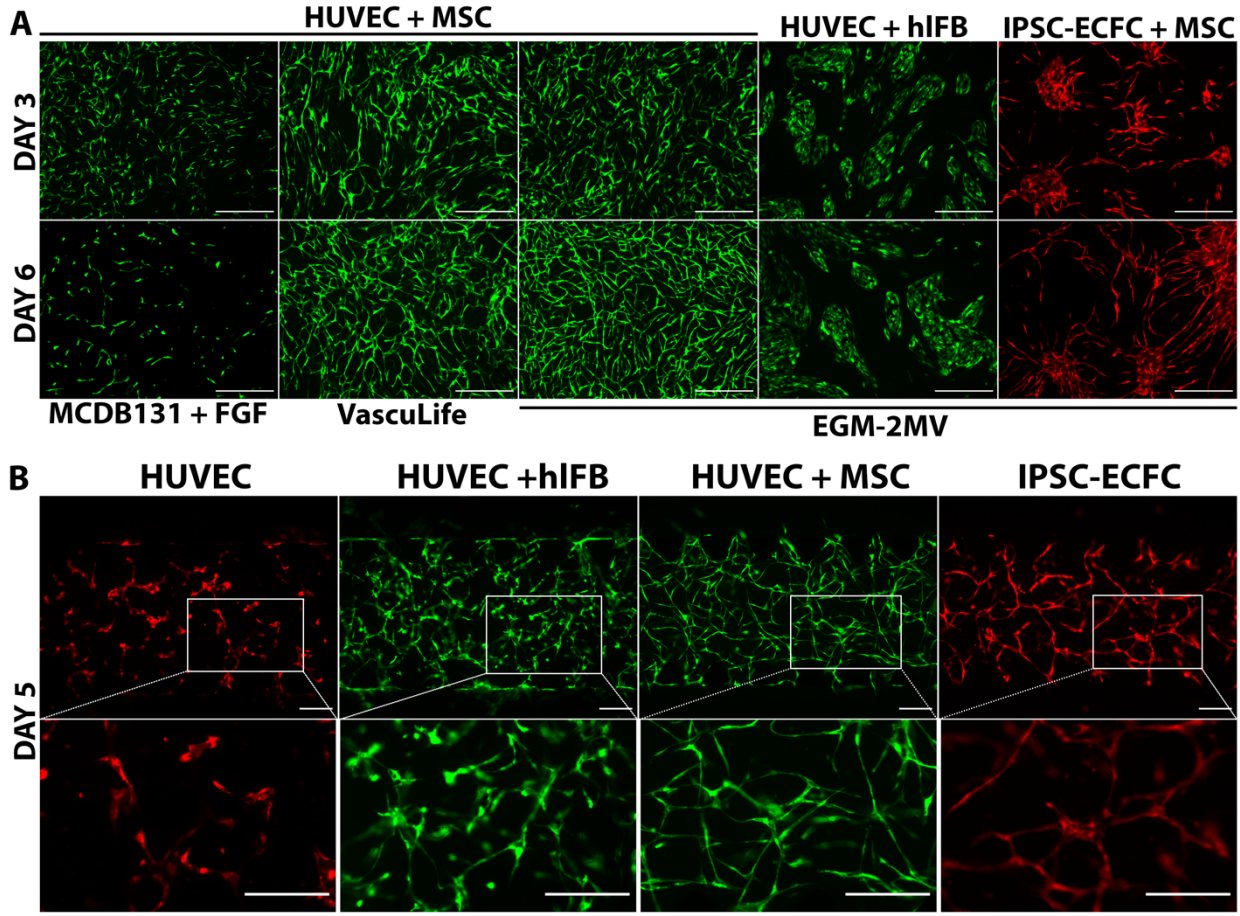


Figure 2. Effect of cell number and cell type ratio on microvascular network.

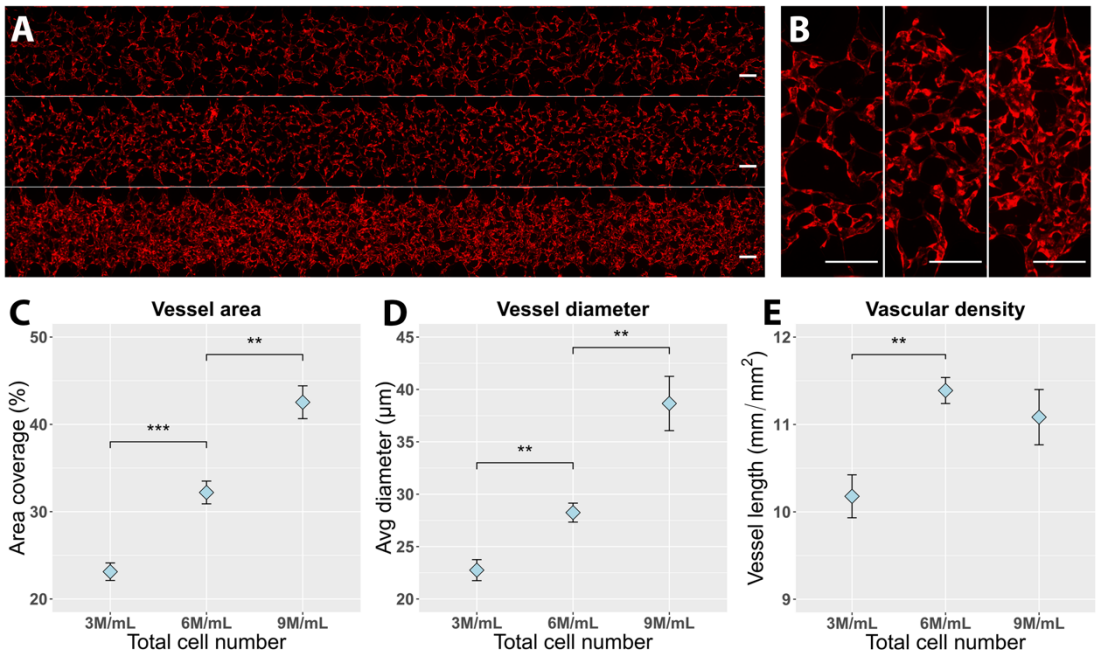


Figure 3. Microvascular networks form perfusable lumens.

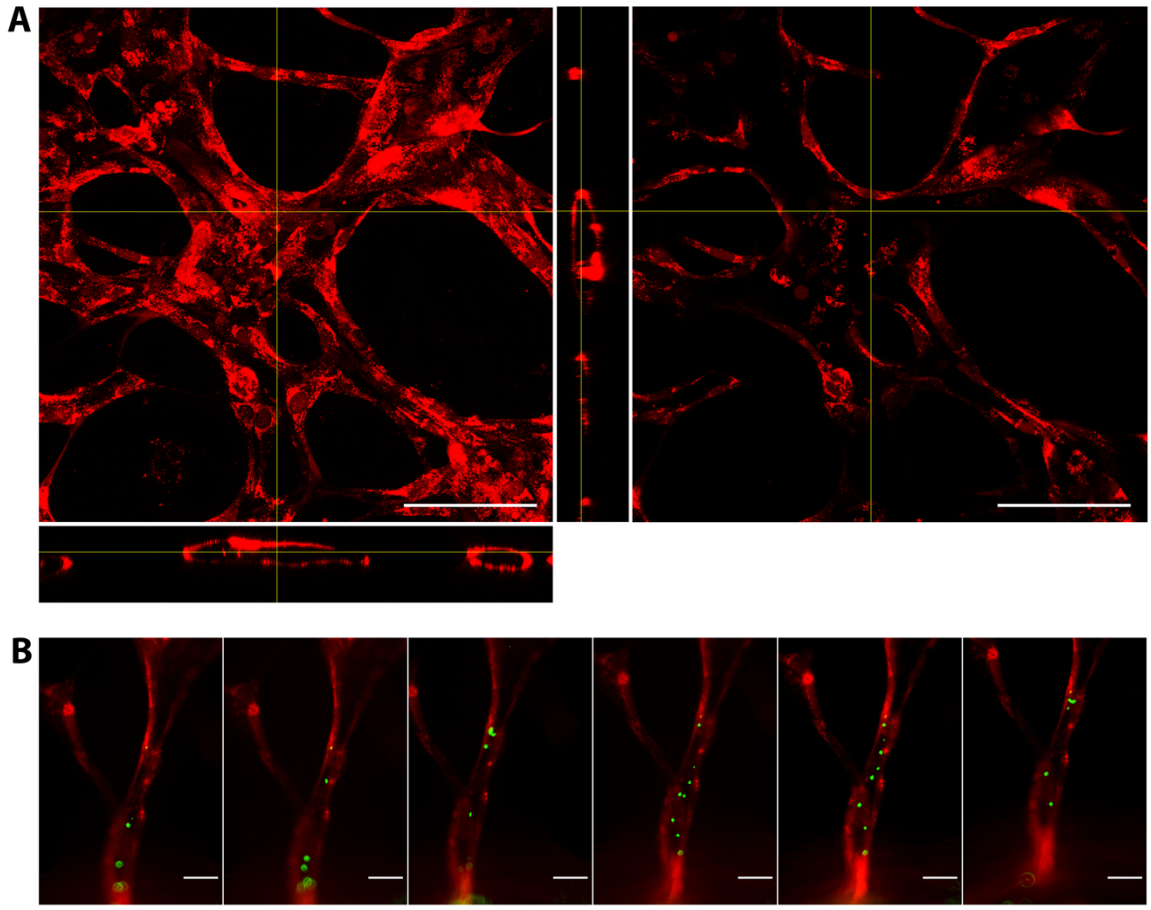
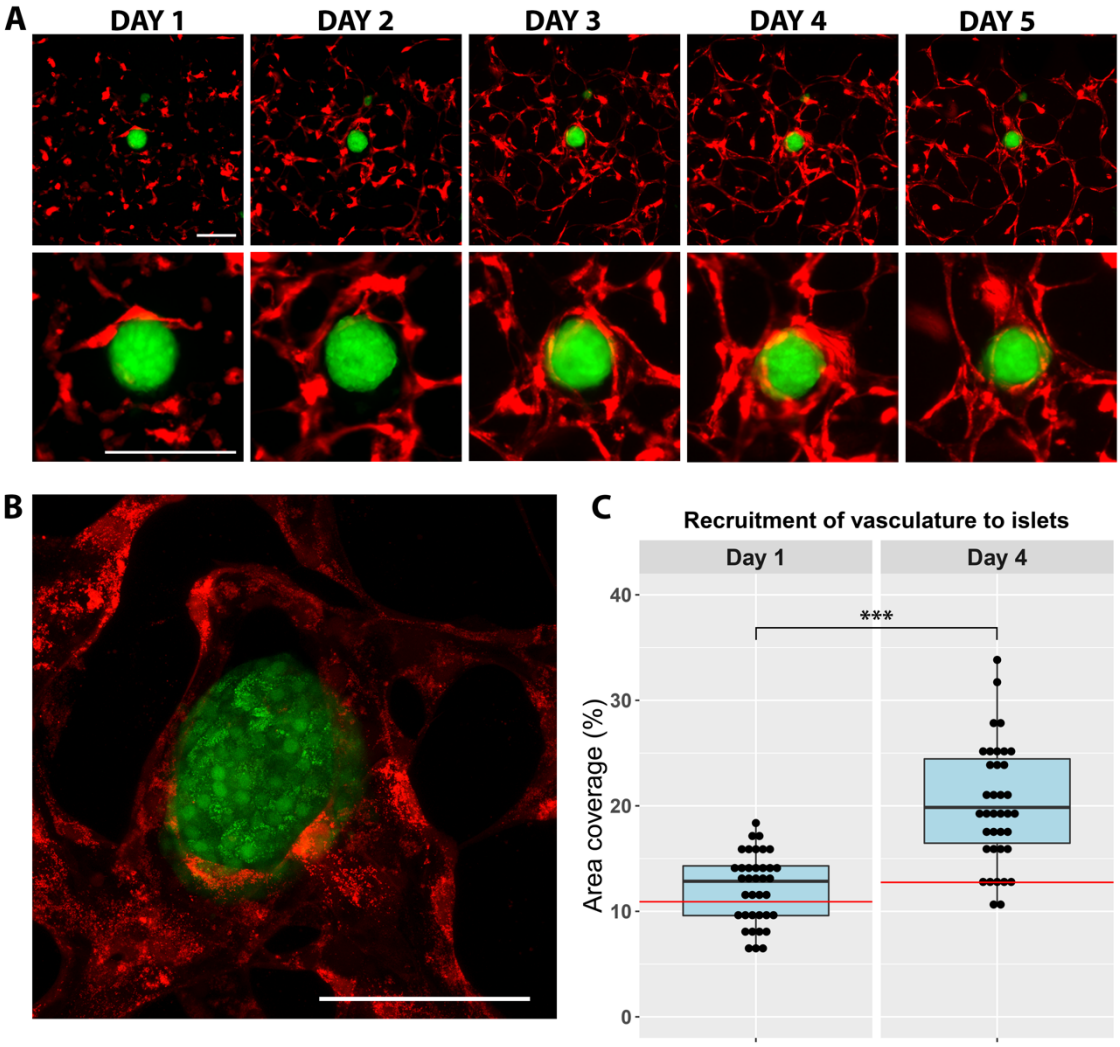


Figure 4. Microvascular network recruitment to islets.



Supplementary figure S1. Colocalization of ACTA2-stained AT-MSCs with HUVECs of the microvascular network after five days of culture.