Prevascularized Bone Tissue Engineering

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Prevascularized Bone Tissue Engineering

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PREVASCULARIZED BONE TISSUE ENGINEERING

DISSERTATION

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on the authority of the rector magnificus,
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on account of the decision of the graduation committee,
to be publicly defended
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by

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born on July 25th, 1979
in Meppel, The Netherlands

Promoter: Prof. Dr. Clemens A. van Blitterswijk

List of publications

Publications related to this thesis

Malda, J., **Rouwkema, J.**, Martens, D.E., Le Comte, E.P., Kooy, F.K., Tramper, J., van Blitterswijk, C.A., and Riesle, J. Oxygen gradients in tissue-engineered PEGT/PBT cartilaginous constructs: measurement and modeling. *Biotechnology and bioengineering* **86**, 9, 2004.

Levenberg, S., **Rouwkema, J.**, Macdonald, M., Garfein, E.S., Kohane, D.S., Darland, D.C., Marini, R., van Blitterswijk, C.A., Mulligan, R.C., D'Amore, P.A., and Langer, R. Engineering vascularized skeletal muscle tissue. *Nature biotechnology* **23**, 879, 2005.

Rouwkema, J., de Boer, J., and Van Blitterswijk, C.A. Endothelial cells assemble into a 3-dimensional prevascular network in a bone tissue engineering construct. *Tissue engineering* **12**, 2685, 2006.

Kruyt, M.C., de Bruijn, J.D., **Rouwkema, J.**, Oner, C., van Blitterswijk, C.A., Verbout, A.J., and Dhert, W.J.A. Effect of density and donor type of bone marrow stromal cells for bone tissue engineering in goats. *Tissue engineering*, Submitted.

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Rouwkema, J., and Van Blitterswijk, C.A. Vascularization in bone tissue engineering. In preparation.

Selected abstracts

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Rouwkema, J., de Boer, J., and van Blitterswijk, C.A. (2006, march) In vitro formation of a three dimensional prevascular network in a bone tissue engineering construct. Chicago (USA), 52nd Annual Meeting of the Orthopaedic Research Society (ORS).

Rouwkema, J., de Boer, J., and van Blitterswijk, C.A. (2006, september) Vascularized bone tissue engineering on a polymer scaffold. Nantes (France), 20th European Conference on Biomaterials (ESB).

Rouwkema, J., Rivron, N.C., de Boer, J., and van Blitterswijk, C.A. (2007, june) In vitro formation of a three dimensional prevascular network in a bone tissue engineering construct: the role of endothelial cells and mesenchymal stem cells. Toronto (Canada), TERMIS North America 2007 Conference (TERMIS NA).

Patents

Levenberg, S., **Rouwkema, J.**, and Langer, R., Engineering vascularized muscle tissue. WO2006084040

Publications not related to this thesis

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Verveen, A.A., and **Rouwkema, J.** The iridescent epidermis of Boa constrictor. *Litteratura Serpentium* **27**, In press, 2007.

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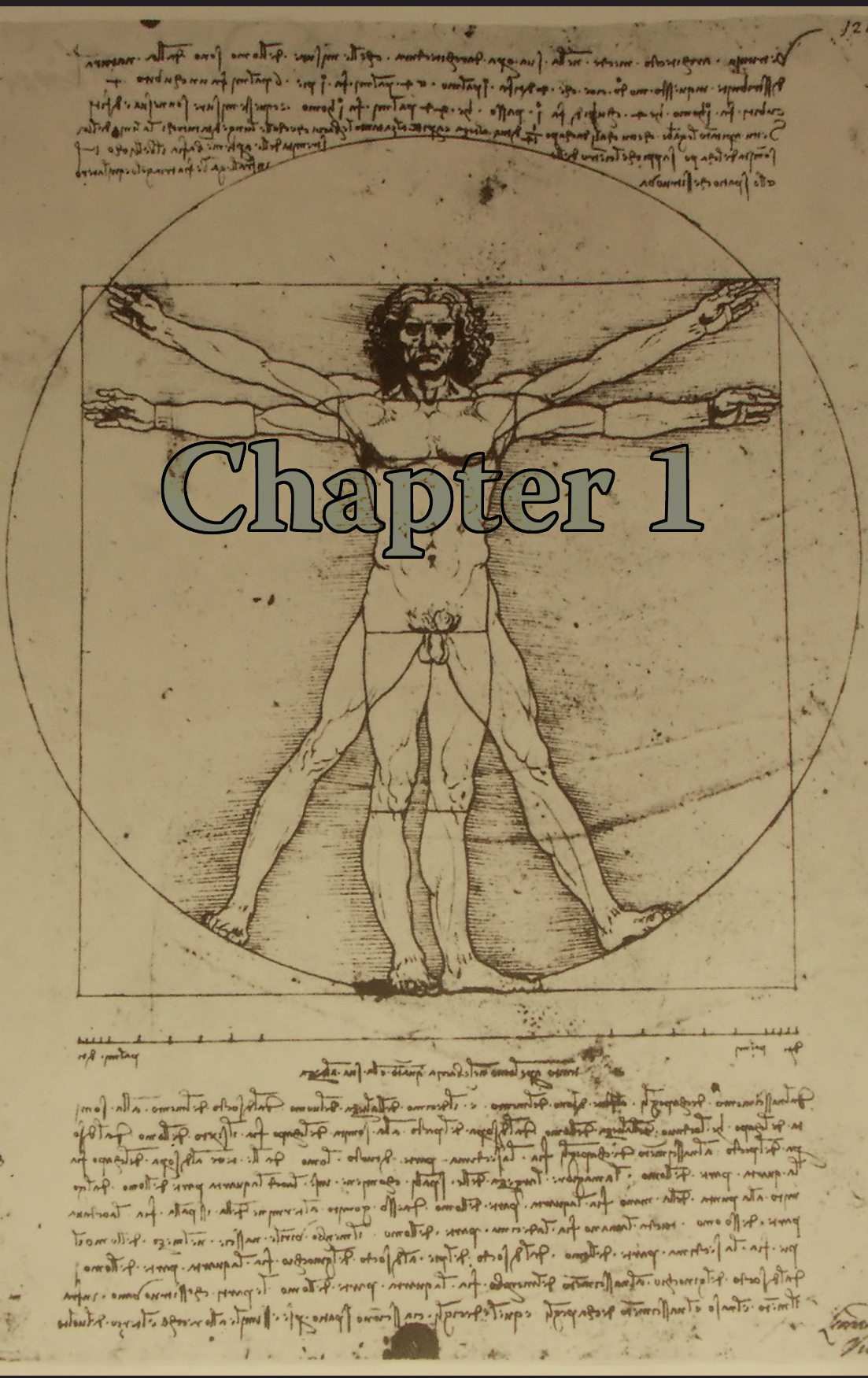

Chapter 1

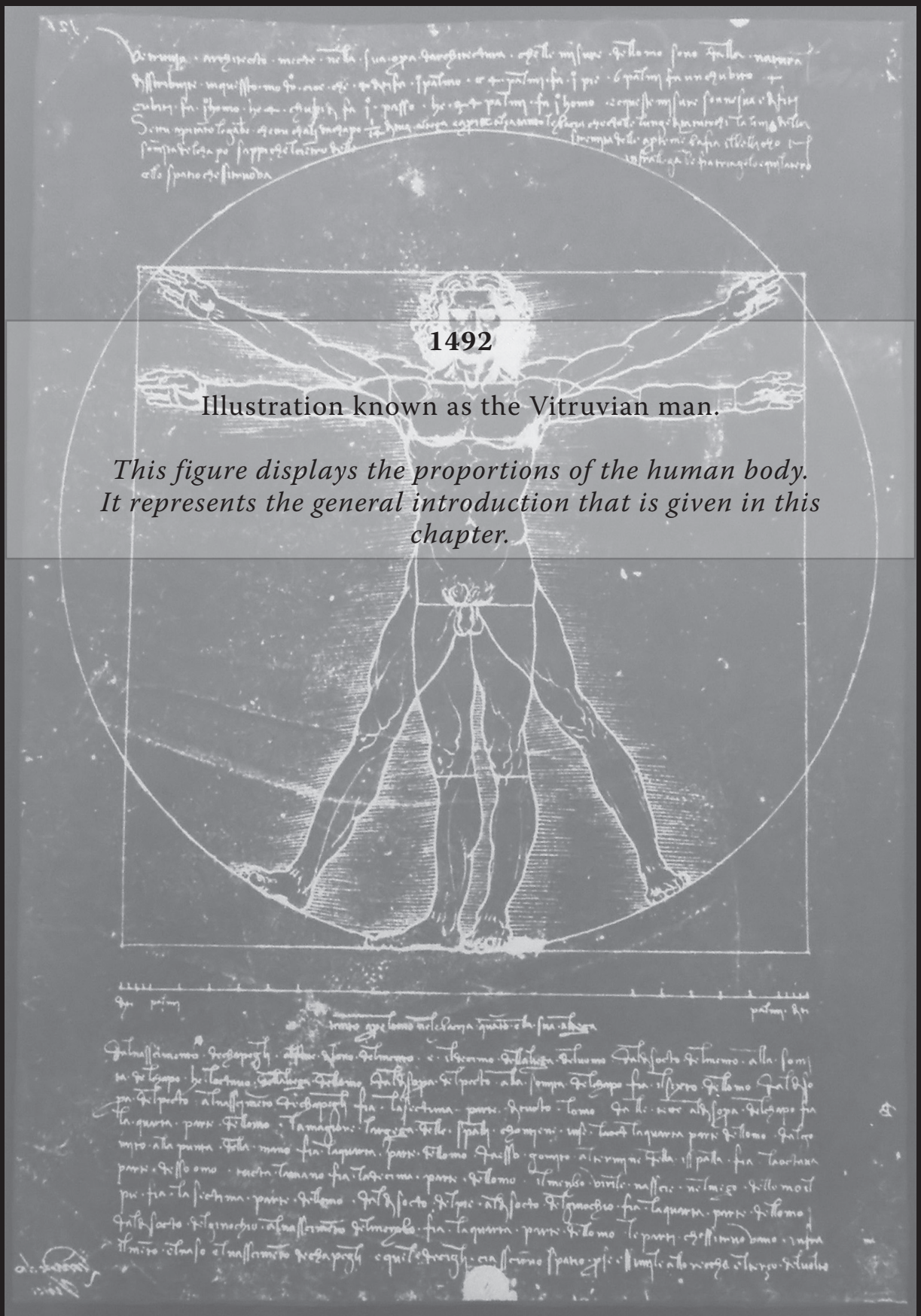

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Illustration known as the Vitruvian man.

This figure displays the proportions of the human body. It represents the general introduction that is given in this chapter.

Chapter 1

General introduction and aims

Bone

Although bones are most widely known as structures that form the internal support system of the body, the functions of this specialized tissue are more diverse. Bone also provides for the attachment of the muscles and tendons essential for locomotion, protects the vital organs of the cranial and thoracic cavities, and encloses the bloodforming elements of the bone marrow. In addition to these mechanical functions, bone plays an important metabolic role as a mobilizable storage of calcium and phosphate, which can be drawn upon when needed in the homeostatic regulation of calcium and phosphate in blood and other fluids of the body.[1] There are different classes of bones, based on their shape. These include long bones (for instance most bones in the limbs), short bones (for instance the bones of the wrist and ankle), flat bones (for instance the bones of the skull) and irregular bones (for instance the bones of the spine).

Morphologically there are two forms of bone tissue: cortical (compact) bone and cancellous (trabecular) bone. Cortical bone, which is rigid and dense, is found mainly in the outer regions of long bones or shells of other bones. Cancellous bone has a highly porous structure (> 75% porosity), which is comprised of a network of flat or needle-shaped trabeculae. This makes the overall organ lighter and allows room for blood vessels and bone marrow. Cancellous bone is mainly found in the inner cavity of long bones.

The microscopic structure of cortical bone consists of repeating units, called Haversian systems or osteons, that generally run parallel to the long axis of the bone. Each osteon has concentric layers of mineralized matrix called lamellae. These are deposited around a central canal, the Haversian canal, containing blood

vessels and nerves that service the bone. Blood vessels of neighboring osteons connect through anastomosing vessels in Volkmann's canals. Osteocytes are found between concentric lamellae and connect to each other and the central canal by cytoplasmic processes through canals called canaliculi. This network permits mechanosensing by the bone cells and the exchange of nutrients and metabolic waste.[2]

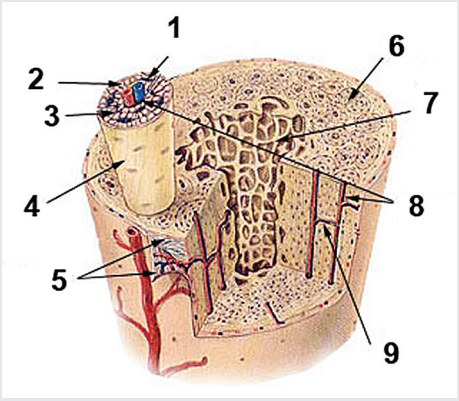

FIG. 1. *Schematic drawing of a long bone. Both cortical (compact) and cancellous (spongy) bone can be distinguished. The osteons of cortical bone are displayed (6), including the Haversian channels (8) that contain blood vessels and nerves. Apart from that, the periosteum (5), which is a highly vascularized membrane that covers the bone surface, can be seen. Other visible structures include the lacunae containing osteocytes (1), lamellae (2), canaliculi (3), osteons (4), trabeculae of spongy bone (7) and Haversian canals (9). Adapted from a figure by the U.S. National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) Program (http://training.seer.cancer.gov/index.html)*

Apart from blood vessels and nerves, bone contains four different cell types. Osteoblasts, osteoclasts and bone lining cells are present on the surface, whereas osteocytes permeate the mineralized interior. Osteoblasts are the fully differentiated cells responsible for the production of the bone matrix and regulation of its mineralization. Osteocytes are mature osteoblasts within the bone matrix and are responsible for the matrix maintenance. Bone lining cells are flat, elongated cells that cover inactive bone surfaces. Bone lining cells are thought to act as osteogenic precursors and to regulate the fluxes of ions between the bone fluid and interstitial fluid compartments for mineral homeostasis.[3] Osteoclasts are large, multinucleated cells, which are capable of resorbing bone mineral.

Bone repair

Bone has an amazing self-healing capacity. It is one of the very few tissues in the adult body that can heal itself without leaving scar tissue. One of the reasons

why bone heals well by itself is that it is constantly remodeling. This means that the machinery to repair or replace bone is constantly active and can thus respond quickly to an injury. Apart from this, bone heals well by itself due to its high level of vascularization. Nevertheless, there are certain occasions where the normal healing capacity of bone is not sufficient, for instance in large fracture gaps, non-unions or after tumor resection. Apart from this, it is sometimes necessary to grow bone at locations where it would normally not grow, for instance in the case of spinal fusion.

Several strategies for the repair of bone defects have been developed over time, each with their own advantages and drawbacks. For long, bone autografts have been considered as the gold standard for treating bone defects owing to the low risk of an adverse immune response.[4] An autograft treatment involves the harvesting of healthy bone from the patient and implanting this bone in the bone defect. Although autografts often yield good results, the drawbacks are that the availability of bone is limited and that a secondary surgery, creating a secondary defect, is necessary. Another strategy that is often used for bone repair is the implantation of allografts. In this case, bone from a human cadaver is harvested and processed to remove the cells and other constituents that may cause adverse effects. Although the amount of bone available for allografts greatly exceeds that of autografts, shortages are still present. Apart from that, the risk of disease transfer, especially of poorly understood diseases like prion based diseases, can not be ruled out completely.[5] Xenografts, typically of bovine or porcine origin, are also used to treat bone defects.

In cases where bone grafts from human or animal sources are not feasible (eg limited supply or insufficient bone volume available), synthetic graft materials (alloplasts) are used. In the past, alloplast materials were materials such as titanium alloys or alumina, which mainly provided mechanical support. However, limitations of these synthetic bone-replacement materials include poor integration with the surrounding tissue, a potential need for future retrieval or replacement, and an inability to adapt to the dynamic bone environment. Another approach has since evolved to employ materials (eg calcium phosphates, bioactive glasses, and biodegradable natural and synthetic polymers) that support natural bone-formation and remoddeling processes. Still, the success of these materials is not always predictable, possibly as a result of its passive approach to bone regeneration. To improve certain limitations of alloplast-based treatments, bone tissue engineering has emerged as a new therapeutic alternative to promote bone healing.

Bone tissue engineering

Tissue engineering has been described as an interdisciplinary field that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function.[6] A concept often used in tissue engineering is the construction of a matrix or a scaffold to provide cells a physical means for attachment. Cells are seeded on this scaffold to produce a tissue precursor *in vitro*. A satisfactory surface is needed to which the cells can attach and on which the cells deposit their extra-cellular matrix. A biodegradable material is often used, which means that after implantation, the cells and their extracellular matrix can replace the scaffold. *In vitro* tissue engineering is often directed to the creation of a tissue precursor, instead of a mature tissue. This means that tissue engineered constructs generally still rely on *in vivo* maturation after implantation. The advantage of tissue engineering is that it produces a living, bioactive implant that can adapt to the implantation environment and can actively participate in the formation of new bone. In this aspect, bone tissue engineering constructs have the potential to yield results similar to autografts. However, tissue engineered constructs are not associated with donor site morbidity and supply limitations, as is the case with autografts. Apart from growing tissues for clinical applications, *in vitro* tissue engineering is often used for the creation of model systems for developmental and medical research.

In the field of bone tissue engineering, biomaterials like ceramics are generally combined with osteogenic cells or osteoprogenitor cells. Numerous groups, including our own, have shown that the combination of artificial scaffolds and osteoprogenitor cells can lead to the formation of new bone in both ectopic and orthotopic sites.[7-14] The mechanism of bone formation in this setting is not yet fully understood. The new bone could be formed by the implanted cells, or by resident cells that are stimulated by the implanted construct. There is evidence that the implantation of osteoprogenitor cells only has an effect on bone formation if the cells are viable,[14] indicating that the implanted cells play an active role in the formation of new bone. However, whether this active role solely comprises the formation of bone by the implanted cells, or also involves the secretion of factors that stimulate bone formation by host cells, remains unknown. Although positive results have been achieved with osteoprogenitor cells in experimental settings, the effect of the use of these cells in clinical bone defects is still unpredictable.[15]

Cells

Several classes of cells for use in tissue engineering can be distinguished, based on their stage of differentiation. The first class consists of terminally differentiated primary cells. In the field of bone tissue engineering, osteoblasts have been used for this purpose.[16-19] Although these cells generally show superior performance regarding tissue-specific characteristics, their use for tissue engineering is often limited by laborious isolation protocols and limited proliferation capacities. Apart from that, *in vitro* de-differentiation due to prolonged proliferation of maturely differentiated cells has been reported.[20]

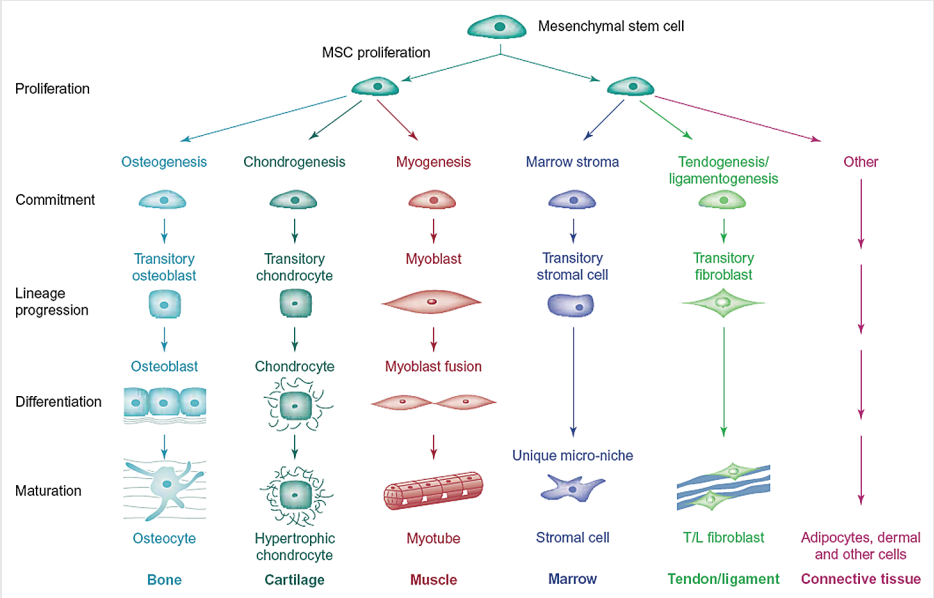

FIG. 2. *Mesenchymal stem cells, as displayed in a review paper published in 2001. This figure schematically depicts the stepwise cellular transitions from the putative mesenchymal stem cell to highly differentiated phenotypes. More recent literature has demonstrated that the differentiation capacities of mesenchymal stem cells is more diverse than the possibilities illustrated here and includes cells outside the mesenchymal lineage. Adapted from Caplan & Bruder.*[141]

Second, specific progenitor cells can be used. Although this may not directly be the case for bone tissue engineering, specific endothelial progenitor cells can be isolated from the blood and are nowadays being used for blood vessel tissue engineering applications.[21-23]

Third, multipotent 'adult' stem cells can be used. For bone tissue engineering, the use of mesenchymal stem cells is the preferred option at the moment. Although bone marrow is the most well known source of mesenchymal stem cells, the isolation from other adult tissues such as fat,[24] hair follicles and scalp subcutaneous tissue,[25] periodontal ligament,[26] thymus and spleen,[27] and peripheral blood[28] has also been reported. Mesenchymal stem cells can be easily isolated from the bone marrow by cell adhesion selection on tissue culture plastic, either with or without a Ficoll density gradient centrifuge step to select for mononuclear cells. Mesenchymal stem cells also display high proliferative capacities, which makes them an interesting candidate for tissue engineering applications. Mesenchymal stem cells do not express specific markers, but rather a complex pattern of molecules, including CD105, CD73, CD106, CD54, CD44, CD90, CD29 and STRO-1. Hemopoietic markers, such as CD45 and CD34, are normally not expressed.[29-33] Mesenchymal stem cells are pluripotent cells that can differentiate along several lineages *in vitro*. Although in early literature it was established that these cells could differentiate towards cells from the mesenchymal lineage like adipocytes, osteoblasts, chondrocytes and myoblasts,[32,34] later literature added neuronal,[35] hepatic,[36] cardiac,[37] endothelial,[38,39] and pancreatic[40] differentiation to the potential of these cells, hereby crossing the mesenchymal lineage boundaries. Differentiation towards osteoprogenitor cells can be achieved by stimulation with for instance BMP-2 or dexamethasone.[41-43]

The fourth class are pluripotent embryonic stem cells. Embryonic stem cells can be regarded as true stem cells due to a combination of two unique properties. First, they have the ability of self-renewal, which is the ability to replicate into identical daughter cells indefinitely. Second, embryonic stem cells, derived from blastocyst-stage early mammalian embryo's, in principal have the ability to form any fully differentiated cell of the body.[44] Human embryonic stem cells express stage-specific embryonic antigens 3 and 4 (SSEA-3 and SSEA-4), high molecular weight glycoproteins TRA-1-60 and TRA-1-81, and alkaline phosphatase.[45] Cell types that have been derived from embryonic stem cells include neural cells,[46-48] insulin secreting cells,[49] cardiomyocytes,[50-52] hematopoietic cells,[53,54] endothelial cells,[55] osteoblasts,[56] hepatocytes,[57] and chondrocytes.[58] Embryonic stem cells have also been used for bone tissue engineering applications.[59-62] Although embryonic stem cells are a valuable cell source to study bone development *in vitro*, there are several drawbacks that make them less fit as a cell source for clinical applications. First, stem cells by nature divide indefinitely, and methods to ensure that they do not retain tumorigenic potential will need to be developed.[63] Second, a treatment with human embryonic stem cells carries the risk of immune rejection, since embryonic stem cell lines are not established from patient own material. This

problem could in theory be solved by somatic cell nuclear transfer (also known as therapeutic cloning), in which the nucleus of the cell from a patient is inserted in an oocyte from which the nucleus has been removed. The resulting blastocyst can then be used for the isolation of 'patient own' embryonic stem cells. Although this strategy may prove useful in the future, it is subject to ethical debate and the only two research papers claiming that somatic cell nuclear transfer worked for human cells later turned out to be fraudulent and were retracted by the publisher.[64,65] The ethical debate regarding embryonic stem cells is not only limited to therapeutic cloning. The creation of human embryonic stem cell lines itself is also a subject of debate, since it generally involves the destruction of excess human embryos from *in vitro* fertilization procedures. This debate, however, may be surpassed by the creation of embryonic stem cell lines from other sources. Recent findings for instance indicate that similar stem cells can be isolated from amniotic fluid.[66]

Biomaterials and scaffolds

In the classical format, tissue engineering often consists of the combination of a biomaterial structure with living cells to obtain a hybrid construct with both mechanical and biological properties that are fit for the treatment of tissue disorders. A suitable surface is needed to which the cells can attach and on which they deposit their extra-cellular matrix (ECM). If the scaffold material is biodegradable, the newly formed tissue consisting of cells and ECM can take over the mechanical properties and replace the biomaterial over time. Although the total number of materials that have been developed and tested for bone tissue engineering is vast, a limited number of classes can be distinguished. These include metals, ceramics, biological and synthetic polymers, and hybrid materials that combine two or more of the previous classes.

Metals like titanium and titanium alloys are often chosen as scaffold material for their mechanical properties, especially for use in load bearing areas. Although these materials are strong enough to withstand the forces in load bearing areas, there is often a mismatch between the stiffness or Young's moduli of the biomaterials and the surrounding bone. Due to this mechanical mismatch, bone surrounding the implant is insufficiently loaded and becomes stress shielded, which can eventually lead to bone resorption and implant loosening.[67-69] However, improved fixation can be achieved by bone tissue growing into a porous matrix of metal. Depending on porosity, moduli can even be tailored to match the modulus of bone more closely, thus reducing the problems associated with stress shielding.[70]

As for biomaterial ceramics, several classes can be distinguished, of which bio-

glass ceramics and calcium-phosphate ceramics are most well known. A common characteristic of these ceramics is that they are bioactive.[71] After implantation, the surface forms a biologically active hydroxyl carbonate apatite (HCA) layer which is structurally and chemically not unlike the mineral phase in bone, and provides the bonding interface with tissue.[72] Apart from performing well in biocompatibility and bone ingrowth, certain classes of calcium-phosphate ceramics are also osteoinductive, meaning that the biomaterial itself can initiate bone formation in an ectopic site.[73,74] Even though these properties are favourable for the use of ceramics in bone tissue engineering applications, their limited mechanical strength currently limits their use to non-load bearing sites.

The range of polymers that have been used and studied for bone tissue engineering applications is vast. To be used for tissue engineering applications, a biomaterial as well as its degradation products, should be biocompatible, meaning that the host response to the material is appropriate for the specific application. Even within a polymer class, mechanical and degradational properties can be fine-tuned by altering the chemical composition or processing of the polymer. This freedom in design makes polymers an interesting candidate for tissue engineering scaffolds. Apart from that, the degradation properties of some classes of polymers makes them fit for the inclusion of bioactive agents like growth factors. By tailoring the degradation characteristics of the material, one can design the release rate and profile of the agents.[75] Although polymer systems offer interesting characteristics for bone tissue engineering, their low mechanical properties make them unfit for application in load bearing sites. Apart from that, the biological activity regarding osteoconduction and osteoinduction is generally less compared to ceramics.

Since the three classes discussed above all have advantages but also disadvantages for bone tissue engineering, hybrid materials have been developed to combine the beneficial properties of individual materials. For instance, calcium phosphate ceramics have been combined with synthetic polymers to decrease their stiffness, while retaining the osteoconductive properties.[76-78] Another example of hybrid materials is the addition of a calcium phosphate coating to metal implants. This enhances the osteo-integration of the metal, while retaining the favourable mechanical properties.[79]

Apart from scaffold composition, scaffold architecture is another important issue in bone tissue engineering. An interconnected porous structure is necessary for bone ingrowth and vascularization. This subject will be dealt with in more detail in chapter two of this thesis.

Blood vessels and vascularization

Blood vessels are part of the circulatory system. They transport blood, and thus nutrients and waste products, to and from almost any part of the body. Three distinct structures can be distinguished in the vascular system. These are the macrovessels (arteries and veins), that branch into microvessels (arterioles and venules) and finally into capillaries. The capillaries facilitate the actual distribution of nutrients to the tissues in the body. They distribute the blood over the tissue while lowering the pressure head, allowing blood to diffuse into the tissue.

Three distinct processes can be distinguished during blood vessel formation; vasculogenesis, angiogenesis and arteriogenesis.[80] Vasculogenesis is the *de novo* vessel-forming process that takes place during early embryonic development. Endothelial cells differentiate from their precursors, the angioblasts, and proliferate

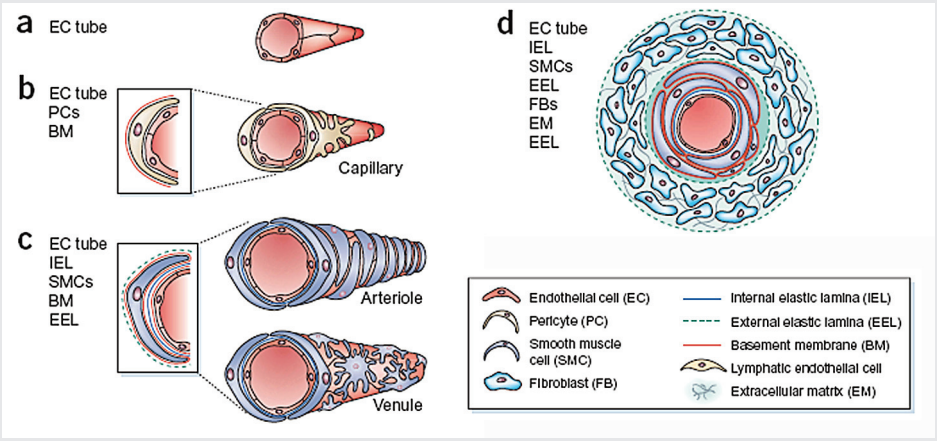

FIG. 3. *Blood vessel compositions.* ***(A)*** *Nascent vessels consist of a tube of endothelial cells (EC). These mature into the specialized structures of capillaries, arteries and veins.* ***(B)*** *Capillaries, the most abundant vessels in our body, consist of EC surrounded by a basement membrane and a sparse layer of pericytes embedded within the EC basement membrane.* ***(C)*** *Arterioles and venules have an increased coverage of mural cells compared with capillaries. Precapillary arterioles are completely invested with vascular smooth muscle cells (SMC) that form their own basement membrane and are circumferentially arranged, closely packed and tightly associated with the endothelium.* ***(D)*** *The walls of larger vessels consists of three specialized layers; an intima composed of EC, a media of SMC and an adventitia of fibroblasts, together with matrix and elastic laminae. The advential layer has its own blood supply, known as vasa vasorum, that extends in part into the media. SMC and elastic laminae contribute to the vessel tone and mediate the control of vessel diameter and blood flow. Additional control of blood flow is provided by arterio-venous shunts that can divert blood away from a capillary bed when necessary. Adapted from Jain.*[99]

within a previously avascular tissue to form a primitive tubular capillary network.[81] Vasculogenesis is followed by angiogenesis, during which this initial vascular network is remodeled into more complex networks through vessel enlargement, sprouting, and bridging.[82] Upon angiogenic stimulation, vascular endothelial cells are activated and begin to degrade their surrounding basement membrane by the expression and release of matrix metalloproteinases (MMPs). Then the endothelial cells migrate into the interstitium, resulting in the formation of capillary buds and sprouts. Endothelial cells behind the migrating endothelium of the sprouts proliferate so that the newly developing blood vessel elongates.[83] Arteriogenesis is the process of structural enlargement and remodeling of preexisting small arterioles into larger collateral vessels.[84] It was long thought that new vessel formation in adults was limited to angiogenesis and arteriogenesis. However, more recent data suggests that the basis for native as well as therapeutic neovascularization is not restricted to angiogenesis but includes postnatal vasculogenesis as well. It has been established that bone marrow derived endothelial progenitor cells are present in the systemic circulation, are augmented in response to certain cytokines and/or tissue ischemia, and home to as well as incorporate into sites of neovascularization.[85-90]

All three classes of blood vessels have an inner lining of endothelial cells, which provides a surface that prevents blood cell attachment and thrombus formation. In capillaries, this endothelial cell layer is surrounded by pericytes that share a common basal lamina with the endothelial cells.[91] The basal lamina consists of collagenous glycoproteins, with type IV collagen being the main component and type V collagen featured to a lesser extent. It also contains non-collagenous glycoproteins such as laminin, fibronectin, heparin sulfate, proteoglycan, entactin, fibronectin, and other glycosaminoglycans.[92] The basal lamina serves as a separation layer between the connective tissue and the endothelium. The role of pericytes is not yet fully understood. Pericytes are, however, functionally significant. When vessels lose pericytes, they become hemorrhagic and hyperdilated, which leads to conditions such as edema, diabetic retinopathy, and even embryonic lethality.[93] Apart from that, pericytes can initiate vasoconstriction and vasodilation within capillary beds to regulate vascular diameter and capillary blood flow.[94] Interestingly, it has also been suggested that pericytes can act as osteoblastic precursors.[95-97] In addition to pericytes, micro- and macrovascular vessels are surrounded by smooth muscle cells and fibroblasts. These cells are responsible for expansion and contraction of the vessel and also serve to stabilize the vessel structure.[98,99]

Vessel maturation, which includes the recruitment of mural cells like pericytes and smooth muscle cells, is an important process in blood vessel formation. Although initially independent of the circulation, the vascular system is later shaped

by forces generated by the circulation.[80] An increased shear stress due to perfusion for instance, results in a strong endothelial cell response, including an upregulated secretion of growth factors such as PDGF-B.[100,101] The secreted PDGF-B in turn acts as a chemo-attractant for mural precursors,[102-104] derived from the mesenchyme surrounding the endothelial tubes.[105,106] Upon contact with endothelial cells, newly recruited mesenchymal cell progenitors are induced towards a mural cell fate, in a process mediated by the activation of TGF-β.[103,107,108] Differentiated pericytes and smooth muscle cells stabilize vessel structures and suppress endothelial cell growth.[102] Vessel growth that is not accompanied by vessel maturation results in disorganized, leaky and hemorrhagic blood vessels,[109] that will generally regress over time in a process called vascular remodeling.[99]

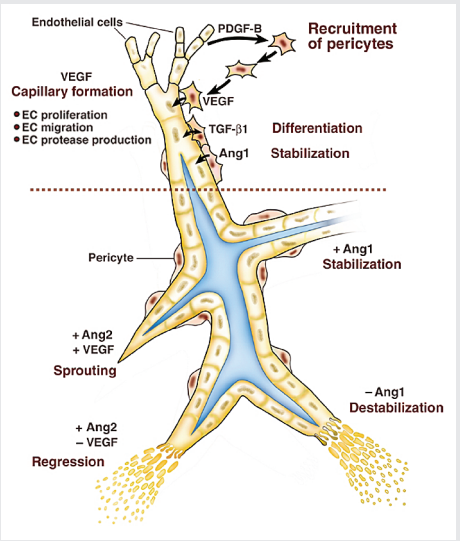

FIG. 4. *Regulation of blood vessel assembly and organization. The upper panel summarizes blood vessel assembly. The formation of a primary capillary network is initiated by VEGF-induced proliferation and migration of endothelial cells (EC). Endothelial secretion of PDGF-B attracts mesenchymal cells, which contact the EC. Contact between the ECs and mesenchymal cells activates TGF-β1, which suppresses endothelial proliferation and migration, induces mural cell differentiation, and is associated with vessel maturation. The lower panel illustrates some processes involved in blood vessel organization. Addition of Ang1 results in stabilization of vessels in the absence of pericytes, whereas addition of Ang2 induces vessel regression in the absence of VEGF (–) and sprouting in the presence of VEGF (+). Adapted from Ramsauer & D'Amore.*[142]

Endothelial cells in established vessels are normally quiescent.[110] In an adult, they have a cell cycle variable from months to years.[111] However, following an injury, cells change their phenotype, migrate and proliferate to heal the lesion in a few days. Endothelial cells can not be regarded as a single cell population. Microarray studies have revealed that there are marked differences in the expression profile of endothelial cells from veins, arteries and microvessels, but also of endothelial cells isolated from different tissues.[112] Apart from that, endothelial cells from different sources differ in their morphological functional aspects and responsiveness to

growth factors.[113,114] All these issues have to be taken into account when using endothelial cells for tissue engineering applications, since *in vitro* culture may result in the loss of specific functions or the introduction of new metabolic characteristics. For instance, the growth rate of endothelial cells *in vitro* largely exceeds the *in vivo* one. Apart from that, it may be important to isolate endothelial cells from the specific tissue type one is trying to repair.

Need for vascularization in tissue engineering

Bone is a highly vascularized tissue that, like most other tissues, relies on the blood vessels to supply the individual cells with nutrients and oxygen. For tissue to grow beyond 100-200 μm (the diffusion limit of oxygen), new blood vessel formation is required.[115,116] The same can be said about tissue engineered constructs. During *in vitro* culture, large volumes of tissue engineered constructs can be supplied with nutrients with for instance perfusion bioreactors.[117,118] However, after implantation of tissue constructs, the supply of oxygen and nutrients to the implant is limited by diffusion processes and the speed of ingrowth of host vessels. In active tissue, sufficient diffusion is confined to 100-200 μm from the next capillary, and the formation of host vessels within the construct takes time.[119] This means that insufficient vascularization can lead to nutrient deficiencies and/or hypoxia in the tissue. Moreover, nutrient and oxygen gradients will be present in the outer regions of the tissue, which could result in non-uniform cell differentiation and integration.[120]

Since vascularization after implantation is a major problem in tissue engineering, the successful use of tissue engineered constructs is as yet limited to thin or avascular tissues like skin and cartilage, where post-implantation neovascularization from the host is sufficient to meet the demand for oxygen and nutrients.[109,121,122] To make the application of tissue engineering for bigger, more active tissues like bone and muscle successful, the problem of vascularization has to be solved.

To date, most approaches in tissue engineering have relied on vascularization by the ingrowth of blood vessels from the host. Several strategies to enhance vascularization have been studied. These strategies include improvement of scaffold architecture to improve blood vessel ingrowth, the inclusion of angiogenic factors, *in vitro* prevascularization and *in vivo* prevascularization or the placement of a vascular pedicle. These strategies, and their implications for bone tissue engineering, will be described in more detail in chapter two of this thesis.

Aims of this thesis and outline

The overall goal of this thesis is to investigate the possible role of *in vitro* prevascularization for bone tissue engineering. This overall goal can be divided into two parts. The first sub-goal is to investigate the potential positive effect of *in vitro* prevascularization in tissue engineering. The second sub-goal is to combine *in vitro* prevascularization and bone tissue engineering.

Bone healing is generally associated with blood vessel formation and ingrowth.[123-129] Studies have shown that fracture healing and ectopic new bone formation can be blocked by the administration of angiogenesis inhibitors,[130,131] while other studies have shown that new bone formation in porous scaffolds was significantly increased by the insertion of a vascular pedicle in the scaffold.[132,133] Apart from that, vascularization is important in tissue engineering for the survival of constructs after implantation. The aim of chapter two is to give an overview of the link between vascularization and bone tissue engineering.

Several strategies have been developed to enhance vascularization in tissue engineering, like optimization of scaffold architecture and angiogenic factor delivery. However, most of these strategies still rely on the ingrowth of host vessels into the construct and therefore it will still take a considerable amount of time before the construct is properly vascularized. The aim of chapter three is to develop a coculture system of endothelial cells, myofibroblasts and smooth muscle cell precursors as a model for *in vitro* prevascularization and to establish a proof of principle for the expected beneficial *in vivo* effects of *in vitro* prevascularization.

The coculture of endothelial cells and mesenchymal stem cells has been described before.[134-137] However, these studies focused mainly on the cellular interactions between the different cells and its effect on the differentiational stage of these cells. The aim of chapter four is to develop a three dimensional coculture system of human mesenchymal stem cells (hMSC) and human umbilical vein endothelial cells (HUVEC) with a focus on the morphogenesis of the endothelial cells, and to give an initial illustration of *in vitro* prevascularization in a bone tissue engineering setting.

Studies have shown that the addition of endothelial cells to tissue constructs can lead to *in vitro* prevascularization.[138-140] However, these studies were in general performed with non clinically relevant endothelial cell sources like HUVEC. If one wants to implement *in vitro* prevascularization in clinical applications, one needs to use an endothelial cell source that can be readily isolated from adult patients in sufficient numbers in an acceptable timeframe. The aim of chapter five is to differentiate hMSC towards endothelial cells and to test the use of these cells and other endothelial (progenitor) cells for prevascularized bone tissue engineering.

The difference of the *in vivo* results as described in chapter three and four points out that different tissue engineering settings will yield different results regarding the success of prevascularization. Prevascularization of the skeletal muscle construct described in chapter three results in massive perfusion of the prevascular structures after implantation. Prevascularization of the bone construct described in chapter four, however, does not. This difference may arise from the multipotency of the mesenchymal stem cells used in the latter system. As was shown in chapter five, mesenchymal stem cells have the potency to differentiate towards endothelial cells. This behavior may interfere with the formation of prevascular structures in this system. The aim of chapter six is to investigate the role of both HUVEC and mesenchymal stem cells in the formation of prevascular structures in a 3D coculture setting.

In chapter seven, general conclusions arising from this thesis are discussed. Chapter seven also outlines some future perspectives.

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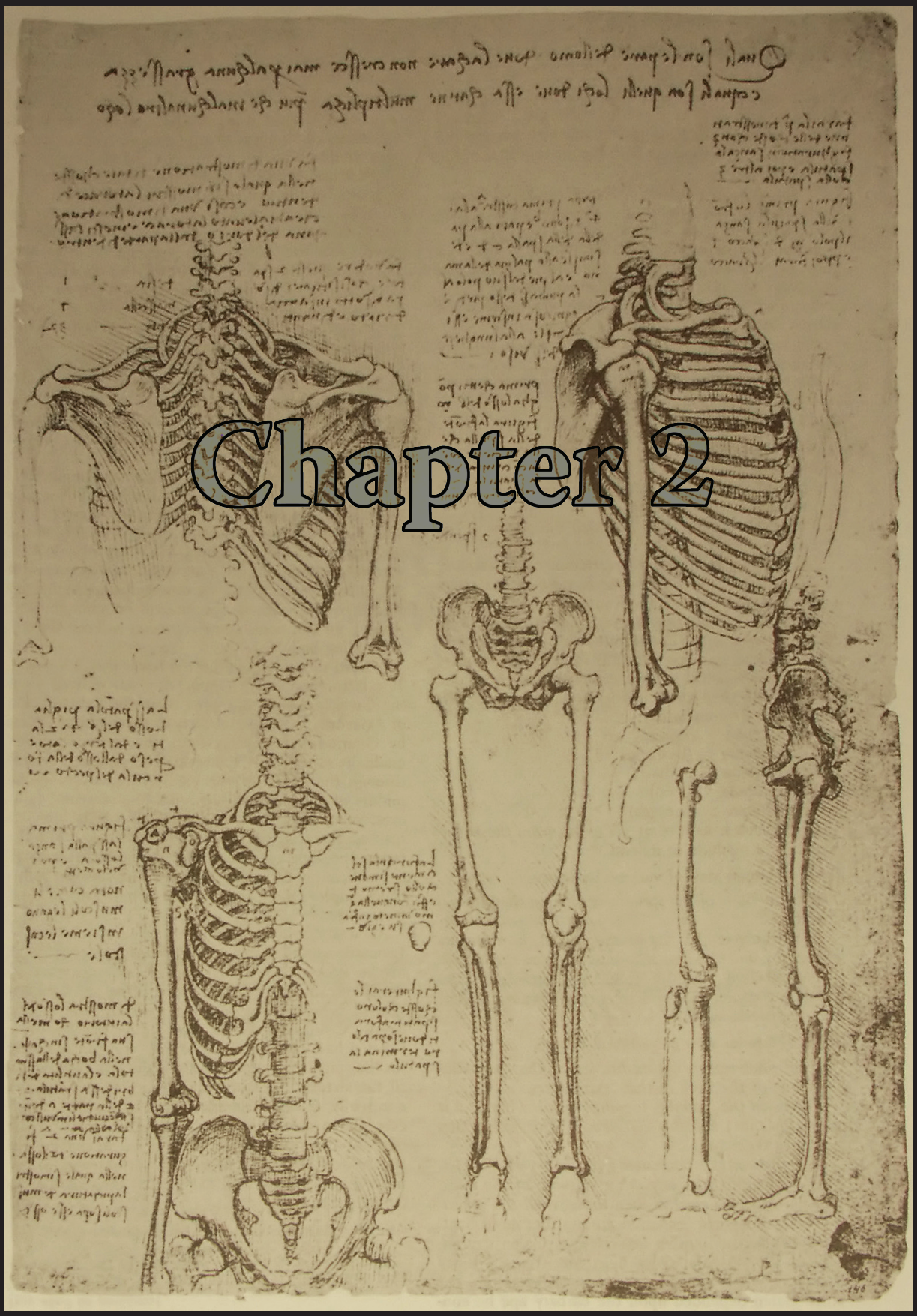
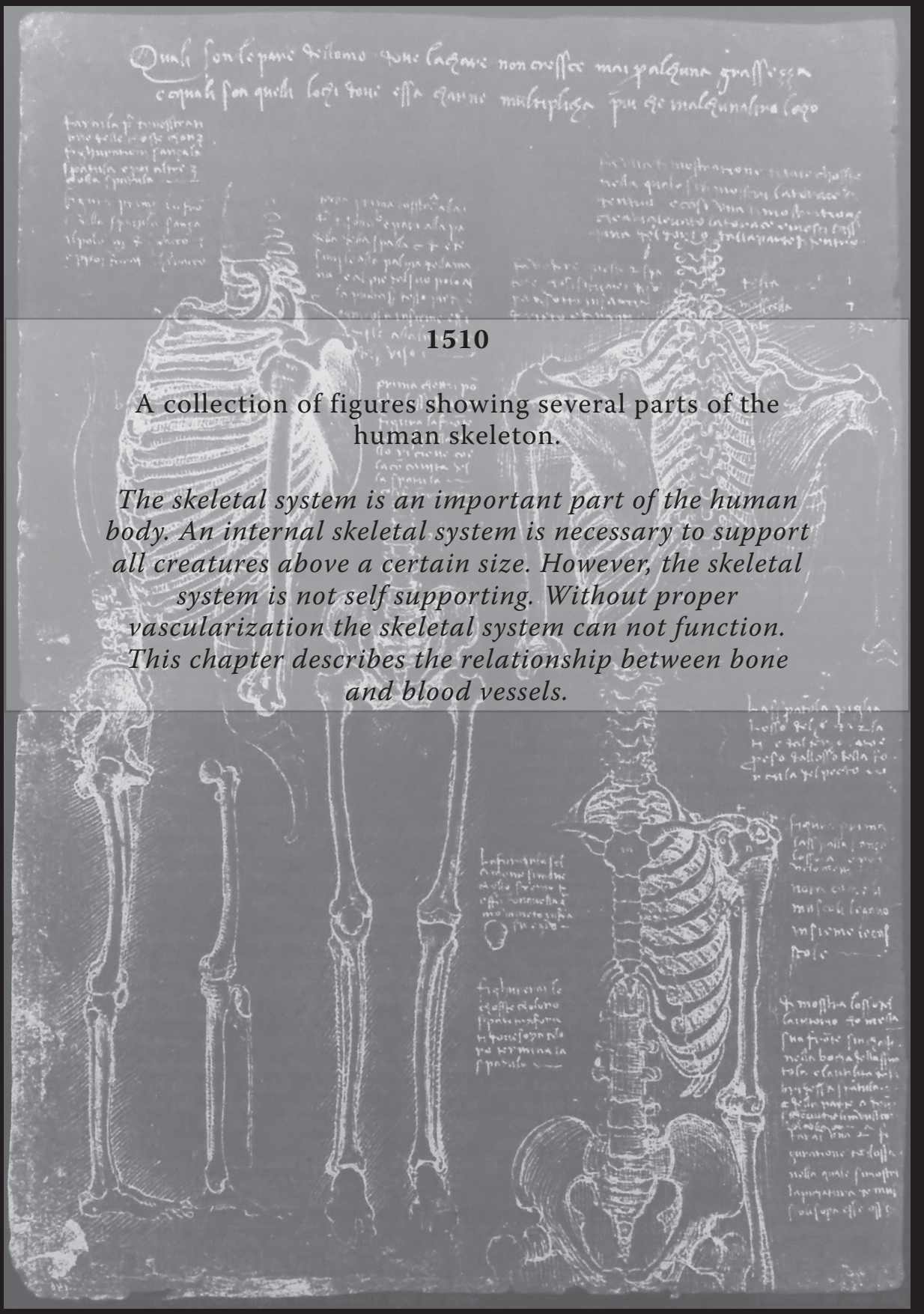
Chapter 2





1510

A collection of figures showing several parts of the human skeleton.

The skeletal system is an important part of the human body. An internal skeletal system is necessary to support all creatures above a certain size. However, the skeletal system is not self supporting. Without proper vascularization the skeletal system can not function. This chapter describes the relationship between bone and blood vessels.

Chapter 2

Vascularization in bone tissue engineering

Abstract

Vascularization is an important process in natural bone development, growth and repair. And even though the exact *in vivo* mechanisms involved in bone tissue engineering are not yet known, the academic field recognizes the importance of vascularization in tissue engineered bone constructs.[1,2] Vascularization of a construct is necessary to keep the cells alive after implantation. If vascularization is inhibited or insufficient, nutrient limitations are likely to occur. This can result in improper function or even death of the implanted cells. Apart from that, vascularization is likely to be important for the delivery of bone forming cells and/or factors to the defect site, and for the differentiation of osteoprogenitor cells.

This chapter will focus on the importance of vascularization in the field of bone tissue engineering. It will discus strategies to enhance vascularization of tissue engineered constructs after implantation. It will furthermore focus on the effect of endothelial cells on the differentiation of osteoprogenitor cells.

Vascularization in bone development and growth

In 1763 Albrecht von Haller wrote: 'the origin of bone is in the artery carrying the blood and in it the mineral elements', indicating the importance of blood vessels in bone formation.[3] Although this claim, at that time, was not yet supported by scientific evidence, future research proved that Von Haller was on the right track. Longitudinal bone growth during vertebrate development takes place through the mechanism of endochondral bone formation. During endochondral bone formation, cartilage, which is an avascular tissue, is replaced by bone in a process called endochondral ossification. In this process, blood vessels play a crucial role.

The centre of longitudinal growth in long bones is the growth plate. Even though the growth plate consists of only one cell type, the chondrocytes, three principal layers can be distinguished. These are the resting zone, proliferative zone, and hypertrophic zone.[4] The chondrocytes in the resting zone replicate at a slow rate. They mainly act as stem-like cells that replenish the pool of proliferative chondrocytes.[5] The chondrocytes in the proliferative zone replicate at a high rate. The daughter cells line up in columns parallel to the long axis of the bone.[5] At a certain point, the cells stop dividing and terminally differentiate into hypertrophic chondrocytes.[6] These cells express higher levels of angiogenic factors, mainly VEGF, that trigger the invasion of blood vessels.[7,8] It is thought that diffusible factors arising from these vessels in turn induce apoptosis and resorption of the hypertrophic chondrocytes.[9] Angiogenesis is a critical process during endochondral ossification, since it permits the degradation of hypertrophic cartilage and calcification of the matrix. This is illustrated by the fact that inhibition of blood vessel invasion results in thickening of the growth plate and impaired bone formation.[10-14]

Vascularization in bone repair

Apart from bone growth, vascularization is also an important factor in bone repair. Studies have shown that fracture healing and ectopic new bone formation can be blocked by the administration of angiogenesis inhibitors,[15,16] while other studies have shown that new bone formation in porous scaffolds was significantly increased by the insertion of a vascular pedicle in the scaffold.[17,18]

The natural repair of fractures occurs in four overlapping phases.[19-21] Damage to a bone generally results in the disruption of blood vessels. The bleeding caused by this disruption activates the coagulation cascade, resulting in a hematoma which encloses the fracture site. This hematoma formation is an important stage

for bone repair. This is illustrated by the fact that removal of the hematoma significantly impairs bone repair, and transplantation of the hematoma results in new bone at the transplantation site.[22,23] The importance of the hematoma is likely to be through it's angiogenic activity.[24,25] During the hematoma stage, inflammatory cells, fibroblasts and stem cells are recruited to the site, and new blood vessels are formed through angiogenesis.[19] During the next stage, granulation tissue forms at the ends of the bones. This tissue is gradually replaced by fibrocartilage to form an internal callus. The formation of this internal callus seems to be related to the vascular pattern at the fracture site.[26] Meanwhile, an external callus is formed by intramembranous ossification of the periosteum. In the next stage, the internal callus becomes mineralized to form a hard callus of woven bone via endochondral ossification. The role of the vasculature in this is thought to be similar to the role during skeletal development and growth.[19,27] Finally, during the remodeling phase of bone regeneration, the fracture callus is replaced by lamellar bone. The size of the callus is reduced to that of the original bone at the fracture site, and the vascular supply reverts to its normal state.[19] Bone repair can also take place via a different mechanism, if the fixation of the fracture is rigid and there is no movement between the different bone fragments. In that case, the fracture heals through direct bone formation, without the classical multistage differentiation of connective tissue and cartilage.[28]

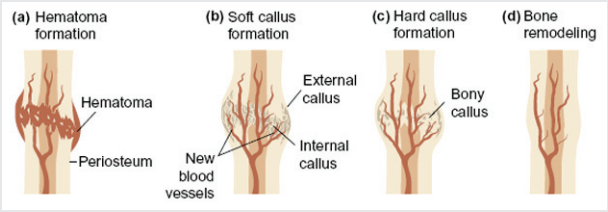


FIG. 1. *The different stages of natural bone repair.* ***(A)*** *Hematoma formation: bone injury generally results in the disruption of blood vessels. This leads to the formation of a hematoma.* ***(B)*** *Soft callus formation: new blood vessels are formed from pre-existing ones via angiogenesis. During this stage, an external callus (intramembranous ossification) and an internal callus (fibrocartilage) are formed.* ***(C)*** *Hard callus formation: during this stage, the callus becomes mineralized, forming a hard callus of woven bone.* ***(D)*** *Bone remodeling: during this stage, the large fracture callus is replaced with lamellar bone, and the vascular supply returns to normal. Adapted from Carano & Filvaroff.*[19]

One of the key factors involved in bone repair is the angiogenic growth factor VEGF. It has been shown that the inhibition of VEGF activity disrupts the repair of femoral fractures and cortical bone defects in mice.[29] VEGF is not only involved in the angiogenic response during bone repair, but also helps to control callus architecture, mineralization and remodeling. To achieve this, VEGF can regulate the recruitment, survival and activity of endothelial cells,[30] but also osteoblasts[14,29,31-33] and osteoclasts.[34-36]

Vascularization in bone tissue engineering

When mesenchymal stem cells are used for bone tissue engineering, new bone is thought to be formed *in vivo* through a process of direct bone formation (intramembranous ossification), and not through endochondral ossification.[37] Therefore, the role of vascularization may be different from the role during natural bone growth and repair. However, recent experiments in our lab suggest that using a different cell source may result in a different mechanism of bone formation. When human embryonic stem cells were differentiated into the osteogenic lineage *in vitro* and then implanted, no *in vivo* bone formation could be detected. However, if the same cells were differentiated into the chondrogenic lineage *in vitro*, bone formation was evident *in vivo*. Further studies indicated that this bone was formed through endochondral ossification (unpublished results). This means that understanding the role of vascularization during endochondral ossification may be critical when using less conventional cell sources for bone tissue engineering.

Even though a direct bone formation process is generally seen in bone tissue engineering constructs, vascularization is still important for the formation of new bone in these settings. It has been shown that cells in bone tissue engineering constructs must be viable after implantation to have a positive effect on bone formation.[38] Therefore, apart from delivering factors that are required for new bone formation, vascularization is important for the survival of the implanted cells. When *in vitro* engineered cellular constructs are implanted, they have to rely on diffusion and vascularization for their metabolic demands. Diffusion is the initial process through which implanted cells receive their nutrients and oxygen. However, diffusion can only provide for cell support within a maximum range of 200 μm into the construct.[39,40] Since initial vascularization is often suboptimal, the survival of cells in the center of large cell-containing constructs is often limited.[41] Indeed, cell labeling experiments performed by Kneser *et al* showed that there is a considerable loss of osteoblasts within the first week following transplantation in porous bone matrices.[42] It is therefore essential, especially for large bone defects, to stimulate vascularization of the graft after implantation.

Strategies to enhance vascularization in tissue engineering

When implanting tissue engineered constructs, spontaneous vascularization of the implant over time is generally seen. This is in part due to an inflammatory wound-healing response, induced by the surgical procedure. Apart from that, the seeded cells often create a hypoxic state in the implant, which stimulates the en-

dogenous release of angiogenic growth factors.[43] However, vessel ingrowth is often too slow or too limited to provide adequate nutrient transport to the transplanted cells. Therefore, additional strategies to enhance vascularization are essential for the survival of large tissue engineered grafts.

Several strategies to enhance vascularization have been studied. These include scaffold design, the inclusion of angiogenic factors, *in vivo* prevascularization and *in vitro* prevascularization. Although all these strategies can in principle enhance vascularization after implantation, their implications for bone tissue engineering are different. Furthermore, the degree to which these strategies can enhance vascularization varies. Both scaffold design and angiogenic factor delivery rely on the ingrowth of host vessels into the entire construct. So even though these strategies can increase the rate of vascularization, it will still take a considerable amount of time before the middle of the implant is perfused. *In vivo* prevascularization can in principle result in the instantaneous perfusion of a construct after implantation at the final site, since the construct is microsurgically anastomosed to the host vasculature. However, before implantation at the final site, a pre-implantation period is necessary. During this stage, the implant has to rely on spontaneous angiogenesis from the vascular axis into the construct. Therefore, nutrient limitations are likely to occur during this stage. *In vitro* prevascularization does not result in the instantaneous perfusion of a construct. Vessels from the host have to grow into the construct until they reach the vascular network that was formed *in vitro*. If anastomosis is then successful, the entire construct can become perfused with blood. In principle this can decrease the time needed for vascularization of the implant dramatically, since host vessels do not have to grow into the entire construct, but only into the outer regions.

Scaffold design

As for scaffold design, the architecture of a scaffold will have a profound effect on the rate of vascularization after implantation. It has been reported that the 3D environment of a scaffold, as opposed to 2D culture, can change the angiogenic activity of incorporated cells.[44] Apart from this, the pore size of the scaffolds is a critical determinant of blood vessel ingrowth. Druecke *et al* showed that vessel ingrowth was significantly faster in scaffolds with pores greater than 250 μm than in those with pores smaller than 250 μm.[45] Not only pore size is important for vascularization, but also pore interconnectivity. Cell migration and thus vascularization will be inhibited if the pores are not interconnected, even if the matrix porosity is high.[46,47]

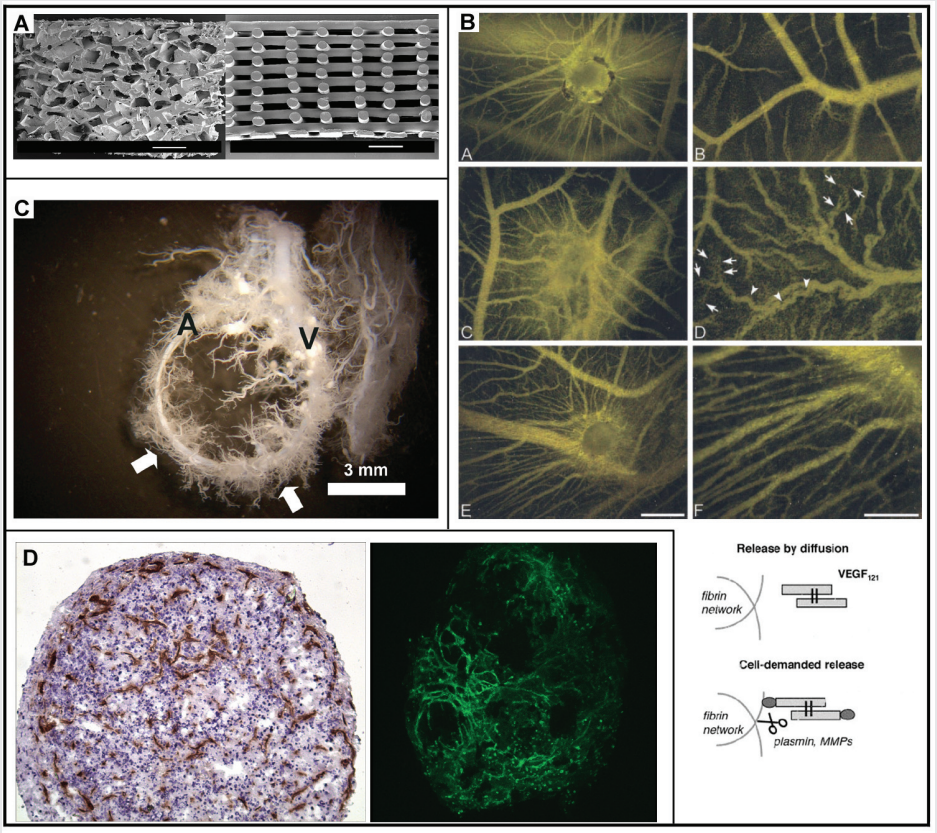


FIG. 2. *Different strategies for the improvement of vascularization in tissue engineering.* ***(A)*** *Scaffold design: the left panel shows a scaffold prepared with compression moulding and salt leaching, the right a scaffold prepared with 3D fiber deposition. Note the more regular, open geometry of the right scaffold. Adapted from Malda et al.*[108] ***(B)*** *Growth factor delivery: fibrin gel matrices were put on a chicken chorioallantoic membrane (a membrane of the chicken egg). The top pictures show the results of an empty gel, the middle of a gel loaded with freely diffusible* $VEGF_{121}$ *and the lower of a gel loaded with* $VEGF_{121}$ *that is released enzymatically by MMPs (cell-demanded release). Note the more regular organization upon cell-demanded release. Adapted from Ehrbar et al.*[72] ***(C)*** *In vivo prevascularization: a loop was prepared by joining an artery (A) and a vein (V). This arteriovenous loop was placed around a bone tissue engineering scaffold and implanted. After 8 weeks of implantation this resulted in a highly vascularized construct as displayed here. Adapted from Kneser et al.*[109] ***(D)*** *In vitro prevascularization: human mesenchymal stem cells (hMSC) were combined with human umbilical vein endothelial cells (HUVEC) in a 3D spheroid coculture system. This resulted in the formation of a three dimensional prevascular network. The left panel shows a cross section of the spheroid stained for the endothelial marker CD31 (brown) with a counterstain of hematoxylin (blue). The right panel shows a 3D image of the entire spheroid stained for CD31 (green). Adapted from Rouwkema et al.*[83]

Conventional scaffold fabrication techniques include amongst others gas foaming, phase separation, freeze drying and particulate leaching. With gas foaming, the material is saturated with a gas at high pressure to achieve high solubility of the gas in the material. The pressure is subsequently lowered, which decreases the solubility of the gas. This results in the formation of gas bubbles of variable size.[48] The phase separation technique is based on thermodynamic demixing of a homogeneous polymer-solvent solution into a polymer-rich phase and a polymer-poor phase. This demixing can be achieved by quickly cooling the polymer solution. Cooling below the freezing point of the solvent results in the formation of a two-phase solid. After sublimation of the solvent, a porous scaffold is formed.[49] As a method to produce a porous scaffold, freeze drying is comparable to phase separation. The difference is that the polymer solution is directly freeze-dried to yield a porous structure.[50] Last, particulate leaching comprises the incorporation of particles into a material solution, where the solvent used is a non-solvent for the particles. After removal of the solvent, the particles can be leached out with a solvent that is a non-solvent for the base material, producing a porous scaffold.[51,52]

The abovementioned fabrication techniques have been widely used to produce 3D scaffolds for tissue engineering applications. Although the shape and the size of the pores can be varied by changing the parameters of these techniques, the organization of the pores is generally random. This results in pore pathways that are not completely interconnected and tortuous, which could impede with nutrient supply and tissue and vessel ingrowth into the scaffold. To better control and design the porosity and interconnectivity of scaffolds, solid free-form fabrication systems are nowadays in the centre of attention.[53,54] These versatile systems are capable of producing complex scaffolds with a well defined architecture and optimal pore interconnectivity. Apart from that, multiple distinct regions can easily be created within a single scaffold.

An example of a solid free-form fabrication system for the production of tissue engineering scaffolds is the rapid prototyping or fiber deposition technology. With this technique, molten polymers, hydrogels or biomaterial pastes are extruded in the form of a fiber. Based on a CAD pattern, the fibers are deposited to form a layer of the scaffold. A complete 3D scaffold can be prepared with a layer-by-layer strategy. This technique can be used to prepare scaffolds consisting of many different materials, including polymers,[55] metals,[56] ceramics,[57] and even gels with encapsulated cells.[58]

Apart from solid free-form fabrication systems to create regular scaffolds that favour tissue and vessel ingrowth, other strategies to enhance vascularization due to scaffold design have been explored. Gafni *et al* for instance designed a system

where a highly degradable biomaterial was used to create a filamentous scaffold. This scaffold was seeded with endothelial cells *in vitro*, resulting in a monolayer of endothelial cells on the filaments. After implantation, the filaments degraded but tubular structures of endothelial cells remained. This resulted in perfused implanted vessels after 2 weeks of implantation.[59]

Angiogenic factor delivery

It is well known from literature that the addition of angiogenic factors to tissue engineered constructs can enhance vascularization after implantation.[60-63] Different stages of blood vessel formation can be stimulated to increase the vascularization of a tissue engineered graft. First, new vessel formation can be stimulated by factors that stimulate the mobilization and recruitment of endothelial (progenitor) cells and thus stimulate the onset of angiogenesis. These include growth factors like VEGF and bFGF. Although the single delivery of these factors generally results in increased angiogenesis, the resulting vessels are often disorganized, leaky and hemorrhagic. Moreover, dosage must be tightly controlled, as excess amounts of VEGF can cause severe vascular leakage and hypotension.[64] Second, growth factors like PDGF can promote the stabilization of new blood vessels by inducing the co-localization of perivascular cells with the immature blood vessels. Since both stages are important for the formation of a functional vascular network in a tissue engineered graft, the dual delivery of factors that stimulate both new blood vessel formation and maturation may be necessary for optimal results. The dual delivery of VEGF and PDGF has been shown to result in the formation of a high number of mature vessels in implanted scaffolds.[60,65]

Apart from the delivery of factors that directly stimulate vessel formation or maturation, indirect approaches have been studied as well. This encompasses the delivery of a factor like Sonic hedgehog (Shh)[66] or BMP-2, -4 or -6[67] that stimulates other cells to produce angiogenic factors. This approach has several advantages when compared to the direct delivery of angiogenic growth factors. First, the secretion of angiogenic factors by the respondent cells is often regulated. This means that the concentration of angiogenic factors released is in the normal physiological range and can be adapted over time according to the needs for different stages of vessel formation. Second, the production of angiogenic factors by respondent cells results in the formation of growth factor micro gradients. It has been shown that these gradients are of importance for capillary morphogenesis.[68] Third, the stimulation with indirect factors often results in the secretion of multiple angiogenic factors that regulate both vessel formation and maturation. Shh for instance can induce interstitial mesenchymal cells to secrete several fac-

tors, including VEGF and angiopoietins-1 and -2. This results in the formation of highly organized, mature vessels.[66]

Several strategies for the delivery of angiogenic factors have been developed. These include the addition of recombinant protein,[69] genes[70] or factor-overexpressing genetically engineered cell transplants.[71] The addition of recombinant proteins to biomaterials is most easily applicable and thus most widely studied. The delivery of growth factors from classic biomaterial matrices is either driven by passive diffusion or coupled to the rate of biomaterial degradation, both of which usually occur independently of and often not in tune with the actual healing process.[64] In this classical setting, the adjustability of the release profile is limited. The magnitude of release can be varied by the amount of growth factor added to the matrix, and the kinetics of release can be varied by altering the material degradation rate via material composition or structure. However, these measures are often insufficient to synchronize growth factor levels with actual cellular demands. A novel approach to solve this problem involves a specific chemical linkage of growth factors to a gel matrix. Penetrating endothelial cells secrete MMPs that degrade the matrix and thus release the growth factors locally. This results in a local cell-demanded release of growth factors. It has been shown that the neovasculature induced by cell-demanded release is better organized than neovasculature induced by non-cell-demanded growth factor release.[72,73]

In vivo prevascularization

Another strategy to enhance vascularization for tissue engineering is *in vivo* prevascularization. This method, also referred to as tissue prefabrication, involves two distinct stages. In the first stage, a bone tissue engineering construct is implanted into a region with a vascular axis suitable for microsurgical transfer. This means that the tissue engineered graft is either wrapped in an axially vascularized tissue like muscle, or that a vascular axis is implanted into the graft. A vascularization period at this initial implant site will result in the formation of a vascular network or pedicle in the tissue engineered construct that is supplied with blood by the vascular axis. During the second stage, the tissue engineered construct is harvested together with the vascular pedicle and re-implanted at the bone defect site. The vascular network can then be connected to the local vasculature using microsurgical techniques of vascular anastomosis.[2] *In vivo* prevascularization has been shown to be feasible and beneficial for bone tissue engineering.[17,18,74] The advantage of this technique is that after implantation at the final site, the construct is immediately perfused due to surgical anastomosis. The drawbacks however, are that two separate surgeries are necessary, a vascular axis has to be removed from

another location, and cells may have to be reseeded after implantation in the bone defect, since nutrient limitations are still likely during the vascularization period at the initial implantation site.

In vitro prevascularization

A strategy to improve vascularization that has gained interest recently is *in vitro* prevascularization. This strategy is based on the fact that endothelial cells can form prevascular structures when they are cultured in the right conditions and environment *in vitro*. The hypothesis of *in vitro* prevascularization is that adding endothelial cells to other tissues *in vitro* will result in the formation of a prevascular network within this tissue. After implantation, this network can then anastomose to the vasculature of the host and supply the construct with nutrients. This means that host blood vessels don't have to grow into the entire construct, but only into the outer regions of the construct. Anastomosis is not as fast as with the previous strategy, since the vascular network is not surgically anastomosed. However, future developments in this field may include the creation of a vascular axis that can be surgically connected to the host vasculature.

The organizational capacity of endothelial cells *in vitro* is remarkable. When seeded on or in a proper matrix, endothelial cells will organize spontaneously into capillary-like structures that often contain lumen.[75] Based on this, tube formation assays, on for instance collagen or Matrigel, are nowadays widely used to study the effect of pro-angiogenic and anti-angiogenic factors.[76] It has been shown that upon implantation, these capillary-like structures can connect to the host vasculature and become functional perfused vessels. However, a study performed by Koike *et al* showed that over time, stabilization of the engineered vessels is critical. When HUVEC alone were cultured in a fibronectin/collagen gel, they did form interconnected capillary-like structures. However, after implantation perfusion was limited and the structures disappeared within 60 days. When on the other hand a mural cell precursor was added during *in vitro* culture, the amount of perfused vessels increased dramatically and the vessels remained stable and functional for one year *in vivo*.[77]

For prevascularized tissue engineering, endothelial cells are generally combined with other cell types to attain a tissue or tissue precursor together with a prevascular network. It is therefore important to find culture conditions that are suitable for the organization of the vascular network, as well as the development of the tissue that is being engineered. This generally means that the use of angiogenic growth factors has to be minimized, since they may negatively influence

the development of the other tissue. *In vitro* prevascularization is a relatively new field of tissue engineering. As such, the amount of tissues that have been used for prevascularization studies is as yet still limited. They include skin,[78-80] skeletal muscle,[81] bone,[82,83] and cardiac muscle.[84,85]

Literature has shown that endothelial cells can form prevascular networks within several tissues *in vitro*. What is most striking, is that the organization of endothelial cells can generally take place in media systems that are designed for the development of the other tissue involved and as such do not contain any added angiogenic factors.[78,80,81,83,85] This is an important finding, since changing medium systems could negatively influence the development of the main tissue. Several studies have shown that the addition of mural cell precursors can result in stabilization and better organization of the vascular structures, without negatively influencing the development of the construct.[81,84] Most importantly, prevascular networks, that have been formed *in vitro*, can connect to the host vascular system after implantation.[79,81] Tremblay *et al* reported that the prevascular network in a skin construct could anastomose to the host vascular system within 4 days, whereas vascularization of a non-prevascularized graft took as much as 14 days.[79] Moreover, Levenberg *et al* reported that prevascularization of a skeletal muscle construct *in vitro* enhanced construct vascularization, perfusion and survival after implantation.[81]

Communication between endothelial cells and osteogenic cells in bone tissue engineering

It is evident that vascularization is an important factor in the field of bone tissue engineering. One of the strategies to enhance vascularization after implantation is *in vitro* prevascularization. The addition of endothelial cells during *in vitro* culture could result in the formation of prevascular structures that can connect to the host vasculature after implantation. However, the combination of endothelial cells and osteoprogenitor cells during *in vitro* culture may have beneficiary effects other than the acceleration of vascularization. Endothelial cells and osteoprogenitor cells are known to secrete an array of growth factors that are beneficial for the growth and differentiation of the other. On the one hand, osteoprogenitor cells secrete VEGF, which is a well known angiogenic growth factor that stimulates endothelial cell growth and organization, as a response to a multitude of factors that play a role in osteogenesis. These factors include 1,25-dihydroxyvitamin D3,[86] BMP-2 and -4,[67] BMP-7,[87] dexamethasone,[88] FGF-1 and -2,[89,90] IGF-1,[91-93] parathy-

roid hormone,[92] PDGF,[94] prostaglandins,[95] and TGF-β.[96] Apart from that, osteoblastic cells secrete VEGF as a response to hypoxia.[97] On the other hand, endothelial cells can enhance the proliferation and differentiation of osteoprogenitor cells by the secretion of osteogenic growth factors like IGf-1,[86] endothelin-1,[86] and BMP-2.[98-100]

Due to the growth factor communication between endothelial cells and osteoprogenitor cells, it can be hypothesized that the addition of endothelial cells to a bone tissue engineering construct will enhance the osteogenic differentiation of the constructs. However, conflicting reports can be found on this subject in literature. Meury *et al* reported that endothelial cells inhibit the differentiation of mesenchymal stem cells into mature osteoblasts and arrest the differentiation at a preosteoblastic stage in coculture systems without direct contact.[101] On the other hand, Kaigler *et al* reported that the addition of endothelial cells increased the osteogenic differentiation of mesenchymal stem cells *in vitro* and resulted in more bone formation after implantation.[102] However, a significant effect was only detected when the cells were cocultured with direct contact and not in indirect coculture systems. Wang *et al* reported an increase in ALP activity in human osteoblast-like cells when cultured in HUVEC-conditioned medium,[86] while Jones *et al* reported a decrease in ALP activity of mesenchymal stem cells when cultured on endothelial cell feeder layers.[103] Villars *et al* showed a decrease of ALP activity when mesenchymal stem cells were cocultured with HUVEC without direct contact, but an increase when the same cells were used in direct contact cocultures.[104] Guillotin *et al* showed an upregulation of ALP activity when osteoprogenitor cells were cocultured in direct contact with different primary endothelial cells.[105]

Several factors could give rise to this discrepancy of results when coculturing endothelial cells and osteoprogenitor cells. However, the absence or presence of direct cell contact between the different cell types in the cocultures seems to be one of the most important factors, as is illustrated by the studies that describe both direct and indirect cocultures.[102,104] Indeed, it has been shown that direct contact between endothelial cells and osteoprogenitor cells has a positive effect on the osteogenic differentiation of these cells.[106,107] Therefore, this is an important factor to take into account when designing experiments to investigate the effect of endothelial cells on osteogenic differentiation in bone tissue engineering.

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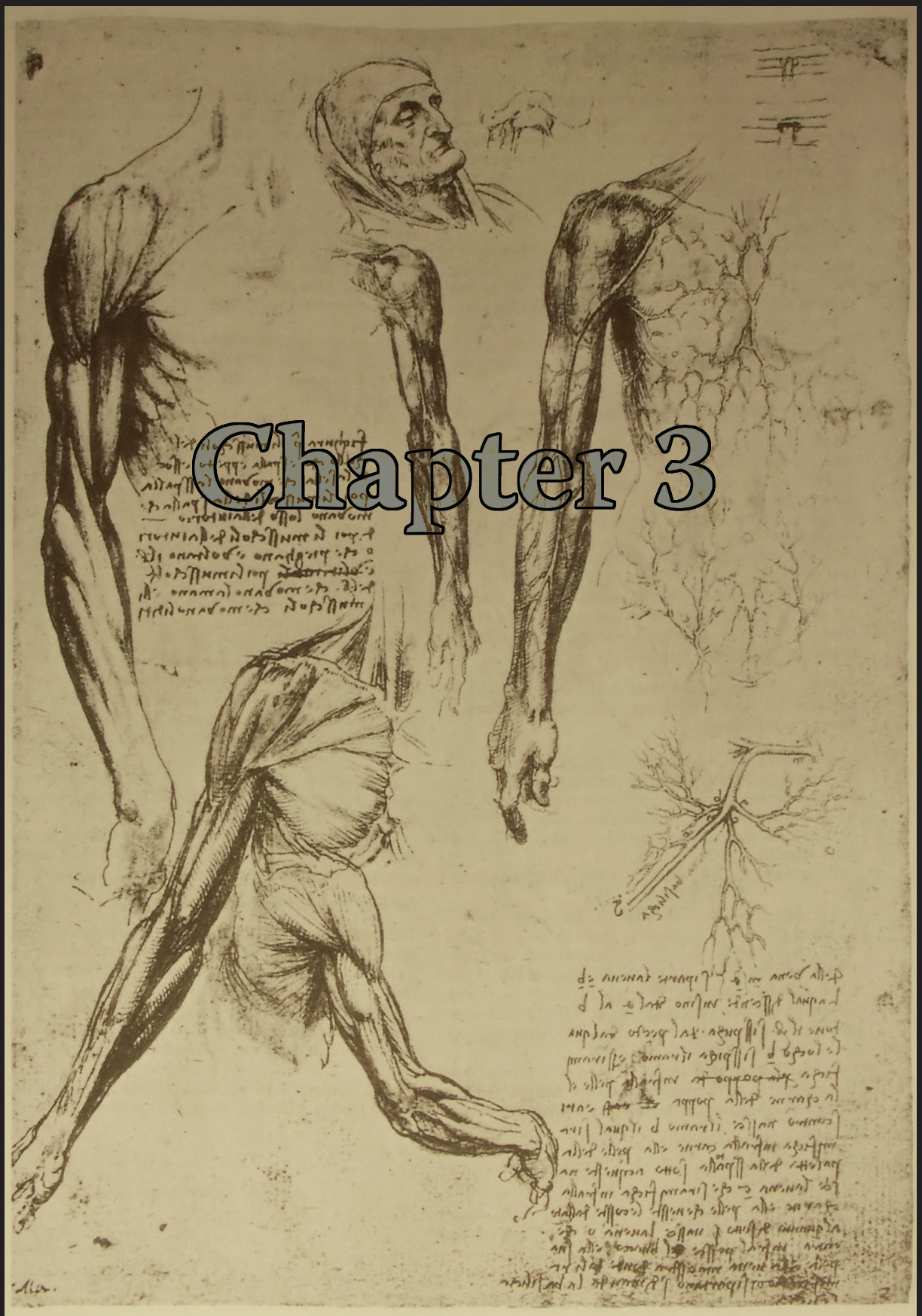
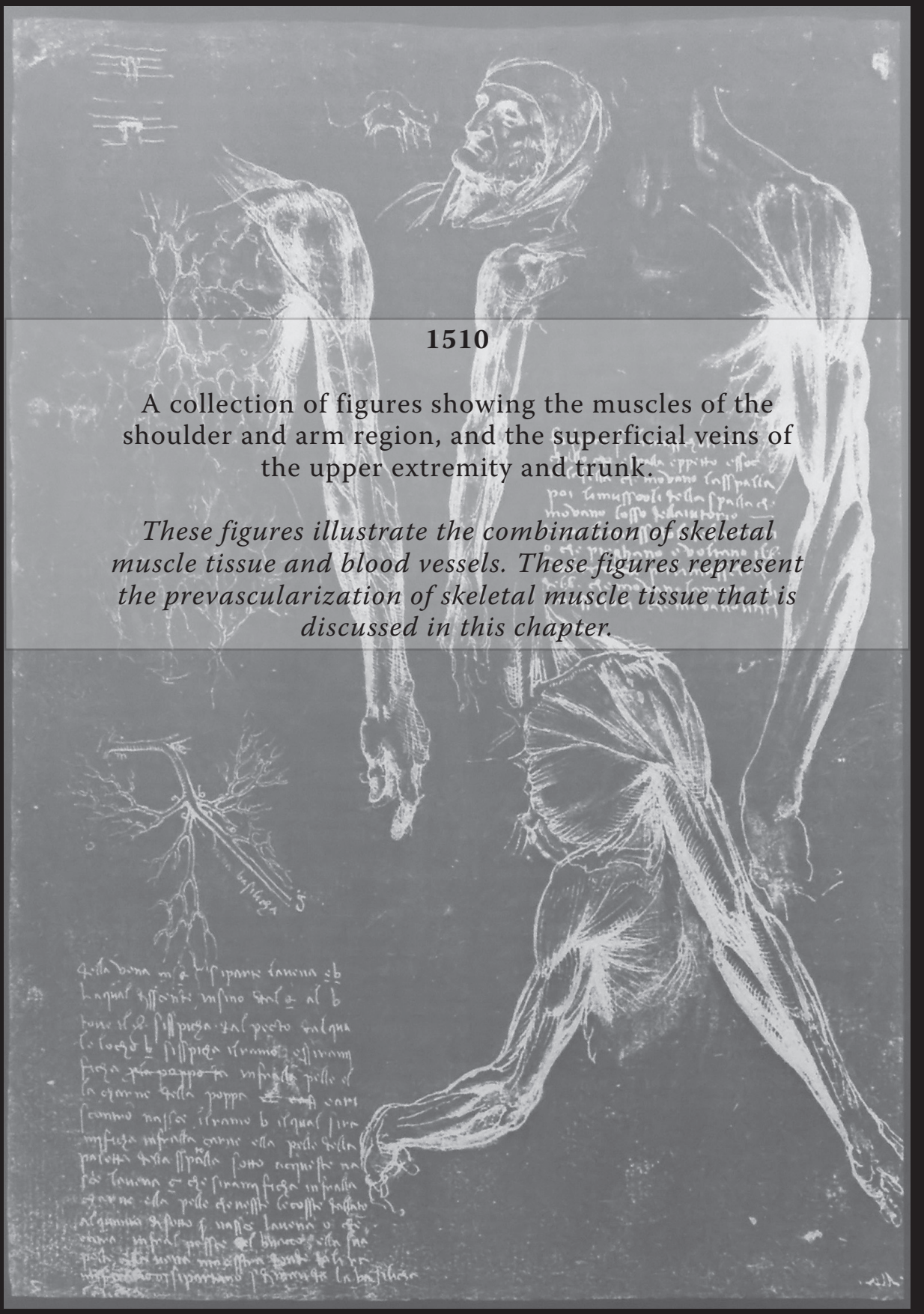


Chapter 3



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A collection of figures showing the muscles of the shoulder and arm region, and the superficial veins of the upper extremity and trunk.

These figures illustrate the combination of skeletal muscle tissue and blood vessels. These figures represent the prevascularization of skeletal muscle tissue that is discussed in this chapter.

Chapter 3

Engineering vascularized skeletal muscle tissue

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Abstract

One of the major obstacles in engineering thick, complex tissues such as muscle is the need to vascularize the tissue *in vitro*. Vascularization *in vitro* could maintain cell viability during tissue growth, induce structural organization and promote vascularization upon implantation. Here we describe the induction of endothelial vessel networks in engineered skeletal muscle tissue constructs using a three-dimensional multiculture system consisting of myoblasts, embryonic fibroblasts and endothelial cells coseeded on highly porous, biodegradable polymer scaffolds. Analysis of the conditions for induction and stabilization of the vessels *in vitro* showed that addition of embryonic fibroblasts increased the levels of vascular endothelial growth factor expression in the construct and promoted formation and stabilization of the endothelial vessels. We studied the survival and vascularization of the engineered muscle implants *in vivo* in three different models. Prevascularization improved the vascularization, blood perfusion and survival of the muscle tissue constructs after transplantation.

Adapted from Levenberg S, *et. al.* Engineering vascularized skeletal muscle tissue. *Nature Biotechnology* **23**, 879-884, 2005.

Introduction

Most approaches to engineering new tissue have relied on the host for vascularization. Although this approach has been useful in many tissues, it has not been as successful in thick, highly vascularized tissues such as muscle.[1–3] Skeletal muscle consists of individual muscle fibers arranged in parallel. Each fiber is a long, cylindrical multinucleated cell that is surrounded by connective tissue. Skeletal muscles have an abundant blood vessel supply, with branches of blood vessels following the connective tissue components of the muscle.[4,5] So far, attempts to engineer skeletal muscle tissue have involved cultivation of skeletal myoblasts only, in some cases using growth factor delivery matrices or genetically engineered myoblasts to provide vascularization factors.[6–8]

We hypothesized that embryonic endothelial cells in the appropriate environment could be used to induce endothelial vessel networks in engineered skeletal muscle tissue *in vitro*.

Materials and methods

Cell culture

Mouse skeletal myoblast cells (C_2C_{12})[11,12] were cultured in DMEM supplemented with 10% FBS, 10% calf serum and 2.5% HEPES buffer. Human umbilical vein endothelial cells (HUVEC) were cultured in endothelial cell medium (EGM-2; Lonza). Mouse embryonic fibroblasts (MEF) (Cell Essentials) were cultured in DMEM supplemented with 10% FBS. HES cell–derived $CD31^+$ endothelial cells were isolated as described[9] and cultured in endothelial cell medium.

Polymer scaffolds

Porous sponges composed of 50% PLLA and 50% PLGA were fabricated as described[10] with pore sizes of 225–500 μm and 93% porosity. The PLGA was selected to degrade quickly (~3 weeks) to facilitate cellular ingrowth, whereas the PLLA was chosen to provide mechanical support to 3D structures. The degradation time of the composed sponges is ~6 months. Biocompatibility of PLLA and PLGA porous scaffolds was previously shown.[13] For seeding, the desired number of cells were pooled and resuspended in 7–15 μl of a 1:1 mixture of culture medium and growth factor-reduced Matrigel (BD Biosciences). This suspension was allowed to absorb into the sponges, after which the sponges were incubated for 30 min at 37

°C to allow solidification of the gel. Culture medium was then added, the sponges were detached from the bottom, and incubated at 37 °C on a XYZ shaker. The medium was changed every other day. At the conclusion of the experiments, samples were fixed in 10% formalin and subsequently embedded in paraffin for sectioning or were transplanted into mice or rats.

Implantation of muscle constructs

Male 5- to 6-week-old SCID mice (CB.17 SCID) were anesthetized with 2.5% isoflurane in balanced oxygen, after which a construct was implanted subcutaneously on each side lateral to the dorsal midline region of each mouse. For intramuscular implantation, constructs were implanted into the outer quadriceps muscle of the right-hand side of 5- to 7-week-old male nude rats. Sutures of 6-0 Prolene in a simple interrupted pattern were used to prevent movement of the constructs from the muscle site, and the skin was closed using surgical staples.

Two to eight weeks after implantation, the mice or rats were killed and the implants were retrieved. Samples were fixed in 10% natural buffered formalin, processed routinely and sectioned at 4 μm before staining. Two assays were involved in perfusion analysis. (i) Lectin perfusion. Lectin HPA (Helix pomatia agglutinin) conjugated to Alexa Fluor 594 (Molecular Probes) (0.5 mg/0.25 ml PBS) was injected into the tail vein of anesthetized animals (20 mg/kg body weight). Circulation was allowed for 2 minutes after which the animals were killed and the implants were retrieved. Samples were snap frozen (liquid nitrogen) in Cryomatrix (Thermo Shandon) and sections of 6 μm were cut with a cryotome. (ii) Luciferase assay (abdominal wall model). Tissue-engineered constructs were placed in 12-well culture dishes in 2 ml of medium and 1.0×10^9 dot blot/cc of AAV-luciferase was added. After 48 hours, the constructs were washed with two volumes of PBS. As a control, luciferin (150 μl of 5mg/ml) was added to each well. After incubation for 11 minutes, the constructs were imaged in the Xenogen IVIS device at a 3-minute exposure. Luminescence was determined by calculating the flux (photons/sec/cm^2) overlying each construct.

Immediately thereafter, the constructs were implanted into isoflurane-anesthetized mice by creation of a 3 mm × 3 mm defect in the anterior abdominal wall of the mice in line with the inferior epigastric artery. The construct was then sutured in place using four 7-0 prolene sutures attached to each corner of the construct. After the ventral skin was sutured, the animal recovered. At various intervals after surgery, the animals were imaged using the Xenogen IVIS device. Mice were anesthetized using an intramuscular injection of ketamine and xylazine. Luciferin (150

µl of 5 mg/ml) was injected subcutaneously on the dorsal surface of the mouse and allowed to circulate for 11 min before the animals were imaged. Luminescence was calculated by determining the photon flux. A 1.0 cm^2 area was chosen arbitrarily as the standard. The ratio of the flux from the tissue-engineered construct relative to the hind limb was calculated. After the final imaging session, the animals were killed and the implants were retrieved and placed in 10% formalin before routine processing and histological sectioning. Unseeded scaffolds were used as controls and showed host-cell infiltration (including fibroblasts and blood vessels) as known from previous studies.[13]

Tissue processing and immunohistochemical staining

Tissue constructs were fixed for 6 hours in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Transverse sections (4 µm) were placed on silanized slides for immunohistochemistry or staining with hematoxylin and eosin.

Immunohistochemical staining was carried out using the Biocare Medical Universal HRP-DAB kit (Biocare Medical) according to the manufacturer's instructions, with prior heat treatment at 95 °C for 20 minutes in ReVeal buffer (Biocare Medical) for epitope recovery. For immunofluorescent staining, the secondary antibodies used were Alexa Fluor (Molecular Probes) and Cy-3 (Jackson Laboratories) followed by DAPI (Sigma) nuclear staining. The primary antibodies were anti-human: CD31 (1:20); desmin (1:150); α-smooth muscle actin (1:50); vWF (1:200) (all from Dako); or vWF (Chemicon) (1:100). Staining with α-smooth muscle actin antibody (as well as other smooth muscle actin markers) to identify fibroblasts differentiation into smooth muscle cells could not be done in the presence of C_2C_{12} cells because of expression of these markers by the C_2C_{12} myoblasts. The vWF antibody was used after deparaffinization and trypsin treatment for epitope recovery.

For labeling implanted myoblasts, myoblast culture medium was supplemented with 10 µm of 5´-bromo-2´-deoxyuridine (BrdU) (Sigma) and applied to 60% confluent dish cultures for 16 hours. Cells were washed and seeded on scaffolds as described. Tissue sections were stained using mouse anti-BrdU antibodies (1:1,000) as described, but with treatment with 2N HCL and 0.5% Triton X-100 for 30 minutes at 37 °C to denature the DNA, before addition of the antibodies. For staining apoptotic cells DeadEnd colorimetric TUNEL system (Promega) was used.

RT-PCR analysis

Total RNA was isolated by an RNEasy Mini Kit (Qiagen), using the isolation-from-tissue protocol. RT-PCR was carried out using a Qiagen OneStep RT-PCR kit with 10 units Rnase inhibitor (Gibco) and 40 ng RNA. To ensure semi quantitative results of the RT-PCR assays the number of PCR cycles for each set of primers was checked to be in the linear range of the amplification. Primer sequences: mouse VEGF, 5′-CCT CCG AAA CCA TGA ACT TTC TGC TC-3′ and 5′-CAG CCT GGC TCA CCG CCT TGG CTT-3′; human PDGF-B, 5′-GGA GCA TTT GGA GTG CGC CT-3′ and 5′-ACA TCC GTG TCC TGT TCC CGA-3′. The amplified products were separated on 1.2% agarose gels with ethidium bromide (E-Gel, Invitrogen). Mean pixel intensities of each band were measured and normalized to mean pixel intensities of glyceraldehyde phosphodehydrogenase band. The values for two experiments (performed in duplicate) were then averaged and graphed with s.d.

Image analysis

Overlapping microscopic pictures were taken at a magnification of 100 × so that the entire area of the sample was covered. An imaging software (AxioVision 3.1, Carl Zeiss) was used to determine the area of endothelial cells, the area of vessels or lumen and the total sample area. The number of structures with lumen was counted manually. For colocalization analysis, 3–6 randomly chosen 20 × fields were analyzed using OpenLab (Density Slice Module) image analysis software (ImproVision) to quantify endothelial cell–positive areas with and without colocalization of smooth muscle cell–positive areas. An endothelial cell–positive area was identified by the presence of a vWF-positive vessel-like structure with a lumen. *P* values were calculated using Student's *t*-test.

Results and discussion

First we developed a threedimensional (3D) coculture system in which mouse myoblasts were mixed with human embryonic endothelial cells (hES cell–derived endothelial cells)[9] or with human umbilical vein endothelial cells (HUVEC) and seeded on highly porous, 3D biodegradable polymer scaffolds. The sponge-like scaffolds were composed of 50 % poly- (L-lactic acid) (PLLA) and 50 % polylactic-glycolic acid (PLGA), with pore sizes of 225–500 μm[10] (Fig. 1a).

As indicated by desmin immunostaining of cross sections of constructs 3 days after seeding of myoblasts, the myoblasts attached to and grew on the scaffolds (Fig. 1b). By 14 days the myoblasts had differentiated and formed partially aligned, elongated and multinucleated myotubes. Some of the myotubes differentiated further and became myogenin positive (36 % ± 7.3 % of total nuclei) (Fig. 1b). When both myoblasts and endothelial cells (either hES cell–derived or HUVECs) were cultured on the scaffolds, the endothelial cells (CD31 positive) organized into tubular structures in between the myoblasts and throughout the construct, forming vessel networks within the engineered muscle tissue *in vitro* (Fig. 1c).

We compared the effects of two media: myoblast medium composed of DMEM with 10 % FCS and 10 % CS with HEPES, and endothelial medium (EGM-2) with 2 % FCS and endothelial growth factor supplements. Myoblast medium promoted both differentiation of the muscle cells (27 % ± 7.6 % myogenin-positive nuclei) and formation of endothelial lumens in the constructs (Fig. 2a), whereas endothelial medium alone did not support differentiation of the muscle cells (2 % ± 7.1 % myogenin-positive nuclei) and inhibited endothelial lumen formation (Fig. 2a). Why the endothelial medium inhibited vessel formation in the muscle constructs is not clear, but it may be related to its inhibitory effect on muscle differentiation and cell signaling in the culture.

Because blood vessels are stabilized by association with pericytes or smooth muscle cells[14–20] and because endothelial cells can induce the differentiation of undifferentiated mesenchymal cells into smooth muscle cells,[21–24] we hypothesized that the formation of vessels characterized by lumen structures in the engineered skeletal muscle tissue would be promoted by embryonic fibroblasts. Addition of embryonic fibroblasts to the cultures, together with myoblasts and endothelial cells, strongly promoted vascularization of the engineered muscle. This was evidenced by increases in the total area of endothelial cells and the number and size of endothelial lumens, compared with constructs seeded with myoblasts and endothelial cells only (Fig. 2a). The effect of the fibroblasts was dependent on medium conditions and the cell ratios (Fig. 2a, tri-cultures), with best vascularization in myoblast medium and addition of 0.2 million fibroblasts per scaffold (1.5

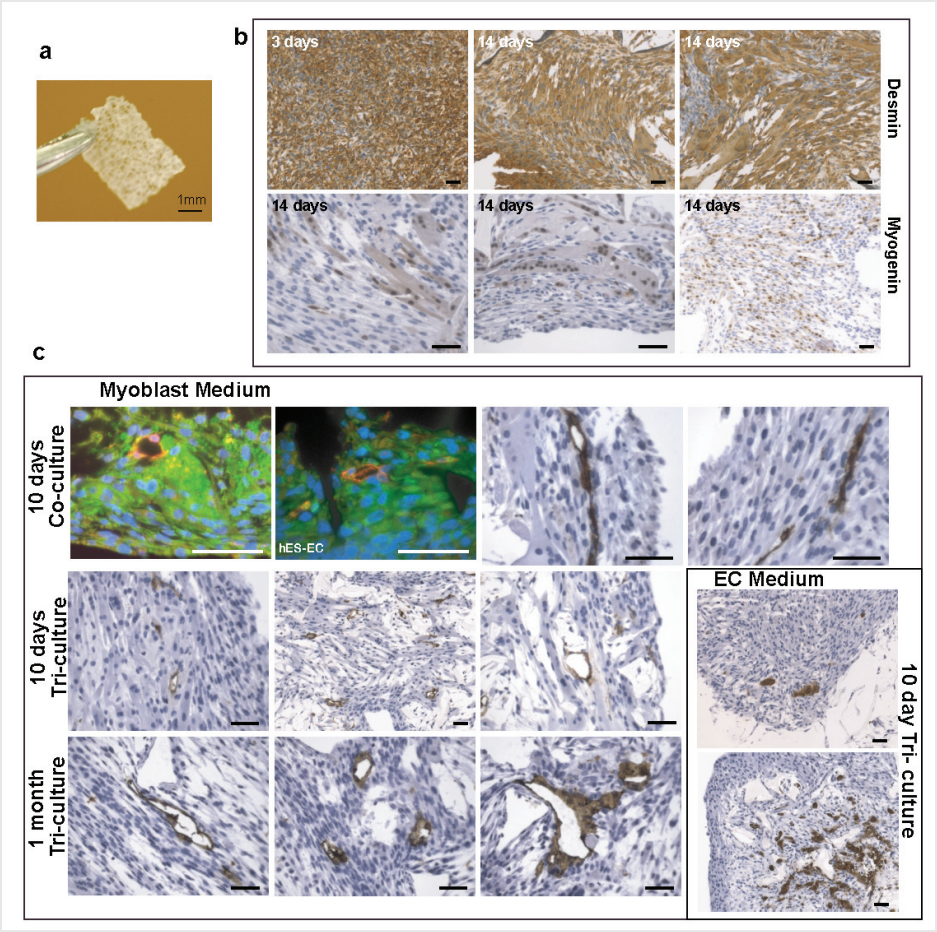


FIG. 1. *In vitro vascularization of engineered skeletal muscle tissue* ***(A)*** *PLLA/PLGA scaffold before cell seeding. Scale bar = 1 mm.* ***(B)*** *Myocyte differentiation on PLLA/PLGA polymer scaffolds. Desmin and myogenin immunostaining of tissue sections taken from 3D scaffolds cocultured with skeletal myoblasts and endothelial cells (HUVEC) and grown for 3 d and 14 d. Scale bar = 50 μm.* ***(C)*** *Vessel-like network formation in vitro in muscle 3D constructs. Endothelial cells (HUVEC or hES cell-derived endothelial cells (hES-EC) when indicated) were coseeded with skeletal myoblasts on polymer scaffolds and grown for 10 days (cocultures). Tissue construct sections were immunofluorescently stained using anti-CD31 antibodies (red), anti-desmin antibodies (green) and DAPI for nuclear staining (blue) (left), or stained using anti-CD31 antibodies alone (brown) and counterstained with hematoxylin (blue) (right). One-month and 10 d tri-cultures, both including mouse embryonic fibroblasts, were grown either in myoblast medium or endothelial medium (EC medium) and stained using anti-CD31 antibodies. Cultured cell numbers (myoblast/endothelial/ fibroblast), 0.5/0.7/0.2 × 10^6; bottom picture in endothelial (EC) medium, 0.5/0.5/0.5 × 10^6. Scale bar = 50 μm.*

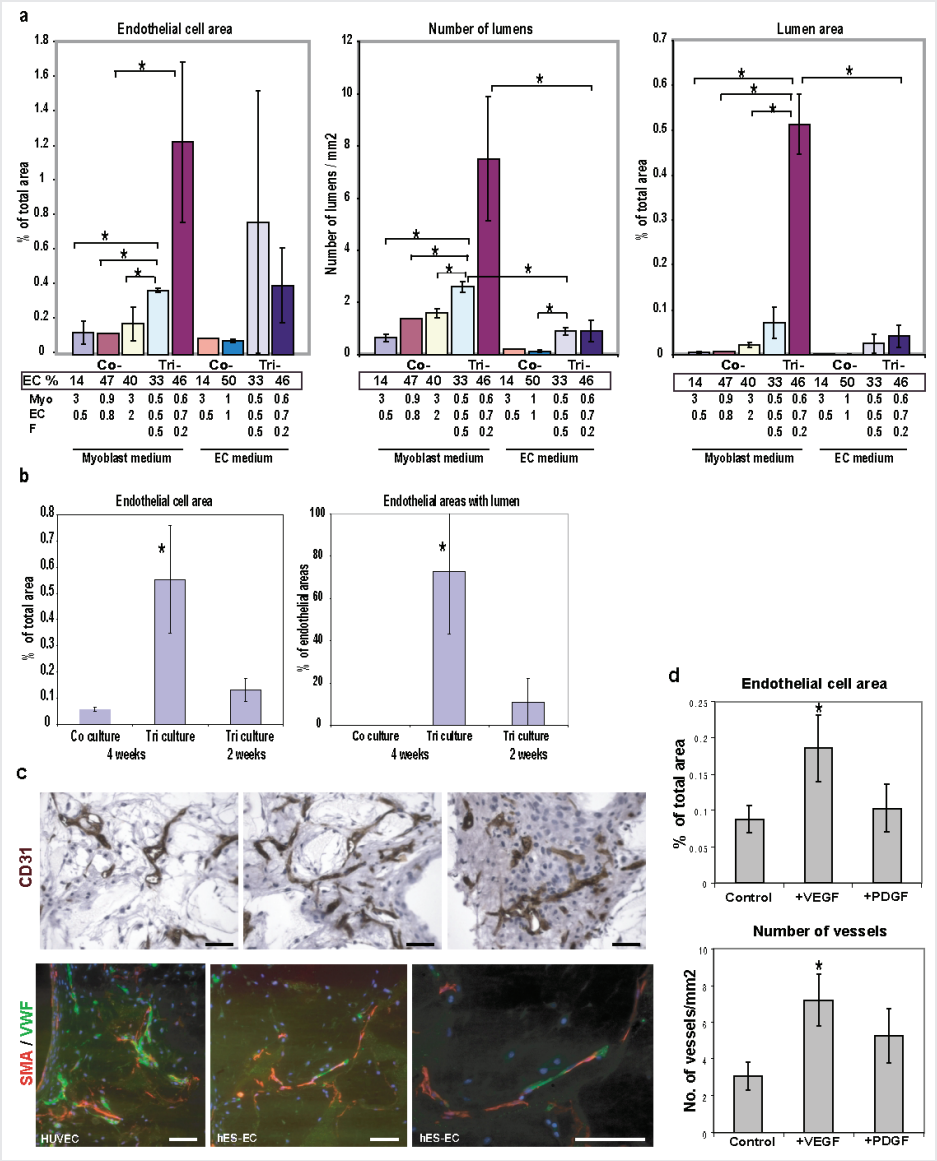


FIG. 2. Quantitative analysis of endothelial vessels in muscle constructs, in vitro. ***(A)*** *Comparison of vessel formation in coculture (Co) and tri-culture (Tri) constructs grown with different cell ratios (cell number × 10^6) and medium conditions (myoblast medium and endothelial medium). Endothelial cell ratio (EC %) is calculated as percentage of the total cell number. Endothelial cell area corresponds to percentages of area positively stained with CD31 antibody within the tissue section. Lumen area shows the total area of all the lumens in the section as percentages of total section area. Myo, myoblasts; EC, endothelial cells (HUVEC); F, mouse embryonic fibroblasts. * denotes statistical significance ($P < 0.05$)*

between the indicated pairs. ***(B)*** *Comparison of vessels in 2-week and 4-week constructs. Cocultures are myoblasts and endothelial cells (0.8 and 0.6 × 10^6 cells per scaffold, respectively). Tri-cultures are myoblasts, fibroblasts and endothelial cells (0.6, 0.2 and 0.6 × 10^6 cells per scaffold respectively). * denotes statistical significance ($P < 0.05$) compared with controls.* ***(C)*** *Effect of embryonic fibroblasts on vessel formation. Constructs seeded with embryonic fibroblasts and endothelial cells (0.5 and 1 × 10^6) were grown for 2 weeks. Construct sections were immunostained with human-specific anti-CD31 antibodies (brown) showing vessel formation throughout the 3D constructs. The lower panel shows colocalization of smooth muscle cells and endothelial cells (HUVEC or hES-EC as indicated). Constructs were immunofluorescently double-stained using anti-vWF antibodies (green), anti-smooth muscle actin antibodies (red) and DAPI for nuclear localization. Note smooth muscle actin–positive cells around elongated endothelial structures. Scale bar = 50 μm.* ***(D)*** *Effect of VEGF or PDGF-B supplementation on endothelial vessel formation. Tri-culture constructs (myoblasts, fibroblasts and endothelial cells (0.6, 0.2 and 0.7 × 10^6 cells per scaffold, respectively)) were incubated with control medium or medium supplemented with VEGF (50 ng/ml) or PDGF-B (5 ng/ml). After 2 weeks, construct sections were immunoassayed using anti-CD31 antibodies and analyzed for endothelial-positive area, and number of endothelial vessels. * denotes statistical significance ($P < 0.05$) compared with controls. The results shown are mean values ± s.d. (n =4).*

million cells total with 40–50 % endothelial cells). The inductive effect of embryonic fibroblasts on endothelial vessels is shown even when comparing tri-culture samples that were seeded with a lower percentage (33 %) of endothelial cells with cocultures of myoblasts and endothelial cells seeded with higher percentages of endothelial cells (40–50 %) (Fig. 2a).

To analyze the stability of the *in vitro* vessel-like structures formed in the muscle constructs, we examined tri-culture constructs (a combination of myoblasts, fibroblasts and endothelial cells) at 2 weeks and 1 month. Large vessel structures (> 1,500 μm^2) were evident only in the 1-month-old tri-culture constructs (Fig. 1c). In addition, tri-cultures grown for 1 month had a twofold increase in the number of endothelial structures, a greater surface area covered by endothelial cells and a higher percentage of vessel-like structures with lumens, compared with 2-week tricultures (Fig. 2b).

These results suggest that addition of embryonic fibroblasts promoted stabilization of the vessel structures over time. Double labeling for von Willebrand factor (vWF) and smooth muscle actin on cross sections of constructs seeded with endothelial cells and embryonic fibroblasts showed that fibroblasts in the cultures became smooth muscle actin positive (suggesting differentiation into smooth muscle cells) and were colocalized around endothelial cells in vessel-like structures (Fig. 2c). Quantitative analysis of the double staining revealed that 65.7

% ± 8.8 % of endothelial vessel-like structures in the constructs had associated smooth muscle cells.

To study the expression of key vasculogenic and angiogenic factors in the 3D muscle constructs, we analyzed the expression of vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF-B) at the mRNA level in the muscle constructs. The RT-PCR results showed that addition of human endothelial cells to myoblast or fibroblast cultures resulted in an increase in mouse VEGF expression. Moreover, tri-cultures that included embryonic fibroblasts had higher levels of VEGF mRNA than myoblast-endothelial cocultures (Fig. 3). The increased VEGF expression is consistent with the increase in the endothelial network observed in the tri-cultures, and could be one of the factors affecting the induction of vascularization of the constructs. Indeed, addition of VEGF to the medium resulted in a larger area covered by endothelial cells and an increase in the number of vessel-like endothelial tubular structures in the constructs (Fig. 2d). Depletion of VEGF from the medium resulted in a decreased number of vessel structures, whereas addition of fibroblasts to cultures without VEGF supplementation restored vessel formation (data not shown).

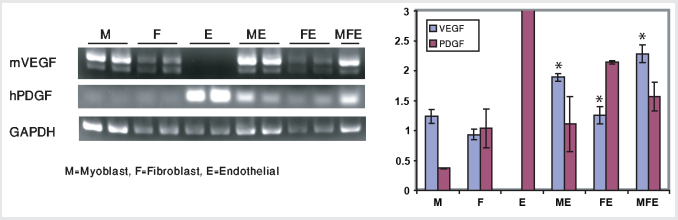


FIG. 3. *VEGF and PDGF-B expression in 3D constructs. Constructs were seeded with myoblasts (M) (0.8 × 10⁶), fibroblasts (F) (0.8 × 10⁶), endothelial cells (E) (0.7 × 10⁶) alone or combination of myoblasts and endothelial cells (ME) (0.8/0.7 × 10⁶), Fibroblasts and endothelial cells (FE) (0.8/0.7 ×10⁶) or tri-culture of myoblasts, fibroblasts and endothelial cells (MFE) (0.6/0.2/0.7 × 10⁶). RNA was isolated from 2 week-old constructs and subjected to RT-PCR analysis using primers for mouse VEGF (mVEGF), human PDGF-B (hPDGF-B) and GAPDH. For each gene, mean pixel intensities of each band (obtained in the linear range of the amplification) were measured and normalized to mean pixel intensities of GAPDH band.*

To assess the therapeutic potential of our approach, we used three models to analyze the survival, differentiation, integration and vascularization of the implant *in vivo*: (i) subcutaneous implantation in the back of severe combined immunodeficient (SCID) mice, (ii) intramuscular implantation into the quadriceps muscle of nude rats, and (iii) replacement of the anterior abdominal muscle segment of nude mice with the construct. In all three models the muscle implant continued to differentiate *in vivo*. The implanted myotubes were elongated and multinucleated

(6–8 nuclei), with a high percentage of myogenin-positive myotubes (67 % ± 9 %) (Fig. 5a). Control implants containing fibroblasts or no cells showed no desmin-positive myotubes or myogenin-positive nuclei within the scaffold area (Fig. 5a). To further ensure that the myotubes observed within the scaffold were derived from implanted cells rather than invading host cells, we incorporated 5-bromodeoxyuridine (BrdU) in the tissue engineered constructs before implantation. BrdU labeling confirmed that the implanted myoblasts had indeed survived and differentiated to populate the scaffold (data not shown). In most cases, particularly in the abdominal muscle, the implanted myotubes were in close contact with the host muscle, with very thin and sometimes barely detectable fibrous tissue around the implant (Fig. 5a). The myotubes in the implanted area were relatively long and thick and in many cases appeared to have reoriented themselves to align with the fibers of the host tissue (Fig. 5a).

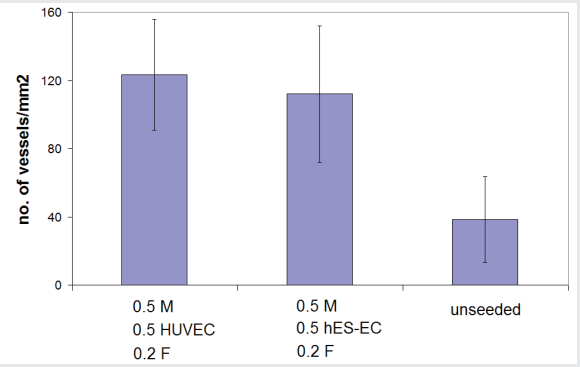
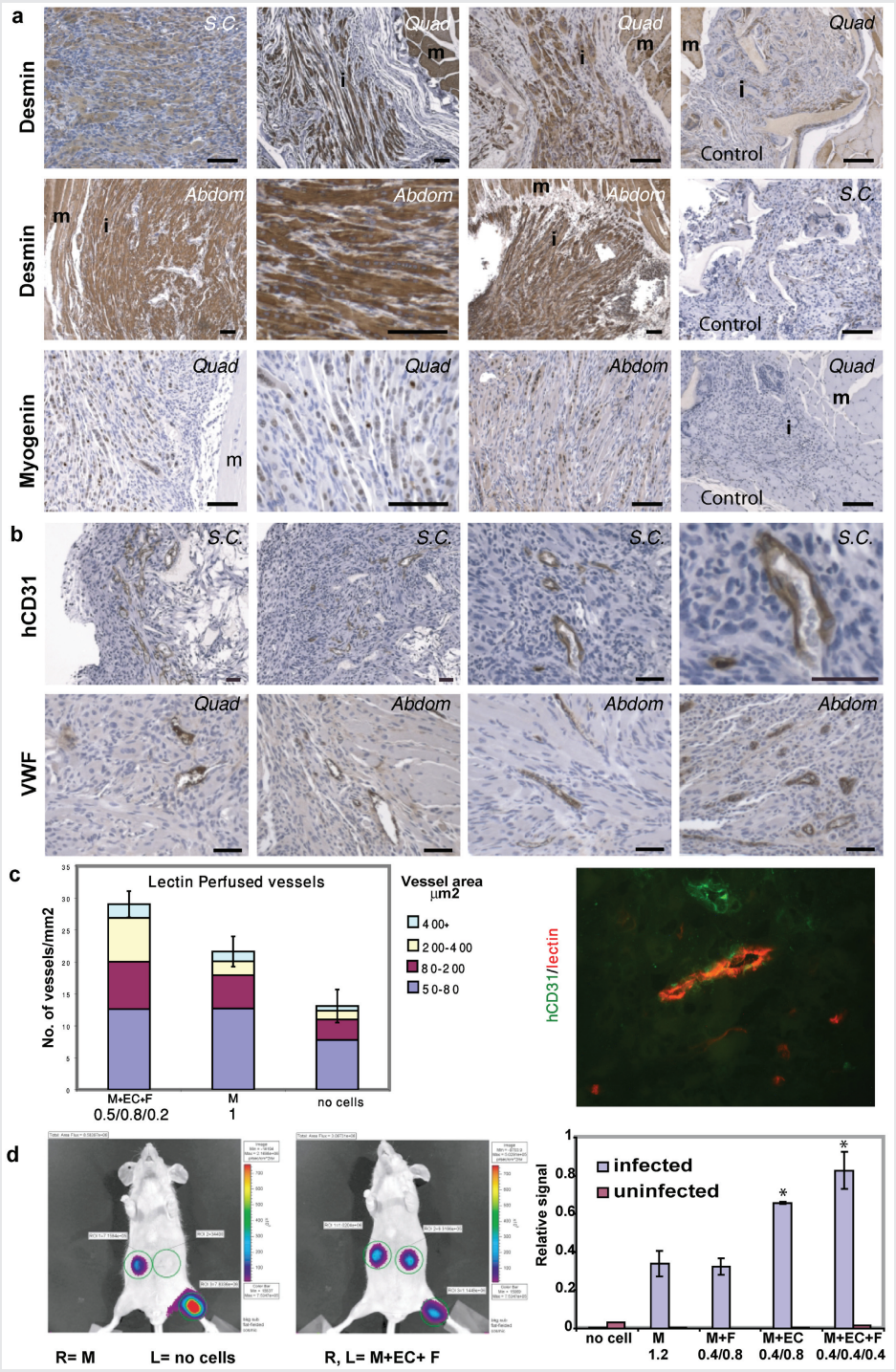


FIG. 4. *Quantitative analysis of number of endothelial vessels in muscle implants seeded with HUVEC or hESC-derived endothelial cells. Vessel formation was compared between tri-culture constructs seeded with myoblasts (M), embryonic fibroblasts (F), and either HUVEC or hESC-derived endothelial cells (hES-EC). Numbers of cells (× 10^6) seeded are indicated. The results are mean values (± s.d.) (n=3).*

The constructs were permeated with host blood vessels (Fig. 5b). Quantification of the number of endothelial vessel-like structures in intramuscular implants 2 weeks after implantation indicated that there was no significant difference between constructs seeded with HUVECs or hES cell–derived endothelial cells (Fig. 4). Staining of subcutaneous implants with anti-human specific endothelial antibodies (anti-CD31) demonstrated the presence of vessels (between elongated myotubes), which were lined by implanted human endothelial cells. Moreover, construct-derived vessels contained intraluminal red blood cells, suggesting that vessels had anastomosed with the host vasculature (Fig. 5b).

To determine whether the vessels were functional, we injected labeled lectin into the tail vein 2 weeks after implantation and counted the perfused vessels. The results indicated that 41 % ± 12 % of human CD31–positive vessels (implant-derived vessels) were perfused with lectin. Quantification of the total number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Desmin
Desmin
Myogenin
S.C.
Quad
Abdom
m
i
Control
b
hCD31
VWF
c
Lectin Perfused vessels
No. of vessels/mm2
Vessel area µm2
400+
200-400
80-200
50-80
M+EC+F
0.5/0.8/0.2
M
1
no cells
hCD31/lectin
d
R= M
L= no cells
R, L= M+EC+ F
Relative signal
infected
uninfected
no cell
M
1.2
M+F
0.4/0.8
M+EC
0.4/0.8
M+EC+F
0.4/0.4/0.4

FIG. 5. *In vivo analysis of engineered muscle constructs* ***(A)*** *Differentiation of engineered muscle in vivo. Two-week-old engineered muscle constructs were implanted either subcutaneously into immunodeficient mice (S.C.), or intramuscularly into rat quadriceps muscle (Quad) or mouse abdominal muscle (Abdom). After 2 weeks in vivo, constructs were sectioned and immunostained using anti-desmin antibodies or myogenin antibodies. For control, constructs without cells were implanted and stained (Control). Note differentiation of myocytes into aligned, multinucleated elongated myotubes. Muscle area, m; implant area, i. Scale bar = 100 μm.* ***(B)*** *Vascularization of engineered muscle in vivo. After 2 weeks in vivo, constructs were sectioned and immunostained using human specific anti-CD31 (hCD31) or anti-vWF antibodies. Scale bar = 50 μm.* ***(C)*** *Functional, lectin-perfused vessels in tissue-engineered muscle implants. After 2 weeks, in vivo constructs were perfused with fluorescently labeled lectin (red). Right, implant's frozen sections were immunofluorescently stained with anti-hCD31 (green), showing implant-derived lectin perfused vessel. Left, quantitative analysis of number and size of lectin-perfused vessels in muscle implants. Vessel formation was compared between tri-culture constructs seeded with myoblasts (M), endothelial cells (HUVEC) (EC), and embryonic fibroblasts (F), and constructs seeded with myoblasts alone or without cells (no cells). (Cell number × 10^6). Standard deviation error bars relate to total number of perfused vessels (n = 3).* ***(D)*** *Microvascular perfusion and survival of tissue-engineered muscle implants. Cells in the constructs were infected with AAV-luciferase for 48 hours before transplantation. Control constructs were not infected with virus. The constructs were then placed in situ in the anterior abdominal muscle walls of nude mice. AAV-luciferase was injected into the left lower extremity of each mouse at the time of surgery to serve as a positive control. Three weeks after surgery, the mice received luciferin to assess perfusion and survival of the tissue engineered implants using luciferase-based in vivo imaging system (IVIS). Quantification of signal detected after 3 weeks. The results are mean values ± s.d. (n = 3). * denotes statistical significance (P < 0.05) compared with myoblasts alone (M) or myoblasts + fibroblasts (M+F). (Cell number × 10^6). Signal intensities are shown on a scale of purple to red (~ 2 × 10^4 to ~ 7.7 × 10^5 p/s/cm^2).*

of perfused vessels (host and implant derived) indicated that muscle constructs seeded with endothelial cells had 30 ± 2 functional vessels per square millimeter compared with 21 ± 2 vessels in constructs seeded with muscle cells only ($n = 3$, $P < 0.01$). The size distribution of functional vessels showed that including endothelial cells in the scaffolds also increased the number of larger or stabilized vessels in the muscle implants (Fig. 5c). These results suggest that pre-endothelialization of the construct, by promoting implant vascularization, can improve blood perfusion to the muscle implant and implant survival *in vivo*.

To further evaluate tissue-engineered muscle construct survival and integration *in vivo*, we used a luciferase-based imaging system. The *in vivo* imaging system (IVIS) works by detecting light generated by the interaction of systemically administered luciferin with locally produced luciferase. Muscle constructs seeded

with or without endothelial cells or fibroblasts, and control constructs were infected with adeno-associated virus (AAV) vector, encoding luciferase, for 48 h before transplantation. Detection of luciferase expression in the constructs indicated no difference among the various muscle constructs *in vitro*. The constructs were then placed *in situ* in the anterior abdominal muscle walls of nude mice. Three weeks after surgery, the mice received luciferin (injected subcutaneously on the dorsal surface) to assess perfusion to the tissue-engineered construct.

A minimal signal was detected in areas that did not receive gene transfer or in constructs that were not seeded with cells. However strong signals were detected from areas either directly transduced with AAV-luciferase (as controls) or transplanted with virally transduced cells, indicating perfused vessels. By using the luciferase system, we were able to noninvasively determine the degree to which different constructs continued to survive (and express luciferase) and maintain vascular connections with the recipient to receive systemically delivered luciferin. The relative signal detected in implants seeded with endothelial cells in cocultures (with myoblasts) and in tri-cultures (with myoblasts and fibroblasts) was higher than in myoblast-only implants (Fig. 5d). Coupled with similar pre-implantation levels of luciferase expression and with the histological evidence of increased functional vascularity, the results suggest that the increased signal in pre-endothelialized samples is related to increased perfusion and survival of the tissue-engineered muscle constructs. Analysis of cell survival in the implant using TUNEL staining indicated a twofold increase in the number of apoptotic cells in muscle-only implants compared with pre-endothelialized implants (36 ± 9 and 19 ± 7 cells per implant cross section, respectively ($n = 3$)).

Conclusion

The approach that we have developed enables formation and stabilization of endothelial vessel networks *in vitro* in 3D engineered skeletal muscle tissue. The overall *in vivo* results show that prevascularization of the implants improves implant vascularization and survival. Unlike previous studies demonstrating endothelial differentiation within fibroblast culture and fibroblast differentiation into pericytes,[14,17,18] this study demonstrates engineering of 3D vascularized skeletal muscle constructs with formation of endothelial networks throughout and in between differentiating skeletal muscle fibers. This study emphasizes the importance of multicell cultures in providing appropriate signals for vascular organization in skeletal muscle tissue. Moreover, it provides evidence for the potential of

endothelial cocultures in promoting *in vivo* vascularization of engineered tissues. Cocultures with endothelial cells may also be important for inducing differentiation of engineered tissues, as embryonic endothelial cells are critical for the earliest stages of organogenesis.[25,26] We believe that this approach could have potential applications in tissue engineering and may provide a tool for the *in vitro* study of multicellular processes such as tissue vascularization.

Acknowledgement

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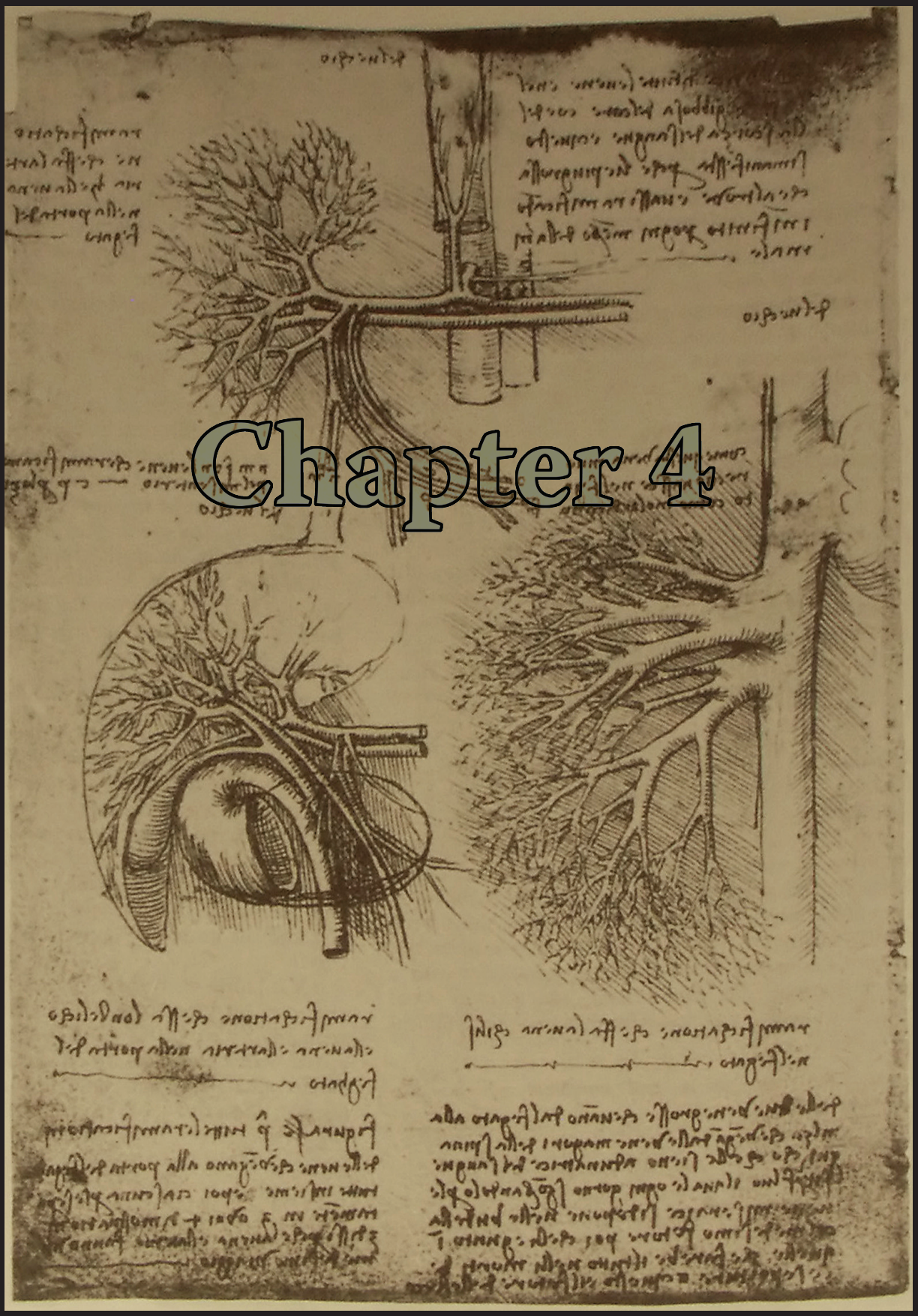
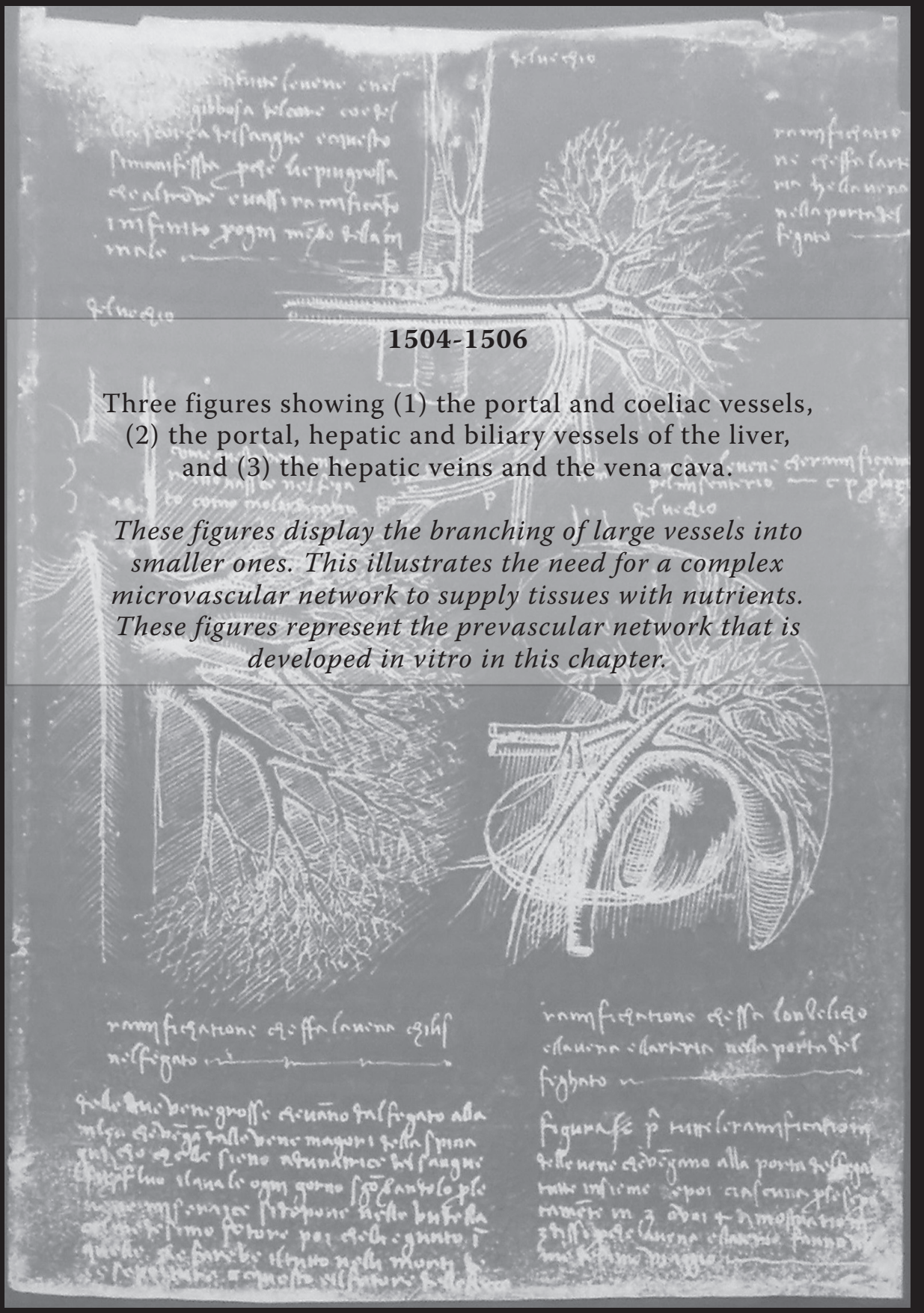


Chapter 4



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Three figures showing (1) the portal and coeliac vessels, (2) the portal, hepatic and biliary vessels of the liver, and (3) the hepatic veins and the vena cava.

These figures display the branching of large vessels into smaller ones. This illustrates the need for a complex microvascular network to supply tissues with nutrients. These figures represent the prevascular network that is developed in vitro in this chapter.

Chapter 4

Endothelial cells assemble into a three dimensional prevascular network in a bone tissue engineering construct

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Abstract

To engineer tissues with clinically relevant dimensions, one must overcome the challenge of rapidly creating functional blood vessels to supply cells with oxygen and nutrients and to remove waste products. We tested the hypothesis that endothelial cells, cocultured with osteoprogenitor cells, can organize into a prevascular network *in vitro*. When cultured in a spheroid coculture model with human mesenchymal stem cells, human umbilical vein endothelial cells (HUVECs) form a 3-dimensional prevascular network within 10 days of *in vitro* culture. The formation of the prevascular network was promoted by seeding 2% or fewer HUVECs. Moreover, the addition of endothelial cells resulted in a 4-fold upregulation of the osteogenic marker alkaline phosphatase. The addition of mouse embryonic fibroblasts or human smooth muscle cells did not result in stabilization of the prevascular network. Upon implantation, the prevascular network developed further and structures including lumen could be seen regularly. However, anastomosis with the host vasculature was limited. We conclude that endothelial cells are able to form a 3-dimensional (3D) prevascular network *in vitro* in a bone tissue engineering setting. This finding is a strong indication that *in vitro* prevascularization is a promising strategy to improve implant vascularization in bone tissue engineering.

Adapted from Rouwkema J, *et. al*. Endothelial cells assemble into a 3-dimensional prevascular network in a bone tissue engineering construct. *Tissue Engineering* **12**(9), 2685-2693, 2006.

Introduction

Vascularization is a critical process during bone growth. The suppression of blood vessel invasion results in thickening of the growth plate and impaired trabecular bone formation.[1] Vascularization is also involved in bone healing, both in natural fractures and in artificial bone implants. Studies have shown that fracture healing and ectopic new bone formation can be blocked by the administration of angiogenesis inhibitors,[2,3] while other studies have shown that new bone formation in porous scaffolds was significantly increased by the insertion of a vascular pedicle in the scaffold.[4,5]

To date, most approaches in tissue engineering have relied on vascularization by the ingrowth of blood vessels from the host. After implantation of tissue constructs, the supply of oxygen and nutrients to the implant is limited by diffusion processes and the speed of ingrowth of host vessels. In active tissue, sufficient diffusion is confined to 150 μm from the next capillary.[6] Furthermore, the formation of host vessels within the construct takes time.[7] This leads to nutrient limitations and/or hypoxia. Moreover, nutrient and oxygen gradients are present in the outer regions of the scaffold,[8] which could result in non-uniform cell differentiation and integration. If tissue engineering is ever to routinely surpass the tissue thickness limit of 100–200 μm, it must overcome the challenge of creating functional blood vessels to supply cells with oxygen and nutrients and to remove waste products.[9]

In the field of bone tissue engineering, bone implant materials such as ceramics are combined with osteoprogenitor cells. Human mesenchymal stem cells (hMSCs) are commonly used as a source for osteoprogenitor cells. These pluripotent cells are isolated from the bone marrow and have the ability to differentiate into adipogenic, chondrogenic, and osteogenic lineages.[10] Differentiation toward osteoprogenitor cells can be achieved by stimulation with, for instance, bone morphogenic protein-2 or dexamethasone.[11–13] Numerous groups, including our own, have shown that the combination of artificial scaffolds and osteoprogenitor cells can lead to the formation of new bone in both ectopic and orthotopic sites.[14–21] Although the mechanism of bone formation in these settings is not yet fully understood, there is evidence that the implantation of osteoprogenitor cells affects bone formation only if the cells are viable,[21] suggesting that the implanted cells play an active role in the formation of new bone. Vascularization is therefore not only necessary for new bone formation; it is also vital for the survival of the implanted cells on the carrier material after implantation.

A strategy to enhance the vascularization of an implant is the delivery of one or more angiogenic molecules directly to the site of interest. Such molecules include, but are not limited to, vascular endothelial growth factor (VEGF), platelet-

derived growth factor-BB (PDGF-BB), and transforming growth factor-β.[9] Indeed, the dual delivery of VEGF and PDGF-BB from a polymer scaffold resulted in the formation of a mature vascular network.[22] However, this technique still relies on the ingrowth of host endothelial and mural cells, and therefore the vascularization will still take considerable time. So, even though the dual delivery of VEGF and PDGF-BB does show a significant increase in vascularization after 2 weeks, it remains uncertain whether there is an effect at earlier time points, which are most crucial for cell survival.

An alternative approach could be the vascularization of engineered tissue constructs before implantation. Endothelial cells, cultured with or without other cell types on scaffolds or in gels, can spontaneously form a capillary-like network *in vitro*.[23–26] Moreover, we recently showed that a prevascular network formed *in vitro* in a muscle construct anastomosed to the host vessels after implantation, resulting in a better vascularization and survival of the implant tissue.[27] Since this approach does not rely on the ingrowth of host endothelial cells into the entire construct but rather only into the outer regions, it may result in much faster vascularization of the implant.

The goal of the current study was to create an endothelial network in a bone tissue engineering construct. We hypothesized that endothelial cells cocultured with osteoprogenitor cells can organize into a prevascular network *in vitro*. Such a network may contribute to the early vascularization of the implant *in vivo*, resulting in better survival of the implanted cells and enhanced bone formation.

Materials and methods

Culture of human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Basel, Switzerland). Cells were grown at 37 °C in a humid atmosphere with 5% carbon dioxide (CO_2) in endothelial growth medium-2 (Lonza). Cells were routinely split at a 1:5 ratio and cultured for fewer than 5 passages. Only HUVECs from passage 3 or 4 were used to seed the coculture experiments.

Isolation and culture of human mesenchymal stem cells

Bone marrow aspirates (5–20 ml) were obtained from 3 donors, aged 36, 43, and 49 years, with written informed consent. Human mesenchymal stem cells

(hMSCs) were isolated and proliferated as described elsewhere.[18] Briefly, aspirates were resuspended by using a 20-gauge needle, plated at a density of $5 \cdot 10^5$ cells/cm^2, and cultured in minimal essential medium (α-MEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Cambrex), 0.2 mM ascorbic acid (AsAP, Sigma, St. Louis, Missouri), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin (Invitrogen), 10 μg/ml streptomycin (Invitrogen), and 1 ng/ml basic fibroblast growth factor (bFGF, Instruchemie, Delfzijl, the Netherlands). Cells were grown at 37 °C in a humid atmosphere with 5% CO_2. Cells were routinely split at a 1:5 ratio and cultured in fewer than 5 passages. hMSCs from passage 3 or 4 were used to seed the coculture experiments.

Culture of mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) were purchased from Cellartis (Göteborg, Sweden). Cells were grown at 37 °C in a humid atmosphere with 5% CO_2 in Dulbecco's modified eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Cambrex), 100 U/mL penicillin (Invitrogen), and 10 μg/ml streptomycin (Invitrogen). Cells were routinely split at a 1:5 ratio.

Culture of human smooth muscle cells

Human smooth muscle cells (hSMCs) were isolated from the umbilical vein and were kindly provided to us by prof. dr. Istvan Vermes. Cells were grown at 37 °C in a humid atmosphere with 5% CO_2 on gelatin coated plastic in DMEM supplemented with 10% fetal bovine serum (FBS, Cambrex), 10% human serum, 25 mm HEPES buffer (Invitrogen), 100 U/ml penicillin (Invitrogen), and 10 μg/ml streptomycin (Invitrogen). Cells were routinely split at a 1:5 ratio.

Proliferation of HUVECs and hMSCs in different media

To assess the proliferation of HUVECs and hMSCs in different media, they were seeded in T25 culture flasks at a density of 2.700 cells/cm^2 and 2.000 cells/cm^2, respectively. Cells were cultured for 4 days in 3 different media: 1) HUVEC medium (EM) (EBM-2, Lonza), 2) osteogenic differentiation medium (ODM) (α-MEM supplemented with 10% FBS, 0.2mM AsAP, 2 mM L-glutamine, 100 U/ml penicillin, 10 μg/ml streptomycin, 10^{-8} M dexamethasone [Sigma], and 0.01 M β-glycerophosphate [Sigma]), and 3) a 1:1 mix of the first 2 media (EODM). Each day, the cells of 3 flasks were counted by using a Coulter counter (Beckman Coulter, Fullerton, California).

HUVECs and hMSCs were cocultured without direct contact in ODM by using cell culture inserts (Becton-Dickinson, Franklin Lakes, NJ). HUVECs were seeded at a density of 2.700 cells/cm^2 in the wells and hMSCs at a density of 2.700 cells/cm^2 in the cell culture inserts. Cells were allowed to proliferate for 3 days, after which pictures were taken to assess the proliferation of the HUVECs.

Generation and culture of HUVEC-hMSC coculture spheroids

Different percentages of HUVECs and hMSCs were pooled to a total of $5 \cdot 10^5$ cells in a round-bottom 10-ml tube (Greiner, Longwood, FL). The cells were resuspended in 4.5 ml of ODM and consequently centrifuged at 1700 rpm for 2 min. The tubes with the cell pellets were incubated at 37 °C in a humid atmosphere with 5% CO_2, which allowed for the spontaneous formation of coculture spheroids. The spheroids were either cultured for 3 days without a medium change, or for 10 days with a medium change at day 5 and day 8.

In vivo organization assay

After 10 days of *in vitro* culture, 4 spheroids seeded with 2% HUVECs and 98% hMSCs were implanted subcutaneously in the dorsal region of 2 male nude mice. The mice were anesthetized with 2.5% isoflurane, after which the spheroids were implanted in separate pockets. Two weeks after implantation, lectin Helix pomatia agglutinin conjugated to Alexa Fluor 594 (Invitrogen) (0.5mg/0.25ml phosphatebuffered saline [PBS]) was injected into the tail vein of anesthetized animals (20mg/kg body weight). Circulation was allowed for 2 min, after which the animals were euthanized and the implants were retrieved. Samples were snap-frozen in Cryomatrix (Thermo Shandon, Waltham, MA).

Immunohistochemical analysis

After harvesting, spheroids were frozen in Cryomatrix at -60 °C. Sections (6 µm) were cut with a cryotome. Sections were fixed in cold acetone (-20 °C) for 5 min and air-dried. Sections were rehydrated for 10 min, after which they were incubated for 30 min with 10% FBS in PBS to block nonspecific background staining. Sections were incubated with mouse-anti-human CD31 (which does not crossreact with mouse tissue) or mouse-anti-human smooth muscle actin (which does cross-react with mouse tissue) primary antibody (Dako, Glostrup, Denmark) for 1 h. Sections were washed in PBS and subsequently incubated with the secondary antibody (horseradish peroxidase conjugated goat-anti-mouse immunoglobulin

antibody, Dako) for 45 min. Slides were developed with diaminobenzidine (Dako) as substrate and were weakly counterstained with hematoxylin (Sigma). For the *in vivo* samples, Alexa Fluor 488 conjugated goat-anti-mouse immunoglobulin antibody (Invitrogen) was used as the secondary antibody. These samples were neither developed nor counterstained.

Whole spheroids were fixed in cold acetone (-20 °C) for 6 min and subsequently rehydrated in tap water for 15 min. Spheroids were incubated in 10% FBS in PBS for 90 min to block nonspecific background staining. Spheroids were incubated with mouse-anti-human CD31 primary antibody (Dako) for 2 h. Spheroids were washed in PBS for 1 hour and subsequently incubated with the secondary antibody (Alexa Fluor 488 conjugated goat-anti-mouse immunoglobulin antibody, Invitrogen) for 2 h.

Image analysis

Images of the entire surface of cross-sections were taken at a magnification of ×100, unless otherwise stated (Eclipse E600, Nikon, Tokyo, Japan). Images were combined to make a single image of the entire cross-section of each sample. Subsequently, CD31-positive areas were pseudo-colored and Bioquant Image Analysis software (Nashville, TN) was used to determine the percentage of the cross-section that stained positive for CD31. Statistical analysis was performed by using the Student *t*-test.

RNA isolation and quantitative polymerase chain reaction

Spheroids were seeded with 100% hMSCs or 95% hMSCs plus 5% HUVECs and subsequently cultured in osteogenic differentiation medium for 10 days *in vitro*. To acquire sufficient RNA per sample, 3 spheroids were pooled and fragmented mechanically. Three samples (9 spheroids) were prepared. Total RNA was isolated by using a RNeasy Mini Kit (Qiagen, Venlo, the Netherlands) and on column DNase treated with 10U RNase free DNase I (Invitrogen) at 37 °C for 30 min. DNAse was inactivated at 72 °C for 15 min. The quality and quantity of RNA were analyzed by spectrophotometry. Two µg of RNA was used for first strand complementary DNA (cDNA) synthesis by using Superscript II (Invitrogen) according to the manufacturer's protocol. One µl of 100× diluted cDNA was used for 18s ribosomal RNA (rRNA) amplification, and 1 µl of undiluted cDNA was used for human alkaline phosphatase. Polymerase chain reaction (PCR) was performed on a Light Cycler real-time PCR machine (Roche, Basel, Switzerland) by

using SYBR Green I Master Mix (Invitrogen). Data were analyzed by using Light Cycler software version 3.5.3, using fit-point method by setting the noise band to the exponential phase of the reaction to exclude background fluorescence. Expression of the alkaline phosphatase gene was calculated relative to 18s rRNA levels by the comparative ΔCT method.[28] Statistical analysis was performed by using the Student t-test. Primer sequences were as follows: human 18s rRNA, 5′-CGGCTACCACATCCAAGGAA-3′and 5′- GCTGGAATTACCGCGGCT-3′; human alkaline phosphatase, 5′-GACCCTTGACCCCCACAAT-3′ and 5′-GCTCGTACT GCATGTCCCCT-3′.

Results

Cell proliferation in different media

To test the behaviour of both HUVECs and hMSCs in different media, they were cultured on tissue culture plastic in 3 different media: EM, ODM, and EODM. The hMSCs proliferated well on tissue culture plastic in all 3 media. Surprisingly, proliferation was highest when hMSCs were cultured in EODM. Proliferation was lowest in ODM, and EM gave an intermediate result. Although proliferation was highest in EODM, the morphology of the cells also changed in this medium (data not shown), indicating that EM influences the differentiation of hMSCs. HUVECs proliferated well on tissue culture plastic when it was cultured in EM. When cultured in ODM, however, HUVECs did not proliferate. When cultured in the mixed medium, HUVECs proliferated, but growth rate was reduced (Fig. 1). To study whether growth factors secreted by hMSCs could help HUVECs to proliferate in

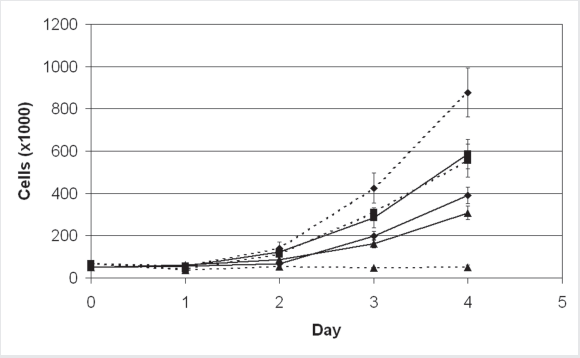

FIG. 1. *Proliferation of human umbilical vein endothelial cells (HUVECs) and human mesenchymal stem cells (hMSCs) in different media. The HUVECs and hMSCs were cultured in HUVEC medium, osteogenic medium, and a 1:1 mixed medium on tissue culture plastic for 4 days. The number of cells was determined every day. Solid line: hMSCs, dotted line: HUVECs, diamond: HUVEC medium, triangle: osteogenic medium, square: mixed medium. Results shown are mean values ± standard deviation (n = 3).*

osteogenic medium, HUVECs were cocultured with hMSCs without direct contact in a cell culture insert assay in ODM. Even though this system allowed the exchange of growth factors between the two cell types, no proliferation of the HUVEC cells could be detected after 3 days.

Spontaneous formation of coculture spheroids

To study the organization of the endothelial cells in a dense 3D cellular environment, a spheroid coculture method was developed. Solid spheroids with a diameter of approximately 1 mm were formed spontaneously between days 3 and 5 with the described method. The spheroids remained intact for the rest of the 10-day culture period (Fig. 2A). Although most cells were incorporated in the spheroids with the method used, smaller cell aggregates were also detected in the medium. The number of these aggregates varied from sample to sample and appeared to be independent of the test parameters. Bone marrow cells from 3 different donors were used to determine whether the formation of cell spheroids was universal or donor specific. The hMSCs of different donors gave similar results regarding spheroid formation (data not shown).

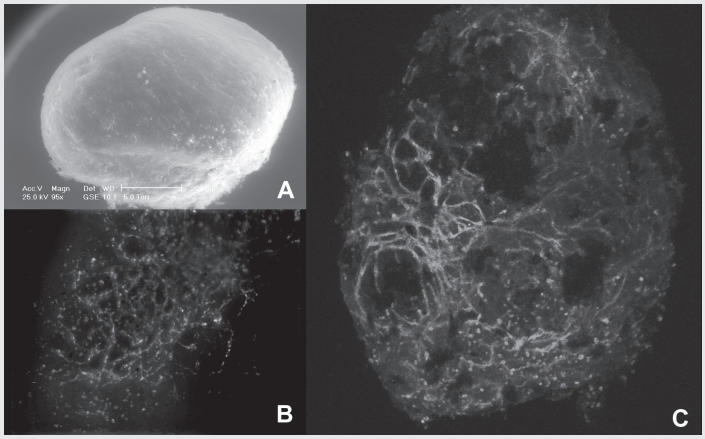

FIG. 2. *(A) Scanning electron microscope picture of coculture spheroids cultured in vitro for 10 days. Scale bar = 200 μm.* ***(B and C)*** *Entire spheroid immunostained with human-specific anti-CD31 antibodies, showing the formation of a 3-dimensional prevascular network. Images were made by using a conventional fluorescence microscope* ***(B)*** *or a convocal fluorescence microscope* ***(C)****.*

Organization of endothelial cells into a 3D prevascular network

After 3 days of pellet coculture, endothelial cells were present as round, individual cells throughout the pellets (data not shown). Image analysis showed that the percentage of endothelial cells had decreased between seeding and day 3 (Fig. 3). Cross-sections of spheroids cultured for 10 days showed that endothelial cells

were still present throughout the spheroids, for all concentrations of HUVECs seeded (Fig. 4). Moreover, endothelial cells had organized into elongated, vessel-like structures. Pictures of whole cell spheroids confirmed that the structures connected to each other and formed a 3D prevascular network of endothelial cells (Fig. 2). Although endothelial cells did organize into a 3D prevascular network, the organization of the network was still primitive, as illustrated by the lack of lumen inside the structures (Fig. 4).

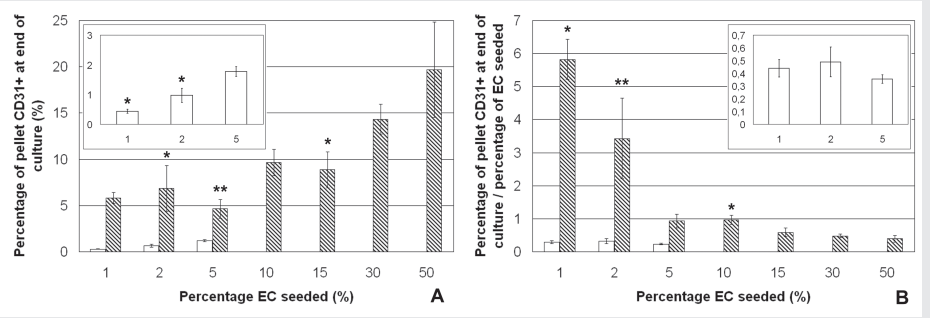

FIG. 3. *Percentages of coculture spheroid cross-section that stain positive for the endothelial marker CD31. Coculture spheroids were seeded with different percentages of human umbilical vein endothelial cells (HUVECs) and cultured in vitro for 10 days. Results shown are mean values ± standard deviation. White bars and insets display the situation after 3 days of culture (n = 4), striped bars show the situation after 10 days of culture (1%: n = 3, 1 donor / 2%: n = 6, 2 donors / 5%: n = 10, 3 donors / 10–50%: n = 3, 1 donor).* ***(A)*** *Percentage of cross-section that stains positive for CD31 after 3 or 10 days of in vitro culture plotted against the percentage of HUVECs seeded.* ***(B)*** *Relative expansion of CD31-positive cells during 3 or 10 days of in vitro culture plotted against the percentage of HUVECs seeded. *p<.05; **p<.005, compared with the group on the right side. EC: endothelial cell.*

Spheroids seeded with different percentages of endothelial cells showed a similar organization of endothelial structures up to 10% of HUVECs seeded. When higher percentages of HUVECs were used, structures appeared less elongated and more endothelial cells could be seen in cell clumps rather than vessel-like structures (Fig. 4).

The area of cross-sections of the spheroids that stained positive for the endothelial marker CD31 was determined. Positive staining was never observed in samples that were seeded with 0% HUVECs. For the samples that were seeded with 50% HUVEC, almost 20% of the cross-section stained positive for the CD31 marker. This percentage dropped with a decrease in the HUVECs seeding density. For samples that were seeded with 5% HUVECs, the area that stained positive for

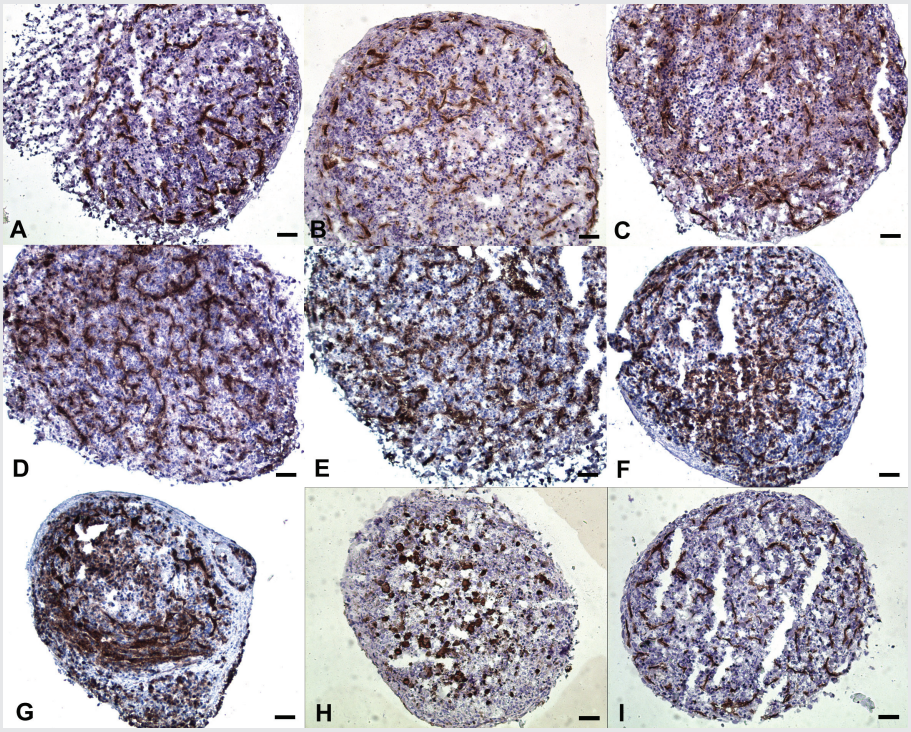

FIG. 4. *Organization of endothelial cells in cocultures seeded with different percentages of human umbilical vein endothelial cells (HUVECs). Spheroids were cultured for 10 days in vitro, and cross-sections were immunostained with human-specific anti-CD31 antibodies (brown) showing the formation of vessel-like structures. Scale bar = 100 μm.* ***(A)*** *1% HUVECs seeded.* ***(B)*** *2% HUVECs seeded.* ***(C)*** *5% HUVECs seeded.* ***(D)*** *10% HUVECs seeded.* ***(E)*** *15% HUVECs seeded.* ***(F)*** *30% HUVECs seeded.* ***(G)*** *50% HUVECs seeded.* ***(H and I)*** *Effect of mouse embryonic fibroblasts (MEFs) on the formation of the prevascular network. Spheroids were seeded with 85% hMSCs plus 5% HUVECs plus 10% MEFs and cultured in vitro for 10 days. Cross-sections were immunostained with human-specific anti-smooth muscle actin* ***(H)*** *and human-specific anti-CD31* ***(I)*** *(brown). Colocalization of smooth muscle actin–positive cells and CD31-positive cells could not be detected.*

the marker was 5%. Interestingly, from that point on the percentage remained a rather constant 5% when the percentage of HUVECs seeded was decreased further up to 1% (Fig. 3A). This means that seeding low percentages of HUVECs has a stimulatory effect on the formation of the prevascular network.

This phenomenon is better demonstrated by plotting the relative expansion of CD31-positive cells against the percentage of HUVECs seeded (Fig. 3B). hMSCs from 3 different donors were used to determine whether the organization of the

endothelial cells was universal or varies with the source of bone marrow cells used. Bone marrow cells of different donors gave similar results regarding endothelial cell organization and proliferation in the cocultures (data incorporated in Fig. 3).

Upregulation of alkaline phosphatase expression in coculture spheroids

The expression of the osteogenic marker alkaline phosphatase was determined in both coculture spheroids and hMSC spheroids. Quantitative PCR analysis showed that the expression of alkaline phosphatase is significantly ($p < .05$; $n = 3$) upregulated with a factor of 4 ± 1.7 by adding 5% HUVECs to the spheroids.

Effect of addition of MEFs on prevascular structures

MEFs were added to the cocultures to test whether these cells can differentiate towards smooth muscle cells and contribute to the stabilization of the prevascular network. When MEFs were added to the hMSC-HUVEC cocultures, smooth muscle actin could be detected in the spheroids, suggesting that cells in these tricultures differentiated towards smooth muscle cells (Fig. 4). Since this phenomenon could not be detected in cocultures of hMSCs and HUVECs alone, it suggests that either the MEF differentiated towards smooth muscle cells or helped other cells in the triculture to differentiate towards smooth muscle cells.

Cells positive for smooth muscle actin were distributed throughout the spheroids, with the exception of the outer perimeter of the spheroids where only a few positive cells were detected. Cells positive for smooth muscle actin did not organize along the vessel-like endothelial structures, and there was no preferred co-localization with these structures. Although MEFs seemed to differentiate toward smooth muscle cells in this system, they did not contribute to stabilization of the vessel-like endothelial structures. This was further confirmed by the fact that triculture spheroids did not show a significant change in the amount and size of vessel-like structures that were formed (data not shown).

Effect of addition of hSMCs on prevascular structures

hSMCs were added to the cocultures to test whether these cells can contribute to the stabilization of the prevascular network. Spheroids were seeded with 5% HUVECs and 0%, 2%, 5%, 10% or 15% hSMCs. When hSMCs were added to the hMSC-HUVEC cocultures, the amount of vessel-like structures that were formed decreased (Fig. 5). Moreover, the organization of the vessel-like structures de-

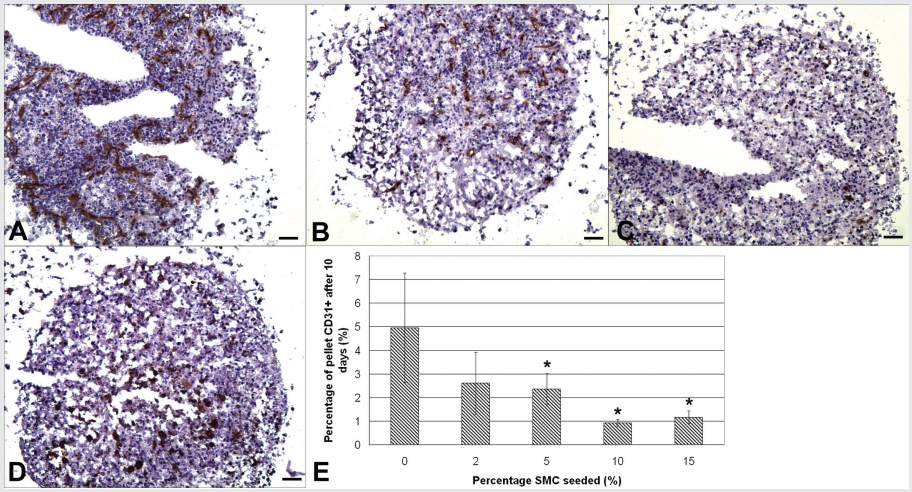

FIG. 5. *Effect of hSMC on vessel-like structure formation in hMSC-HUVEC coculture spheroids. Spheroids were seeded with 5% HUVEC and different percentages of hSMC. Spheroids were cultured for 10 days in vitro, and cross-sections were immunostained with human-specific anti-CD31 antibodies (brown) showing the formation of vessel-like structures. Scale bar = 100 μm.* ***(A)*** *0% hSMCs seeded.* ***(B)*** *5% hSMCs seeded.* ***(C)*** *10% hSMCs seeded.* ***(D)*** *10% hSMCs seeded, immunostained with anti-smooth muscle actin instead of anti-CD31.* ***(E)*** *Percentages of coculture spheroids that stain positive for the endothelail marker CD31 when different percentages of hSMCs were added. Results shown are mean values ± standard deviation (n = 3). *p < .05, compared with 0% hSMCs.*

creased when increasing the percentage of hSMCs. When 10% or 15% of hSMCs were added to the cocultures, $CD31^+$ structures generally consisted of single cells or small cell clumps and did not form elongated vessel-like structures. Cells positive for smooth muscle actin were distributed as round single cells throughout the spheroids (Fig. 5D). These cells did not organize along the vessel-like endothelial structures, and there was no preferred co-localization with these structures.

Stability and organization of the prevascular network in vivo

Coculture spheroids cultured *in vitro* for 10 days were implanted subcutaneously in nude mice to assess the stability and potential anastomosis of the prevascular network *in vivo*. Because of migration of the samples or complete incorporation in the mouse tissue, only 3 of 4 spheroids could be retrieved after 2 weeks of implantation. Cross-sections of the retrieved spheroids confirmed that vessel-like structures were still present after 14 days of subcutaneous implantation. The structures had developed further, and lumen could now frequently be seen inside the vessel-like structures (Fig. 6).

To determine whether the implanted prevascular network had anastomosed and become functional, we injected labeled lectin into the tail vein of the mice before explantation. Lectin perfusion showed that locally, there was costaining of human CD31 and labeled lectin (Fig. 6), indicating either that there was anastomosis of the human vessel-like structures with the blood system of the host or that single or multiple human endothelial cells were incorporated in remodeling or growing blood vessels of the host. Costaining was seen infrequently, only in small structures and only at the edge of the spheroids. No perfused human vessel structures could be detected further away from the periphery of the implant.

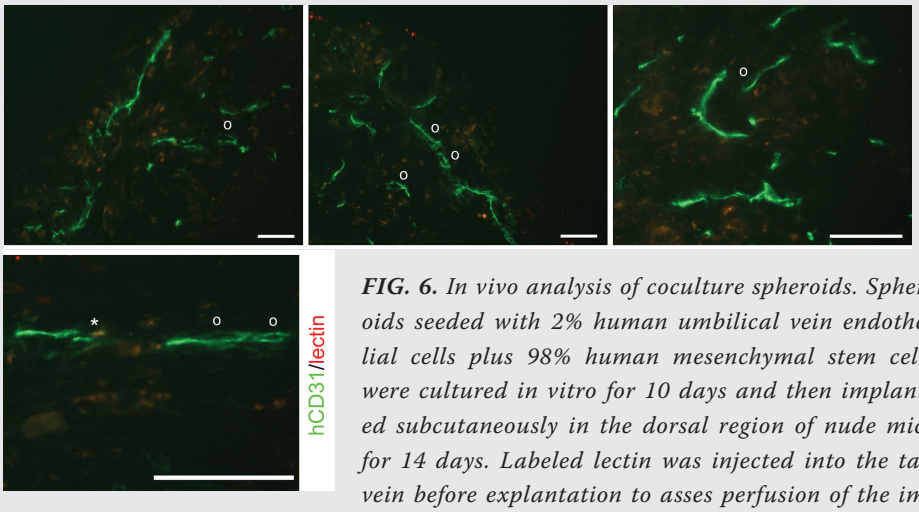

FIG. 6. *In vivo analysis of coculture spheroids. Spheroids seeded with 2% human umbilical vein endothelial cells plus 98% human mesenchymal stem cells were cultured in vitro for 10 days and then implanted subcutaneously in the dorsal region of nude mice for 14 days. Labeled lectin was injected into the tail vein before explantation to asses perfusion of the implant (red). Cross-sections were immunostained with human-specific anti-CD31 antibodies (green). Note the presence of lumen in the vessel structures (o) and the local costaining of lectin and anti-human-CD31 (asterisk). Scale bar = 50 μm.*

Discussion

Rapid vascularization is critical in most cell-based tissue engineering applications to ensure optimal cell survival and implant integration. Several strategies to improve vascularization have been investigated. Most strategies, however, rely on the ingrowth of blood vessels from the host, meaning that vascularization still takes considerable time. A potential strategy to circumvent this is to combine the implant with a prevascular network *in vitro* that can connect to the blood system of the host after implantation, resulting in the fast formation of a vasculature in the implant.

In this study, we investigated the hypothesis that an *in vitro* coculture strategy with osteoprogenitor cells and endothelial cells can result in a prevascular network for the application in bone tissue engineering. We showed that endothelial cells in the presented spheroid coculture model form a 3D prevascular network *in vitro*. The organization of the endothelial cells into a network was generally seen in all samples, but was promoted by seeding 2% HUVECs or less. Addition of MEFs did not result in stabilizing smooth muscle cells or in an increase in the amount of vessel-like structures. Addition of hSMC did not result in stabilization of the vessel-like structures, but rather decreased the amount of structures that were formed. Upon implantation of the coculture spheroids, the prevascular network developed further and lumen could be seen regularly inside the vessel-like structures. Although there was evidence that human endothelial cells or structures in the periphery of the spheroids were incorporated in host vessels, the implanted prevascular network did not yet become extensively perfused.

To our knowledge, we report for the first time the formation of a 3D prevascular network combined with osteoprogenitor cells for the use in bone tissue engineering. Spheroidal cocultures of osteoprogenitor cells and endothelial cells have been reported before,[29,30] but the formation of a 3D prevascular network inside the spheroids had not been further addressed. In contrast, Stahl *et al*.[29] report that coculture spheroids differentiate spontaneously to organize into a core of osteoblasts and a surface layer of endothelial cells. The differences in endothelial cell organization may arise from the fact that these authors used only 500 cells per spheroid and performed the cocultures with a 1:1 ratio of osteoblasts and endothelial cells; as we have demonstrated, this is not an optimal ratio for endothelial cell organization.

Why low percentages of endothelial cells positively affect the formation of the prevascular network remains unclear. After 3 days of culture, there was no statistically relevant difference between the groups seeded with low percentages of HUVEC, indicating that this phenomenon is not caused by differences in seeding efficiencies. Most likely, the communication between the different cell types is more optimal when low percentages of HUVECs are seeded, allowing for a better proliferation and organization of these cells. However, the possibility that a subfraction of hMSCs differentiates toward endothelial cells and gets incorporated into the prevascular structures can not yet be ruled out.

The hMSCs are commonly used as a source for osteoprogenitor cells. These pluripotent cells are isolated from the bone marrow and have the ability to differentiate into adipogenic, chondrogenic, and osteogenic lineages.[10] To stimulate osteogenic differentiation of the hMSCs, osteogenic medium was chosen as the

coculture medium, even though HUVECs were unable to proliferate in this medium on cell culture plastic. Surprisingly, proliferation of HUVECs was seen in this medium in the coculture spheroids. Previous studies have shown that hMSCs secrete growth factors that enhance endothelial cell proliferation.[31,32] However, an indirect coculture model of HUVECs and hMSCs in osteogenic differentiation medium could not restore the proliferation of endothelial cells. This suggests that the effect of the hMSCs on the proliferation of HUVECs in the coculture spheroid model occurs not only via the excretion of growth factors but also via direct cell-cell contact. This direct coupling between endothelial cells and osteoprogenitor cells has been reported before.[33]

Blood vessels are stabilized by association with pericytes or smooth muscle cells.[24,27,34,35] Moreover, endothelial cells can induce the differentiation of undifferentiated embryonic fibroblasts into smooth muscle cells.[24,36] We therefore hypothesized that the formation of vessel-like structures in the spheroid cocultures could be promoted by the addition of undifferentiated embryonic fibroblasts. The addition of MEFs to the spheroids, however, did not result in stabilization of the vessel-like structures, even though cells differentiated toward smooth muscle cells as indicated by the expression of smooth muscle actin. Whether the smooth muscle actin-positive cells arise from hMSCs or MEFs remains uncertain. It has been reported that hMSCs can express smooth muscle actin and that direct coculture with endothelial cells enhances the expression of smooth muscle actin by hMSCs.[37] With the staining techniques used in these studies, however, clear positive staining for smooth muscle actin was not detected in the coculture spheroids but rather only in the triculture spheroids, including the embryonic fibroblasts. This finding indicates that the embryonic fibroblasts give rise to the smooth muscle actin-positive cells. It is unclear why the smooth muscle actin-positive cells did not co-localize with or stabilize the endothelial structures.

The addition of hSMCs to the spheroids had a negative effect on the amount and organization of the vessel-like structures. *In vivo*, blood vessels are stabilized by the recruitment of mural cell precursors, that differentiate towards pericytes or smooth muscle cells after colocalization with the endothelial structures.[38] Therefore, mature smooth muscle cells are less likely to stabilize vessel-like structures *in vitro*. However, the reason for the negative effect remains unclear. It is possible that hSMCs disturb the communication between hMSCs and HUVECs and thus prevent the organization of the endothelial structures.

Previous research has shown that human endothelial networks cultured *in vitro* can anastomose to the host vasculature within 2 weeks after implantation.[27,35] In the present study, however, integration of the prevascular network with the host

vasculature was limited. It is uncertain whether this was due to insufficient differentiation of the prevascular network *in vitro*, the lack of stabilizing smooth muscle cells, or the potential presence of other factors that prevent anastomosis. Even though the present data do not show the formation of an extensive functional vascular network arising from the implanted endothelial cells, they do indicate that the prevascular network formed *in vitro* is stable after implantation and organizes further *in vivo*.

The combination of endothelial cells and osteoprogenitor cells could have beneficiary effects other than the acceleration of vascularization. Endothelial cells and osteoprogenitor cells are known to secrete an array of growth factors that are beneficial for the growth and differentiation of the other. Osteoprogenitor cells are known to secrete VEGF in quantities high enough to enhance the survival and differentiation of endothelial cells.[31,32,39] Endothelial cells, on the other hand, can enhance the proliferation and differentiation of osteoprogenitor cells by the secretion of osteogenic growth factors such as insulin growth factor-1, endothelin-1[40] and bone morphogenic protein-2.[41–43] Moreover, it has been reported that not only growth factors, but also direct contact with endothelial cells, has a positive effect on the alkaline phosphatase activity of osteoblasts.[29,44] This study shows that in this spheroid coculture system, the addition of endothelial cells also upregulates the expression of the osteogenic marker alkaline phosphatase. Although more research is necessary to better understand the differentiation of the hMSCs in this system, our study indicates that the addition of endothelial cells is likely to have a positive effect on the osteogenic differentiation.

Conclusion

In summary, we have demonstrated the *in vitro* formation of a 3D prevascular network in combination with osteoprogenitor cells. Moreover, the addition of endothelial cells to hMSCs is likely to enhance the osteogenic differentiation of these cells, as was shown by the upregulation of alkaline phosphatase expression. Even though contribution of the prevascular network to the vascularization of the spheroid *in vivo* is still limited in this study, these findings indicate that *in vitro* prevascularization is a promising strategy to improve implant vascularization in the field of bone tissue engineering. This approach may also provide a tool for the *in vitro* study of bone vascularization.

Acknowledgement

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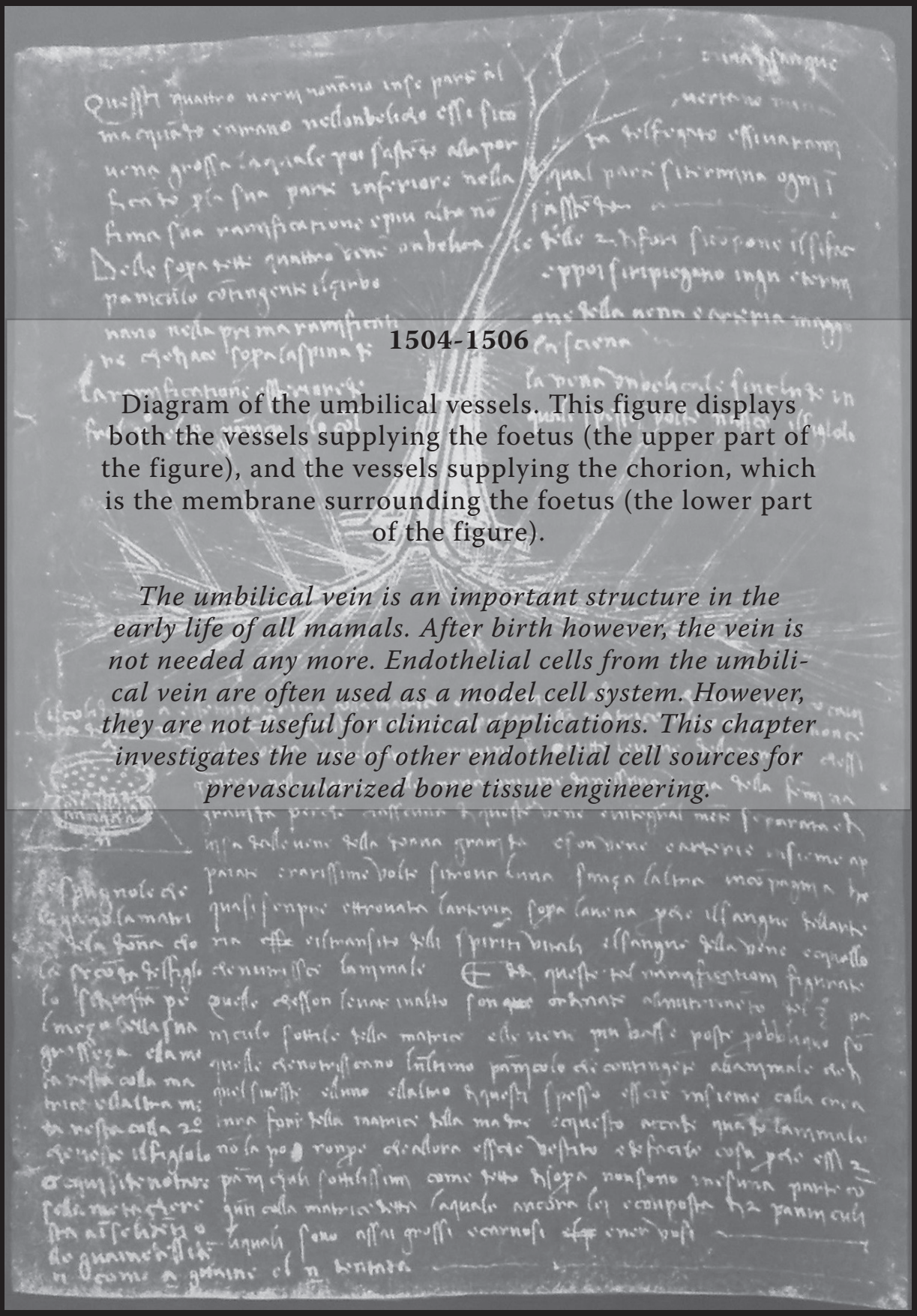
Chapter 5



1504-1506

Diagram of the umbilical vessels. This figure displays both the vessels supplying the foetus (the upper part of the figure), and the vessels supplying the chorion, which is the membrane surrounding the foetus (the lower part of the figure).

The umbilical vein is an important structure in the early life of all mamals. After birth however, the vein is not needed any more. Endothelial cells from the umbilical vein are often used as a model cell system. However, they are not useful for clinical applications. This chapter investigates the use of other endothelial cell sources for prevascularized bone tissue engineering.

Chapter 5

The use of endothelial progenitor cells from blood and bone marrow for prevascularized bone tissue engineering

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Abstract

One of the major limitations of tissue engineering is the inability to provide sufficient blood supply in the initial phase after implantation. *In vitro* prevascularization can be a strategy to solve this problem. Although recent publications show promising results, these studies were generally performed with clinically irrelevant endothelial cell model systems. We tested the use of endothelial progenitor cells (EPC) from the blood and bone marrow for their use in a prevascularized bone tissue engineering setting. Human mesenchymal stem cells (hMSC) were differentiated towards endothelial cells. They formed capillary-like structures containing lumen, stained positive for CD31, attained the ability to take up acetylated low density lipoproteins (acLDL) and formed perfused vessels *in vivo*. However, in a 3D coculture setting with undifferentiated hMSC, the cells dedifferentiated and did not form prevascular structures. EPC from the cord blood were able to form prevascular structures in the same coculture setting, but only when the state of endothelial differentiation was mature. The amount of prevascular structures formed when using EPC was less than when human umbilical vein endothelial cells (HUVEC) or human dermal microvascular endothelial cells (HMVEC) were used. The degree of organization, however, was higher. We conclude that EPC can be used for complex tissue engineering applications, but that the differentiation stage of these cells is of importance.

Adapted from Rouwkema J, *et. al.* The use of endothelial progenitor cells from blood and bone marrow in a complex tissue engineering setting. *Stem Cells, Submitted*

Introduction

In the last few years, the focus in tissue engineering has become more complex. Instead of growing a single cell source on a uniform scaffold, one is now trying to mimic the natural tissue as close as possible by using the several cell types that make up a tissue and scaffolds with specific regions for specific organization of the tissue. However, one of the major limitations of tissue engineering is still the inability to provide sufficient blood supply in the initial phase after implantation. As long as a proper vascularization has not been established, the implant has to rely on diffusion for the supply of nutrients and the removal of waste. This can lead to nutrient limitations, which can result in improper integration or even death of the implant. Since vascularization is an important issue in tissue engineering, the inclusion of endothelial (progenitor) cells to tissue engineered constructs has become a point of focus in tissue engineering.[1]

In the field of bone tissue engineering, bone implant materials like ceramics are combined with osteoprogenitor cells. Human mesenchymal stem cells (hMSC) are commonly used for this purpose. These pluripotent cells can be isolated from the bone marrow and have the ability to differentiate into adipogenic, chondrogenic and osteogenic lineages.[2] Differentiation towards osteoprogenitor cells can be achieved by stimulation with for instance BMP-2 or dexamethasone.[3-5] Numerous groups, including our own, have shown that the combination of artificial scaffolds and osteoprogenitor cells can lead to the formation of new bone in both ectopic and orthotopic sites.[6-10] Vascularization is vital for the survival of the implanted cells on the carrier material after implantation. Apart from that, vascularization is a critical process during bone growth and repair. Studies have shown that fracture healing and ectopic new bone formation can be blocked by the administration of angiogenesis inhibitors, [11,12] while others have shown that new bone formation in porous scaffolds was significantly increased by the insertion of a vascular pedicle in the scaffold.[13,14]

Previous studies have shown that endothelial cells can form vascular structures *in vitro*[15-17] that can anastomose to the vasculature of the host after implantation.[18,19] Prevascularization of a tissue can enhance the implant perfusion and survival.[19] We previously reported the formation of a prevascular network *in vitro*, by combining hMSC and human umbilical vein endothelial cells (HUVEC).[20] These results were promising for prevascularized bone tissue engineering, since the co-culture did not negatively influence the osteogenic differentiation of the hMSC. The aforementioned studies however, were in general performed with non-clinically applicable endothelial cell sources like HUVEC. If one wants to implement *in vitro* prevascularization in clinical applications, one should be able to use an

endothelial cell source that can be readily isolated from adult patients in sufficient numbers in an acceptable timeframe. Here we tested the potential use of endothelial progenitor cells isolated from blood and differentiated from mesenchymal stem cells in comparison with both microvascular and macrovascular mature endothelial cells.

There are studies indicating that mesenchymal stem cells can differentiate towards endothelial cells *in vitro*.[21-23] Since mesenchymal stem cells are already being used for bone tissue engineering, endothelial cells derived from this cell source would be ideal for prevascularized bone tissue engineering. Endothelial differentiation of mesenchymal stem cells would allow for prevascularized bone tissue engineering from one single cell source.

Another possible endothelial cell source for prevascularized bone tissue engineering are endothelial progenitor cells (EPC). EPC originate from the bone marrow and can be mobilized upon secretion of angiogenic factors like SDF-1α and VEGF by ischemic tissue.[24] EPC then circulate in the blood and can differentiate into functional endothelium.[25] EPC can be derived from hematopietic stem cells or from angiogenic monocytes.[24,26] EPC can be isolated and differentiated from peripheral blood using *ex vivo* culture.[25] These EPC display endothelial phenotypical characteristics and can enhance neovascularization by incorporation and differentiation, and by the secretion of angiogenic factors affecting resident endothelium.[27-29] EPC seeding of tissue engineered small diameter vascular grafts resulted in formation of an endothelial layer capable of fully covering the luminal surface *in vitro*.[30,31] Importantly, clinical application of *ex vivo* cultured EPC has proven to be safe and feasible, as autologous EPC cultured from blood have been applied in an experimental setting to treat patients with acute myocardial infarction.[32] With promising results *in vitro* and *in vivo* and easy accessibility, EPC are an attractive candidate for prevascularized tissue engineering strategies.

The goal of the current study is twofold: 1) to develop a new method to differentiate human MSC towards endothelial (progenitor) cells, and 2) to investigate the potential role of these cells and endothelial progenitor cells from the blood for prevascularized bone tissue engineering. We investigate whether these cells are capable of forming or inducing three dimensional prevascular networks in a coculture with undifferentiated hMSC. If endothelial progenitor cells in coculture with hMSC do indeed form a prevascular network, they would be an easily accessible and practical cell source for prevascularized bone tissue engineering.

Materials and methods

Culture of HUVEC and HMVEC

HUVEC and human dermal microvascular endothelial cells (HMVEC) were purchased from Lonza (Basel, Switzerland). Cells were grown at 37 °C in a humid atmosphere with 5% carbon dioxide (CO_2) in endothelial growth medium-2 (EGM-2, Lonza). Cells were routinely split at a 1:5 ratio and cultured < 5 passages. Only cells from passage 3 or 4 were used to seed the coculture experiments.

Isolation and culture of hMSC

Bone marrow aspirates were obtained from two donors, aged 27 and 65, with written informed consent. Aspirates were resuspended using a 20G needle and plated at a density of $5 \cdot 10^5$ mononucleated cells/cm^2. Cells were grown in MSC proliferation medium (Minimal essential medium (α-MEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Cambrex), 0.2 mM ascorbic acid (AsAP, Sigma), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin (Invitrogen), 10 µg/ml streptomycin (Invitrogen), and 1 ng/ml basic fibroblast growth factor (bFGF, Instruchemie, Delfzijl, The Netherlands)) at 37 °C in a humid atmosphere with 5% CO_2. Cells were characterized by FACS and tested > 90% positive for CD29, CD44, CD105 and CD166. Cells tested negative for CD31. Cells were routinely split at a 1:5 ratio and cultured < 5 passages. MSC from passage 3 or 4 were used to seed the coculture experiments. All results in this paper were similar for both donors.

Endothelial induction of hMSC

hMSC from passage 4 were used for the endothelial induction assays. Cells were seeded on tissue culture plastic and cultured in MSC proliferation medium without bFGF supplemented with 50 ng/ml hrVEGF$_{165}$ (Sigma) for 10 days. Cells were split at a 1:6 ratio at sub-confluence. For induction on Matrigel, the wells of a 6-well plate were coated with 0,9 ml growth factor reduced Matrigel (BD Biosciences) per well. Cells were seeded at a density of $2 \cdot 10^4$ cells per well. Cells were grown in EGM-2 at 37 °C in a humid atmosphere with 5% CO_2. The formation of capillary-like structures was followed over time using an inverted microscope (Nikon Eclipse TE300). At day 10, pieces of Matrigel were frozen in Cryomatrix (Thermo Shandon, Waltham, MA) at -60°C. Cross sections (6 µm) were cut with a cryotome and stained with hematoxylin (Sigma). For Immunohistochemical analysis, hMSC were seeded on Matrigel coated cover slips in stead of 6-well plates. hMSC induced on Matrigel for 10 days were used for coculture experiments (EC-

MSC)

Acetylated low-density lipoproteins (acLDL) uptake assay

hMSC that were cultured on Matrigel in EGM-2 for 9 days were removed by incubating in a 1:1 mixture of 0,25% trypsin and dispase for 15 minutes. Recovered cells were seeded on cover slips and cultured in EGM-2 for one more day. Subsequently, cells were incubated in EGM-2 supplemented with 10 µg/ml DiI-labeled acLDL (DiI-acLDL; Invitrogen) for 4 hours at 37 °C in a humid atmosphere with 5% CO_2. Finally, cells were washed with phosphate buffered saline (PBS) 3 times for 5 minutes.

In vivo assay

hMSC were cultured on Matrigel in EGM-2 for 14 days to allow them to organize into capillary-like structures. Four pieces of Matrigel containing these structures were then implanted subcutaneously in the dorsal region of 2 male nude mice. The mice were anesthetized with 2,5% isoflurane, after which the samples were implanted in separate pockets. Two weeks after implantation, the animals were euthanized and the implants were retrieved. Samples were snap-frozen in Cryomatrix.

Isolation and culture of human EPC

Mononuclear cells (MNC) were isolated from umbilical cord blood by Ficoll density gradient separation (Histopaque 1077; Sigma, St. Louis, USA). MNC were plated on gelatin (Sigma) coated 6-wells plates at a density of 10^7 cells per well in induction medium (M199 medium (Invitrogen) containing 20% fetal calf serum (Invitrogen), 0.05 mg/ml bovine pituitary extract (Invitrogen), 10 units/ml heparin (Leo Pharma, Breda, the Netherlands), and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml; Invitrogen)). Medium was changed after 4 days, washing non-adherent cells away. After 7 days, cells had adopted a spindle-shape morphology and proliferated minimally (low-proliferative spindle-shaped EPC, LP-SS-EPC).

Subsequently, medium was changed to differentiation medium (EGM-2 medium, supplemented with 20% fetal calf serum (Invitrogen) and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml; Invitrogen)), facilitating differentiation towards high-proliferative 'cobblestone'-shaped EPC (HP-CS-EPC). Twice weekly,

half of the medium was removed and replaced with fresh medium. Upon reaching confluence, HP-CS-EPC were passaged onto fibronectin (Becton Dickinson) coated wells in regular EGM-2 medium, which was used for further culture. The total culture period for obtaining HP-CS-EPC was four weeks on average.

LP-SS-EPC cultured for a similar period of time without switching to differentiation medium retained the spindle-shaped morphology. These cells, late-outgrowth LP-SS-EPC, were also used for coculture experiments.

EPC characterization

EPC were evaluated for the ability to bind FITC-labeled Ulex Europeus Lectin-1 (UEA-1; Vector, Burlingame, USA) and to take up DiI-acLDL (Invitrogen). For this, EPC cultured on fibronectin-coated coverslips were washed in PBS and placed in EGM-2 with 1:100 diluted FITC-labeled UEA-1 for 1 hour at 37 °C, followed by a 2 hour incubation in EBM-2 with 1:400 diluted DiI-acLDL at 37 °C. Finally, cell nuclei were stained with DAPI and cover slips were mounted on glass slides using Vectashield (Vector laboratories, Burlingame, CA, USA).

The presence of CD31 and Von Willebrand Factor (vWF) was identified in EPC cultured on coverslips, fixed in 4% paraformaldehyde, and permeabilized (only for vWF staining) using 0.1% saponin in PBS. Coverslips were incubated with anti-CD31 or anti-vWF antibody (Dako), followed by a FITC-labeled goat-anti-mouse secondary antibody (BD Pharmingen) and DAPI. Isotype-stained sections served as controls. Staining was evaluated by fluorescence microscopy.

Labeling with 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE)

Cells were detached using trypsin-EDTA and resuspended in PBS to a final concentration of $1 \cdot 10^6$ cells/ml with 10 μM CFSE (Sigma). After 10 minutes incubation at 37 °C, cells were washed in PBS, resuspended in 5 ml of medium, and again incubated for 5 minutes at 37 °C to allow excess CFSE to wash out. Cells were washed with PBS twice before being used for further experiments.

Generation and culture of coculture spheroids

Coculture spheroids were prepared as described before.[20] In brief, different percentages of endothelial (progenitor) cells and hMSC were pooled to a total of $5 \cdot 10^5$ cells in a round-bottom 10 ml tube (Greiner). The cells were re-suspended in 4.5 ml osteogenic differentiation medium (α-MEM supplemented with 10% FBS,

0.2 mM AsAP, 2 mM L-glutamine, 100 U/ml penicillin, 10 µg/ml streptomycin, 10^{-8} M dexamethasone (Sigma), and 0.01 M β-glycerophosphate (Sigma)) and consequently centrifuged at 1600 rpm for 2 minutes. The tubes with the cell pellets were incubated at 37 °C in a humid atmosphere with 5% CO_2, which allowed for the spontaneous formation of coculture spheroids. The spheroids were cultured for 10 days with a medium change at day 5 and day 8.

Immunohistochemical analysis

After harvesting, spheroids were frozen in Cryomatrix (Thermo Shandon) at -60 °C. Sections (6 µm) were cut with a cryotome. Sections were fixed in cold acetone for 5 minutes and air dried. Before staining, sections were rehydrated for 10 minutes, after which they were incubated for 30 minutes with 10% FBS in PBS to block non-specific background staining. Sections were then incubated with mouse-anti-human CD31 (does not cross-react with mouse tissue) primary antibody (Dako) for 1 hour. Sections were washed in phosphate-buffered saline and subsequently incubated with the secondary antibody (horseradish peroxidase conjugated goat-anti-mouse immunoglobulin antibody, Dako) for 45 minutes. Slides were developed with diaminobenzidine (Dako) as substrate, and weakly counterstained with hematoxylin (Sigma). For fluorescent staining, Alexa Fluor 594 conjugated goat-anti-mouse immunoglobulin antibody (Invitrogen) was used as the secondary antibody. These samples were neither developed nor counterstained.

Image analysis

Images of the entire surface of cross sections were taken at a magnification of 100x, unless otherwise stated (Nikon Eclipse E600). Images were combined to make a single image of the entire cross section of each sample. Subsequently, CD31 positive areas were pseudo colored and Bioquant image analysis software (Bioquant image analysis corp.) was used to determine the percentage of the cross section that stained positive for CD31. Statistical analysis was performed using the Student's t-test.

Results

Endothelial induction of hMSC

The addition of 50 ng/ml VEGF to hMSC resulted in the expression of the endothelial marker CD31 in approximately all the cells after 10 days as assessed by

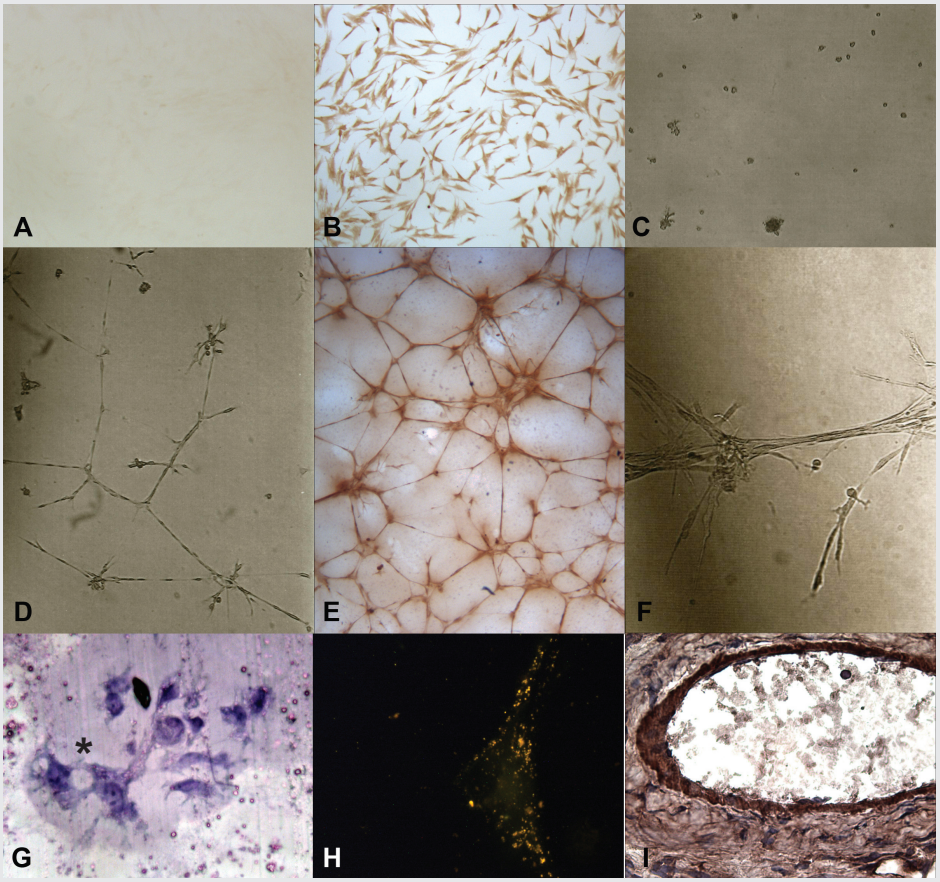

FIG. 1. *Endothelial induction of hMSC.* ***(A)*** *CD31 immunostaining of hMSC cultured in MSC proliferation medium for 10 days.* ***(B)*** *CD31 immunostaining of hMSC cultured in MSC proliferation medium without bFGF, with 50 ng/ml $VEGF_{165}$ for 10 days.* ***(C)*** *hMSC cultured on Matrigel for 7 days in MSC proliferation medium.* ***(D)*** *hMSC cultured on Matrigel for 4 days in EGM-2 medium.* ***(E)*** *CD31 immunostaining of hMSC cultured on Matrigel for 4 days in EGM-2 medium.* ***(F)*** *hMSC cultured on Matrigel for 7 days in EGM-2 medium.* ***(G)*** *Cross section of hMSC cultured on Matrigel in EGM-2 medium for 10 days. Note the formation of lumen-like structures (asterix).* ***(H)*** *Uptake of ac-LDL by hMSC cultured on Matrigel in EGM-2 medium for 9 days and subsequently on a glass coverslip for 1 day.* ***(I)*** *human specific CD31 immunostaining (brown) of structures that were implanted subcutaneously in nude mice for 2 weeks.*

immunostaining (Fig. 1B). For improved endothelial differentiation, hMSC were cultured on Matrigel in EGM-2. This resulted in the formation of capillary-like structures within four days, whereas cells that were cultured on Matrigel in MSC proliferation medium retained a round morphology and did not organize or proliferate (Fig. 1C and D). Visual observation revealed that > 90% of the cells present were incorporated in these structures. Immunohistochemical analysis showed that the structures that were formed expressed the endothelial marker CD31 after 4 days of culture (Fig. 1E). After 7 days, the capillary-like structures had organized further into tubes containing multiple cells (Fig. 1F). Cross sections showed that lumina were present (Fig. 1G). Cells that were cultured on Matrigel in EGM-2 for nine days were removed from the gel and reseeded on tissue culture plastic. Although their morphology returned to a fibroblast-like morphology, they were able to take up acLDL (Fig. 1H), although uptake was limited when compared to HUVEC. Four samples of Matrigel containing capillary-like structures were implanted subcutaneously in nude mice. Because of resorption of the Matrigel, only two pieces of samples could be retrieved together with surrounding tissue after 14 days of implantation. Cross sections stained for human specific CD31 revealed a limited number of vessels consisting of implanted human cells (Fig. 1I). The vessels were perfused, as was shown by the presence of erythrocytes.

Endothelial progenitor cells

Within hours after initial plating, MNC in part clustered and attached to the well surface (Fig. 2); from the adherent clusters spindle-shaped cells grew within 4 to 7 days (early-outgrowth LP-SS-EPC; Fig. 2). Depending on culture conditions, LP-SS-EPC either maintained their spindle-shaped morphology, or were induced to undergo a phenotypical change and change in proliferation rate while adopting a 'cobblestone' morphology (HP-CS-EPC; Fig. 2). Importantly, HP-CS-EPC did not appear to originate from distinct foci of proliferation in the well, but rather from a generalized transition in phenotype of the LP-SS-EPC (Fig. 2), with subsequent proliferation to confluence. Late-outgrowth LP-SS-EPC migrated out of the initial clusters to become dispersed throughout the well, but proliferated minimally and never reached confluence.

Both spindle- and cobblestone-shaped EPC bound UEA-1 and took up acLDL (Fig. 3), which are considered typical EPC-characteristics. Interestingly, vWF expression, which is a highly specialized characteristic of endothelial cells, was not observed in early or late outgrowth LP-SS-EPC. In contrast, HP-CS-EPC did show vWF expression, although expression was not as high as in HUVEC (Fig. 3). These observations are consistent with an immature endothelial phenotype in early- and late outgrowth LP-SS-EPC versus a more mature phenotype in HP-CS-EPC.

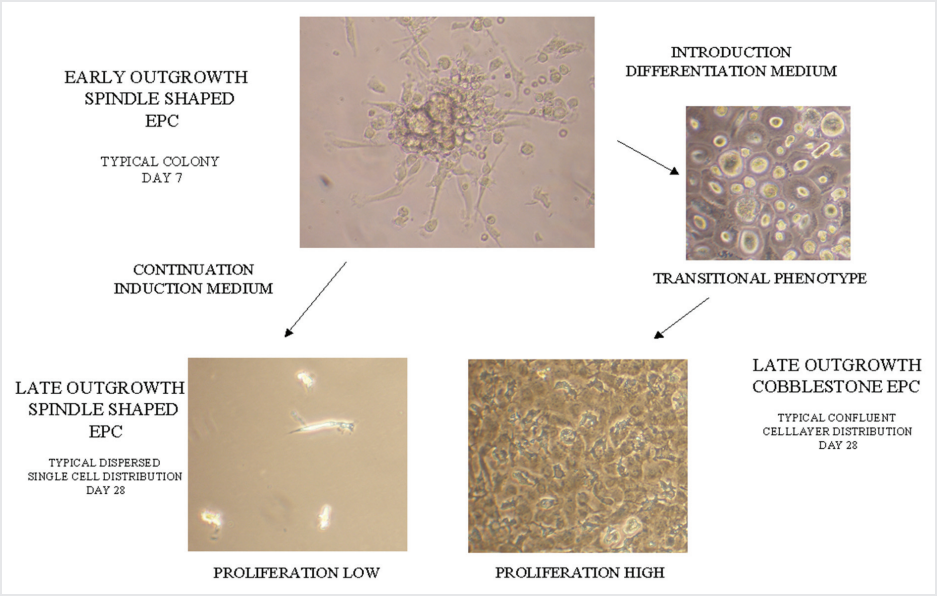

FIG. 2. *EPC morphology by light microscopy. At day 7 after plating blood mononuclear cells in EPC induction medium, typical colonies of early-outgrowth spindle shaped EPC were observed with the spindle shaped cells predominantly located at the colony edges. Continuation of the serum and cytokine-rich induction medium in the EPC culture resulted in late-outgrowth spindle shaped EPC, which were found dispersed throughout the well and did not have the proliferative capacity to reach confluence. In contrast, introducing the EGM-medium based differentiation medium induced morphological changes in the EPC, resulting in a high-proliferative EPC type with cobble-stone morphology that became confluent after approximately four weeks total culture time.*

Coculture spheroids

The formation of coculture spheroids was similar as reported before.[20] Solid spheroids with a diameter of approximately 1 mm were formed spontaneously between day 3 and day 5 with the method described. These spheroids remained intact for the remainder of the 10 day culture period. This current study revealed that the different endothelial (progenitor) cells used did not alter the formation of coculture spheroids.

Prevascular network formation

Different endothelial (progenitor) cells were used to study their potential for the formation of a prevascular network in a bone tissue engineering setting. Im-

munostaining for the endothelial marker CD31 revealed that samples that were seeded with EC-MSC, early outgrowth LP-SS-EPC or late outgrowth LP-SS-EPC did not contain CD31 positive cells after 10 days of coculture (Fig. 4J). However, round unorganized CFSE labeled endothelial progenitor cells were still present (Fig. 4K), indicating that these cells had dedifferentiated during the course of co-culture.

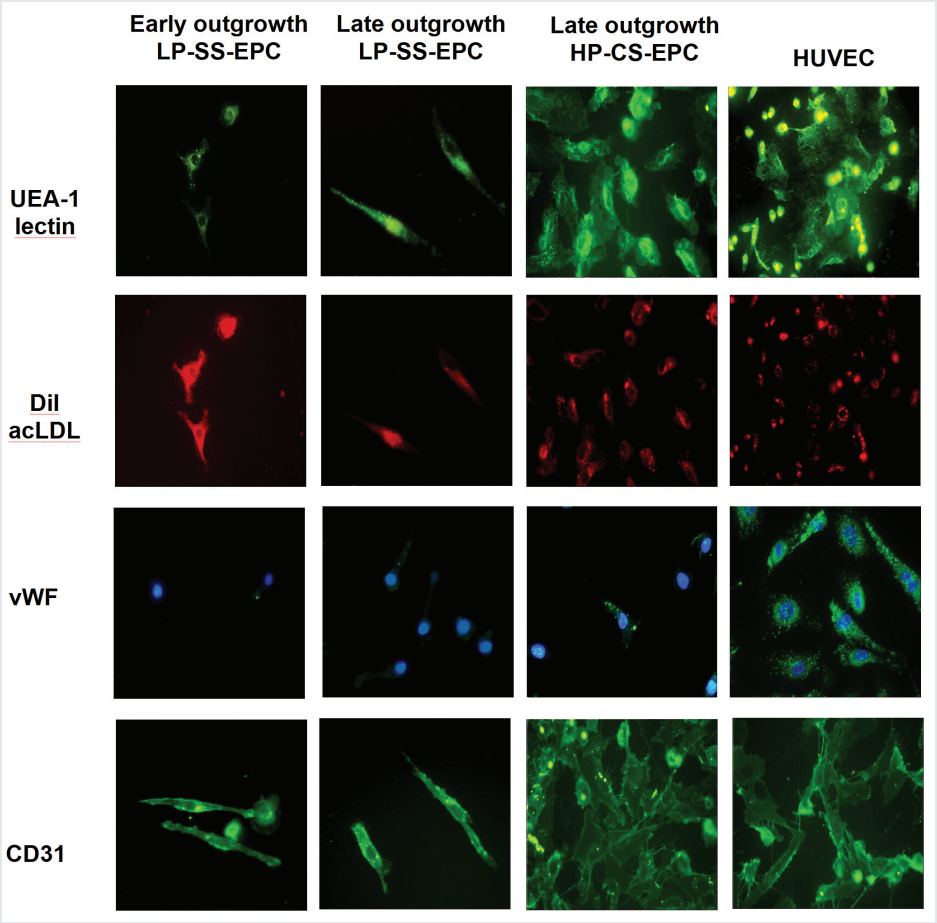

FIG. 3. *EPC characterization. Early and late outgrowth LP-SS-EPC and late outgrowth CS-HP-EPC bound FITC-labeled UEA-1 lectin, took up DiI-labeled acetylated LDL and stained positive for CD31, similar to mature endothelial cells (HUVEC). However, expression of von Willebrand Factor (vWF), a protein highly specific to endothelial cells, was not observed in SS-LP-EPC, in contrast to CS-HP-EPC that contained some vWF, although not in such high levels as HUVEC.*

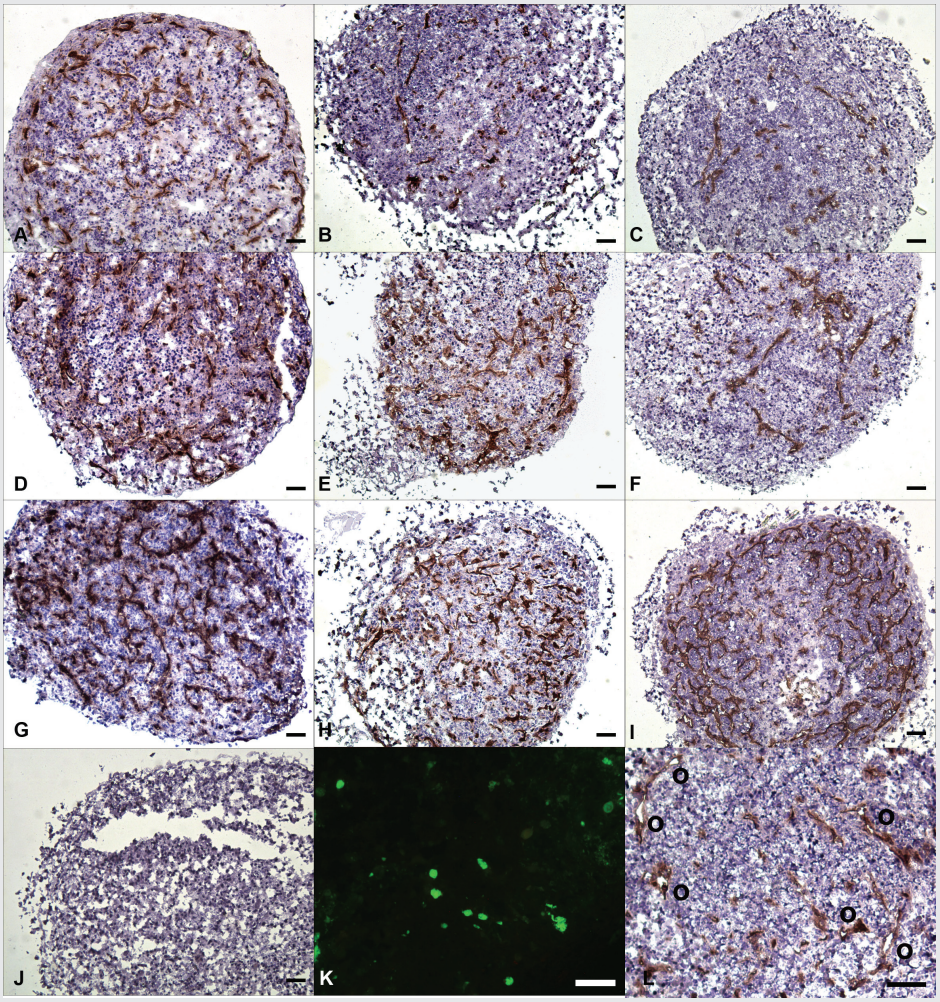

FIG. 4. *CD31 staining (brown) of coculture spheroids cultured in osteogenic differentiation medium for 10 days. Counterstained with hematoxylin. Scale bar = 100 μm.* ***(A, D and G)*** *Cocultures of HUVEC and hMSC. 2%, 5% and 10% HUVEC seeded respectively.* ***(B, E and H)*** *Cocultures of HMVEC and hMSC. 2%, 5% and 10% HMVEC seeded respectively.* ***(C, F and I)*** *Cocultures of HP-CS-EPC and hMSC. 2%, 5% and 10% HP-CS-EPC seeded respectively.* ***(J)*** *Coculture of late outgrowth LP-SS-EPC and hMSC. No positive staining for CD31 can be detected. Results are similar for early outgrowth LP-SS-EPC and EC-MSC.* ***(K)*** *Coculture of CFSE (green) labeled late outgrowth LP-SS-EPC and hMSC stained for CD31 (red). No CD31-positive staining can be detected, even though CFSE labeled cells are still present. Results are similar for early outgrowth LP-SS-EPC and EC-MSC.* ***(L)*** *Close up of co-culture seeded with 5% HP-CS-EPC and hMSC. Note the presence of lumen (o) in the prevascular structures.*

Samples seeded with HP-CS-EPC, HMVEC or HUVEC did contain CD31 positive prevascular structures after 10 days (Fig. 4). When different cells were used, the amount of CD31 positive structures varied. The amount of CD31 positive structures was quantified as the percentage of a cross section of the spheroid that stained positive for CD31. Quantification showed that seeding low percentages of HUVEC has a positive effect on the survival/proliferation of these cells (Fig. 5A). When 5, 10 or 15 percent HUVEC was seeded, the area that stained positive after 10 days of coculture was less than 5, 10 or 15 percent respectively. When 1 or 2 percent of HUVEC was seeded however, 5,8 ± 0,6% and 6,9 ± 2,4% of the area stained positive for CD31 respectively. When HMVEC was used for the coculture, results were similar to HUVEC for 5 or 10 percent endothelial cells seeded. Contrary to HUVEC however, seeding low percentages of HMVEC hardly had a positive effect on the survival/proliferation of these cells. When 1 or 2 percent of HMVEC was seeded, 1,7 ± 1,0% and 1,7± 0,8% of the area stained positive for CD31 respectively. HP-CS-EPC performed comparable to HUVEC and HMVEC for 5, 10 and 15 percent endothelial cells seeded. When lower percentages of EPC were seeded however, there was no positive effect on the survival/proliferation of these cells. Seeding 1 or 2 percent EPC resulted in 0,22 ± 0,26% and 0,71± 0,56% of the area staining positive for CD31 respectively.

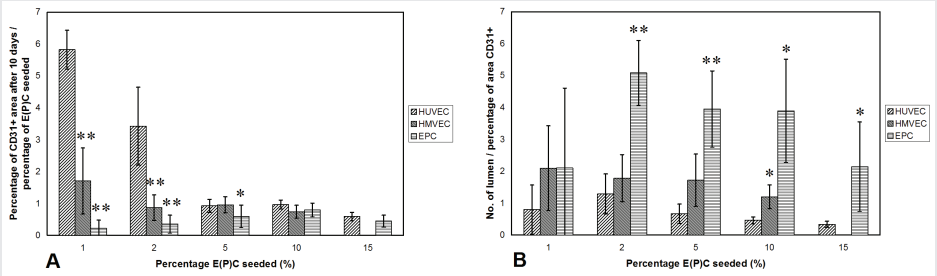

FIG. 5. *Quantification of the prevascular structures. Spheroids were seeded with different percentages of HUVEC, HMVEC and HP-CS-EPC and cultured in vitro for 10 days. Results shown are mean values ± standard deviation (n=4).* ***(A)*** *Percentage of coculture spheroid cross-section that stains positive for the endothelial marker CD31. The relative expansion of CD31-positive cells is plotted against the percentage of E(P)C seeded.* ***(B)*** *Number of lumen per cross section. The relative number of lumen (standardized for the percentage of CD31⁺ area) is plotted against the percentage of E(P)C seeded. *p < .05; **p < .005, compared to HUVEC.*

As for the morphology of the CD31⁺ structures, a difference was observed when either HUVEC, HMVEC or HP-CS-EPC were used for the cocultures. Whereas HUVEC tended to organize more in cell clumps in stead of prevascular structures

at higher seeding densities, HP-CS-EPC and to a lesser extent HMVEC organized into more elongated prevascular structures. Apart from that, the number of lumen was higher when HP-CS-EPC were used (Fig. 4L and 5B). The number of lumen when using HMVEC also seemed higher compared to HUVEC. However, a statistical difference was only evident when 10 percent of endothelial cells were seeded.

Discussion

Vascularization is recognized as an important factor in tissue engineering. It has been shown that the addition of endothelial cells to tissue cultures *in vitro* can result in the formation of prevascular structures.[33,18,19] Moreover, we recently showed that prevascularizing a tissue *in vitro* can enhance tissue vascularization and survival *in vivo*.[19] However, most of these studies were performed with endothelial cell model systems which are not directly clinically applicable. For clinical use, an endothelial cell source should be found that can be easily isolated from the patient and can be expanded to sufficient numbers.

A potential source of endothelial cells are endothelial progenitor cells from the blood. These cells can be isolated relatively easy and can be expanded to clinically significant numbers. Several isolation protocols for EPC have been established. In this study we chose to isolate EPC without prior sorting. Mononuclear cells from the cord blood were directly seeded on gelatin and cultured under different conditions. This yielded three distinct groups of EPC in different stages of endothelial differentiation, which allowed us to investigate the effect of the stage of endothelial differentiation for use in a complex tissue engineering application.

EPC from the blood have successfully been tested for their use in tissue engineering applications.[34-36] However, the complexity of most models was limited. In general, the EPC were used as the sole cell source, or only combined with a single mature cell type. This means that optimal culture conditions and media could be used to sustain the EPC, or that the secretion of disturbing growth factors by other cell types was limited. Tissue engineering however, is focusing more and more on complex culture systems using multiple (progenitor) cell types to mimic the *in vivo* situation.[37,38] It is likely that these situations will be more challenging for the cells, since this requires the use of suboptimal culture conditions and intermediate medium systems to allow all cell types to perform simultaneously. Apart from that, the use of multiple progenitor cells is likely to give rise to cellular interactions that could influence the differentiation of the progenitor cells used.

When labeled EC-MSC, early outgrowth LP-SS-EPC or late outgrowth LP-SS-EPC were cocultured with undifferentiated hMSC, no positive staining for CD31 could be seen after 10 days. However, labeled cells were still present in the spheroid cross sections. The cells had not organized and were present as round, single cells. The lack of CD31 staining is an indication that the endothelial progenitor cells de-differentiated during the course of coculture. HP-CS-EPC, as well as HUVEC and HMVEC, on the other hand, were capable of forming well organized CD31 positive prevascular structures during ten days of coculture with hMSC. Since only HP-CS-EPC, the EPC used with the most mature endothelial phenotype, and the mature HUVEC and HMVEC are capable of forming prevascular structures, these results clearly demonstrate the difference between the use of immature and mature EPC in this complex tissue engineering setting. In the presence of alternate cell types, EPC appear to de-differentiate and loose their capacity for neovascularization. With this, we have identified a degree of EPC pre-differentiation to be a prerequisite for successful application in complex tissue engineering strategies. vWF expression might be a potential marker for an adequate state of differentiation.

When comparing the morphology of the prevascular structures while using HP-CS-EPC, HUVEC or HMVEC, distinct differences can be seen. HMVEC and especially HP-CS-EPC form more elongated structures, containing more lumen as compared to HUVEC. Similar results were obtained in a study by Sieminski *et al*. They compared the formation of microvascular networks in collagen gels by EPC from the blood and several vessel-derived endothelial cells. The amount of structures and lumen was highest when EPC were used.[39] It is interesting to note that in this system, the rate of organization is the opposite from the amount of structure formation when the different E(P)C are compared. Whether there is a direct relation between the amount of prevascular structures and the organization of these structures can not be concluded from this data. However, it is known that differentiation of cells is often accompanied by a decrease in proliferation.[40] Why HP-CS-EPC, the cells with the lowest degree of maturation, produce the best organized prevascular structures remains unknown. It could be that a lower degree of maturation is accompanied by a higher degree of plasticity, enabling the cells to adapt better to new environments.

This study focuses on prevascularized bone tissue engineering. Multipotent MSC from the bone marrow are widely used as osteoprogenitor cells for bone tissue engineering. It is well established that these cells have the ability to differentiate into adipogenic, chondrogenic and osteogenic lineages.[2,41] Few publications indicate that mesenchymal stem cells can also differentiate into the endothelial lineage.[21-23] Here we investigate for the first time the use of endothelial differenti-

ated bone marrow mesenchymal stem cells for use in prevascularized bone tissue engineering. If mesenchymal stem cells from the bone marrow could be used for this purpose, it means that one can prepare a prevascularized bone tissue engineering construct from a single, easily accessible, bone marrow biopsy.

We showed that hMSC from the bone marrow can differentiate towards endothelial cells *in vitro*. The bone marrow derived mesenchymal stem cells used in this study were > 90% positive for CD29, CD44, CD105 and CD166 and negative for CD31, which makes them consistent with MSC, yet distinct from MAPC.[42] The addition of 50 ng/ml VEGF to MSC proliferation medium without bFGF resulted in the expression of CD31 in approximately all the cells after 10 days as assessed by immunostaining. However, the expression was minimal when assessed with FACS analysis (data not shown), which is consistent with another report in literature.[23] Apart from that, the morphology of the cells did not change drastically, indicating that differentiation towards endothelial cells was limited in this setting. Culturing the cells on Matrigel in EGM2 resulted in enhanced endothelial differentiation. Visual observation revealed that > 90% of the cells present were incorporated in CD31 positive capillary-like structures, indicating that this behaviour was not caused by the differentiation of a small subset of cells. Subcutaneous implantation of the capillary-like structures showed that some human vessels were present and perfused after two weeks. Although a quantitative analysis of this *in vivo* study could not be performed due to resorption of the Matrigel, these results do show that hMSC have the possibility to differentiate towards endothelial cells *in vitro* and can form functional perfused vessels after implantation.

The direct *in vitro* differentiation of mesenchymal stem cells has been reported before.[21-23] The state of differentiation as reported in these papers is comparable to the state achieved with the method described in this paper. Although these papers mention the promising possibilities of endothelial cells from mesenchymal stem cells for tissue engineering, they do not investigate the use of these cells for this purpose. To our knowledge, this is the first report of the use of endothelial progenitors from mesenchymal stem cells in a (complex) tissue engineering application.

Conclusion

In summary, we have demonstrated a new method to differentiate human MSC towards endothelial cells *in vitro*. Although the differentiation was such that the cells formed capillary-like structures, stained positive for the endothelial marker

CD31, were able to incorporate acLDL and were able to form perfused vessels *in vivo*, the cells were not able to retain their endothelial characteristics when they were put in a 3D coculture environment with undifferentiated hMSC. The same was seen when two immature EPC types isolated from cord blood were put in this coculture environment. Although the cells were still present after 10 days, the cells did not organize and dedifferentiated into cells not expressing CD31. On the other hand, a more mature EPC isolated from the cord blood was able to form prevascular structures in the 3D coculture with hMSC. Although the amount of structures was lower as compared to HUVEC and HMVEC, the degree of organization was higher. This study shows that endothelial progenitor cells have potential for use in complex tissue engineering applications like prevascularized bone tissue engineering. However, the endothelial phenotype should be mature enough to prevent dedifferentiation of the cells.

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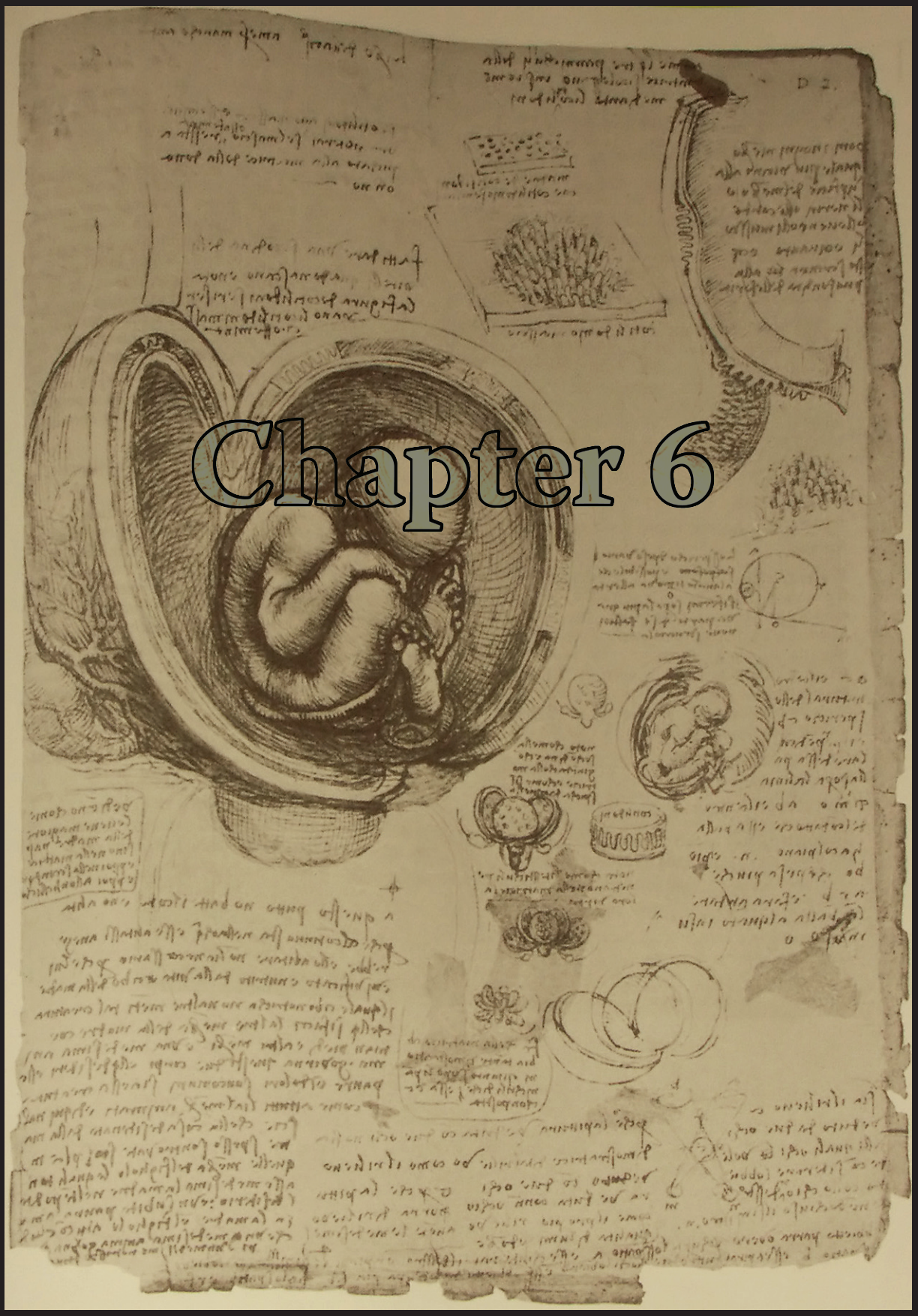
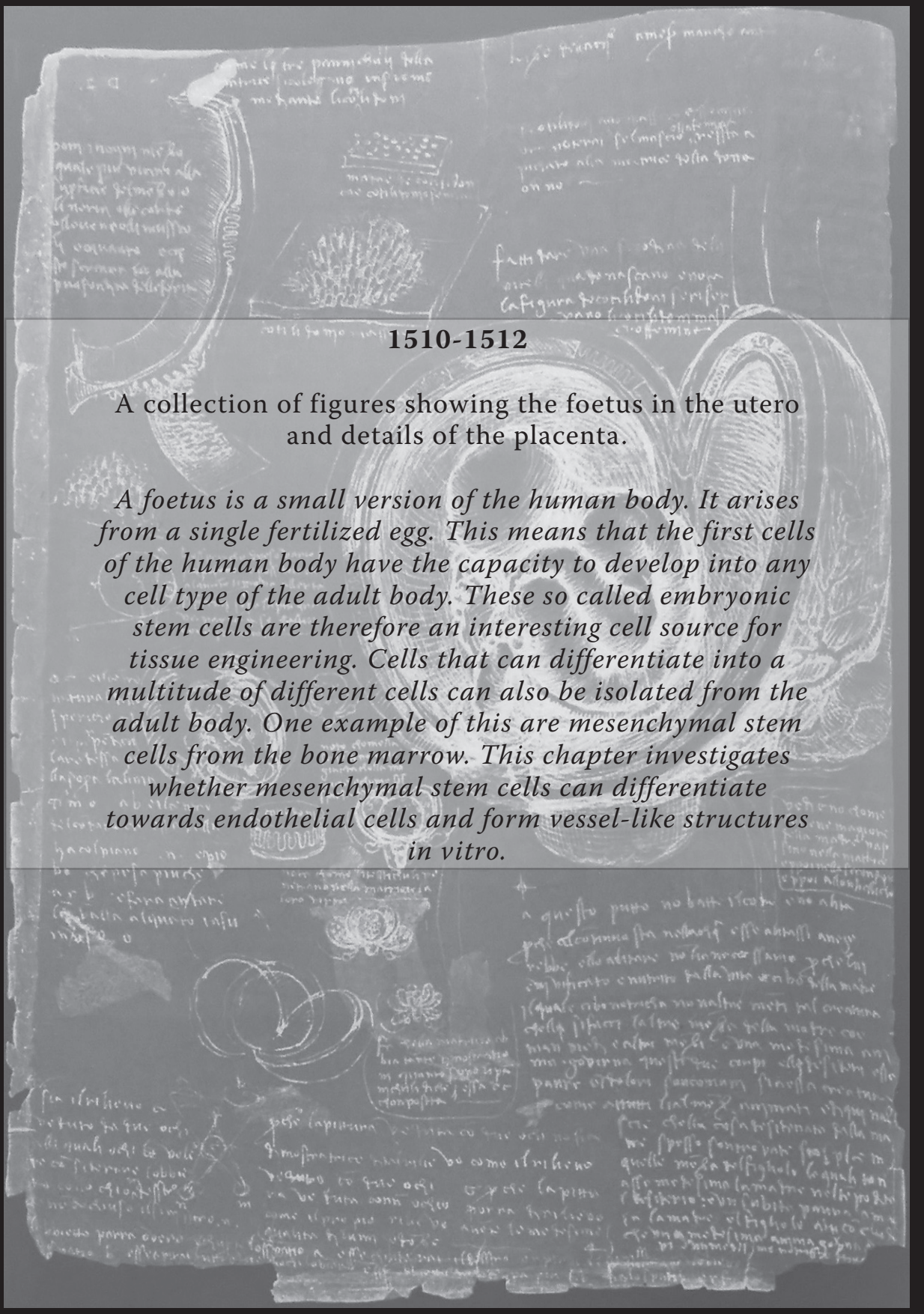


Chapter 6



1510-1512

A collection of figures showing the foetus in the utero and details of the placenta.

A foetus is a small version of the human body. It arises from a single fertilized egg. This means that the first cells of the human body have the capacity to develop into any cell type of the adult body. These so called embryonic stem cells are therefore an interesting cell source for tissue engineering. Cells that can differentiate into a multitude of different cells can also be isolated from the adult body. One example of this are mesenchymal stem cells from the bone marrow. This chapter investigates whether mesenchymal stem cells can differentiate towards endothelial cells and form vessel-like structures in vitro.

Chapter 6

Endothelial cells induce mesenchymal stem cells to form prevascular structures in a three dimensional coculture setting

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Abstract

Rapid vascularization after implantation is essential for the survival of cells in tissue engineered constructs. We recently developed a coculture system of human mesenchymal stem cells (hMSC) and human umbilical vein endothelial cells (HUVEC) to study *in vitro* prevascularization as a method to enhance vascularization in the field of bone tissue engineering. This resulted in the formation of an interconnected prevascular network. However, due to the multipotent nature of the hMSC used in this system, it remained unclear which cells were involved in the formation of this network. In this paper, the role of both cell types in the formation of the prevascular structures was studied in more detail. Labeling with CFSE showed that HUVEC did not organize into prevascular structures in a 3D coculture system, but remained present as round cells. Labeling of hMSC with BrdU on the other hand showed that hMSC were able to differentiate towards cells expressing both CD31 and vWF and got incorporated in the prevascular network. Prevascular network formation was not seen in indirect cocultures of HUVEC and hMSC, and was inhibited by the addition of anti-VEGF neutralizing antibodies, indicating that both direct cell contact and diffusible growth factors like VEGF are important for the formation of the prevascular network and the differentiation of hMSC towards endothelial cells. We conclude that hMSC have the potential to differentiate towards endothelial cells and get incorporated in prevascular networks *in vitro*. This is an important finding for prevascularized bone tissue engineering, since this indicates the possibility of prevascularized bone tissue engineering from a single cell source.

Adapted from Rouwkema J, *et. al*. Endothelial cells induce mesenchymal stem cells to form prevascular structures in a three dimensional co-culture setting. *In preparation*

Introduction

Tissue engineering has been described as an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function.[1] In the field of bone tissue engineering, a bone implant material such as ceramics is often combined with osteoprogenitor cells to engineer a construct that can be used for the treatment of bone defects. Human mesenchymal stem cells (hMSC) from the bone marrow are commonly used as a source for osteoprogenitor cells. Numerous groups, including our own, have shown that the combination of artificial scaffolds and osteoprogenitor cells can lead to the formation of new bone in both ectopic and orthotopic sites.[2-4] Although the exact mechanism of bone formation by the implanted cells is not yet known, there is evidence that the implantation of mesenchymal stem cells only has an effect on bone formation if the cells are viable.[5] However, after implantation the cells often have to rely on diffusion for the supply of nutrients and oxygen, since a vascular network for the transport of these substances is not yet present. For large constructs, diffusion alone is not sufficient for the supply of nutrients and therefore nutrient limitations and cell death are common in large constructs after implantation. To ensure the survival of the implanted cells on the carrier material after implantation, rapid vascularization is essential.

Previous studies have shown that endothelial cells can form vascular structures *in vitro*[6-9] that can anastomose to the vasculature of the host after implantation.[10,11] It has also been shown that this prevascularization enhances the implant perfusion and survival.[10] Therefore, *in vitro* prevascularization is a promising technique to enhance vascularization and thus implant integration and survival in the field of tissue engineering. We recently developed a coculture system to study the *in vitro* formation of a prevascular network in a bone tissue engineering setting.[12] Cocultures of human umbilical vein endothelial cells (HUVEC) and hMSC in a spheroid coculture system resulted in the formation of an interconnected three dimensional prevascular network throughout the spheroid. It remained unclear however, which cells were involved in the formation of this network.

Mesenchymal stem cells from the bone marrow are pluripotent cells that can differentiate along several lineages *in vitro*. Although it was early established that these cells could differentiate towards cells from the mesenchymal lineage like adipocytes, osteoblasts, chondrocytes and myoblasts,[13,14] literature later added neuronal,[15] hepatic,[16] cardiac[17] and pancreatic[18] differentiation to the potential of these cells, hereby crossing the mesenchymal lineage boundaries. Apart from the aforementioned differentiation capacities, there is evidence from literature that

mesenchymal stem cells from the bone marrow can also differentiate towards endothelial cells *in vitro*.[19-21] This is an interesting factor for prevascularized bone tissue engineering. If mesenchymal stem cells from the bone marrow can indeed be differentiated towards endothelial cells, this would enable the acquisition of both cell types that are needed; osteoprogenitor cells and endothelial cells, from a single bone marrow biopsy.

Mesenchymal stem cells are known to secrete angiogenic growth factors like VEGF that stimulate angiogenesis during for instance bone healing.[22,23] Therefore, VEGF is expected to play a role in the formation of a prevascular network in cocultures of HUVEC and hMSC. On the other hand, Oswald *et al* showed that VEGF can induce MSC to differentiate towards endothelial cells.[19] Therefore, in cocultures of hMSC and HUVEC, VEGF may not only be involved in the stimulation of the endothelial cells, but could also have a direct effect on the differentiation of hMSC. Apart from that, it is known from literature that direct cell contact is an important factor in the interaction between endothelial cells and osteoprogenitor cells.[24,25] In this paper we attempt to get more insight in the role of both VEGF and direct cell contact in the formation of prevascular structures in HUVEC-hMSC cocultures.

In this current study we investigate if hMSC can differentiate towards endothelial cells and get incorporated in the prevascular network that is formed *in vitro* in a 3D spheroid coculture system. To get insight in the mechanisms that are involved in the formation of the prevascular network, spheroid cocultures of HUVEC and hMSC are compared with indirect cocultures of HUVEC and hMSC, and spheroid cocultures in the presence of neutralizing antibodies for VEGF. Apart from that, we study the fate of the formed networks after implantation to see if *in vitro* prevascularization has a positive effect on *in vivo* vascularization in this bone tissue engineering setting.

Materials and methods

Culture of HUVEC

HUVEC were purchased from Lonza (Basel, Switzerland). Cells were grown at 37 °C in a humid atmosphere with 5% carbon dioxide (CO_2) in endothelial growth medium-2 (EGM-2) (Lonza). Cells were routinely split at a 1:6 ratio and cultured < 5 passages. Only HUVEC from passage 3 or 4 were used to seed the coculture experiments.

Isolation and culture of hMSC

Bone marrow aspirates (10-20ml) were obtained from two donors, aged 27 and 65, with written informed consent. hMSC were isolated and proliferated as described previously.[26] Briefly, aspirates were resuspended using a 20G needle, plated at a density of $5 \cdot 10^5$ cells/cm^2 and cultured in MSC proliferation medium (minimal essential medium (α-MEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Cambrex), 0.2 mM ascorbic acid (AsAP, Sigma, St. Louis, MI), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin (Invitrogen), 10 µg/ml streptomycin (Invitrogen), and 1 ng/ml basic fibroblast growth factor (bFGF, Instruchemie, Delfzijl, The Netherlands)). Cells were characterized by FACS and tested > 90% positive for CD29, CD44, CD105 and CD166. Cells tested negative for CD31. Cells were grown at 37 °C in a humid atmosphere with 5% CO_2. Cells were routinely split at a 1:6 ratio and cultured < 5 passages. hMSC from passage 3 or 4 were used to seed the coculture experiments. All results in this paper were similar for both donors.

Labeling with 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE)

Cells were removed from the culture plastic using trypsin and resuspended in phosphate buffered saline (PBS) to a cell concentration of $2 \cdot 10^6$ cells/ml. An equal volume of 20 µM CFSE (Sigma) in PBS was added and the cells were incubated for 10 minutes at 37 °C. 8 ml of PBS was added and cells were spun down. Cells were resuspended in 2 ml of growth medium and incubated for 5 minutes at 37 °C. 8 ml of PBS was added and cells were spun down. Cells were washed with PBS once more before they were used for further experiments. To check the efficiency of CFSE labeling, labeled cells were seeded on a microscopic slide. These cells were fixed with ethanol after 3 hours and the percentage of CFSE positive cells was determined by counterstaining with DAPI. This revealed that 100% of the cells were labeled with CFSE.

Labeling of cells with 5-bromo-2´-deoxyuridine (BrdU)

When cells in a culture flask reached ± 70% confluence, they were put in proliferation medium containing 10 µmol/l BrdU (Invitrogen). The cells were incubated in this medium for 24 hours, after which the medium was removed and the cells were used for seeding coculture experiments. To check the efficiency of BrdU-uptake, labeled cells were seeded on a microscopic slide. These cells were fixed with ethanol after 3 hours and the percentage of BrdU positive cells was determined by

immunostaining for BrdU combined with DAPI staining. This revealed that 90 ± 5% of the cells were labeled with BrdU.

2D coculture assay

A mix of 5% HUVEC and 95% hMSC was seeded in a chamber slide system (Lab Tek) at a density of $2 \cdot 10^4$ cells/cm^2. Cells were incubated in osteogenic differentiation medium (ODM) (α-MEM supplemented with 10% FBS, 0.2 mM AsAP, 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin (Invitrogen), 10 µg/ml streptomycin (Invitrogen), 10^{-8} M dexamethasone (Sigma), and 0,01 M β-glycerophosphate (Sigma)) at 37 °C in a humid atmosphere with 5% CO_2. Samples were grown for 6 or 10 days with a medium change every other day. Cells were then fixed with ethanol for 2 minutes.

Generation and culture of coculture spheroids

HUVEC (5%) and hMSC (95%) were pooled to a total of $5 \cdot 10^5$ cells in a round-bottom 10 ml tube (Greiner). The cells were re-suspended in 4.5 ml ODM and subsequently centrifuged at 1600 rpm for 2 minutes. The tubes with the cell pellets were incubated at 37 °C in a humid atmosphere with 5% CO_2, which allowed for the spontaneous formation of coculture spheroids. The spheroids were either cultured for 3, 5, 7 or 10 days with a medium change at day 5 and 7.

Indirect coculture essay

Spheroids consisting of only hMSC were prepared as described in the previous paragraph. After three days of culture, when the spheroids were solid enough to handle, they were transferred to a non-tissue culture treated 6-well plate and cell culture inserts (Becton Dickinson) containing a near-confluent layer of HUVEC were added to the wells. In this system, spheroids were cultured for an additional 7 days in ODM (supplied both to the well and the insert) with a medium change after 2 and 5 days.

VEGF neutralizing essay

Spheroids consisting of 5% HUVEC and 95% hMSC were prepared as described before. Four spheroids were prepared and cultured in ODM, and four spheroids in ODM medium supplemented with 2 µg/ml neutralizing anti-rhVEGF antibod-

ies which bind both to $VEGF_{165}$ and $VEGF_{121}$ (R&D systems, Abingdon, United Kingdom). The spheroids were cultured for 10 days with a medium change at day 5 and 7.

Porous polymer construct preparation

Porous blocks were produced by a compression moulding and salt-leaching method, as previously described.[27] In brief, 1000PEGT70PBT30 (PolyActive™, IsoTis S.A.) powders (PEGT/PBT ratio of 70/30 and a PEGT molecular mass of 1000 g/mol) of less than 600 µm were homogeneously mixed with sodium chloride grains. The grain size was 500–600 µm, and the amount of the salt was adjusted to a final volume percentage of 80%. The mixture was compression moulded into a block, and then the block was immersed in demineralized water to remove sodium chloride. Cylindrical scaffolds (diameter 4 mm) were cored out of 2 mm thick porous blocks and cut in half. To improve cell attachment, scaffolds were treated under an argon plasma (0.1–0.2 mbar) for 30 min. Scaffolds were immersed in 70% ethanol overnight for sterilization. Samples were subsequently air-dried and washed with phosphate buffered saline 6 times to remove the ethanol. Samples were incubated in culture medium for 1 hour prior to cell seeding.

In vivo assay

Previously sterilized scaffolds were put in a six-well plate and seeded statically with $1 \cdot 10^6$ cells per scaffolds. The cells were suspended in 30 µl of ODM, applied to the scaffold and allowed to attach for 2,5 hours. After this time, 5 ml of ODM was added to the well and the scaffolds were incubated at 37 °C in a humid atmosphere with 5% CO_2 for 10 days with a medium change every other day. Four groups of samples were produced; empty scaffolds, scaffolds seeded with 5% HUVEC and 95% hMSC, scaffolds seeded with 10% HUVEC and 90% hMSC, and scaffolds seeded with hMSC alone. After 10 days of *in vitro* culture, one scaffold of each group was implanted subcutaneously in the dorsal region of 12 male nude mice. The mice were anesthetized with 2.5% isoflurane, after which the scaffolds were implanted in separate pockets. One week (n = 6) or two weeks (n = 6) after implantation, lectin HPA (Helix pomatia agglutinin) conjugated to Alexa Fluor 488 (Invitrogen) (0.5 mg/0.25 ml PBS) was injected into the tail vein of anesthesized animals (20 mg/kg body weight). Circulation was allowed for 2 minutes after which the animals were euthanized and the implants were retrieved. Samples were snap frozen in Cryomatrix (Thermo Shandon, Waltham, MA) in liquid nitrogen.

Immunohistochemical analysis

After harvesting, spheroids were frozen in Cryomatrix (Thermo Shandon) at -60 °C. Sections (6 µm) were cut with a cryotome. Sections were fixed in cold acetone (-20 °C) for 5 minutes and air dried. Samples were rehydrated for 10 minutes, after which they were incubated for 30 minutes with 10% FBS in PBS to block non-specific background staining. Sections were incubated with mouse-anti-human CD31 (does not cross-react with mouse tissue) or vWF primary antibody (Dako, Glostrup, Denmark) for 1 hour. Sections were washed in PBS and subsequently incubated with the secondary antibody (horseradish peroxidase conjugated goat-anti-mouse immunoglobulin antibody, Dako) for 45 minutes. Slides were developed with diaminobenzidine (Dako) as substrate, and weakly counterstained with hematoxylin (Sigma). For fluorescent staining, AlexaFluor 594 conjugated goat-anti-mouse immunoglobulin antibody (Invitrogen) was used as the secondary antibody. These samples were neither developed nor counterstained. These samples were mounted with DAPI containing mounting medium (Vectashield, Vector Laboratories). Samples that were stained for both BrdU and CD31 or vWF were first rehydrated and then incubated for 30 minutes in 2N hydrochloric acid (Sigma). Sections were then washed in PBS and subsequently stained for CD31 or vWF according to the standard protocol. Afterwards, samples were incubated for 30 minutes with 10% FBS in PBS and subsequently with AlexaFluor 488 conjugated mouse-anti BrdU primary antibody (Invitrogen) for 1 hour.

Results

2D cocultures of hMSC and CFSE labeled HUVEC

Cocultures of hMSC and CFSE labeled HUVEC were seeded on tissue culture plastic slides at a high density of $2 \cdot 10^4$ cells/cm^2. After one day of coculture, a confluent cell layer covered the surface of the slide. After 6 days of coculture, CFSE positive cells started to organize into CD31 positive multicellular structures (Fig 1A). Organization continued over the next 4 days. After 10 days of coculture, CD31 positive structures were more elongated and resembled pre-capillary structures (Fig 1B). After 10 days of coculture, CD31 positive structures were positive for CFSE, although the signal was weak due to dilution through cell division. However, it could be concluded that the structures consisted of HUVEC.

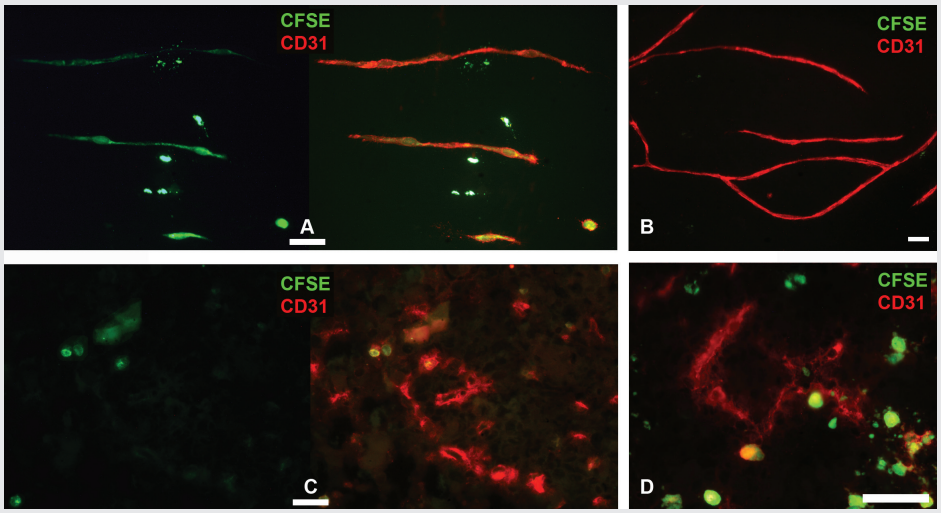

FIG. 1. *Cocultures of hMSC and CFSE labeled HUVEC (green). Scale bar = 100 μm.* ***(A and B)*** *Confluent 2D cocultures on tissue culture plastic.* ***(C and D)*** *3D spheroid cocultures. A and C were cocultured for 6 days, B and D for 10 days. Samples were immunostained with anti-CD31 antibodies (red) showing the formation of vessel-like structures. A and C are split up in two panels to better visualize CFSE. Left panel is CFSE, right panel is CFSE plus CD31.*

Coculture spheroids and prevascular network formation

The formation of coculture spheroids was similar as reported before.[12] In brief, solid spheroids with a diameter of approximately 1 mm were formed spontaneously between day 3 and day 5 with the method described in 'Materials and Methods'. These spheroids remained intact for the remainder of the 10 day culture period. Cross sections of the spheroids revealed that $CD31^+$ prevascular structures were present after coculture, as reported before.

3D coculture of hMSC and CFSE labeled HUVEC

CD31 positive structures could be seen in cross sections of hMSC-HUVEC coculture spheroids that had been cultured for 6 days (Fig 1C). Although CFSE labeled HUVEC were often seen close to or incorporated in CD31 positive structures, they did not organize into these structures. CFSE labeled HUVEC were present as round, unorganized cells and the CD31 positive structures were mainly composed of unlabeled cells. Coculture spheroids that were cultured for 10 days showed similar results. CD31 positive structures could be seen throughout the spheroid. Although labeled HUVEC were seen in or close to CD31 positive structures, the majority of the structures were composed of unlabeled cells (Fig 1D).

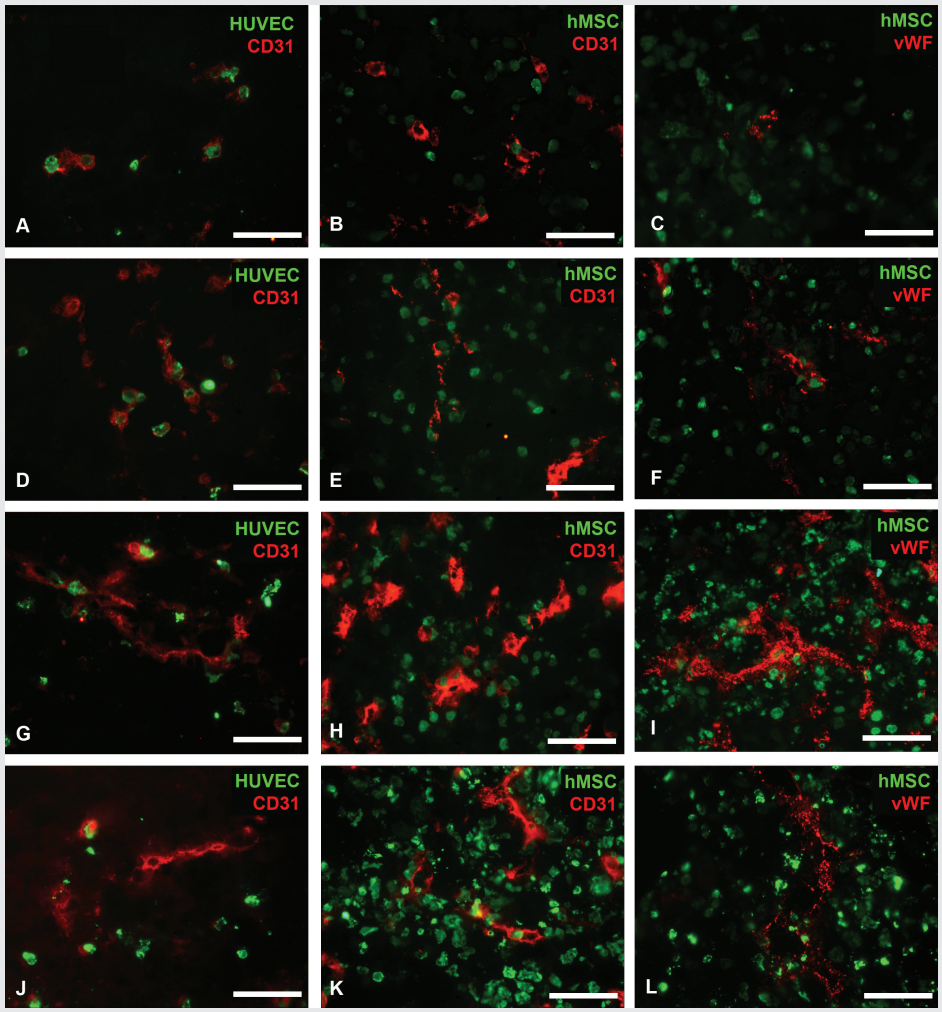

FIG. 2. *3D spheroid coculture of hMSC and HUVEC with BrdU labeling (green). Scale bar = 100 μm.* ***(A, B and C)*** *were cocultured for 3 days.* ***(D, E and F)*** *were cocultured for 5 days.* ***(G, H and I)*** *were cocultured for 7 days.* ***(J, K and L)*** *were cocultured for 10 days. The left row displays samples seeded with BrdU labeled HUVEC and stained for CD31. The middle row displays samples seeded with BrdU labeled hMSC and stained for CD31. The right row displays samples seeded with BrdU labeled hMSC and stained for vWF. Note the presence of non-labeled cells in the CD31 positive structures in the left row. Note the presence of labeled cells (hMSC) that stain positive for CD31 and vWF in the middle and right row respectively.*

3D coculture of hMSC and BrdU labeled HUVEC

Cross sections of spheroid cocultures of hMSC and BrdU labeled HUVEC that were cultured for 10 days showed results similar to cocultures of hMSC and CFSE labeled HUVEC. BrdU labeled HUVEC were present throughout the spheroid (Fig 2). Although some HUVEC were present in CD31 positive structures, the structures mainly consisted of non-labeled cells. Opposed to CFSE, which is a cytoplasmic marker, BrdU did not give any information on the morphology of HUVEC, since it is a nuclear marker.

3D coculture of BrdU labeled hMSC and HUVEC

Coculture spheroids seeded with BrdU labeled hMSC and HUVEC were cultured for 3, 5, 7 and 10 days. Cross sections were made and stained for CD31 and vWF. After 3 days of coculture, $Cd31^+$ and vWF^+ cells could be seen in the cross sections. Positively stained cells were mainly present as single cells, although some 'structures' containing a limited number of cells could be seen (Fig 2B and C). Although no BrdU labeled cells (hMSC) stained positive for vWF, a small number of labeled cells did stain positive for CD31 (Fig 2B). After 5 days of coculture, $CD31^+$ and vWF^+ cells had started to organize into more complex structures. Both $CD31^+$ and vWF^+ BrdU labeled cells (hMSC) were present in these structures (Fig 2E and F). Between day 5 and 10, prevascular structures organized further. Both $CD31^+$ and vWF^+ BrdU labeled cells (hMSC) remained present in the prevascular structures over time (Fig 2H, I, K and L).

3D indirect coculture

hMSC spheroids were cultured in indirect cocultures with HUVEC to investigate the role of direct cell contact on the endothelial differentiation of hMSC. After three days of culture, hMSC spheroids were solid enough to transfer them to a 6-well plate. Culturing the spheroids in a well for the remaining 7 days did not alter the macroscopic morphology of the spheroids when compared with spheroids that were cultured in a tube for the full ten days. The amount of HUVEC in the cell culture insert decreased during the 7 days of indirect coculture. However, sufficient HUVEC was still present at the end of the 7 day period. Cross sections of the hMSC spheroids that were cocultured with HUVEC in this indirect system did not show any CD31 (Fig 3C) or vWF (data not shown) positive staining at the end of the 10 day period.

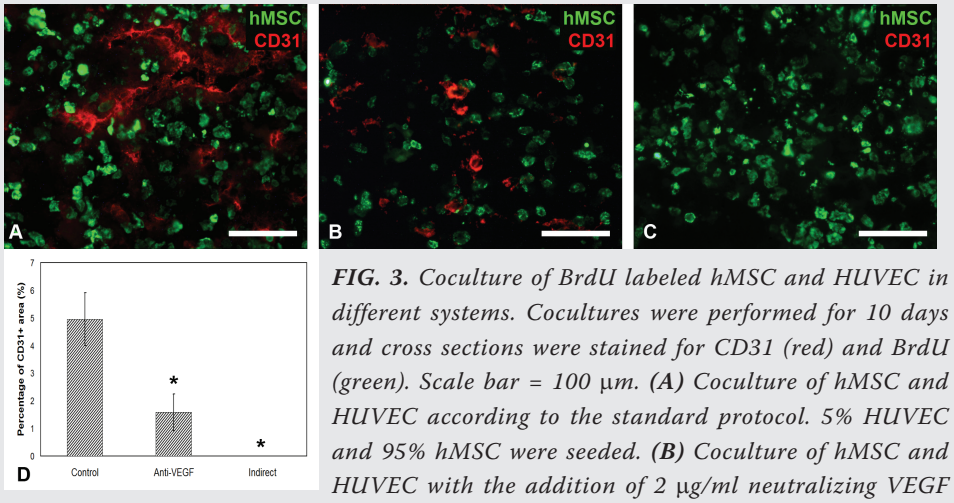

FIG. 3. *Coculture of BrdU labeled hMSC and HUVEC in different systems. Cocultures were performed for 10 days and cross sections were stained for CD31 (red) and BrdU (green). Scale bar = 100 μm.* ***(A)*** *Coculture of hMSC and HUVEC according to the standard protocol. 5% HUVEC and 95% hMSC were seeded.* ***(B)*** *Coculture of hMSC and HUVEC with the addition of 2 μg/ml neutralizing VEGF antibody to the medium. 5% HUVEC and 95% hMSC were seeded.* ***(C)*** *Cross section of a hMSC spheroid after indirect coculture with HUVEC.* ***(D)*** *Quantification of the prevascular structures in the different settings. The graph shows the percentage of a cross section that stains positive for CD31. Results shown are mean values ± standard deviation (n=4). *p < .0005, compared to control.*

VEGF neutralizing assay

To investigate the role of VEGF in the formation of the prevascular structures and differentiation of hMSC, coculture spheroids seeded with BrdU labeled hMSC were cultured in the presence of neutralizing anti-rhVEGF antibodies. The antibodies used bind both to $VEGF_{165}$ and $VEGF_{121}$. Cross sections of coculture spheroids revealed that although CD31 positive cells were present in the spheroid, they were generally seen as single cells and did not organize into prevascular structures. Moreover, CD31 positive cells did not stain positive for BrdU (Fig 3B).

Implantation of the prevascular network

During seeding of the scaffolds, the cells coagulated due to the high cell density and formed a gel-like cell mass in and around the scaffold. During the subsequent culture time of the scaffold, the cell mass became more dense. A thin capsule of cells remained around the scaffold during the entire *in vitro* culture period. Histology after 10 days of *in vitro* culture revealed that a high density of cells was present both in the scaffold and as a capsule surrounding the scaffold (Fig 4A). Immunostaining for CD31 showed that CD31 positive structures, comparable to

those formed in coculture spheroids, were present throughout the scaffold and capsule.

All implanted scaffolds were retrieved after 1 or 2 weeks of implantation. After 1 week of implantation, perfused blood vessels were seen in the periphery of the implant in the proximity of implanted human prevascular structures (Fig 4B). The prevascular structures had developed further and lumen could frequently be seen inside the structures. Penetration of perfused vessels was limited to the outer capsule of the implant. No perfused human prevascular structures could be seen. Although more prevascular structures of human origin could be detected in samples seeded with 10% HUVEC as opposed to 5% HUVEC, results were similar between these two groups. No positive staining for human CD31 could be seen in samples seeded only with hMSC, nor in empty scaffolds.

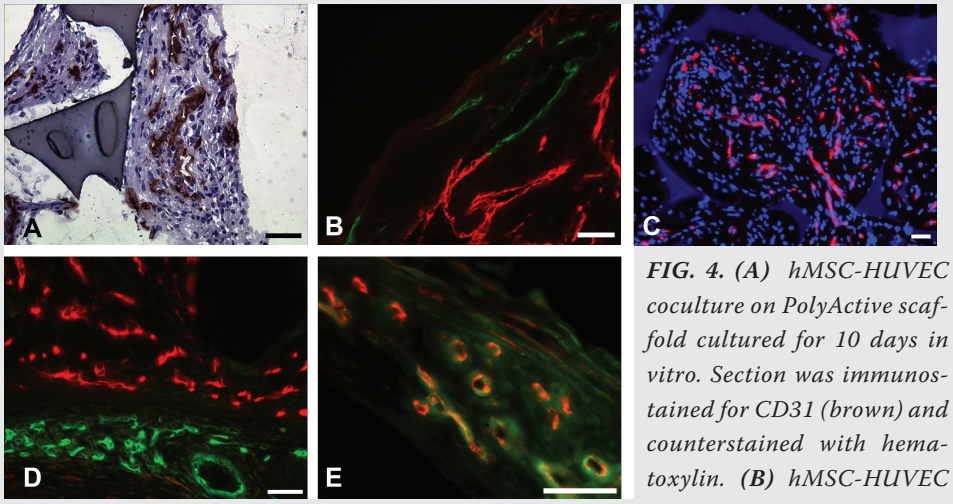

FIG. 4. (A) *hMSC-HUVEC coculture on PolyActive scaffold cultured for 10 days in vitro. Section was immunostained for CD31 (brown) and counterstained with hematoxylin.* ***(B)*** *hMSC-HUVEC coculture on PolyActive scaffold. Samples were cultured in vitro for 10 days and then implanted subcutaneously for 1 week. Before explantation, mice were perfused with labeled lectin (green). Section were immunostained for CD31 (red).* ***(C, D and E)*** *hMSC-HUVEC coculture on PolyActive scaffold. Samples were cultured in vitro for 10 days and then implanted subcutaneously for 2 weeks. Before explantation, mice were perfused with labeled lectin (green). Section were immunostained for CD31 (red).* ***(C)*** *Picture from the middle of the implant after 2 weeks of implantation, counterstained with DAPI (blue). Scale bar = 100 μm.*

After 2 weeks of implantation, a highly vascularized capsule was observed surrounding the implants (Fig 4D). Penetration of perfused vessels into the implant was limited for seeded samples as opposed to the empty scaffolds. Human prevascular structures could still be seen throughout the entire scaffold in coculture

samples (Fig 4C). Perfused human vascular structures could be seen in the periphery of the implant, but not deeper into the construct (Fig 4E). Although more prevascular structures of human origin could be detected in samples seeded with 10% HUVEC as opposed to 5% HUVEC, results were similar between these two groups. No positive staining for human CD31 could be seen in samples seeded only with hMSC, nor in empty scaffolds.

Discussion

Vascularization is nowadays recognized as an important aspect in the field of tissue engineering. For cells in a tissue engineered construct to survive after implantation, fast vascularization is critical. We recently developed a 3D coculture system of HUVEC and hMSC as a model system for prevascularized bone tissue engineering.[12] Although an interconnected 3D prevascular network was formed in this setting, the question remained which cell type was forming the network. In this study we performed several labeling experiments to determine the fate of the two cell types used in this coculture system. Labeling of HUVEC with the cytoplasmic marker CFSE revealed that after coculture, most HUVEC could be seen as round, single cells and that the prevascular structures mainly consisted of non-labeled cells. Labeling of hMSC with the nuclear marker BrdU showed that labeled hMSC were present in the prevascular structures that stained both positive for CD31 and vWF. Moreover, vWF was localized in structures resembling Weibel Palade bodies, which are specific for vascular endothelium.[28] Prevascular networks were not seen in spheroids with only hMSC, even when in indirect coculture with HUVEC. The formation of prevascular structures could be inhibited by the addition of a neutralizing antibody for VEGF. This suggests that direct contact between HUVEC and hMSC initiates a series of events that stimulates hMSC to differentiate towards endothelial cells and form prevascular structures in this three dimensional coculture system.

In this study we show that hMSC can differentiate towards endothelial cells in a 3D coculture system. The differentiation is such that the cells get incorporated in the prevascular network and express the endothelial markers CD31 and vWF. Interestingly, this differentiation is achieved in a medium that is generally used for osteogenic differentiation of hMSC and does not contain any added angiogenic factors. The exact mechanism of endothelial differentiation in this system remains unknown. However, since positive staining for CD31 and vWF was never seen in samples with only hMSC, and HUVEC are often seen in or near the prevascular

structures, we hypothesize that the endothelial differentiation of hMSC in this setting is caused by an interaction with HUVEC. Positive staining for CD31 and vWF was absent in hMSC spheroids that were cocultured with HUVEC in an indirect coculture system, indicating that the differentiation of hMSC is not merely achieved by the secretion of growth factors, but is at least in part initiated by a direct interaction with HUVEC. However, as we have shown before, HUVEC do not survive well in ODM without direct cell contact with hMSC.[12] This leaves open the possibility that endothelial cells can induce the differentiation of hMSC through growth factor secretion, but that endothelial cells need direct contact with hMSC to actually secrete the growth factors involved in this system. The fact that mesenchymal stem cells can differentiate towards other cell types by direct contact with these cells has been reported before. Wang *et al* reported that hMSC can differentiate towards smooth muscle cells or cardiomyocytes by direct contact with these cells. Indirect cocultures or cultures with conditioned medium on the other hand, did not result in this differentiation behaviour.[29]

Although direct cell contact between HUVEC and hMSC seems to be an important factor in the endothelial differentiation of hMSC in this system, experiments using neutralizing VEGF antibodies showed that diffusible growth factors are also involved. It is known from literature that hMSC can secrete VEGF which stimulates endothelial cell recruitment and proliferation and functions as an angiogenic factor in for instance bone healing.[22,23] However, VEGF may also have a direct effect on the endothelial differentiation of hMSC.[19] At the moment it remains unclear whether VEGF acts directly on hMSC for the endothelial differentiation, or whether VEGF mainly stimulates HUVEC which in result leads to the differentiation of hMSC by another mechanism in this system.

Since the differentiation of hMSC is seen in the 3D coculture setting, but not in the 2D setting, it can be concluded that not only the contact with HUVEC is important for the differentiation of hMSC, but also the 3D culture environment. Although the difference between the 2D and 3D system is striking and as yet not fully explainable, a difference in cell function when comparing 2D to 3D systems is not new. For instance, Huang *et al* reported that the secretion of VEGF by MSC was strongly increased when the cells were cultured in a 3D in stead of a 2D environment.[30] Different explanations for the difference between the 2D and 3D system can be thought of. First, it could be hypothesized that a difference in differentiation is caused by a difference in cell shape, as is illustrated by the fact that the differentiation of human mesenchymal stem cells can be varied from osteogenic to adipogenic by solely altering the shape of the cells.[31] Second, secreted extracellular matrix, that is generally more abundant in 3D systems as compared to 2D systems, could also play a critical role for the differentiation of MSC to-

wards endothelial cells in this system.[32] Third, it is known that 3D cell cultures can result in gradients of nutrients and hypoxia for the cells.[33] This could also trigger an alternative differentiation of the MSC.[34]

The *in vitro* differentiation of hMSC towards endothelial cells, by adding high concentrations or a mix of angiogenic growth factors, has been reported before.[19-21] However, we recently showed that these cells can de-differentiate when they are used in a complex 3D coculture setting (see chapter 5). So even though differentiation protocols for the *in vitro* differentiation of MSC towards endothelial cells are present, these protocols are as yet not capable of producing endothelial cells that are mature enough for the formation of prevascular networks in this complex tissue engineering setting. Alternatively, it has been indicated that endothelial progenitor cells can directly be isolated from the bone marrow.[35] However, the isolation protocols used are often laborious. Moreover, only small numbers of endothelial progenitor cells can be isolated from the bone marrow, which makes them as yet unfit for clinical applications. Since hMSC can be easily isolated and expanded to large numbers in a short time, the differentiation of hMSC towards endothelial cells has a clear advantage over the isolation of endothelial progenitor cells from the bone marrow.

CD31 positive hMSC were already detectable after three days of coculture. vWF positive hMSC however, were only present after 5 days of coculture, indicating that the differentiation of hMSC towards endothelial cells in this system comprises different stages. This is consistent with data from endothelial progenitor cells in the blood that generally express CD31 before they start expressing vWF.[36] It should be noted that the time needed for differentiation in this system is short when compared to previous reports on the differentiation of hMSC towards endothelial cells.[19-21] The time needed for differentiation in these reports varied from 7 to 21 days, depending on the concentration of angiogenic factors applied.

The stability and function of the prevascular structures *in vivo* was assessed by subcutaneous implantation in nude mice. Even though the anastomosis of prevascular structures was successful in our previous prevascularized muscle tissue engineering setting,[10] anastomosis after implantation is limited in this current setting. It has been reported before that the addition of endothelial cells to bone tissue engineering constructs did not increase vascularization after implantation and that the number of perfused implanted vessels was limited.[37] However, bone formation was still enhanced by the endothelial cells in this report. Why the effect of prevascularization as determined by the amount of anastomosis is limited in this case, whereas it is successful in other tissues, remains unknown. However, this current study points out that not only HUVEC, but also hMSC are incorpo-

rated in the prevascular structures. This may have an effect on the maturation of the structures and thus on the anastomosis after implantation.

Conclusion

In summary, we have demonstrated that HUVEC induce hMSC to differentiate towards an endothelial cell type and get incorporated in prevascular structures in a three dimensional *in vitro* coculture setting. This finding is especially important for the field of bone tissue engineering. It demonstrates the possibility of prevascularized bone tissue engineering from a single cell type. In the current protocol a mature endothelial cell type is still needed to induce the endothelial differentiation of the hMSC. However, future research will focus on identification of the factors that are involved in the endothelial induction of hMSC. If these factors are known, they may be substituted by non-cellular additives that achieve the same results. This will simplify the protocol for prevascularized bone tissue engineering, which means that the applicability of this technique increases. Apart from that, endothelial cells derived from hMSC may prove to be a useful cell source for other tissue engineering applications.

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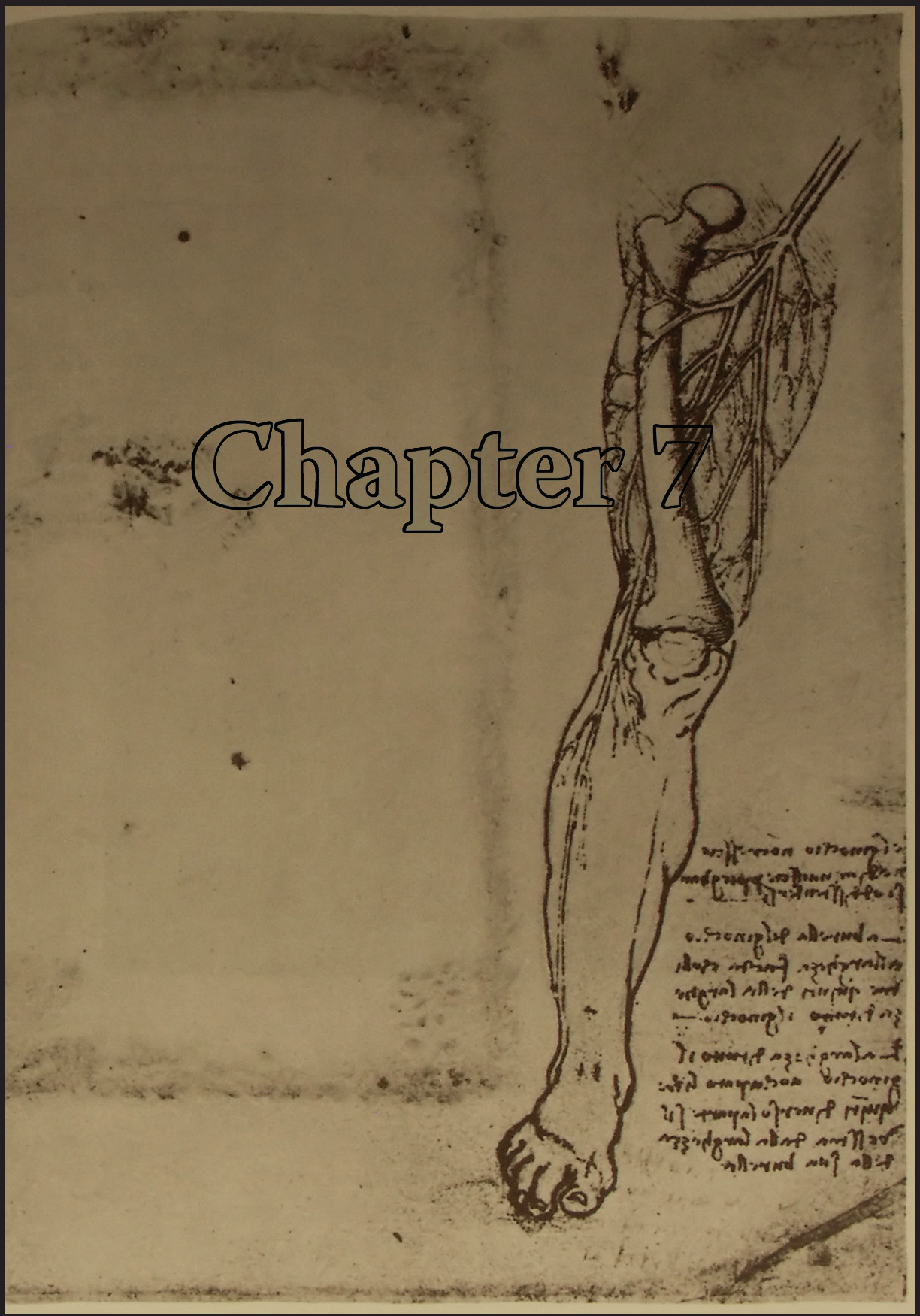
Chapter 7



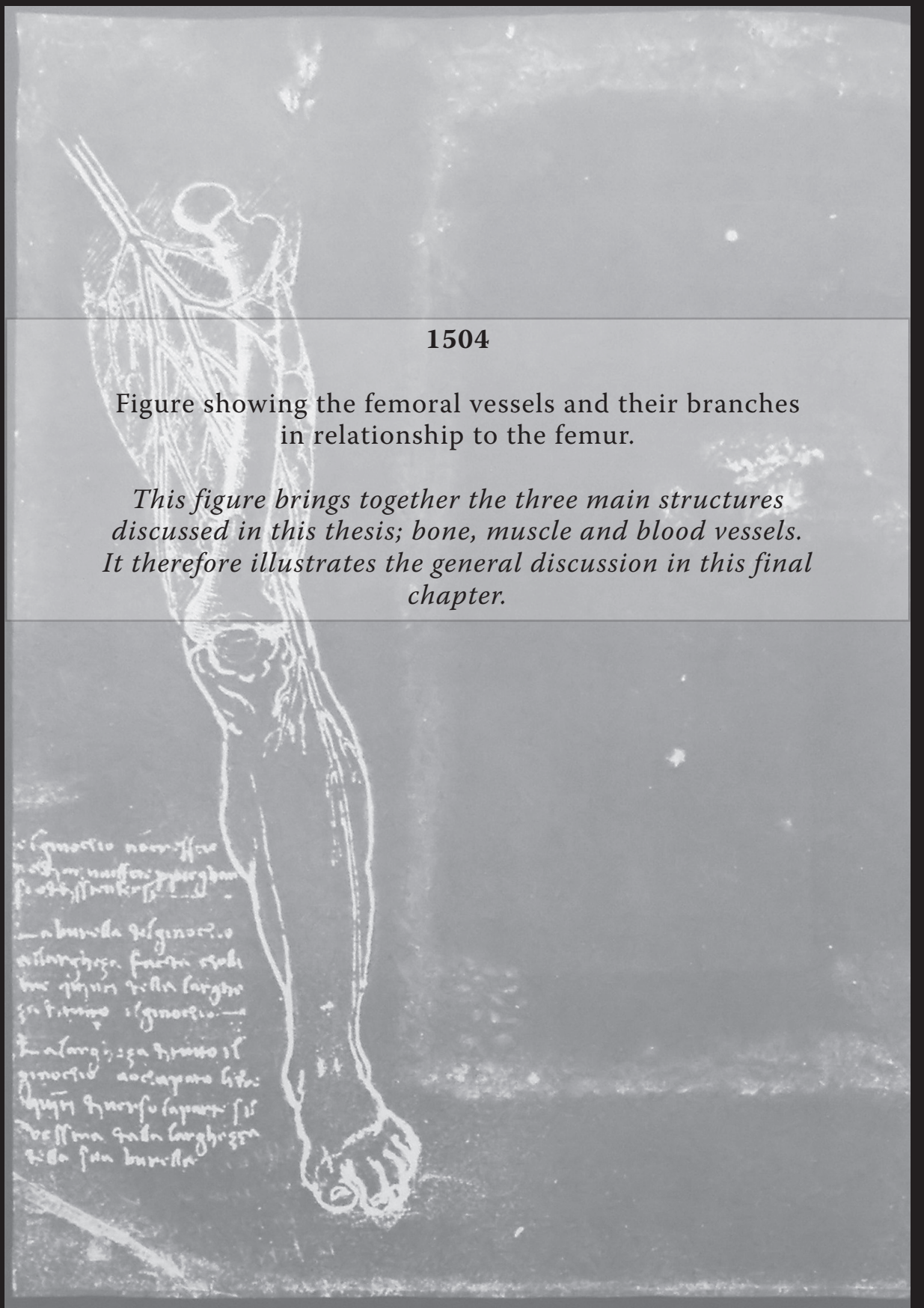


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Figure showing the femoral vessels and their branches in relationship to the femur.

This figure brings together the three main structures discussed in this thesis; bone, muscle and blood vessels. It therefore illustrates the general discussion in this final chapter.

Chapter 7

General conclusions and discussion

Conclusions and discussion

Vascularization is attracting more and more attention in the field of tissue engineering. This is illustrated by the fact that during the last ten years, the amount of papers that are published on this subject per year has steadily increased (Fig. 1). Vascularization is nowadays recognized as one of the main hurdles that have to be overcome to translate tissue engineering research to clinical application on a broad scale.[1-4] This is especially the case for large tissue engineered constructs containing active cells, since nutrient limitations after implantation are most likely to occur in these constructs.

In chapter one it is stated that the overall goal of this thesis is to investigate the possible role of *in vitro* prevascularization for bone tissue engineering. This overall goal can be divided into two parts. The first sub-goal is to investigate the potential positive effect of *in vitro* prevascularization in tissue engineering. The second sub-goal is to combine *in vitro* prevascularization and bone tissue engineering.

Although the first sub-goal is discussed throughout this thesis, the most important conclusions in favour of *in vitro* prevascularization can be found in chapter three. In chapter three the successful integration of skeletal muscle tissue engineering and *in vitro* prevascularization is described. Endothelial cells developed into vessel-like structures *in vitro* without disturbing the differentiation of the

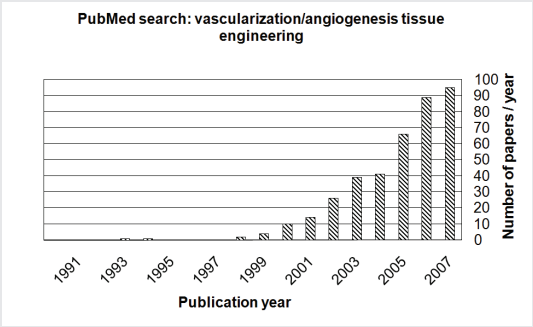

FIG. 1. *The number of papers published on vascularization/angiogenesis in tissue engineering per year. Two searches were performed in PubMed and the results were combined. The first search was performed with 'vascularization tissue engineering' as keywords, the second with 'angiogenesis tissue engineering'. The value for 2007 is a predictive value based on the amount of papers listed on 06-07-2007.*

skeletal muscle tissue. Moreover, the addition of smooth muscle cell precursors resulted in the co-localization of these cells with the endothelial structures and stabilization of these structures. After implantation, the vessel-like structures anastomosed to the host vascular network. This improved the perfusion and survival of the implant. The following conclusions can be drawn from this chapter:

- Endothelial cells can form vessel-like structures *in vitro* in a skeletal muscle tissue engineering setting, without the addition of angiogenic factors.
- Smooth muscle precursors can stabilize the vascular structures that are formed during *in vitro* prevascularization in a skeletal muscle tissue engineering setting.
- The addition of endothelial cells and smooth muscle precursors does not negatively influence the development of skeletal muscle tissue *in vitro*.
- *In vitro* prevascularization of a skeletal muscle construct can enhance perfusion and survival of the construct after implantation.

From chapter three it can thus be concluded that in vitro prevascularization is a promising strategy to enhance vascularization in the field of tissue engineering.

Chapter four, five and six are mainly directed towards the second sub-goal, which is to combine *in vitro* prevascularization and bone tissue engineering. In chapter four, a coculture system of hMSC and HUVEC is described that results in the formation of prevascular structures in a bone tissue engineering setting.

However, the organization of the endothelial structures was limited and lumen-containing structures were generally not formed *in vitro*. Moreover, the addition of smooth muscle cells or smooth muscle precursor cells did not result in stabilization and better organization of the structures, which was the case in the skeletal muscle tissue engineering construct described in chapter three. After implantation, the prevascular structures of the bone tissue engineering construct organized further into lumen-containing structures. However, anastomosis to the host vasculature was limited.

The HUVEC used in chapter four is a model endothelial cell system that is often used in research. However, these cells are not fit for clinical application. Apart from that, it is known that endothelial cells isolated from different locations can behave differently *in vitro*. Therefore, chapter five explores the use of several endothelial cells and endothelial progenitor cells in prevascularized bone tissue engineering. hMSC were differentiated towards endothelial cells as a cell source that is fit for clinical application. Apart from that, the use of endothelial progenitor cells isolated from the cord blood was investigated. HUVEC and human dermal microvascular endothelial cells are used to investigate the behaviour of endothelial cells isolated from different types of vessels. Although hMSC could be differentiated to endothelial-like cells that formed $CD31^+$ capillary like structures, incorporated ac-LDL and formed perfused vessels *in vivo*, the cells de-differentiated and did not form prevascular structures in a 3D coculture system with undifferentiated hMSC. As for the endothelial progenitor cells, only the type with a high degree of differentiation, which adopts a cobblestone-like morphology on culture plastic, was able to initiate the formation of prevascular structures in the coculture setting. Two other, less mature, endothelial progenitor cell types dedifferentiated and did not form any structures. When the prevascular structures formed in cocultures with HUVEC, HMVEC or cobblestone-like endothelial progenitor cells were compared, a difference in morphology could be seen. Although HUVEC resulted in the highest amount of prevascular structures, the organization was limited and the amount of lumen-like structures was minimal. Cobblestone-like endothelial progenitor cells on the other hand formed less structures, but with a higher degree of organization and more lumen-like structures. HMVEC gave intermediate results, both for the amount and organization of the structures.

Chapter four and five showed that endothelial cells and mature endothelial progenitor cells in a three dimensional coculture system with undifferentiated hMSC can result in the formation of a prevascular network. However, the nature of the cells that are involved in the formation of the prevascular structures remains unclear. In chapter five it was indicated that hMSC can differentiate towards endothelial cells *in vitro*. It is thus possible that mesenchymal stem cells are actu-

ally involved in the formation of the prevascular network. Chapter six describes labeling studies that were performed to study the fate of HUVEC and hMSC in both a 2D and 3D coculture system. Labeling of HUVEC with the cytoplasmic labeling agent CFSE revealed that in the 2D coculture system, HUVEC organized into tube-like structures. In the 3D system however, HUVEC remained present as round cells and the prevascular structures mainly consisted of non-labeled cells. Labeling of hMSC with BrdU revealed that hMSC differentiated into cells that expressed CD31 and vWF and were incorporated in the prevascular structures. Indirect coculture of a hMSC spheroid and HUVEC did not result in endothelial differentiation of hMSC. Apart from that, neutralizing anti-VEGF antibody inhibited the formation of prevascular structures in the cocultures and the endothelial differentiation of hMSC. This indicates that both direct cell contact between HUVEC and hMSC, and the secretion of diffusible factors like VEGF are important for the development of the prevascular structure and the endothelial differentiation of hMSC in this system.

Chapter four, five and six describe several aspects of the implementation of *in vitro* prevascularization in bone tissue engineering. Apart from that, chapter five and six give clear indications that hMSC can differentiate towards endothelial cells *in vitro*. This is not only interesting for prevascularized bone tissue engineering. Due to the accessibility of hMSC and their proliferative capacities, endothelial cells from hMSC may prove to be an interesting cell source for numerous tissue engineering applications in the future. The following conclusions can be drawn from chapter four, five and six:

- Interconnecting prevascular structures can be formed in 3D cocultures of hMSC and endothelial cells without the addition of angiogenic growth factors.
- The prevascular structures are not stabilized by the addition of smooth muscle cells or smooth muscle cell precursors.
- The addition of endothelial progenitor cells to hMSC spheroids can result in the formation of a prevascular network, but only if the degree of differentiation is sufficient.
- hMSC can differentiate towards endothelial cells on Matrigel *in vitro*.
- hMSC can differentiate into $CD31^+$ and vWF^+ cells that get incorporated in the prevascular structures in spheroid cocultures of hMSC and HUVEC.
- The contribution of *in vitro* prevascularization to *in vivo* vascularization is limited in this current bone tissue engineering setting.

From chapter four, five and six it can thus be concluded that although in vitro prevascularization holds promising possibilities for bone tissue engineering, the contribution of in vitro prevascularization to vascularization after implantation of bone tissue engineered constructs is as yet limited.

When comparing chapters four, five and six with chapter three, it is clear that the success of *in vitro* prevascularization depends on the tissue that is prevascularized and the settings and cells that are used to study prevascularization. In skeletal muscle tissue, endothelial cells organized into capillary-like structures containing lumen that were stabilized by smooth muscle cells. After implantation, the structures anastomosed to the host vasculature and contributed to implant vascularization and survival. In bone tissue on the other hand, endothelial cells did organize into elongated prevascular structures, but organization was more immature as indicated by the limited amount of lumen. The structures were not stabilized by smooth muscle cells and anastomosis after implantation was limited. It remains uncertain what exactly causes the difference between these two tissue settings, but several reasons can be hypothesized.

There is a clear difference between the cells that are used in the two different systems. This means that the interaction between the different cell types in a system is different as well. The development of bone and blood vessels is intimately linked, whereas the development of muscle and blood vessels is not. Apart from that, hMSC are known to secrete an array of growth factors.[5] Therefore, more interaction between the cells can be expected in the bone tissue engineering setting. As a consequence, it may be more challenging to orchestrate this interaction in such a way that the organization of the endothelial cells is optimal.

Another complexity of the bone tissue engineering setting is the fact that hMSC can and do differentiate towards endothelial cells. This means that in the muscle tissue one has to optimize the organization of endothelial cells, whereas in the bone tissue one has to optimize both the differentiation of hMSC towards endothelial cells, and the subsequent organization of these cells and the originally seeded endothelial cells into vascular structures.

It is known that the micro-environment is an important factor for the organization of endothelial cells. This includes not only diffusible factors, but also the extracellular matrix. In the muscle tissue engineering setting, scaffolds were seeded with cells suspended in Matrigel. This means that an extracellular matrix, which is known to stimulate angiogenesis,[6] was present from the start. In the bone tissue

engineering setting, the cells were seeded without the addition of an extracellular matrix. This may also partly explain the difference in organization and maturation of the vessel-like structures between the two systems.

Future directions

Even though *in vitro* prevascularization was successful in a skeletal muscle tissue engineering setting, the *in vivo* results of prevascularized bone constructs were limited. When the skeletal muscle and bone constructs are compared, a difference in maturation of the prevascular structures is already evident *in vitro*. In the skeletal muscle construct the endothelial cells organize into vessel-like structures containing lumen. In the bone construct the endothelial cells do form an interconnected network of elongated endothelial cells, but lumen is not generally seen *in vitro*. It is likely that the maturity of the prevascular structures will determine their ability to anastomose to the host vasculature after implantation. Therefore, the maturation of prevascular structures *in vitro* may be a good indicator for the *in vivo* results of *in vitro* prevascularization. If *in vitro* maturation indeed determines *in vivo* anastomosis, future research should focus on maturation of the prevascular structures *in vitro*. Future directions of research may include:

Study the endothelial differentiation of hMSC in 3D cocultures with endothelial cells

This thesis shows that mesenchymal stem cells can differentiate towards endothelial cells and get incorporated in the prevascular structures that are formed in cocultures of hMSC and endothelial cells. More research is needed to identify the factors that are involved in the endothelial differentiation of hMSC in this system, since understanding this differentiational behaviour may be critical for the improvement of the maturation of the prevascular structures.

Study the role of extracellular matrix

Extracellular matrix is an important factor in the organization of blood vessels *in vivo*. Therefore, it is likely that extracellular matrix has an effect on the organization of prevascular networks in a tissue engineering construct *in vitro* as well. Sottile has published a review describing the effects of a multitude of extracellular matrix proteins on the behaviour of endothelial cells and blood vessel formation.[7]

This review may be a good initial guide for the selection of extracellular matrix proteins. Proteins of specific interest are proteins that either promote angiogenesis or stabilize vessels. These proteins may be added to cocultures of hMSC and endothelial cells to determine whether this results in an improved organization or maturation of the prevascular structures.

Study the incorporation of smooth muscle cells in prevascular structures in a bone tissue engineering setting

It is evident, both from literature and chapter three of this thesis, that mural cells and mural cell precursors play a crucial role in vascularization, both *in vitro* and *in vivo*. As yet, the addition of smooth muscle cells and smooth muscle cell precursors to the bone tissue engineering construct as described in chapter four, did not result in stabilization of the vessel-like structures. The apparent lack of communication between the endothelial structures and the mural cell (precursors) may be one of the important reasons why the organization and anastomosis of the vessel-like structures in the bone tissue engineering setting is less compared to the skeletal muscle tissue engineering setting. Future research should focus on the communication between endothelial cells and added smooth muscle precursors in a bone tissue engineering setting. Apart from that, it is indicated in literature that mesenchymal stem cells have the ability to differentiate towards smooth muscle cells.[8] However, in our cocultures of hMSC and HUVEC, differentiation towards smooth muscle cells was generally not detected. Additional stimuli may be necessary to initiate this differentiation. Although the addition of differential stimuli to the coculture system may prove to yield negative results, since they may negatively influence the differentiation of the osteoprogenitor cells, studies in this direction are worthwhile and can yield clues on the maturation of the prevascular structures in this bone tissue engineering system.

Study applicable endothelial cell sources for prevascularized bone tissue engineering

An important factor for the application of *in vitro* prevascularization is the source of the cells involved. If prevascularization is to be used in clinical procedures, the endothelial cells should be isolated from the patient in sufficient numbers and in an acceptable time frame. Since mesenchymal stem cells are already frequently used as the source of osteoprogenitor cells for bone tissue engineering, these cells are an interesting cell source for endothelial cells. This thesis already describes the differentiation of mesenchymal stem cells from the bone marrow to-

wards endothelial cells. However, we were still unsuccessful in using these cells for prevascularized bone tissue engineering, without the addition of mature endothelial cells. Future research should focus on refining the endothelial differentiation protocols for mesenchymal stem cells *in vitro*. Apart from that, other sources of endothelial cells should be investigated. These may include the isolation of endothelial progenitor cells directly from bone marrow biopsies or from the peripheral blood, or adult stem cells isolated for instance from adipose tissue.

Study alternative protocols for prevascularization in bone tissue engineering (temporal and spatial separation of different cell types)

The work in this thesis is restricted to one possible strategy of *in vitro* prevascularization. Endothelial cells are mixed and seeded simultaneously with the other cell type and development of both the prevascular structures and the other tissue takes place at the same time. Alternative strategies include both temporal and spatial separation of the different cell types. This allows the different cell types to develop more independent of each other and therefore alternative strategies could contribute to the organization of the prevascular structures. Studies should thus be performed where the different cell types are seeded in specific regions or patterns or in different time frames.

Focus on creation of a vascular axis inside a tissue engineered construct in vitro that can be microsurgically anastomosed to host blood vessels

In vitro prevascularization has the potential to accelerate vascularization after implantation. However, blood vessels from the host still have to grow into the outer regions of the construct until they meet and can anastomose with the prevascular network. Vascularization can even be faster when the prevasculature can be microsurgically connected to the host blood system, as is the case with *in vivo* prevascularization which is described in chapter two of this thesis. Future research should therefore focus on the creation of a vascular axis in the prevascularized construct, that can be microsurgically anastomosed upon implantation.

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Summary

Tissue engineering has been an active field of research for several decades now. However, the number of successful clinical applications in the field of tissue engineering are limited and can mainly be found in thin or avascular tissues like skin and cartilage. One of the current limitations of tissue engineering is the inability to provide sufficient blood supply in the initial phase after implantation. Insufficient vascularization after implantation can result in nutrient limitations, which can result in suboptimal integration of, and cell death in tissue engineered constructs. Several strategies to improve vascularization after implantation have been studied in the past. These include angiogenic growth factor delivery, scaffold design to facilitate angiogenesis, and *in vivo* prevascularization. This thesis explores a relatively new method to improve vascularization; *in vitro* prevascularization. The rationale of this method is that endothelial cells can form a prevascular network in another tissue during *in vitro* culture. After implantation of the resulting construct, the prevascular network can anastomose to the vasculature of the host and thus become a functional, perfused vascular network that provides the construct with nutrients. This will result in a faster vascularization and thus a better survival of the implant.

Chapter three describes a proof of principle for the concept of *in vitro* prevascularization. In this chapter C_2C_{12} mouse myoblast cells are combined with human umbilical vein endothelial cells (HUVEC) for prevascularized skeletal muscle tissue engineering. This chapter shows that the endothelial cells organized into prevascular structures *in vitro*, without negatively influencing the differentiation of the muscle tissue. Moreover, smooth muscle precursor cells colocalized with the endothelial cells in the prevascular structures, which resulted in stabilization and better organization of these structures, as indicated by an increase in the amount of lumen formation. After implantation, the skeletal muscle tissue integrated properly with the surrounding tissue. Moreover, the prevascular structures connected to the host vasculature and became functional perfused vessels. This resulted in increased perfusion and survival of the tissue engineered construct.

Bone formation and vascularization are intimately linked, both in bone development and repair. Therefore, vascularization in bone tissue engineering is not only important for the survival of the implanted cells, but also for proper bone formation. Chapter two gives an overview of the relation between bone formation and vascularization. During bone development and growth, cartilage of the growth plate goes into hypertrophy. Hypertrophic chondrocytes secrete angiogenic factors, which results in vascularization of the hypertrophic cartilage. Vascularization is essential for the delivery of osteogenic cells, the removal of hypertrophic chondrocytes, and mineralization of the matrix. Inhibition of vascularization of the hypertrophic zone in the growth plate results in impaired or no bone formation. The relationship between osteogenesis and angiogenesis is also evident when the interactions between osteogenic and angiogenic cells are studied. Osteogenic cells secrete the angiogenic growth factor VEGF in response to a multitude of osteogenic factors including 1,25-dihydroxyvitamin D_3, BMP-2 and dexamethasone. On the other hand, endothelial cells can enhance the proliferation and differentiation of osteoprogenitor cells by the secretion of osteogenic growth factors like IGf-1 and BMP-2. Apart from that, it has also been shown that direct contact between endothelial cells and osteoprogenitor cells stimulates the osteogenic differentiation of the latter.

Chapter four describes a model system for prevascularized bone tissue engineering. This system consists of spheroid cocultures of human mesenchymal stem cells (hMSC) and HUVEC. It is shown that a three-dimensional interconnected prevascular network was formed in these constructs within 10 days of *in vitro* culture, without the addition of angiogenic growth factors. However, maturation of the prevascular structures *in vitro* was limited and lumen formation was minimal. The formation of the prevascular network was promoted by seeding less than 5% HUVEC. Moreover, the addition of endothelial cells resulted in an upregulation of the osteogenic marker alkaline phosphatase, which is an indication that the addition of endothelial cells does not negatively influence the osteogenic differentiation of the construct. The addition of smooth muscle cells or smooth muscle cell precursors did not result in stabilization of the prevascular network. After implantation of the coculture spheroids, the prevascular network developed further and lumen was formed regularly. However, anastomosis to the host vasculature was limited.

Since the HUVEC that are used in chapter four are a model system that is not fit for clinical applications, chapter five explores the use of several endothelial progenitor cells (EPC) for prevascularized bone tissue engineering. First, hMSC from the bone marrow were differentiated towards endothelial cells. Differentiation was such that they formed capillary-like structures containing lumen, stained

positive for CD31, attained the ability to take up acetylated low density lipoproteins (acLDL) and formed perfused vessels *in vivo*. However, in a 3D coculture setting with undifferentiated hMSC, the cells dedifferentiated and did not form prevascular structures. Second, the use of EPC isolated from umbilical cord blood for prevascularized tissue engineering was investigated. Three groups of EPC, in different stages of endothelial differentiation, were used for prevascularized bone tissue engineering. Only the most mature EPC resulted in the formation of prevascular structures in a bone tissue engineering setting, whereas the other two dedifferentiated and did not form prevascular structures. The amount of prevascular structures formed when using EPC was less than when HUVEC or human dermal microvascular endothelial cells (HMVEC) were used. The degree of organization, however, was higher.

Chapter five demonstrates that hMSC can differentiate towards endothelial cells. Therefore, it is possible that hMSC are directly involved in the formation of the prevascular network in cocultures of hMSC and endothelial cells. Chapter six describes several labeling studies that investigate the role of hMSC in the formation of the prevascular network. Cocultures of CFSE labeled HUVEC and hMSC showed that in a 2D environment, HUVEC organized into capillary-like structures. In a 3D environment on the other hand, labeled HUVEC could mainly be seen as unorganized round cells. Although these cells were often close to or part of the prevascular structures that were formed in these constructs, the structures mainly consisted of unlabeled cells. Three-dimensional cocultures of HUVEC and BrdU labeled hMSC showed that hMSC were differentiated into cells expressing both CD31 and vWF and were incorporated in the prevascular structures. Further data in chapter five shows that both direct cell contact between HUVEC and hMSC and VEGF played an important role in the differentiation of hMSC and the formation of the prevascular structures.

In conclusion, this thesis shows that *in vitro* prevascularization can be a promising strategy to enhance vascularization in the field of tissue engineering. However, it is likely that the success of *in vitro* prevascularization will vary between different tissues and settings. This thesis shows that in a skeletal muscle tissue engineering setting, *in vitro* prevascularization enhances vascularization and survival of the construct after implantation. In a bone tissue engineering setting however, the contribution of *in vitro* prevascularization to *in vivo* vascularization is as yet minimal. However, this does not mean that *in vitro* prevascularization is not useful for bone tissue engineering. It is indicated in chapter three, as well as known from literature, that endothelial cells can enhance the osteogenic differentiation of hMSC. This means that the addition of endothelial cells to bone tissue engineering constructs could enhance *in vivo* bone formation, even if the direct

contribution to vascularization is limited. Furthermore, future research may identify the factors that are needed to improve anastomosis of the prevascular network in these bone tissue engineering constructs after implantation. Apart from that, the differentiation of hMSC towards endothelial cells is a promising finding. The ability of hMSC to differentiate towards endothelial cells is not only interesting for prevascularized bone tissue engineering. Due to the accessibility of hMSC and their proliferative capacities, endothelial cells form hMSC may prove to be an interesting cell source for numerous tissue engineering applications in the future.

Samenvatting

Weefselkweek is al tientallen jaren een actief veld van onderzoek. Het aantal succesvolle klinische toepassingen van weefselkweek is echter nog beperkt. De toepassingen die er zijn, bestaan voornamelijk uit dunne of minimaal doorbloede weefsels als huid en kraakbeen. Eén van de grote limitaties van weefselkweek is de vorming van een volwaardige bloedvoorziening na implantatie. Onvoldoende vascularisatie na implantatie kan resulteren in een tekort aan voedingsstoffen, wat er weer voor kan zorgen dat de cellen in het gekweekte weefsel niet goed integreren of zelfs sterven. In het verleden zijn er meerdere methodes ontwikkeld om de vascularisatie na implantatie te verbeteren. Enkele voorbeelden hiervan zijn de toediening van angiogene groeifactoren, scaffold design om de ingroei van bloedvaten te vergemakkelijken en *in vivo* prevascularisatie. Dit proefschrift onderzoekt een relatief nieuwe methode om vascularisatie te verbeteren, namelijk *in vitro* prevascularisatie. Het idee achter deze methode is dat endotheelcellen prevasculaire netwerken kunnen vormen in andere weefsels tijdens de *in vitro* kweek. Na implantatie kunnen deze netwerken dan aansluiten op het bloedvatsysteem van de patiënt, wat er voor zorgt dat het netwerk functioneel wordt en het construct kan voorzien van voedingsstoffen. Dit resulteert uiteindelijk in snellere vascularisatie en dus betere overleving van het construct.

Hoofdstuk drie beschrijft een 'proof of principle' voor *in vitro* prevascularisatie. In dit hoofdstuk worden muis myoblast cellen (C_2C_{12}) gecombineerd met humane endotheelcellen geïsoleerd uit de navelstreng (HUVEC) voor de kweek van geprevasculariseerd skeletspierweefsel. Dit hoofdstuk laat zien dat de endotheelcellen zich tijdens de kweek organiseerden in prevasculaire structuren, zonder de differentiatie van het spierweefsel negatief te beïnvloeden. Daarnaast zorgde de toevoeging van voorlopers van gladde spiercellen voor een stabilisatie en betere organisatie van de structuren. Na implantatie was het spierweefsel goed in staat om te integreren met het omliggende weefsel. Bovendien sloten de prevasculaire structuren aan op het bloedvatsysteem van het proefdier, waardoor ze doorstroomd werden met bloed. Dit resulteerde uiteindelijk in een betere doorbloeding

en overleving van het implantaat.

Botvorming en vascularisatie zijn nauw met elkaar verbonden, zowel tijdens botgroei als tijdens de genezing van bot. Hierdoor is vascularisatie niet alleen belangrijk voor de overleving van cellen bij bot weefselkweek, maar ook voor de correcte vorming van nieuw botweefsel. Hoofdstuk twee geeft een overzicht van de relatie tussen botvorming en vascularisatie. Tijdens botontwikkeling en botgroei wordt het uiteinde van het kraakbeen van de groeiplaat hypertroof. De hypertrofe chondrocyten scheiden vervolgens angiogene factoren uit, wat leidt tot vascularisatie van het hypertrofe kraakbeen. Vascularisatie is essentieel voor de toevoer van osteogene cellen, de verwijdering van hypertrofe chondrocyten en de mineralisatie van de matrix. Het remmen van de vascularisatie in de hypertrofe zone van de groeiplaat leidt tot verslechterde of zelfs geen botvorming. De relatie tussen bot- en bloedvatvorming wordt ook duidelijk wanneer de interacties tussen osteogene en angiogene cellen bestudeerd worden. Als reactie op osteogene groeifactoren als 1,25-dihydroxyvitamine D_3, BMP-2 en dexamethason, produceren osteogene cellen angiogene groeifactoren als VEGF. Aan de andere kant kunnen endotheelcellen de proliferatie en differentiatie van osteogene cellen stimuleren door de uitscheiding van groeifactoren als Igf-1 en BMP-2. Daarnaast is het ook bekend dat direct contact tussen endotheelcellen en osteogene cellen de osteogene differentiatie van deze cellen stimuleert.

Hoofdstuk vier beschrijft een modelsysteem voor de kweek van geprevasculariseerd bot. Dit systeem bestaat uit bolvormige cokweken van humane mesenchymale stamcellen (hMSC) uit het beenmerg en HUVEC. Hoofdstuk vier laat zien dat in deze constructen binnen 10 dagen *in vitro* kweek een driedimensionaal prevasculair netwerk werd gevormd. De rijping van deze prevasculaire structuren was echter beperkt en holle structuren waren slechts beperkt aanwezig. De vorming van de prevasculaire structuren werd gestimuleerd door het zaaien van 5% of minder HUVEC. Daarnaast resulteerde de toevoeging van endotheelcellen in een verhoging van de expressie van alkaline phosphatase, wat een indicatie is dat de toevoeging van endotheelcellen de osteogene differentiatie van het construct niet negatief beïnvloedt. De toevoeging van gladde spiercellen of voorlopercellen hiervan zorgde niet voor stabilisatie van het prevasculaire netwerk. Na implantatie ontwikkelde het prevasculaire netwerk zich verder, wat resulteerde in de vorming van holle structuren. De aansluiting op het vasculaire systeem van het proefdier was echter beperkt.

De HUVEC die gebruikt worden in hoofdstuk vier zijn een model celsysteem die niet bruikbaar is voor klinische toepassing. Hoofdstuk vijf bestudeert daarom het gebruik van enkele endotheel voorlopercellen (EPC) voor geprevascullariseerd

bot weefselkweek. Eerst werden hMSC uit het beenmerg gedifferentieerd richting endotheelcellen. De cellen vormden capillaire structuren, kleurden positief voor de endotheelmarker CD31, waren in staat om acLDL op te nemen en vormden met bloed doorstroomde vaten na implantatie. Wanneer deze cellen echter in een driedimensionale cokweek werden gebracht met ongedifferentieerde hMSC, de-differentieerden de cellen weer en werden er geen prevasculaire structuren gevormd. Daarnaast werden EPC geïsoleerd uit navelstrengbloed. Drie groepen EPC, in verschillende stages van differentiatie, werden gebruikt voor geprevasculariseerd bot weefselkweek. Enkel het gebruik van de meest gedifferentieerde EPC resulteerde in de vorming van prevasculaire structuren. Bij de overige twee de-differentieerden de cellen en werden er geen prevasculaire structuren gevormd. Het aantal prevasculaire structuren bij gebruik van EPC was lager dan bij gebruik van HUVEC of humane microvasculaire endotheelcellen uit de huid (HMVEC). De mate van organisatie was echter hoger.

Hoofdstuk vijf laat zien dat hMSC richting endotheelcellen kunnen differentiëren. Het is daarom mogelijk dat de hMSC direct betrokken zijn bij de vorming van prevasculaire structuren in cokweken van hMSC en endotheelcellen. Hoofdstuk zes beschrijft enkele labeling studies die de rol van hMSC bij de vorming van prevasculaire structuren onderzoeken. Cokweken van CFSE gelabelde HUVEC en hMSC lieten zien dat HUVEC zich in een tweedimensionale omgeving organiseerden in capillaire structuren. Echter, in een driedimensionale omgeving bleven HUVEC voornamelijk aanwezig als niet georganiseerde, ronde cellen. Hoewel ze veelvuldig deel uitmaakten van de prevasculaire structuren die gevormd werden in deze cokweken, bestond het merendeel van de structuren uit niet-gelabelde cellen. Driedimensionale cokweken van HUVEC en BrdU gelabelde hMSC lieten zien dat hMSC differentieerden naar cellen die positief waren voor de endotheelmarkers CD31 en vWF en opgenomen werden in de prevasculaire structuren. Verder laat hoofdstuk zes zien dat zowel direct contact tussen HUVEC en hMSC en de groeifactor VEGF een belangrijke rol spelen bij de differentiatie van hMSC en de vorming van prevasculaire structuren.

Dit proefschrift laat zien dat *in vitro* prevascularisatie een veelbelovende strategie kan zijn voor de verbetering van vascularisatie in weefselkweek toepassingen. Het is echter aannemelijk dat het succes van deze strategie afhankelijk is van het weefsel dat gekweekt wordt en het systeem dat daarvoor gebruikt wordt. Dit proefschrift toont aan dat *in vitro* prevascularisatie resulteert in een betere vascularisatie en overleving van gekweekt skeletspierweefsel. In het bestudeerde bot weefselkweek systeem is de bijdrage van *in vitro* prevascularisatie voor *in vivo* vascularisatie echter beperkt. Dit betekent niet dat *in vitro* prevascularisatie niet zinvol is voor bot weefselkweek. De literatuur geeft namelijk aan dat endotheelcel-

len de osteogene differentiatie van hMSC kunnen bevorderen. Dat betekent dat de toevoeging van endotheelcellen een positief effect kan hebben op de vorming van bot na implantatie, ook als de directe bijdrage aan vascularisatie beperkt is. Tevens is er de mogelijkheid dat toekomstig onderzoek de factoren kan identificeren die nodig zijn voor een correcte aansluiting van de prevasculaire structuren op het vasculaire systeem van de patiënt. Daarnaast is de differentiatie van hMSC naar endotheelcellen een veelbelovende vondst. Het vermogen van hMSC om te differentiëren tot endotheelcellen is niet alleen interessant voor geprevasculariseerd bot weefselkweek. Vanwege de toegankelijkheid en de hoge groeicapaciteit van deze cellen, hebben hMSC de potentie om een interessante bron van endotheelcellen te zijn voor een groot aantal weefselkweektoepassingen in de toekomst

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Curriculum vitae

Jeroen Rouwkema was born on the 25th of July 1979 in Meppel, The Netherlands. He grew up in Smilde until he graduated from Dr Nassau College (atheneum). He then went to Wageningen to study Bioprocess Engineering, for which he received his masters degree cum laude in January 2003. During his studies he performed traineeships at IsoTis in collaboration with the Department of Bioprocess Engineering in Wageningen (Dr. ir. J. Malda and Dr. ir. D.E. Martens), the Department of Organic Chemistry in Wageningen (Dr. C.A.G.M. Weijers and Dr. M.C.R. Franssen), and at the Department of Chemical Engineering of the Massachusetts Institute of Technology at Cambridge, USA (Dr. S. Levenberg and Prof. dr. R.S. Langer). In February 2003 he started as a PhD student at the Department of Tissue Regeneration of the University of Twente under the supervision of Prof. dr. C.A. van Blitterswijk. The subject of his research was prevascularization in bone tissue engineering and the results are described in this thesis. From September 2007, he will continue his scientific work at the department of Tissue Regenration of the University of Twente.