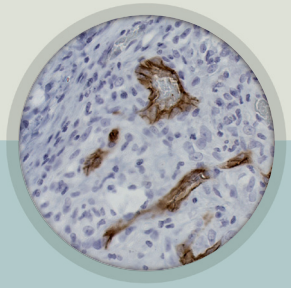
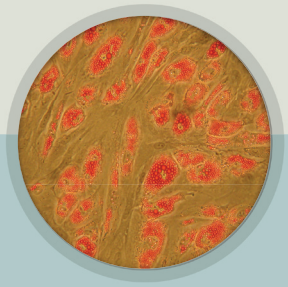
ENGINEERING VASCULARIZED ADIPOSE TISSUE

FEMKE VERSEIJDEN





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CHAPTER 1

General introduction

SOFT TISSUE DEFECTS AND CURRENT SURGICAL TREATMENT OPTIONS

A large portion of the plastic and reconstructive surgical procedures performed each year is aimed at repairing soft tissue defects, which result for example from traumatic injury or tumor resections [1,2]. Large soft tissue defects, lead to a change in function and 'normal' body contour, which in its turn can lead to disability and emotional stress [2]. Currently used methods to repair soft tissue defects include the use of autologous adipose tissue transplants and synthetic implants. Both procedures have their own advantages and drawbacks [3,4,5,6].

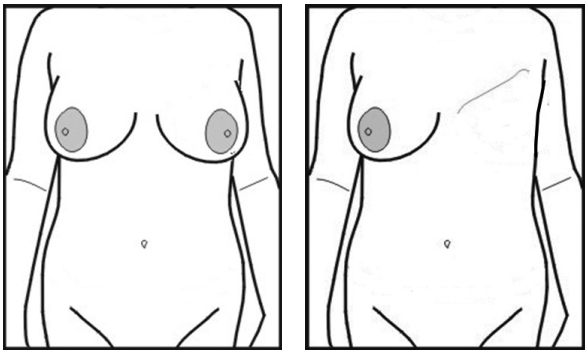

Figure 1. Example of a large soft tissue defect due to tumor resection. Drawing of an adult female before (left) and after (right) mastectomy. Adapted from figures at www.microsurgeon.org

Autologous adipose tissue transplants have the advantage for a low risk of an adverse immune response and have been reported to restore specific soft tissue defects [7,8,9]. However, donor-site morbidity remains the principal drawback of large autologous adipose tissue transplants [10]. In addition, the success of non-vascularized autologous adipose tissue transplants is unpredictable, as they have the tendency to lose 40 to 60 percent of the volume over time [10,11,12,13].

Synthetic materials such as silicone implants have the advantage of an endless supply and often yield good results. However, limitations of these synthetic filler materials include rupture, leakage, dislocation and suboptimal biocompatibility [14,15,16]. To address limitations of current reconstructive surgical procedures, adipose tissue engineering has emerged as a new alternative to repair soft tissue defects.

ADIPOSE TISSUE

Adipose tissue is a highly specialized connective tissue that is found in two forms: white and brown adipose tissue. Brown adipose tissue is so called because of its color,

which is due to its high vascularity and numerous mitochondria (containing colored cytochromes) in the cells. Brown adipose tissue has a limited distribution throughout the body and functions primarily as a heat source [1,17,18]. During aging, brown adipose tissue is gradually replaced by white adipose tissue.

White adipose tissue is widely distributed throughout the entire body and is most widely known for its role as energy reservoir. However, the function of white adipose tissue is much more diverse. It also shapes the body contour, helps to keep organs into place, contributes to insulation/thermoregulation of the body and can serve as a mechanical cushion (think of adipose tissue in the hand palm/foot sole). Besides that, white adipose tissue also functions as a secretory organ. It synthesizes various factors (for example cytokines and leptin) that exert their effect locally or are transported by the blood to a distant organ [18,19,20].

Macroscopically, adipose tissue is subdivided into incomplete lobules. These lobules are units of 10^2-10^3 adipose cells, which are separated by fibrous septa containing a rich vascular supply and nerves [21]. Apart from fibroblasts, vascular endothelial cells and neurocytes, adipose tissue is mainly composed of two cell types: adipocytes and adipose precursor cells. Adipocytes are differentiated cells with a diameter varying from 50 to 150μm and an eccentric, flattened nucleus. They are specialized in storing energy as lipid. Adipose precursor cells are mesenchymally derived spindle-formed cells with small lipid inclusions that can readily proliferate and can differentiate into adipocytes [22,23,24,25].

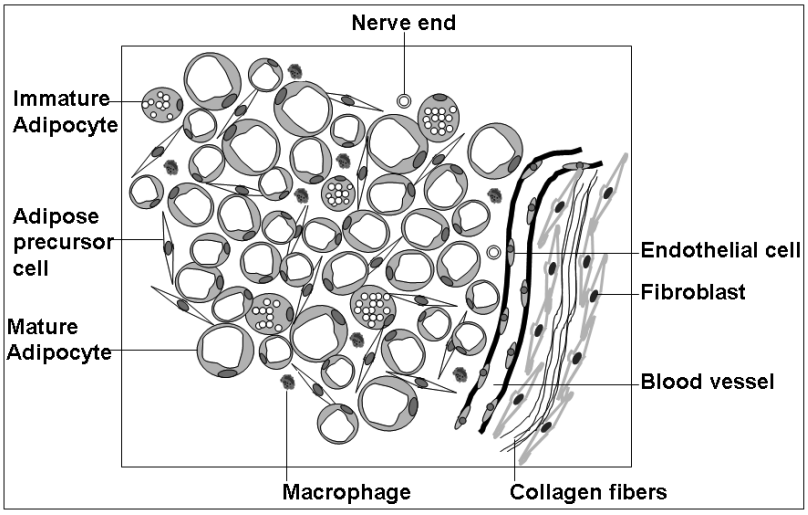

Figure 2. Schematic drawing of the cellular composition of adipose tissue. Adipose tissue is mainly composed of (im)mature adipocytes, adipose precursor cells, adipose tissue matrix (every adipocyte is surrounded by a lamina basalis and a network of thin collagen fibers), fibroblasts and endothelial cells. Immune cells such as macrophages are also present in adipose tissue.

ADIPOSE TISSUE ENGINEERING

Tissue engineering is a relatively new multidisciplinary field that combines engineering and life sciences to improve, repair or restore tissue function [26]. To date, tissue engineering techniques have been developed for several tissues of which some have already translated to the clinical bedside. Typically, in tissue engineering living cells are combined with nonliving biomaterials to 'construct' a tissue equivalent in vitro. The nonliving biomaterials allow three-dimensional tissue formation and provide structural support for the cells until they have synthesized their own extracellular matrix.

In vitro engineered tissue equivalents should be customizable, immune-compatible and able to adapt to the implantation environment similar to autologous tissue transplants. However, in vitro engineered tissue equivalents will not be associated with donor site morbidity, as is the case with larger autologous tissue transplants.

In the field of adipose tissue engineering two approaches have been used. In the first approach, a scaffold is combined with bioactive factors that induce migration and differentiation of cells from the surrounding tissue, which is referred to as in situ adipogenesis. The second approach is cell-based and combines living adipogenic or adipogenic precursor cells with biodegradable matrices/scaffolds ex vivo to allow the development of a three-dimensional tissue structure. This approach is referred to as cell-based or de novo adipogenesis [27,28].

SUITABLE CELLS

Different cell types with different stages of differentiation can be distuinguished for use in adipose tissue engineering.

MATURE CELLS

Firstly, fully differentiated cells, adipocytes, can be used. Adipocytes have optimal tissue-specific characteristics and can readily be attained from clinical procedures; however they have low expandibility and poor ability for volume retention after in vivo implantation [2,28,29].

STEM CELLS

Secondly, stem cells can be used. Two broad types of stem cells can be distinguished: embryonic stem cells and adult stem cells. Embryonic stem cells are isolated from the inner cell mass of blastocysts. They have the unique attributes of endless self-renewal and pluripotency, that is, the ability to form all fully differentiated cell types in the adult body. Although embryonic stem cells have been successfully used for studying adipogenesis in vitro [30], they currently have several drawbacks that make them less suitable for clinical application. Firstly, they are allogenic and will face a substantial risk

of immune-rejection in vivo. In theory this can be circumvented by somatic cell nuclear transfer (i.e. therapeutic cloning or SCNT). By transfer of a nucleus from a somatic cell into an enucleated oocyte, the oocyte can form a blastocyst upon stimulation, from which embryonic stem cells can be isolated that are genetically matched to the donor [31]. No such human embryonic stem cell lines however, have been obtained yet. Secondly, they retain tumorigenic potential as it is still difficult to regulate their proliferation and differentiation. Finally, debates relating to the ethical aspect of embryonic stem cell use are ongoing. Some of the disadvantages of embryonic stem cells (immune rejection and ethical issues) may be surpassed by the recent creation of induced pluripotent stem cells (iPS cells= mature cells that have been genetically reprogrammed to an embryonic stem cell–like pluripotent state by being forced to express genes and factors important for maintaining the defining properties of embryonic stem cells) [32,33,34]. However, iPS cells will hold embryonic stem cells' disadvantages of pluripotency (difficulty to regulate their differentiation) and tumorigenicity.

Adult stem cells can be isolated from multiple adult tissues (bone marrow, adipose tissue, blood, muscle, blood vessels, skin, hair follicles, intestine, brain, liver) and have the ability to differentiate into a restricted amount of cell types (multipotent). For adipose tissue engineering the use of mesenchymal stem cells derived from adipose tissue is preferred and has received the most attention [2,23,35,36]. Adipose tissue-derived mesenchymal stem cells (ASC), also commonly referred to as adipose tissue-derived stromal cells, are highly expandable and have the potential to differentiate into various cells of the mesodermal and endodermal lineage in vitro (such as adipocytes, chondrocytes, osteoblasts myoblasts, endothelial cells and hepatocytes) [37,38,39]. Adipose tissue-derived mesenchymal stem cells do not express specific cell surface markers, but rather a complex pattern of cell surface markers including stromal cell (CD105, CD90, CD146), endothelial cell (CD31, CD34) and hemopoietic markers (CD45) [40,41,42,43]. They can be isolated from adipose tissue by collagenase digestion followed by cell selection through centrifugation (to separate the floating adipocytes from the stromal-vascular fraction) and adherence to cell culture plastic. Differentiation towards adipocytes can be induced with for instance insulin, dexamethasone, indomethacin and isobutylmethylxanthin [40,44,45,46,47,48].

BIOMATERIALS

Biomaterials play an important role in adipose tissue engineering strategies. Apart from providing a temporary three-dimensional architecture, they also have to mediate cellular attachment and viability and must allow lipid accumulation inside the construct after implantation [49]. Moreover, engineered adipose tissue equivalents should be easy to manipulate, while being soft and flexible, in order to avoid discomfort after implantation.

A large variety of biomaterials have been tested for human adipose tissue engineering purposes, which can be divided into biologically derived materials and synthetic materials.

Biologically derived materials (e.g. purified protein components derived from animal or human tissues such as collagen, fibrin, alginate, Matrigel™ or hyaluronic acid) are advantageous because of their inherent properties of being biocompatible and biodegradable. Synthetic materials however, provide a greater control over materials properties such as microarchitecture. In addition, the absence of animal or human substances reduces the risk of pathogen transmission [50,51].

Despite the successful generation of adipose tissue in both biologically derived (fibrin, hyaluronic acid, collagen, alginate and Matrigel™) and synthetic materials (poly lactic-co-glycolic acid and poly glycolic acid), tissue engineered adipose tissue equivalents have not made it into the clinical realm, yet. A critical point is construct size owing to limitations in blood supply [49].

BLOOD VESSELS AND BLOOD SUPPLY

Blood vessels carry blood to and from virtually every tissue in the body, assuring metabolic homeostasis by delivering nutrients and oxygen and removing waste products. They are essential for tissue maintenance, growth and repair. There are 3 types of blood vessels: Vessels that carry blood away from the heart (arteries/arterioles), vessels that facilitate the exchange of nutrients, oxygen and waste products between the blood and the tissues (the capillaries) and vessels that carry blood from the capillaries towards the heart (venules and veins).

All types of blood vessels consist of an inner endothelial cell lining surrounded by mural cells and a basal membrane. Endothelial cells form a barrier between the underlying tissue and the blood. They react with physical and chemical stimuli within the circulation and are involved in the immune response, coagulation, production of extracellular matrix components, and regulation of blood vessel tone. In addition, endothelial cells are pivotal in vessel growth.

Studies have shown that not all endothelial cells are alike. Endothelial cells can vary between tissues and between arteries and veins, expressing different markers and responding differently to similar stimuli [53,54,55,56,57].

Mural cells associate with and coat the endothelial tube. They play an important role in blood vessel formation by stabilizing vessel structures and suppressing endothelial cell growth. Mural cells are commonly subdivided into vascular smooth muscle cells and pericytes. Vascular smooth muscle cells are generally associated with arteries and veins. They are not in direct contact with the endothelial cells but are separated from

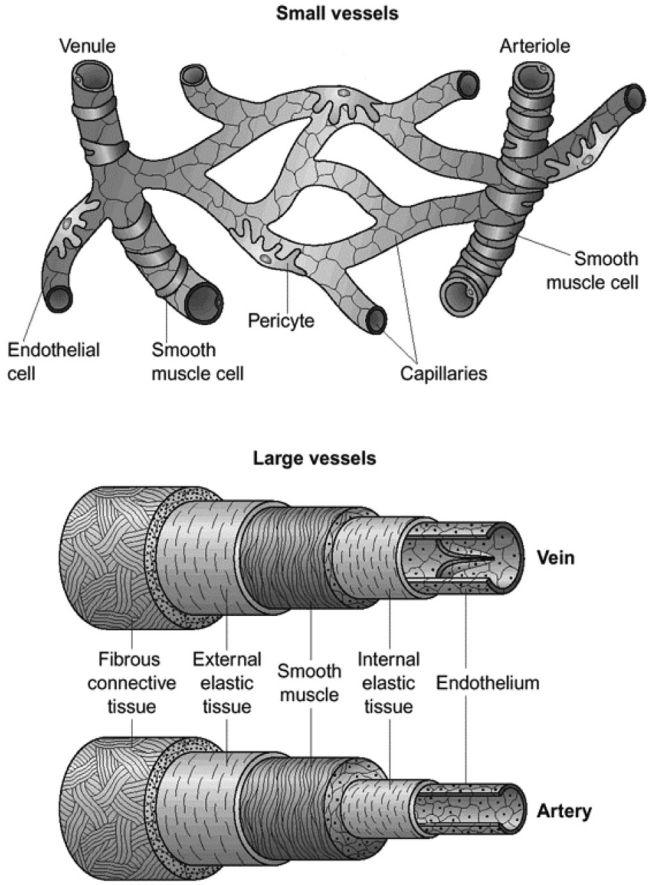

Figure 3. Composition of different types of blood vessels. Capillaries (top) consist of endothelium surrounded by a basement membrane. They are covered loosely by pericytes. Arterioles and venules have an increased coverage of mural cells compared with capillaries. Arterioles are completely covered by vascular smooth muscle cells. Larger vessels (bottom) are composed of multiple layers: an intima, which consists of endothelium, basement membrane and an internal elastic layer; a media, which consists of a thick layer of smooth muscle with reticular fibers (collagen III), elastin and proteoglycans; and an adventitia, which consists of fibroblasts with elastic fibers and collageneous matrix. Veins are characterized by valves preventing backflow of blood, whereas arteries have strong elastic vessel walls for withstanding the high blood pressures. Adapted from Ondine Cleaver & Douglas A. Melton [52].

the endothelium through the basal membrane. Pericytes are generally associated with smaller diameter blood vessels (capillaries) and share their basal membrane with the endothelium [58,59]. The basal membrane surrounds the vascular cells and provides structural support. Its major constituents are type IV collagen, laminin, heparin-sulphate proteoglycans and nidogen/entactin. Due to different isoforms, quantity and assembly of these proteins the basal membrane composition varies from one tissue to another [60,61,62].

VESSEL GROWTH AND MATURATION

Vessels can grow in several ways. Vasculogenesis refers to de novo formation of blood vessels from endothelial progenitors, angiogenesis and arteriogenesis refer to sprouting and subsequent stabilization of the nascent vascular bed, and collateral growth refers to the growth and formation of collateral bridges between arterial networks [63,64,65]. During vasculogenesis, endothelial progenitors assemble into a primitive vascular labyrinth of small capillaries [65]. It was thought for many years that vasculogenesis only occurred during blood vessel development in the embryo. Recent data, however, indicate that endothelial progenitors also contribute to vessel growth in the adult. Even more, endothelial progenitors can be therapeutically used to stimulate vessel growth in ischemic tissues [66,67,68]. Vasculogenesis leads to an immature, poorly functional vasculature. It is followed by angiogenesis for growth and remodeling of the primitive vascular network into a complex network. During angiogenesis, endothelial cells from existing vessel walls degrade their surrounding basement membrane, proliferate and migrate into the interstitium resulting in the formation of sprouts [69,70,71].

Vessel maturation is an essential process in blood vessel formation. It involves both the maturation of the vessel wall and the maturation of the vessel network. Maturation of the vessel wall includes recruitment of mural cells, generation of surrounding matrix and elastic laminae and organ specific specialization of endothelial cells/mural cells/matrix. Maturation of the vessel network includes patterning of the nascent vessel network by expansion, branching and pruning to meet local demands. Vessel maturation is regulated by a myriad of cell-cell and cell-matrix signaling molecules as well as by mechanical forces generated by the circulation. When vessel growth is not followed by vessel maturation, a disorganized leaky vasculature will be formed that will generally regress over time due to the process of vascular remodeling [72,73].

BLOOD SUPPLY: A PREREQUISITE FOR SURVIVAL OF ENGINEERED TISSUES

Living tissues quickly starve without the delivery of oxygen and nutrients to the cells by the blood. Any tissue (except for the avascular cornea and cartilage) that is more than 100-200 μm thick (the oxygen diffusion limit) needs a vascular network to ensure that every cell within the tissue is close enough to capillaries to exchange oxygen, nutrients and waste products. Hence, engineered adipose tissue constructs that are more than a few cells thick require an integrated vasculature for in vivo survival [69,73,74].

During in vitro culture, engineered tissues that have a size above the diffusion limit can be supplied with nutrients using perfusion bioreactor systems or microfluidic technologies [75,76,77]. After implantation, however, the spontaneous ingrowth of

vessels is often too slow to provide adequate nutrient transport to the cells inside the constructs. Therefore, additional strategies to improve vascularization are crucial to ensure the survival of thick tissue-engineered constructs [73,78,79].

In the past few years a number of approaches to enhance vascularization within engineered tissue constructs have been devised, which will be briefly explained below: (1) angiogenic factor delivery, (2) improving scaffold design, (3) in vivo prevascularization and (4) in vitro prevascularization.

ANGIOGENIC FACTOR DELIVERY

Several studies have shown that controlled delivery of angiogenic factors to engineered-tissue constructs can enhance vessel ingrowth after implantation [16,80,81]. Direct (such as VEGF, FGF, PDGF) and indirect (such as Sonic hedgehog) angiogenic agents have been used [80,81,82,83] and several strategies for the supply of these agents have been developed (such as the addition of recombinant protein, gene transfer and angiogenic factor-overexpression) [84,85,86,87]. Despite the development of new delivery strategies and the combined use of multiple agents however, vessels induced by angiogenic factor delivery are often immature and leaky [73]. A suboptimal mix of agiogenic agents and/or a suboptimal fashion of delivery likely cause this. Finding the optimal cocktail of molecules and delivering them in the optimal fashion remain therefore major challenges in this area of research.

IMPROVING SCAFFOLD DESIGN

The architecture of the scaffold used in tissue constructs significantly affects blood vessel ingrowth. Highly porous scaffolds with pore sizes of $\geq$ 250 μm and high pore interconnectivity facilitate cell migration and vascularization [88,89,90]. Other than designing porous scaffolds, different strategies to enhance vascularization due to scaffold design have been employed. For instance, microfabrication techniques (such as Micro Electro Mechanical Systems or MEMS) have been used to fabricate microchannel networks within degradable biomaterials. Such microchannel networks can be seeded with endothelial cells in vitro, resulting in a capillary network that can be perfused in vivo [16,91,92]. In addition, scaffolds can be coated with angiogenic matrices such as fibrin and Matrigel™ [93,94,95,96]. The main purpose of a scaffold in tissue engineering is supporting attachment and growth of tissue cells. Since the requirements for supporting tissue vascularization and tissue development do not necessarily correspond, designing a scaffold that promotes both vascularization and development/growth of cells is a challenging task.

IN VIVO PREVASCULARIZATION

This method involves wrapping a vascularized soft-tissue flap around the tissue construct or inserting a vascular pedicle or loop within the tissue construct. Following an in vivo incubation period, the tissue construct is vascularized by a process of angiogenic outgrowth. Then, the vascularized tissue construct can be transferred to the desired location and its vascular network connected to the local vasculature using microsurgical techniques [83,97,98,99]. An advantage of this in vivo technique is that when the tissue construct is implanted at its final location, it can be perfused immediately due to surgical anastomosis. The limitations of this technique however, are that two surgical procedures are necessary, a vascular loop is removed from its original location and extra cells may need to be seeded at the defect site, since oxygen/nutrient deficiencies are likely during the vascularization period at the initial implantation site [79].

IN VITRO PREVASCULARIZATION

This method involves prefabrication of vascular structures within tissue constructs prior to implantation. When cultured under the right conditions and in the appropriate environment, endothelial cells are able to form vascular structures in vitro. For in vitro prevascularization, target cells and cellular components of vessel walls (endothelial cells and perivascular cells) are cocultured in a three-dimensional matrix, to obtain an engineered tissue equivalent with a primitive vascular network. This vascular network can then anastomose to ingrowing host blood vessels, reducing the time for vascularization of the entire construct [73,79,100,101].

The first three approaches mentioned above solely rely on spontaneous vessel ingrowth postimplantation. During this time, oxygen/nutrient transport to the cells is completely dependent on diffusion, which is inadequate to support cells deeper (that is deeper than the diffusion limit of 100 to 200 μm) within the implant. The in vitro prevascularization approach that includes a vascular network within the tissue construct in vitro, potentially overcomes this limitation. In this approach however, rapid anastomosis of the engineered vascular structures and those of the host is critical.

THESIS AIM AND OUTLINE

The aim of this thesis is twofold:

- To explore the angiogenic and vasculogenic potential of ASC in different tissue engineering settings and
- To investigate the potential positive effect of in vitro prevascularization on the vascularization of ASC-based adipose tissue constructs.

Chapter 2: Although multiple studies have shown that ASC can release factors that stimulate blood vessel growth [102,103,104], detailed information about the effects of adipogenic differentiation on the angiogenic potential of ASC remains largely unknown. In this chapter we started out investigating the expression and secretion of a large panel of angiogenic factors during ASC differentiation and evaluated the effects of ASC-conditioned medium on endothelial cells.

Chapter 3: Another parameter influencing ASC behavior and vascularization of ASC-based adipose tissue constructs is the extracellular environment. Various biomaterials have been combined with ASC for adipose tissue engineering purposes [49]. Considering limitations associated with synthetic and exogenous matrix-based tissues, we were interested in the use of scaffold/biomaterial-free ASC constructs for adipogenesis. In this chapter, we compared scaffold-free ASC-based adipose tissue constructs with another promising method that makes use of fibrin as a scaffold material. We evaluated in vivo construct adipogenic differentiation and vascularization. Additionally, we determined the impact of scaffold-free and fibrin-based environments on ASC adipogenic differentiation and their secretion of angiogenic factors in vitro.

Chapter 4: In this chapter we explored the supportive role of ASC in de novo vessel formation in vitro. We hypothesized that ASC can support the formation of vascular structures in engineered tissue constructs. Using a three-dimensional coculture system of ASC and endothelial cells we assessed the ability of ASC to support the formation of vascular-like structures from endothelial cells in vitro. In addition, we studied the mechanisms leading to the support of the formation of vascular structures in vitro.

Chapter 5: In the past few years a number of new approaches to promote the vascularization of implanted tissue constructs have been devised, such as optimization of scaffold architecture, the controlled release of angiogenic factors and the prefabrication of vascular structures within tissue constructs in vitro, also referred to as prevascularization. Although prevascularization has yielded promising results in skin, skeletal muscle and bone tissue constructs [100,105,106], the contribution of prevascularization in adipose tissue constructs has not been tested yet. The aim of this chapter was to generate prevascularized adipose tissue constructs by coculturing ASC and endothelial cells in spheroids, and to establish a proof of principal for the expected positive effect of prevascularization on in vivo vascularization of adipose tissue constructs.

Chapter 6: The in vivo results as described in chapter 5 pointed out that prevascularization holds promising possibilities for adipose tissue engineering. Although the

small spheroid tissue constructs used in chapter 5 have been shown to form bigger tissue patches of mm^3 scale [107,108], we reasoned that it is likely that the use of a scaffold material is needed to produce prevascularized adipose tissue equivalents of more clinically relevant dimensions (cm^3 scale). Fibrin-based constructs can easily be scaled-up, and as described in chapter 4, positively affect ingrowth of host blood vessels. The aim of this chapter was to generate prevascularized fibrin-based adipose tissue constructs and to compare the in vivo vascularization of these constructs with the in vivo vascularization of non-prevascularized ASC-only fibrin-based constructs.

Chapter 7: In this final chapter the main results described in this thesis are discussed and suggestions for further research are offered.

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CHAPTER 2

Angiogenic capacity of human adipose-derived stromal cells during adipogenic differentiation: an in vitro study

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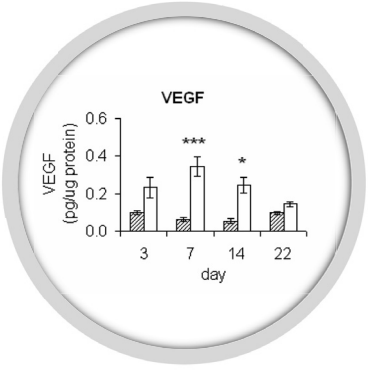


ABSTRACT

Background: Improving vascularization of engineered adipose tissue constructs is a major challenge in the field of plastic surgery. Although human adipose–derived stromal cells (hASCs) are known to release factors that stimulate new blood vessel formation, detailed information about the effects of adipogenic differentiation on the angiogenic potential of hASCs remains largely unknown. In the present study, we studied the expression and secretion of a large panel of angiogenic factors during hASC differentiation and evaluated the effects of hASC-conditioned medium (hASC-CM) on endothelial cells (EC).

Methods: hASC were cultured on adipogenic medium or basal medium. Conditioned medium was collected and cells were harvested following 0, 3, 7, 14 and 22 days of culture. The stage of adipogenic differentiation of hASC was assessed using Oil Red O staining, fatty acid binding protein-4 gene expression (FABP4) and glycerol-3-phosphate dehydrogenase (GPDH) activity.

Results: Gene expression of vascular endothelial growth factor (VEGF), placental growth factor (PGF), angiopoietin-1 (ANGPT1), angiopoietin-2 (ANGPT2) and protein secretion of VEGF significantly increased during short-term adipogenic differentiation of hASCs. Moreover, conditioned medium from differentiated hASCs strongly enhanced EC numbers compared to conditioned medium from undifferentiated hASCs

Conclusion: In vitro adipogenic differentiation of hASCs improves their ability to support endothelial viable cell numbers and suggests that hASCs differentiated for a short period potentially improve angiogenic responses for in vivo implantation.

INTRODUCTION

The restoration of soft tissue contour defects remains a major challenge in plastic surgery. Soft tissue defects result, for example, after oncological resection or complex trauma or from congenital or acquired adipose atrophy [1,2]. Current reconstructive methods all have intrinsic disadvantages: large tissue transfers give donor site morbidity; foreign material injections or implants give unpredictable host responses; autografting of fat pads or injections of adipocyte cell suspensions give unsatisfactory results due to limited fat graft survival and necrosis [3,4]. Adipose tissue engineering using mesenchymal stem cells has been suggested as a promising technique for reconstruction of soft tissue defects. Mesenchymal stem cells derived from adipose tissue, also referred to as preadipocytes or adipose-derived stromal cells (ASC) are considered a feasible cell source for adipose tissue regeneration as they are easily obtained, expanded and differentiated to adipocytes [5,6]. Indeed, various studies have shown that in vivo implantation of hASCs is useful for fat pad formation [7,8,9,10]. However, long-term maintenance of tissue-engineered adipose tissue still remains elusive. Similar to autologous fat transplantation procedures, there is an apparent resorption of engineered adipose tissue over time. The absence of an adequate blood supply is thought to be the main cause for this progressive resorption of adipose tissue constructs [7,11,12,13].

Recently, several groups demonstrated that the ASC fraction of human adipose tissue is able to secrete numerous factors involved in new blood vessel formation [14,15,16,17]. In vitro adipogenic differentiation of these cells before implantation may influence their angiogenic activity as shown for the murine 3T3-F442A cell line [18,19], and subsequently affect their ability to stimulate blood vessel ingrowth in tissue engineered constructs. Details about the angiogenic activity of human primary ASCs during adipogenic differentiation are unknown. The aim of this study was to investigate the changes in expression and secretion of important angiogenic factors during adipogenic differentiation of hASCs and to evaluate whether differences between undifferentiated and differentiated cells affect endothelial viable cell numbers in vitro.

MATERIALS AND METHODS

CHEMICALS AND MEDIA

Dulbecco's modified Eagle Medium 1g/l glucose (LG-DMEM) and 4.5 g/l glucose (HG-DMEM), human endothelial-SFM medium, penicillin/streptomycin, collagenase type I and trypsin/ ethylenediaminetetraacetic acid (EDTA) were purchased from Invitrogen (Carlsbad, California). Fetal bovine serum (FBS) was purchased from PAA Laboratories

Gmbh (Cölbe, Germany). Human serum was purchased from Lonza (Verviers, Belgium). Bovine serum albumin (BSA) and other chemicals were from Sigma-Aldrich (Zwijndrecht, The Netherlands). Insulin was from Eli Lilly (Indianapolis, Indiana) and Fibroblast growth factor (FGF-2) and epidermal growth factor (EGF) were from Peprotech EC Ltd. (London, United Kingdom).

ISOLATION AND CULTURE OF HASCS

For this study, hASCs were isolated from breast or abdominal adipose tissue that was obtained from 4 donors, aged 23 (donor 1), 45 (donor 2), 58 (donor 3) and 37 (donor 4) and with BMI values ranging from 24.8-30.4. Adipose tissue was received with approval of the Medical Ethical Committee (# MEC-2005-157). Excised human adipose tissue was washed with 70% (v/v) ethanol and LG-DMEM, minced and digested in sterile-filtered 0.1% collagenase type I solution in the presence of 1% bovine serum albumin. The mixture was suspended in LG-DMEM at 37˚C for 60 minutes in an orbital shaker. Human ASCs were isolated by differential centrifugation, washed with medium (LG-DMEM, 10% FCS, 1% penicillin/streptomycin, 0.5% gentamycin), filtered through a 100 µm mesh and grown in basal medium; i.e. medium supplemented with 10^{-12} M dexamethasone and 10^{-5} M ascorbic acid.

After 24 hours, cultures were washed with PBS with 2% FBS to remove erythrocytes and replenished with basal medium. The isolated hASCs were cultured at 37ºC in a humid atmosphere with 5% CO_2 until they reached 90% confluence. At 90% confluence, adherent cells were released with 0.5% trypsin-EDTA and then either replated at a density of 5.7 x 10^3 cells/cm^2 or used in the experimental set-up.

IN VITRO ADIPOGENIC DIFFERENTIATION EXPERIMENTAL SET UP

First and second passage hASCs used in the experimental set-up were phenotypically characterized and plated at confluence with a density of 2.0 x 10^4 cells/cm^2 in basal medium and allowed to adhere overnight. Subsequently, cells were either replenished using basal medium or adipogenic medium (HG-DMEM, 10% FBS, 1µM dexamethasone, 0.01 mg/ml insulin, 0.2 mM indomethacin, 0.5 mM 3-isobutyl-I-methyl-xanthine, 1% penicillin/streptomycin and 0.5% gentamycin). Cells were grown at 37ºC in a humid atmosphere with 5% $CO_{2.}$ Medium was refreshed every 2-3 days and 24 hours before each measurement. Cell and medium samples were collected at day 0, 3, 7, 14 and 22 and adipogenic differentiation was assessed using Oil Red O staining, glycerol-3-phosphate dehydrogenase (GPDH) activity and the gene expression levels of fatty acid binding protein 4 (FABP4).

In addition, differences in angiogenic capacity were evaluated by the gene expression of vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (basic) (FGF-2), placental growth factor (PGF), platelet derived growth factor B (PDGFB),

transforming growth factor beta 1 (TGFß1), angiopoietin 1 (ANGPT1) and angiopoietin 2 (ANGPT2) using quantitative RT-PCR, by the protein secretion of VEGF, PGF, ANGPT1 and ANGPT2 using sandwich ELISAs and by the ability of the conditioned medium to stimulate endothelial cell survival using the MTT assay.

FLOW CYTOMETRIC CHARACTERIZATION OF HASCS

Early passage hASCs were washed and labeled for 30 minutes on ice with mouse anti-human monoclonal fluorescently labeled antibodies directed against CD34, CD105, CD90, CD144 (dilutions according to manufacturer's instructions, R&D systems, Abingdon, United Kingdom) and CD31 (Chemicon, Huissen, The Netherlands). Cells were directly analyzed on a FACS-Calibur instrument (Becton-Dickinson, Franklin Lakes, New Jersey, www.bd.com).

OIL RED O STAINING

A 0.5% (w/v) stock solution of Oil Red O in isopropanol was diluted 3:2 with distilled water to prepare an Oil Red O working solution. Following 3, 7, 14 and 22 days of culturing, hASCs were rinsed with PBS, fixed in 10% formalin in PBS for 10 minutes and stained for 15 minutes with 1 ml Oil Red O working solution. Then, cells were washed with 1ml distilled water to remove excess of Oil Red O solution. The adipogenic cells were identified by accumulation of cytoplasmatic lipid vesicles. Representative pictures of Oil Red O stained cells within three different fields of one well of a 6-well tissue culture plate were taken using an inverted microscope (Nikon, Badhoevedorp, The Netherlands, www.nikon.com) at a 200x magnification. These pictures were used to determine the number of hASCs with Oil Red O staining as percentage of the total number of cells within each picture using NIH ImageJ software. Statistical comparisons were performed with the two-way ANOVA (SAS software, SAS Institute Inc., Cary, North Carolina).

GLYCEROL-3-PHOSPHATE DEHYDROGENASE ACTIVITY

Glycerol-3-phosphate dehydrogenase (GPDH) activity was determined using the GPDH activity assay kit (Takara Mirus Bio, Otsu, Shiga, Japan) according to manufacturer's instructions with slight modifications: Briefly, cells were lysed with enzyme extraction buffer and centrifuged at 4ºC to separate the aqueous fraction from the fat fraction. Immediately after separation the aqueous fraction was stored at 4ºC and a 2-8 times serial dilution of the aqueous fraction with dilution buffer (supplemented with 0.1M ß-mercaptoethanol) was prepared. The calibration curves were made using a 1% GPDH (Roche, Mannheim, Germany) solution in dilution buffer. GPDH activity was measured under zero-order kinetics at 25ºC in a Victor 2 Wallac spectrophotometer (PerkinElmer, Gouda, The Netherlands, www.perkinelmer.com). The reaction was initiated by addi-

tion of 25 µl of the samples to 100 µl of substrate solution. The rate of NADH oxidation was measured by a decrease in absorbance at 340nm. One unit of GPDH activity corresponded to oxidation of 1 µmol NADH per minute. After assessing the GPDH activity, the aqueous fraction of the cell lysate was used to determine total protein with the Quant-it protein assay (Invitrogen, Carlsbad, California). GPDH activity values were corrected for total cellular protein and are expressed as mean ± SEM. Statistical analysis was performed using the mixed model ANOVA (SAS software)

RNA ISOLATION AND COMPLEMENTARY DNA (CDNA) SYNTHESIS

RNA was extracted from non-differentiating and differentiating hASCs cultured for 0, 3, 7, 14 and 22 days using Qiazol Lysis Reagent (Qiagen Benelux, Venlo, The Netherlands). Total RNA extraction was performed by adding 1/5 volume of chloroform per 1 ml of Qiazol followed by 2-propanol precipitation.

Total RNA was quantified by spectrophotometric analysis at wavelengths of 260nm and 280nm and RNA purity was determined by calculating the 260:280 ratio. One microgram RNA was reverse transcribed into cDNA using the Fermentas RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to manufacturer's protocol. A 20 nanogram RNA equivalent was used for amplification.

QUANTITATIVE PCR

The mRNA levels of FABP4 and angiogenic factors were analyzed with the ABI PRISM® 7000 Sequence Detection System and 7000 System SDS software (ABI, Foster City, California, www.appliedbiosystems.com), using Taqman®Gene Expression Assays for FABP4 (Hs00609791_m1), VEGF (Hs00173626_m1), FGF-2 (Hs00266645_m1), PGF (Hs00182176_m1), PDGFB (Hs00234042_m1), TGFß1 (Hs00171257_m1), ANGPT1 (Hs00181613_m1) and ANGPT2 (Hs 00169867) (ABI) according to the manufacturer's instructions. Two-step thermal cycling parameters were 10 minutes at 95 ˚C, followed by 40 cycles of 15 seconds at 95 ˚C and 1 minute at 60 ˚C. Beta-2-microglobulin (B2M) mRNA levels were analyzed with the qPCR Master Mix Plus for SYBR® Green I dTTP (Eurogentec, San Diego, California) and the following, gene-specific primers set: Fw 5'-TGCTCGCGCTACTCTCTCTTT -3', Rv 5'-TCTGCTGGATGACGTGAGTAAAC-3'). As the expression of this gene was stable between experimental conditions, we used B2M for data normalization. These delta-Ct (dCt) values were then normalized to their Ct values in the samples taken at day 0 to obtain the delta delta-Ct (ddCt) values. Statistical analysis was performed by using the mixed model ANOVA (SAS software).

HASCS CONDITIONED MEDIA COLLECTION, PREPARATION AND ANALYSIS

On days 2, 6, 13 and 21 of culture hASCs were switched to fresh, low serum (0.5% FCS) basal medium or adipogenic medium and incubated at 37˚C and 5% CO_2 for 24 hours. Then, conditioned media were collected and centrifuged at 400xg for 5 minutes at 4˚C. The concentration of hASCs-secreted angiogenic factors in the conditioned medium was measured using commercially available sandwich ELISA kits according to manufacturer's instructions (VEGF, PGF, ANGPT1 and ANGPT2; R&D systems). After media collection, cells were lysed with PBS with 0.1% Triton and total protein determined with the MicroBCA protein assay (Pierce, Pittsburgh, Pennsylvania). ELISA values are expressed as mean ± SEM picograms of the secreted factor per microgram total cellular protein. Statistical analysis was performed by using the mixed model ANOVA (SAS software).

EVALUATION OF BIOACTIVITY OF HASC CONDITIONED MEDIA

Isolated human umbilical vein endothelial cells (HUVEC) at passage 4 or less were seeded at a density of 5000 cells per well in fibronectin coated 96-well plates and cultured for 24 hours in human endothelial-SFM medium supplemented with 20% FBS, 10% human serum, 20 ng/mL FGF-2, and 100 ng/mL EGF. The next day, these cells were starved with HG-DMEM supplemented with 0.5% FBS. Twenty-four hours later, the HUVEC were refreshed with either fresh low serum (0.5%) basal or adipogenic medium, or conditioned low serum basal or adipogenic medium that had been harvested previously from hASCs. After 24 hours endothelial viable cell numbers were determined with the MTT viability assay based on the Mossman's protocol [20]. Briefly, 3 hours before the end of the incubation 50 µl MTT reagent (3mg/ml) was added to the medium.

After incubation the medium was removed and the formazan crystals were dissolved in 100 µl DMSO and the optical density was read at 510nm in the Victor 2 Wallac spectrophotometer (PerkinElmer). The optical densities obtained from low serum unconditioned media HUVEC cultures used as a control was set to 100% for comparative purposes. The optical densities of HUVEC cultured on conditioned media were expressed as a percentage of the optical densities measured in the corresponding unconditioned media. Statistical comparisons were performed with the Mixed model ANOVA (SAS software).

RESULTS

CHARACTERIZATION OF HASCS

Since isolation and culture can affect the immunophenotype of primary hASCs [21,22], flow cytometry analysis of the hASCs used in this study was performed. FACS analysis demonstrated that the percentage of cells positive for the stromal mesenchymal cell markers CD105 (97.6% ± 1.8%) and CD90 (74% ± 15.3%) and the hematopoietic progenitor marker CD34 (85.1% ± 16%) was high. The percentage of cells positive for the endothelial markers CD144 (14.7% ± 7.8%) and CD31 (7.4 ± 2.8%) on the other hand, was low (data not shown).

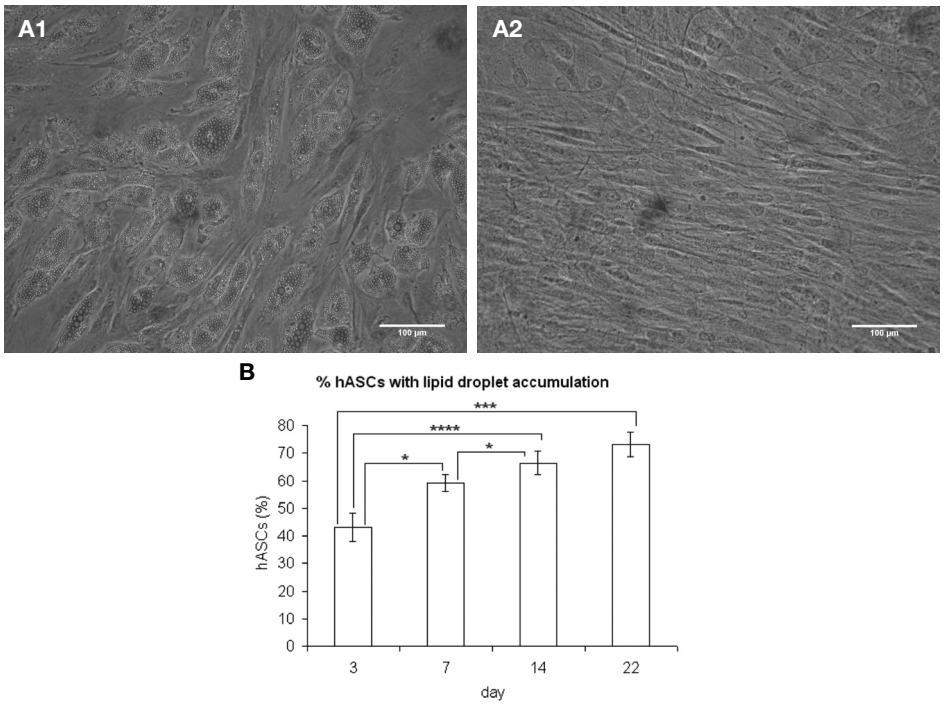


Figure 1. HASCs differentiated toward the adipogenic lineage accumulate intracellular lipid droplets and display adipocyte-specific GPDH activity and FABP4 expression. **(A)** Images of differentiated and undifferentiated hASCs after fixation and staining with Oil Red O at day 14 of culture (magnification: 200x). Differentiating hASCs **(A.1)** showed a round morphology and accumulation of intracellular red stained lipid droplets, while non-differentiating hASCs **(A.2)** maintained their spindle like shape and had minimal to no intracellular lipid droplet formation. **(B)** The percentage of hASCs with lipid droplet accumulation was determined by counting the cells positively stained with Oil Red O in pictures taken of representative fields within a well of a 6-well tissue culture plate (see Materials and Methods section). Until day 14, a significant increase in the amount of cells with lipid droplet accumulation was observed. The hASC % was calculated as percentage of the total number of cells within each field. Values are expressed as mean ± SEM; n= 4 donors. Statistical comparisons were performed with the two-way ANOVA.*P<0.05, ***P<0.0005, ****P<0.0001. (see color section for a full-color version)

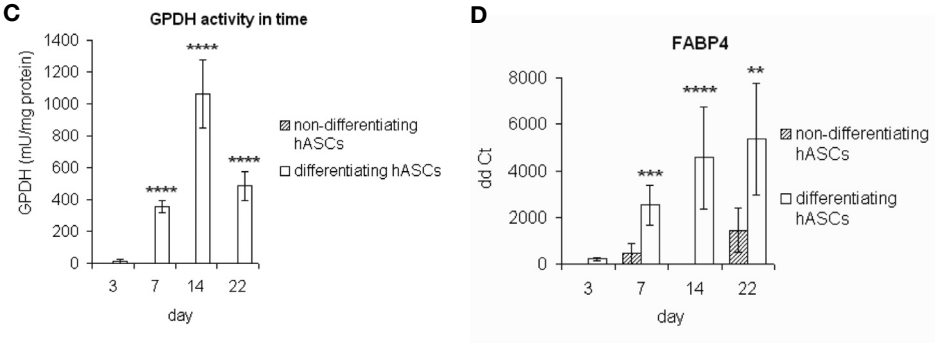
HASCS CULTURED IN ADIPOGENIC MEDIUM SHOW ADIPOCYTE-SPECIFIC CHARACTERISTICS

OIL RED O STAINING

Accumulation of lipid-containing vesicles in the cytoplasm is widely believed to be a prominent characteristic of adipogenic differentiation. After 3 days of differentiation, intracellular lipid droplet accumulation was first observed in 38.5% ± 5.6% of the hASCs in all donors (Fig.1A.1), while the hASCs that were cultured on basal medium kept their spindle like shape and accumulated no small lipid droplets in the cytoplasm (Fig.1A.2). Until day 14, there was a significant increase in the amount of cells with lipid droplet accumulation (Fig.1B).

GLYCEROL-3-PHOSPHATE DEHYDROGENASE ACTIVITY.

Another important feature of differentiated adipocytes is the secretion of the cytosolic enzyme GPDH, the activity of which in differentiating hASCs of one donor (= donor 2) was observed as early as 3 days after the start of differentiation. For the other donors, GPDH activity was first observed in hASCs at the measurement on day 7. Highest GPDH activity in hASCs of all donors was seen at day 14. This peak was then followed by a decrease in enzyme activity at day 22 (Fig.1C). No detectable GPDH activity was measured at any time point in hASCs cultured on basal medium.



(C) The GPDH activity in hASCs differentiated in tissue culture was directly measured by the rate of NADH oxidation (see Materials and Methods section). The hASCs cultured on adipogenic medium displayed prominent NADH oxidation from day 7 until day 22, while NADH oxidation in undifferentiated hASCs was not detectable. Values were corrected for total cellular protein and are presented as mean ± SEM; n= 4 donors. Statistical comparisons were performed with the Mixed model ANOVA.****P<0.0001 (compared to undifferentiated hASCs). **(D)** Quantitative PCR was used to measure the expression levels of the adipocyte specific gene FABP4. Differentiating hASCs displayed high expression levels of FABP4 that increased in time, while non-differentiating hASCs displayed very low FABP4 expression levels. Expression levels are relative to beta-2-microglobulin positive-control housekeeping gene and to the expression level at day 0 (ddCt). Values are presented as mean ± SEM; n= 4 donors. Statistical comparisons were performed with the Mixed model ANOVA. **P<0.01, ***P<0.0005, ****P<0.0001 (compared to undifferentiated hASCs).

FABP4 EXPRESSION

PPARγ activation is considered to be an absolute requirement for the differentiation of adipose tissue [23,24]. The adipocyte-specific FABP4 gene is a downstream target of PPARγ activation and is the most widely used adipocyte differentiation marker [25]. We used the expression of the FABP4 gene to further confirm the differentiation of the hASCs cultured on adipogenic medium. The expression of FABP4 in differentiating hASCs was first observed at day 7 and increased in time. In contrast, FABP4 expression was not observed in non-differentiating hASCs, except for one donor (= donor 2) and here only at days 7 and day 22 (Fig.1D)

ASSESSMENT OF ANGIOGENIC FACTORS IN DIFFERENTIATING AND PROLIFERATING HASCS

GENE EXPRESSION

To study the effect of differentiation on the angiogenic capacity of hASCs, expression of a series of important angiogenic factors was measured in both differentiating and non-differentiating hASCs. Expression in differentiating hASCs was increased for ANGPT1 and ANGPT2, with elevated peaks at days 7 and 14, and for VEGF and PGF, starting from day 7. No changes in the expression profile between differentiating and non-differentiating hASCs was found for FGF-2, PDGFB and TGFβ1 (Fig.2).

PROTEIN SECRETION

To determine whether the increase on mRNA levels of VEGF, PGF, ANGPT1 and ANGPT2 in differentiating hASCs resulted in a similar increase in corresponding polypeptides, the concentrations of these proteins were measured in 24 hour conditioned media. The amount of VEGF secreted by non-differentiating hASCs was decreased at days 7 and 14, while the secretion of VEGF in differentiating hASCs was elevated at day 7.

These changes resulted in a 5-fold higher VEGF secretion in differentiating hASCs compared to non-differentiating hASCs at day 7 [0.344 ± 0. 086 pg/μg protein versus 0.061 ± 0.033 pg/μg protein, ($P<0.0002$, $n=3$)] and day 14 [0.244 ± 0.086 pg/μg protein versus 0.053 ± 0.054 pg/μg protein, ($P<0.0122$, $n=3$)). Unlike to VEGF, the different transcript abundances of PGF, ANGPT1 and ANGPT2 in differentiating and non-differentiating hASCs were not reflected in the protein secretion profiles. Enhanced secretion of ANGPT2 became apparent at day 22 [0.068± 0.045 pg/μg protein versus 0.011 ± 0.021 pg/μg protein in non-differentiating hASCs ($P<0.0051$, $n=3$) of culture while the gene expression of ANGPT2 in differentiating hASCs was enhanced at day 7 and 14 of culture. Also, the secreted amounts of PGF and ANGPT1 between differentiating and non-differentiating hASCs did not differ significantly (Fig.3). None of the angiogenic factors were detected in unconditioned basal culture media (data not shown).

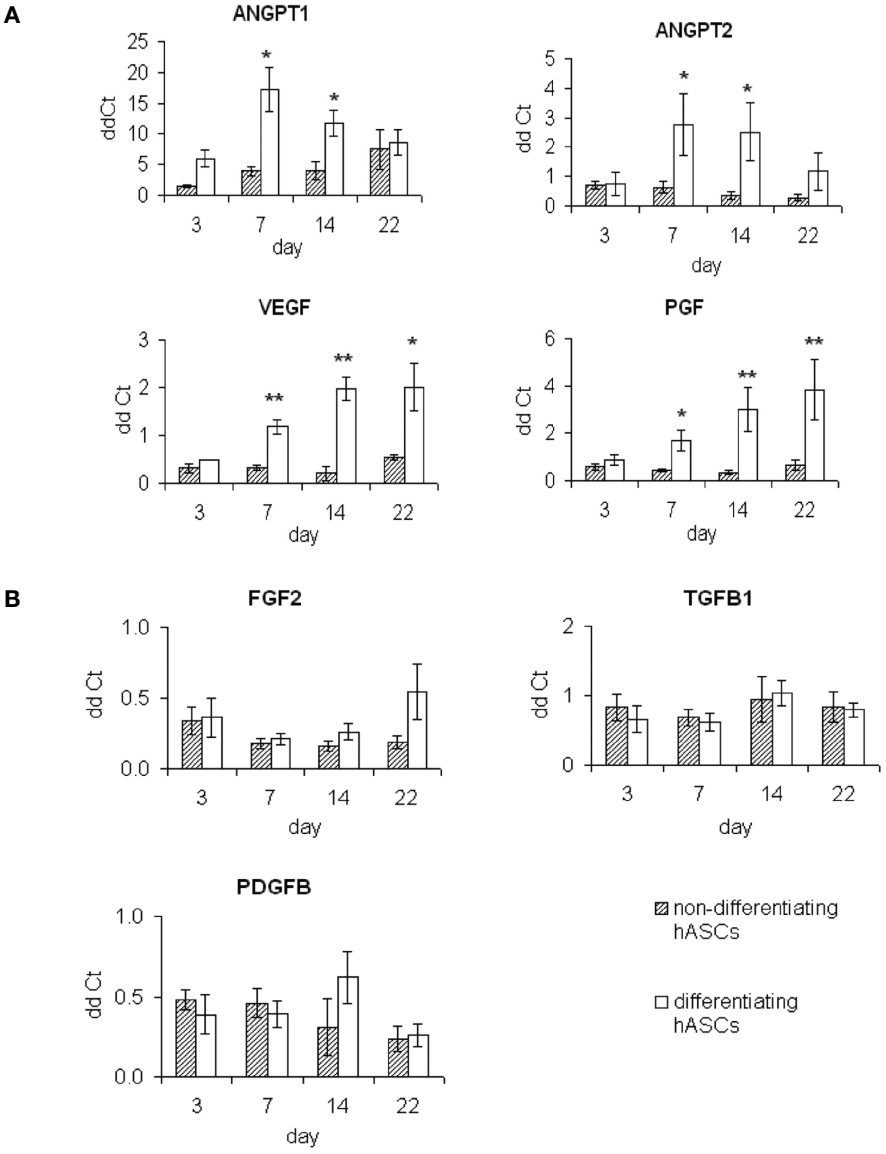


Figure 2. Differentiating hASCs express higher levels for VEGF, PGF, ANGPT1 and ANGPT2 compared to non-differentiating hASCs. Real-time quantitative PCR was used to compare the expression patterns of a series of angiogenic genes between differentiating and non-differentiating hASCs. **(A)** Differentiated hASCs had significantly higher expression levels of ANGPT1 and ANGPT2 at days 7 and 14 after induction and of VEGF and PGF at days 7, 14 and 22 after induction. **(B)** The expression levels of FGF-2, TGFβ1 and PDGFB did not significantly differ between differentiating and non-differentiating hASCs. Expression levels are relative to beta-2-microglobulin positive-control housekeeping gene and to the expression level at day 0 (ddCt). Values are means ± SEM; n= 4 donors. Statistical comparisons were performed with the mixed model ANOVA. *P<0.05, **P<0.01, ***P<0.0005 (compared to undifferentiated hASCs).

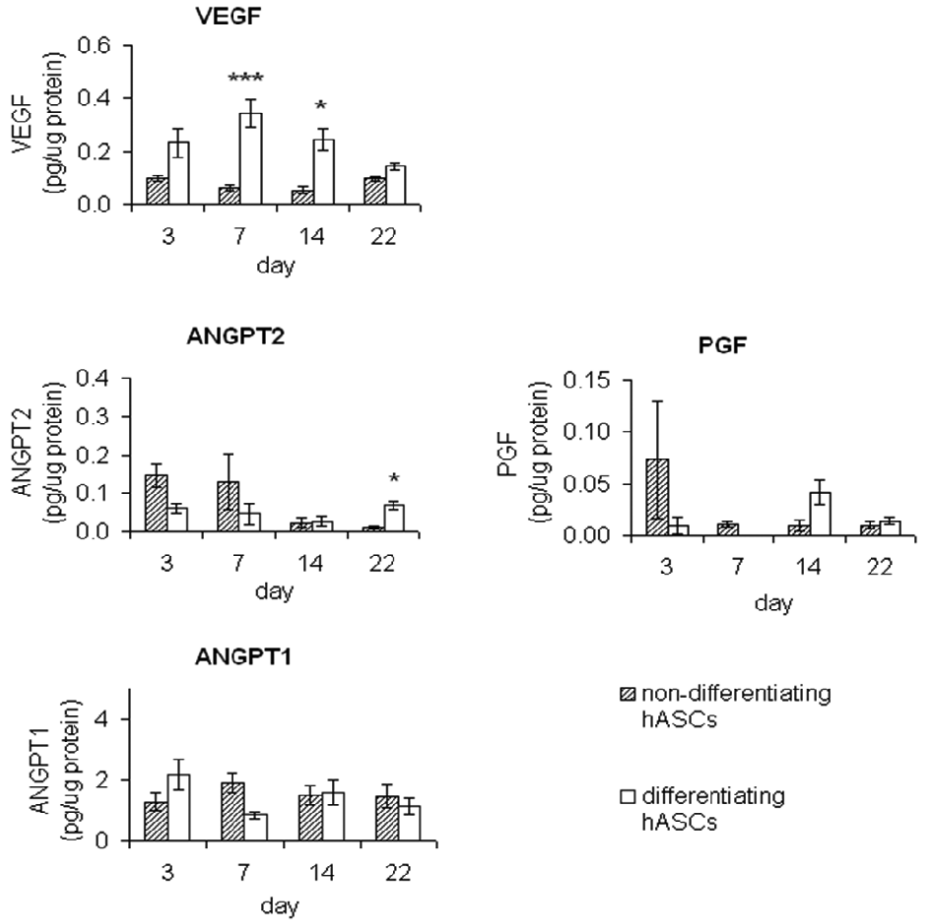


Figure 3. Increased secretion of VEGF by differentiating hASCs at day 7 and 14 of culture.
The secretion of VEGF, PGF, ANGPT1 and ANGPT2 by differentiating and non-differentiating hASCs over 24 hours was measured by ELISA at day 0, 3, 7, 14 and 22 of culture. **(A)** Differentiated hASCs secrete a significantly higher amount of VEGF at day 7 and 14 of culture than undifferentiated hASCs. **(B)** Except for the increased ANGPT2 secretion of differentiating hASCs at day 22 of culture, the differences in the secretion of PGF, ANGPT1 and ANGPT2 between differentiating and non-differentiating hASCs were not significant. Values were corrected for total cellular protein and are presented as mean ± SEM; n= 3 donors. Statistical comparisons were performed with the Mixed model ANOVA. *P<0.05, ***P<0.0005 (compared to undifferentiated hASCs).

EFFECTS OF HASC-CONDITIONED MEDIUM ON ENDOTHELIAL VIABLE CELL NUMBERS

To verify the activity of angiogenic factors secreted in the conditioned medium, the effects of hASC-conditioned medium (hASC-CM) on human endothelial cell growth were measured. Conditioned medium of non-differentiating hASCs collected at day 3 gave equal viable cell numbers compared to unconditioned medium. Also, non-

differentiating hASC-CM from days 7 and 14 slightly diminished endothelial cell numbers. In contrast, differentiating hASC-CM harvested at days 3, 7 and 14 resulted in higher endothelial cell numbers compared to unconditioned medium (Table 1). These findings indicate that hASC adipogenic differentiation for 3 to 14 days positively affects endothelial viable cell numbers.

Table 1. Percentage viable HUVEC after 24 hours of incubation with conditioned medium from differentiating and non-differentiating hASCs.

	Conditioned medium[a]			
	3 days	7 days	14 days	22 days
Differentiating hASCs	136% ±13%[b]	119% ± 5.6%[b]	122% ± 8.4%[b]	110% ± 10%
Non-differentiating hASCs	99% ± 5.2%	91% ± 4.9%	79% ± 5%	100% ± 5%

a) Low serum conditioned medium (CM) of differentiating and non-differentiating hASCs collected after 3, 7, 14 and 22 days of culture. Viable cell numbers were measured using the MTT assay. Effects of conditioned medium of differentiating and non-differentiating hASCs on endothelial viable cell numbers are shown as a percentage of the viable cell numbers measured in the corresponding unconditioned media (percentages are expressed as mean ± SEM; n=4 donors).
b) $P<0.01$ (versus percentage HUVEC cultured in CM of non-differentiating hASCs of the same culture day).

DISCUSSION

In the present study, we demonstrated that multiple angiogenic factors are expressed and secreted by both non-differentiating and differentiating human adipose-derived stromal cells in vitro. Unlike previous studies we were specifically interested in determining how much better, if at all, differentiating hASCs could express and secrete these angiogenic factors when compared with non-differentiating hASCs. In addition, we aimed to evaluate the effects of non-differentiating and differentiating hASC- conditioned medium on endothelial viable cell numbers in vitro.

In order to be able to relate hASC adipogenic differentiation and hASC angiogenic capacity, we evaluated adipogenic differentiation of hASCs for the first time in a timeline manner using three different parameters of adipogenic differentiation [i.e. intracellular lipid droplet accumulation (as detected by Oil Red O), activity of GPDH and expression of FABP4 mRNA]. Using our isolation procedure, and culture conditions, we found that our CD34+/ CD90+/ CD105+ hASCs consistently differentiated into adipocytes following 3 days of adipogenic induction. In one donor FABP4 was also expressed in non-differentiating hASCs on day 7 and 22. It is known that basal transcription levels of FABP4 in undifferentiated hASCs differ between donors and even clones [26]. This may

be an explanation for the relatively high basal FABP4 expression in the undifferentiated hASCs cultures of this one donor.

The in vivo induction and control of neovascularization is complicated, as a myriad of angiogenic factors is needed for sprouting and subsequent vessel stabilization [27]. We therefore analyzed the expression of several angiogenic factors that are involved in both the sprouting [VEGF, PGF, FGF-2, ANGPT2] and the maturation of vessels [PDGFB, $TGF\beta_1$, ANGPT1] by hASCs, before and during differentiation. In addition, we analyzed the secretion of VEGF, PGF, ANGPT1 and ANGPT2 during hASC differentiation. Gene expression analysis showed that adipogenic induction of hASCs for 7 and 14 days significantly increased the expression levels of both the sprouting factors VEGF, PGF and ANGPT2 and the maturation factor ANGPT1. Gene expression and protein secretion are not necessarily correlated. Discrepancies may be due to regulatory mechanisms at the (post-) transcriptional or the secretory level of the protein under investigation. Nonetheless, the protein analyses of hASC-conditioned medium demonstrated that the secretion of the VEGF protein was significantly higher after 7 and 14 days of adipogenic induction.

The above-described results suggested that short-time in vitro differentiated hASCs may have a higher angiogenic capacity than undifferentiated hASCs. To investigate this further, we obtained conditioned medium from cultured hASCs before and during adipogenic differentiation and found that conditioned medium of hASCs differentiated for 3, 7 and 14 days significantly improved endothelial viable cell numbers in vitro, compared to undifferentiated hASCs. Since only VEGF secretion was significantly increased in differentiated hASCs when compared to undifferentiated hASCs, it is likely that this growth factor is mainly responsible for the higher viable endothelial cell numbers after incubation in conditioned medium obtained from differentiated hASCs. However, we can not exclude the influence of other growth factors and metabolites that were not monitored in this study (such as hepatocyte growth factor, granulocyte macrophage colony-stimulating factor and 1-butyryl glycerol [14,28].

Taken together, our results indicate for the first time that short, adipogenic differentiation of primary human ASCs is beneficial for endothelial viable cell numbers in vitro. This is in agreement with studies directed at in vivo adipogenesis. In these studies, ASC differentiation (in mice) was linked to the sprouting of new blood vessels from the preexisting vasculature in intact living adipose tissue [29]. Also, impaired differentiation of hASCs, by suppression of PPARγ activation, has been shown to prevent angiogenesis [30].

Adipose tissue formation, survival and especially vascularization are the most important requirements for achieving clinical success in cell-based adipose tissue engineering. Our study indicates an increase in endothelial viable cell numbers when cultured on conditioned medium of shortly differentiated hASCs, suggesting that short-term adipogenic differentiation of hASCs may be beneficial with respect to stimulation

of the vascularization of the transplant. However, the possibility of improving in vivo vascularization of hASC implants by short in vitro adipogenic differentiation of hASCs needs to be confirmed by further in vivo studies, which was beyond the scope of the present project.

The development and clinical application of in vitro engineered adipose tissue constructs is still a futuristic goal. For engineered adipose tissue constructs to become a viable option for future clinical use, they must be able to survive and maintain their shape in vivo. For this, the presence of an adequate blood supply is a prerequisite. Several approaches to improve vascularization in tissue-engineered constructs have been used and suggested, including: surgical insertion of a pre-existing vessel network into the construct [31], surrounding the tissue construct with a highly vascularized tissue fragment (i.e. omentum) [32], and in vitro assembly of a vessel network in the tissue construct by co-culturing with endothelial cells [33,34]. We believe that combining such techniques with a short in vitro adipogenic differentiation of hASCs may improve the long-term survival of adipose tissue constructs.

CONCLUSION

In summary, our data demonstrate that adipogenic differentiation of hASCs for a period of 7 to 14 days improves their angiogenic capacity, as demonstrated by increased gene expression of VEGF, PGF, ANGPT1, ANGPT2 and increased protein secretion of VEGF. Additionally, conditioned medium of shortly differentiated hASCs enhanced the endothelial viable cell numbers in vitro, suggesting that short adipogenic-differentiated hASCs are potentially the better choice for adipose tissue engineering applications.

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CHAPTER 3

Comparing scaffold-free and fibrin-based adipose-derived stromal cell constructs for adipose tissue engineering

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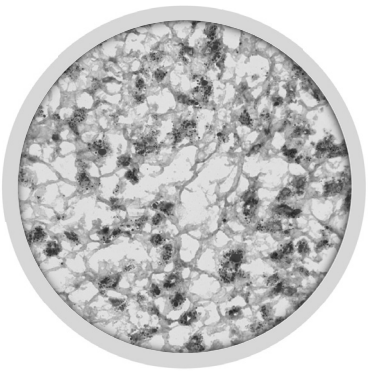


ABSTRACT

Success of adipose tissue engineering for soft tissue repair has been limited by insufficient adipogenic differentiation, an unfavorable host response and insufficient vascularization. In this study, we examined how scaffold-free spheroid and fibrin-based environments impact these parameters in human adipose-derived stromal cell (ASC)-based adipose constructs. ASC were differentiated in spheroids or fibrin-based constructs. After 7 days, conditioned medium was collected and spheroids/fibrin-based constructs were either harvested or implanted subcutaneously in athymic mice. Following 7 days of implantation, the number of blood vessels in fibrin-based constructs was significantly higher than in spheroids [93±45 versus 23±11 vessels/mm2], and the inflammatory response to fibrin-based constructs was less severe. The reasons for these results were investigated further in vitro. We found that ASC in fibrin-based constructs secreted significantly higher levels of the angiogenic factors VEGF and HGF, and lower levels of the inflammatory cytokine IL8. Furthermore, ASC in fibrin-based-constructs secreted significantly higher levels of leptin and showed a 2.5-fold upregulation of the adipogenic transcription factor PPARG and a 4-to 5-fold upregulation of the adipocyte-specific markers FABP4, perilipin and leptin. Based on these results, we conclude that fibrin-based ASC-constructs are more suitable for ASC-based adipose tissue reconstruction than scaffold-free spheroids.

INTRODUCTION

Soft tissue defects due to tumor resection, trauma, deep burns or congenital deformities require surgical replacement of lost soft tissue to regain shape and/or physiological function. Current surgical procedures to repair soft tissue defects include the use of synthetic implants and adipose tissue transplants. Improved patient outcomes notwithstanding [1,2,3,4], both procedures have their limitations including implant dislocation and toxicity [5], donor-site morbidity or deformity [6] and an unsatisfactory long-term outcome due to resorption [5,7,8,9].

Adipose-derived stromal cell (ASC)-based reconstruction after soft tissue loss, promises to restore the shape and volume of the affected site. New human adipose tissue has been formed in immune-compromised rodents by injection of ASC [10] or delivery of ASC in tissue-like structures [6,11,12,13,14,15], but poor survival after transplantation due to ischemia, insufficient adipogenic differentiation and adverse effects of scaffold materials currently hinder the use of these techniques.

Scaffold–based tissue engineering, though promising, faces several challenges: (1) synthetic scaffolds and extracellular matrix–based xenograft materials elicit a foreign body response, (2) scaffold degradation products may release potentially toxic substances and (3) scaffold compliance is often different from adipose tissue [16,17] .

The generation of three-dimensional tissues that are composed only of cells and their own secreted matrix, also called scaffold-free tissue engineering, addresses the limitations associated with synthetic and exogenous matrix-based tissues [18,19]. Scaffold-free human ASC cell sheets can be created and stacked to form adipose-like substitutes [20,21]. We recently created three-dimensional spherical scaffold-free constructs composed of human ASC (ASC spheroids) of up to 600µm in diameter [22]. To date, it is unclear whether the use of scaffold-free ASC constructs is advantageous for adipogenesis and/or in vivo-engraftment when compared to scaffold-based constructs.

To investigate this, we wanted to compare our scaffold-free methods with another promising method that makes use of scaffolds. Various scaffold materials have been combined with ASC for adipose tissue engineering purposes [16]. Along with collagen [23,24], hyaluronic acid [25], poly(lactic-co-glycolic-acid) (PLGA) [14,26], polyglycolic acid (PGA) [27], Matrigel™ [28] and alginate[11], fibrin has shown a promising scaffold material for generation of adipose tissue [29,30,31,32]. Besides that, fibrin is especially an attractive material for clinical use since it could be produced from a patient's own blood, thereby avoiding the risk of a foreign body response [33].

Since the in vivo engraftment of ASC constructs is most of all dependent on ASC survival, a potential successful ASC construct not only supports adipogenesis but also encourages angiogenesis to minimize ASC ischemia and death. As the extracellular

environment influences cell behavior [34,35], it is likely that the scaffold (-free) environment will impact important ASC biological features for in vivo-engraftment, such as adipogenic differentiation potential and the secretion of adipokines, which include angiogenic and inflammatory factors.

In the current study we compared the in vivo engraftment of scaffold-free ASC spheroids with fibrin–based ASC constructs. We investigated in vivo construct survival, vascularization, and adipogenic differentiation. Complimentary to the in vivo experiments, we evaluated the impact of spheroid and fibrin-based environments on ASC adipogenic differentiation and their secretion of adipokines in vitro.

MATERIALS AND METHODS

ISOLATION AND CULTURE OF ASC

Subcutaneous abdominal adipose tissue was obtained as leftover material from donors undergoing breast reconstruction surgery with approval of the Erasmus MC Medical Ethical Committee (# MEC-2005-157) and according to the Code of Conduct: "Proper Secondary Use of Human Tissue" (http://www.federa.org). Leftover adipose tissue was only used from donors who did not opt-out to such use.

ASC from 3 different donors of 56, 58 and 62 years of age were isolated from the adipose tissue using collagenase type I (Invitrogen, Carlsbad, California) as previously described [22,36] and grown on basal medium (Dulbecco's Modified Eagle Medium 1g/l glucose (Invitrogen), 10% FCS (PAA Laboratories, Pasching, Austria), 10^{-12} M dexamethasone, 10^{-5} M ascorbic acid (both from Sigma-Aldrich, St. Louis, Missouri), 1% penicillin/streptomycin, 0.5% gentamycin (both from Invitrogen)). The isolated primary ASC were grown until they reached 90% confluence. At 90% confluence, adherent cells were released with 0.05% trypsin/ ethylenediaminetetraacetic acid (EDTA) and then stored in liquid nitrogen until further use. ASC at second passage were used in this study.

FORMATION OF SPHEROIDS

ASC were pooled to a total of $0.5*10^6$ cells in 10 ml polypropylene tubes (Techno Plastic Products, Trasadingen, Switzerland) in 350µl culture medium: human endothelial-SFM (Invitrogen) supplemented with 5% FCS, 20ng/ml fibroblast growth factor 2 (FGF2), 100ng/ml epidermal growth factor (EGF), 1% penicillin/streptomycin and 0.5% gentamycin. After centrifugation at 150 x g for 5 minutes, cell pellets were incubated overnight in 37ºC at 5% CO_2 in a humidified atmosphere to allow spheroid formation. After overnight incubation, the culture medium of the spheroids was replaced by 350µl of a 1:1 mixture of culture medium and differentiation medium (Dulbecco's modified

Eagle Medium 4.5g/l glucose (Invitrogen), 10% FCS, 1μM dexamethasone (Sigma-Aldrich), 0.01 mg/ml insulin (Eli Lilly, Houten, The Netherlands) 0.2 mM indomethacin (Sigma-Aldrich), 0.5mM 3-isobutyl-I-methyl-xanthine (Sigma-Aldrich), 1% penicillin/streptomycin and 0.5% gentamycin) from now on called adipogenic medium. Medium was refreshed every other day. At day 7, spheroids were collected for RNA isolation or implantation, or cultured for another 48 hours on fresh adipogenic medium to analyze secreted angiogenic and inflammatory factors.

FORMATION OF FIBRIN-BASED CONSTRUCTS

Fibrin-based constructs were prepared with materials from the fibrin in vitro angiogenesis assay® (Millipore Corporation, Massachusetts) according to manufacturer's instructions with some slight modifications. In short, ASC were suspended in 30 μl of fibrinogen solution at a density, of $1.67*10^4$ cells/μl (= total of $0.5*10^6$ cells per construct). After addition of 20μl of thrombin solution, the cultures were immediately placed in individual wells of a 96-wells plate (Costar®, Corning Inc.). After gel formation, the plates were incubated at 37ºC in 5% CO_2 in a humidified atmosphere for 15 minutes to ensure polymerization of the gels. Following polymerization, 350μl of culture medium was added to each well. After overnight equilibration, the culture medium was replaced by 350μl adipogenic medium. Medium was refreshed every other day. At day 7, fibrin constructs were collected for RNA isolation or implantation, or cultured for another 48 hours on fresh adipogenic medium to analyze secreted angiogenic and inflammatory factors.

RNA ISOLATION AND COMPLEMENTARY DNA (CDNA) SYNTHESIS

For each of the three ASC donors $0.5*10^6$ second passage undifferentiated ASC were harvested for RNA isolation. In addition, 9 spheroid and 9 fibrin constructs were prepared for each of the three ASC donors. The spheroids were fragmented with a pestle on ice and subsequently sheared using an insulin syringe. The fibrin cultures were snap frozen and processed using the Mikro-Dismembrator S® (B. Braun Biotech International GmbH, Melsungen, Germany). Total RNA was extracted from undifferentiated ASC, spheroids and fibrin cultures using Qiazol Lysis Reagent (Qiagen Benelux, Venlo, The Netherlands) and further purified using the RNeasy Micro Kit (Qiagen, Venlo, The Netherlands) with on-column DNA-digestion. Total RNA was quantified using a NanoDrop™ 1000 spectrophotometer (Nanodrop technologies, Wilmington, DE) according to manufacturer's instructions and 250 nanogram RNA was reverse transcribed into cDNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany).

QUANTITATIVE POLYMERASE CHAIN REACTION (Q-PCR)

The mRNA levels of the adipogenic markers peroxisome proliferator-activated receptor gamma (PPARG), fatty acid binding protein 4 (FABP4), perilipin 1 (PLIN 1) and leptin (LEP) together with beta-2-microglobulin (B2M) were analyzed with the ABI PRISM® 7000 Sequence Detection System and 7000 System SDS software (ABI, Foster City, USA, www.appliedbiosystems.com), using the Taqman®Gene Expression Assays for PPARG (Hs01115510_m1), FABP4 (Hs01086177_m1), PLIN1 (Hs01106927_m1), LEP (Hs00174877_m1) and B2M (Hs00984230_m1) (ABI) according to the manufacturer's instructions. The B2M mRNA levels were analyzed for data normalization.

CONDITIONED MEDIA COLLECTION AND ANALYSIS

On culture day 7, spheroid- and fibrin-based ASC cultures were switched to fresh adipogenic medium and incubated at 37°C and 5% CO_2 for 48 hours. Then, conditioned media were collected and assayed for the concentration of ASC-secreted inflammatory cytokines, angiogenic factors and leptin.

ANALYSIS OF INFLAMMATORY CYTOKINES AND ANGIOGENIC FACTORS

A human cytokine-inflammation multiplex chemiluminescent ELISA kit (cat. no.110451HU, Quansys Biosciences, West Logan, Utah) and a human angiogenesis multiplex chemiluminescent ELISA kit (cat. no.150251HU; Quansys Biosciences) were used to assay the conditioned medium for the release of the following inflammatory cytokines and angiogenic factors: interleukin 1 alpha (IL1 alpha), interleukin 1 Beta (IL1 beta), interleukin 2 (IL2), interleukin 4 (IL4), interleukin 6 (IL6), interleukin 8 (IL8), interleukin 10 (IL10), tumor necrosis factor alpha (TNF alpha) interferon gamma (IFN gamma), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), angiopoietin 2 (ANG2), fibroblast growth factor 2 (FGF2), hepatocyte growth factor (HGF), and the tissue inhibitors of metalloproteinases (TIMP): TIMP1, and TIMP2. These multiplex chemiluminescent ELISA kits were performed according to manufacturer's instructions. The plate was imaged using the Kodak Digital Science ™ Image Station 440CF system (NEN Life Science Products Inc, Boston, Massachusetts) with custom software. Q-view software® (Quansys Biosciences) was used for calculation of angiogenic factor concentration using linear regression of the standard curve.

LEPTIN QUANTIFICATION

The concentration of leptin in the conditioned medium was measured using a sandwich ELISA kit (Leptin Human ELISA Kit®, cat no. KAC2281, Invitrogen) according to manufacturer's instructions. The plate was read at 450 nm using a microplate reader (Versamax®, Molecular Devices, Sunnyvale, California). The amount of leptin was determined from standard curves provided with the kit.

IN VIVO IMPLANTATION

For in vivo implantation, three spheroid- and fibrin -based constructs, each initially seeded with $0.5*10^6$ ASC, were created from each of the three donors, resulting in a total of 9 ASC spheroids and 9 fibrin-based constructs. After 7 days of in vitro culture, the spheroids and fibrin-based constructs were implanted subcutaneously in the left and right scapular area of 9 nine-weeks-old athymic male nude mice (NMRI-nu/nu, Taconic, Hudson, New York). The mice were placed under general anesthesia with 2.5% isoflurane after which two separate 0.5 cm incisions were made through the dorsal skin. Next, two separate subcutaneous pockets were prepared by blunt dissection of the subcutaneous tissue. One pocket was filled with an ASC spheroid and the other pocket was filled with an ASC seeded fibrin-based construct. Pockets were closed with discontinuous sutures using Mersilk 5-0 (Ethicon, Somerville, New Jersey). Seven days after implantation, the mice were sacrificed and the constructs retrieved. All animal procedures were approved by the institutional Animal Experiments Committee of the ErasmusMC (EUR. 1292). (i.e. adherence to the rules prescribed in the national Animals Act, which implements the 'Guidelines on the protection of experimental animals' by the Council of Europe, 1986: Directive 86/609/EC).

HISTOLOGY

Spheroids and fibrin-based constructs harvested before or after 7 days of implantation were embedded in Tissue-Tek (Sakura, Finetek Europe, Zoeterwoude, The Netherlands) and snap frozen or fixed in 10% formalin in PBS followed by embedding in paraffin. Paraffin sections (5 µm) were deparaffinized and rehydrated. Cryo-sections (5 µm) were fixed with 3.7% formalin in deionised water for 1 hour.

VIMENTIN STAINING

To distinguish human tissue from mouse tissue in paraffin and cryo-sections of implanted spheroids and fibrin-based constructs, a monoclonal mouse anti-human vimentin antibody (Clone V9, cat no. V6630, 1:40 dilution in PBS/ 1% BSA, Sigma-Aldrich) was used. To reduce unspecific binding of the secondary goat anti-mouse antibody (Dako, Glostrup, Denmark) to mouse IgGs, the mouse-on mouse HRP-Polymer Kit (Biocare Medical, Concord, California) was used, according to manufacturer's instructions with some slight modifications. In short, antigen retrieval was performed through incubation in Rodent Decloaker® (Biocare Medical) for 60 minutes at 95˚C. Non-specific binding sites were blocked with Rodent Block M® (Biocare Medical) and sections were stained overnight with vimentin at 4˚C. The MM-polymer-HRP® secondary antibody (Biocare Medical) was used, followed by incubation in diaminobenzidine (Sigma-Aldrich) to visualize vimentin expression. The slides were weakly counterstained with hematoxylin, dehydrated through graded alcohols and mounted with Permount (VWR International, Amsterdam, The Netherlands).

KI-67 STAINING

A monoclonal mouse anti-human Ki-67 antibody (Clone MIB-1, cat no. M7240, Dako) was used to identify actively proliferating cells in cryo-sections of spheroids and fibrin-based constructs after 7 days of culture. Sections were fixed in acetone for 10 minutes, washed in PBS and incubated for 60 minutes with Ki-67 antibody (1:200 dilution in PBS /1% BSA) at room temperature. Subsequently, a secondary biotin-conjugated goat anti-mouse antibody (1:200 dilution in PBS/1% BSA, cat no. E0433, Dako) was used for 30 minutes followed by incubation with streptavidin-horseradish peroxidase (1:300 dilution in PBS/1% BSA, cat no. P0397, Dako) for 30 minutes. Diaminobenzidine (Sigma-Aldrich) was used to visualize Ki-67 expression. The slides were weakly counterstained with hematoxylin, dehydrated through graded alcohols and mounted with Permount (VWR International). Cell proliferation was quantified on 2-3, high resolution (0.23 μm/pixel), low magnification (40×) digital micrographs (Nanozoomer HT, Hamamatsu Photonics, Hamamatsu City, Japan) covering complete Ki-67 stained cross-sections of each construct using NIH ImageJ software (http://rsb.info.nih.gov/ij/). The mitotic index was determined on 4 fields of view per construct cross-section and calculated as the percentage of Ki-67 positively stained cells to total cells.

OIL RED O STAINING

Accumulated lipid in spheroids and fibrin constructs were determined after 7 days of in vitro culture and after 7 days of implantation. In short, a 0.5% (w/v) stock solution of Oil Red O in triethyl-phosphate (Sigma-Aldrich) was diluted 3:2 with deionised water to prepare an Oil Red O working solution. After fixation, cryo-sections were rinsed in deionised water. Subsequently, the sections were immersed in the Oil Red O working solution for 30 minutes. Hereafter, sections were washed with three exchanges of deionised water for 3 times. Then, sections were counterstained with hematoxylin for 1 minute. Finally, sections were rinsed with running tap water for 10 minutes and covered with Imsolmount (Klinipath, Zevenaar, The Netherlands).

QUANTIFICATION OF CONSTRUCT VASCULARIZATION AND INFLAMMATION

H&E staining was performed to evaluate vascularization and inflammation after implantation. High resolution (0.23 μm/pixel), low magnification (40×) digital micrographs covering 3 complete H&E stained cross-sections taken from top, middle, and bottom sections of each retrieved construct were made with a Nanozoomer HT (Hamamatsu Photonics) for analysis.

The rate of vascularization was estimated by counting the total number of vessel lumens per cross-section area using NIH ImageJ software. Likewise, the average vessel diameter was determined by measuring the diameter of the vessel lumens in each

cross-section. The infiltration of inflammatory cells was scored on a scale from 0 to 3; a score *0* indicated no or only few inflammatory cells present (minimal inflammation), a score *1* indicated groups of inflammatory cells around the construct and no or only few cells inside the construct (mild inflammation), a score 2 indicated groups of inflammatory cells around and inside the construct (severe inflammation) and a score *3* indicated inflammatory cells throughout the construct and the surrounding tissue (intense inflammation).

STATISTICAL ANALYSIS

The mitotic indices and all gene expression and protein secretion data were analyzed with the Kruskal-Wallis test followed by post hoc Dunn's multiple comparison tests and are expressed as median (interquartile range, IQR). All vascularization data were analyzed with the unpaired t-test with Welch correction and are expressed as mean± standard deviation. For statistical comparisons of the inflammation scores, the number of spheroids and fibrin-based constructs with score 0-1 (indicating minimal to mild inflammation) were compared to the number of spheroids and fibrin constructs with score 2-3 (indicating severe to intense inflammation) using the Fisher's exact test. Statistical significance was defined as $P<0.05$. Statistical analysis was performed using SPSS 15.0 (SPSS Inc., Chicago, Illinois).

RESULTS

IN VIVO PERFORMANCE OF ASC TISSUE CONSTRUCTS

Following 7 days of culture, spheroids and fibrin-based constructs were implanted subcutaneously in athymic mice, to determine construct in vivo engraftment. Seven days after implantation, we could identify 8 out of 9 engrafted spheroids and 8 out of 9 engrafted fibrin-based constructs. Gross examination revealed that both spheroids and fibrin-based constructs had a spherical to oval shape, appeared light yellow in color and were invaded by vessels (Fig.1A).

H&E stained cross-sections of the retrieved constructs showed that host blood vessels were present at the periphery of all constructs while 4 out of 8 spheroids and 5 out of 8 fibrin-based constructs contained host blood vessels within the constructs (Fig.1B). To provide more insight in the vascularization of these spheroids and fibrin-based constructs, we determined the number of vessel lumens per cross-section and their average diameter. In fibrin-based constructs the number of vessel lumens per cross-section [93± 45 lumens/mm^2] was significantly higher when compared to spheroids [23±11 lumens/mm^2], while the average vessel lumen diameter was comparable [spheroids: 10±2μm versus fibrin constructs: 10±1μm].

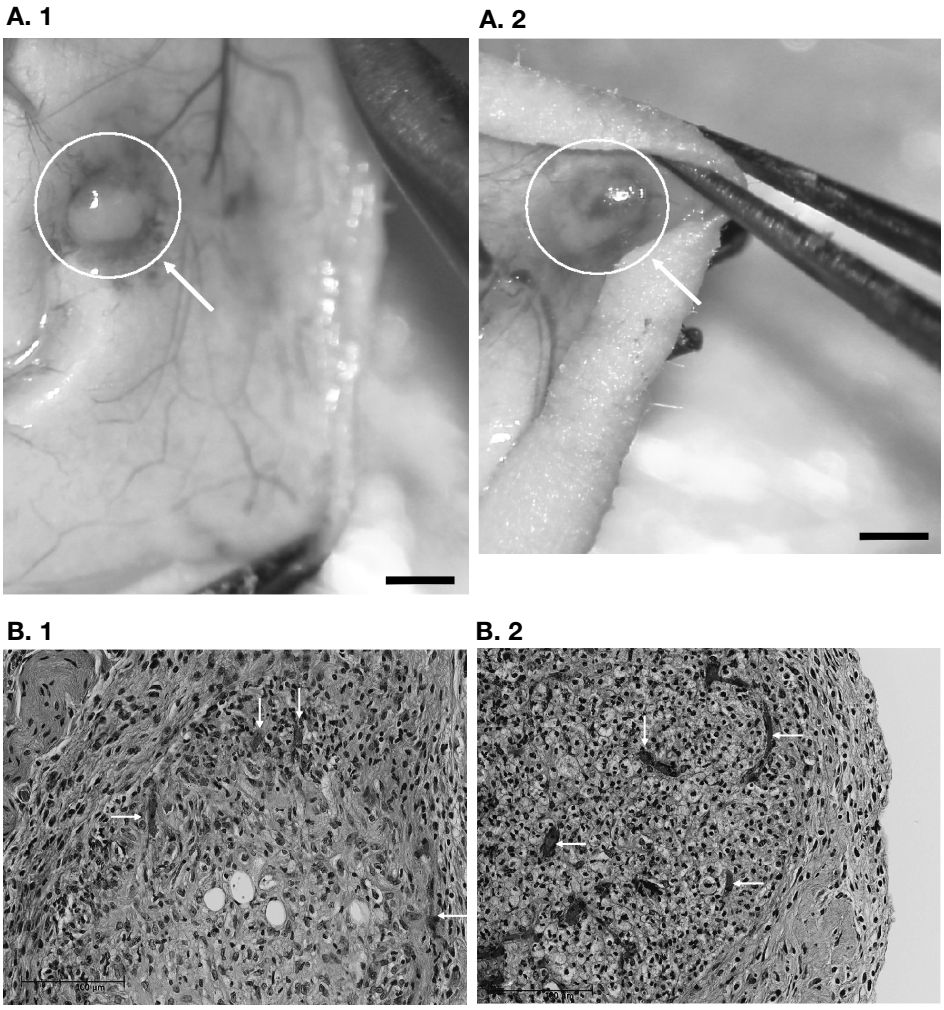


Figure 1. Host blood vessels invade spheroids and fibrin-based constructs. Spheroids and fibrin-based constructs were cultured for 7 days in vitro, and then implanted subcutaneously in the right and left scapular region of athymic mice for 7 days. Representative images of **(A.1)** a spheroid and **(A.2)** a fibrin-based construct taken at day 7 postimplantation. Scale bars= 1mm. **(B)** Construct cross-sections were stained with hematoxylin (blue) & eosin (pink). **(B.1)** spheroid cross-section and **(B.2)** fibrin-based construct cross-section, showing invasion of host blood vessels (see white arrows). Scale bars = 100μm. (see color section for a full-color version)

A profound inflammatory response was generally noted in cross-sections of spheroids (minimal to mild inflammation: 1 construct; severe to massive inflammation; 7 constructs), whereas the inflammatory response in fibrin construct cross-sections was grossly mild (minimal to mild inflammation: 6 constructs; severe to intense inflammation: 2 constructs) (Fig.2). In addition, immunostaining of construct cross-sections for human-vimentin demonstrated no to minor positive staining in the severe to intense inflamed constructs

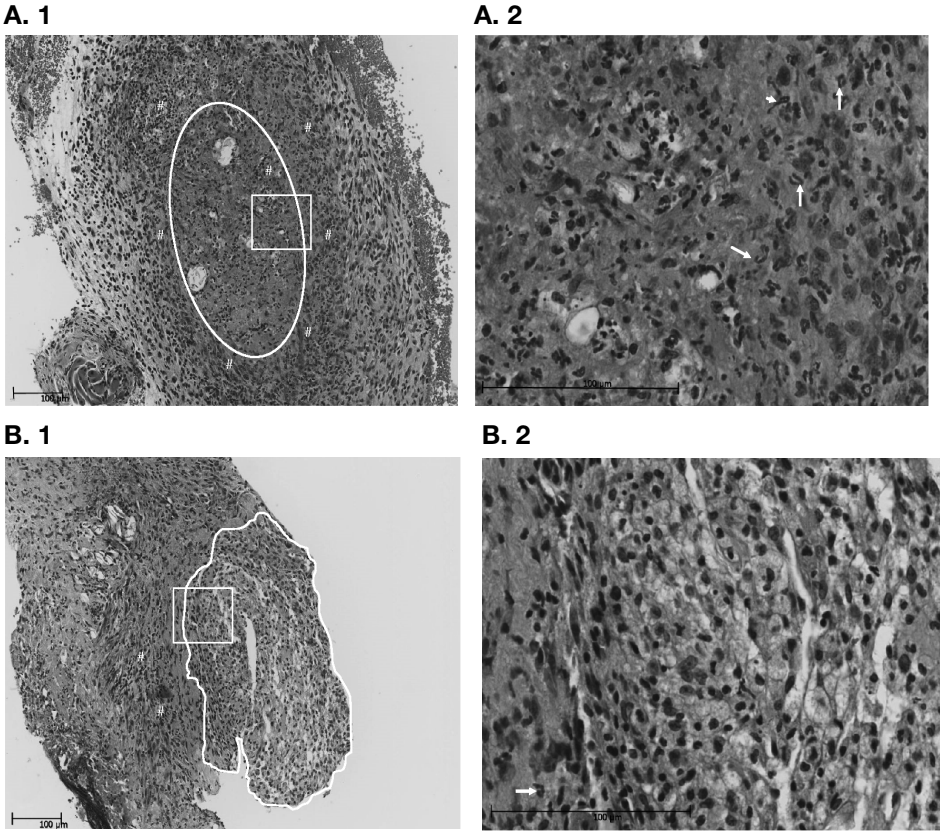


Figure 2. Upon 7 days of implantation fibrin-based constructs elicit a less severe inflammatory response than spheroids. Representative hematoxylin (blue) & eosin (pink) stained sections of spheroids and fibrin-based constructs after 7 days of implantation in athymic mice. **(A.1)** Overview of a spheroid cross-section and **(A.2)** high magnification image of selection in (A.1) (see white square). **(B.1)** Overview of a fibrin-based construct cross-section and **(B.2)** high magnification image of selection in (B.1) (see white square). Areas with inflammatory cell infiltration (see white #) and inflammatory cells (see arrows) are indicated. Scale bars= 100µm. **(C)** Scoring of the inflammatory response to the constructs. High inflammatory response to spheroids (score 0-1= minimum to mild inflammation; 1 construct, score 2-3= severe to intense inflammation; 7 constructs) was noted while the inflammatory response to fibrin-based constructs was grossly mild (score 0-1= minimum to mild inflammation; 6 constructs, score 2-3= severe to intense inflammation; 2 constructs). (see color section for a full-color version)

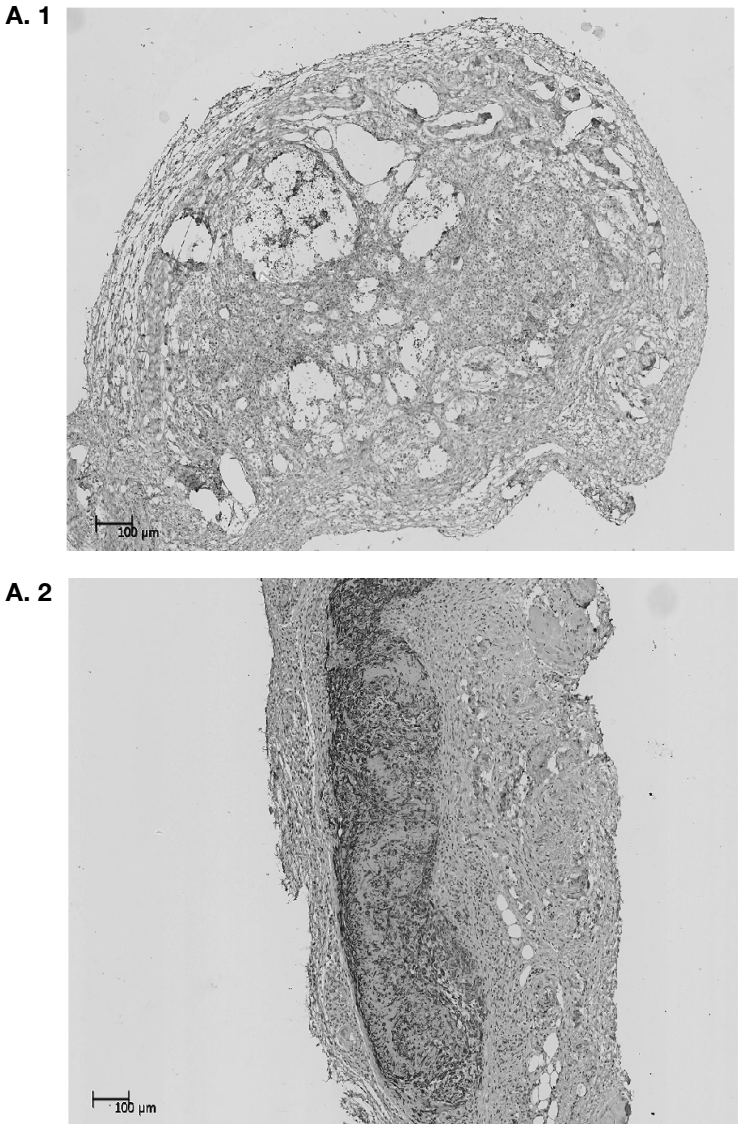
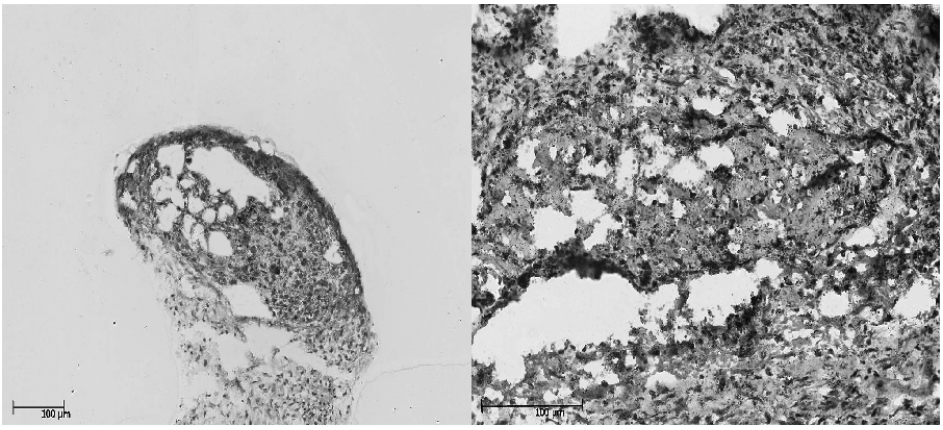


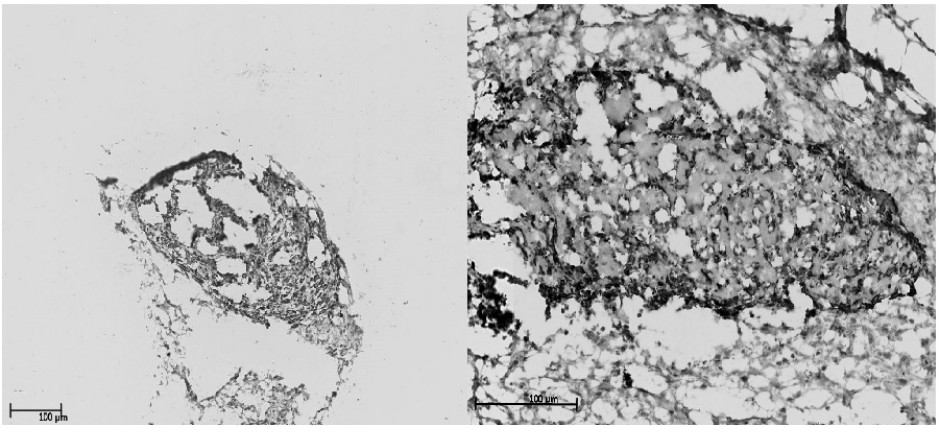
Figure 3. Assessment of ASC presence and lipid accumulation in transplanted spheroids and fibrin-based constructs. To detect ASC in spheroids and fibrin-based constructs after 7 days of implantation, cross-sections were stained with human specific vimentin antibody (brown) and counterstained with hematoxylin (blue). **(A)** Representative images of anti-human vimentin-stained cross-sections of a spheroid **(A.1)** and a fibrin-based construct **(A.2)** after 7 days of implantation. Note the limited vimentin staining in the inflamed spheroid when compared to the fibrin-based construct. **(B)** To detect lipid accumulation in constructs after 7 days of implantation, cross-sections were stained with Oil Red O (red) and counterstained with hematoxylin (blue). **(B.1)** Spheroid and **(B.2)** fibrin-based construct. **(C)** Anti-human vimentin (brown) stained consecutive cross-section of the same spheroid **(C.1)** and fibrin-based construct **(C.2)** as shown in (B.1) and (B.2). Note that Oil Red O-positive cells and vimentin-positive cells are located within the same area. Scale bars= 100µm. (see color section for a full-color version)

(mainly spheroids) whereas the minimal to mild inflamed constructs (mainly fibrin-based constructs) were strongly positive, indicating that cells of human origin were only sparsely present in the severely inflamed constructs (Fig.3A). Furthermore, Oil Red O –positive cells were present in both spheroids and fibrin-based constructs. Notably, the Oil-Red O positive cells are located within the same area as vimentin-positive cells in consecutive cross-sections of the same spheroid and fibrin-based constructs, suggesting that the Oil Red O-positive cells were from human origin (Fig.3B-C).

B. 1 **B. 2**



C. 1 **C. 2**



ANGIOGENIC FACTOR SECRETION BY ASC IN SPHEROIDS AND FIBRIN-BASED CONSTRUCTS

The ability of ASC to secrete angiogenic factors may have beneficial effects on the vascularization and survival of ASC constructs in vivo. To assess the ability of ASC to

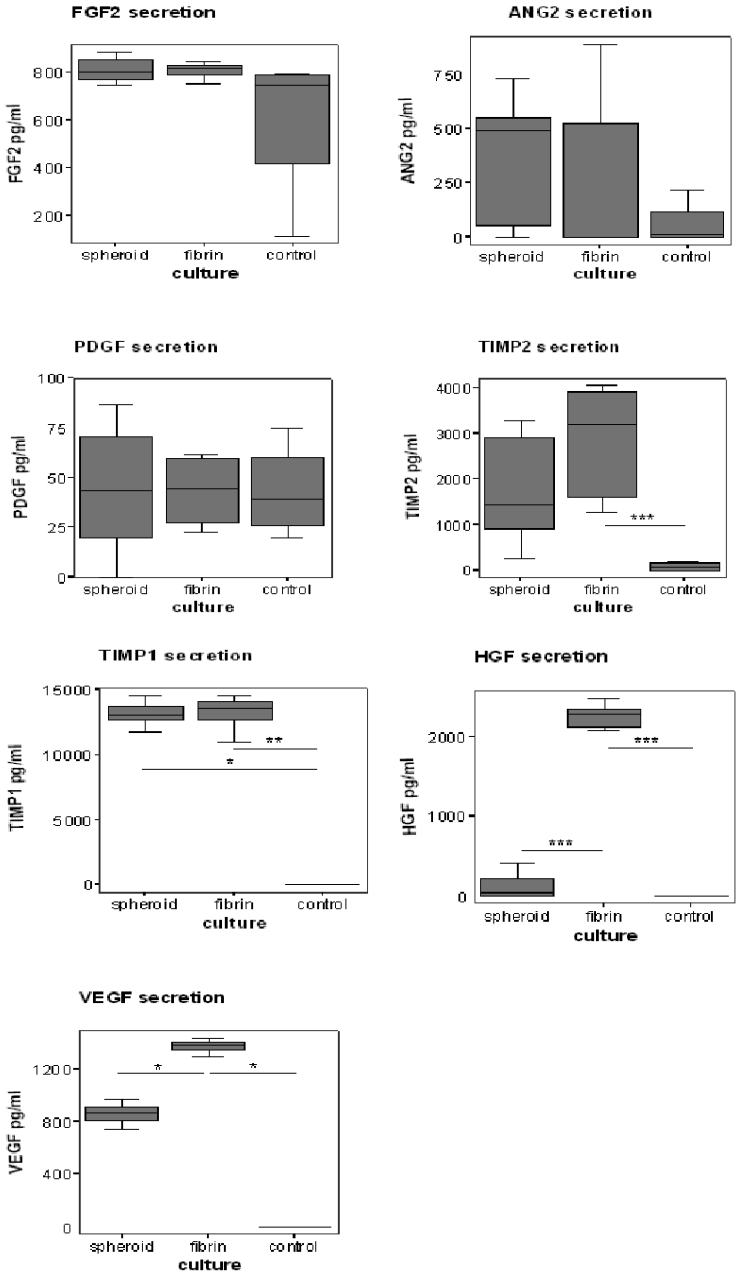


Figure 4. ASC in fibrin-based constructs secrete higher levels of the angiogenic factors VEGF and HGF than ASC in spheroids. The secretion of the angiogenic factors FGF2, ANG2, PDGF, TIMP2, TIMP1, HGF and VEGF by ASC in spheroids and fibrin-based constructs over 48 hours was measured by multiplex chemiluminescent ELISA at day 7. Unconditioned adipogenic medium was used as control condition. Values are presented as median (interquartile range); n= 5, 3 ASC donors. *= P≤ 0.05, **= P≤ 0.01, ***= P≤ 0.001 (fibrin-based construct conditioned medium versus spheroid conditioned medium and control).

secrete angiogenic factors, we analyzed the release of several angiogenic factors into the culture medium over a 48-hour period. Figure 4 illustrates the data from several important angiogenic factors assayed by multiplex chemiluminescent ELISA at day 7. Of these, FGF2 was added to the culture medium, which masks any differences in the levels of FGF2 between the conditioned media and unconditioned medium.

No significant differences in the levels of ANG2 and PDGF between conditioned and unconditioned medium were observed. In contrast, the levels of TIMP2 in conditioned medium from fibrin constructs and the levels of TIMP1 in conditioned medium from both spheroids and fibrin-based constructs were significantly higher when compared to unconditioned medium. Moreover, the levels of HGF and VEGF in conditioned medium from fibrin-based constructs were significantly higher when compared to spheroid conditioned and unconditioned medium.

INFLAMMATORY CYTOKINE SECRETION BY ASC IN SPHEROIDS AND FIBRIN-BASED CONSTRUCTS

The secretion of a vast amount of pro-inflammatory cytokines may have a negative effect on the in vivo-engraftment of ASC constructs. To assess if culture of ASC in spheroids or fibrin-based constructs modulated the secretion of inflammatory cytokines we analyzed the release of several inflammatory cytokines into the culture medium over a 48-hour period. Figure 5 illustrates the data from 9 different proteins assayed by multiplex chemiluminescent ELISA at day 7. No statistically significant differences in the secretion of the cytokines IL1α, IL2, IL4 and IL10 were observed between conditioned media of spheroids and fibrin-based constructs when compared to unconditioned medium (= control). In contrast, IL1β, IL6, IFNγ and TNFα were secreted in significant amounts in both spheroids and fibrin-based constructs when compared to unconditioned medium. In addition, the levels of IL8 in spheroid-conditioned medium were significantly higher when compared to conditioned medium from fibrin-based constructs and unconditioned medium.

ASC ADIPOGENIC DIFFERENTIATION IN SPHEROIDS AND FIBRIN-BASED CONSTRUCTS

Previous studies have shown that adipogenic differentiation of ASC in vitro can improve adipogenesis in vivo [37,38]. Important characteristics of adipogenic differentiation are the accumulation of lipid, the expression of adipogenesis-related markers and the secretion of leptin [39,40]. To qualitatively determine lipid accumulation in ASC in spheroids and fibrin-based constructs after 7 days of culture, we stained cryo-sections with Oil Red O. Oil Red O positive cells were evident in both spheroids and fibrin-based constructs (Fig.6A).

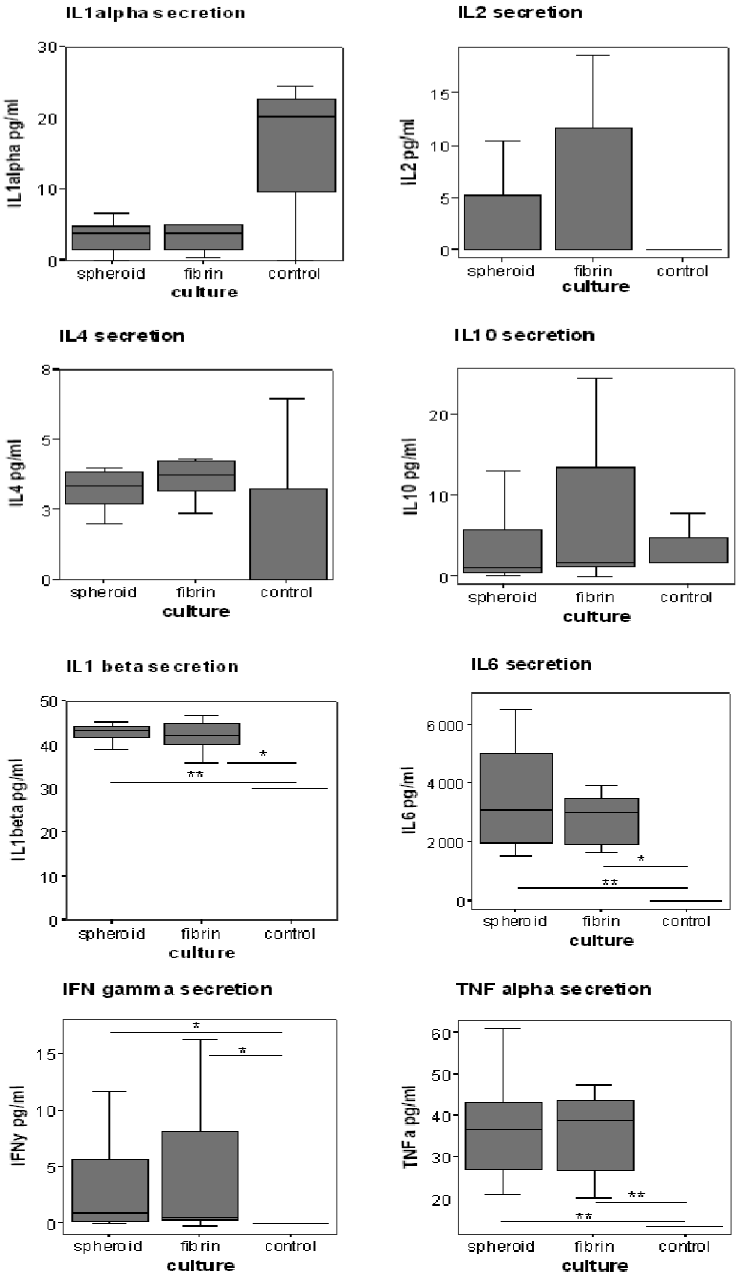


Figure 5. ASC in fibrin-based constructs secrete lower levels of the cytokine interleukin 8 than ASC in spheroids. The secretion of the inflammatory cytokines IL1 alpha, IL2, IL4, IL10, IL1 beta, IL6, TNF alpha, IFN gamma and IL8 by ASC in spheroids and fibrin-based constructs over 48 hours was measured by multiplex chemiluminescent ELISA at day 7. Unconditioned adipogenic medium was used as control condition. Values are presented as median (interquartile range); n= 5, 3 ASC donors. *= P≤ 0.05, **= P≤ 0.01, ***= P≤ 0.001 (spheroid and fibrin-based construct conditioned medium versus each other and control).

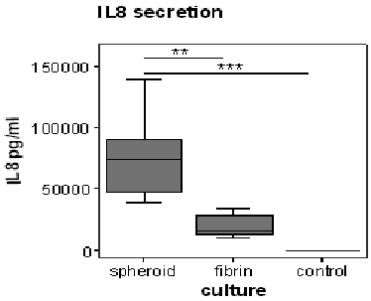


Figure 5. (continued)

To quantitatively assess adipogenic differentiation of the ASC in spheroids and fibrin-based constructs, we measured the gene expression of the adipogenic markers PPARG, PLIN1, FABP4 and LEP after 7 days of culture. In addition, we measured the release of leptin into the culture medium over a 48-hour period. Q-PCR analysis showed that all four adipogenic markers were expressed in ASC cultured in spheroids or fibrin-based constructs. Notably, only ASC in fibrin-based constructs showed a significantly higher expression of these adipogenic markers when compared to undifferentiated ASC harvested at day 0 (= control) (Fig.6B). Moreover, the levels of leptin in conditioned medium from fibrin-based constructs were significantly higher when compared to conditioned medium from spheroids and unconditioned medium (=control) (Fig.6C).

To determine the proliferative activity of differentiating ASC in spheroid and fibrin-based constructs Ki-67 staining was used. Mitotic indices were calculated. Comparison of the mitotic index revealed no significant difference in ASC proliferative activity between spheroids and fibrin-based constructs (spheroid mitotic index median: 3%, [IQR: 9.7-1.1%] versus fibrin median: 3.5%, [IQR: 8.9-0.7%]).

DISCUSSION

The results of our study suggest that fibrin-based ASC constructs better support in vivo engraftment than scaffold-free ASC spheroids, as indicated by a less severe inflammatory response and a higher degree of vascularization after in vivo transplantation. In addition, fibrin-based ASC constructs better support ASC adipogenesis as indicated by the higher expression levels of adipocyte-specific markers and the increased secretion of leptin.

An important parameter determining the success of ASC constructs for soft tissue repair is an unfavorable host response. In our study the inflammatory response after subcutaneous transplantation in nude mice was more profound in spheroids than in

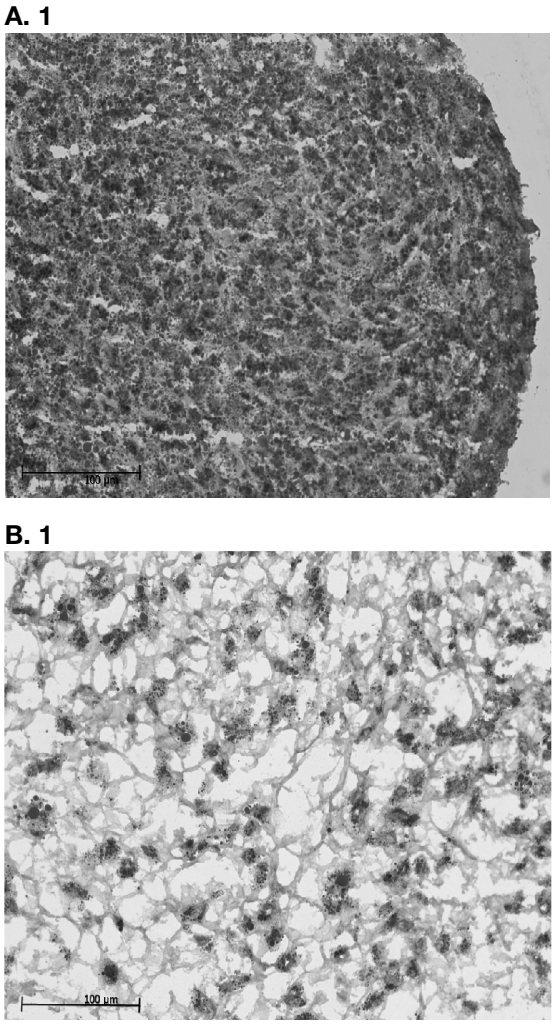


Figure 6. ASC in fibrin-based constructs display increased adipogenic differentiation when compared to ASC in spheroids. **(A)** ASC in spheroids and fibrin-based constructs were cultured for 7 days in vitro in adipogenic medium. Cross-sections were stained with Oil Red O (red, showing intracellular lipid) and counterstained with hematoxylin (blue). **(A.1)** Spheroid. **(A.2)** Fibrin-based construct. Scale bars= 100 µm. **(B)** Q-PCR was used to measure the expression levels of the adipocyte-specific markers PPARGγ, PLIN, FABP4 and LEP. Expression levels are relative to beta-2-microglobulin positive-control housekeeping gene (delta Ct). Values are presented as medians (interquartile range). For each of the three ASC donors 9 spheroids, 9 fibrin-based constructs and $0.5*10^6$ undifferentiated ASC (=control) were prepared and assayed. *= P≤ 0.05, **= P≤ 0.01 and ***= P≤ 0.001 (relative expression in fibrin-based constructs compared to spheroids and control). **(C)** The secretion of leptin by ASC in conditioned medium of spheroids and fibrin-based constructs over 48 hours was measured by sandwich ELISA at day 7. Unconditioned adipogenic medium was used as control condition. Values are presented as median (interquartile range); n= 9, 3 ASC donors. **= P≤ 0.01, ***= P≤ 0.001 (fibrin-based construct conditioned medium versus spheroid conditioned medium and control). (see color section for a full-color version)

fibrin-based constructs. In line with this finding, ASC cultured in spheroids secreted higher amounts of the pro-inflammatory mediator IL8 than ASC cultured in fibrin-based constructs. Taken together, these findings indicate that the ASC in spheroids induce a higher inflammatory response than ASC in fibrin-based constructs, which likely results from a higher secretion of IL8. However, the possibility that other than the investigated factors contributed to the higher inflammatory response in spheroids, such as more cell death due to less sufficient diffusion of oxygen and nutrients, cannot yet be ruled out.

Another important parameter determining the success of ASC constructs is vascularization [41,42,43]. We quantified the vascularization of construct cross-sections upon transplantation and found that the number of vascular lumens-per construct-area was

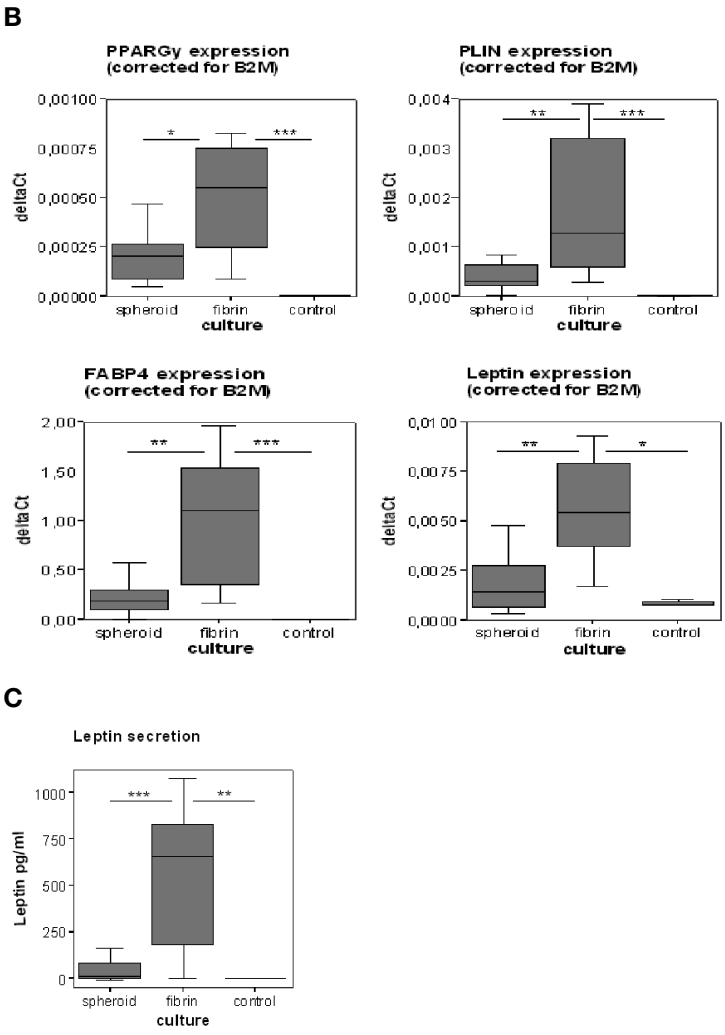


Figure 6. (continued)

significantly higher in fibrin-based constructs when compared to spheroids, indicating augmented ingrowth of mouse vessels. In line with this finding, we found that ASC in fibrin-based constructs secreted higher amounts of the prominent pro-angiogenic factors VEGF and HGF when compared to ASC in spheroids. In all, these findings indicate that fibrin favors the vascularization of ASC constructs upon transplantation. Such a positive effect of fibrin on in vivo vascularization has also recently been shown for bone marrow-derived stromal cell/ fibrin-based constructs by Huang et al. [44].

Although previous studies demonstrate that scaffold-free spheroid culture provides favorable conditions for reconstruction of liver [45], pancreas [46], blood vessel [47], myocardial muscle [48], ganglion [48], cartilage [49] and bone tissue [50,51], its use for reconstruction of adipose tissue has not been investigated until now. In our study the adipogenic differentiation of ASC was clearly increased in fibrin-based cultures when compared to scaffold-free spheroid cultures. Therefore, we conclude that the spheroid culture environment is less favorable for ASC adipogenic differentiation than the fibrin culture environment. Possibly, differences in cell density accounted for the observed differences in ASC adipogenic differentiation between spheroid and fibrin-based cultures. In fibrin-based cultures the ASC are more loosely packed than in the spheroid cultures, as can be seen in Figures 6A-B. Mueller-Klieser et al. [52], and others [53,54] showed that the relatively high cell density of especially larger (>150-200μm) spheroids limits the diffusion of many molecules to individual cells inside the spheroids, leading to nutrient deprivation. It could therefore be hypothesized that the observed difference in adipogenic differentiation between spheroids and fibrin-based cultures are due to a difference in nutrient delivery to individual cells.

Collectively, our in vitro and in vivo experiments with fibrin are promising and demonstrate that fibrin provides favorable conditions for adipose tissue reconstruction. Further studies should determine the in vivo survival of fibrin-based adipose constructs in the long term, as well as attempt to develop fibrin-based adipose constructs of appropriate size and predictable forms to repair soft tissue defects.

A potential strategy that could benefit the in vivo survival of especially larger fibrin-based adipose constructs is the prefabrication of a vascular network within the tissue construct, as previously demonstrated by Levenberg et al. [55] for engineered skeletal muscle tissue. Furthermore, advanced techniques such as inkjet printing and magnetically influenced self-assembly can be used to alter the geometry of the fibrin-based constructs into appropriate and predictable forms [56,57,58,59].

CONCLUSION

The use of fibrin-based ASC constructs leads to increased expression of adipocyte-specific markers, a higher secretion of angiogenic factors and a lower secretion of the cytokine IL8 when compared to scaffold-free ASC spheroids. In addition, fibrin-based

ASC constructs display a higher degree of vascularization and elicit a less severe inflammatory response than ASC spheroids upon transplantation. Therefore, we conclude that fibrin-based constructs are more suitable for ASC-based adipose tissue reconstruction than scaffold-free ASC spheroids.

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CHAPTER 4

Adult human bone marrow-and adipose tissue-derived stromal cells support the formation of prevascular-like structures from endothelial cells in vitro

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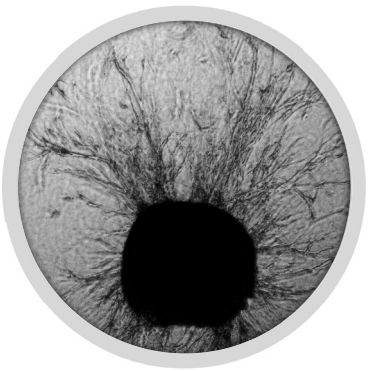


ABSTRACT

Inadequate vascularization of in vitro-engineered tissue constructs after implantation is a major problem in most tissue engineering applications. In this study we evaluated whether adipose tissue-derived stromal cells (ASC), similar to bone marrow-derived stromal cells (BMSC), can support the organization of endothelial cells into prevascular-like structures using an in vitro model. In addition, we investigated the mechanisms leading to the support of endothelial organization by these cells. We cultured human umbilical vein endothelial cells (HUVEC), ASC and BMSC either alone or in combination in fibrin embedded spheroids for 14 days. We found that BMSC and ASC formed cellular networks that expressed alpha smooth muscle actin, and in the case of ASC also CD34. Furthermore, BMSC and ASC secreted hepatocyte growth factor (HGF) and tissue inhibitor of metalloproteinase (TIMP) 1 and -2. In addition, ASC conditioned medium induced HUVEC outgrowth whereas BMSC-conditioned medium and HGF-supplemented medium did not. Finally, both BMSC and ASC supported HUVEC organization into prevascular-like structures when co-cultured. Our results suggest that both BMSC and ASC can support the formation of prevascular-like structures in vitro. Furthermore, our findings indicate that cell-cell contacts and reciprocal signaling play an important role in the formation of these prevascular structures.

INTRODUCTION

The realization of a viable and adequate vascular supply in engineered replacement tissues or organs is a prerequisite for their survival after implantation and has become one of the major challenges in tissue engineering. The lack of an adequate blood supply in the core of tissue engineered constructs thicker than approximately 150μm results in hypoxia and an inadequate supply of nutrients, eventually leading to cell death [1,2].

For new blood vessels to be formed in tissue-engineered constructs, a balanced array of angiogenic factors involved in both angiogenesis and vessel maturation needs to be delivered in the right way at the right time, and at the site of implantation [3]. Finding this cocktail of angiogenic factors for the development of functional vessels is an elaborate and demanding task and forms a major hurdle in tissue engineering.

In vitro studies demonstrated that bone marrow-derived stromal cells (BMSC) as well as adipose tissue-derived stromal cells (ASC) are able to express many angiogenic factors that can stimulate endothelial and smooth muscle cells to proliferate and migrate [4,5,6,7,8], and can even differentiate into cells that express markers of the endothelial lineage [6,9,10,11,12,13,14]. Several in vivo studies also indicated that both cell types could be used in therapeutic revascularization of ischemic tissues [6,9,11,15,16].

The requirements for therapeutic revascularization and the vascularization of engineered tissues share many similarities. BMSC and ASC may therefore not only be beneficial for therapeutic revascularization of ischemic tissues, but may also represent suitable cell sources to support new vessel formation in engineered tissue constructs.

Another approach to promote the in vivo vascularization of tissue constructs would be to already incorporate endothelial cells into the engineered tissues in vitro [17,18,19,20]. Recently, Rouwkema et al.[19] showed that BMSC were able to support the formation of endothelial cell networks in a bone tissue-engineered construct. Similar to BMSC, ASC have multilineage potential and can be used in various tissue-engineering applications [21,22,23,24,25]. ASC might even be preferred over BMSC since they require a less invasive harvesting procedure and, are generally present in higher numbers in adipose tissue than BMSC are present in bone marrow [26]. The idea that not only BMSC but also ASC could support the formation of prevascular-like structures in an in vitro tissue-engineering context is therefore very attractive.

We hypothesized that stromal derived cells of adipose tissue origin, similar to those of bone marrow origin, can be used to support the formation of prevascular-like structures within engineered tissues in vitro. To test this hypothesis, we assessed the ability of both ASC and BMSC to support the formation of prevascular-like structures from HUVEC using an in vitro model. In addition, we studied the mechanisms leading to the support of the formation of prevascular structures in vitro.

Since vessel growth *in situ* frequently takes place in a microenvironment promoting vascularization, we cultured HUVEC-, ASC-, and BMSC-spheroids in fibrin, using endothelial growth medium supplemented with VEGF. We then assessed their organization capabilities and the release of several factors known to be involved in vessel formation. In addition, we determined the expression of the vascular markers CD34 and alpha smooth muscle actin (αSMA) by ASC and BMSC. Furthermore; we tested the effect of factors secreted by ASC or BMSC on HUVEC outgrowth and organization. Finally, we labeled HUVEC with superparamagnetic iron oxide particles (SPIO) and co-cultured them with different ratios of ASC and BMSC, to assess their organization and collective three-dimensional (3D) assembly.

MATERIALS AND METHODS

MATERIALS

Dulbecco's modified Eagle's medium containing either 1 g/L glucose (LG-DMEM) or 4.5 g/L glucose (HG-DMEM), human endothelial serum free medium (SFM), penicillin/ streptomycin, fungizone, collagenase type I, and trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Invitrogen (Carlsbad, California).

Fetal bovine serum (FBS) for ASC was purchased from PAA Laboratories (Cölbe, Germany) and FBS for BMSC was purchased from Invitrogen. Both fetal bovine sera were pre-selected from a batch to maintain multipotential capacities of the cells. Human serum was purchased from Lonza (Verviers, Belgium). Insulin was from Eli Lilly (Indianapolis, Indiana), and vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF-2), epidermal growth factor (EGF) and hepatocyte growth factor (HGF) were obtained from Peprotech EC (London, United Kingdom). Monoclonal Anti-HGF antibody was obtained from R&D systems (R&D systems Europe, Abingdon, United Kingdom). Bovine serum albumin (BSA) and other chemicals were from Sigma-Aldrich (St. Louis, Missouri) unless stated otherwise.

METHODS

ISOLATION AND EXPANSION OF HUMAN BONE MARROW-DERIVED STROMAL CELLS (BMSC)

BMSC of four donors (two men/ two women, 31-61/ 42-53 years old) were obtained during total hip arthroplasty after informed consent and with approval of the local medical ethical committee (METC 2004-142). Bone marrow aspirates were taken from the greater trochanter. Heparinized aspirates were seeded at a density of 30-90 $*10^6$ nucleated cells per T175 culture flask. After 24 hours, non-adherent cells and cell de-

bris were washed out. BMSC were further expanded using a standard culture medium; LG-DMEM, with 10% FBS, 0.5% gentamycin and 0.015% fungizone, 10^{-4}M L-ascorbic acid 2-phosphate (Instruchemie B.V.Delfzijl, The Netherlands) and 1 ng/ml FGF-2. Cells were cultured at 37ºC under humidified conditions in air containing 5% carbon dioxide. Medium was changed twice a week. When cultures reached near confluence, they were trypsinized using 0.05% trypsin-EDTA and stored in liquid nitrogen until further use. BMSC from passage 2 or 3 were thawed and used in the experiments after reaching near confluence.

ISOLATION AND CULTURE OF HUMAN ADIPOSE TISSUE-DERIVED STROMAL CELLS (ASC)

For this study, ASC from four female donors 43, 46, 49 and 56 years of age were isolated from subcutaneous abdominal adipose tissue. Adipose tissue was received with approval of the Medical Ethics Committee (# MEC-2005-157). Excised human adipose tissue was washed with LG-DMEM, minced, and digested in sterile-filtered 0.1% collagenase type I solution in the presence of 1% BSA. The mixture was suspended in LG-DMEM at 37˚C for 60 min in an orbital shaker. ASC were isolated by differential centrifugation, washed with medium (LG-DMEM, 10% FBS, 1% penicillin/streptomycin, and 0.5% gentamycin), filtered through a 100 µm mesh, and grown in basal medium supplemented with 10^{-12}M dexamethasone and 10^{-5} M ascorbic acid. After 24 hours, cultures were washed with phosphate-buffered saline (PBS) with 2% FBS to remove erythrocytes and replenished with the supplemented basal medium. Cells were cultured at 37ºC under humidified conditions in air containing 5% carbon dioxide. Medium was changed twice a week. When cultures reached near confluence, they were trypsinized using 0.05% trypsin-EDTA and stored in liquid nitrogen. ASC from passage 1 or 2 were thawed and used in the experiments after reaching near confluence.

HUMAN UMBILICAL VEIN ENDOTHELIAL (HUVEC) CELL CULTURE

We obtained HUVEC isolates from four donors. Each isolate was derived from an individual umbilical vein. HUVEC at passage 4 or less were seeded at a density of $1*10^4$ cells/cm^2 in culture flasks and cultured in endothelial growth medium (EGM) consisting of human endothelial-serum free medium supplemented with 10% FBS, 20ng/ml FGF2 and 100ng/ml EGF. Medium was changed twice a week. Cells were subcultured at near confluence with 0.05% trypsin/EDTA.

LABELING OF HUVEC WITH IRON OXIDE NANOPARTICELS

HUVEC at passage 5 or less were labeled with the so-called superparamagnetic iron oxide (SPIO) particles. These SPIO particles have a mean particle diameter of 150nm,

consist of an iron oxide core of 4nm and are coated with a low-molecular-weight dextran [27,28]. Labeling of HUVEC with SPIO was performed at near confluence using Endorem (Guerbet S.A., Paris, France) and Lipofectamine 2000 (Invitrogen). Briefly, 40µl of both Endorem and Lipofectamine 2000 were mixed separately with 1 ml of Opti-MEM (Invitrogen). The two solutions were then combined and incubated for 20 minutes at room temperature. HUVEC at passage 4 or less were cultured to near confluence in a T175 culture flask. After reaching near confluence, the cells were washed three times with phosphate buffered saline (PBS) and the culture medium was replaced by 20 ml Opti-MEM. The total volume of SPIO-Lipofectamine suspension (equivalent to 460.5µg iron content) was added drop-wise to 20 ml Opti-MEM. Subsequently, the cells were incubated at 37°C/5%CO_2 for 24 hours. Following 24 hours of incubation the cells were rinsed three times in PBS and incubated with 10%FBS for 1 hour to remove loose SPIO-Lipofectamine complexes. The SPIO-labeled HUVEC were then trypsinized and used in the experiments.

FORMATION AND CULTURE OF SPHEROIDS IN FIBRIN MATRIX

We used an in vitro fibrin based culture model, slightly modified from models developed by Vernon and Sage [29] and Xue [30] et al.

To form spheroids of cells for culture in a fibrin matrix, we used the hanging droplet method. A cell suspension of 500,000 cells per ml EGM was prepared. Forty microliters of this suspension (containing 20,000 cells) was pipetted onto the inside of the lid of a six-well culture plate coated with sterile parafilm. The lid was then overturned and placed on top of the six-well culture plate containing sterile PBS. These hanging droplets were left for two days to form coherent spheroids. For spheroid formation, HUVEC, BMSC and ASC were used separately or the SPIO-labeled HUVEC were mixed with different percentages of BMSC or ASC (60%, 80% and 95%). For the single culture experiments, 3 different donors were used to generate 18 BMSC, ASC or HUVEC spheroids. Since little variation between donors was seen in these experiments, cells from a single donor were used to generate 6 spheroids per co-culture condition in the co-culture experiments.

To form the fibrin gels, human fibrinogen (6mg/ml, Sigma-Aldrich) was dissolved in serum free HG-DMEM and was sterile filtered through a 0.2µm filter. Human plasma thrombin (0.32U/ml, Sigma-Aldrich) was added to 200µl of the fibrinogen, and this mixture was immediately placed in individual wells of a 48-well culture plate. After gel formation, the spheroids were placed carefully onto these gels and another 200µl fibrinogen solution was added on top of the spheroids and lower gel. In this way the spheroid was fully immersed in the fibrin matrix. After gel formation, EGM supplemented with 50ng/ml vascular endothelial growth factor (VEGF) was added to each well. The spheroids were cultured for 14 days and medium was changed every other

day. Medium was collected and stored at -80ºC. After 14 days, immersed spheroids were fixed in 10% formalin in PBS and embedded in paraffin.

HISTOMORPHOMETRY OF BMSC, ASC, HUVEC/ BMSC AND HUVEC/ASC SPHEROIDS

Histomorphometric analysis was used to quantify length, width and density of the cellular strands growing out of the BMSC and ASC (co-) culture spheroids. Low magnification (40X) digital micrographs of randomly selected spheroids were made with the Olympus U-CMAD3 camera (Olympus Soft Imaging Solutions, Münster, Germany) coupled to a light microscope (Olympus) at day 3, 7, 10 and 14 of culture for the analysis. Four spheroids per experiment group were analyzed. Subsequently, NIH ImageJ software (http://rsb.info.nih.gov/ij/) was used to measure the length, width and density of the cellular strands. The mean density was assessed by counting the number of cellular strands in three separate areas of 40 μm^2 in each image. The mean width was determined by measuring the widths at the middle of 10 separate strands in each image. The mean maximum length of the cellular strands was determined by measuring the lengths of the three longest strands originating from the center of an individual spheroid.

HISTOLOGY

IMMUNOCYTOCHEMISTRY OF CULTURED BMSC AND ASC BEFORE SPHEROID FORMATION:

To determine the expression of CD34 and alpha smooth muscle actin (αSMA) in BMSC and ASC after passage 1 or 2 expansion and before spheroid formation, BMSC and ASC were suspended in fibrin ($0.5*10^6$ cells/50µl fibrin) in a 96-wells plate. Gels were then directly fixed in 10% formalin/PBS and embedded in paraffin.

IMMUNOHISTOCHEMISTRY OF CD34 AND αSMA:

Paraffin sections of cultured BMSC and ASC or of fibrin immersed BMSC and ASC spheroids were deparaffinized and rehydrated.

Antigen retrieval for the mouse anti-human CD34 antibody (Biocare Medical, Concord, California) was performed through boiling in BORG Decloacker (Biocare Medical) at 95ºC for 45 minutes, while antigen retrieval for the mouse anti-human αSMA antibody (clone 1A4, Sigma-Aldrich) required incubation in 0.1% pronase at 37ºC for 30 minutes. Endogenous peroxidase was blocked with 3% H_2O_2 in PBS. Non-specific binding sites were blocked with 10% goat serum in PBS/1%BSA and sections were stained overnight at 4ºC with primary antibodies recognizing CD34 (1:50), or αSMA (1:20,000). Sections were incubated with goat anti-mouse secondary antibody diluted in PBS/1%BSA 1:200

(DAKO, Glöstrup, Denmark) for 30 minutes followed by incubation with streptavidin-horseradish peroxidase (DAKO) diluted in PBS/1%BSA 1:300 for 30 minutes. To visualize bound antibodies di-aminobenzidine was used as a substrate. The slides were weakly counterstained with hematoxylin, dehydrated through graded alcohols and mounted with Permount (VWR International B.V., Amsterdam, The Netherlands).

PERL'S IRON STAIN:

To localize SPIO-labeled HUVEC in mixed stromal cell/labeled HUVEC spheroids, Perl's stain (Klinipath, Duiven, The Netherlands) was used for iron detection. Slides were incubated with Perl's reagent (2% potassium ferrocyanide/ 2% hydrochloric acid, 50:50, vol/vol) for 15 minutes at room temperature and then washed three times in deionised water. Nuclear fast red was used as a counter stain. After staining, the slides were dehydrated through graded alcohols and mounted with Permount.

MULTIPLEX ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

A multiplex chemiluminescent ELISA (Quansys Biosciences, West Logan, Utah) was used to assay 48-hours conditioned medium of the 100% BMSC, 100% ASC and 100% HUVEC spheroid cultures for the release of the angiogenic factors: platelet derived growth factor B (PDGFB), VEGF, angiopoietin 2 (ANG2), FGF2, thrombopoietin (TPO), tumor necrosis factor alpha (TNFα), hepatocyte growth factor (HGF), and the tissue inhibitors of metalloproteinases (TIMP): TIMP1, and TIMP2 at day 14 of culture. This multiplex chemiluminescent ELISA was performed according to the manufacturer's instructions and provided values in pg/ml based on standard curves of samples provided with the kit.

EFFECT OF BMSC AND ASC CONDITIONED MEDIUM ON HUVEC OUTGROWTH AND ORGANIZATION

HUVEC spheroids were cultured in 48-hour BMSC or ASC conditioned medium collected at day 14 of culture, or in EGM +50ng/ml VEGF (control medium). Control HUVEC spheroids were cultured in control medium. Cultures were continued for 14 days and images were taken at 3, 7, 10 and 14 days of culture.

EFFECT OF HGF ON BMSC, ASC AND HUVEC OUTGROWTH

To examine the role of HGF on HUVEC outgrowth, HUVEC spheroids were exposed for 14 days to control medium, control medium supplemented with 14ng/ml HGF (an amount equal to the maximum amount of HGF measured in BMSC and ASC conditioned medium) or control medium supplemented with 100ng/ml HGF. The autocrine/ paracrine role of endogenous HGF on BMSC and ASC outgrowth was tested by culturing BMSC and ASC spheroids in control medium, and in control medium supplemented with 0.04 and 0.3µg/mL anti-HGF. The neutralization dose$_{50}$ (ND$_{50}$) for this antibody was

0.1–0.3µg/ml of the antibody in the presence of 100ng/ml of recombinant human HGF. Images of spheroids were taken at 3, 7, 10 and 14 days of culture.

STATISTICAL ANALYSIS

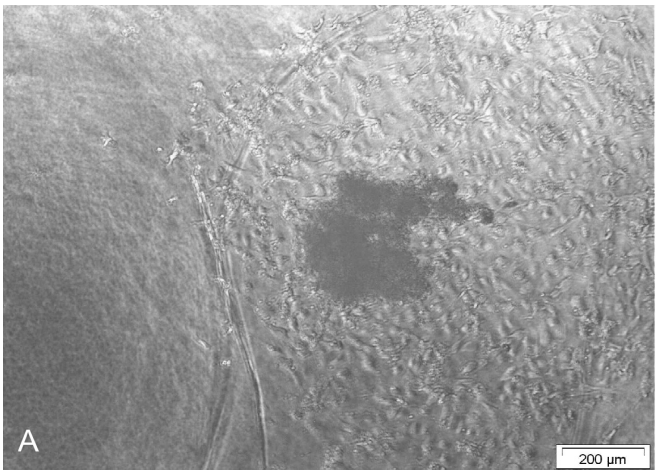
Statistical analysis was performed using Graphpad Prism 5.01 (Graphpad Software, San Diego, California). All values are expressed as mean ± standard deviation. The levels of secreted factors in BMSC conditioned medium, ASC conditioned medium, HUVEC conditioned medium or unconditioned medium were compared with a one-way analysis of variance (ANOVA) followed by a post hoc Tukey-Kramer test. The cellular strand length, width and density of BMSC, ASC, HUVEC/BMSC and HUVEC/ASC spheroids were compared with a Kruskall-Wallis test followed by post hoc Dunn's multiple comparison tests. Values with $P<0.05$ or less were considered significant.

RESULTS

HUVEC, IN CONTRAST TO BMSC AND ASC, DO NOT GROW OUT IN CELLULAR STRANDS TO FORM AN ORGANIZED NETWORK IN FIBRIN.

Upon 14 days of culture we observed in 100% HUVEC spheroids only very minor cellular outgrowth and organization. Occasionally, HUVEC did form small colonies in a monolayer around the spheroids (Fig.1A). In contrast, both the BMSC and ASC spheroids were capable of forming cellular strands already after 3 days of culture. These cellular strands organized into a complex branched interconnecting network over time (Fig.1B-C). Cellular strands emanating from the BMSC spheroids organized into a less dense network than the cellular strands emanating from the ASC spheroids [1.1 ± 0.12 cellular strands/mm^2 in BMSC spheroids versus 1.6 ± 0.1 cellular strands/mm^2 in ASC spheroids ($P<0.05$)] (Fig.1D-F). Contrary to this difference in strand density, strand length [863 µm ± 386 µm in BMSC spheroids versus 806 µm ± 326 µm in ASC spheroids] and width [8.8 µm ± 2.7 µm in BMSC spheroids versus 5.2 µm ± 1.8 µm in ASC spheroids] did not differ significantly between BMSC and ASC spheroids.

Before use in the spheroid culture, BMSC did not express CD34 whereas 6.0 ± 4.8% of ASC expressed CD34. Cellular networks subsequently growing out of the BMSC spheroids did not express the vascular marker CD34 (Fig.2A) whereas CD34 expression was found in the center of the ASC spheroids, as well as in the emanating cellular networks, especially at the periphery (Fig. 2B). Expanded BMSC and ASC expressed low levels of αSMA [BMSC 10% ± 17.2%; ASC 5.3% ± 4.7%] before spheroid culture. Surprisingly, expression of αSMA was abundantly found in the organized cellular networks as well as in the center of both BMSC- and ASC containing spheroids (Fig.3). The HUVEC, BMSC and ASC of different donors gave similar results regarding cellular organization.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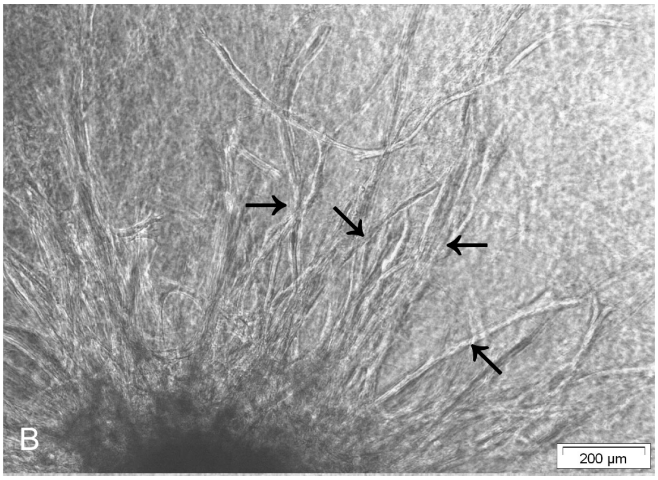
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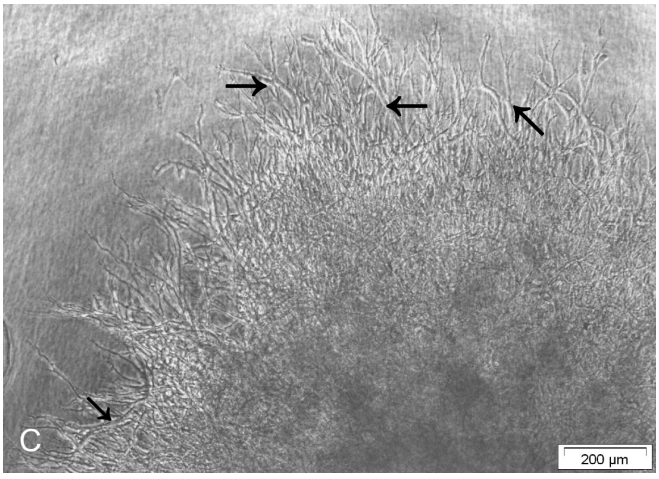
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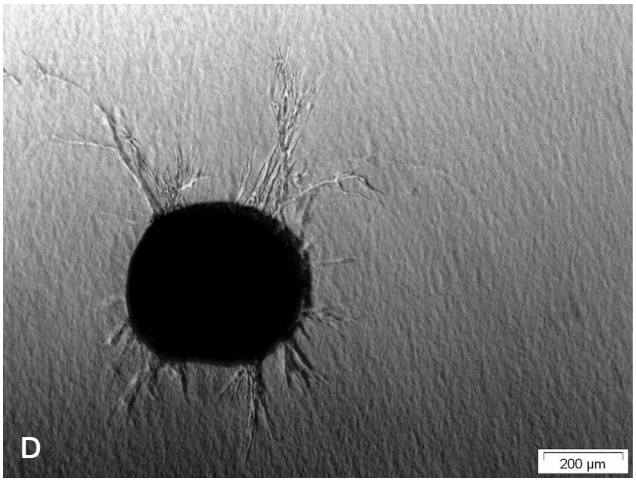
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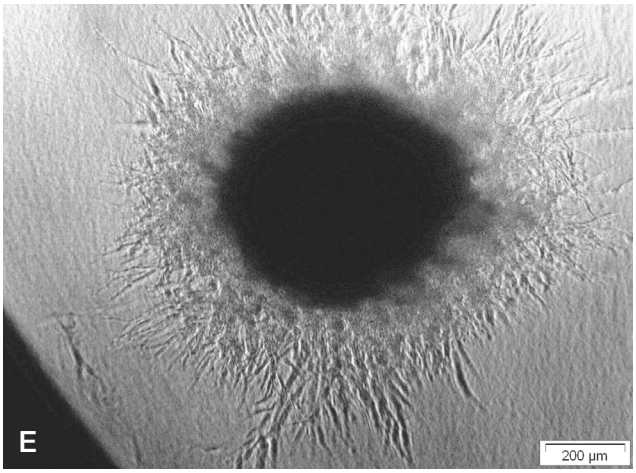
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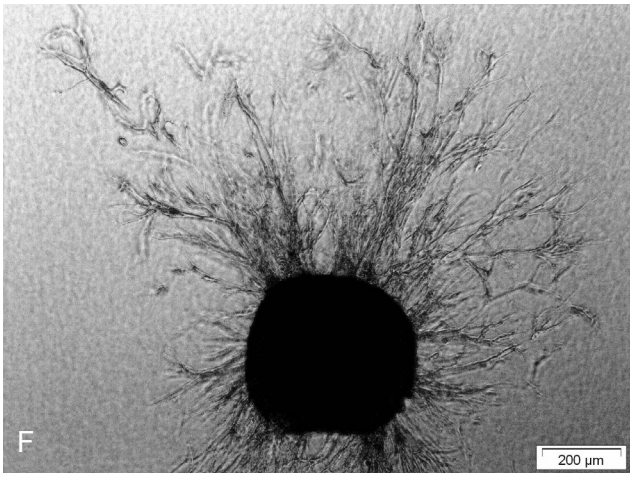
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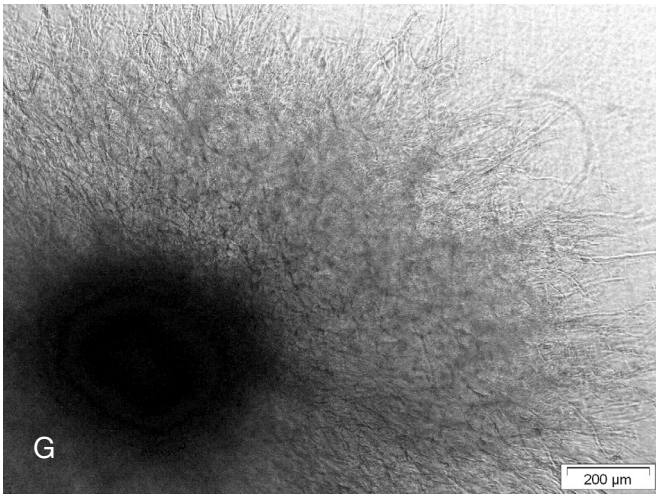


Figure 1. Bone marrow-derived stromal cell (BMSC) and adipose tissue-derived stromal cell (ASC) spheroids but not human umbilical vein endothelial cell (HUVEC) spheroids form an interconnected cellular network in fibrin. Images of 100% HUVEC, 100% BMSC and 100% ASC spheroids in fibrin cultured for 3, 7 or 14 days (magnification 40x). **(A)** One hundred percent HUVEC spheroid at day 14 of culture. HUVEC showed very minor organization. **(B)** BMSC and **(C)** ASC spheroids grow out in cellular strands resembling tubular structures (day 14 of culture, arrows indicate tube-like structures). **(D**, day 3; **F**, day 7**)** Cellular strands emanating from 100% BMSC spheroids organize into a less dense network than cellular strands emanating from 100% ASC spheroids **(E**, day 3; **G**, day 7**)**.

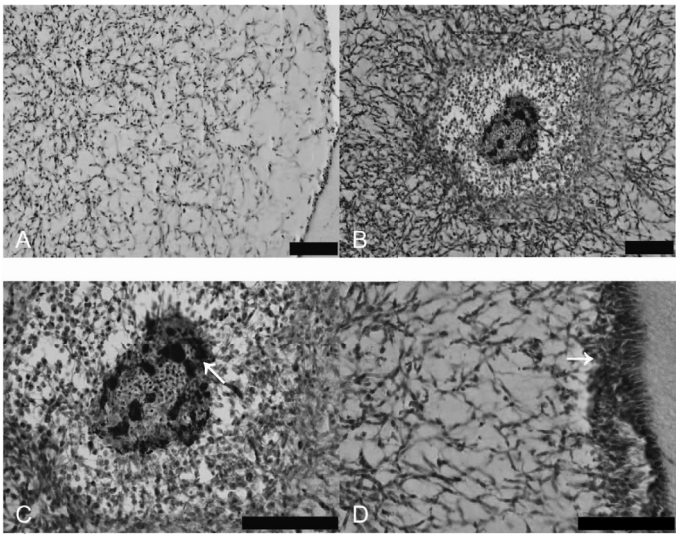


Figure 2. Cellular networks of ASC but not BMSC express CD34. One hundred percent BMSC and 100% ASC spheroids were cultured in fibrin in vitro for 14 days, and cross-sections were immunostained with anti-human CD34 antibody (brown). Scale bar = 200µm. **(A)** BMSC networks do not express CD34. **(B)** Cell clusters in the center of ASC spheroids, as well as ASC networks express CD34 (arrows indicate CD34 positive cell clusters in spheroid core and outer rim). (see color section for a full-color version)

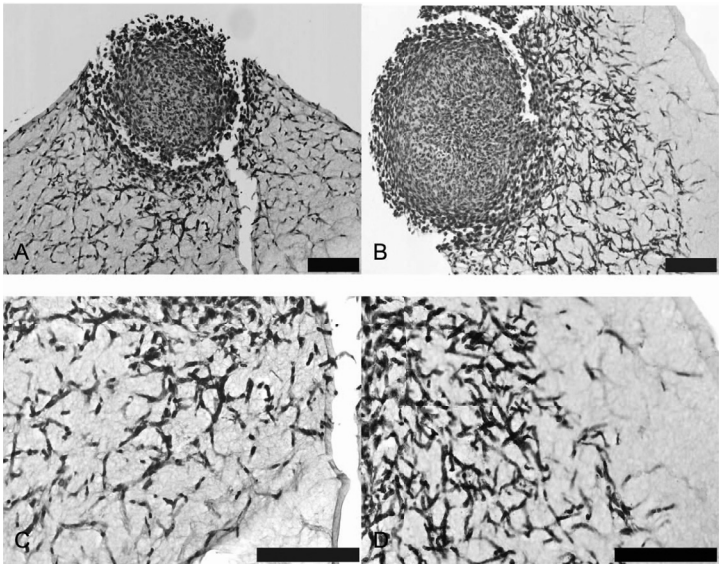


Figure 3. Cellular networks of ASC and BMSC both express α smooth muscle actin (αSMA). One hundred percent BMSC and 100% ASC spheroids were cultured in fibrin in vitro for 14 days, and cross-sections were immunostained with anti-human αSMA antibody (brown). Scale bar = 200μm. αSMA expression was found in the center of BMSC **(A)** and ASC spheroids **(B)** as well as in the cellular networks formed by BMSC **(C)** and ASC **(D)**. (see color section for a full-color version)

BMSC AND ASC CONDITIONED MEDIUM; ANGIOGENIC FACTORS AND ITS EFFECT ON HUVEC OUTGROWTH AND ORGANIZATION.

To further assess the ability of BMSC and ASC to support the formation of vessel-like structures, we analyzed the release of several angiogenic factors and TIMPs into the culture medium. Figure 4 illustrates the data from 9 different proteins assayed by multiplex chemiluminescent ELISA at day 14.

Of these, VEGF and FGF2 were added to the culture medium, as can be observed from the analysis of unconditioned medium. No significant differences in the secretion of the angiogenic factors ANG2, PDGFB, TNFα and TPO were observed between conditioned media of BMSC- and ASC spheroids when compared to unconditioned and HUVEC conditioned medium.

In contrast, TIMP1, TIMP2 and HGF were secreted in significant amounts when compared to unconditioned medium and HUVEC conditioned medium. There was no significant difference in the release of these factors between BMSC spheroids and ASC spheroids.

To examine whether BMSC/ASC conditioned medium or secreted HGF levels influenced HUVEC outgrowth, HUVEC spheroids were cultured on BMSC/ASC conditioned medium or medium supplemented with HGF. When cultured on BMSC conditioned medium or medium supplemented with HGF, no additional effect on HUVEC outgrowth

over the control spheroids was observed (Fig.5A-C). In contrast, when cultured on ASC conditioned medium, enhanced organization of HUVEC into cellular strands was observed over the controls (Fig. 5D). In addition, we examined whether blocking HGF in BMSC and ASC spheroid cultures inhibited ASC or BMSC outgrowth. Anti-HGF antibody did not inhibit the outgrowth of BMSC and ASC spheroids (Fig.5E-F).

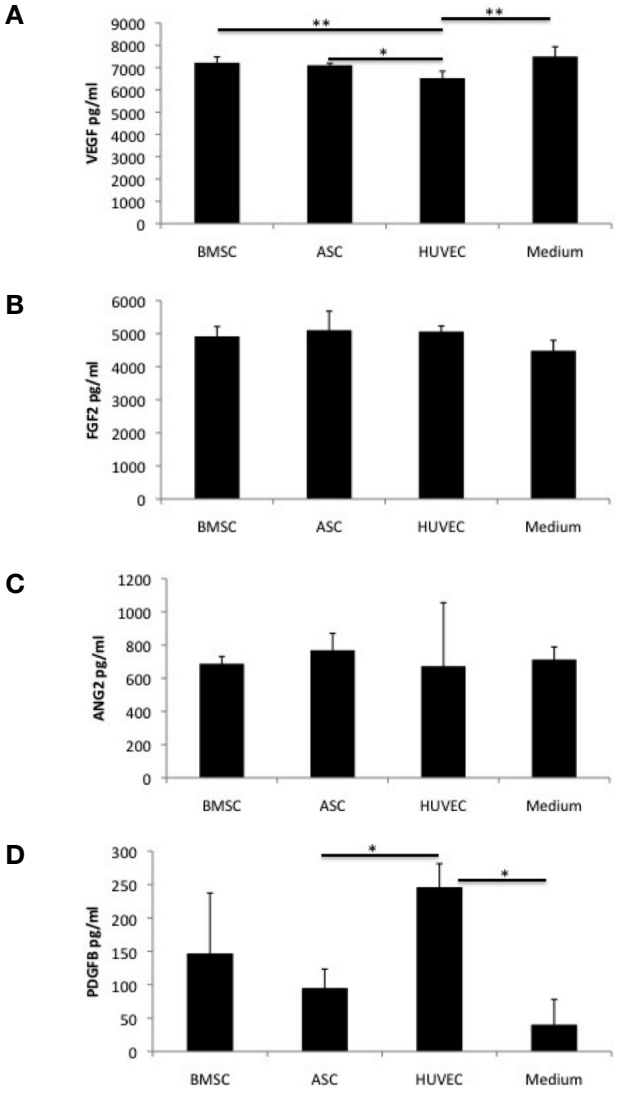
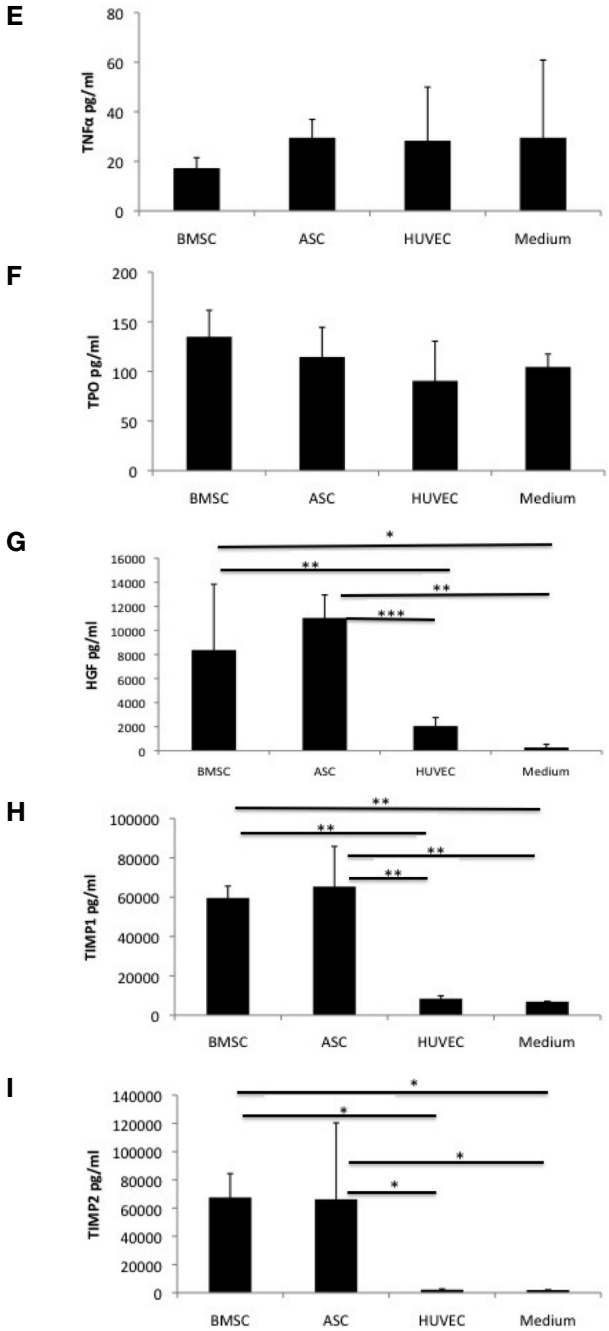


Figure 4. Release of pro- and anti-angiogenic factors by BMSC and ASC. The secretion of vascular endothelial growth factor (VEGF) **(A)**, fibroblast growth factor (FGF2) **(B)**, angiopoietin 2 (ANG2) **(C)**, platelet-derived growth factor B (PDGFB) **(D)**, tumor necrosis factor α (TNFα) **(E)**, thrombopoietin (TPO) **(F)**, hepatocyte growth factor (HGF) **(G)**, tissue inhibitor metalloproteinase 1 (TIMP)1 **(H)** and TIMP2 **(I)** by 100% BMSC, 100%ASC and 100%HUVEC spheroids over 48 hours was measured



by multiplex ELISA at day 14 of culture. The secretion of 100% BMSC- and 100% ASC spheroids were compared with HUVEC conditioned and unconditioned medium. Conditioned medium of three different HUVEC, BMSC and ASC donors were assayed in duplicate. Error bars represent standard error of the mean. Statistical comparisons were performed with the one way analysis of variance (ANOVA) followed by the Tukey-Kramer posttest. *P<0.05, **P<0.01, ***P<0.001.

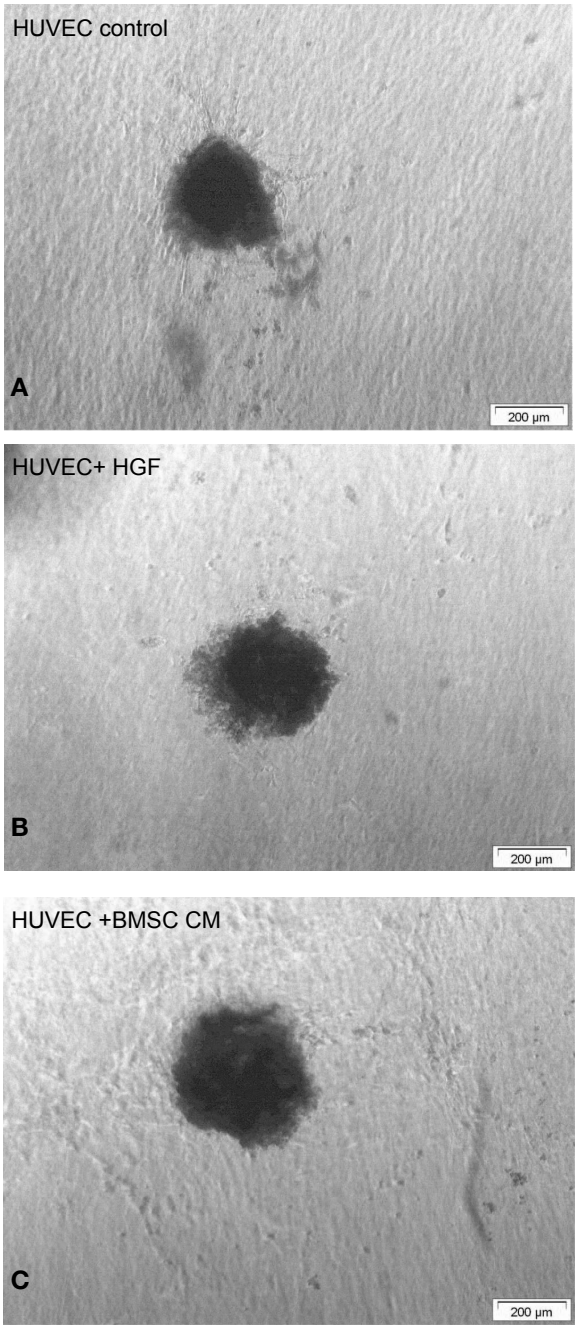
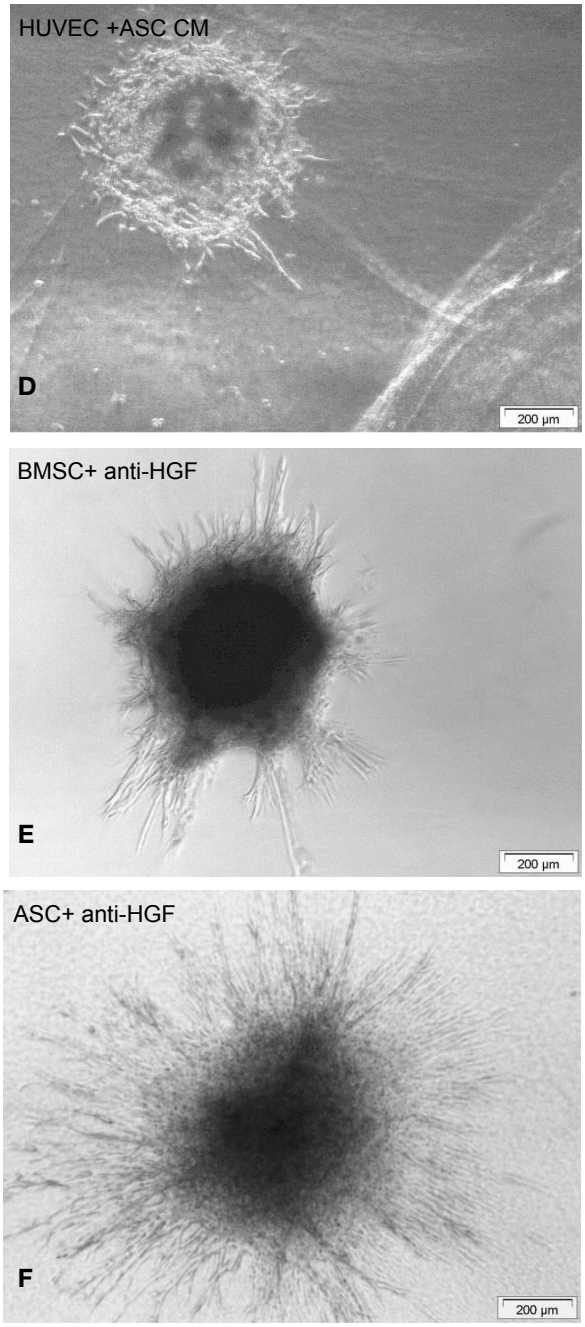


Figure 5. Culture of HUVEC spheroids on ASC conditioned medium but not BMSC conditioned medium or hepatocyte growth factor (HGF) supplemented medium affects HUVEC outgrowth. Images of HUVEC, BMSC or ASC spheroids in fibrin at 3 days of culture. Scale bar = 200µm. **(A)** HUVEC spheroid cultured in endothelial cell growth medium supplemented with 50ng/ml vascular endothelial growth factor (VEGF) (control medium). **(B)** HUVEC spheroid cultured in control medium supple-



mented with 100ng/ml HGF. **(C)** HUVEC spheroid cultured on 48-hour BMSC conditioned medium collected at day 14. **(D)** HUVEC spheroid cultured on 48-hour ASC conditioned medium collected at day 14. **(E)** BMSC spheroid cultured on control medium supplemented with anti-HGF antibody (0.3 µg/mL). **(F)** ASC spheroid cultured on control medium supplemented with anti-HGF antibody (0.3 µg/mL).

BMSC AND ASC SUPPORT HUVEC OUTGROWTH IN FIBRIN

Increasing percentages of BMSC or ASC (60%, 80% and 95%) were co-cultured with SPIO-labeled HUVEC in fibrin embedded spheroids, to assess their organization and collective 3D-assembly. By labeling HUVEC intracellularly with SPIO particles, we were able to track these specific cells within the pellets by performing iron stains on the sections. HUVEC organization was similar in both the HUVEC/BMSC and HUVEC/ASC spheroid co-cultures. After 14 days of co-culture, a large portion of the HUVEC was located in clusters of variable size in the periphery of the spheroid cores. Originating from these peripheral HUVEC clusters, spindle-shaped cells grew out in cellular strands and formed cord-like structures, integrated and surrounded by the interconnecting cellular network of ASC or BMSC. HUVEC outgrowth independent of ASC or BMSC was not observed in our cultures. In addition, we were unable to observe the formation of a lumen in the combined HUVEC/ASC or HUVEC/BMSC cell strands (Fig.6A-B). The outgrowing cellular strands in these cultures therefore resemble mostly prevascular structures.

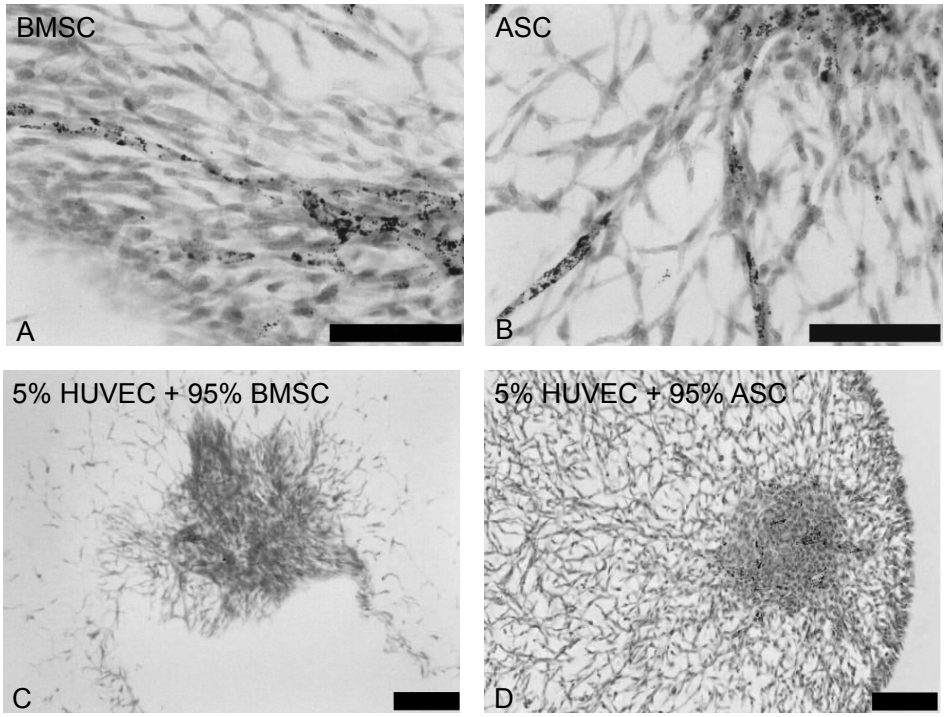
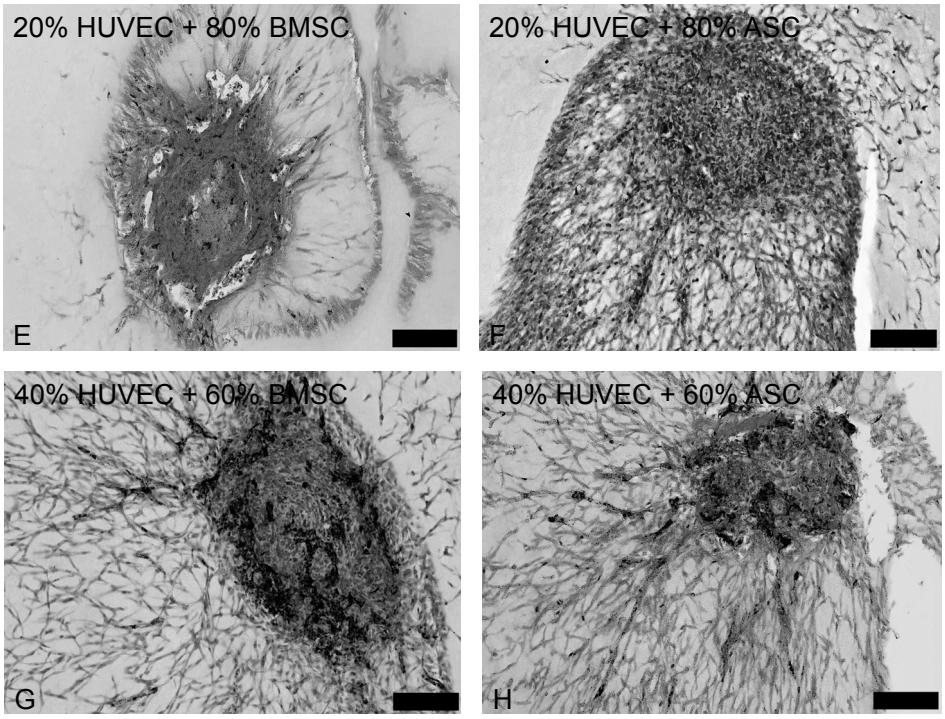


Figure 6. HUVEC organize into prevascular-like structures when co-cultured with BMSC and ASC. HUVEC/BMSC and HUVEC/ASC co-culture spheroids were cultured in fibrin in vitro for 14 days, and cross-sections were stained with Perl's iron stain to detect superparamagnetic iron oxide (SPIO) labeled HUVEC (blue). Nuclear fast red was used as a counter stain. Images at the left show HUVEC/BMSC co-culture spheroids. Images at the right show HUVEC/ASC spheroids. Scale bar = 200µm. **(A-B)** (see color section for a full-color version)



Blue stained prevascular-like structures from SPIO-labeled HUVEC alongside of BMSC or ASC networks. **(C-D)** Five percent HUVEC seeded. **(E-F)** 20% HUVEC seeded. **(G-H)** 40% HUVEC seeded.

Organization of HUVEC in clusters was independent of the amount of BMSC and ASC added to the co-culture spheroids, however, lower proportions of BMSC or ASC in the co-culture spheroids resulted in the formation of more HUVEC clusters in the core of the spheroids. Furthermore, outgrowing HUVEC structures were only observed in co-culture spheroids containing 80% or 60% of BMSC or ASC (Fig.6C-H). HUVEC structures in the co-culture spheroids expressed the vascular marker CD34 (Fig.7). In addition, αSMA was expressed by cells in the center and in the periphery as well as in the outgrowing cellular networks of the co-culture spheroids (Fig.8). Moreover, co-staining of αSMA and Perl's iron stain showed that outgrowing SPIO-labeled HUVEC structures were surrounded by αSMA-expressing cells (Fig.9).

The co-culture of 60% BMSC or 60% ASC with HUVEC affected the organization of the outgrowing cellular strands. Compared to the 100% BMSC and 100% ASC spheroids, cellular strands emanating from 40% HUVEC/60%BMSC and 40%HUVEC/60%ASC co-culture spheroids organized into a denser network [3.2 ± 0.9 cellular strands/mm^2 in 40%HUVEC/60%BMSC versus 2.1 ± 1.0 cellular strands/ mm^2 in 100% BMSC ($P<0.05$); 4.1 ± 0.8 cellular strands/ mm^2 in 40%HUVEC/60%ASC versus 3.4 ± 0.4 cellular strands/mm^2 in 100% ASC ($P<0.05$)]. Little change in the organization of the cellular strands was observed in co-culture spheroids containing 95%

or 80% BMSC or ASC [2.3 ± 0.9 and 2.6 ± 0.6 cellular strands/mm^2 in 5% HUVEC/95% BMSC and 20% HUVEC/80% BMSC versus 2.1 ± 1.0 cellular strands/mm^2 in 100% BMSC; 3.5 ± 0.34 and 3.2 ± 0.6 cellular strands/mm^2 in 5% HUVEC/95% ASC and 20% HUVEC/80% ASC versus 3.4 ± 0.4 cellular strands/mm^2 in 100% ASC]. (Fig.10).

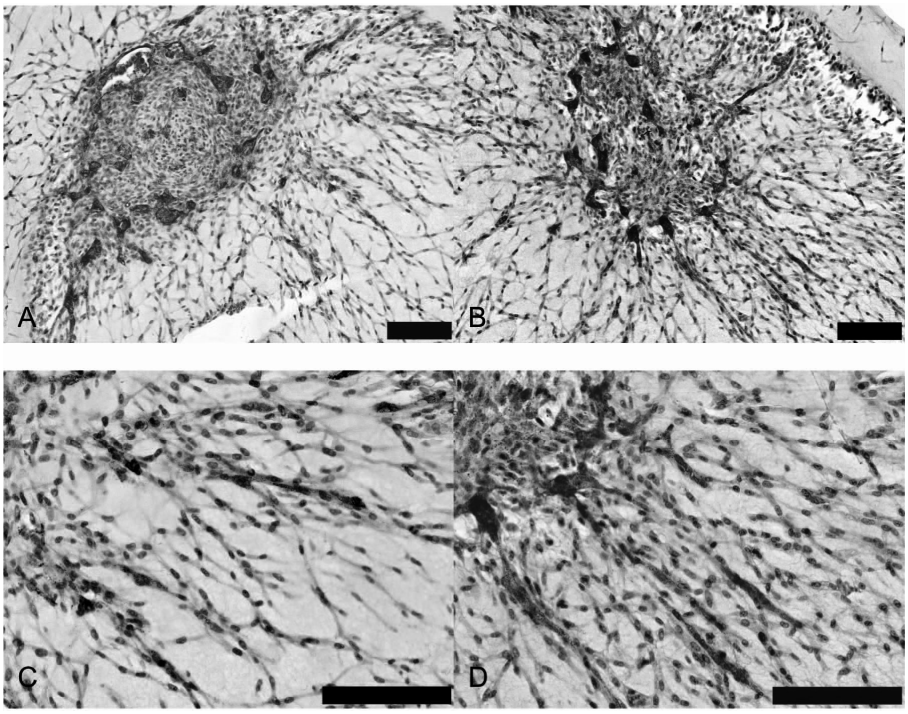


Figure 7. Prevascular-like structures in HUVEC/BMSC and HUVEC/ASC co-culture spheroids express CD34. HUVEC/BMSC and HUVEC/ASC co-culture spheroids were cultured in fibrin in vitro for 14 days, and cross-sections were immunostained with anti-human CD34 antibody (brown). Scale bar = 200μm. CD34 positive vascular-like structures in HUVEC/BMSC **(A, C)** and HUVEC/ASC spheroids **(B, D)**. (see color section for a full-color version)

DISCUSSION

The results of our study suggest that ASC similar to BMSC support the formation of prevascular-like structures by HUVEC in vitro. This is based on our finding that in vitro cultured spheroids with 100% HUVEC did not form any organized structures, while co-culture spheroids of HUVEC with ASC or BMSC did form elongated cord-like structures in fibrin. In addition, our study showed that BMSC and ASC displayed several proneovascular features: both the BMSC spheroids and the ASC spheroids generated a branched interconnected cellular network that abundantly expressed αSMA and in the case of ASC spheroids also CD34.

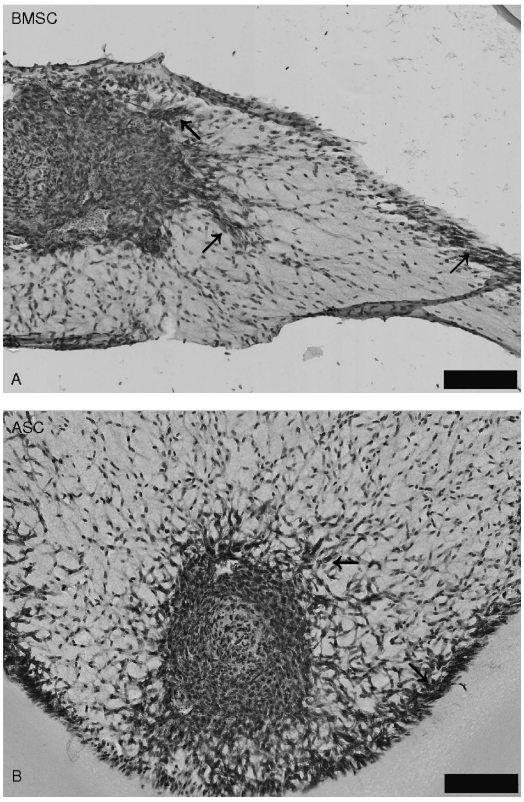


Figure 8. αSMA is expressed in both HUVEC/BMSC and HUVEC/ASC co-culture spheroids. Forty percent HUVEC/ 60%BMSC and 40% HUVEC/ 60% ASC co-culture spheroids were cultured in fibrin in vitro for 14 days, and cross-sections were immunostained with anti-human αSMA antibody (brown). Scale bar = 200μm. Similar to 100% BMSC and 100% ASC spheroids (Figure 3), αSMA was expressed by cells in the center and in the periphery as well as in the outgrowing cellular networks of the HUVEC/BMSC **(A)** and HUVEC/ASC **(B)** co-culture spheroids (arrows indicate outgrowing aSMA-positive cells). (see color section for a full-color version)

Our finding that only low levels of αSMA were expressed by ASC and BMSC prior to use in the spheroid cultures suggests an affiliation of BMSC and ASC to the smooth muscle lineage under our culture conditions. Moreover, these αSMA-expressing cells surrounded the endothelial cord-like structures in the co-culture spheroids, suggesting a stabilizing role for the BMSC and ASC in this setting. To investigate the mechanism by which the BMSC and ASC support the formation of endothelial outgrowth in our cultures in more detail, we investigated the secretion of several factors known to influence angiogenesis. We found significantly higher amounts of HGF, TIMP1 and TIMP2 in the conditioned medium of BMSC and ASC spheroid cultures when compared to HUVEC conditioned medium or unconditioned medium. In contrast, the levels of ANG2, TPO, PDGFB and TNFα in BMSC and ASC conditioned medium did not significantly differ from HUVEC conditioned or unconditioned medium.

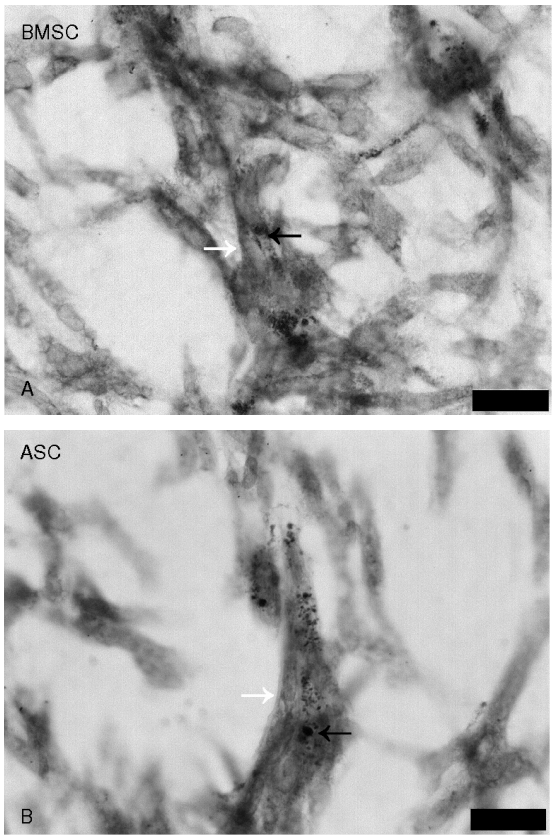


Figure 9. αSMA expressing BMSC and ASC surround outgrowing HUVEC strands in the co-culture spheroids. Forty percent HUVEC/ 60%BMSC and 40% HUVEC/ 60% ASC co-culture spheroids were cultured in fibrin in vitro for 14 days, and cross-sections were co-stained with anti-human αSMA antibody (brown) and Perl's iron stain (blue) to identify simultaneously αSMA-positive cells and SPIO-labeled HUVEC. Scale bar = 20μm. **(A)** Blue stained SPIO-labeled HUVEC (see black arrow) are surrounded by brown stained αSMA expressing BMSC (see white arrow). **(B)** Blue stained SPIO-labeled HUVEC (see black arrow) that co-localize with brown stained αSMA-expressing ASC (see white arrow). (see color section for a full-color version)

Secretion of these factors, however, could be significant but too little to be discriminated from the levels already present in the unconditioned medium. HGF, TIMP1 and TIMP2 are known to be involved in new vessel formation: HGF stimulates endothelial cell proliferation and induces angiogenesis [31], whereas TIMPs are known to inhibit the activities of matrix metalloproteinases (MMPs) [32,33]. Although HGF is known to be an important angiogenic factor, experiments using varying concentrations of HGF to stimulate outgrowth in HUVEC spheroids showed that HGF on its own was not able to initiate outgrowth and organization of HUVEC in this system. Given the elevated levels of TIMPs in the medium, it would have been interesting to also analyze the activity of MMPs in our cultures by zymography. However, MMPs are abundantly present in

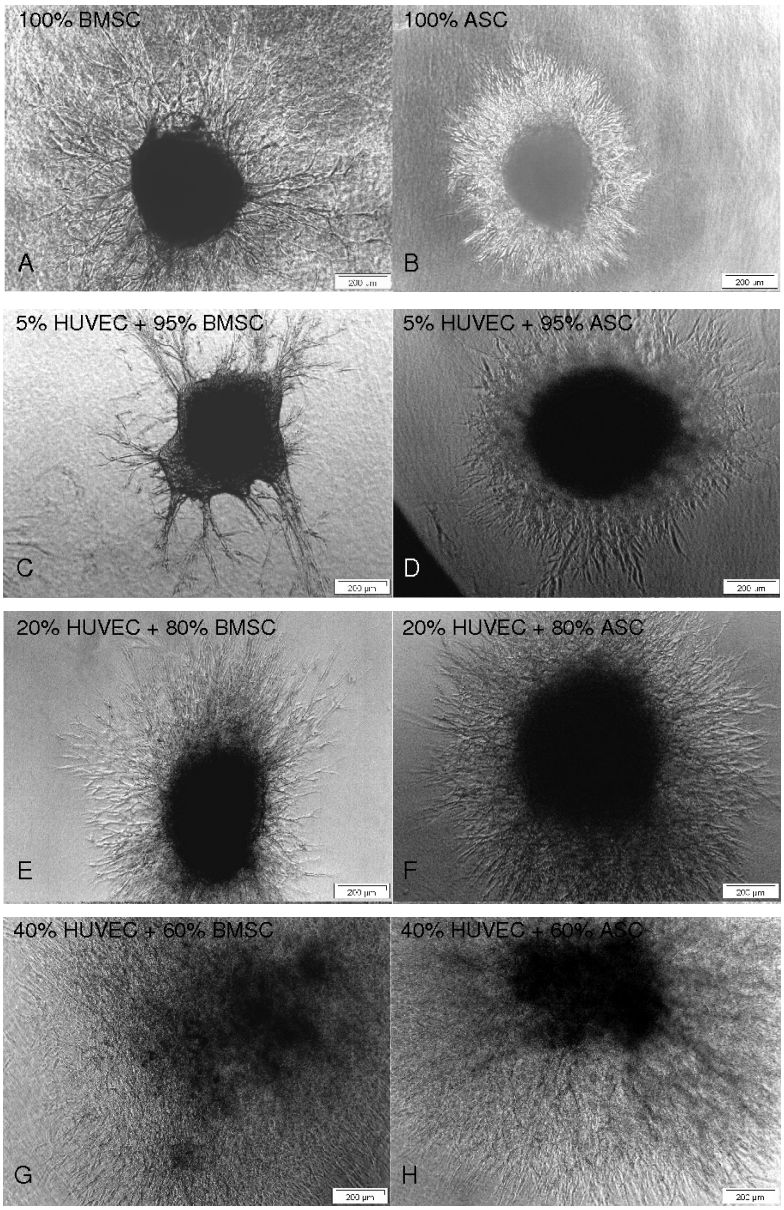


Figure 10. Co-culture of 60% BMSC or ASC with HUVEC affect cellular outgrowth. Images of (co-) culture spheroids in fibrin at 3 days of culture. Scale bar = 200µm. Images at the left show spheroids containing BMSC. Images at the right show spheroids containing ASC. **(A)** 100% BMSC **(B)** 100% ASC **(C)** 5% HUVEC + 95% BMSC **(D)** 5% HUVEC + 95% ASC **(E)** 20% HUVEC + 80% BMSC **(F)** 20% HUVEC +80% ASC **(G)** 40% HUVEC + 60% BMSC **(H)** 40% HUVEC + 60% ASC. Cellular strands emanating from HUVEC/BMSC or HUVEC/ASC co-culture spheroids, containing 60% BMSC or 60% ASC, organized into a denser network when compared to 100% BMSC or 100% ASC spheroids. No significant change in network density was measured in HUVEC/BMSC and HUVEC/ASC spheroids containing 95% and 80% BMSC or ASC.

serum and given the high levels of serum in our medium, this would have obscured measuring the secreted MMPs specifically by the stromal cells.

Despite the high TIMP levels however, we observed removal of much of the fibrin within the network (as observed histologically, data not shown), suggesting that enough endoproteases (MMPs or other factors such as tissue plasminogen activator) were secreted to facilitate fibrin degradation and subsequent cell migration.

The secretion of angiogenic factors by BMSC and ASC in vitro has been reported before [4,5]. To our knowledge however, this is the first study in which both the angiogenic factor secretion by BMSC and ASC and their interaction with HUVEC can be compared in a 3-D setting in vitro. Although we did not find significant differences in the secretion of the measured angiogenic factors between BMSC and ASC, ASC conditioned medium did have a positive effect on HUVEC outgrowth and organization (Figure 5D) while no such effect was observed for HUVEC spheroids cultured on BMSC conditioned medium (Figure 5C). These results suggest that other, yet uncharacterized trophic factors or metabolites were secreted by ASC.

Despite this difference in the effect of ASC and BMSC conditioned medium on HUVEC outgrowth and organization, BMSC and ASC both supported HUVEC outgrowth in the direct co-culture, indicating that HUVEC outgrowth, particularly in the BMSC co-culture spheroids, is not merely achieved by the secretion of growth factors. The exact mechanism by which BMSC and ASC support HUVEC outgrowth in this system remains unknown. However, since both BMSC and ASC formed integrated cellular networks with the outgrowing HUVEC, we hypothesize that direct cell-cell contacts and reciprocal signaling play an important role.

The outgrowth and organization of HUVEC into prevascular-like structures in HUVEC/BMSC and HUVEC/ASC spheroid co-cultures was seen in samples that contained 80% or 60% stromal cells. No organized endothelial structures were observed in 5% HUVEC/95% stromal cell co-cultures. In contrast, Rouwkema et al.[19] reported that spheroid co-cultures of BMSC and HUVEC containing 98% BMSC or more, promoted the formation organized endothelial structures. It is uncertain whether this difference is due to the use of a higher total cell number (500,000 cells instead of 20,000 cells), a different method of spheroid formation (centrifugation instead of the hanging droplet method) or the use of osteogenic culture medium. All these factors, however, might affect the interaction of the HUVEC with the stromal cells. For example, spheroids obtained by centrifugation of cells, are more densely packed when compared to those obtained by the hanging droplet method. This, in combination with a higher total cell number, may create a more hypoxic environment in the core of the spheroids, hereby affecting HUVEC-stromal cell interactions.

Mimicking the complete physiological process of vessel formation in vitro for tissue engineering purposes still poses a future challenge. For example, not all HUVEC in our

co-culture grew out and formed cellular strands. In addition, the outgrowing HUVEC structures in our cultures did not yet include a lumen. Possible reasons for this could be an insufficient culture time, and/or suboptimal interaction between the stromal cells and the endothelial cells. The signals required for an optimal interaction between stromal cells and endothelial cells are mostly unknown, but likely include specific cell-cell contacts as well as paracrine- (direct angiogenic factors and indirect angiogenic factors such as HIF-1alpha), mechanical- (shear stress) and physical (oxygen tension) stimuli. In this light, several strategies might be considered to improve the supportive role of stromal cells in the formation of organized endothelial structures in vitro. For example, the selected culture medium in combination with the supplementary growth factors most certainly plays an essential role in vascular network formation of HUVEC-stromal cell co-cultures in vitro. In this study, we used endothelial growth medium supplemented with VEGF, a medium that stimulates endothelial cell growth and sprouting. Although endothelial proliferation is an important step in new vessel formation, endothelial cell differentiation, organization and stabilization are at least equally important. Moreover, the culture medium should also support the tissue that actually is being constructed. Finding a culture medium that supports tissue-specific needs and enables BMSC and ASC to provide the critical support for endothelial differentiation, organization and stabilization should therefore be a matter of further investigation. Likewise, in vivo, tissue-hypoxia is a potent stimulus triggering expansion of the nascent vessel network via upregulation of many angiogenic factors through hypoxia inducible factors [34]. In addition, culture of BMSC and ASC under hypoxic conditions in vitro has shown to augment the expression of a wide array of angiogenic factors [4,35,36,37]. Exposure of stromal cell-HUVEC co-cultures to hypoxic conditions may therefore possibly induce the appropriate angiogenic milieu, promoting the formation of more mature vascular-like structures.

CONCLUSION

In summary, our results suggest that ASC, similar to BMSC, can support the formation of prevascular-like structures in vitro partly through the release of trophic factors but also by the formation of direct cell-cell contacts. Thus, ASC may be an attractive alternative source of cells for the use in complex tissue engineering applications.

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CHAPTER 5

Prevascular structures promote vascularization in engineered human adipose tissue constructs upon implantation

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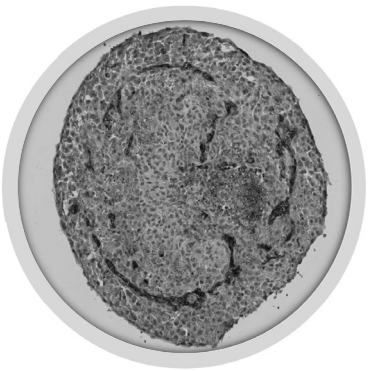


ABSTRACT

Vascularization is still one of the most important limitations for the survival of engineered tissues after implantation. In this study, we aim to improve the in vivo vascularization of engineered adipose tissue by pre-forming vascular structures within in vitro-engineered adipose tissue constructs that can integrate with the host vascular system upon implantation. Different cell culture media were tested and different amounts of human adipose tissue-derived mesenchymal stromal cells (ASC) and human umbilical vein endothelial cells (HUVEC) were combined in spheroid co-cultures to obtain optimal conditions for the generation of prevascularized adipose tissue constructs. Immunohistochemistry revealed that prevascular structures were formed in the constructs only when 20% ASC and 80% HUVEC were combined and cultured in a 1:1 mixture of endothelial cell medium and adipogenic medium. Moreover, the ASC in these constructs accumulated lipid and expressed the adipocyte-specific gene fatty acid binding protein-4. Implantation of prevascularized ASC/HUVEC constructs in nude mice resulted in a significantly higher amount of vessels (37±17 vessels/mm^2) within the constructs compared to non-prevascularized constructs composed only of ASC (3± 4 vessels/mm^2). Moreover, a subset of the pre-formed human vascular structures (3.6±4.2 structures/mm^2) anastomosed with the mouse vasculature as indicated by the presence of intravascular red blood cells. Our results indicate that pre-formed vascular structures within in vitro-engineered adipose tissue constructs can integrate with the host vascular system and improve the vascularization upon implantation.

INTRODUCTION

Despite various levels of clinical success, currently used surgical procedures to repair soft tissue defects are not optimal. Autologous adipose tissue grafts suffer from donor-site morbidity [1] and have a tendency to lose volume over time [2,3,4]. Synthetic implants also have significant drawbacks such as infection, rejection, leakage and/or dislocation [4].

The in vitro development of autologous adipose tissue substitutes using human adipose tissue-derived mesenchymal stromal cells (ASC) may offer a future alternative to these current treatments [5,6,7,8,9]. However, limited survival of in vitro-engineered adipose tissue after in vivo implantation is still a major hurdle for clinical use [1,10]. The reason for this limited survival is that ingrowth of blood vessels from the host into the engineered adipose tissue construct can take several days [11], which is too slow and fatal for many of the cells in the construct. A sufficiently rapid in vivo vascularization, preventing cell-death in the core of the implanted engineered adipose tissue constructs, has not been realized yet.

We and others have shown that endothelial cells (EC) co-cultured with stromal cells derived from bone marrow, skin, cardiac muscle, skeletal muscle or adipose tissue can form primitive vascular-like structures in vitro [12,13,14,15,16,17,18,19,20]. Few studies also indicate that such in vitro preformed vascular-like structures (or prevascular structures) are able to connect to the host vessels after implantation [17,18,19,21] . The ability of prevascular structures to promote the in vivo vascularization of engineered adipose tissue constructs, however, has not been tested yet.

In this study, we generate prevascularized adipose tissue constructs using three-dimensional (3D) spheroids (about 0.6 mm in diameter) containing co-cultured ASC and endothelial cells (EC) and test this hypothesis. Specifically, we investigated the following questions: (1) can we induce in vitro prevascularization of the 3D spheroid HUVEC/ASC constructs while simultaneously enabling adipogenic differentiation of the ASC? (2) Can the in vitro generated prevascular structures integrate with the host vasculature?

MATERIALS AND METHODS

CELL CULTURE

Subcutaneous abdominal adipose tissue was obtained from donors with approval of the Medical Ethical Committee (# MEC-2005-157). ASC from 3 different donors of 47, 54 and 59 years of age were isolated from the adipose tissue as previously described [22] and grown on basal medium (Dulbecco's Modified Eagle Medium 1g/l glucose

(Invitrogen, Carlsbad, California), 10% FCS (PAA Laboratories, Pasching, Austria), 10^{-12} M dexamethasone, 10^{-5} M ascorbic acid (both from Sigma-Aldrich, St. Louis, Missouri), 1% penicillin/streptomycin, 0.5% gentamycin (both from Invitrogen)).

Commercially derived pooled human umbilical vein endothelial cells (HUVEC) (Lonza, Verviers, Belgium) were cultured in endothelial growth medium consisting of human endothelial serum-free medium (Invitrogen), supplemented with 20% FCS, 10% human serum (PAA Laboratories), 20ng/ml fibroblast growth factor 2 (FGF2) and 100ng/ml epidermal growth factor (EGF) (both from Peprotech EC, London, United Kingdom). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2, and the medium was replenished every 3 days. Passage 2 ASC and passage 5 or less HUVEC were used in this study.

MEDIUM SELECTION: PROLIFERATION OF ASC AND HUVEC IN DIFFERENT MEDIA

The proliferation of ASC and HUVEC in different media was tested in 6-well (ASC) and 24-well (HUVEC) plates (Costar®, Corning Inc., Corning, New York) seeded with 1042 cells/cm^2 and 5000 cells/cm^2, respectively. ASC and HUVEC were seeded onto the culture plates in basal medium and endothelial growth medium respectively and incubated overnight at 37ºC in a humid atmosphere with 5% CO_2 to ensure attachment of the cells. The next day, these media were removed and the cells were washed with PBS and replenished with 3 different test media: *(1)* endothelial cell medium consisting of endothelial-serum free medium supplemented with 5% FCS, 20ng/ml FGF-2 and 100ng/ml EGF; *(2)* adipogenic medium consisting of Dulbecco's modified Eagle Medium 4.5g/l glucose (Invitrogen), 10% FCS, 1µM dexamethasone, 0.01 mg/ml insulin (Eli Lilly, Houten, The Netherlands) 0.2 mM indomethacin (Sigma-Aldrich), 0.5mM 3-isobutyl-I-methyl-xanthine (Sigma-Aldrich), 1% penicillin/streptomycin and 0.5% gentamycin; *(3)* 1:1 mixture of endothelial cell medium and adipogenic medium (endothelial/adipogenic mix medium). The culture media were refreshed every 3 days and cell numbers of duplicate wells were counted twice with a Casy® cell counter (Innovatis, Bielefeld, Germany) at 0, 3, 7 and 10 days of culture.

FORMATION OF SPHEROIDS

Spheroids containing only ASC or ASC with different amounts of HUVEC (5% HUVEC/95% ASC, 20% HUVEC /80% ASC, 40% HUVEC /60% ASC and 80% HUVEC /20% ASC) were prepared in 10 ml polypropylene tubes (Techno Plastic Products, Trasadingen, Switzerland) in 0.5 ml endothelial cell medium. ASC or HUVEC/ASC suspensions containing a total of $2.5*10^5$ cells were added to the tubes and subsequently centrifuged at 150 x g for 5 minutes. The tubes with the resulting cell pellets were then incubated overnight at 37ºC with 5% CO_2 in a humidified atmosphere to allow

the formation of spheroids. After overnight incubation, the endothelial cell medium of the ASC/HUVEC co-culture spheroids was replaced by endothelial/adipogenic mix medium and in ASC spheroids by endothelial/adipogenic mix medium or adipogenic medium. Medium was replenished every 2 days.

FLOW CYTOMETRIC ANALYSIS OF CO-CULTURE SPHEROIDS

Following 2, 7 and 14 days of culture, HUVEC/ ASC co-culture spheroids were harvested for flow cytometric analysis. To obtain a single cell suspension, spheroids were washed twice with PBS and digested in a sterile-filtered collagenase type I solution (1000 units/ml in PBS) for 20 minutes at 37˚C. After digestion, cell suspensions were washed with FACS buffer (Hanks' Balanced Salt Solution (Invitrogen), 2% BSA, 0.05% sodium azide (both from Sigma-Aldrich) and 2% heat-inactivated human serum (PAA Laboratories)) and labeled for 30 minutes on ice in the dark with mouse anti-human CD31 fluorescently labeled antibody (dilution 1:1 in FACS buffer, BD Biosciences Pharmingen (cat no.555446), Erembodegem, Belgium). Cells were washed again, resuspended in 300µl of FACS buffer and directly analyzed on a FACS-Calibur instrument (Becton-Dickinson Biosciences, Franklin Lakes, New Jersey).

RNA ISOLATION, COMPLEMENTARY DNA (CDNA) SYNTHESIS AND QUANTITATIVE POLYMERASE CHAIN REACTION (Q-PCR)

ASC spheroids were cultured in endothelial/adipogenic mix medium or adipogenic medium for 7 days in vitro. Six spheroids were prepared from each ASC donor, and 3 pools of 2 spheroids from each donor were used for RNA isolation. The spheroids were fragmented with a pestle on ice and sheared using an insulin syringe.

Total RNA was extracted from ASC spheroids using Qiazol Lysis Reagent (Qiagen, Venlo, The Netherlands). RNA was further purified using the RNeasy Micro Kit (Qiagen) with on-column DNA-digestion. Total RNA was quantified using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware) according to manufacturer's instructions and 250 ng RNA was reverse transcribed into cDNA using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany).

The mRNA levels of the adipogenic marker fatty acid binding protein 4 (FABP4), were analyzed using the Taqman®Gene Expression Assay for FABP4 (Hs00609791_m1) (ABI) according to the manufacturer's instructions. Beta-2-microglobulin (B2M) mRNA levels were analyzed for normalization with the Q-PCR MasterMix Plus for SYBR® Green I dTTP (Eurogentec, San Diego, California) and the following, gene-specific primers set: Fw 5'-TGCTCGCGCTACTCTCTCTTT -3', Rv 5'-TCTGCTGGATGACGTGAGTAAAC-3'). Q-PCR was performed with an ABI PRISM® 7000 Sequence Detection System and analyzed using 7000 System SDS software (ABI, Foster City, California).

IN VIVO IMPLANTATION

Four spheroids consisting of ASC and four spheroids consisting of 80% HUVEC /20% ASC were created from each of the three donors, resulting in a total of twelve ASC spheroids and twelve 80% HUVEC /20% ASC spheroids. After 7 days of in vitro culture, the spheroids were implanted subcutaneously in the left and right scapular area of six nine-weeks-old athymic male nude mice (NMRI-nu/nu, Taconic, Hudson, New York). The mice were placed under general anesthesia with 2.5% isoflurane after which two separate 0.5 cm incisions were made through the dorsal skin. Next, two separate subcutaneous pockets were prepared by blunt dissection of the subcutaneous tissue. One pocket was filled with two ASC spheroids and the other pocket was filled with two 80% HUVEC/20% ASC spheroids. Pockets were closed with discontinuous sutures using Mersilk 5-0 (Ethicon, Somerville, New Jersey). The content of each pocket was regarded as one construct.

Seven days after implantation, the mice were sacrificed and the constructs retrieved. All procedures were approved by the animal ethical committee (EUR. 1292).

HISTOLOGY

Constructs harvested 7 days after culture or 7 days after implantation were fixed in 10% formalin in PBS and embedded in paraffin, or directly embedded in Tissue-Tek (Sakura, Finetek Europe, Zoeterwoude, The Netherlands) and snap frozen.

Paraffin-embedded sections (5 µm) were deparaffinized and rehydrated. Cryosections (5 µm) were fixed with 3.7% formalin in deionised water for 1 hour.

CD31 STAINING OF IN VITRO CULTURED SPHEROIDS

To determine HUVEC organization in spheroid cultures, a monoclonal mouse anti-human CD31 antibody (Clone JC70A, Dako (cat no. M0823), Glostrup, Denmark) was used. Antigen retrieval was achieved by heating at 95˚C for 15 minutes in Dako Cytomation Target Retrieval Solution high pH. After overnight incubation with CD31 antibody (1:40 dilution in PBS /1% BSA) at 4˚C, a secondary biotin-conjugated goat anti-mouse antibody (1:200 dilution in PBS/1% BSA, Dako (cat no. E0433)) was used for 30 minutes at room temperature followed by incubation with streptavidin-horseradish peroxidase (1:300 dilution in PBS/1% BSA, Dako (cat no. P0397)) for 30 minutes. Diaminobenzidine (Sigma-Aldrich) was used to visualize CD31 expression. The slides were weakly counterstained with hematoxylin, dehydrated through graded alcohols and mounted with Permount (VWR International B.V., Amsterdam, The Netherlands).

CD31 AND VIMENTIN STAINING IN SPHEROID CONSTRUCTS POST-IMPLANTATION

To distinguish human tissue from mouse tissue and to determine the number and relative area occupied by human vascular structures in construct sections post-implantation,

monoclonal mouse anti-human vimentin antibody (Clone V9, 1:40 dilution in PBS/ 1% BSA, Sigma-Aldrich (cat no. V6630)) and monoclonal mouse anti-human CD31 antibody (Dako) were used, respectively. To reduce unspecific binding of the secondary goat anti-mouse antibody (Dako) to mouse IgGs, the mouse-on mouse HRP-Polymer Kit (Biocare Medical, Concord, California) was used, according to manufacturer's instructions with some slight modifications. In short, antigen retrieval was performed through incubation in Rodent Decloaker® (Biocare Medical) for 60 minutes at 95˚C. Non-specific binding sites were blocked with Rodent Block M® (Biocare Medical) and sections were stained overnight with CD31 or vimentin at 4˚C. The MM-polymer-HRP® secondary antibody (Biocare Medical) was used, followed by incubation in diaminobenzidine (Sigma-Aldrich) to visualize CD31 or vimentin expression, respectively. The slides were weakly counterstained with hematoxylin, dehydrated through graded alcohols and mounted with Permount (VWR International B.V., Amsterdam, The Netherlands).

OIL RED O STAINING

Accumulated lipid in ASC in spheroids was detected after 7 days of in vitro culture and after 7 days of implantation. In short, a 0.5% (w/v) stock solution of Oil Red O in triethyl-phosphate (Sigma-Aldrich) was diluted 3:2 with deionised water to prepare an Oil Red O working solution. Subsequently, cryo-sections were fixed, rinsed and immersed in Oil Red O working solution for 30 minutes. Hereafter, sections were washed with deionised water and counterstained with hematoxylin for 1 minute. Finally, sections were rinsed with running tap water for 10 minutes, and covered with Imsolmount (Klinipath, Zevenaar, The Netherlands).

HISTOMORPHOMETRY

High resolution (0.23 µm/pixel), low magnification (40×) digital micrographs covering 2-to-3 complete vimentin/CD31 immunostained cross-sections of each construct were made with a Nanozoomer HT (Hamamatsu Photonics, Hamamatsu City, Japan) for analysis. Subsequently, NIH ImageJ software (http://rsb.info.nih.gov/ij/) was used to count the number of CD31 positive prevascular structures, the area occupied by these structures and the complete area of human tissue in the constructs.

STATISTICAL ANALYSIS

All data are expressed as mean ± standard deviation, except a part of the in vivo vascularization data, which are expressed as median (IQR). The FACS data were analyzed with the mixed model analysis of variance using SAS software (SAS Institute, Cary, North Carolina). The FABP4 expression data were analyzed with the unpaired t test with Welch correction using Graphpad Prism 5.01 software (Graphpad Software,

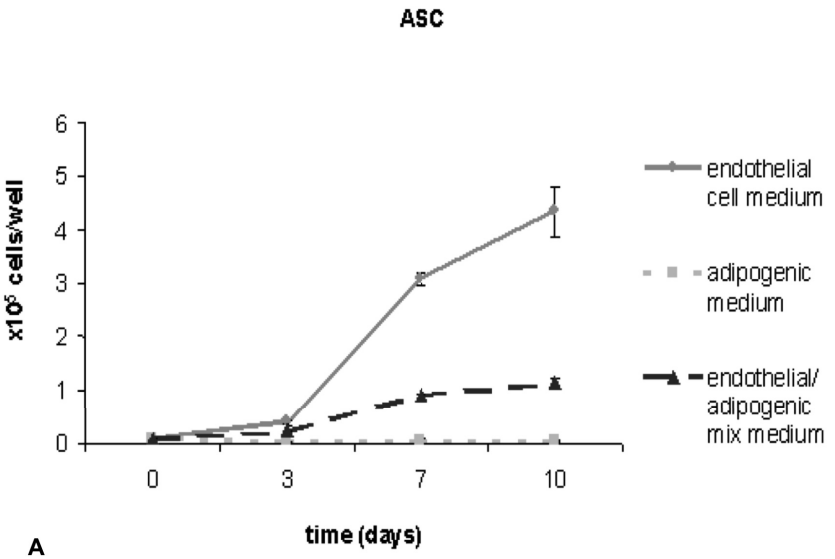
San Diego, California). Differences in the number of (mouse) vascular lumens per cross-sectional area between prevascularized and non-prevascularized constructs were analyzed with the nonparametric Mann-Whitney test using Graphpad Prism 5.01 software. Values with $P \leq 0.05$ were considered significant.

RESULTS

HUVEC AND ASC PROLIFERATION IN DIFFERENT MEDIA

To find the appropriate culture conditions for our HUVEC/ASC spheroid co-cultures we determined the proliferation potential of both ASC and HUVEC in 3 different media: endothelial cell medium, adipogenic medium and endothelial/adipogenic mix medium.

ASC and HUVEC proliferated on both endothelial cell medium and endothelial/adipogenic mix medium, while neither cell type proliferated in adipogenic medium (Fig.1). ASC and HUVEC proliferation was higher on endothelial cell medium than on endothelial/adipogenic mix medium. However, ASC had a more spindle-like morphology and accumulated no lipid on endothelial cell medium when compared to the ASC cultured in endothelial/adipogenic mix medium (data not shown), indicating that endothelial/adipogenic mix medium had a more positive effect on the adipogenic differentiation of ASC. Since endothelial/adipogenic mix medium supported both ASC and HUVEC proliferation and was more likely to support ASC adipogenic differentiation than endothelial cell medium, subsequent co-culture experiments were conducted in endothelial/adipogenic mix medium.



A

FORMATION OF SPHEROIDS

Following the described method, ASC and HUVEC/ASC co-cultures formed solid 3D spheroids with a diameter of 0.63 ± 0.12mm. These spheroids were stable during the total subsequent culture period of 14 days.

FLOW CYTOMETRIC ANALYSES OF CD31-POSITIVE CELLS IN HUVEC/ ASC SPHEROID CO-CULTURES

To determine whether the amount of HUVEC seeded in the spheroid co-cultures remained stable during culture we determined the percentage of CD31-positive cells in the co-culture spheroids after 2, 7 and 14 days of culture using flow cytometric analysis. The percentage of CD31-positive cells decreased in all spheroids during culture (Fig.2A). Especially between days 2 and 7 there was a significant decline in the percentage of CD31-positive cells in almost all spheroids. However, after 14 days of culture all spheroids still contained CD31-positive cells, with the spheroids seeded with 80% of HUVEC still containing almost 25% of CD31-positive cells. This percentage decreased with decreasing HUVEC seeding densities (Fig.2A-E). Spheroids containing no HUVEC also contained a small percentage (0.77-3.21%) of CD31-positive cells.

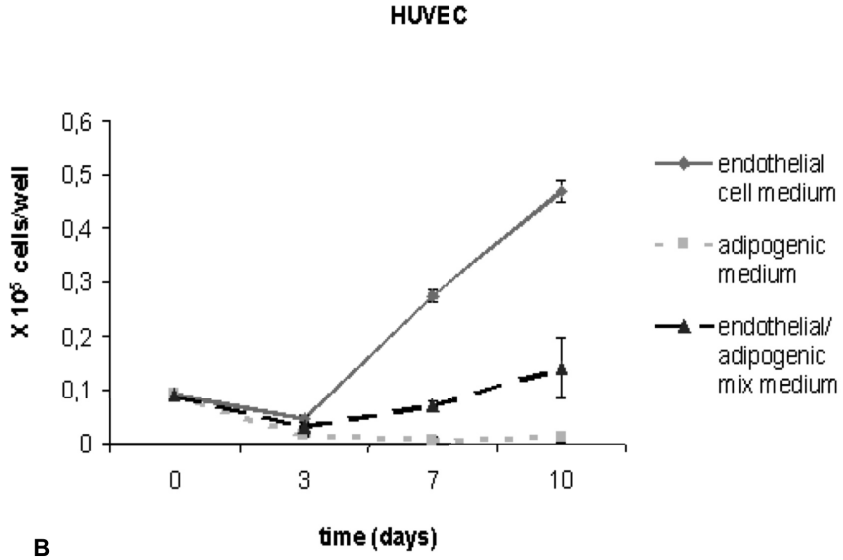


Figure 1. Proliferation of ASC and HUVEC in tissue culture flasks in different media. **(A)** ASC and **(B)** HUVEC were cultured in tissue culture flasks for 10 days in three different media; endothelial cell medium, adipogenic medium and a 1:1 mixture of endothelial cell medium and adipogenic medium (endothelial/adipogenic mix medium). The number of cells was determined at days 3, 7 and 10 of culture. Results are shown as mean ± standard deviation. Proliferation of ASC was determined for 3 different donors. HUVEC and ASC numbers were assayed in duplicate wells.

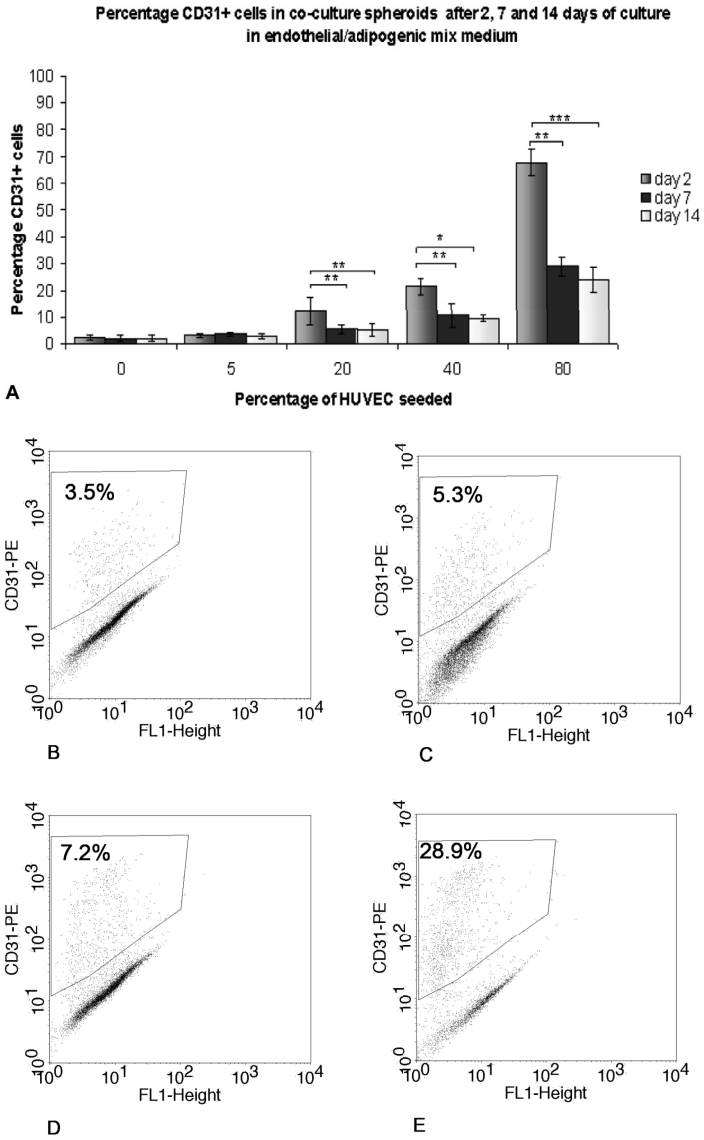


Figure 2. Flow cytometric analysis of CD31-positive cells in HUVEC/ASC spheroid co-cultures. The percentage of CD31-positive cells in co-culture spheroids seeded with different percentages of HUVEC and ASC was determined using flow cytometric analysis after 2, 7 and 14 days of culture. **(A)** Graph depicting the percentage of CD31+-cells in co-culture spheroids seeded with 0%, 5%, 20%, 40% and 80% HUVEC after 2, 7 and 14 days of culture. Values are shown as mean ± standard deviation. For each of the three ASC donors two spheroid co-cultures seeded with 0%, 5%, 20%, 40% and 80% HUVEC were assayed. Statistical comparisons were performed with the mixed model analysis of variance. *P<0.05, **P<0.01, ***P<0.001 (compared to day 7 and 14 of culture). **(B-E)** Representative dot-plots, depicting the expression of CD31 in co-culture spheroids seeded with (B) 5% HUVEC (C) 20% HUVEC (D) 40%HUVEC and (E) 80% HUVEC after 7 days of culture. The percentages in the gated areas represent the percentage of CD31-positive cells

HUVEC ORGANIZATION IN HUVEC/ASC SPHEROID CO-CULTURES

After 7 days of culture in endothelial/adipogenic mix medium, cross-sections of spheroids co-cultures were stained with CD31 to determine endothelial organization. CD31-positive cells in ASC spheroids seeded with 5, 20, and 40% HUVEC were present as round cell clusters dispersed throughout the spheroids (Fig.3A-C).

In ASC spheroids seeded with 80% HUVEC however, the CD31-positive endothelial cells aligned and formed prevascular structures amidst the ASC (Fig.3D). This endothelial organization was observed in spheroids of all 3 donors. Although HUVEC aligned and formed loops, no lumen formation was observed.

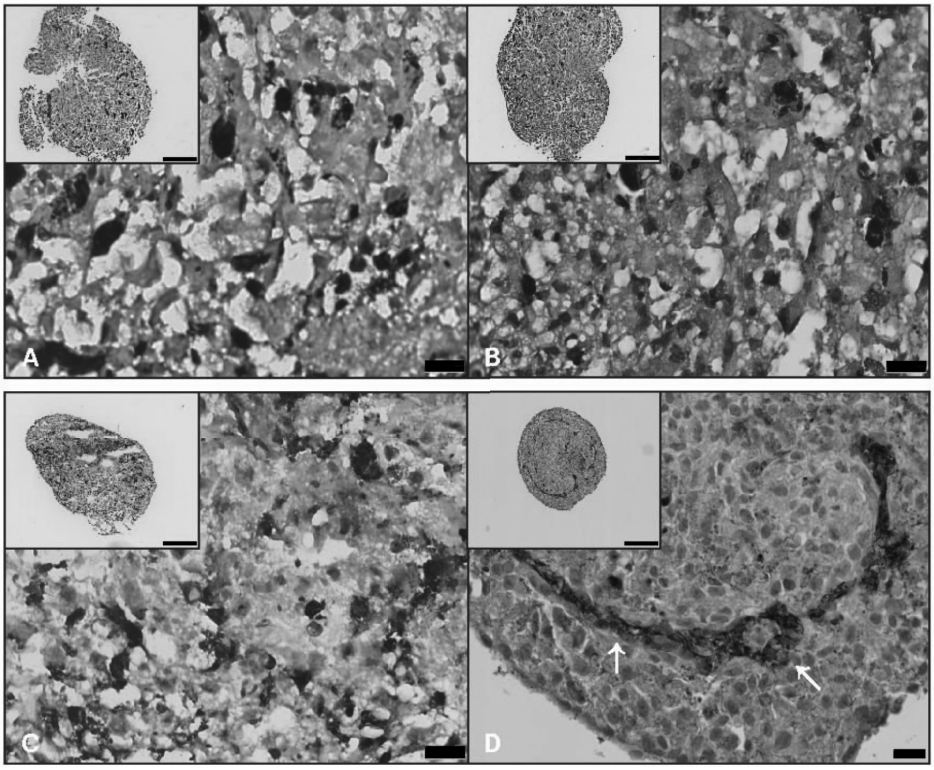


Figure 3. HUVEC elongate and form organized prevascular structures in 80%HUVEC/20%ASC spheroid co-cultures. Spheroid co-cultures seeded with different percentages of HUVEC were cultured for 7 days in vitro, and immunostained with anti-human CD31 (brown), showing organization of HUVEC. Cross-sections were counterstained with hematoxylin (blue). **(A)** 5% HUVEC seeded **(B)** 20% HUVEC seeded **(C)** 40% HUVEC seeded **(D)** 80% HUVEC seeded (white arrows point at strand and loop formation). Scale bars= 20 μm. Insets show general overview of cross-sections. Inset scale bars= 200μm. (see color section for a full-color version)

ADIPOGENIC DIFFERENTIATION OF ASC IN SPHEROID CULTURES IN VITRO

To assess adipogenic differentiation of the ASC in the spheroids, we measured the expression of the adipogenic marker FABP4 in ASC spheroids after 7 days of culture. Q-PCR analysis showed that FABP4 is expressed by ASC in spheroids cultured in endothelial/adipogenic mix medium, albeit significantly lower than in the ASC cultured in adipogenic medium (Fig.4A).

To further characterize adipogenic differentiation, we evaluated the ability of ASC to accumulate lipid after 7 days of culture in endothelial/adipogenic mix medium. Oil Red O staining showed that Oil Red O-positive cells were evident in ASC in these spheroid cultures (Fig.4B).

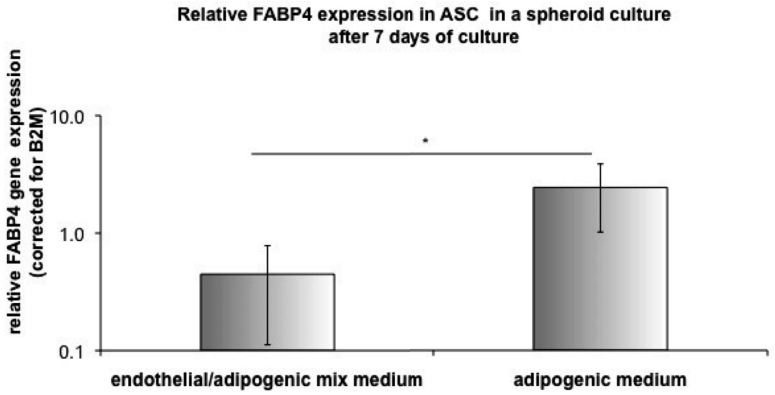
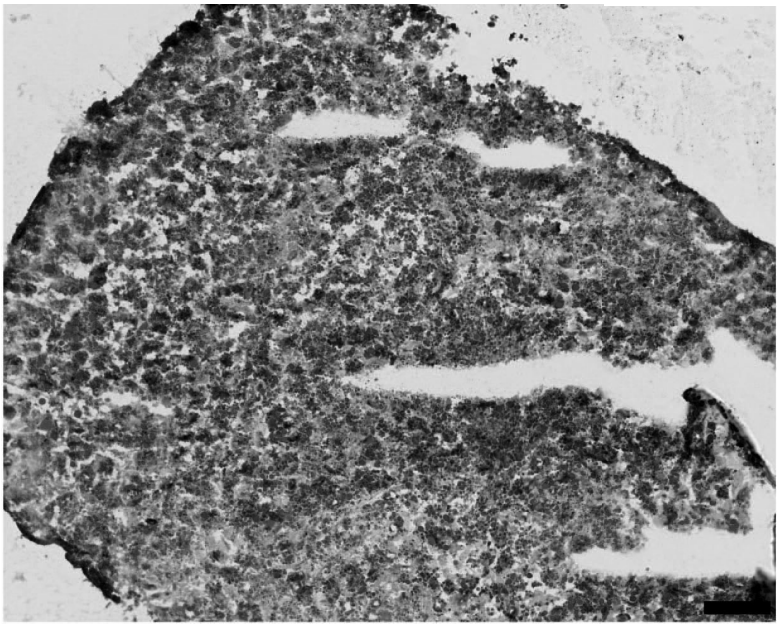


Figure 4. ASC in spheroid cultures express the adipocyte specific gene fatty acid binding protein 4 (FABP4) and stain positive for Oil Red O. ASC spheroid cultures were cultured for 7 days in vitro in endothelial/adipogenic mix medium or in adipogenic medium. **(A)** Q-PCR was used to measure the expression levels of FABP4. Expression levels are relative to beta-2-microglobulin positive-control housekeeping gene (dCt). Values are means ± standard deviations. Six ASC spheroid cultures were prepared from each of the three ASC donors, and three pools of two spheroids from each donor were assayed. Statistical comparisons were performed with the unpaired t test with Welch correction. *P<0.05 (compared to adipogenic medium).

ASSESSMENT OF HUMAN VASCULAR STRUCTURES IN HUVEC/ASC SPHEROIDS FOLLOWING IMPLANTATION IN NUDE MICE

Following 7 days of culture, the prevascularized 80% HUVEC/20% ASC spheroids and non-prevascularized ASC spheroids were implanted subcutaneously in nude mice, in order to determine if preformed human vascular structures were able to promote in vivo vascularization. After 7 days the spheroid constructs were retrieved. Gross examination revealed that the constructs still had a spherical shape and were invaded by vessels (Fig.5).

To determine whether human vascular structures were present inside the retrieved constructs, we performed immunostaining with anti-human CD31. Cross-sections of



(B) Cross-section of an ASC spheroid stained with Oil Red O (red) and counterstained with hematoxylin (blue). Note the presence of red-stained lipid droplets. Scale bar= 50μm

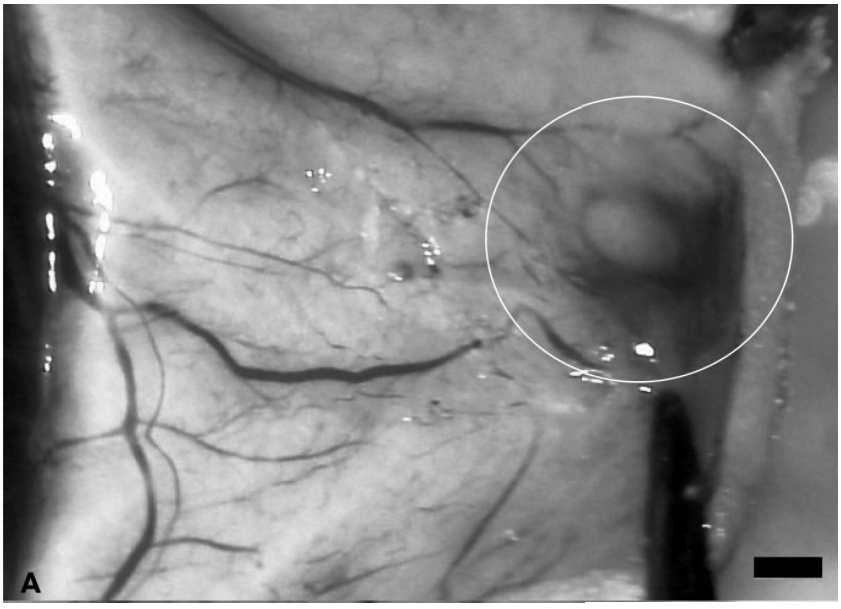


Figure 5. Macroscopic view of spheroid constructs 7 days after implantation. Representative images of **(A)** ASC spheroid construct and **(B)** 80% HUVEC/20%ASC spheroid construct taken at day 7 postimplantation. Scale bar= 1mm. (see color section for a full-color version)

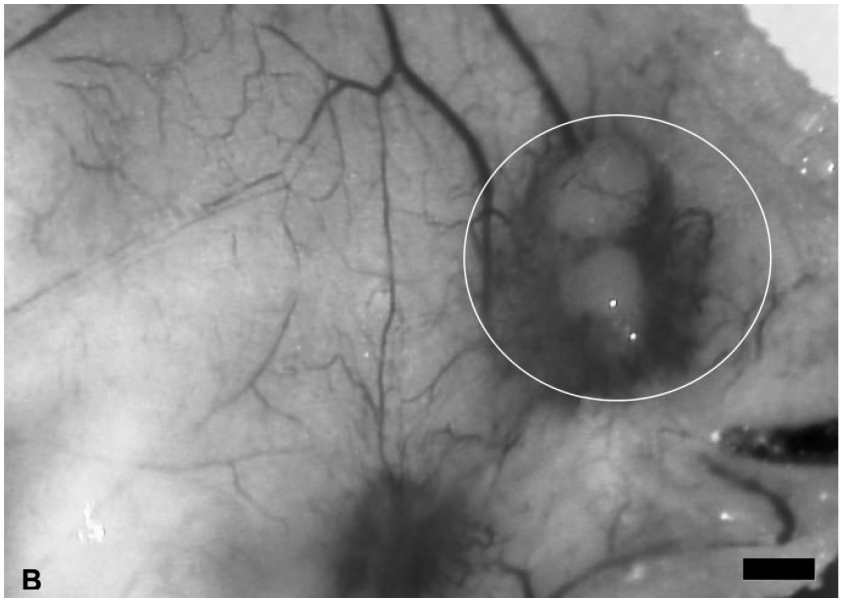


Figure 5. (continued)

the retrieved spheroid constructs showed that none of the ASC spheroid constructs and 4 out of the 6 80% HUVEC /20% ASC spheroid constructs contained human CD31-positive structures. To provide insight in the vascularization of the constructs, we measured three parameters: (1) the number and (2) density of vascular lumens of human origin and (3) the percentage of human CD31-positive cells per cross section. The number of human vascular lumens per cross-section ranged from 13 lumens/mm^2 to 45 lumens/mm^2 (average 23±15 lumens/mm^2) with a lumen density that ranged from 0.1 to 4.5% (average 1.8±2.0%) and a human CD31-positive-cell-density of 6.9±4.5%. The number of human vascular lumens and the lumen density were variable in cross-sections of different spheroid constructs of the same ASC donor as well as between ASC donors.

Importantly, the human CD31-positive vascular structures showed an increased organization when compared to the constructs prior to implantation (compare Fig.3D with Fig.6A) and frequently contained lumen. A subset of human vascular lumens (3.6±4.2 lumens/mm^2) with a lumen density of 1.0±1.1% was filled with red blood cells indicating that these lumens were perfused (Fig.6A-B).

The number of vascular lumens of mouse origin (lumens not stained with CD31) and the total number of vascular lumens (lumens not stained with CD31 plus the CD31-positive lumens) per tissue section were also evaluated for the ASC and 80%HUVEC/20%ASC constructs. Both the median values of the number of mouse vascular lumens/mm^2 and the total number of vascular lumens were significantly higher in 80%HUVEC/20%ASC constructs when compared to ASC constructs (number of

mouse vascular lumens/mm^2 median: 17 [IQR: 14-17] versus median: 0 [IQR: 0-6], $p<0.05$; total number of vascular lumens median 38 [IQR: 35-47] versus median: 0 [IQR: 0-6], $p<0.05$).

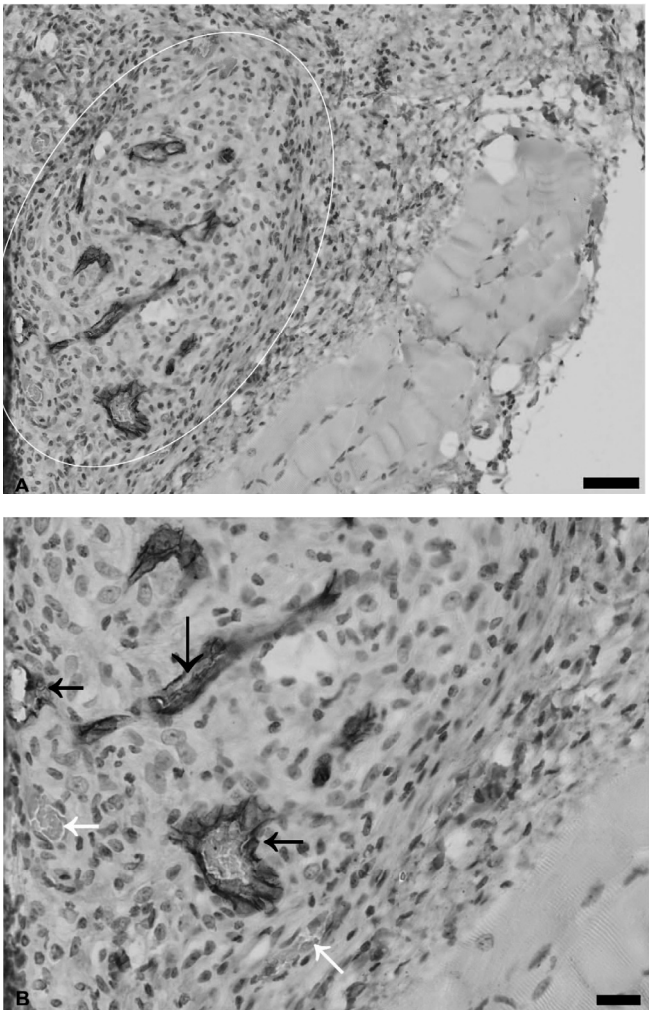


Figure 6. In vivo organization of prevascular structures in spheroid constructs. Spheroid co-cultures seeded with 80% HUVEC /20% ASC were cultured for 7 days in vitro, and then implanted subcutaneously in the right and left scapular region of nude mice for 7 days. Cross-sections were immunostained with human specific CD31 antibody (brown) and counterstained with hematoxylin (blue). **(A)** Overview of a cross-section, showing human CD31-positive vascular structures (spheroid located in white oval). Scale bar = 50µm. **(B)** High-magnification image of human CD31-positive lumina (see black arrows) and unstained mouse vessels (see white arrows). Scale bar = 20µm. Note the presence of red blood cells in human CD31-positive lumina. (see color section for a full-color version)

ASSESSMENT OF LIPID ACCUMULATION IN SPHEROIDS FOLLOWING IMPLANTATION IN NUDE MICE

To evaluate whether ASC in spheroid constructs also contained lipids after implantation, cross-sections of retrieved constructs were stained with Oil Red O. Similar to the in vitro cultures, Oil Red O-positive cells were evident in spheroid constructs (Fig.7A). Vimentin staining of consecutive cross-sections of the same spheroid constructs showed that Oil Red O-positive cells and vimentin-positive cells coincide in the same area, indicating that the Oil Red O-positive cells were from human origin (Fig.7B).

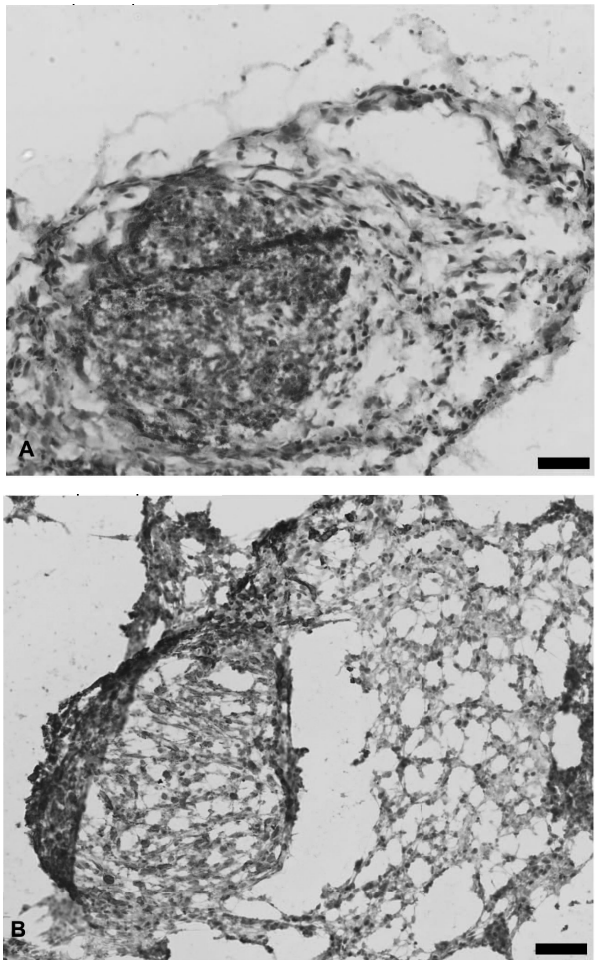


Figure 7. Accumulated lipid in HUVEC/ASC spheroid constructs 7 days after implantation. Spheroid co-cultures seeded with 80% HUVEC and 20% ASC were cultured for 7 days in vitro, and then implanted subcutaneously in the right and left scapular region of nude mice for 7 days. **(A)** Cross-sections were stained with Oil Red O (red) or **(B)** immunostained with vimentin (brown) and counter-stained with hematoxylin (blue). Scale bar = 50μm. Note that Oil Red O-positive cells and vimentin-positive cells coincide in the same area. (see color section for a full-color version)

DISCUSSION

The present study demonstrates that our HUVEC/ASC spheroid co-culture system leads to the formation of prevascular structures while simultaneously enabling adipogenic differentiation of ASC in vitro, as indicated by the accumulation of lipid and the expression of FABP4. Moreover, our study shows that the prevascular structures within the implanted constructs are able to continue their development in vivo and can be incorporated into the host vasculature, thereby contributing to the vascularization of the constructs.

After 7 days of implantation of the spheroids, we found that most of the 80% HUVEC /20% ASC spheroid constructs contained human CD31-positive vascular structures. These vascular structures regularly included lumen, indicating stability and advanced development of the vascular structures in vivo. In contrast, ASC spheroid constructs did not contain any human CD31-positive vascular structures, demonstrating that the addition of HUVEC to ASC spheroids is necessary for the formation of human vascular structures in vivo.

In vitro formed vascular structures have been shown to be able to connect to host vessels upon implantation [17,18,19,21]. In our study, we observed red blood cells in a subset of human CD31-positively stained lumina, indicating a connection of the HUVEC-derived vascular structures with the mouse vasculature. In addition, we found a higher total number of vascular lumens in cross- sections of 80% HUVEC/20%ASC constructs when compared to the total number of lumens in ASC constructs. This higher total number of vascular lumens in 80% HUVEC/20%ASC constructs is partly due to the presence of HUVEC-derived lumens, but also the result of a higher number of mouse-derived lumens, indicating augmented ingrowth of mouse vessels. This increased ingrowth of host (mouse) vessels likely results from paracrine signalling induced by the implanted HUVEC/ASC co-culture spheroids. Taken together, these findings indicate that the in vitro formation of prevascular structures in spheroid cultures can contribute to the development of vascular structures in vivo. Such a contribution of in vitro prevascularization on in vivo vascularization has also recently been shown in HUVEC/fibroblast fibrin-based co-cultures by Chen et al. [23].

Spheroids composed of combinations of tissue-derived stromal cells and endothelial cells for the purpose of prevascularization have been used before [18,24,25]. To our knowledge however, this is the first study in which ASC are combined with HUVEC in a spheroid co-culture. We found that only spheroids seeded with a high (80%) percentage of HUVEC lead to the formation of prevascular structures. In contrast, Rouwkema et al. [18], showed that the formation of prevascular structures in HUVEC/bone marrow-derived stromal cell (BMSC)/ spheroid co-cultures, was positively affected by low (2%) percentages of HUVEC seeding. It is uncertain whether this difference is due to the use of ASC instead of BMSC, or the use of a different culture medium (endothelial/ adipogenic mix medium instead of osteogenic medium).

Although HUVEC are an easily accessible source of endothelial cells, they will not be able to be used in a clinical setting due to their incompatibility with the recipient's immune system. A potential alternative is the use of microvascular endothelial cells derived from adipose tissue [26]. Microvascular endothelial cells have already been demonstrated to be able to form vascular structures in cultured skin substitutes after implantation [27]. In addition, endothelial progenitor cells derived from blood [28,29] are a promising alternative.

An optimal engineered tissue is assembled from multiple tissue-specific cells, has a typical tissue architecture and function and supports a vasculature compatible with the native vascular system [30,31,32]. Endothelial cell/ASC spheroid constructs may be appropriate tissue units for engineering vascularized adipose tissue equivalents. We have shown that the ASC in our spheroid constructs obtain adipocyte-specific characteristics whereas the HUVEC remain able to form prevascular structures that can integrate with the host (mouse) vasculature in vivo. Spheroid constructs have also been shown to be able to form coherent macrotissue patches (mm^3 scale), indicating that spheroid constructs can be scaled-up and used for the generation of larger-sized tissue constructs [24]. Further studies should determine the in vivo survival of prevascularized adipose tissue constructs in the long term, as well as attempt to engineer thicker prevascularized adipose tissue equivalents. Potential strategies to produce prevascularized adipose tissue equivalents with clinically relevant dimensions could be the integration of spheroid constructs with biomaterials such as photocrosslinkable gels and advanced techniques such as microfluidics [33,34].

CONCLUSION

Based on our results, we conclude that a spheroid co-culture of ASC with endothelial cells is suitable for the engineering of prevascularized adipose tissue constructs and that prevascularization improves the vascularization of engineered adipose tissue constructs in vivo. Integration of these prevascular structures in the host vasculature might make the difference between cell survival and cell death in especially thicker (>0.6mm in diameter) adipose tissue constructs following the initial period after implantation.

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CHAPTER 6

Vascularization of prevascularized and non-prevascularized fibrin-based human adipose tissue constructs after implantation in nude mice

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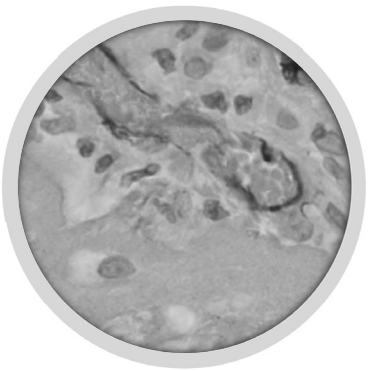

ABSTRACT

Adipose regeneration strategies have been hampered by the inability to supply an adequate vascular supply following implantation. Vascularization in vitro, also called prevascularization, is a promising method that could promote the vascularization of engineered adipose tissue constructs upon implantation. Herein, we compared the ability of prevascularized-to-non-prevascularized fibrin-based human adipose tissue to promote vascularization. Human adipose tissue-derived stromal cells (ASC) and different mixtures (1:1, 1:2 and 1:5) of ASC with human umbilical vein endothelial cells (HUVEC) were cultured in fibrin at two different densities ($1.0*10^6$ and $10*10^6$ cells/ml) for 7 days. Histological analysis revealed that prevascular structures formed in 1:5 ASC/HUVEC fibrin-based constructs seeded with a total of $10*10^6$ cells/ml. These constructs and ASC-only constructs were implanted subcutaneously in athymic mice for 7 days and generated lipid-containing grafts. The number and density of blood vessels within the ASC/HUVEC constructs was similar to that of ASC-only constructs. Furthermore, immunostaining studies demonstrated human-derived vasculature within few of the ASC/HUVEC and ASC-only constructs. A subset of this human-derived vasculature contained erythrocytes, indicating integration with the host vasculature. In conclusion, our study indicates no difference in the rate of vascularization of prevascularized ASC/HUVEC and non-prevascularized ASC-only fibrin-based constructs, suggesting that prevascularization of these fibrin-based constructs does not promote vascularization. Our results further indicate that not only endothelial cells, but also ASC may contribute to the formation of vascular lumens upon implantation. This finding is interesting since it demonstrates the possibility of vascularized adipose tissue engineering from a single cell source.

INTRODUCTION

For patients with soft tissue defects, soft tissue repair with autologous in vitro engineered adipose tissue could be a promising alternative to current surgical therapies. Unfortunately, the inability to maintain larger masses of living cells in vivo currently limits the size of in vitro engineered adipose tissue constructs. Insufficient vascularization is thought to be the main reason for this obstacle [1,2,3].

Any tissue that is more than a few hundred microns thick needs a vascular network to ensure that every cell is close enough to the blood supply to absorb oxygen and nutrients by diffusion [2,4,5,6,7]. The lack of an adequate vascular network will result in hypoxia and an inadequate supply of nutrients, eventually leading to cell death. Hence, promoting rapid in vivo-vascularization is crucial for the survival of large engineered adipose tissue constructs.

One method that can accelerate the process of vascularization upon transplantation is to preform vascular networks within engineered tissue constructs in vitro, also referred to as prevascularization [6,8,9,10]. We recently reported the formation of three-dimensional prevascularized adipose tissue constructs by co-culturing adipose tissue-derived stromal cells (ASC) and human umbilical vein endothelial cells (HUVEC) in spheroids and showed that the preformed vascular networks within these constructs (with a diameter of approximately 600 microns) could integrate with the host vascular system and improve the vascularization upon transplantation [11]

Although spheroids have been shown to be able to form bigger macrotissue patches (mm^3 scale) [12], it is likely that the use of a scaffold material is needed to produce prevascularized adipose tissue equivalents with more clinically relevant dimensions (cm^3 scale). For this, a scaffold material is needed that can easily be scaled-up and used in a clinical setting. Fibrin has shown a successful scaffold material for generation of adipose tissue [13,14,15,16] and is especially useful for clinical aims since it can be derived from a patient's own blood thereby avoiding a foreign body response [17]. In addition, fibrin-based constructs can easily be scaled-up and molded into any desired shape [18]

In the current study we generate prevascularized fibrin-based adipose tissue constructs by coculturing ASC and endothelial cells (EC) in fibrin gel and compare the vascularization of these fibrin-based ASC/HUVEC constructs with the vascularization of ASC-only fibrin-based constructs following subcutaneous implantation in nude mice. We demonstrate that the rate of vascularization in currently used (1.3 ± 0.5 mm in diameter) prevascularized ASC/HUVEC fibrin-based constructs and ASC-only constructs is comparable. Moreover, our results suggest that ASC can be incorporated in vascular lumens perfusing the implanted constructs. This indicates the possibility of vascularized adipose tissue engineering from a single cell type.

MATERIALS AND METHODS

ISOLATION AND CULTURE OF ASC

Subcutaneous abdominal adipose tissue was obtained as leftover material from donors undergoing breast reconstruction surgery with approval of the Erasmus MC Medical Ethical Committee (# MEC-2005-157) and according to the Code of Conduct: "Proper Secondary Use of Human Tissue" (http://www.federa.org). Leftover adipose tissue was only used from donors who did not opt-out to such use.

ASC from 3 different donors of 47, 54 and 59 years of age and with a BMI of 25.7, 25.2 and 23.2 were isolated from adipose tissue using collagenase type I (Invitrogen, Carlsbad, California) as previously described [19,20] and grown on basal medium (Dulbecco's Modified Eagle Medium 1g/l glucose (Invitrogen), 10% FCS (PAA Laboratories, Pasching, Austria), 10^{-12} M dexamethasone, 10^{-5} M ascorbic acid (both from Sigma-Aldrich, St. Louis, Missouri), 1% penicillin/streptomycin, 0.5% gentamycin (both from Invitrogen). The isolated primary ASC were grown until they reached 90% confluence. At 90% confluence, adherent cells were released with 0.05% trypsin/ ethylenediaminetetraacetic acid (EDTA, Invitrogen) and then stored in liquid nitrogen until further use. ASC at second passage were used in this study.

CULTURE OF HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVEC)

Commercially derived pooled HUVEC (Lonza, Verviers, Belgium) were seeded at a density of $1*10^4$ cells/cm^2 in culture flasks and cultured in endothelial growth medium consisting of human endothelial-serum free medium (Invitrogen), supplemented with 5% FCS (PAA Laboratories, Cölbe, Germany), 20ng/ml fibroblast growth factor (FGF2) and 100ng/ml epidermal growth factor (EGF) (both from Peprotech EC, London, United Kingdom). Cells were released at near confluence with 0.05% trypsin/EDTA and subcultured. Passage 2 HUVEC were stored in liquid nitrogen until further use. HUVEC at passage 5 or less were used in this study.

CO-CULTURE OF ASC AND HUVEC IN FIBRIN

Previous work with ASC/HUVEC spheroid co-cultures showed that an ASC: HUVEC ratio of 1:5 was optimal for the formation of prevascular structures in vitro [11]. However, the fibrin culture environment is different from a spheroid culture environment; therefore we tested 3 different ASC: HUVEC ratios (1:1, 1:2 and 1:5) in fibrin. Besides that, we also tested a low ($1.0 *10^6$ cells/ml) and a high ($10*10^6$ cells/ml) cell seeding density. Constructs with only ASC were used as control.

Fibrin matrices were prepared with the fibrin in vitro angiogenesis assay® (Millipore Corporation, Billerica, Massachusetts) according to manufacturer's instructions with some slight modifications. In short, ASC and different mixtures of ASC with HUVEC

(1:1, 1:2 and 1:5) were each suspended in 30 µl of fibrinogen solution at two different densities ($1.0*10^6$ cells/ml and $10*10^6$ cells/ml) leading to a total of $5*10^4$ cells or $5*10^5$ cells per fibrinogen solution. After addition of 20µl of thrombin solution, the cultures were immediately placed in individual wells of a 96-wells plate.

After gel formation, the plates were incubated at 37ºC in 5% CO_2 in a humidified environment for 15 minutes to ensure polymerization of the gels. Following polymerization, 200µl of endothelial growth medium was added to each well. After overnight equilibration, the culture medium was replaced by 200µl of a 1:1 mixture of endothelial growth medium and adipogenic medium (consisting of Dulbecco's modified Eagle Medium 4.5g/l glucose (Invitrogen), 10% FCS, 1µM dexamethasone, 0.01 mg/ml insulin (Eli Lilly, Houten, The Netherlands) 0.2 mM indomethacin (Sigma-Aldrich), 0.5mM 3-isobutyl-I-methyl-xanthine (Sigma-Aldrich), 1% penicillin/streptomycin and 0.5% gentamycin) from now on referred to as endothelial/adipogenic mix medium. Endothelial/adipogenic mix medium was changed every 2 days.

IN VIVO IMPLANTATION

Nine ASC fibrin-based constructs and 9 ASC/ HUVEC (ratio 1:5) fibrin-based constructs each seeded with a total of $5*10^5$ cells were obtained by generating 3 ASC fibrin-based constructs and 3 ASC/HUVEC fibrin-based constructs with cells of each of the 3 donors. Since in previous experiments, cell-free fibrin-based constructs could not be identified and retrieved after 7 days of subcutaneous implantation in nude mice (data not shown), cell-free fibrin-based constructs were not included as a control group in this study. The mice were placed under general anesthesia with 2.5% isoflurane after which two separate 0.5 cm incisions were made through the dorsal skin. Next, two separate subcutaneous pockets were prepared by blunt dissection of the subcutaneous tissue. One pocket was filled with an ASC fibrin-based construct and the other pocket was filled with an ASC/HUVEC fibrin-based construct. Pockets were closed with discontinuous sutures using Mersilk 5-0 (Ethicon, Somerville, New Jersey). Seven days after implantation, the mice were sacrificed and the constructs retrieved. Six ASC fibrin-based constructs and 6 ASC/ HUVEC fibrin-based constructs (consisting of 2 ASC fibrin-based constructs and 2 ASC/HUVEC fibrin-based constructs generated with cells from each of the 3 donors) were embedded in paraffin and used for histomorphometry, whereas the remaining 3 ASC fibrin-based constructs and 3 ASC/ HUVEC fibrin-based constructs (and thus containing 1 ASC fibrin-based construct and 1 ASC/ HUVEC fibrin-based construct generated with cells of each of the 3 donors) were embedded in Tissue-Tek (Sakura, Finetek Europe, Zoeterwoude, The Netherlands) and snap frozen for Oil Red O and vimentin (immuno)staining. All animal procedures were approved by the institutional Animal Experiments Committee of the ErasmusMC (EUR. 1292). (i.e. adherence to the rules prescribed in the national Animals Act, which

implements the 'Guidelines on the protection of experimental animals' by the Council of Europe, 1986: Directive 86/609/EC).

HISTOLOGY

Fibrin-based constructs harvested 7 days after culture or 7 days after implantation were embedded in Tissue-Tek (Sakura, Finetek Europe, Zoeterwoude, The Netherlands) and snap frozen or fixed in 10% formalin in PBS followed by embedding in paraffin. Paraffin-embedded sections (5 µm) were deparaffinized and rehydrated. Cryo-sections (5 µm) were fixed with 3.7% formalin in deionised water for 1 hour.

CD31 STAINING OF IN-VITRO CULTURED FIBRIN-BASED CONSTRUCTS

To determine HUVEC organization in the fibrin-based ASC/HUVEC constructs, a monoclonal mouse anti-human CD31 antibody (Clone JC70A, Dako (cat no. M0823), Glostrup, Denmark) was used. All sections were treated with 3% hydrogen peroxide in PBS to block endogenous peroxidase activity. Antigen retrieval was achieved by boiling at 95˚C for 15 minutes in Dako Cytomation Target Retrieval Solution high pH (Dako, Glostrup, Denmark). Non-specific binding of the monoclonal mouse anti-human CD31-antibody was reduced by blocking with 1%BSA/10% goat serum in PBS for 30 minutes. This was followed by an overnight incubation with CD31 (1:40 dilution in PBS/ 1%BSA) at 4˚C. Sections were incubated with goat anti-mouse secondary antibody (1:200 dilution in PBS/1% BSA, Dako, cat no. E0433) for 30 minutes followed by incubation with streptavidin-horseradish peroxidase (1:300 dilution in PBS/1% BSA, Dako, cat no. P0397) for 30 minutes. Diaminobenzidine (Sigma-Aldrich) was used to visualize CD31 expression. The slides were weakly counterstained with hematoxylin, dehydrated through graded alcohols and mounted with Permount (VWR International, Amsterdam, The Netherlands).

CD31 STAINING AND VIMENTIN STAINING IN FIBRIN-BASED CONSTRUCTS POST- TRANSPLANTATION

To distinguish seeded human cells from mouse tissue and to determine the number and relative area occupied by human vascular structures in construct sections post-transplantation, monoclonal mouse anti-human vimentin antibody (Clone V9, 1:40 dilution in PBS/ 1% BSA, Sigma-Aldrich, cat no. V6630) and monoclonal mouse anti-human CD31 antibody (Dako) were used, respectively. To reduce unspecific binding of the secondary goat anti-mouse antibody (Dako) to mouse IgGs, the mouse-on mouse HRP-Polymer Kit (Biocare Medical, Concord, California) was used, according to manufacturer's instructions with some slight modifications. In short, antigen retrieval was performed through incubation in Rodent Decloaker® (Biocare Medical) for 60 minutes at 95˚C. Non-specific binding sites were blocked with Rodent Block M®

(Biocare Medical) and sections were stained overnight with CD31 or vimentin at 4˚C. The MM-polymer-HRP® secondary antibody (Biocare Medical) was used, followed by incubation in diaminobenzidine (Sigma-Aldrich) to visualize vimentin or CD31 expression, respectively. The slides were weakly counterstained with hematoxylin, dehydrated through graded alcohols and mounted with Permount (VWR International).

OIL RED O STAINING

Accumulated lipid in ASC in fibrin-based constructs was detected after 7 days of in vitro culture and after 7 days of implantation using Oil Red O staining as previously described [11,19]. In short, cryo-sections were fixed, rinsed and immersed in Oil Red O working solution for 30 minutes. Hereafter, sections were washed with deionised water and counterstained with hematoxylin for 1 minute. Finally, sections were rinsed with running tap water for 10 minutes, and covered with Imsolmount (Klinipath, Zevenaar, The Netherlands).

HISTOMORPHOMETRY

High resolution (0.23 μm/pixel), low magnification (40×) digital micrographs covering 2-to-3 complete vimentin or CD31 immunostained cross-sections taken from middle sections of each retrieved construct were made with a Nanozoomer HT (Hamamatsu Photonics, Hamamatsu City, Japan) for analysis. Subsequently, the total number of vessel lumens (mouse- and human-derived), the number of human CD31-positive vascular structures, the area occupied by these structures and the complete area of human tissue in the constructs was quantified in each entire cross-section using NIH ImageJ software (http://rsb.info.nih.gov/ij/). From these data we calculated four parameters: (1) the total number and (2) density of all lumens and (3) the number and (4) density of all human CD31-positive lumens per cross section area.

STATISTICAL ANALYSIS

Differences in the number and density of (mouse- and human-derived) vascular lumens per cross-sectional area between ASC/HUVEC and ASC-only fibrin-based constructs were analyzed with the unpaired t-test with Welch correction and are expressed as mean± standard deviation. Statistical significance was defined as $P<0.05$. Statistical analysis was performed using Graphpad Prism 5.01 software (Graphpad Software, San Diego, California).

RESULTS

HUVEC ORGANIZATION IN ASC/HUVEC FIBRIN-BASED CONSTRUCTS IN VITRO

Cross sections of fibrin-based constructs seeded with a low cell density and cultured for 7 days showed that CD31-positive endothelial cells were highly dispersed throughout the fibrin matrix at all ASC: HUVEC ratios, showing no formation of organized structures (Fig.1A-C). In cross-sections of fibrin-based constructs seeded with a high cell density more CD31-positive endothelial cells were present in cell clumps. Only in 1:5 fibrin-based constructs the formation of lumen-consisting prevascular structures was seen (Fig.1D). No CD31-positive endothelial cells were present in the fibrin-based ASC-only constructs (Fig.1E).

ASSESSMENT OF SURVIVAL AND IN VIVO ADIPOGENESIS

High cell density ($10*10^6$ cells/ml) ASC-only fibrin-based constructs were implanted subcutaneously in nude mice. In addition, we implanted the fibrin-based constructs that were seeded with a high cell density ($10*10^6$cells/ml) and a 1:5 ratio of ASC and HUVEC, since these constructs had already formed prevascular structures in vitro.

Seven days following implantation, we could readily identify all implanted fibrin-based constructs both by visual inspection and by microscopic examination. Remaining traces of the biodegradable fibrin could be found within some implanted tissue-constructs (H&E staining Fig.2A).

Oil Red O staining of both ASC (/HUVEC) construct cross-sections revealed the presence of red-stained lipid containing cells within the implanted constructs (Fig.2B). Similarly, anti-human vimentin staining showed that numerous human vimentin-positive cells were still present within the implanted constructs, suggesting that lipid containing cells might be from human origin (Figure 2C-D).

HISTOLOGICAL ASSESSMENT OF CONSTRUCT VASCULARIZATION

To assess construct vascularization upon implantation, cross-sections of the retrieved constructs were stained with H&E to determine the total vascularization (both mouse- and human-derived lumens) (Fig.3A), or stained with anti-human specific CD31 antibody to determine human-specific vascularization (Fig.3B-D). Four quantitative parameters of construct vascularization were measured in stained cross-sections: (1) the total number and (2) density of all lumens and (3) the number and (4) density of all human CD31-positive lumens per cross section area. Total graft vascularization (mouse- and human-derived lumens) was not significantly different between ASC/HUVEC and ASC-only fibrin-based constructs. In ASC/HUVEC fibrin-based constructs, both the number of lumens per cross-section area (19 ± 32 lumens/mm^2) and the lu-

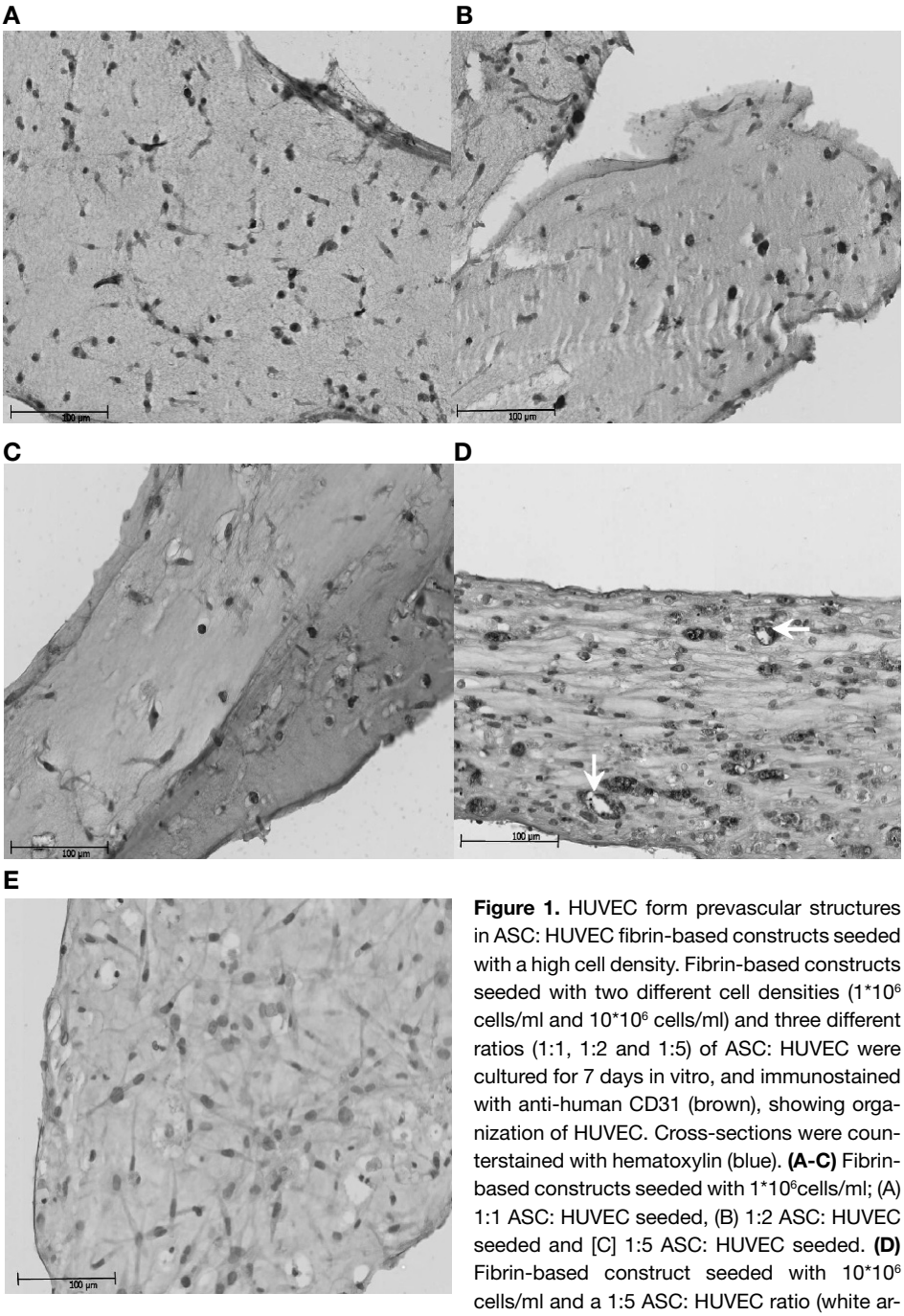

Figure 1. HUVEC form prevascular structures in ASC: HUVEC fibrin-based constructs seeded with a high cell density. Fibrin-based constructs seeded with two different cell densities ($1*10^6$ cells/ml and $10*10^6$ cells/ml) and three different ratios (1:1, 1:2 and 1:5) of ASC: HUVEC were cultured for 7 days in vitro, and immunostained with anti-human CD31 (brown), showing organization of HUVEC. Cross-sections were counterstained with hematoxylin (blue). **(A-C)** Fibrin-based constructs seeded with $1*10^6$cells/ml; (A) 1:1 ASC: HUVEC seeded, (B) 1:2 ASC: HUVEC seeded and [C] 1:5 ASC: HUVEC seeded. **(D)** Fibrin-based construct seeded with $10*10^6$ cells/ml and a 1:5 ASC: HUVEC ratio (white arrows point at prevascular structures). **(E)** Fibrin-based construct seeded with $10*10^6$cells/ml and ASC-only. Note the absence of anti-human CD31 staining (brown). Scale bars= 100 µm.

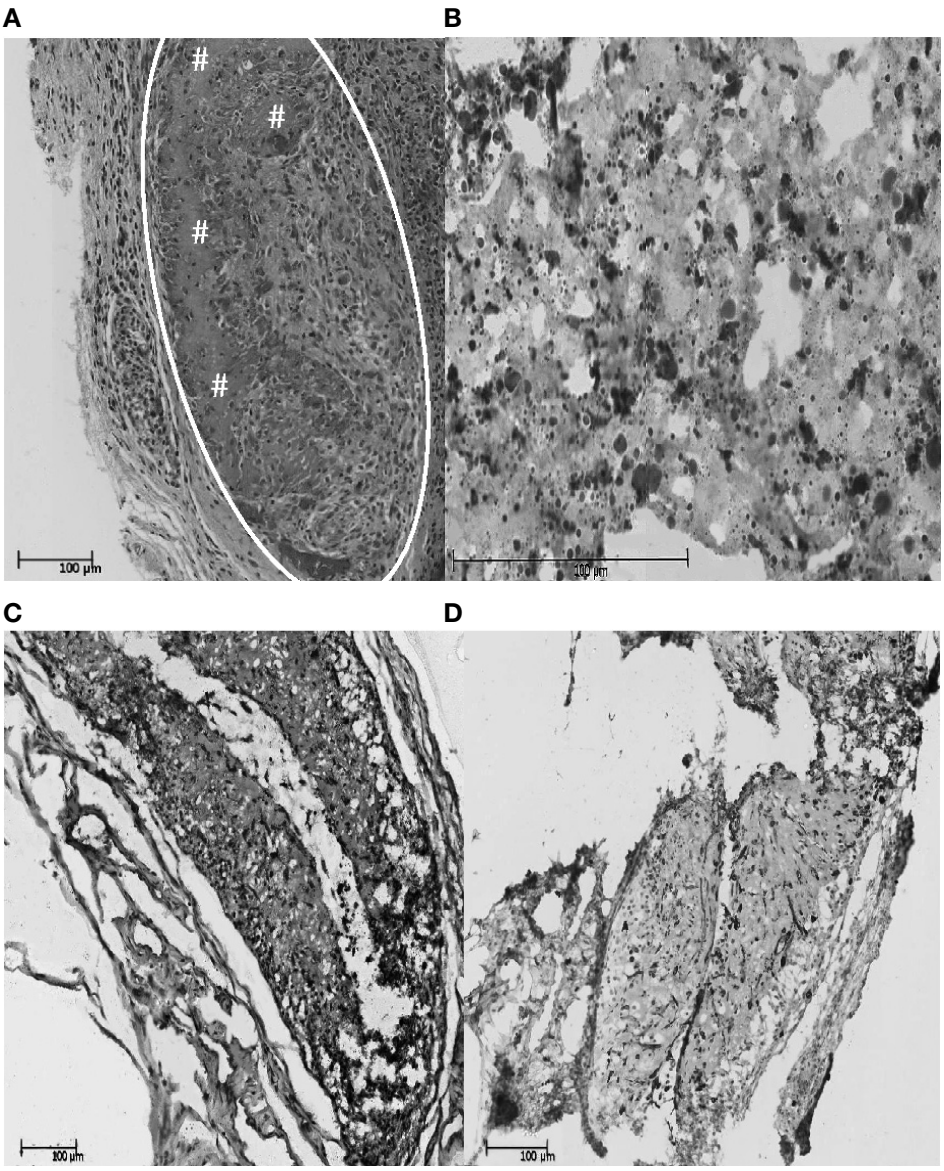

Figure 2. Histological analysis of the fibrin-based constructs 7 days postimplantation. Fibrin-based constructs were cultured for 7 days in vitro, and then implanted subcutaneously in the right and left scapular region of nude mice for 7 days. **(A)** H&E-stained cross-section of an ASC: HUVEC fibrin-based construct containing some fibrin remnants (indicated by #). **(B)** High magnification image of a cross-section of an ASC/HUVEC fibrin-based construct stained with Oil Red O (red). **(C)** Low magnification image of a cross-section of another ASC/HUVEC fibrin-based construct stained with Oil Red O (red). **(D)** Consecutive cross-section of ASC/HUVEC construct shown in [C] immunostained with vimentin (brown) and counterstained with hematoxylin (blue). Oil Red O-positive, lipid-containing cells are present in the constructs. Note that Oil Red O-positive cells and vimentin-positive cells are located within the same area. Scale bars= 100 µm. (see color section for a full-color version)

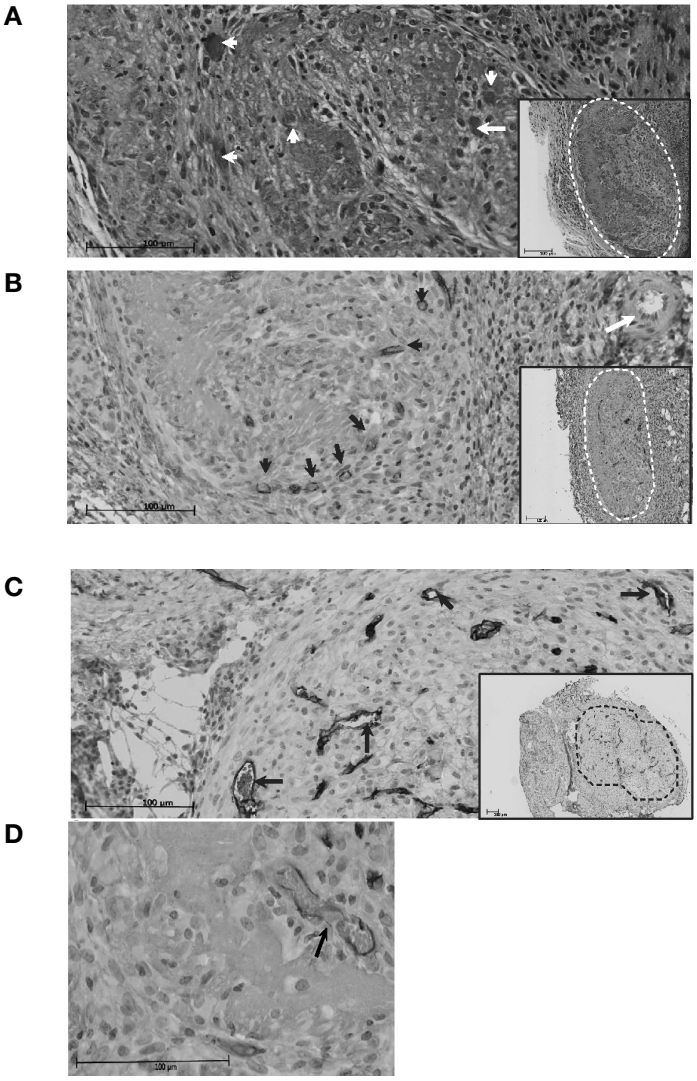

Figure 3. Vascularization of fibrin-based constructs 7 days postimplantation. Fibrin-based constructs seeded with ASC or a 1:5 mixture of ASC: HUVEC were cultured for 7 days in vitro, and then implanted subcutaneously in the right and left scapular region of nude mice for 7 days. Cross-sections were stained with H&E to determine the total amount of (mouse-and human-derived) vascular lumens or immunostained with human specific CD31 antibody (brown) and counterstained with hematoxylin (blue) to determine human-derived vascular lumens. **(A)** Vascular lumens could easily be identified within H&E stained cross-sections of fibrin-based constructs (white arrows point at some vascular lumens). Inset shows general overview of H&E stained cross-section. Inset scale bar= 100µm. **(B-C)** Images of human CD31-positive lumina (see black arrows) in an ASC-only (white arrow points at unstained mouse vessel) (B) and (C) an ASC: HUVEC fibrin-based construct. **(D)** High magnification image of human CD31-positive lumen containing erythrocytes (see black arrow) in an ASC-only fibrin-based construct. Scale bar = 100µm. (see color section for a full-color version)

men density (1.4%±1.5%) were not significantly different from ASC-only fibrin-based constructs (54±67 lumens/mm^2 and 3.5%±1.8% respectively). We next assessed the relative contribution of human-specific vascularization. Surprisingly, only 1 out of the 6 ASC/HUVEC fibrin-based constructs contained human CD31-positive lumens (6 lumens/mm^2 and a lumen density of 0.3%), whereas 2 out of 6 ASC-only fibrin-based constructs contained human CD31-positive lumens (20±35 lumens/mm^2 and a lumen density of 0.6%±0.1%). In both ASC/HUVEC and ASC-only constructs a subset of the human vascular lumens (1.2 and 11.5±0.5 lumens/mm^2 respectively; lumen density 0.3% and 1.3%±1.3% respectively) was filled with erythrocytes, indicating that these lumens were perfused (Fig.3D).

DISCUSSION

The results of our study show that, in contrast to ASC constructs without HUVEC, lumen-consisting prevascular structures are formed in adipose ASC/HUVEC fibrin-based constructs in vitro. The presence of these human prevascular structures within the adipose fibrin-based constructs however, does not promote vascularization of the constructs after implantation. Moreover, our study shows that neither the addition of HUVEC, nor the presence of human prevascular structures in vitro is necessary for the generation of human CD31 positive-vascular structures in adipose fibrin-based ASC-constructs in vivo.

Fibrin-embedded combinations of tissue-derived stromal cells and HUVEC for the purpose of prevascularization have been used before [21,22]. To our knowledge however, this is the first study in which ASC are suspended with endothelial cells (EC) in fibrin gels without the use of a secondary carrier material. Such carrier materials are often not-clinically applicable (e.g. microcarrier beads). We found that prevascular structures were formed in ASC/ HUVEC co-cultures with a 1:5 ratio of ASC to HUVEC. This is in line with the findings of Chen et al [23], who used a similar ratio (1:5) of HUVEC/human lung fibroblasts. Upon implantation, the prevascular structures in the ASC/HUVEC constructs however, did not contribute to a higher number or density of vascular lumens when compared to the ASC-only constructs. This is in contrast to our previous results. In previous experiments, using similar cells and the same culture medium but using spheroid-based constructs, we showed that co-culture of ASC/HUVEC actually can improve the amount and density of vascular lumens after implantation [11].

Clearly, the specific fibrin environment is of influence on the different outcome of our current experiments. Fibrin may change the paracrine signaling and/or interaction between the ASC and HUVEC in this experimental setup, hereby causing the different

results compared to our former experiments with spheroids. Although the difference between the outcome of our current fibrin-based experiments and our former spheroid experiments is as yet not fully explainable, a difference in ASC function when comparing different ASC formulations or delivery methods is not entirely new. For instance, Amos et al. (Amos et al., 2010) recently reported that ASC spheroids had an increased in vivo therapeutic capacity and an increased secretion of trophic factors when compared to ASC suspensions. Alternatively, fibrin itself may induce vessel ingrowth, thereby masking any pro-angiogenic effects induced by the ASC/HUVEC co-culture.

After 7 days of implantation, we found that human-derived CD31-positive lumens were not only present in ASC/HUVEC fibrin-based constructs, but also in ASC-only fibrin-based constructs, demonstrating that prevascularization, or even the addition of HUVEC to ASC in fibrin-based constructs is not necessary for the formation of human vascular structures in vivo. Moreover, we found that a subset of the human-derived lumens contained erythrocytes, indicating that these human-derived lumens were connected to the host vasculature and contributed to construct perfusion. Since we found no CD31-positive cells in the ASC-only fibrin-based constructs prior to implantation, the ASC themselves may have differentiated to CD31 expressing cells and may have contributed to vessel formation. Previously, Scherberich et al. [24] also showed that human CD31-positive vessels can be generated within ASC-only constructs after implantation, albeit these researchers used osteogenic constructs. The number of constructs that contained human-derived CD31-positive lumens however was low, implying that most detected lumens were host(mouse)-derived. Upon further investigation, it appeared that all our ASC/HUVEC and ASC-only fibrin-based constructs that generated human-derived vessels in vivo were derived from the same ASC donor. This indicates a substantial variation amongst the ASC donors used. Heterogeneity in ASC plasticity between multiple donors has been reported before [25], and may reflect differences in the composition of the adipose tissue between the donors.

The results of our study show that in vitro formed prevascular structures did not contribute significantly to vascularization upon implantation. In the current study however, we still used relatively small (1.3 ± 0.5 mm in diameter) ASC/HUVEC and ASC-only fibrin-based tissue constructs. It may be that this construct size is too small to reveal any differences in the vascularization potential between prevascularized ASC/HUVEC and nonprevascularized ASC-only fibrin-based constructs upon implantation. Further in vivo studies, using bigger, more critically sized fibrin-based tissue constructs (e.g. cm^3 scale), should reveal whether any positive effects of prevascularization can be observed.

CONCLUSION

In summary, our study indicates a similar rate of vascularization of small-sized (≤1.8mm in diameter) ASC/HUVEC as of ASC-only fibrin-based constructs, suggesting that prevascularization of these fibrin-based ASC constructs with HUVEC prior to implantation does not promote the vascularization. This may be because of the pro-angiogenic influence of fibrin [26], either directly on the host vasculature, or indirectly through affecting the paracrine signaling and/or interaction between the ASC and HUVEC. Our results further indicate that not only endothelial cells, but also ASC may contribute to the formation of vascular lumens upon implantation of adipose fibrin-based ASC constructs. This finding is interesting since it demonstrates the possibility of vascularized adipose tissue engineering from a single cell source. In addition, our results illustrate variation amongst ASC donors with regard to their ability to support vascularization. This finding merits further investigation; a better understanding of which (subpopulation) of ASC can support vascularization is important for improving vascularization of engineered adipose tissue after implantation. Altogether it is clear that the success of prevascularization depends on multiple factors. These factors amongst others include the adipose tissue engineering environment (e.g. biomaterial) and the cells (e.g. ASC donor) used. Both these factors can influence the interaction between ASC and endothelial cells and the interaction between the implanted constructs and host tissue

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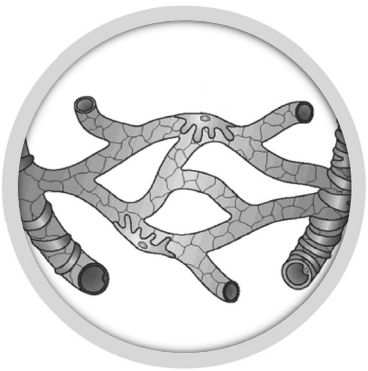
CHAPTER 7

Summary & General discussion



ADIPOSE TISSUE ENGINEERING AND VASCULARIZATION

Reconstruction of soft tissue defects represents critical challenges for contemporary plastic surgery practice. Currently, autologous microvascular soft tissue transplants or synthetic materials are used for reconstruction of soft tissue defects. Because of optimal tissue characteristics and low risk of immunologic rejection, microvascular autologous adipose tissue transplants are common practice. Despite advances in the transplantation of adipose tissue, donor site morbidity continues to be an intrinsic drawback of this procedure. Therefore, scientists have been encouraged to engineer adipose tissue equivalents. In the field of adipose tissue engineering, biomaterials are generally combined with adipose progenitor cells. Human mesenchymal stem cells derived from adipose tissue also referred to as adipose-derived stromal cells (ASC), are commonly used for this purpose as they can be readily obtained, expanded and differentiated to adipocytes [1,2] (CHAPTER 1). Indeed, various studies including our own have shown that in vivo implantation of ASC can lead to the formation of new adipose tissue [3,4,5]. However, a critical obstacle in the engineering of tissue equivalents is the ability to maintain larger masses of living cells in vivo. To date, the size of most engineered tissues is limited owing to the difficulty of providing an adequate blood supply upon implantation. Several strategies to enhance vascularization in adipose tissue engineering are currently being explored such as the delivery of angiogenic factors [6,7,8], improving scaffold design [8,9,10,11] and in vivo prevascularization [12,13,14,15]. In this thesis, we investigated the angiogenic potential of adipose tissue-derived stromal cells (ASC) in relevant (adipose) tissue engineering settings (CHAPTER 2 AND 3). Besides that, we investigated the potential beneficial effects of in vitro prevascularization on in vivo vascularization of ASC-based adipose tissue constructs (CHAPTER 4, 5 AND 6).

ANGIOGENIC POTENTIAL ASC

ASC have been reported to promote ischemic tissue revascularization [16,17,18,19]. Two mechanisms on how these cells promote vascularization have been put forward: (1) via endothelial trans-differentiation and incorporation of ASC in ingrowing vessels and (2) via secretion of diffusible molecules (e.g. angiogenic factors) that stimulate vascularization [19,20,21,22]. In particular the latter mechanism has been shown to play a key role in the beneficial effects of ASC in ischemic tissue revascularization [19,20]. Since ischemic tissue revascularization and vascularization of engineered tissue constructs share many similarities, we hypothesized that the ability of ASC to secrete angiogenic factors could also be beneficial for promoting vascularization in

ASC-based adipose tissue constructs. However, little is known on the secretion of angiogenic factors by ASC in an adipose tissue engineering setting. This led us to investigate the angiogenic potential of ASC in adipose tissue engineering settings. First, we investigated whether adipogenic differentiation influenced the gene expression and protein secretion of angiogenic factors by ASC (CHAPTER 2). We examined changes in expression and secretion of important angiogenic factors during adipogenic differentiation of ASC and evaluated whether differences between undifferentiated and differentiated cells affect endothelial cell numbers in vitro. We showed that expression and secretion of the angiogenic factor VEGF was increased in short-term (7-14 days) adipogenic differentiated ASC when compared to undifferentiated ASC. Moreover, conditioned medium from differentiated ASC strongly enhanced endothelial cell numbers in vitro compared to conditioned medium from undifferentiated ASC. From these in vitro experiments, it can be concluded that in vitro adipogenic differentiation of ASC for a period of 7-14 days improves their in vitro angiogenic potential, (as compared to undifferentiated ASC) and may be beneficial with respect to vascularization of ASC-based constructs. In addition to this, Weiser et al.[23] showed that short-term in vitro differentiation of ASC is also sufficient for in vivo formation of adipose tissue and even improves engineered adipose tissue survival. Taken together, these findings indicate that short in vitro adipogenic-precultivation of ASC is potentially the better choice for adipose tissue engineering applications.

Next we investigated the angiogenic potential of ASC constructs in vivo. A construct for in vivo implantation can be generated with and without a scaffold material. We were interested in the effect of the scaffold environment on ASC-based construct vascularization and angiogenic factor secretion (CHAPTER 3). Numerous studies have used a scaffold/extracellular matrix-based tissue engineering approach for the generation of adipose tissue. Although the effect of the extracellular environment on stem cell differentiation has recently gained interest [24,25,26] there remains much to be learned on the role of the extracellular environment on stem cell behavior. To date, little is known about the influence of the extracellular environment on ASC angiogenic potential. In CHAPTER 3 we compared ASC-based constructs with and without the addition of the extracellular matrix fibrin. We showed that a fibrin environment clearly improved vessel ingrowth in vivo and also enhanced ASC adipogenic differentiation and secretion of angiogenic factors (i.e.VEGF and HGF) in vitro. Our results indicate that the fibrin environment impacts ASC angiogenic potential. Besides that, our study indicates that fibrin is a very promising material for adipose tissue engineering. This is not only because it improves ASC angiogenic potential but also because it supports ASC adipogenic differentiation. The positive effect of the fibrin environment on ASC-based construct vascularization may be partly explained by the improved ASC angiogenic potential. However, being a naturally occurring scaffold in wound healing

and tissue repair fibrin itself may have also contributed to increased vessel ingrowth through its angiogenic properties (such as binding of growth factors, facilitating cell migration, interacting with endothelial cells through VE-cadherins) [27,28]. In spite of its positive effect on adipogenesis and vascularization fibrin has limited structural stability, which can complicate the formation of volume-stable adipose tissue. A method to compensate for the poor mechanical properties of fibrin could be the combined use of more rigid supportive structures with fibrin as demonstrated by Cho et al [29,30].

IN VITRO PREVASCULARIZATION

Although the rate of blood vessel ingrowth in implanted ASC-based adipose tissue constructs may be increased by short in vitro adipogenic precultivation of ASC or the use of fibrin as a scaffold, vessel ingrowth will probably not be enough to supply thick implants with a suitable blood supply in a timely manner. For this reason, a tissue engineering approach that includes a vascular network within the tissue construct is likely to be necessary for thick implants.

CHAPTER 4, 5 AND 6 are mainly directed towards our second aim, which is to investigate the potential positive effect of in vitro prevascularization on the in vivo vascularization of ASC-based adipose constructs.

Recently, Rouwkema et al.[31] demonstrated that bone marrow-derived stromal cells (BMSC) can support the organization of endothelial cells into a three-dimensional prevascular network in vitro. BMSC and ASC share many similarities. We therefore speculated that ASC, similar to BMSC, could support the organization of endothelial cells into prevascular structures in vitro. Although indirect and direct co-cultures of ASC and endothelial cells have been described before, these studies mainly focused on the interactions between ASC and endothelial cells [32,33,34]. In CHAPTER 4 we specifically focused on the ability of ASC to support the de novo formation of vascular structures from endothelial cells in vitro. We compared angiogenic factor secretion and endothelial organization between BMSC/endothelial cell and ASC/endothelial cell co-cultures. Our experiments showed that both ASC conditioned medium as well as direct co-culture of ASC with human umbilical vein endothelial cells (HUVEC) supported the organization of HUVEC into vascular-like structures in vitro. In contrast, only the direct co-culture of BMSC with HUVEC was sufficient to support the formation of prevascular structures. Furthermore, we found no significant differences in the secretion of the measured angiogenic factors between BMSC and ASC. Although the exact mechanism by which BMSC and ASC support endothelial organization remains unknown, our conditioned medium and direct co-culture experiments suggest that apart from the secretion of angiogenic factors also direct cell interactions (through cell-cell contacts

and/or reciprocal signaling) play an important role. In order to optimize the formation of vascular-like structures in BMSC/endothelial cell and ASC/endothelial cell co-cultures more research is necessary to better understand these direct cell interactions.

The results described in CHAPTER 4 illustrate that ASC can promote the organization of endothelial cells into vascular-like structures in vitro. The ability to form vascular-like structures in ASC-based constructs in an adipose tissue engineering setting was further examined in CHAPTER 5 AND 6.

In CHAPTER 5 we investigated if it was possible to induce the formation of vascular-like structures in three-dimensional spheroid ASC/HUVEC co-cultures while simultaneously enabling adipogenic differentiation of the ASC. In addition, we examined whether these in vitro generated prevascular structures could integrate with the host vasculature in vivo. For this, we first determined what co-culture medium is suitable for both HUVEC and ASC, and what ratio of HUVEC/ASC is optimal for the formation of vascular-like structures. Several media (adipogenic medium, endothelial cell medium, 1:1 mix of adipogenic and endothelial medium) and several combinations of ASC and HUVEC (95% ASC/5%HUVEC, 80% ASC/20% HUVEC, 60% ASC/40% HUVEC and 20% ASC/80% HUVEC) were tested. We showed that a 20% ASC/80% HUVEC co-culture in 1:1 endothelial cell/adipogenic mix medium leads to the formation of vascular-like structures while simultaneously enabling ASC adipogenic differentiation in vitro. Next, we implanted these 20% ASC/80% HUVEC co-cultures subcutaneously in athymic mice and determined if in vitro-formed vascular-like structures could integrate with the host's vascular system. In spite of limited organization of vascular-like structures in vitro, we did find human-derived lumen-containing vascular structures in vivo. Moreover, a subset of these human-derived lumens (3.6±4.2 lumens/mm2 of the total amount of 23 ±15 lumens/mm2) contained red blood cells, indicating that these lumens were able to anastomose to the host vasculature and contribute to construct vascularization. Apart from this increase in vascularization through human-derived lumens, we also showed an increase in vascularization through enhanced host vessel ingrowth in the in vitro prevascularized ASC/HUVEC constructs when compared to non-prevascularized ASC-only constructs. Collectively, these results demonstrate that the formation of vascular structures in vitro is a promising strategy to promote the in vivo vascularization of adipose ASC-based constructs. However, these results also raise new questions for future investigation. For example, it is unclear why only a limited number of the human-derived lumens were perfused upon implantation in vivo. Although Rouwkema et al [31] reported similar results for in vitro prevascularized (BMSC/HUVEC) bone tissue constructs, Levenberg et al.[35] described a high percentage (~41%) of perfused human-derived lumens within in vitro prevascularized skeletal muscle tissue constructs. It is uncertain what caused these differences, however several reasons can be proposed:

(1) Difference in endothelial organization in vitro. In our study and the study of Rouwkema et al.[31] the endothelial cells mainly formed interconnected strands within the constructs, whereas in the study of Levenberg et al.[35] lumen formation was commonly seen in vitro. This could indicate that the vascular structures formed in the study of Levenberg et al. were more mature, increasing their ability to anastomose with host vessels. This difference in endothelial organization could be caused by the use of different cells and/or the use of different culture media.
(2) Difference in extracellular environment. In the skeletal muscle tissue constructs, myoblasts were suspended in Matrigel and then seeded on poly-l-lactic-acid (PLLA)/poly lactic glycolic acid (PLGA) scaffolds, whereas in the adipose tissue and bone tissue constructs no scaffolds or matrices were used. As mentioned earlier (CHAPTER 3) the extracellular environment can affect cell behavior and cell interactions. Besides that, Matrigel is known to stimulate angiogenesis [36]. This may also have caused differences in endothelial organization and vessel ingrowth between the different tissue constructs.

Even though cellular spheroids as used in CHAPTER 5 have been shown to be able to form bigger macrotissue patches of mm^3 scale [37], the use of a biomaterial is likely needed to produce in vitro prevascularized adipose tissue constructs with more clinically relevant dimensions (cm^3 scale). As shown in CHAPTER 3 fibrin has a positive effect on adipogenic differentiation and vascularization of ASC-based constructs. Besides that, fibrin can easily be scaled-up and used in a clinical setting [38,39]. Therefore, fibrin was chosen as a biomaterial in subsequent prevascularization experiments described in CHAPTER 6.

In CHAPTER 6 we determined the optimal co-culture conditions (i.e. cell seeding density and ASC/HUVEC ratio) for in vitro prevascularization of fibrin-based ASC constructs and compared the ability of in vitro prevascularized- and non-prevascularized fibrin-based adipose tissue constructs to promote vascularization. Different mixtures (1:1, 1:2 and 1:5) of ASC with HUVEC were cultured in fibrin at two different densities ($1.0*10^6$ and $10*10^6$ cells/ml) for 7 days. We found that prevascular structures solely formed in 1:5 ASC/HUVEC fibrin-based constructs seeded with a total of $10*10^6$ cells/ml. After implantation of these constructs and ASC-only fibrin-based constructs, we found that there was no difference in the rate of vascularization of ASC/HUVEC and ASC-only fibrin-based constructs in vivo. This outcome is in contrast to the results obtained in the spheroid experiments (CHAPTER 5), where we showed that in vitro-prevascularization of spheroid constructs increased the amount and density of vascular lumens after implantation. Clearly, the specific fibrin environment is of influence on this difference in outcome. Our former experiments of CHAPTER 3 already showed that the

fibrin environment can influence ASC paracrine signaling and thus also may influence ASC-HUVEC interactions and vessel ingrowth.

We further demonstrated that human-derived vasculature formed in both prevascularized and non-prevascularized fibrin-based constructs upon in vivo implantation. This exciting finding (combined with the absence of endothelial cells in ASC-only constructs prior to implantation) supports the notion that ASC not only secrete angiogenic factors that stimulate angiogenesis, but also can differentiate towards endothelial cells and contribute to the formation of human vascular structures in vivo. This finding is especially exciting since it demonstrates the possibility of engineering vascularized adipose tissue from a single cell source. However, in the current experiments the amount of constructs containing human-derived vascular structures was low. Additionally, all fibrin-based ASC constructs that generated human-derived vasculature in vivo were derived from the same ASC donor. These results illustrate that a better understanding of which (subpopulation of) ASC differentiate(s) into endothelial cells and what factors are involved in their endothelial differentiation is indispensible for optimizing this vasculogenic potential of ASC. Altogether it is clear that the success of prevascularization depends on multiple factors. These factors amongst others include the adipose tissue engineering environment (e.g. biomaterial, culture media) and the cells (e.g. ASC donor) used. Both these factors can influence the interaction between ASC and endothelial cells and the interaction between the implanted constructs and host tissue.

CONCLUSIONS AND FUTURE PERSPECTIVES:

The aim of this thesis was to explore the angiogenic potential of ASC in different adipose tissue engineering settings and to investigate the potential positive effect of in vitro-prevascularization on the vascularization of ASC-based adipose tissue constructs. The work presented in this thesis showed that ASC have marked angiogenic potential in adipose tissue engineering settings. This angiogenic potential not only involves the secretion of angiogenic factors (CHAPTER 2, 3 AND 4) but also involves the ability to incorporate into new vessels (CHAPTER 6). Strategies that can promote ASC angiogenic potential are short (7-14 days) in vitro adipogenic cultivation (CHAPTER 2) and the use of fibrin as extracellular matrix (CHAPTER 3). Although these strategies can improve the natural vascularization response, vascular ingrowth will probably not be sufficient to supply thick adipose tissue constructs with the appropriate number of new blood vessels in a short period of time. Our studies showed that in vitro-prevascularization of engineered ASC-based adipose constructs is a promising strategy to overcome this problem (CHAPTER 5). However, our results also pointed out that the contribution of

prevascular structures to vascularization of adipose tissue constructs is as yet limited and needs to be improved. Besides that, it was also shown that different adipose tissue engineering environments yield different results regarding the success of in vitro prevascularization (CHAPTER 6). Hence, further research is required to improve the contribution of in vitro-formed prevascular structures to the vascularization in vivo and to determine the right adipose tissue engineering environment for both in vitro-prevascularization and clinical translation. Future directions of research may include:

OPTIMIZE PROTOCOLS FOR IN VITRO PREVASCULARIZATION

In this thesis ASC and HUVEC were co-cultured and seeded together to form prevascularized adipose tissue constructs. Although this approach was effective to some extent, alternative seeding and culturing protocols should be tested to optimize the formation and organization of prevascular structures in vitro. Preliminary work of Kang et al. [40] already showed that seeding HUVEC and ASC together delayed HUVEC growth. Next to seeding order, spatial cell organization can also greatly effect tissue development [41]. In order to find the optimal in vitro prevascularization method, studies should be conducted in which ASC and HUVEC are seeded in different arrangements and/or at different time points.

FOSTER THE CONNECTION OF ENGINEERED PREVASCULAR STRUCTURES WITH HOST VESSELS

Anastomosis of the engineered vessels and those of the host is critical for the in vivo-success of in vitro-prevascularization. In order to be able to foster their connection, a comprehensive understanding of the cross talk between host tissue and implanted cells is needed. Besides that, it seems more likely for mature prevascular structures to anastomose to host vessels than for immature prevascular structures. In vascular and skeletal muscle tissue engineering settings, mural-precursor cells (e.g. embryonic fibroblasts) have been shown to enhance the maturation and stability of prevascular structures in vitro, resulting in a high functionality of these structures in vivo [35,42]. Future studies should therefore test whether the addition of mural cells/ mural-precursor cells is also beneficial for the in vitro-maturation and in vivo-anastomosis of prevascular structures in our adipose tissue engineering setting. Interestingly, we found in ASC/HUVEC co-cultures (described in CHAPTER 4) that ASC expressed the smooth muscle marker alpha-smooth muscle actin, suggesting an affiliation of ASC to the smooth muscle lineage. In line with this, other studies showed that ASC could differentiate towards smooth muscle cells in vitro [43,44]. It may be worthwhile to examine the use of ASC-derived smooth muscle cells for improving the maturation of prevascular structures in our adipose ASC/HUVEC co-cultures.

STUDY THE LONG-TERM FATE AND FUNCTION OF PREVASCULAR STRUCTURES IN ASC-BASED ADIPOSE TISSUE CONSTRUCTS IN VIVO

This thesis (CHAPTER 5 and 6) shows that a subset of human-derived lumens in ASC-based adipose tissue constructs is perfused upon 7 days of implantation. In future studies it would be interesting to examine what happens to the perfused and non-perfused human-derived lumens in the long-term. Besides that, more research is crucial to assess human-derived blood vessel maturation and function in vivo. Important parameters of vascular function are: vascular perfusion, vascular permeability and tissue oxygenation/survival. In order to determine the vascular function of human-derived vasculature these parameters should be examined both in the short and the long-term. Levenberg et al. [35] already demonstrated techniques to qualify vascular perfusion (intravenous injection with fluorescently labeled lectin visualized by Xenogen IVIS device) and tissue survival (viral transfection of implanted cells with luciferase combined with injection of luciferin for determining cell survival and TUNEL staining for determining apoptotic cells). Additionally, oxygen microelectrodes or porphyrin-based oxygen sensitive dyes could be used to give an indication of tissue oxygenation [45,46,47,48].

STUDY SUITABLE ENDOTHELIAL CELL SOURCES FOR PREVASCULARIZED ADIPOSE TISSUE ENGINEERING

As mentioned earlier HUVEC are not suitable for clinical use due to their incompatibility with the recipient's immune system. Apart from being compatible with the recipient's immune system, the ideal endothelial cell source for in vitro prevascularization must be available in sufficient quantities, be able to form prevascular structures in engineered tissue and be capable of being harvested with minimal donor site morbidity. At present, it is unclear which source of mature endothelial cells or stem/ progenitor cells is most suitable for clinical application. As shown in CHAPTER 6 of this thesis ASC themselves can contribute to vessel formation in vivo. However, the number of constructs in which ASC contributed to vessel formation in vivo was low. Future research should attempt to identify which (subpopulation of) ASC contribute to vessel formation and what factors play a role in this vasculogenic potential of ASC, since a better understanding may provide clues on how to stimulate this potential. In addition, it may be also worthwhile to differentiate a selection of ASC towards endothelial cells in vitro (as has been previously shown [22,49,50]) and then use them for prevascularized adipose tissue engineering. Next to this, other potential alternatives for HUVEC should be investigated. These may include mature microvascular endothelial cells derived from adipose tissue or vascular progenitors derived from the blood or bone marrow.

STUDY ASC DONOR VARIATION

Our results in CHAPTER 6 of this thesis pointed out that there was marked heterogeneity in the ability of ASC populations to form vascular structures in vivo. Similarly, differences in adipogenic potential amongst ASC populations have been reported [51]. Multiple factors could potentially influence variations among ASC donors. Among these are age, gender, health status and genetic background of the donor. In addition, variations could also be due to differences in harvesting and culturing protocols or due to variations in the composition of ASC populations at the time of harvest. To date, there are no specific parameters that allow quick and reliable selection of ASC with clinical potential (e.g. successful formation of adipose tissue/vascular structures in vivo). ASC consist of a heterogeneous population of cells, and culture of ASC without selection for well-defined subpopulations may reinforce already existing variations in the composition of ASC derived from different donors. Future experiments using selected subpopulations of ASC with different phenotypes will need to be conducted to determine their potential for clinical application. These experiments could lead to optimized protocols for isolating and culturing ASC, resulting in better defined/characterized cell populations and less variability. Such a better definition/characterization of cells and cell-based products will also be needed to get EMEA approval for future clinical application of engineered ASC-based products. In the new European Medicines Evaluation Agency (EMEA) regulations (in effect from September 2008, www.emea.europa.eu/pdfs/human/cpwp/41086906enfin.pdf) it is stated that tissue engineered products intended for future clinical application require an extensive characterization of cells and used scaffold/matrices. Thus, to be able to meet the new EMEA regulations and to be able to qualify ASC populations for future clinical application, further research is needed to establish adequate characterization and to develop relevant efficacy essays.

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CHAPTER 8

Nederlandse samenvatting

VET WEEFSELKWEEK EN BLOEDVOORZIENING

Herstel van defecten in zachte weefsels zoals spier, bind- of vetweefsel na bijvoorbeeld trauma of tumorverwijdering vormt een reconstructieve uitdaging in de plastische chirurgie. Naast synthetische implantaten zoals siliconen wordt lichaamseigen vetweefsel vaak gebruikt voor het herstel van weke delen weefseldefecten. Het voordeel van het gebruik van lichaamseigen vetweefsel is dat het een natuurlijk zacht weefsel is, en daardoor optimale eigenschappen heeft voor opvulling van zachte weefsel defecten. Bovendien is het risico op afstoting van dit lichaamseigen weefsel nihil. Echter, het oogsten van grote hoeveelheden vetweefsel van de donorlocatie (ook wel donor site morbidity genoemd) is één van de belangrijkste nadelen van het gebruik van lichaamseigen vetweefsel voor het herstel van zachte weefsel defecten, ondanks verbetering van de huidige transplantatie technieken. Om deze reden zoeken wetenschappers een manier om levensvatbaar vetweefsel buiten het lichaam te ontwikkelen door middel van weefselkweek (ook wel vet tissue engineering genoemd). Voor het kweken van vetweefsel worden in het algemeen vet voorloper cellen gecombineerd met implantaat materialen. Humane mesenchymale stamcellen geïsoleerd uit vetweefsel, ook wel adipose-derived stem cells (ASC) genoemd, worden vaak gebruikt als vet voorloper cellen. Deze cellen zijn eenvoudig te isoleren uit vetweefsel en kunnen gemakkelijk in kweek vermeerderd worden. Bovendien kunnen zij gestimuleerd worden om zich te ontwikkelen tot vetcellen (vet differentiatie) (HOOFDSTUK 1). Verschillende studies, inclusief onze eigen studies (HOOFDSTUK 5 en 6) hebben aangetoond dat onderhuidse implantatie van deze gekweekte ASC inderdaad kan leiden tot de vorming van nieuw vetweefsel. Echter het volume/omvang van het gevormde vetweefsel na implantatie is tot op heden beperkt. Het ontbreken van een volwaardige bloedvoorziening na implantatie wordt gezien als de belangrijkste oorzaak hiervoor. Immers, onvoldoende doorbloeding van ASC in geïmplanteerde vetweefselconstructen resulteert in een tekort aan voedingsstoffen, wat er uiteindelijk voor zorgt dat de cellen in de constructen afsterven.

Verschillende methoden om de bloedvoorziening van gekweekte vetweefsel constructen te verbeteren worden momenteel onderzocht, zoals de lokale toediening van middelen die de ingroei van bloedvaten stimuleren, aanpassing van het construct ontwerp om de ingroei van bloedvaten te vergemakkelijken en het chirurgisch omleggen van bestaande bloedvaten door het vetweefselconstruct. In dit proefschrift onderzochten we de mogelijkheid van ASC (de vet voorloper cellen) om de ingroei van bloedvaten in vetweefselkweken te stimuleren (HOOFDSTUK 2 en 3). Daarnaast hebben we de mogelijk positieve effecten van het in kweek realiseren van primitieve bloedvaten in vetweefsel constructen op de doorbloeding van deze constructen na implantatie onderzocht (HOOFDSTUK 4, 5 en 6).

DE MOGELIJKHEID VAN ASC OM BLOEDVATINGROEI TE STIMULEREN (ASC ANGIOGEEN POTENTIEEL)

Onderzoek heeft uitgewezen dat ASC in staat zijn de doorbloeding van slecht of matig doorbloed weefsel (bijvoorbeeld na een infarct) te bevorderen. Men denkt dat ASC dit doen via twee mechanismen: (1) via inbouw van ASC in ingroeiende bloedvaten en (2) via de uitscheiding van eiwitten die de ingroei van bloedvaten stimuleren (angiogene factoren). Vooral het laatst genoemde mechanisme speelt een belangrijke rol in de gunstige effecten van ASC op de doorbloeding van slecht of matig doorbloed weefsel. Aangezien het stimuleren van de bloedvoorziening in slecht of matig doorbloed weefsel en het stimuleren van de bloedvoorziening in gekweekte weefsels veel overeenkomsten vertoont, veronderstelden we dat het vermogen van ASC om angiogene factoren uit te scheiden ook gunstig zou zijn voor het bevorderen van de doorbloeding van gekweekte vetweefsel constructen. Er is echter weinig bekend over de uitscheiding van angiogene factoren door ASC in vetweefselkweken. Dit deed ons besluiten hier onderzoek naar te doen.

In HOOFDSTUK 2 hebben we eerst in kweek onderzocht of vet differentiatie van ASC de uitscheiding van angiogene factoren beïnvloed. Hoofdstuk 2 laat zien dat een korte (7-14 dagen) vet differentiatie van ASC, zorgt voor een hogere uitscheiding van de angiogene factor vascular endothelial growth factor (VEGF) in vergelijking met ASC zonder vet differentiatie. Verder laat hoofdstuk 2 zien dat kweekvloeistof afkomstig van ASC met een korte (7-14 dagen) vet differentiatie bevorderend is voor de kweek van bloedvatcellen. Aan de hand van deze resultaten stellen wij dat korte (7-14 dagen) vet differentiatie van ASC in kweek gunstig is voor hun angiogeen potentieel.

In HOOFDSTUK 3 onderzochten we het effect van het implantaat materiaal fibrine op de vet differentiatie en het angiogeen potentieel van ASC in kweek. Ook hebben we het effect van fibrine op de doorbloeding van ASC-vetweefselconstructen onderzocht. Dit hoofdstuk laat zien dat de doorbloeding van ASC-vetweefselconstructen verbeterd door toevoeging van het implantaat materiaal fibrine. Verder laat dit hoofdstuk zien dat fibrine de uitscheiding van angiogene factoren en de differentiatie van ASC naar vetcellen in kweek bevorderd. Hieruit stellen wij dat fibrine het angiogeen potentieel van ASC positief beïnvloed en bovendien een geschikt implantaat materiaal is voor vetweefselkweken.

DE VORMING VAN BLOEDVAT STRUCTUREN IN ASC-VETWEEFSEL CONSTRUCTEN

Hoewel de natuurlijke ingroei van bloedvaten in ASC-vetweefselconstructen na implantatie zal kunnen worden bevorderd door stimulatie van het angiogeen potentieel van ASC, zal deze respons waarschijnlijk niet voldoende zijn om in korte tijd grote vetweefselconstructen van voldoende bloed te voorzien. Een relatief nieuwe methode om de doorbloeding van weefselconstructen te bevorderen is het (in kweek) ontwikkelen van primitieve bloedvaten in de weefselconstructen. Het idee achter deze nieuwe methode is dat cellen van de bloedvatwand een netwerk van primitieve bloedvaten kunnen vormen in weefselconstructen tijdens kweek. Als dit netwerk van primitieve bloedvaten na implantatie snel kan aansluiten op ingroeiende bloedvaten van de patient, wordt doorbloeding van het weefselconstruct sneller tot stand gebracht en wordt weefselverlies door cel sterfte voorkomen.

HOOFDSTUK 4, 5 EN 6 zijn voornamelijk gericht op het tweede doel van dit proefschrift; namelijk het onderzoeken van het mogelijke positieve effect van in kweek gerealiseerde primitieve bloedvaten op de doorbloeding van ASC-vetweefsel constructen.

Humane mesenchymale stamcellen uit beenmerg (BMSC) zijn tijdens kweek in staat de vorming van primitieve bloedvaten uit cellen van de bloedvat wand te ondersteunen. BMSC en ASC hebben veel gemeenschappelijke eigenschappen. We veronderstelden daarom dat ASC, net als BMSC, de vorming van primitieve bloedvaten uit cellen van de bloedvat wand zouden kunnen ondersteunen tijdens kweek. In HOOFDSTUK 4 vergeleken we de capaciteit van ASC en BMSC om de vorming van primitieve bloedvaten uit cellen van de bloedvat wand te ondersteunen gebruikmakende van ASC-bloedvatwand cel en BMSC-bloedvatwand cel co-kweken. In Hoofdstuk 4 hebben we laten zien dat ASC, net als BMSC, in staat zijn de vorming van bloedvat structuren uit cellen van de bloedvatwand te ondersteunen. Verder laat hoofdstuk 4 zien dat zowel de uitscheiding van angiogene factoren (in vooral ASC-bloedvatwand cel kweken) als direct cel-cel contact (in vooral BMSC-bloedvatwand cel kweken) een belangrijke rol spelen bij de vorming van deze bloedvat structuren tijdens kweek.

Of cellen van de bloedvatwand ook in staat zijn bloedvat structuren te vormen in ASC-vetweefsel constructen is onderzocht in HOOFDSTUK 5 en HOOFDSTUK 6.

HOOFDSTUK 5 beschrijft een bolvormig kweeksysteem van ASC en cellen uit de bloedvatwand voor de kweek van bloedvatrijk vetweefsel. In dit systeem onderzochten we of gelijktijdige vorming van bloedvat structuren door cellen uit de bloedvatwand en differentiatie van ASC richting vetcellen mogelijk was. Daarnaast onderzochten we of in kweek gevormde bloedvat structuren in staat zijn aan te sluiten op ingroeiende bloedvaten van de gastheer na implantatie. Hoofdstuk 5 laat zien dat cellen van de bloedvat wand zich tijdens kweek konden organiseren in bloedvat structuren, terwijl ASC dif-

ferentieerden richting vetcellen. De vorming van bloedvat structuren werd echter alleen waargenomen in kweken samengesteld uit 20% ASC en 80% bloedvat wand cellen. Daarnaast was de mate van organisatie van de in kweek gevormde bloedvat structuren beperkt. Na onderhuidse implantatie van de constructen in immuungecomprimeerde muizen echter, ontwikkelden de bloedvat structuren zich verder, wat resulteerde in aansluiting van een deel van de bloedvat structuren op ingroeiende bloedvaten. De conclusie was derhalve dat in kweek gevormde bloedvat structuren in staat zijn na implantatie bij te dragen aan de doorbloeding van een vetweefsel construct.

Hoewel de kleine bolvormige weefsel constructen, zoals beschreven in hoofdstuk 5, kunnen worden opgeschaald naar grotere weefselconstructen met een volume van mm^3, is het gebruik van een implantaat materiaal waarschijnlijk nodig om bloedvatrijk vetweefsel te maken met meer klinisch relevante afmetingen (cm^3 schaal). Hoofdstuk 3 heeft laten zien dat fibrine een geschikt implantaat materiaal is voor vetweefselkweken en bovendien een positief effect heeft op het angiogeen potentieel van ASC en de doorbloeding van ASC-vetweefsel constructen. Daarnaast kan de vorm en grootte van een fibrine construct eenvoudig worden aangepast.

In HOOFDSTUK 6 hebben we daartoe ASC en cellen uit de bloedvatwand gecombineerd in fibrine voor de kweek van bloedvatrijk vetweefsel. In dit hoofdstuk vergeleken we de doorbloeding van ASC/bloedvatwand cel-fibrine constructen met de doorbloeding van ASC -fibrine constructen na onderhuidse implantatie in immuungecomprimeerde muizen. Zeven dagen na implantatie ontdekten we dat er geen significant verschil was in de doorbloeding van ASC/bloedvatwand cel en ASC -fibrine constructen Verder vonden we na implantatie bloedvat structuren van humane oorsprong in zowel ASC/bloedvatwand cel fibrine-vetweefselconstructen als ASC -fibrine constructen. Dit doet vermoeden dat een deel van de ASC in de ASC/bloedvatwand cel-fibrine constructen gedifferentieerd zijn richting bloedvatwand cellen en bloedvatstructuren hebben gevormd.

Concluderend laat dit proefschrift zien dat ASC in vetweefselkweken een duidelijk angiogeen potentieel hebben. Dit angiogeen potentieel van ASC uit zich met name door de secretie van angiogene factoren. Mogelijke strategieën om het angiogeen potentieel van ASC te verhogen in een vetweefselkweek setting zijn korte (7-14 dagen) differentiatie van ASC richting vet en het zaaien van ASC in een fibrine mal. Hoewel de natuurlijke ingroei van bloedvaten in vetweefselconstructen zou kunnen worden bevorderd door toepassing van deze strategieën, zal deze respons hoogstwaarschijnlijk niet voldoende zijn om in korte tijd grote vetweefselconstructen van voldoende bloed te voorzien. Dit proefschrift laat zien dat het realiseren van primitieve bloedvatstructuren in vetweefselkweken voor implantatie een veelbelovende strategie is voor verbetering van de doorbloeding na implantatie. Echter in het bestudeerde vetweefselkweeksysteem is de bijdrage van de in kweek gevormde bloedvat structuren aan de doorbloeding

na implantatie nog beperkt. Bovendien lijkt het succes van deze strategie afhankelijk te zijn van het kweeksysteem dat daarvoor gebruikt wordt. Toekomstig onderzoek is nodig om de factoren te identificeren die nodig zijn voor een hogere bijdrage van bloedvat structuren aan de doorbloeding na implantatie. Daarnaast is meer onderzoek nodig om de optimale (kweek)omstandigheden te vinden voor zowel de vorming van bloedvat structuren in kweek als uiteindelijke toepassing van het gerealiseerde vetweefselconstruct in de kliniek.

DANKWOORD

Na viereneenhalf jaar onderzoek is mijn proefschrift af! En nu het zover is, zou ik graag alle mensen die mij hierbij geholpen hebben willen bedanken.

Allereerst wil ik mijn promotor, **Prof. dr. S.E.R. Hovius** bedanken. Professor Hovius, bedankt voor de mogelijkheid om op de afdeling Plastische & Reconstructieve Chirurgie mijn promotieonderzoek te mogen verrichten, wat geleid heeft tot dit proefschrift. Bedankt voor de klinische en wetenschappelijke begeleiding in de afgelopen jaren en zeker ook voor uw enthousiasme.

Vervolgens natuurlijk mijn copromotoren **Dr. Gerjo van Osch** en **Dr. Stefan Hofer MD**.

Gerjo, jouw enorm positieve instelling en enthousiasme heeft me vele keren geholpen om een experiment wat ik niet meer zag zitten toch maar uit te voeren. Ik herinner me onze wekelijkse brainstorming sessies nog goed evenals onze trips naar Londen en Porto. Bedankt voor de laagdrempelige begeleiding en de vrijheid die je me gaf. Bedankt ook dat je altijd beschikbaar was voor raad en advies, niet alleen op wetenschappelijk maar ook op meer persoonlijk vlak. Jouw enorme vakkennis en kundigheid, niet alleen op gebied van tissue engineering, maakt je tot een groot voorbeeld. Jammer dat we nu minder intensief samenwerken, daar moeten we toch snel iets voor verzinnen:).

Stefan, als initiatiefnemer van het vetonderzoek heb jij de basis gevormd en een belangrijke rol gespeeld in mijn promotieonderzoek. Enorm bedankt voor je begeleiding in mijn eerste twee jaar. Zoals ik je wel verteld heb toen je wegging naar Canada, leverde jij voor mij een sterke stimulans om het vetonderzoek voort te zetten. Je nooit aflatende steun, goede bereikbaarheid en vertrouwen in mij heb ik als erg prettig ervaren. Bedankt ook voor de mogelijkheid die je me bood om mijn (wetenschappelijke) horizon te komen verruimen in Toronto. Wie weet komt het er nog van….

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Dr. Roeland Hanemaaijer en **Dr. Reinout Stoop** hartelijk dank voor de mogelijkheid om angiogenese experimenten uit te voeren met MVECs in het TNO-lab.

Dr. Eric Farrell (or shall I say CLB ☺), you and I both know we have totally different personalities. Despite our differences however, I enjoyed every minute of our collaboration. I'd say we were a hell of a team and produced two nice papers together. Would love to pull the same trick once again in the future! Besides that, I thank you for your persistence in inviting me over and over and over again to the many cometogethers after work.

Natuurlijk wil ik ook de vele collega's met wie ik in de loop van de jaren heb gewerkt bedanken, in het bijzonder diegenen die op enig moment direct of indirect betrokken zijn geweest bij mijn onderzoek. Als eerste mijn collega's werkzaam in het lab van de Plastische & Reconstructieve chirurgie:

Sandra Posthumus-van Sluijs, met jou heb ik een heel groot deel van het onderzoek samen gedaan. Het uitvoeren van experimenten samen met jou was altijd gezellig en we waren een goed team. Ik heb veel van je nauwkeurige manier van werken geleerd en wil je bedanken voor al je geduld en hulp.

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Charlotte de Saint Aulaire, als ik iemand ken die alles uit het leven haalt wat er in zit ben jij het wel. KNO-arts in opleiding, OIO, trouwen en dan ook nog een prachtige dochter. Je enthousiasme en energie zijn aanstekelijk en ondanks je zo volle agenda maak je altijd tijd vrij voor een praatje. Ik heb erg genoten van je verhalen over je klinische ervaringen en heb je sollicitatiehulp als heel waardevol ervaren. Fijn dat je mijn collega was, ik hoop dat ik voor KNO-advies later nog bij je terecht kan ☺.

Urville Djasim, als kamergenoot heb ik mogen genieten van de mooie zelfgemaakte foto's op jouw desktop. Naast deze mooie foto's hebben jouw enthousiasme voor onderzoek en je droge humor me keer op keer opgevrolijkt. Bedankt ook nog voor je feedback en voor het ongewild oplossen van heel wat van mijn computer/software gerelateerde problemen…..

Miao Tong, you were a wonderful roommate and colleague. I always enjoyed our talks and discussions (whether it was about research or Urville's hair ☺). I wish you all the best finishing your own thesis.

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CURRICULUM VITAE

Femke Verseijden was born on the 10th of March 1980 in Alkmaar, The Netherlands. After finishing High school in 1998, she went to Amsterdam to study Economics and received her Bachelor's degree with honors in September 1999. She then started with the study Veterinary Medicine at the Faculty of Veterinary medicine (Utrecht University). After receiving her Master degree in August 2005, she started from December 2005 as a PhD student at the Department of Plastic & Reconstructive Surgery of the ErasmusMC, University Medical Center Rotterdam under the supervision of Dr. G.J.V.M.van Osch, Dr. S.O.P. Hofer M.D. and Prof.dr. S.E.R. Hovius M.D.. The subject of her research was adipose tissue engineering with a special focus on vascularization of adipose tissue constructs and the results are presented in this thesis. From May 2010, she started her clinical internship in the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University under supervision of Prof.dr. L.J. Hellebrekers.

PHD PORTFOLIO SUMMARY

Name PhD student:	Femke Verseijden
Erasmus MC Department:	Plastic & Reconstructive Surgery
PhD period:	December 2005- May 2010
Promotor:	Prof. dr. S. E. R. hovius
Co-promotors:	Dr. G. J. V. M. van Osch, dr. S. O. P. Hofer M.D.

PhD training	**Year**
In-depth courses	
• Cell-based Therapies and Tissue Engineering Short-Course (Case Western Reserve University in Cleveland, Ohio).	2007
• Basic Course in Microsurgery (EUR)	2007
• Laser Scanning Microscopy (EUR)	2008
• Scientific Writing in English for publication-writing to be read (EUR)	2009
(Inter)national conferences, poster presentations	
• Angiogenic factors in human adipose-derived stromal vascular cells during adipogenic differentiation at the Tissue Engineering and Regenerative Medicine Society TERMIS-EU in Rotterdam, The Netherlands.	2006
• Angiogenic factors in human adipose-derived stromal vascular cells during adipogenic differentiation at the Nederlandse Vereniging voor Biomaterialen en Tissue Engineering (NBTE) in Lunteren, The Netherlands.	2006
• In vitro investigations to improve vascularization in adipose tissue constructs at the NBTE in Lunteren, The Netherlands.	2007
• In vitro model of angiogenesis by adult bone marrow-derived stromal cells and adipose-derived stromal cells in a fibrin matrix at the TERMIS-EU in Porto, Portugal.	2008
• The use of adipose tissue-derived stromal cells for soft tissue generation at the 2nd International Symposium Stem cells, Development and Regulation (SCDD) in Amsterdam, The Netherlands.	2008
• Engineering vascularized human adipose tissue at the TERMIS -EU in Galway, Ireland.	2010

(Inter)national conferences, podium presentations

- Keynote presentation: Vascularized adipose tissue engineering at the TERMIS-EU in London, United Kingdom. 2007
- Engineering human vascularized adipose tissue at the NBTE in Lunteren, The Netherlands. 2008
- Engineering human vascularized adipose tissue at the European Conference of Scientists & Plastic Surgeons (ECSAPS) in Rotterdam,The Netherlands. 2009
- Human bone marrow- and adipose-derived stromal cells support prevascularization at the NBTE in Lunteren, The Netherlands 2009
- Adipose tissue engineering: comparing scaffold-free and fibrin-based constructs at the TERMIS-EU in Galway, Ireland. 2010

Nominations

- Nominated for the best oral presentation at the ECSAPS 2009

Other

- Chair: Skin tissue engineering session at the TERMIS-EU in London, United Kingdom. 2007
- Chair: Angiogenesis/Vascularization session at the ECSAPS in Rotterdam, The Netherlands. 2009

Teaching activities

- Lecture adipose-derived stem cells and tissue engineering, 3rd year medical students 2006
- Lecture adipose-derived stem cells and tissue engineering, 3rd year medical students 2007
- Lecture adipose-derived stem cells and tissue engineering, 3rd year medical students 2008
- Supervising stem cell isolation experiments, 6th year medical student 2010

LIST OF PUBLICATIONS

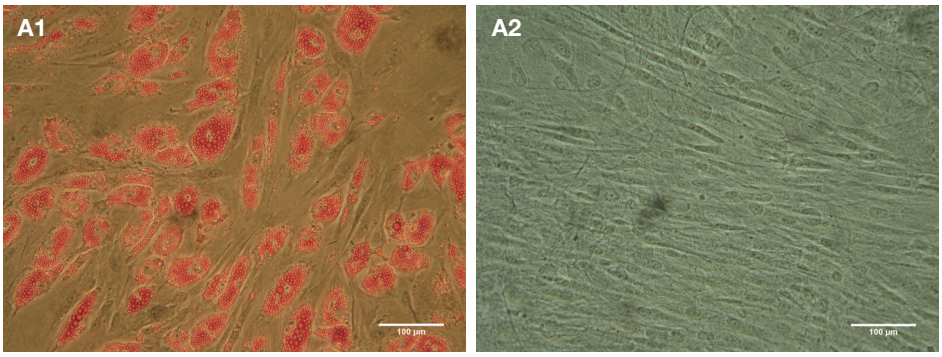
Prevascular structures promote vascularization in engineered human adipose tissue constructs upon implantation. **Femke Verseijden**, Sandra J. Posthumus-van Sluijs, Eric Farrell, Steven E.R. Hovius, Johan W. van Neck, Stefan O.P. Hofer, Gerjo J.V.M. van Osch. **Cell Transplantation** 2010 [Epub ahead of print].

Adult human bone marrow- and adipose tissue-derived stromal cells support the formation of primitive vascular-like structures from endothelial cells in vitro. **Femke Verseijden**, Sandra J. Posthumus-van Sluijs, Predrag Pavljasevic, Gerjo J.V.M. van Osch, Eric Farrell. Tissue Engineering Part A. 2010, 16(1): 101-114.

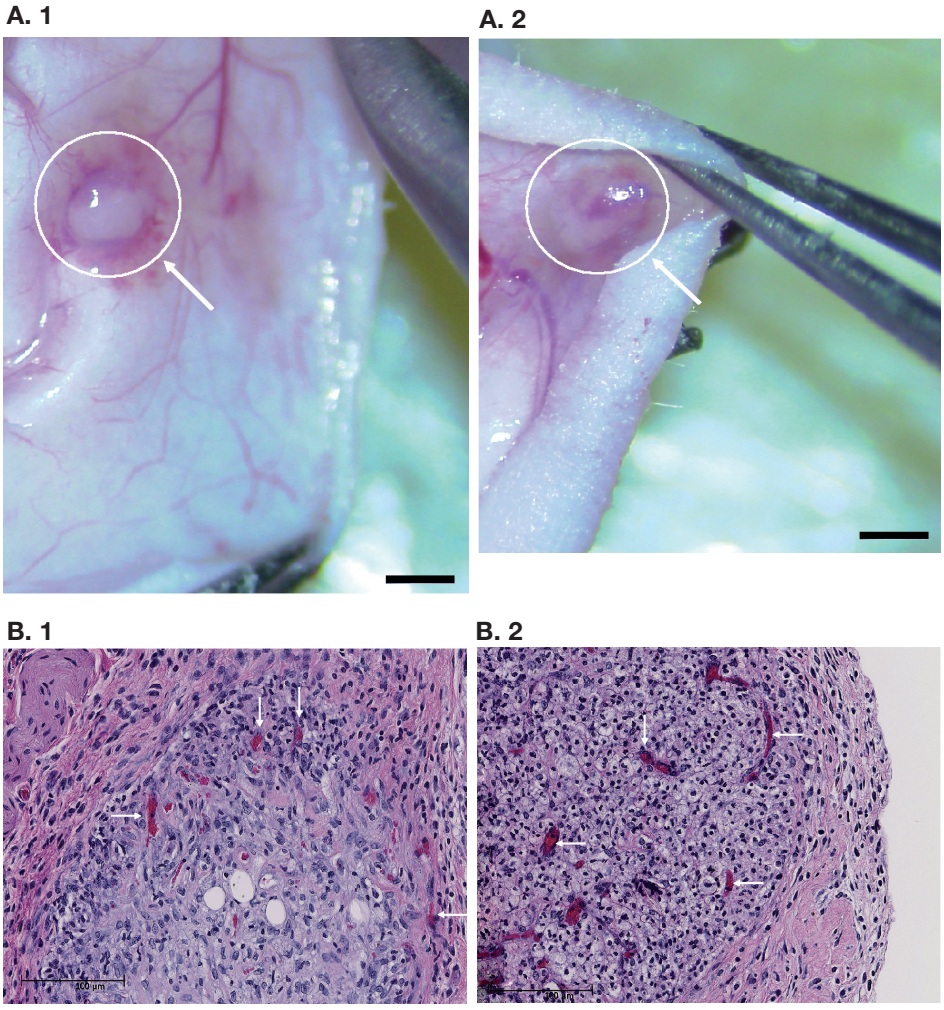
Angiogenic capacity of human adipose-derived stromal cells during adipogenic differentiation: An in vitro study. **Femke Verseijden**, Holger Jahr, Sandra J. Posthumus-van Sluijs, Timo L. Ten Hagen, Steven E.R. Hovius, Ann L.B. Seynhaeve, Johan W. van Neck, Gerjo J.V.M. van Osch, Stefan O.P. Hofer. Tissue Engineering Part A. 2009, 15(2): 445-452.

Androgen receptor CAG repeat polymorphisms in canine prostate cancer. Chen-Li Lai, Henry L'Eplattenier, René van den Ham, **Femke Verseijden**, Astrid Jagtenberg, Jan A. Mol, Erik Teske. Journal of Veterinary Internal Medicine 2008, 22(6):1380-1384.

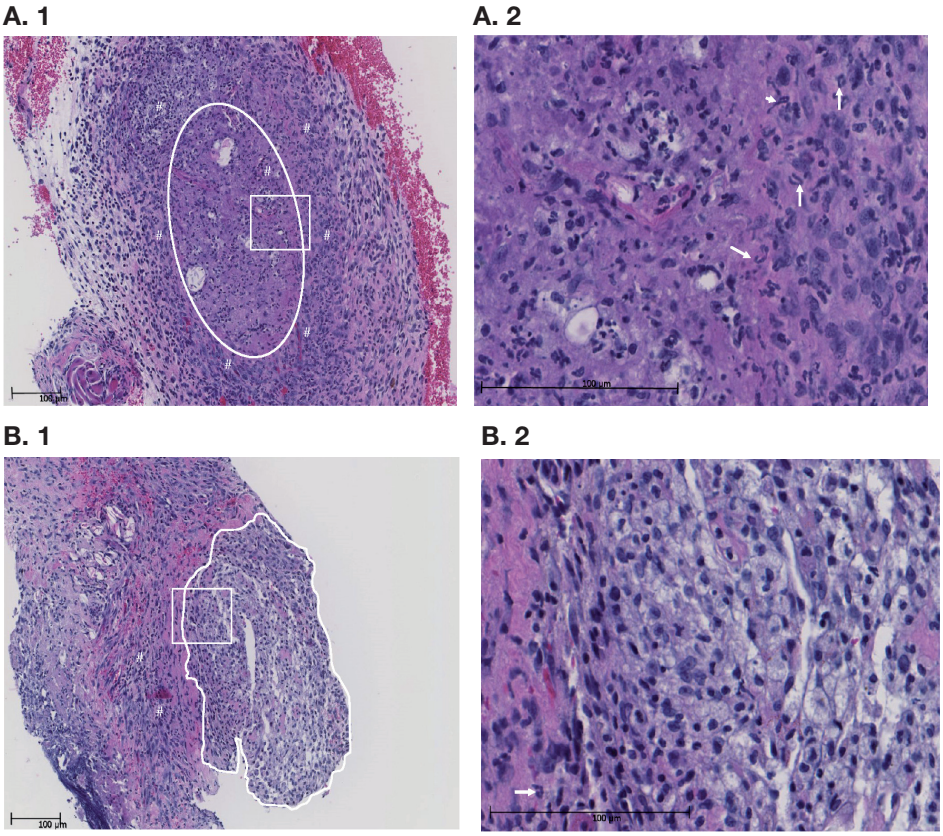
COLOR FIGURES



Chapter 2 Figure 1.



Chapter 3 Figure 1.



Chapter 3 Figure 2.

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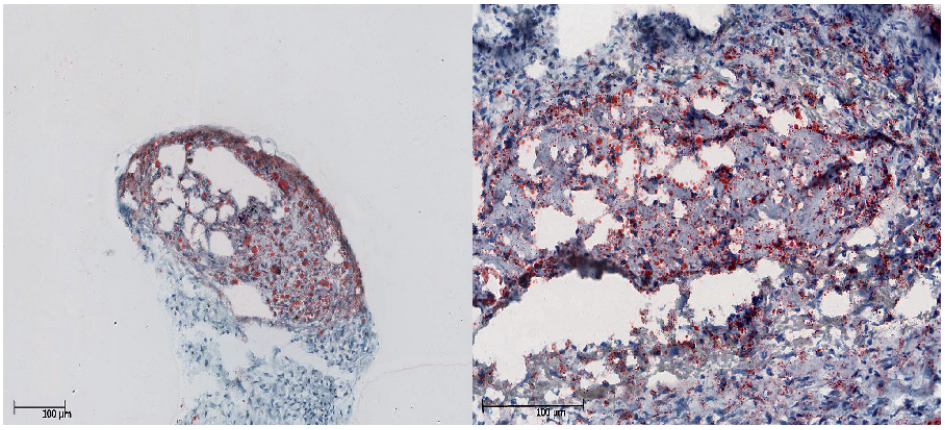
B. 2



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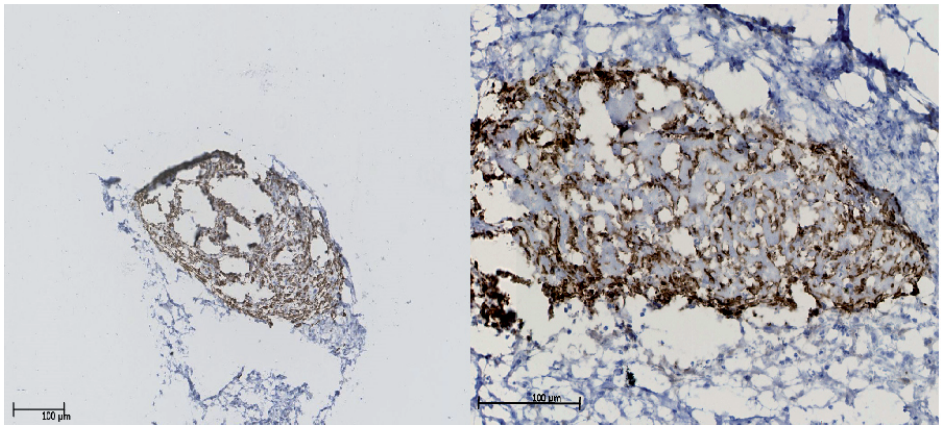

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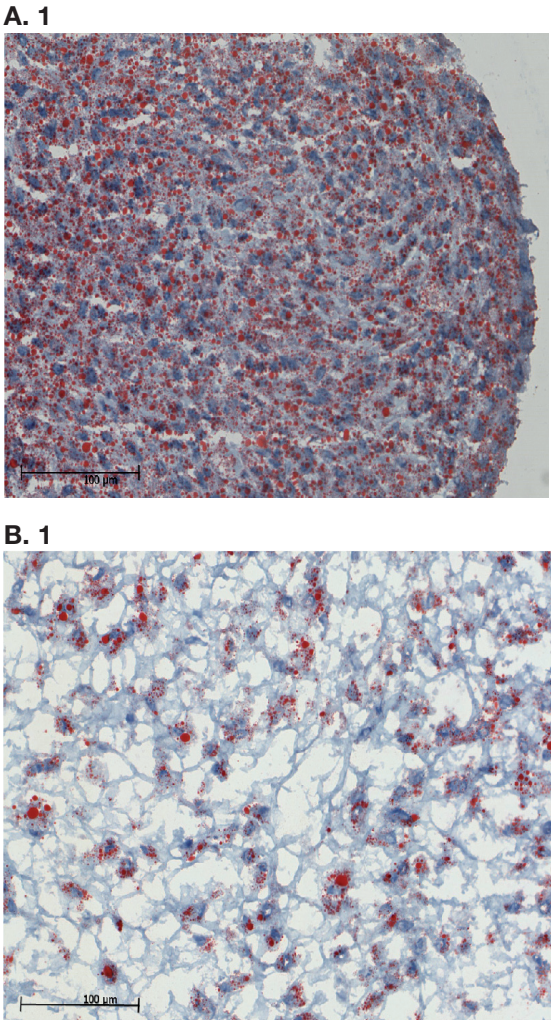

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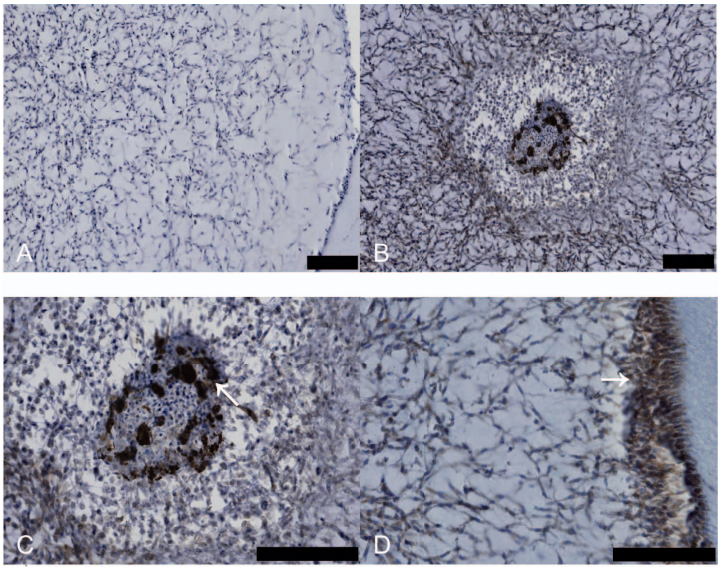

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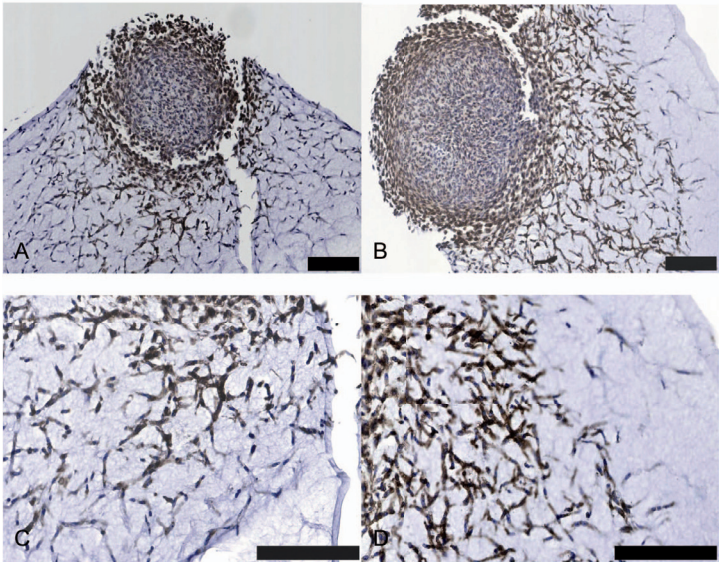

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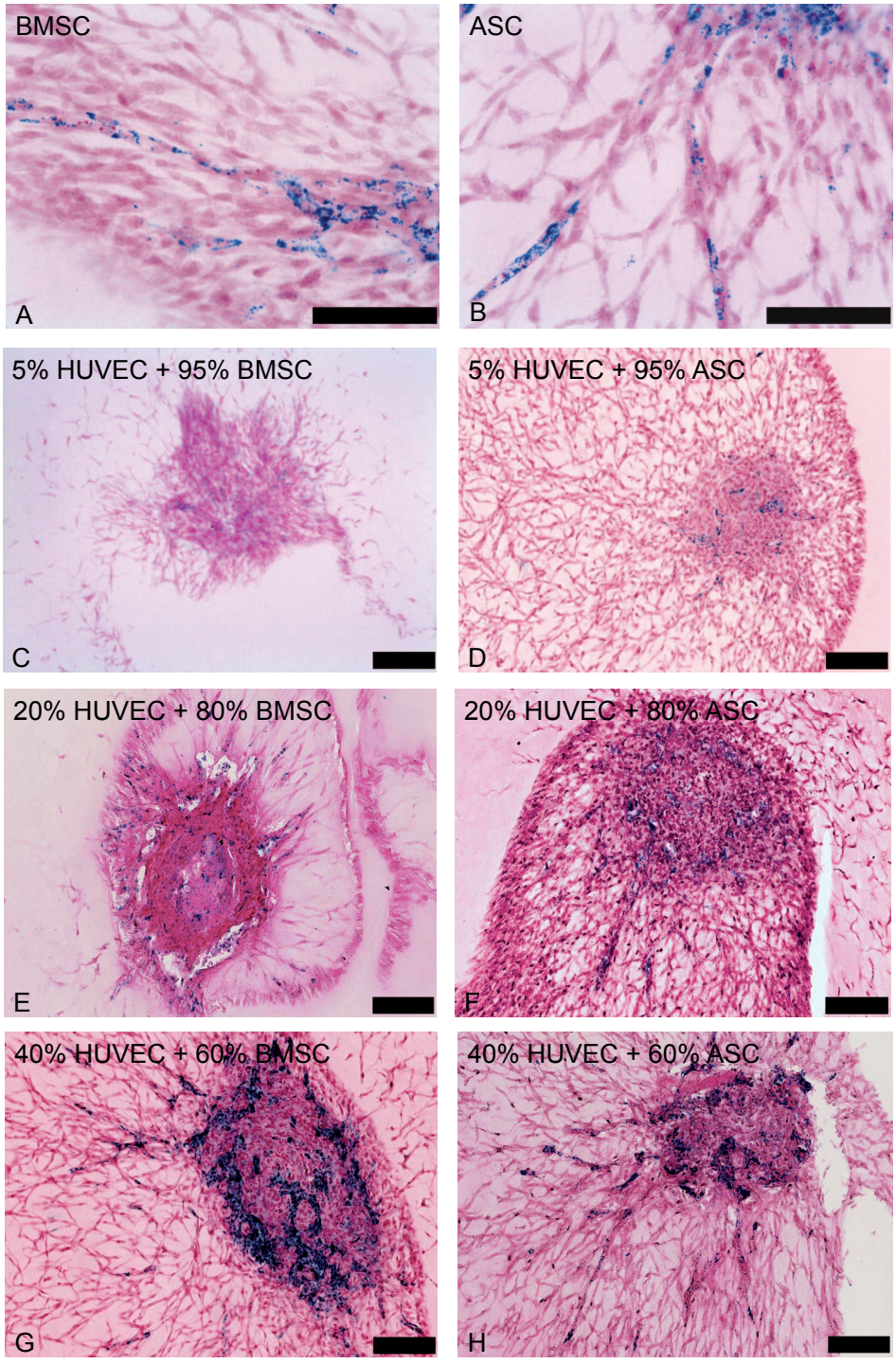

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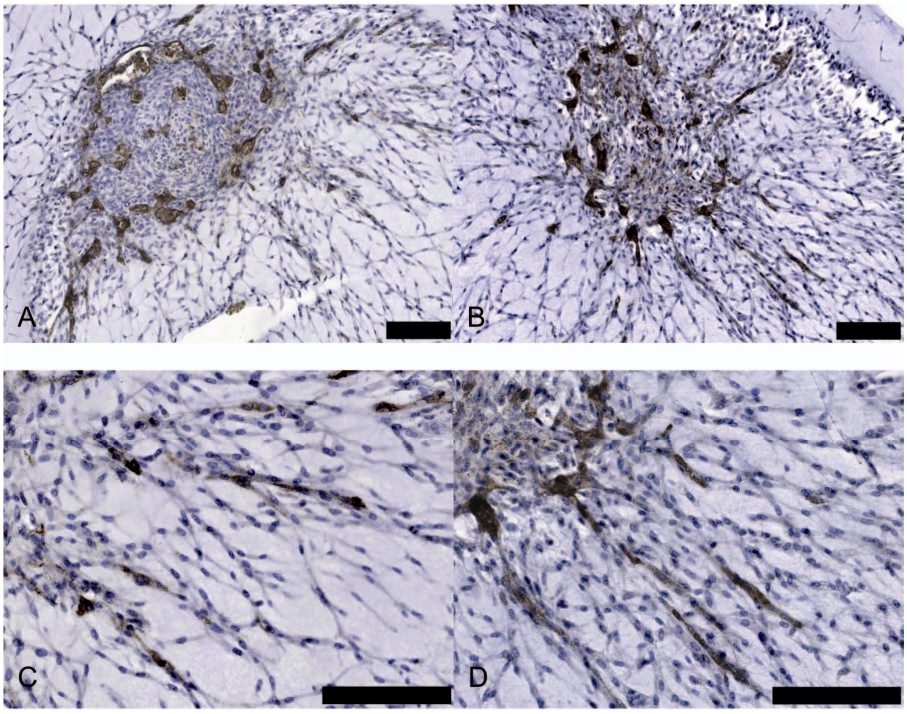

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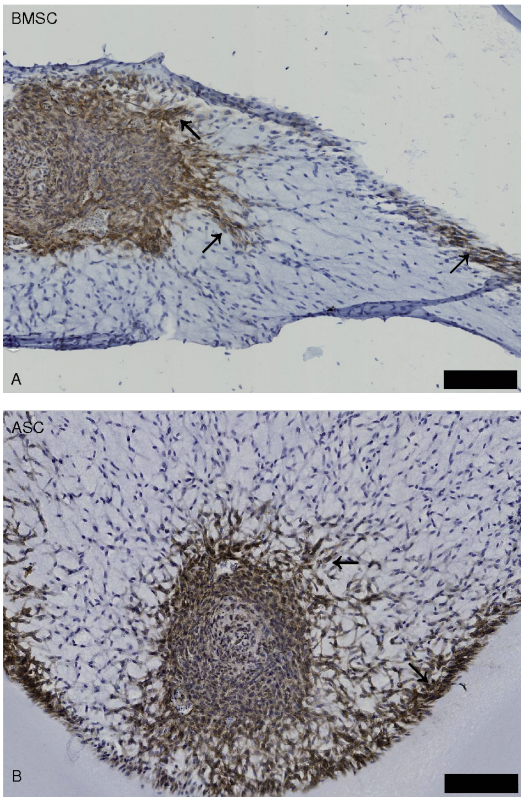

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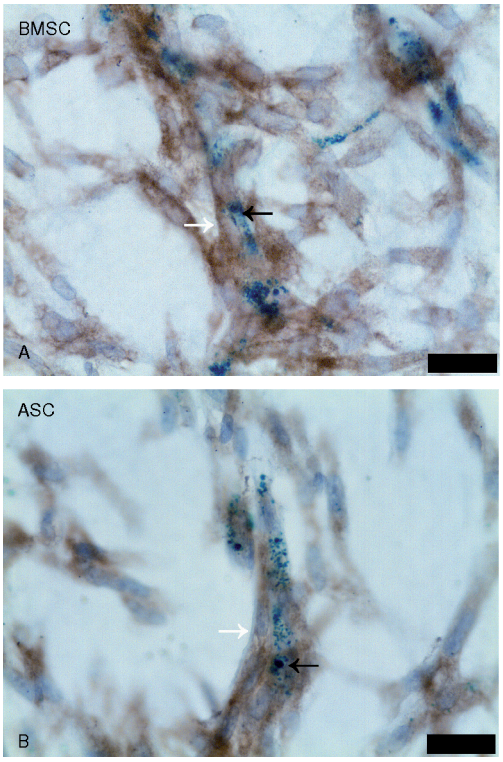

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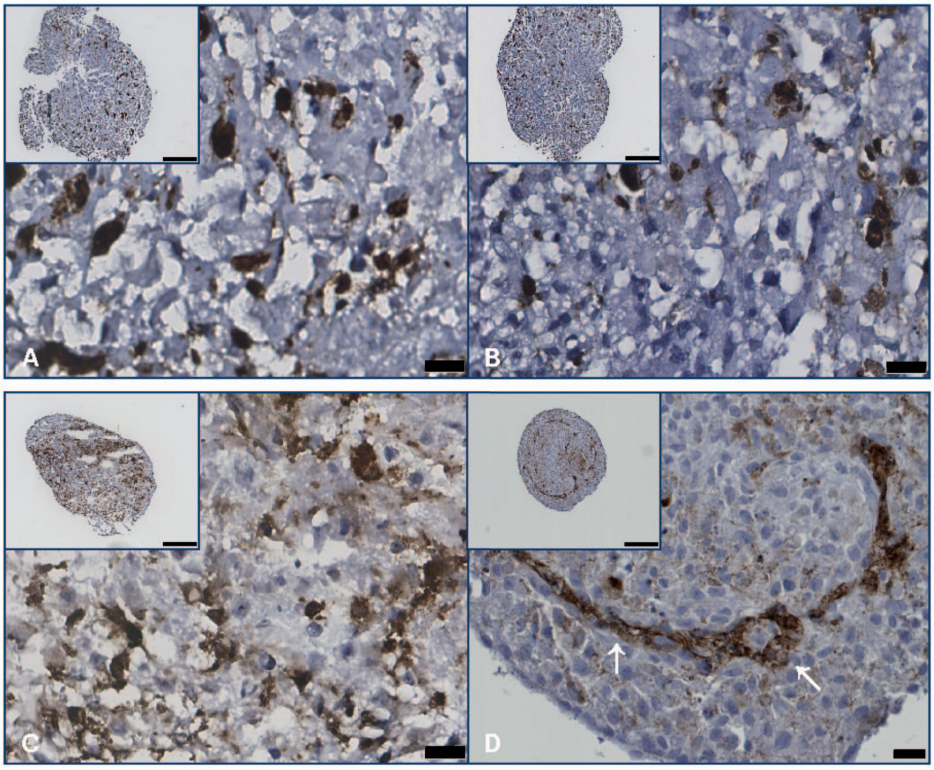

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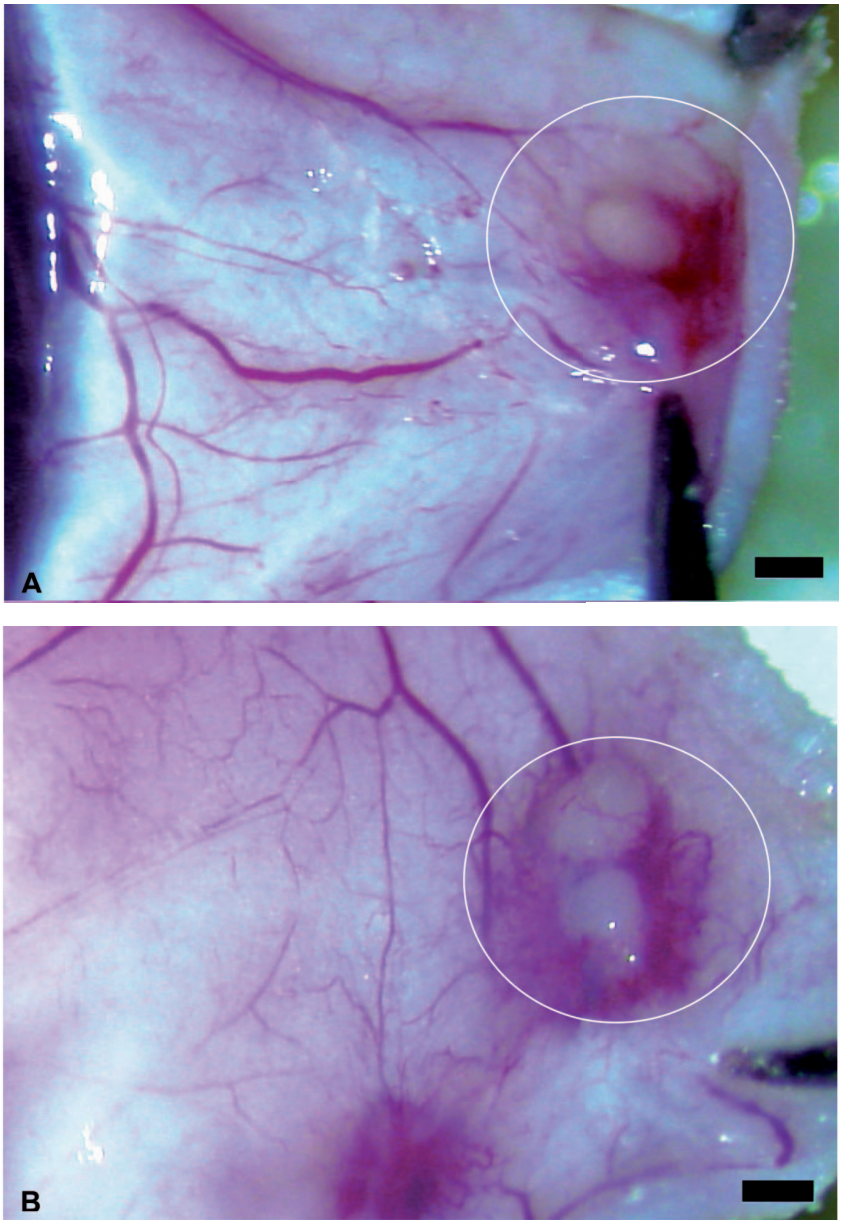

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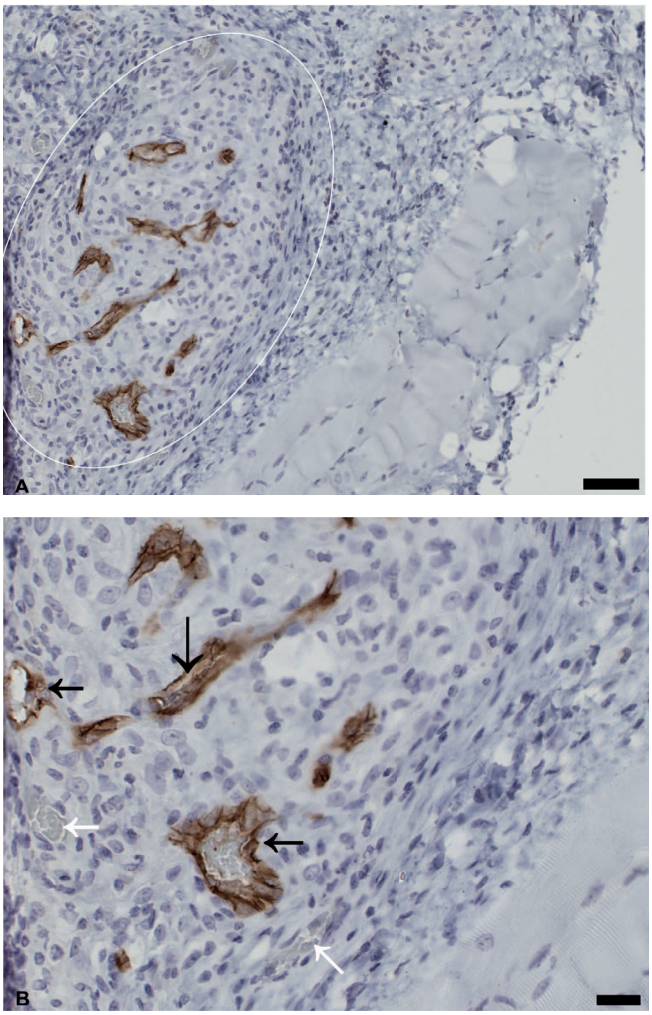
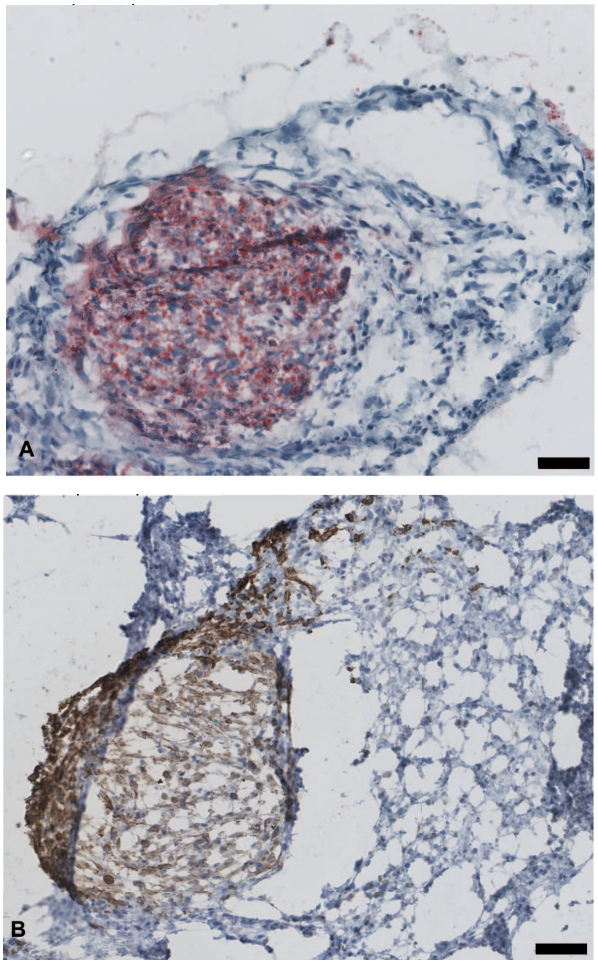
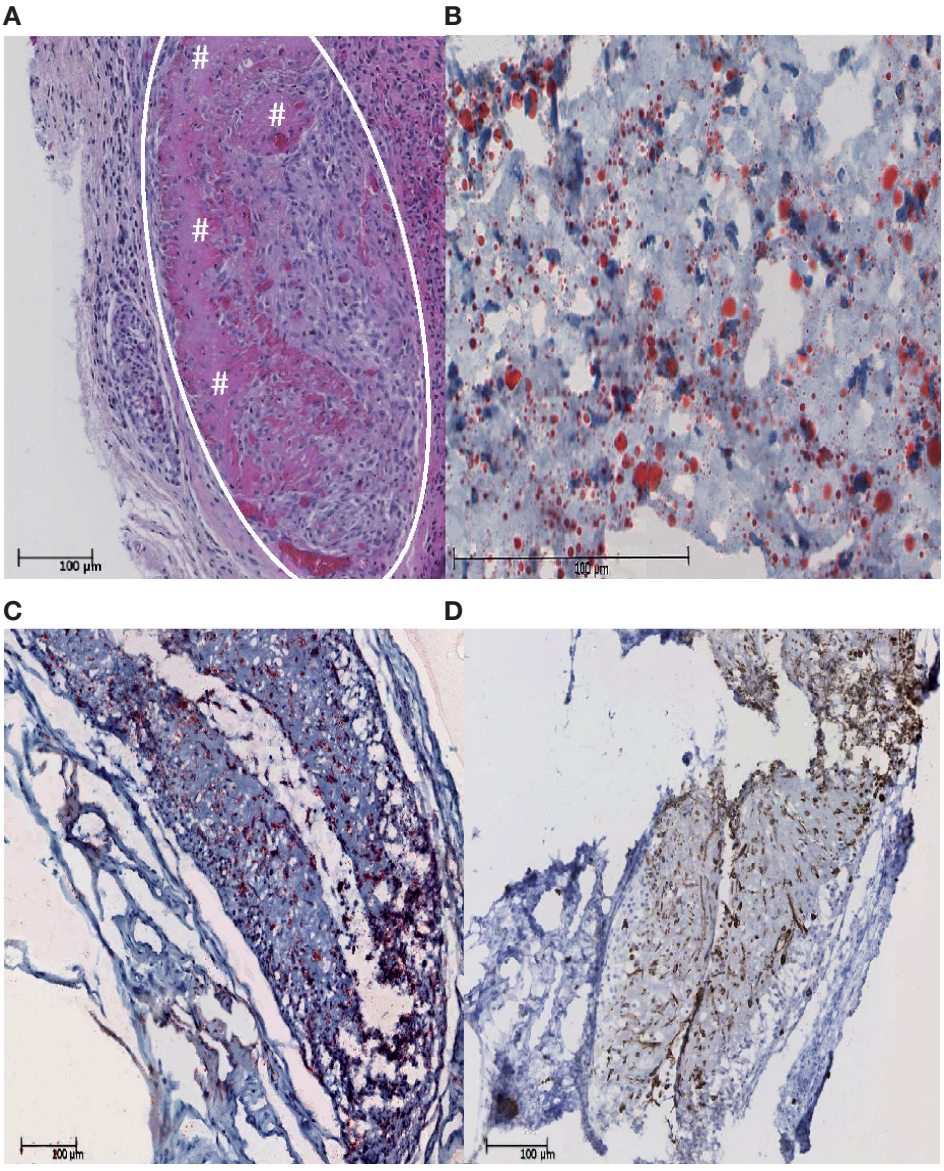


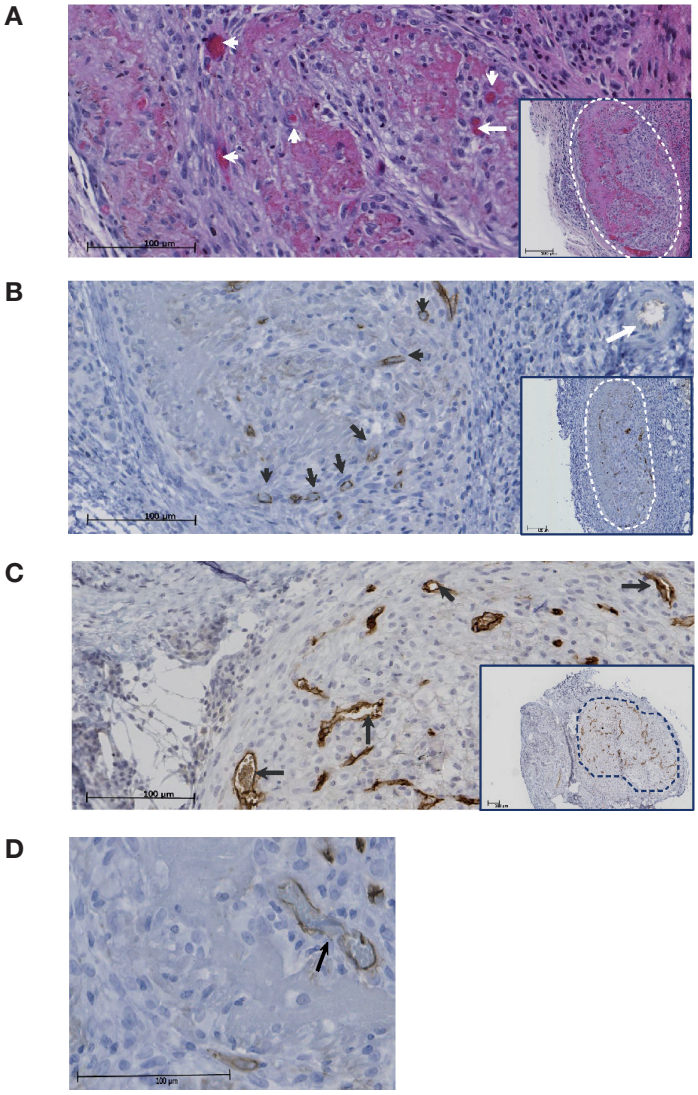
Chapter 5 Figure 6.



Chapter 5 Figure 7.



Chapter 6 Figure 2.



Chapter 6 Figure 3.