Adipose Tissue Engineering - *In vitro* Development of a Subcutaneous Fat Layer and a Vascularized Adipose Tissue Construct utilizing Extracellular Matrix Structures

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Etwas Kurzgesagtes kann die Frucht und Ernte von vielem Langgedachten sein.

Friedrich Nietzsche

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1. Introduction and goals of this thesis

A lack of the subcutaneous fat layer resulting from severe injuries, post-operative or post-traumatic loss often requires adipose tissue restauration to improve the patient's physical and emotional well-being [Bauer-Kreisel 2010, Patrick 2001]. The American society of plastic surgeons has reported a large increase in reconstruction procedures in recent years [ASPS 2012]. Moreover, reconstructive plastic surgeries, which improve functions and appearances of abnormal structures, also increased 5 % in 2011 in the USA [ASPS 2012]. Therefore, the demand for adipose tissue in reconstructive and plastic surgery has substantially increased. Current strategies comprise transplantation of autologous adipose tissue or composite tissue flaps, as these are available, biologically functional and biocompatible [Markey 2000, Nahabedian 2002]. However, the amount of adipose tissue needed, the unpredictable survival rate and the donor site morbidity require another source of adipose tissue.

Tissue engineering strategies have been investigated to develop adipose tissue to overcome the current limitations in plastic and reconstructive medicine. A predominant strategy to generate three-dimensional adipose tissue is to use an autologous cell source on a scaffold system which is biodegradable and biocompatible [Yang 2006, Burd 2007, Tabata 2009]. Adipose-derived mesenchymal stem cells (ASC) isolated from lipoaspirations or liposections are readily available autologous cells. ASC are promising for adipose tissue engineering as it has been shown that they proliferate rapidly and differentiate into adipocytes *in vitro* and *in vivo* [Sterodimas 2010, Philips 2012]. There are various applications for adipose tissue in plastic and reconstructive surgery, and different strategies may be utilized to engineer the best fitting and easiest to apply adipose tissue. This thesis focuses on two specific applications of adipose tissue:

I. Engineering an adipose tissue construct for subcutaneous fat layer reconstruction to functionalize and cushion hypertrophic scar tissue in revision surgery after full-thickness burns or complex injuries.

II. Generating vascularized adipose tissue *in vitro* with a longterm perspective for large-volume augmentation in plastic and reconstructive surgery or for basic research of adipose tissue and as a substitute for animal models in pharmacological trials.

1.1 Subcutaneous adipose tissue layer

Complex injuries and massive burns in the upper and lower extremities frequently result in extensive and hypertrophic scar formation. This, in turn, may cause severe pain and constrains the range of motion through adhesion of scar tissue to mobile structures like nerves, blood vessels or tendons [Moore 2009, Gabriel 2011]. To avoid the adhesion of these structures a substitute for the missing adipose tissue is desirable, which adds volume and also cushions the scarred tissue to regain tissue elasticity. A promising scaffold material is collagen, as it is available in large amounts, biocompatible and has been shown to possess a regenerative potential. In many surgical applications collagen demonstrated revascularization and recellularization *in vivo*. It was shown in several tissue engineering studies that many cell types readily adhere and proliferate on these scaffolds [Arca 2012, Jarman-Smith 2004, Mulier 2011, Elgharably 2013]. A combination of these scaffold materials as cell carrier and autologous ASC harvested from liposuctions may result in a thin subcutaneous fat construct which is easy to dose and to handle in regard of location control. The idea was to employ ASC and a thin, commercially available collagen scaffold to engineer a functional subcutaneous tissue construct for the use in plastic and reconstructive surgery. Therefore, the first goal of this thesis was to develop an adipose tissue layer

in vitro that may serve as the basis for further *in vivo* applications in the upper and lower extremities.

The relevant data is described in chapter 4.1/ 5.1 and will be published in part in "Werner K., Jakubietz M., Jakubietz R., Schmidt K., Muhr C., Bauer-Kreisel B., Blunk T. Towards reconstruction of the subcutaneous fat Layer utilizing adipose-derived stromal cell-seeded collagen matrices. *Cytotherapy*, accepted (09.06.2014)".

1.2 Vascularized adipose tissue

The vascularization is a major factor in rebuilding large volumes of adipose tissue after tumor resection, traumatic injuries or generally generating natural adipose tissue. Due to the fact that insufficiently supplied tissue undergoes necrosis, the augmentation with non-vascularized tissue would result in volume loss and deformation [Reilly 2007, Bucky 2008]. Therefore, the engineered construct has to be highly vascularized similar to nature tissue, where every adipocyte is at least attached to one capillary [Schwegler 2011]. The integration of a vascular pedicle to anastomose the nutrient and oxygen supply may be a useful strategy for clinical use or *in vitro* perfusion in a bioreactor system.

The decellularized porcine jejunum established by Walles and coworkers is a promising tool for engineering vascularized tissues *in vitro* [Mertsching 2009, Scheller 2013]. It provides an arterial and venous inlet, a decellularized vascular structure throughout the construct which can be reseeded with endothelial cells, and an extracellular matrix structure which can function as a scaffold for the combination with other cell types. The extracellular matrix (ECM) mainly consists of collagen type I/III and elastin which were demonstrated to support cell adhesion and proliferation [Mertsching 2009, Pusch 2011]. This collagen-elastin structure can also be

reorganized by newly formed tissue. Thus, the second goal of this thesis was to establish, for the first time, a vascularized adipose tissue *in vitro* on the basis of the decellularized porcine jejunal segment and a co-culture of ASC and microvascular endothelial cells.

2. State of art

2.1 Adipose tissue engineering

Tissue engineering is an interdisciplinary field which deals with the establishment of natural tissue under artificial conditions. The focus of this study lies on generating two kinds of adipose tissue *in vitro*, a thin subcutaneous fat layer and a vascularized adipose tissue construct.

2.1.1 Characteristics of adipose tissue

The construction of a functional fat substitute as therapeutic alternative requires knowledge about structure, mechanical properties, biological function, and typical characteristics of the natural tissue.

Adipose tissue is a special type of connective tissue which mainly consists of fat cells and is specialized to synthesize and store triglyceride within a network of fibers. Mature fat cells (adipocytes) exist in two forms in the human body, brown adipose tissue (BAT) and white adipose tissue (WAT) [Minuth 2003]. BAT is composed of multivacuolated adipocytes and is especially abundant in infants. Its primary function is to convert energy, stored in small lipid droplets, into body heat. This adipose tissue is highly vascularized due to the demand for oxygen and contains a high number of mitochondria which stain the tissue brown, due to their cytochrome [Minuth 2003, Cannon 2004, Rosen 2006].

This work focuses on WAT. WAT fulfills many different functions in the body, and it is the principal type of adipose tissue in adults. In WAT the adipocytes exist as univacuolated cells. They consist of one large fat droplet and the cytoplasm with the nucleus being pressed to the membrane of the cell. The fat vacuole and the flattened cell nucleus on one side give the cells the classical-termed signet ring appearance

[Minuth 2003, Napolitano 1963]. White adipose tissue is not as vascularized as brown adipose tissue, but each adipocyte is connected to at least one blood vessel, thereby nutrients can be delivered and metabolites removed.

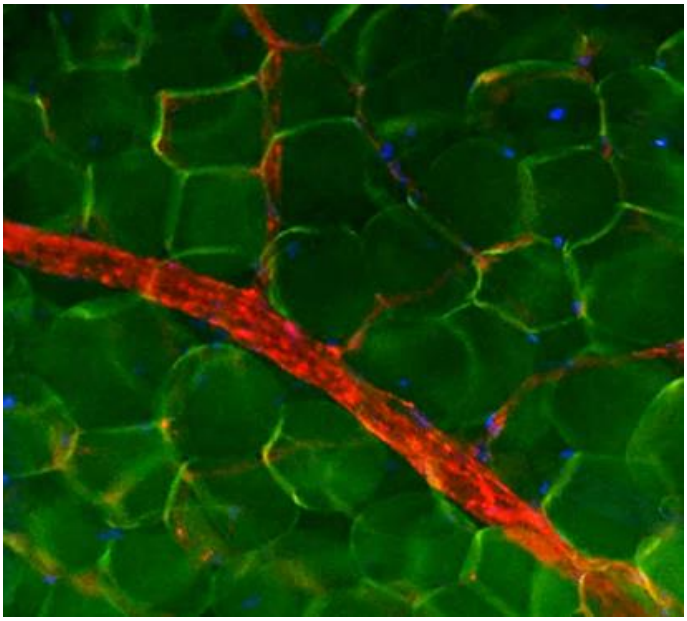


Fig. 1: White adipose tissue.
A typical morphology of white adipocytes displays a central fat vacuole and a nucleus pressed against the cell membrane. White adipose tissue is highly vascularized, with at least one vessel connected to each adipocyte. This native adipose tissue is stained with BODIPY (green) for triglycerides in the vacuole of adipocytes, with Cy3-labeled antibody (red) against CD31, an integral membrane protein expressed on the surface of endothelial cells, and DAPI for cell nuclei.

Adipose tissue is dispersed over the whole body, mainly as subcutaneous and as visceral fat [Rosen 2006, Napolitano 1963, Ailhaud 1992, Wronska 2012]. In addition to the adipocytes, adipose tissue contains stromal vascular cells including fibroblasts, endothelial cells, macrophages, stem cells and preadipocytes [Ailhaud 1992, Wronska 2012]. The energy balance is modulated through the nutrition supply and storage, which in turn is regulated by number and size of the adipocytes. Adipose tissue mass can be increased by two mechanisms: first through hyperplasia, by increasing the cell number, and second through hypertrophy, which is an increase of the cell size by elevated fat storage. In the lipid metabolism the triglycerides can be stored or mobilized as lipid droplets which are increased and enlarged or decreased and diminished within the cell [Ailhaud 1992, Wronska 2012, Gregoire 1998].

Adipocytes are formed by mesenchymal stem cells throughout a life-span. Fat cell development can be divided into two phases, determination and differentiation. In the first step a multipotent mesenchymal stem cell develops into a unipotent adipocyte progenitor cell which still can proliferate, but is already committed to the adipogenic lineage. With the corresponding environmental stimuli the preadipocytes differentiate and mature into adipocytes [Rosen 2006, Napolitano 1963, Gregoire 1998].

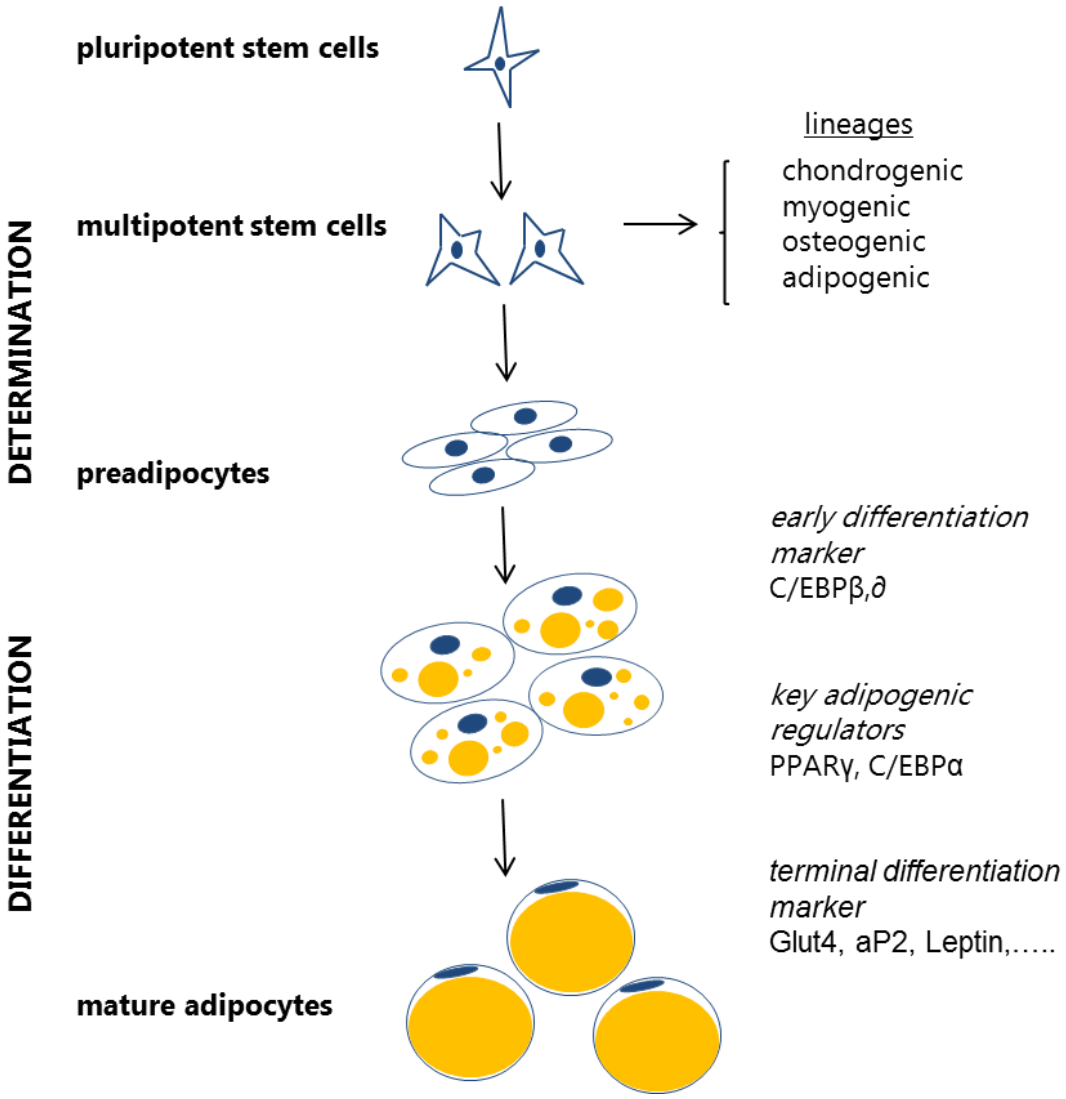


Fig. 2: Schema of adipogenesis.
In the determination phase, stem cells are committed to one specific cell type, which can proliferate and are defined as progenitor cells. With the adequate stimuli the preadipocytes differentiate into mature adipocytes. Some adipogenic transcription factors and marker genes, associated with the various stages in adipogenic differentiation, are noted here. (C/EBPα/β/∂ = CCAAT/enhancer binding protein α/β/∂, PPARγ = peroxisome proliferator-activated receptor γ, Glut4 = glucose transporter 4, aP2 = fatty acid binding protein). The scheme was modified after Flynn 2008/b and Ailhaud 1992.

The differentiation is a complex multi-step process which is well characterized by *in vitro* studies of, e.g., 3T3-L1 mouse preadipocyte cell lines [Green 1975, Fischbach 2004/a, Avram 2007]. The adipogenesis is primarily controlled by the transcription factors CCAAT/enhancer binding proteins (C/EBPα, β and *∂*) and peroxisome proliferator activated receptor γ (PPARγ), which act cooperatively to promote adipocyte differentiation. C/EBPβ and C/EBP*∂* are expressed very early in adipogenesis and stimulate the reciprocal up-regulation of PPARγ and C/EBPα, the key regulators in adipogenic differentiation. Both regulators initiate directly and indirectly the expression of adipogenic target genes, e.g. encoding glucose transporter 4 (Glut4) or fatty-acid-binding protein (FABP4 respectively aP2) [Rosen 2006, Ailhaud 1992, Gregoire 1998, Lowe 2011, Farmer 2006, Henry 2012]. Simultaneously, the appearance of the cell changes from the fibroblastic morphology to the signet ring shape. Although the mature adipocytes are very fragile, they function as structural and storage fat and have to withstand serious mechanical strains. Therefore, the extracellular matrix (ECM) is also altered during adipogenesis. For example, collagen type I is decreased and collagen type IV is increased. To stabilize the adipose tissue, a fibrillar network of collagen IV, V and laminin is built during adipogenesis as outer sceleton to protect mature adipocytes from mechanical disruption [Gregoire 1998, Henry 2012, Flynn 2010, Nakaijma 1998, Divoux 2011].

Besides its function as energy depot and isolation material adipose tissue has been established to be an immunological and active endocrine organ, which secretes cytokine and complement factors, also known as adipokines. Adipocytes are involved in insulin regulation, glucose balance, vascular homeostasis and through the lipid metabolism in energy balance [Henry 2012, Guerre-Millo 2002, Halberg 2008, Trayhurn 2008]. This thesis will focus on the function as structural fat, which determines the body contours and the mechanical protection. It has a cushioning effect to mobile structures and provides tissue elasticity in the extremities as well.

2.1.2 Strategies of tissue engineering

Adipose tissue engineering aims to construct a fat equivalent with the mechanical structure and biological functions of the natural adipose tissue to remodel body contours and to gradually regenerate the artificial tissue into autologous tissue.

Regardless which approach is chosen, the constructs have to be mechanically fitted to the application area *ex vivo* and integratable into the surrounding tissue *in vivo*. A specific volume and shape have to be constructed dependent on the application. Additionally, a sufficient vascularization has to be provided to avoid tissue resorption. There are two main strategies to generate tissue constructs, differing essentially whether cells are seeded on the constructs or not [Bauer-Kreisel 2010].

In the first strategy, a scaffold with adipose tissue inducing-substances is implanted to induce a de novo adipogenesis *in vivo*. These factors stimulate the migration of cells into the scaffold, the adipose differentiation and ideally also the ingrowth of blood vessels. The integration of a pre-existing vasculature is an option to accelerate the *in vivo* development of vascularized adipose tissue.

In the second and commonly used strategy, cell-based constructs are implanted to generate adipose tissue (Fig. 3). Cells used in this approach are mainly harvested from donor tissue and are expanded until the desired number is reached. Afterwards, these cells can be seeded onto or into a matrix which functions as a cell carrier. The cell-seeded matrix can be either transplanted directly or after further cultivation and adipogenic differentiation *in vitro*. To enhance angiogenesis in the artificial tissue *in vivo,* growth factors or blood vessels can be integrated into the constructs before implantation [Bauer-Kreisel 2010].

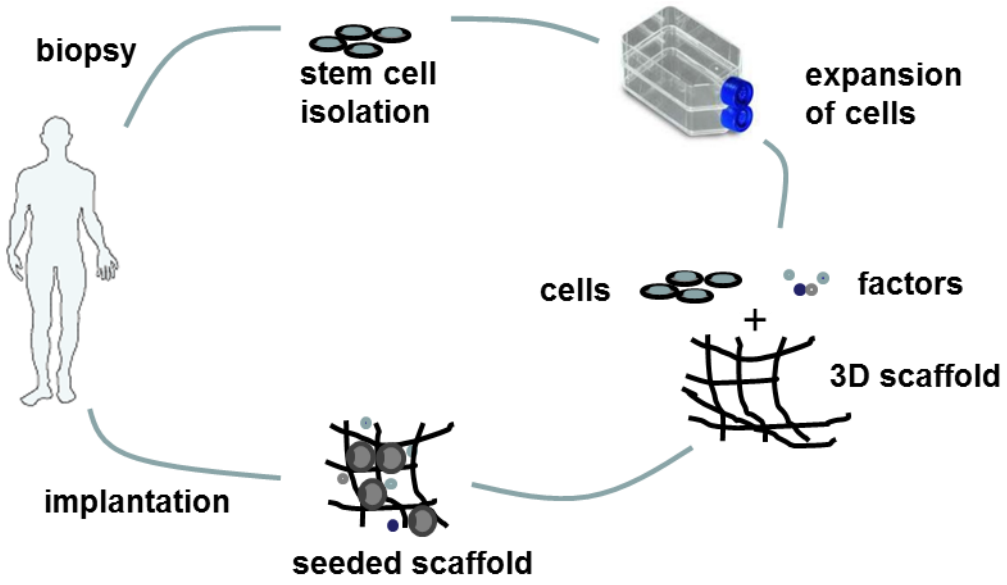


Fig. 3: Concept of cell-based adipose tissue engineering.
Cells are isolated from biopsies of patients, then expanded to obtain the sufficient amount of cells. After seeding the cell-matrix construct is either cultured *in vitro* with various stimuli or implanted directly into the patient.

2.1.3 Scaffolds

To initiate adipose tissue engineering a scaffold has to be selected and certain aspects have to be considered. The scaffolds provide the shape, the three-dimensionality and the mechanical properties of the designed tissue equivalent. Thus, the template choice depends on the physical and mechanical environment of the application area.

As in all kinds of tissue engineering projects the general requirements, like biocompatibility, cell adhesion ability and biodegradable matrix material, are valid for adipose tissue engineering as well [Bauer-Kreisel 2010, Flynn 2008/b, Gomillion 2011, Casadei 2012]. Additionally, a 'natural feel' is favored as adipose tissue functions subcutaneously as cushioning and form-giving material. Therefore, it is necessary to adjust the mechanical properties of the material in order to reach the appropriate stability. The microstructure and the porosity are important as they enable vascular ingrowth and diffusion of nutrients, or cells to adhere, proliferate and differentiate

into mature adipocytes [Gomillion 2011]. The pore size has to be adequately chosen, as during maturation, changes in size from 10 µm to approximately 100 µm of the mature adipocyte take place [Bauer-Kreisel 2010, von Heimburg 2003]. For implantation, the engineered constructs have to possess some additional properties. These materials need to be biodegradable with nontoxic by-products, biocompatible, sterilizable and easy to handle during surgical procedures [Gomillion 2011]. Moreover, the scaffold should promote cell ingrowth and neovascularization of the engineered construct in the patient.

One possible option is to use synthetic materials such as **polylactic acid** (PLA), **polyglycolic acid** (PGA) and their copolymers. These materials are already widely used in tissue engineering as they are biodegradable, absorbable polymers and have proven to support the development of many different tissue types including adipose tissue. The great advantage of synthetic material is the uniformity in the material, the predictable degradation and the variety of shapes, as they are produced under controlled conditions [Gomillion 2011, Casadei 2012].

A polyglycolic acid construct seeded with 3T3-L1 preadipocytes showed a full coherent fat pad *in vitro* after 5 weeks cultured under adipogenic conditions. But as PGA has the disadvantage of fast dissolving (3-4 weeks), the original size could not be sustained [Fischbach 2004/b, Weiser 2008]. **Poly(lactic-co-glycolic) acid** (PLGA) was seeded with ASC and subcutaneously implanted into rats. After 5 weeks, these constructs showed differentiated adipocytes and some newly formed blood vessels in the engineered tissue. However, after a long-term implantation of PLGA constructs, the support structure as well as the adipose tissue was resorbed [Patrick 2002]. The **polyethylene glycol** (PEG)-based hydrogel is another synthetic material used in tissue engineering approaches. Anderson and coworkers established a promising tissue forming construct *in vitro* by using cell adhesion ligands and metalloproteinase-degradable peptide sequences embedded in a thiol-ene photopolymerizable PEG hydrogel. Human mesenchymal stem cells encapsulated in

these hydrogel proliferated and differentiated under adipogenic conditions into adipocytes [Anderson S.B. 2011].

Natural materials like hyaluronic acid, fibrin, collagen or even decellularized tissue are also possible and already used as scaffold systems in tissue engineering. They are beneficial for the use in engineering tissue because they are enzymatically degradable without toxic by-products and they are known to have high cell affinity for many cell types including mesenchymal stem cells.

Natural **hyaluronic acid** (HA) is highly soluble and rapidly decomposing *in vivo*, but after esterification the material becomes insoluble and can be fabricated into sponges and meshes [von Heimburg 2001/c, Halbleib 2003]. Injectable forms of HA are used cosmetically as contour correction and wrinkle fillers, as they provide structural and mechanical characteristics of natural adipose tissue [Gutowska 2001, Newman 2009]. Another advantage of HA hydrogel are the angiogenesis enhancing degradation products *in vivo* [West 1989, Nair 2006]. **Fibrin** gels are already used in adipose tissue engineering and are clinically approved [Schoeller 2001]. The rapid degradation through proteases is a handicap of fibrin, but the structure and stability can be altered by the concentration of fibrinogen, pH-value and other factors [Helgerson 2004, Meinhart 1999, Eyrich 2007]. As fibrin has poor mechanical stability, a combination with a supporting structure demonstrated an advantage in forming adipose tissue [Cho 2005, Cho 2007, Wittmann 2013]. **Collagen** also represents a widely used material in tissue engineering as it is a major extracellular matrix component [Lequeux 2012]. Ibrahimi et al. reported the improvement of adipogenesis of precursor cells through collagens [Ibrahimi 1992]. The biocompatibility, enzymatic degradation, the seeding ability and the mechanical properties after cross-linking of collagen scaffolds, account for the wide use in tissue engineering, as demonstrated by e.g. Tabata et al. and von Heimburg et al. for adipose tissue formation [Hiraoka 2006, von Heimburg 2001/b].

Using the entire **natural extracellular matrix** (ECM) after decellularization is an alternative approach [Flynn 2008/b, Badylak 2009]. Decellularized tissue provides a

natural composition of a three-dimensional matrix, which can be reseeded with autologous cells. ECM is biocompatible, non-inflammatory and can be remodeled by the surrounding cells *in vivo*. After dehydration and powdering it can be shaped in various ways or used as a whole. Flynn et al. demonstrated that decellularized ECM of adipose tissue supports adipogenesis of ASC. They also showed adipo-inductive properties *in vitro* and *in vivo* with decellularized porous adipose tissue foams [Flynn 2012, Flynn 2006, Flynn 2008/a].

2.1.4 Cells

Cells are an important parameter of tissue engineering. The difficulty is to find an appropriate cell source. Ideally, the cells should be autologous or non-immunogenic, available in sufficient quantities, proliferative without losing their adipogenic potential and the harvest should be minimally invasive, so that the donor site morbidity is minimized.

Mature adipocytes seem to be the obvious choice for adipose tissue engineering, however they are very fragile and easily damaged during cell isolation. Adipocytes can be cultured in ceiling or floating culture but they have been reported to dedifferentiate under these conditions [Zhang 2000, Dodson 2008, Poulos 2010]. Dedifferentiated cells are again able to proliferate and can be differentiated into several cell types including adipocytes [Dodson 2008, Poulos 2010]. The limited life span in culture and the low proliferation potential of these cells is a poor basis to build large volumes of adipose tissue. Another experimental cell source are **preadipocyte cell lines**, like the 3T3-L1 or other murine adipogenic progenitor cell lines. These cell lines are well characterized, commercially available, easy to expand and the adipogenic differentiation is well specified in 2D and 3D models [Green 1975, Fischbach 2004/a, Avram 2007]. This is an effective model system to pursue basic research of adipogenic biology, but these cell lines are not suitable for clinical

applications as they are aneuploid and of xenogenic origin [Poulos 2010, Fischbach 2004/a, Avram 2007, Kang 2005].

Stem cells from various sources have some favorable features: they are able to proliferate several times and are capable of self-renewal; they are immature, not yet specialized and have the potential to differentiate. Embryonic stem cells are pluripotent, which means they are capable to produce any phenotype from blood cells, over neural cells and muscle cells, to adipocytes and many other cell types [Gomillion 2006, Dani 1999]. However, the use of embryonic cells is limited by ethical concerns and legal constraints in all areas of biomedical research [Spencer 2011]. Adult stem cells, such as bone marrow-derived mesenchymal stem cells (BMSC) or adipose-derived stem cells (ASC), do not have the potency as embryonic stem cells, but are still mulitpotent and able to differentiate into several different cell lineages [Gomillion 2006]. BMSC are used for years in tissue engineering applications, not only because of their differentiation potential, but also since they are isolated and easily expanded *in vitro* [Hong 2005, Minguell 2001, Barry 2004]. They are obtained from bone marrow aspirates. The BMSC are separated from the hematopoetic fraction through their plastic-adherence, and the number of cells has to be increased by *ex vivo* expansion of these small samples [Colter 2000, Ogawa 1993]. Disadvantages may be the difficult and painful harvest from the donor and the small amount of stem cells available in the donor tissue [Auquier 1995, De Ugarte 2003]. A weak point shared with ASC may be the donor-, the donor site-, the age- and the BMI-dependent behavior regarding proliferation and differentiation potential [Zuk 2002]. But in contrast, adipose-derived stem cells have the advantage, that sufficient amounts of cells can be obtained. Adipose tissue can be easily gained in large amounts from lipoaspirates or abdominoplastic procedures. The obtained adipose tissue has to be digested enzymatically, then handled with cell fraction centrifugation, and afterwards ASC are isolated from the stromal vascular fraction (SVF) through their potential to adhere on plastic surfaces. Large quantities of stem cells can be harvested through this method from one adipose tissue sample and even be further expanded [De

Ugarte 2003, Bunnell 2008, Wilson 2011, Gimble 2007, Locke 2009]. These cells are multipotent and can differentiate into the chondrogenic, myogenic, osteogenic and especially adipogenic lineage when cultured with the specific stimuli [Gimble 2007, Locke 2009].

2.1.5 Pre-implantation strategies

Many adipose tissue engineering approaches, with adipogenic stimulation before the *in vivo* implantation, showed a beneficial outcome concerning the adipogenesis of the engineered constructs [Bauer-Kreisel 2010]. A typical pre-implantation stimulus is an adipogenic induction cocktail which consists of insulin, a glucocorticoid (dexamethasone or cortisol), an agent to increase the intracellular cAMP (e.g. isobutylmethylxanthine, IBMX, a phosphodiesterase inhibitor) and a PPARγ agonist, like indomethacin (Fig. 4) [Farmer 2006, Hauner 1987, Hauner 1989].

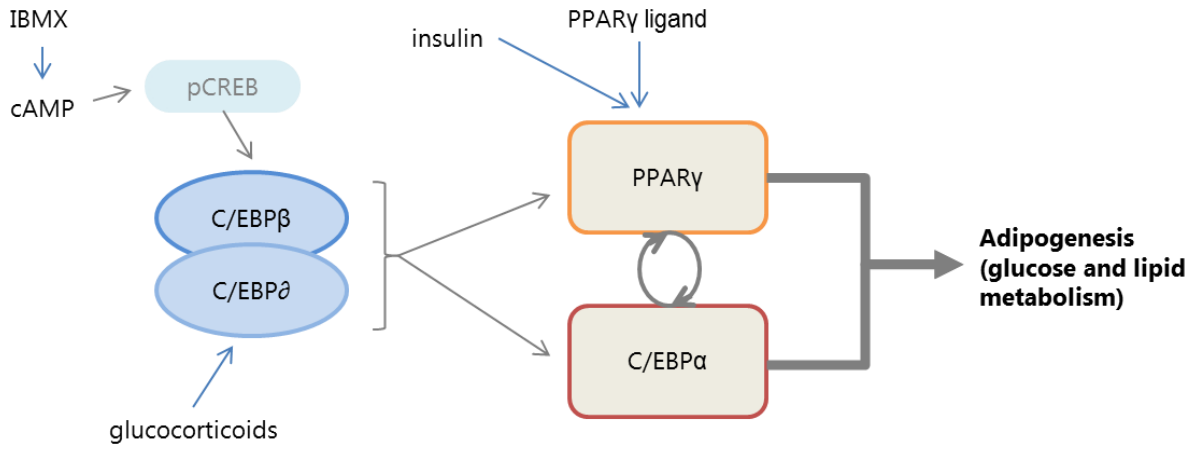


Fig. 4: Induction of adipogenesis by exposure of preadipocytes to a hormonal cocktail.
Adipogenesis is typically induced by a cocktail comprised of insulin, glucocorticoids, agents that elevate cAMP (e.g. IBMX) and a PPARγ ligand, which activates PPARγ. In the early differentiation C/EBPβ and C/EBPδ coactivate PPARγ and C/EBPα. Once activation of the key regulators, PPARγ and C/EBPα, has occurred both factors oversee the terminal adipogenic differentiation by interdependent regulation. (C/EBPα/β/δ = CCAAT enhancer binding protein α/β/δ, PPARγ = peroxisome proliferator activated receptor γ). This induction scheme was modified after Farmer 2006 and Rosen 2006.

Considering the extent of adipogenesis *in vivo*, not only the induction cocktail, but also the moment of implantation and the chosen cell passage of ASC are important factors to increase fat accumulation in the cells [Bauer-Kreisel 2010]. An additive or other strategy favors co-culture systems with e.g. endothelial cells or other neoangiogenesis promoting approaches, like incorporating a pre-existing blood vessel [Rouwkema 2008, Kaully 2009, Verseijden 2010, Strassburg 2013, Cronin 2007, Walton 2004, Dolderer 2007, Lin 2008]. Engineered adipose tissue with a functional vascular system throughout the construct would be likely better supported by nutrients and therefore have a better survival rate *in vivo*. However, to date, there is no such adipose construct engineered *in vitro* with a functional vascular system to connect to the host vasculature.

2.2 Subcutaneous adipose tissue

As already described in chapter 2.1.1, adipose tissue is distributed over the whole body, around the organs in the abdominal cavity and in the mediastinum as visceral fat, and under the body surface in the hypodermis of the skin as subcutaneous fat (SAT) [Wronska 2012, Sbarbati 2010]. Tissue engineering of subcutaneous fat is interested in remodeling or regaining body contours and, even more important, in the cushioning function of the adipose tissue in the extremities to preserve or recover tissue elasticity in revision surgery after severe injuries.

2.2.1 Subcutaneous adipose tissue characteristics

Two histologically and anatomically distinguishable SAT structures have been found; a ‘superficial SAT’ with smaller adipocytes surrounded by a fibrous shell and

characterized by a well-defined mechanical function, and a 'deep SAT' with only a thin ECM matrix surrounding the large adipocytes with important metabolic function [Sbarbati 2010, Smith 2001, Wronska 2012, Walker 2007]. Generally, SAT has a higher amount of small cells due to the high differentiation potential, a higher density of vasculature and a higher angiogenic potential than visceral adipose tissue [Walker 2007, Gealekman 2011]. The metabolic activity of SAT is supposed to be less than in the visceral adipose tissue. Macrophage infiltration and production of pro-inflammatory cytokines are not as high [Cancello 2006]. But it was also shown that SAT synthesized most of the leptin, a most prominent adipokine, which greatly affects energy regulation, and that it is the primary long-term triglyceride storage organ in the body [Van Harmelen 1998, McQuaid 2010].

2.2.2 Medical need in scar revision surgery

Complex injuries of the upper and lower extremities often result in instable scarring. Due to extensive damage of the subcutaneous fat layer a constant risk of adhesion formation of nerves, tendons and blood vessels is present. These conglutinations of the mobile structure result in severe pain and restricted ability of motion [Branski 2012, McHugh 1997, Moore 1996]. Adipose tissue as filling material is a standard application in congenital or traumatic volume defects. The bigger the adipose tissue defect, the more challenging is the surgical procedure, for instance in complex traumatic injuries of the upper and lower extremities. There are various procedures in scar revision surgery to cushion the adipose tissue defects and to regain the desired elasticity. Removed tissue is preferably replaced by autologous adipose tissue. Current strategies for full-thickness wounds are autologous free fat transfers, composite tissue flaps or the use of artificial replacements, if there is no adequate autologous tissue available [Kreymerman 2011, Lindford 2012].

Often an autologous free fat transfer is performed, if the defect is not too extensive [Ellenbogen 2000, Wetterau 2012, Coleman 2006, Mojallal 2009, Klinger 2008]. The advantage of this method is the availability of a sufficient amount of autologous fat tissue to obtain desired volume. But this strategy has its limits. Volume stability and reconstruction success are hard to predict. Due to the fragile structure of the mature adipocytes many of them are lost during preparation. Also, local necrosis and resorption of the augmented tissue volume occur. Therefore, repeated surgeries are necessary. In some cases connective tissue is developed and painful deformations appear. Absorption and oily cyst formation are described in adipose tissue augmentation as well [Klinger 2008, Niechajev 1994, Ersek 1991, von Heimburg 2001/a, Karacalar 2000, Peer 1950, Moscona 1989]. In many cases no subcutaneous layer is left to inject autologous tissue when defects in the upper and lower extremities are too severe. Furthermore, this injection technique is demanding in dosage and localization.

Surgeons use pedicle and free flaps or dermal full-thickness substitutes for extensive and full-thickness defects, as well as in cases of hypertrophic scarring after complex injuries or burns. A pedicle flap is harvested from the donor site and rotated around the intact pedicles to the defect site. Free flaps are explanted with associated blood vessels and nerves from the donor site and afterwards reconnected to the vasculature of the defect site. Using flaps incorporating fat, vasculature, muscle, and skin represents the gold-standard for complex reconstruction. This method uses a vascularized tissue of a dispensable area of the patient to be transplanted onto the defect. The disadvantages are: the number of disposable flaps is finite, the risk of unaesthetic results in donor site and reconstructed defect site is existent, and the surgery is often technically challenging, long and cost intensive [Beahm 2003, Médard de Chardon 2009, Avery 2010, Branski 2012, Lukas 2008]. As a résumé, the reconstruction with autologous fat transferes suffers from scarcity of available donor tissue and aesthetic results, but here artificial replacements and tissue engineered constructs can be introduced.

Resurfacing of full-thickness wounds is currently performed using dermal substitutes [Burke 1981, Heimbach 2003, Ryssel 2010, Schneider 2009]. But without the hypodermis, which provides the elasticity and the flexibility of the mobile structure, and the newly formed epidermis, the chance of adhesion formation is still present. To date, there is no adequate matrix or tissue available for the reconstruction of the subcutaneous fat layer, especially for atrophic and marginally perfused scar tissue, where microsurgical tissue transfer may not be the ideal option. Functional capacity and aesthetics are the key attributes for the desired adipose tissue transplants. To overcome the aesthetic limitations, the insertion of a laminar matrix seeded with stem cells which induce a thin fat layer on top of the matrix, would be an appealing treatment.

For the use in revision surgery the scaffolds need to be stable, available on a large scale, transportable and easy to handle. In terms of tissue engineering, the matrix has to support the proliferation and differentiation of used cells *in vitro* and be easily vascularized *in vivo*. Natural collagen materials are characterized through their superior biocompatibility and have shown to support revascularization and recellularization *in vivo*. Moreover, from the surgical point of view, naturally derived decellularized collagen materials have shown promising results in a range of clinical applications, like abdominal wall and breast reconstruction. *In vitro* tissue engineering approaches with these matrices have proven to foster the adherence and proliferation of different cell types [Zhang 2011, Silverman 2011, Slavin 2012, Ellis 2012, Ng 2004, Nie 2012]. Adipose-derived stem cells (ASC) are a readily available autologous cell source in the adipose tissue, which can be obtained by minimally invasive surgical procedure. The immature precursor cells can easily be expanded and differentiated into adipocytes *in vitro* and *in vivo*. These preassigned preadipocytes are more resistant to mechanical damage and ischemic conditions than mature adipocytes [Bunnell 2008, Zuk 2002]. The combination of a laminar scaffold and ASC promises an easy handling, like better dosage and control of location, and a better

cell survival than implanted cells alone, and a better host integration through recellularization and remodeling of the degradable matrix [Bauer-Kreisel 2010].

On this basis, the first goal of this thesis was to develop a thin adipose tissue layer employing commercially available collagen matrices and human ASC *in vitro* for further *in vivo* applications in scar revision surgeries of the upper and lower extremities.

2.3 Vascularization in adipose tissue

To generate large volumes of adipose tissue the vascularization has to be considered, as nutrition and oxygen can only diffuse in a small range, and cells further than 150-200 µm away from a vessel are not sufficiently supplied. Without a functional vascular network an unhomogeneous and gradient distribution of nutrients occur in large volumes of fat tissue, which then result in necrosis and volume loss in the middle of the constructs [Patrick 1998].

2.3.1 Vascularization in native tissue

As mentioned above, adipose tissue is nerved by an extensive vascular network, in which every adipocyte is attached at least to one small capillary. The capillary network has to be inevitably adjusted by regional and temporal neovascularization as adipose tissue is in continuous remodeling [Nishimura 2007, Christiaens 2010, Crandall 1997, Hausman 2004, Rupnick 2002]. In hypoxic conditions growing adipose tissue secretes angiogenic factors, endothelial cells become activated, start to proliferate and vessel branching takes place. It has been described that ASC and (pre)adipocytes secrete

several pro-angiogenic factors, like vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), transforming growth factor β (TGF β) and angiopoietins [Hausman 2004, Cao 2007, Lijnen 2008, Rehman 2004, Kilroy 2007]. During neovascularization, preadipocytes migrate alongside the evolving capillary endothelial cells. Meanwhile, the extracellular matrix has to be reorganized. In this extracellular proteolysis, many matrix metalloproteinases (MMP), including MMP-2 and MMP-9, are involved, which are excreted by adipose tissue and stromal vascular cells [Hausman 2004, Chavey 2003, Mawuoi 2002]. During adipose tissue remodeling and expansion, membrane-bound VEGF is released. This, and the VEGF secreted by ASC, which is induced by hypoxia and insulin, is necessary to initiate the formation of immature vessels through vasculogenesis and angiogenic sprouting. Further angiogenic factors released by (pre)adipocytes, angiopoietins (e.g. Ang 1, Ang 2), are reciprocally regulating the vessel branching and length. So Ang 1 is stabilizing and Ang 2 is destabilizing the vessel, to prevent excessive branching and sprouting [Liekens 2001, Papetti 2002, Yancopoulos 2000]. Also other adipokines like leptin, seem to be involved in angiogenesis. Sierra-Honigmann et al. demonstrated that leptin supports HUVEC (human umbilical vein endothelial cell) migration, proliferation and tube formation. Other studies could show that leptin is involved in the upregulation of VEGF, activation of MMP-2 and MMP-9, and facilitates angiogenesis [Cao 2007, Lijnen 2008, Sierra-Honigmann 1998, Cao 2001].

2.3.2 Vascularization in tissue engineering

There are many different strategies to vascularize tissue, i.e. controlled release of pro-angiogenic factors through functionalized scaffolds, bioreactor systems, co-culture systems and *in vivo* systems [Lovett 2009]. A classical approach is to supplement scaffolds with pro-angiogenic factors like VEGF and FGF-2. These growth factors,

immobilized in the scaffold, were found to enhance an early mature vascularization [Zisch 2003/b, Radisic 2006, Zisch 2003/a, Huang 2005, Sarkar 2006, Anderson 2011, Nillesen 2007]. A bilayered construct of different hydrogels demonstrated to be supportive towards the differentiation of ASC into the vascular phenotype on the one hand, and the dermal fibroblast phenotype on the other hand [Natesan 2011]. Kimura et al. utilized a sustained release of bFGF from microspheres resulting in a small vascularized adipose tissue *in vivo* [Kimura 2010]. But in large constructs a delay of vessel ingrowth from the host tissue into the construct center would entail necrosis of the engineered tissue. Another strategy is to use a bioreactor system which perfuses the engineered porous scaffolds by rotation. These systems provide oxygen and nutrition transport through the tissue to maintain cell viability. Endothelial cells showed cord formation and capillary structures in a co-culture in a 5 week perfusion experiment [Cartmell 2003, Dutt 2003]. Valuable co-culture systems demonstrated spontaneous formation of tubular structures in the engineered constructs [Black 1998, Griffith 2005, Wenger 2005, Borges 2003]. Co-cultures with ASC even showed interactive beneficial effects on endothelial maturation and sprouting. The endothelial cells reciprocally have a stimulation effect on preadipocyte proliferation and differentiation [Neels 2004, Aoki 2003, Sarkanen 2012]. However, the nutrition supply troughout the entire construct still relies on vessel ingrowth and integration of the tubular structures to host vasculature *in vivo*. *In vivo* strategies imply arterial-venous shunts, loops and ligated vascular pedicles, which are incorporated in the construct during surgery [Dolderer 2013, Mian 2000, Chiu 2011, Lokmic 2007, Dolderer 2001, Lokmic 2008]. This can be managed by the sandwich strategy, where the vessel is placed intermediate of two halves of the construct, or by fixing the vessel loop into a chamber and adding a hydrogel with or without cells subsequently. Overall, with these techniques vascularization and adipose tissue development have been achieved *in vivo* [Dolderer 2007, Wittmann 2013]. Due to the complex surgical procedure and the lacking rapide vascularization within the entire construct the search for suitable vascularization strategies is not complete yet.

2.3.3 New strategy in generating large vascularized adipose tissue

So far there is no *in vitro* engineered adipose tissue construct with discrete, intrinsic vascularization, which can anastomose into the host vasculature in clinical applications. A decellularized biological material with pre-existing vasculature, which can be reseeded with cells would be ideal to generate such a vascularized construct. With an existing vessel pedicle the whole construct could be connected to a perfusion bioreactor, and the issue of the nutrient and oxygen supply of large volume constructs *in vitro* may be solved. To address these challenges, a decellularized ECM of e.g. small intestine comes into consideration. This tissue has an arterial and venous inlet which can be used for the connection to the host vasculature. The ECM of the small intestine exhibits also a pre-existing vascular network and a structure of collagen, elastin and other important extracellular matrix components, which are useful in tissue engineering. Schanz et al. demonstrated that a porcine jejunum is suitable for engineering a liver construct *in vitro* [Schanz 2010]. In other tissue engineering studies Walles and coworkers could show that this system is applicable for other engineering approaches, such as thrachea or bowl constructs [Mertsching 2005, Pusch 2011, Schanz 2010]. A combination of ASC and endothelial cells on this matrix seems to be a promising way to engineer a clinically usable vascularized adipose tissue. Additionally, a perfusion bioreactor system could provide a physiological pulsatile flow in the reseeded porcine jejunal segment and thereby ensure a sufficient supply of nutrients within the construct.

Therefore, the second goal of this thesis was to engineer, for the first time, a vascularized adipose tissue *in vitro,* utilizing a perfusion bioreactor system and a decellularized porcine jejunal segment reseeded with a co-culture of ASC and MVEC.

3 Material and Methods

3.1 Material

3.1.1 Chemicals

Name	Manufacturer, Head office
3-Isobutyl-1-methylxanthine (IBMX)	Serva Electrophoresis, Heidelberg, Germany
Ambion RNaseZAP	Invitrogen, Karlsruhe, Germany
Antibody diluent	DAKO, Hamburg, Germany
4,4-Difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene-8-propionic acid (BODIPY 493/503)	lifetechnologies, Invitrogen, Karlsruhe, Germany
Bovine serum albumin (BSA)	Sigma Life Science, Sigma Aldrich, Steinheim, Germany
Calcium chloride ($CaCl_2$)	Sigma Aldrich, Steinheim, Germany
Collagenase NB4	Serva Electrophoresis, Heidelberg, Germany
Collagen matrices Collagen Cell Carrier Hypro-Sorb OptiMaix Permacol Strattice	 Viscofan BioEngineering, Weinheim, Germany Hypro Otrokovice, Otrokovice, Czech Republic Matricel, Herzogenrath, Germany Covidien, Norwalke, CT, USA LifeCell Corp., Branchburg, NJ, USA
DAPI mounting medium	DAKO, Hamburg, Germany
Dexamethasone	Sigma Aldrich, Steinheim, Germany
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, Steinheim, Germany
Disodium hydrogen phosphate (Na_2HPO_4)	Sigma Aldrich, Steinheim, Germany
Distilled water (DNAse/RNAse free)	GIBCO, Invitrogen, Karlsruhe, Germany
DNase	Roche, Mannheim, Germany

Dulbecco`s modified eagle`s medium, F12 nutrient mixture (HAM)[+]L-glutamine (DMEM/F12)	GIBCO, Invitrogen, Karlsruhe, Germany
Dulbecco`s PBS	PAA Laboraties, Pasching, Austria
EGM™-2 BulletKit™ (endothelial cell growth medium-2 with growth factors, cytokines and supplements)	Lonza, Walkersville, USA
Ethanol	Sigma Aldrich, Steinheim, Germany
Fetal bovine serum 500ml (FBS)	GIBCO, Invitrogen, Karlsruhe, Germany
Formaldehyde 37%	Merck, Darmstadt, Germany
Gentamycin	Invitrogen, Karlsruhe, Germany
Glucose	Sigma Life Science, Sigma Aldrich, Steinheim, Germany
Glycergel mounting medium	DAKO, Hamburg, Germany
Hematoxylin	Sigma Aldrich, Steinheim, Germany Bio Optica, Milan, Italy
4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma Life Science, Sigma Aldrich, Steinheim, Germany
Hydrochloric acid (HCl)	Roth, Karlsruhe, Germany
Hydrogen peroxide (H_2O_2)	Sigma-Aldrich, Steinheim, Germany
ImProm-II™ reverse transcriptation system Kit	Promega, Madison, USA
Indomethacin	Sigma Aldrich, Steinheim, Germany
Insulin	Sigma Aldrich, Steinheim, Germany
Isopropanol	Sigma Aldrich, Steinheim, Germany
Life/Dead cell staining kit II (life/dead assay)	PromoKine, Heidelberg, Germany
Liquid DAB substrate pack	Biogenex, Fremont, CA, USA
Liquid nitrogen	Linde, Munich, Germany
Magnesium sulfate ($MgSO_{4*}$ 7 H_2O)	Roth, Karlsruhe, Germany
MESA green qPCR™ mastermix plus for Sybr® assay (Sybr Green)	Eurogentec, Seraing, Belgium
Microvascular endothelial cells (MVEC)	Lonza, Walkersville, USA
MINI26-1KT PKH26 red fluorescent linker	Sigma Aldrich, Steinheim, Germany

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay)	Applichem, Darmstadt, Germany
Oil red O (ORO)	Sigma Aldrich, Steinheim, Germany
Penicillin/Streptomycin (P/S)	GIBCO, Invitrogen, Karlsruhe, Germany
Phosphate buffered saline (PBS) PBS^- (without Ca^{2+}, Mg^{2+})	Oxoid, Thermo Fisher Scientific, Waltham, USA
Potassium chloride (KCl)	Sigma Life Science, Sigma Aldrich, Steinheim, Germany
Potassium hydrogen phosphate (KH_2PO_4)	Roth, Karlsruhe, Germany
Preadipocyte basal medium (PBM-2^{TM})	Lonza, Walkersville, USA
Primary antibody mouse anti-human CD31	DAKO, Hamburg, Germany
Quanti Tech Primer Assay aP2 (FABP4) (QT01667694) C/EBPα (QT00203357) GAPDH (QT00079247) PPARγ (QT00029841)	Quiagen, Hilden, Germany
RNAseZAP	Invitrogen, Karlsruhe, Germany
Secondary antibody Cy3-conj. donkey anti mouse IgG	DAKO, Hamburg, Germany
Secondary antibody rabbit anti-mouse HRP	DAKO, Hamburg, Germany
Sodium chloride (NaCl)	Sigma Aldrich, Steinheim, Germany
Sodium deoxycholate ($(C_{24}H_{39})$ Na O_4)	Roth, Karlsruhe, Germany
Sucrose	Sigma life science, Sigma Aldrich, Steinheim, Germany
Terralin Liquid® disinfection	Schülke, Norderstedt, Germany
Tissue Tek® O.C.T.	Sakura, Finetek, Zoeterwonde, Netherlands
TritonX 100	Sigma Aldrich, Steinheim, Germany
TRIzol® Reagent	Invitrogen, Karlsruhe, Germany
Trypsin (0.25% Trypsin-EDTA (1x))	GIBCO, Invitrogen, Karlsruhe, Germany
VascuLife® VEGF complete (endothelial cell basal medium with supplements)	CellSystems, Troisdorf, Germany

3.1.4 Used materials and equipment

Name	Manufacturer, Head office
Accu-jet® pro	Brand, Wertheim, Germany
Bottle Top filter	Roth, Karlsruhe, Germany
Cell Crown (cutom made)	Öhrlein, Würzburg, Germany
cellSens Dimension microscopy Imaging Software	Olympus, Hamburg, Germany
Centrifuge Rotina 420R	Hettich, Tuttlingen, Germany
Coverslip 24x60 mm	MENZEL, Braunschweig, Germany
Cryovials 2 ml sterile	Hartenstein, Wuerzburg, Germany
Culture flask	Greiner bio-one, Frickenhausen, Germany
Fat Pan Liquid Blocker Super Pap Pen Mini	Daido Sangyo Co., Tokyo, Japan
Filter Mini sart High Flow 0,2 µm	Sartorius stedim biotech, Hartenstein, Germany
Greiner tubes (50 ml, 15 ml)	Greiner, Frickenhausen, Germany
Heating block Thermomixer comfort MTP	Eppendorf, Hamburg, Germany
Incubator, Nuaire, Auroflow IR direct heat CO_2 Incubator	IBS Integra Bioscience, Fernwald, Germany
Kryotom CM 3050 S	Leica, Solms, Germany
Laminar flow Type HS 18	Heraeus, Buckinghamshire, England
Mastercycler gradient (Thermocycler)	Eppendorf, Hamburg, Germany
Microplate MRX ELISA reader	Dynatec Laboratories, Chantilly, USA
Microscope BX51	Olympus, Hamburg, Germany
Microscope IX 51	Olympus, Hamburg, Germany
NanoDrop 2000 C spectrophotometer	Thermo Fisher Scientific, Waltham, USA
Hemacytometer (Neubauer counting chamber)	Precicolor HBG, Giessen-Luetzellinden Germany
Opticon™ 2 DNA Engine	MJ Research, Waltham, USA
PCR reaction stripes 8 tubes 0.2 ml	Roth, Karlsruhe, Germany
PCR TW 8-tube stripes 0.1 ml wite	Biozym Biotech Trading GmbH
pH-meter HI 2210	Hanna Instruments, Kehl am Rhein,

	Germany
Pipettes (1-10 µl; 10-100 µl; 100-1000 µl)	Eppendorf Research, San Diego, CA, USA
Prism 6	GraphPad, La Jolla, CA, USA
SafeSeal Micro Tubes 1.5 ml/2.0 ml	Sarstedt, Nümbrecht, Germany
Sample container 2.5 ml	Hartenstein, Würzburg, Germany
Scale, Ohaus Pioneer™	Ohaus, Parsippany, NJ, USA
Scalpel (Disposable scalpel No. 11, 15 and 21)	Feather®, Osaka, Japan
Serological pipette (5 ml, 10 ml, 25 ml, 50 ml)	Cellstar, Greiner bio-one, Frickenhausen, Germany
Shaking device Unimax 1010	Heidolph, Schwabach, Germany
Special accuracy weighing machine Feinwaage ALJ 220-4	Kern, Balingen, Germany
Spectra Mesh 100µm, Macroporous Filters	SpectrumLabs, Breda, Netherlands
Sterile biopsy punches	Stiefel Laboratories, Sligo, Ireland
Super Frost® plus glas slide	R. Langenbrinck, Emmendingen, Germany
TECAN Genios Pro plate reader	Tecan, Crailsheim, Germany
Tissue culture plates	Greiner bio-one, Frickenhausen, Germany
Tissuelyser	Qiagen, Hilden, Germany
Ultrasonic device, SonoPlus	Bandelin, Berlin, Germany
Vortex, IKA® MS3 basic	IKA®, Staufen, Germany
Water bath	Memmert, Schwabach, Germany
XC30 camera and DP71 camera	Olympus, Hamburg, Germany

3.1.5 Media and buffers

Storage medium	
DMEM/F12	
FBS	10%
P/S	1%
DMSO	5%

Proliferation medium	
DMEM/F12	
FBS	10%
P/S	1%

Growth medium – stem cells (PGM)	
PBM	
FBS	10%
P/S	1%

Growth medium – endothelial cells (VascuLife)	
VascuLife basal medium	
VEGF	5 ng/ml
EGF	5 ng/ml
FGF basic	5 ng/ml
IGF-1	15 ng/ml
L-glutamine	10 mM
Hydrocortisone hemisuccinate	1.0 µg/ml
Heparin sulfate	0.75 U/ml
Ascorbic acid	50 µg/ml
FBS	2%

Adipogenic differentiation medium – mono-culture	
Growth medium	
Insulin	1.7 µM
Dexamethasone	1.0 µM
Indomethacin	200 µM
IBMX	500 µM

Adipogenic differentiation medium – co-culture	
Growth medium – stem cells	½ medium-volume
Growth medium – endothelial cells without EGF	½ medium-volume
Insulin	1.7 µM
Dexamethasone	1.0 µM
Indomethacin	200 µM
IBMX	500 µM

Decellularization solution – jejunal section	
ddH_2O	ad 500 ml
Sodium deoxychelate	45 g

Decellularization solution – jejunal segment	
ddH_2O	ad 1000 ml
Sodium deoxychelate	34 g

DNase solution	
PBS	300 ml
DNase	100 mg
P/S	1%

PBS$^-$ buffer, pH 7.2	
NaCl	140 mM
KCl	2 mM
KH_2PO_4	1.5 mM
Na_2HPO_4 x 2 H_2O	8.5 mM
ddH_2O	ad 1000 ml

Collagenase buffer, pH 7,4	
ddH_2O	
HEPES	0.1 M
NaCl	120 mM
KCl	50 mM
$CaCl_2$	1 mM
Glucose	5 mM
BSA	1.5%
Collagenase NB4	0.1%

PBST	
PBS	
TritonX 100	0.3%

Blocking buffer	
PBST	
BSA	1.5%

3.2 Methods

3.2.1 Collagen matrices - Cross examination in culture medium

For a seeding and handling experiment five different collagen scaffolds were tested. Three porcine-derived and two bovine-derived biological meshes were utilized in the study of subcutaneous fat layer engineering. Porcine collagen: Permacol™ (Covidien, Norwalke, CT, USA), OptiMaix (Matricel, Herzogenrath, Germany) and Strattice™ (LifeCell Corp., Branchburg, NJ, USA); bovine collagen: Hypro-Sorb (Hypro Otrokovice, Otrokovice, Czech Republic) and Collagen Cell Carrier (Viscofan BioEngineering, Weinheim, Germany). The matrix of Permacol™ is chemically cross-linked (Patent Number WO8505274), all other matrix samples are not chemically cross-linked.

These five choosen scaffolds have to be form- and size-stable and easy to handle to be useable in cell culture and later in *in vivo* applications. Therefore, all collagen scaffolds were cut in discs with a diameter of 5mm with a biopsy punch, then they were soaked in medium to test their size and shape stability. Shape and size were observed for two weeks under culture conditions and after 14 days in medium the mechanical stability was tested by transferring the scaffold to another culture plate with tweezers.

3.2.2 Preparation of a decellularized porcine jejunum

With the use of natural tissue to engineere vascularized adipose tissue a decellularization of the matrix is necessary, as natural tissue of xenogenic origin is biological incompatible and not suitable for implantation. Therefore, all porcine jejunal material was completelly decellularized and sterilized before application.

3.2.2.1 Explantation and decellularization of jejunal sections

For general tests like cell adhesion, culture conditions and differentiation 1 cm^2 sections of the jejunal matrix were used.

To gain the jejunal sections the whole porcine jejunum was explanted from German landrace pigs (3 months, 15-25g). All animals received human care in compliance with the guide for care and use of laboratory animals published by the National Institutes of Health (NIH publication No 85-23, revised 1996 [Mertsching 2005]). The animals were heparanized and killed by an overdose of narcotics shortly before explantation. After opening the abdomen the intestine section including duodenum and jejunum was exposed to find the artery and vein pairs for preparation. Before arteries and veins were connected to cannulae (Braunüle) all associated lymph nodes were removed. Through the cannulae all attached vessels were then rinsed with physiological NaCl solution. For the preparation of jejunal sections, an approximately 1.9 m jejunum was explanted and then moved into a solution of PBS^- and 1% gentamycin on ice for transportation. Decellularization started on the same day following a modified protocol of Meezan E. et al. [Meezan 1975, Moll 2013].

The explanted whole porcine jejunum was washed with cold PBS^- + 1% gentamycin+ 1% penicilin/streptomycin to remove blood and excrements from the matrix. To perform cell lysis a decellularization solution containing sodium desoxycholate (0.2 M) was perfused through the lumen by hand and through the vascular system by a pump system with a pressure control (pressure about 80mm Hg). The rinsing step was done until the tissue was swelling and started to lose its fleshy color. On the next day, cell debris and the rest of the decellularization solution were removed by perfusion with PBS^- for 1-2 hours. The remaining matrix was cut into 6-8 cm pieces and the serosa was removed before they were placed into a flask with DNase solution (100mg in 300ml PBS) and incubated at 4°C overnight. After another washing step the jejunal sections were sterilized with Gamma-radiation and stored at 4°C in PBS until further decrease in size to 1 cm^2 sections and the use in cell culture.

3.2.2.2 Explantation and decellularization of a jejunal segments

In the custom made bioreactor system a jejunal segment is used. In contrast to the jejunal section the jejunal segment is 8-10 cm long with intact vascularization and lumen cavity.

The explantation procedure of the jejunal segment was done as described above only with small variations; this time not the 1.9 m jejunum was explanted, but 5-10 cm pieces with an artery influx and a venous efflux each. For later preparation and to see wether the vessel structure was still intact the artery influx and the venous efflux were connected to cannulae and rinsed by physiological NaCl solution, before moving these segments into a solution of PBS^- and 1% gentamycin.

To start the decellularization the vascular structure and the matrix lumen were washed with cold PBS^- supplemented with 1% gentamycin and 1% penicillin/streptomycin to remove blood residue from the artery and veins and excrements from the jejunal lumen. The next step was performed in a bioreactor system which can pump solution through the vascular system with a defined pressure. In this reactor the jejunal segment (5-10 cm) was perfused with a pressure of 80-100 mm Hg (~ 2-3 ml/min) with a solution containing sodium desoxycholate (0.08 M) to set the cell lysis. The tissue was flushed until the color of the jejunum changed from flesh to clear and nearly white. It is important that no air is pumped into the pipe system because it would disrupt the small vascular vessels in the jejunal segment. On the next day cell debris and the rest of the decellularization solution were removed by a perfusion with PBS^- for 1-2 hours. The remaining matrix is nearly white and was then moved to a flask with DNase solution and incubated on an orbital shaker at 4°C overnight. After another washing step, the jejunal segment was sterilized with Gamma-radiation and stored at 4°C in PBS until use.

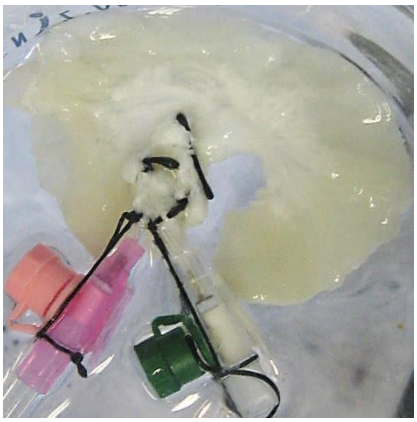


Fig. 5: Decellularized porcine jejunal segment.
A porcine jejunal segment with an arterial influx (pink Braunüle) and a venous efflux (transparent-green Braunüle). After a successful decellularization, the remaining exracellular matrix of the jejunal segment is completely white.

3.2.3 Cell isolation

3.2.3.1 Isolation of the stromal vascular fraction

Human adipose-derived stem cells were isolated from subcutaneous adipose tissue obtained in liposuction procedures of healthy donors (age 45 ± 25 years and BMI 28 ± 5) according to methods described in literature [Dubois 2008, Bunnell 2008]. The study was approved by the ethics committee of the University of Wuerzburg, Germany.

In brief, the fat tissue was digested with fresh and sterile collagenase buffer (Hepes 0.1M, NaCl 0.12M, KCl 0.05M, $CaCl_2$ 0.001M, glucose 0.005M, 1.5% bovine serum albumin (BSA) and 0.1% collagenase NB4) for 2 h at 37°C on an orbital shaker. The resulting suspension was filtered through 100 µm mesh (Spectra/Mesh Macroporous Filters, SpectrumLabs) and centrifuged at 300 g for 10 min. Floating fat was aspirated and pelleted cells were resuspended in the remaining serum by gently shaking. The solution was then again filtered through a 100 µm mesh (Cell Strainer, BD Falcon) and washed with PBS and 300g for 10 min in the centrifuge. Supernatant was removed

and cells were resuspended in proliferation medium (DMEM/F12 medium supplemented with 1% penicillin/streptomycin and 10% FBS) and supplemented with 5% DMSO for storage. Cells were then frozen in liquid nitrogen and stored at -80°C until further use. One sample was seeded in a T175 flask with approximately 5,000 cells/cm^2 and cultured in maintenance medium for some weeks to monitor the cell behaviour for consistency in proliferation and appearance.

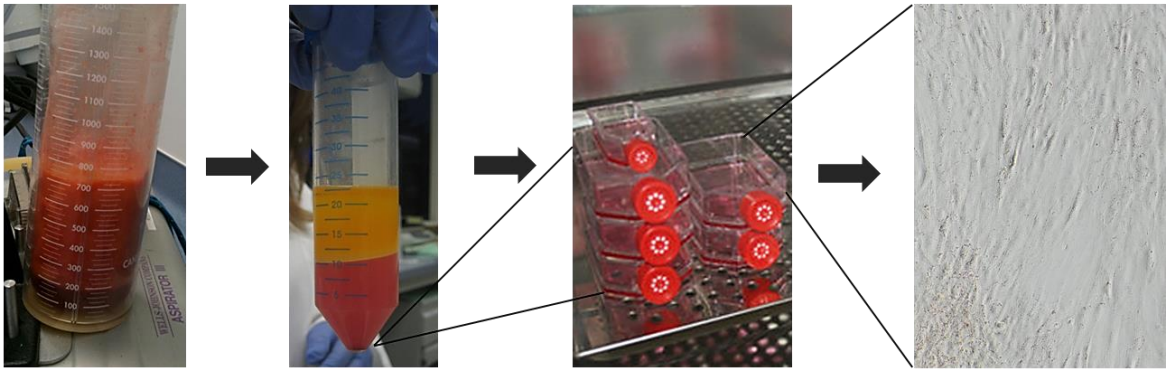


Fig. 6: Important steps of the isolation of hASC.

3.2.4 Cell culture

3.2.4.1 2D culture

To determine the favorable conditions for an adipogenic co-culture system, stem cells were cultured in a 2D model with four different media: PGM, a preadipocyte basal medium; VascuLife, an endothelial cell medium, and a mixture of both media with and without EGF (endothelial growth factor).

Cells of the stromal vascular fraction were thawed and then seeded in tissue culture flasks in a humidified atmosphere at 37°C and 5% CO_2 for 24 h. To remove non-

adherent cells all medium was renewed and the cell layer was washed twice with PBS. The adherent cells, human adipose-derived stem cells (hASC), were cultured at a sub-confluent level. After the expansion to passage 2 to 4 in DMEM/F12 medium, confluent cells were trypsinized and seeded in 24-well plates at a density of 50,000 cells/cm^2. Surplus cells were frozen in liquid nitrogen and stored in -80°C until further use. After two days, to establish basal culture conditions, in one of the following media, PGM, VascuLife, or 1:1 PGM/VascuLife ± EGF, hASC were induced to undergo adipogenic differentiation (with 1.7 µM insulin, 1 µM dexamethasone, 200 µM indomethacin and 500 µM methyl-isobutylxanthine). Adipogenesis and proliferation of the hASC in these four media were examined during two weeks of culture.

3.2.4.2 Engineering a cell-matrix construct

- **Collagen matrices as basis for engineering subcutaneous adipose constructs**

Engineering a thin subcutaneous adipose construct includes a scaffold which supports cell adherence and adipogenesis.

All size and shape stable collagen scaffolds were cut by a biopsy punch with a diameter of 5 mm and then seeded with hASC of passage 2-4 at densities of 30,000, 100,000 and 300,000 cells per disc in growth medium (PGM supplemented with 10% FBS and 1% penicillin/streptomycin). After 24 h of incubation, scaffolds were moved to 24-well plates with adipogenic differentiation medium (growth medium supplemented with insulin, dexamethasone, indomethacin and 3-isobutyl-1-methylxanthine) or with growth medium, as the negative control. The whole culture was carried out in a humified atmosphere with 37°C and 5% CO_2. Cells were cultured up to 21 days and culture medium was exchanged every two to three days.

Subsequently, to investigate a short term induction protocol for better clinical use, Permacol™ was seeded with 300,000 cells per disc, incubated overnight in growth medium as described above and then differentiated with four different protocols. The first group, the negative control, received growth medium for the complete time of culture. The second group, the positive control, was cultured with differentiation medium for the complete culture time. Group three and four was cultured with differentiation medium for two or four days, and followed by growth medium for the rest of the culture.

Constructs were harvested on day 0, day 7 and day 14 (day 21), because fat accumulation started on day 7 to be visible and quantifiable with histological and biochemical analysis. For quantitative RT-PCR analysis, samples were harvested on day 0, day 2 and day 7, as the increase of the gene expression occurs earlier than the visible effects within the cell.

- Decellularized jejunum as basis for an engineered vascular adipose tissue

- Small jejunal section for cell culture under static conditions

Before seeding the whole jejunal segment with its vascular structure, a small jejunal section was tested for hASC adherence, the support of adipogenesis and the co-culture of hASC and endothelial cells.

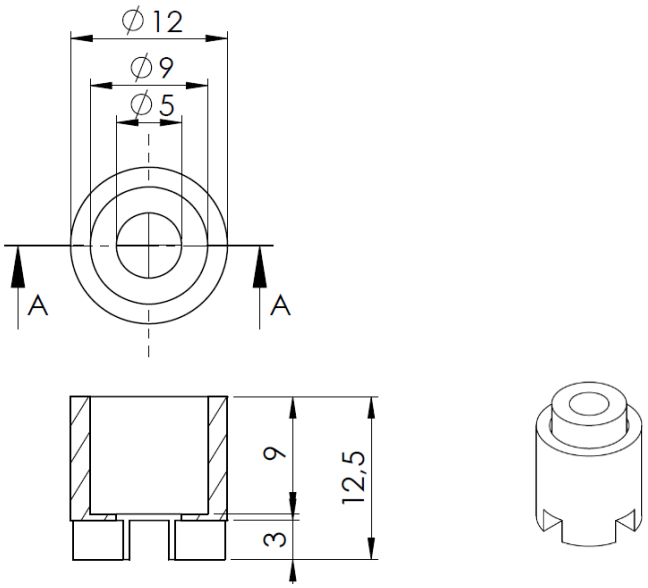
The decellularized jejunal section was cut in 1 cm^2 pieces. Each one was mounted over the inner piece of a cell crown (Fig. 7) and orientated with the submucosa on the upper side before the outer coverage of the cell crown was put on top to fixate the small jejunal section. Before seeding, all small jejunal sections were cultured in medium for 2 h in an incubator. For a monoculture, cell crowns were flipped so that

the inner jejunum side was turned upwards. ASC were now seeded on the villi with a density of 300,000 cells/cm^2 and cultured with PGM medium.

For a co-culture, human microvascular endothelial cells (MVEC, Lonza, Walkersville, USA) were seeded a day before the ASC on the submucosa side of the porcine matrix with the same density as the ASC (300,000 cells/cm^2) and cultured with EGM2 medium. After 20-24h cell crowns were flipped and the ASC were seeded as described before on the inner side of the lumen, on the villi. The whole culture was then carried out in a 1:1 PGM: EGM medium mixture without EGF in a humidified atmosphere with 37°C and 5% CO_2.

After another day medium was exchanged to a maintenance or adipogenic induction medium supplemented with insulin, dexamethasone, indomethacin and IBMX for further cultivation. Samples were harvested on day 0, day 2, day 7 or on day 0, day 7 and day 14, depending on the analysis.

Fig. 7: Schema of a cell crown and its dimensions (in mm).
The cell crowns were cutom made form Öhrlein Company.



- Small jejunal section for cell culture in a small flow-through bioreactor

All experiments with porcine jejunal sections and segments in a flow-through bioreactor system were performed in the Department of Tissue Engineering and Regenerative Medicine, head: Prof. Dr. Heike Walles.

To determine the effect of flow conditions in a co-culture system, 1 cm^2 decellularized jejunal sections were fixed in a two chamber bioreactor (Fig. 8). The bioreactor is constructed in a way that the jejunal section is like a membrane between two chambers. Hereby assigned is the possibility to have two different culture conditions (flow or static) for two different cell types (MVEC and hASC), which can be seeded on and are separated by the scaffold (jejunal section) as membrane.

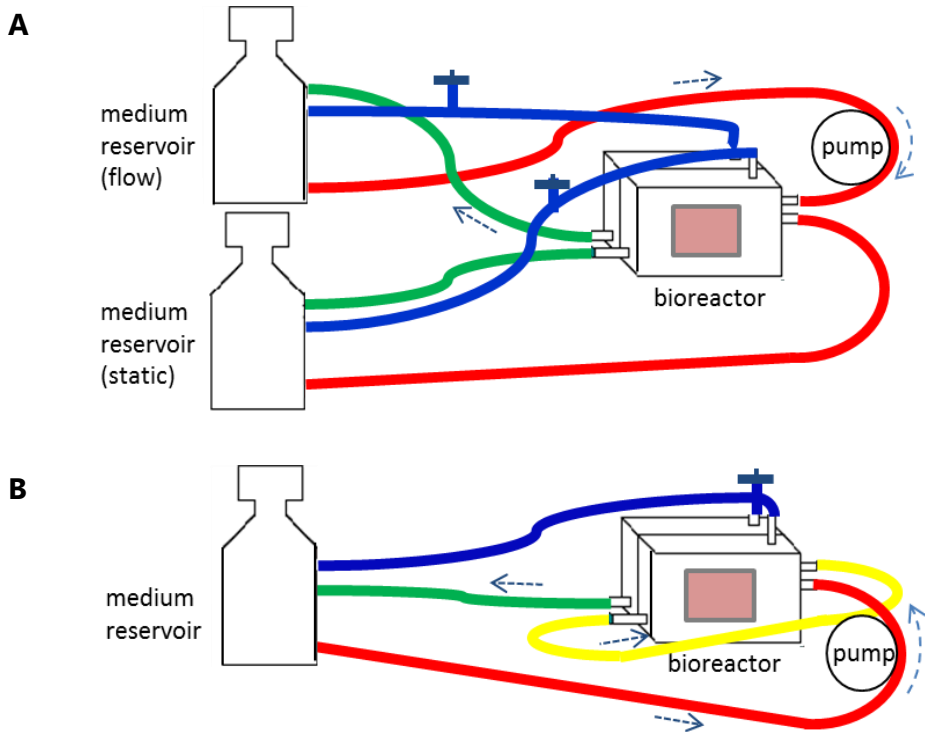


Fig. 8: Schema of small bioreactor systems.
A: flow-through bioreactor for a co-culture system with two different culture conditions, on the one side with a flow (side with the direction arrows) for the MVEC and on the other side without a flow for the hASC. Arrows indicate the flow direction. Red is the influx, green the efflux and blue is the ventilation .
B: flow-through bioreactor for a co-culture system for flow culture condition. The 1:1 PGM/VascuLife medium first runs through the chamber with the MVEC, then circuits (yellow line) to the other chamber with the hASC and goes back to the medium flask with allways the same flow of 3.8 ml/min. Arrows indicate the flow direction. Red is the influx, green the efflux and blue is the ventilation [Moll 2013].

First the jejunal sections were kept for two hours in growth medium, then the MVEC were seeded with a density of 400,000 cells/cm^2 into the chamber with the submucosa side of the jejunal section. In a static overnight culture with VascuLife medium MVEC could adhere. On the following day a PGM medium-cell suspension with a density of 400,000 hASC/cm^2 was added into the other chamber with the inner lumen side. After the cells did adhere, flow conditions with a flow of 3.8 ml/min were applied: In the first group a flow for both cell types was applied, see Fig. 8B and the second group was cultured with a flow in the chamber with MVEC, and a static culture for the hASC (Fig. 8A). As a control, a third group with static conditions for both cell types was cultured in cell crowns. After 24 h medium was exchanged to an adipogenic differentiation medium containing insulin, dexamethasone, indomethacine and methyl-isobutylxanthine.

- The whole jejunal segment with its vascular system and lumen in a bioreactor system as basis for a vascularized adipose tissue construct

With the intention to engineer a vascularized adipose tissue construct, the whole jejunal segment (6-8 cm long) with its vessel system had to be reseeded, the vascular structure with endothelial cells and lumen with hASC. For coculturing this construct a large flow-through bioreactor system (Fig. 9) was used, fostering the vascularization by physiological perfusion of the vessel system.

To reseed the whole jejunal segment, the reactor system had to be filled with medium (VascuLife) so that the medium level in glass flasks and glass reactor was at least 2 cm and no bubbles were present in the pipe with the pressure sensor. The jejunal segment was moved into the reactor, then the reseeding of the vasculature with MVEC was started in a two-stage process with a 5 ml syringe. 3 Mio MVEC in 2 ml VascuLife were injected into the arterial inlet. After 4 h adherence, the porcine

matrix was turned and, again, 3 Mio MVEC in 2 ml VascuLife were injected, to seed the other side of the vasculature. The construct was statically cultured for 3 h, then a slow flow over the arterial inlet was applied. On the next day the flow was changed to a computer-controlled perfusion of 80-120 mm Hg. For the next two weeks half of the medium was exchanged twice a week.

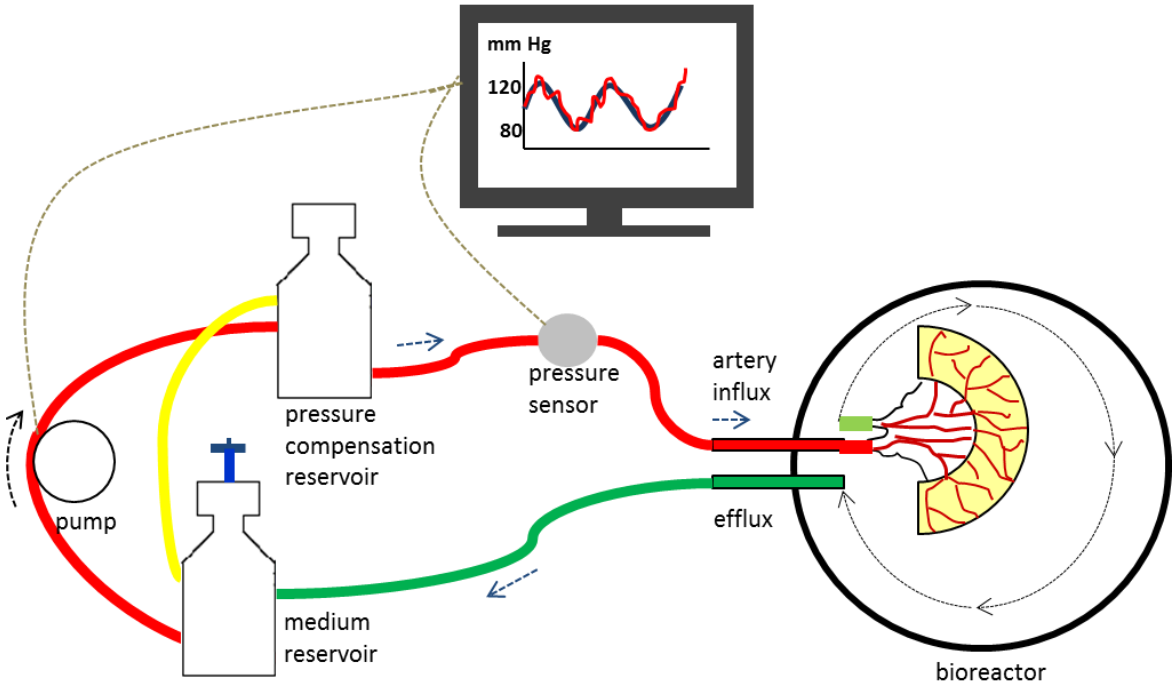


Fig. 9: Schema of settings of the bioreactor system to foster vascularization.
From medium reservoir (bottle with air-strainer) medium runs over the pump to the pressure-compensation bottle to the pressure sensor (gray filled circle) into the artery of the porcine jejunal segment. From there the medium gets into the glass reactor, at a certain level the medium flows back to the medium reservoir bottle. Over the pump and the pressure sensor the pulse and pressure in the vascular system can be computer controlled. Arrows indicate the flow direction. Red is the influx, green the efflux and blue is the ventilation [Schanz 2010].

On day 14 of culture, the whole medium was exchanged to a 1:1 VascuLife/PGM medium without EGF. Jejunal segments were moved to a petri dish and the jejunal lumen was sealed on one side with a clamp. Then the lumen was filled with 1 ml cell suspension with 2-5 Mio ASC according to the volume of the lumen and afterwards clamped on the open side to seal the construct. The vasculature was reconnected to the computer-controlled perfusion and cultured overnight. After removal of the clamps the matrix was cultured for 6 days in 1:1 VascuLife/PGM medium without EGF.

On day 21 of the whole culture, the medium was exchanged to a 1:1 medium without EGF, but with induction supplements (insulin, dexamethasone, indomethacin and IBMX) to differentiate the stem cells adipogenically (day 0 of induction). For another three weeks, half of the medium was exchanged twice a week and the construct was cultured in 37°C and 5% CO_2 incubator. Tissue samples were harvested at day 14, day 21 (day 0 of induction), day 30 (day 9 of induction) and day 42 (day 21 of induction) for histology, PCR and whole mount staining.

3.2.5 Histological and immunohistochemical analysis of engineered tissue

3.2.5.1 Cryosections

Scaffolds were harvested, washed twice with PBS at room temperature and fixed with 4% formalin for 1 h at 4°C. After rinsing with PBS, the matrix was immediately embedded in Tissue Tec®, then frozen with liquid nitrogen and stored at -80°C until use. The cell-matrix construct was vertically sectioned in 8 µm slices at a chamber temperature of -23°C and an object temperature of -25°C.

3.2.5.2 Oil Red O-Hematoxylin-Eosin staining

Standard hematoxylin and eosin (H-E) staining were used to examine density and distribution of cells within the cell-matrix construct. Hematoxylin stains basophile structures, like DNA in nuclei, blue and eosin a acidic dye, stains basic protein

structures in cytoplasm pink. In order to follow the synthesis of triglycerides on slices, the amount of lipid droplets in adipogenically induced cells were stained with hematoxylin and the lipophilic dye oil red O (ORO). Cryosections were washed with PBS, then they were stained with ORO for 7 min at room temperature and the nuclei were counterstained with hematoxylin for 1 min and 10 min blueing before they were covered with Glycer gel and a glass cover. Sections were analysed by bright field using an Olympus BX microscope.

3.2.5.3 Antibody staining (CD31)

For the immunohistochemical detection of MVEC, cryosections were washed with PBS and endogenous peroxidase activity was blocked by incubation with 1% H_2O_2. After rinsing, sections were incubated in 5% BSA/PBS solution for 20 min to prevent non-specific antibody binding. Subsequently, the primary antibody (mouse anti-human CD31 Dako; diluted 1:400 in antibody-diluent) was applied over night at room temperature. After washing, slides were incubated with the secondary antibody (rabbit anti-mouse HRP) for an hour, washed again and then developed with diaminobenzidine (liquid DAB Substrate Pack, Biogenex, Fremont, CA, USA). Sections were counterstained with ORO and hematoxylin, mounted with Glycer gel and imaged by bright field using an Olympus BX microscope.

3.2.5.4 Whole mount staining

In order to visualize the 3D structures in the cell-seeded constructs, the seeded jejunal segment samples were incubated with cold 100% methanol for 30 min at RT. The matrices were then washed with PBS for 1h on a rocking board. To block non-

specific binding the tissue was incubated in a 3% blocking buffer (PBS+ 0.3% TritonX 100 +1.5% BSA) for 20h on a rocking board. After the removal of the blocking buffer at the next day, the constructs were washed with PBS + 0.3% TritonX 100 (PBST) for 2h. The primary antibody (mouse anti-human CD31, diluted 1:400 in antibody-diluent) was added in a volume to immerse the tissue entirely. At the following day the matrices were washed 1.5 h with PBST and 1.5 h with 3% blocking buffer on a rocking board. The tissue samples were then incubated with the second antibody (Cy3-conj. donkey anti mouse IgG, diluted 1:400 in antibody-diluent) for 2 h and afterwards washed with a 1:1 dilution of 3% blocking buffer and PBST overnight on a rocking board. To visualize the fat accumulation the constructs were incubated with BODIPY (dilution 1:100 in PBS) for 30 min on a rocking board. Thereafter, samples were washed with PBS for 1 h twice on a rocking board. The stained tissue was then mounted on a microscope slide and covered with DAPI mounting medium, to stain the nuclei, and a coverslip. The samples were examined immediately under the fluorescent microscope, Olympus BX.

3.2.5.6 Live/dead staining

The live/dead assay (PromoKine) was performed to assess the ratio of viable and dead cells on the scaffolds. The constructs were washed with PBS and then incubated with staining solution (1ml PBS with 1µl calcein and 2µl ethidium homodimere-III) for 30 min at room temperature. The calcein-acetoxymethylester is hydrolyzed by biesterases to a green fluorescent calcein stain in viable cells. The ethidium homodimere III is a fluorescent, highly affine to nucleic acids, which can only pass through the damaged outer membrane of dead cells and will stain them red. After two PBS washing steps live cells shine green and dead cells red under the fluorescent microscope. Thicker tissue samples were sectioned with a cryostat (described above) and then examined under a microscope. Different filters were used for the imaging of

calcein and ethidium bromide (ex./em. 460-490 nm/520 nm and ex./em. 510-550 nm/590 nm). The resulting images were overlaid.

3.2.6 Quantitative analysis of engineered tissue

3.2.6.1 Quantitative real time-polymerase chain reaction (qRT-PCR) analysis

End point RT-PCR was conducted to characterize the gene expression of ASC in differentiation medium during culture. Total RNA was harvested from homogenized cell-matrix constructs with Trizol reagent (Invitrogen, Karlsruhe, Germany) and isolated according to the manufacturer's instructions. To synthesize first strand cDNA, total RNA was transcribed by using the IMPROM-II^{TM} reverse transcription system (Promega (Madison, USA). The gene-specific quantitative PCR was performed with MESA GREEN MasterMix Plus for SYBR® Assay (Eurogentec, Seraing, Belgium) with an MJ Research Opticon2 (BioRad) following the scheme: initial denaturation 95 °C for 15 min, amplification: 95 °C for 15 s, 55 °C for 30 s, 72 °C for 30 s (40 cycles) and the melting curve analysis was carried out at 74 °C to 89 °C for each set of primer. The following primers were used: PPARγ (QT00029841), C/EBPα (QT00203357), aP2 (QT01667694) and as housekeeping gene: glyceraldehyde-3-phosphate dehydrogenase (GAPDH)(QT00079247). Expression levels for each gene were normalized to the house keeping gene and the increase of the gene expression was determined by the $2^{-\Delta\Delta CT}$ method. (All melting curves were carefully checked and the efficiency of each primer was calculated for a quality control.)

3.2.6.2 DNA Assay

For a cell number analysis, cell-matrix-constructs were added to 1 ml PBS and homogenized with a frequency of 20 s^{-1} for 2 min with a homogenizer (Tissuelyzer, Quiagen). After exposure to an ultrasonic device (SonoPlus, Bandelin) 10 µl cell debris suspension was mixed with 200 µl dye solution (Hoechst33258) and fluorescence was measured at 340 nm and 465 nm with a Tecan fluorometer (GENios Pro, Tecan).

3.2.6.3 Quantitative analyse of triglyceride content (ORO-Assay)

In order to measure the coverage of stained fat accumulation per well or on top of cell matrix constructs, the images of oil red O (ORO)-stained cells were taken, using an Olympus IX50 microscope. Processed images were analysed for percent coverage using the Cellsense software (Olympus). For an optical density (OD) analysis, the maintenance medium was removed and 1 ml isopropanol 100% was added to each well or construct for 10 min to extract the ORO from the cells. The OD value was measured at 510 nm using a NanoDrop2000c spectrometer (Thermo Scientific, Waltham, USA) and then normalized to the cell number in each well or on each construct to determine the triglyceride content within the cells.

3.2.6.4 MTT Assay - Investigation of cell survival

To detect the viable cells in the decellularized jejunal segment, a section of the matrix was moved into a solution of 2 ml medium and 1 ml water-soluble dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) and incubated for one

hour. In viable cells the yellow MTT will be reduced to blue-violet water-insoluble formazan. After washing, the viable cells on the construct are visible to the eye.

3.2.8 Statistical evaluation

The obtained data of the triglyceride accumulation within the cells and the PCR values were statistically analyzed by a two-way ANOVA (analysis of variance) followed by Tukey´s post-hoc test (GraphPad Prism 6). The statistical significance level was set to $p<0.05$.

4 Results

4.1 Engineering a subcutaneous fat layer using a collagen matrix

For the development of a subcutaneous adipose construct, three main criteria were considered for the selection of a collagen matrix: First, collagen scaffolds already used in clinics or in cell culture, so biocompatibility and/or cell adhesion is ensured. Second, collagen scaffolds, which are biodegradable at a rate, at which cells are rebuilding their own extracellular matrix and third, commercially available collagen material, which is not exceeding the height of 1.5 mm. This height should not be exceeded, so that the engineered subcutaneous cushioning layer is not compromising the cosmesis in revision surgery of the upper and lower extremities. Five products on porcine or bovine collagen basis were chosen for closer investigation in this study.

4.1.1 Selection and handling of collagen matrices

Permacol™, a chemically crosslinked porcine collagen scaffold which is applied in breast reconstruction and hernia repair, has already been used in cell culture, e.g. fibroblasts or stem cell culture [Kulig 2013, Jarman-Smith 2004, Arca 2012]. The supplier Covidien guarantees a degradability rate of approximately 6 months. It provides a biomechanical strength for a long time and also has been shown to enable ingrowth of cells, neovascularization and consecutively, remodeling and integration with the host tissue [covidien 2011]. Strattice™, a competitive product to Permacol™, is also a porcine collagen matrix, but without chemical cross-linkage. Strattice™ is also applied in breast reconstruction and hernia repair. This matrix shows minimal

inflammatory response, and biodegradability at the same rate as the body remodels autologous tissue. OptiMaix™, a porcine, and Collagen Cell Carrier™, a bovine matrix, are thin collagen materials, which are mostly utilized in cell culture. They were shown to be stable for many days and suitable for cell adhesion and proliferation for many cell types [Savkovic 2012]. HyproSorb™ is a bovine atelocollagen scaffold with two different structures on each side. On one side it has a rough surface suitable for bone material integration, and on the other side it has a smooth structure for adhesion and healing of adipose tissue. HyproSorb™ is often used in dentistry with good wound healing properties; it displays good cell adhesion, good biocompatibility and a complete resorption after 6 months. Characteristics of these five selected collagen matrices are summarized below in Table 1.

Table 1: Properties of the chosen commercially available collagen matrices.

	Collagen matrices				
	Permacol™	Strattice™	OptiMaix®	Collagen Cell Carrier®	Hypro-Sorb®
Origin	porcine	porcine	porcine	bovine	bovine
Chemical cross-linking	yes	no	no	no	no
Thickness	0.5 mm	± 1.3 mm	0.5 mm	0.02 mm	0.8 mm

4.1.2 Cell seeding

The five described collagen matrices were tested for stability and handling in cell culture. All scaffolds were cut into discs with 5 mm diameter by a biopsy punch, without a deformation on the edges. After moistening with growth medium, all these materials were transferred to a culture well with 100 µl PGM growth medium and cultured in 5% CO_2, at 37°C and humidified atmosphere. After 2.5 h a cell suspension

with 300,000 hASC was added. Cells penetrating the material deeper than the outer structure were undesired in this study. Only the top surface of the material should be seeded, to act as a layer between skin and mobile structures enabling them to shift. The rest of the material, without cells, should function as persisting padding while cells from surrounding tissue *in vivo* repopulate and remodel the matrix. HyproSorb™ was seeded on the smoother, suitable side for adipose tissue. After the overnight culture, all matrices were transferred again to new well plates and further cultivated for 14 days. Four collagen scaffolds, Permacol™, Strattice™, OptiMaix™ and Collagen Cell Carrier™, were, as seeded constructs, stable in form and size at transport, but HyproSorb™ matrices were, populated with cells, slightly gelatinous. During culture, the differences in size and form stability were apparent (Fig. 10). HyproSorb™ lost almost 50% of its size and the disc was deformed with no similarity to the original form after 7 days in culture. In the following 7 days HyproSorb™ continued to decrease in volume. Collagen Cell Carrier™ and OptiMaix™ were rolling up on two sides of the scaffolds within the 14 days of culture as shown in Fig. 10. Strattice™ was stable in shape, but showed slight shrinkage until day 14. Only Permacol™ stayed stable in size and form during the complete culture.

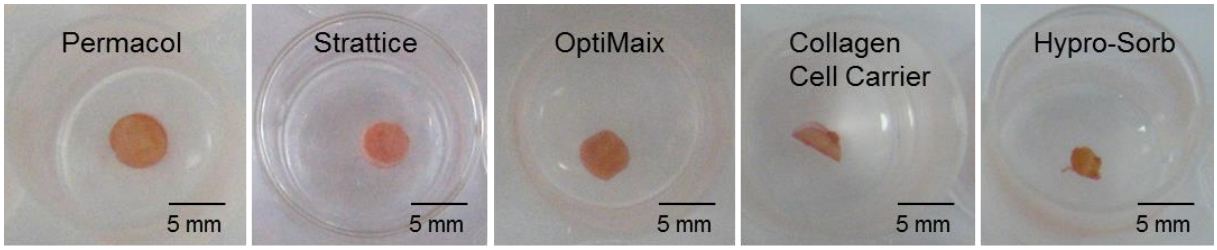


Fig. 10: Comparison of the chosen collagen matrices.
Size and shape stability of adipose-derived stem cell-seeded collagen matrices after 14 days of culture. Initially, all matrix discs had a 5 mm diameter. Only Permacol™ maintained its size and shape during culture.

Distribution of the ASC was analyzed by histological sections. The uneven distribution of the ASC on the Collagen Cell Carrier™ was apparent. On one side of the surface area was a large cell accumulation and on the entire remaining surface, on top of the

matrix, only a few cells were found. In contrast, HyproSorb™, showed cells randomly distributed in the whole construct. An infiltration of stem cells into the outer regions of the scaffold was found on the OptiMaix™ material. Only Strattice™ and Permacol™ displayed a coherent monolayer of hASC on top of the collagen matrix, with a small amount of cells penetrating the outermost edge of the scaffold. Thereby these two materials seemed to be most promising scaffolds for engineering a subcutaneous fat layer, as they were form and size stable in culture, and could be seeded with hASC as a monolayer on top of the matrix. Hence, in all following experiments only Permacol™ and Strattice™ were used. All characteristics of the five collagen matrices and the handling and seeding results are summarized in Table 2.

Table 2: Second part - stability in culture and cell adhesion of the chosen collagen matrices.

	Collagen matrices				
	Permacol™	Strattice™	OptiMaix®	Collagen Cell Carrier®	Hypro-Sorb®
Origin	porcine	porcine	porcine	bovine	bovine
Chemical cross-linking	yes	no	no	no	no
Thickness	0.5 mm	± 1.3 mm	0.5 mm	0.02 mm	0.8 mm
Size stability	stable	slight shrinkage	slight shrinkage	stable	shrinkage
Shape stability	stable	stable	curling up	curling up	dissolving
Cell adhesion	good	good	good	fair	good
Cell location	layers on top	layers on top	slightly into scaffold	incoherent on top	scattered in scaffold

Further, it was of interest to vary the thickness of the cell layer on the construct, to fit it into the surrounding tissue without loss of cosmesis. Therefore different cell concentrations were seeded on top of the collagen matrices to investigate whether more than one layer could be achieved. After cutting scaffolds in standard 5 mm diameter discs, collagen matrices were incubated for 2 h in growth medium. Then, scaffolds were seeded with cell suspensions of 30,000, 100,000 and 300,000 cells per disc and incubated overnight. All matrices were harvested, fixed with formalin, embedded and cryo sectioned. Cell nuclei were stained with hematoxylin or DAPI to visualize the cell layer on top of the scaffold material. The cell concentration and the number of cell layers on the matrices correlated: (a/d) 30,000 cells per disc showed an incomplete cell layer on the surface, (b/e) with 100,000 cells seeded, a complete monolayer of hASC on top of the matrix was observed, and (c/f) 300,000 cells per disc exhibited a coherent multilayer of stem cells, all shown in Fig. 11.

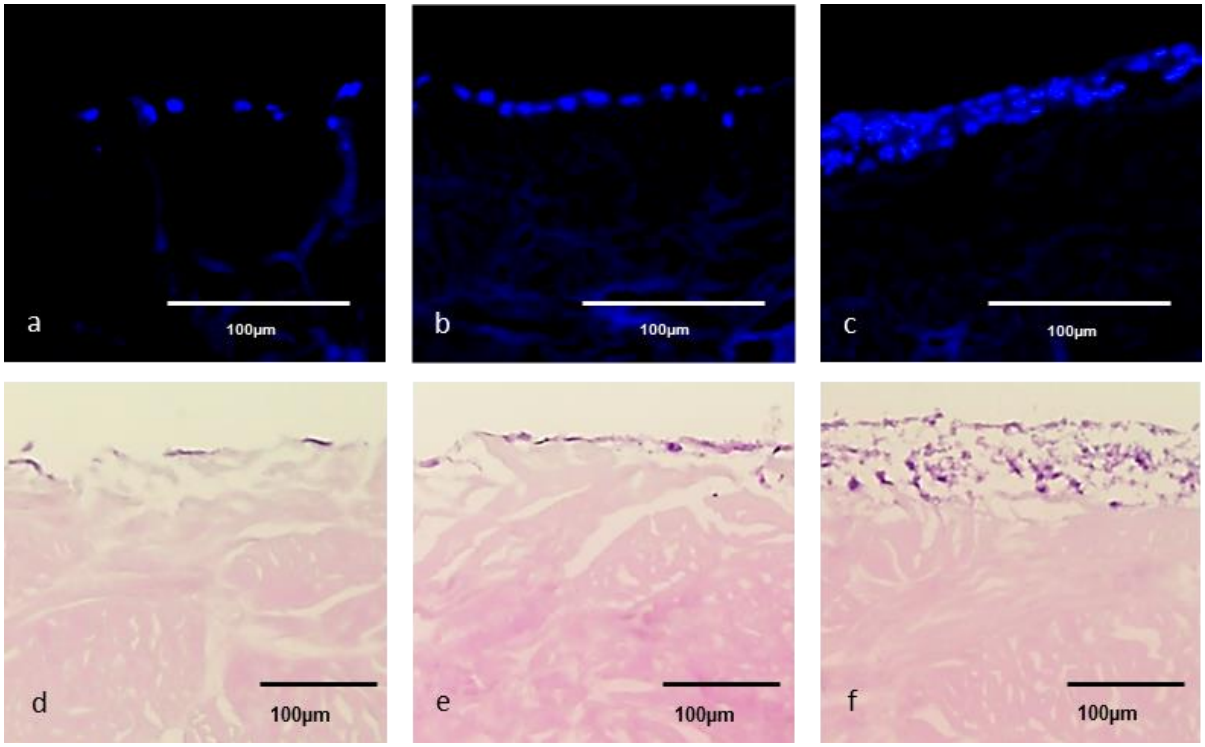


Fig. 11: Cell seeding with different cell densities on disc-shaped (ø 5mm) Permacol™ matrix. a/d: 30,000 cells; b/e: 100,000 cells; and c/f: 300,000 cells seeded on top of the matrix; constructs were harvested one day after seeding. Histological sections were stained with DAPI (fluorescent staining for nuclei; top row) and with hematoxylin and eosin (for nuclei and collagen; bottom row). A cell density of 100,000 cells per disc was needed to yield a single layer, whereas 300,000 cells per disc resulted in a multilayer of cells.

4.1.3 Adipogenic differentiation - Triglyceride synthesis

To test adipogenic differentiation potential of the stem cell layer on Permacol™ and Strattice™, collagen discs were again seeded with 300,000 hASC per disc. For adhesion, constructs were cultured overnight in an incubator. On the next day (day 0), growth medium was exchanged to differentiation medium, with a common adipogenic hormonal cocktail for all experimental groups. In a control group, basal growth medium was continued. For molecular analysis, samples were harvested on day 0, 2 and 7; for a triglyceride quantification assay and histological staining constructs were harvested on day 0, 7 and 14.

On top view images, fat accumulation could be demonstrated in the cytoplasma of differentiated ASC seeded on both collagen constructs, cultured in differentiation medium for 14 days. A distinct contrast to the non-induced control group was observed (Fig 12 A a-d). Also, on histological sections of day 7, a pronounced difference between cell-collagen constructs cultured with induction cocktail and samples of the control group could be detected (data not shown) and grew even stronger on samples of day 14 (see Fig 12 A e-f). After the end of culture, a thin fat layer was observed on top of the collagen matrices when induced. A small part of cells migrated into the upper layers of the scaffolds while most cells stayed as a multilayer on top of the matrix. The histological results were confirmed by the quantification assay of the triglyceride content of the cells. Corresponding to the histological observations, a triglyceride production in the cells was detected after adipogenic induction already on day 7 which further increased by the end of the culture on day 14. In contrast to the induced samples, the control group displayed only a small increase of triglyceride level in the cells during the whole culture. The quantitative triglyceride accumulation within the cells is summarized in Fig. 12 B.

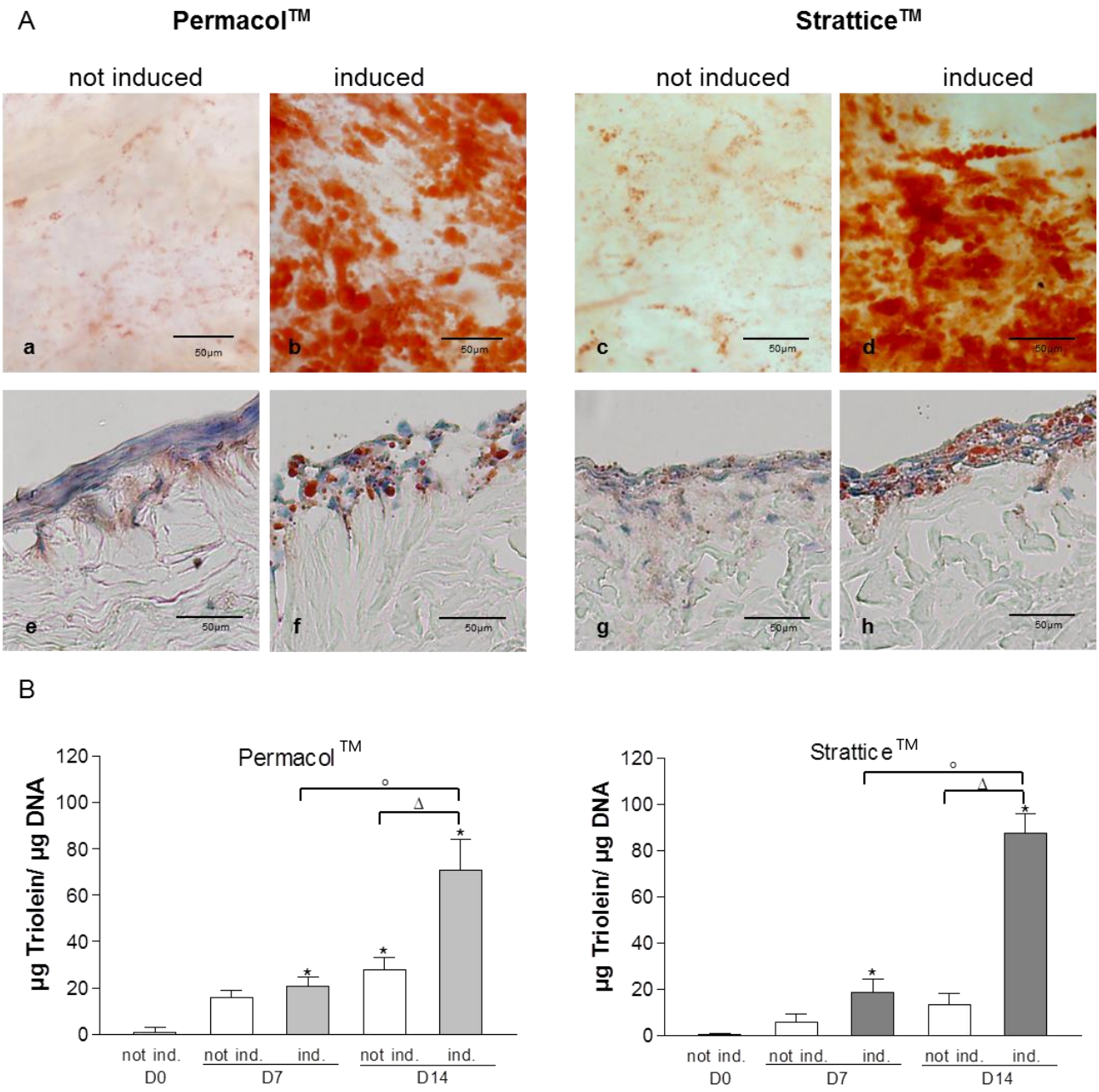


Fig. 12: Differentiation of hASC on collagen matrices after adipogenic induction.
hASC were cultured on top of Permacol™ and Strattice™ with an adipogenic induction cocktail for up to 14 days.
A: Substantial adipogenic differentiation was observed for the induced constructs, with a distinct contrast to not induced controls. a-d: Top view of seeded matrices stained with oil red O for lipids after 14 days of cultivation. e-h: Histological sections of matrices stained with oil red O and hematoxylin after 14 days of cultivation.
B: Quantification of accumulated triglycerides within the cells on day 0, day 7, and day 14 for induced constructs and not induced controls. Values are expressed as mean ± standard deviation (n=3). Statistically significant differences are indicated by * (to D0 'not ind.'), Δ (at the same day between 'not ind.' and 'ind.' samples) and ° (between samples with the same type of induction on different days). Three independent experiments were conducted, representative results of one experiment are shown here.

4.1.4 Molecular adipogenic differentiation – Gen expression

To confirm these results with molecular data, a quantitative PCR of three important adipogenic marker genes was performed. All findings in histology and quantitative assays were supported by the PCR results (Fig. 13). Gene expression was normalized to GAPDH, the obtained values were normalized to values of samples on day 0. The expression level of the transcription factors PPARγ and C/EBPα, two key factors of adipogenesis, as well as the transporter gene aP2 (FABP4) showed an increase from day 0 to day 2 and further increased to day 7 of induction. Results were similar on both Permacol™ and Strattice™. Interestingly, on the molecular level, also a small, but distinct increase in expression level of the adipogenic markers was found in the control constructs on day 7 in relation to day 0. As noticed before, this was also observed on histological stainings and in the triglyceride assay for both collagen scaffolds.

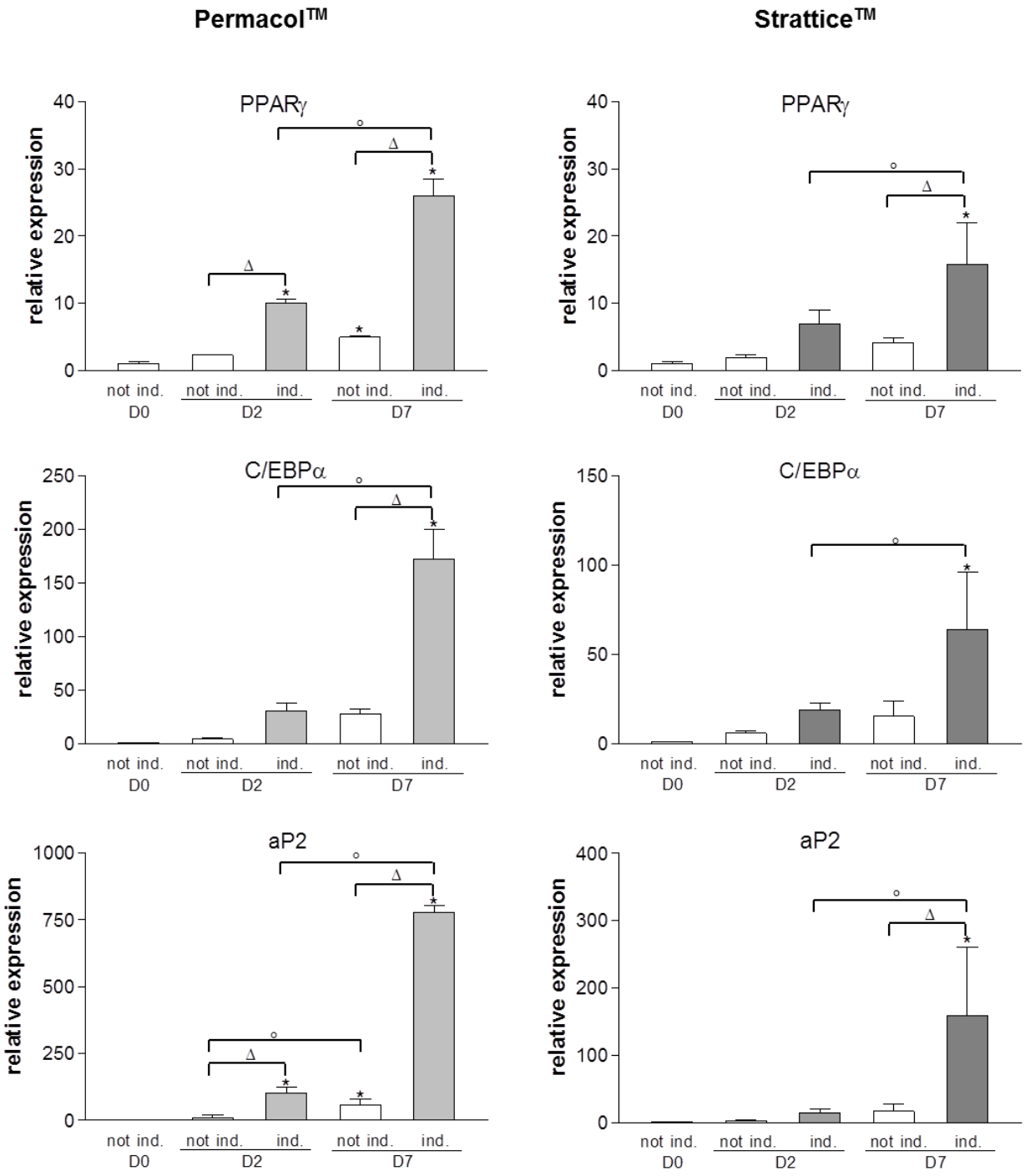


Fig. 13: Adipogenic marker gene expression of hASC on collagen matrices as determined by qRT-PCR.

Results up to day 7 of culture for Permacol™ and Strattice™ are shown. Gene expression was normalized to GAPDH, the obtained values were normalized to values of samples on day 0. Values are expressed as mean ± standard deviation (n=3). Statistically significant differences are indicated by * (to D0 'not ind.'), Δ (at the same day between 'not ind.' and 'ind.' samples) and ° (between samples with the same type of induction on different days). Two independent experiments were conducted, representative results of one experiment are shown here.

4.1.5 Testing cells isolated from different donors and donor sites

In order to investigate the general applicability of the presented approach, cells from different donors and different donor sites were investigated on the Permacol™ matrix. Cells were isolated from the abdomen of a woman age 52 with a BMI of 26, the thigh of another woman age 35 and a BMI of 30, or from the abdomen of a man age 72 and a BMI of 27. As described before, 300,000 stem cells were seeded on a 5 mm diameter disc and adipogenically induced by a hormonal cocktail, or cultured in growth medium (PGM), as a control.

The stem cells of all sources displayed an increase of adipogenic differentiation over time as expected; they differed only in the intensity of adipogenic differentiation (Fig. 14).

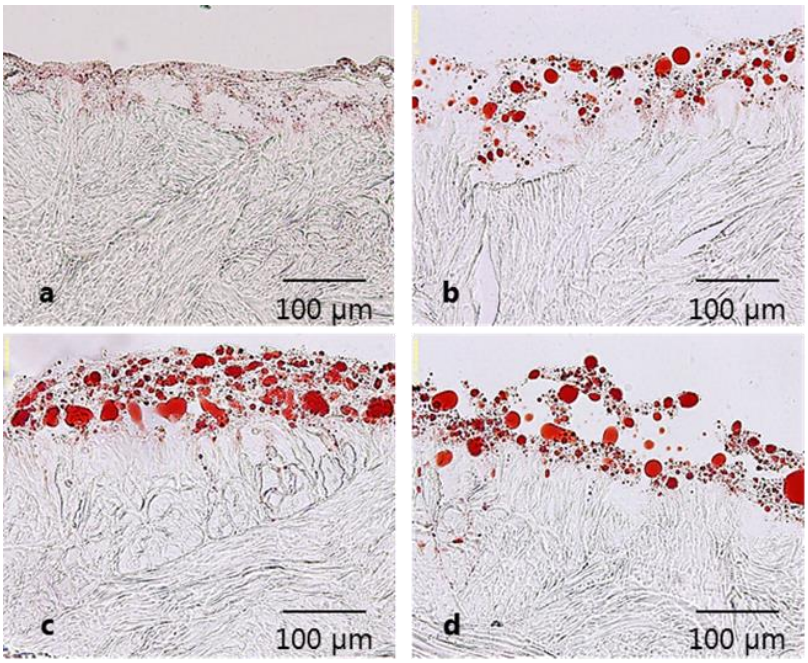


Fig. 14: Adipogenic differentiation of different cell sources.
Cells were cultured on top of Permacol™ with an adipogenic induction cocktail for up to 14 days. **A:** Oil red O staining for lipid droplets within the cells at day 14 after induction. a) Non-induced control; the other histological sections show adipogenically induced hASC, isolated from b) the abdomen of a woman age 52 with a BMI of 26; c) the thigh of another woman age 35 and a BMI of 30; d) the abdomen of a man age 72 and a BMI of 27

These observations could be confirmed by a quantitative triglyceride assay (Fig. 15).

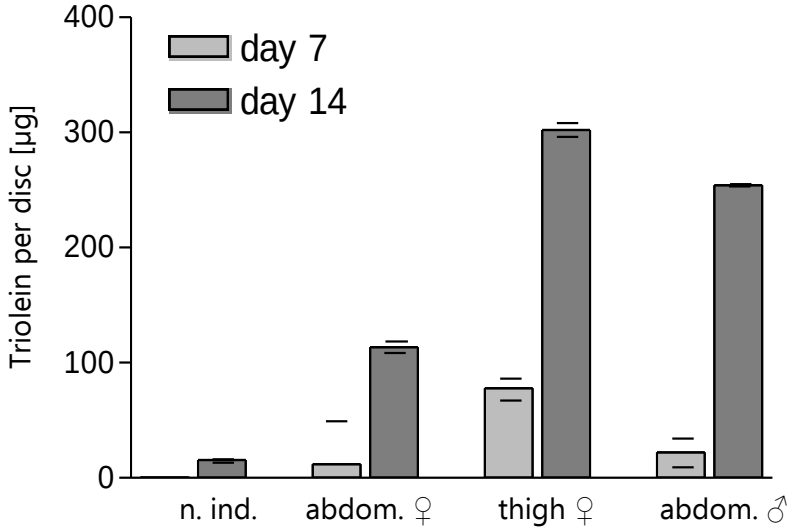


Fig. 15: Adipogenic differentiation of different cell sources.
Cells were cultured on top of Permacol™ with an adipogenic induction cocktail for up to 14 days. Quantification of triolein amount per disc at day 7 and 14 (n=2) after adipogenic induction of different hASC sources. Triolein content of day 7 of the non-induced control was below the detection limit (not shown). Dashes, above and below represent the individual values of both biological replicates.

4.1.6 Varying the adipogenic induction protocol on the collagen scaffolds

In an approach to improve the adipogenic induction protocol, the induction time *in vitro* was shortened. This may be valuable for clinical application, as it could save time between the liposuction and the implantation of the fat-collagen construct. As done before, 300,000 hASC were seeded on 5 mm discs of Permacol™ and Strattice™, then cultured in growth medium overnight so cells could adhere. Constructs were transferred on the next day to either induction medium or growth medium as the control. Constructs were divided into four groups: the first group was cultured with growth medium for the whole culture as a negative control, the second group was induced for the first two days and then received growth medium for the rest of the

culture; the third group was induced for four days and received growth medium for the rest of culture. The last group was cultured in the adipogenic induction cocktail for the whole culture as a positive control (Fig. 16 A).

After 14 days in culture, histological staining was done. The short-term induction for two days (group two) did not stimulate adipogenic differentiation enough to achieve an adequate fat layer. However, the short-term induction for four days (group three) showed a substantial fat accumulation in the cells on top of the scaffolds. Adipogenesis was less than in the positive control with permanent application of adipogenic inducers, but increased strongly compared to the non-induced negative control or to the samples with short-term induction for two days (Fig. 16 B).

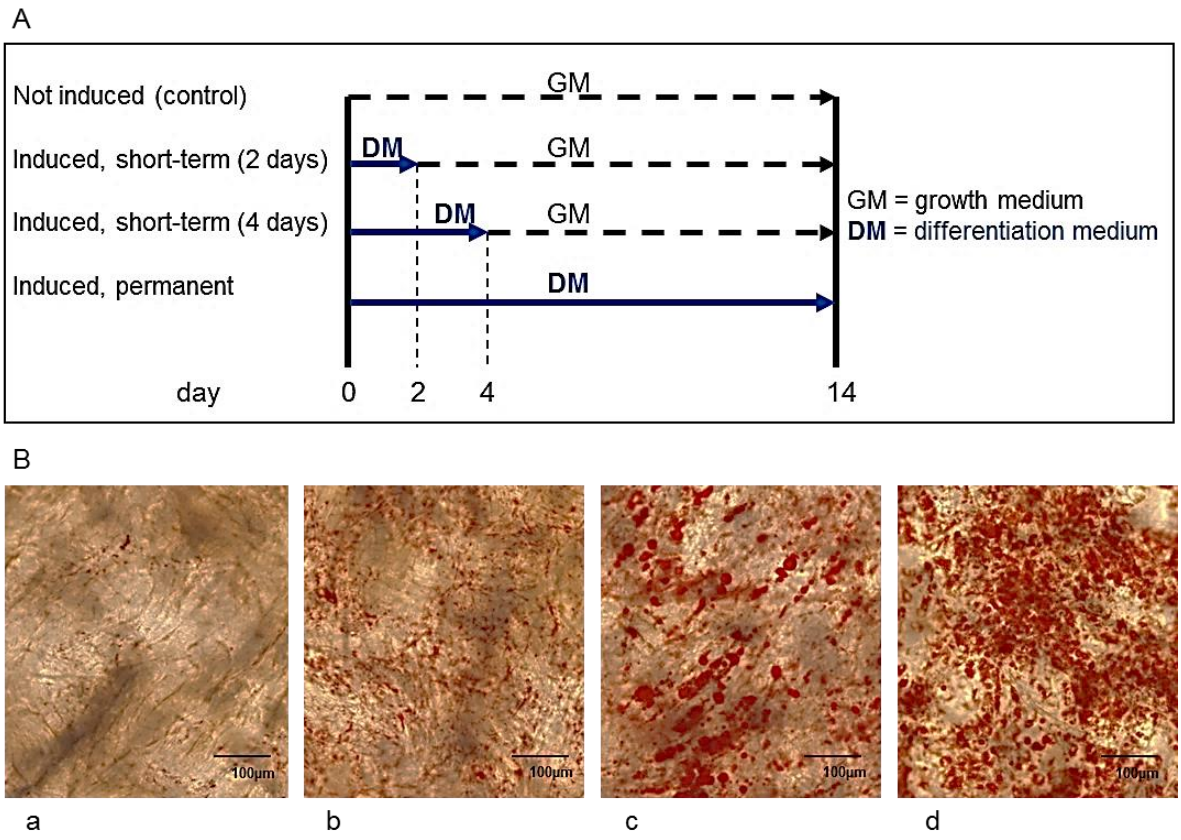


Fig. 16: Variation of the adipogenic induction protocol.
A: Scheme shows the different adipogenic induction protocols used for ASC differentiation on Permacol™ matrix.
B: Top view of seeded matrices stained with oil red O for lipids after 14 days of cultivation according to different protocols. a) not induced control, growth medium for the entire culture time; b) 2 days differentiation medium, then 12 days growth medium; c) 4 days differentiation medium, then 10 days growth medium; and d) permanent induction with differentiation medium for 14 days. A short-term induction for 4 days (c) already resulted in substantial adipogenesis.

4.2 Engineering vascularized adipose tissue

The second goal of the presented thesis was to engineer a vascularized fat tissue *in vitro*. The idea was to use a natural ECM with a pre-existing vascular stucture and pedicles to connect the vessels to the reservoir with medium.

To this end a porcine jejunum was decellularized and used as scaffold for cell seeding. At the vascular pedicle of the jejunum, the cannulization of the artery and the vein can be easily achieved. In the outer area of the jejunal segment a multi-branched vessel system is available, which can be reseeded with human endothelial cells via the artery inflow. The lumen of the jejunal segment provides a large surface to be seeded with human stem cells, which can then be adipogenically differentiated.

4.2.1 Stem cell seeding of a porcine jejunal section

To investigate if the extracellular matrix of the jejunal section is at all suitable for seeding progenitor cells from human fat tissue, various cell densities were seeded on top of the inner side of jejunal sections. The porcine scaffold was cut into pieces of 1 cm^2 and then mounted over a cell crown with an inner seeding diameter of 5 mm. Afterwards the cell crown was incubated in growth medium and humidified atmosphere. After 2 h different cell concentrations were seeded onto the inner side of the cell crown-fixed jejunal section and incubated overnight, so cells could adhere on the scaffold. On day 7 of the culture, samples were harvested and histologically stained with hematoxylin. Stem cell layers, with varying thickness according to the cell seeding densities, were found in the villi structure of the jejunum. Noticable, stem cells seeded onto the inner side of the matrix did not migrate further into the ECM than to the muscularis mucosa. When 50,000 cells were seeded a monolayer around and on top of the villi was visible. When 300,000 cells were seeded, a multilayer of

stem cells was found on the inner side of the jejunal section, at which the villi could not be distinguished from the mucosa anymore (Fig. 17).

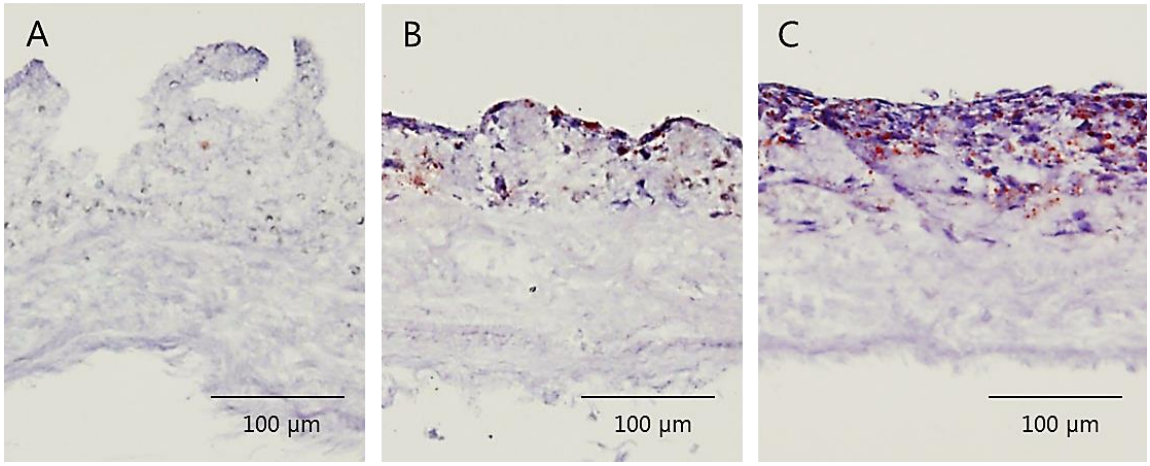


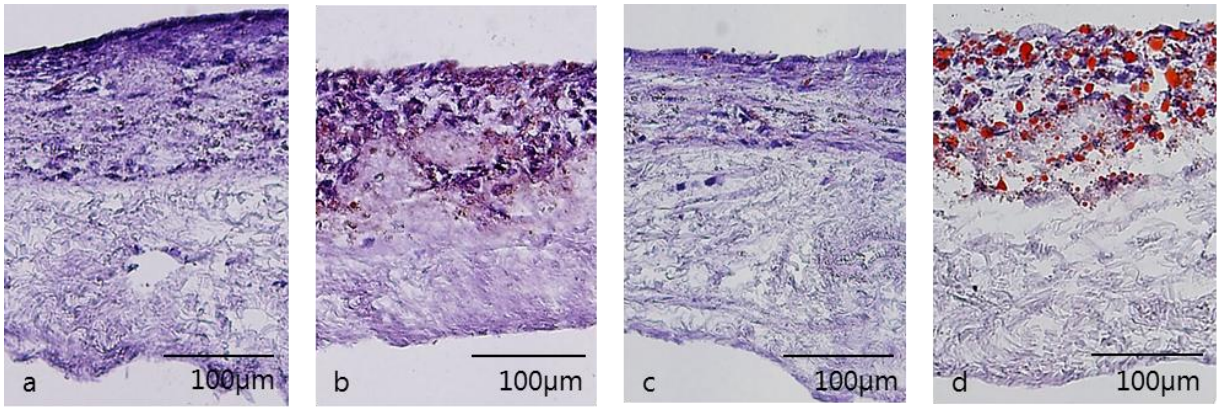
Fig. 17: Decellularized porcine jejunal sections (diameter 5mm) seeded with different densities of hASC.
Cells were adipogenically induced for 7 days. Histological sections stained with hematoxylin and oil red O (lipid droplets). A: without cells, B: seeded with 50,000 cells, a monolayer between and on top of the villi was detectable, and C) seeded with 300,000 cells, a multilayer of stem cells was visible on the inner side of the porcine jejunum.

4.2.2 Adipogenic differentiation

To investigate adipogenesis on the jejunal section, 300,000 hASC were seeded onto the porcine ECM and adipogenically induced by a hormonal cocktail on the following day. Some of the constructs were cultivated with growth medium as a control group. On day 0, 7 and 14, constructs were harvested from each group for histological analysis and for triglyceride assay. Samples for PCR analysis were taken on day 0, 2 and 7, as the expression level increase occurs earlier than the visual effects in the cell. An increase of fat droplet accumulation over the culture period was found on the histological sections in constructs with permanent adipogenic induction compared to the negative control group. Triglyceride synthesis in cells was quantified after staining with oil red O by leaching of the dye that was bound to triglycerides in the cells, and then normalized to the cell number on each jejunal section. From day 7 till day 14,

the triglyceride amount in cells of the group with differentiation medium had doubled (Fig. 18 B). Compared to the induced constructs distinctly less oil red O was recognizable on the histological sections of the control group (Fig 18 A), and only a small increase of triglyceride was measured in these cells (Fig. 18 B).

A



B

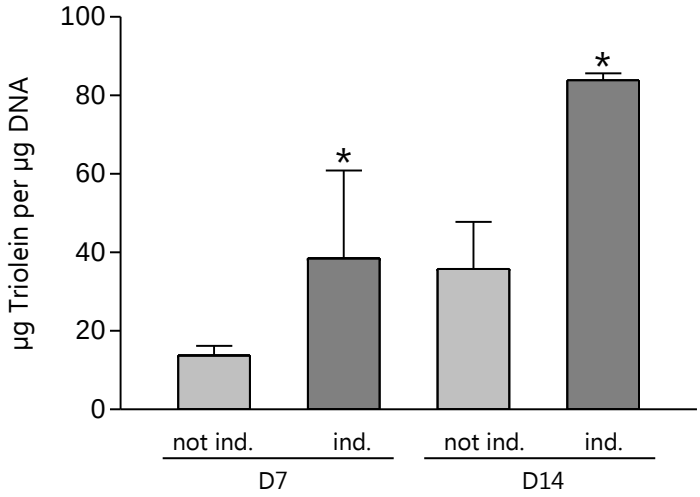


Fig. 18: Differentiation of hASC on the jejunal sections after adipogenic induction.
300,000 hASC were seeded on the inner side of the jejunal sections and cultured with adipogenic stimuli for up to 14 days.
A: a/c: uninduced controls and b/d samples after adipogenic induction. Constructs were harvested on day 7 (a/b) and day 14 (c/d). An adipogenic development of the induced constructs and a distinct contrast of lipid droplet accumulation versus the non-induced control could be shown. Cell nuclei were stained with hematoxylin and lipid droplets with oil red O.
B: Quantification of accumulated triglycerides within the cells on day 7 and day 14. Values are expressed as mean ± standard deviation (n=3). Statistically significant differences are indicated by * (at the same day to 'not ind.' samples). Three independent experiments were conducted; representative results of one experiment are shown here.

The PCR data of important adipogenic marker genes corroborated the results of the histological and quantitative analyses of triglycerides within maturing hASC. Samples cultured with differentiation medium showed an increase of the expression level of PPARγ and C/EBPα, the two key transcribtion factors of adipogenesis, and of aP2 (FAB4), a transporter gene in adipogenic development. On the other hand, samples cultured without adipogenic induction displayed only a small increase of the expression levels of these genes (Fig. 19). All obtained data indicated that hASC can be seeded and adipogenically differentiated on the jejunal section.

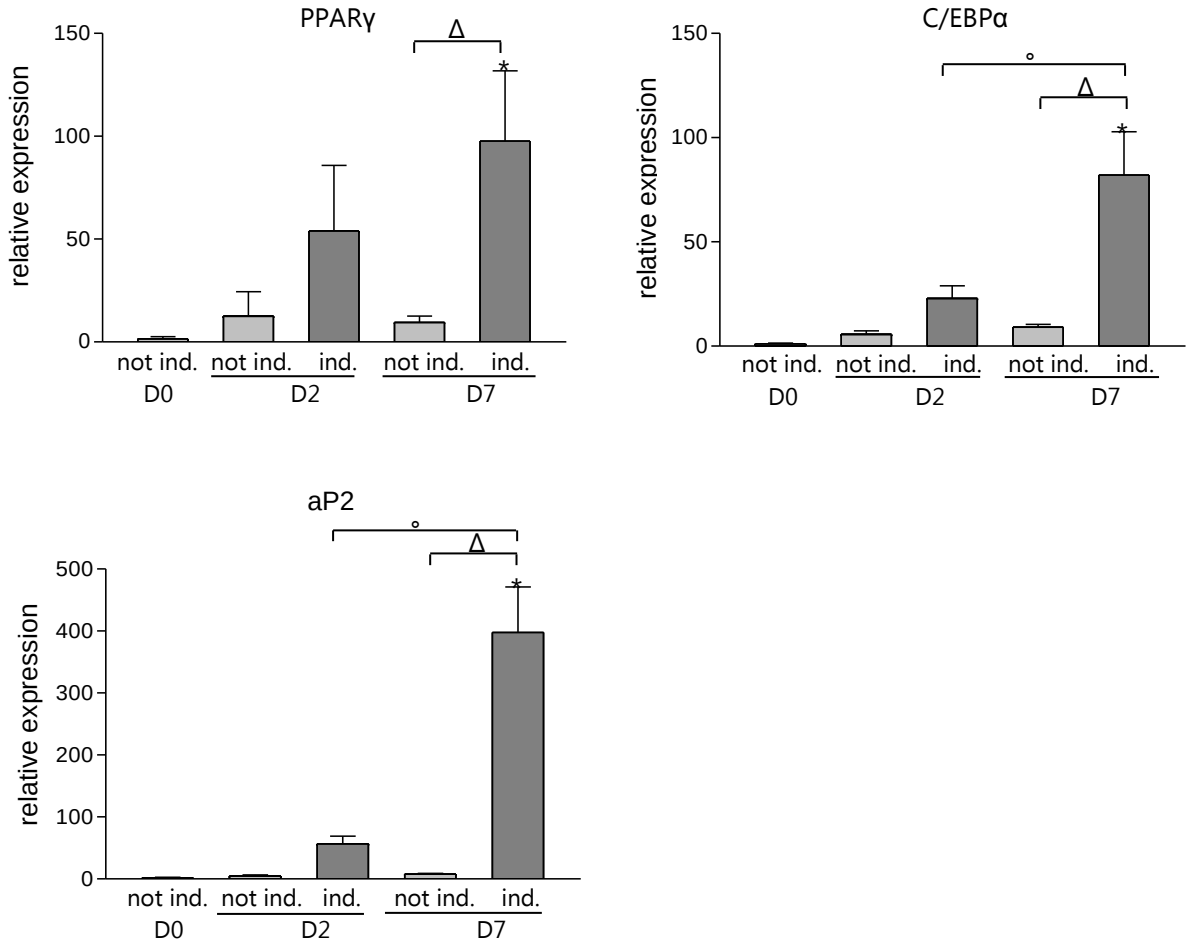


Fig. 19: Adipogenic marker gene expression of hASC on porcine jejunal sections as determined by qRT-PCR.

Results up to day 7 of culture are shown. Gene expression was normalized to GAPDH, the obtained values were normalized to values of samples on day 0. Values are expressed as mean ± standard deviation (n=3). Statistically significant differences are indicated by * (to D0 'not ind.'), Δ (at the same day between 'not ind.' and 'ind.' samples) and ° (between samples with the same type of induction on different days). Two independent experiments were conducted; representative results of one experiment are shown here.

4.2.3 Co-culture of endothelial cells and ASC on a jejunal section

For the engineering of vascularized fat tissue, not only ASC are needed, but also endothelial cells. Therefore, commercially available microvascular endothelial cells were tested in a co-culture system on the jejunal section. To provide adequate conditions for both cell types, the medium had to be modified. Therefor, adipogenesis was tested in a 2D culture in well plates with a medium mixture of endothelial cell medium (VascuLife) and progenitor specific cell medium (PGM). To start with, 50,000 ASC were seeded into each well of a 24-well plate and then cultured with different medium compositions. The experiment was divided into four groups, one was cultured with a 1:1 mixture of PGM/VascuLife with adipogenic inducers and all growth factors of VascuLife, the next group got a 1:1 mixture of PGM/VascuLife with adipogebic inducers and growth factors of VascuLife, but without endothelial growth factor (EGF), a supplement growth factor of the VascuLife medium (EGF had previously been reported to affect adipogenesis (Harrington 2007 and Hauner 1995)). Another group was treated with PGM medium with adipogenic inducers as the positive control, and the last group was cultured with PGM medium without hormonal induction as a negative control. An increase of the triglyceride content in the cells was measured in all groups with adipogenic hormonal cocktail and only a negligible triglyceride content was found in the negative control. The 1:1 medium without EGF displayed stronger adipogenesis than the 1:1 medium with EGF, but the best adipogenesis was still found in the positive control, with PGM and the hormonal cocktail alone (Fig. 20). The data of the triglyceride assay was confirmed by bright field microscopy (data not shown).

All in all, a distinct adipogenic differentiation of the ASC was observed in the 1:1 medium without EGF. At the same time the endothelial cells still proliferated and showed the endothelial-typical surface marker and appearance, which has been shown previously [Muhr 2013]. Hence, the 1:1 VascuLife/PGM medium without EGF was further employed as the co-culture medium.

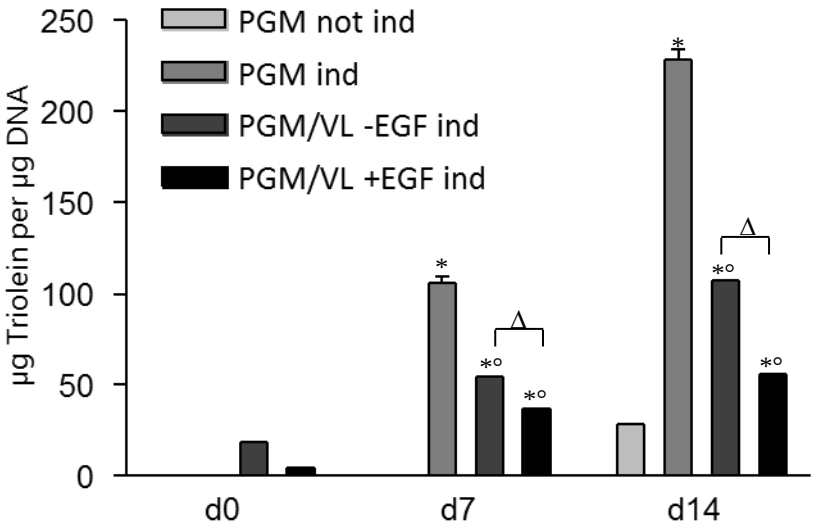


Fig. 20: Determination of adipogenic maturation using different culture media.
hASC were seeded in a 2D monolayer culture using different media as indicated. At day 0, day 7 and day 14 samples were harvested and the triolein amount per cell was quantified. Statistically significant differences are indicated by * (to D0 PGM 'not ind'.), ° (to the same day PGM 'ind'.) and Δ (between induced samples with and without EGF at the same days). PGM/VL = 1:1 PGM VascuLife medium.

The next step was to determine whether the co-culture system with microvascular endothelial cells and hASC could be also converted into a 3D system on a piece of jejunum. First, 300,000 endothelial cells were seeded on top of the outer side of the mucosa, by turning the cell crown upside down and cultivating the endothelial cells in VascuLife overnight. The cell crowns were turned upright on the next day, after the adherence of the endothelial cells was completed. Then, 300,000 ASC were seeded on the inner side of the jejunal section and cultured for 14 days in co-culture medium. Histological sections performed after ASC seeding showed the cell distribution. On the inner side of the villi all stem cells were settled, and on the other side, separated by the mucosa and the muscularis mucosa, the endothelial cells were located as a monolayer (MVEC were immunohistologically stained brown with CD31-antibody) (Fig. 21). During culture ASC were migrating over the muscularis mucosa towards the endothelial cells as shown in Fig. 21.

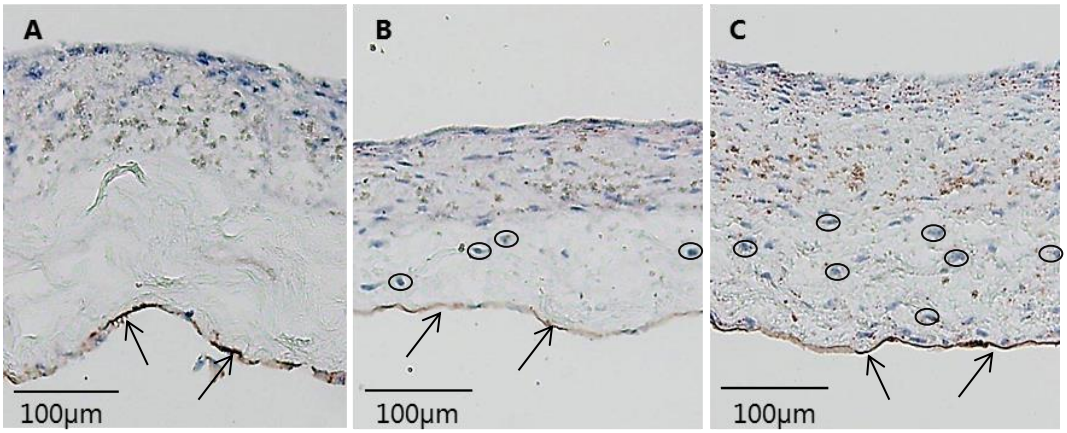


Fig. 21: Co-culture of ASC and MVEC on jejunal sections with 1:1 PGM/VascuLife medium without adipogenic induction.
The decellularized jejunal sections were seeded with 300,000 human ASC on the inner side and with the same amount of human MVEC on the outer side. For this co-culture a 1:1 medium based on PGM and VascuLife was established. Histological sections were stained with hematoxylin and CD31-antibody (endothelial cell marker, arrows). Samples were harvested on A) day 0, B) day 7 and C) day 14. A fraction of ASC was shown to migrate towards MVEC during culture (labeled with black circles). The thickness of the constructs in cross sections varies, as in nature the jejunum is not even and some pieces get more stretched than others when placing the jejunal section onto the cell crown.

For analysis of adipogenesis in this co-culture system, the culture was started as described before. After seeding the ASC and one night in 1:1 co-culture medium without EGF, all constructs were divided into two groups. One group was permanently cultured in 1:1 co-culture medium without EGF and without induction, the other group received 1:1 co-culture medium without EGF, but with an adipogenic hormonal cocktail. Adipogenesis was observed on histological sections of the co-culture constructs. Histological sections, stained with oil red O, hematoxylin and CD 31, displayed an increase of fat droplets on the constructs during the culture. No fat was observable on day 0, but on day 14, small fat droplets were detected, which enlarged until day 21. In the negative control group, no fat accumulation was apparent during the whole culture phase (Fig. 22). These results demonstrated that a co-culture is possible on these scaffolds employing 1:1 co-culture medium. A less distinct migration of ASC towards MVEC was noticeable in the induced group compared to the non-induced group during the whole culture (Fig. 22).

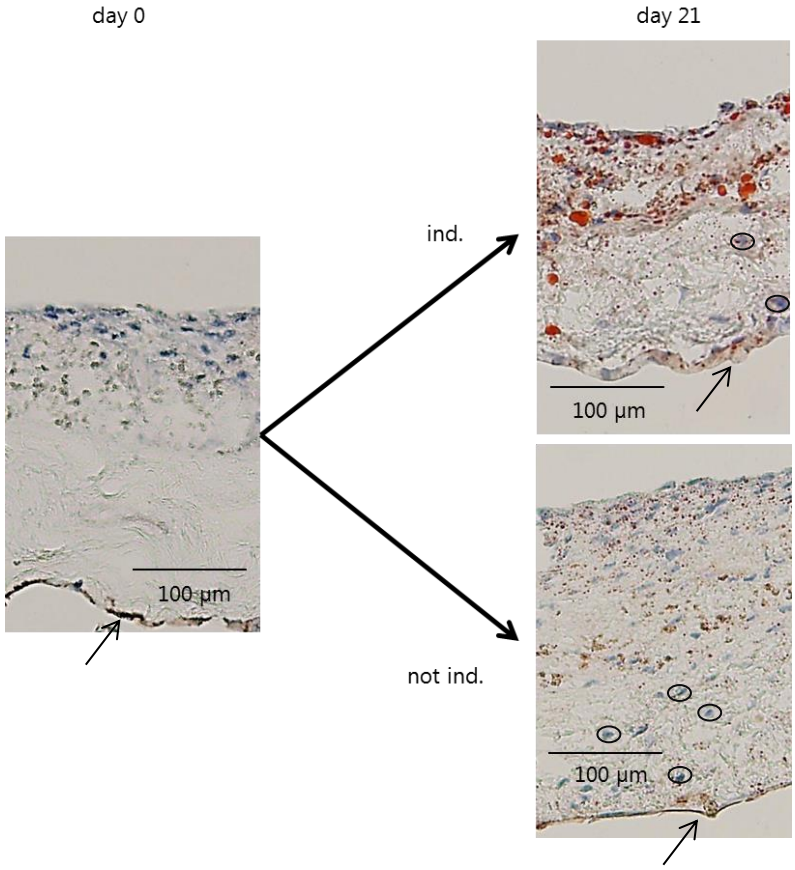


Fig. 22: Adipogenesis of a co-culture with ASC and MVEC in 1:1 co-culture induction medium. The decellularized jejunal sections were seeded with 300,000 human ASC on the inner side and with the same amount of human MVEC/cm^2 on the outer side. For this co-culture a 1:1 medium based on PGM and VascuLife was established and adipogenically induced. Histological sections were stained with hematoxylin and CD31-antibody (endothelial cell marker, small arrow). Migrating ASC are labeled with a black circles.

4.2.4 Small bioreactor - flow conditions

The next step towards the complete, custom-made flow-through bioreactor system was to test the flow conditions in a small flow reactor, thereby investigating the adipogenic differentiation of ASC under flow conditions. At first, 1 cm^2 pieces of jejunum were cut and fixed into the two-chamber system of the flow reactor. Then 400,000 endothelial cells were seeded onto the outer side and cultured overnight in VascuLife. On the next day 400,000 adipose derived stem cells were seeded onto the inner side of the jejunal section and cultured overnight in 1:1 co-culture medium. The day after, a flow was applied and the medium was changed to the 1:1 co-culture

medium. For this experiment, three settings were chosen: first, a reactor with flow on both sides; second, a reactor with flow on the endothelial cell side and static conditions on the stem cell side; and the last condition was a static culture on both sides. After the flow conditions were applied, the adipogenic differentiation was started by adding a hormonal cocktail. On day 7 and 14, constructs were harvested and histologically examined. There were no differences between all three flow culture conditions concerning adipogenesis. All constructs displayed a good adipogenesis and a nearly coherent endothelial cell monolayer on the opposite side. The difference in thickness of the tissue could be explained with the naturally non-homogeneous intestine wall. In static culture the scaffold had to be spanned onto the cell crown, which sometimes causes tension and adds to a deformation of the tissue (Fig. 23). The results suggested that all conditions were suitable for adipogenesis in co-culture in the custom-made bioreactor system.

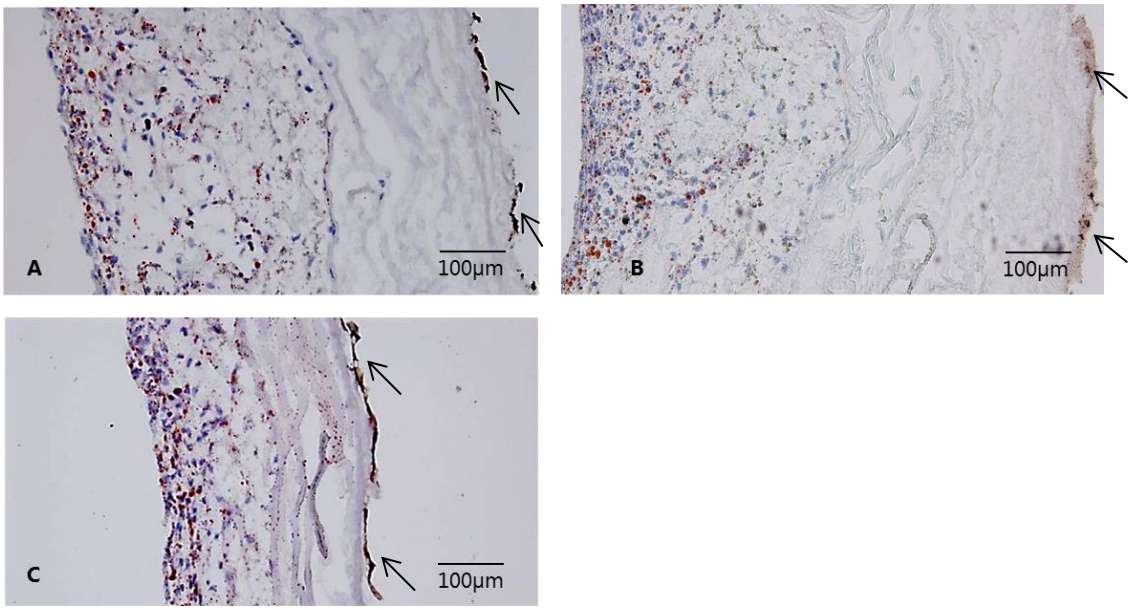
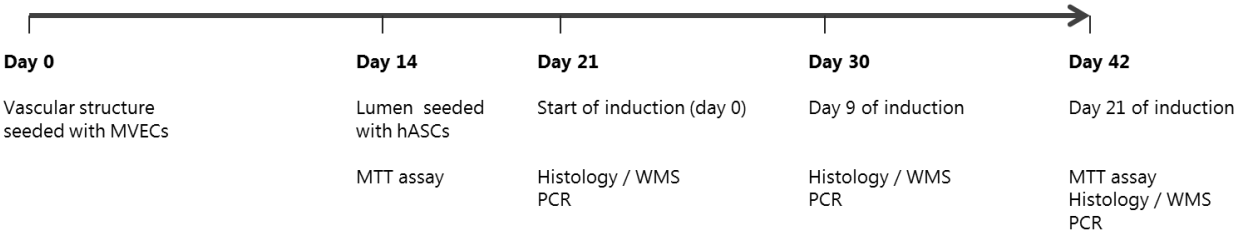


Fig.23: Co-culture of ASC and MVEC on jejunal sections after 14 days in a small flow bioreactor system.
The decellularized jejunal sectionts were seeded with $4*10^5$ human ASC/cm^2 on the inner side and with the same amount of human MVEC/cm^2 on the outer side. In the bioreactor system, the following cultivation conditions were applied: A) a flow on both sides, B) ASC cultured statically and MVEC cultured dynamically and C) a control was cultured statically in a cell crown. Histological sections were stained with oil red O, CD31 antibody and hematoxylin. Arrows indicate human MVEC stained brown with CD 31 antibody.

4.2.5 Using a custom-made bioreactor system to foster vascularization

In the next step a whole decellularized jejunal segment was used with its arterial and venous inlet, the decellularized vascular structure, which had to be reseeded with MVEC, and the lumen, which had to be reseeded with stem cells. As cells in the vessel structures are physiologically exposed to shear stress through the blood stream, an external medium flow was applied through the arterial inlet. In this study, as a prototype, one piece of a whole jejunal segment with the length of 8 cm was adipogenically induced in a custom-made bioreactor system.

Fig. 24: Experimental set-up



The pre-existing vascular structure was reseeded with 6 Mio. MVEC in two portions into the arterial system, and cultured with VascuLife medium. After seeding, cells were statically cultured overnight, and then a physiological flow with a pressure of 80-120 mmHg was applied. After 14 days, the distribution of viable endothelial cells in the vessel structure was monitored by a MTT-assay. A fine network of vessels within the vascular structure of the jejunal segment was stained blue by water-insoluble formazan indicating viable cells (Fig. 25A). The medium was then exchanged with a 1:1 co-culture medium without EGF, and the lumen of the jejunal segment was seeded with 5 Mio. ASC. The endothelial cells were still cultured with a flow and a pressure of 80-120 mmHg; ASC within the lumen were not directly exposed to the

fluid flow. After another 7 days in 1:1 co-culture medium without EGF, adipogenic inducers were added and the culture was continued for another 21 days in co-culture differentiation medium. At the end of the culture, the MTT-assay was repeated. Now there were not only living endothelial cells in the vessels, but also adipogenically differentiated ASC in the lumen (Fig. 25 B).

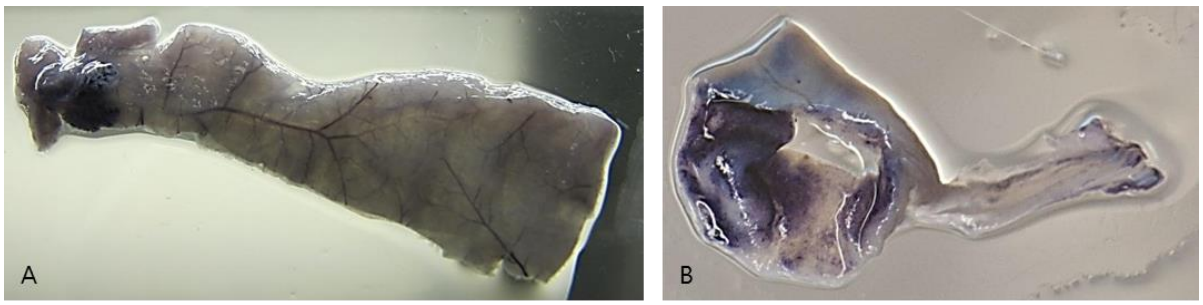


Fig.25: Reseeded jejunal segment cultured in a custom-made flow-through bioreactor system.
A: Construct after 14 days of culture, MTT staining (dark blue) indicates living MVEC (vascular structure)
B: Construct after 42 days of culture, MTT staining indicates living cells in co-culture of ASC (lumen) and MVEC (vascular structure)

During the culture, samples were harvested on day 21 (day 0 of induction), day 30 (day 9 of induction) and day 42 (day 21 of induction) for histological staining, whole mount staining and PCR analysis. In the whole mount-stained samples, a fine branched vessel system was observed which grew wider and subsequently densified over the culture periode (Fig. 26). The immunohistological sections confirmed the vascular structure present in the submucosa, and, thus, in the immediate vicinity of the ASC (Fig. 26).

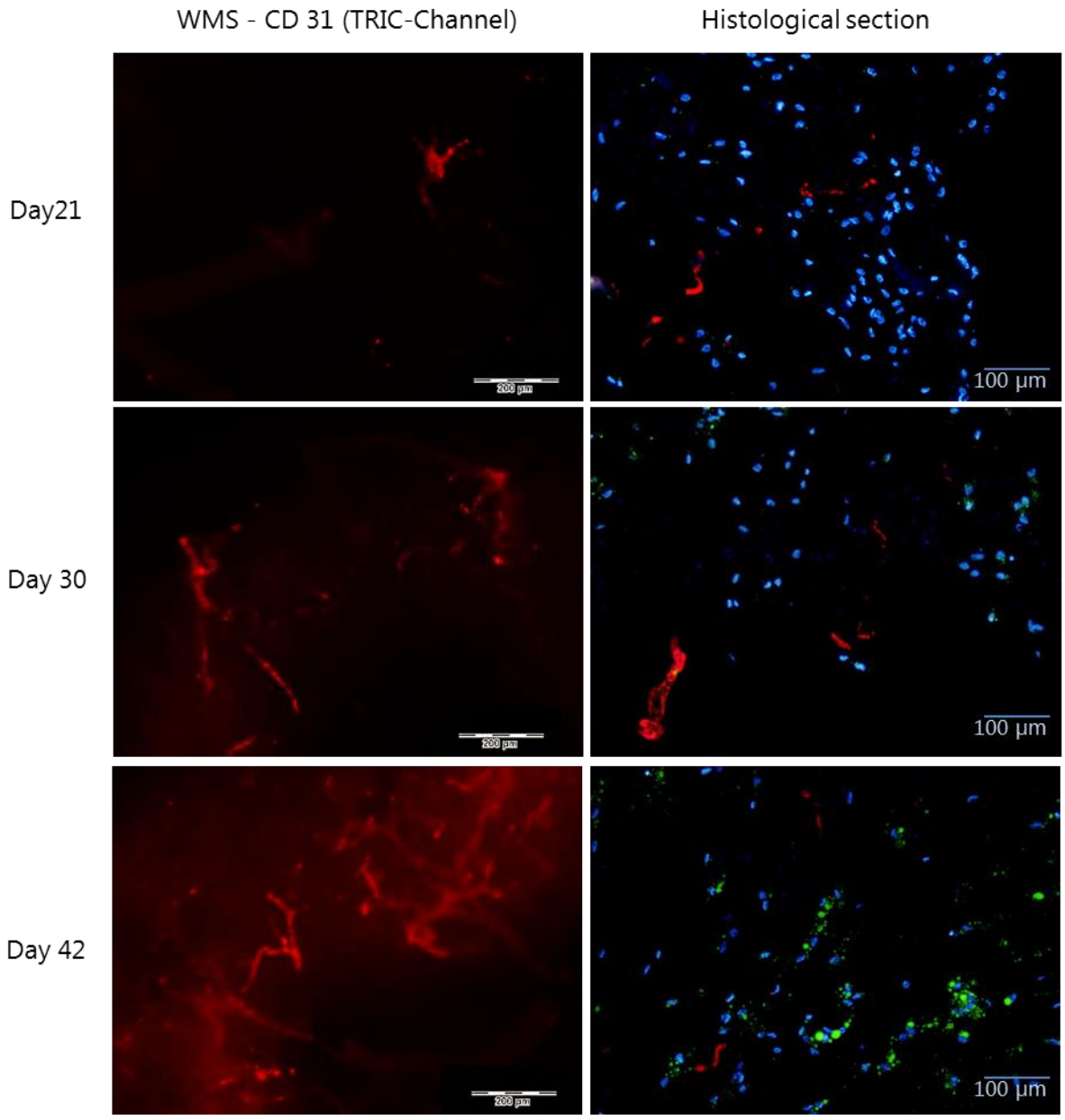


Fig. 26: Reseeded porcine jejunal segment cultured in a custom-made bioreactor for up to 42 days.
After three weeks in culture a fine network of vessels was observed which grew wider and densified during culture. (day 21, 30 and 42 of culture = day 0, 9 and 21 of adipogenic induction)
WMS: samples were stained red with CD31 antibody and secondary antibody labeled with Cy3 for MVEC.
Histological sections: MVEC were stained red with CD31 antibody and secondary antibody labeled with CY3, lipid droplets were stained green with BODIPY and the cell nuclei were stained blue with DAPI.

By BODIPY staining of the whole mount samples, the increase of the fat droplets in the tissue over time could be visualized (Fig. 27). It appeared that the vascular structure was reaching into the fat accumulations of the ASC on day 21 of induction (Fig. 27). On histological sections, the progressing adipogenesis could also be observed. The increase of the fat droplets in the cells was displayed with an oil red O

staining for triglycerides. Only very small fat droplets were found on day 0 of induction, but during the culture the fat accumulation in the cells increased from day 0 to day 21 of induction (Fig. 28).

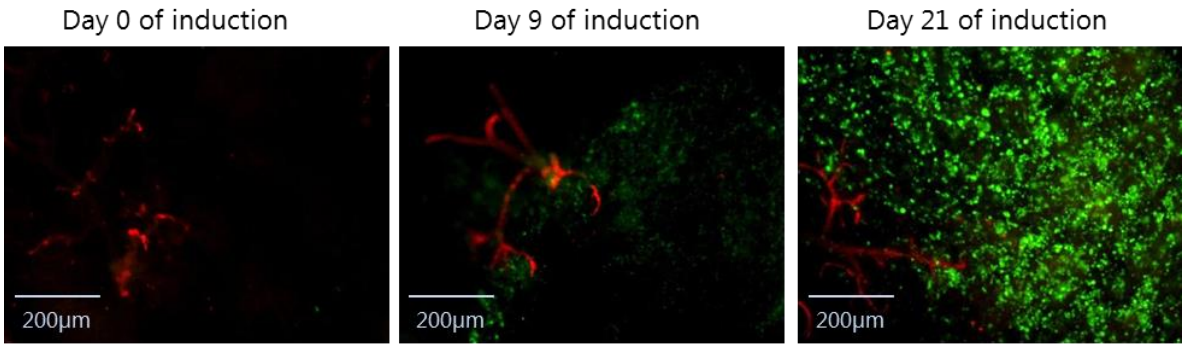


Fig. 27: Adipogenesis of ASC/MVEC co-culture on a reseeded jejunal segment cultured in bioreactor – whole mount staining.
Whole mount staining samples were stained with CD 31 antibody and secondary antibody labeled with Cy3 for MVEC (red) and BODIPY for lipid droplets within ASC (green). (day 0, 9 and 21 of adipogenic induction = day 21, 30 and 42 of culture)

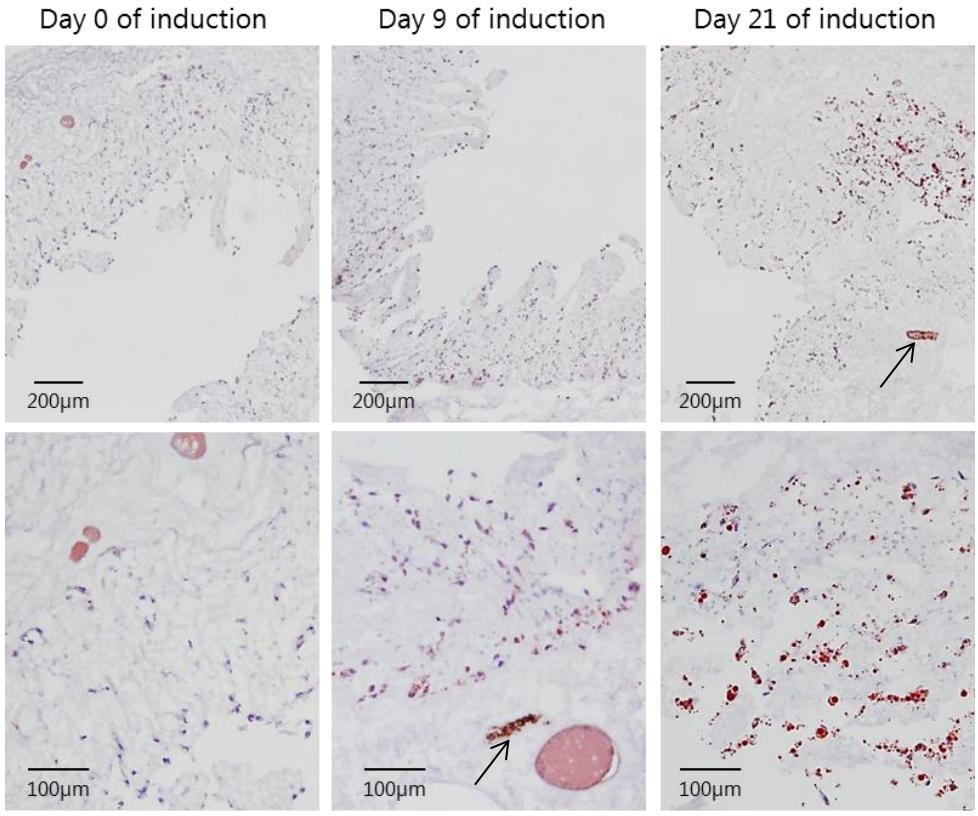


Fig. 28: Adipogenesis of ASC/MVEC co-culture on a reseeded jejunal segment cultured in bioreactor – histological staining.
Histological sections were stained with hematoxylin for cell nuclei (blue), CD31 antibody for MVEC (brown, arrows), and with oil red O for lipid droplets within ASC (red). (day 0, 9 and 21 of adipogenic induction = day 21, 30 and 42 of culture)

To determine adipogenesis on the molecular level and to quantify the expression levels, a quantitative real-time PCR for important adipogenic marker was done. The expression levels of the adipogenic markers PPARγ, C/EBPα and aP2 distinctly increased compared to day 0 of induction (day 21 of culture) (Fig. 29). It has to be kept in mind that the gene expression in the whole construct was measured, i.e., in ASC and MVEC: the expression levels were calculated for all cells taken together, but only a part of the cells were able to undergo adipogenic differentiation. That means, if only ASC had been analyzed (technically not possible), an even stronger increase of the expression levels of adipogenic marker would likely have been measured.

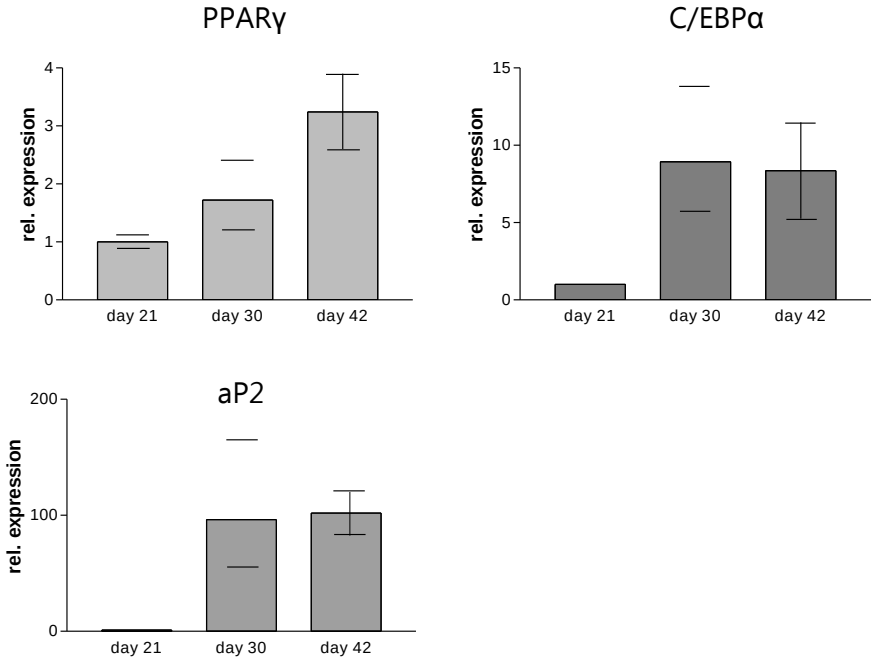


Fig. 29: Gene expression of adipogenically induced cells in bioreactor on day 21, 30, and 42 of culture (day 0, 9, and 21 of adipogenic induction).
Gene expression was determined by qRT-PCR; values were normalized to GAPDH for each group and time point, and further normalized to values at day 21. Dashes represent the individual values of two biological replicates and error-bars represent standard deviation of biological replicates (n=3).

Taken together, the development of a vascular network and progressing adipogenesis over the culture period was observed. The merge of vascular development and adipogenic differentiation at day 42 of culture indicated a successful generation of vascularized adipose tissue *in vitro* (Fig. 30).

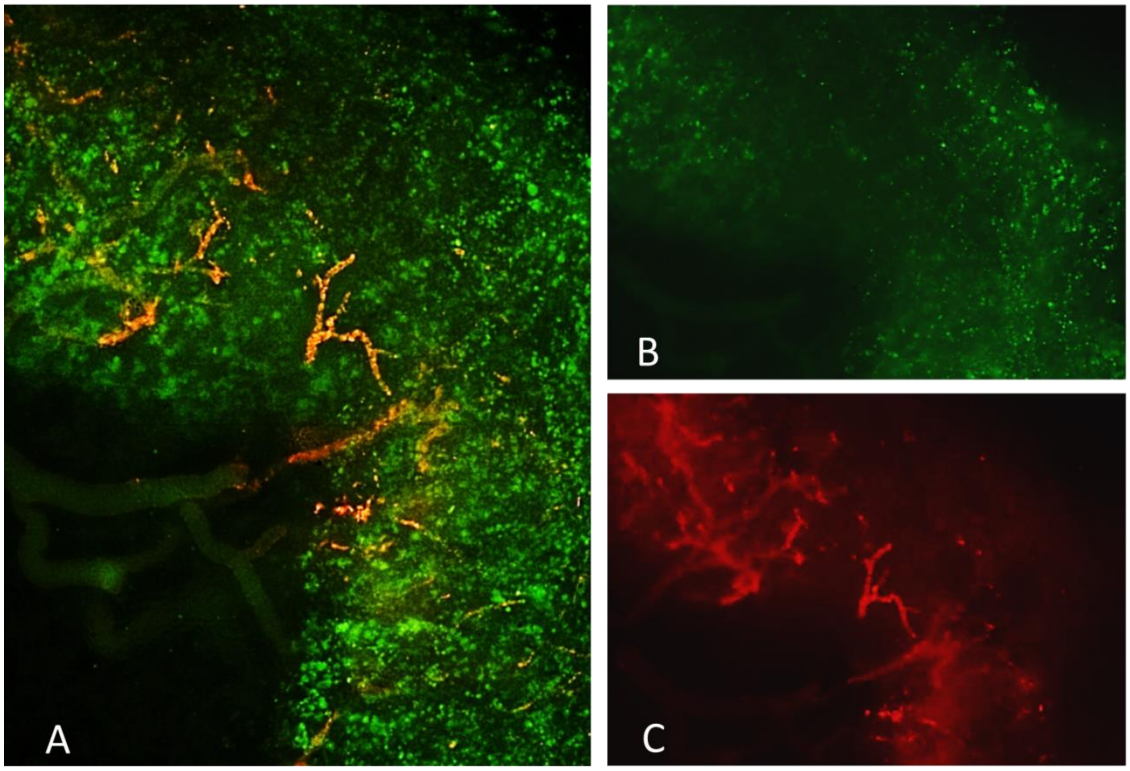


Fig. 30: Vascularized fat tissue construct engineered *in vitro*.
Whole mount staining (WMS) of a reseeded porcine jejunal segment cultured in a bioreactor after 42 days in culture.
A: The overlay shows a successful development of vascularized adipose tissue, with a capillary structure in the immediate vicinity of adipogenically differentiated ASC.
B: Adipogenically differentiated ASC in the lumen, lipid droplets were stained green with BODIPY.
C: Capillary structures, MVEC stained red with CD31 antibody and secondary antibody labeled with Cy3.

5 Discussion

Adipose tissue, generated by methods of tissue engineering, represents a considerable possibility to overcome the limitations of autologous fat grafts and synthetic implants in reconstructive surgery. The constantly increasing demand for adequate soft tissue substitutes can be addressed by adipose tissue engineering, as it provides autologous adipose tissue, by employing a small amount of autologous stem cells and utilizing scaffolds as cell carriers for specific applications. Ideally, engineered adipose constructs can be tailored to a specific application in using different types of matrices. For example, in a surgical strategy for hand reconstruction a thin fat layer construct would be a better choice than a large-volume adipose tissue substitute possibly used in augmentation of tumor defects. To use a thin and stable matrix is a key factor for generating a subcutaneous fat layer on the upper and lower extremities. In other cases the vascularization ability of scaffolds is necessary for building a large-volume fat substitute.

5.1 Engineering a subcutaneous fat layer

In cases of full-thickness defects after extensive resection of lesions, traumas, burn injuries or excision of hypertrophic scars on the upper and lower extremities, tissue flap grafts or full-thickness skin grafts are so far the surgeon´s choice to introduce a new subcutaneous fat layer [Saint-Cyr 2012, Lukas 2008, Kaufman 2007, Orgill 2013]. These methods are limited by donor site availability and morbidity, technically demanding and time consuming procedures, high risk for complications and aesthetic drawbacks. Treatments with split-thickness skin grafts or dermal substitutes have drawbacks as well; mainly insufficient cushioning, decreased elasticity and scar

contracture [Haik 2012]. A solution would be a thin subcutaneous fat layer to underlay the dermal substitute in reconstructed wounds, to improve function and aesthetics likewise.

To engineer such a subcutaneous fat layer, a suitable scaffold has to be found, as it simplifies the application and the control of location. An ideal material for soft tissue engineering would be strong, easy to handle and biocompatible while it supports cell adherence and new tissue growth. One logical consequence is to use collagen, as many collagen types are present as main components in natural extracellular matrices in many tissues [Choi 2010]. Collagen matrices have been shown to be biocompatible and to display a supportive structure for cell adherence and new tissue growth. They are well established in many tissue engineering applications [von Heimburg 2003, Hiraoka 2006, Neuss 2008] and in some studies collagen matrices showed a tremendous potential in adipose tissue development [von Heimburg 2003, Vashi 2006, Gentleman 2006]. Thereby, collagen is well characterized and can be fabricated in many ways. Some further criteria for the scaffold of subcutaneous fat layer constructs are: easy handling for implantation, thin sheets (not thicker than 1.5 mm), and clinical or cell biological approval. Acellular dermal matrices are widely used in clinical fields, for example in thorax surgery, hernia and breast reconstruction, and consist of a complete extracellular matrix structure. These matrices demonstrate to have a regenerative potential, to be minimally inflammatory; cells migrate into the dermal surrogate, remodel and replace it by autologous tissue [Hammond 2008, Liyanage 2006, O´Brien 2011, Hirsch 2012]. For tissue engineering purposes, these acellular dermal scaffolds seem to be excellent candidates for a basis-structure supporting cell proliferation and differentiation. For an adipose tissue engineering approach, the matrix has to be size and shape stable under culture conditions, and furthermore, has to support cell adhesion and adipogenic differentiation in order to result in a thin fat layer.

Therefore, five selected commercially available collagen matrices were investigated, initially for handling, shape and size stability under culture conditions with cell culture

medium. Meeting essential demands, Permacol™, the only chemically cross-linked collagen material, possessed the desired size and shape stability. The chemical cross-linking of the collagen by hexamethylene diisocyanate (HMDI) seems to be beneficial for long-term stability in culture. Strattice™, a non-cross-linked porcine dermal collagen matrix, on the other hand, showed excellent shape stability, but lost a bit of its size through slight shrinkage. All other matrices were considerably affected by culture conditions with regard to size and/or shape. (Chapter 4.1/Table 2)

With the intentsion to create a cell-seeded tissue substitute, the best possible cell source has to be chosen. ASC can be derived from autologous adipose tissue and have been demonstrated to differentiate into multiple tissue lineages including adipose phenotype. An additional feature of these cells is the wide availability, and the abundant and easy access as waste product in liposuctions [Bauer-Kreisel 2010, Gomillion 2006]. Preliminary cell-seeding experiments showed that ASC readily adhere as a homogeneous thin cell layer on top of the collagen matrices Permacol™ and Strattice™, but migrate into deeper levels of the construct on OptiMaix and Hypro-Sorb, or were allocated incoherently on the Collagen Cell Carrier scaffold. A thin cell layer on top of the matrix is desired, as one side can serve as cushion and the other, non-seeded side, enables connection to the surrounding tissue and ingrowth of host cells and vessels after implantation. The thickness of the cell layer on top of Permacol™ and Strattice™ could be varied depending on the seeding density of the ASC (Fig. 11). This is beneficial considering the diversity of subcutaneous tissue thickness and the demand for aesthetic shape remodeling. Thus, it is possible to construct thicker or thinner subcutaneous cushion layers as required by varying the cell seeding density.

In general, hASC have the ability to proliferate and differentiate into the adipogenic lineage readily, and were also reported to accelerated wound healing in several cases, e.g. in hernia repair [Nie 2012, Hirsch 2012, Altman 2010, Liu 2011]. An improved stability and an enhanced vascularization incorporated in the construct seeded with ASC, compared to a non-seeded dermal matrix, was demonstrated *in vivo* by Orbay

et al. [Orbay 2011]. Additionally, ASC could be differentiated into a thin fat layer on top of the dermal matrix which would be an advantage for cushioning. However, to date, no adipogenic differentiation of stem cells on top of thin acellular dermal matrices has been described.

Using a hormonal cocktail, ASC can be differentiated into mature adipocytes *in vitro*, but the cell-matrix interaction can additionally influence the differentiation ability of cells [Discher 2009]. The ability of Permacol™ and Strattice™ to facilitate ASC maturation to adipocytes had to be determined. Using a permanent induction with adipogenic stimuli, a substantial adipogenic differentiation could be achieved in the cell layer on top of Permacol™ and Strattice™. Oil red O staining, hematoxylin staining and the quantification of triglyceride accumulation per cell proved the ability of Permacol™ and Strattice™ to support adipogenic differentiation of ASC cultured with induction medium (Fig. 12). From a molecular point of view, elevated expression levels of important adipogenic marker genes like PPARγ, C/EBPα and aP2 confirmed these observations (Fig. 13). Interestingly, constructs without adipogenic stimulation displayed a slight adipogenic differentiation as well, demonstrated on the cellular and the molecular level. This indicated that these collagen matrices per se provide an inductive environment for adipogenesis of hASC. In a previous study, Flynn et al. could demonstrate that the acellular ECM isolated from human adipose tissue also provided an inductive microenvironment for the adipogenic maturation of hASC. Gene expression of the master regulators in adipogenesis PPARγ and C/EBPα were sufficiently induced without the need of exogeneous adipogenic stimuli [Flynn 2010].

Several studies have reported that patient factors, such as age and body mass index (BMI), can have an impact on the proliferation and viability of ASC [Wronska 2012, Wilson 2011, Buschmann 2013]. Here, first studies using ASC from different donors suggested that, in principle, engineering of a thin fat layer on top of the Permacol™ matrix was possible independent of donor age, BMI and donor site. In future studies, the extent of adipogenic differentiation may be evaluated further with more donors and donor sites.

For clinical use it may be beneficial to implant ASC while being in an immature development status, yet committed to the adipogenic phenotype, as they withstand necrosis under ischemic conditions and are mechanically more stable. Compared to mature adipocytes, preadipocytes have a lower oxygen demand, which may readily enable them to overcome the hypoxic conditions after implantation *in vivo* [Weiser 2008]. Another noteworthy feature of immature adipocytes is the secretion of angiogenic factors, which may lead to a faster vascularization *in vivo* [Crandall 1997, Bouloumie 2002]. Further, utilizing premature ASC, as fat layer on top of the matrix, could reduce the ex vivo cultivation time and could reduce the therapy cost.

Following these goals variations of the induction protocol were tested. Reducing the *in vitro* adipogenic differentiation time, but still facilitating sufficient promotion of adipogenic maturation after implantation *in vivo* would be desirable.

In this study, a short-term induction of two days only led to emergence of adipogenic differentiation, whereas an initial induction period of four days resulted in a substantial adipogenesis, close to the adipogenic differentiation of the permanently induced constructs (Fig. 16). Hence, a 4 day precultivation time may be sufficient to overcome time and cost issues for clinical use and still generate a layer of committed preadipocytes on top of the matrix to fulfill the cushion requirements of the engineered substitute.

Further investigations in an *in vivo* model have to elucidate the potential of these engineered constructs, and the minimal period of *ex vivo* cultivation required to achieve a mature fat layer *in vivo*. A possible animal model could be implantation of the engineered construct between the carnial plate and skin of a rat, or between skin and Achilles tendon of a rabbit. The advantage of the rat model is the easier and less cost-intensive housing and the possibility to use immune-incompetent rats. The advantage of a rabbit is the size, as a lesion can be introduced above the Achilles tendon and the adhesion can be simulated. In this model the biocompatibility, the integrity and the ingrowth of the surrounding tissue into the construct may be demonstrated.

5.2 Engineering vascularized adipose tissue *in vitro*

Adipose tissue serves as energy storage and building block for the contours of the human body. In regenerative medicine, adipose tissue is needed to reconstruct the function of the defect site and the outer appearance of the patient's body, lost through, e.g., trauma, tumor resection or genetically caused defects. The rebuilding does not only help to regain the normal function and appearance of the treated area, but also the subjective well-being of the patient [Wetterau 2012, Coleman 2006, Saint-Cyr 2012]. In addition to the function in maintaining the body contours and providing mechanical protection, adipose tissue is also a source of endocrine secretion [Wronska 2012, Trayhurn 2008]. For basic research on adipose tissue and for medical testing, an *ex vivo* model of a certain size is necessary, as animal models should be reduced. With adequately engineered vascularized adipose tissue, metabolism studies or drug testing in a simple *in vitro* human test system may be possible.

Vascularized adipose tissue generated by tissue engineering methods should exhibit a homogeneous cell distribution, withstand volume loss and necrosis. In literature, different strategies to vascularize adipose tissue constructs are described. One approach of is a controlled release of angiogenic growth factors, such as basic fibroblast growth factor, from microspheres or from the cell carrier itself. The effect that is described is a neo-vascularization and a better adipogenesis *in vivo* [Kimura 2010, Ito 2012]. Another strategy is to use a co-culture system of endothelial cells and ASC. A formation of vessel-like structures was characterized, but no adequate vascularization for the engineered construct could be gained [Kang 2009, Verseijden 2012]. The integration of a loop or vessel bundle from the host into the engineered adipose tissue constructs during surgery is a third approach. Using this method, a better vascularization and stronger adipogenesis of the newly formed adipose tissue *in vivo* could be demonstrated [Wiggenhauser 2012, Debels 2013, Wittmann 2013]. However, until today, in *in vivo* studies, vascularized adipose tissue rarely developed

volumes larger than 2 ml [Bauer-Kreisel 2010]. *In vitro*, up to now there was not one study that presented constructs with a fully developed vascular network and an ability to be connected to a supply system to avoid tissue necrosis. An eligible candidate for engineering a vascularized tissue would be a natural ECM, nerved by a vessel system and provided with pedicles, which allow a connection of the tissue to the culture medium supply. The acellular porcine jejunum introduced by Walles et al. as a biological vascularized scaffold appeared to be appropriate for this approach [Mertsching 2005, Schanz 2010]. Utilization of the decellularized jejunal segment enables a physiological supply of nutrients, through the arterial and venous pedicles, and provides a stable scaffold. This ECM consists of natural elastin and collagen, where cells can adhere to and remodel the scaffold, simultaneous to the development of the newly formed tissue. By combination of this scaffold system and hASC, an autologous and patient fitted implant could be designed.

Therefore, the property of the jejunum ECM to facilitate cell adhesion was tested by seeding ASC on this scaffold (chapter 4.2). The thickness of the layer could be modified by varying the cell number. This can be beneficial, as the layer can be adjusted to the de novo vascularization of the construct. Several studies reported the qualification of decellularized jejunum as a scaffold in tissue and organ engineering, supporting cell adhesion for cell types like hepatocytes, caco-2-cells and endothelial cells, confirming the findings in this study [Mertsching 2005, Mertsching 2009, Pusch 2011, Schanz 2010, Linke 2007].

The adipogenic induction of stem cells on the porcine jejunum had not yet been analyzed. Thus, at first, ASC were adipogenically differentiated on small sections of the decellularized jejunal by a permanent induction protocol. Cells clearly started triglyceride storage over the cultivation time of 14 days, indicating the commitment to the adipogenic phenotype (Fig. 18), as determined histologically and by a quantitative enzymatic assay. Adipogenesis was further confirmed on the molecular level by qRT-PCR (Fig. 19).

With the objective to build vascularized fat tissue consisting at least of endothelial cells and ASC, committed to adipogenic maturation, co-culture conditions had to be verified. In preceeding work of our group, it had been demonstrated that the 1:1 mixture of an endothelial medium (EGM) without endothelial growth factor (EGF), and preadipocyte growth medium (PGM), did not affect endothelial cell proliferation, their "cobblestone" appearance and CD 31 expression [Muhr 2013]. Here the proliferation and differentiation of a mono-culture of ASC in 1:1 VascuLife (another endothelial cell medium) and PGM was examined. The 1:1 mixture containing all growth factors and inducers compromised the adipogenic differentiation potential of ASC. In contrast, an elimination of EGF resulted in a sufficient adipogenic induction. Taken together, these results suggest that the 1:1 VascuLife and PGM without EGF mix is a suitable medium for a co-culture system of ASC and endothelial cells. Also, they confirm earlier reports in literature that EGF can interfere with adipogenesis [Harrington 2007, Hauner 1995, Muhr 2013]. In subsequent preliminary co-culture studies, the jejunal sections were demonstrated to enable adipogenic differentiation of ASC and simultaneously the preservation of the endothelial phenotype, as indicated through histological staining for triglycerides and CD 31 expression. Notably, an increasing migration of ASC through the ECM towards the MVEC was apparent during the culture period on non-induced constructs (Fig. 21). Based on these results, the jejunal section cultured with 1:1 mixture of VascuLife and PGM without EGF appeared to support the maturation of fat tissue constructs *in vitro* and was, therefore, used in further co-culture studies.

As described in numerous studies, flow conditions in culture have an impact on the functionality, vitality and differentiation of cells [Liu M 2013, Zhang C 2013]. To stay close to physiological requirements, culture conditions were varied by using a bioreactor with two chambers and the possibility for dynamic culture. Physiologically, endothelial cells are subjected to shear forces through the flow of the blood in the vessels, whereas ASC are not directly affected by a flow [Yamamoto 2011, Califano 2010, Iatridis 2003, Wronska 2012]. A dynamic culture would mimic the natural conditions for endothelial cells and a static culture the natural environment for ASC.

Previous studies of Walles et al. demonstrated a better survival and functionality of the endothelial cells cultured under specific flow conditions (personal communication). Here the comparison of flow condition for the ASC revealed no apparent differences in adipogenesis. Therefore, in all following experiments the conditions in the custom-made bioreactor fostering vascularization were chosen to be dynamic in the vessel system and static for the ASC in the lumen.

In the custom-made bioreactor, the advantages of the decellularized porcine jejunum as scaffold and vascular structure system can be utilized. It is possible to connect the vascular network over arterial and venous pedicles to the culture medium supply. Through this circulation an optimal exchange of nutrients and metabolites is allowed. In the present work, for the first time it was possible to engineer a vascularized fat tissue *in vitro*, utilizing the custom-made bioreactor system in a six week culture. The successful repopulation of the vessel structure was shown by a MTT-assay after two weeks of endothelial cell seeding (Fig. 25). As expected, the adipogenic differentiation increased during induction, but noteworthily, simultaneously the vessel network appeared to densify, as observed on whole mount staining of the tissue samples (Fig. 26). Histological sections confirmed vascularization by immuno-histochemical staining for CD 31 and adipogenic maturation by oil red O staining or BODIPY staining (Fig. 27/28). The increase of adipogenic differentiation was also determined by gene expression analysis of adipogenic marker genes.

Taken together, for the first time, a vascularized adipose tissue construct was engineered *in vitro*, utilizing a co-culture of hASC and MVEC on the decellularized jejunal segment in a custom-made bioreactor system (Fig 30). Additionally, through the pedicles it can support anastomosis in reconstructive surgery and can be kept viable *in vitro* for metabolic testing. In fact, besides the intended use in reconstructive surgery, the system may be used for basic research to further study the close interplay between angiogenesis and adipogenesis. Adipose tissue is known for the secretion of angiogenic factors and ASC to promote vessel growth [Nishimura 2007, Rehman 2004, Kilroy 2007]. Sarkanen et al. reported even that hASC are able to

contribute to vessel maturation by differentiation into vessel supporting structures [Sarkanen 2003].

Further investigations should include the comparison of adipogenic differentiation in non-induced and induced culture. To evaluate the importance of the supply through the vascular network in the adipogenic differentiation culture, it is further necessary to compare reseeded and non-reseeded vessel structures. Another study in line may be the use of cells from the stromal vascular fraction (SVF) in the lumen. As a multicellular fraction, it may support and increase the adipogenic differentiation and the vascularization in the construct. A further possibility is to use a hydrogel incorporated with ASC and/or SVF cells to fill the lumen of the jejunal segment. It is very likely that this would simplify the handling. Nevertheless, the thickness of the hydrogel has to be taken into consideration as neo-vascularization into the hydrogel has to occur to guarantee nutrient supply in the middle of the construct.

6 Summary and conclusion

Each year millions of plastic and reconstructive procedures are performed to regenerate soft tissue defects after, for example, traumata, deep burns or tumor resections. Tissue engineered adipose tissue grafts are a promising alternative to autologous fat transfer or synthetic implants to meet this demand for adipose tissue. Strategies of tissue engineering, especially the use of cell carriers, provide an environment for better cell survival, an easier positioning and supplemented with the appropriate conditions a faster vascularization *in vivo*. To successfully engineer an adipose tissue substitute for clinical use, it is crucial to know the actual intended application. In some areas, like the upper and lower extremities, only a thin subcutaneous fat layer is needed and in others, large volumes of vascularized fat grafts are more desirable. The use and interplay of stem cells and selected scaffolds were investigated and provide now a basis for the generation of fitted and suitable substitutes in two different application areas.

Complex injuries of the upper and lower extremities, in many cases, lead to excessive scarring. Due to severe damage to the subcutaneous fat layer, a common sequela is adhesion formation to mobile structures like tendons, nerves, and blood vessels resulting in restricted motion and disabling pain [Moor 1996, McHugh 1997]. In order to generate a subcutaneous fat layer to cushion scarred tissue after substantial burns or injuries, different collagen matrices were tested for clinical handling and the ability to support adipogenesis. When testing five different collagen matrices, Permacol™ and Strattice™ showed promising characteristics; additionally both possess the clinical approval. Under culture conditions, only Permacol™, a cross-linked collagen matrix, exhibited an excellent long-term stability. Ranking nearly on the same level was Strattice™, a non-cross-linked dermal scaffold; it only exhibited a slight

shrinkage. All other scaffolds tested were severely compromised in stability under culture conditions. Engineering a subcutaneous fat layer, a construct would be desirable with a thin layer of emerging fat for cushioning on one side, and a non-seeded other side for cell migration and host integration. With Permacol™ and Strattice™, it was possible to produce constructs with ASC (adipose derived stem cells) seeded on one side, which could be adipogenically differentiated. Additionally, the thickness of the cell layer could be varied. Thereby, it becomes possible to adjust the thickness of the construct to the surrounding tissue. In order to reduce the pre-implantation time *ex vivo* and the costs, the culture time was varied by testing different induction protocols. An adipogenic induction period of only four days was demonstrated to be sufficient to obtain a substantial adipogenic differentiation of the applied ASC. Thus, seeded with ASC, Permacol™ and Strattice™ are suitable scaffolds to engineer subcutaneous fat layers for reconstruction of the upper and lower extremities, as they support adipogenesis and are appropriately thin, and therefore would not compromise the cosmesis.

For the engineering of large-volume adipose tissue, adequate vascularization still represents a major challenge. With the objective to engineer vascularized fat pads, it is important to consider the slow kinetics of revascularization *in vivo*. Therefore, a decellularized porcine jejunum with pre-existing vascular structures and pedicles to connect to the host vasculature or the circulation of a bioreactor system was used. In a first step, the ability of a small decellularized jejunal section was tested for cell adhesion and for supporting adipogenic differentiation of hASC mono-cultures. Cell adhesion and adipogenic maturation of ASC seeded on the jejunal material was verified through histological and molecular analysis. After the successful mono-culture, the goal was to establish a MVEC (microvascular endothelial cells) and ASC co-culture; suitable culture conditions had to be found, which support the viability of both cell types and do not interfere with the adipogenic differentiation. After the elimination of EGF (epidermal growth factor) from the co-culture medium, substantial

adipogenic maturation was observed. In the next step, a large jejunal segment (length 8 cm), with its pre-existing vascular structures and arterial/venous pedicles, was connected to the supply system of a custom-made bioreactor. After successful reseeding the vascular structure with endothelial cells, the lumen was seeded with ASC which were then adipogenically induced. Histological and molecular examinations confirmed adipogenic maturation and the existence of seeded vessels within the engineered construct. Noteworthily, a co-localization of adipogenically differentiating ASC and endothelial cells in vascular networks could be observed. So, for the first time a vascularized fat construct was developed *in vitro*, based on the use of a decellularized porcine jejunum. As this engineered construct can be connected to a supply system or even to a patient vasculature, it is versatile in use, for example, as transplant in plastic and reconstruction surgery, as model in basic research or as an *in vitro* drug testing system.

To summarize, in this work a promising substitute for subcutaneous fat layer reconstruction, in the upper and lower extremities, was developed, and the first, as far as reported, *in vitro* generated adipose tissue construct with integrated vascular networks was successfully engineered.

7 Zusammenfassung und Schlussbetrachtung

Jedes Jahr werden Millionen von plastischen und wiederherstellenden Eingriffe durchgeführt, um zum Beispiel nach Traumata, hochgradigen Verbrennungen oder Tumorekonstruktionen, die natürliche Erscheinung und Funktion im Bereich von Weichgewebsdefekt wiederherzustellen. Gezüchtete Fettgewebskonstrukte sind eine vielversprechende Alternative zu autologen Fettgewebstransfers oder synthetischen Implantaten, um dem Bedarf an Fettgewebe gerecht zu werden. Die Strategien der Gewebezüchtung, besonders das Verwenden von Zellträgern, schaffen eine Umgebung für besseres Zellüberleben, eine einfachere Positionierung und - versehen mit den entsprechenden Eigenschaften - eine schnellere Vaskularisierung *in vivo*. Um erfolgreich einen Fettgewebe-Ersatz für die klinische Anwendung herzustellen, ist es notwendig das spätere Anwendungsgebiet zu kennen. In manchen Bereichen, wie in den oberen und unteren Extremitäten, braucht man nur eine dünne Unterhautfettschicht, und in anderen Bereichen wiederum ist ein großes Volumen an vaskularisiertem Fettgewebskonstrukt anzustreben. Die Nutzung und das Zusammenspiel von Stammzellen und ausgewählten Zellträgern wurden untersucht und legen nun eine Basis für die Herstellung von passendem und zweckmäßigem Ersatzgewebe zweier unterschiedlicher Anwendungsgebiete.

Komplexe Verletzungen der oberen und unteren Extremitäten führen oftmals zu beträchtlicher Narbenbildung. Eine häufige Folgeerscheinung, hervorgerufen durch eine schwere Beschädigung des Unterhautfettgewebes, ist die Adhäsion zwischen mobilen Strukturen wie Sehnen, Nerven und Blutgefäßen. Dies resultiert dann in eingeschränkter Beweglichkeit und lähmenden Schmerzen [Moor 1996, McHugh 1997]. Um eine subkutane Fettschicht herzustellen, die das vernarbte Gewebe nach schwerer Verbrennung oder Verletzung polstert, wurden verschiedene

Kollagenmaterialien auf die klinische Handhabung und die Unterstützung der Adipogenese untersucht. In der Untersuchung von fünf verschiedenen Kollagenmatrices zeigten Permacol™ und Strattice™ vielversprechende Eigenschaften. Beide besitzen außerdem die klinische Zulassung. Permacol™, eine chemisch quervernetzte Kollagenmatrix, zeigte unter Kulturbedingungen hervorragende Langzeitstabilität. Fast ebenso gute Eigenschaften konnten bei Strattice™, einem nicht vernetzten dermalen Gerüstmaterial, beobachtet werden; es zeigte lediglich leichte Schrumpfung. Alle sonst getesteten Kollagenmaterialien waren unter Kulturbedingungen stark in ihrer Stabilität beeinträchtigt. Zur Herstellung einer subkutanen Fettschicht wäre ein Konstrukt wünschenswert mit einer dünnen, gerade entstehenden Fettschicht für die Polsterung auf der einen Seite und einer nicht besiedelten anderen Seite für die Zelleinwanderung und die Integration in das umliegende Gewebe. Mit Permacol™ und Strattice™ war es möglich Konstrukte herzustellen, welche auf einer Seite mit ASC (aus dem Fettgewebe isolierte Stammzellen) besiedelt und anschließend adipogen differenziert werden konnten. Zusätzlich konnte die Dicke der Zellschicht hierbei variiert werden. Somit ist es möglich die Dicke des Konstruktes an das umliegende Gewebe anzupassen. Um die Preimplantationszeit *ex vivo* zu verkürzen und damit auch die Kosten zu senken, wurde die Kulturzeit variiert, indem verschiedene Induktionsprotokolle getestet wurden. Eine adipogene Induktionsperiode von nur vier Tagen erwies sich als ausreichend, um eine substantielle adipogene Differenzierung der eingesetzten ASC zu erreichen. Das heißt, die mit ASC besiedelten Permacol™ und Strattice™ Matrices sind zweckdienliche Zellträgermaterialien, um eine subkutane Fettschicht für die oberen und unteren Extremitäten herzustellen, da sie die Adipogenese unterstützen und durch die nur geringe und anpassbare Dicke die Kosmesis nicht beeinträchtigen.

Für die Entwicklung von großvolumigem Fettgewebe stellt die adäquate Vaskularisierung noch immer eine große Herausforderung dar. Mit dem Ziel ein vaskularisiertes Fettkonstrukt herzustellen, ist es wichtig die langsame Kinetik der

Revaskularisierung *in vivo* zu berücksichtigen. Daher wurde hier ein dezellularisiertes Schweinedarmsegment mit schon vorhandenen Gefäßstrukturen und Gefäßanschlüssen für die Verbindung zum Kreislaufsystem des Patienten oder eines Bioreaktor-Systems verwendet. Im ersten Schritt wurden auf einem kleinen dezellularisierten Schweinedarm-Stück die Zelladhäsion und die adipogene Differenzierung der ASC in Monokultur getestet. Die Zelladhäsion und die adipogene Reifung konnte mittels histologischer und molekularer Analysen auf dem jejunalen Material nachgewiesen werden. Nach der erfolgreichen Monokultur musste die Co-Kultur von MVEC (micro vaskuläre Endothelzellen) und ASC etabliert werden. Um dieses Ziel zu erreichen, wurden geeignete Kulturbedingungen gesucht, die die Lebensfähigkeit beider Zelltypen unterstützen und gleichzeitig die adipogene Differenzierung nicht beeinträchtigen. Nach dem Ausschluss von EGF (epidermaler Wachstumsfaktor) aus dem Co-Kulturmedium wurde eine substantielle adipogene Reifung der ASC beobachtet. Im nächsten Schritt wurde ein großes dezellularisiertes jejunales Darmsegment (Länge 8 cm) mit der schon existenten Gefäßstruktur und dem arteriellen und venösen Gefäßstiel an den spezialangefertigten Bioreaktor angeschlossen. Nach der erfolgreichen Wiederbesiedelung der Gefäßstrukturen mit Endothelzellen wurde das Darmlumen mit ASC besiedelt, welche anschließend adipogen induziert wurden. Histologische und molekulare Untersuchungen konnten die adipogenen Reifung und die Existenz von besiedelten Gefäßen im hergestellten Konstrukt bestätigen. Besonders erwähnenswert ist die Beobachtung der Co-Lokalisierung von adipogen differenzierenden ASC und Endothelzellen in vasculären Netzwerken. Somit wurde zum ersten Mal - basierend auf einem dezellularisierten Schweinedarm - ein vaskularisiertes Fettgewebskonstrukt *in vitro* hergestellt. Da dieses Konstrukt an das Versorgungssystem angeschlossen oder mit dem Blutkreislauf des Patienten verbunden werden kann, ist es vielfältig einsetzbar, zum Beispiel in der plastisch-rekonstruktiven Chirurgie, als Modell in der Grundlagenforschung oder als ein *in vitro* Medikamenten-Testsystem.

Zusammengefasst, wurde in der vorgelegten Arbeit ein vielversprechendes Ersatzmaterial für die Rekonstruktion des Unterhautfettgewebes für die unteren und oberen Extremitäten entwickelt, und zum ersten Mal erfolgreich, so weit in der Literatur bekannt, ein Fettgewebskonstrukt mit integriertem vaskularisiertem Netzwerk *in vitro* generiert.

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Appendix

List of Abbreviation

2D	2-dimensional
3D	3-dimensional
Ang1 /Ang2	angiopoietines 1 and 2
aP2 /FABP4	fatty-acid-binding protein
ASC	adipose derived stem cell(s)
BAT	brown adipose tissue
bFGF	basic fibroblast growth factor
BMI	body mass index
BMSC	bone marrow derived mesenchymal stem cells
BSA	bovine serum albumin
C/EBPα, β and ∂	CCAAT/enhancer binding protein alpha, beta and delta
cAMP	cyclic adenosine 3´,5´´-monophosphate
cDNA	complementary deoxyribonucleic acid
Cy3	cyanine 3
DAPI	4´,6-diamino-2-phenylindole
DMEM	Dulbecco`s modified eagle`s medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ECM	extracellular matrix
EGF	epidermal growth factor
EGM	endothelial cell growth medium
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

Glut4	facilitated glucose transporter 4
h...	human...
HA	hyaluronic acid
HGF	hepatocyte growth factor
HMDI	hexamathylene diisocyanate
HUVEC	human umbilical vein endothelial cell(s)
IBMX	3-isobuthyl-1-methylxanthine
mm Hg	millimeters of mercury
MMP	matrix metalloproteinase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVEC	microvascular endothelial cell(s)
OD	optical density
ORO	oil red O
PBS	phosphate buffered saline
RT-PCR	real time polymerase chain reaction
PEG	polyethylene glycol
PGA	polyglycolic acid
PGM	preadipocyte growth medium
PLA	polylactic acid
PLGA	poly(lactic-co-glycolic) acid
PPARγ	peroxisome proliferator activated receptor γ
RNA	ribonucleic acid
rpm	revolution per minute
SAT	subcutaneous adipose tissue
SEM	scanning electron microscopy
SVF	stromal vascular fraction

TGF β	transforming growth factor beta
VEGF	vascular endothelial growth factor
WAT	white adipose tissue
WMS	whole mount staining

List of Figures and Tables

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