Establishment of defined culture conditions for the differentiation, long-term maintenance and co-culture of adipose-derived stem cells for the setup of human vascularized adipose tissue

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Abbreviations

2D	Two-dimensional
3D	Three-dimensional
A2P	Ascorbic acid-2-phosphate
AcLDL	Acetylated low density lipoprotein
ACLY	ATP citrate lyase
AdipoQ	Adiponectin
AGT II	Angiotensinogen
Ang I/II	Angiopoietin I/II
ASC	Adipose-derived stem cell
ATP	Adenosin triphosphate
BAT	Brown adipose tissue
bFGF	Basic fibroblast growth factor
BM	Bone marrow
cAMP	Cyclic adenosine monophosphate
CBM	Cellulose-based material
CD	Cluster of differentiation
CoM	Defined co-culture medium
Ctrl	Control
C/EBP-α, -β, -δ	CCAAT/enhancer-binding protein-α, -β, -δ
DAPI	4, 6-diamidin-2-phenylindol
Def-Diff	Defined differentiation medium
Def-M	Defined maintenance medium
Diff	Differentiation medium
DIFF	Adipogenic differentiation mix
DiffASC	Adipogenic differentiated ASCs
Dex	Dexamethasone
DLK-1	Delta-like non-canonical Notch ligand-1
DMEM	Dulbecco's Modified Eagle Medium
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EPC	Endothelial progenitor cell
FA	Fatty acid
FABP-4	Fatty acid binding protein-4
FAS	Fatty acid synthase
FATP-1/-4	Fatty acid transport protein-1/4

G3P	Glycerol-3-phosphate
GC	Glucocorticoid
GLUT-4	Glucose transporter-4
GMP	Good manufacturing practice
HC	Hydrocortisone
HGF	Hepatocyte growth factor
HS	Human serum
HSL	Hormone-sensitive lipase
HUVEC	Human umbilical cord vein endothelial cell
IBMX	Isobutylmethylxanthine
IGF	Insulin-like growth factor
IL-6	Interleukin-6
IRS-1	Insulin receptor substrate-1
LDL	Low-density-lipoprotein
LPL	Lipoprotein lipase
MA	Mature adipocyte
MAIN	Adipocyte maintenance mix
MSC	Mesenchymal stem cell
MvEC	Microvascular endothelial cell
NG2	Neuron glial antigen-2
NO	Nitric oxide
PAI-1	Plasminogen activator inhibitor-1
PDGF-β	Platelet-derived growth factor-β
PECAM-1	Platelet and endothelial cell adhesion molecule-1
PGI2	Prostaglandin-I2
PPAR-γ	Peroxisome proliferator-activated receptor-γ
SC	Serum-containing
SF	Serum-free
SREBP-1	Sterol regulatory element-binding protein-1
TCPS	Tissue culture polystyrene
TE	Tissue engineering
TG	Triglyceride
TGF- β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
VEGF-A/C/D	Vascular endothelial growth factor A, C, D
VWF	Von Willebrand factor
WAT	White adipose tissue
XF	Xeno-free

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1. Summary / Zusammenfassung

1.1. Summary

Most current attempts in engineering adipose tissue are based on the supplementation with human or animal-derived sera. However, especially the use of animal-derived serum is linked to many disadvantages, like potential contaminations, ethical issues and in general the missing identification of many ingredients. Therefore, serum supplementation impedes the actual application of engineered adipose tissue constructs as implants, to substitute lost tissue after tumor resection, severe burnings or trauma. Equally, due to a potential cover up of the cellular response by unidentified components, it impairs the *in vitro* use of such models as test systems to elucidate mechanisms of disease development, screen for new drugs or generally assess pharmaceutical safety levels. To be capable for functional anastomosis with the host tissue after implantation and for the use in time- and maturation-dependent *in vitro* purposes, engineered constructs have to exhibit a minimum sustainability.

So far, only few authors addressed the serum-free, defined differentiation of adipocytes. And there are hardly any trials available on the defined maintenance of adipocytes. In this study, the development of a defined culture medium for the adipogenic differentiation of primary human adipose-derived stem cells (ASCs) was aimed. Based on the addition of specific factors for the replacement of serum, ASCs were differentiated to viable and characteristic adipocytes for 14 days, which was proven through the accumulation of lipids, the expression of perilipin A and by the release of leptin and glycerol. Furthermore, a defined maintenance medium was developed, which supported the maturation and stability of cells for a long-term period of additional 42 days until day 56.

In order to pursue the goal of a physiological tissue substitute of relevant size, the integration of a vascular component is of fundamental importance to allow sufficient nutrient supply of all peripheral tissue areas. For this purpose, a natural vascular system based on a cellular component would be ideal. Due to the lack of an adequate co-culture medium, a major challenge in adipose tissue vascularization is represented by the setup of an adipocyte/endothelial cell (EC) co-culture. In this study, the development of a tissue-tailored co-culture medium based on adipocyte- and EC-factors was developed. Thereby the critical role of epidermal growth factor (EGF) and hydrocortisone (HC) in adipocyte/microvascular (mv)EC co-culture was determined.

Through the adjustment of their supplementation, a functional co-culture of adipocytes and mvECs was achieved. In there, mvECs maintained the cell-specific expression of von Willebrand factor (vWF) and cluster of differentiation 31 (CD31). Additionally, cells kept their ability to incorporate acetylated low density lipoprotein (acLDL). By combining the experiences from both mentioned attempts, a defined adipocyte/EC co-culture medium was developed. Next to the maintenance of functional and characteristic adipocytes, the medium facilitated the formation of vascular-like structures in the direct co-culture.

To be able to establish tissue constructs of relevant size, current *in vitro* attempts have to be transferred to a three-dimensional (3D) environment. In this trial, a 3D adipose tissue model was set up based on the differentiation of ASCs in a collagen type I hydrogel in co-culture with mvECs for 21 days in total. The comparison of these models with native adipose tissue demonstrated high accordance in the gene expression levels related to differentiation and fatty acid metabolism. Some deviations were found mostly in maturation-dependent genes linked to tissue functionality and angiogenic mediation.

Differentiation and the maintenance of a homeostatic tissue state highly rely on the physical and chemical characteristics of the applied scaffold. As another part, the influence of a novel cellulose-based material (CBM) on defined adipogenic differentiation of ASCs and the defined maintenance of mvECs was investigated in this thesis. An accelerating effect of CBM on the defined differentiation of ASCs was proven by enhanced release of leptin and the increased expression of perilipin A. CBM was further shown to facilitate the formation of vascular-like structures by mvECs under defined conditions in the absence of another supporting cell type. Additionally, the successful co-culture of adipocytes and mvECs was demonstrated on CBM under defined conditions.

Summarized, defined culture media for the differentiation, maintenance and co-culture of primary ASC and mvECs were developed. The supporting effect of CBM on the defined establishment of cultures was proven. Further the successful setup of a 3D adipocyte/mvEC co-culture model with a high predictive power was shown. Combined these achievements can be used for the *in vitro* setup of a 3D vascularized adipose tissue under defined conditions.

1.2. Zusammenfassung

Die meisten aktuellen Ansätze zum Aufbau eines künstlichen Fettgewebekonstruktes basieren auf dem Einsatz von Seren, tierischen oder humanen Ursprungs. Speziell die Verwendung von tierischem Serum ist jedoch mit vielen Nachteilen verbunden. Dazu zählen potentielle Kontaminationen, große Variationen zwischen den Chargen und die vielen, nicht identifizierten Komponenten. Die Supplementierung mit Serum behindert so den Einsatz von künstlichen Fettgewebekonstrukten als Implantat zum Ersatz von nativem Gewebe nach einer Tumorentfernung, schweren Verbrennungen oder Traumata. *In vitro* Ansätze z.B. zur Aufklärung von Krankheitsentstehungen, der Entwicklung oder Sicherheitseinstufung von Medikamenten, basieren auf der zellulären Antwort, welche ebenfalls durch Serum-Komponenten verschleiert werden kann. Zudem sollten Fettgewebekonstrukte eine grundsätzliche Stabilität aufweisen um nach der Implantation eine ausreichende Anastomose mit dem Empfängergewebe und die Nutzung für zeit- und reifeabhängige *in vitro* Fragestellungen zu ermöglichen. Die definierte serumfreie Differenzierung von Adipozyten wurde bisher nur von wenigen Autoren adressiert. Zum definierten Erhalt von Adipozyten sind kaum Studien vorhanden. In dieser Arbeit wurde die Entwicklung definierter Kulturmedien für die adipogene Differenzierung humaner primärer Stammzellen aus dem Fettgewebe (adipose-derived stem cells, ASCs) angestrebt. Durch die Zugabe von spezifischen Faktoren als Alternative zu Serum, erfolgte eine 14-tägige Differenzierung zu viablen und charakteristischen Adipozyten, was sich durch die Einlagerung von Lipiden, der Expression von Perilipin A und der Freisetzung von Leptin und Glycerol bestätigen ließ. Weiterhin wurde ein definiertes Erhaltungsmedium entwickelt, welches die Reifung und Stabilität der Adipozyten über einen langfristigen Kulturzeitraum von 42 Tagen bis zu Tag 56 unterstützte.

Zur ausreichenden Versorgung, auch peripher gelegener Geweberegionen, ist die Integration einer vaskulären Komponente beim Aufbau eines physiologischen Gewebeersatzes in relevanter Größe fundamental. Idealerweise sollte das vaskuläre System aus einer natürlichen, zellulären Komponente bestehen. Durch den Mangel eines adäquaten Co-Kulturmediums, stellt der Aufbau einer Adipozyten/Endothelzell (endothelial cell, EC) Co-Kultur bei der Vaskularisierung von Fettgewebe eine zentrale Herausforderung dar. In dieser Arbeit wurde ein gewebespezifisches Co-Kulturmedium mit Adipozyten- und EC-Faktoren entwickelt. Hierbei zeigte sich die

kritische Rolle vom epidermalen Wachstumsfaktor (epidermal growth factor, EGF) und Hydrocortison (HC) in der Co-Kultur von Adipozyten und mikrovaskulären (mv)EC. Durch die Supplementanpassung ließ sich eine funktionelle Adipozyten/mvEC Co-Kultur aufbauen. Darin erhielten die mvECs die zellspezifische Expression des von Willebrand Faktors (vWF) und des Oberflächenmarkers 31 (Cluster of differentiation, CD31). Außerdem behielten sie die Fähigkeit zur Aufnahme von acetyliertem Lipoprotein niedriger Dichte (acetylated low density liporotein, acLDL). Durch die Erkenntnisse aus beiden Ansätzen ließ sich ein definiertes Adipozyten/EC Co-Kulturmedium entwickeln. Neben dem Erhalt funktioneller und charakteristischer Adipozyten, unterstütze das Medium in direkter Co-Kultur die Ausbildung vaskulärer Strukturen.

Zum Aufbau von Gewebekonstrukten relevanter Größe ist die Überführung der aktuellen Ansätze in eine dreidimensionale (3D) Umgebung notwendig. In dieser Thesis wurde ein 3D Fettgewebekonstrukt mit differenzierten ASCs in einem Kollagen Typ I Hydrogel in Co-Kultur mit mvECs über insgesamt 21 Tage aufgebaut. Im Vergleich der Modelle mit nativem Gewebe zeigte sich eine größtenteils übereinstimmende Expression von Genen, die mit der Differenzierung und dem Fettstoffwechsel verbunden sind. Einige Abweichungen wurden hingegen bei zumeist reifeabhängigen Genen, die im Zusammenhang mit der Gewebefunktionalität und der Koordination von angiogenen Prozessen stehen, festgestellt.

Die Differenzierung und eine homeostatische Gewebeerhaltung hängen maßgeblich von den physikalischen und chemischen Eigenschaften des eingesetzten Biomaterials ab. In einem weiteren Teil dieser Thesis wurde der Einfluss eines neuartigen Cellulose-basierten Materials (CBM) auf die definierte adipogene Differenzierung und die definierte mvEC Erhaltung untersucht. Die erhöhte Ausschüttung von Leptin und die Expression von Perilipin A zeigte einen beschleunigenden Effekt von CBM auf die Differenzierung von ASCs. Weiterhin ermöglichte CBM die Ausbildung vaskulärer Strukturen in der definierten Kultur ohne die Unterstützung weiterer Zelltypen. Schließlich gelang die definierte Co-Kultur von Adipozyten und mvECs auf CBM.

Zusammengefasst wurden in dieser Arbeit definierte Medien zur Differenzierung, Erhaltung und Co-Kultur von primären ASCs mit mvECs entwickelt. CBM zeigte einen unterstützenden Effekt im definierten Ansatz dieser Kulturen. Außerdem gelang der Aufbau einer 3D Adipozyten/mvEC Co-Kultur. In Kombination können diese Ergebnisse zum Aufbau eines vaskularisierten 3D Fettgewebekonstruktes unter definierten Bedingungen genutzt werden.

2. Introduction

2.1. Human adipose tissue

2.1.1. Categories, location and structure

Fatty tissue comprises about 12 % to 25 % of the human body and can be divided into brown (BAT) and white adipose tissue (WAT), with regard to its functionality [1]. BAT is mainly responsible for heat production and mostly found in newborns. WAT serves alongside with other functions as energy reservoir, heat insulation and mechanical protection of organs [2, 3]. WAT may be further subdivided into visceral and subcutaneous fatty tissue. While visceral fatty tissue is located next to and between internal organs, subcutaneous fatty tissue is placed adjacently to the dermal layer on top of connective and muscle tissue [4]. Visceral fat is known to produce more pro-inflammatory cytokines while subcutaneous fat e. g. produces hormones like leptin [5]. Within WAT, several adipocytes are organized in lobuli, which are in turn separated with reticular connective tissue [6, 7]. Human adipose tissue is a highly vascularized organ, whereby each adipocyte is thought to be connected to the vascular system through at least one capillary [8, 9].

2.1.2. Functions

The best known function of adipose tissue is the storage of energy in form of triglycerides (TG), consisting of fatty acids (FA) and glycerol [10]. Next to this, adipose tissue stabilizes the position of the inner organs and the musculoskeletal system as support material, prevents shock damages and thermally insulates vital organs [10]. Adipose tissue additionally fulfills an endocrine function, which remained undetected for a long time [11] leading to the neglection of the influence of adipose tissue on metabolic processes of the body. Today, it is known that adipose tissue does not only secrete lipids and free FAs but moreover releases hormones and thereby communicates via an endocrine, paracrine or autocrine manner. The hormones leptin and adiponectin (adipoQ), secreted by adipose tissue, e. g. regulate energy expenditure and food intake, while apolipoprotein E and low-density lipoprotein (LDL) mediate the transport of lipids. Adipose tissue is known to secrete cytokines and other immunologically active factors like tumor necrosis factor- α (TNF-α), interleukin-6 (IL-6) or resistin, known to modulate inflammation. Factors coordinating the reaction of the

innate immune system, like adipsin or visfatin also originate from adipose tissue [2, 12].

2.2. Cells of human adipose tissue

Adipose tissue comprises a heterogeneous population of cell types (**Figure 1**), embedded in an extracellular matrix (ECM). Mononuclear cells like macrophages mediate adipose tissue inflammation [13]. Fibroblasts are found in the stroma and participate in matrix production and tissue organization [14]. The characteristic functions of adipose tissue like endocrine signaling and lipid storage are implemented and mediated by the main cell types of adipose tissue, namely mature adipocytes (MA), endothelial cells (EC) and their progenitors like adipose-derived stem cells (ASC).

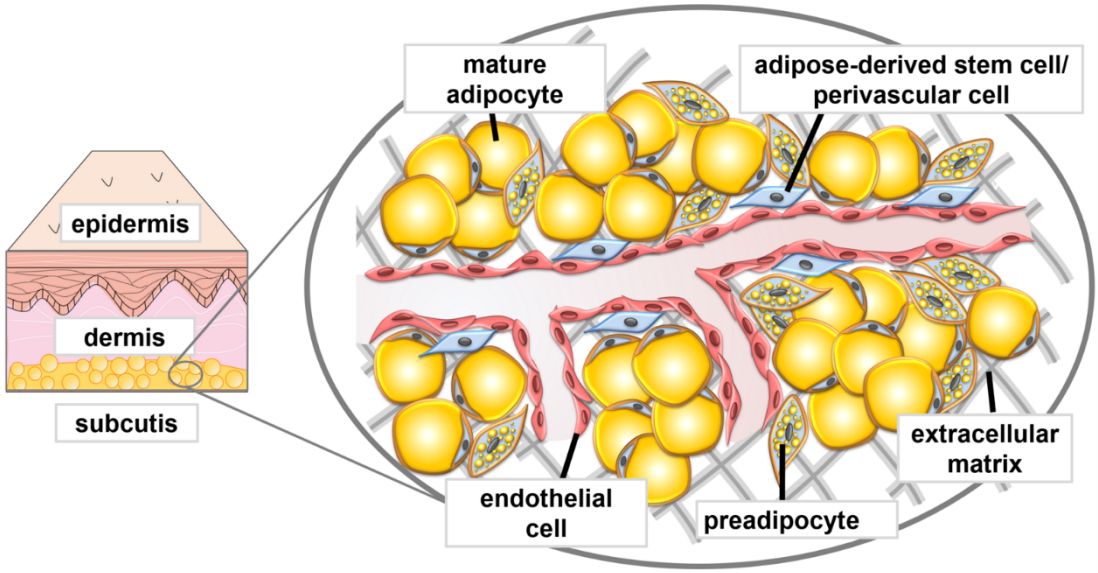

Figure 1: Structure of human adipose tissue. Subcutaneous adipose tissue (subcutis) is localized as third layer of the skin, below the epidermis and the dermis. Adipose tissue comprises MAs and preadipocytes, organized in lobuli. Blood vessels including ECs cross through the tissue. In the vasculogenic zone, perivascular cells respectively ASCs are situated. Cells are embedded into the adipose tissue-specific ECM.

2.2.1. Adipocytes

With 30 % to 50 %, MAs are the most common cell type of adipose tissue [15]. Adipocytes are surrounded by an outer basal lamina which is produced by themselves and consists of laminin, collagen type IV and heparansulfate-proteoglycanes, like perlecan [16-18]. They include an unilocular vacuole [19] and can reach diameters of 50 µm to 100 µm, when terminally differentiated [15]. Depending on the nutritional

status, adipocyte size may further increase up to pathophysiological levels of 200 µm, known as hypertrophy [20]. The incorporated lipid vacuole claims about 95 % of the cell's volume, wherefore the cytoplasm including the cell nucleus is displaced to the cell wall [21]. A phospholipid-monolayer, including TGs and cholesterol esters, thereby separates the lipid vacuole from the cytoplasm [22].

MAs are unable to proliferate [23] but may e. g. increase their lipid stores by lipogenesis and decrease them by lipolysis or lipid release depending on the energy status [24]. Lipogenesis is triggered by insulin, which stimulates the intake of glucose from blood into the cells via the glucose transporter-4 (GLUT-4), (compare **Figure 2**). Following pyruvate dehydrogenase and the cyclic adenosine monophosphate (cAMP) phosphodiesterase are activated [25]. The incorporated glucose is used to produce glycerol-3-phosphate (G3P) via glycolysis. Equally, glucose is metabolized to pyruvate and following citrate, which is used in the *de novo* lipogenesis of FAs by the adenosin triphosphate (ATP) citrate lyase (ACLY) and the FA synthase (FAS) [26, 27]. Subsequently, TGs are esterified based on either these produced or on free FAs, derived from diet or metabolic processes of the liver, in combination with G3P [24]. FAs are thereby provided through FA binding protein-4 (FABP-4), lipoprotein lipase (LPL) and FA transport protein-1 (FATP-1) and -4, which mediate the uptake of FAs from the blood stream [28-30].

In contrast, the degradation of TGs, namely lipolysis is initiated by catecholamines, like epinephrine, binding to β-adrenergic receptors. Following adenylyl cyclase, which is elevating the intracellular concentration of cAMP, is activated. CAMP is responsible for the phosphorylation of the hormone-sensitive lipase (HSL) which moves to the membrane of lipid vacuoles and facilitates the hydrolysis of TGs to G3P and free FAs [24]. Before this step is possible, perilipin A, a membrane protein covering about 20 % of the lipid vacuole of adipocytes and preventing the hydrolysis of TGs [31], is equally phosphorylated and separates from the lipid vacuole membrane, pathing the way for HSL. Next to the fluctuating increase and decrease of incorporated lipids, adipocytes may also dedifferentiate to preadipocytes accompanied by the release of all lipids [32], or they may become apoptotic. To compensate this reduction, new adipocytes are formed. Consequently, adipose tissue undergoes a turnover rate of about 10 % per year [20].

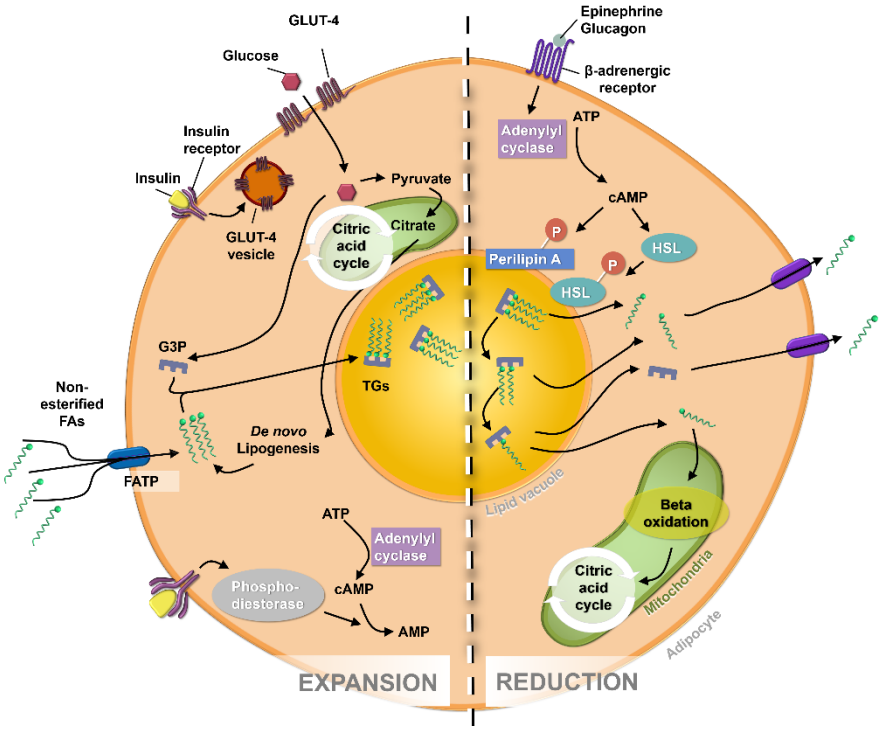

Figure 2: Metabolic processes of human adipocytes during expansion and reduction of lipid stores.

Adipocytes indicate the level of their stored lipids via the release of hormones, like adipoQ or leptin. In a period of active lipogenesis, released leptin levels are proportional to accumulated lipids and high leptin levels indicate a big volume of stored lipids per cell. In contrast, adipoQ release indicates low lipid levels and stimulates increased energy uptake and lipogenesis [33, 34]. However, increased leptin levels might equally be initiated by glucocorticoids (GC) and insulin, showing a multi-dependent production of the hormone [35].

2.2.2. Adipose-derived stem cells

Next to MAs, adipose tissue comprises adipose-derived stem cells (ASC). They are classified as mesenchymal stem cells (MSC) and thereby share many surface antigens with bone-marrow-derived (BM)-MSCs. According to the International Society for Cellular Therapy, these cells show the expression of cluster of differentiation (CD) 73, 90 and 105, plastic adherence and a proliferative capacity. Additionally, ASCs respectively MSCs are capable to differentiate along the mesodermal germ layer, giving rise to not only adipocytes but also to chondrocytes, osteoblast and smooth muscle cells [36].

In vivo, ASCs are thought to reside within the vessel wall. However, it has not been elucidated whether their actual bed is rather in the vasculogenic zone in between media and adventitia or in the perivascular niche in direct contact with ECs [37-39]. There is evidence for the ability of ASCs to differentiate into perivascular cells and take over their position and function [40]. Some voices even classify one cell type as a subcategory of the other [41]. In bigger vessels, the perivascular niche is known to be occupied by smooth muscle cells [42].

ASCs can give rise to MAs. The transcription factors peroxisome proliferator-activated receptor-γ (PPAR-γ), CCAAT/enhancer-binding protein-α (C/EBP-α), C/EBP-β, C/EBP-δ and sterol regulatory element-binding protein-1 (SREBP-1) play key roles during adipogenic differentiation [43, 44]. Within adipogenic differentiation, insulin receptor substrate 1 (IRS-1) is partly responsible for the activation of these adipogenic regulators [45]. In the determination phase, preadipocytes commit to the adipogenic lineage. Although, their morphology remains the same, cells lose their ability to proliferate. In the early terminal phase of differentiation, cell's morphology is changed with the beginning lipid accumulation. In the remaining terminal phase, adipocytes show increased lipid accumulation and express adipocyte-specific antigens [44]. In contrast, delta-like non-canonical Notch ligand-1 (DLK-1) is a known negative regulator of adipogenesis [46].

2.2.3. Endothelial cells

The vascular system of the human body is covered with ECs [47]. *In vivo* microvascular (mv)ECs show a flat, elongated morphology [48]. The endothelium mainly represents a regulator of vascular homeostasis and by the manifestation of close cell-cell contacts it functions as a barrier, which separates the circulating blood from the surrounding tissues [49]. The cell-contact based barrier consists of tight junctions, which e. g. consist of platelet and EC adhesion molecule-1 (PECAM-1), also known as CD31 [50]. Through gap junctions, ECs facilitate an intercellular exchange of substances [51]. Additionally, ECs regulate the vessel tonus e. g. by the release of nitric oxide (NO), or prostaglandin-I2 (PGI2) [52]. Further they mediate blood coagulation [53] e. g. via the release of von Willebrand-Factor (vWF) [54]. The assembly of new blood vessels, their reconstruction and their degradation is mediated by additional cell types (see chapter 3, [55] "3.3 Adipogenesis is linked to angiogenesis") via various stimuli, like

vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor-β (TGF-β), platelet-derived growth factor-β (PDGF-β), plasminogen activator inhibitor-1 (PAI-1), or angiopoietin I (Ang I) and II [56].

2.3. Adipose tissue engineering

The need for artificially engineered adipose tissue arises from various conditions, like congenital deformities, acquired infections, traumata or the loss of tissue after tumor resection, respectively the suffering of severe burnings [57]. The current gold standard is to treat such conditions by the transfer of autologous fat with the Coleman's technique or cell-assisted lipotransfer from intact body sites [58-60]. With this technique, the transplantation of small tissue pieces has been performed successfully [61]. Nonetheless, outcomes of adipose tissue transfer are unpredictable as grafts are under high risk to become necrotic over time and their retention rate varies from 10 % to 90 % [62]. This condition is mainly related to the insufficient nutrient supply in large tissue defects and the only slowly progressing neovascularization [63]. Hence, the *in vitro* development of fatty tissue equivalents is urgently needed to treat defects and replace lost tissue by designing physiological constructs, which facilitate fast tissue anastomosis and cell survival. Tissue engineering (TE) describes an interdisciplinary field, which pursues to construct, repair and replace tissue by combining cells and biomaterials with specific factors [64].

Adipose tissue interacts with many other organs by endocrine and paracrine signals and resulting gives rise to or participates in many diseases, like diabetes or Morbus Crohn [65-67]. Currently, e. g. spheroids are designed to model adipose tissue and elucidate the mechanism behind the development and heritage of such related diseases and for the exploration of new potential drugs [68]. However, lifelike adipose tissue models are thought to deliver predictions that are more reliable and are urgently needed [67, 69]. Additionally, adipose tissue sequestrates and thus influences the bioavailable levels of different drugs and may serve in the investigation of safe and effective drug doses [67].

2.3.1. *In vitro* vascularization

In vivo most cells are found in a maximum distance of 200 µm to the next blood vessel. Oxygen, nutrients and waste products are known to be able to diffuse across this

distance between the vasculature and the peripheral tissue. Engineered tissue constructs, which exceed these dimensions, consequently have to include a supplying component [70]. The high vascularization level in adipose tissue is considered to be a key factor in WAT mass and function [71, 72]. Supply systems for adipose TE have to be permeable and cytocompatible. That is why cellular components are included often to mimic the native function of blood vessel as good as possible [73].

2.3.2. Cell sources

In contrast to BM-MSCs, ASCs are available in high quantities from adipose tissue, their harvest shows little donor site morbidity and is cheaper, safer and less invasive compared to BM aspiration [37]. In contrast to embryonic stem cells, ASCs are less associated to immune reactions [74] and free of ethical concerns [75].

ASCs' capacity to undergo adipogenic differentiation is well described [76, 77] and may be initiated *in vitro* via the application of adipogenic initiators e. g. GCs, like dexamethasone (Dex) or hydrocortisone (HC), insulin, isobutylmethylxanthine (IBMX) and others [44] (see chapter 3 "Adipogenic differentiation", [55]). ASCs do not only give rise to MAs [78], but also induce and support the adipogenic differentiation of other progenitor cell types [79]. ASCs and adipogenic differentiated ASCs (diffASC) were confirmed to produce ECM components *in vitro* [80]. Additionally, ASCs have been demonstrated to take up the position of perivascular cells *in vitro* and modulate and support the growth of new vascular structures [81, 82]. Adipose tissue has been modelled successfully *in vitro* in two-dimensional (2D) and three-dimensional (3D) mono- and co-culture attempts, based on ASCs. However, only very few data is available on the sustainability of formed systems *in vitro* and the cellular phenotype therein [83]. In recent publications, MAs are variously defined as preadipocytes differentiated for five to 14 days *in vitro* [84, 85], although a further adipogenic development of cells has been reported at least until day 28 [86, 87].

Directly isolated primary MAs show the great advantage of an already existing physiological cell functionality. Cells exhibit the characteristic adipogenic cell morphology, size and metabolic activity as found in native adipose tissue *in vivo*. MAs are non-proliferating cells and therefore cannot be expanded following their isolation. Their rapid dedifferentiation *in vitro* due to a lack of knowledge on their specific culture requirements is another major drawback. Starting already few days after isolation, cells

begin to organize their lipids in several, smaller vacuoles [18, 88, 89], first resulting in multivacuolar and finally fibroblast-like, dedifferentiated cells [90]. Some very promising studies have recently been published on their successful culture and application to build up adipose and full skin tissue models *in vitro* through the optimization of media and matrix properties [91-93]. Nonetheless, MAs are still used very rarely in adipose TE attempts and receive little to no attention [57].

To be able to supply the engineered adipose tissue via a natural system, an EC type has to be integrated into the *in vitro* attempt. ECs are present in almost every tissue of the human body but are less concentrated compared to stromal cells [94]. Endothelial progenitor cells (EPCs) show the advantage of a high proliferative activity and therefore the ability to give rise to high proliferative colonies, capable of forming blood vessels [95]. However, they are relatively rare in the human body and only found to low percentages e. g. with 0.01 % of circulating mononuclear cells in peripheral blood or in the BM [96]. A non-invasive source of ECs is the umbilical cord, which is usually discarded as medical waste. Human umbilical cord ECs (HUVEC) exhibit a relatively high proliferation capacity and are often used in *in vitro* attempts [97]. Especially in the past decade, concerns on the use of HUVECs in TE attempts increased, as fundamental differences in their behavior compared to adult ECs were revealed [98]. Adult mvECs only show a limited ability to expand, however the cells are still capable to form capillary-like structures [99] and may be isolated from adult human skin, which is also available as medical waste from skin tightening surgeries [100]. ECs, as many other cell types, are known to vary phenotypically depending on their tissue of origin [101]. In order to modulate the microenvironment specifically, the donor and target tissue should match at best. Therefore, mvECs derived from human WAT represent the most appropriate choice when targeting vascularization of adipose tissue constructs.

2.3.3. Current attempts in adipose tissue engineering

In vivo subcutaneous adipose tissue exhibits a soft viscoelasticity with moduli of about 1 kPa - 2 kPa [102] and it is known that rather soft materials favor adipogenesis and the maturation of adipocytes [103, 104]. *In vivo* conditions are presumed to be best simulated by the use of natural decellularized matrices, which already exhibit the appropriate properties like stiffness, pore size, topography and matrix components

[105]. To mimic these properties, hydrogels based on collagen type I, fibrinogen, hyaluronic acid, or gelatin have been often used next to the classic tissue culture polystyrene (TCPS) to set up adipose tissue constructs (reviewed in [106]). However, adipocyte generation was also successfully performed on stiff materials like polyethersulfone [107]. Similarly, the successful culture of ECs and the formation of vascular-like structures are associated to materials with low stiffness [108]. Next to collagen and fibrin, synthetic scaffolds like polyacrylate have been used successfully through the functionalization with RGD (Arg-Gly-Asp) [73]. A relatively new material in the field is represented by bacterial cellulose [109-111]. The material exhibits a natural porosity, biocompatibility and biodegradability [109, 112] and was shown to support adipogenic differentiation [113, 114] and stimulate an activated state in ECs [115].

The functionality and sustainability of tissue-engineered constructs highly depends on the nutritional status of cellular components. Good achievements have been made by the application of a dynamic system to transport nutrients to and waste products away from the tissue sites via the flow of a liquid phase [107, 116, 117]. Nevertheless, for the generation of physiological tissue conditions, a vascular component has to be integrated into the system. Vascularization still represents one of the key challenges in TE [118]. According to current attempts, tissue-engineered constructs, may either be vascularized by implanting them *in vivo*, whereby the host tissue guides vascularization of the external constructs, or culture cells *in vitro* by providing some support to facilitate the formation of vascular structures [119]. *In vitro* scaffold-based strategies, e. g. with natural decellularized matrices [120], or the artificially formed biorap system [73], provide guiding geometrics and facilitate the arrangement of ECs in a branched system. *De novo* angiogenesis on the other hand relies on the intrinsic formation of vascular structures by the included ECs [121]. Current strategies for *de novo in vitro* vascularization include cell seeding, cell sheet and spheroid formation [122].

In order to facilitate *de novo in vitro* adipose tissue vascularization the setup of a functional co-culture consisting of an endothelial and an adipose cell type is an inevitable prerequisite. Several works have been published on the functional co-culture of ECs with either adipogenic differentiated MSCs or MAs [123, 124]. Dynamic culture was shown to not only facilitate tissue nutrition, but also particularly support characteristic endothelial morphology and the physiological organization of angiogenic and vasculogenic processes, as it resembles the native conditions of the EC niche

[125]. Wiesner *et al.* highlighted the positive effect of dynamic culture conditions on the parallel seeding and culture of ECs and adipocyte progenitor cells in a decellularized natural matrix for the generation of large-scale tissue substitutes [126]. Equally, Abbott *et al.* reported enhanced adipocyte functionality under dynamic conditions [127]. Furthermore, Aubin *et al.* and Abbott *et al*. managed to maintain engineered adipose tissue *in vitro* for a long-term period of four weeks including MAs [128] and eleven weeks based on adipogenic differentiated (diff)ASCs [129], while Bellas *et al*. sustained adipose tissue constructs with integrated vascular cells under dynamic conditions [83].

2.3.4. Current challenges in adipose tissue engineering

Despite the various attempts, *in vitro* vascularization of adipose tissue is still not achieved sufficiently. In the setup of a functional co-culture for vascularized adipose TE, the choice of the co-culture medium plays a fundamental role. Current attempts mostly use 1:1 mixtures of adipocyte and EC media or are, presumably due to the higher susceptibility of ECs, completely based on EC media [130-135]. In order to allow physiological tissue development in *in vitro* co-culture setups, including adipocyte generation and vascularization, co-culture conditions, like the used medium have to be adjusted specifically to the cellular requirements of the integrated cell types.

Media for the culture of mammalian cells are most often composed with a basal medium including ingredients with low cell specificity. Due to that, basal media are designed and can be used for a range of different cell types [136]. Depending on the definition, they include e. g. buffering agents and salts for the osmotic balance and the maintenance of the membrane potential [137], carbohydrates as main energy source [138], amino acids for anabolic processes [139] and vitamins as general support of metabolic and antioxidative pathways [136] (see yellow section in **Table 1**). On top of these basic ingredients, most cell types rely on the availability of additional ingredients, like transport or adhesion proteins for the delivery of hormones and also lipids and vitamins [138, 140] for the transduction of signals, to store energy or as structural element [141] (see orange section in **Table 1**). Hormones, growth factors and cytokines may therein fulfill general roles in cell proliferation, growth and survival, but may also act very cell-specifically, promoting differentiation and the maintenance of unique cell functions [142-144]. In **Table 1** cell specificity of the factors generally increases with

their position in the table from the top to the bottom. Serum is a versatile source of different factors like proteins, hormones, growth factors, cytokines, lipids, vitamins and carbohydrates [136]. In consequence, it serves as an adequate supplier for a wide range of different cell types and is used as universal supplement in many cell- and tissue culture approaches [136, 142]. Serum containing (SC) media are nevertheless associated to some fundamental issues concerning the sought application of TE products as *in vitro* test systems or clinical implants. First, sera pose the risk of potential contaminations [145]. Moreover, the composition of sera, independent of their animal or human origin is not completely identified and may vary from batch to batch [146]. These drawbacks have to be eliminated prior to a possible production of TE products under good manufacturing practice (GMP)-conform conditions, which is an important prerequisite for their clinical application.

Table 1: Cell Culture media components and their functions.

	Component	Function
Basic constituents included in the basal medium	**Buffers** *(e. g. $NaHCO_3$)*	Maintain pH-value [136]
	Phenol red	Indicator of pH-value [147]
	Salts *(e. g. with Na^+, K^+, Ca^{2+}, Cl^-, HCO_3^-)*	Osmotic balance and membrane potential [137]
	Keto acids *(e. g. pyruvate & oxaloacetate)*	Carbohydrate metabolism, amino acid metabolism [136]
	Non-protein nitrogen compounds *(e. g. urea, purines, creatinine)*	Cell growth, amino acid metabolism
	Carbohydrates *(e. g. glucose, galactose, fructose)*	Energy source [138]
	Amino acids *(e. g. glutamine, methionine)*	All anabolic processes like growth and proliferation [139]
	Vitamins *(e. g. ascorbic acid, biotin)*	Growth and proliferation, antioxidant [136]
	Trace elements *(e. g. iron, copper, selenium)*	Cell growth, basic biological processes [139]
Supplemental constituents, classically delivered by serum	**Transport-, and adhesion proteins** *(e. g. albumin, transferrin, fibronectin)*	Transport of water, salts, FAs, hormones and vitamins, cell protection, cell attachment [138, 140]
	Hormones *(e. g. insulin, prostaglandins)*	Various cell-type specific functions including growth, proliferation, survival, differentiation adhesion or migration [142]
	Growth factors and cytokines *(e. g. PDGF, VEGF, ILs, interferons)*	Various cell-type specific functions including growth, proliferation, survival, differentiation adhesion or migration [143, 144]
	Lipids *(e. g. oleic acid, cholesterol)*	Signal transduction, energy storage, structure [141]

Additionally, robustness and reliability of *in vitro* test systems substantially depend on the consistency of the medium composition, as tissue development and homeostasis strongly rely on the crosstalk between cells [148-150]. Unidentified serum components might interfere with the underlying cell signaling and influence test outcomes extensively. The defined setup of engineered vascularized adipose tissue and TE products in general, is a central challenge to facilitate their actual application as implant or *in vitro* test system. Nonetheless, the exclusion of serum is often related to impaired differentiation, reduced cell adherence and loss of cell-specific features [141]. For this reason, the replacement of serum with adequate alternatives is sought. Serum replacement may take place to different levels (see **Figure 3**). Human serum (HS) may be used in xeno-free (XF) attempts, in contrast to serum-free (SF) attempts. For the completely defined setup of cell culture media, Van der Valk *et al.* provided a general strategy [141]. Based on a basal medium like Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12, supplements are added empirically to optimize a cell culture medium cell-specifically. According to his pyramid, cell specificity increases starting rather unspecific with the basal medium and the pre-coating of culture vessels with adhesion factors over the addition of (un)specific growth factors, hormones and lipids, over antioxidants (like β-mercaptoethanol or vitamins) up to very cell specific vitamins like retinoic acid [141].

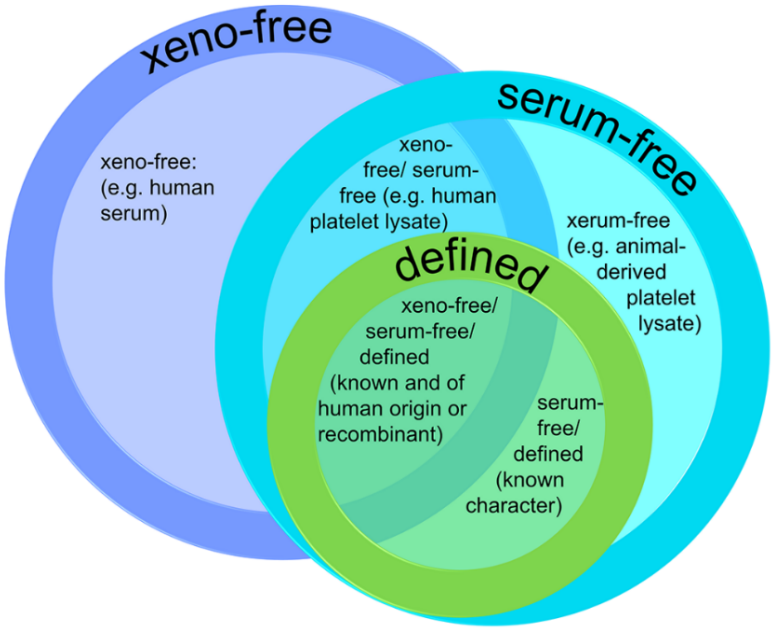

Figure 3: Categories of cell culture media supplements
(taken from chapter 4 [151]).

There are some current attempts addressing defined WAT engineering e. g. by Rajala *et al.*, who successfully performed an adipogenic differentiation under defined conditions, however without allocating the efficiency of the differentiation [152].

To allow for time-dependent investigations *in vitro* and facilitate host anastomosis after implantation, engineered adipose tissue constructs have to be sustainable for a long-term period of at least several weeks. Vascularization represents an important element for the achievement of tissue maintenance. Huttala *et al.* managed the setup of a defined co-culture of ECs and ASCs [153]. However the defined setup of an adipocyte/EC co-culture has not been addressed till now. Defined media are urgently needed for the efficient adipogenic differentiation of ASCs and the maintenance of adipocytes and ECs in mono- and co-culture. An important determining factor, not only limited to defined cell culture, is the choice of culture matrix, which on the one hand should support tissue development and maturation but on the other hand should endure long-term periods without intense degradation.

2.4. Aim of the study

The aim of this dissertation was the development of novel cell-specifically composed culture media for the adipogenic differentiation of ASCs, the long-term maintenance of the received adipocytes and mvECs and the functional co-culture of the different cell types. In contrast to previous approaches, the media were sought to be developed completely defined by the exclusion of sera. Furthermore, a novel cellulose-based material (CBM) was to be evaluated concerning its usability to support the sustainable maintenance of an adipocyte/EC co-culture under defined conditions. Moreover, the transfer of an adipose tissue co-culture model to a 3D environment was sought. Therefore the following hypotheses were evaluated:

(H1) The supplementation with defined media additives facilitates the efficient adipogenic differentiation of ASCs and the maintenance of the received adipocytes in SF conditions.

(H2) The maintenance of adipocyte and mvEC characteristics is achieved by the variation of the concentrations of EGF and HC in co-culture.

(H3) The supplementation with defined media additives facilitates the functional co-culture of adipocytes and mvECs.

(H4) The CBM supports the defined adipogenic differentiation of ASCs, the defined maintenance of mvECs and the defined adipocyte/mvEC co-culture.

(H5) A lifelike 3D adipose tissue model can be set up based on co-cultured ASCs *in vitro*.

Summarized, this dissertation provides an approach to transfer vascularized adipose TE to an advanced level by adjusting its setup to defined conditions. Thereby it was pursued to accelerate and reinforce the application of engineered adipose tissue constructs in regenerative medicine and *in vitro* investigative approaches.

2.5. Structure of the thesis

In the following, the peer-reviewed publications, which constitute the main methodical and experimental sections of this dissertation, are integrated. The published review article “Adipose-derived stem cell differentiation as a basic tool for vascularized adipose tissue engineering” gives some general background on the use of ASCs in the setup of vascularized WAT in chapter 3 [55]. The published research articles “Completely serum-free and chemically defined adipocyte development and maintenance” in chapter 4 [151] and “EGF and hydrocortisone as critical factors for the co-culture of adipogenic differentiated ASCs and endothelial cells” in chapter 5 [87] address the investigation of H1 and H2, respectively. The articles “Completely defined co-culture of adipogenic differentiated ASCs and microvascular endothelial cells” in chapter 6 [154] and “A cellulose-based material for vascularized adipose tissue engineering” in chapter 7 [155] equally relate to the examination of H3 and H4. Chapter 8 deals with the analysis of H5 and offers an interrelated summary and discussion of the complete results. Chapter 9 resumes the main findings and following chapter 10 provides an outlook towards the next sought steps. Furthermore, the cited literature is included in chapter 11.

3. Adipose-derived stem cell differentiation as a basic tool for vascularized adipose tissue engineering

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Abstract

The development of *in vitro* adipose tissue constructs is highly desired to cope with the increased demand for substitutes to replace damaged soft tissue after high graded burns, deformities or tumor removal. To achieve clinically relevant dimensions, vascularization of soft tissue constructs becomes inevitable but still poses a challenge. Adipose-derived stem cells (ASCs) represent a promising cell source for the setup of vascularized fatty tissue constructs as they can be differentiated into adipocytes and endothelial cells *in vitro* and are thereby available in sufficiently high cell numbers.

This review summarizes the currently known characteristics of ASCs and achievements in adipogenic and endothelial differentiation *in vitro*. Further, the interdependency of adipogenesis and angiogenesis based on the crosstalk of endothelial cells, stem cells and adipocytes is addressed at the molecular level. Finally, achievements and limitations of current co-culture conditions for the construction of vascularized adipose tissue are evaluated.

4. Completely serum-free and chemically defined adipocyte development and maintenance

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Abstract

Background aims: *In vitro* engineered adipose tissue is in great demand to treat lost or damaged soft tissue or to screen for new drugs, among other applications. However, today most attempts depend on the use of animal-derived sera. To pave the way for the application of adipose tissue–engineered products in clinical trials or as reliable and robust *in vitro* test systems, sera should be completely excluded from the production process. In this study, we aimed to develop an *in vitro* adipose tissue model in the absence of sera and maintain its function long-term.

Methods: Human adipose tissue–derived stem cells were expanded and characterized in a xeno- and serum-free environment. Adipogenic differentiation was induced using a completely defined medium. Developed adipocytes were maintained in a completely defined maturation medium for additional 28 days. In addition to cell viability and adherence, adipocyte-specific markers such as perilipin A expression or leptin release were evaluated.

Results: The defined differentiation medium enhanced cell adherence and lipid accumulation at a significant level compared with the corresponding negative control. The defined maturation medium also significantly supported cell adherence and functional adipocyte maturation during the long-term culture period.

Conclusions: The process described here enables functional adipocyte generation and maintenance without the addition of unknown or animal-derived constituents, achieving an important milestone in the introduction of adipose tissue–engineered products into clinical trials or *in vitro* screening.

5. EGF and hydrocortisone as critical factors for the co-culture of adipogenic differentiated ASCs and endothelial cells.

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Abstract

In vitro composed vascularized adipose tissue is and will continue to be in great demand e.g. for the treatment of extensive high-graded burns or the replacement of tissue after tumor removal. Up to date, the lack of adequate culture conditions, mainly a culture medium, decelerates further achievements. In our study, we evaluated the influence of epidermal growth factor (EGF) and hydrocortisone (HC), often supplemented in endothelial cell (EC) specific media, on the co-culture of adipogenic differentiated adipose-derived stem cells (ASCs) and microvascular endothelial cells (mvECs). In ASCs, EGF and HC are thought to inhibit adipogenic differentiation and have lipolytic activities. Our results showed that in indirect co-culture for 14 days, adipogenic differentiated ASCs further incorporated lipids and partly gained an univacuolar morphology when kept in media with low levels of EGF and HC. In media with high EGF and HC levels, cells did not incorporate further lipids, on the contrary, cells without lipid droplets appeared. Glycerol release, to measure lipolysis, also increased with elevated amounts of EGF and HC in the culture medium. Adipogenic differentiated ASCs were able to release leptin in all setups. MvECs were functional and expressed the cell specific markers, CD31 and von Willebrand factor (vWF), independent of the EGF and HC content as long as further EC specific factors were present. Taken together, our study demonstrates that adipogenic differentiated ASCs can be successfully co-cultured with mvECs in a culture medium containing low or no

amounts of EGF and HC, as long as further endothelial cell and adipocyte specific factors are available.

6. Completely defined co-culture of adipogenic differentiated adipose-derived stem cells and microvascular endothelial cells

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Abstract

Vascularized adipose tissue models are in high demand as alternatives to animal models to elucidate the mechanisms of widespread diseases, screen for new drugs or assess drug safety levels. Animal-derived sera such as fetal bovine serum (FBS), which are commonly used in these models, are associated with ethical concerns, risk of contaminations and inconsistencies of their composition and impact on cells. In this study, we developed a serum-free, defined co-culture medium and implemented it in an adipocyte/endothelial cell (EC) co-culture model.

Human adipose-derived stem cells were differentiated under defined conditions (diffASCs) and, like human microvascular ECs (mvECs), cultured in a defined co-culture medium in mono-, indirect or direct co-culture for 14 days. The defined co-culture medium was superior when compared to mono-culture media and facilitated the functional maintenance and maturation of diffASCs including perilipin A expression, lipid accumulation, and also glycerol and leptin release. The medium also allowed mvEC maintenance, confirmed by the expression of CD31 and von Willebrand factor (vWF), and by acetylated low-density lipoprotein (acLDL) uptake. Thereby mvECs showed strong dependence on EC-specific factors. Additionally, mvECs formed vascular structures in direct co-culture with diffASCs.

The completely defined co-culture system allows for the serum-free culture of adipocyte/EC co-cultures and thereby represents a valuable and ethically acceptable tool for the culture and study of vascularized adipose tissue models.

7. A cellulose-based material for vascularized adipose tissue engineering

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The article is included with a different wording compared to the original publication.

Abstract

In vitro adipose tissue constructs could on the one side be used as models to screen for new drugs or elucidate metabolic pathways and on the other side could serve as tissue implants to treat deep wounds or adipose tissue traumata. The integration of a vascular component would allow the lasting sustainability of larger constructs but is currently missing which prevents the actual clinical use of artificial adipose tissue. With this trial, we sought to evaluate a novel material based on bacterial cellulose (CBM) concerning its influence on adipogenic differentiation of human adipose-derived stem cells (ASCs), the maintenance of differentiated adipocytes (diffASCs) and human microvascular endothelial cells (mvECs) in mono- and co-culture under serum-free, defined conditions. Defined adipogenic differentiation was slightly supported by CBM compared to tissue culture polystyrene (TCPS). With regard to the maintenance of generated adipocytes, CBM and TCPS showed a comparable influence. Compared to TCPS, the formation of vascular-like structures was significantly supported by CBM in the mvEC mono-culture. In the co-culture attempt, CBM and TCPS equally facilitated the development and maintenance of vascular-like structures. Summarizing, CBM supports engineering of vascular adipose tissue in several respects. CBM does not only have the potential to speed up the generation of artificial adipose tissue constructs in future attempts, but also simplify their setup requirements.

8. Results and Discussion

8.1. Defined adipocyte generation

The application of engineered tissues as implants or *in vitro* test systems would be carried forward substantially by methods and materials facilitating their defined setup in the absence of sera. Consequently, potential contaminations in their clinical implementation could be reduced markedly [146]. Besides that, the reliability, robustness and the predictive power of *in vitro* test systems and models could be enhanced, generating a powerful tool, which represents the human body more accurately [136]. The transfer of products to a defined setup is pursued in various fields of TE. It especially poses a challenge in the differentiation and development of diverse cell types, as the formation and conversion of cellular and tissue components relies on a broad range of signals and factors. Within adipogenic differentiation, cellular commitment is followed by extensive accumulation of lipids in vacuoles [44], which finally comprise a multiple of the cell's original volume [15].

As engineered adipose tissue is urgently needed in regenerative medicine and in *in vitro* investigations, defined adipogenic differentiation has been addressed in various attempts before. Hauner *et al.* generated the earliest available results already in 1989 [156]. In current attempts, Rodriguez *et al*. and Körner *et al*. e. g. successfully performed an adipogenic differentiation with a defined differentiation mix and received characteristic adipocytes [157, 158]. Today, most studies still include undefined components in the differentiation process of the cells [159, 160]. In addition, many defined approaches still include serum or animal-derived components in the cell expansion phase, which represents a major drawback in terms of their applicability. In contrast, Rajala *et al*. used allogenic HS for the expansion phase and developed a defined and XF method to differentiate human ASCs adipogenically [152]. The medium was nevertheless developed for the parallel differentiation of several cell types like induced pluripotent stem cells. Hence, its composition was not tailored specifically to ASCs' requirements and included a wide range of probably non-essential factors. To minimize costs and variations of a system and to keep an easy transferability to other applications, defined culture media should consist of as few components as possible. As a general drawback, the current studies only provide a proof of concept and almost none of them includes a comparison of the results, generated under the different defined conditions to classically applied SC attempts. By that, an adequate reference

to evaluate and classify the results comprehensively is detained. On top, the available results do not allow to deduce a conclusion on the differentiation rates and the general efficiency.

With this approach a SF adipogenic differentiation medium, completely defined in its composition, should be developed. Further, the efficiency of the differentiation was to be compared to classically used SC attempts to be able to rate them. Based on a well-established SC medium a SF and defined adipogenic differentiation medium was developed. Therefore, literature was searched for factors known to generally support SF cell culture or specifically promote adipogenic differentiation or maintenance. Based on the found information various combinations were evaluated in preliminary experiments to find the optimal formulation. Following the selection of the most suitable formulation, this cell type-tailored defined differentiation medium (Def-Diff) including differentiation specific factors (DIFF) and the defined supplement A, was proven to allow for an efficient adipogenic differentiation. Thereby viable adipocytes with a high rate of cell adherence of about 100 % were generated in contrast to the negative control (Ctrl-Diff), which only contained the DIFF mix [151]. With about 80 % of the cells expressing the adipocyte-specific factor perilipin A and released leptin, defined differentiated cells showed a rate close to the SC positive control (SC-Diff). In a further analysis the adipogenic proteins CEB/P-α, PPAR-γ and adipoQ were demonstrated to be expressed to an extent close to SC-Diff (**Figure 4**). The comparable expression of CEB/P-α and PPAR-γ are in line with the high percentage of perilipin A-expressing cells. These results indicate a comparable initiation of adipogenic differentiation in the presence of serum and the defined supplement. However, the reduced lipid

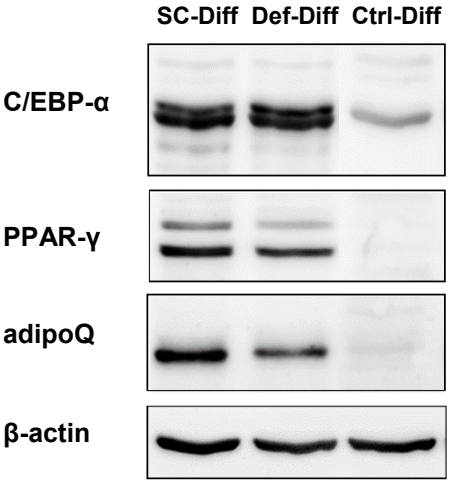


Figure 4: Protein expression analysis of diffASCs. DiffASCs, differentiated for 14 days, analyzed for the expression of the adipogenic proteins C/EBP-α, PPAR-γ and adipoQ via a Western Blot, β-actin is displayed as a reference gene; positive control (SC-Diff) containing the differentiation initiators (DIFF) and serum, defined attempt (Def-Diff) containing DIFF and a defined supplement A, negative control (Ctrl-Diff) containing solely DIFF, n=1.

accumulation in Def-Diff indicates a shortage of available factors, to support *de novo* lipogenesis or the FA uptake from the extracellular surrounding.

The high leptin levels and percentage of perilipin A-positive cells in the defined attempt by that highlight the high differentiation rate compared to the supplement-free Ctrl-Diff, close to SC-Diff. Nonetheless, the reduced lipid accumulation indicates a retarded maturation process. Hence, the developed differentiation medium did not keep up with serum supplementation completely and obviously there were still some serum components which have not been met in the supplement and have to be addressed in future attempts. Still, for the first time adipogenic differentiation was performed under completely defined conditions *in vitro* with proven efficiency*,* subsequently to a SF and XF expansion phase with human primary ASCs. Bottom line, the method represents a valuable tool in the setup of WAT constructs under defined conditions and may serve as a reference to rank future outcomes.

As a major limitation, available trials provide no proof of the long-term stability of the generated adipocytes. It is known that MAs dedifferentiate *in vitro* in case of insufficiently adjusted culture conditions [32, 93]. The long-term maintenance of differentiated preadipocytes has been demonstrated by Fischbach *et al*. with a 3T3-model for 35 days continuously to the differentiation initiation [161] and Bellas *et al.* based on human ASCs for six months [83]. Despite, adipogenic differentiation of MSCs may also be followed by dedifferentiation *in vitro* depending on the composition of the medium and other culture parameters [87, 162]. For the implementation as *in vitro* system, tissue sustainability is of substantial importance to allow for time- and maturation-dependent investigations. Further, the successful implantation of engineered tissue substitutes heavily depends on an adequate host anastomosis and long-term implant survival *in vivo* requires tissue stability of at least several weeks [63]. So far, no results on the long-term maintenance under defined conditions are available. With this study, the long-term maintenance of adipocytes, differentiated under defined conditions, was to be proven via the composition of a defined maintenance medium (Def-M), including a defined supplement B next to the maintenance mix (MAIN) for at least 28 days post-differentiation. While the positive control SC-M included serum instead of supplement B, the negative control (Ctrl-M) solely included MAIN as supplement. In contrast to Ctrl-M, Def-M was shown to maintain cell adherence comparably to SC-M, indicating an adequate supplementation with specific components allowing cell-material junctions. Although lipid accumulation,

which is an indicator of the developmental state of MAs, were still reduced in the defined attempt compared to the SC-M, leptin levels showed comparable values and confirmed a similar level of cell functionality and maturation. Low glycerol levels highlighted the reduced lipolytic activity of adipocytes in the defined approach. The equal percentage of perilipin A-expressing cells validates the adequate differentiation rate, achieved in the defined attempt. Compared to day 0 (directly after the 14-day differentiation period) the rate of differentiated adipocytes was decreased independent of the supplementation with serum or the defined mix in supplement B, apparent in the reduced rate of perilipin A-expressing cells. Coincidently leptin and lipid levels increased. Inhibited adipogenesis of ASCs in the presence of MAs has been shown before and was assigned to the release of angiontensin II [163]. This effect might be explained by the self-organized adjustment of a homeostatic equilibrium of differentiated and dedifferentiated cells and is in accordance with the results received in this study. As many differentiated cells were present after some days in culture, dedifferentiation of some diffASCs was presumably induced through a negative feedback loop.

To test whether the sustainability of adipocytes may continue during an elongated *in vitro* culture period, defined adipogenic diffASCs were maintained for additional 14 days in the defined maintenance medium until day 42 post differentiation (day 56 after the start of defined differentiation). Similar to day 28, diffASCs including incorporated lipid droplets and perilipin A were sustained in Def-M and SC-M (**Figure 5**). Although the size of lipid vacuoles and the level of accumulated lipids was again reduced in Def-M with 61.02 (± 13.79) % compared to the SC-M with 104.25 (± 26.50) %, the rate of present adipocytes was still comparable. This was visible through the ratio of cell nuclei stained with 4, 6-diamidin-2-phenylindol (DAPI) in blue to visible perilipin A-lined vacuoles in green. Ctrl-M exhibited values of 12.33 (± 3.13) %. Furthermore, no significant reduction in the percentage of perilipin A-expressing cells was observed within the different media between day 28 and day 42 (SC-M: 76.77 (± 37.32) %, Def-M: 64.94 (± 22.77) % and Ctrl-M: 28.77 (± 25.37) %), displaying maintained adipocyte stability and supporting the thesis of an adjusted balance of cell ratios.

Concluding, the defined adipogenic differentiation medium enables for the generation of a functional culture of MAs. Furthermore, the defined maintenance medium

facilitates the sustainable long-term *in vitro* culture for at least six weeks. Subsequently, H1 was positively confirmed.

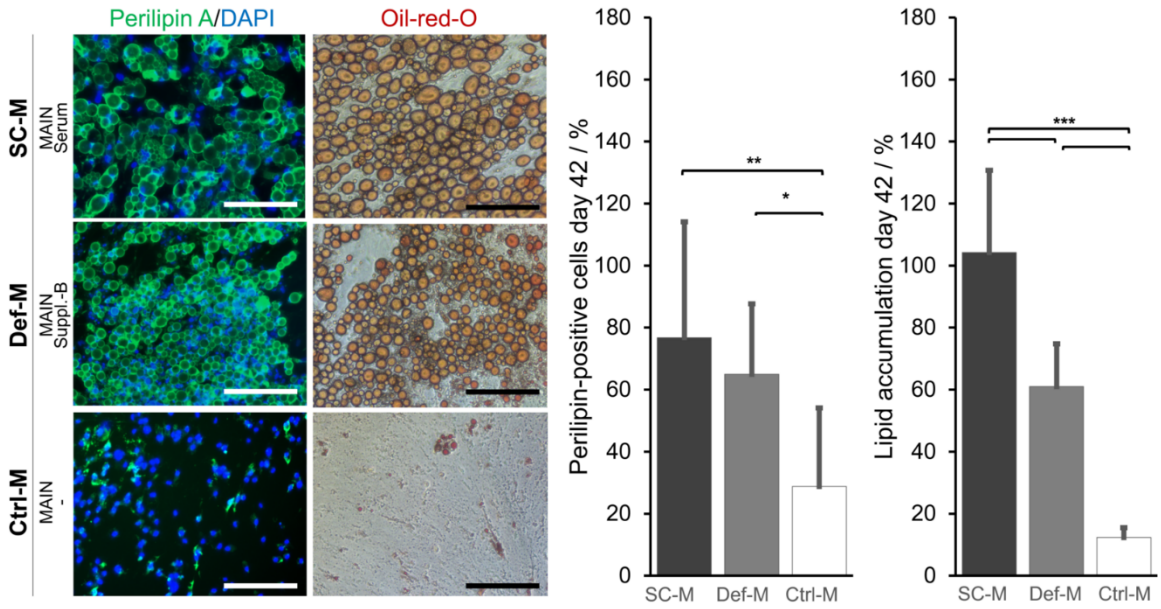


Figure 5: Maintained diffASCs on day 56 (42 after differentiation). Perilipin A/DAPI immunofluorescence staining of diffASCs: perilipin A is shown in green cell nuclei in blue, Oil-red-O staining, quantitative analysis of perilipin A expressing cells by IF staining, normalized to SC-Diff day 14, quantitative analysis of lipid accumulation by Oil-red-O staining, positive control (SC-M) containing the maintenance factors (MAIN) and serum, defined attempt (Def-M) containing MAIN and a defined supplement B, negative control (Ctrl-M) containing solely MAIN; scale bar: 200 µm,

8.2. Functional (defined) adipocyte endothelial cell co-culture

Engineered adipose tissue constructs have to be produced in an appropriate size, in order to push forward their application in regenerative medicine [23]. The enlargement of present products inevitably entails the necessity of a vascular system, which allows for the simultaneous nutrition of internal and peripheral parts of the tissue construct. Current attempts include the endothelialization of vascular geometries in artificially produced scaffolds or decellularized tissue pieces [70, 118]. The most physiological model is nonetheless produced by ECs and their associated cells via the self-organized *de novo* formation of capillary-like structures. A fundamental milestone in the setup of vascularized WAT is the functional co-culture of ECs and adipocytes respectively ASCs, which in turn heavily relies on the use of an appropriate culture medium. The medium must meet the requirements of both cell types adequately, which might deviate considerably. In terms of adipose TE, adipocytes should be enabled to maturate, secrete hormones and function in terms of lipid and glucose metabolism. ECs should

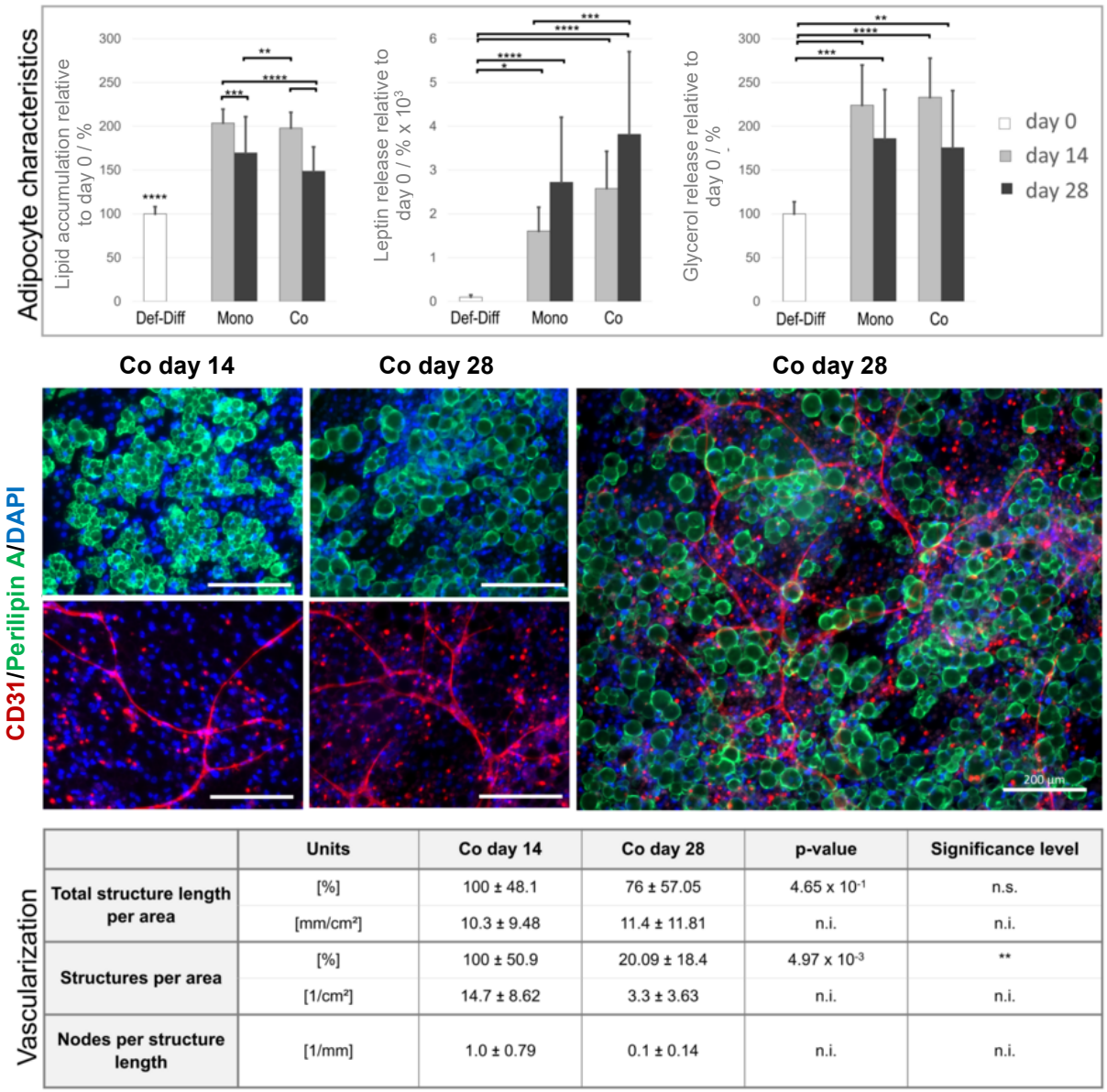
also display cell-specific characteristics like the expression of CD31 and vWF and function physiologically e. g. detectable by the uptake of acLDL. Since tissue supply depends on the development and maintenance of a vascular system, the medium should further facilitate the setup of vascular-like structures via angiogenic processes. This developmental process substantially benefits from the interplay of adipocytes, ASCs and ECs (reviewed in [55]). An adequate culture medium thereby should be designed to support the functionality of the used cell types without covering their natural crosstalk. Human primary mvECs have been mono-cultured successfully *in vitro* by the use of cell type-specific media [73, 92, 164]. In those, ECs were provided with VEGF and basic fibroblast growth factor (bFGF), as potent mitogens [71, 165-168], insulin-like growth factor (IGF) for physiological EC metabolism, migration and vessel formation (reviewed in [169]) ascorbic acid-2-phosphate (A2P) promoting EC growth [170] and EGF as a potent contributor to cell migration [171]. Specific media are equally available for adipocytes and include factors supporting lipid metabolism, lipogenesis and other anabolic processes like insulin, GCs or vitamins like panthothenate and biotin [93, 158, 163, 172, 173]. Due to the complexity of an adequate composition, there is currently no adipocyte/EC co-culture medium available. Currently performed co-culture attempts are therefore mostly based on either the EC or the adipocyte medium or a simple 1:1 mixture of them [126, 131, 174, 175]. In order to compose a functional co-culture medium for diffASCs and mvECs in this study, each factor used in the mono-culture attempt was reviewed previous to its addition. It was found that the role of GCs like HC and Dex are discussed controversially regarding their effect on adipocyte and EC functionality. In adipocytes pro- and antilipolytic effects were detected so far. Thereby a dose-dependent impact has been held responsible [35]. Equally, the impact of EGF on adipogenic differentiation and adipocyte functionality is discussed controversially with a reported pro- and anti-adipogenic effect within differentiation as well as a lipogenic and a lipolytic effect on differentiated adipocytes [176]. Within this study, the effect of EGF and HC and the general dependency of mvECs and diffASCs on classically supplemented factors were evaluated in mono- and co-culture setups [87]. It was confirmed that mvECs depend on the presence of the cell specific factors VEGF, IGF, bFGF and A2P. It was shown before, that (diff)ASCs are able to provide several essential EC factors like VEGF, hepatocyte growth factor (HGF) or bFGF [177]. Despite that, the available levels in the co-culture attempt were obviously not high enough, since the tissue functionality was

not yet fully developed. DiffASCs were not affected visibly by the addition of EC-specific factors. In contrast, the further maturation of diffASCs appeared to profit from the EC-factors likewise. Nonetheless, as an important result, diffASC maturation was impaired by the presence of EGF and HC in a dose-dependent manner. These results are in line with the work of Werner *et al.* [178], who reported a reduced adipogenic differentiation of stem cells and Huber *et al*. who showed an increased lipolytic behavior of MAs in the presence of EGF and HC [92]. The effect is most likely associated to a proliferative and anti-adipogenic effect of EGF and high doses of GCs [35, 179, 180]. MvECs however appeared to be sufficiently supplied with a minimum available amount of EGF and HC. Based on these results it was concluded that an adequate adipocyte/EC co-culture medium has to include essential EC factors, like VEGF and bFGF and has to be reduced in EGF and HC to keep cell functionality. Accordingly, the maintenance of adipocytes and mvECs can be optimized in co-culture by the reduction of EGF and HC to 10 % of the original concentration in classical EC media, which supports H2.

With a view to the sought application of the vascularized adipose tissue construct as *in vitro* test system or *in vivo* implant, the transfer of the co-culture attempt to a defined system was aimed. Up to date, the development or usage of a defined co-culture medium for the setup of vascularized adipose tissue was not reported. Therefore, defined adipocyte/EC co-culture media (CoM) were designed based on the knowledge gained during the development of the media for defined adipocyte generation (Def-M) and the composition of an appropriate SC co-culture medium. In the course of this, cell-specific factors for adipocytes and ECs were combined with pre-evaluated serum substitutes. After the selection procedure in preliminary experiments, comparable to the preparatory phase mentioned in chapter 8.1, the media were evaluated in mono-, indirect and direct co-cultures of diffASCs and mvECs. Therein, ASCs were differentiated adipogenically under defined conditions for 14 days prior to the co-culture phase, as described in chapter 4 [151]. Based on CoM, a functional co-culture of adipocytes and mvECs was achieved under defined conditions for the first time. Adipocytes exhibited high cell viability, perilipin A expression and lipid accumulation. Additionally, they increased their leptin release at moderate lipolytic levels (chapter 6 [154]). Simultaneously, mvECs showed the characteristic expression of CD31 and the ability to take up acLDL. Further, they formed vascular-like structures in co-culture. Thus, the developed defined medium may be categorized as appropriate to maintain

and maturate an adipocyte/EC co-culture under defined conditions and thereby led to the achievement of an important milestone in the development of vascularized adipose tissue constructs *in vitro*.

To evaluate the suitability of the defined CoM to maintain the co-culture for an expanded long-term culture period, additional experiments were performed in which diffASCs and mvECs were co-cultured for 28 days in total (**Figure 6**). Comparably to



Vascularization	Units	Co day 14	Co day 28	p-value	Significance level
Total structure length per area	[%]	100 ± 48.1	76 ± 57.05	4.65×10^{-1}	n.s.
	[mm/cm²]	10.3 ± 9.48	11.4 ± 11.81	n.i.	n.i.
Structures per area	[%]	100 ± 50.9	20.09 ± 18.4	4.97×10^{-3}	**
	[1/cm²]	14.7 ± 8.62	3.3 ± 3.63	n.i.	n.i.
Nodes per structure length	[1/mm]	1.0 ± 0.79	0.1 ± 0.14	n.i.	n.i.

Figure 6: DiffASCs and mvECs in defined mono- and direct co-culture on day 14 and 28 on TCPS. Adipocyte characteristics: quantitative analysis of lipid accumulation, quantitative analysis of leptin release and quantitative analysis of glycerol release of diffASCs in defined mono- and co-culture; values of diffASCs immediately after differentiation (day 0) were set as 100 %; n=3. Perilipin A/CD31/DAPI immunofluorescence staining: specific protein expression of diffASCs and mvECs in mono- and co-culture on day 28 is shown in green and red, nuclei were stained in blue. Quantitative analysis of vascularization on day 14 and 28 of mvECs in co-culture: values of vascular-like structures on day 14 in co-culture were set as 100 %; n=2; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, n.s = non-significant, n.i. = not investigated, scale bar: 200 µm.

day 14, adipocytes continued to express perilipin A. In addition, despite mono- and co-cultured attempts showed minimal reductions in their lipid stores (day 0: 100 (± 8.1) %, day 14: 204 (± 15.9) % and 198 (± 18.0) %, day 28: 170 (± 41.2) % and 149 (± 27.9) % respectively for mono- and co-culture), lipid values were still found to be around 150 % on day 28 compared to day 0. The co-culture with ECs has been connected to both, pro- and anti-adipogenic effects on ASCs and MAs [123, 130, 174, 175]. Wnt 1 and 4 signaling has been held responsible for a shift towards a perivascular and thereby non-adipogenic cell type in ASCs, which then might support vascular structures built by ECs [174]. On the other hand, ECs have been shown to support adipogenic processes [87, 130]. In this study, we detected enhanced lipid accumulation of diffASCs in indirect contact with mvECs, which however only became visible in the SC medium [87]. Direct mvECs/diffASCs contact did not result in enhanced lipid accumulation.

Leptin levels did further increase until day 28 compared to day 0 in all attempts (day 0: 100 (± 47.7) %, day 14: 1608 (± 543.1) % and 2581 (± 846.2) %, day 28: 2723 (± 1484.0) % and 3819 (± 1888.1) % respectively for mono- and co-culture). In co-culture this effect was tendentially enhanced, suggesting intensified adipocyte signaling in the presence of mvECs. The lipolytic rate of diffASCs did not change substantially between day 14 and 28 independent of the attempt (day 0: 100 (± 13.8) %, day 14: 224 (± 46.0) % and 233 (± 44.8) %, day 28: 186.0 (± 55.9) % and 176 (± 65.2) % respectively for mono- and co-culture). In brief, the gained results are best explained by the development of an equilibrium between adipocytes and de- or non-differentiated ASCs. In summary, it can be concluded, that the defined CoM is also suitable to maintain functional co-cultures of diffASCs and mvECs during a long-term period of at least four weeks.

Vasculogenic and angiogenic processes in WAT are known to strongly rely on the presence of a supportive cell type. Equally, the expansion of adipose tissue by the proliferation of progenitor cells or the enlargement of existing adipocytes by additional lipid accumulation depend on the crosstalk with ECs [55]. Adipogenesis and angiogenesis have to be considered as interconnected processes [148-150].

Adipocytes and ASCs may both function to support the development, maturation and maintenance of vascular structures via soluble factors, like HGF, VEGF and TGF-β [132, 135, 181] and direct cell-cell contacts [135, 182]. Thereby the range and concentration level of released factors may slightly deviate between adipocytes and

ASCs [183]. In this study, a supporting effect of present (diff)ASCs on the cell-specific protein expression and functionality of mvECs was detected under defined conditions in indirect co-culture (chapter 6 [154]). Obviously, the defined conditions allowed for the signaling and mutual support of the different cell types through soluble factors. Furthermore, the setup of vascular structures was facilitated based on the presence of (diff)ASCs in direct co-culture. As this behavior was not detected in indirect conditions in either the SC or the defined attempt (chapter 6 [154]), it was concluded, that this effect is rather based on direct cell-cell contacts or the interaction with produced extracellular components than the communication via soluble factors. MvECs might have released PDGF-β for the purpose of perivascular cell recruitment in order to stabilize formed vascular structures [135, 167, 184, 185]. PDGF-β, locally immobilized to surrounding ECM components, might have facilitated mural cell attachment [167]. This is e. g. confirmed by the results of Merfeld-Clauss and his coworkers, who detected enhanced expression of α-smooth muscle actin, a characteristic protein of perivascular cells like pericytes and smooth muscle cells, in ASCs following co-culture with ECs [184, 186]. More evidence is delivered by Rohringer *et al*., who reported on the expression of neuron glial antigen 2 (NG2) by ASCs after direct contact to ECs [135]. As ASCs have been cultured for more than 14 days prior to the addition of mvECs, they most likely formed an interstitial network consisting of ECM components, like laminin, fibronectin or collagen type IV [124, 187]. Angiogenic processes are facilitated through the presence of such ECM components [188-190]. The availability of compatible binding sites for cell attachment and sequences for the degradation through matrix metalloproteinases most likely contributed to the enhanced development and maintenance of vascular-like structures substantially. In short, a defined adipocyte/EC co-culture medium was developed for the first time, which supports the maintenance and maturation of functional adipocytes and mvECs. Additionally, the defined system represents an appropriate platform to facilitate adipocyte/EC crosstalk, which is the most important prerequisite in the setup of physiological vascularized adipose tissue constructs *in vitro*.

Altogether the specific composition of the defined CoM allowed for functional co-culture of mvECs and (diff)ASCs including the formation of vasculuar-like structures and the long-term maintenance of the adipogenic and endothelial structures which confirms H3.

8.3. Bacterial cellulose for the defined setup of vascular adipose tissue

Next to a suitable cell source and the adjustment of the biochemical surrounding via the media composition, scaffolds play an important role in the setup of TE products, as they may provide varying mechanical properties and geometries and may thereby influence cellular behavior substantially [191]. It was shown before that adipogenic differentiation is favored by soft biomaterials [103, 104]. The underlying mechanism was attributed to reduced cell spreading on soft materials, leading to low cell tension and thereby to the blockage of the Rho/ROCK pathway and connected the Wnt/β-catenin signaling pathway [192]. Equally, the enhanced formation of vascular structures on soft materials was confirmed [108].

Regardless, adipogenic differentiation was also successfully performed on relatively stiff materials like polyethersulfone [107] or TCPS [174]. Natural materials like collagen type I or gelatin possess the advantage of included binding sites for the cells. Bacterial cellulose is a natural biopolymer e. g. synthesized by *gluconacetobacter xylinus.* With 99 % water it possesses characteristics of a hydrogel. Its modulus was detected at 0.33 mPa [193]. Thereby the material shows higher stiffness compared to native subcutaneous adipose tissue [103]. Anyway, the material was reported to transfer cells into a quiescent state, thereby supporting their specific characteristics and functionalities [115]. Aiming for the long-term culture of developed vascularized adipose tissue constructs, such an influence could bring substantial benefits in the physiological maintenance of adipocytes and ECs. Especially when SF defined culture conditions are sought, reduced cell adherence, impaired differentiation and loss of cell-specific features are an issue, due to the absence of potential non-identified serum components [141].

In this trial, the influence of CBM on the defined culture of mvECs was to be investigated in order to categorize the material with regard to its suitability for the use in vascularized adipose TE. In the present work, both, CBM and TCPS facilitated the maintenance of EC characteristics like the expression of CD31 and the capability to take up acLDL [155]. It was nevertheless remarkable, that mvECs formed vascular-like structures on CBM in the absence of any supporting cell type at all. The structures were still present on day 28 of the defined culture attempt. This effect was hardly detected at all on TCPS. Again, the softer material properties might be held responsible for this effect [108]. On the other hand, an enhanced matrix formation of mvECs in consequence to the quietening effect of CBM is feasible [194]. The built ECM

components might have supported angiogenic processes and the maintenance of generated structures.

An enhancing effect of nanocellulose on lipid accumulation and adipogenic gene expression was detected before for adipocytes in a 3D setup [114]. Next to the material's effect on vascularization, the influence of CBM on the adipogenic differentiation of ASCs and the maintenance of functional adipocyte features was to be evaluated in this study. In preliminary experiments addressing ASC long-term culture, CBM but not TCPS facilitated the sustainable culture of an intact cell sheet without the detachment of the culture surface even after 42 days of ASC expansion (**Figure 7**).

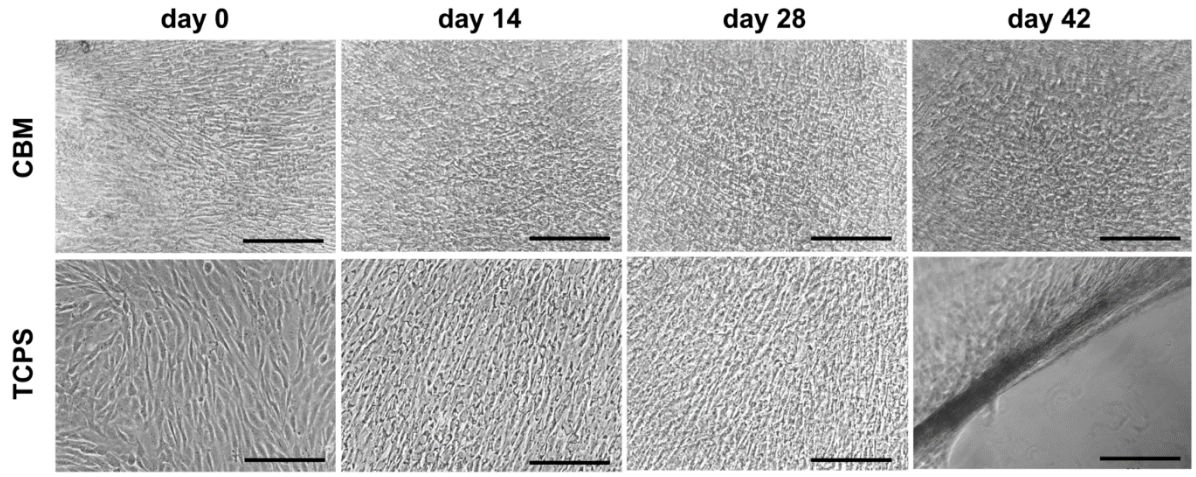


Figure 7: ASCs maintained on CBM or TCPS till day 42 post-confluence. Phase contrast images of ASC cultures in SF and XF MSC growth medium for up to 42 days on either CBM or TCPS, scale bar: 200 µm, n=3.

By addressing the differentiation and maintenance of adipocytes under defined conditions, we achieved considerable adipogenic development (chapter 4 [151]). Despite this, a slight deceleration in lipid accumulation was received and differentiated adipocytes exhibited a minor reduced maturation level. Based on the gained results, CBM positively influenced the defined adipogenic differentiation compared to TCPS, visible through a markedly enhanced leptin release and the intensified expression of perilipin A. As the molecular basis of cellulose does not indicate the potential of a biochemically induced enhancement of adipogenic differentiation, it was concluded, that cell density and the orientation of ASCs in the environment with reduced cell tension were responsible for this supportive effect, as it was described before in other attempts [195, 196]. The supporting effect of CBM however was somehow compensated in the consecutive maintenance phase by TCPS. It is likely that CBM,

despite its supporting effect on adipogenic differentiation, did not especially favor the maintenance of diffASCs over TCPS. Furthermore, (diff)ASCs might have secreted and integrated ECM components in their environment in both attempts equally, covering the effect of CBM through the additional supportive layer with adipose tissue specific properties [124]. This would equally explain the comparable extent of vascular-like structure formation, found in the co-culture attempts on either CBM or TCPS in contrast to the results in the mono-culture attempt, which clearly indicated a beneficial effect of CBM. Concluding, H4 is considered to be approved concerning a supporting effect on defined adipogenic differentiation and defined mvEC maintenance. However, an additional promotion of adipocyte maintenance and adipocyte/EC co-culture was not detectable in this study.

8.4. 3D setup of adipose tissue

The native microenvironment of adipose tissue has to be modeled at the best to facilitate a physiological response of the cellular components [197, 198]. Next to the provision of essential factors, the presence of associated cell types and a tissue-specific matrix, the orientation of cells plays a fundamental role. The later mentioned is substantially affected by the 2D or 3D environment, the cells are exposed to [199, 200]. Additionally, the 3D setup is the prerequisite for the generation of tissue models and substitutes of relevant size. In this trial, the *in vitro* system based on human diffASCs was transferred to a 3D setup to further adjust the microenvironment to native conditions. As shown in the preceding chapters 5 [87], 6 [154] and 7 [155], mvECs were integrated into the system with regard to the sought vascularization of the model. For the evaluation of *in vitro* models suitability to represent and replace native WAT, the models were compared to native subcutaneous WAT.

After the integration of human primary ASCs in collagen type I hydrogels and their differentiation for 14 days, 3D models were either indirectly co-cultured with human primary mvECs seeded in 2D in a SC medium or mono-cultured in a SC adipocyte medium as described in chapter 5 [87]. The co-culture was continued for seven days until day 21 after the 3D arrangement of diffASCs. Co-cultured mvECs thereby equally built and maintained a dense monolayer and cell sheet integrity as shown in **Figure 8.** Furthermore, they kept their ability to take up acLDL. Encapsulated ASCs were viable after the differentiation process on day 1 and after the co- and mono-culture phase on

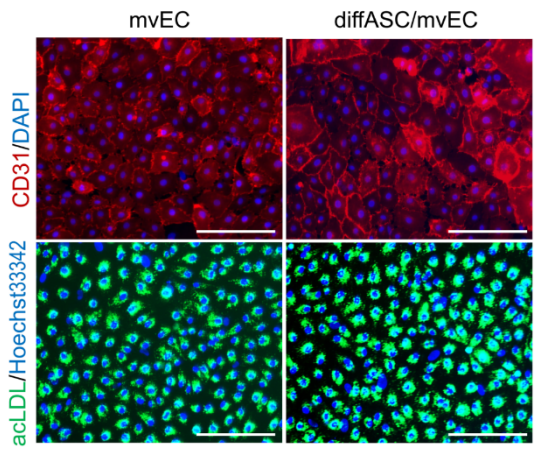


Figure 8: MvECs after seven days in mono- and co-culture with diffASCs in 3D collagen type I gels. CD31/DAPI immunofluorescence staining: CD31 shown in red and cell nuclei stained with DAPI in blue, acLDL assay: with accumulated acLDL in green and cell nuclei stained with Hoechst 33342 and displayed in blue, scale bar 200 µm, n=3.

day 7 and expressed perilipin A (**Figure 9**). Lipid vacuoles did not reach the diameters as found in native adipose tissue, however an increase of their volume was received between day 1 and 7 mainly in the co-culture attempt. The ratio of cells to matrix volume was lower in the *in vitro* models compared to native adipose tissue.

To be able to further compare engineered adipose tissue co-culture attempts to native adipose tissue, gene expression levels of different genes associated to adipogenic differentiation, FA metabolism, adipocyte functionality and angiogenesis were

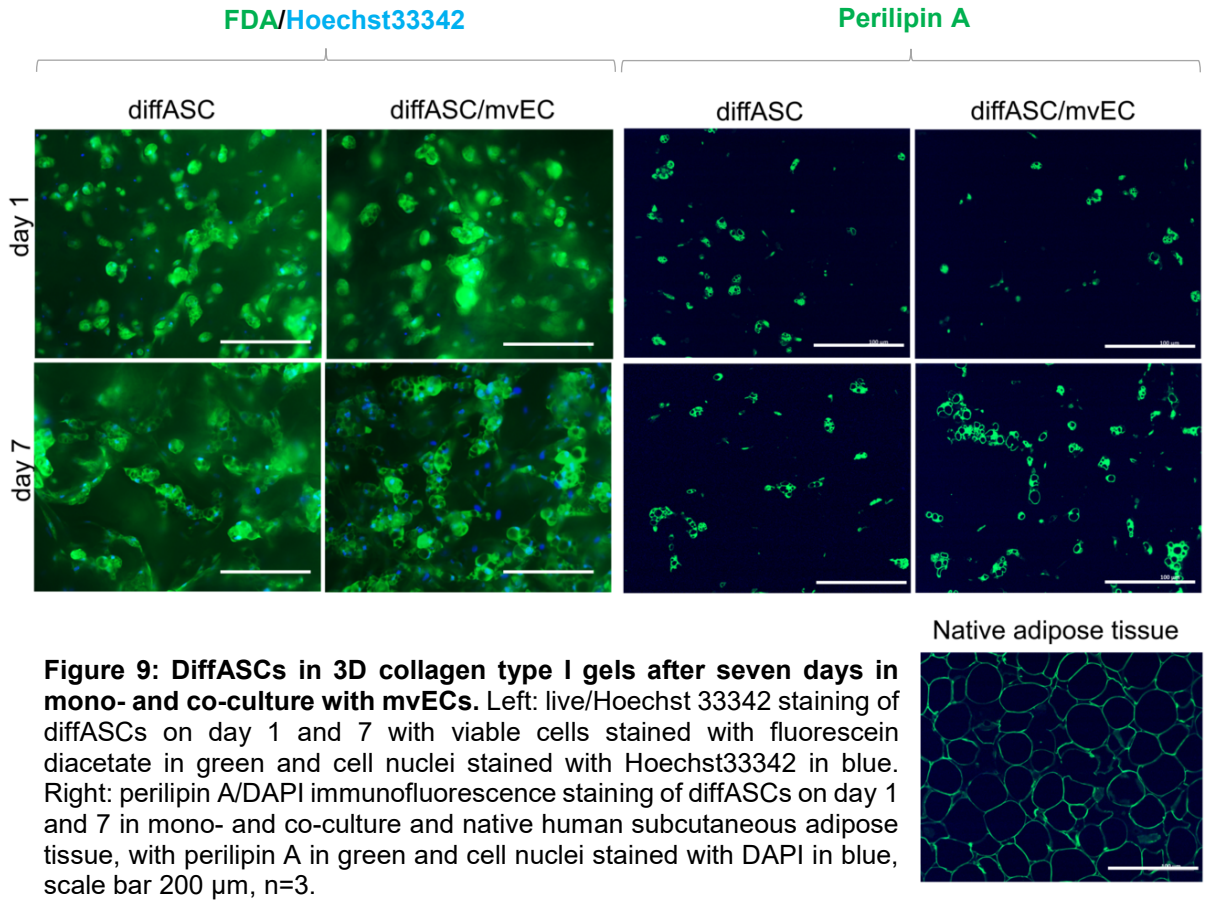


Figure 9: DiffASCs in 3D collagen type I gels after seven days in mono- and co-culture with mvECs. Left: live/Hoechst 33342 staining of diffASCs on day 1 and 7 with viable cells stained with fluorescein diacetate in green and cell nuclei stained with Hoechst33342 in blue. Right: perilipin A/DAPI immunofluorescence staining of diffASCs on day 1 and 7 in mono- and co-culture and native human subcutaneous adipose tissue, with perilipin A in green and cell nuclei stained with DAPI in blue, scale bar 200 µm, n=3.

analyzed via the available mRNA levels with a Taqman Low Density Array and compared to the levels found in native adipose tissue.

While levels of adipogenic differentiation and FA metabolism were found to be consistent with the physiological state *in vivo* in most cases, genes associated to adipocyte functionality and angiogenesis were found to deviate occasionally (**Figure 10**). As seen in the first part of **Figure 10**, genes involved in adipogenesis, like CEBP-α, PPAR-γ, CEBP-β and -δ or IRS-1 and angiotensinogen (AGT) [201, 202] are especially increased in the mono-culture attempts, while inhibiting genes, like TGF-β2 [203] were tendentially elevated in co-culture. Taken together, these results indicate an increased level of adipogenic differentiation in the absence of mvECs. As co-cultures however exhibit a noticeably increased level of leptin it is to be assumed, that adipogenic

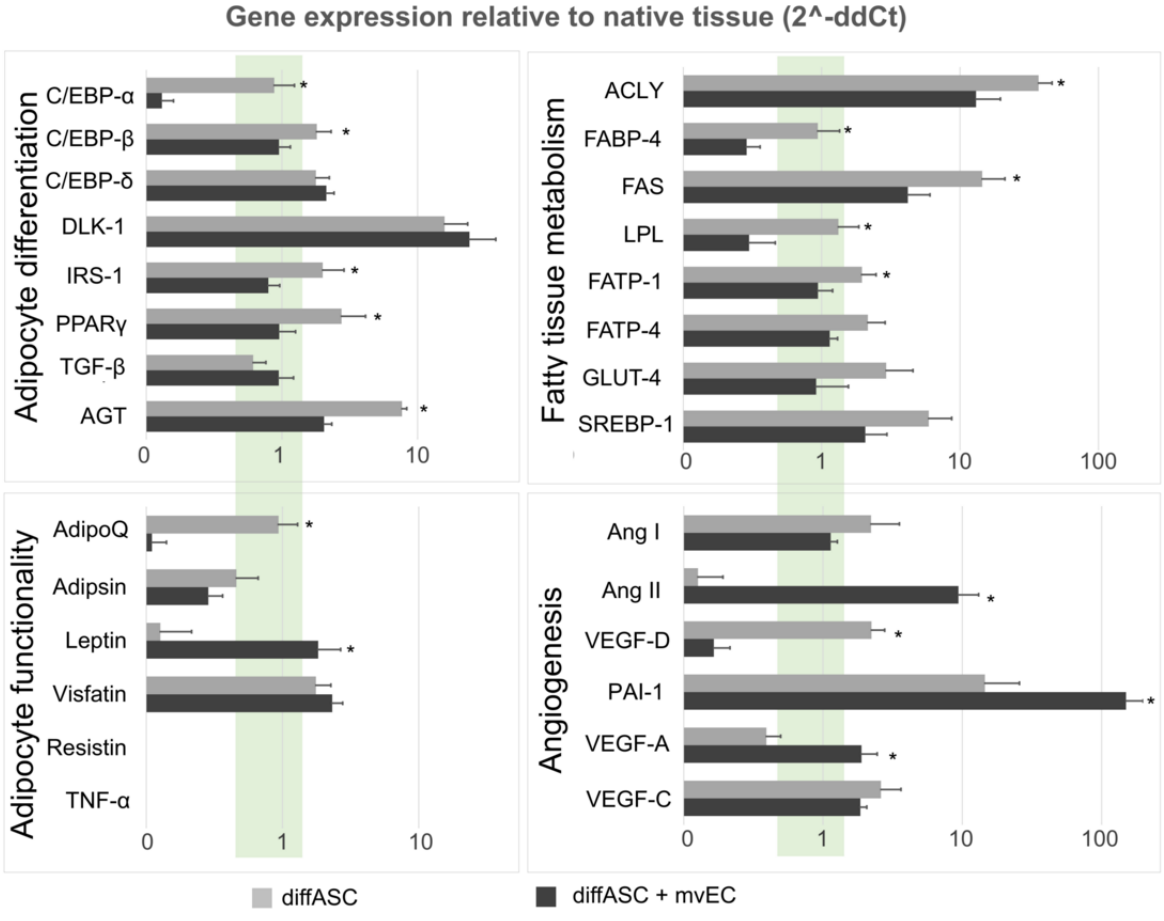


Figure 10: Gene expression analysis of diffASCs in 3D collagen type I gels after seven days in mono- and co-culture with mvECs. Gene expression was analyzed for genes associated to adipogenic differentiation, fatty acid metabolism, adipocyte functionality and angiogenesis based on the available mRNA and compared to native subcutaneous adipose tissue for diffASCs in mono-culture and in co-culture with mvECs after seven days in a 3D microenvironment. Expression of genes was referred to the level of human adipose tissue (=1). Green areas indicate an expression level in between 50 % and 150 % compared to human adipose tissue respectively, *p < 0,05, n=3.

differentiation was pushed in an earlier stage and the displayed snapshot on day 7 of co-culture already shows a state of beginning deregulation, probably initiated by mvECs. Until day 7, mvECs proliferated and formed a confluent monolayer, which shares characteristics of a vascular network in terms of cell-cell contacts. Presumably, mvECs released signals to promote the attraction of mural cells and thereby inhibited further adipogenic differentiation [167, 182]. AdipoQ is an indicator of low lipid stores and thereby considered as a promoter of glucose uptake and adipogenic differentiation [2, 33]. As it is expressed contrary to leptin in mono- and co-cultures, this hypothesis is supported. The upregulation of the negative regulator of adipogenesis DLK-1 is attributed to the immature state of diffASCs [204]. Its general upregulation indicates an immature state in all attempts independent of the presence of ECs.

When looking at genes responsible for FA metabolism, the stated effect of reduced adipogenic differentiation by mvECs is confirmed as all of the associated genes are expressed to a higher extent in the mono- compared to the co-culture trials. The reduced expression might again be induced by mvECs in a deregulation of adipogenic differentiation towards a homeostatic state of the setting. SREBP-1 is the master regulator of FA metabolism. ACLY and FAS participate in the generation of FAs [26, 27]. FABP-4, LPL and FATP-1 and -4 play an important role in the uptake of FAs from the surrounding [28-30]. GLUT-4 facilitates glucose uptake from the extracellular space [205]. It is remarkable that genes, responsible for the *de novo* synthesis of FAs like GLUT-4, are rather expressed compared to those addressing the uptake of FAs like LPL. Apparently, *de novo* lipogenesis is the preferred metabolic process in lipid accumulation taking place in diffASCs in mono- and co-culture in this event.

Next to adipoQ and leptin, adipsin, visfatin, resistin and TNF-α were classified as indicators of adipocyte functionality. Adipsin is responsible for the suppression of infectious agents in the innate immune system [206], TNF-α and resistin are equally linked to inflammatory processes [12, 207]. The low expression of these genes might be led back to the simplified and sterile environment generated *in vitro*. Additionally, the absence of other tissue cell types like macrophages, which play an essential role in processes within the generation of an immune response, may be responsible for this condition. The elevated levels of visfatin may be attributed to facilitated glucose uptake and the adipocyte-mediated initiation of vessel maturation [208]. Both processes are in accordance to the actual state of the *in vitro* setup, in which adipose tissue functionalities are still developing.

Within angiogenic factors, Ang II is known to facilitate the loosening of mural cell/EC contacts and the promotion of proliferation and migration of cells in the presence of VEGF-A, while Ang I in contrary supports vessel maturation via mural cell attachment [167, 209]. DiffASCs in co-culture presumably receive paracrine signals from mvECs and are thereby informed about their presence. Consequently, they might try to attract them to non-vascularized tissue areas through the release of factors with a reconstructive function, like Ang II or VEGF-A. Conclusively, VEGF-D, which is connected to EC proliferation, is increased in the mono-culture attempt, where paracrine signaling from mvECs is not available [210]. The intense upregulation of PAI-1, for which only a few functions are known apart from the support of angiogenesis [211], is striking. It might be linked to the high available concentration of insulin *in vitro* in all attempts [212], or the hypoxic state of diffASCs located in internal areas of the 3D constructs.

This attempt represents a first time comparison of an *in vitro* engineered 3D adipose tissue model based on diffASCs and mvECs with native human WAT. In short, the adipose tissue model exhibits high viability and shares many features and functionalities with native adipose tissue. Deviating expressed genes are mostly attributed to the immature state of the diffASCs after three weeks of *in vitro* culture. Other differences like in the expression of genes associated to adipose tissue functionalities are presumably owed to the absence of other cell types or the organoid network in contrast to the *in vivo* situation. Future attempts should address the adjustment of the underlying reasons of the elucidated divergences, like the addition of other cell types and the setup of direct co-cultures with refined cell ratios, to optimize the system to a more reliable model of human adipose tissue *in vivo*. Concluding, H5 is only partially applicable. DiffASCs represent native adipose tissue well *in vitro* in terms of differentiation and FA metabolism and partly within the interaction in angiogenic processes. Nonetheless, the hypothesis cannot be verified completely until further adjustments like the inclusion of additional cell types, dynamic culture conditions and an elongation of the culture period are implemented to further optimize adipocyte functionality and physiological cell-cell interaction.

9. Conclusion

The use of serum as cell culture supplement is associated to potential contaminations and the cover up of the cellular response *in vitro*. However, inadequate supplementation results in dedifferentiation, loss of cell integrity and apoptosis. In this thesis, the SF generation and maintenance of adipocytes was aimed. By the addition of cell-specific factors, a completely defined adipogenic differentiation medium was developed, which facilitated the differentiation of functional and characteristic adipocytes based on human primary ASCs. Additionally, another supplementation allowed for the sustainable maintenance of the generated adipocytes through a long-term culture period of up to 56 days in total.

To be able to engineer physiological and sustainable adipose tissue constructs of relevant size, a vascular component has to be integrated into the system. With regard to the addition of a natural vascular system to adipose tissue models, the functional co-culture of adipocytes and ECs is an important prerequisite. In this study, the composition of a suitable co-culture medium for human primary mvECs and diffASCs was sought. By the optimization of a medium based on adipocyte- and EC-specific factors, with particular regard to the controversially discussed factors EGF and HC, an improved co-culture medium was developed. The medium adequately supported the maturation and maintenance of diffASCs in co-culture without affecting mvEC behavior.

For the defined setup of vascularized adipose tissue constructs, the development of a defined co-culture medium for adipocytes and mvECs represented a subsequent objective of this thesis. Based on the gained experience from the defined adipogenic differentiation and the adjustment of the SC co-culture medium, a SF and defined co-culture medium was developed for adipocytes and mvECs. The medium supported functional adipocyte and mvECs maintenance. Further, the medium facilitated the formation of interconnected vascular-like structures in the direct co-culture setup of adipocytes and mvECs. The structures were maintained for up to 28 days.

It is known, that physiological response of primary adipocytes and ECs strongly relies on their structural environment. Another focus of this study was the examination of the influence of a novel CBM on the setup of vascularized adipose tissue under defined conditions. An accelerating effect of CBM on the lipid accumulation during defined adipogenic differentiation was detected. Moreover, CBM led to the formation and

stabilization of vascular-like structures by mvECs in the absence of another supportive cell type. CBM additionally facilitated the defined direct co-culture of mvECs and diffASCs for 14 days including the formation and maintenance of vascular structures comparably to TCPS.

To further adjust the *in vitro* environment to native conditions and receive substitutes of relevant size, TE attempts have to be transferred to a 3D setup. In a last step, the establishment of a 3D adipose tissue model in co-culture with mvECs was addressed. The model's features, functionalities and gene expression profiles were compared to native adipose tissue to allow a classification of its imitative power. A functional and characteristic 3D adipose tissue model was established. The engineered models altogether showed natural expression levels of adipocyte genes. A partly deviating expression of differentiation and functionality genes was mainly ascribed to the immature state of diffASCs and the isolated and artificial *in vitro* culture setup.

This thesis provides a tool set for the defined setup of vascularized adipose tissue constructs based on human primary ASCs and human primary mvECs. The results gained through the adjustments in the structural surrounding by matrix and cell orientation may be used to further optimize *in vitro* adipose tissue constructs. With the developed defined media the entry of corresponding products into the clinical application as implant and the *in vitro* application as predictive instrument can be accelerated considerably. Furthermore, the experiences gained on the development of defined media in this study may be transferred and adjusted to other tissues and applications to generally reduce the dependency on (animal-derived) sera in cell culture.

10. Outlook

In this thesis, the defined development of functional adipocytes including the accumulation of lipids, the expression of perilipin A and the release of leptin and glycerol was performed successfully. Nonetheless, the level of differentiation of SC attempts was only achieved to 80 % for perilipin A expressing cells and about 50 % concerning the lipid accumulation. Further investigations have to search for additional, possibly so far unidentified factors, which support general and cell specific functions. Following, the set up systems have to be analyzed in comparison to SC attempts with regard to their reliability, robustness and predictive power. Finally, their validation as *in vitro* models has to prove their suitability to mimic native adipose tissue.

The successful long-term culture of adipocytes and the co-culture with mvECs under defined conditions was shown within this thesis. To further increase the chance of adequate anastomosis of implants with the host tissue, this long-term culture period should be further elongated to at least three months. Therefore, a dynamic culture of the system with a circulating liquid component, transporting nutrients and waste products shows high potential.

Through the 3D setup of adipose tissue models, tissue substitutes close to native tissue were engineered. The 3D arrangement under defined conditions promises to combine the advantages of both attempts and might underline its superior features. In future attempts, the procedure could further be transferred to an additive manufacturing process to allow its production with high throughput techniques.

The beneficial influence of CBM on the defined adipogenic differentiation and vascularization under defined conditions were demonstrated in this trial. Thereby the suitability of CBM for the setup of vascularized adipose tissue was highlighted. The application of CBM in 3D setups would be highly desirable. Future attempts have to address the modification of CBM to facilitate its application in individual free-form attempts. Furthermore, CBM could be modified concerning its stiffness and binding sites, to resemble adipose tissue ECM more naturally.

Substantial *de novo* vascularization was achieved in this thesis in mono- and co-culture attempts under defined conditions for the first time. With regard to the setup of enlarged tissue constructs, *de novo* vascularization might not be sufficient to supply all peripheral tissue areas adequately before cell death occurs, caused by nutrient deficiency. Therefore, the combination of *de novo* vascularization with predefined vascular geometries for the larger vessels are conceivable. This combined approach

would also allow for an appropriate connection to the dynamic flow of a bioreactor. Furthermore, the miniaturization of this dynamic system to a fat-on-a-chip model would allow scaling up the number of investigated variables in *in vitro* approaches.

As a further adjustment to mimic native adipose tissue, additional cell types, like macrophages have to be integrated into the construct to be able to recreate tissue specific processes, like adipose tissue inflammation.

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12. Danksagung

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