Development of culture media for the construction of vascularized adipose tissue and vascularized 3D full-skin equivalents *in vitro*

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Abbreviations

3D	Three-dimensional
acLDL	Acetylated low density lipoprotein
AM	Adipocyte maintenance medium
ANG	Angiopoietin
ASC	Adipose-derived stem cell
ATP	adenosine triphosphate
BAEC	bovine aortic endothelial cell
BCE	bovine capillary endothelial cell
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CAL	Cell-assisted lipotransfer
cAMP	cyclic adenosine monophosphate
CD	Cluster of differentiation
CNP	C-type natriuretic peptide
CRP	C-reactive protein
DAPI	4',6-diamidino-2-phenylindole
DFAT	Dedifferentiated fat cell
DiI	1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DiO	3,3'-Dilinoleyloxacarbocyanine perchlorate
DMEM	Dulbecco´s Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
EET	Epoxyeicosatrienoic acid
EGF	Epidermal growth factor
EGM	Endothelial growth medium
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial NO Synthase
EtOH	Ethanol
FCS	Fetal calf serum
FDA	Fluorescein diacetate
FFA	Free fatty acid
FGF	Fibroblast growth factor
Gly-3-P	Glycerol-3-phosphate
GM	Methacrylated gelatin
GPDH	Glycerol-3-phosphate dehydrogenase
HC	Hydrocortisone
HDMEC	Human dermal microvascular endothelial cell
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HKGS	Human keratinocyte growth supplement
HO-1	Hem oxygenase -1
HSL	Hormone-sensitive lipase
HUVEC	Human umbilical vascular endothelial cell
ICAM	Intercellular adhesion molecule
IGF-1	Insulin-like growth factor-1
IL	Interleukin
KGM	Keratinocyte growth medium supplement
LAP	Lithium phenyl-2,4,6-trimethylbenzoylphosphinate
LPL	Lipoprotein lipase
MAAnh	Methacrylic anhydride

MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NO	Nitric oxide
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PEG	Polyethylene glycol
PG	Prostaglandin
PI	Propidium iodide
PI-3-K	Phosphoinositol-3-kinase
RGD	Arginine-glycine-aspartic acid
RT	Room temperature
SMC	Smooth muscle cell
TCPD	Tissue culture petri dish
TGF-beta	Transforming growth factor beta
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF-alpha	Tumor necrosis factor alpha
VCAM	Vascular cell adhesion molecule
VE-Cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand Factor
WST	Water soluble tetrazolium

Structure of the dissertation

The dissertation starts with an English and a German summary in chapter 1 *Summary/Zusammenfassung*. Chapter 2 *Introduction* gives a basic background related to the work and illustrates the state of the art as well as it introduces the aim of the study. The following Chapters 3 to 7 represent five peer-reviewed manuscripts, which constitute the methodical and experimental sections of this dissertation. Chapter 3 *Decelerating mature adipocyte dedifferentiation by media composition* was published in “Tissue Engineering Part C Methods” in 2015. Chapter 4 *Understanding the effects of mature adipocytes and endothelial cells on fatty acid metabolism and vascular tone in physiological fatty tissue for vascularized adipose tissue engineering* is a review article published in “Cell and Tissue Research” in 2015. Chapter 5 *Influence of epidermal growth factor (EGF) and hydrocortisone on the co-culture of mature adipocytes and endothelial cells for vascularized adipose tissue engineering* was accepted at “Cell Biology International” in 2016. Chapter 6 *Integration of mature adipocytes to build-up a functional three-layered full-skin equivalent* was published in “Tissue Engineering Part C” in 2016. Chapter 7 *Methacrylated gelatin and mature adipocytes are promising components for adipose tissue engineering* was published in the “Journal of Biomaterials Applications” in 2015. Chapter 8 *Results and Discussion* offers an interrelated discussion of the experimental results and findings. Chapter 9 *Conclusion* resumes the main findings of this dissertation, followed by an *Outlook* in Chapter 10. Chapter 11 includes all cited *References* and Chapter 12 contains the *Table of figures.*

Furthermore, a *CV* and a *list of publications and contributions* describing the candidates’ activities including own peer-reviewed publications and the presentation of the data at international conferences are presented in the Chapters 13 and 14.

1. Summary/Zusammenfassung

1.1. Summary

The development of media for the *in vitro* culture of mature adipocytes alone as well as in co-culture with endothelial cells or fibroblasts and keratinocytes are highly important for adipose and skin tissue engineering. Over the past decades, research focused on the use of stem cells to compose *in vitro* adipose tissues which need to be differentiated into the adipogenic lineage. Mature adipocytes have been rarely considered since the up to now established culture conditions lead to dedifferentiation of the cells. During the dedifferentiation, these cells release their lipid droplet, gain an elongated morphology and are able to proliferate again. Besides that, mature adipocytes show important advantages over stem cells. They can be isolated in high amounts from adipose tissue and are immediately ready to use and fully functional.

A major challenge in tissue engineering is the composition of a vascularized three-layered full-skin equivalent. So far, there are hardly any studies available on the construction of a full-skin equivalent, whereas the functionality of the adipose tissue layer is still insufficient. Only a few authors have reported on the perfusion culture of dermis/epidermis equivalents, whereas there is no data available on the dynamic supply of a skin equivalent including adipose tissue through a vascularization system. Therefore, the aim of this study was to find a medium for the successful *in vitro* culture of mature adipocytes. It was shown that adipocytes hold in an optimized medium supplemented with factors reducing lipolysis and favoring lipogenesis stayed viable and functional for up to 21 days, which was proven by the release of glycerol and leptin, as well as due to the staining of perilipin A.

Another culture medium for the co-culture of mature adipocytes with endothelial cells which is important for the fabrication of a vascularized adipose tissue equivalent is presented in this thesis. Here, an adipocyte medium was supplemented with factors important for endothelial cell viability and functionality, like vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and hydrocortisone among others. Cell morphology and functionality were evaluated by fluorescein diacetate staining and leptin release of mature adipocytes as well as von Willebrand Factor (vWF) staining and the uptake of acetylated lipoprotein (acLDL) of endothelial cells. It could be confirmed that an optimized medium containing a reduced amount of hydrocortisone allowed the successful co-culture of mature adipocytes and endothe-

lial cells. A seeding protocol for the endothelialization of tubular vascular structures made of a synthetic polyacrylate or methacrylated gelatin was established. The presence of a continuous cell monolayer was proven by a 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl-tetrazolium Bromide- (MTT) assay and acLDL uptake. For the dynamic culture of these endothelialized vascular structures as well as surrounding tissue, a fluid flow bioreactor was developed. It could be shown that endothelial cells cultured under fluid flow aligned along the medium flow. Moreover, fibroblasts introduced into a hydrogel could be successfully supplied through a gelatin tube, which was determined by a live-dead-staining.

Within this thesis, a culture medium for the co-culture of mature adipocytes, fibroblasts and keratinocytes was developed and a three-layered full-skin equivalent was composed which was functional on day 14. The optimized culture medium consisted of an adipocyte basal medium supplemented with ascorbic acid and calcium chloride. A stratified epidermis could be determined by hemalaun-eosin staining as well as staining of cytokeratin 10 and 14. The functionality of the mature adipocytes could be proven by measuring the production of the adipokines leptin, adipsin, visfatin and plasminogen activator inhibitor-1 (PAI-1). The epidermis of the full-skin equivalents was able to withstand a deep penetration of the cytotoxic surfactant Triton X. For the construction of vascularized full-skin equivalents, first results on the co-culture of endothelial cells with adipocytes, fibroblasts and keratinocytes are presented. It was demonstrated that a co-culture was possible and the epidermis was already stratified on day 7.

As a proof-of-concept to enable the future fabrication of full-skin equivalents using additive manufacturing methods, mature adipocytes were introduced into a printable methacrylated gelatin matrix. By adjusting the curing time, the stiffness of the hydrogels could be adapted to that of native fatty tissue. Further, large tissue equivalents could be produced by a layer-by-layer method and cells stayed viable for up to 24 hours in this construct which was proven by a live-dead-staining.

Taken together, culture media are now available for the *in vitro* culture of adipose tissue constructs consisting of mature adipocytes as well as vascularized adipose tissue composites which additionally include endothelial cells. Functional three-layered full-skin equivalents can be composed and cultured by using an optimized medium. Additionally, it could be shown that mature adipocytes can be introduced into a bioink.

1.2. Zusammenfassung

Die Entwicklung von Medien für die *in vitro* Kultur von reifen Adipozyten alleine sowie deren Co-Kultur mit Endothelzellen oder Fibroblasten und Keratinozyten ist essentiell für den Aufbau von Fettgewebe und Vollhaut im Labor. In den letzten Jahrzehnten fokussierte die Forschung auf die Verwendung von differenzierten Stammzellen, um Fettgewebe aufzubauen. Reife Adipozyten wurden bisher kaum verwendet, da die etablierten Kulturbedingungen zur Dedifferenzierung der Zellen führen. Während der Dedifferenzierung verlieren die Adipozyten ihre Lipidvakuolen, erhalten eine längliche Morphologie und können proliferieren. Reife Adipozyten haben jedoch wichtige Vorteile gegenüber den Stammzellen. Sie können in großen Mengen aus dem Fettgewebe isoliert und direkt verwendet werden, da sie voll funktional sind.

Eine große Herausforderung im Tissue Engineering ist der Aufbau eines vaskularisierten dreischichtigen Vollhaut-Äquivalents. Bis heute gibt es nur wenige Studien, die sich mit dem Aufbau von Vollhaut-Äquivalenten beschäftigen, wobei die Funktionalität der Fettgewebsschicht immer noch unzureichend ist. Einzelne Autoren haben von der erfolgreichen Perfusion von Dermis/Epidermis-Äquivalenten berichtet, wobei es noch keine Daten zur dynamischen Kultivierung von Haut-Äquivalenten mit Fettgewebsschicht gibt, die über ein vaskuläres System versorgt werden.

Das Ziel dieser Arbeit war es deshalb, ein Medium für die erfolgreiche *in vitro* Kultur für reife Adipozyten zu finden. Es konnte gezeigt werden, dass Adipozyten, welche in einem optimierten Medium mit Faktoren, die die Lipolyse hemmen und die Lipogenese fördern, kultiviert wurden, für bis zu 21 Tagen viabel und funktionell blieben. Das konnte durch die Freisetzung von Glycerol und Leptin, sowie aufgrund der Färbung von Perilipin A bestätigt werden.

In dieser Arbeit wurde ein weiteres Kulturmedium für die Co-Kultur von reifen Adipozyten mit Endothelzellen entwickelt, welches wichtig für den Aufbau von vaskulären Fettgewebs-Äquivalenten ist. Hierfür wurde ein Adipozytenmedium mit Faktoren angereichert, die wichtig für die Endothelzellviabilität und –funktionalität sind, wie unter anderem der vaskuläre endotheliale Wachstumsfaktor (*vascular endothelial growth factor*; VEGF), der fibroblastäre Wachstumsfaktor (*fibroblast growth factor*; FGF) und Hydrokortison. Die Zellmorphologie und –funktionalität wurden mittels Färbung mit Fluoreszeindiazetat und Leptinfreisetzung der reifen Adipozyten sowie der von Willebrand- (vWF) Färbung und der Aufnahme von acetyliertem Lipoprotein niederer Dichte (*acetylated low density lipoprotein*; acLDL)

von Endothelzellen bewertet. Es konnte bestätigt werden, dass ein optimiertes Medium mit einem reduzierten Gehalt an Hydrokortison die erfolgreiche Co-Kultur von reifen Adipozyten mit Endothelzellen erlaubt.

Es wurde ein Protokoll für die Besiedlung von tubulären Gefäßstrukturen mit Endothelzellen aus einem synthetischen Polyacrylat bzw. methacrylierter Gelatine erstellt. Die Anwesenheit eines durchgehenden Zell-Monolayers wurde mittels 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromid- (MTT) Assay und der acLDL-Aufnahme bestätigt. Für die dynamische Kultur dieser endothelialisierten Strukturen wie auch dem umgebenden Gewebe wurde ein Durchflussbioreaktor entwickelt. Es konnte gezeigt werden, dass sich unter Fluss kultivierte Endothelzellen entlang des Mediumflusses ausrichteten. Ferner konnten Fibroblasten, welche in einem Hydrogel eingebracht waren, erfolgreich durch ein Gelatineröhrchen versorgt werden, was mittels einer Lebend-Tot-Färbung nachgewiesen wurde.

In dieser Arbeit wurde ein Kulturmedium für die Co-Kultur von reifen Adipozyten, Fibroblasten und Keratinozyten entwickelt. Es war möglich, ein dreischichtiges Vollhautmodell aufzubauen, welches an Tag 14 funktional war. Das optimierte Kulturmedium bestand aus einem adipozytenspezifischen Basalmedium und war mit Ascorbinsäure und Calciumchlorid angereichert. Eine ausdifferenzierte und funktionale Epidermis konnte mittels Hemalaun-Eosin-Färbung sowie Färbung der Cytokeratine 10 und 14 nachgewiesen werden. Die Epidermis der Vollhaut-Äquivalente konnte ein tiefes Eindringen des zytotoxischen Tensids Triton X verhindern. Die in der Fettgewebsschicht befindlichen Adipozyten waren in der Lage, die Adipokine Leptin, Adipsin, Visfatin und den Plasminogenaktivator-Inhibitor (*plasminogen activator inhibitor-1*; PAI-1) freizusetzen. Zum Aufbau von vaskularisierten Vollhaut-Äquivalenten konnten in dieser Thesis erste Ergebnisse zur Co-Kultur von Endothelzellen mit Adipozyten, Fibroblasten und Keratinozyten dargestellt werden. Es wurde gezeigt, dass eine Co-Kultur möglich ist und die Epidermis schon nach sieben Tagen ausdifferenziert war.

Im Rahmen einer Machbarkeitsstudie um die zukünftige Herstellung der Vollhaut-Äquivalente durch additive Fertigungsverfahren zu ermöglichen, wurden reife Adipozyten in eine verdruckbare methacrylierte Gelatinematrix eingebracht. Durch die Anpassung der Vernetzungszeit konnte die Festigkeit der Hydrogele an die von nativem Fettgewebe angeglichen werden. Ferner konnten große Fettgewebs-Äquivalente durch einen schichtweisen Aufbau hergestellt werden und die Zellen

blieben für bis zu 24 h viabel, was mit einer Lebend-Tot-Färbung bestätigt werden konnte.

Es ist nun ein Kultivierungsmedium zum Aufbau von Fettgewebs-Äquivalenten mit reifen Adipozyten sowie von vaskularisierten Fettgewebs-Konstrukten vorhanden, welche zusätzlich Endothelzellen enthalten. Funktionelle dreischichtige Vollhaut-Äquivalente können aufgebaut und mit einem optimierten Medium kultiviert werden. Zusätzlich konnte gezeigt werden, dass sich reife Adipozyten in eine Biotinte einbringen lassen.

2. Introduction

2.1. Skin and subcutaneous adipose tissue

2.1.1. Skin

Native skin covers an area of around two square meters and is therefore the largest and heaviest organ of the human body. Besides the mechanical barrier to the outer environment it is involved in thermoregulation, water balance and skin pigmentation. Further, the skin is important as a first barrier to the environment, protects the body from bacteria and UV light, transmits sensory stimuli and is involved in wound healing as well as the synthesis of growth factors and vitamins (1). Human skin consists of three layers, the underlying subcutaneous fatty tissue, the dermis and the outer protective layer, the epidermis (Figure 1B). A hemalaun-eosin staining of native human skin tissue is shown in Figure 1C.

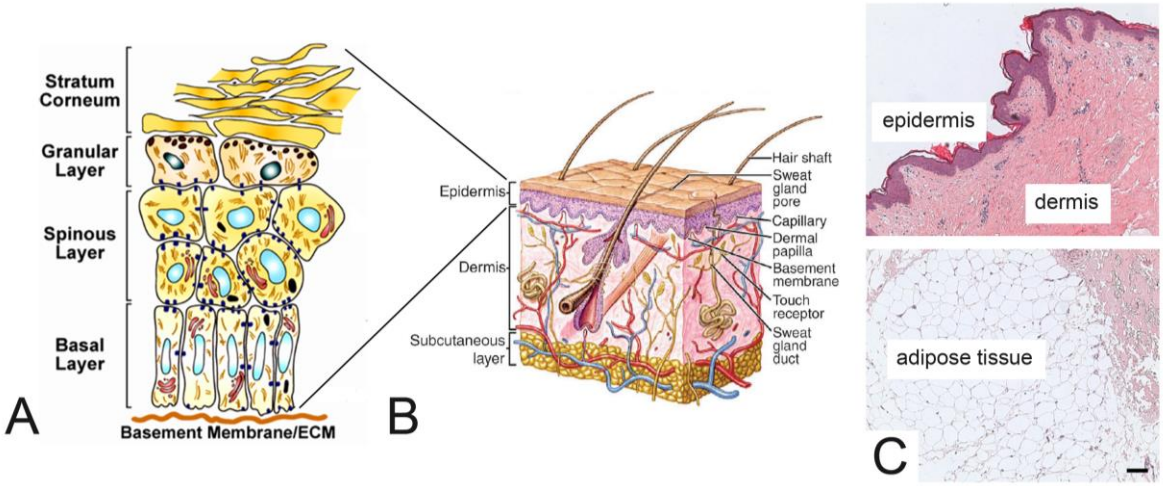

Figure 1: Structure of native human skin. A) A schematic of the epidermis. A monolayer of viable and proliferative keratinocytes is settled on the basement membrane. These cells have a columnar shape. During differentiation, these cells migrate to the spinous layer and then to the granular layer, where they reach a roundish and afterwards elongated morphology. In these stages, cells start to lose their organelles. The *stratum corneum* consists of dead and stratified keratinocytes, which compose the outer boundary to the environment (image slightly modified from (2)). B) Schematic structure of the full-skin containing epidermis, dermis and subcutaneous adipose tissue. Underneath the epidermis, a connective tissue, the dermis, can be found. Here, several receptors, like the touch receptor, or the sweat gland ducts are anchored. The dermis contains small blood capillaries and nerves, which supply the dermis as well as the epidermis. The subcutaneous layer underlies the dermis and consists mainly of mature adipocytes. Here, large blood vessels and nerves are embedded (image slightly modified from (3)). C) Hemalaun-Eosin staining of native human skin. Hemalaun stains the cell nuclei blue, and eosin interacts with eosinophilic structures like the cytoplasm for it to be shown in red. The overlying cornified epidermis defines the outer border to the environment. The connective tissue of the dermis is stained in red. The underlying adipose tissue has the typical lattice-like structure (image slightly modified from Chapter 6 (4)). Scale bar: 100 µm.

With a thickness of around 0.04 to 0.4 mm the **epidermis** is a multilayered squamous epithelium, which consists of around 90 % keratinocytes (5). These keratinocytes pass through several differentiation stages from basal to apical where they form corneocytes, stratify and then drop off. According to the differentiation status, the epidermis is arranged in four layers, which is shown in Figure 1A: The *stratum basale* is a monolayer of columnar viable keratinocytes settled on a basal membrane covering the dermis. These cells are proliferative and express cytokeratin 5 and 14 in a 1:1 ratio (6). Cytokeratins are an essential component of the cytoskeleton of keratinocytes and are responsible for the tensile strength of the epidermis. Besides keratinocytes, pigment forming melanocytes and the Merkel cells which function as mechanoreceptors can be found in the *stratum basale* (5). The overlying *stratum spinosum* consists of a four to eight layered cell sheet with polygonal post-mitotic but metabolically active keratinocytes and wide intercellular spaces. These cells express cytokeratin 10 but also involucrin, which is a precursor of the protein cover of maturating keratinocytes (6). In this layer also dendritic cells (Langerhans cells) of the immune system can be found (5). The *stratum granulosum* consists of a few cell layers of flattened keratinocytes. Here, the transformation of living keratinocytes into dead corneocytes without cell organelles takes place, called keratinization. Corneocytes are enriched with keratohyalin granules which consist mainly of the protein profillagrin. It is then modified to fillagrin which causes an irreversible aggregation and polymerization of keratin filaments responsible for the stratification of the cells (5,7). The outermost stratified layer is the *stratum corneum* which is the protection layer in touch with the environment and responsible for cell rejection. Corneocytes are dead and immobile cells without a nucleus or cell organelles. The cells are dehydrating and the plasma membrane is replaced by a cornified envelope. The latter is a 15 nm thick and dense network consisting of proteins like loricrin, profillagrin and involucrin. The intracellular space is closed by nonpolar lipids, ceramides, keratin and cholesterol (8).

The epidermis is separated from the **dermis** by a basal membrane. The thickness of the dermis depends on its location in the human body (5). Here, mainly fibroblasts are located. They are encapsulated into a self-made network of connective tissue, mainly consisting of collagen, elastin and proteoglycans, which are important for the stretchability and elasticity of the skin. Further, the blood and nerve supply is located in the dermis as well as cells of the immune system, like mast cells or macrophages

(5). Perspiratory glands and hair roots are located in the dermis. The dermis consists of two areas: the apical *stratum papillare* is connected to the epidermis. This layer is enriched with fibroblasts and blood capillaries which provide the epidermis with nutrients and oxygen. It consists mainly of collagen type I and III which are connected to the basal membrane by collagen VII. Underneath this layer, the *stratum reticulare* can be found. Here, mainly thick bundles of collagen type I and elastic fibres arranged parallel to the epidermis which are important for the tensile strength of this layer (9).

The **subcutaneous layer** consists of adipose tissue. This layer connects the dermis and epidermis to the inner structures like fascia and periosteum (9). A detailed description of the subcutaneous layer can be found in chapter 2.1.2.

2.1.2. Subcutaneous adipose tissue

Native human adipose tissue is a highly metabolic active and vascularized organ. It is involved in the storage of energy in form of triacylglycerides, heat isolation and mechanical support for inner organs. Further, adipose tissue is known as an endocrine organ and releases a number of factors involved in energy metabolism and homeostasis, inflammation, angiogenesis, fatty tissue growth and extracellular matrix remodeling (10).

Fatty tissue can be classified into brown and white tissue. Brown adipose tissue is mainly available in newborns where it is responsible for heat production. It is later replaced by white adipose tissue (11). In adults, white adipose tissue is either located subcutaneously underneath the skin or viscerally around inner organs (11,12). Subcutaneous adipose tissue consists of around 30 to 50 % mature adipocytes, which can reach diameters of 50 to 100 µm, depending on the energy status (13). Mature adipocytes are organized in lobuli, whereas each lobuli is surrounded by reticular connective tissue (9). It is thought that every adipocyte is connected to at least one capillary (14,15). Further, nerve tracts as well as cells from the immune system can be found in adipose tissue. Adipose-derived stem cells and preadipocytes are important for tissue regeneration. Fibroblasts are involved in extracellular matrix production. The structure of subcutaneous adipose tissue is shown in Figure 2.

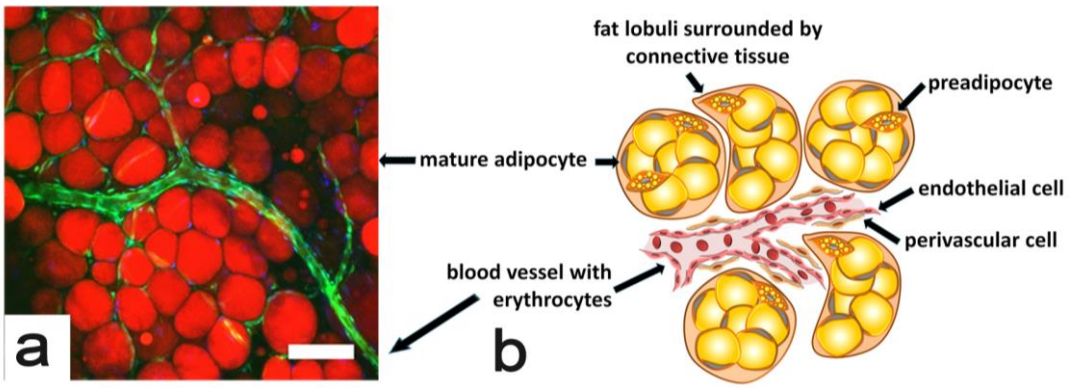

Figure 2: Structure of native adipose tissue. A) Human adipose tissue consists of mature adipocytes incorporating one huge lipid droplet (Nile Red; red). A network of blood vessels is seen in adipose tissue. Vital cells are shown in green (fluorescein diacaetate) and nuclei are stained with Hoechst 33342 in blue. Scale bar: 100 µm. B) Schematic overview of native adipose tissue. Mature adipocytes are organized in lobuli, which are surrounded by connective tissue. Adipose tissue is a highly vascularized organ. It is thought that every adipocyte is connected to one capillary (taken from Chapter 4 (16)).

2.2. Adipose and full-skin tissue engineering

There is a high demand of tissue engineered adipose and full-skin constructs as test systems and for the treatment of patients suffering from various diseases or skin deformities. Up to date, patients suffering from burns or shallow tissue defects only affecting the dermal and epidermal layer are treated by transplantation of body´s own skin tissue. This method is limited to the availability of sufficient donor sites, donor site morbidity and scar formation (17). Additionally, several wound covers as dermis and epidermis substitutes are already on the market. They are used with cells (e. g. Apligraf®, Dermagraft®, EpidermFT™) or without cells (e.g. Biobrane®, Integra®) (18). These methods are both neglecting the subcutaneous adipose tissue which is important for skin mobility and the treatment of contour defects. Small soft tissue defects can be cured by the transplantation of autologous tissue according to the Coleman´s technique or the cell-assisted lipotransfer (CAL), which have already shown promising results in clinic (19–22). Briefly, with the Coleman´s technique, a liposuction is followed by centrifugation of small intact tissue fragments to remove them from non-viable components. Adipocyte lobuli are then injected in minuscule portions to allow maximal surface contact to the host tissue to ensure rapid neo-vascularization with host endothelial cells and therefore reduced adipocyte apoptosis (19,21). During CAL, the adipose tissue fragments are additionally supplemented

with cells of the stromal vascular fraction. This mixture is then injected. The stromal vascular fraction is thought to enhance revascularization as well as it provides precursors for adipocytes (22). However, these methods lack sufficient nutrient supply when used to cure large tissue defects. Hence, for the treatment of high-graded and deep burn wounds, tumor removal, diabetic foot, congenital malformations or skin deformities after trauma, which are also affecting the subcutaneous adipose tissue, a satisfying curing method is highly needed. First efforts have been made in the composition of three-layered tissue constructs *in vitro* whereby there are not yet satisfactory results concerning long-term stability and functionality of the constructs (23–26). In the following sections the mainly used cell types for the construction of adipose tissue and full-skin are discussed.

2.3. Cell sources for adipose tissue engineering

2.3.1. Mature adipocytes

Mature adipocytes are terminally differentiated cells and the main actors in adipose tissue. They arise from adipose-derived stem cells which differentiate into preadipocytes storing their lipids in several droplets and then terminate into non-proliferative mature adipocytes (27). The cytoplasm of these cells is filled with one huge lipid vacuole, pressing the nucleus to the outer cell membrane and giving them an univacuolar phenotype (28). The lipid vacuole consists of triacylglycerides and cholesterol esters, which are wrapped by a phospholipid-monolayer and associated proteins, like perilipin A (29). Each mature adipocyte is surrounded by an outer basal lamina (30,31). It consists of laminin, collagen type IV and heparansulfate-proteoglycan and is produced by the adipocytes itself (32).

An often cited drawback of mature adipocytes is their dedifferentiation due to insufficient knowledge about optimal *in vitro* culture conditions. After around one week, cells are re-organizing their lipids into several lipid vacuoles (33–35). After two weeks, a cell population of multivacuolar adipocytes as well as cells with an elongated fibroblast-like shape could be seen. These cells have reduced their cell volume and were called “dedifferentiated fat cells” (DFAT) (36). They are then able to proliferate again, show multilineage potential and express stem cell specific markers (37,38). Mature adipocytes were rarely used in the past, probably because of dedifferentiation during *in vitro* culture. Since the 1990´s no studies have been performed with mature adipocytes concerning adipose tissue engineering

(33,36,39,40). In literature, several reasons were discussed to promote dedifferentiation. It was thought that hypoxia might be responsible for dedifferentiation (35). Other authors suggest that a high cell density similar to that in native adipose tissue is indispensable for a successful *in vitro* culture. It was seen that cells cultured with close cell-cell-contacts showed reduced dedifferentiation compared to cells which were not able to interconnect with other adipocytes (39). We suggested that an optimized culture medium, which contains factors important for the activation of lipogenesis and reduction of lipolysis can decelerate adipocyte dedifferentiation (Chapter 3 (41)).

2.3.2. Stem cells

Stem cells are important for tissue regeneration and can be isolated in high amounts from adipose tissue (adipose-derived stem cells; ASCs) or bone marrow (mesenchymal stem cells; MSCs). These cells are multipotent and can differentiate into several lineages, like the adipogenic, osteogenic or chondrogenic lineage. Defining the expression profile of ASCs and MSCs is still a challenge. Dominici *et al.* have set minimal criteria to specify MSCs (42). It is thought that they should be able to adhere to cell culture plastic, be positive for CD105, CD73 and CD90 as well as negative for markers like CD45 and CD34 among others. Further, they need to show multipotent differentiation potential (42). The definition of ASCs is still not clear, but literature agrees that they express stem cell markers similar to MSCs (Volz *et al.,* 2016 (43)).

Up to date, several approaches have been followed with stem cells in adipose tissue engineering (44–46). Here, they were differentiated into the adipogenic lineage and cultured on several substrates, like fibrin, collagen, polyethylene(glycol) or decellularized matrices (47,48). Yet, no promising study is available to proof the long-term stability of differentiated stem cells *in vitro* (49).

2.3.3. Fibroblasts and keratinocytes

Fibroblasts are the fundamental cells of the dermis and all connective tissues. They have a spindle-shaped morphology and are responsible for the production of all types of fibres and the ground substance to compose extracellular matrix (5). **Keratinocytes** are the main cell type of the epidermis. Keratinocyte stem cells

govern the renewal of mammalian keratinocytes and stratified epithelia. It is thought that several types of stem cells are located in different niches within the epidermis but the exact distribution of the stem cells is not yet clear (50,51). The morphology and differentiation status of keratinocytes is dependent on the epidermal layer they are observed in. In the basal layer, they have a cubical shape and are anchored to the basement membrane by hemidesmosomes. In higher layers they receive an elongated morphology and stratify (5). Keratinocyte differentiation *in vitro* was studied extensively and air-lift culture was introduced several decades ago to initiate keratinocyte differentiation (52). Moreover, co-culture models of fibroblasts and keratinocytes to compose a dermis/epidermis equivalent are already partly standardized and used as wound covers or *in vitro* test systems (53–57).

2.4. Methods for the fabrication of 3D adipose and skin tissue

The fabrication of accurately fitting three-dimensional (3D) implants to replace tissues or organs is still a technological barrier to overcome. Computer-based additive manufacturing techniques are gaining more and more interest in medical engineering and medicine since they might be able to directly transform digital data into physical objects through a layer-by-layer material deposition or material solidification (58). The most commonly used methods are based on inkjet printing, bioplotting and laser-assisted bioprinting (59). Bioplotting is the extrusion of continuous filaments and provides a resolution of several hundred micrometers and can therefore be used for the fabrication of large constructs in the mm- to cm-range. Cell viability is reported to be around 40 to 80 %. Inkjet printing deposits ink drops in a layer-by-layer manner. It is the most commonly used technique for tissue engineering. This method is fast, cheap and has a resolution of around 50 to 200 µm. Cell survival is thought to be higher than 85 %. With the laser-assisted bioprinting, constructs with a high resolution can be built. Here, photosensitive materials are deposited and patterned while curing with a high-resolution laser. Cell survival is measured higher than 95 % (58,59).

The materials used in bioprinting technologies are obliged to have specific material properties. It needs to be printable and therefore should have an appropriate viscosity and gelation behavior. For medical applications, the material has to be biocompatible and biodegradable to allow remodeling and replacement of the tissue with the cells own extracellular matrix. Further, the material should be able to mimic

the structural, mechanical and functional properties of the replacing tissue (58). Here, the latest literature focuses on UV-crosslinkable or heat-induced gelling materials, e.g. methacrylated gelatin or decellularized matrices (45,60).

2.5. Aim of the study

The purpose of this dissertation was to provide culture media for the successful *in vitro* culture of mature adipocytes as well as co-culture with endothelial cells or fibroblasts and keratinocytes. Further, it should be shown that mature adipocytes can be used with novel 3D bioprinting technologies. Within this thesis, the following hypotheses should be evaluated:

1.) Mature adipocytes are known to lose their stored lipids and functionality while they dedifferentiate under *in vitro* conditions. It was hypothesized that adipocytes can be cultured *in vitro* when using an optimized culture medium consisting of factors which allow lipogenesis and inhibit lipolysis.
2.) Current studies mainly focus on the use of stem cells for the composition of vascularized adipose tissue constructs, which is very time-, material- and cost-intensive. Alternatively, it was hypothesized that mature adipocytes can be successfully co-cultured with endothelial cells. Therefore, factors enhancing endothelial cell survival, proliferation and functionality need to be added to the optimized culture medium for adipocytes.
3.) It was hypothesized that mature adipocytes can also be used for full-skin engineering and therefore be co-cultured with fibroblasts and keratinocytes. It was proposed that an adipocyte culture medium needs to be composed of factors, which inhibit adipocyte dedifferentiation and favor stratification of the epidermis.
4.) In a last step, it was hypothesized that mature adipocytes can be used with bioprinting technologies and can be introduced into a printable matrix consisting of methacrylated gelatin.

Taken together, this dissertation should offer a basic approach to construct a nature-mimicking bio-printed vascularized adipose tissue as well as a vascularized full-skin equivalent based on the use of mature adipocytes in the fatty tissue layer.

3. Decelerating mature adipocyte dedifferentiation by media composition

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4. Understanding the effects of mature adipocytes and endothelial cells on fatty acid metabolism and vascular tone in physiological fatty tissue for vascularized adipose tissue engineering

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5. Influence of epidermal growth factor (EGF) and hydrocortisone on the co-culture of mature adipocytes and endothelial cells for vascularized adipose tissue engineering

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6. Integration of mature adipocytes to build-up a functional three-layered full-skin equivalent

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7. Methacrylated gelatin and mature adipocytes are promising components for adipose tissue engineering

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8. Results and Discussion

8.1. Mature adipocytes are very promising for adipose tissue engineering

In the present study, mature adipocytes should be used for several applications like the construction of vascularized adipose tissue or three-layered full-skin equivalents. For the composition of adipose tissue, up to date, mainly stem cells were isolated from the bone marrow or adipose tissue and expanded *in vitro*. Then, they were differentiated into the adipogenic lineage and encapsulated into several scaffolds (44–46). This process is quite material-, time- and cost-intensive. Additionally, it is known that only around 42 % of the stem cells differentiate, probably due to the fact that these cells are an inhomogeneous population (63). Here, mature adipocytes are a very promising cell type, since they can be isolated in high amounts and are immediately ready to use. These cells are still rarely used, mainly because of their often cited drawbacks like vulnerability, non-proliferation and dedifferentiation. A method was established in our lab which allows a gentle isolation of mature adipocytes from subcutaneous adipose tissue reaching a high cell yield. In this study, the improvement of the culture conditions of mature adipocytes to prevent them from dedifferentiation was attained (Chapter 3 (41)). It was shown before by several authors that mature adipocytes start to organize their single lipid droplet into several small lipid vacuoles during dedifferentiation. After that, they release their lipids completely and receive an elongated, fibroblast-like shape (33,38,40,64,65). Matsumoto *et al.* and Ono *et al.* have seen that these cells are then able to proliferate again and have multilineage potential (37,38). Up to date, mature adipocytes were mainly cultured in DMEM/Ham´s F-12 and 10 % FCS, whereby it is known that a high amount of FCS favors dedifferentiation (39,66). The aim of this study was to find a culture medium which decelerates the dedifferentiation of mature adipocytes *in vitro* (Chapter 3 (41)). Therefore, two commercially available media were evaluated: Adipocyte Maintainance Medium-1 (AM-1) from ZenBio and Adipocyte Nutrition Medium from PromoCell, which are thought to be used for the culture of differentiated stem cells. These media contain factors which favor lipogenesis and inhibit lipolysis, like insulin, dexamethasone, biotin and pantothenate (67–71). It was proven that both media reduced the dedifferentiation of mature adipocytes and the majority of these cells kept their lipid droplets and functional status. In the control medium (DMEM/Ham´s F-12 and 10 % FCS) cells received an elongated morphology and

proliferated extensively. As a sign of reduced lipolysis, cells held in the two test media released less glycerol compared to the control. At the same time, they released higher levels of leptin, a hormone only produced by functional mature adipocytes to control energy homeostasis in the body. Further, a high number of perilipin A-positive adipocytes could be found in the test media, whereas the number of CD73-positive dedifferentiated fat cells was higher in the control medium (Chapter 3 (41)). Perilipin A can be found in the membrane of lipid vacuoles in mature adipocytes and controls lipolysis and lipogenesis, whereas CD73 is expressed from cells with multilineage character, like dedifferentiated fat cells (29,37). To determine which of these test media is superior, the total cell survival was evaluated. Here, this study proved that cells cultured in the Adipocyte Nutrition Medium from PromoCell showed a drastic initial cell death on day 1, whereas cells cultured in AM-1 from ZenBio stayed viable for up to 21 days. Therefore, it can be concluded that AM-1 medium provided by ZenBio can be successfully used for the culture of viable and functional mature adipocytes *in vitro* for up to 21 days in a collagen type I hydrogel (Chapter 3 (41)).

8.2. Construction of a vascularized adipose tissue

Large adipose tissue constructs are highly needed to treat skin tissue defects occurring after high-graded burns, tumor removal or abnormal deformities. To supply these constructs, a functional vascularization system is indispensable. Up to date, several methods, like the stimulation of angiogenesis or endothelialization of artificial capillaries or decellularized vessels, are already available to generate and culture blood vessel systems (72). During angiogenesis, endothelial cells and endothelial-cell-associated cells, like pericytes, are stimulated to proliferate and migrate to form capillary-like structures in a tissue (73). Several authors have already cultured this self-assembled vascularization system under fluid flow in micro-chips (74–76). Yet, no studies have been performed where a vessel system built through angiogenesis processes was used to supply surrounding tissue cells. This method is limited since the neo-vascularization of large tissue constructs might be too slow. Additionally, no useful method is available to connect this self-assembled blood vessel system to a bioreactor for dynamic perfusion. Miller *et al.* used the lost template strategy (77). Briefly, they printed a vascular structure with carbohydrate glass. This glass lattice was then encapsulated into a hydrogel with living cells. After hydrogel polymerization,

the glass structure was washed out; the remaining channels were seeded with endothelial cells and cultured under flow. In another attempt, Groeber *et al.* have used decellularized vessels from porcine jejunum, seeded them with endothelial cells and then connected them to a fluid flow bioreactor to supply surrounding tissue (78). Sekine *et al.* followed a similar approach, where they supplied cell sheets through a vascular bed taken from a rat femoral muscle (79). For implantation, these structures may raise ethical concerns since animals are involved and the tissues cannot be standardized. Further, a method needs to be established to remove xenogeneic materials sufficiently to prevent immune responses from the human body. Several attempts have been rising to produce artificial vascular structures *in vitro*. Here, methods like bioprinting and electrospinning have been used (80,81). In this study, a UV-cured synthetic polymer material (made of (co-BPA-co-IBA-co-IL)-polyacrylate and kindly provided by Fraunhofer IAP and ILT) and a dip-coated methacrylated gelatin tube (kindly provided by Fraunhofer IGB; department GTM) were evaluated for endothelial cell attachment and use in a fluid flow bioreactor to supply a surrounding tissue with nutrients and oxygen (Huber *et al.*, 2016 (82)). Macroscopic images of both tubes can be found in Figure 3. The inner diameter of the tubes was 1.2 mm.

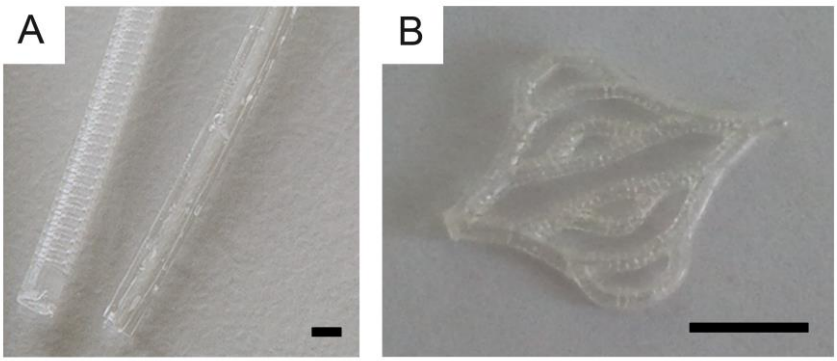

Figure 3: Macroscopic images of manufactured capillary-like structures. A) The (co-BPA-co-IBA-co-IL)-polyacrylate tube with pore size of 200 µm (left) was kindly provided by Fraunhofer IAP and ILT. The tube made of methacrylated gelatin (right) was received from Fraunhofer IGB, department GTM. Tubes had an inner diameter of approx. 1.2 mm. Scale bar: 1 mm. B) Branched tube made of the polyacrylate (kindly provided by Fraunhofer ILT). Scale bar: 5 mm

The basic requirements for biomaterials are their non-cytotoxicity, biocompatibility and their sterilizability. Gelatin tubes could be successfully sterilized in 70 % EtOH

and then washed in PBS. For the cured polyacrylate a washing protocol has been developed in order to remove unreacted monomers from the crosslinked polymers to achieve cytocompatibility (Huber *et al.*, 2016 (82)). Ethanol (70 % w/v) was used as a rinsing solvent in order to simultaneously achieve disinfection of the polyacrylate material for subsequent application in *in vitro* cell-culture. The cytocompatibility of the rinsed polymer was evaluated according to DIN ISO 10933-5 by culture of human fibroblasts within material extracts for 24 h. Figure 4 shows the increasing cytocompatibility of the polyacrylate extracts with prolonged duration of rinsing in ethanol. Washing the polyacrylate for four to five days significantly increased the relative cell viability compared to tissue culture petri dishes (TCPD) from 31 % ± 6.7 % on day one to 97 % ± 7.2 % on day five. As the cytocompatibility was nearly 100 % on day five, this washing protocol was used for all upcoming experiments.

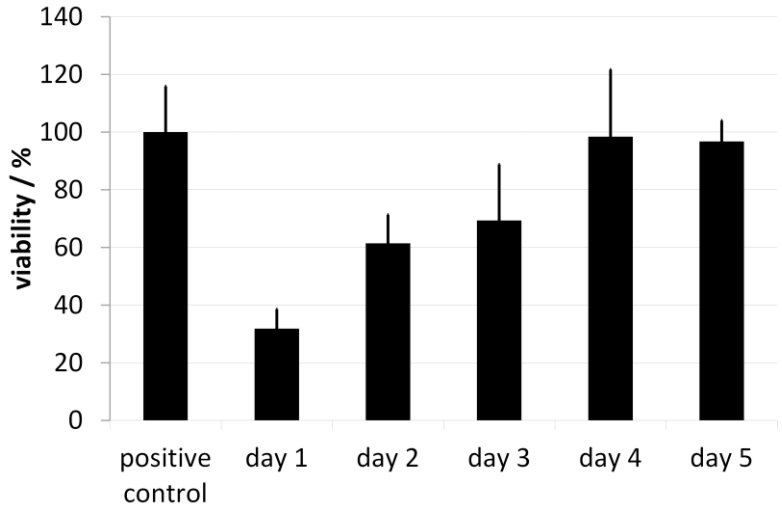

Figure 4: Viability of human fibroblasts cultured with polyacrylate material extracts for 24 h. Polyacrylate material was rinsed in 70 % ethanol for up to five days and material extracts were tested according to DIN ISO 10933-5. After four days of washing, cell viability was nearly 100 %. Toxic material was washed out completely after this time. Positive control = tissue culture petri dish (Huber *et al.*, 2016 (82)).

To enhance cyto- and hemocompatibility of synthetic materials, biofunctionalization and endothelial cell adhesion are considered to be promising techniques. For the reduction of thrombogenicity, the immobilization of the anticoagulant heparin is a widely used approach to prevent platelet adhesion and reduce thrombus formation at the blood-material interface (83–85). Pan *et al.* have shown that heparin immobilized to titanium surfaces showed a reduced platelet attachment and activation. Further,

they have seen an improved endothelial cell adhesion and spreading compared to pristine titanium (86). Devine *et al.* performed an *in vivo* study resulting in better patency rates of heparin-bounded Dacron prostheses after three years compared to non-modified heparin, but no longer significant after five years (84). Furthermore, anti-viral, antibacterial and tumor-inhibiting properties of heparin have been reported (87–89).

Endothelial cell adhesion was already improved by coating the luminal wall with extracellular matrix proteins such as collagen, fibronectin or laminin or short peptide sequences (RGD, REDV, YIGSR) from these proteins, whereas the RGD sequence is the most frequently used (90–92). To enhance endothelial cell adhesion as well as hemocompatibility, the polyacrylates were covalently functionalized using a modified form of heparin and RGD. Cell-material interactions of endothelial cells with non-functionalized and functionalized polyacrylate were investigated by the evaluation of endothelial cell adhesion and proliferation after 24 h on planar material samples (Figure 5). On polyacrylates which had been functionalized with both, thio-modified heparin and RGD, a confluent endothelial cell monolayer had formed comparable to that on tissue culture petri dishes reference surfaces (Huber *et al.*, 2016 (82)). On non-functionalized polyacrylates and on those surfaces that had been treated with the RGD peptides or the thio-modified heparin separately, non-confluent cell colonies were found. These data show that a biofunctionalization with thio-modified heparin/RGD promoted endothelial cell adhesion on a polyacrylate surface.

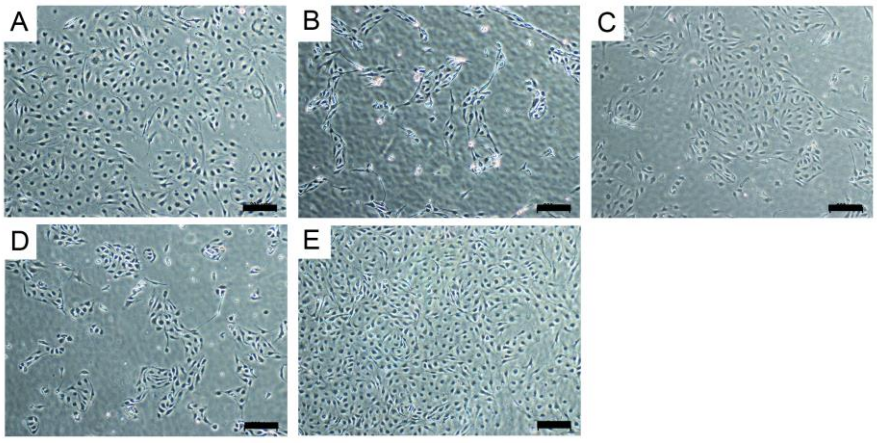

Figure 5: Human dermal endothelial cells cultured for 24 h on biofunctionalized polyacrylate materials. A) Tissue culture petri dish as a positive control. B) Untreated polyacrylate. C) Polyacrylate functionalized with thio-modified heparin, D) with RGD, E) with thio-modified heparin/RGD. Polyacrylate material was functionalized and endothelial cells were cultured for 24 hours. Only few cells attached on the non-functionalized polyacrylate as well as on the polyacrylate treated

with thio-modified heparin or RGD. The functionalization with thio-modified heparin/RGD resulted in a confluent cell monolayer, what comparable to the standard tissue culture control surface. Scale bar: 200 µm (Huber *et al.*, 2016 (82)).

In a next step, a seeding procedure for tubular structures was established. Here, thio-modified heparin/RGD-modified polyacrylate tubes were filled with a suspension of endothelial cells and cultured using either a static regime or rotating the tube at 0.5 rpm at 37 °C. A non-confluent layer of endothelial cells was detected after statically seeding the tubes, wherefore a rotating seeding protocol was established (Figure 6). Functionalized polyacrylate tubes seeded rotating for four hours showed a homogenous degree of endothelialization on the whole inner capillary wall (Huber *et al.*, 2016 (82)). Opposing to that, non-functionalized tubes showed a low cell attachment after four hours of rotating seeding. For the seeding of a biofunctionalized porous material the protocol was further improved, since the cell suspension was leaking out of the pores. Here, tubes were seeded under rotation for one hour and then cultured statically for another four days to allow cell proliferation. As shown in Figure 6D, a confluent monolayer could be established.

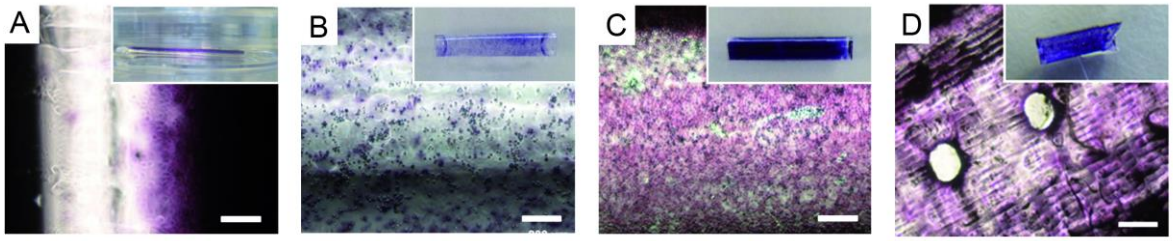

Figure 6: Endothelialization of tubular polyacrylate substrates. The effectiveness of cell seeding to the inner wall of polyacrylate tubes was compared applying the cell suspension in a static way or by rotating the tube at 0.5 rpm for 4 h at 37 °C. Cell density and viability was detected by MTT. A-C) Non-porous tubes. D) Porous tube with pore size of approx. 200 µm. A) Static seeding led to non-confluent monolayers on one side of the tube. B) Rotating seeding of non-functionalized tubes resulted in low cell attachment all over the tube. C) Rotating cell seeding of tubes functionalized with thio-modified heparin/RGDC resulted in confluent monolayers on the inner surface. D) For the seeding of porous materials, an improved protocol was used. Cells were allowed to attach while the tube rotated for 1 hour at 0.5 rpm and 37 °C. Subsequent static culture for 4 days allowed cell spreading and proliferation to create a confluent monolayer. Scale bar: 200 µm (Huber *et al.*, 2016 (82)).

It is well known that endothelial cells adhere to and proliferate on gelatin coated surfaces (93). Therefore, for the endothelialization of gelatin tubes, no biofunctionalization was needed. In this study it was shown that gelatin tubes can be successfully seeded with endothelial cells when using the protocol established for porous polyacrylate tubes (Figure 7).

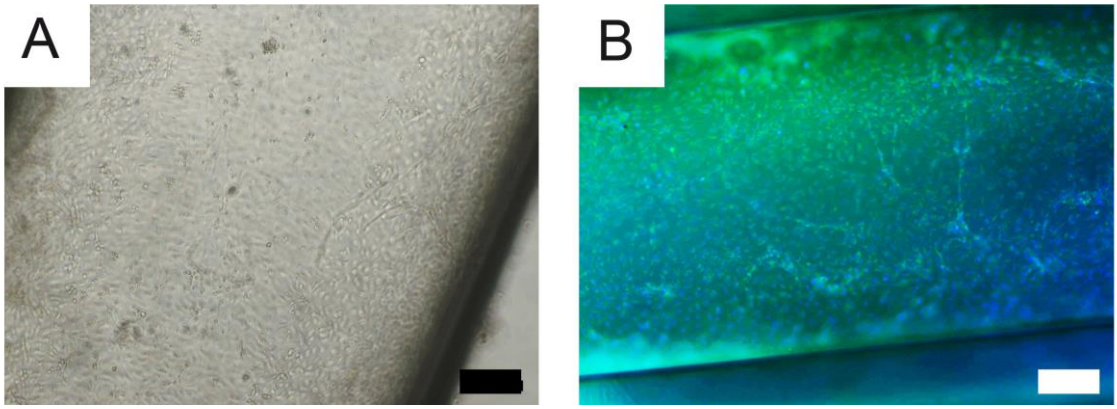

Figure 7: Gelatin tubes can be successfully seeded with functional endothelial cells. Endothelialization took place under rotation for one hour at 37 °C with 0.5 rpm. Tubes were then cultured statically for four days to allow cell spreading and proliferation. A) Lightmicroscopic image. A confluent monolayer of endothelial cells could be found. B) Endothelial cells stayed functional and incorporated acLDL (green). Nuclei are shown in blue (Hoechst 33342). Scale bar: 200 µm

For the dynamic culture of endothelial cells in tubular constructs and supply of vascularized tissue constructs, a fluid flow bioreactor is needed. In cooperation with Unitechnologies SA (Switzerland) a closed-loop perfusion system was established to incorporate an artificial capillary for delivery of nutrients and oxygen. The construction was based on previous studies of other authors (79,94,95). In Figure 8 macroscopic images of the bioreactor chambers as well as the bioreactor system connected to the tubing, pump and medium reservoir flask are shown. The bioreactor itself consisted of an outer and an inner chamber. The outer chamber was used to connect the capillary system to removable and commercially available one-way cannulas via silicon adhesive. This system allowed the use of artificial capillaries with a wide range of diameters in the same bioreactor. The cannulas were then connected to the bioreactor by micro-connectors which enable a tight fixation. In the inner chamber, the tissue was cultured and supplied by the artificial capillary.

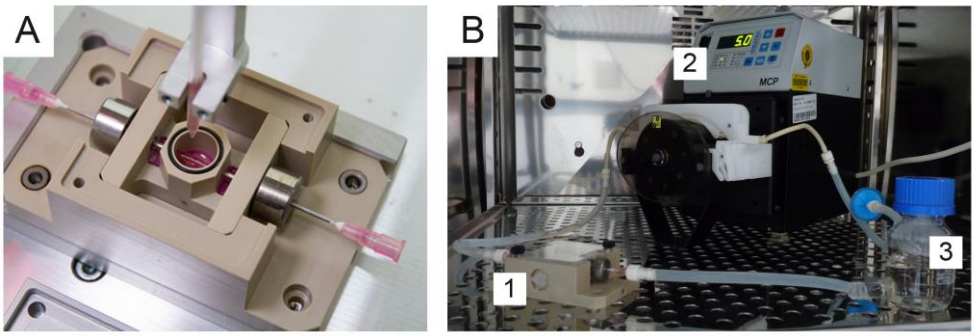

Figure 8: Bioreactor design. A) Dispensing a matrix solution into the bioreactor containing a perfusable artificial capillary. The bioreactor was planned and manufactured in cooperation with Unitechnologies SA (Switzerland). The inner chamber allowed the culture of a vascularized tissue. Vascular structures could be fixed to cannulas in the outer chamber. Cannulas were then connected to a tubing system. B) Bioreactor (1) connected to a pump system (2) which allowed medium circulation through the tubing system. Sufficient medium was stored in the medium reservoir flask (3).

Several authors have already shown that endothelial cells align with their cell axis along the medium flow and receive an elongated cell shape under fluid flow conditions (96–98). This phenomenon should be confirmed in the polyacrylate tubes by culturing endothelial cells in a fluid flow bioreactor (Huber *et al.*, 2016 (82)). A shear stress rate of 1 N m^{-2} was used which is comparable to native capillaries (99). After cell attachment, endothelial cells in non-porous polyacrylate tubes were cultured in a fluid flow bioreactor for seven days. The flow speed of the medium was raised slowly until the resulting wall shear stress of 1 N m^{-2} was achieved and cells were then cultured for 48 hours. It could be confirmed that endothelial cells aligned in the direction of the medium flow (Figure 9). They changed their morphology to an elongated shape, while control cells cultured on tissue culture petri dishes conserved the cobblestone-like morphology which is commonly observed in endothelia in static cultures. The specific markers for endothelial cells, CD31 and vWF, were expressed in both, the static and the dynamic regime (Figure 9).

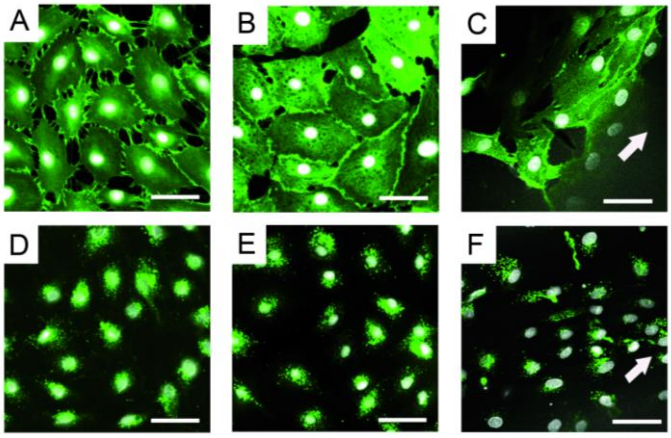

Figure 9: Dynamic culture of human endothelial cells in tubular polyacrylate materials. A, B, C) CD31. D, E, F) vWF. A, D) Static culture of endothelial cells on a tissue culture petri dish as a positive control. B, E) Static culture of endothelial cells on the polyacrylate functionalized with thio-modified heparin/RGD. C, F) Dynamic culture of endothelial cells in a polyacrylate tube functionalized with thio-modified heparin/RGD. Arrow shows the direction of flow. Endothelial cells were cultured with fluid flow in a bioreactor (constructed in cooperation with Unitechnologies, Switzerland). Cells experienced a wall shear stress of 0.012 N m^{-2} for 24 h. The shear stress was then increased at a rate of 0.012 N m^{-2} h^{-1} up to the maximum of 1 N m^{-2}, which is comparable to the shear stress experienced by endothelial cells in native capillaries. Endothelial cells expressed CD31 and vWF under static and dynamic conditions. Dynamic culture resulted in characteristic cell lining along the medium flow. Scale bar: 50 µm (Huber *et al.*, 2016 (82)).

The angiogenic sprouting of endothelial cells and perivascular cells from the artificial vascular system into the interiors of the engineered tissue might be indispensable, since biocompatible and functional capillaries with diameters in the µm-range cannot be manufactured yet. Here, besides endothelial cells, perivascular cells play an important role in the development and maintenance of blood vessels (100). They are associated to capillaries and located inside the basal lamina. During angiogenesis processes, perivascular cells start new vessel outgrowth and later on stabilize functional vessels (101). Perivascular cells might be important to lead the non-orientated endothelial cells through VEGF-release (102,103). The attachment of perivascular cells to the vascular structures might be a result of PDGF-beta release from endothelial cells (104–106). Additionally, Dore-Duffy *et al.* and Dar *et al.* showed that perivascular cells have stem cell character and are able to differentiate into several lineages (107,108). In a recently published study by us, we isolated perivascular cells from human adipose tissue and cultured them in a perivascular cell specific medium (PromoCell) compared to a stem cell medium (Lonza) to evaluate,

which medium holds these cells in their perivascular niche *in vitro* (109). Here, we showed that perivascular cells kept their typical morphology when cultured in pericyte medium. In stem cell medium, the cells seemed to leave their perivascular niche and showed a more intensified differentiation into several lineages (109).

In a next step, perivascular cells were co-cultured with endothelial cells to promote angiogenesis. Besides the co-culture itself, several authors have already shown that the generation of angiogenic structures can be initiated by the application of FGF and VEGF *in vitro* (110–112). It is known that VEGF and FGF stimulate the endothelial secretion of proteases and plasminogen activators what causes the degradation of the vessel basement membrane and therefore cell invasion into the surrounding matrix (113,114). In this study, this approach was evaluated with primary human endothelial cells and perivascular cells in a collagen type I hydrogel and medium supplementation of VEGF and bFGF. After three days, endothelial cells organized themselves into capillary-like structures and perivascular cells started to attach to these structures (Figure 10).

This self-assembled vascularization system was already successfully cultured under fluid flow in micro-chips but without supply of surrounding tissue (74–76). These authors could further detect perfusable lumina. In a next step, angiogenesis and vessel outgrowth needs to be stimulated from vascular analogues, like the polyacrylate or gelatin tube, cultured under fluid flow in a bioreactor to supply the interiors of large tissue constructs sufficiently.

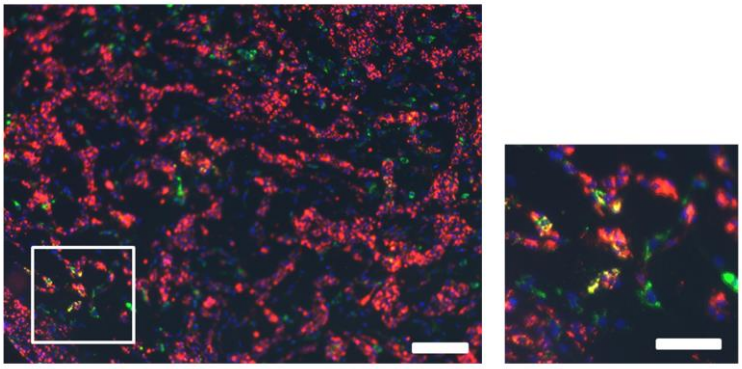

Figure 10: Co-culture of perivascular cells and endothelial cells resulted in the formation of vascular structures on day 3. Endothelial cells were stained with DiI (red) and perivascular cells with DiO (green), nuclei are shown in blue. Both cell types were encapsulated in a collagen type I hydrogel and cultured for 3 days in an endothelial cell growth medium (EGM-2; Lonza) supplemented with 10 ng mL^{-1} VEGF and bFGF. Endothelial cells organized themselves to capillary-like structures (left).

Pervascular cells attached to these structures (right). Scale bar: 200 µm (left), 100 µm (right).

For the composition of vascularized tissue, a vascular system was embedded in a hydrogel containing tissue cells. This setup was tested with a gelatin tube embedded in a collagen type I hydrogel with encapsulated fibroblasts. The cells were cultured in the bioreactor for two days with a medium flow of 3.7 mL min^{-1}. As seen in Figure 11A, cells in the construct could be successfully supplied with nutrients comparable to the static control (Figure 11B). Many living cells could be found under static and dynamic culture (seen in green) whereas only a few dead cells (stained in red) were available.

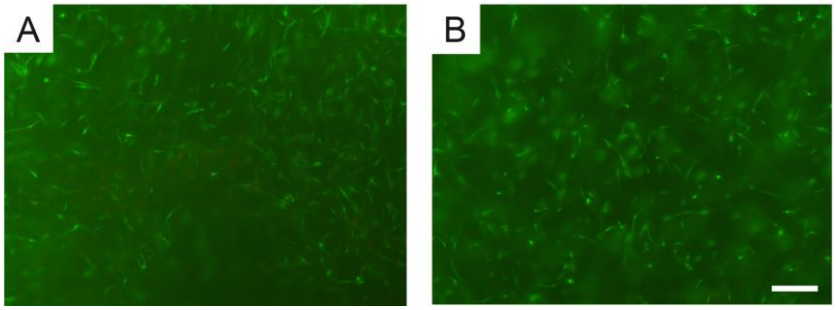

Figure 11: Dynamic culture of a collagen type I hydrogel incorporating fibroblasts supplied through a gelatin tube. A) Fibroblasts encapsulated into a collagen type I hydrogel and cultured for 2 days with a medium flow of 3.7 mL min^{-1}. Cells stayed alive (green; fluorescein diacetate), hardly any dead cells could be found (red; propidium jiodide). B) Live-Dead staining of a static culture of fibroblasts encapsulated into a collagen type I hydrogel and cultured for 2 days as a control. Mainly living cells (green) were seen. Living cells (green), dead cells (red). Scale bar: 200 µm.

For the composition of a vascularized adipose tissue, a suitable culture medium is highly needed to co-culture mature adipocytes and endothelial cells (Chapter 4 (16) and Chapter 5 (61)). In native adipose tissue, mature adipocytes and endothelial cells are in close contact to each other and communicate via soluble factors (Chapter 4 (16)). Here, endothelial cells are able to influence lipolysis and lipogenesis of mature adipocytes. Opposing, mature adipocytes affect the vascular tone of blood vessels and hereby regulate nutrient supply and energy transport to other organs in form of free fatty acids. For an optimal culture medium which maintains the functions and phenotype of endothelial cells and mature adipocytes without allowing excessive growth and cell dedifferentiation, an appropriate balance of supplemented factors is needed. Up to date, no culture medium is available which can serve the needs of

mature adipocytes and endothelial cells (Chapter 4 (16)). In this study, several culture media for the co-culture of mature adipocytes and endothelial cells were evaluated (Chapter 5 (61)). As a basal medium, the adipocyte specific medium AM-1 from ZenBio (Chapter 3 (41)) was used. Commercially available media for endothelial cells are often supplemented with factors like VEGF and FGF to enhance cell proliferation, cell survival and migration (111,113,115). IGF-1 is important for angiogenesis, while ascorbic acid sustains cell growth and maintains the characteristic cobblestone-like morphology of endothelial cells (116). Therefore, the culture medium was supplemented with several factors of a commercially available endothelial cell growth medium (EGM-2; Lonza). Endothelial cell media are often additionally supplemented with hydrocortisone and EGF. Hydrocortisone is known to inhibit lipogenesis in mature adipocytes what could then enhance adipocyte dedifferentiation (117,118). The effect of EGF on adipocytes has been discussed controversial. Some studies say that it stimulates lipolysis and is even able to inhibit preadipocyte differentiation and lipid accumulation (119–121). Others suggest that EGF has an antilipolytic activity on mature adipocytes (122–124). Here, the culture media were supplemented with variable amounts of EGF and hydrocortisone. Then, the influence on endothelial cells and mature adipocytes in mono-culture and indirect co-culture was evaluated (Chapter 3 (41)). In mono-culture, it was proven that all culture media increased the proliferation of mature adipocytes, as a sign of dedifferentiation. It is known that mature adipocytes are terminally differentiated and non-proliferative cells, whereas dedifferentiated fat cells are able to proliferate again (37). Endothelial cell proliferation was comparable to the control, whereas it decreased when cultured with a reduced amount of hydrocortisone but a high level of EGF. In co-culture, it was seen that a high amount of adipocytes dedifferentiated when cultured in an endothelial cell growth medium (Lonza). A reduction of hydrocortisone resulted in less dedifferentiation but maintenance of functional adipocytes, whereas EGF in all concentrations had no negative impact on mature adipocytes. Endothelial cells expressed vWF and were able to uptake acLDL in all setups. Taken together, it was shown that mature adipocytes and endothelial cells can be successfully co-cultured. Here, for the composition of a vascularized adipose tissue construct, an optimized culture medium, consisting of an adipocyte specific basal medium (AM-1; ZenBio) supplemented with factors of an endothelial cell growth medium (Lonza) and high or low levels of EGF but reduced amounts of

hydrocortisone should be used to maintain the functions and phenotype of endothelial cells and mature adipocytes *in vitro* (Chapter 3 (41)).

To summarize, the data of this study showed that a synthetic polyacrylate tube as well as a gelatin tube could be successfully seeded with an endothelial cell monolayer, whereas a biofunctionalization with thio-modified heparin/RGD is needed for the polyacrylate material prior to endothelialization. A bioreactor system could be established which is suitable for the culture of endothelialized vascular structures. Here, it could be confirmed that endothelial cells align along the medium flow. Further, the established bioreactor can be used for the culture of vascularized tissues. In this study, fibroblasts could be supplied through a gelatin tube under dynamic conditions. For the composition of a vascularized adipose tissue, now an appropriate culture medium is available.

8.3. Composition of a functional three-layered full-skin equivalent

Co-cultures of fibroblasts and keratinocytes to construct dermis/epidermis equivalents are already well established and used as *in vitro* test systems or implants to cure shallow skin defects (18,57). These constructs are still neglecting the subcutaneous adipose tissue resulting in reduced skin mobility and low success to treat contour defects. First studies have been performed in composing three-layered tissue substitutes *in vitro* (23–26). Still, up to date, no satisfactory model is available since the subdermal layer lacks a sufficient amount of functional adipocytes. In this study, mature adipocytes were used to compose the subcutis, because they are immediately functional and do not need time-consuming differentiation processes (Chapter 6 (4)). For the co-culture of mature adipocytes, fibroblasts and keratinocytes still no appropriate culture medium is available which allows sufficient differention of keratinocytes to build an epidermal layer and simultaneously keeps mature adipocytes and fibroblasts in their differentiated and functional status. The aim of this study was to compose a functional three-layered full-skin equivalent. Therefore, mature adipocytes were seeded into a collagen type I hydrogel in inserts of 24 well plates. Fibroblasts were also encapsulated in collagen type I and seeded on top. These constructs were overlaid by a monolayer of keratinocytes. Adipocyte medium AM-1 (ZenBio) containing $CaCl_2$ and ascorbyl-2-phosphate was used as a basal medium (=Air). This Air medium was supplemented with HKGS (human keratinocyte growth supplement, Life Technologies) and named Air-HKGS or with KGM

(keratinocyte growth medium supplement without the supplied $CaCl_2$; PromoCell) and named Air-KGM (Chapter 6 (4)).

It was shown that a similar tissue morphology compared to native skin could be established in all three culture media after two weeks. The epidermis was stratified and the adipose tissue layer showed the known lattice structure. Mature adipocytes in all tissue equivalents expressed laminin and perilipin A. Laminin could be further found in the epidermal basal layer as well as in the dermis of full-skin equivalents cultured in Air and Air-HKGS. No laminin expression of fibroblasts and in the basal membrane of the epidermal layer was found when cells were co-cultured in Air-KGM. Cytokeratin 10 expression could be found in the corneum of skin equivalents held in Air and Air-HKGS and allover the epidermis when cultured in Air-KGM. Cytokeratin 14 was expressed in the lower parts of the native epidermis as well as in the epidermis of the full-skin equivalents. It was proven that mature adipocytes in all skin equivalents showed functionality and released adipsin, leptin, visfatin and PAI-1, whereas cells co-cultured in Air-KGM released less amounts of leptin and adipsin compared to the control, where mature adipocytes were held in mono-culture. Adipocytes co-cultured in Air-HKGS released less leptin than mature adipocytes cultured alone. The epidermis was comparably able to withstand a deep penetration of the cytotoxic Triton X in all setups.

Taken together, in this study it was proven that mature adipocytes can be successfully co-cultured with fibroblasts and keratinocytes to compose a functional 3D full-skin equivalent. For the co-culture, prefereably the Air-medium should be used since functional adipocytes, fibroblasts and keratinocytes are hardly available when cultured in Air-KGM and adipocytes released slightly lower levels of leptin when cultured in Air-HKGS (Chapter 6 (4)).

For the composition of a vascularized full-skin equivalent, a co-culture of at least four cell types, adipocytes, fibroblasts, keratinocytes and endothelial cells, is indispensable. Some research has already been done with vascularization of skin constructs consisting of a dermis and an epidermis by co-culturing endothelial cells with fibroblasts in the dermal layer and using keratinocytes to build a stratified epidermis (125–129). Up to date, no studies have been performed with the addition of adipocytes in a subcutaneous layer.

The first *in vitro* vascularized dermis/epidermis equivalent was composed by Black *et al.* in 1998 (125). They seeded a chitosan-collagen-biopolymer with fibroblasts and

human umbilical vein endothelial cells (HUVECs) and cultured it for 10 days. Afterwards, keratinocytes were seeded on top and the construct was cultured for another 7 days submers to allow keratinocyte proliferation. The subsequent airlift culture enabled keratinocytes to differentiate and build a stratified epidermis. Black *et al.* have shown that endothelial cells arranged themselves into capillary-like structures and deposited components of the basement membrane, like laminin and collagen type IV. Similar studies have been then performed by other working groups (126–128). An interesting approach was done by Gibot *et al.* (127). The aim of their study was to compose a vascularized skin-equivalent without using exogenous angiogenic growth factors or scaffolds but basing the construct on autologous cells and their own extracellular matrix (127). Therefore, they cultured fibroblasts for 14 days to let them produce extracellular matrix and build a cell sheet. These sheets were then seeded with HUVECs or HMVECs and stacked. After 7 days of culture, keratinocytes were seeded on top and cultured submers for keratinocyte proliferation. Airlift culture allowed the stratification of the epidermis. Like Black *et al.* they were also able to find a stratified epidermis as well as capillary-like structures surrounded by laminin. In a further step, Gibot *et al.* implanted their constructs into athymic mice. They were able to see that already after four days host and graft capillaries anastomosed. Additionally, they found an active invasion of host endothelial cells into the graft dermis (127). A first study on the integration of a lymphatic and a blood vessel system into a dermis/epidermis construct was done by Marino *et al.* (129). They used HDMECs which they proposed to be an inhomogeneous cell population consisting of $Prox1^+$ lymphatic endothelial cells and $Prox1^-$ blood endothelial cells. These cells were co-cultured with fibroblasts in a fibrin hydrogel covered by a layer of keratinocytes, which started to stratify. They have found $Prox1^+/CD31^+$ lymphatic vessels as well as $Prox1^-/CD31^+$ blood vessels. These two vessel systems were never found to anastomose *in vitro*. Further, they were able to show that the lymphatic vessel system was functional and able to uptake a dye from the extracellular space. After implantation into rats the vessels were able to anastomose with the equivalent host vessel.

For the *in vitro* composition of vascularized full-skin equivalents containing the underlying subcutis, an adequate culture medium is indispensable. For the culture of fibroblasts mostly DMEM with 10 % FCS is used. Endothelial cells and keratinocytes are in need of further factors which allow them a proper functionality and

differentiation, especially when in co-culture with other cells (Chapter 5 (61) and Chapter 6 (4)). Commercially available media for endothelial cells are often supplemented with factors like VEGF, FGF, IGF-1, ascorbic acid and heparin. Keratinocytes are in need of similar factors. Here, commercially available media are usually containing FCS, insulin and EGF, which are important for cell migration and proliferation (130,131), whereas epinephrine and hydrocortisone are involved in differentiation (132–134). In the above presented studies the co-culture medium was also based on factors important for endothelial cells as well as keratinocytes, e.g. hydrocortisone, insulin, ascorbic acid and EGF (125,127,128). For mature adipocytes a medium is needed which prevents them from dedifferentiation (Chapter 3 (41)).

Based on the presented studies and our own observations in co-culturing mature adipocytes, fibroblasts and keratinocytes we evaluated the same three media for the additional culture of endothelial cells. A full-skin equivalent was generated in 24 well inserts according to our previous study (Chapter 6 (4)) while endothelial cells were additionally seeded on the well bottom. Constructs were cultured for seven days in the three culture media Air, Air-HKGS and Air-KGM. It was shown that endothelial cells can be successfully co-cultured with a full-skin equivalent. Endothelial cells were viable and expressed the cell-specific marker CD31 on day 7. Only very sporadic $CD31^-$ cells could be detected (Figure 12). Mature adipocytes formed the typical lattice-like structure of native adipose tissue. Comparable to the results on the co-culture of mature adipocytes, fibroblasts and keratinocytes (Chapter 6 (4)), the full-skin equivalents co-cultured with endothelial cells exhibited a fully differentiated and stratified epidermis (Figure 12).

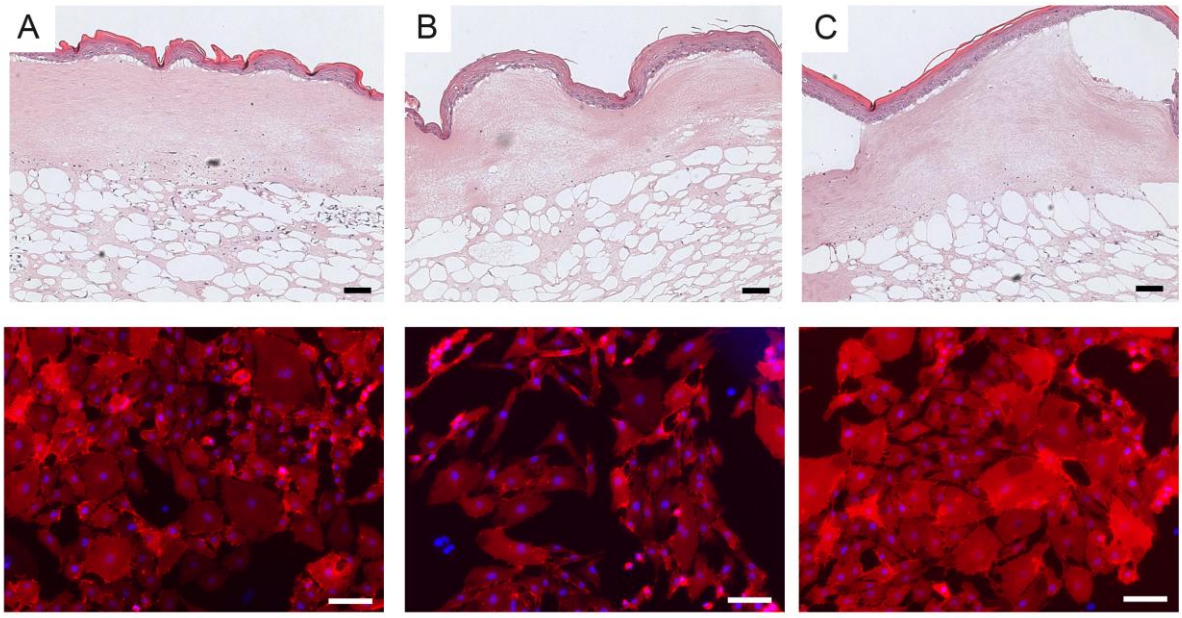

Figure 12: Co-culture of full-skin equivalents with endothelial cells. A full-skin equivalent was composed in inserts in a collagen type I hydrogel, where mature adipocytes built the subcutis, fibroblasts the dermal layer and keratinocytes were seeded on top to stratify into an epidermal layer. Endothelial cells were seeded on the well bottom. A-C) Hemalaun-Eosin staining of full-skin equivalents co-cultured with endothelial cells for seven days (top). CD31 expression (red) of endothelial cells (bottom). Nuclei were stained in blue (DAPI). Used culture media: A) Adipocyte medium AM-1 (ZenBio) containing $CaCl_2$ and ascorbyl-2-phosphate was used as a basal medium (=Air). B) Air medium was supplemented with HKGS (human keratinocyte growth supplement, Life Technologies). C) Air was supplemented with KGM (keratinocyte growth medium supplement without the supplied $CaCl_2$; PromoCell). Scale bar: 100 µm.

8.4. Bioprinting of adipose tissue and skin

Additive manufacturing methods are gaining more and more interest in medical engineering and medicine. First approaches have been made in the printing of adipose tissue, where stem cells were incorporated into a matrix and then differentiated into the adipogenic lineage (45,135,136). Here, Pati *et al.* followed an interesting approach. After decellularization of human adipose tissue, the extracellular matrix was milled and resolved in PBS to allow heat-induced gelation. ASCs were seeded into this matrix and differentiated into the adipogenic lineage for 14 days (45). Up to date, no study is available concerning the printability of mature adipocytes in a photo-sensitive matrix. Photopolymer-based materials for injekt bioprinting are very promising since they can be used to shape complex 3D structures. Therefore, our approach was to incorporate mature adipocytes into

methacrylated gelatin which was then UV-crosslinked (Chapter 7 (62)). It was shown that neither methacrylated gelatin nor the used photoinitiator LAP were cytotoxic. For the hydrogel preparation, several curing times were used to evaluate cell survival and adapt the material stiffness to native fatty tissue. It was proven that the hydrogels which were cured for 1 min contained many viable cells, had a stiffness similar to native tissue and could be cultured for at least two weeks while cells still expressed cell-specific markers like perilipin A or laminin. Moreover, the tissue had a similar lattice structure like native adipose tissue. As a proof-of-concept, a layer-by-layer technique was used to produce adipose tissue equivalents with a height of around 7 mm. In this constructs mature adipocytes still stayed viable after 24 hours (Chapter 7 (62)). Taken together, it was proven that mature adipocytes can be successfully encapsulated into methacrylated gelatin and adipose tissue equivalents with a comparable stiffness to native adipose tissue can be fabricated. Moreover, large adipose tissue equivalents could be produced using a layer-by-layer technique. These results are highly valuable for use of mature adipocytes and methacrylated gelatin in bioprinting technologies.

Some approaches have already been done in bioprinting skin tissue (45,137–140). Several authors incorporated fibroblasts into collagen and seeded keratinocytes on top (137,138,140). Rimann *et al.* used a light-induced bioink polymerization. Here, fibroblasts were encapsulated into an extracellular-matrix-like bioink and covered with a monolayer of keratinocytes (139). These constructs were successfully cultured for up to seven weeks.

Up to date, only a few studies have been performed in supplying skin equivalents through perfusion in a fluid flow bioreactor (141,142). Again, the subcutaneous adipose tissue was missing. Sun *et al.* co-cultured fibroblasts and keratinocytes on several matrices. Tissue was cultured under perfusion, while the structure was hold under airlift conditions. They have shown that the cell viability was higher when constructs were cultured under perfusion compared to static culture (142). A similar approach was done by Helmedag *et al.* who cultured fibroblasts and HUVECs in a fibrin gel with keratinocytes on top. The dermis was perfused with medium. They found a stratified epidermis and surprisingly, they have seen a higher density and longer capillary-like structures in the static cultures compared to the dynamic one (141). In a next step, instead of perfusion, a capillary-like network should be established to supply the surrounding tissue with oxygen and nutrients.

9. Conclusion

In the present thesis a culture medium was provided for the successful *in vitro* culture of mature adipocytes. It is well-known that mature adipocytes dedifferentiate under *in vitro* conditions. Here, they organize their one lipid droplet into several droplets before they release their fat storages completely. Cells receive then an elongated shape and are able to proliferate again. It could be shown that a culture medium which contains factors favoring lipogenesis and inhibiting lipolysis, like biotin, pantothenate, insulin and dexamethasone, could be used for the successful *in vitro* culture of mature adipocytes for up to 21 days.

Another focus of this work was the establishment of culture conditions for the co-culture of mature adipocytes with endothelial cells and the subsequent vascularization of a tissue. For the co-culture, a specific medium was compiled, which consists of factors inhibiting adipocyte dedifferentiation, but still allows endothelial cell proliferation and functionality. This medium consisted of an adipocyte basal medium and was then added by factors typically used in commercially available endothelial cell media, like vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and insulin-like growth factor-1 (IGF-1) among others. The amount of hydrocortisone was reduced to prevent adipocyte dedifferentiation. No negative impact on endothelial cells was observed when co-cultured with mature adipocytes.

To compose a vascularization system, artificial capillary structures should be endothelialized. Here, a biofunctionalization protocol with thio-modified heparin and RGD was established for synthetic polyacrylate materials. Additionally, a method to seed these synthetic polyacrylate tubes as well as gelatin tubes with endothelial cells was developed. To dynamically culture these structures, a fluid flow bioreactor was engineered. Endothelialized tubes were successfully cultured in the bioreactor and an endothelial cell alignment along the medium flow was observed. In a next step, a gelatin tube was placed in the bioreactor and embedded in a collagen type I hydrogel which incorporated fibroblasts. These cells could be supplied sufficiently with nutrients and oxygen under fluid flow through the gelatin tube.

This thesis further focused on the construction of a functional three-layered full-skin equivalent. A culture medium for the co-culture of mature adipocytes, fibroblasts and keratinocytes was established. The optimized medium consists of an adipocyte specific basal medium which is supplemented with the factors needed for the

stratification of the epidermis, calcium chloride and ascorbic acid. The full-skin equivalents could be cultured for two weeks while building a differentiated epidermis. Viable and functional adipocytes were detected on day 14. For the composition of vascularized full-skin equivalents, these cells need to be co-cultured with endothelial cells. It was shown that endothelial cells can be successfully co-cultured with full-skin equivalents and a stratified epidermis was already seen on day 7.

In a last step, to prove future fabrication of full-skin equivalents with additive manufacturing methods, mature adipocytes were introduced into a printable matrix of methacrylated gelatin. The matrix stiffness was adapted to that of native adipose tissue and viable cells were detected after 14 days of culture. In a first proof-of-concept, a layer-by-layer method was used to produce high adipose tissue equivalents.

This study shows the usability and potentials of mature adipocytes which are up to now neglected in research studies. Thereby the results of this dissertation offer a basis to compose a nature-mimicking bio-printed vascularized adipose tissue as well as a vascularized full-skin equivalent based on the use of mature adipocytes in the fatty tissue layer.

10. Outlook

Mature adipocytes are a promising source for the fabrication of a vascularized adipose tissue and full-skin tissue engineering. In this thesis, a medium for the culture of mature adipocytes *in vitro* could be found. Still, further knowledge should be gained about the triggers of the dedifferentiation process of mature adipocytes. These understandings might help to adapt the culture medium to hold these cells viable and functional for more than 21 days.

A medium for the co-culture of mature adipocytes with endothelial cells is now available. However, both cells need to be co-cultured in a bioreactor under fluid flow conditions, where endothelial cells reside as a monolayer in the artificial vascular structure and adipocytes build the surrounding tissue. For the construction of large vascularized adipose tissue constructs, neo-angiogenesis arising from the existing vascular structure might be useful to supply the interiors of the tissue which cannot be reached by the large artificial vascular system. Therefore, a direct co-culture of endothelial cells, perivascular cells and adipocytes in a hydrogel is indispensable. It needs to be evaluated if the co-culture medium should be adapted (e.g. by the addition of growth factors like VEGF or bFGF) or if the mature adipocytes release sufficient signals to promote the formation of capillary-like structures by endothelial cells and perivascular cells. Optimally, these newly built vascular structures which are connected to the larger artificial vessel system have perfusable lumina. This study then needs to be followed by a long-term culture study.

Up to date, linear tubes as artificial vascular systems were evaluated. These structures fabricated of variable materials need to be further enhanced by branching and minimization. For vessel systems with smaller diameter, the endothelialization protocol might be adapted since small caliber vessels are susceptible for cell clogging.

A full-skin equivalent can be composed of mature adipocytes, fibroblasts and keratinocytes. However, up to date there are no data available of a vascularized three-layered full-skin equivalent. In this thesis it was shown that endothelial cells can be co-cultured with a three-layered full-skin equivalent. Similar to the vascularized adipose tissue, these constructs now need to be cultured in a fluid-flow bioreactor and supplied through an artificial vascular system. Here, an endothelialized tube needs to be embedded into an adipose tissue layer and endothelial cells should be attracted to sprout into the dermal layer to compose small capillaries and allow

successful supply with nutrients and oxygen. Here, it is important that the epidermis is sufficiently supplied but still be hold under air-lift conditions to allow appropriate epidermal differentiation.

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12. Table of Figures

13. List of Publications and contributions

1. **Birgit Huber**, Kirsten Borchers, Günter E. M. Tovar, Petra J. Kluger (2015); *Methacrylated gelatin and mature adipocytes are promising components for adipose tissue engineering*; J Biomater Appl, 30:699-710.
2. **Birgit Huber**, Ann-Cathrin Volz, Petra J. Kluger (2015); *How do culture media influence in vitro perivascular cell behavior?;* Cell Biol Int, 39:1395-407.
3. **Birgit Huber**, Ann-Cathrin Volz, Petra J. Kluger (2015); *Understanding the effects of mature adipocytes and endothelial cells on fatty acid metabolism and vascular tone in physiological fatty tissue for vascularized adipose tissue engineering*; Cell Tissue Res, 362:269-79.
4. **Birgit Huber**, Petra J. Kluger (2015); *Decelerating mature adipocyte dedifferentiation by media composition;* Tissue Eng Part C, 21:1237-4.
5. Ann-Cathrin Volz, **Birgit Huber**, Petra J. Kluger (2016); *ASC differentiation as a basic tool for vascularized adipose tissue engineering;* Differentiation, pii: S0301-4681.
6. **Birgit Huber**, Alina M. Czaja, Petra J. Kluger (2016); *Influence of epidermal growth factor (EGF) and hydrocortisone on the co-culture of mature adipocytes and endothelial cells for vascularized adipose tissue engineering;* Cell Biol Int, 40:569-78.
7. **Birgit Huber**, Antonia Link, Kirstin Linke, Sandra Gehrke, Marc Winnefeld, Petra J. Kluger (2016); *Integration of mature adipocytes to build-up a functional three-layered full-skin equivalent*. Tissue Eng Part C Methods, accepted for publication
8. **Birgit Huber**, Sascha Engelhardt, Wolfdietrich Meyer, Hartmut Krüger, Annika Wenz, Veronika Schönhaar, Günter E. M. Tovar, Petra J. Kluger, Kirsten Borchers (2016); *Blood-vessel mimicking structures by stereolithographic fabrication of small porous tubes using cytocompatible polyacrylate elastomers, biofunctionalization and endothelialization;* J Funct Biomater, 7:11.

No.	Accepted for publication	No of all authors	Position of the candidate in list of authors	Scientific ideas of candidate (%)	Data generated by candidate (%)	Analysis and interpretation by candidate (%)	Paper writing by candidate (%)
1	Yes	4	1	50	100	80	90
2	Yes	3	1	80	20	80	80
3	Yes	3	1	review	review	review	90
4	Yes	2	1	90	100	90	100
5	Yes	3	2	review	review	review	30
6	Yes	3	1	90	40	90	100
7	Yes	6	1	90	20	80	90
8	Yes	9	1	20	30	30	50

Oral presentations

Birgit Huber, Eva Hoch, Günter E. M. Tovar, Kirsten Borchers, Petra J. Kluger; *Optimized culture conditions for mature adipocytes in 3D Adipose Tissue Engineering*; Euro BioMat in Weimar, May 2015

Birgit Huber, Eva Hoch, Günter E. M. Tovar, Kirsten Borchers, Petra J. Kluger; *Methacrylated gelatin and mature adipocytes: promising components for Adipose Tissue Engineering*; International Federation for Adipose Therapeutics and Science (IFATS) 12th annual meeting in Amsterdam/Netherlands, November 2014

Birgit Huber, Eva Hoch, Günter E. M. Tovar, Kirsten Borchers, Petra J. Kluger; *Fatty tissue equivalents – build up with mature adipocytes in a gelatin hydrogel;* Jahrestagung der dt. Gesellschaft für Biomaterialien (DGBM) in Dresden, November 2014

Birgit Huber, Eva Hoch, Günter E. M. Tovar, Kirsten Borchers, Petra J. Kluger; *Fatty tissue equivalents – build up with mature adipocytes in a gelatin hydrogel;* Tagung der European Society for Biomaterials (ESB) in Liverpool/United Kingdom, September 2014

Birgit Huber, Esther Novosel, Wolfdietrich Meyer, Annika Wenz, Veronika Schönhaar, Günter E. M. Tovar, Petra J. Kluger, Kirsten Borchers; *Biofunctionalization and endothelialization of new cytocompatible scaffolds based on multifunctional heparin derivates for vascularized tissue constructs;* Symposium of the Tissue Engineering and Regenerative Medicine International Society (TERMIS) in Genua/Italy, June 2014

Birgit Huber, Petra J. Kluger, Kirsten Borchers; *Synthetische Blutgefäße für den Einsatz im vaskulären Tissue Engineering*; VDI-Wissensforum in Friedrichshafen, May 2014

Poster presentations

Ann-Cathrin Volz, **Birgit Huber**, Petra Kluger; *Characterization of human fatty tissue pericytes and evaluation of their angiogenic potential*; Euro BioMat in Weimar, April 2015

Birgit Huber, Wolfdietrich Meyer, Sascha Engelhardt, Alexander Fischer, Lisa Sewald, Annika Wenz, Iván Calderon, Esther Novosel, Anke Hoppensack, Thomas Hirth, Günter E. M. Tovar, Kirsten Borchers, Petra J. Kluger; *Biofunctionalisation and endothelialisation of synthetic biocompatible scaffolds for vascularised tissue constructs;* First International Symposium on Vascular Tissue Engineering in Leiden/Netherlands, May 2013

Birgit Huber, Wolfdietrich Meyer, Sascha Engelhardt, Alexander Fischer, Esther Novosel, Annika Wenz, Heike Walles, Thomas Hirth, Kirsten Borchers, Petra J. Kluger; *Biofunctionalisation and endothelialisation of biocompatible Artificial Blood Vessels;* Euro BioMAT in Weimar, April 2013

Birgit Huber, Nadine Klechowitz, Kirsten Borchers, Heike Walles, Thomas Hirth, Petra J. Kluger; *Isolation and culture of primary human subcutaneous adipocytes and construction of a fatty tissue-equivalent;* Jahrestagung der deutschen Gesellschaft für Biomaterialien (DGBM) in Hamburg, November 2012

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15. Eidesstattliche Erklärung

Ich erkläre hiermit, dass ich die zur Promotion eingereichte Arbeit mit dem Titel:

„Development of culture media for the construction of vascularized adipose tissue and vascularized 3D full-skin equivalents *in vitro*“

selbstständig verfasst, nur die angegebenen Quellen und Hilfsmittel benutzt und wörtlich oder inhaltlich übernommene Stellen (alternativ: Zitate) als solche gekennzeichnet habe. Ich versichere, dass diese Angaben wahr sind und dass ich nichts verschwiegen habe.

Stuttgart, den 1. August 2016

Birgit Huber