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

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Abstract
Vascularized adipose tissue models are highly demanded as alternative to existing animal models to elucidate the mechanisms of widespread diseases, screen for new drugs or assess corresponding safety levels. Standardly used animal-derived sera therein, are associated to ethical concerns, the risk of contaminations and many uncertainties in their composition and impact on cells. Therefore, their use should be completely omitted. In this study, we developed a serum-free, defined co-culture medium and implemented it to set up an adipocyte/endothelial cell (EC) co-culture model.

Human adipose-derived stem cells were differentiated under defined conditions (diffASCs) and, like human microvascular ECs (mvECs), cultured in a developed defined co-culture medium in mono-, indirect or direct co-culture for 14 days. The developed defined co-culture medium was superior to compared mono-culture media and facilitated the functional maintenance and maturation of diffASCs including perlinin A expression, lipid accumulation and also glycerol and leptin release. The medium equally allowed mvEC maintenance, confirmed by the expression of cluster of differentiation 31 (CD31) and von Willebrand factor (vWF) and acetylated low-density lipoprotein (acLDL) uptake. Thereby mvECs showed a strong dependency on EC-specific factors. Additionally, the development of vascular structures by mvECs was facilitated when directly co-cultured with diffASCs.

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

Keywords: xeno-free, serum-free, vascularized adipose tissue engineering, adipocytes, vascularization

1 Introduction

Cell and tissue based in vitro models keep gaining importance in the replacement of animal trials. As a major drawback, the current models heavily rely on the use of animal-derived sera like fetal bovine serum (FBS). As FBS is collected from fetuses, it is discussed to cause animal harm and is generally associated to ethical concerns (van der Valk et al., 2004). Thereby the pursuit of the overall goal aimed in the 3R concept of Russel is remained rather questionable on this background (Russell and Burch, 1959). Additionally, sera include the risk of potential contaminations, and may vary in terms of their exact constitution from batch to batch (van der Valk et al., 2004; Gstraunthaler, 2003; Gstraunthaler et al., 2013). Both, potential contaminations and an unstable composition and therefore fluctuation in the product quality are considered as major challenges to overcome before the engineered models may be produced under Good Manufacturing Practice or Good Cell Culture Practice confrom conditions and furthermore be applied as human tissue implants (Hartung et al., 2002). Independent of the animal or human origin, the composition of blood serum is not completely identified – neither qualitatively nor quantitatively. To overcome the mentioned drawbacks the setup of in vitro engineered cell and tissue models has to be performed under completely defined conditions, excluding serum and all animal-derived components like e. g. bovine brain extract. A defined medium by definition only contains factors of known character, like recombinant growth factors and hormones and has to exclude complex proteins or hydrolysates (van der Valk et al., 2010).

Adipose tissue (AT) has long been neglected as target tissue in regenerative medicine and too little attention has been paid to the tissue’s influence on body functions and its participation in different diseases. However, the last decades brought light to the importance of AT’s metabolic function and its relation to diseases like obesity, diabetes, pancreatitis and Morbus Crohn (Peyrin-Biroulet et al., 2007; Xu et al., 2003; Navina and Singh, 2015). A representative model of AT is highly needed to further elucidate the existing pathways during the healthy and diseased states and uncover further involvement of the tissue in other processes. In vitro engineered tissue models have not yet reached a dimension of significance, which is strongly connected to the inability to engineer an adequate vascular support system. The formation of new blood vessels
(vasculogenesis) and the extension of existing blood vessels (angiogenesis) are of fundamental importance for the development, enlargement or homeostasis of almost any tissue in the living body. The integration of a vascular component is especially of high importance in the setup of AT. In vivo AT is highly vascularized whereby each adipocyte is in contact with at least one blood vessel (Silha et al., 2005). The efficient accumulation and release of lipids and the transport of tissue-related hormones like leptin or adiponectin rely on the presence of a vascular system (Coelho et al., 2013). Additionally, the endothelial component is critical to allow constant tissue functionality and homeostasis of the tissue components (Cao, 2007, 2010). Likewise, in vitro engineered tissues rely on the integration of a vascular component. A vascular system is not only needed to allow for a scale-up and in consequence a sufficient volume of the tissue fragment (Yao et al., 2013a), but as well for some sought in vitro investigations which specifically rely on the presence of a vascular component, e.g. to study tissue’s interaction with the endothelium.

The integration of a vascular supply system in AT engineering attempts is still challenging. Current strategies for the in vitro vascularization of AT include vascular guiding geometries, like they are still present in naturally-derived decellularized matrix (Baptista et al., 2011) or the artificial biopair system (Huber et al., 2016b). Next to form-guided vascularization, de novo angiogenesis based on integrated cells is addressed in many attempts. For both, the introduction of angiogenic growth factors is feasible to support cell proliferation, migration and functionality (Nomi et al., 2006). In case of sought de novo vascularization, supportive perivascular cells, which guide endothelial cells (ECs) and support newly formed vessels, are needed (Tang et al., 2008). There are various trials, addressing the vascularization of AT constructs in vitro (Aubin et al., 2015; Huttala et al., 2018; Yao et al., 2013b).

Independent of the underlying strategy, the setup of a functional adipocyte/EC co-culture still represents a major milestone in the artificial vascularization of AT. The dependency of cells’ response on media ingredients has frequently been shown before, also for AT attempts (Aubin et al., 2015). Within the adjustment of the culture conditions like cell ratio, static vs. dynamic culture or the three-dimensional (3D) orientation of cellular components, the development of an adequate culture medium is of fundamental importance to keep the different cell types characteristic and functional and allow for tissue homeostasis as important prerequisite for long-term maintenance. We have shown before that e.g. hydrocortisone and EGF play a fundamental role in the co-culture of ECs and adipocytes – whether mature or developed of adipose-derived stem cells (ASCs) (Volz et al., 2017; Huber et al., 2016a). Presumably due to the higher sensitivity of ECs, compared to adipogenic differentiated ASCs (diffASCs), current attempts are in most cases based on either the addition of adipocyte factors to EC medium or on a 1:1 mixture of EC and adipocyte media (Pellegrinelli et al., 2013; Yao et al., 2013a,b; Aubin et al., 2015; Arnal-Pastor et al., 2016). Promising results like the supportive effect of ASC co-culture on the formation of tubular structures by ECs have been generated (Bellas et al., 2013).

The homeostasis of functional adipose tissue strongly relies on the crossstalk of the incorporated cell types (reviewed in (Volz et al., 2016)). Human and animal-derived sera include many different constituents (e.g. growth factors, hormones or cytokines) which might interfere with cell signaling based on soluble factors. Vascularization and adipocytes’ influence therein e.g. rely on the available factors in the culture medium (Moya et al., 2010; Aubin et al., 2015; Yao et al., 2013b; Rajashekar et al., 2008; Aoki et al., 2003). The existing inconsistency within the published results concerning mutual interference between the cell types might at least partially be led back to variations in the added sera. In general, the results generated in in vitro models or test systems are under high risk to be influenced through unknown components, thereby impairing their reliability and reproducibility, known as the most important features of test systems in general.

The exclusion of sera or all unidentified components from cell culture media is of high relevance for all tissue engineering trials. In a very recent work, serum-free adipocyte/human umbilical cord vein ECs co-culture was addressed at least in some phases with serum-free EC medium, which additionally underlines the currency of the topic (Huttala et al., 2018). However, so far no completely serum-free (SF) adipocyte/EC co-culture models including a defined custom-made co-culture medium for vascularized adipose tissue engineering has been reported. In a previous trial, we successfully developed defined media for the differentiation and maintenance of adipocytes derived from ASCs (Volz and Kluger, 2018). With this study, we aimed to develop an adequate adipocyte/EC co-culture system, free of serum and based on a completely chemically defined medium composition.

2 Material and Methods

ASC isolation and expansion

All research was carried out in accordance with the rules for investigation of human subjects as defined in the Declaration of Helsinki. As described previously, ASCs were isolated out of human fatty tissue samples (Klinik Charlottenhaus, Stuttgart) of plastic surgeries received from Dr. Ziegler (Volz et al., 2017; Huber et al., 2015a). Patients gave a written agreement according to the permission of the Landesärztekammer Baden-Württemberg (F-2012-078; for normal skin from elective surgeries).

Abbreviations

3D, three-dimensional; AcLDL, acetylated low-density lipoprotein; ACM, defined adipocyte maintenance medium; ASC, adipose-derived stem cell; AT, adipose tissue; CD31, cluster of differentiation 31; CoM, defined adipocyte/endothelial cell co-culture medium; DAPI, 4',6-diamidino-2-phenylindole; DiffASC, adipogenic differentiated adipose-derived stem cell; EC, endothelial cell; EGCM, endothelial cell basali medium; ECM, defined endothelial cell growth medium; EGM-2mv, Endothelial cell growth medium 2, microvascular; FBS, fetal bovine serum; FDA, fluorescein diacetate; IF, immunofluorescence; MAIN, defined adipocyte maintenance mix; MSCGM, Mesenchymal stem cell growth medium; MvEC, microvascular endothelial cell; PBS, phosphate buffered saline; PI, propidium iodide; RT, room temperature; SF, serum-free; VEGF, vascular endothelial cell growth factor; vWF, von Willebrand Factor; XF, xeno-free
Initially, ASCs were seeded with a density of $5 \times 10^3$ cells/cm² in chemically defined Mesenchymal Stem Cell Growth Medium (MSCGM-CD, Lonza, Switzerland) containing 2% fetal bovine serum (FBS) (Lonza Switzerland). Medium exchange was performed twice a week. After the first passage the medium was switched to xeno-free (XF) and SF MSCGM (XF/SF-MSCGM, PELOBiotech, Germany) containing 5% human platelet lysate. ASCs were used up to passage three. For the experiments, ASCs of three different donors were evaluated separately, to allow for the recognition of donor dependent effects.

**MvEC isolation and expansion**

Dermal microvascular ECs (mvECs) were isolated from adult human skin (Klinik Charlottenhaus, Stuttgart) as described by us before (Volz et al., 2017). Briefly, human dermis was dissected into small pieces and digested in dispase solution (2 U/ml; Serva Electrophoresis, Germany) overnight at 4 °C. After the removal of the epidermis, mvECs were detached from the dermal layer by incubation with 0.05% trypsin in ethylenediaminetetraacetic acid (Life Technologies, Germany) for 40 min at 37 °C and isolated mechanically into microvascular Endothelial Cell Growth Medium-2 (EGM-2mv; Lonza, Switzerland). For cell expansion, mvECs were seeded with $5 \times 10^3$ cells/cm² and grown until they reached about 90% confluency. MvECs were used up to passage three.

### 2.3 Composition and preevaluation of co-culture media in mono-culture

The composition of the defined adipocytes-EC co-culture medium (CoM) was developed based on defined mono-culture media for adipocytes (adipocyte maintenance medium, ACM, developed by us (Volz and Kluger, 2018)) or ECs (EC growth medium, ECM, PELOBiotech, Germany) (Tab. 1). CoM was based on EC basal medium (ECBM), whereby additional adipocyte- and EC-specific factors, like growth factors, hormones and vitamins were added. For adipocytes a defined adipocyte maintenance mix (MAIN) was added. In all attempts, EGF and HC were reduced to 5 ng/ml and 0.2 µg/ml respectively as confirmed to be beneficial in previous studies by us (Volz et al., 2017; Huber et al., 2016a).

To test their general suitability for either adipocytes or ECs the media were first evaluated in mono-culture. Therefore 24 well plates were coated with 300 µl of 250 µg/ml collagen type I (rat tail, kindly provided by the Fraunhofer IGB, Germany) solution in 0.1% acetic acid. Excess solution was aspirated and plates were dried at room temperature (RT) for about 1 h.

ASCs were seeded in XF/SF-MSCGM with $2.5 \times 10^4$ cells/cm² in 24-well plates. After three days of expansion until confluency, adipogenic differentiation was initiated through the addition of defined adipogenic differentiation medium as described before. Differentiation was continued for 14 days with three medium exchanges a week (Fig. 1).

MvECs were seeded with $3.5 \times 10^3$ cells/cm² in 24-well plates and expanded for three additional days in EGM-2mv.

On day 3 of mvEC expansion, respectively day 14 of adipogenic differentiation of ASCs, the medium was changed to the different test media.

Cultures were continued for 14 days while the medium exchange took place twice a week. The culture attempts were evaluated on day 0, 7 and 14 in the test media.

### Tab. 1: Media composition

<table>
<thead>
<tr>
<th>Basal Medium</th>
<th>ACM</th>
<th>CoM</th>
<th>ECM</th>
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<tbody>
<tr>
<td>Adipocyte Suppl.</td>
<td>x</td>
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<tr>
<td>EC Suppl.</td>
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**Fig. 1: Setup of defined media evaluation in mono-culture and (in)direct co-culture**
2.4 Evaluation of co-culture media in (in)direct diffASC/EC co-culture
For indirect co-culture ASCs were cultured and differentiated analogously to the mono-culture attempt. On day 13 of differentiation, mVECs of three different donors respectively were seeded in collagen-coated inserts of 0.4 µm pore size with 1.3 x 10^5 cells/cm² in EGM-2-IV. After 24 h of cell attachment, inserts were transferred into the well plates with diffASCs and cultured in the different defined culture media for additional 14 days. Next to the co-culture attempts diffASCs and mVECs were held in mono-culture in their specific media ACM or ECM respectively as controls. Medium was exchanged twice a week. For direct co-culture mvECs were directly seeded on top of diffASCs in 24-well plates with 8 x 10^3 cells/cm² and cultured in CoM for 14 days. Next to the co-culture attempts, diffASCs and mVECs were held in mono-culture in ACM and ECM respectively as controls.

2.5 Viability evaluation
Cells’ viability in the different medium attempts was evaluated based on a live/dead staining on day 0, 7 and 14. Before the staining solution, consisting of 10 µg/ml fluorescein diacetate (FDA, Sigma Aldrich, Germany) and 5 mg/ml propidium iodide (PI, Sigma Aldrich, Germany) in Dulbecco’s Modified Eagle Medium (Biochrom, Germany), was added for 15 min at 37 °C, cells were washed in phosphate buffered saline (PBS, Biochrom, Germany). Finally, cells were imaged in PBS at RT with an Axio Observer microscope (10x objective) and an Axiocam 506 mono using the software ZEN (all Zeiss, Germany).

2.6 Oil-red-O staining
On day 0, 7 and 14 an Oil-Red-O staining was performed to evaluate the level of accumulated lipids in the diffASCs. The staining was evaluated quantitatively and qualitatively as described by us before (Volz et al., 2017). Images were taken with an Axiovert 135 microscope (10x objective) and an Axiocam 105 color using the software ZEN (all Zeiss, Germany).

2.7 Immunofluorescence staining
Perilipin A, as a membrane protein located on adipocytes’ lipid vacuole, as well as cluster of differentiation (CD) 31 and von Willebrand factor (vWF) as endothelial proteins, were evaluated as markers of cell specificity on day 0, 7 and 14 with an immunofluorescence (IF) staining. Therefore, cells were fixed in 4 % paraformaldehyde for 10 min. For the evaluation of the intracellular markers vWF and perlipin A, cells were permeabilized for 10 min with 0.1 % Triton X (Sigma, Germany). Following, cells were incubated in blocking solution, consisting of 3 % bovine serum albumin (Biomol, Germany) in 0.1 % Triton X for 30 min to block unspecific binding sites. The primary antibody against perlipin A was rabbit-derived (P1998, lot: 09544801V, Sigma Aldrich, Germany) and applied in the ratio 1:300. The antibodies against CD31 (M082301, lot: B0117, Santa Cruz, Germany) were both derived from mouse and applied in the ratio 1:50. All antibodies were diluted in blocking solution and incubated for 3 h at RT. For perlipin A, a goat-derived Alexa Fluor™488-conjugated secondary antibody (ab150077, lot: GR322463, Abcam, GB) was applied with 1:250 in blocking solution for 30 min at RT, while an anti-mouse Cy3-conjugated antibody from goat (115-165-003, lot: 118666, dianova, Germany) was incubated 1:250 and used to visualize CD31 and vWF. Cell nuclei were counterstained with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI, Serva Electrophoresis, Germany) in PBS for 15 min. Finally, cells were imaged in PBS using a 10x objective. Z-stack images were taken with an Axios Observer microscope and an Axiocam 506 mono using the software ZEN (all Zeiss, Germany) and combined using the function Extended depth of focus.

2.8 Quantitative analysis of vascular-like structures
Vascular-like structures were analyzed based on the IF images of the antibody staining against CD31 with Image J. The received structures were evaluated concerning total vessel length, number of structures, and number of nodes per 1 mm structure.

2.9 Leptin ELISA
Leptin release was measured using an ELISA Kit (Pepro Tech, Germany) according to the manufacturer’s protocol based on cells’ 24 h culture supernatants in a 1:1 dilution. To each well 100 µl 3,3’,5,5’-tetramethylbenzidine substrate (Sigma-Aldrich, Germany) were added for approx. 20 min at RT for color development. The wells were read out at 650 nm with a wavelength correction set at 470 nm (Omega Fluostar; BMG Labtech, Germany).

2.10 Glycerol assay
To determine the adipocytes’ lipolytic rate, glycerol release was evaluated based on cells’ 24 h supernatants with a glycerol quantification kit (Randox, Ireland). Briefly, 55 µl samples of each attempt were used in a 1:5 dilution, mixed with 100 µl reagent and incubated for 20 min at RT in duplicates. Color development was read out at 520 nm (Omega Fluostar; BMG Labtech, Germany).

2.11 Acetylated low-density lipoprotein intake
By usage of the scavenger pathway mvECs are able to take in acetylated low-density lipoprotein (acLDL). To test this EC-functionality, Alexa Fluor™488-labeled acLDL (Invitrogen, Germany) was mixed 1:1000 in EGM-2 and cells were incubated for 4 h at 37 °C. After a rinsing step with PBS, nuclei were counterstained with 0.1 µg/ml Hoechst 33342 (Sigma Aldrich, Germany) for 15 min at 37 °C. Finally, cells were imaged in PBS at RT with an Axio Observer microscope (10x objective) and an Axiocam 506 mono using the software ZEN (all Zeiss, Germany).

2.12 Statistics
All experiments were repeatedly performed, using cells from at least three different biological donors. Outliers were identified by a Grubbs’ test and excluded from the data set. The obtained data was compared by a one-way analysis of variance (ANOVA)
with repetitive measurement and a Tukey post-hoc test using OriginPro 2017. Vascularization was evaluated with a Wilcoxon test. Statistic significances were stated as $p < 0.05$, very significant as $p < 0.01$ and highly significant as $p < 0.001$.

### 3 Results

The development of vascularized AT equivalents to replace soft tissue and to generate *in vitro* test systems is still a major challenge. A vascular network is indispensable for the sufficient supply of a construct. The prerequisite for a successful setup of the model are suitable culture conditions. In this study, we evaluated the suitability of a developed defined co-culture medium for the setup of an adipocyte/EC co-culture model with regard to cell maintenance in diffASCs and mvECs mono-cultures, as well as in an indirect and direct co-culture attempt of both cell types.

#### 3.1 Defined co-culture medium facilitates functional diffASC mono-culture

To test and compare their general suitability ACM, CoM and ECM were used to mono-culture diffASCs for 14 days. Media’s influence on cell viability was evaluated with a live/dead staining and an IF staining against perilipin A was performed to test for adipocyte specific characteristics. Additionally, accumulated lipids were visualized and analyzed quantitatively via an Oil-red-O staining (Fig. 2).

Although diffASCs’ viability was high in all approaches, ECBM-based media (CoM and ECM) led to a higher viability compared to ACM (Fig. 2A-C). In all culture attempts, many perilipin-A-positive cells could be found. However, each attempt as well exhibited some non-expressing cells, indicating that not all of the present cells committed to the adipogenic lineage or that some of the cells dedifferentiated during the maintenance phase (Fig. 2D-F). The level of perilipin A expression was found to be the lowest in ACM. Additionally, in contrast to the ECBM-based media some of the diffASCs in ACM still exhibited an elongated, fibroblast-like morphology and incorporated both less and smaller lipid vacuoles, compared to cells in the other test media (Fig. 2G-I).

Lipid storage of diffASCs after 14 days of differentiation (day 0) was set as 100 %. The quantitative analysis demonstrated an increase in incorporated lipids in all media after 7 and 14 days with the lowest content observed in ACM with 178 ($\pm$ 31.5) % and 230 ($\pm$ 75.6) % (Fig. 2J). Comparable results have been obtained during the culture in ECM (198 ($\pm$ 27.2) % and 271 ($\pm$ 72.4) %) and CoM (208 ($\pm$ 39.6) % and 320 ($\pm$ 84.4) %). CoM was significantly higher compared to all other attempts except for ECM on day 14, indicating a better adipogenic development in the composed co-culture medium (overview of detailed data in Tab. S1, S2, S3).

![Fig. 2: DiffASC mono-culture on day 14](image)

(A-C) FDA/PI viability staining: living cells are shown in green, dead cells in red. (D-F) Perilipin A/DAPI staining: specific marker expression is shown in green, nuclei are stained in blue. (G-I) Oil-red-O staining: lipids were stained with Oil-Red-O, visible in red. (J) Quantitative analysis of lipid accumulation of diffASCs on day 7 and day 14 in mono-culture; lipid storage of diffASCs immediately after differentiation (day 0) was set as 100 %. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, scale bar: 200 μm. Data were evaluated from three independent donors in duplicates. Quantitative data are displayed as means. Representative images were selected from one donor.

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3.2 Defined co-culture medium facilitates functional mvEC mono-culture

To evaluate cell morphology and prove the expression of EC-specific markers, CD31 and vWF were evaluated next to a live/dead staining for mvECs on day 14 in the test media (Fig. 3). In the mono-cultures of mvECs, ACM alone did not allow for proper cell attachment. In contrast, visible mvECs were viable on day 14 in both, CoM and ECM and build a confluent monolayer including the EC-specific cobblestone-like morphology as well as vWF and CD31 expression. The acLDL uptake of mvECs was analyzed to evaluate adequate EC functionality. After 14 days of maintenance, mvECs in all media but ACM remained their ability to take up acLDL.

Fig. 3: MvEC monoculture on day 14
(A-C) FDA/PI - viability staining: living cells are shown in green, dead cells in red. (D-F) CD31/DAPI staining: CD31 expression is shown in red, nuclei are stained in blue. (G-I) VWF/DAPI staining: vWF expression is shown in red, nuclei are stained in blue. (J-L) AcLDL assay: acLDL incorporated in cells is shown in green, nuclei were stained in blue, scale bar: 200 μm. Data were evaluated from three independent donors in duplicates. Representative images were selected from one donor.

3.3 Defined co-culture medium supports indirect diffASC/mvEC co-culture

To test diffASCs’ and mvECs’ behavior in the media in a more physiological co-culture attempt and elucidate a possible influence of each other cell type through soluble factors, an indirect co-culture study was performed for 14 days.

As seen in the mono-culture attempt, the IF staining and the Oil-red-O staining demonstrated a higher perilipin A expression, a bigger amount of lipid vacuoles and a more roundish cell morphology in the ECBM-based media compared to ACM (Fig. 4G). In all approaches, the diffASCs showed an increase in lipid accumulation between day 0 and day 7 but then in contrast to the results received in the mono-culture attempt (displayed in 3.2) decreased their lipid levels non-significantly until day 14 (Fig. 4G and S2). The lowest lipid accumulation was seen in ACM mono-culture (197 (± 103.9) % and 130 (± 27.3) %).

To determine the functionality and lipolytic rate of diffASCs, the release of leptin and glycerol were measured in the cell supernatants on day 7 and 14 of indirect diffASCs-mvECs co-culture. The levels immediately after differentiation were set as 100 %, respectively (Fig. 4G-I).

On day 7 leptin release of diffASCs significantly increased, compared to diffASCs immediately after differentiation (day 0) in all test media. This supports the overall hypothesis that diffASCs further gained adipocyte-specific functions during the continuing culture period. Comparable levels of leptin release were reached in ACM and ECM (Fig. 4H and S2). The highest levels were detected in CoM with 3766 (± 2642.3) % and 2195 (± 1579.9) % and the lowest in ACM mono-culture with 858 (± 622.7) % and 76 (± 124.9 %). In general, on day 14 the leptin release declined in all media. However, the percentages were still situated above the base level on day 0, which is consistent with the decreasing lipid content between day 7 and day 14.
14. CoM led to the highest values in lipid accumulation and leptin release in all attempts, highlighting its superior influence on adipocyte development. This conclusion is especially supported on day 7 when CoM led to significantly increased leptin values compared to the other media. While lipid accumulation in ACM was observed to be independent of the presence of ECs, leptin release was increased in the presence of mvECs, indicating a higher diffASCs functionality.

Glycerol release was comparable in ACM, in CoM, and ECM (Fig. 4I and S2). In general glycerol levels decreased in all attempts between day 7 and day 14 (significantly for ACM in mono-culture with 14 (± 12.6)% on day 14, which was primarily preceded by a remarkable rise to 137 (± 198.8)% on day 7.

After 14 days in indirect co-culture with diffASCs, mvECs in CoM and ECM again orientated themselves in even monolayers, throughout expressed CD31 and vWF and were able to take up acLDL (Fig. 5). As well in line with the results displayed in 3.2, mvECs in ACM showed poor cell attachment in ACM and were unable to build an even monolayer. However, in contrast to mvECs mono-cultured in ACM, some cells were still adherent on day 14 and showed some extent of marker expression, even if no functional acLDL uptake was measurable.

3.4 Direct co-culture supports the maintenance of diffASCs and mvECs and leads to the formation of vascular-like structures in defined conditions

DiffASCs and mvECs were co-cultured in CoM for 14 days to evaluate possible benefits based on a direct cell-cell interaction (Fig. 6).

After 2 weeks in direct co-culture most diffASCs still exhibited perilipin A expression and accumulated lipids, visible by the Oil-red-O staining. However, some cells without associated adipocyte markers were observed. In both, the mono- and the co-culture attempt, the levels of accumulated lipids increased highly significant to 280 (± 98.7)% and 277 (± 93.6)% on day 7, respectively, compared to 100% (± 4.8)% on day 0. Until day 14, only a small, non-significant decrease was recorded (Fig. 6A and S3).

Glycerol release increased from 100 (± 9.1)% on day 0 to 148 (± 47.5)% on day 7 and to 236 (± 79.1)% on day 14 in mono-culture. In the co-culture attempt, glycerol increased to 229 (± 78.1)% on day 7 and stayed at 229 (± 89.3)% on day 14 (Fig.
6C and S3). Thereby all attempts significantly increased and only the mono-culture attempt on day 7 showed relatively low levels of released glycerol.

MvEcS exhibited a strong expression of CD31. Remarkably, mvEcS formed vessel-like structures, which were organized in a vascular-like network in-between the diffASCs. Such structures were not observed in the mono-culture of mvEcS with the same donors at all (see Table on vascularization in Fig. 6I). The developed vascular structures in the co-culture attempt were evaluated quantitatively in terms of the total structure length per area, the number of structures per area and eventually existing nodes within the structures. Structure lengths in co-culture showed absolute values of 4.9 (± 7.24) mm/cm². With values normalized to 100 % within the donors they showed a standard deviation of ±31.8 %. The amount of structures was 7.6 (± 7.16) /cm² in average, respectively 100 (± 32.5 %). Within the found structures, nodes were detected with 1.1 (± 2.57) nodes/mm structure in co-culture. In the higher magnifications of Fig. 6H, both, close proximity of mvEcS with diffASCs and with undifferentiated ASCs are visible by stained nuclei with and without a surrounding perilipin A expression.

In the co-culture attempt some cells with stored acLDL were visible. However, their orientation suggests that these cells did not match with the cells of the vascular-like structures at all events.

**Fig. 5: MvEcS in indirect co-culture with diffASCs on day 14**

(A-C) CD31/DAPI staining: CD31 expression is shown in red, nuclei are stained in blue. (D-F) VWF/DAPI staining: vWF expression is shown in red, nuclei are stained in blue. (G-I) AcLDL assay: acLDL incorporated in cells is shown in green, nuclei were stained in blue, scale bar: 200 μm. Data were evaluated from three independent donors in duplicates. Representative images were selected from one donor.

### 4 Discussion

Previously reported data like the setup of a functional vascularized adipose tissue constructs by Sorrell et al. (Sorrell et al., 2011) showed great progress towards the actual application of tissue engineered constructs. In this study, we aimed to develop a defined co-culture medium, which has the potential to bring great benefit to many existing attempts of vascularized adipose tissue engineering without ethical concerns associated to the use of FBS.

Within the analysis of the general suitability of the media, ECBM-based media showed a superior effect on diffASCs’ survival and differentiation compared to the adipocyte-specific medium ACM. CoM resulted in the best adipocyte maturation. ACM and CoM both contain the adipocyte-specific factors (MAIN) and resulted in significantly different levels of lipid accumulation. According to this, it can be concluded that ECM is supplemented adequately to not only fulfill mvEcS’ but as well diffASCs’ requirements, independent of the cell type. It seemed as diffASCs are not completely dependent on the classical adipogenic factors in ACM as long as the basal medium is well composed and they are supplied with a variety of growth factors. However, the factors and nutrients in ECM and the AC–specific factors synergistically support adipocyte maintenance.

A functional EC culture is, amongst others, characterized by the expression of EC-specific markers like vWF and CD31, the ability to take up acLDL and the formation of dense monolayer, including tight cell-cell contacts. Especially the later represents an important feature in vivo to maintain ECs barrier function (Bazzoni and Dejana, 2004). The adipogenic supplements, added in ACM and CoM, did not have a visible positive or negative effect on the behavior of mvEcS. However, ACM alone did not support a functional EC culture, seen by almost complete cell detachment on day 14. This clearly highlights...
a strong reliability of mvECs on the presence of EC factors like vascular endothelium growth factor (VEGF) and insulin-like growth factor, which we were already confirmed before in studies based on serum-containing media by us (Volz et al., 2017; Huber et al., 2016a). Concluding, based on the mono-culture evaluation, CoM and ECM both represent suitable SF and defined media for a diffASC/mvEC co-culture attempt, whereby CoM somewhat lies ahead with regard to diffASC culture.

To test a mutual influence on cells’ reaction in the different test media via paracrine signaling, diffASCs and mvECs were co-cultured in an indirect setting for 14 days post differentiation. In the main, the results obtained in the mono-culture attempts were reproduced here. Lipid accumulation, perilipin A expression and leptin release were again higher in CoM and ECM compared to ACM in the indirect co-culture setting, which confirms these two as the most suitable media with regard to adipocyte development. The direct comparison of mono- and co-cultured diffASCs did not reveal any differences in lipid accumulation and thereby no visible support of adipogenic differentiation by the presence of mvECs was observed, as shown by others and a study by ourselves before (Yao et al., 2013a; Fukumura et al., 2003; Volz et al., 2017). However, others substantiated an anti-adipogenic effect of ECs in crosstalk (Rajashekhar et al., 2008). Ultimately, ECs’ role in adipocyte development appears to depend on deviating conditions. The significantly increased leptin levels in ACM co-culture compared to the mono-culture trial suggest a supportive role of mvEC signaling in diffASCs functionality. This hypothesis is supported by the significantly elevated glycerol levels in ACM mono-culture on day 7, which indicate an early induction of lipolysis possibly induced by dedifferentiation (Marcinkiewicz et al., 2006; Huber and Kluger, 2015). Following, the very low glycerol levels in ACM mono-culture on day 14 might be explained by the low remaining lipid levels in the culture due to early dedifferentiation in the absence of ECs.

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**Fig. 6: DiffASCs and mvECs in direct co-culture on day 14**

(A) Quantitative analysis of lipid accumulation. (B) Quantitative analysis of leptin release of diffASCs. (C) Quantitative analysis of glycerol release; values of diffASCs immediately after differentiation (day 0) were set as 100 %. (D) Oil-red-O staining: lipids were stained with Oil-red-O and are visible in red. (E) AcLDL assay: acLDL incorporated in cells is shown in green, nuclei were stained in blue. (F-H) Perilipin A/CD31/DAPI staining: specific marker expression is shown in green and red, nuclei were stained in blue. (I) Quantitative analysis of vascularization on day 14; values of vascular-like structures on day 14 in co-culture were set as 100 %, *p < 0.05, **p < 0.01, ***p < 0.001, scale bar: 200 μm, n.i. = not investigated, n.d. = not detectable. Data were evaluated from three independent donors in duplicates. Quantitative data are displayed as means. Representative images were selected from one donor.
In contrast to the mono-culture trials, lipid contents decreased in between day 7 and 14 in the co-culture attempts and the mono-culture control. This difference might be explained by the altered medium volumes, which were three times as high in the indirect co-culture compared to the mono-culture attempts. Adipogenic differentiation and lipogenesis is known to be dependent on autocrine stimulation e.g. with angiopoietin II, insulin-like growth factor-I (IGF-I), and VEGF or adiponectin (reviewed in (Huber et al., 2015c; Volz et al., 2016)), which might have been concentrated lower due to the increased medium volume. However, the incorporated lipids and leptin even more stayed at levels clearly above those of day 0, while glycerol decreased to values below the base level in all attempts on day 14, indicating a low lipolytic state of the adipocytes in general (Galic et al., 2010). Thereby the results show a well-developed cell functionality, especially in CoM and support the hypothesis of a self-equilibrating state of homeostasis which developed over the 14 days of culture.

In contrast to the mono-culture attempt in ACM, displayed in chapter 3.2, some mvECs were still preserved and adherent in the co-culture attempt on day 14. The maintained cells exhibited EC-specific marker expression of CD31 and vWF. DiffASCs potentially produced some pro-endothelial or pro-angiogenic factors. Feasible supportive factors might have been angiopoietin II, basic fibroblast growth factor, hepatocyte growth factor, VEGF and leptin (Volz et al., 2016). However, the levels were not sufficient to compensate the absence of endothelial-specific factors, which were only available in ECM and CoM in which mvECs showed adequate maintenance. To sum up, CoM is as well suitable for the indirect co-culture of diffASCs and mvECs. The mutual crosstalk therein has to be part of future investigations. Furthermore, a possible support of diffASCs by soluble mvECs factors in the adipocyte factor-free ECM has to be examined.

CoM also proved to be very functional in the direct co-culture of diffASCs and mvECs. DiffASCs maintained and further increased their accumulated lipids as well as their perlipin A expression and leptin release, while mvECs kept their cell-specific marker expression. Additionally, the conditions including the culture medium facilitated and supported the formation of vascular-like structures in the presence of diffASCs.

Glycerol and lipid levels uniformly increased after day 0 in all attempts and confirm a further development of the diffASCs. The elevated glycerol levels are in accordance with early findings correlating lipid stores with glycerol release (Jacobsson and Smith, 1972) and suggest an increase in the basal lipolytic rate. Leptin again showed a disproportional elevation in the direct culture, 20 times above the values detected on day 0. Very high leptin levels hereby indicate a fundamental increase in cells’ functionality. The absence of noticeable differences between the mono- and co-culture attempts do not suggest a possible beneficial influence of mvECs on diffASCs differentiation. From other groups, both a pro-differentiating (Yao et al., 2013a; Aubin et al., 2015) and a triggered dedifferentiating effect on adipocytes by the presence of ECs has been reported (Hutley et al., 2001; Rajashekhar et al., 2008; Aoki et al., 2003; Moya et al., 2010). Future investigations are necessary to evaluate the endothelial-derived effects (beneficial or adverse) on a more molecular level. Further optimizations in the physiology of the model might allow to intensify EC’s effect and permit its detection more easily. The maintenance of not only lipid and glycerol, but also leptin levels might be led back to a homeostatic state of the culture, which was adjusted in a self-organized manner between day 0 and 7 and maintained until day 14.

In direct co-culture, cells with a noticeable acLDL uptake were present. The assignment of the functionality to mvECs was however difficult. We detected a noticeable acLDL incorporation in mono-cultures of diffASCs in CoM in another setup (data not shown). This functionality has been reported for asc before, particularly after ASC culture in EC medium or as a response to the application of shear stress, but to some extent as well in the undifferentiated state (Ikegame et al., 2011; Chen et al., 2014). In the mentioned studies, this function has been attributed to the endothelial differentiation of ASCs. So far acLDL uptake has neither been associated to adipogenic differentiated ASCs nor to mature adipocytes. However, we now received the result of acLDL uptake by diffASCs, evident by the simultaneously visible lipid droplets. Further investigations will have to uncover, whether this occurrence is conditioned by a starting trans-differentiation of diffASCs or an independently existing property of these cells.

In the direct co-culture setting, a marked beneficial effect towards the formation of vascular-like structures has been observed. It has been shown before that ASCs support the formation of vascular-like structures and their maturation and maintenance through soluble factors like fibroblast growth factors, VEGFs, hepatocyte growth factor or transforming growth factor-β and Angiopoietin I and II (Zhang et al., 2015; Lin et al., 2016; Xue et al., 2017; Xie et al., 2016; Strassburg et al., 2016). The results obtained from mono- and indirect co-culture confirm the presence of non-differentiated or dedifferentiated ASCs in all attempts and support the hypothesis of a mutual beneficial effect of ASCs on the formation of vascular-like structures. However, the development of vascular-like structures was not detected in the indirect co-culture setting; despite the paracrine signaling would have been possible through the shared culture medium. Therefore, angiogenic processes must have been based on a direct cellular interaction e.g. by functioning as perivascular cells and supporting long-term maintenance of formed structures as described by Rohringer et al. and others (Merfeld-Clauss et al., 2010; Rohringer et al., 2014; Cun et al., 2015). A possible way of interaction between ECs and perivascular cells has been reported via the expression of platelet derived growth factor β by ECs and its subsequent binding to the extracellular matrix and by mural cell types earlier by Gaengel et al. (Gaengel et al., 2009). This condition may additionally serve as explanation for the absent support of adipocyte function in co-culture as found in the indirect attempt. MvECs might have still supported adipocyte development in direct co-culture. However, they equally triggered the development of supporting perivascular cells of (diff)ASCs, which led to a compensation of the effect. Furthermore, as diffASCs were seeded and differentiated 2 weeks prior to mvECs, they were probably able to synthesize and embed different tissue specific extracellular matrix components like collagen type IV, laminin or fibronectin as described before (Sorrell et al., 2011; Kubo et al., 2000). In fact, the found cellular structures have spread over several levels in the culture attempt, which became evident through microscopic imaging over hundreds of µm. This observation supports the assumption of the development of a 3D-like arrangement of matrix and cellular structures in the culture. Some of these matrix components might not only have facilitated enhanced EC attachment, but as well mediated EC migration, sprouting and vessel stabilization during angiogenetic processes as elsewhere (Strassburg et al., 2016; Strassburg et al., 2016; Strassburg et al., 2016). These assumptions are supported by beneficial effects on angiogenic processes by decellularized AT e.g. shown by Sorrell et al. (2011). Additionally, the enhanced binding and presentation of angiogenic factors, derived from the medium or released by diffASCs, might have supported angiogenic processes of ECs (reviewed in (Neve et al., 2014)).
determination of the concrete components, responsible for the enhanced formation of vascular-like structures has to be part of
continuative trials. To avoid a possible overlap of beneficial influencing by matrix components and cellular signals, solely the
influence of decellularized matrix components on angiogenetic processes should be evaluated. Independent of the direct or
indirect pathway, the developed defined co-culture medium facilitated a sufficient level of crosstalk between the included cell
types to allow the formation of vascular-like structures and their maintenance and may therefore be rated as appropriate for the
set-up of adipocyte/EC co-cultures in terms of engineering vascularized AT. As the 3D arrangement was as well confirmed to
support adipogenic differentiation next to angiogenetic developments, future attempts have to address a 3D co-culture, in
constructs of several mm in each dimension (Miyamoto et al., 2017). Consecutively, the 3D arrangement promises a further approach to the in vivo situation.

5 Conclusion and Outlook

With this current work, we developed a suitable SF and defined adipocyte/EC co-culture medium, which not only keeps
diffASCs and mvECs characteristic and functional over time but as well allows for sufficient adipocyte/endothelial crosstalk
and in consequence supportive effects on each other cell type including the formation of vascular-like structures. Based on this,
we managed to build and maintain a vascularized AT model under completely defined medium conditions. Through the
replacement of serum at maintained ability of de novo angiogenesis by the cellular components, the system thereby represents
a very valuable alternative to classically used serum-based attempts. Furthermore, the model promises higher robustness,
reproducibility and reliability of the achieved results. In future attempts, the system’s ability to represent native human tissue
adequately has to be further evaluated, to allow for its validation as in vitro model. Consequently, the model may be applied
alternatively to currently used animal trials for the elucidation of disease mechanisms, the screening for new drugs and the
assessment of corresponding safety levels. In combination with existing attempts to engineer epidermis and dermis, the defined
adipose tissue model may furthermore be used to set up a full skin equivalent, which is also urgently needed as in vitro test
system. The developed CoM may additionally be used for the setup of other tissue approaches after some cell specific
adjustments and thereby contribute to a general reduction of the use of FBS in cell and tissue culture. Taken together, the
displayed results concerning a defined co-culture medium may reduce the number of required animals by both, the reduction of the use of FBS in vitro and the replacement of currently performed animal trials as such by the use of in vitro models with high predictive power.

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Conflict of interest statement
The authors declare no conflict of interest.