# Cell-derived Extracellular Matrix - a promising Biomaterial for in vitro-vascularization in Adipose Tissue Engineering

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**Abstract**

Tissue constructs of physiologically relevant scale require a vascular system to maintain cell viability. However, in vitro vascularization of engineered tissues is still a major challenge. Successful approaches are based on a feeder layer (FL) to support vascularization. Here, we investigated whether the supporting effect on the self-assembled formation of vascular-like structures by microvascular endothelial cells (mvECs) originates from the FL itself or from its extracellular matrix (ECM). Therefore, we compared the influence of ECM, either derived from adipose-derived stem cells (ASCs) or adipogenic differentiated ASCs, with the classical approaches based on a cellular FL. All cell-derived ECM (cdECM) substrates enable mvEC growth with high viability. Vascular-like structure formation was visualized by immunofluorescence staining of endothelial surface protein CD 31 and can be observed on all cdECM and FL substrates but not on control substrate collagen I. On adipogenic differentiated ECM longer and higher branched structures can be found compared to stem cell cdECM. An increased concentration of pro-angiogenic factors can be found in cdECM substrates and FL approaches compared to controls. Finally, expression of proteins associated with tube formation (E-selectin and thrombomodulin) was confirmed. These results highlight cdECM as promising biomaterial for in vitro vascularization in adipose tissue engineering.



## 1. Introduction

Adipose tissue is a highly metabolic and vascularized tissue. In native tissue, a dense capillary network provides the supply of the residing cells with nutrients and inspiratory gases and the removal of waste products. Since the diffusion limit of oxygen is less than 200 µm (Olive, Vikse, & Trotter, 1992; Thomlinson & Gray, 1955), the centers of large tissue constructs experience necrosis and volume loss without a functional vascular network. Consequently, there is an urgent need for fast vascularization after implantation of adipose tissue implants to maintain tissue mass and viability. In addition, for the *in vitro*use of tissue constructs, for example as a testing system, a stable functional vascular system would be desirable to allow constructs of a larger size and to maintain comprehensive cell behavior. Furthermore, such vascularized tissue constructs would allow *in vitro*investigations regarding the development and therapy of vascular diseases. The inclusion of a functional vascular system remains one of the biggest challenges in three- dimensional (3D) tissue engineering approaches. To date there are several strategies to vascularize engineered 3D tissue constructs, e.g. functionalized scaffolds, perfusion bioreactors, co-culture and *in vivo* approaches (Lovett, Lee, Edwards, & Kaplan, 2009). Pro-angiogenic factors immobilized in the scaffold material were found to enhance vascularization (Laschke et al., 2008; Yoon, Chung, Lee, & Park, 2006). For example, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which are known to induce vessel formation and platelet-derived growth factor (PDGF) β which supports stabilization of the newly formed vessels (Gaengel, Genove, Armulik, & Betsholtz, 2009). Different co-culture systems, using monolayer or spheroid cultures, demonstrated the spontaneous formation of vascular-like structures (Walser et al., 2013; Wenz, Tjoeng, Schneider, Kluger, & Borchers, 2018). In particular, the co-culture of

endothelial cells (ECs) with adipose-derived stem cells (ASCs) showed a beneficial effect on vessel maturation and sprouting (Verseijden et al., 2012; Volz, Hack, Atzinger, & Kluger, 2018). ECs have a reciprocal effect on pre-adipocyte proliferation and differentiation (Aoki, Toda, Sakemi, & Sugihara, 2003). With these techniques, vascularization has been achieved directly*in vivo* or after implantation of the engineered tissue constructs (prevascularization) (Laschke, Strohe, et al., 2009; Laschke, Vollmar, & Menger, 2009). So far there is no successful *in vitro* approach with a physiological and functional vascular system, which ensures adequate stability and reproducibility. In most approaches, some type of feeder cells are used to support the formation of vascular-like structures by ECs. This living cellular part impedes a commercial application due to the difficult handling and storage. In contrast, lyophilized acellular biomaterials can be stored for long periods of time. In addition, acellular biomaterials evoke far fewer concerns regarding their admission for regenerative medicine compared to the approaches including living cells. However, during dehydration of natural materials changes in structure and composition may occur. Thus, it has to be clarified if the processing of the biomaterial affects its ability to influence cellular behavior. To address this issue, next to the effect of the wet hydrogel-like form, the effect of the dehydrated materials on cellular behavior should be investigated.

A critical requirement for engineering tissue constructs is the use of a suitable scaffold that provides appropriate biological and physicochemical properties. The cell surrounding material also plays an important role in vascularization. There are several synthetic and natural scaffold materials used for vascularized tissue engineering approaches, e.g. polylactic acid, polyethylene glycol, collagen or hyaluronic acid. However, the extracellular matrix (ECM) as the natural environment of the cells *in vivo* represents the most physiological biomaterial. A variety of ECM-hybrid materials and pure decellularized ECM were investigated towards their ability to support stem cell differentiation and (neo)vascularization *in vivo* and*in vitro* (Adam Young, Bajaj, & Christman, 2014; Badylak, Freytes, & Gilbert, 2009; L. Flynn, Prestwich, Semple, & Woodhouse, 2009; L. E. Flynn, 2010). All these studies were performed with decellularized ECM derived from native tissue. For several years, another source of natural ECM moves to the fore. *In vitro*generated cell-derived ECM (cdECM) from different cell-types (e.g. fibroblasts and ASCs) was isolated and used as a biomaterial in a variety of applications (Lu, Hoshiba, Kawazoe, & Chen, 2011; Lu, Hoshiba, Kawazoe, Koda, et al., 2011; Sart et al., 2016; Schenke-Layland et al., 2009; Wolchok & Tresco, 2010). Several studies show that cdECMs, obtained from different cell-types, can induce adipogenic, chondrogenic and also osteogenic differentiation of ASCs indicating its influence on cell fate (Dzobo et al., 2016; Guneta, Loh, & Choong, 2016; Guneta et al., 2017; Guo et al., 2013).

Our previous study revealed the spontaneous formation of vascular-like structures by mvECs in co-culture with adipogenic differentiated ASCs (Volz et al., 2018). In this study, we aimed to analyze whether this effect has to be attributed to cell-cell or cell-matrix interactions. Consequently, we investigated the effect of dry and wet cdECM regarding its ability to support the formation of vascular-like structures by mvECs. Furthermore, we tested whether there is a difference between ECM derived from stem cells and adipogenic differentiated cells regarding their capability to induce vascular-like structure formation.

## 2. Materials and Methods

All research was carried out in accordance with the rules for the investigation of human subjects as defined in the Declaration of Helsinki. Patients provided written agreement in compliance with the Landesärztekammer Baden-Württemberg (F-2012- 078, for normal skin from elective surgeries).

### 2.1. Cell isolation and expansion

ASCs were isolated from human tissue samples obtained from patients undergoing plastic surgery (Dr. Ziegler; Klinik Charlottenhaus, Stuttgart, Germany) as described before (Huber, Borchers, Tovar, & Kluger, 2016). ASCs were initially seeded at a density of 5x103 cells/cm2 in serum-free Mesenchymal Stem Cell (MSC) Growth Medium (MSCGM, PELOBiotech) containing 5 % human platelet lysate. ASCs were used up to passage three.

Dermal microvascular ECs (mvECs) were isolated from juvenile foreskins (Dr. Yurttas, Stuttgart, Germany)

as described before (Volz, Huber, Schwandt, & Kluger, 2017). Briefly, dermis was cut into small pieces and digested in a dispase solution (2 U/ml; Serva Electrophoresis, Germany) overnight at 4 °C. After the removal of the epidermis, mvECs were isolated from the dermal layer by incubation with 0.05 % trypsin in ethylenediaminetetraacetic acid (EDTA; Life Technologies, Germany) for 40 min at 37 °C and mechanically isolated in mvEC Growth Medium-2 (EGM-2mv; Lonza, Switzerland). For cell expansion, mvECs were seeded with 5 x $10^3$ cells/$cm^2$. MvECs were used up to passage three.

2.2. Generation of cell-derived extracellular matrix substrates and ASC feeder layer

ASCs were seeded into 8-well chamber slides (ibidi, Germany) and 24-well plates respectively at a density of 25 x103 cells/cm2 in serum-free MSCGM containing 5 % human platelet lysate. At confluency, medium was changed to either serum-containing GM (Dulbecco's Modified Eagle Medium (DMEM) with 10 % fetal calf serum (FCS) = scdECM) or adipogenic differentiation medium (DMEM with 10 % FCS, 1 µg/mL insulin, 1 µM dexamethasone, 100 µM indomethacin, 500 µM 3-isobutyl-1- methylxanthine = acdECM) both supplemented with 50 µg/mL Na-L-Ascorbate. The medium was changed every other day. At day 7, cells were lysed using hypotonic ammonium hydroxide solution and ECM was washed with ultrapure water. For dry ECM approaches (= dry), ECM was dried at room temperature (RT) and for wet ECM approaches (= wet), ECM was stored in ultrapure water until seeded with mvECs. Cellular substrates (= FL) were seeded with mvECs without lysis of ASCs (Figure 1).

2.3 Microscopic pictures and degree of swelling

Macroscopic pictures of wet cdECM substrates were taken directly after cell removal. To investigate the water uptake, respectively the degree of swelling, lyophilized cdECMs was weighed to determine the dry weight [weight (dry cdECM)]. Subsequently, cdECMs were swollen in demineralized water for 24h at RT and weighed again [weight (swollen cdECM)].

The degree of swelling was calculated as:

$$\text{Degree of swelling } [\%] = \frac{\text{weight (swollen cdECM)} - weight\ (dry\ cdECM)}{weight\ (dry\ cdECM)} \text{x } 100 \quad (1)$$

2.4 Immunofluorescence staining of fibronectin and picro sirius staining of ECM substrates

For immunofluorescence (IF) staining of fibronectin, cdECM substrates were fixed in 4 % paraformaldehyde (Carl Roth, Germany) for 10min followed by incubation with blocking solution, consisting of 3 % bovine serum albumin (Biomol, Germany) in 0.1 % Triton X (Sigma Aldrich, Germany) for 30 min to block unspecific binding sites. Subsequently, the primary antibody (mouse anti-fibronectin, Santa Cruz, Germany; 1:200) was incubated for 1h at RT and secondary antibody (anti-mouse Cy3, Dianova, Germany; 1:250) was incubated for 30 min at RT. Both were diluted in blocking solution. For the histological picro sirius staining, fixed cdECM samples were dehydrated and blocked in paraffin. Histological sections (5 µm) were produced and stained with picro sirius according to manufacturer's protocol (Morphisto, Germany). Images were taken with an Axio Observer microscope and Axiocam 506 mono using the software ZENblue (Carl Zeiss, Germany).

2.5 Seeding of mvECs on cell-derived ECM and feeder layer

Isolated dry and wet cdECM substrates were re-seeded with mvECs at a density of 1 x 104 cells/cm2 in a defined mvEC adipocyte co-culture medium 9. For FL approaches mvECs were directly seeded on top of adipogenic differentiated and undifferentiated ASCs at a density of 1 x 104 cells/ cm2 in defined co-culture medium, developed by us earlier 9. Cells were cultured for 14 days and the medium was changed every other day (Figure 1). As a control, all experiments were performed on collagen I (rat tail; 250 µg/mL in 0.1 % acetic acid) coated tissue culture polystyrene (COL I) and uncoated tissue culture polystyrene (TC). All media were supplemented with 1 % penicillin/streptomycin.

2.6 Cytocompatibility

Cytocompatibility of the cdECM substrates was shown by the analysis of lactate dehydrogenase (LDH) in the cell culture supernatant. At day 3 after seeding, an LDH assay (TaKaRa Bio Europe, France) was

performed according to the manufacturer's instructions. To exclude the remaining LDH from cell lysis, LDH concentration from supernatant from cdECM substrates without mvECs was determined. Values were subtracted from the LDH concentrations measured from mvECs on the different cdECM substrates. On day 14, live-dead staining was performed to assess the viability of cultured cells. Before staining the cells were washed in phosphate-buffered saline (PBS, Biochrom, Germany) and subsequently treated with staining solution, consisting of 200 ng/ml fluorescein diacetate (FDA, Sigma Aldrich, Germany) and 20 µg/mL propidium iodide (PI, Sigma Aldrich, Germany) in DMEM, for 15 min at 37 °C. Finally, cells were imaged in PBS with calcium and magnesium at RT with an Axio Observer microscope and an Axiocam 506 mono camera using the software ZEN (Carl Zeiss, Germany).

2.7 Immunofluorescence staining of cell-specific proteins

For IF staining of cell-specific proteins, cells were fixed in 4 % paraformaldehyde for 10 min and permeabilized for 10 min with 0.1 % Triton X in PBS. Following, cells were incubated in blocking solution, consisting of 3 % bovine serum albumin in

0.1 % Triton X for 30 min to block unspecific binding sites. Primary antibodies (mouse anti-CD31, 1:50, Dako, Germany; rabbit anti-CD31, 1:200, abcam, GB; goat anti-E-selectin, 1:200, R&D Systems, USA; sheep anti-thrombomodulin, 1:200, R&D Systems, USA) were diluted in blocking solution and incubated for 2 h at RT. Secondary antibodies (anti-rabbit Alexa FluorTM 488, abcam, GB; anti-mouse Cy3, Dianova, Germany; donkey anti-sheep Alexa FluorTM 647, abcam, GB; donkey anti-goat Alexa FluorTM 594, abcam, GB) were diluted 1:250 incubated 30 min at RT. All antibodies were diluted in blocking solution.

2.8 Enzyme-linked immunosorbent assay

For characterization of cdECM substrates regarding growth factors composition, substrates were washed 3 days in culture medium. For the characterization of FL, medium from day 3 was collected. Determination of growth factors VEGF, bFGF and PDGFβ were performed using enzyme-linked immunosorbent assays (ELISA) (all PEPROTech, Germany) according to the manufacturer's instructions. The converted TMB was read out at 450 nm with a wavelength correction set at 620 nm (TECAN Saphire II, Tecan, Switzerland)

2.9 Statistics

All experiments were repeatedly performed, using cells from at least three different biological donors of ECs. The obtained data were compared by a one-way analysis of variance (ANOVA) with repetitive measurement and a Tukey post-hoc test using OriginPro 2018b. Statistical significances were stated as *p [?] 0.05, very significant as **p [?] 0.01 and highly significant as ***p [?] 0.001.

3. Results

3.1 Macroscopic pictures and degree of swelling

Macroscopic pictures show that wet scdECM and acdECM substrates exhibit a transparent gel-like appearance on the bottom of the petri dish (Figure 2). Determination of the degree of swelling of the different cdECM substrates revealed a higher water uptake capacity of acdECM (2357.6 (± 201.1) %) compared to the scdECM (1624.3 (± 96.4) %). IF staining of fibronectin revealed smaller pores in the scdECM substrates compared to the acdECM substrates. Images of the picro sirius staining showed more densely packed collagen fibers in the scdECM substrate compared to the acdECM substrate.

3.2 Acellular and cellular substrates are cytocompatible for mvECs

Cytocompatibility of the materials was determined by the measurement of the release of LDH after seeding with mvECs. LDH is an enzyme that is released during cell death and therefore can be used to quantify cytotoxicity. LDH release of mvECs seeded onto the different substrates was determined 3 days after seeding (Figure 3, A). The values of TC were set as 100 (± 3.5) %. For cdECM substrates, values were normalized to TC. Results showed no significant increase of released LDH of mvECs when seeded on COL I coating (89.4 (± 13.8) %), dry scdECM (113.7 (± 31.0) %); dry acdECM (108.0 (± 29.0) %), wet scdECM (96.3 (±

33.4) %), or wet acdECM (93.4 (± 29.0) %). For the stem cell and adipogenic differentiated FL substrates, values were normalized to stem cell FL approach without mvECs (FL stem cell), which was set as 100 (± 3.3) %. For adipogenic differentiated FL (FL adipogenic: 157.9 (± 13.4) %) approach, a higher LDH release was found compared to stem cell FL. As in the cdECM approaches, no significant increase in LDH release was observed when mvECs were seeded onto the FL for stem cell and adipogenic differentiated cells (FL stem +mvECs: 126.1 (± 15.8) %; FL ad +mvECs: 176.8 (± 25.0) %).

The viability of the mvECs cultured on the different substrates was assessed on day 14 after seeding with mvECs by live-dead staining with FDA and PI (Figure 3, B). Results show that mvECs were viable on all acellular and FL substrates on day 14 and only a few dead cells could be found.

3.3 Cell-derived ECM substrates support the formation of vascular-like structures by mvECs

To investigate the effect of cdECM substrates on the formation of vascular-like structures by mvECs, CD31 was visualized by IF staining (Figure 4). CD31 is a specific endothelial surface protein mainly localized on cell-cell connections and mainly responsible for the control of leukocyte transmigration *in vivo* (Piali et al., 1995). The staining pattern showed that mvECs grew to a confluent cell layer on all acellular substrates. The degree of structure formation on the different substrates was analyzed and quantified using ImageJ on basis of the CD31 IF images. The formation of vascular-like structures by mvECs was detected on all tested substrates in contrast to the controls (TC and COL I) on which no structure formation was observed. Quantification of structure lengths revealed longer structures for wet acdECM (433.5 (± 293.1) µm) substrate compared to both scdECM substrates (dry: 235.9 (± 100.0) µm; wet: 232.9 (± 183.8) µm). Dry acdECM (297.2 (± 149.1) µm) substrates exhibited a slightly but not significantly higher structure length compared to dry and wet scdECM substrates. Vascular structure lengths of mvECs cultured on the adipogenic FL (FL adipogenic: 483.5 (± 287.4) µm) were significantly longer than those of all other approaches except for wet acdECM substrate. Vascular structures on stem cell FL exhibited an average length per structure of 302.1 (± 168.7) µm. Another essential criterion for the maturation of a functional vascular network is the formation of nodes. Therefore, the number of nodes formed by the mvECs on the different substrates was quantified. No nodes could be detected on the controls TC and COL I. In adipogenic approaches (dry acdECM: 1.2 (± 1.1); wet acdECM: 1.7 (±1.2); FL adipogenic: 6.7 (± 3.9)) the number of nodes was higher compared to the stem cell approaches (dry scdECM: 0.7 (± 0.4); wet scdECM: 0.2 (± 0.6); FL stem cell: 1.7 (± 1.5)) for all substrates. Furthermore, the number of nodes on wet acdECM was slightly but not significantly higher compared to the dry acdECM and comparable to stem cell FL. The significantly highest number of nodes could be observed in the adipogenic FL approach. In sum, on scdECM substrates, many short structures were identified whereas on acdECM substrates longer and more branched structures were formed. By co-culture with the stem cell FL, the mvECs formed islets of a confluent layer within the ASCs and vascular-like structures sprouting from these islets were apparent. Long and highly branched vascular-like structures were formed by mvECs on adipogenic FL.

3.4 Quantification of pro-angiogenic factors on substrates

To confine which ECM components are responsible for its pro-angiogenic effect, the relative concentration of growth factors VEGF, bFGF and PDGFβ were determined in the supernatant after washing the acellular substrates for 3 days (Figure 5). Values were normalized to TC. Determination of VEGF revealed significantly higher concentrations for dry and wet acdECM substrates compared to all other acellular substrates (TC: 1 (± 0.2)fold; COL I: 0.9 (± 0.2)fold; dry scdECM: 1 (± 0.1)fold; dry acdECM: 1.4 (± 0.1)fold; wet scdECM: 1.1 (± 0.1)fold; wet acdECM: 1.5 (± 0.2)fold). For quantification of growth factor concentration on FL approaches cell culture supernatant from day 3 (according to the 3 days of washing of acellular substrates) was collected. A 10-fold higher concentration of VEGF can be found on FL approaches with no difference between stem cell FL and adipogenic FL (FL stem cell: 10.0 (± 0.3)fold; FL adipogenic: 10.0 (± 0.2)fold). For bFGF significantly higher concentrations can be found on cdECM substrates compared to controls (TC: 1 (±0.1)fold; COL I: 1.1 (±0.3)fold; dry scdECM: 2.6 (±0.2)fold; dry acdECM: 2.2 (±0.2)fold; wet scdECM: 2.4 (±0.1)fold; wet acdECM: 2.5 (±0.2)fold) and on FL approaches higher concentration can be found compared to all other substrates (FL stem cell: 6.1 (±1.2)fold; FL adipogenic: 7.0 (±0.7)fold). For PDGFβ a significantly

higher concentration can be found on cdECM substrates (dry scdECM: 3.6 (± 0.7)fold; dry acdECM: 3.5 (± 0.2)fold; wet scdECM: 3.5 (± 0.4)fold; wet acdECM: 3.3 (± 0.3)fold) compared to the controls TC (1 (± 0.2)fold) and COL I (0.9 (± 0.5)fold). Between the different cdECM substrates no difference in PDGFβ concentration can be measured. On FL substrates, a higher concentration of PDGFβ can be found compared to acellular substrates but no difference between stem cell and adipogenic differentiated approach (FL stem: 5.5 (± 0.3)fold; FL ad: 6.3 (± 1.0)fold) was observed.

3.5 Expression of proteins associated with tube formation in newly formed vascular structures

Recent studies showed that the expression of adhesion molecules E-selectin and thrombomodulin in ECs is associated with the tube formation of new blood vessels (Oh et al., 2007; Pan et al., 2017). Therefore, we investigated the expression of these proteins in the newly formed vascular-like structures (Figure 6). Results of the IF staining revealed the expression of neither E-selectin nor thrombomodulin in mvECs cultured on TC or COL I. However, all newly formed vascular-like structures showed expression of E- selectin and thrombomodulin on all cdECM substrates. E- selectin and thrombomodulin expression of the vascular-like structures were comparably high in the FL approaches. E-selectin and thrombomodulin staining corresponded to the CD31 staining pattern of the vascular-like structures (Supplementary figure 1).

4. Discussion

The implementation of a functional vascular system into an engineered tissue construct would address one of the major bottlenecks in tissue engineering and regenerative medicine. In the present study, we aimed to investigate the supportive effect of cdECM on the self-assembled formation of vascular-like structures by mvECs for its use as a biomaterial for adipose tissue engineering compared to the well-established application of a supportive FL.

Determination of the degree of swelling revealed higher capacity of water uptake of the adipogenic ECM compared to stem cell ECM. This effect can be explained by the higher pore size in adipogenic ECM shown by fibronectin and collagen staining. The higher pore size of adipogenic ECM may also be able to enhance the degree of vascular structure formation by ECs. Chui et al. showed that higher pore size is associated with a higher degree of neovascularization in an *in vitro* PEG hydrogel model (Chiu et al., 2011). Furthermore, Artel et al. proposed an agent-based model indicating that pores of larger size support vascularization in a polymer scaffold (Artel, Mehdizadeh, Chiu, Brey, & Cinar, 2011).

Analysis of LDH release of mvECs on the substrates revealed no cytotoxic effects of the cdECM substrates, the FL cells or the controls (COL I coating and TC). Even on day 14 after seeding, a confluent viable monolayer of mvECs was observed which indicates a good cytocompatibility of the cdECM substrates and their possible use in tissue engineering. For comprehensive toxicological and immunogenic characterization further analysis is required, e.g. the analysis of the ECM impact on the metabolic activity of the mvECs and for the intended *in vivo* use, biocompatibility of the ECM has to be evaluated.

Visualization of mvECs on day 14 after seeding by staining of the specific surface protein CD31 showed the self-assembled formation of vascular-like structures on all substrates except for the controls COL I and TC. Structure formation on the adipogenic FL approach was in line with our previous study 9 as a lower degree of structure formation was found on the stem cell FL approach. In addition, on dry and wet cdECM approaches, the degree of structure formation on adipogenic ECM substrates was higher compared to the corresponding stem cell approach, which is reflected by longer structures and a higher number of nodes. The effect of enhanced structure formation on adipogenic substrates could be explained by the different secretomes of ASCs and (pre-)adipocytes (Kapur & Katz, 2013). It is well known that ASCs secrete a broad spectrum of pro-angiogenic proteins and they were often used as a delivery system of growth factors and cytokines in vascularization approaches (Kondo et al., 2009; Liu et al., 2011; Moon et al., 2006; Nakagami, Maeda, Kaneda, Ogihara, & Morishita, 2005; Rehman et al., 2004). For example, Matusda et al. showed that conditioned cell culture medium of ASCs positively influenced EC proliferation and the formation of new vessels *in vivo* (Matsuda et al., 2013). During adipogenic differentiation, ASCs secret further pro-angiogenic factors like leptin. Leptin is known to be upregulated during adipogenic differentiation and was

shown to exhibit a pro-angiogenic effect itself but also upregulates the secretion of VEGF (Cao, Brakenhielm, Wahlestedt, Thyberg, & Cao, 2001). By secreting their specific set of proteins, ASCs and (pre-)adipocytes not only condition their cell culture medium but also their ECM which we use in this study as a biomaterial for induction of vascular formation by mvECs. Thus, cdECM does not only contain a set of specific factors, but a broad spectrum pro-angiogenic factors with its synergistic effects needed for the successful formation of vascular structures by ECs. Especially acdECM induces the formation of vascular-like structures and seems to be able to stabilize the newly formed structures.

The two most important pro-angiogenic factors are VEGF and bFGF. Results revealed higher VEGF concentrations released from acdECM substrates compared to scdECM approaches. On FL approaches high amounts of VEGF were found, most likely produced by FL cells. These results are in line with the degree of vascular-like structure formation. On acdECM approaches, longer and more branched structures were formed whereas on FL approaches the highest degree of structure formation occurred. Determination of the bFGF concentration in the different substrates revealed a higher concentration from cdECM substrates compared to controls and the highest bFGF concentration from FL approaches. Both factors – VEGF and bFGF – are able to induce the formation of new vascular structures (Marra et al., 2008; Murakami & Simons, 2008; Nissen et al., 2007; Tomanek, Hansen, & Christensen, 2008). Therefore, in our study, the induction of the formation of vascular-like structures may among other events, be attributed to the synergistic effect of available VEGF and bFGF. We further investigated the amount of pro-angiogenic factor PDGFβ from cdECM substrates. It is secreted by ECs during angiogenesis to attract perivascular cells, which stabilize the newly formed vessels 6. Further, PDGFβ was shown to induce vascular structure formation by modulating proliferation and tube formation of ECs (Battegay, Rupp, Iruela-Arispe, Sage, & Pech, 1994). PDGFβ can be found in all cdECM substrates as well as FL approaches. The PDGFβ concentration from FL approaches is higher compared to the other substrates which is in line with the higher degree of structure formation. *In vivo* , these growth factors are known to be partially bound to ECM after their secretion (Ostman, Andersson, Betsholtz, Westermark, & Heldin, 1991). To date, there are no studies investigating their binding capacity and protein half-life in *in vitro* generated cdECM.

A critical step in the formation of a new vascular system is the formation of a lumen in the vascular structure to enable perfusion with blood i*n vivo* and culture medium *in vitro* . Recent studies show that the adhesion proteins E-selectin and thrombomodulin are associated with tube formation. *In vivo* , E- selectin is mainly contributing to the binding of immune cells by mediating adhesive interactions of circulating leukocytes with the endothelium (Ley & Tedder, 1995). Nevertheless, it also plays a role in the homing of endothelial progenitor cells (EPCs) and therefore promotes neovascularization. Studies showed that E-selectin potentiates angiogenesis in ischaemic tissue, by mediating EPC- endothelial interactions (Oh et al., 2007). During this process of neovascularization, EPCs are mobilized from the bone marrow into the circulation and recruited to new sites of vascularization, using cues that resemble an inflammatory response. Therefore, E-selectin plays a crucial role in EPC homing and following neovascularization and tube formation. In this study, we use this protein as an indicator for a functional vascular structure. IF staining of E-selectin revealed specific expression of E- selectin almost exclusively on the newly formed vascular-like structures. Thrombomodulin is a transmembrane molecule expressed on ECs acting as an anticoagulant (Dahlback & Villoutreix, 2005; Dittman & Majerus, 1990). The fourth and fifth region of an epidermal growth factor (EGF)-like region of thrombomodulin (TME45) was shown to stimulate proliferation of human umbilical vein ECs and to promote tube formation and angiogenesis (Ikezoe et al., 2017). Therefore, we used this protein as an indicator for functionality of the newly formed vascular-like structures. In line with the results obtained by E-selectin staining, thrombomodulin was found to be expressed mainly in the mvECs contributing to the structure formation. Thus, we suggest that newly formed vascular-like structures exhibit promising characteristics to develop a functional vascular system. The expression of E-selectin and thrombomodulin and their function in tube formation and neovascularization *in vivo* represent promising characteristics when considering implantation of prevascularized constructs.

The chemical composition and physical properties of the ECM are highly tissue specific. Different components of the ECM, like collagens, fibronectin and laminin, were investigated towards their ability to mimic the

natural ECM (Huber et al., 2016; Lv, Bu, Kayser, Bausch, & Li, 2013; Rammelt et al., 2006). However, the highly complex composition of natural ECM could not be copied by a combination of the different components. Furthermore, ECM is biocompatible and can be remodeled by the residing cells (Badylak, 2007). In addition to the biological activity, like the induction of ASC differentiation (Guneta et al., 2017) and vascular-like structure formation by ECs shown in this study, there are several further advantages of cdECM. The amount of autologous ECM is limited to the donated tissue and allogenic ECM suffers from donor variability. Furthermore, donors vary in age, gender, BMI and different donor sites may also influence the chemical and physical properties which might impact the cell-ECM interaction. In contrast, the production of cdECM is scalable, as producing cells can be expanded in advance. Native ECM is usually derived from adult tissue state. AcdECM however, could be obtained from the developmental state of the tissue specific cells and therefore may provide more supportive signals for tissue development and regeneration. These signals can promote the differentiation of stem cells and support the formation of vascular structures by ECs. Also scdECM provides the ability to induce vascular-like structure formation by ECs and can be used for approaches addressing other tissues. For example evaluating its usability in vascularized tissue engineering of tissues producing low amounts of ECM by itself, e.g. liver, would be promising.

One critical point in the commercial application of biomaterials is the maintenance of their biological impact after processing and storage. The most common processing for preserving biomaterials is drying. In this study, it could be shown that dry cdECM partly maintains its biological properties regarding the induction of the self-assembled vascular-like structure formation of ECs with some restrictions. However, drying of the cdECM would be a conceivable method for improving storage possibilities when necessary.

## 5. Conclusion

In the present study, we demonstrated that cdECM (as a dry coating and as a wet hydrogel-like form) is able to induce the self-assembled formation of vascular-like structures by mvECs and helps to support their maintenance. Thereby, this study revealed a promising application of acdECM as a biomaterial in adipose tissue engineering approaches. The present study clearly confirms acdECM as a promising material for vascularized adipose tissue engineering by supporting the formation of vascular structures. In addition, also scdECM provides the ability to induce vascular-like structure formation and can be used for approaches addressing other tissues. Further investigations regarding other lineage-specific cdECMs and the transfer from 2D cell culture to 3D cell culture should be pursued. Thereby the combination with other materials, allowing the direct adjustment of geometry and relevant physical parameters should be addressed.

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## Conflicts of interest

The authors declare no conflict of interest.

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**Figure legends**

**Figure 1: Schematic overview of the study procedure.** The pro-angiogenic potential of different acellular and cellular substrates was analyzed. ASCs were cultured in growth and adipogenic differentiation medium respectively for 7 days. For acellular ECM substrates (cdECM), ASCs were removed and the remaining ECM was dried or stored under wet conditions. For cellular substrates (FL), ASCs were not removed. MvECs were seeded onto the different substrates. Cytocompatibility was determined at day 3 and structure formation was determined at day 14 of cell culture.

**Figure 2: Degree of swelling of ECM substrates and staining of fibronectin and collagen.** A: Macroscopic pictures of cdECM substrates show a transparent gel-like ECM on the bottom of the petri dish. The degree of swelling was calculated in percent. Results reveal a higher swelling rate of acdECM compared to the scdECM (Diameter of petri dish is 35 mm). B: Fixed cdECM samples were stained for fibronectin and histological sections of the cdECM samples were stained for collagen fibers using picro sirius staining. Fibronectin staining revealed smaller pores in the scdECM substrate compared to the acdECM substrate (Fibronectin indicated in red; scale bar: 200 µm). Picro sirius staining showed denser packed collagen fibers (red staining) in the scdECM compared to the acdECM (Scale bar: 100 µm)

**Figure 3: Biocompatibility of the acellular and feeder layer substrates** . 1 x 104 cells/cm2 mvECs were seeded in a defined medium onto the different substrates. A: Relative LDH release was measured at day 3 after seeding with mvECs. For acellular substrates values were normalized to TC. No significant increase in LDH release can be observed on COL I coating or dry and wet cdECM for both, scdECM and acdECM. For FL approaches, values were normalized to stem cell FL without mvECs (FL stem cell). None of the FL approaches (stem cell and adipogenic differentiated) exhibit a significant increase of released LDH after seeding of mvECs (FL +mvECs), (n.s. = not significant; ** p [?] 0.01 ) B: Live-dead staining (FDA, indicating alive cells displayed in green/PI, indicating dead cells, displayed in red) was performed at day 14 after seeding with mvECs. A confluent layer of viable cells was observed in all approaches. Scale bar represents 200 µm.

**Figure 4: Formation of vascular-like structures by mvECs on cellular and acellular ECM substrates** . 1 x 104 mvECs /cm2 were seeded in defined co-culture medium onto the different substrates and were cultured for 14 days. Medium was changed three times a week. For determination of newly formed vascular- like structures, IF staining of CD31 (indicated in red) was performed at day 14 after seeding with

mvECs. On controls (TC and COL I) a confluent layer of mvECs could be observed without any structure formation. On cdECM substrates, formation of vascular-like structures could be observed with the strongest manifestation on wet acdECM. The highest degree of structure formation could be observed on adipogenic FL. On stem cell FL cluster formation of mvECs can be found and a considerably lower degree of structure formation compared to the adipogenic approach was detected. Length per structure and number of formed nodes was quantified using ImageJ. Analysis revealed a significantly higher structure length of mvECs on wet acdECM substrate compared to dry acdECM and stem cell approaches (dry and wet scdECM) and comparable to FL approaches. Structure length on adipogenic FL was significantly higher compared to all approaches except wet acdECM. On adipogenic FL, a significantly higher number of nodes can be observed compared to all other approaches. (Scale bar: 200 µm; * p [?] 0.05; ** p [?] 0.01; *** p [?] 0.001)

**Figure 5: Pro-angiogenic factors concentration on cellular and acellular ECM substrates** . Relative concentration from released acellular and cellular substrates. For the determination of VEGF, bFGF and PDGFβ from substrates, supernatant from day 3 was investigated regarding the concentration of the growth factors using ELISA. For statistical analysis, values on TC were set as 1 and data were normalized to TC. For VEGF a significantly higher amount can be found in acdECM substrates (dry and wet) compared to all other acellular substrates including controls. FL substrates exhibit a 10-fold higher concentration of VEGF compared to acellular substrates. For bFGF a higher concentration can be found in cdECM substrates compared to controls. On FL approaches, a higher concentration can be found compared to all other approaches. For PDGFβ a significantly higher (3-fold) amount can be found in all cdECM substrates compared to the controls TC and COL I. Between the individual cdECM substrates no difference in remaining PDGFβ can be found. FL substrates exhibit significantly higher PDGFβ concentrations compared to acellular substrates. (* p [?] 0.05; ** p [?] 0.01; *** p [?] 0.001).

**Figure 6: Expression of E-selectin and thrombomodulin by mvECs forming vascular-like structures.** 1 x 104 mvECs /cm2 were seeded in defined co-culture medium onto the different substrates and were cultured for 14 days. Medium was changed three times a week. For both proteins no specific staining was observed on controls (TC and COL I coating). For all acellular and cellular substrates, specific staining of E-selectin and thrombomodulin (both indicated in red) can be found mainly on the newly formed vascular-like structures. (Scale bar: 200 µm)

**Supplementary Figure 1:** Expression of CD31 by mvECs. CD31 staining of samples stained for E-selectin. and thrombomodulin. Both corresponding to Figure 6. (Scale bar: 200µm; green: CD31, white: Nuclei)