

ARTICLE

Adipose stem cell-derived extracellular matrix represents a promising biomaterial by inducing spontaneous formation of prevascular-like structures by mvECs

Svenja Nellinger¹ | Isabelle Schmidt² | Simon Heine¹ | Ann-Cathrin Volz¹ | Petra J. Kluger²

¹Reutlingen Research Institute, Reutlingen University, Reutlingen, Germany

²School of Applied Chemistry, Reutlingen University, Reutlingen, Germany

Correspondence

Petra J. Kluger, Reutlingen University, Applied Chemistry, Alteburgstr. 150, 72762 Reutlingen, Germany.
Email: petra.kluger@reutlingen-university.de

Funding information

Ministry of Science, Research and the Arts (Baden-Württemberg, Germany), Landesgraduiertenförderung, Program: Intelligent Process and Material Development in Biomateriomics (University Tuebingen and Reutlingen)

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 prevascular-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 adipogenically differentiated ASCs, with the classical cell-based FL. All cell-derived ECM (cdECM) substrates enabled mvEC growth with high viability. Prevascular-like structures were visualized by immunofluorescence staining of endothelial surface protein CD31 and could be observed on all cdECM and FL substrates but not on control substrate collagen I. On adipogenically differentiated ECM, longer and higher branched structures could be found compared with stem cell cdECM. An increased concentration of proangiogenic factors was found in cdECM substrates and FL approaches compared with controls. Finally, the expression of proteins associated with tube formation (E-selectin and thrombomodulin) was confirmed. These results highlight cdECM as promising biomaterial for adipose tissue engineering by inducing the spontaneous formation of prevascular-like structures by mvECs.

KEYWORDS

adipose-derived stem cells, biomaterials, extracellular matrix, prevascular-like structures, tissue engineering

1 | INTRODUCTION

Adipose tissue is a highly metabolic and vascularized tissue. In native tissue, a dense capillary network provides the supply of nutrients and inspiratory gases to the residing cells and removes their waste products. Since the diffusion limit of oxygen is <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 following 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

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2020 The Authors. *Biotechnology and Bioengineering* Published by Wiley Periodicals LLC

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. To date, there are several strategies to vascularize engineered 3D tissue constructs, for example, functionalized scaffolds, perfusion bioreactors, cocultures, and *in vivo* approaches (Laschke & Menger, 2016). Proangiogenic 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) are known to induce vessel formation and platelet-derived growth factor (PDGF) β supports stabilization of the newly formed vessels (Gaengel, Genove, Armulik, & Betsholtz, 2009). Different coculture systems, including monolayer or spheroid cultures, demonstrated spontaneous formation of vascular-like structures (Walser et al., 2013; Wenz, Tjoeng, Schneider, Kluger, & Borchers, 2018). In particular, the coculture of endothelial cells (ECs) with adipose-derived stem cells (ASCs) showed a beneficial effect on prevascular-like structure formation (Verseijden et al., 2012; Volz, Hack, Atzinger, & Kluger, 2018). Abovementioned techniques help to prevascularize a construct, but the complete vascularization is only achieved post implantation *in vivo* (Laschke, Strohe et al., 2009; Laschke, Vollmar, & Menger, 2009). So far, there is no successful *in vitro* approach to create 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 difficulties in handling and storage. In contrast, lyophilized acellular biomaterials can be stored for long periods. Moreover, they evoke far fewer concerns regarding their application in regenerative medicine compared with the approaches including living cells. However, changes in structure and composition may occur during dehydration of natural materials. Thus, it has to be clarified if the processing of a 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, for example, 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; Flynn, 2010; Flynn, Prestwich, Semple, & Woodhouse, 2009). All these studies were performed with decellularized ECM derived from native tissue. For the past years, another source of natural ECM moves to the fore. *In vitro* generated

cell-derived ECM (cdECM) was isolated from different cell-types (e.g., fibroblasts and ASCs) 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 osteogenic differentiation of ASCs indicating its influence on cell fate (Dzobo et al., 2016; Guneta et al., 2017; Guneta, Loh, & Choong, 2016; Guo et al., 2013).

Our previous study revealed a spontaneous formation of prevascular-like structures by microvascular endothelial cells (mvECs) in a coculture with adipogenically differentiated ASCs (Volz et al., 2018). In the following the term “prevascular-like structures” defines the aggregation/alignment of CD31-positive mvECs to fiber- or network-like structures, which stand out from the rest of the cellular monolayer. This term was previously used by Verseijden, Posthumus-van Sluijs, Farrell et al. (2010) and Verseijden, Posthumus-van Sluijs, Pavljasevic et al. (2010) to describe the alignment of ECs in spheroids without lumen formation. The formation of vacuoles within ECs and subsequent lumenogenesis (tube formation) of prevascular-like structures requires the activation of cellular pathways and the transcription of different genes (Bayless & Davis, 2002). Furthermore, the expression of E-selectin and thrombomodulin was shown to contribute to tube formation (Oh et al., 2007; Pan et al., 2017). In this study, we aimed to analyze whether the formation of prevascular-like structures by mvECs has to be attributed to cell–cell or cell–matrix interactions. The maintenance of the biological impact after processing and storage represents an important feature regarding the commercial application of biomaterials. The most common processing method for the preservation of biomaterials is drying. Consequently, we directly compared the effect of the hydrogel-like, wet cdECM and the dried cdECM as a coating regarding its ability to support the formation of prevascular-like structures by mvECs. The formation of vascular structures is rather a developmental process than a maintenance phenomenon as it can, for example, be found during (adipogenic) differentiation rather than in the stem cell niche. Thus, we tested whether there is a difference between cdECM derived from stem cells and adipogenically differentiated cells regarding their capability to induce prevascular-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 5×10^3 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.

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 5×10^3 cells/cm². 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 25×10^3 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's 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 and 1% penicillin/streptomycin. 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 at 4°C until seeded with mvECs. Cellular substrates (feeder layer [FL]) were seeded with mvECs without lysis of ASCs (Figure 1).

2.3 | Macroscopic pictures and degree of swelling

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

The degree of swelling was calculated as

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

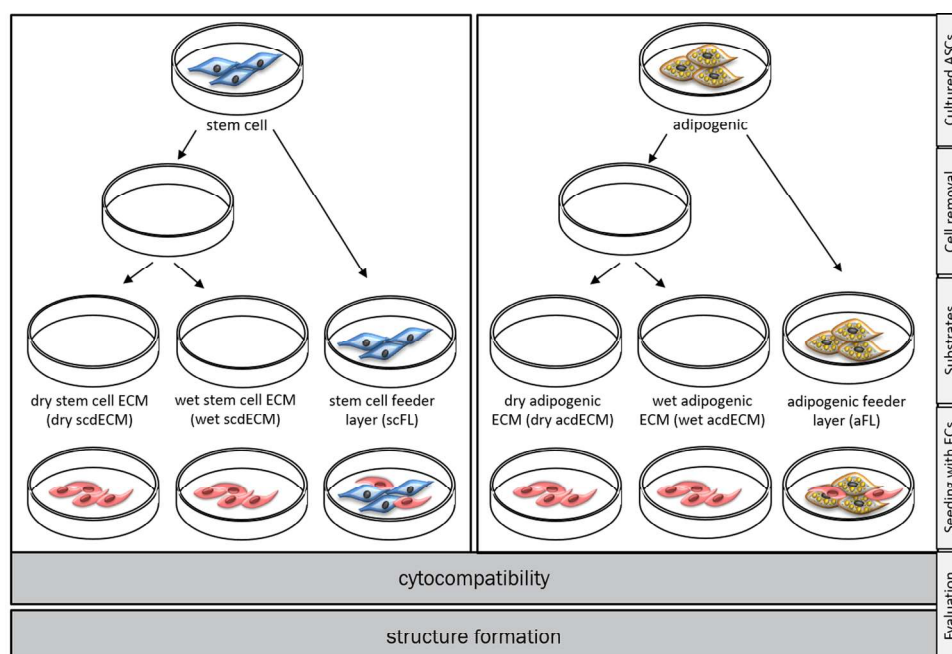


FIGURE 1 Schematic overview of the study procedure. The proangiogenic potential of different acellular and cellular substrates was analyzed. ASCs were cultured in growth and adipogenic differentiation medium respectively for 7 days. For acellular ECM substrates (cdECM), ASCs were removed and the remaining cdECM 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 prevascular-like structure formation was determined at Day 14 of cell culture. ASCs, adipose-derived stem cells; cdECM, cell-derived extracellular matrix; FL, feeder layer; MvECs, microvascular endothelial cells [Color figure can be viewed at wileyonlinelibrary.com]

2.4 | Immunofluorescence staining of fibronectin and quantification of pore size

For immunofluorescence (IF) staining of fibronectin, cdECM substrates were fixed in 4% paraformaldehyde (Carl Roth, Germany) for 10 min followed by incubation with blocking solution, consisting of 3% bovine serum albumin (BSA; 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 1 hr at RT. After washing three times with 0.1% Tween-20 (Sigma Aldrich, Germany) in phosphate-buffered saline (PBS), secondary antibody (anti-mouse Cy3, Dianova, Germany; 1:250) was incubated for 30 min at RT. Both were diluted in blocking solution. Images were taken with Axio Observer microscope and Axiocam 305 color using ZENblue software (Carl Zeiss, Germany). The pore size of the different cdECM substrates was quantified using ImageJ based on the IF images. The 100 largest pores for each image were determined and results are given as the mean.

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

Isolated dry and wet cdECM substrates were reseeded with mvECs at a density of 1×10^4 cells/cm² in a defined mvEC adipocyte co-culture medium (Volz et al., 2018). For FL approaches, mvECs were directly seeded on top of adipogenically differentiated and undifferentiated ASCs at a density of 1×10^4 cells/cm² in defined co-culture medium, developed by us earlier (Volz et al., 2018). 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 (COL 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 demonstrated 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 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 the Axio Observer microscope and Axiocam 305 color camera using ZENblue software (Carl Zeiss, Germany). The number of dead and viable cells was quantified using the software ImageJ and results were depicted as a percentage.

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% BSA 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; sheep anti-thrombomodulin, 1:200, R&D Systems) were diluted in blocking solution and incubated with samples for 2 hr at RT. Secondary antibodies (antirabbit Alexa FluorTM 488, abcam, GB; antimouse Cy3, Dianova, Germany; donkey anti-sheep Alexa FluorTM 647, abcam, GB; donkey anti-goat Alexa FluorTM 594, abcam, GB) were diluted 1:250 in blocking solution and incubated with samples for 30 min at RT.

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. Quantification of growth factors VEGF, bFGF, and PDGFβ was performed using enzyme-linked immunosorbent assays (ELISA) (all PEPROTech, Germany) according to the manufacturer's instructions. The converted 3,3',5,5'-tetramethylbenzidine was read out at 450 nm with a wavelength correction set at 620 nm (TECAN Sapphire II, Tecan, Switzerland).

2.9 | Statistical analysis

All experiments were performed at least three times, using cells from at least three different biological donors of ECs. The obtained data were compared by a one-way analysis of variance with repetitive measurement and a Bonferroni post hoc test using OriginPro 2018b. Statistical significances were stated as * $p \leq .05$, very significant as ** $p \leq .01$, and highly significant as *** $p \leq .001$.

3 | RESULTS

3.1 | Macroscopic pictures and degree of swelling

Macroscopic pictures showed that wet scdECM and acdECM substrates exhibited a transparent gel-like appearance on the bottom of

a petri dish (Figure 2). Determination of the degree of swelling of the different cdECM substrates revealed a higher water uptake capacity of acdECM (2,357.6 \pm 201.1%) compared with the scdECM (1,624.3 \pm 96.4%). Quantification of the pore size in the IF staining of fibronectin revealed smaller pores in the scdECM substrates compared with the acdECM substrates.

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 by mvECs seeded on the different substrates was measured 3 days after seeding (Figure 3a). The values of TC were set as 100 (\pm 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 \pm 13.8%), dry scdECM (113.7 \pm 31.0%); dry acdECM (108.0 \pm 29.0%), wet scdECM (96.3 \pm 33.4%), or wet acdECM (93.4 \pm 29.0%). For the stem cell and adipogenic FL substrates, values were normalized to stem cell FL approach without mvECs (FL stem cell), which was set as 100 (\pm 3.3)%. For adipogenic FL (FL adipogenic: 157.9 \pm 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 adipogenically differentiated cells (FL stem + mvECs: 126.1 \pm 15.8%; FL ad + mvECs: 176.8 \pm 25.0%).

The viability of mvECs cultured on the different substrates was assessed on Day 14 after seeding with mvECs by live-dead staining with FDA and PI (Figure 3b). Results showed that mvECs were viable on all acellular and FL substrates on Day 14 and only a few dead cells could be found. Quantification of the percentage of dead cells revealed <1% of dead cells on all acellular substrates (TC: 0.14 \pm 0.08%; COL I: 0.29 \pm 0.21%; dry scdECM: 0.46 \pm 0.11%; dry acdECM: 0.61 \pm 0.13%; wet scdECM: 0.25 \pm 0.23%; and wet acdECM: 0.45 \pm 0.26%). On FL substrates higher amounts of dead cells were found in adipogenic approaches compared to the stem cell approach (FL stem cell: 0.25 \pm 0.08%; FL adipogenic: 4.58 \pm 0.19%; FL stem cell + mvECs: 0.37 \pm 0.18%; FL adipogenic + mvECs: 3.25 \pm 1.66%).

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

To investigate the effect of cdECM substrates on the formation of prevascular-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 revealed 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 prevascular-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. Higher magnification of the cellular monolayer or the

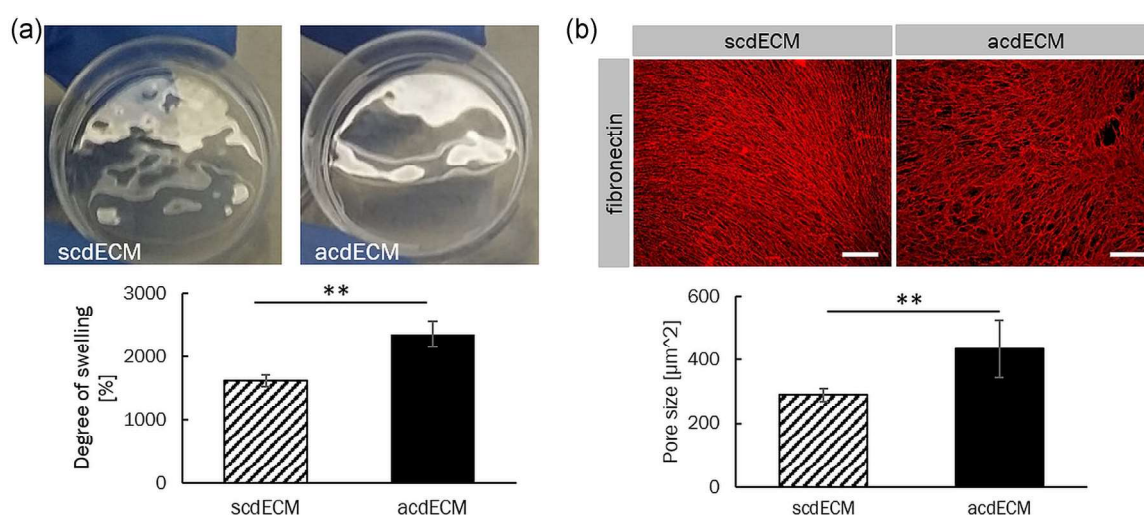


FIGURE 2 Degree of swelling of ECM substrates and staining of fibronectin and quantification of pore size. (a) Macroscopic pictures of cdECM substrates show a transparent gel-like cdECM on the bottom of the petri dish. The degree of swelling was calculated in percent. Results revealed a higher swelling rate of acdECM compared with the scdECM (diameter of the petri dish is 35 mm). (b) Fixed cdECM samples were stained for fibronectin. Quantification of the pore sizes in fibronectin staining revealed smaller pores in the scdECM substrate compared with the acdECM substrate (fibronectin indicated in red; scale bar = 200 μm). AcECM, adipogenic cell-derived extracellular matrix; scdECM, stem cell-derived extracellular matrix. (** $p \leq .01$) [Color figure can be viewed at wileyonlinelibrary.com]

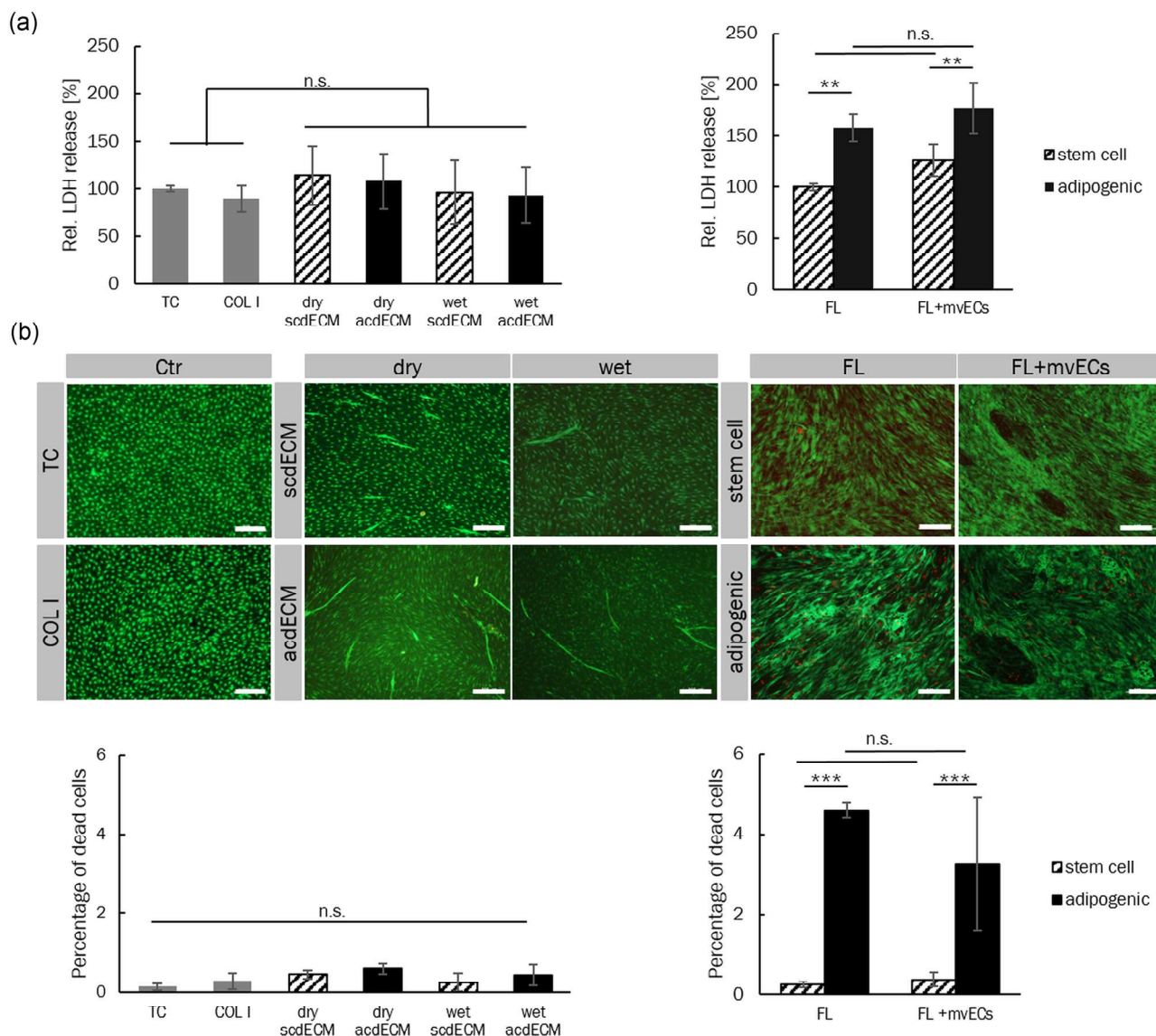


FIGURE 3 Cytocompatibility of the acellular and feeder layer substrates. About 1×10^4 cells/cm² mvECs were seeded in a defined medium onto the different substrates. (a) Relative LDH release was measured at Day 3 after seeding with mvECs. For acellular substrates, values were normalized to TC. No significant increase in LDH release could 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) exhibited a significant increase of released LDH after seeding of mvECs (FL + mvECs). (b) Live-dead staining (FDA, indicating alive cells displayed in green/PI, indicating dead cells, displayed in red) was performed on Day 14 after seeding with mvECs. A confluent layer of viable cells was observed in all approaches. For each approach, the percentage of dead cells was quantified using ImageJ. The analysis revealed an amount of <1% of dead cells for all acellular substrates with no significant differences between the different substrates. In FL approaches, a higher number of dead cells could be found in adipogenic approaches compared to stem cell approaches. Scale bar represents 200 μ m. AcdECM, adipogenic cell-derived extracellular matrix; cdECM, cell-derived extracellular matrix; COL I, collagen I; FDA, fluorescein diacetate; FL, feeder layer; LDH, lactate dehydrogenase; mvECs, microvascular endothelial cells; ns, not significant; PI, propidium iodide; scdECM, stem cell-derived extracellular matrix; TC, tissue culture. (** $p \leq .01$, *** $p \leq .001$) [Color figure can be viewed at wileyonlinelibrary.com]

prevascular-like structures shows the typical localization of CD31 at cell-cell contacts. Quantification of the structures' lengths revealed longer structures in wet acdECM ($433.5 (\pm 293.1) \mu$ m) substrate compared with both scdECM substrates (dry: $235.9 (\pm 100.0) \mu$ m and wet: $232.9 (\pm 183.8) \mu$ m). Dry acdECM ($297.2 (\pm 149.1) \mu$ m) substrates exhibited a slightly but not significantly higher structure length compared with dry and wet scdECM substrates. The lengths of

prevascular-like structures found on the adipogenic FL (FL adipogenic: $483.5 (\pm 287.4) \mu$ m) were significantly longer than those of all other approaches except for wet acdECM substrates. Prevascular-like structures on stem cell FL exhibited an average length per structure of $302.1 (\pm 168.7) \mu$ m. Another essential criterion for the maturation of a vascular network is the formation of nodes. Therefore, the number of nodes formed by the mvECs on the different

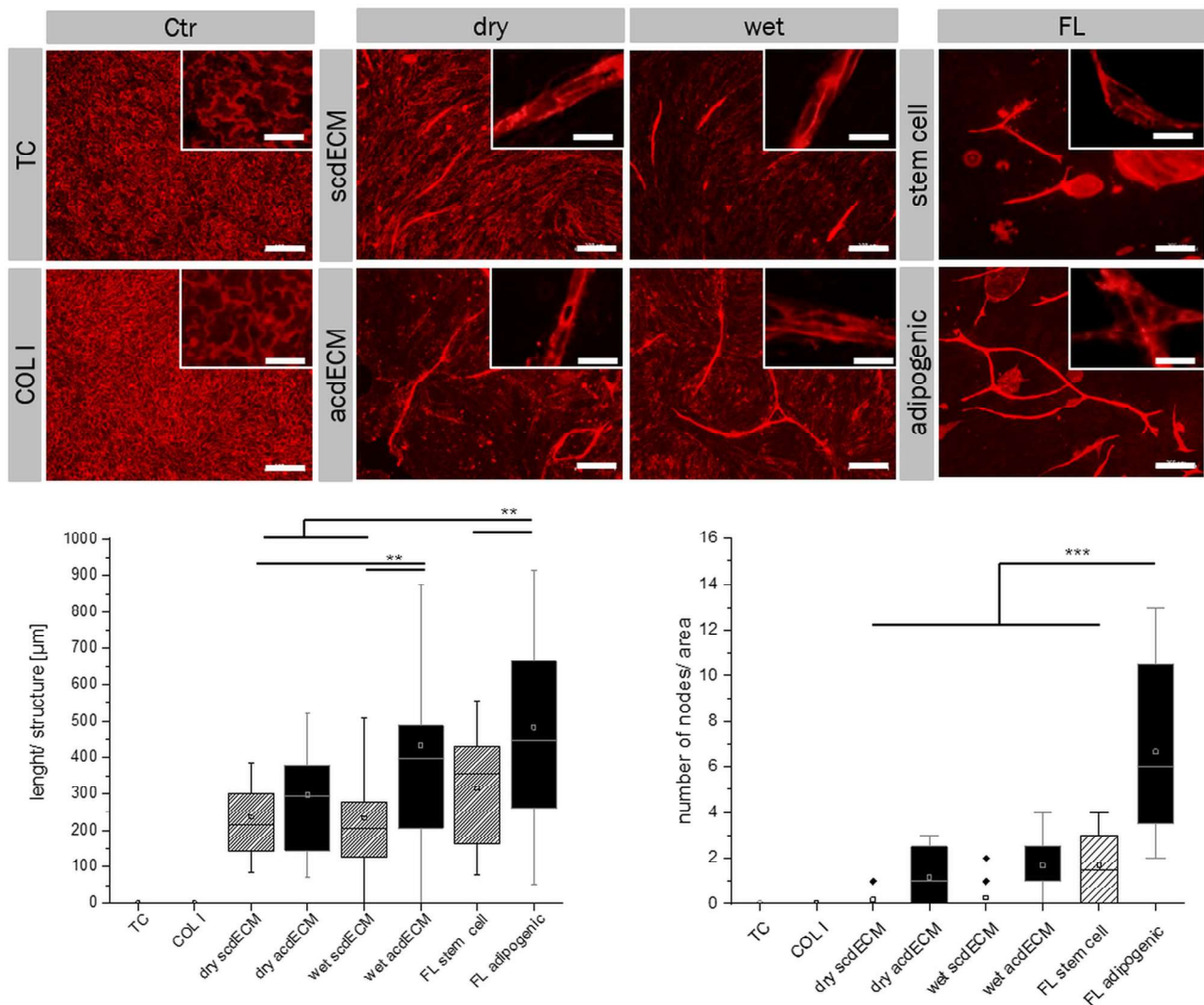


FIGURE 4 Formation of prevascular-like structures by mvECs on cellular and acellular ECM substrates. About 1×10^4 mvECs/cm² were seeded in defined coculture medium onto the different substrates and were cultured for 14 days. Medium was changed three times a week. For determination of newly formed prevascular-like 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, the formation of prevascular-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 could be found and a considerably lower degree of structure formation compared to the adipogenic approach was detected. For each representative overview image, a magnified section of the cellular monolayer or the prevascular-like structures is pictured in the upper right corner to show the localization of CD31 at cell-cell contacts. 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 sdcECM), comparable with FL approaches. Structure length on adipogenic FL was significantly higher compared with all approaches except wet acdECM. On adipogenic FL, a significantly higher number of nodes could be observed compared with all other approaches. acdECM, adipogenic cell-derived extracellular matrix; cdECM, cell-derived extracellular matrix; COL I, collagen I; TC, tissue culture. (Scale bar overview image: 200 μm; scale bar magnified section: 25 μm; * $p \leq .05$; ** $p \leq .01$; *** $p \leq .001$) [Color figure can be viewed at wileyonlinelibrary.com]

substrates was quantified. No nodes could be detected on the controls TC and COL I. In adipogenic approaches (dry acdECM: $1.2 [\pm 1.1]$; wet acdECM: $1.7 [\pm 1.2]$; and FL adipogenic: $6.7 [\pm 3.9]$) the number of nodes was higher compared with the stem cell approaches (dry sdcECM: $0.7 [\pm 0.4]$; wet sdcECM: $0.2 [\pm 0.6]$; and FL stem cell: $1.7 [\pm 1.5]$) for all substrates. Furthermore, the number of nodes on wet

acdECM was slightly but not significantly higher compared with the dry acdECM and comparable with stem cell FL. The significantly highest number of nodes could be observed in the adipogenic FL approach. To summarize, many short structures were identified on sdcECM substrates, whereas on acdECM substrates the structures were longer and more branched. By coculture with the stem cell FL,

mvECs formed islets of a confluent layer within the ASCs and prevascular-like structures sprouting from these islets were apparent. Long and highly branched prevascular-like structures were formed by mvECs on adipogenic FL.

3.4 | Quantification of proangiogenic factors on substrates

To confine which cdECM components are responsible for its proangiogenic effect, the relative concentrations of growth factors VEGF, bFGF, and PDGF β were determined in the supernatants after washing the acellular substrates for 3 days (Figure 5). Values were normalized to TC. The VEGF concentration was significantly higher for dry and wet acdECM substrates compared with all other acellular substrates. For quantification of growth factor concentrations on FL approaches, cell culture supernatant from Day 3 (corresponding to 3 days of washing of acellular substrates) was collected. A 10-fold higher concentration of VEGF could be found in FL approaches with no difference between stem cell FL and adipogenic FL significantly higher concentrations of bFGF could be found in cdECM substrates compared with controls and on FL approaches a higher concentration could be found compared with all other substrates. For PDGF β a significantly higher concentration could be found on cdECM substrates compared with the controls. Between the different cdECM substrates no difference in PDGF β concentration could be measured. On FL substrates, a higher concentration of PDGF β could be found

compared with acellular substrates but no difference between stem cell and adipogenically differentiated approach was observed.

3.5 | Expression of proteins associated with tube formation in newly formed prevascular-like structures

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). To get an indication if lumenogenesis occurs to any extent, we investigated the expression of these proteins in the newly formed prevascular-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 prevascular-like structures showed expression of E-selectin and thrombomodulin on all cdECM substrates. Furthermore, E-selectin and thrombomodulin expression of the prevascular-like structures were found in the newly formed prevascular-like structures on both FL approaches. E-selectin and thrombomodulin staining corresponded to the CD31 staining pattern of the prevascular-like structures (Figure S1).

4 | DISCUSSION

The implementation of a functional vascular system into an engineered tissue construct would address one of the major

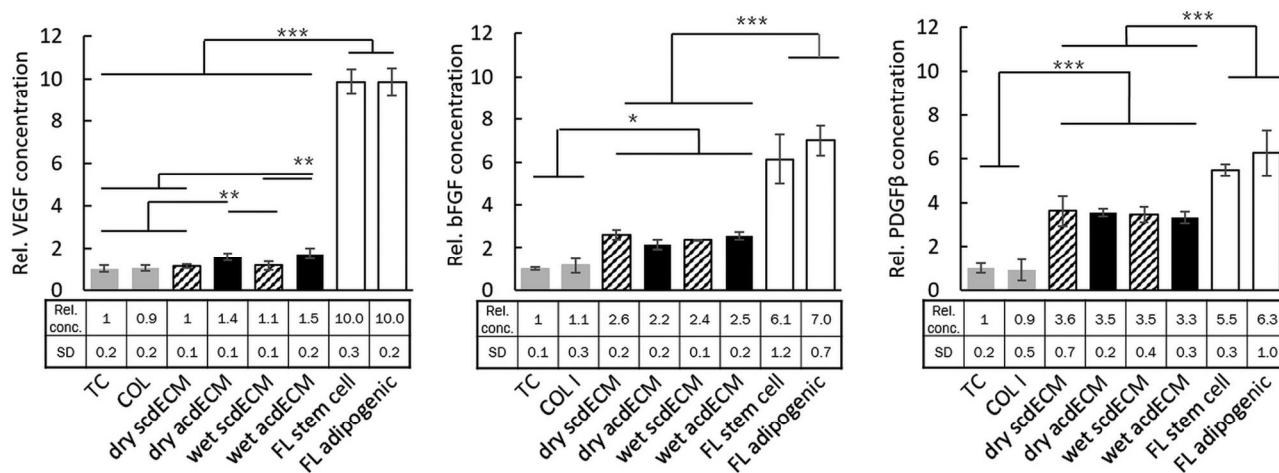


FIGURE 5 Proangiogenic factor concentrations on cellular and acellular ECM substrates. For the determination of VEGF, bFGF, and PDGF β from the different substrates, supernatant from Day 3 was investigated regarding the concentration of the growth factors using ELISA. For statistical analysis, values on TC were set as 1 and data were normalized to TC. For VEGF, a significantly higher amount could be found in acdECM substrates (dry and wet) compared with all other acellular substrates including controls. FL substrates exhibited a 10-fold higher concentration of VEGF compared with acellular substrates. For bFGF, a higher concentration could be found in cdECM substrates compared with controls. On FL approaches, a higher concentration could be found compared with all other approaches. For PDGF β , a significantly higher (three-fold) amount could be found in all cdECM substrates compared with the controls TC and COL I. Between the individual cdECM substrates no difference in remaining PDGF β could be found. FL substrates exhibited significantly higher PDGF β concentrations compared with acellular substrates. acdECM, adipogenic cell-derived extracellular matrix; bFGF, basic fibroblast growth factor; cdECM, cell-derived extracellular matrix; COL I, collagen I; ELISA, enzyme-linked immunosorbent assay; FL, feeder layer; PDGF β , platelet-derived growth factor beta; scdECM, stem cell-derived extracellular matrix; TC, tissue culture; VEGF, vascular endothelial growth factor. (* $p \leq .05$; ** $p \leq .01$; *** $p \leq .001$)

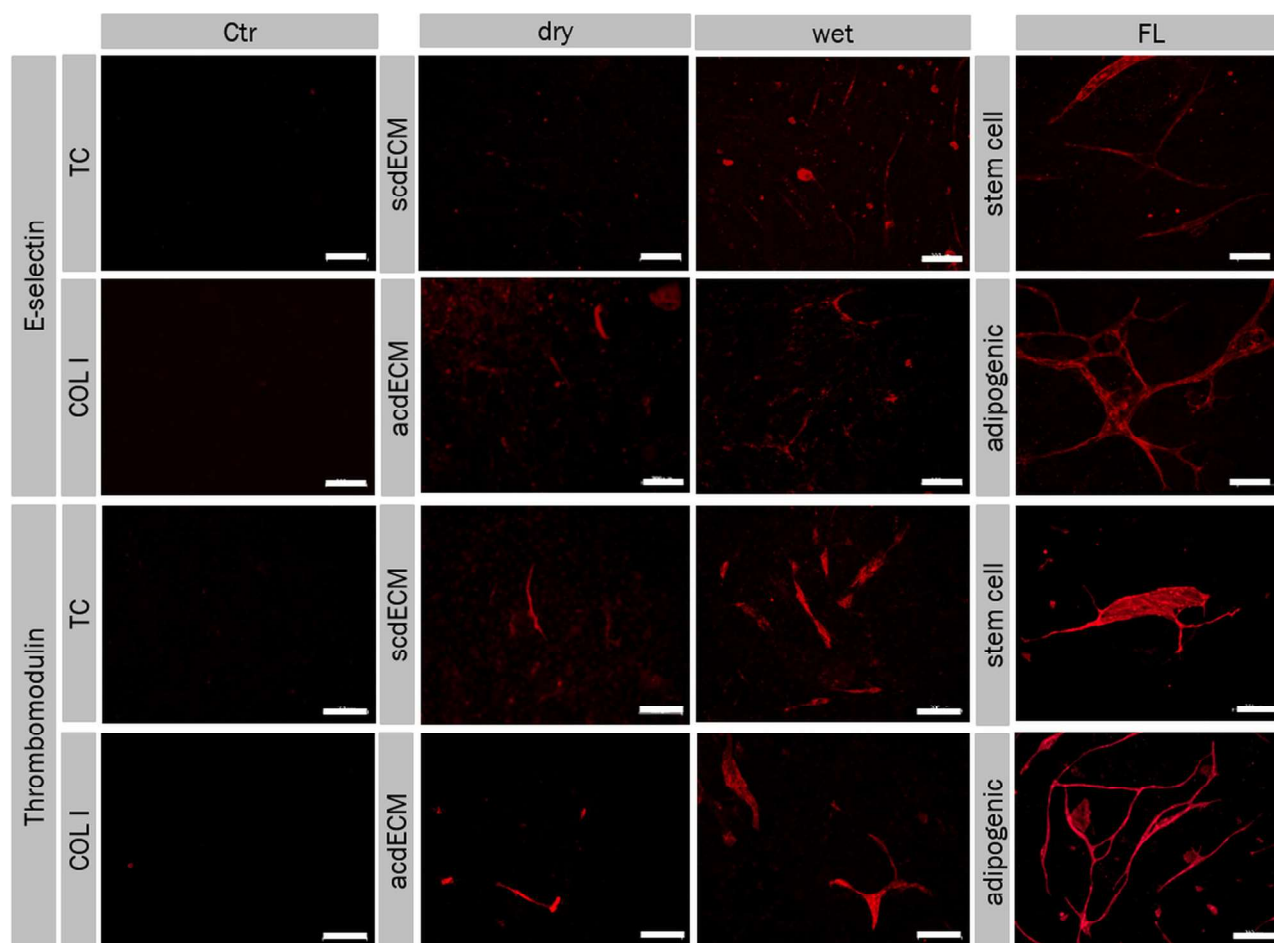


FIGURE 6 Expression of E-selectin and thrombomodulin by mvECs forming prevascular-like structures. About 1×10^4 mvECs/cm² were seeded in defined coculture medium onto the different substrates and were cultured for 14 days. Medium was changed three times a week. For both proteins, no specific staining 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) could be found mainly on the newly formed prevascular-like structures. (Scale bar = 200 μ m). acdECM: adipogenic cell-derived extracellular matrix; COL I, collagen I; FL, feeder layer; mvECs, microvascular endothelial cells; scdECM, stem cell-derived extracellular matrix; TC, tissue culture [Color figure can be viewed at wileyonlinelibrary.com]

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 prevascular-like structures by mvECs for its use as a biomaterial for adipose tissue engineering compared with the well-established application of a supportive FL.

Determination of the degree of swelling revealed a higher capacity of water uptake of the adipogenic ECM compared with stem cell ECM. This effect can be explained by the larger pore size in acdECM shown by fibronectin staining. The development of larger pores in acdECM may be attributed to morphological changes of ASCs during adipogenesis. By incorporating lipids, adipogenically differentiated ASCs develop a more spherical shape compared with the stem cells and substantially increase their volume (Moldovan et al., 2019). During adipogenesis, ASCs further stop to proliferate which results in a lower total cell number in adipogenic approaches (Fajas, 2003). Thus, the amount of cdECM in stem cell approaches may be higher which leads to more densely packed collagen fibers

and smaller pores. The larger pores found in adipogenic ECM might also be able to enhance the degree of prevascular-like structure formation by mvECs. Chui et al. (2011) showed that larger pore size is associated with a higher degree of neovascularization in an in vitro PEG hydrogel model. Furthermore, Artel, Mehdizadeh, Chiu, Brey, and Cinar (2011) proposed an agent-based model indicating that pores of larger size support vascularization in a polymer scaffold.

Analysis of LDH release of mvECs on the substrates revealed no cytotoxic effect 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. Nearly no dead mvECs were observed during the quantification of the live/dead staining on acellular substrates. For the stem cell FL approach, <1% of dead cells could be found. However, on the adipogenic FL approach around 4% of dead cells could be found for FL with and without mvECs. Adipogenically differentiated ASCs may be more sensitive to the change to the defined coculture

medium compared with the nondifferentiated ASCs which leads to an increase of cell death. For comprehensive toxicological and immunogenic characterization further analysis is required, for example, the analysis of the cdECM impact on the metabolic activity of the mvECs and for the intended *in vivo* use, biocompatibility of the cdECM 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 prevascular-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 (Volz et al., 2018) 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 prevascular-like structure formation on adipogenic ECM substrates was higher compared with 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 proangiogenic 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, Matsuda et al. (2013) showed that conditioned cell culture medium of ASCs positively influenced EC proliferation and the formation of new vessels *in vivo*. During adipogenic differentiation, ASCs secrete further proangiogenic factors like leptin. Leptin is known to be upregulated during adipogenic differentiation and was shown to exhibit a proangiogenic 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 prevascular-like structure formation by mvECs. Thus, cdECM does not only contain a set of specific factors, but a broad spectrum of proangiogenic factors with its synergistic effects needed for the successful formation of prevascular-like structures by ECs. Especially acdECM supports the formation of prevascular-like structures and seems to be able to stabilize the newly formed structures.

The two most important proangiogenic factors are VEGF and bFGF. Results revealed higher VEGF concentrations released from acdECM substrates compared with 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 prevascular-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 with 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 prevascular-like structures might among other events, be attributed to the synergistic effect of available VEGF and bFGF. We further investigated the amount of the proangiogenic factor PDGF β from cdECM substrates. It is secreted by ECs during angiogenesis to attract perivascular cells, which stabilize the newly formed vessels (Gaengel et al., 2009). Furthermore, PDGF β was shown to induce vascular structure formation by modulating proliferation and tube formation of ECs (Battegay, Rupp, Iruela-Arispe, Sage, & Pech, 1994). PDGF β can be found in all cdECM substrates as well as FL approaches. The PDGF β concentration from FL approaches is higher compared with 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 *in vivo* and culture medium *in vitro*. 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 ischemic 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. Thrombomodulin is a transmembrane protein expressed on ECs acting as an anticoagulant (Dahlback & Villoutreix, 2005; Dittman & Majerus, 1990). The fourth and fifth region of an EGF-like region of thrombomodulin (TME45) was shown to stimulate the proliferation of human umbilical vein ECs and to promote tube formation and angiogenesis (Ikezo et al., 2017). In this study, we use these proteins as indicators for the development of the prevascular-like structure towards a tubular vascular structure with a lumen. IF staining of E-selectin and thrombomodulin revealed specific expression of E-selectin almost exclusively on the newly formed prevascular-like structures. Thus, we suggest that newly formed prevascular-like structures exhibit promising characteristics to develop a lumen. 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.

Compared with ECM derived from native tissue, cdECM exhibited a variety of advantages, which confirms it as a promising biomaterial for tissue engineering. For example, the possibility of autologous production without harvesting high amounts of

autologous tissue and the generation of ECM from different developmental stages. This study confirms a supportive effect of cdECM on the spontaneous formation of prevascular-like structures by mvECs. Furthermore, it could be shown that dry cdECM partly maintains its biological properties regarding the induction of self-assembled prevascular-like structure formation of mvECs with some restrictions. Drying of the cdECM would be a convenient method to improve storage possibilities when necessary. Due to the relatively low amounts of cdECM, which can be produced with current methods, this study is limited to 2D approaches, which insufficiently reflect physiological conditions. Further studies should focus on the upscaling of the generation of cdECM to enable the setup of continuative experiments in 3D constructs consisting of cdECM, which would better reflect the situation in vivo.

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 prevascular-like structures by mvECs and helps to support their maintenance. Mainly acdECM was confirmed as a promising material for adipose tissue engineering by supporting the formation of prevascular-like structures. In addition, scdECM also provides the ability to induce prevascular-like structure formation and can be used for approaches addressing other tissues. In future investigations regarding other lineage-specific cdECMs, the upscaling of cdECM generation and the transfer from 2D cell culture to 3D cell culture should be pursued.

ACKNOWLEDGMENTS

This study was financially supported by the Landesgraduiertenförderung by the Ministry of Science, Research, and the Arts (Baden-Württemberg, Germany) under the program "Intelligent Process and Material Development in Biomateriomics" (University of Tuebingen and Reutlingen University).

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

ORCID

Svenja Nellinger  <http://orcid.org/0000-0001-7281-2196>

REFERENCES

- Adam Young, D., Bajaj, V., & Christman, K. L. (2014). Award winner for outstanding research in the PhD category, 2014 Society for Biomaterials annual meeting and exposition, Denver, Colorado, April 16-19, 2014: Decellularized adipose matrix hydrogels stimulate in vivo neovascularization and adipose formation. *Journal of Biomedical Materials Research. Part A*, 102(6), 1641-1651. <https://doi.org/10.1002/jbm.a.35109>
- Artel, A., Mehdizadeh, H., Chiu, Y. C., Brey, E. M., & Cinar, A. (2011). An agent-based model for the investigation of neovascularization within porous scaffolds. *Tissue Engineering. Part A*, 17(17-18), 2133-2141. <https://doi.org/10.1089/ten.TEA.2010.0571>
- Badylak, S. F., Freytes, D. O., & Gilbert, T. W. (2009). Extracellular matrix as a biological scaffold material: Structure and function. *Acta Biomaterialia*, 5(1), 1-13. <https://doi.org/10.1016/j.actbio.2008.09.013>
- Battegay, E. J., Rupp, J., Iruela-Arispe, L., Sage, E. H., & Pech, M. (1994). PDGF-BB modulates endothelial proliferation and angiogenesis in vitro via PDGF beta-receptors. *Journal of Cell Biology*, 125(4), 917-928. <https://doi.org/10.1083/jcb.125.4.917>
- Bayless, K. J., & Davis, G. E. (2002). The Cdc42 and Rac1 GTPases are required for capillary lumen formation in three-dimensional extracellular matrices. *Journal of Cell Science*, 115(Pt 6), 1123-1136.
- Cao, R., Brakenhielm, E., Wahlestedt, C., Thyberg, J., & Cao, Y. (2001). Leptin induces vascular permeability and synergistically stimulates angiogenesis with FGF-2 and VEGF. *Proceedings of the National Academy of Sciences of the United States of America*, 98(11), 6390-6395. <https://doi.org/10.1073/pnas.101564798>
- Chiu, Y. C., Cheng, M. H., Engel, H., Kao, S. W., Larson, J. C., Gupta, S., & Brey, E. M. (2011). The role of pore size on vascularization and tissue remodeling in PEG hydrogels. *Biomaterials*, 32(26), 6045-6051. <https://doi.org/10.1016/j.biomaterials.2011.04.066>
- Dahlback, B., & Villoutreix, B. O. (2005). The anticoagulant protein C pathway. *FEBS Letters*, 579(15), 3310-3316. <https://doi.org/10.1016/j.febslet.2005.03.001>
- Dittman, W. A., & Majerus, P. W. (1990). Structure and function of thrombomodulin: A natural anticoagulant. *Blood*, 75(2), 329-336.
- Dzobo, K., Turnley, T., Wishart, A., Rowe, A., Kallmeyer, K., van Vollenstee, F. A., ... Parker, M. I. (2016). Fibroblast-derived extracellular matrix induces chondrogenic differentiation in human adipose-derived mesenchymal stromal/stem cells in vitro. *International Journal of Molecular Sciences*, 17(8), <https://doi.org/10.3390/ijms17081259>
- Fajas, L. (2003). Adipogenesis: A cross-talk between cell proliferation and cell differentiation. *Annals of Medicine*, 35(2), 79-85. <https://doi.org/10.1080/07853890310009999>
- Flynn, L., Prestwich, G. D., Semple, J. L., & Woodhouse, K. A. (2009). Adipose tissue engineering in vivo with adipose-derived stem cells on naturally derived scaffolds. *Journal of Biomedical Materials Research. Part A*, 89A(4), 929-941. <https://doi.org/10.1002/jbm.a.32044>
- Flynn, L. E. (2010). The use of decellularized adipose tissue to provide an inductive microenvironment for the adipogenic differentiation of human adipose-derived stem cells. *Biomaterials*, 31(17), 4715-4724. <https://doi.org/10.1016/j.biomaterials.2010.02.046>
- Gaengel, K., Genove, G., Armulik, A., & Betsholtz, C. (2009). Endothelial-mural cell signaling in vascular development and angiogenesis. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 29(5), 630-638. <https://doi.org/10.1161/ATVBAHA.107.161521>
- Guneta, V., Loh, Q. L., & Choong, C. (2016). Cell-secreted extracellular matrix formation and differentiation of adipose-derived stem cells in 3D alginate scaffolds with tunable properties. *Journal of Biomedical Materials Research. Part A*, 104(5), 1090-1101. <https://doi.org/10.1002/jbm.a.35644>
- Guneta, V., Zhou, Z., Tan, N. S., Sugii, S., Wong, M. T. C., & Choong, C. (2017). Recellularization of decellularized adipose tissue-derived stem cells: Role of the cell-secreted extracellular matrix in cellular differentiation. *Biomaterials Science*, 6(1), 168-178. <https://doi.org/10.1039/c7bm00695k>
- Guo, Y., Zeng, Q. C., Yan, Y. X., Shen, L., Liu, L., Li, R. X., ... Huang, S. J. (2013). Proliferative effect and osteoinductive potential of extracellular matrix coated on cell culture plates. *SpringerPlus*, 2, 303. <https://doi.org/10.1186/2193-1801-2-303>
- Huber, B., Borchers, K., Tovar, G. E., & Kluger, P. J. (2016). Methacrylated gelatin and mature adipocytes are promising components for adipose tissue engineering. *Journal of Biomaterials Applications*, 30(6), 699-710. <https://doi.org/10.1177/0885328215587450>
- Ikezoe, T., Yang, J., Nishioka, C., Pan, B., Xu, K., Furihata, M., ... Yokoyama, A. (2017). The fifth epidermal growth factor-like region of

- thrombomodulin exerts cytoprotective function and prevents SOS in a murine model. *Bone Marrow Transplantation*, 52(1), 73–79. <https://doi.org/10.1038/bmt.2016.195>
- Kapur, S. K., & Katz, A. J. (2013). Review of the adipose derived stem cell secretome. *Biochimie*, 95(12), 2222–2228. <https://doi.org/10.1016/j.biochi.2013.06.001>
- Kondo, K., Shintani, S., Shibata, R., Murakami, H., Murakami, R., Imaizumi, M., ... Murohara, T. (2009). Implantation of adipose-derived regenerative cells enhances ischemia-induced angiogenesis. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 29(1), 61–U167. <https://doi.org/10.1161/Atvbaha.108.166496>
- Laschke, M. W., & Menger, M. D. (2016). Prevascularization in tissue engineering: Current concepts and future directions. *Biotechnology Advances*, 34(2), 112–121. <https://doi.org/10.1016/j.biotechadv.2015.12.004>
- Laschke, M. W., Rucker, M., Jensen, G., Carvalho, C., Mulhaupt, R., Gellrich, N. C., & Menger, M. D. (2008). Incorporation of growth factor containing Matrigel promotes vascularization of porous PLGA scaffolds. *Journal of Biomedical Materials Research. Part A*, 85(2), 397–407. <https://doi.org/10.1002/jbm.a.31503>
- Laschke, M. W., Strohe, A., Scheuer, C., Eglin, D., Verrier, S., Alini, M., ... Menger, M. D. (2009). In vivo biocompatibility and vascularization of biodegradable porous polyurethane scaffolds for tissue engineering. *Acta Biomaterialia*, 5(6), 1991–2001. <https://doi.org/10.1016/j.actbio.2009.02.006>
- Laschke, M. W., Vollmar, B., & Menger, M. D. (2009). Inosculation: Connecting the life-sustaining pipelines. *Tissue Engineering. Part B, Reviews*, 15(4), 455–465. <https://doi.org/10.1089/ten.TEB.2009.0252>
- Ley, K., & Tedder, T. F. (1995). Leukocyte interactions with vascular endothelium. New insights into selectin-mediated attachment and rolling. *Journal of Immunology*, 155(2), 525–528.
- Liu, S., Zhang, H., Zhang, X., Lu, W., Huang, X., Xie, H., ... Jin, Y. (2011). Synergistic angiogenesis promoting effects of extracellular matrix scaffolds and adipose-derived stem cells during wound repair. *Tissue Engineering. Part A*, 17(5–6), 725–739. <https://doi.org/10.1089/ten.TEA.2010.0331>
- Lu, H. X., Hoshiba, T., Kawazoe, N., & Chen, G. P. (2011). Autologous extracellular matrix scaffolds for tissue engineering. *Biomaterials*, 32(10), 2489–2499. <https://doi.org/10.1016/j.biomaterials.2010.12.016>
- Lu, H. X., Hoshiba, T., Kawazoe, N., Koda, I., Song, M. H., & Chen, G. P. (2011). Cultured cell-derived extracellular matrix scaffolds for tissue engineering. *Biomaterials*, 32(36), 9658–9666. <https://doi.org/10.1016/j.biomaterials.2011.08.091>
- Marra, K. G., DeFail, A., Clavijo-Alvarez, J. A., Badylak, S. F., Taieb, A., Schipper, B., ... Rubin, J. P. (2008). FGF-2 enhances vascularization for adipose tissue engineering. *Plastic and Reconstructive Surgery*, 121(4), 1153–1164. <https://doi.org/10.1097/01.prs.0000305517.93747.72>
- Matsuda, K., Falkenberg, K. J., Woods, A. A., Choi, Y. S., Morrison, W. A., & Dilley, R. J. (2013). Adipose-derived stem cells promote angiogenesis and tissue formation for in vivo tissue engineering. *Tissue Engineering. Part A*, 19(11–12), 1327–1335. <https://doi.org/10.1089/ten.TEA.2012.0391>
- Moldovan, L. M. Y., Lustig, M., Naftaly, A., Mardamshina, M., Geiger, T., Gefen, A., & Benayahu, D. (2019). Cell shape alteration during adipogenesis is associated with coordinated matrix cues. *Journal of Cellular Physiology*, 234(4), 3850–3863. <https://doi.org/10.1002/jcp.27157>
- Moon, M. H., Kim, S. Y., Kim, Y. J., Kim, S. J., Lee, J. B., Bae, Y. C., ... Jung, J. S. (2006). Human adipose tissue-derived mesenchymal stem cells improve postnatal neovascularization in a mouse model of hindlimb ischemia. *Cellular Physiology and Biochemistry*, 17(5–6), 279–290. <https://doi.org/10.1159/000094140>
- Murakami, M., & Simons, M. (2008). Fibroblast growth factor regulation of neovascularization. *Current Opinions in Hematology*, 15(3), 215–220. <https://doi.org/10.1097/MOH.0b013e3282f97d98>
- Nakagami, H., Maeda, K., Kaneda, Y., Ogihara, T., & Morishita, R. (2005). Novel autologous cell therapy in ischemic limb disease through growth factor secretion by cultured adipose tissue-derived stromal cells. *Hypertension*, 46(4), 867.
- Nissen, L. J., Cao, R., Hedlund, E. M., Wang, Z., Zhao, X., Wetterskog, D., ... Cao, Y. (2007). Angiogenic factors FGF2 and PDGF-BB synergistically promote murine tumor neovascularization and metastasis. *Journal of Clinical Investigation*, 117(10), 2766–2777. <https://doi.org/10.1172/JCI32479>
- Oh, I. Y., Yoon, C. H., Hur, J., Kim, J. H., Kim, T. Y., Lee, C. S., ... Kim, H. S. (2007). Involvement of E-selectin in recruitment of endothelial progenitor cells and angiogenesis in ischemic muscle. *Blood*, 110(12), 3891–3899. <https://doi.org/10.1182/blood-2006-10-048991>
- Olive, P. L., Vikse, C., & Trotter, M. J. (1992). Measurement of oxygen diffusion distance in tumor cubes using a fluorescent hypoxia probe. *International Journal of Radiation Oncology, Biology, Physics*, 22(3), 397–402. [https://doi.org/10.1016/0360-3016\(92\)90840-E](https://doi.org/10.1016/0360-3016(92)90840-E)
- Ostman, A., Andersson, M., Betsholtz, C., Westermark, B., & Heldin, C. H. (1991). Identification of a cell retention signal in the B-chain of platelet-derived growth factor and in the long splice version of the A-chain. *Cell Regulation*, 2(7), 503–512. <https://doi.org/10.1091/mbc.2.7.503>
- Pan, B., Wang, X., Nishioka, C., Honda, G., Yokoyama, A., Zeng, L., ... Ikezoe, T. (2017). G-protein coupled receptor 15 mediates angiogenesis and cytoprotective function of thrombomodulin. *Scientific Reports*, 7(1), 692. <https://doi.org/10.1038/s41598-017-00781-w>
- Piali, L., Hammel, P., Uherek, C., Bachmann, F., Gisler, R. H., Dunon, D., & Imhof, B. A. (1995). CD31/PECAM-1 is a ligand for alpha v beta 3 integrin involved in adhesion of leukocytes to endothelium. *Journal of Cell Biology*, 130(2), 451–460. <https://doi.org/10.1083/jcb.130.2.451>
- Rehman, J., Traktuev, D., Li, J., Merfeld-Clauss, S., Temm-Grove, C. J., Bovenkerk, J. E., ... March, K. L. (2004). Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation*, 109(10), 1292–1298. <https://doi.org/10.1161/01.CIR.0000121425.42966.F1>
- Sart, S., Yan, Y. W., Li, Y., Lochner, E., Zeng, C. C., Ma, T., & Li, Y. (2016). Crosslinking of extracellular matrix scaffolds derived from pluripotent stem cell aggregates modulates neural differentiation. *Acta Biomaterialia*, 30, 222–232. <https://doi.org/10.1016/j.actbio.2015.11.016>
- Schenke-Layland, K., Rofail, F., Heydarkhan, S., Gluck, J. M., Ingle, N. P., Angelis, E., ... Heydarkhan-Hagvall, S. (2009). The use of three-dimensional nanostructures to instruct cells to produce extracellular matrix for regenerative medicine strategies. *Biomaterials*, 30(27), 4665–4675. <https://doi.org/10.1016/j.biomaterials.2009.05.033>
- Thomlinson, R. H., & Gray, L. H. (1955). The histological structure of some human lung cancers and the possible implications for radiotherapy. *British Journal of Cancer*, 9(4), 539–549. <https://doi.org/10.1038/bjc.1955.55>
- Tomanek, R. J., Hansen, H. K., & Christensen, L. P. (2008). Temporally expressed PDGF and FGF-2 regulate embryonic coronary artery formation and growth. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 28(7), 1237–1243. <https://doi.org/10.1161/ATVBAHA.108.166454>
- Verseijden, F., Posthumus-van Sluijs, S. J., Farrell, E., van Neck, J. W., Hovius, S. E. R., Hofer, S. O. P., & van Osch, G. J. V. M. (2010). Prevascular structures promote vascularization in engineered human adipose tissue constructs upon implantation. *Cell Transplantation*, 19(8), 1007–1020. <https://doi.org/10.3727/096368910X492571>
- Verseijden, F., Posthumus-van Sluijs, S. J., van Neck, J. W., Hofer, S. O., Hovius, S. E., & van Osch, G. J. (2012). Comparing scaffold-free and fibrin-based adipose-derived stromal cell constructs for adipose tissue engineering: An in vitro and in vivo study. *Cell Transplantation*, 21(10), 2283–2297. <https://doi.org/10.3727/096368912X653129>

- Verseijden, F., Posthumus-van Sluijs, S. J., Pavljasevic, P., Hofer, S. O. P., van Osch, G. J. V. M., & Farrell, E. (2010). Adult human bone marrow- and adipose tissue-derived stromal cells support the formation of prevascular-like structures from endothelial cells in vitro. *Tissue Engineering. Part A*, 16(1), 101–114. <https://doi.org/10.1089/ten.tea.2009.0106>
- Volz, A. C., Hack, L., Atzinger, F. B., & Kluger, P. J. (2018). Completely defined co-culture of adipogenic differentiated ASCs and microvascular endothelial cells. *Altex-Alternatives to Animal Experimentation*, 35(4), 464–476. <https://doi.org/10.14573/altex.1802191>
- Volz, A. C., Huber, B., Schwandt, A. M., & Kluger, P. J. (2017). EGF and hydrocortisone as critical factors for the co-culture of adipogenic differentiated ASCs and endothelial cells. *Differentiation*, 95, 21–30. <https://doi.org/10.1016/j.diff.2017.01.002>
- Walser, R., Metzger, W., Gorg, A., Pohlemann, T., Menger, M. D., & Laschke, M. W. (2013). Generation of co-culture spheroids as vascularisation units for bone tissue engineering. *European Cells & Materials*, 26, 222–233. <https://doi.org/10.22203/ecm.v026a16>
- Wenz, A., Tjoeng, I., Schneider, I., Kluger, P. J., & Borchers, K. (2018). Improved vasculogenesis and bone matrix formation through coculture of endothelial cells and stem cells in tissue-specific methacryloyl gelatin-based hydrogels. *Biotechnology and Bioengineering*, 115(10), 2643–2653. <https://doi.org/10.1002/bit.26792>
- Wolchok, J. C., & Tresco, P. A. (2010). The isolation of cell derived extracellular matrix constructs using sacrificial open-cell foams.

Biomaterials, 31(36), 9595–9603. <https://doi.org/10.1016/j.biomaterials.2010.08.072>

- Yoon, J. J., Chung, H. J., Lee, H. J., & Park, T. G. (2006). Heparin-immobilized biodegradable scaffolds for local and sustained release of angiogenic growth factor. *Journal of Biomedical Materials Research. Part A*, 79A(4), 934–942. <https://doi.org/10.1002/jbm.a.30843>

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Nellinger S, Schmidt I, Heine S, Volz A-C, Kluger PJ. Adipose stem cell-derived extracellular matrix represents a promising biomaterial by inducing spontaneous formation of prevascular-like structures by mvECs. *Biotechnology and Bioengineering*. 2020;117:3160–3172. <https://doi.org/10.1002/bit.27481>