Differential behavior of pericytes and adipose stromal cells in vasculogenesis and angiogenesis

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Abstract

Perfusable vascularization of tissue-engineered systems remains one of the main hurdles in the field of providing large-scale functional tissues. Blood vessel formation by endothelial cells is dependent on supporting cells such as stromal cells. From them, the pericyte is the most relevant due to its close interaction with the endothelial cells, protecting them from external harm and architecting new vessels and connections. However, the identity of this cell type and its relation with other mesodermic cells, such as the mesenchymal stromal/stem cells, is highly debated. In this study, we delve on the distinct behavior of stromal cells and pericytes in the context of vascularization. The protein MCAM/CD146 was proposed as a specific marker for pericyte selection from adipose-derived stromal cells. By employing microfluidic chips that consist of an internal chamber flanked by two parallel media channels, we replicated the primary processes involved in vessel formation: vasculogenesis, which refers to the de novo vessel formation, and angiogenesis, the sprouting of new vessels from existing ones. In vasculogenesis, ASCs promoted the formation and proliferation of endothelial structures similar to the embryonic capillary plexus, whereas selected pericytes reduced their size and forced them to form vessel-like structures. In angiogenesis, on the other hand, pericytes promoted significantly more sprouting than the ASCs. Furthermore, this pericytic MCAM-enriched cell population exhibited an intimate interaction with endothelial cells leading to the synthesis of a shared basement membrane between these cell types, which was rarely observed for its unselected counterpart. These results shed light on the role of pericytes in *in vitro* vascularization and confirm the candidate marker MCAM/CD146 as a reliable marker for mural cell selection. In addition, the adipose pericyte is proposed as a very promising cell type to advance the field of tissue-engineered vascularization.

Keywords

Vascularization, MCAM/CD146, mesenchymal stem cells, microfluidic chip.

Introduction

The circulatory system is the interconnected and hierarchical network of vessels that irrigate most human tissues, nourishing the cells with nutrients and oxygen, removing waste products and serving as a connection for the endocrine and immune systems to swiftly reach any part of the body (Yang et

al. 2020). All vessels are lined with endothelial cells. In the capillaries, the smallest branch of the circulation, endothelial cells are lined externally by a mural cell type known as pericyte, which supports its homeostasis, protects it from stromal debris by phagocytosis and regulates the blood flow (Zhao and Chappell 2019).

For tissue engineering, the size of avascularized bioengineered organs is limited to a few hundred micrometers (Chandra and Atala 2019). Any thicker tissue will eventually lead to cell death without irrigation of a blood-mimicking fluid due to the lack of oxygen and nutrient diffusion to the innermost cells, causing the formation of a necrotic core. However, achieving vascularization in *in vitro* systems is still a challenge due to the complexity of the process and variety of cells involved (Zhang and Radisic 2020).

Human adipose tissue is an easily obtainable biological source, which can be digested using collagenases to extract a heterogeneous mixture of cells, known as stromal vascular fraction (SVF) (Sun et al. 2019). The spindle-shaped cells produced by seeding the SVF on cell culture-treated plastic are often referred as adipose-derived mesenchymal stromal/stem cells or adipose stromal cells (ASCs) (Bourin et al. 2013), and have been widely reported to have immunomodulatory properties (Melief et al. 2013) and *in vitro* multipotency (Brooks et al. 2019). Within this heterogeneous collection, we can find a wide variety of stromal cells such as fibroblasts, pre-adipocytes, and the previously mentioned pericytes (Sun et al. 2019). The Melanoma Cell Adhesion Molecule (MCAM), also known as Cluster of Differentiation (CD) 146, can act as a mural cell marker for bone marrow-derived stromal cells (Blocki et al. 2013) and has been proposed as an specific membrane protein for adipose-resident pericyte isolation (Wang et al. 2019). Importantly, MCAM is not exclusive for pericytes and can be expressed in other vascular-related cell types such as endothelial cells and lymphocytes, but these cells can be depleted due to their incapability or inefficiency to attach and divide on cell culture-treated plastic surfaces (Wang et al. 2020). The identity of pericytes *in vivo* and, specially, *in vitro*, are however widely debated (Caplan 2017; Blocki et al. 2013). So is their relationship with mesenchymal stem cells, their contribution to vascularization processes and their use in cell therapies and tissue engineering (Blocki et al. 2018; Kosyakova et al. 2020; Zhao and Chappell 2019; Souza et al. 2016; Guimarães-Camboa et al. 2017).

In recent years, microfluidic devices have allowed the creation of organ-on-chip technologies, also known as microphysiological systems (Leung et al. 2022). These platforms have been demonstrated as powerful tools for the study of tissue development, cell-cell and cell-matrix interaction, and vascularization (Vila Cuenca et al. 2021; Alimperti et al. 2017). Here, we use a microfluidic platform to mimic vasculogenic and angiogenic processes, comparing how pericytes and ASCs interact with endothelial cells to form the vessels and promote their growth. The present study is based on two hypotheses, (1) that CD146 serves as a reliable marker for the selection of pericytes from heterogenous adipose stromal cells, and (2) that pericytes can be cultured *in vitro* without losing the capacity to act as mural cells, promoting more vessel formation than their unselected counterpart, ensheating themselves and the associated endothelial cells into a shared basement membrane.

Materials and methods

Cell isolation and culture

ASCs and pericytes were isolated from adipose tissue kindly provided by the Clinic for Plastic, Hand and Burns Surgery (University Hospital Aachen). First, the adipose layer was separated from the dermis and manually minced until achieving a homogenous slurry. The minced fat was added to gentleMACS C-Tubes (Miltenyi), mixed with the same volume of collagenase I (1 mg/mL; Sigma-Aldrich), and placed

in a gentleMACS Octo Dissociator to achieve complete homogeneization and digestion at 37°C with consecutive stirring steps for 15 minutes. The mixture is left for another 30 minutes at 37°C in soft agitation on a roller mixer. The digested SVF is then filtered using a 100 µm cell strainer (Falcon), mixed with sterile PBS and centrifuged at 300 g. The supernatant is removed and the pellet is re-suspended in PBS for two more washing steps. To expand the ASCs, three quarters of the SVF suspension is seeded in a cell culture-plastic flask and cultured in Mesenpan media (PAN-Biotech) supplemented with 2% (v/v) fetal bovine serum (FBS; Gibco) and 1% (v/v) antibiotic/antimycotic solution (ABM; Gibco).

The other part of the SVF is diluted 1:10 in Erythrocyte Lysis Buffer (PAN-Biotech), briefly vortexed and incubated at room temperature (RT) for 10 minutes. The mixture is washed another 3 times as previously described. The resulting pellet is resuspended in 10 µL anti-CD146 magnetic microbeads (Miltenyi) and 90 µL blocking buffer (Miltenyi), and incubated for 15 minutes at 4°C. The mixture is washed again three times to remove unattached magnetic microbeads. A MACS LS column (Miltenyi) is attached to a magnetic separator (Miltenyi) and prepared by rinsing it with PBS. The labeled cell suspension is added to the column and rinsed three times with Mesenpan to remove the $CD146^-$ cells. The column is then separated from the magnetic holder, filled with media and pressed with a sterile plunger. The resulting $CD146^+$ cell suspension is considered as pericytes, and will be cultured as previously described in the same conditions as the ASCs.

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords using collagenase type I (Sigma-Aldrich, 400 U/mL) to separate the cell layer surrounding the vein lumen. The detached cells were subsequently expanded in endothelial cell growth medium 2 (EGM2; Promocell) supplemented with 1% (v/v) ABM. The human umbilical cords were generously provided by the Clinic for Gynecology and Obstetrics at RWTH Aachen University Hospital and were used within 24 hours after delivery.

HUVECs, ASCs and pericytes were cryopreserved in liquid nitrogen using a freezing solution consisting of 60% (v/v) DMEM, 20% (v/v) FBS, and 20% (v/v) DMSO. Prior to the device experiment, HUVECs were thawed and expanded up to passage 3 in EGM2. ASCs and pericytes were cultured up to passage 4 in Mesenpan.

Preparation of the vascularization assays in microfluidic devices

HUVECs, ASCs and pericytes were detached by trypsin/EDTA (Pan-Biotech) digestion. Cells were then suspended in tris-buffered saline (TBS) solution containing thrombin (Sigma-Aldrich) and calcium chloride ($CaCl_2$; Sigma-Aldrich). This mixture was combined with a fibrinogen solution (VWR) pre-cooled on ice and either 10 µL were injected into the central chamber of the idenTx 3 microfluidic chip (AIM Biotech) for the chip experiments or 350 µL were directly deposited into 24-well plate wells for the bulk gels. The resulting gel contained 5 mg/mL fibrinogen, 3 IU/mL thrombin and 3.75 mM $CaCl_2$ dissolved in 50 µg/mL TBS.

For imaging after the preparation, cells were pre-stained with vybrant dyes (Invitrogen) by adding 5 µL of cell-labeling solution to a 1 x 10^6 cell/mL cell suspension in serum-free cell media. The mixture was vortex and incubated for 20 minutes at 37°C. Then, cells were centrifuged at 300 g and washed with PBS three times to remove residual dye.

For the vasculogenesis chip set-up, 10 x 10^6 HUVECs/mL and 2 x 10^6 supporting cells/mL were pre-mixed in the injected solution. For the bulk vasculogenesis gel, 10 x 10^6 HUVECs/mL and 5 x 10^6 supporting cells/mL were added. For the angiogenesis chip set-up, only the supporting cells were suspended in the polymerizing solution. In all cases, the fibrin gels were incubated for 20 min at room temperature and another 20 min at 37°C and 5% CO_2 in a humidified atmosphere to achieve the complete polymerization of the fibrin. For the angiogenesis assay, the bottom channel was filled with

a solution of 50 µg/mL fibronectin in EGM microvascular vessel 2 (EGM-MV 2, PromoCell) immediately after the polymerization and incubated for 40 minutes at 37°C. The fibronectin was washed away with EGM-MV 2 and a solution of 2 x 10^5 HUVECs/mL was injected in the coated channel.

Chips of both set-ups were filled with EGM-MV 2 supplemented with 1% (v/v) ABM, adding 100 µL to the bottom left well, 80 µL to the bottom right well, 60 µL to the top left well and 40 µL to the top right well, to achieve gravity driven flow both inside each of the channels and between them through the hydrogel chamber. The chips were placed in humidified chambers with the bottom exposed to air, in a 5% CO2 and 37°C atmosphere. The media was changed every 24 hours for 10 days. For the bulk gels in 24-well plates, 500 µL of EGM-MV 2 was added and changed every 48-72 hours. Three biological replicates (independent donors) were used.

For fixation, the medium was removed from the channels and substituted with ice-cold methanol at -20°C. All fibrin gels were incubated at 4°C for 30 minutes and then washed three times with PBS.

Immunofluorescence imaging

The fixed chips were first stained with mouse anti-CD31 (PECAM-1, 1:100; Sigma-Aldrich) solved in antibody diluent made of 3% Bovine Serum Albumin (BSA) and 1% ABM for 48 hours at 37°C. Then, the samples were washed three times. Afterwards, a mixture of Alexa Fluor® 594 goat anti-mouse IgG (1:400, Invitrogen) and Phalloidin-iFluor™ 488 Conjugate (Cayman Chemical) was added to the samples and incubated for 48 hours at 37°C. The chips were washed, stained with 0.4 µg/mL of a DAPI solution for 2 hours at 37°C, washed again and stored at 4°C until imaging. Microscopy was done with a LSM 980 with Airyscan 2 (Zeiss) confocal laser scanning microscope, creating stacks of the whole gel thickness, 200 µm, with a step size of 3 µm. The resulting composite was processed with Imaris 10 (Oxford Instruments), creating 3D surfaces of the $CD31^+$ vessel-like objects. The volume of these objects was measured.Only in the case of the angiogenesis model, the structures were considered tubular and homogenous enough to assess them as cylinders, to approximate the diameter based on the area and volume data. The number of branching and sprouting points was manually counted. The vessel-like objects were subtracted from the F-actin channel to show only the supporting cells. From this, 3D surfaces were again created to measure the distance of these cells from the $CD31^+$ structures.

The data was analyzed for normality with the Shapiro-Wilk test. Except for the “distance to vessel” data of the angiogenesis assay, all results were normally distributed and unpaired T-test were performed. For the “distance to vessel” data, a non-parametric Mann-Whitney Test was used.

Transmission electron microscopy (TEM) imaging

For TEM analysis, the bulk fibrin gels were fixed in a 3% glutaraldehyde solution for at least 24 hours. They were then rinsed in 0.1 M Soerensen’s phosphate buffer (Merck) and fixed with 1% osmium tetraoxide (Carol Roth) in a 17% sucrose buffer (Merck). After a dehydration process with a series of increasing ethanol concentrations, the samples were immersed in propylene oxide, then in a 1:1 mixture of Epon resin (Serva) and propylene oxide for one hour, followed by a final one-hour incubation in pure Epon resin. The samples were then embedded in pure Epon at 90 °C for two hours.

Ultrathin sections between 70 to 100 nm were obtained using an ultramicrotome (Reichert Ultracut S, Leica) equipped with a Diatome diamond knife. These sections were placed onto Cu/Rh grids (HR23 Maxtaform, Plano). To enhance contrast, the sections were stained with a 0.5% uranyl acetate and 1% lead citrate solution (both from EMS). The final observation was performed using a Zeiss Leo 906 (Carl Zeiss) transmission electron microscope, operated at an acceleration voltage of 60 kV.

Results and discussion

Microfluidic chips serve as a platform for the study of vessel formation through vasculogenesis and angiogenesis

Vascularization can be achieved by vasculogenesis, in which ECs organize themselves into interconnected vessels, or by angiogenesis, where new vessels originate from pre-existing ones (Figure 1A). To compare both mechanisms and how pericytes and ASCs act in both of them, we used a three-channel microfluidic chip (Figure B). For mimicking vasculogenesis, both the ECs and the supporting cells are added into the hydrogel in the middle chamber, while for the angiogenesis only the supporting cells are added in the middle chamber and one of the channels is coated with ECs (Figure C). The pre-staining of the cells with Vybrant dyes allows to confirm the deposition of the cells after gel injection. Furthermore, imaging of the chips 24 hours after the preparation show the endothelial cells attached to the hydrogel-media interface in the angiogenesis condition, marking the beginning of angiogenesis (Figure C, right image).

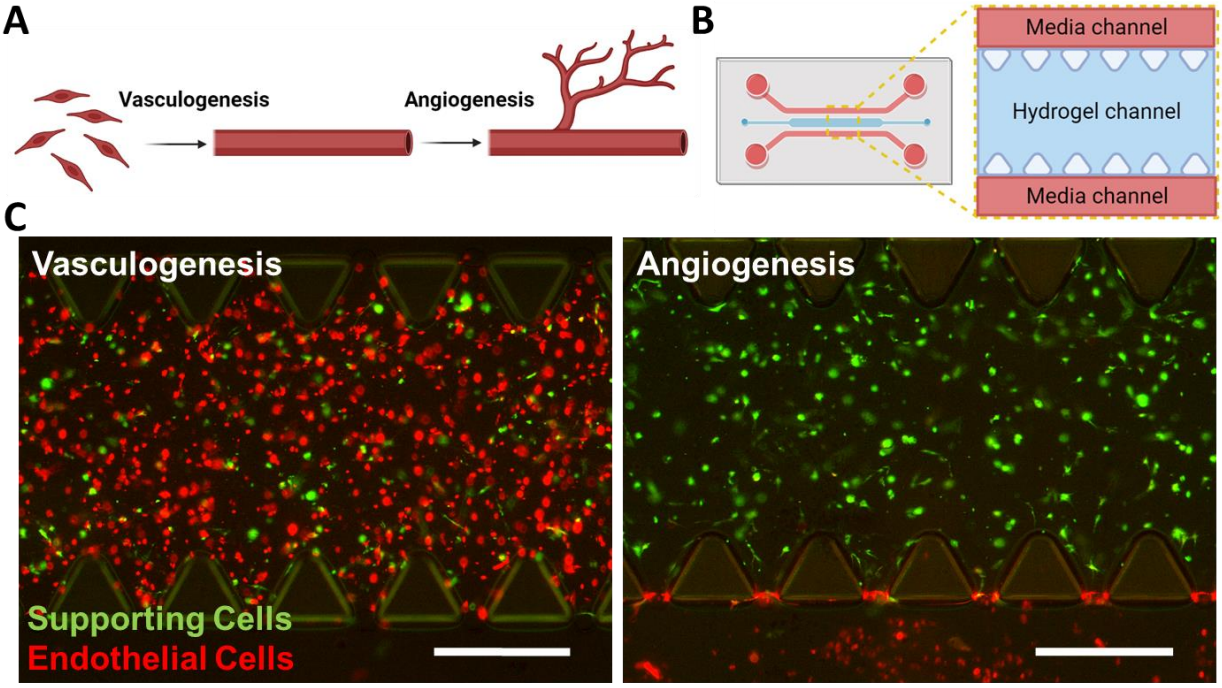

Figure 1. A) Main mechanisms of vessel formation in the human body. **B)** Schematic drawing showing the internal hydrogel chamber (blue) and the surrounding parallel media channels (red). **C)** Fluorescence image showing endothelial cells (Vybrant DiD, red) and supporting cells (Vybrant DiO, green), which can be either ASCs or pericytes, in two different set-ups to study vasculogenesis and angiogenesis. Scale bars: 500 µm.

MCAM/CD146-enriched adipose ASCs act as pericytes *in vitro* and show enhanced pro-angiogenic potential

We utilized the MCAM/CD146 protein as a specific marker for pericytes in heterogeneous ASC populations. Immunofluorescence images of the microfluidic device comparing pericytes and the unselected ASCs in vasculogenesis show the capacity of both cell types to support endothelial cell coalescence, elongation and tubulogenesis (Figure 2A). The quantification of the vessel-like structures shows a greater average volume and number of branching points in the gels co-cultured with ASCs,

but these differences are not statistically significant (Figure 2B and C). Also, both supporting cell types show a similar average distance to the vascular-like structures (Figure 2D).

The endothelial structures formed in the presence of the ASCs resemble the bloated and disorganized networks of the primitive plexus typical from the vasculogenesis processes that occur in the early embryo (Udan et al. 2013). In contrast, pericytes seem to inhibit this process and promote the formation of structures more similar to the vessels found in later stages of development. At the same time, pericytes seem to arrest the proliferative capacity of the endothelial cells, leading to smaller final endothelial volume.

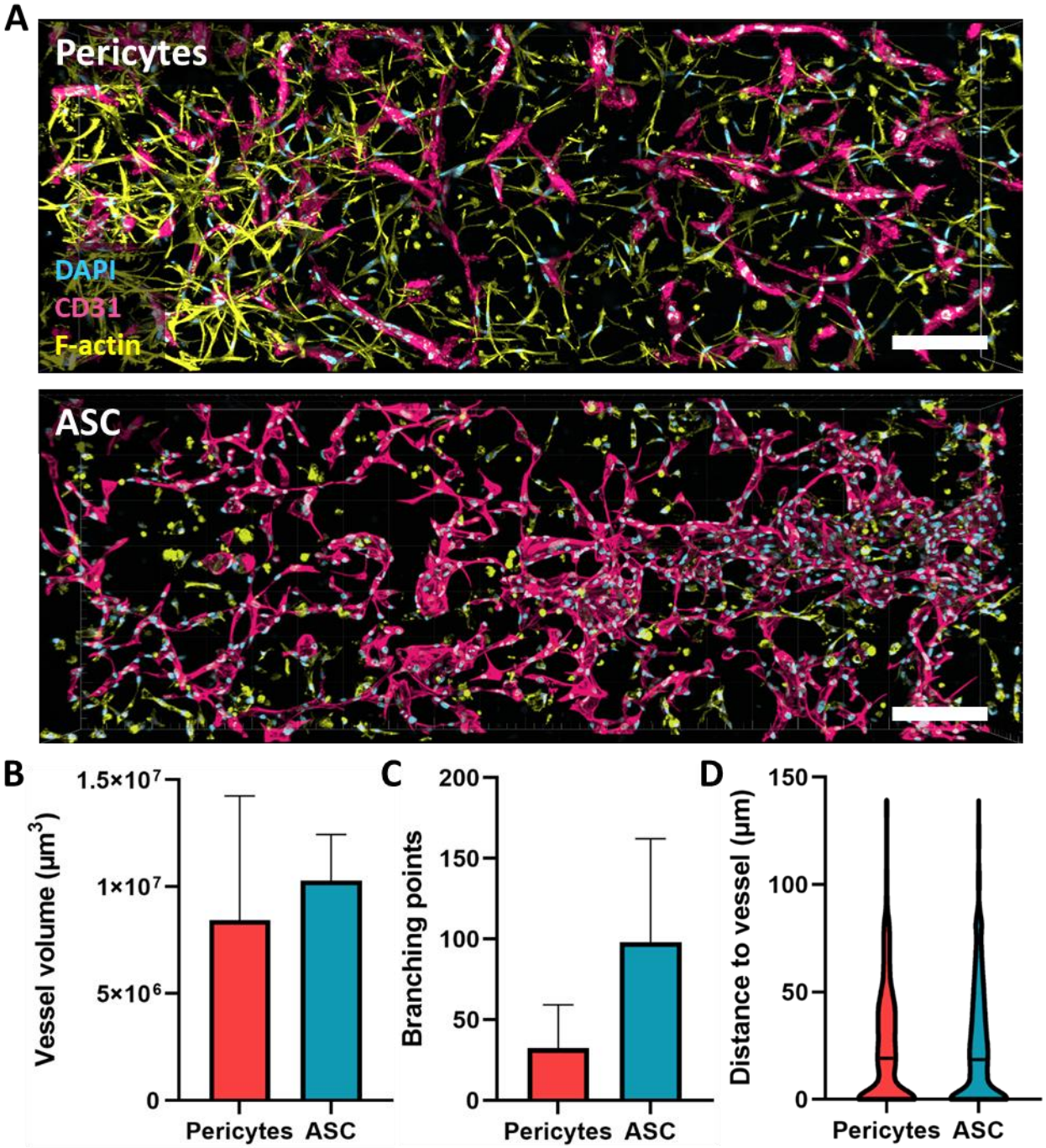

Figure 2. A) Immunofluorescence images of the vasculogenesis process within the microfluidic chips co-cultured with either pericytes or unselected ASCs, stained for nuclei (DAPI; cyan), CD31 showing the endothelial vessel-like structures (magenta) and the supporting cells' actin cytoskeleton (Phalloidin;

yellow). Scale bars: 200 µm. **B)** Comparison of total vessel volume between both co-culture conditions. **C)** Comparison of the total number of branching points between conditions. **D)** Comparison of the distance between each type of supporting cell and the nearest vessel-like structure. The trunked violin plots depict summary statistics and the kernel density estimation to show the frequency distribution of each condition. The middle line represents the median. (B, C and D: n = 3, t-test, *p < 0.05).

In the angiogenesis assay, on the other hand, pericytes are clearly promoting more vessel sprouting than the unselected ASCs (Figure 3A). Statistically significant differences can be found in both the vessel volume (p = 0.035) and branching points (p = 0.0329) (Figure 3B and D). These results are not correlated to any remarkable difference in vessel diameter (Figure 3C). As it can also be observed in the augmented immunofluorescence image, the pericytes tend to be significantly closer or even directly associated with the vessels, while the ASCs remain homogenously distributed in the gel (p < 0.0001) (Figure 3A and E).

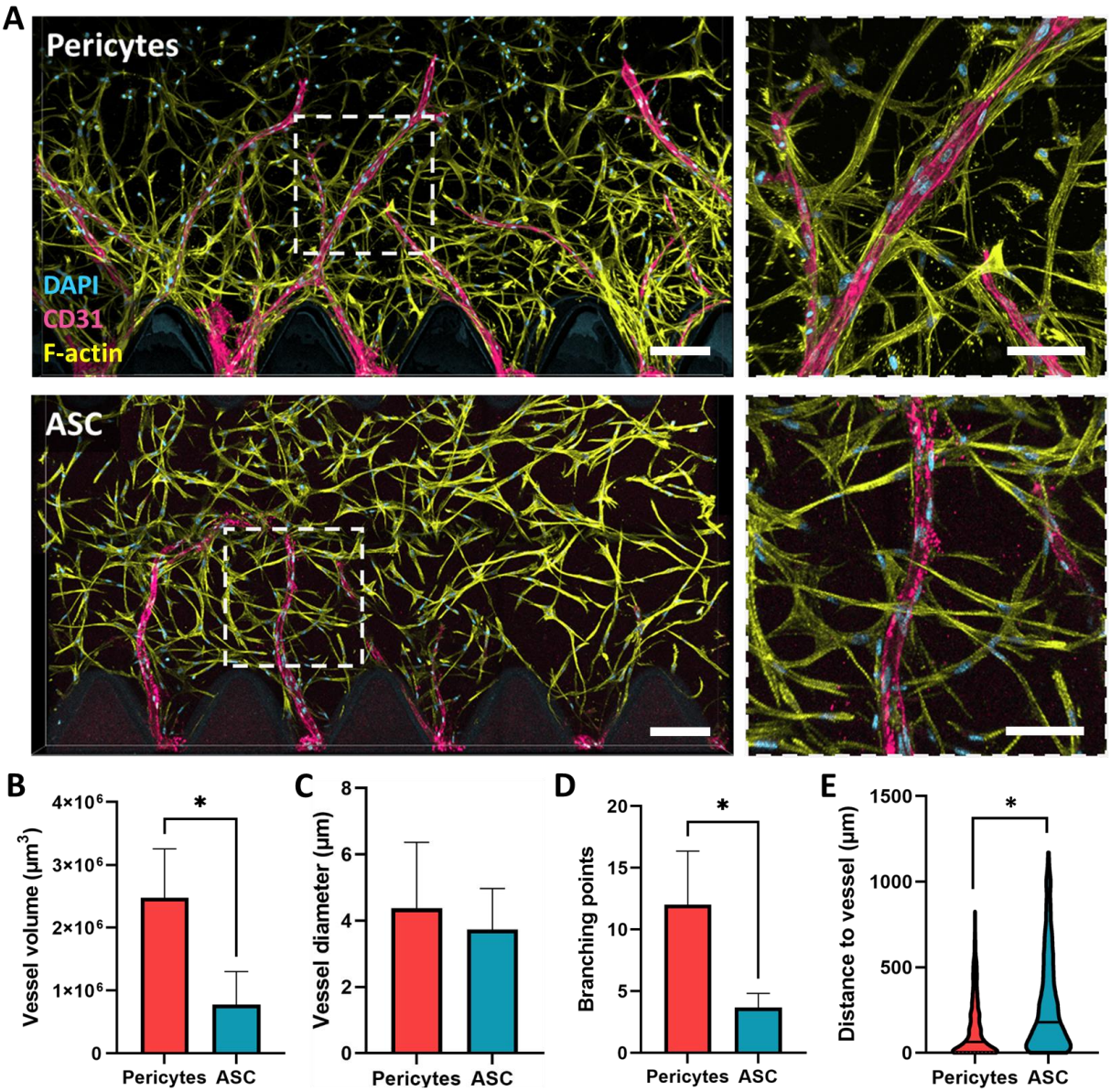

Figure 3. A) Immunofluorescence images of the angiogenic sprouting within the microfluidic chips co-cultured with either pericytes or unselected ASCs, stained for nuclei (DAPI; cyan), CD31 showing the endothelial vessel-like structures (magenta) and the supporting cells actin cytoskeleton (Phalloidin; yellow). Scale bar: 200 µm (left), 100 µm (right). **B)** Comparison of total vessel volume between both

co-culture conditions. **C)** Comparison of average vessel diameter. **D)** Comparison of the total number of branching points between conditions. **E)** Comparison of the distance between each type of supporting cell and the nearest vessel-like structure. The trunked violin plots depict summary statistics and the kernel density estimation to show the frequency distribution of each condition. The middle line represents the median. (B, C and D: n = 3, t-test; E: n = 3, Mann-Whitney test; *p < 0.05).

The main identifier of *in vivo* pericytes is the presence of a dense proteic fiber network shared with the endothelial vessels and enwrapping both of them, which is known as basement membrane. Confirming the previous findings, TEM reveals a close association of these pericytes with the endothelial vessels, with them forming a common basement membrane of shared matrix fibers (Figure 4). Such an interaction is rarely observed between the ASCs and the ECs, which only present a loose association without a remarkable amount of shared fibers.

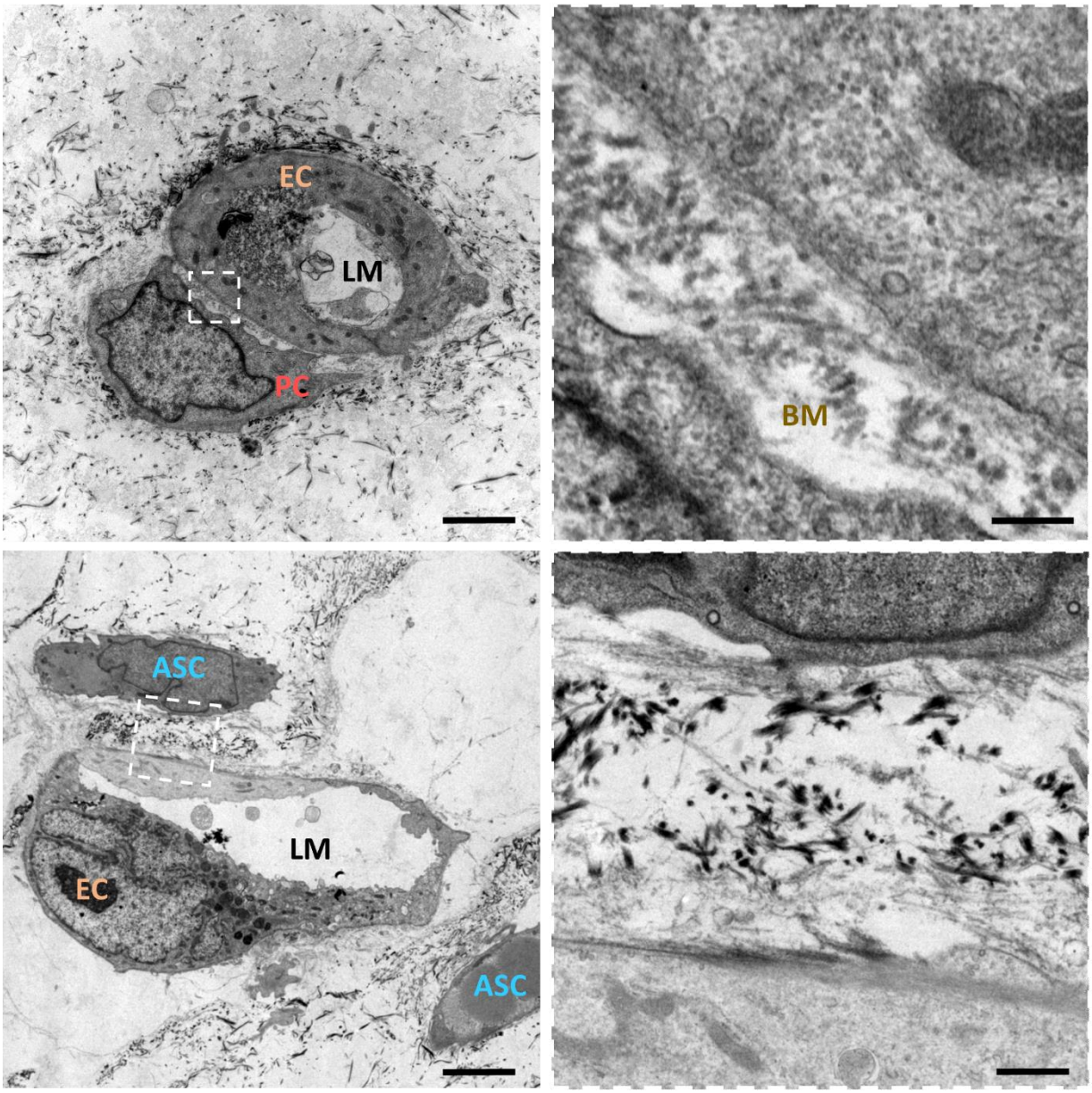

Figure 4. TEM image of the contact between the supporting cells and the vessels. Only pericytes possess a common basement membrane matrix with the endothelial cells. EC: Endothelial Cell; PC: Pericyte; ASC: Adipose stromal cell; BM: Basement membrane; LM: Lumen. Scale bars: 2500 nm (top left), 5000 nm (bottom left), 250 nm (right).

The relation of pericytes and mesenchymal stromal/stem cells has been widely debated in the last years, ranging from considering them as two completely independent populations to suggesting the latter as an activated form of the pericyte (Blocki et al. 2013; Souza et al. 2016; Blocki et al. 2018; Guimarães-Camboa et al. 2017). It has also been discussed if the pericytic identity can be maintained in culture or if subsequent passages growing outside of the body would lead to a loss of its characteristics (Blocki et al. 2013). Here, we demonstrate that pericytes can be isolated from adipose tissue using the MCAM marker as a specific protein for their selection. We also proof that this MCAM-enriched population of plastic-adherent cells acts as a mural cell *in vitro*, showing different behaviors in vasculogenesis and angiogenesis. Overall, we propose them as powerful pro-angiogenic supporting cells for tissue engineering purposes.

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