Optimizing Blood Vessel-on-a-Chip Models: The Impact of Mesenchymal Stem Cells on Endothelial Monolayer Integrity

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Abstract

This study aimed to create a structurally correct bilayered blood vessel-on-chip model with a mesenchymal stem cell (MSC) support layer surrounding a human umbilical vein endothelial cell (HUVEC) monolayer. The developed model was used to investigate the effect of MSCs on the integrity of the endothelial monolayer. A microfluidic platform was fabricated and microchannels were coated with a collagen matrix. A circular lumen in the collagen matrix was created with the template removal method, after which the lumen was seeded with MSCs prior to HUVECs. Additional experiments were performed on a plate to determine the effects of co-culture in a collagen gel without the limitations of on-chip experiments. In the research, challenges were encountered such as incomplete staining and non-confluent cell layers, which hindered clear conclusions on MSC and HUVEC layer mixing. Nonetheless, co-culturing MSCs with HUVECs seemed to enhance endothelial junction formation, suggesting MSCs may support endothelial integrity. Seeding cells onto microfluidic chips showed that achieving uniform cell attachment throughout the lumen was difficult. Cells primarily attached to the bottom surface of the lumen, emphasizing the need for protocol improvements to enhance cell adhesion within the lumen. While this study established a foundational model, further optimization is necessary to achieve clinically relevant and reproducible results. The results help to understand the interaction of MSCs with HUVECs within microfluidic systems and show the critical need for advanced techniques to further study the complex process of metastasis.

1 Introduction

Every year, about 18.000 women in the Netherlands receive the diagnosis breast cancer (BC). Of these women, 6% are already at stadium IV [1], which means the cancer has left the tumorsite and spread to other parts of the body. The process behind tumorcell migration into distant tissues is called metastasis. Relapse of a patient is often caused by a newly found metastasis rather than a local reoccurrence of BC, highlighting the critical need to target the metastatic process, especially for later stages of cancer progression [2].

Blood vessels are central in the process of metastatic spreading and are characterized by their structure consisting of multiple concentric layers of vascular cells surrounding a central lumen [3]. Endothelial cells form the innermost layer, creating a continuous monolayer that serves as an active barrier to circulating cells and other blood components. Perivascular cells surround the endothelial monolayer seperated by a thin basal lamina, as shown in Figure 1. Perivascular cells, including pericytes, provide structural support, communicate with endothelial cells, ensure the tightness of the vascular barrier and are crucial for maintaining endothelial integrity.

Extravasation of circulating tumor cells (CTCs) from the blood vessel is a main event during the process of metastasis, as shown in Figure 2 [4]. During metastasis, tumor cells intravasate into the blood stream and circulate through the body, after which CTCs arrest on the vascular endothelium and migrate across the endothelium into the underlying tissue. CTCs can either get trapped in smaller capillaries or adhere to larger venes when the flow is relatively low [5]. Hindering the extravasation of CTCs would reduce metastasis and significantly improve survival rates [6]. Therefore, understanding the underlying pathways of tumor cell behavior during extravasation and their interaction with vascular endothelium is essential for developing potential therapeutics that target metastasis.

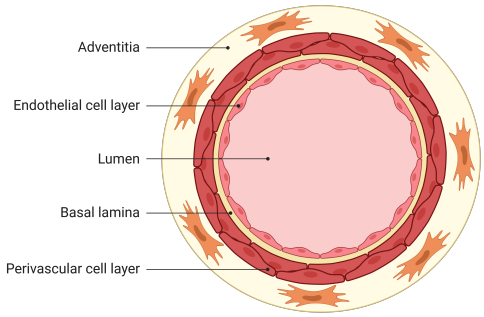

Fig. 1: Cross-section of blood vessel, consisting of multiple concentric layers of cells. The outermost layer is formed by perivascular cells, surrounding the innermost layer of endothelial cells, separated by a thin basal lamina. Created with BioRender.com

To investigate extravasation of CTCs, several in vivo and ex vivo experiments have been performed in mouse models [7] or in zebrafish embryos [8]. However, the interaction and communication among human cancer cells, human endothelial cells and human underlying tissues is missing in these models [9]. Furthermore, animal models have a low throughput due to limitations such as cost and time. Additionally, the extravasation of CTCs into intact organs is a challenging process to study, since it is a rare and only brief event [10].

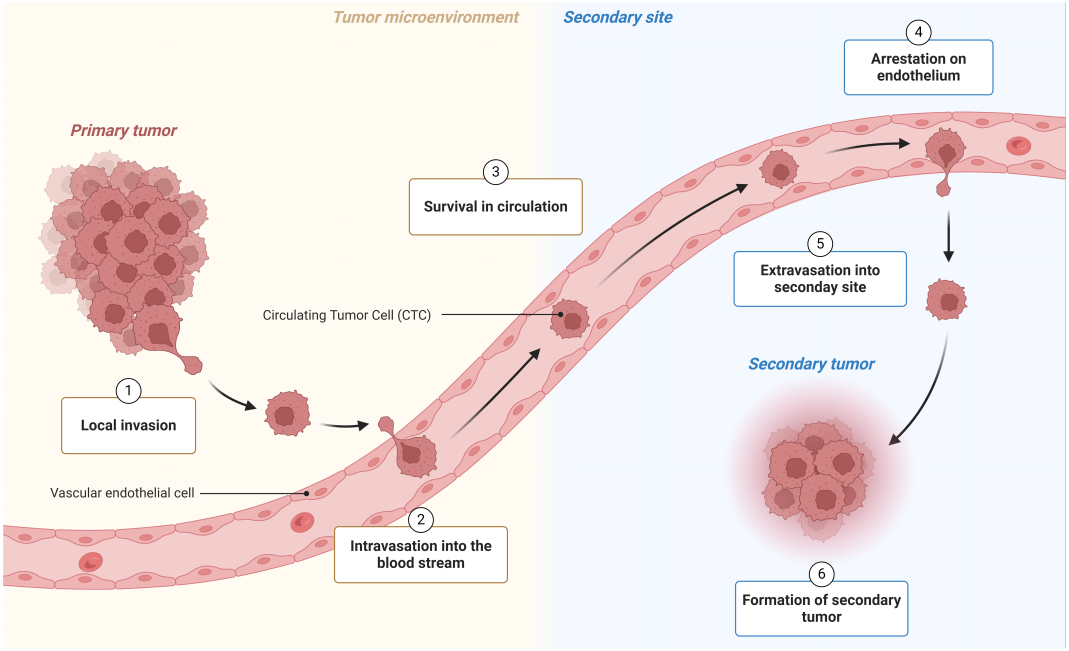

Fig. 2: Schematic representation of the metastatic cascade. Steps include tumor cell intravasation into the blood stream, circulation and extravasation from the blood vessel into the underlying tissue at the secondary site. Created with BioRender.com

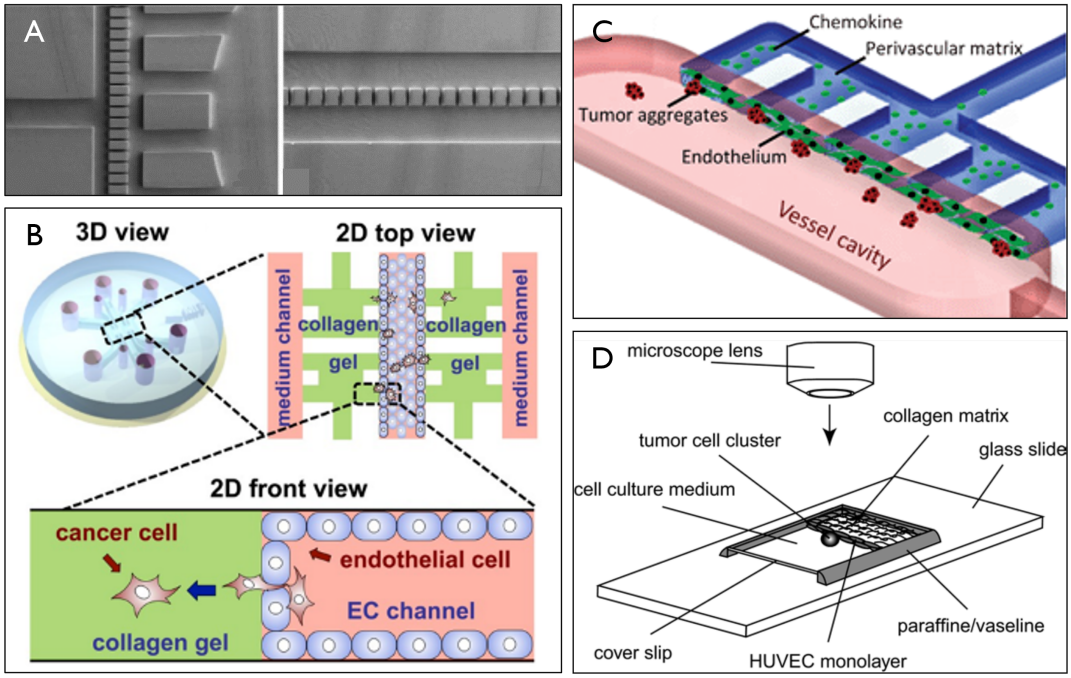

Fig. 3: Overview of current studies involving extravasation on chip research. A) Microgaps to be coated with a monolayer of endothelial cells replicating the basal lamina [11]. B) Square-shaped vessel channel surrounded next to a collagen matrix [12]. C) Monolayer epithelium separating a vessel cavity from the perivascular matrix [13]. D) Monolayer epithelium again separating a tumor cell cluster in a cell culture medium compartment from a collagen matrix [14].

Potential solutions for some of these limitations are offered by in vitro models, which use human cells within a highly controlled environment [15]. However, these models are often oversimplified, lacking the complex three-dimensional (3D) arrangement and essential interactions between cells and the extracellular matrix (ECM) necessary for modelling in vivo responses.

Therefore, there is a clear necessity for an in vitro model that includes the complex 3D structures of the in vivo human microenvironment. Microfluidics have the potential to overcome some of the technical constraints of 2D in vitro assays, offering novel models for investigating complex processes in a precisely controlled 3D environment [9]. Another advantage of these so called Organ-on-a-chip models is the possiblity of

high-resolution real-time imaging. Additionally, controlled flow and shear stress can be applied to more accurately mimic in vivo conditions.

Several microfluidic devices have been developed to investigate the CTC extravasation. Common techniques are microgaps coated with endothelial cells [11], square-shaped vessel channels [12] or a simple straight endothelial monolayer sheet [13, 14] (Figure 3). However, most of these do not incorporate an accurate blood-vessel model.

Although multiple blood vessel-on-chip models have been created, only few studies aim to reconstruct the multiple concentric layers of in vivo bloodvessels. In one study, perivascular cells were randomly embedded in a collagen matrix surrounding a channel seeded with endothelial cells [16]. Even though the barrier properties and cell junctions were improved with the inclusion of perivascular cells, this model did not replicate a perivascular layer closely surrounding the endothelial layer. A different method is the Viscous Finger Patterning technique, which brought the perivascular cells near the endothelial cells without mixing the two cellular layers [17]. However, it did not allow for precise control of the vessel geometry, potentially leading to irregularities when studying flow-related aspects. In another study, bone marrow stromal cells were seeded in a circular lumen and allowed to adhere to the collagen matrix [18]. After that, the endothelial cells were seeded into the same vessel, creating a double concentric layered vessel. However, the used chip design does not allow for the inclusion of a separately modeled tissue specific metastatic niche, which is necessary to distinguish the secondary site from the immediate surroundings of the blood vessel. Accurately modeling this niche is essential, as determining the tissue-specific metastatic potential of BC cells can improve diagnosis and aid in selecting the most effective therapy [19].

There is a strong need for an accurate blood-vessel-on-chip model to investigate tumor cell behaviour during extravasation. The aim of this study was to create a structurally correct bilayered blood vessel-on-chip model, while incorporating the possibility of studying CTC extravasation towards a tissue specific metastatic niche. The effect of a Mesenchymal Stem Cell (MSC) layer on the integrity of an endothelial cell layer in the blood vessel-on-chip model was investigated. In a study by de Souza et al. [20], it was suggested that MSCs are adventitial cells present in the most outer perivascular cell layer (Figure 1) . Therefore, we hypothesized that the inclusion of a MSC support layer will result in a more accurate blood vessel-on-a-chip model, by supporting the formation of tight junctions in the endothelial cell layer and thereby reducing permeability and ultimately extravasation of circulating tumor cells (CTCs) compared to a model with only endothelial cells.

A microfluidic platform was fabricated and microchannels were coated with a collagen matrix. A circular lumen in the collagen matrix was created with the template removal method [21], after which the lumen was seeded with MSCs prior to endothelial cells. With immunostaining and celltracker the cells were visualized and the vessel integrity could be analysed. Additional experiments were performed on a plate to determine the effects of co-culture in a collagen gel without the limitations of on-chip experiments. The results contribute to the understanding of MSC and HUVEC interactions within microfluidic systems and emphasize the need for optimized techniques to further study the process of extravasation.

2 Materials and Methods

2.1 Chip design

In a previous study, a blood vessel-on-chip design was made and developed to study extravasation [22]. It consists of four channels, each with a separate inlet and outlet and connected by micropillars. The height of the channels equals to 0.40 mm and all channels are 6.00 mm in length. The main channel (Figure 4 in blue; width = 0.50 mm) is elongated on both sides with guidance channels (width = 0.30 mm) to properly insert and position the wire within the main channel. Other channels are the metastatic niche channel (Figure 4 in green; width = 0.50 mm) where a tissue specific environment could possibly be created, and the two outermost side channels which are used for medium perfusion (Figure 4 in red; width = 0.30 mm).

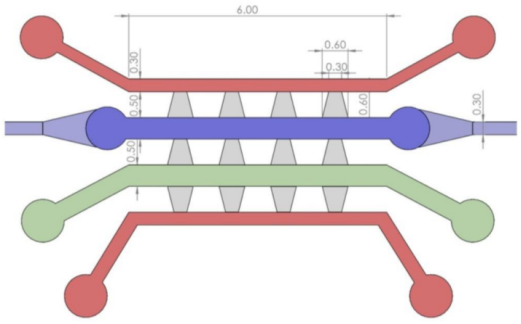

Fig. 4: Chip design by Wippert et al. [22] consisting of two side channels for medium perfusion (red), the main channel elongated on both sides with guidance channels (blue) and the metastatic niche channel (green). Measurements are given in mm.

The space between all channels is 0.60 mm and includes micropillars, creating four trapezoidal connections (height 0.20 mm). The broad side of the connection is 0.60 mm in length while the narrow side is only 0.30 mm. The broad side is directed towards the main and metastatic niche channel while the narrow side is directed to the medium channels to allow for proper hydrogel filling and medium perfusion. The connections between the main and metastatic niche channel have the narrow side directed towards the main channel to further optimalize hydrogel filling.

2.2 Chip fabrication

The chips were fabricated from a PDMS mixture, consisting of a cross-linker (SYLGARD 184; Merck) and a non-crosslinked PDMS prepolymer (SYLGARD) combined in a 1:10 weight ratio. The mixture was poured onto the mold and degassed for 1 hour in a vacuum chamber to eliminate air bubbles, after which it was baked for at least 4 hours at 65 ℃. The PDMS cast was carefully taken out of the mold before all chips were cut separate and covered with tape to prevent dust from collecting on the chips. Fluidic inlets and outlets were punched with a 1 mm Ø biopsy puncher. The PDMS chips were bonded to microscope slides (VWR; 26x76x1.0mm) by oxygen plasma surface treatment using CUTE Plasma System plasma cleaner (Femto Science). To ensure the PDMS-to-glass bonding, an additional overnight baking step at 65 ℃ was performed. Finally, the chips were sterilized with UV light in the laminar flow hood for a total duration of 1 hour, at 15 minute intervals per side.

2.3 Lumen formation

To enhance collagen matrix adhesion, the main channel and metastatic niche channel were coated with a 2 mg/mL polydopamine (PDA; Sigma Aldrich) solution in 10 mM Tris-HCl. The solution was filtered using a 0.22 μmeter filter and 1 mL syringe. After filtration, the solution was slowly pipetted into the two central channels and incubated for 1 hour at room temperature. Chips were washed three times with PBS before taking out all liquids and leaving them to dry in the incubator at 37℃ for at least 2 hours.

Once completely dry, a circular lumen is formed in the collagen matrix with the template removal method. A 0.25 mm Ø nylon wire (Decathlon) was UV sterilized for 1 hour and inserted in the main channel through the guidance channels. The position of the wire was checked with a microscope to ensure it was in the middle of the channel. A collagen solution (2.5 mg/mL) was prepared from 3 mg/mL Cellmatrix type I-P stock solution (Nitta Gelatin), 10x PBS and neutralizing buffer with respective ratio of 16:2:1. The buffer consists of 262 mM $NaHCO_3$, 20 mM HEPES and 0.05 M NaOH in distilled water [23]. All components were placed on ice to prevent polymerization. The collagen solution was quickly, but carefully pipetted into the main and metastatic niche channel to prevent bubbles from forming. Chips were then incubated for 30 minutes at 37℃ and 5% CO_2 to boost polymerization. After that, PBS was put in the side channels together with PBS filled pipette tips. Additionally, a drop of PBS was placed on the inlets and outlets of the main and metastatic niche channel to prevent drying out the collagen. At last, chips were left in the incubator at 37℃ and 5% CO_2 overnight to allow full polymerization of the collagen.

Next, the pipette tips were removed and the side channels were filled with new PBS. Then the wire was slowly pulled out of the main channel with a pair of tweezers and chips were examined with a microscope for bubbles or leakage. All liquid was removed from the chip before entirely closing off the guidance channel with baysilone-paste (GE Bayer Silicones).

2.4 Cell culture

Immortalized human umbilical vein endothelial cells (HUVEC/tert2) were obtained from CliniSciences and cultured in Endothelial Cell Basal Medium (EBM; Lonza; CC-3121) with 0.2% bovine brain extract, 5 ng/ml rh EGF, 10 mM L-glutamine, 0.75 Units/ml heparin sulfate, 1 μg/ml hydrocortisone, 50 μg/ml and 10% fetal bovine serum, hereafter referred to as EBM. HUVEC culture flasks for were coated with EmbryoMax 0.1% Gelatin Solution (Merck/Sigma Aldrich) for at least 30 min at 37℃ and 5% CO_2. Mesenchymal stem cells were obtained from ATCC, LGC-standards and cultured in α Minimum Essential Medium (αMEM; Gibco; 22571020) with 10% fetal bovine serum (FBS; Gibco), 100 U/mL of penicillin and 100 μg/mL of streptomycin, hereafter referred to as αMEM. All experiments were performed with HUVECs at passage 15 to 19 and MSCs at passage 8 to 10. Morphology of cells during regular culture and experiments was studied using phase contrast microscopy (Nikon).

2.5 Immunofluorescent staining

Cells were fixated for 15 minutes with 4% paraformaldehyde (PFA) at room temperature, after which they were washed with PBS three times. Permeabilization of the cells was done with 0.1% Triton-X 100 in PBS solution for 20 minutes. After washing three times with PBS, cells were blocked with 1% w/v bovine serum albumin (BSA) in PBS for 1 hour at room temperature. Then, cells were incubated with primary antibodies overnight at 4℃. Primary antibodies used were rabbit anti-vascular endothelial cadherin (VE-cadherin; adherens junctions; Invitrogen) at 1:100 dilution from a 0.25 mg/mL stock solution or mouse anti-zona occludens-1 (ZO-1; tight junctions; Invitrogen) at 1:100 dilution from a 0.5 mg/mL stock solution. Samples were washed three times between each addition of any further staining.

The secondary antibodies used are: goat anti-mouse Alexa Fluor 488 (GFP; Invitrogen) at 1:400 dilution from a 2 mg/mL stock (Section 2.8); goat anti-rabbit Alexa Fluor 647 (Cy5; Invitrogen) at 1:200 dilution from a 1 mg/mL stock (Section 2.8); goat anti-rabbit Alexa Fluor 488 (GFP; Invitrogen) at 1:400 dilution from a 2 mg/mL stock (Sections 2.10-2.12) To stain the nuclei, cells were incubated for 15 minutes with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen) at 1:200 dilution from a 1 mg/mL stock. To stain the actin filaments of all cells, samples were incubated for 15 minutes with Texas-Red-X Phalloidin (Invitrogen) at 1:200 dilution from 200 U/mL stock solution. Images were acquired with EVOS FL Imaging System (Invitrogen) fluorescence microscope and Zeiss LSM 880 confocal microscope. After acquisition, images were further analysed using Fiji ImageJ software [24].

2.6 2D viability assay

HUVECs and MSCs were each seeded in a 96 wells plate at 10,000 cells/cm^2 for co-culture or 20,000 cells/cm^2 for single culture controls. All conditions were performed in triplo. Cells were cultured in EBM, αMEM or a mix of EBM and αMEM in 1:1 volume ratio. After three days of incubation at 37 ℃ and 5% CO_2, a Presto Blue assay (Invitrogen) was performed and measured with Victor plate reader with D560/10 X (Chroma) excitation filter nm and D590/10 M (Chroma) emission filter.

2.7 Bilayer imaging on plate

A 96 wells plate was coated with the same collagen solution used in the chips (section 2.3) and incubated for 30 min at 37℃ and 5% CO_2. For staining with Cell Tracker, a working solution with final dye concentration of 15 μM was prepared in serumfree medium and warmed to 37℃. The harvested cells were centrifugated, resuspended in the working solution and incubated for 30 minutes at 37℃ and 5% CO_2. After incubation, the cell suspension was centrifugated and the supernatant was aspirated. Stained cells were then resuspended in regular EBM. MSCs stained with Cell Tracker Red (Invitrogen) were seeded with 5,000 cells/well and cultured overnight in EBM. After 24h, HUVECs stained with Cell Tracker Green (Invitrogen) were seeded with 10,000 cells/well and cultured for 3 days at 37℃ and 5% CO_2. Both co-culture and single culture conditions were included in triplo. After incubation, cells were fixated and stained for DAPI, followed by imaging, as described in 2.5.

2.8 Endothelial junction formation on plate

A 96 wells plate was coated with the same collagen solution used in the chips and incubated for 30 minutes at 37℃ and 5% CO_2. MSCs were seeded with 5,000 cells/well and cultured overnight in EBM. After that, HUVECs were seeded with 10,000 cells/well and cultured for three days at 37℃ and 5%

CO_2. Both co-culture as single culture conditions were included in triplo. As control, HUVECs were also seeded without coating and 0.1% gelatin coating. After incubation, cells were fixated and stained for Vascular Endothelial cadherin (VE-cadherin), Zona occulens 1 (ZO-1) and DAPI, followed by imaging, as described in 2.5.

2.9 Cell seeding on chip

To ensure a proper flow through the created lumen, the main channel was first carefully filled with EBM. A pipette tip was filled with 12 μL of medium and placed in the inlet, subsequently the medium was slightly aspirated with an empty pipette tip from the outlet to fill the entire lumen with medium. This was done on all chips before continuing with cell seeding, to allow a few minutes of incubation with medium in the main channel to establish a cell appropriate environment. Unless otherwise indicated, HUVECs were seeded at a density of 10 x 10^6 cells/mL and MSCs were seeded at a density of 2 x 10^6 cells/mL. In co-culture experiments, the chips were first seeded with MSCs and incubated overnight, followed by seeding with HUVECs after overnight incubation (day 1).

To seed cells, a pipette tip with 12 μL of cell suspension was placed in the inlet while EBM was still in the main channel. As previously described, the cell suspension was evenly spread throughout the lumen by slight aspiration with an empty tip from the outlet. The tips with cell suspension were left in the inlet and outlet during incubation, by storing the chips in an empty pipette tip box with an additional mini petridish filled with PBS to add extra humidity. First, the chips were incubated at 37℃ and 5% CO_2 upside down for 30 minutes to allow adherence of the cells to the top of the lumen. Next, the chips were flipped upright and incubated at 37℃ and 5% CO_2 for another 30 minutes to allow adherence to the bottom of the channel. After the total 1 hour of incubation, medium was placed in the side channels by filling a pipette tip with 15 μL EBM, placing it in the inlet and aspirating the medium slightly from the outlet with an empty tip. Again, tips with medium and cell suspension were left during incubation at 37℃ and 5% CO_2 overnight.

The next day (day 1) all tips were removed and the chips were studied with a microscope to visualize the formed single layer vessel. In single culture conditions, EBM was refreshed in both side channels and the main channel with the same technique as described before. In co-culture conditions, side channels were emptied and HUVECs were seeded into the main channel to create a bilayered vessel. In that case, EBM refreshment and a second round of chip inspection took place after overnight incubation with both MSCs and HUVECs seeded on the chip (day 2).

2.10 Bilayered vessel visualization

MSCs and HUVECs were seeded on the chips following described steps for co-culture cell seeding. On day 4, the cells in the chip were fixated and stained for Phalloidin, VE-cadherin and DAPI, followed by imaging, as described in 2.5.

2.11 On-chip bilayer imaging

MSCs stained with Cell Tracker Red (Invitrogen) and HUVECs stained with Cell Tracker Green (Invitrogen), as described in 2.7, were seeded on the chips following described steps for co-culture cell seeding. On day 2, the cells in the chips were fixated and stained for DAPI, followed by imaging, as described in 2.5.

2.12 On-chip endothelial junction formation

MSCs stained with Cell Tracker Red (Invitrogen), as described in 2.7, and HUVECs were seeded on chips following described steps for co-culture cell seeding. Additionally, some chips were seeded with only HUVECs in single culture on day 1. On day 2, the cells in the chips were fixated, stained for VE-cadherin and DAPI, followed by imaging, as described in 2.5.

3 Results

3.1 2D viability assay

To find the most suitable medium for co-culture conditions, a Presto Blue cell viability assay was performed. Cell morphology and metabolism was examined after three days of single or co-culture in three different medium conditions. The used medium conditions were HUVEC medium (EBM), MSC medium (αMEM) and a 1:1 volume ratio mix of EBM and αMEM (Combi). Results are given in Figures 5 and 6.

Figure 5 shows the fluorescent values measured of each conditions after the Presto Blue assay. The highest fluorescent value corresponds to the highest metabolism, which is in EBM for all cell culture conditions. It is apparent that the metabolism for MSCs in EBM is much larger than the metabolism in αMEM. Metabolism for HUVECs in αMEM is close to zero, which corresponds with the clumps of dead cells seen in Figure 6B. Additionally, co-culture conditions show metabolism in between MSC and HUVEC metabolism for each medium condition. Similarly, the 1:1 mixed combination medium shows fluorescent values in between EBM and αMEM values. After this experiment EBM was used for all co-culture conditions.

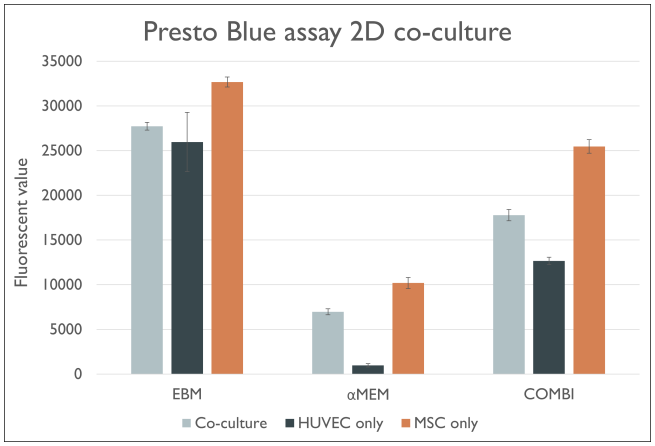

Fig. 5: The fluorescent value measured with Victor Plate reader after Presto Blue assay. single culture and co-culture of HUVECs and MSCs were cultured for three days in EBM, αMEM or combination medium (1:1 mix). Data represents mean ± SD, n = 3.

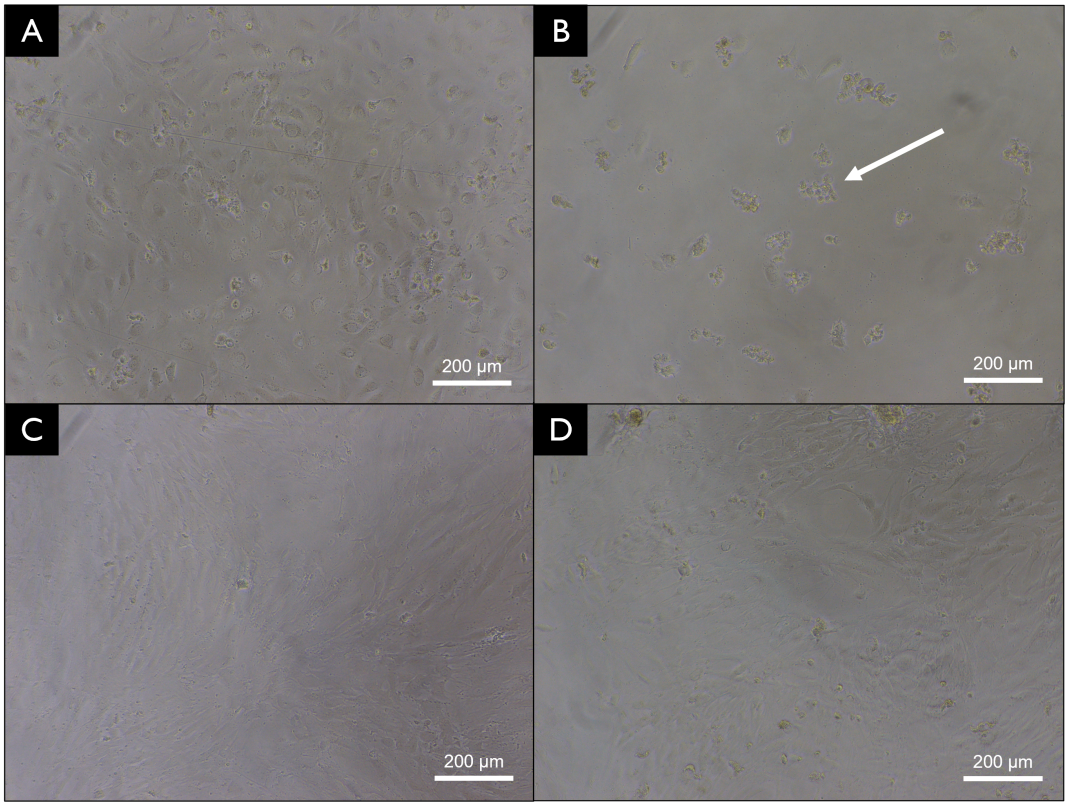

Fig. 6: Representative phase contrast microscopic images of (A) HUVECs single culture in EBM, (B) HUVECs single culture in αMEM, (C) MSCs in single culture in EBM and (D) HUVECs and MSCs in co-culture in EBM. HUVECs cultured in αMEM showed clumps of dead cells, visible at the arrow.

Figure 6 shows the morphology of single culture HUVECs in EBM, single culture HUVECs in αMEM, single culture MSCs in EBM and co-culture of HUVECs with MSCs in EBM. HUVECs cultured in αMEM show clumps of round cells, while HUVECs in EBM form a confluent layer over the entire surface. MSC single culture in EBM shows spread out MSCs over the surface forming a confluent layer of cells. In the co-culture of HUVECs with MSCs, distinguishing different cell types is not possible, but different morphological cells are visible compared to single culture conditions.

3.2 Bilayer imaging on plate

To see whether the two cell type layers mix, an experiment in collagen gels was conducted. Both cell types were stained with Cell Tracker to be able to distinguish easily between cell types. MSCs were stained with Cell Tracker red and seeded on the collagen gel 24 hours prior to HUVECs, which were stained with Cell Tracker green. After three days of culture, cell were fixated and stained for DAPI. Images were taken with EVOS to analyse the separate or mixed layers (Figure 7).

With DAPI staining, it became visible that the Cell Tracker did not fully stain all cells, as more nuclei are visible than red or green signals. The HUVECs, stained with green Cell Tracker were visible with the GFP filter, but also with the RFP filter used for MSCs stained with red Cell Tracker, meaning that there is spectral bleedthrough between the red and green dye. The RFP filter was interchanged with a Texas Red filter to visualize the red MSCs without disturbance of the green HUVECs in the image.

Figure 7 shows MSC single culture, HUVEC single culture and MSC with HUVEC co-culture. Both cell types were unable to form a fully confluent cell layer in all experimental conditions. In co-culture (Figure 7C) both green and red signal is visible in the same plane of focus and no clear separate layers of color were visible.

3.3 Endothelial junction formation on plate

To see whether co-culture with MSCs stimulates the formation of endothelial junctions between HUVECs, another experiment in collagen gels was conducted. MSCs were seeded on the collagen 24 hours prior to HUVECs. After three days of culture, cells were fixated and stained for ZO-1 (tight junctions), VE-cadherin (adherens junctions) and DAPI (nuclei). Images were taken with EVOS to visualize the endothelial junctions (Figure 8)

In all experimental conditions, there is little to no signal of ZO-1. MSCs in single culture (Figure 8A) also do not show VE-cadherin signal, while HUVECs in single culture (Figure 8B) show small network structures for VE-cadherin. MSCs and HUVECs in co-culture (Figure 8C) show larger network structures for VE-cadherin with a more intense signal at the periphery of the cell. The controls with HUVECs on a non-coated surface (Figure 8D) or the standard gelatin coating (Figure 8E) show large spots of VE-cadherin but do not form network structures as seen in the collagen gel conditions. Additionally, VE-cadherin signal is more visible at parts of the cell membrane in contact with other cells.

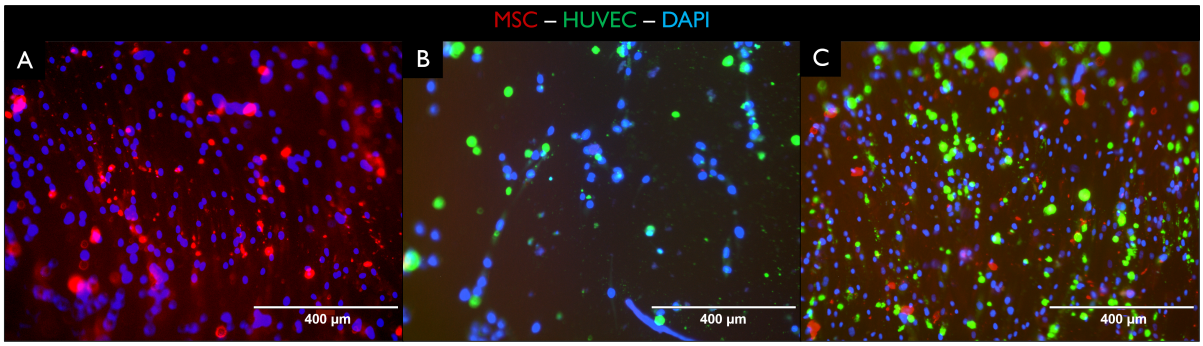

Fig. 7: Representative EVOS overlay microscopic images of (A) MSCs stained with red Cell Tracker in single culture, (B) HUVECs stained with green Cell Tracker in single culture and (C) MSCs (red) and HUVECs (green) in co-culture. Red MSC signal and green HUVEC signal is visible in the same plane of focus in co-culture conditions. Samples were cultured in collagen gel for three days, fixated and stained for DAPI Used filters are Texas Red, GFP and DAPI with 10x objective.

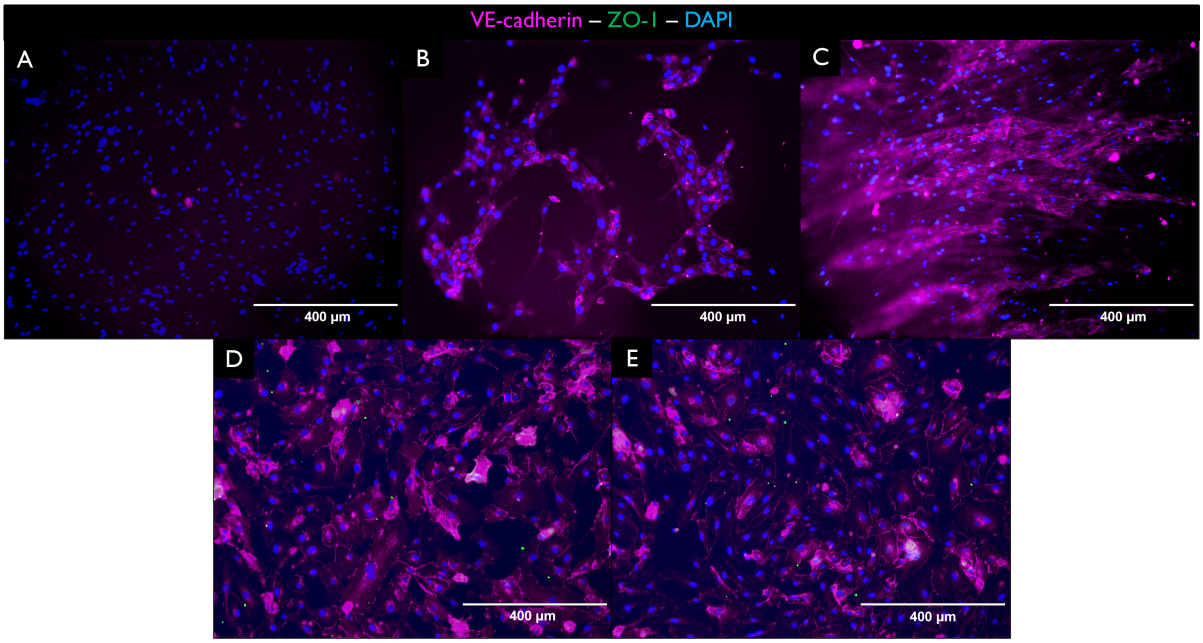

Fig. 8: Representative EVOS overlay microscopic images of HUVEC and MSC single cultures and co-culture in collagen gel after three days of culture, fixation and staining of the nuclei for DAPI, tight junctions for ZO-1 and adherens junctions for VE-cadherin. ZO-1 was not clearly visible in any experimental condition. (A) MSCs in single culture. No VE-cadherin signal is visible. (B) HUVECs in single culture. For VE-cadherin, small network structures are visible. (C) MSCs and HUVECs in co-culture. For VE-cadherin, large network structures are visible with a strong signal at the periphery of the cell. (D) HUVECs in single culture on a non-coated surface. (E) HUVECs in single culture on 0.1% gelatin coated surface. Both conditions without the collagen gel show large spots of magenta signal, seemingly staining the entire cell membrane. A small amount of signal is found at the periphery of the cell, mostly at the cell membrane contacting with neighboring cells. Used filters are Cy5, GFP and DAPI with 10x objective.

3.4 Bilayered vessel visualization

To start on-chip experiments, the protocol for seeding MSCs and HUVECs was sligthly improved. Pipetting technique, method of wire-removal, upside down incubation time and time between seeding was varied, which resulted in the more optimal seeding protocol as described in 2.9. After that, MSCs and HUVECs were succesfully seeded together on-chip to form a bilayered blood vessel-on-chip. MSCs were seeded 24 hours prior to HUVECs on day 0. To visualize the formation of a bilayered vessel, the cells were cultured for 4 days, fixated and stained for phalloidin (actin filements), VE-cadherin (adherens junctions) and DAPI (nuclei). Images taken with phase contrast microscope, EVOS and Zeiss confocal microscope are shown in Figures 9, 10 and 11.

Figure 9 shows several chips seeded with with MSCs and HUVECs. The lumen seeded with MSCs only (Figure 9A) shows that MSCs lay neatly in the lumen, forming a thin layer which is specifically visible at the lateral sides. The lumen seeded with both MSCs and HUVECs shows that MSCs have a more stretched out morphology within the collagen matrix, as seen in Figure 9B. HUVECs are more spread within the entire lumen and better visible in the middle of the lumen, compared to the MSCs. Figure 9C shows escaping cells outside of the lumen between the micropillars. After fixation, includ-

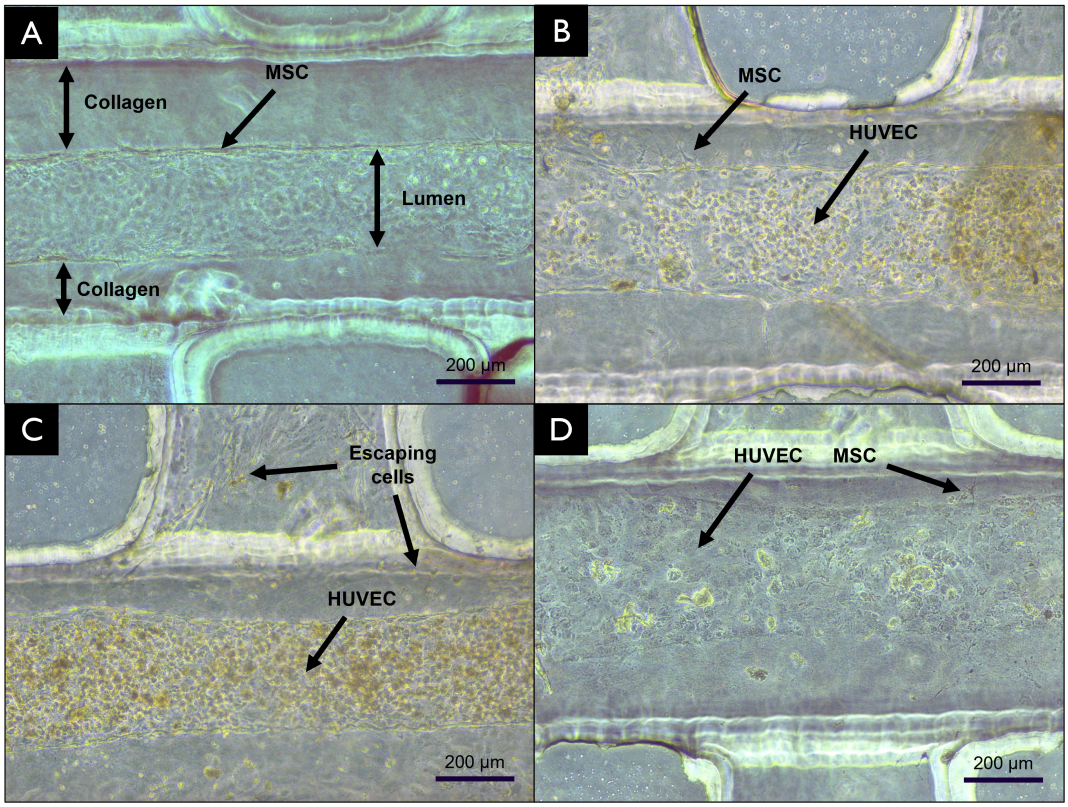

Fig. 9: Representative phase contrast microscopic images of several seeded chips. (A) Seeded lumen with MSCs on day 1. (B) Seeded lumen with MSCs and HUVECs on day 4 (different chip than A). (C) Seeded lumen with MSCs and HUVECs before fixation on day 4. Escaping cells are visible between the micropillars. (D) Seeded lumen from (C) after fixation. It is visible that less cells are present in the lumen after fixation compared to the lumen before fixation.

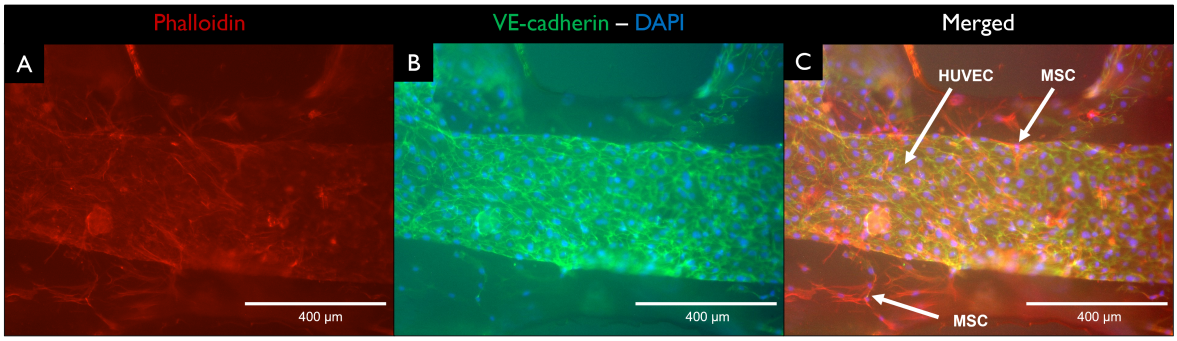

Fig. 10: Representative EVOS microscopic images of lumen seeded with both MSCs and HUVECs after four days of culture, fixation and staining.(A) Actin filaments of MSCs and HUVECs stained for Phallloidin (RFP-filter). MSCs are layed around the lumen, but do not form a vessel-like structure. (B) Overlay of nuclei stained for DAPI (DAPI-filter) and adherens junctions stained for VE-cadherin (GFP-filter). (C) Merged image of (A) and (B) showing phalloidin stained MSCs spreading along the vessel created by HUVECs forming adherens junctions. Used filters are Texas Red, GFP and DAPI with 10x objective.

ing three times washing with PBS, less cells are visible in the lumen, but present cells seem properly attached (Figure 9D).

The chip in Figure 9B after fixation and staining is shown in Figure 10. Phalloidin stains the actin filaments of both HUVECs and MSCs in red (Figure 10A). However, the actin filaments are more strongly stained in the MSCs, resulting in a clear image of the MSC morphology. MSCs to be laying along the side of the lumen without forming a vessel-like structure. They show a stretched out morphology, spanning a large surface of the vessel created by HUVECs (Figure 10C). VE-cadherin in green shows a vessel is formed out of a single layer of HUVECs connecting to each other by adherens junctions (Figure 10B). VE-cadherin signal is highest at the periphery of the cells.

Microscopic images taken with a confocal microscope are shown in Figure 11. VE-cadherin shows clearly at the periphery of the cell and MSCs visible lay around the lumen with a stretched out morphology. However, further investigation showed that only the bottom of the lumen was seeded with cells, meaning that a full vessel was unable to be formed.

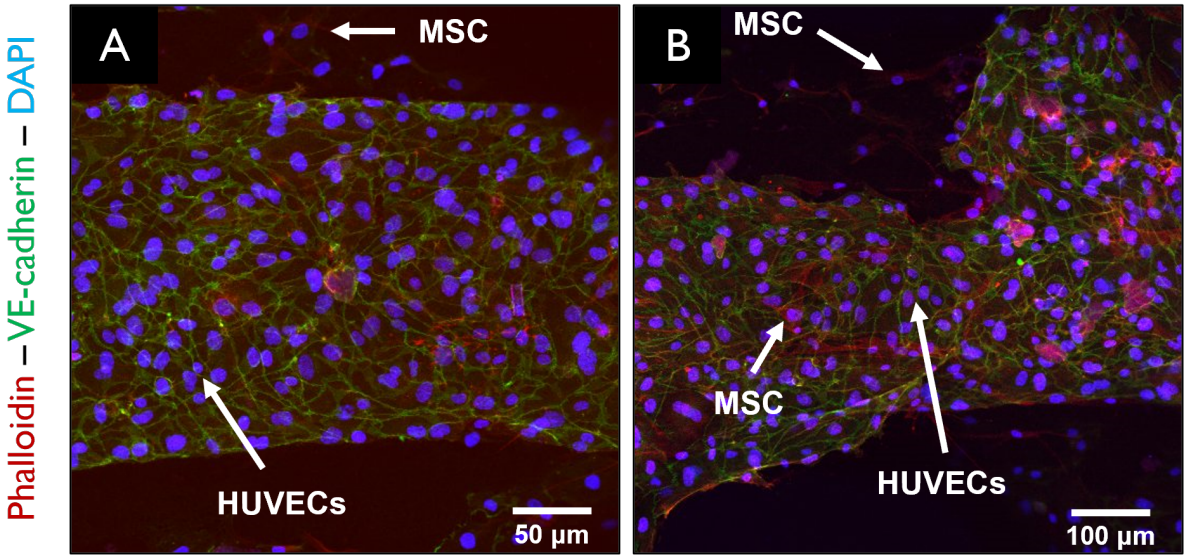

Fig. 11: Representative confocal microscopic images of lumen seeded with both MSCs and HUVECs after four days of culture, fixation and staining. VE-cadherin is most intens at the periphery of the cell showing the adherens junctions formed by HUVECs. MSCs are visibly laying around the lumen in the collagen matrix with a stretched out morphology. Fluorescent images were acquired with 10x objective.

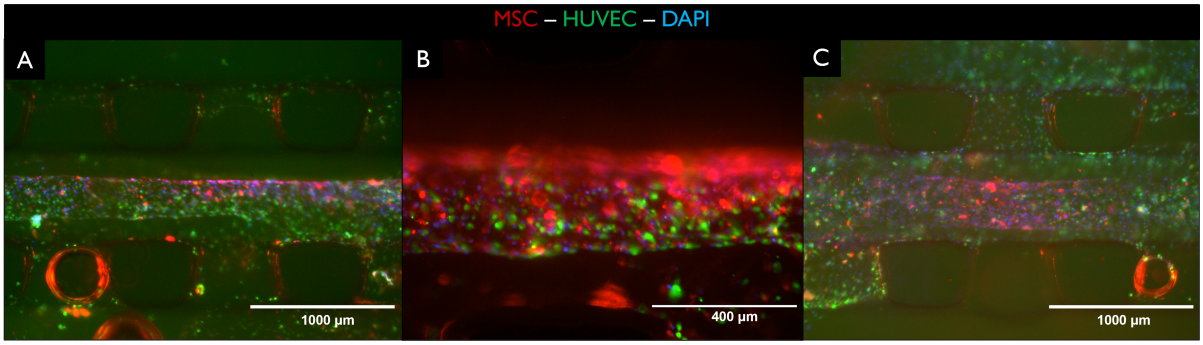

Fig. 12: Representative EVOS microscopic images of lumen seeded with both MSCs and HUVECs after one day of culture, fixation and staining for DAPI. MSCs are stained with red Cell Tracker and HUVECs are stained with green Cell Tracker. (A) Seeded lumen with HUVECs and MSCs. (B) Seeded lumen with HUVECs and MSCs at higher magnification, where MSCs seem more numerous on one lateral side of the vessel. (C) Seeded lumen with HUVECs and MSCs. MSCs are present in lumen, but HUVECs have also spread throughout the rest of the chip. Used filters are Texas Red, GFP and DAPI with 4x (A,C) or 10x (B) objective.

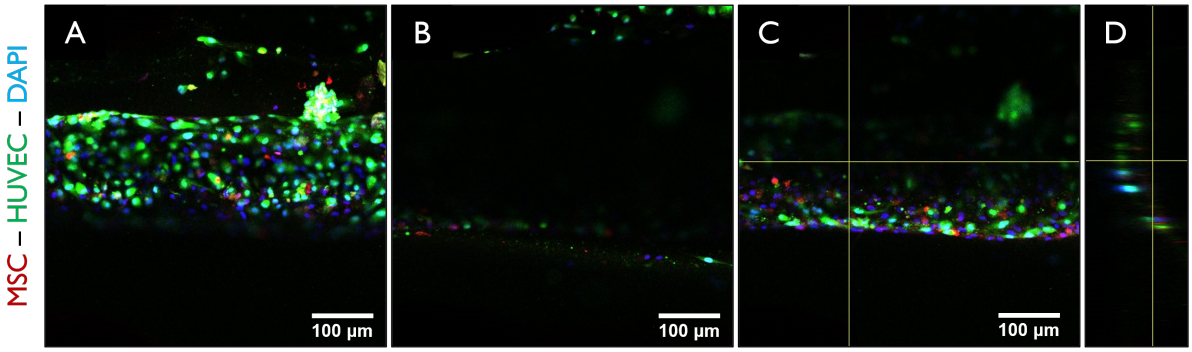

Fig. 13: Representative confocal microscopic images of lumen seeded with both MSCs and HUVECs after one day of culture, fixation and staining for DAPI. MSCs are stained with red Cell Tracker and HUVECs are stained with green Cell Tracker. Bottom (A), top (B) and lateral sides (C) were inspected to analyse the integrity of the seeded lumen. Orthogonal views of the lumen (D) revealed cells were only present at the bottom of the lumen. Fluorescent images were acquired with 10x objective.

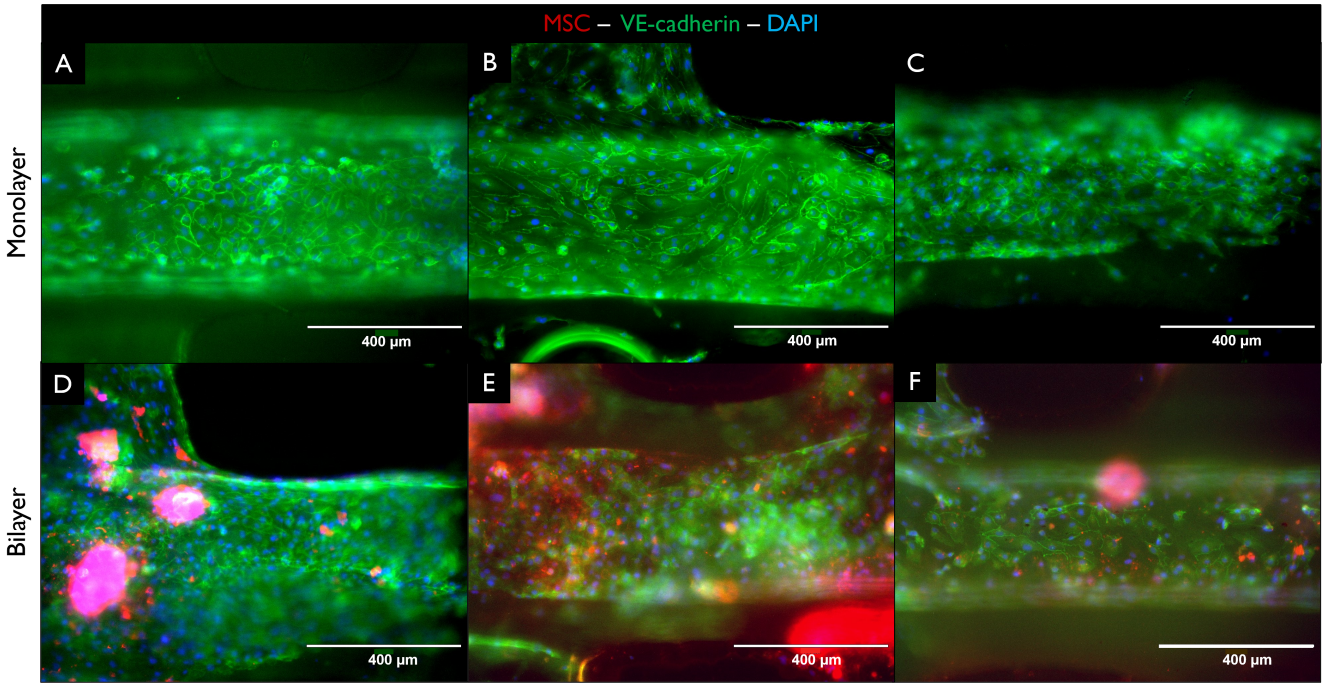

Fig. 14: Representative EVOS microscopic images of lumen seeded with both MSCs and HUVECs after one day of culture, fixation and staining for DAPI and VE-cadherin. MSCs are stained with red Cell Tracker. (A-C) Lumens seeded with HUVECs and (D-F) lumens seeded with both MSCs and HUVECs. No lumen is covered with a fully confluent layer of HUVECs. Monolayered vessels show a little amount of cells outside of the lumen. Bilayered vessels show a large amount of HUVECs between the micropilars, outside of the lumen. Used filters are Texas Red, GFP and DAPI with 10x objective.

3.5 On-chip bilayer imaging

To see whether the two cell type layers mix on-chip, the bilayer imaging experiment on a plate was performed on a chip as well. Both cell types were stained with Cell Tracker to be able to distinguish easily between cell types. MSCs (red) were seeded on the chip 24 hours prior to HUVECs (green). After one day of culture, cell were fixated and stained for DAPI. Images were taken with EVOS and Zeiss confocal microscope to analyse the layers of the formed vessel (Figure 12 and 13).

A lumen was seeded and imaged with 4x and 10x objectives (Figure 12A-B). The higher magnification seems to show more MSCs towards one lateral side of the lumen. In other chips, the HUVECs were not only located in the lumen, but spread towards other channels of the chip, as seen in Figure 12C. The MSCs do seem to have attached to the sides of the lumen, but this is only visible in a small part of the lumen.

The lumen in Figure 12A and B was images with Zeiss confocal to investigate the formed structure. Figure 13 shows a seeded bottom layer (A), but the top layer (B) is mostly empty. Only on one lateral side cells were present (C) and orthogonal view (D) reveals merely the bottom of the lumen was seeded, forming approxiametly one-third of the vessel structure.

3.6 On-chip endothelial junction formation

To see whether co-culture with MSCs stimulates the formation of endothelial junctions between HUVECs in a vessel, another on chip experiment was conducted. MSCs were stained with Cell Tracker red and seeded 24 hours prior to HUVECs. After one day of culture, cell were fixated and stained for DAPI and VE-cadherin. Images were taken with EVOS and Zeiss confocal microscope to visualize the endothelial junctions formed in monolayered or bilayered vessels (Figure 14, 15 and 16).

Figure 14A-C shows lumens seeded with HUVECs only. VE-cadherin shows the adherens junctions between the HUVECs forming a vessel-like structure. However, HUVECs are not only visible in the lumen, but also between the micropillars (B). The cells do not seem to be forming a fully confluent layer. This is also visible in Figure 15, which shows confocal images of bottom (A) and top (B) of the lumen. Especially the top layer is not confluent enough to cover the surface of the lumen. The lateral sides (C) are clearly seeded with cells. Orthogonal views reveal that cells are present all around the lumen.

Figure 14D-F shows lumens seeded with both MSCs and HUVECs. VE-cadherin shows that HUVECs seem to be forming vessel-like structures. Again, a lot of HUVECs are also visible troughout the whole main channel (outside the lumen), between the micropillar and even in the side channel. MSCs are visible in the lumen, but also in little amounts between the micropillars. Similarly to the monolayered vessel, the lumen is seeded with cells but there is not a confluent layer formed. This can be seen better in Figure 16. The bottom (A) looks almost confluent, but the top (B) shows a very little amount of cells. Lateral sides (C) are clearly seeded with cells, but a small additional vessel-structure is formed next to the luminal vessel. Orthogonal views reveal that mainly the bottom half of the vessel is formed. Another observation was that a large number of cells seemed to have moved all the way to the top or bottom of the main channel, forming a monolayer of cells across the width of the whole channel (data not shown).

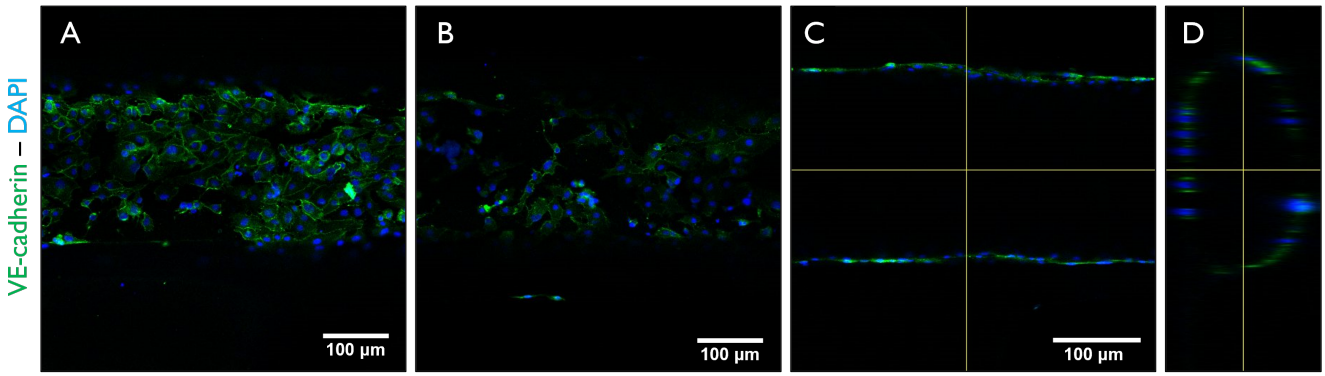

Fig. 15: Representative confocal microscopic images of lumen seeded with HUVECs after one day of culture, fixation and staining for DAPI and VE-cadherin. Bottom (A), top (B) and lateral sides (C) were inspected to analyse the integrity of the seeded lumen. Top and bottom views show cells are not fully covering the surface of the lumen. Orthogonal views of the lumen (D) revealed cells were present all around the lumen. Fluorescent images were acquired with 10x objective.

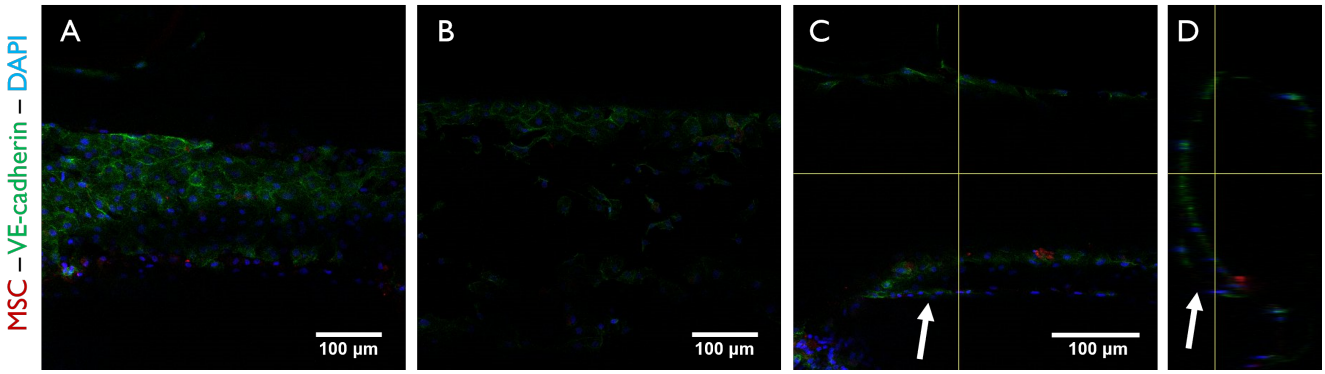

Fig. 16: Representative confocal microscopic images of lumen seeded with both MSCs and HUVECs after one day of culture, fixation and staining for DAPI and VE-cadherin. MSCs are stained with red Cell Tracker. Bottom (A), top (B) and lateral sides (C) were inspected to analyse the integrity of the seeded lumen. Top and bottom views show cells are not fully covering the surface of the lumen, especially on the top. Lateral view indicates a small additional vessel structure formed outside of the lumen, visible at the arrows. Orthogonal views of the lumen (D) revealed cells were mostly covering the bottom of the lumen. Fluorescent images were acquired with 10x objective.

4 Discussion

The extravasation of circulating tumor cells is a challenging process to study due to its brief and rare occurence. Since it is of great clinical importance to understand more about this step in the metastatic cascade, several in vivo and in vitro studies have been performed [9]. These studies include multiple microfluidic devices, but these often fail to implement an accurate blood vessel model consisting of multiple concentric layers. In this study, the aim is to create a bilayered blood vessel-on-chip model consisting of a MSC support layer surrounding a HUVEC monolayer.

First, the most suitable medium for co-culture needed to be found. In Figure 5 the results of the viability assay are given. It is apparent that the metabolism of HUVECs in αMEM is extremely low. Dead HUVECs are also visible in Figure 6B. A plausible reason would be that αMEM simply lacks the essential growth supplements present in EBM (e.g. EGF) which are needed for endothelial proliferation. It is also visible that MSCs' metabolism is much higher in EBM than in αMEM, while αMEM is the regular culture medium for MSCs. EBM is enriched with necessary growth supplements for endothelial proliferation. These supplements include rh EGF, L-glutamine, heparin sulfate and hydrocortisone, and could potentially influence the proliferation or differentiation of MSCs as well. It is suggested that rh EGF stimulates the proliferation of MSCs without affecting the differentiation process or potential [25]. Similarly, L-glutamine promotes MSC proliferation, inhibits the secretion of pro-inflammatory cytokines IL-1β and IL-6, and stimulates the secretion of anti-inflammatory cytokines IL-10 and TGF-β [26]. In contrast, hydrocortisone negatively affects the MSC proliferation and also inhibits the osteogenic differentiation while stimulating early adipogenic differentiation [27].

Overall, it was concluded that EBM is the most suitable medium for co-culture conditions. It could be useful to study potential differences in gene expression between MSCs cultured in EBM or αMEM, ensuring the enhanced MSC proliferation in EBM does not affect the differential properties of MSCs.

To see whether the two cell type layers would mix, an experiment in collagen gels on a plate was conducted. Figure 7 shows the EVOS images of the bilayer imaging experiment on a plate. However, as mentioned in Section 3.2, the Cell Tracker staining failed to stain all cells properly, as not all nuclei showed a surrounding green or red signal. We suspect

three possible causes for the partially failed staining. Firstly, Cell Tracker fluorescent probes are able to pass through the cell membrane, but become cell-impermeant products by reacting with thiol groups in the cytoplasm [28]. Serum can prematurely react with the probes, disabling entry into the cell [29]. This is why staining should be performed in serum-free medium. If there was a small amount of serum left with the cell pellet, this could have partially inactivated the staining. Secondly, the concentration of the dye in the working solution significantly affects the amount of fluorescence, especially when cells are cultured for 72 hours or longer. If the concentration was too low, the signal could have faded over the three day culture period, resulting in little signal after fixation. At last, during the staining procedure it was observed that cells sedimented to the bottom of the tube during the incubation in the Cell Tracker working solution. This could have resulted in the solution not being able to reach all cells properly, since they were closely packed together.

To analyse whether the two cell layers will mix, it is important that confluent cell layers are formed. Otherwise, gaps in the cell layers would allow cells from one type to easily break through and be visible in between the other cell layer. Unfortunaly, it does not seem like there is a confluent cell layer formed in any condition during the experiment. In combination with the incomplete staining, this means that no clear conclusions can be drawn about the mixing of the two cell type layers even though red and green signal is visible in the same focus plane.

If this experiment were to be repeated, the Cell Tracker staining should be improved and a more confluent cell layer will have to be formed (e.g. by seeding more cells). Also, it would be most suitable to use confocal microscopy to analyze the two cell layers.

To see whether co-culture with MSCs stimulates the formation of endothelial junctions between HUVECs, another experiment in collagen gels was conducted. The formation of endothelial junctions on a plate is shown using EVOS images (Figure 8) A first observation is that ZO-1 was poorly visible in all experimental conditions. In some conditions it showed vaguely throughout the entire cell cytoplasm and slightly more intense in the nucleus, while it is expected to stain tight-junctions on the cell membrane. To get better ZO-1 signal around the periphery of the cell, a confluent layer of cells needs to form and culture for another minimal 24 hours [30]. It is likely that the HUVECs did not form a confluent enough layer, inhibiting the cells from forming mature tight-junctions.

The VE-cadherin staining is most intense at the cell membrane in contact with neighboring cells and shows that larger network structures are formed by HUVECs in co-culture with MSCs, compared to HUVECs in single culture (Figure 8). Other studies have used MSCs since they have the ability to promote vessel formation by producing pro-angiogenic cytokines such as VEGF, IL-1β, IL-6, Il-8, TGF-β [31]. Studying the difference in gene expression between co-cultures and single cultures specifically for pro-angiogenic cytokines and growth factors, could give useful insights in the effects of MSCs on endothelial junction formation between HUVECs. This experiment also showed that the collagen gel created an appropriate environment for HUVECs to form network structures through adherens junctions, since the control conditions with gelatin coating and without coating showed less adherens junctions. These controls also showed large spots of VE-cadherin signal, staining the entire cell.

After some slight optimization of the seeding protocol, cells were seeded on chips. Figure 10 shows a seeded lumen with both HUVECs and MSCs, where HUVECs seem to have formed a vessel-like structure. However, further analysis with Zeiss confocal microscope (Figure 11) revealed only the bottom of the lumen was seeded with cells. Cells seem to attach more easily to the bottom of the lumen, so further optimization of the incubation protocol is necessary. Leaving the chips upside down first for a longer time before flipping them upright could be a potential solution. Additionally, HUVECs are very likely to have moved throughout the rest of chip instead of having stayed in the lumen, resulting in less cells being left in the lumen to properly attach to the collagen matrix surface. Pipetting the cell suspension into the main channel should be done extra carefully. Also, taking out the previous cell suspension to seed with the second cell type could make it easier for the second cell suspension to stay in the main channel. However, taking out the first suspension would result in more detachment of the first cell type.

The MSCs showed a stretched out morphology along the side of the lumen, but this could be influenced by flow through the main channel and connecting the chip to a circulatory system would be more accurate. Unfortunately, it was observed that a lot of cells were already washed away during the fixation process, which includes multiple times washing with PBS (Figure 9). It should be investigated how the cells could attach more strongly to the luminal surface, to make sure that the vessel structure can handle flow through the lumen without losing cells.

The bilayer imaging experiment on a plate was performed on chip as well. Analysis with confocal microscope revealed that cells were mainly on the bottom side of the lumen and were not forming a confluent monolayer of cells around the lumen (Figure 13). Similarly to the plate experiment, without the formation of a confluent layer it is not possible to draw clear conclusions about the mixing of two cell layers. Cell Tracker staining showed less clearly the formation of the vessel, since it stains the entire cytoplasm. Since the MSCs and HUVECs are likely to be in close proximity, staining with VE-cadherin for HUVECs and a different specific marker for MSCs could give a more clear image. Specific markers could be CD271/NGF R and STRO-1 [32, 33]. However, these markers should be previously tested on the cells used in the experiment, since they are specific only towards certain MSC lines and could potentially show overlap with endothelial cells.

Next, chips were seeded with only HUVECs or both MSCs and HUVECs to compare the formation of endothelial junctions in monolayered and bilayered vessels, respectively. Figure 14 shows both monolayered and bilayered seeded lumens. In bilayered chips it was noted that a large number of cells were present all the way at the top or bottom of the main channel outside of the lumen, forming a monolayer of cells across the entire width of the channel (data not shown). Again, there were also a lot of cells present in between the micropillars and in the side channels. It seems to be a problem that the cells are moving to unwanted locations outside of the lumen, which was more frequently observed in chips that

were seeded with both cell types. This is seen as well in Figure 16, where there is an additional vessel structure formed next to the lumen.

A possible explanation could be the ability of MSCs to remodel the extra cellular matrix (ECM). Matrix metalloproteinases (MMPs) are enzymes involved in degrading ECM components such as collagen, and their expression by MSCs can be upregulated under certain conditions [34]. This remodeling process likely plays a role in facilitating the migration of endothelial cells [35]. Recreating the basal lamina might limit the direct effects of matrix remodeling by separating the MSC and HUVEC layers. Therefore, MSCs would initially remodel the outer regions of the collagen rather than the luminal surface, potentially reducing its permeability to HUVECs and thereby preventing HUVEC migration.

Confocal microscopy revealed that the monolayered vessel (Figure 15) had cells seeded all around the lumen, although the surface was not fully covered with HUVECs. The bilayered vessel (Figure 16) showed that mainly the bottom part of the lumen was seeded with cells. It is possible that the MSCs are interfering with the adherens of HUVECs to the surface of the lumen, mainly with the top side. Whether this is a direct effect of the MSCs or a consequence of the additional steps of the seeding procedure is unclear. MSCs could also be embedded in the collagen gel during chip preparation [16], to protect them from being washed away during the seeding of HUVECs and to allow HUVECs to adhere better to the surface of the lumen. This creates a bit more distance between the two cell layers, but as seen in Figure 10 and 11, the MSCs are also laying deeper in the collagen gel with the current seeding procedure. Additionally, separating the two cell layers could reduce the potential effects of matrix remodeling by MMPs as mentioned previously.

Once the seeding procedure is further optimized to create all around seeded lumens with both cell types, some additional research can be performed. The integrity of the vessel can be analysed with VE-cadherin staining, but the permeability of the vessel should be further analysed with for example a FITC-dextran leakage assay [17]. Within this study different ratios of the two cell types could be implemented to see the effect of the number of HUVECs on the vessel permeability in the presence of MSCs. In this study, the recommended ratio of 1:5 (MSC:HUVEC) is used [31], but ratios of 1:1 and 1:10 have also been frequently studied [36, 37]. This ratio could also influence the conservation of the separate two cell layers, since a more confluent HUVEC monolayer could prevent MSCs from breaking through [37].

The eventual goal of this blood vessel-on-chip model is to study the process of extravasation towards a tissue specific niche. Experiments could be performed with circulating fluorescent CTCs to study their transendothelial migration [10] while implementing tissue specific factors in the metastatic niche channel [38].

5 Conclusion

To conclude, this study aimed to create a structurally correct bilayered blood vessel-on-chip model with a mesenchymal stem cell (MSC) support layer surrounding a human umbilical vein endothelial cell (HUVEC) monolayer. The developed model was used to investigated the effect of MSCs on the integrity of the endothelial monolayer.

The findings showed that EBM significantly enhanced the proliferation of both HUVECs and MSC compared to alphaMEM, suggesting it is the most suitable medium for co-culture conditions. However, incomplete staining and non-confluent cell layers hindered clear conclusions on MSC and HUVEC layer mixing. Nonetheless, co-culturing MSCs with HUVECs seemed to enhance endothelial junction formation, suggesting MSCs may support endothelial integrity, which is possibly mediated by pro-angiogenic cytokines.

Achieving uniform cell attachment throughout the lumen was found to be difficult during the cell seeding on the microfluidic chips. Cells primarily attached to the bottom surface of the lumen, which highlights the need for improvements in the protocol to enhance cell adhesion within the lumen and prevent cell loss during fixation or under flow conditions. Embedding MSCs in the collagen matrix during chip preparation could be a possible solution to improve HUVEC adhesion.

While this study established a foundational model, further optimization of the seeding procedure is necessary to achieve clinically relevant and reproducible results. The results help to understand the interaction of MSCs with HUVECs within microfluidic systems and show the critical need for advanced techniques to further study the complex process of metastasis.

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References

[1] Nederland IK. Incidentie borstkanker; 2024. [Online; accessed 17. Jun. 2024]. Available from: https://iknl.nl/kankersoorten/borstkanker/registratie/incidentie.

[2] Belkacemi Y, Hanna NE, Besnard C, Majdoul S, Gligorov J. Local and Regional Breast Cancer Recurrences: Salvage Therapy Options in the New Era of Molecular Subtypes. Front Oncol. 2018 Apr;8:363245. doi:10.3389/fonc.2018.00112.

[3] Ricard N, Bailly S, Guignabert C, Simons M. The quiescent endothelium: signalling pathways regulating organ-specific endothelial normalcy. Nat Rev Cardiol. 2021;18(8):565. doi:10.1038/s41569-021-00517-4.

[4] Strell C, Entschladen F. Extravasation of leukocytes in comparison to tumor cells. Cell Commun Signal. 2008 Dec;6(1):1-13. doi:10.1186/1478-811X-6-10.

[5] Katt ME, Wong AD, Searson PC. Dissemination from a Solid Tumor: Examining the Multiple Parallel Pathways. Trends in Cancer. 2018 Jan;4(1):20-37. doi:10.1016/j.trecan.2017.12.002.

[6] Ganesh K, Massagué J. Targeting metastatic cancer. Nat Med. 2021 Jan;27(1):34. doi:10.1038/s41591-020-01195-4.

[7] Naumov GN, Wilson SM, MacDonald IC, Schmidt EE, Morris VL, Groom AC, et al. Cellular expression of green fluorescent protein, coupled with high-resolution in vivo videomicroscopy, to monitor steps in tumor metastasis. J Cell Sci. 1999 Jun;112(12):1835-42. doi:10.1242/jcs.112.12.1835.

[8] Stoletov K, Kato H, Zardouzian E, Kelber J, Yang J, Shattil S, et al. Visualizing extravasation dynamics of metastatic tumor cells. J Cell Sci. 2010 Jul;123(13):2332. doi:10.1242/jcs.069443.

[9] Bersini S, Jeon JS, Dubini G, Arrigoni C, Chung S, Charest JL, et al. A Microfluidic 3D In Vitro Model for Specificity of Breast Cancer Metastasis to Bone. Biomaterials. 2014 Mar;35(8):2454. doi:10.1016/j.biomaterials.2013.11.050.

[10] Humayun M, Ayuso JM, Brenneke RA, Virumbrales-Muñoz M, Lugo-Cintrón K, Kerr S, et al. Elucidating cancer-vascular paracrine signaling using a human organotypic breast cancer cell extravasation model. Biomaterials. 2021;270:120640. doi:10.1016/j.biomaterials.2020.120640.

[11] Chaw KC, Manimaran M, Tay EH, Swaminathan S. Multi-step microfluidic device for studying cancer metastasis. Lab Chip. 2007 Jul;7(8):1041-7. doi:10.1039/B707399M.

[12] Jeon JS, Zervantonakis IK, Chung S, Kamm RD, Charest JL. In Vitro Model of Tumor Cell Extravasation. PLoS One. 2013;8(2). doi:10.1371/journal.pone.0056910.

[13] Zhang Q, Liu T, Qin J. A microfluidic-based device for study of transendothelial invasion of tumor aggregates in realtime. Lab Chip. 2012 Jul;12(16):2837-42. doi:10.1039/C2LC00030J.

[14] Heyder C, Gloria-Maercker E, Entschladen F, Hatzmann W, Niggemann B, Zänker K, et al. Realtime visualization of tumor cell/endothelial cell interactions during transmigration across the endothelial barrier. J Cancer Res Clin Oncol. 2002 Oct;128(10):533-8. doi:10.1007/s00432-002-0377-7.

[15] Jeon JS, Bersini S, Gilardi M, Dubini G, Charest JL, Moretti M, et al. Human 3D vascularized organotypic microfluidic assays to study breast cancer cell extravasation. Proc Natl Acad Sci USA. 2015 Jan;112(1):214-9. doi:10.1073/pnas.1417115112.

[16] Zheng Y, Chen J, Craven M, Choi NW, Totorica S, Diaz-Santana A, et al. In vitro microvessels for the study of angiogenesis and thrombosis. Proc Natl Acad Sci USA. 2012 Jun;109(24):9342-7. doi:10.1073/pnas.1201240109.

[17] Delannoy E, Tellier G, Cholet J, Leroy AM, Treizebré A, Soncin F. Multi-Layered Human Blood Vessels-on-Chip Design Using Double Viscous Finger Patterning. Biomedicines. 2022 Mar;10(4):797. doi:10.3390/biomedicines10040797.

[18] Alimperti S, Mirabella T, Bajaj V, Polacheck W, Pirone DM, Duffield J, et al. Three-dimensional biomimetic vascular model reveals a RhoA, Rac1, and N-cadherin balance in mural cell–endothelial cell-regulated barrier function. Proc Natl Acad Sci USA. 2017 Aug;114(33):8758-63. doi:10.1073/pnas.1618333114.

[19] Firatligil-Yildirir B, Bati-Ayaz G, Tahmaz I, Bilgen M, Pesen-Okvur D, Yalcin-Ozuysal O. On-chip determination of tissue-specific metastatic potential of breast cancer cells. Biotechnol Bioeng. 2021 Oct;118(10):3799-810. doi:10.1002/bit.27855.

[20] de Souza LEB, Malta TM, Kashima Haddad S, Covas DT. Mesenchymal Stem Cells and Pericytes: To What Extent Are They Related? Stem Cells Dev. 2016 Dec;25(24):1843-52. doi:10.1089/scd.2016.0109.

[21] Yan L, Dwiggins CW, Gupta U, Stroka KM. A Rapid-Patterning 3D Vessel-on-Chip for Imaging and Quantitatively Analyzing Cell–Cell Junction Phenotypes. Bioengineering. 2023 Sep;10(9):1080. doi:10.3390/bioengineering10091080.

[22] Wippert A. Developing a model for studying the extravasation of breast cancer cells from the blood stream into the bone metastatic niche. 2023 May.

[23] Pauty J, Usuba R, Cheng IG, Hespel L, Takahashi H, Kato K, et al. A Vascular Endothelial Growth Factor-Dependent Sprouting Angiogenesis Assay Based on an In Vitro Human Blood Vessel Model for the Study of Anti-Angiogenic Drugs. eBioMedicine. 2018 Jan;27:225-36. doi:10.1016/j.ebiom.2017.12.014.

[24] Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012 Jul;9:676-82. doi:10.1038/nmeth.2019.

[25] Tamama K, Kawasaki H, Wells A. Epidermal Growth Factor (EGF) Treatment on Multipotential Stromal Cells (MSCs). Possible Enhancement of Therapeutic Potential of MSC. J Biomed Biotechnol. 2010;2010. doi:10.1155/2010/795385.

[26] Dos Santos GG, Hastreiter AA, Sartori T, Borelli P, Fock RA. L-Glutamine in vitro Modulates some Immunomodulatory Properties of Bone Marrow Mesenchymal Stem Cells. Stem Cell Rev Rep. 2017 Aug;13(4):482-90. doi:10.1007/s12015-017-9746-0.

[27] Shipunova NN, Petinati NA, Drize NI. Effect of hydrocortisone on multipotent human mesenchymal stromal cells. Bull Exp Biol Med. 2013 May;155(1):159-63. doi:10.1007/s10517-013-2102-8.

[28] Thermofisher. CellTracker fluorescent probes product information sheet; 2018. Rev. B.0.

[29] Thermofisher. Cell Tracing and Tracking Support—Troubleshooting - NL; 2024. [Online; accessed 9. Jul. 2024]. Available from: https://www.thermofisher.com/nl/en/home/technical-resources/technical-reference-library/cell-analysis-support-center/cell-tracing-tracking-support/cell-tracing-tracking-support-troubleshooting.html.

[30] Gottardi CJ, Arpin M, Fanning AS, Louvard D. The junction-associated protein, zonula occludens-1, localizes to the nucleus before the maturation and during the remodeling of cell-cell contacts. Proc Natl Acad Sci USA. 1996 Oct;93(20):10779. doi:10.1073/pnas.93.20.10779.

[31] Shafiee S, Shariatzadeh S, Zafari A, Majd A, Niknejad H. Recent Advances on Cell-Based Co-Culture Strategies for Prevascularization in Tissue Engineering. Front Bioeng Biotechnol. 2021;9. doi:10.3389/fbioe.2021.745314.

[32] Álvarez Viejo M, Menéndez-Menéndez Y, Otero-Hernández J. CD271 as a marker to identify mesenchymal stem cells from diverse sources before culture. World Journal of Stem Cells. 2015 Mar;7(2):470. doi:10.4252/wjsc.v7.i2.470.

[33] Ning H, Lin G, Lue TF, Lin CS. Mesenchymal Stem Cell Marker Stro-1 is a 75kd Endothelial Antigen. Biochem Biophys Res Commun. 2011 Sep;413(2):353. doi:10.1016/j.bbrc.2011.08.104.

[34] Han Y, Yang J, Fang J, Zhou Y, Candi E, Wang J, et al. The secretion profile of mesenchymal stem cells and potential applications in treating human diseases. Sig Transduct Target Ther. 2022 Mar;7(92):1-19. doi:10.1038/s41392-022-00932-0.

[35] Almalki SG, Agrawal DK. Effects of matrix metalloproteinases on the fate of mesenchymal stem cells. Stem Cell Res Ther. 2016 Dec;7(1):1-12. doi:10.1186/s13287-016-0393-1.

[36] Rao RR, Peterson AW, Ceccarelli J, Putnam AJ, Stegemann JP. Matrix Composition Regulates Three-Dimensional Network Formation by Endothelial Cells and Mesenchymal Stem Cells in Collagen/Fibrin Materials. Angiogenesis. 2012 Jun;15(2):253. doi:10.1007/s10456-012-9257-1.

[37] Sierra-Parraga JM, Merino A, Eijken M, Leuvenink H, Ploeg R, Møller BK, et al. Reparative effect of mesenchymal stromal cells on endothelial cells after hypoxic and inflammatory injury. Stem Cell Res Ther. 2020 Aug;11(1):352. doi:10.1186/s13287-020-01869-3.

[38] Marturano-Kruik A, Nava MM, Yeager K, Chramiec A, Hao L, Robinson S, et al. Human bone perivascular niche-on-a-chip for studying metastatic colonization. Proc Natl Acad Sci USA. 2018 Feb;115(6):1256-61. doi:10.1073/pnas.1714282115.