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ABSTRACT

The interrelationship between angiogenesis and lymphangiogenesis is inadequately researched. Endothelial cells are key role players in both processes of vascular formations. The purpose of this project was to use *in vitro* 3D sprouting assay to investigate the contribution of lymphatic endothelial cells to blood endothelial cell's angiogenesis. The hope is that the project will contribute to a better understanding of the interaction between the two endothelial cells during vessel formation. The approach used in effort to achieve this was culturing both lymphatic endothelial cells (LEDs) and blood endothelial cells (ECs). Further, the LECs and ECs were incorporated onto microcarriers in three different ratios (1:1, 1:2 and 2:1). The three different ratios were imbedded in a fibrin gel. Their development from day one to day four was documented. Additionally, the ECs had been modified to express green fluorescence enabling us to verify that the incorporation of a ratio was successful. The 2:1 and 1:2 ratios resulted in a similar, albeit lower, sprouting rate than that of the 1:1 ratio. These results suggest that the process of angiogenesis and lymphangiogenesis might be more effective when equal amounts of ECs and LECs are incorporated to the beads. Ultimately, more research is needed to understand how LECs contributes and interacts with ECs during angiogenesis. This knowledge could greatly impact the development of treatments for diseases linked to this process, such as cancer.

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INTRODUCTION

Cardiovascular diseases (CVDs) are the leading cause of mortality in the world, with heart attacks and strokes as the largest percentage (World Health Organisation, 2021). Over the last decades there have been great advances in medicine to improve treatment of CVDs (Kandaswamy & Zuo, 2018). Despite these advances there are still several ischemic vascular diseases that are considered as undertreated. The stimulation of new blood vessels has therefore gained interest over the years as it could be a very useful strategy for treating patients suffering from CVDs. (World Health Organisation, 2021). There are two specific processes of blood vessel formation, vasculogenesis and angiogenesis. In recent time, research in the field of angiogenesis has accelerated due to the recognition that it is a defining characteristic of over 50 pathological conditions, including CVDs (Shah et al., 2019). Similarly, studies of how the lymphatic system contributes to some of the same pathological conditions have been conducted, due to findings that cardiac lymphatics play a role in ischemic cardiac disease. Therefore, lymphangiogenesis may also be a source of treatment for diseases involving angiogenesis (Liu & Oliver, 2023). As a result of this, the development of new therapies to tackle and understand the pathological processes of diseases like CVDs are important. In order to accomplish this, there is a need to create models for research as they contribute to isolate and better understand specific processes occurring in sick patients. There are different *in vivo* and *in vitro* models created, one *in vitro* method is 3D sprouting assay.

The main aim of this thesis is to investigate the lymphatic endothelial cell's (LECs) contribution to blood endothelial cell's (ECs) angiogenic sprouting. This is to better understand how angiogenesis and endothelial cells can help develop treatment methods for ischemic diseases. To achieve this aim an *in vitro* 3D sprouting assay was utilized. In this thesis three different ratios of ECs and LECs were incorporated to the microcarriers before being embedded in a fibrin gel. The ECs were modified to express green fluorescence in order to differentiate between the two endothelial cells. This particular study is of interest as no prior studies have examined the contribution of LECs on the ECs sprouting by incorporating them at different ratios and examining them under a fluorescent microscope. It is therefore the first step of trying to understand the contribution of LECs on the EC sprouting while using a 3D sprouting assay.

GENERAL BACKGROUND

To understand angiogenesis there are some main components and factors that needs to be introduced. The focus of this chapter is to first present the key concepts of angiogenesis, endothelial and various of the key players of angiogenesis. It will then explain the process of lymphangiogenesis, as both ECs and LECs were cultured examined in this project. Lastly, there will be description of the 3D sprouting assay method and its advantages and limitations.

Angiogenesis

Angiogenesis is a term that is used to describe the formation and stabilization of new blood vessels from pre-existing ones. It plays a critical role in both physiological and pathological conditions, as it ensures sufficient supply of nutrients to tissues. Angiogenesis creates new blood vessels by either sprouting or by splitting existing blood vessels, see Figure 1 (Otrock et al., 2007). This process of angiogenesis involves a complex cascade of events, including endothelial cell activation, recruitment and proliferation followed by interacting with other cell types, enabling stabilization of the initially immature new vasculature (Nishida et al., 2006). Cells are heavily dependent on gas exchange and nutrients of which blood vessels supply, thus angiogenesis occurs throughout adult life (Alberts et al., 2002). For instance, tissues can become ischemic (hypoxia) and demand oxygen as it's growing. In these cases angiogenic factors are released and sprouting angiogenesis is triggered (Pauty et al., 2017). Cells will therefore never be further away than 100 μm of a capillary (Alberts et al., 2002). As a result of this, angiogenesis' involvement in some physiological processes such as muscle growth will be vital for providing oxygen. However, angiogenesis is not limited to physiological processes, it can also resume in cases of pathological conditions. Cardiovascular diseases, diabetic retinopathy and cancers are examples of diseases which are angiogenesis dependent. Particularly well studied is tumour growth, as cancer cells are dependent on blood supply (Kubis & Levy, 2003)

Endothelial cells

Endothelial cells (ECs) play a pivotal role in both the circulatory system and lymphatic systems. In the body, ECs form a monolayer called the vascular endothelium which creates a

cellular lining encircled by smooth muscle present in all blood vessels. The membrane of ECs is in direct contact with circulating cells and blood constituents, making them a key component in providing all tissues with nutrients. The ECs are known for their ability to migrate and starting new sprouts, allowing them to repair existing blood vessels and create new ones (Alberts et al., 2002). A specific type of endothelial cell, tip cells, lead the angiogenic sprouting or splitting. Tip cells are capable of high migration rates, the following cells are named stalk cells which are not as migratory (Palm et al., 2016). The tip cell will respond to signals from Vascular Endothelial Growth Factors (VEGF) and start migrating (Siemerink et al., 2013). As new blood vessels form, the stalk endothelial cells go through the process of lumenogenesis, which is the formation of tube shaped compartments, lumen, that allows blood flow (Ucuzian et al., 2010). In Figure 1 the tip cells are illustrated in red color visualizing how they migrate towards VEGF. Stalk cells follow and form lumen.

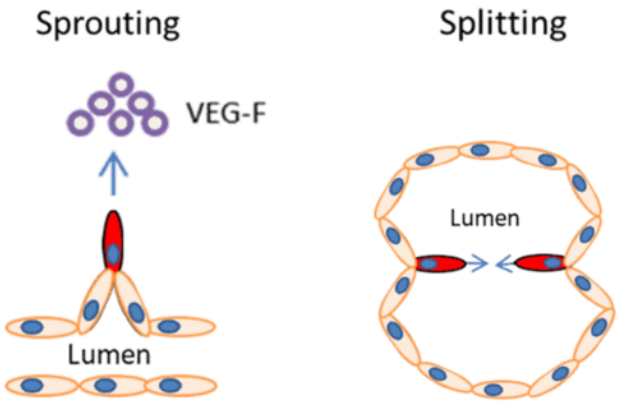

Figure 1: *Illustrates the two different methods of angiogenesis, sprouting and splitting. Retrieved from IT, 2021.*

Lymphatic endothelial cells (LECs) make up the lymphatic system, which complements the circulatory system in transporting cells and critical factors. Similarly, to the circulatory system, the lymphatic system is also made up of a single layer of endothelium lining the interior of vessels, this type of endothelial cell is called the LEC. The lymphatic vessels are found in tissues and LECs line the sinuses of lymph nodes which makes them crucial participants in numerous elements of the immune system (Lucas & Tamburini, 2019). The lymphatic system compliments the circulatory system by ensuring proteins and other macromolecules which cannot be absorbed directly into the blood can be transported from peripheral tissue by the lymphatic vessels (*Lymfesystemet*, n.d.).

Blood endothelial cells (ECs) and LECs are essential for the formation of the circulatory and lymphatic vascular system, respectively, but serve different basic functions. ECs are a key component of blood vessels in the closed circulatory system, while the LECs form the one-way passage for fluid and molecules like leukocytes in the lymphatic vascular system, which ultimately is drained back into the blood circulation (Alitalo, 2011). Despite their close similarities, ECs and LECs have distinct differences due to their unique and somewhat complementary basic functions. The main function of the ECs is supplying oxygen and nutrients to and regenerating tissues and organs, while the focus of the LECs is primarily on the immune responses and clearing excess fluids from tissue (Przysinda et al., 2020).

Regulation of angiogenesis

There are several factors that regulate angiogenesis. These factors are needed in order for angiogenesis to be activated and initiate vessel growth. The extracellular matrix (ECM) plays a key role in regulating angiogenesis by providing essential structural support and acting as a scaffold for cytokines. Angiogenesis is a tightly regulated process that alternates between activation and inhibition of different factors and physical and chemical constraints provided by the local ECM. The ECM is remodeled by enzymes secreted by activated endothelial tip cells. The creation of new ECM structures and breaking down existing ones allow for cell migration and proliferating, thus the formation of new blood vessels (Senger & Davis, 2011).

Angiogenic Growth Factors (i.e. soluble secreted signaling proteins) are a group of molecules that play a pivotal role in angiogenesis. Particularly, Vascular Endothelial Growth Factor (VEGF) has a fundamental role in modulating angiogenesis by specifically targeting endothelial cells and inducing their proliferation and migration. Used *in vitro,* VEGF will stimulate the degradation of ECM as well as induce ECs proliferation, migration and tube formation(Liekens et al., 2001). There are several isoforms of VEGF and among these isoforms, one of them play a crucial role in angiogenesis – VEGF-A (Perrot-Applanat, 2012). VEGF-A regulates angiogenesis by binding to and activating Vascular Endothelial Growth Factor Receptors (VEGFR), specifically VEGFR-1 and VEGFR-2 (Shibuya, 2011). In particular, the signaling between VEGF-A and VEGFR leads to an increase of permeability of the microvascular network which according to Dvorak et al., 1995 is an important step for the

initiation of angiogenesis. VEGF-A/VEGFR-2 signaling is the most prominent signal, though the activation of both receptors VEGFR-1 and VEGFR-2 induce cellular responses in ECs, VEGF-A/VEGFR-2 produce a much stronger mitogenic signal and survival signal compared to VEGF/VEGFR-1, making it the more influential part of initiating vessel growth (Abhinand et al., 2016).

While VEGF is one of the most critical growth factors involved in angiogenesis, other factors play a role during new blood vessel formation that are secreted when local tissues sense low oxygen tension (hypoxia) (Bhutta et al., 2023). If an area reaches hypoxia it will activate hypoxia inducible factors (HIFs) to avoid necrosis, tissue death. HIFs, like HIF-1α, will regulate the oxygen level by rising the transcription level of growth factors (Khalid & Azimpouran, 2023, Ziello et al., 2007). VEGF and other growth factors like Fibroblast Growth Factor (FGF), Epidermal Growth Factor (EGF) and angiopoietins will then stimulate the ECs through their membrane receptors inducing important processes like proliferation, migrating and thus angiogenesis (Geindreau et al., 2022).

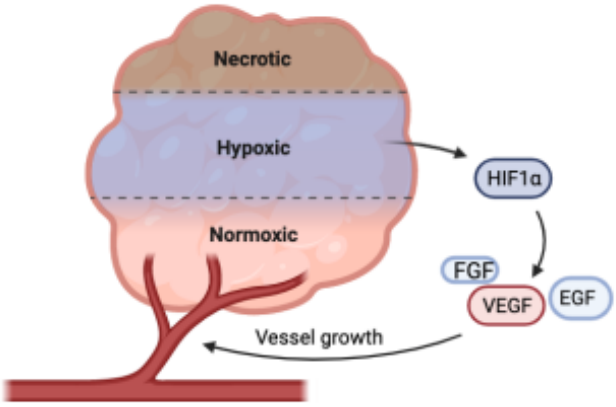

Figure 2: *Illustrating how hypoxia, exemplified in a tumor, will trigger the transcription level of VEGF and ultimately induce vessel growth. Created in biorender.com.*

Lymphangiogenesis

The process of developing new lymphatic vessels from pre-existing vessels is called lymphangiogenesis. Lymphangiogenesis is present during the embryonic development and only resumes in adult life during a variety of pathological conditions such as tumor growth and tissue repair (Pepper & Skobe, 2003). It is a process that has been understudied, however, over recent years the scope of lymphangiogenic factors has expanded. This is because it has come to light that various factors, including angiogenic growth factors are also involved in the

regulation of lymphangiogenesis. For instance, in a study done by R. Cao et al., 2012 it was found that the subfamily of FGF, namely FGF-1, which also involves angiogenesis, possibly could be identified as a potent inducer of lymphangiogenesis. Furthermore, Prox1, has proved to be the main regulator of lymphangiogenesis, although precisely how it regulates the formation of new lymphatic vasculature has yet to be fully described (Grimm et al., 2023).

Endothelial cell types: blood vs. lymphatic

These two types of endothelial cells have been shown to respond distinctively to different growth factors. For example, the LECs have two specific growth factors, VEGF-C and VEGF-D, which trigger lymphangiogenesis in cases of pathological conditions like inflammatory diseases, by activating the receptor VEGFR-3 (Lee et al., 2010). The activation of VEGFR-3 leads to lymphatic migration and proliferation. This signalling to VEGFR-3 is the main pathway for lymphangiogenesis (Yan et al., 2017). VEGF-C and VEGF-D are also growth factors found in ECs, but in a much lower quantity than LECs (Kriehuber et al., 2001). By binding to VEGFR-2 they can contribute to angiogenesis in ECs, yet the response they receive in comparison to activating VEGFR-3 in LECs is much weaker as the VEGFR-3 is expressed at higher levels in LECs (Y. Cao et al., 1998). A molecular marker that can be used to distinguish LECs from ECs is the transcription factor Prox1 (Norgall et al., 2007).

In vitro 3D sprouting assay

In vitro 3D sprouting assays are valuable tools for studying and replicating angiogenesis and lymphangiogenesis. Various *in vitro* models have been described, but one specifically *in vitro* method described in 1995 by Nehls and Herrmann has been consistently used over the years since (Nehls & Drenckhahn, 1995). This in vitro model of angiogenesis is based on coated microcarrier beads (MCs) with ECs. This method has since been modified and used over the past two decades, an improved protocol that supports the sprouting of HUVECs was reported by Nakatsu et al., 2003. The basic principle of this method involves coating ECs on Cytodex beads, suspending them in a fibrinogen solution and then incorporate it with a thrombin solution, making the fibrinogen polymerize, thus mimicking the *in vivo* environment for the ECs (Staton et al., 2009). In studies over the past decade MCs have been coated with ECs and

cultured in endothelial growth medium (EGM-2/EGM-2MV, Lonza) enhanced with various cytokines. This lead to a significant increase in both the number of sprouts and their length (Morin & Tranquillo, 2013).

Advantages and limitations concerning *in vitro* 3D sprouting assay

The in vitro 3D sprouting assay offers several advantages and some limitations in studying angiogenesis. Amongst its advantages, this assay displays a pretty close representation of the *in vivo* environment angiogenesis occurs in, making it more physiologically relevant than other 2D assays (Edmondson et al., 2014). Additionally, this *in vitro* assay gives an advantage of being able to isolate and adapting the study to a specific process related to EC's angiogenesis like matrix degradation, migration, proliferation or tube formation. This method of studying is usually not as expensive as *in vivo*, and the animal handling skills are not necessary. Furthermore, it can be used to screen for potential angiogenic or anti-angiogenic compound in a high-throughput manner (Goodwin, 2007). However, there are also limitations to the *in vitro* 3D sprouting assay. It lacks the other cell types and components found *in vivo,* causing the behaviour of the ECs to vary depending on the conditions they're cultured in, still or flowing. The *in vitro* assay cannot fully capture the physiological relationship occurring in an *in vivo* environment (Staton et al., 2004).

Research gap and study rationale

Understanding the relationship between endothelial cells (ECs) and lymphatic endothelial cells (LECs) could be crucial for finding new treatment for cardiovascular diseases. More specifically, there is a lack of knowledge in understanding the role of LECs in EC sprouting. Gaining insight into this relationship could provide critical information for the development of novel therapeutic approaches in various diseases, such as cancer, cardiovascular disorders and inflammatory conditions. Angiogenesis has been extensively investigated, while lymphangiogenesis has received increased attention in recent years. However, the current understanding of lymphangiogenesis lags behind that of angiogenesis, especially the understanding of the relationship between the LECs and the ECs (Sweat et al., 2014).

Objectives

This thesis is a first step for trying to understand the contribution of LECs on the ECs sprouting while using a 3D sprouting assay.

MATERIALS AND METHODS

Cell culture

Three primary cells were tested in this project, including Human Umbilical Vein Endothelial Cells (HUVECs), Human lung Microvascular Lymphatic Endothelial Cells (HMLEC-dLy) (LECs) and Human dermal Microvascular Endothelial Cells (HMVEC-d) previously transfected to express green fluorescent protein (GFP). The cells were originally purchased from Lonza and were cultured according to the supplier's guidelines. The protocols for cell culturing were followed by using aseptic techniques in a cell culture room. The aseptic technique prevents other bacteria and microorganisms form being introduced to the cell culture. The cells were all stored in liquid nitrogen and had to be broken out of cryopreservation to get ready for use. All cells were cultured in T75 flasks, HUVECs in EGM™-2 BulletKit Medium (Lonza, #CC-3156), LECs and GFPs in EGM™-2MV medium (Lonza, #CC-4147). The three cell types were seeded for subculturing according to the recommended seeding density – 5,000 viable cells/cm^2.

Subculture to confluent monolayer and Maintenance

Cells ranging from passage 4 and 6 were used in all experiments and all cells were maintained in a 37°C humidified 5% CO_2 atmosphere. All cells were subject to passage when they were at a 70%-85% confluency and contained many mitotic figures throughout the flask (important to not let confluency reach 90%-100%). Specifically, they were passaged by first using 2 mL trypsin/EDTA (2.21mM, Corning 25-3053 CI) 0.25% in each T75 flask which was aspirated before adding new 5 mL of trypsin/EDTA to each flask to then be incubated at 37 °C for 6-7 minutes. The trypsin will detach the cells from the flask. To check that all cells have detached they were studied under the microscope. 5 mL of media was added to all flasks before the cells were collected into a 15 mL colonial tube to be centrifuged at 300 rpm for 6 minutes. The supernatant was aspirated, and the cell pellet gently resuspended in 6 mL of media, then transferred 2 mL of cells into T75 flasks with 8 mL media.

3D Fibrin Sprouting Assay

Cytodex™ 3 microcarrier (MC) (Amersham Biosciences) were used in this 3D fibrin sprouting assay. According to the vendor, each 1g of dry microcarrier amount have around 3

million microcarriers. One fully confluent microcarrier can accommodate around 400 endothelial cells. This protocol was optimized to achieve a minimal of 200 cells/microcarrier. MCs were first hydrated in PBS for three hours (~0.2 ml/mg of dry microcarriers) at room temperature. The PBS supernatant was decanted and replaced with fresh PBS (~0.1 mL/mg of dry microcarriers) and microcarriers were sterilized by autoclave (liquid cycle). Let the beads settle and aspirated the supernatant and resuspended the beads with 3 mL of fresh media, adding 1 mL of beads into the three Eppendorf tubes marked with 1:1, 1:2 and 2:1. Trypsin was then added to both the LECs and GFP cells, they were centrifuged, supernatant aspirated and reconstituted the pellet cells with 0.75 mL of EGM-2MV ($1x10^6$ cells/mL). Cells were mixed with MCs, and placed together in the 2mL Eppendorf centrifuge tube. To achieve the three different ratio conditions of 1:1, 1:2 and 2:1 of GFPs and LECs respectively, the 1:1 ratio had 0.375 mL of both LECs and GFPs. The 1:2 ratio had 0.1875 mL of GFP and 0.25 ml of LECs and the 2:1 ratio had 0.25 mL of GFPs and 0.1875 mL of LECs. Incubate for 4 hours at 37 °C, gently flick and inverting the tube every 20 minutes. Each solution was then transferred to a 15 mL tube, when the beads had settled the supernatant was aspirated and new 15 mL of EGM-2MV was added. The 15 mL solution was divided on three 6-well plates where they could be studied under the microscope to observe the cell incorporation to the beads. The next day a random well was used for being embedded in the fibrin gel.

Fibrin gel

For the fibrin gel formulations, the following percentages by volume were used: (solution A) Fibrinogen solution (4 mg/mL, dissolved in 0.9 g NaCl, Sigma Aldrich F3879) 68.2%, Aprotinin (500 μg/mL, dissolved in PBS, Sigma Aldrich A4529) 9.1%, microcarriers with cells in solution 22.7%. Solution B – Thrombin (22.72 units/mL, Sigma Aldrich T6884) 8.3% and PBS 91.7%. Solution A and B were mixed in a 5:4 ratio, here 0.25 mL of A was introduced to each well (one and a half 24 well plate), then introduced 0.20 mL of B by carefully mixing the solution with A inside the well without making air bubbles. The two 24 well plate containing the gel solution was then left in room temperature for 20 min and then placed in the incubator (37 °C, 5% CO_2) and allowed for gelation for 30 minutes. Each well with the 3D fibrin gel was then topped with 0.8mL of fresh media and incubated (37 °C, 5% CO_2) for 3 days with daily media changes.

RESULTS AND DISCUSSION

3D sprouting angiogenesis assays of three different ratios

Angiogenesis plays an important role in enabling pathological conditions, such as tumor growth, therefore it may also be the way to treat such diseases. Likewise, lymphangiogenesis has also been proven to play a part in some of the same pathological cases, for instance in tumor growth (Nagahara et al., 2022). Although the specific mechanisms of how the ECs and LECs precisely work together needs more research, the study conducted here could be a start in understanding whether these two distinct types of endothelial cells have a positive or negative impact on each other's vessel formation. Consequently, this information could be used to treat related diseases.

To explore the role of lymphatic endothelial cells into the sprouting of blood endothelial cells, an *in vitro* 3D sprouting assay was used, incorporating ECs and LECs onto microcarriers in three different ratios (1:1, 1:2 and 2:1). While there were three different ratios, the overall number of cells for these ratios was kept constant. The three different ratios were each embedded in a fibrin gel in 12 wells of 24 well plate, see Figure 3. We chose these ratios to examine whether changes in the proportion of ECs to LECs would influence the overall amount of sprouting. Furthermore, the ECs were transfected to express green fluorescent protein, thereby enabling the identification of the ECs that are most likely to initiate or contribute to the sprouting process. Pictures were taken through the microscope on day one and day four of each ratio in three different magnifications. A random 2:1 well was pictured on day four using a fluorescent microscope, helping visualize the sprouting of ECs.

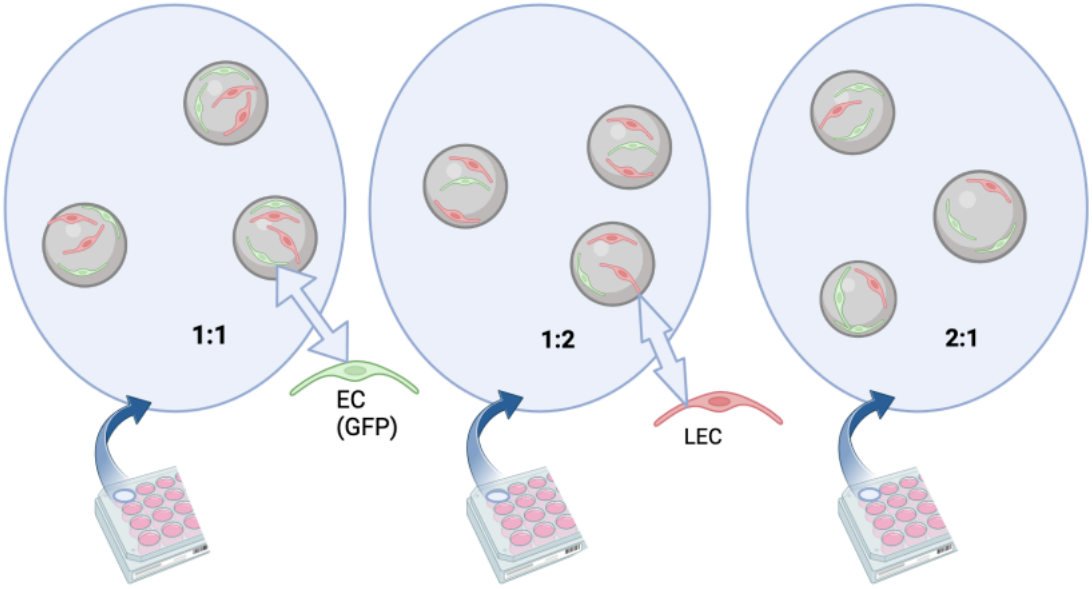

Figure 3: *Illustration of how the different ratios of ECs and LECs were incorporated to the beads. Created with biorender.com.*

Comparison of *in vitro* 3D angiogenic sprouting per ratio

The MCs coated with cells were pictured 24 hours after being embedded in a matrix composed of Fibrinogen, Aprotinin and Thrombin, Figure 4 shows three images of the MCs with a 1:1 ratio of ECs and LECs, respectively. Figure 4A provides a general and representative perspective of the each well. Although, it is not possible to definitively determine the lineage contribution of the sprouts in either ECs or LECs, the observed sprouting within a single day of being introduced to the gel suggests that the environmental conditions of the gel are favorable for their growth. Furthermore, it seems that the co-culture of the two types of endothelial cells did not inhibit their respective. Although, which type of endothelial cell or if both are contributing to the *in vitro* sprouting cannot be determined from these micrographs.

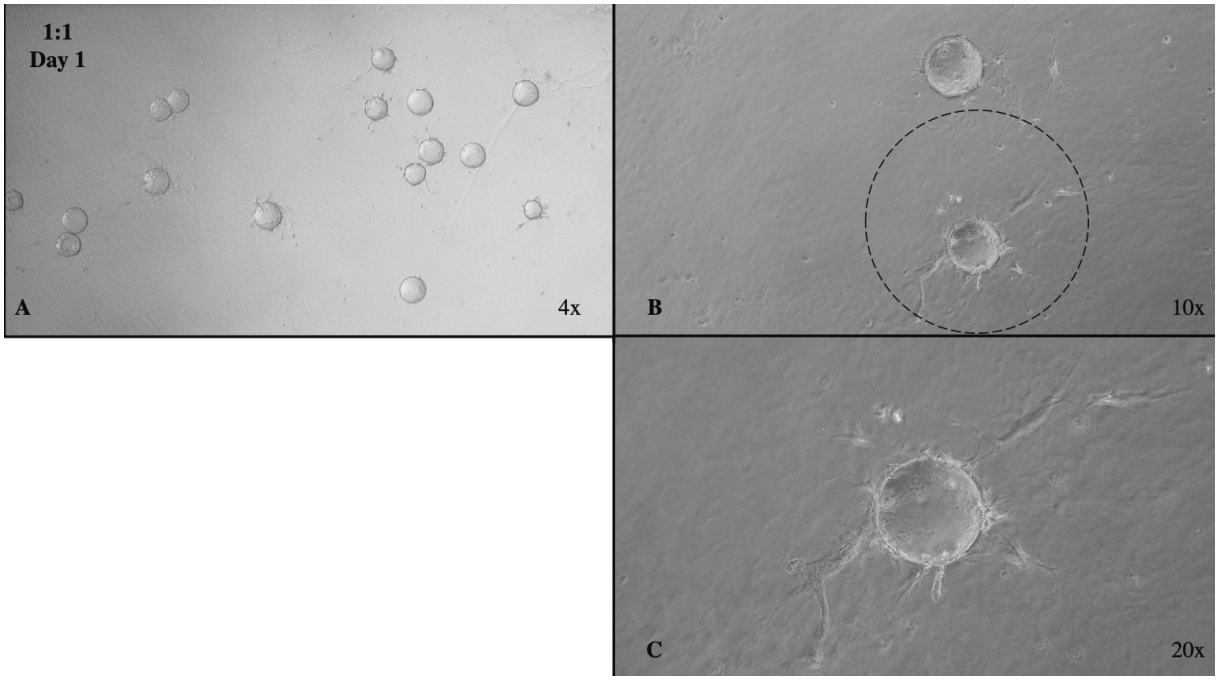

Figure 4: *Display of a set of micrographs capturing coated microcarriers imbedded in a 3D fibrin gel on Day 1. Beads have been coated with two distinct types of endothelial cells, namely ECs and LECs, at a 1:1 ratio, respectively. The beads are embedded in a fibrin gel in a 3D angiogenic sprouting assay. The images have been acquired through a microscope on day 1 and are presented in three magnifications: A) 4x, B) 10x and C)20x. The encircled bead in B is shown in C at 20x.*

Over the next days the cells were observed, and daily media changes were done. On the fourth day new pictures were taken in the magnifications 4x, 10x and 20x of the 1:1 ratio well plate. One sprout has been defined when two or more cells are still connected to microcarriers and are sprouting away from the microcarrier. That said, it is possible to observe sprouts as well as cells simply migrating away from the microcarrier. Specifically, Figure 5A is directly comparable to Figure 4A, since they both represent the identical region of interest. It is possible to observe a significant increase in the number of sprouts by day four when compared to day one. Figure 5C displays a MC at 20x magnification offering a more detailed view of the sprouts. Although, the ECs have yet to form stabilized vessels, they have started the sprouting process. On day four, the number of sprouts per bead was manually counted and quantified. For the ratio of 1:1, the average count was 0.847 sprouts per bead. It is noteworthy that we used microvascular endothelial cells in this study, whereas many other studies, such as Kannan et al., 2022, use Human Umbilical Vein Endothelial Cells (HUVECs). HUVECs are known to exhibit a higher sprouting ability than the LECs and ECs used here.

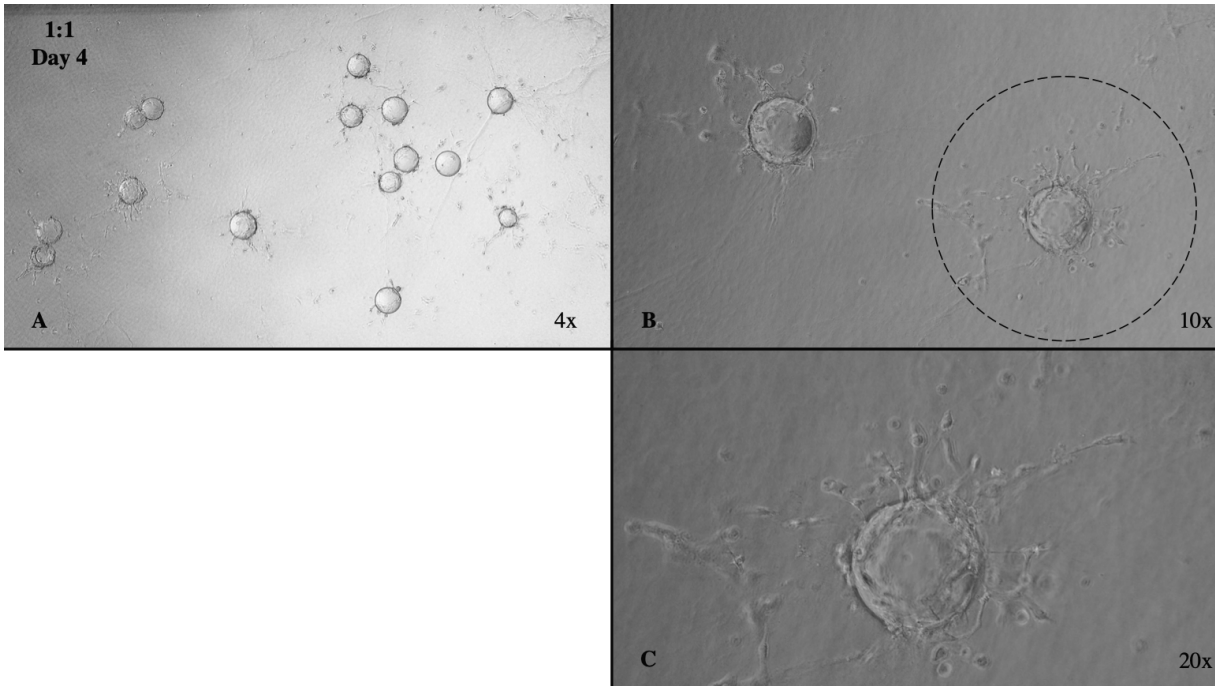

Figure 5*: Depiction of the same set of beads as shown in Figure 4, imaged on day 4. Picture A) presents a region of beads captured at 4x magnification. B) highlights an individual bead imaged at 10x magnification, the highlighted bead is presented at a 20x magnification in picture C.*

The images in Figure 6 were all taken on day one, the early phase of sprouting angiogenesis, after the MCs were incorporated with the endothelial cells with a 1:2 ratio of ECs and LECs, respectively. Figure 6A provides an overview of the beads in a 4x magnification. The chosen area exhibits the beads at a noticeable distance from each other, in contrast to circa 50% of the other wells, where the beads were observed to be situated more clustered together, exemplified in Figure 7A. Figure 6B highlights a specific bead, which is further magnified to 20x in figure 6C. Analogous to the MCs shown in Figure 4, the ECs in Figure 6 have initiated the sprouting process. Although, on day four they were pictured again, see Figure 7, and as anticipated there had occurred more sprouting.

By day four, as shown in Figure 7 it was observed more sprouting as compared to day 1. The image seen in Figure 7A portrays the positioning of the beads to be closer together compared to Figure 6A. The position of the MCs will influence their ability to form sprouts and make it harder to see and thus, count the sprouts. However, there were still a few beads placed in a beneficial position, see Figure 7B,C, making the number of sprouts relatively close to those seen in Figure 5. The average number of sprouts per bead was 0.720, indicating the ratio of 1:2 is not as sufficient for angiogenic sprouting. Although, had the MCs been spread out more

evenly throughout all 12 wells, it may have resulted in a higher number of sprouts within the wells containing the ECs ratio of 1:2.

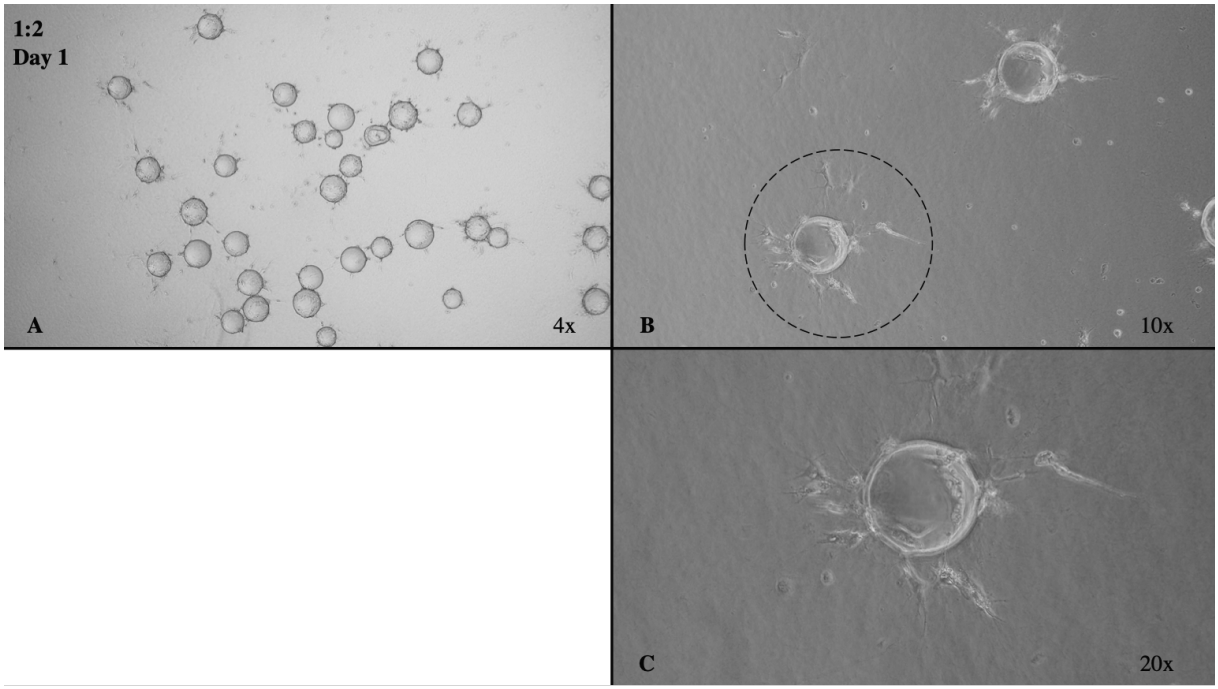

Figure 6: *Image that displays three photographs taken on day 1. depicting microcarrier beads coated with ECs and LECs, at a 1:2 ratio, respectively. The beads are embedded in a fibrin gel for a 3D angiogenesis sprouting assay. A) displays a 4x magnified view of the beads, while B) shows a separate field in 10x magnification, with one selected bead marked by a circle. C) The circled bead is exhibited in a higher magnification of 20x.*

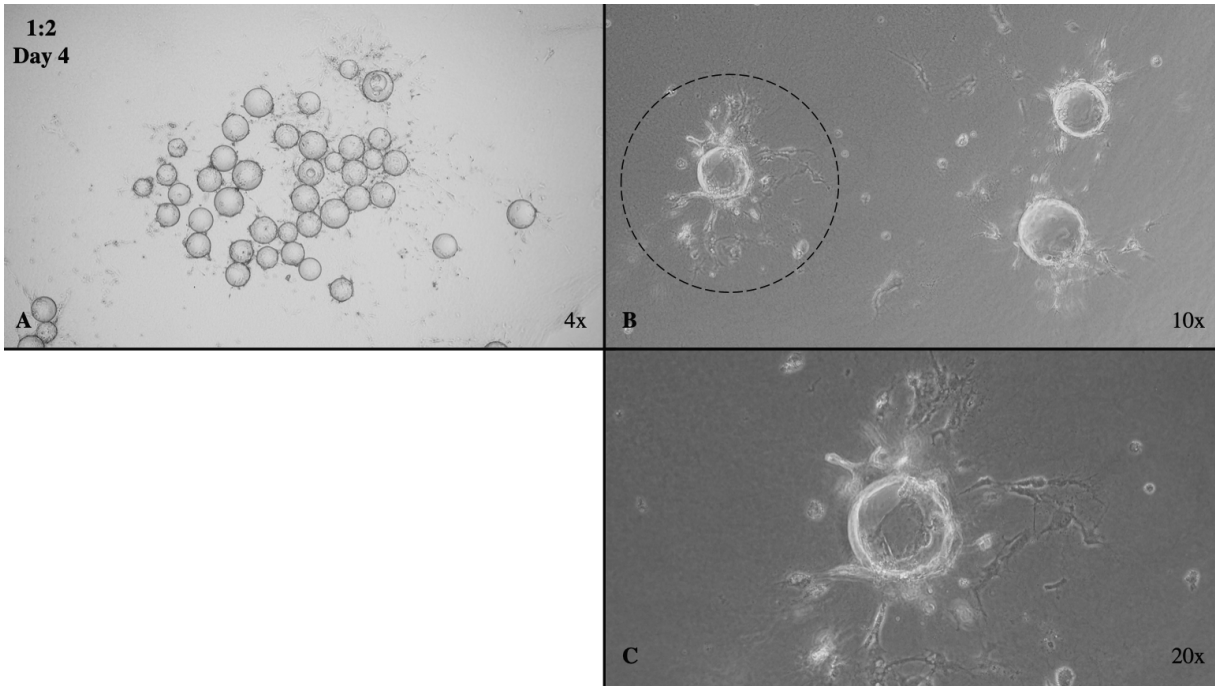

Figure 7: *showcase of a series of three micrographs taken of the same beads as depicted in Figure 6, here on day 4. A) exhibits a 4x magnified view of the microcarriers, while panel B captures a group of microbeads imaged at 10x magnification, with a specific bead encircled. C displays a closer look at the circled bead with a magnification of 20x.*

The incorporation of ECs on the MCs with a ratio of 2:1, ECs:LECs, were also pictured through the microscope about 24 h after gelation, see Figure 8. A representative image was selected to portray the beads from all twelve wells on day one, seen in Figure 8A with a magnification of 4x. The endothelial cells incorporated on the bead highlighted in Figure 8B have made notable progress in the sprouting process, further examination of Figure 8C gives a more detailed image of the sprouts.

The images displayed in Figure 9 were all taken of the 2:1 ratio on the fourth day after gelation, an image in magnification 4x that represents the typical findings was selected and can be seen in Figure 9A. Figure 9B and 9C show more detailed images of a representative bead. This ratio seemed to have a bit more of migration rather than sprouting on day four, see Figure 9. These findings correlate with a study done by Nakatsu et al., 2003. Despite them conducting the study on HUVECs embedded in fibrin gel, the authors observed a higher number of migrating cells on and after day four. Additionally, the supplementing of skin fibroblasts (SF) on top of the gel, resulted in a significant decrease in the number of migrating cells. Here, there were no components growing on top of the gel, perhaps if there were, the microvascular cells would migrate less, thus form more sprouts. Furthermore, as seen in

Figure 9A, though the MCs are not as clustered as the ones in Figure 7A, there are still some beads connected in pairs. This positioning could effect the amount of sprouts counted and possibly lead to migration, as the ECs could prefer the matrix environment apart from the clustered beads. The 12 wells containing the ratio of 2:1 had the overall lowest average number of sprouts per bead, at 0.718. Based on the numbers obtained, it appears that there is no significant difference between these wells containing a 2:1 ratio and those with a 1:2 ratio. However, the beads were more scattered and conveniently placed for beneficial sprouting in those with the 2:1 ratio. These findings could be due to the ECs not being as active, as they were slow growers in the preparation period and needed more time to reach optimal confluency.

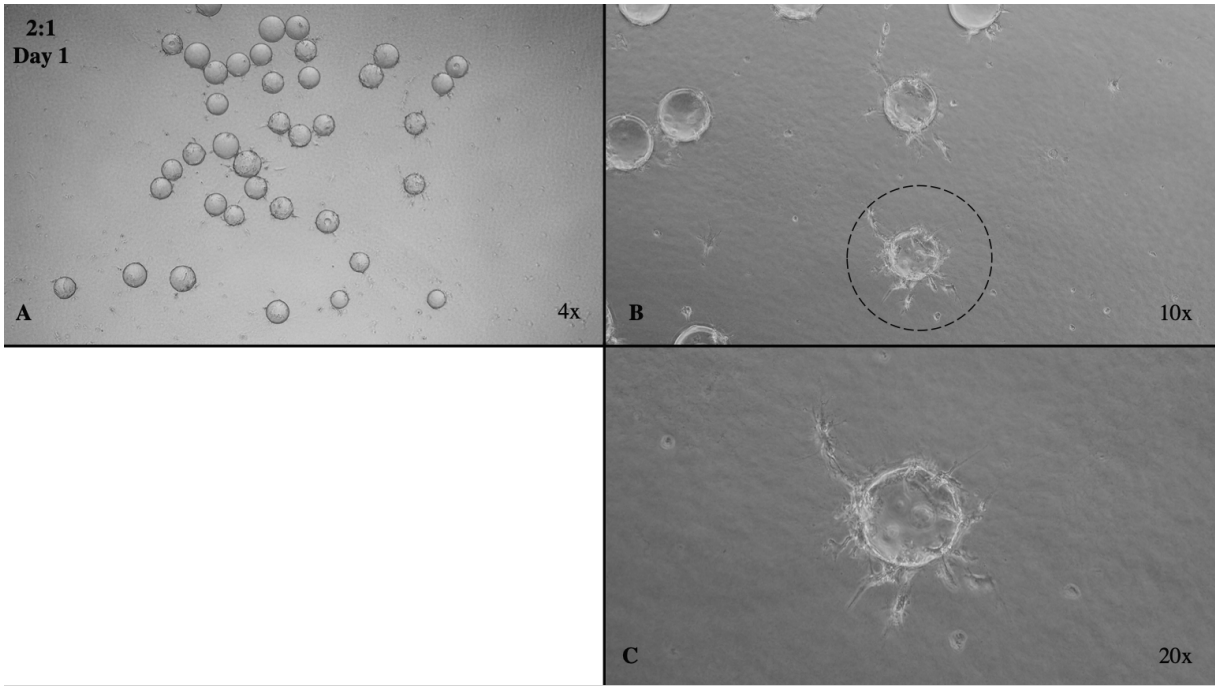

Figure 8: *A series of three pictures taken of beads coated in two types of ECs, ECs and LECs with a 2:1 ratio, respectively. All images were depicted on day one after incorporating the cells with MCs, through a microscope. A) An area of the beads at a 4x magnification, while B displays a separate area of the beads in a 10x magnification, it has one bead encircled. The encircled bead is shown in C at a 20x magnification.*

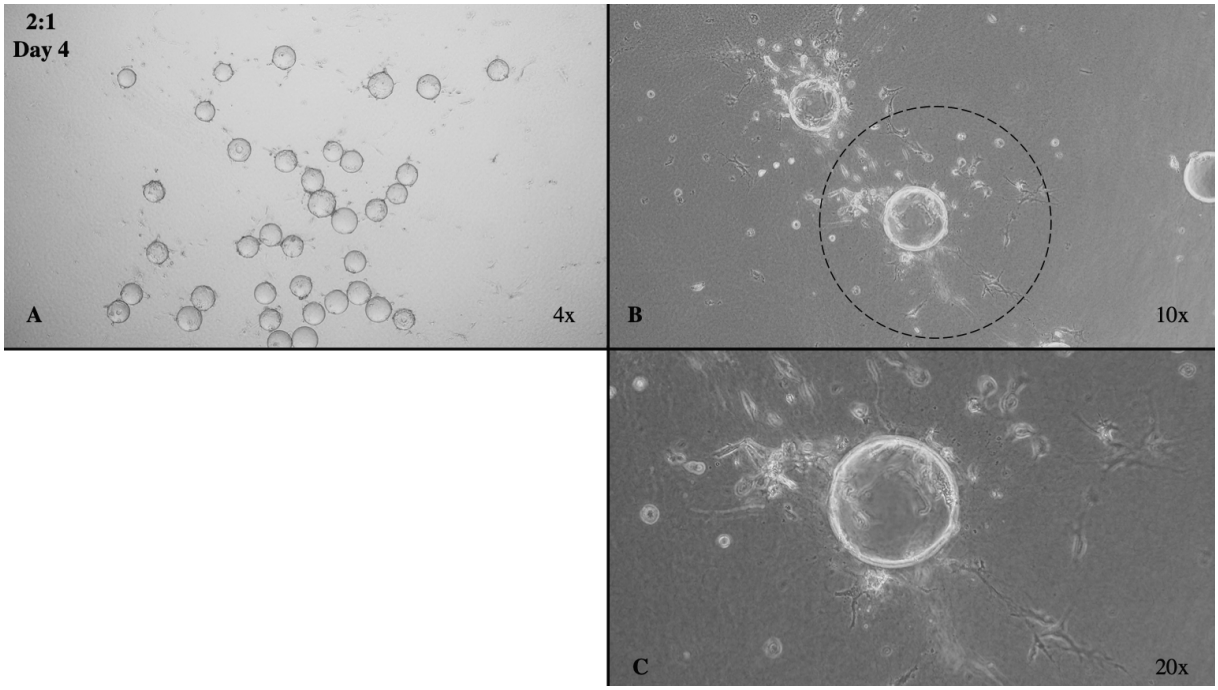

Figure 9: *Display of three micrographs captured on the fourth day, depicting the same beads as shown in Figure 8. A) displays a 4x magnified view of the MCs. B) Captures a distinct region of the beads imaged at a higher magnification of 10x, featuring one specific bead marked by a circle. C) Presents a 20x magnified view of the circled bead, offering a detailed observation.*

The blood endothelial cells were transfected with GFP enabling the visualisation of which endothelial cell type is the biggest contributor to the sprouting. On day four a random well from the 2:1 ratio well plate was depicted with a microscope using the Differential Interference Contrast (DIC) technique, see Figure 10A. The GFPs were detected and pictured at a magnification of 15.75x, shown in Figure 10B. In Figure 10A we clearly see more cells than those expressing green in Figure 10B, giving an indication that we did the ratio successfully.

ECs fluorescing green are seen on two of the three beads in focus, see Figure 10A. Even though this image was taken on day four, the ECs have only started forming two sprouts. However, there are few ECs attached to the beads. The fact that most of the ECs in this image have not started sprouting, could indicate that there are no growth stimuli present in this region. Another possibility could be that they would need more time to form matured sprouts as they were slow growers in the start of this study. Moreover, there are LECs present on all beads imaged as not all the cells express green, a few of them have migrated and especially the beads at the top of Figure 10A contains only LECs.

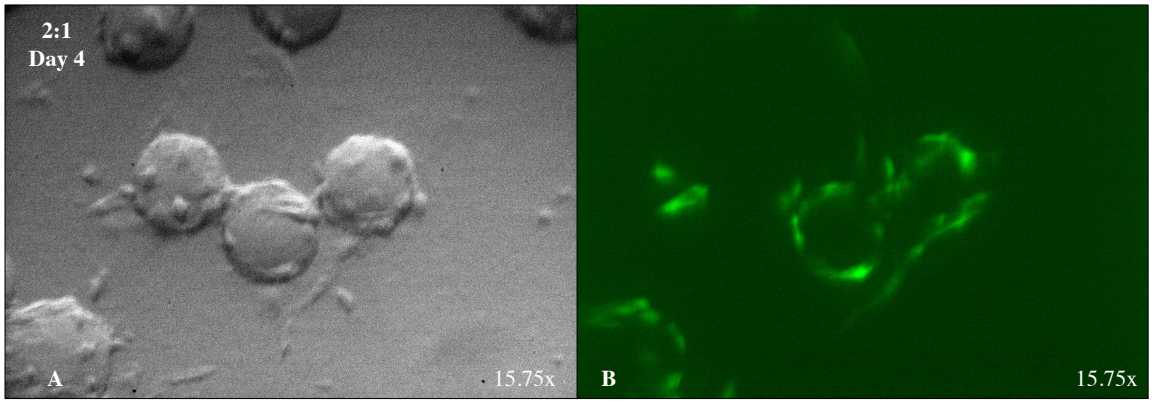

Figure 10: *Two images taken on the fourth day of MCs coated with EC and LECs at a 2:1 ratio, respectively. Both images are of the same area with the magnification of 15.75x. A) DIC image of the beads. B) shows the ECs, transfected with GFP, fluorescing green.*

Figure 10 presents an area that was further magnified to 6x in Figure 11, focusing on a region with a high concentration of ECs. Due to time constraint, not all wells were imaged using both the fluorescence microscope and Differential Interference Contrast (DIC) imaging; ideally, all wells would have been examined. In the event of the images being studied as intended, there are a few occurrences that could possibly happen. One may argue that it could be possible to observe that ECs could serve as tip cells with LECs and ECs in combination following, or there may be no contribution form the LECs to angiogenic sprouting at all. Alternatively, if LECs were found to act as tip cells with ECs following, this would suggest that LECs could initiate the sprouting process. Such a finding would have significant implications and warrant further investigation.

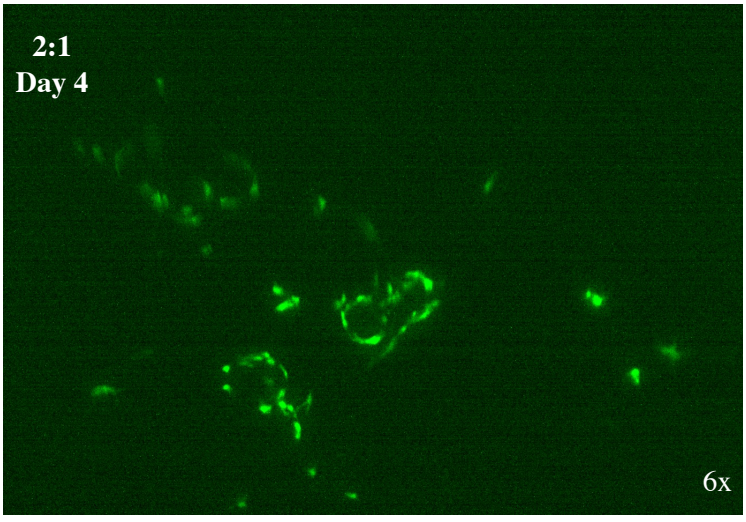

Figure 11: *displays the same area as figure 10 in a magnification of 6x. The ECs transfected with GFP can be seen fluorescing green.*

Quantification of sprouts

Figure 12 describes the sprout quantification using a bar plot. The data seems to suggest that the ration of ECs to LECs could play a role in influencing the sprouting process, but no statistical differences were observed. The 1:1 ratio yielded the highest average number of sprouts per bead, perhaps suggesting that a more balanced ratio of these two cell types might be optimal for the initiation of sprouting in the *in vitro* model.

The 2:1 and 1:2 ratios resulted in a similar, albeit lower, sprouting rate. This finding could indicate that the process of angiogenesis and lymphangiogenesis might be more effective when both cell types are equally present. The observation that the 2:1 ratio resulted in the lowest average could suggest that an overrepresentation of either cell type might somewhat inhibit the sprouting process. Taking together these results, however, should be interpreted with caution. Additional factors such as the spatial distribution of the cells and the specific growth conditions could also play a role in influencing the sprouting. furthermore, more in-depth investigations, such as fluorescent imaging or confocal microscopy, could shed light on the individual roles of the ECs and the LECs during the sprouting process, providing a more detailed understanding of the dynamics between these cell types.

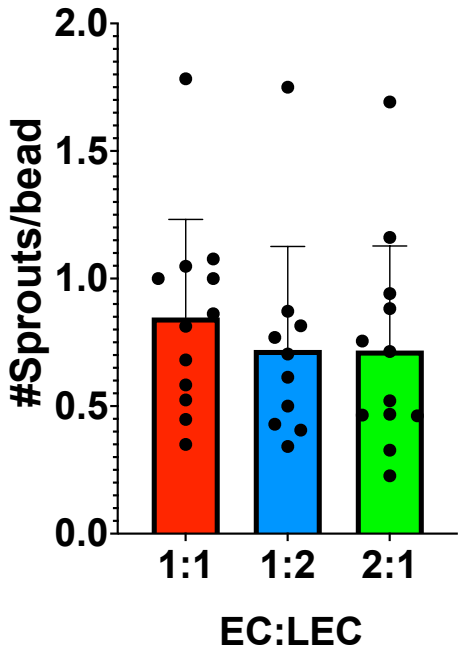

***Figure 12**: Quantification of average sprout number of 3D angiogenic sprouts per microcarrier, presenting the three ratio conditions of 1:1, 1:2, 2:1 of blood ECs and LECs, respectively.*

CONCLUSION

In conclusion, this study provides a preliminary insight into the relationship between blood ECs and LECs during the process of angiogenesis and lymphangiogenesis, using an in vitro 3D sprouting assay. Overall, these findings offer an important starting point for understanding the relationship between ECs and LECs in the process of angiogenesis and lymphangiogenesis. Further research is warranted to uncover the complex interplay between these cell types, which could have significant implications for the development of therapies targeting diseases associated with these processes, such as cancer.

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APPENDIX

Table 1: *Raw data of number of beads and counted sprouts on day 4, used to calculate the average number of sprouts per bead. Rows marked, were statistical outliers and therefore removed.*

1:1	number of MCs	number of sprouts day 4	sprout/bead
well 1	23	41	1,783
well 2	42	44	1,048
well 3	29	25	0,862
well 4	58	26	0,448
well 5	63	33	0,524
well 6	47	32	0,681
well 7	16	13	0,813
well 8	13	14	1,077
well 9	20	7	0,350
well 10	7	7	1,000
well 11	12	7	0,583
well 12	10	10	1,000
1:2			
well 1	54	38	0,704
well 2	47	41	0,872
well 3	54	44	0,815
well 4	9	28	3,111
well 5	32	16	0,500
well 6	14	34	2,429
well 7	31	19	0,613
well 8	16	28	1,750
well 9	38	13	0,342
well 10	32	13	0,406
well 11	39	30	0,769
well 12	35	15	0,429
2:1			
well 1	47	22	0,468
well 2	31	36	1,161
well 3	34	32	0,941
well 4	13	22	1,692
well 5	88	20	0,227
well 6	65	30	0,462
well 7	56	40	0,714

well 8	49	37	0,755
well 9	43	20	0,465
well 10	49	16	0,327
well 11	48	25	0,521
well 12	17	15	0,882

Table 2: *overview of the ratios and their average number of sprouts per bead.*

ratio	average sprout/bead
1:1	0,847
1:2	0,720
2:1	0,718