IN VITRO CO-CULTURE MODELS OF PERIPHERAL BLOOD DERIVED CELLS AND ENDOTHELIAL CELLS IN ANGIOGENESIS

Master's Thesis

University of Turku

MSc Degree Programme in Biomedical Sciences

Drug Discovery and Development

May 2021

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UNIVERSITY OF TURKU
Institute of Biomedicine, Faculty of Medicine

SABAPATHI, NANDITA: *In vitro* co-culture models of peripheral blood derived cells and endothelial cells in angiogenesis
Master's Thesis, 54 pages.
Drug Discovery and Development
May 2021

Poor vascularization of tissue-engineered constructs is a common challenge in regenerative medicine and there is a need to find an optimal cell source that is proangiogenic and aids in the neovascularization process.

Endothelial progenitor cells (EPCs) are cells that participate in new blood vessel formation and regeneration of blood vessel endothelium in ischemic and hypoxic conditions. Two major types of EPCs are Myeloid Angiogenic Cells (MACs) and Endothelial Colony Forming Cells (ECFCs). MACs promote formation of new blood vessels via a paracrine mode of action.

Co-cultures of mesenchymal stem cells (MSCs) with peripheral blood derived mononuclear cells (MNCs) have been shown to possess angiogenic differentiation potential by inducing the differentiation of MACs.

This project aimed to test the functionality of MACs in a co-culture model with human umbilical vein endothelial cells (HUVECs). MACs with surface markers CD14 and CD31 were isolated from MSC-MNC co-cultures using magnetic-activated cell sorting and allowed to grow in a Transwell® setup with HUVECs. Optimisation of culture conditions with MSCs and HUVECs was also done to see if tube formation is affected by fibronectin coating and the type of culture media used.

Results show that co-cultures of MACs and HUVECs give rise to looping and branching tubular structures, such as those seen in *de novo* vascularization and that tube formation is favoured when cells were cultured in Endothelial Growth Media and on fibronectin coated surfaces.

These kind of *in vitro* assays will aid in assessing the proangiogenic capabilities of MACs. Further studies elucidating which paracrine vasoactive factors affect tube formation in angiogenesis will help in producing clinically applicable tissue-engineered constructs with better vascularization.

Key words: endothelial cells, angiogenesis, mononuclear cells, mesenchymal stem cells, co-culture

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1. INTRODUCTION

1.1 Angiogenesis and vasculogenesis

The process of new blood vessel formation from an already existing vascular network is known as angiogenesis. On the other hand, vasculogenesis refers to the *de novo* formation of a primordial blood vessel network that is triggered by the differentiation of precursor angioblasts into endothelial cells. During early embryogenesis, both angiogenesis and vasculogenesis contribute to blood vessel formation. However, in a healthy adult, angiogenesis is limited to few physiological conditions, such as endometrium and placenta formation, wound healing, and hair follicle vascularization (Li et al., 2005).

Angiogenesis is also seen in certain pathological conditions such as cancers, ophthalmological diseases, and chronic inflammatory diseases (Polverini, 1995). There is also evidence to suggest that post-natal vasculogenesis is possible due to the presence of endothelial cells and endothelial progenitor cells in the circulatory system (Ribatti et al., 2001).

The dynamic process of angiogenesis involves several sequential steps (Figure 1). When tissue injury or neoplastic changes take place, the pre-existing vascular structure is disrupted. Angiogenesis is then initiated due to the cytokine secretion from monocytes, platelets, and fibroblasts (Bauer et al., 2005). Endothelial cells (ECs) which are a type of haematopoietic vascular stem cell are then recruited to the site of angiogenesis by growth factors such as vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and placental growth factor (PGF). Activated ECs then proliferate and produce outward sprouts in the direction of growth stimuli through the vascular basement membrane. Capillary sprout extension is then facilitated by adhesion molecules such as integrins that attach the cells to the surrounding extracellular matrix. Matrix metalloproteases (MMPs) are then released by the newly formed capillary tips thus dissolving the extracellular matrix (ECM) at the vascular front. Specialised ECs called ‘tip cells’ then form filopodia like structures that help in guiding the movement of extending vessels. These structures use specific receptors to respond to both attractive (eg: VEGF) as well as repulsive (eg: Sema3A) cues in their surroundings. Vascular loops and branching tube-like structures are then formed by interactions between cell-cell and

cell-ECM. Vessels are then stabilised by smooth muscle cells and pericytes forming a covering around the newly formed capillaries, new basement membrane is formed and finally blood flow takes place (Li et al., 2005).

During angiogenesis, pericytes are recruited by endothelial cells of newly formed vessels by secretion of PDGF-β. These cells with a prominent nucleus and several long processes are implicated in vasculogenesis by taking part in extracellular matrix modulation, paracrine signalling, and direct interaction with ECs. Pericytes are $CD146^{+}$ and $CD34^{-}$ and they might represent a subpopulation of mesenchymal stem cells (MSCs) in the bone marrow and aid in development of early capillary sprouts (Loibl et al., 2014). Pericytes are technically not necessary during the initial stages of vasculature development but they induce vessel maturation as well as regulate microvessel integrity, structure, and function (Blocki et al., 2013).

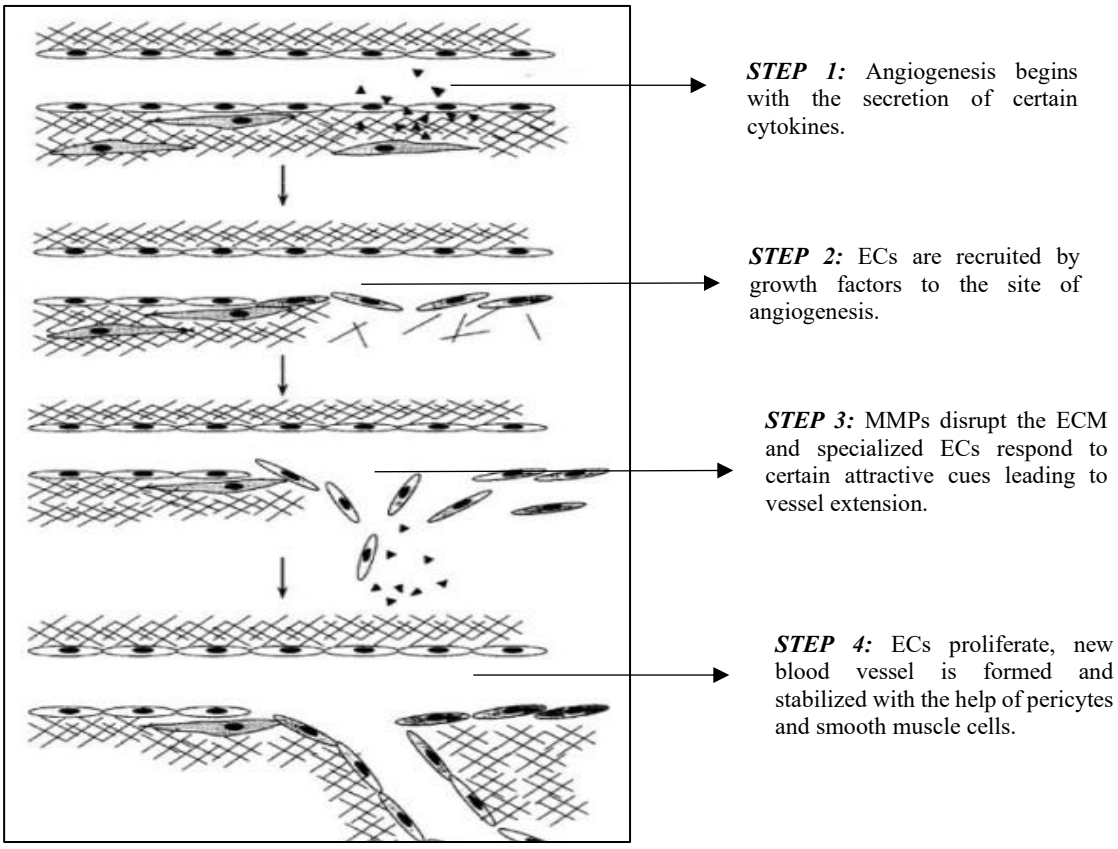


Figure 1. Schematic diagram of angiogenesis process. Angiogenesis starts with the release of certain angiogenic cytokines. Growth factors facilitate recruitment of endothelial cells (ECs). Activated ECs then produce capillary sprouts, which then release matrix metalloproteases (MMPs) that dissolve the basement membrane. Specialised ECs then take part in vessel extension by responding to certain cues. Finally, neovessel formation takes place (Adapted from Bauer et al., 2005).

Vasculogenesis was originally thought to take place only during foetal development but recent studies show that vasculogenesis also occurs in adults. In foetal vasculogenesis, endothelial progenitor cells (EPCs) known as angioblasts differentiate into ECs, thus forming a primitive vascular network. Postnatal vasculogenesis, on the other hand, is initiated by the differentiation of multipotent adult progenitor cells (MAPCs) into early EPCs (Figure 2).

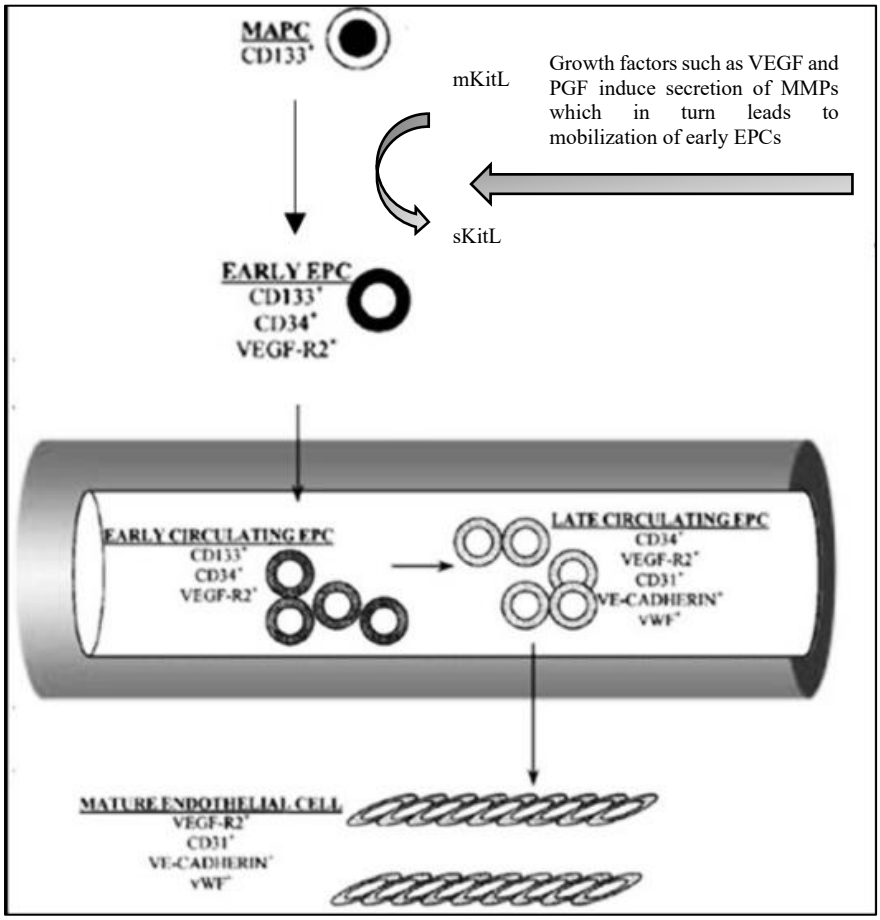


Figure 2. Diagrammatic illustration of steps involved in postnatal vasculogenesis Adult vasculogenesis starts with the differentiation of multipotent adult progenitor cells (MAPCs) into early EPCs. Growth factors such as VEGF and placental growth factor (PGF) then induce MMP secretion. This leads to conversion of membrane bound Kit ligand (mKitL) to soluble Kit ligand (sKitL), which in turn leads to mobilisation of early EPCs. These early EPCs in circulation then differentiate into late EPCs with specific EC markers. Late EPCs then further differentiate and give rise to mature ECs (Adapted from Bauer et al., 2005).

Post-natal vasculogenesis is either triggered by local ischemic conditions or is injury driven. It is believed to take place when MAPCs present in the peripheral blood or bone marrow differentiate into early EPCs. These early EPCs express specific haematopoietic cell surface markers such as CD34, CD133 and VEGFR2. EPCs are then mobilised from the bone marrow into circulation by means of cytokine mediated pathways such as VEGF and stromal cell derived factor 1 (SDF-1). Increased levels of these growth factors act as activators and attach to their specific cell receptors and subsequently lead to increased secretion of MMPs. MMPs then facilitate the conversion of membrane bound Kit ligand (mKitL) to soluble Kit ligand (sKitL). This entire process is what sets off the mobilisation of early EPCs into circulation. Later these early EPCs differentiate into late EPCs with specific EC markers such as CD34, CD31, VEGFR2. These late circulating EPCs then arrive at the site of neovessel formation, act as sources for proangiogenic soluble factors or further differentiate and develop into mature ECs (Bauer et al., 2005).

1.1.1 Angiogenesis and vasculogenesis as a therapeutic target

Abnormal or improper angiogenesis and vasculogenesis can lead to several diseases. Therefore, angiogenesis itself can be used as a diagnostic or prognostic indicator in clinical applications. For instance, impaired angiogenesis has been linked to conditions such as coronary artery disease (CAD), cardiovascular diseases, and compromised wound healing, while increased angiogenesis is often seen in inflammatory diseases, such as atherosclerosis, malignant tumors, and diabetic retinopathy (Pandya et al., 2006).

Angiogenesis and vasculogenesis are both indispensable processes for successful wound healing and tissue regeneration. Several diseases ranging from CAD to diabetic ulcers result in ischemia. Current treatment for these conditions ranges from invasive procedures such as stenting/surgery for CAD to palliative care for recurring diabetic wounds. Hence, there is a clear unmet clinical need for providing restoration of blood flow to ischemic areas through the generation of new blood vessels (Gianni-Barrera et al., 2020).

Angiogenesis also plays a key role in bone tissue engineering. Bone tissue has certain inherent regenerative properties; however, sufficient endothelialisation of constructs is necessary for successful bone healing. Vascularization of engineered constructs before transplantation would greatly help in producing functional tissue engineered grafts. Insufficient vascularization of these constructs is an ever-present medical challenge.

Several researchers have tried to show that implanting endothelial cells and pericytes into these constructs may provide a way to induce *de novo* vascular network formation (Jain et al., 2005).

Currently various methods by which vascularization of tissue engineered constructs can be improved are being studied by researchers. These methods include but are not limited to, modification of chemical composition of scaffolds, incorporation of proangiogenic cytokines at the site of implantation and seeding of constructs with vasculogenic cells (Laschke and Menger, 2012). This technique of cell seeding in particular can help augment vascularization by a few different mechanisms. Seeded cells can either stimulate angiogenesis by releasing certain proangiogenic growth factors or in the case of stem cells, they can differentiate into vascular cells capable of *de novo* vascularization. For instance, Schumann et al., have demonstrated that when both osteoblast-like cells and bone marrow derived mesenchymal stem cells were seeded onto poly(L-lactide-co-glycoid) (PLGA) scaffolds, accelerated vascularization took place in the scaffolds due to increased expression of VEGF (Schumann et al., 2009). Liu et al., have shown that when mouse models were implanted with co-cultured umbilical cord blood-derived EPC/MSC scaffold grafts, increased ectopic bone formation was observed *in vivo* (Liu et al., 2013). Despite all these findings, inadequate vascularization remains a challenge in the field of tissue engineering and regenerative medicine and finding solutions for this issue can help produce more clinically translatable constructs.

1.2 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent cells capable of differentiation into osteocytes, adipocytes, and chondrocytes both *in vitro* and *in vivo* (Oswald et al., 2004). These cells have been isolated from various tissue sources, such as adipose tissue, bone marrow, dental tissue pulp and umbilical cord blood (Ibraheim et al., 2017).
The golden standard source for MSCs is the bone marrow. Bone marrow derived MSCs are key players in blood vessel formation, stabilisation, and regulation (Watt et al., 2013).

Depending on the tissue they are isolated from, MSCs show variations in their differentiation capacity, immunophenotype and immunomodulatory activity. These differences confer different MSCs with specific characteristics and features (Guo et al.,

2020). There are certain prerequisites that MSCs should meet and these criteria are defined by the International Society for Cellular Therapy (ISCT). The first requirement is that MSCs show plastic adherence when cultured *in vitro*. Second, MSCs should express certain specific cell surface markers such as CD73, CD90, and CD105 and lack the expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR. Finally, MSCs should differentiate into three separate cell lineages (osteoblasts, chondrocytes, adipocytes) when cultured under specific conditions and supplemented appropriately (Hmadcha et al., 2020).

MSCs are a lucrative cell source within the field of tissue engineering and regenerative medicine. Researchers have tried to harness the mesodermal differentiation potential of MSCs for various indications such as autoimmune diseases, musculo-skeletal defects, and cardiovascular diseases (Gomez-Salazar et al., 2020). The therapeutic potential of MSCs is mainly facilitated by their innate ability to migrate towards the sites of injury. Transplanted MSCs produce certain paracrine soluble biomolecules such as cytokines and growth factors that promote angiogenesis, cell survival and tissue regeneration (Hmadcha et al., 2020). Several studies have shown that both myeloid cells and MSCs enhance the vasculogenic properties of endothelial colony forming cells (ECFCs) in *in vivo* as well as *in vitro* models, showing the formation of *de novo* vasculature network (Watt et al.,2013).

The exact mechanisms by which MSCs contribute to tissue repair during injury are not known. One hypothesis is that these cells play a key role in the recruitment of macrophages and fibroblasts to the site of injury by secreting certain paracrine growth factors such as VEGF, keratinocyte growth factor, insulin-like growth factor and angiopoietin-1. This in turn promotes angiogenesis and collagen production thus reducing the risk of scar formation (Ibraheim et al., 2017). MSCs further enhance differentiation of ECs via VEGF secretion. VEGF is an important growth factor that is explicitly implicated in the differentiation of ECs as well as blood vessel formation (Sunitha et al., 2019).

1.3 Peripheral blood-derived mononuclear cells

Peripheral blood-derived mononuclear cells (PB-MNCs) are a heterogeneous population of blood cells, such as lymphocytes, monocytes, natural killer cells, and dendritic cells. They are identified by their round nucleus and as their name indicates, can be isolated from peripheral blood. They are obtained by minimally invasive procedures and are hence an easy and accessible source. MNCs have been used by researchers for various purposes ranging from immunomodulatory studies to cytokine secretion studies (Kleiveland C.R., 2015). Their differentiation potential into multiple cell types, such as endothelial cells, cardiomyocytes, osteoblasts etc. makes them an attractive cell source in the field of regenerative medicine (Zhang and Huang, 2012).

1.3.1 Endothelial progenitor cells

In 1997, Asahara et al. provided a new insight that peripheral blood contains a cell population that is capable of differentiating into endothelial progenitor cells and since then EPCs derived from peripheral blood have been shown to be of various subtypes. These putative EPCs were isolated for the first time by means of magnetic bead separation based on surface antigens expressions.

EPCs are generally isolated either from *ex vivo/in vitro* culture of PB-MNCs or by direct flushing of bone marrow and then expanding the cells in endothelial cell culture conditions (George et al., 2011). The term EPC is not an all-encapsulating term and generally refers to any cell that can differentiate into an endothelial cell line (George et al., 2011). Studies have shown that EPCs are capable of promoting revascularisation and this makes them ideal candidates for cell-based therapy strategies for ischemic diseases (Medina et al., 2011).

In order to harness the full potential of EPCs for cellular therapy, appropriate validation of these cells and their secretome should be studied (Edwards et al., 2018). There is a lot of variation in the phenotypic markers used to identify EPCs. Based on the methodology, differences in expression of cell surface markers have been noted. EPCs that were quantified using flow cytometry techniques were shown to express CD34 and VEGFR2 (Medina et al., 2017). Since CD34 and VEGFR2 are also expressed by circulating endothelial cells isolated from vasculature, some research groups have proposed that these cells also express CD133 as an additional progenitor marker (Medina et al., 2017).

However, this remains a controversial opinion. There is contrasting evidence that $CD34^{+}CD133^{+}$ cells give rise to endothelial cells but also $CD34^{+}CD133^{+}VEGFR2^{+}$ cells have been shown to remain haemopoietic instead of giving rise to endothelium (Medina et al., 2017).

Interestingly, when EPCs were isolated using cell culture-based technologies, two distinct type of EPC subset populations with vasoreparative properties were observed (Medina et al., 2017). These two major classes of cells based on their phenotypic lineage are cells of haematopoietic lineage and cells of endothelial lineage (Table 1). Myeloid Angiogenic Cells (MACs) belong to the first class, while Endothelial Colony Forming Cells (ECFCs) belong to the latter class. Other cells that fall under the group of haematopoietic lineage are circulating angiogenic cells, early EPCs, early outgrowth EPCs, haematopoietic EPCs, small EPCs, and myeloid EPCs. On the other hand, outgrowth endothelial cells, blood outgrowth endothelial cells, endothelial outgrowth cells, late EPCs, late outgrowth EPCs, non-haematopoietic EPCs and large EPCs are of endothelial lineage (Medina et al., 2017).

Table 1. Cell types classified based on their phenotypic lineage (Adapted from Medina et al., 2017).

Haematopoietic Lineage	**Endothelial Lineage**
Myeloid Angiogenic Cells (MACs)	Endothelial Colony Forming Cells (ECFCs)
Circulating Angiogenic Cells (CACs)	Outgrowth Endothelial Cells (OECs)
Early EPCs	Blood Outgrowth Endothelial Cells (BOECs)
Early outgrowth EPCs	Late outgrowth EPCs
Myeloid EPCs	Late EPCs

Researchers have shown that EPCs that were first cultured *in vitro* and then transplanted into *ex vivo* models augmented the neovascularisation process even if these cells were not supplemented with any external proangiogenic factors (Asahara and Kawamoto, 2004). These types of findings help to propagate the idea that perhaps EPCs are capable of acting as a stable source of ECs and may be able to supplement pre-existing ECs in the vascular networks. In addition, genetic modification of these EPCs may be another avenue to look into for therapeutic purposes. Genetically altering EPCs to express and produce more angiogenic growth factors may even help address the limitations of EPC transplantation strategies (Asahara and Kawamoto,2004).

1.3.2 Myeloid angiogenic cells

Early EPCs are also known as circulating angiogenic cells (CACs). However, sufficient proof is not available to show that these CACs would exist *in vivo*. It has been suggested that CACs can be generated *in vitro* when PB-MNCs are cultured in endothelial cell culture conditions. These cultured cells are then termed as myeloid angiogenic cells (MACs). It is thought that MACs augment angiogenesis/tubulogenesis through a paracrine mode of action by secreting certain paracrine vasoactive biomolecules, while ECFCs boost both *in vitro* and *in vivo* tube formation capacity due to their intrinsic vasoreparative and vasculogenic potential. ECFCs act as foundational cells capable of promoting new vascular tube formation as well as aid in repair of vascular structures (Medina et al., 2017).

Medina et al., have also highlighted the distinction between MACs and ECFCs based on their cell surface markers. MACs are cells that are positive for CD45, CD14 and CD31 surface markers. They do not express CD146 and CD34 markers. ECFCs are those cells that express CD31, CD105 and CD146 markers but are negative for CD45 and CD14.

Sieveking et al. (2008) showed that early EPCs do not participate in *de novo* tubule formation however when these same early EPCs were co-cultured with endothelial cells and fibroblasts in a Transwell® setup, they were shown to stimulate the tubulogenesis process in a dose-dependent manner, suggesting that these cells have a paracrine effect.

It should be noted that MACs are neither endothelial nor progenitors in nature, they are simply monocytic, myeloid cells that are of haematopoietic origin and capable of stimulating angiogenesis (Medina et al., 2017).

1.4 Pericytes and endothelial cells

The entire blood vascular network is lined by a single thin layer of endothelial cells and a few scattered pericytes. Pericytes are the cells present on the basement membrane of endothelial cell tubes. These cells interact through transmembrane and perform two major functions: provide structural support and integrity to vascular structures as well as

maintain constant microvasculature blood flow (Ribatti et al., 2011). There is also an interplay between pericytes and endothelial cells that takes place.

Pericytes help in EC proliferation, migration, and stabilisation and in turn ECs aid in the activation of pericyte precursor population (Ribatti et al., 2011). These cells are key players during the process of angiogenesis and new capillary tubes are formed from existing endothelial cells by means of sprouting. This new sprout then hollows out to form tubular blood vessels. These capillaries then connect with other capillaries and anastomose thus facilitating blood circulation (Alberts et al., 2002).

Human umbilical vein endothelial cells (HUVECs) are endothelial cells isolated from the umbilical vein and are a widely used model to study angiogenesis and tube formation (Kocherova et al., 2019). These cells are readily available commercially, easy to sustain *in vitro* and proliferate well. HUVECs can be used in *in vitro* assays to mimic the body's vasculature network and to assess how soluble proangiogenic factors can affect the ability of these endothelial cells to form tubular structures.

1.5 Cell-cell communication in angiogenesis and vasculogenesis

Cell communication are of various types i.e., autocrine, endocrine, paracrine, and direct cell-cell contact (Herzog et al., 2014). Understanding the cellular crosstalk and molecular mechanisms behind angiogenesis will help to pinpoint which soluble factors and cells play a key role in vascularization process. Co-culture setups, in particular, are very useful for researchers as it helps us to study how cells communicate with each other based on soluble growth factors, cellular contact, and extracellular matrix components (Kirkpatrick et al., 2011).

Co-culture models have also been used to find *in vivo* strategies that can help enhance endothelial tube formation or aid in neovascularisation process. These type of synergistic models are in particular very useful in the field of bone tissue engineering, where production of a fully functional and vascularised bone tissue construct is a major issue. They can further also be extrapolated for other tissue engineering purposes.

In order to tackle the issue of insufficient vascularization of tissue engineered constructs, it is pertinent to understand how cells interact with one another and influence each other.

Cells can also be co-cultured using a permeable Transwell® insert, which allows diffusion of soluble factors secreted by the cells. Here, the insert contains a specific cell population while the well contains another population, thus, preserving the cellular polarity. Such insert co-culture systems, which do not allow direct cell-cell contact can help researchers to study what type of paracrine signaling takes places between two or more cell populations (Renaud and Martinoli, 2016).

Various cells have been cultured in a co-culture setup to study and understand the cellular mechanisms behind angiogenesis. Both MSCs and ECs have been heavily implicated as key players in neovascularisation. During angiogenesis, ECs give rise to primordial vascular plexus which are later remodelled into larger vessels (Lamalice et al., 2007). MSCs on the other hand are capable of differentiating into ECs which can take part in postnatal vascularization and secrete certain proangiogenic cytokines (Tao et al., 2016). MSCs have further also been shown to stabilize EC formed structures both *in vivo* and *in vitro* (Chen et al., 2018). Due to these reasons, MSCs and ECs are often investigated in co-culture models to understand how their interactions can help augment angiogenesis.

For instance, Bidarra et al. (2011) have shown that MSCs co-cultured with HUVECs show significant improvement in proliferation rates and increased osteogenic differentiation potential. Oki et al. (2018) demonstrated that when MSCs were co-cultured with HUVECs, lumen like structures were formed within 72 hours. They also showed that MSCs co-cultured with HUVECs produced tubular structures but HUVECs treated with VEGF alone did not produce any such vessels. Hyun Kim et al. (2013) showed that when $CD31^+$ cells isolated from peripheral blood were co-cultured with HUVECs they showed higher potential of tube formation compared to $CD31^-$ or MNC alone culture groups. Xu et al. (2020) showed that when MSCs and ECs are co-cultured in a Transwell® setup, the paracrine signalling between these two cell types has a significant effect on the upregulation and osteogenic differentiation potential of MSCs.

Several other studies have also shown that direct cellular contact with MSCs influence the formation of tubular structures by ECs. Similarly, when endothelial cells and pericytes are co-cultured, they very obviously interact with each other to promote and support

vessel assembly, growth control as well as normal microvasculature functions (Hirsch and D'amore, 1997; Peters, 2018). Additionally, both HUVECs and MSCs are suggested to secrete specific growth factors that can support vascularization. Piard et al. (2019) have recently shown that the media in which HUVECs and MSCs were cultured tended to promote tube formation, which is a later stage of angiogenesis.

1.6 Prior research using co-culture models of PB-MNCs and MSCs

Our lab has recently shown that spindle shaped EC-like cells are found in co-cultures of PB-MNCs and MSCs and these cells were further identified as MACs, capable of producing and enhancing formation of tube-like structures, when co-cultured with HUVECs in both 2D and 3D culture setups (Uusitalo-Kylmälä et al., 2021). However, in this study, the exact proangiogenic factors that mediated the formation of tube-like structures were not identified. Previously, our lab has also shown that when MSCs and MNCs were co-cultured and cultures were supplemented with exogenous VEGF, osteoblastic differentiation and bone formation by MSCs was improved (Joensuu et al., 2015).

These studies provide an insight that MSC-MNC co-cultures do produce an endothelial cell like population that is capable of enhancing neovascularisation and that MACs do function in a paracrine manner to promote tube formation *in vitro*. Since these studies already assessed the functionality of MACs when grown in direct co-culture setups, there was a potential to study how MACs interact with HUVECs when they were cultured in a Transwell® setup. Hence, this master's thesis project was carried out.

1.7 Aim of the project

This project can be divided into three parts. The first aim was to optimize the culture conditions for co-culturing MSCs and HUVECs. Then, we aimed to assess the cell-cell interactions and effect of MSCs on HUVECs to form tubular structures when cultured in a Transwell® setup. Finally, the major aim of this study was to test the functionality of MACs in a co-culture model with HUVECs.

Our lab has previously shown that when MACs and HUVECs are co-cultured in direct contact with each other, branching loops and tube-like structures similar to those seen during vasculogenesis are formed, suggesting that MACs are capable of supporting angiogenesis *in vitro*. This project therefore focused on studying if MACs secrete proangiogenic soluble factors, which will enhance tube formation, as well as to study if the MACs and HUVECs require cell-cell contact in order to secrete soluble factors.

We hence hypothesized that MACs in a Transwell® setup will interact with HUVECs and induce them to form tube-like structures. The aim was to evaluate if tube formation is dependent on cell-cell contact or paracrine growth factors secreted by the cell populations, or both. MACs with surface markers CD14 and CD31 were isolated from MSC-MNC co-cultures by magnetic-activated cell sorting and cultured in a Transwell® setup with HUVECs.

Our research is mainly significant from a tissue-engineering perspective. Although tissue engineering is a promising avenue in the field of tissue healing and regeneration, we are still lacking the final clinical breakthrough. A major challenge is the inadequate vascularisation of the engineered constructs leading to poor survival of cells and tissue necrosis. Understanding the cellular crosstalk and molecular mechanisms behind neovascularization will help in construction of clinically stable tissue engineered constructs embedded with a strong vascular network. These would avoid the problems of ischemic cell death due to insufficiently vascularised tissue constructs.

2 RESULTS

2.1 Endothelial Growth Medium (EGM) and fibronectin promote network formation in co-cultures of MSCs and HUVECs

In this part, we attempted to identify which culture conditions promote optimal tube-like structure formation when human umbilical vein endothelial cells (HUVECs) and mesenchymal stem cells (MSCs) are co-cultured. Our results show that when cells were cultured on plastic (i.e., not on fibronectin coated plates) or in Endothelial Growth Medium 2 (EGM 2), poor growth of cells, as well as minimal tube formation was

observed (Fig 3A, 3C and 3D). However, when HUVECs were co-cultured with MSCs in Endothelial Growth Medium (EGM) and in the presence of fibronectin coating, clear tube formation was seen (Fig 3B). The cells also survived well even after a week of culturing. By quantification, we noticed that the culture media used and fibronectin coating made a clear difference in the number of tubes formed (Fig 4A), the length of tubes (Fig 4B) as well as number of branchpoints of the network (Fig 4C).

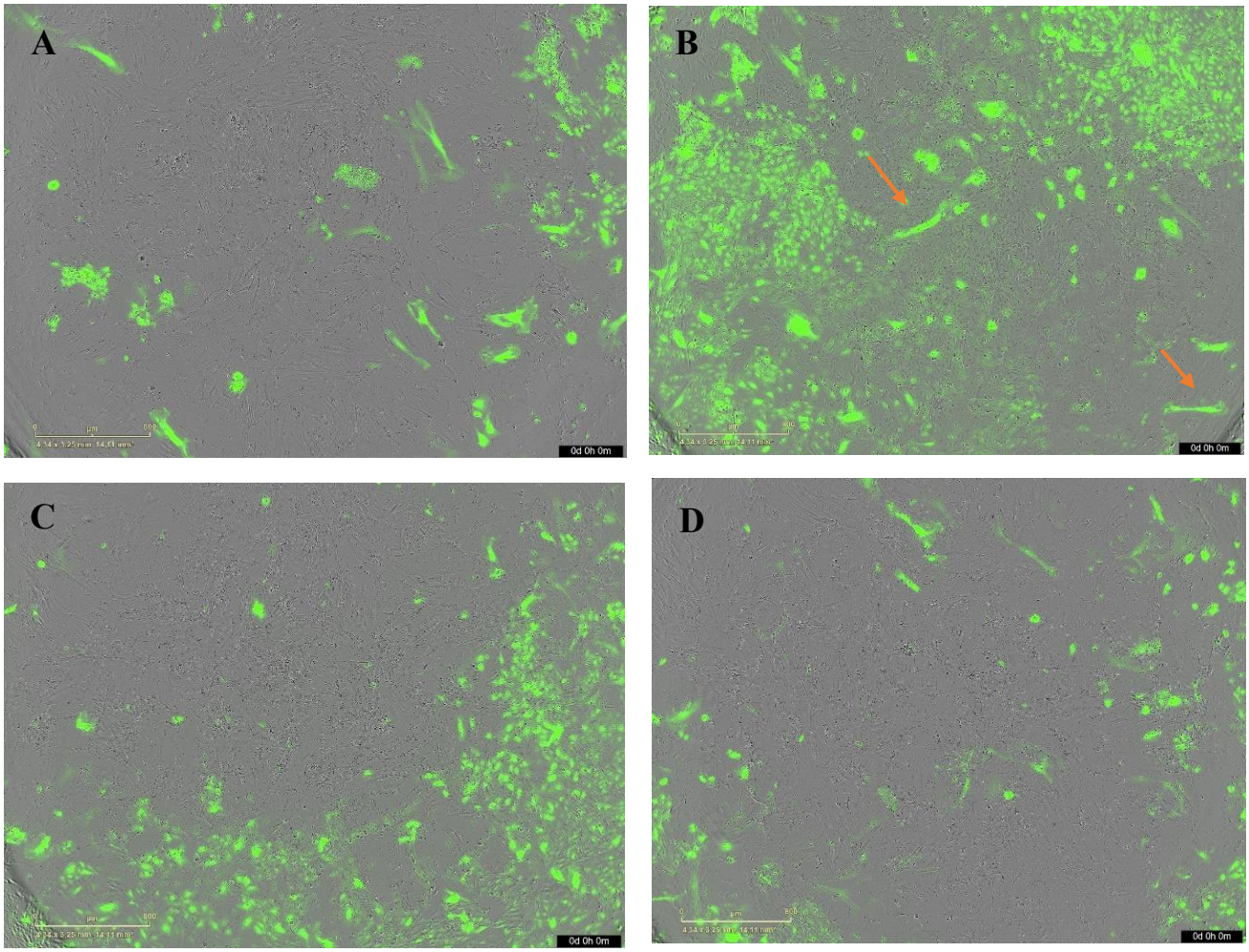


Figure 3. Images acquired from IncuCyte® S3 after a week of co-culturing MSCs and HUVECs in a 96-well culture plate with different culture setups. A: MSC and HUVEC cultured in EGM without fibronectin; B: MSC and HUVEC cultured in EGM with fibronectin; C: MSC and HUVEC cultured in EGM 2 without fibronectin and D: MSC and HUVEC cultured in EGM 2 with fibronectin. Red arrows indicate formation of tube-like structures.

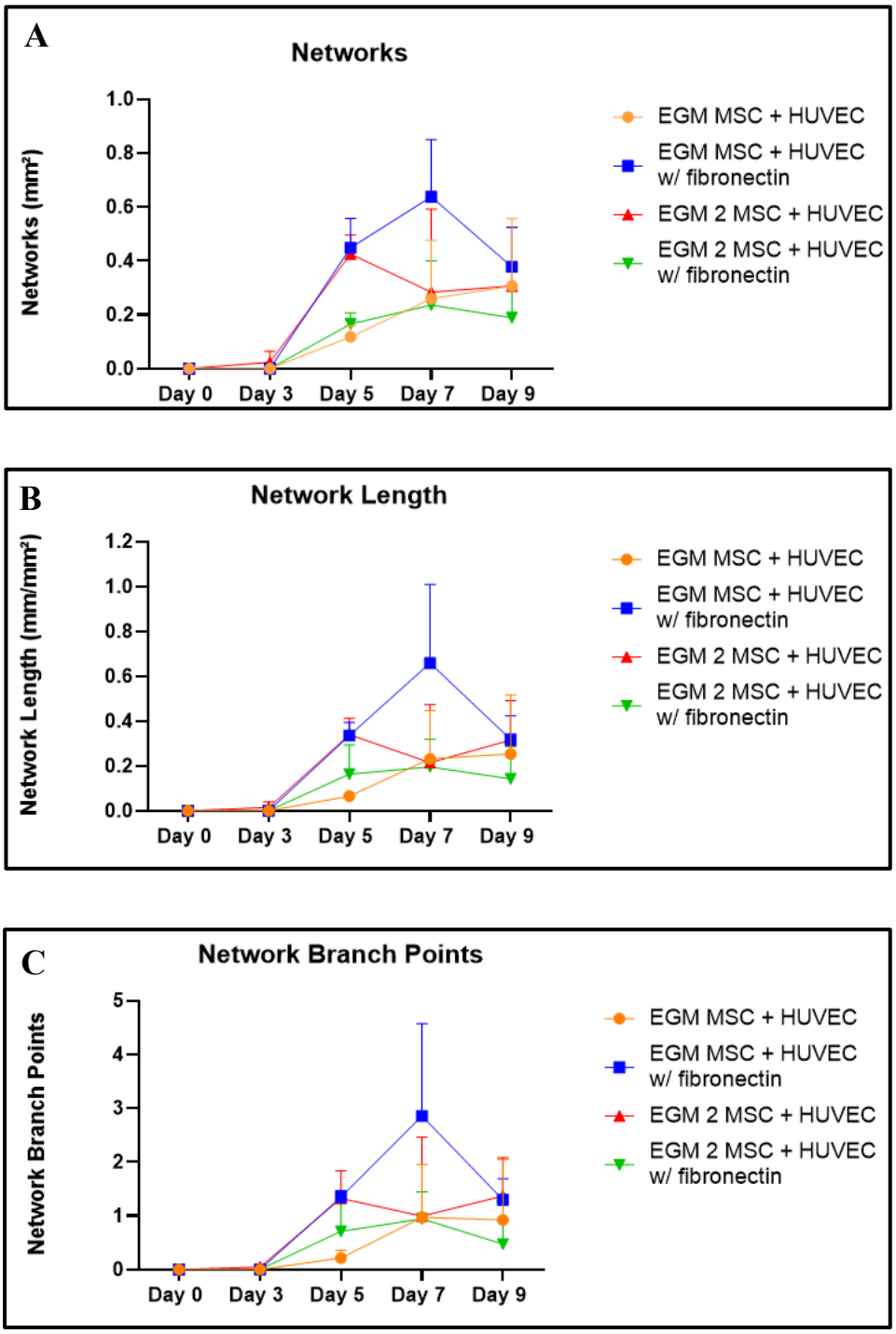


Figure 4. Quantification of HUVEC networks using numerical data obtained from IncuCyte® Angiogenesis Analysis Software. HUVECs co-cultured with MSCs in EGM with fibronectin coating show the greatest number of networks (A) as well as the highest network length (B) and network branch points (C).

2.2 MSCs and HUVECs form tube-like structures when cultured in Transwell® setup

Results from MSCs and HUVECs co-cultured in Transwell® setup were quite similar to the previous optimisation experiment. Experimental groups which contained HUVECs in the well and MSCs in the Transwell® showed increased network formation per mm^2 (Fig 5A) and increased network length per mm/mm^2 (Fig 5B). However, maximal network branch points were seen when HUVECs were cultured with both MSCs and HUVECs in the cell culture insert (Fig 5C).

Further imaging with both normal light microscope (Fig 6) and multichannel fluorescence microscope (Fig 7) showed morphological changes and both short as well as elongated tubular structures. HUVECs cultured alone were used as control and presence of few elongated tube-like structures were observed in this group (Fig 6A and 7A). Microscopy imaging showed that when MSCs and HUVECs were co-cultured, elongated tubes were formed (Fig 6 B and 7B). When the experimental groups containing HUVECs in the well and MSCs in the cell culture insert (Fig 6C and 6D) as well as HUVECs with both MSCs and HUVECs in the cell culture insert (Fig 7C and 7D) were observed under the microscope, the presence of both short, as well as elongated and branched, tube-like structures were seen. Experiments were done twice and the microscopy images are from the first set of experiment, while the quantification results are from the second set, since there were technical issues with IncuCyte® imaging in the first experiment and the obtained images were not clear enough for analyses.

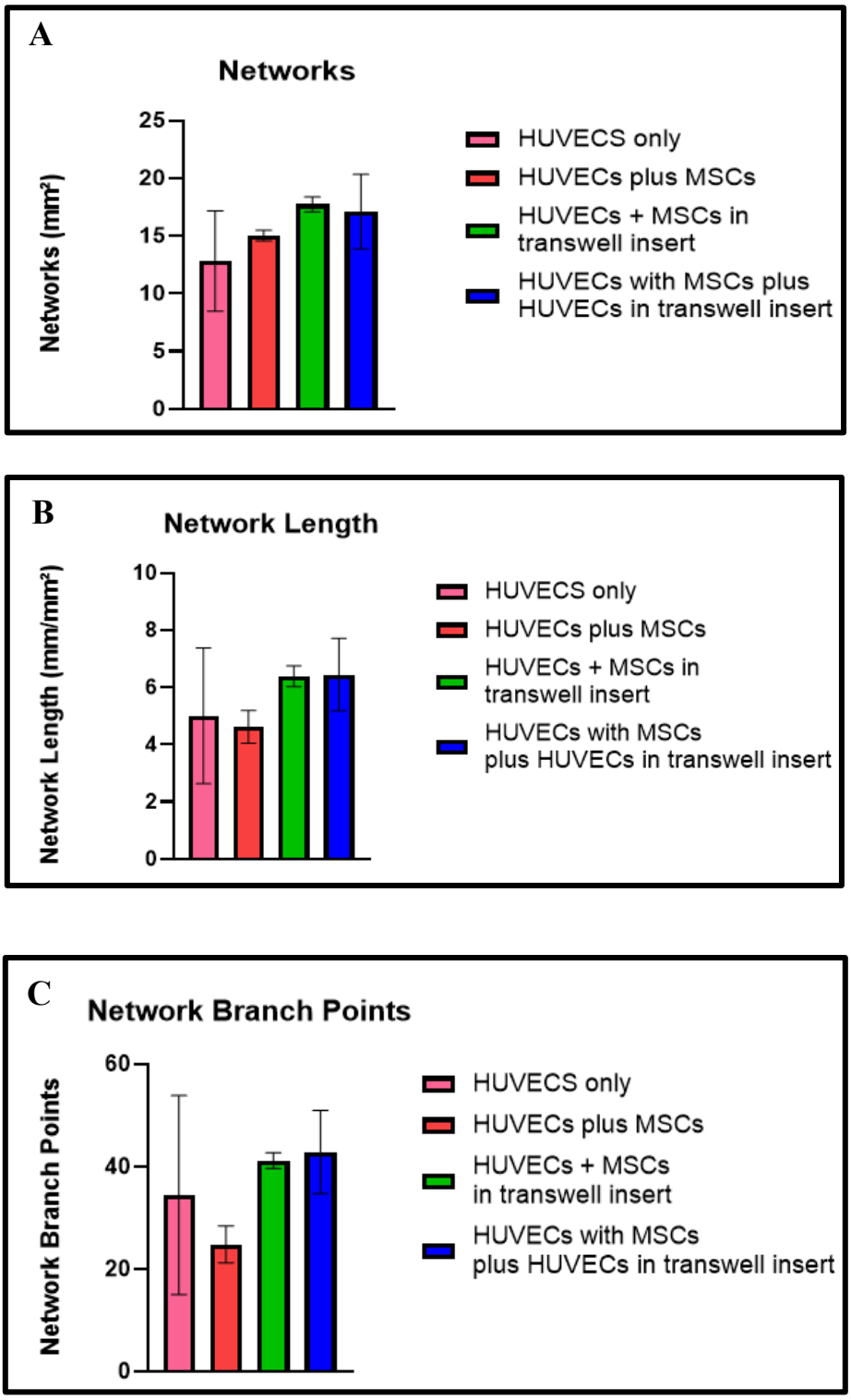


Figure 5. Quantification of HUVEC networks on day 7 from experiments where MSCs and HUVECs were co-cultured in Transwell®. When cells were co-cultured in the insert (blue bars), trends for better network formation per mm^2, network length per mm/mm^2 and network branch points were seen.

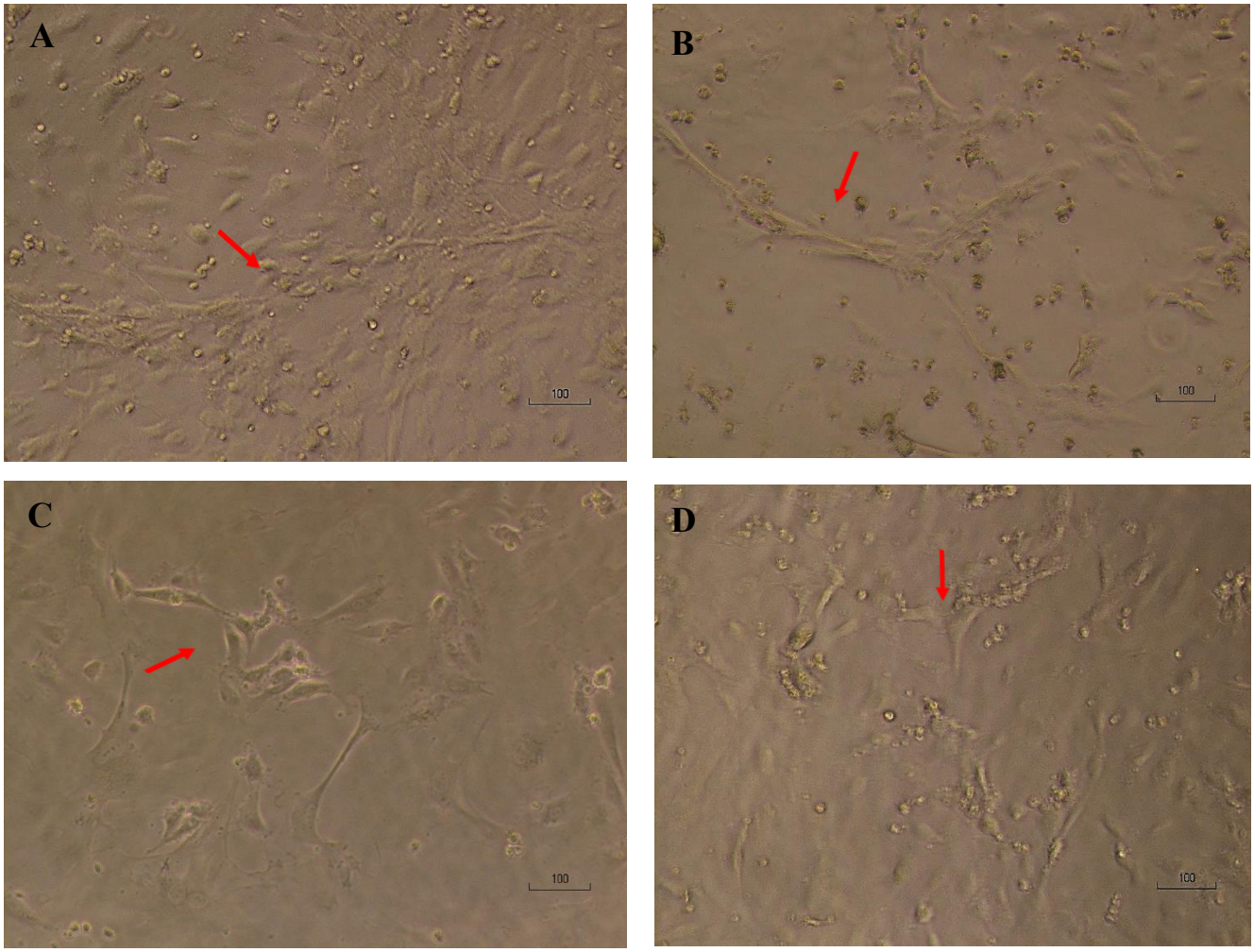


Figure 6. Images acquired from light microscope showing formation of tube like structures by the co-cultured cells. A: HUVECs cultured alone; B: HUVECs and MSCs cultured together; C: HUVECs in the bottom of the well and MSCs alone in the Transwell® insert; D: HUVECs in the bottom of the well and HUVEC plus MSCs together in the Transwell® insert. Red arrows indicate the tube-like structures. Magnification 10x.

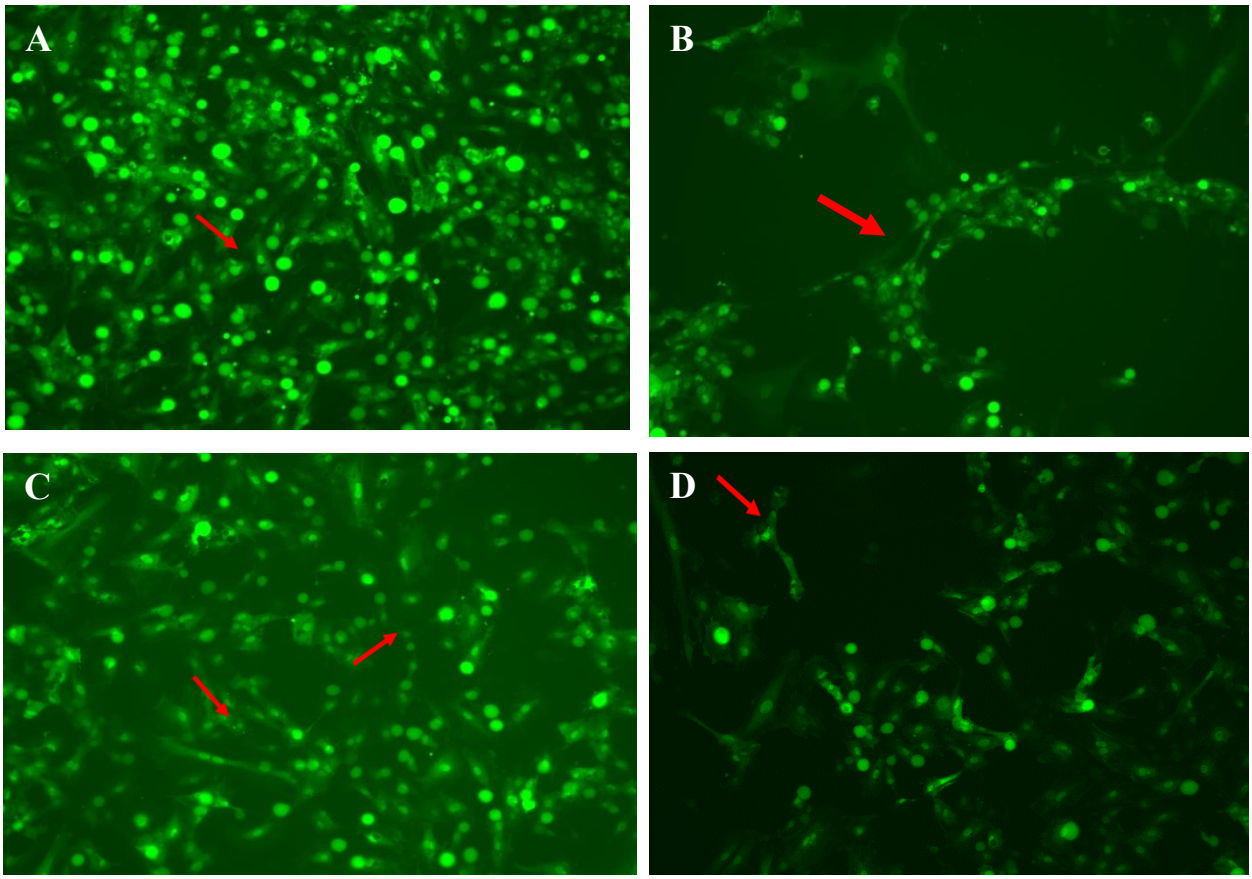


Figure 7. Images acquired from multichannel fluorescence microscope showing formation of elongated tubes (red arrows) by the co-cultured cells. A: HUVECs cultured alone; B: HUVECs and MSCs cultured together; C: HUVECs in the bottom of the well and MSCs alone in the Transwell insert; D: HUVECs in the bottom of the well and HUVEC plus MSCs together in the Transwell insert. Magnification 10x.

2.3 MACs and HUVECs co-cultured in Transwell® setup give rise to looping and branching tube-like structures similar to those seen in *de novo* vascularization

Upon co-culturing Myeloid Angiogenic Cells (MACs) isolated from MSC-MNC cultures with HUVECs for up to 14 days, we observed the formation of circular loops and branching tubular structures that resemble those seen in neovascularisation processes (Fig 8 and 9). Experiments were carried out in triplicates to minimise variation. The microscopy images in (Fig 8 and 9) are from the third experiment. Quantificiation of the tubular structures was done by using data from the IncuCyte® S3 Angiogenesis module. Variations in the time points that were used to quantify data are due to the fact that it was not always possible to consistently obtain good images with the IncuCyte®. Quantification results shown below (Fig 10 and 11) are from the first and third experiment. Images and quantification data from the second experiment are omitted here as there were issues with MACs isolation, which in turn led to very poor and inconsitent results.

In the first attempt, the most number of networks per mm^2 and network length per mm/mm^2 were seen when HUVECs were co-cultured with MACs and HUVECs in a Transwell® insert (Fig 10A and 10B). Results from the first set of experiments also show that network formation peaks around day 4 for all groups. This may be due to the secretion of certain paracrine growth factors by the cells. In the third experiment, maximal network formation per mm^2 and network length per mm/mm^2 were seen when HUVECs were co-cultured with only MACs in the insert (Fig 11A and 11B). This indicates that MACs could secrete some soluble proangiogenic factors that promote tube formation.

However, in both the first and third experiments, not much difference was seen between the groups when the number of network branch points were compared (Fig 10C and 11C). HUVECs cultured alone and HUVECs cultured with MACs without inserts were used as control. No tube-like structures were seen when the HUVECs only group was observed under the microscope, although, few circular loop like structures were seen when the HUVECs cultured with MACs without inserts were imaged using a light microscope (Fig 9 B).

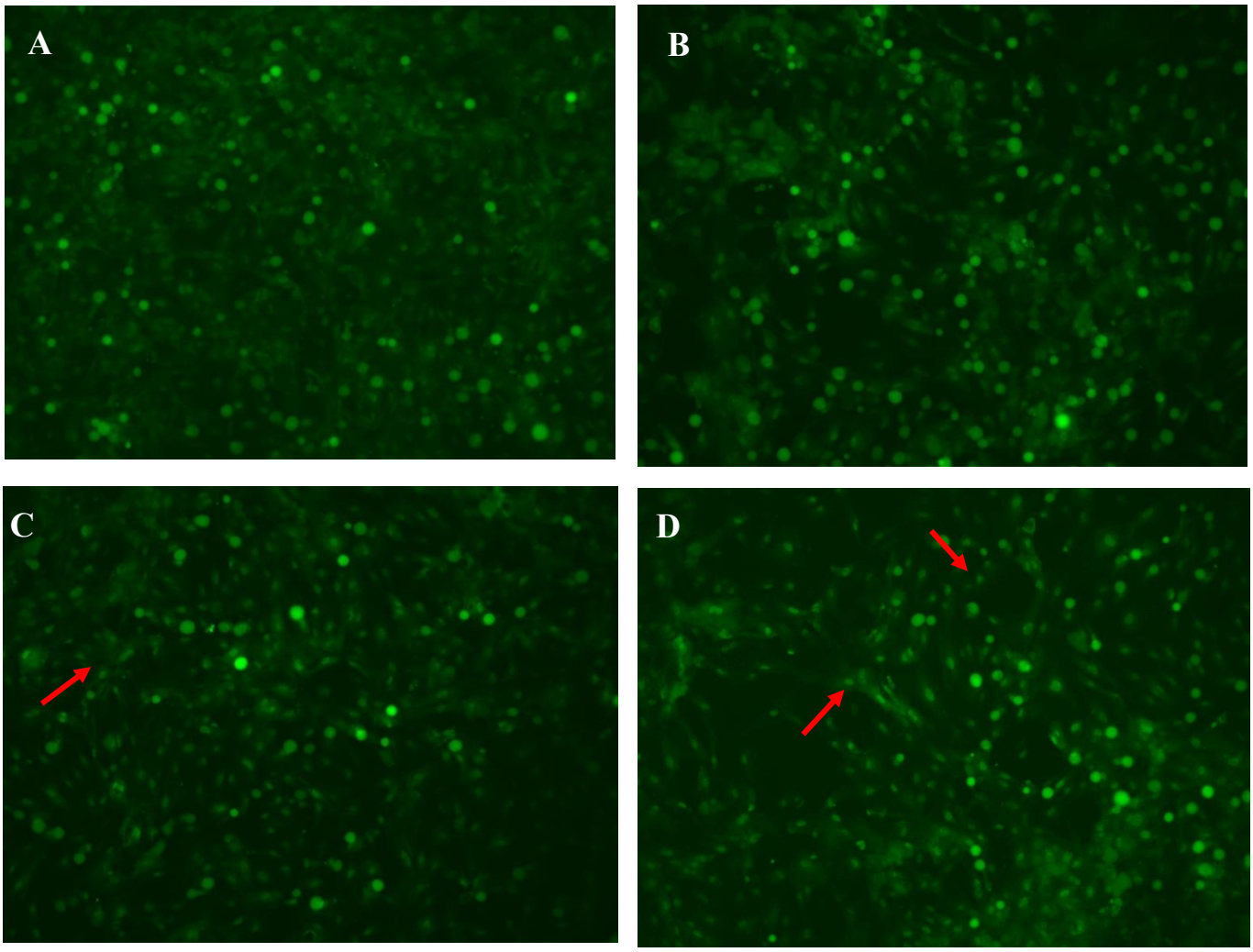


Figure 8. Images acquired by multichannel fluorescence microscopy showing formation of elongated tubes (red arrows) by the co-cultured cells. A: HUVECs cultured alone; B: HUVECs and MACs cultured together; C: HUVECs in the bottom of the well and MACs alone in the Transwell® insert; D: HUVECs in the bottom of the well and HUVEC plus MACs together in the Transwell® insert. HUVECs cultured alone and HUVECs cultured with MACs without inserts were used as control. Magnification 10x.

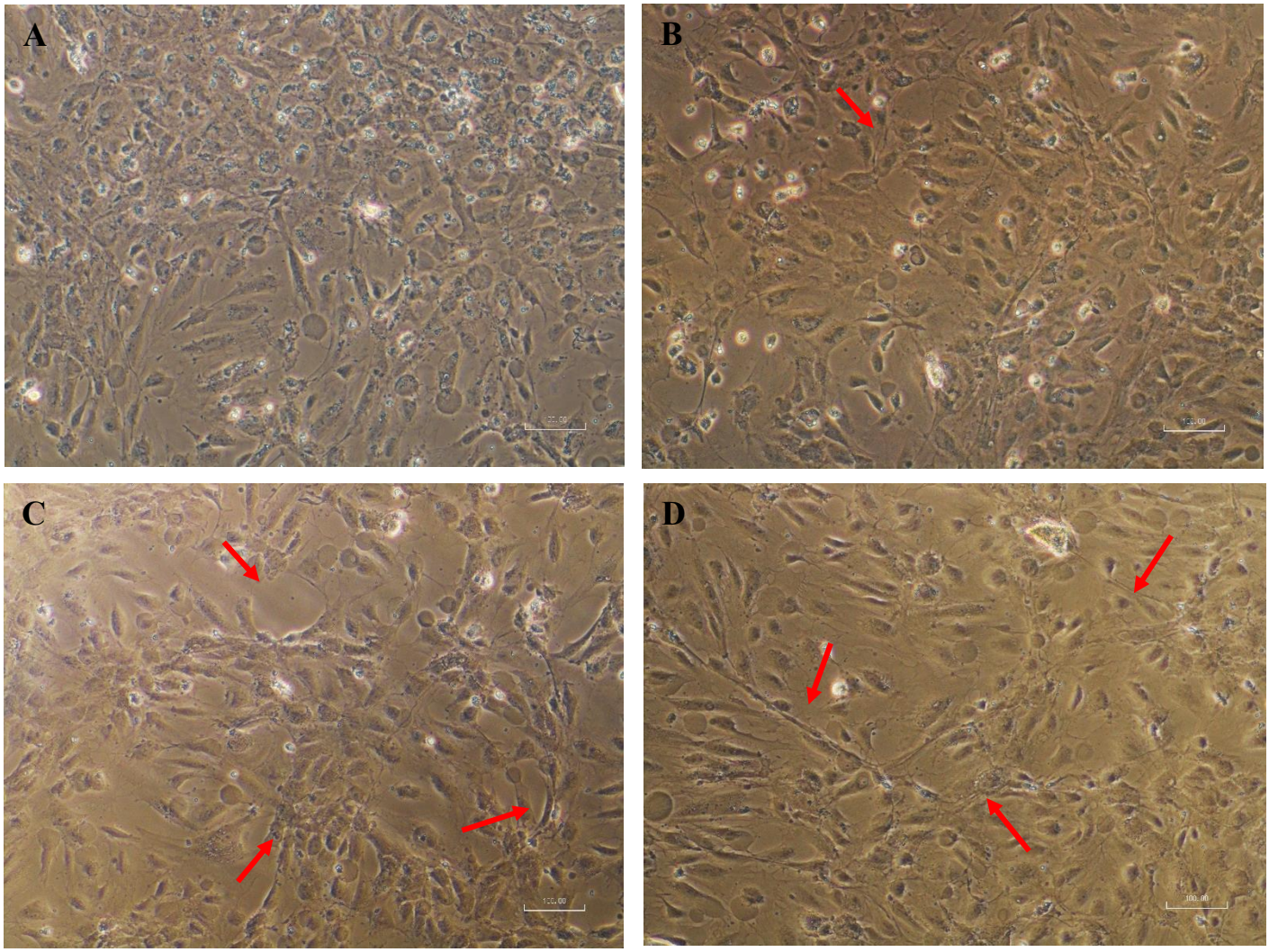
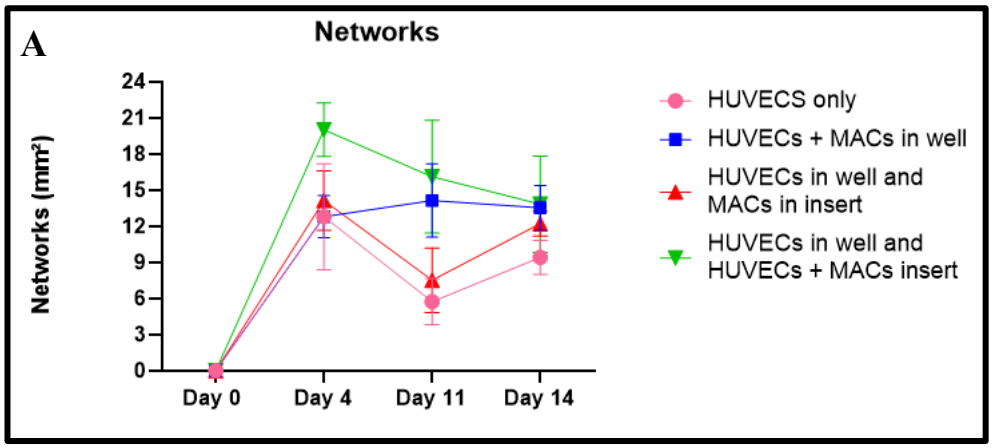
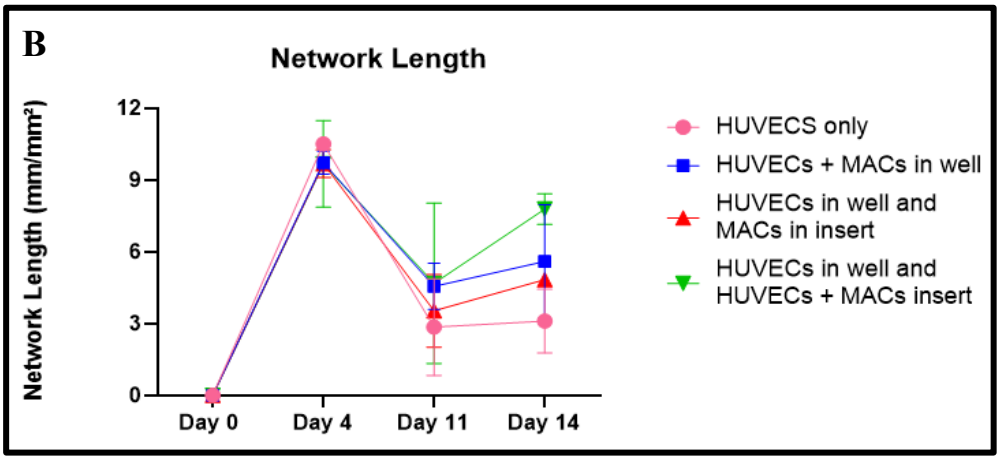


Figure 9. Images acquired by light microscopy showing formation of circular loops and branching elongated tubes (red arrows) by the co-cultured cells. A: HUVECs cultured alone; B: HUVECs and MACs cultured together; C: HUVECs in the bottom of the well and MACs alone in the Transwell® insert; D: HUVECs in the bottom of the well and HUVEC plus MACs together in the Transwell® insert. HUVECs cultured alone and HUVECs cultured with MACs without inserts were used as control. Magnification 10x.





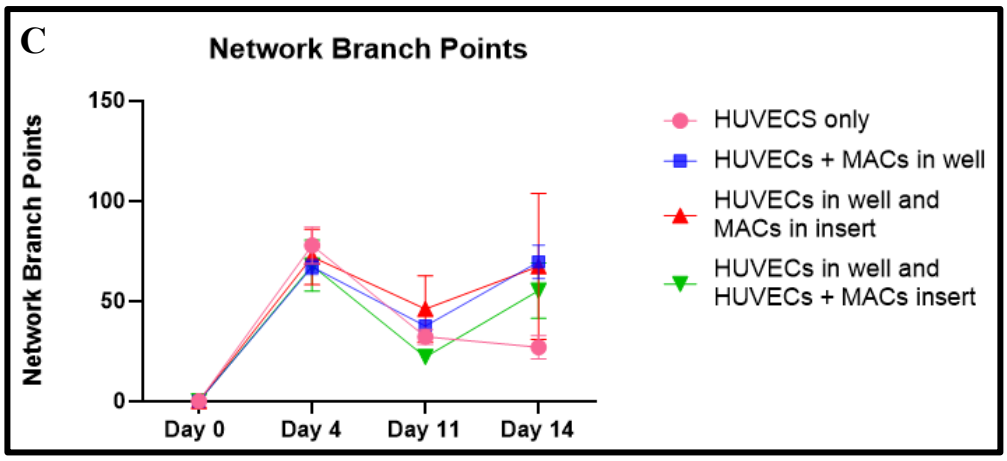


Figure 10. Quantification results from the first experiment of MACs and HUVECs co-cultured in Transwell. Network formation seems to peak at day 4 for all groups. However, increased networks per mm^2 and increased network lengths per mm/mm^2 at all time points are the highest, when HUVECs were co-cultured with MACs + HUVECs in the insert. No significant difference is seen between the groups when network branch points are considered.

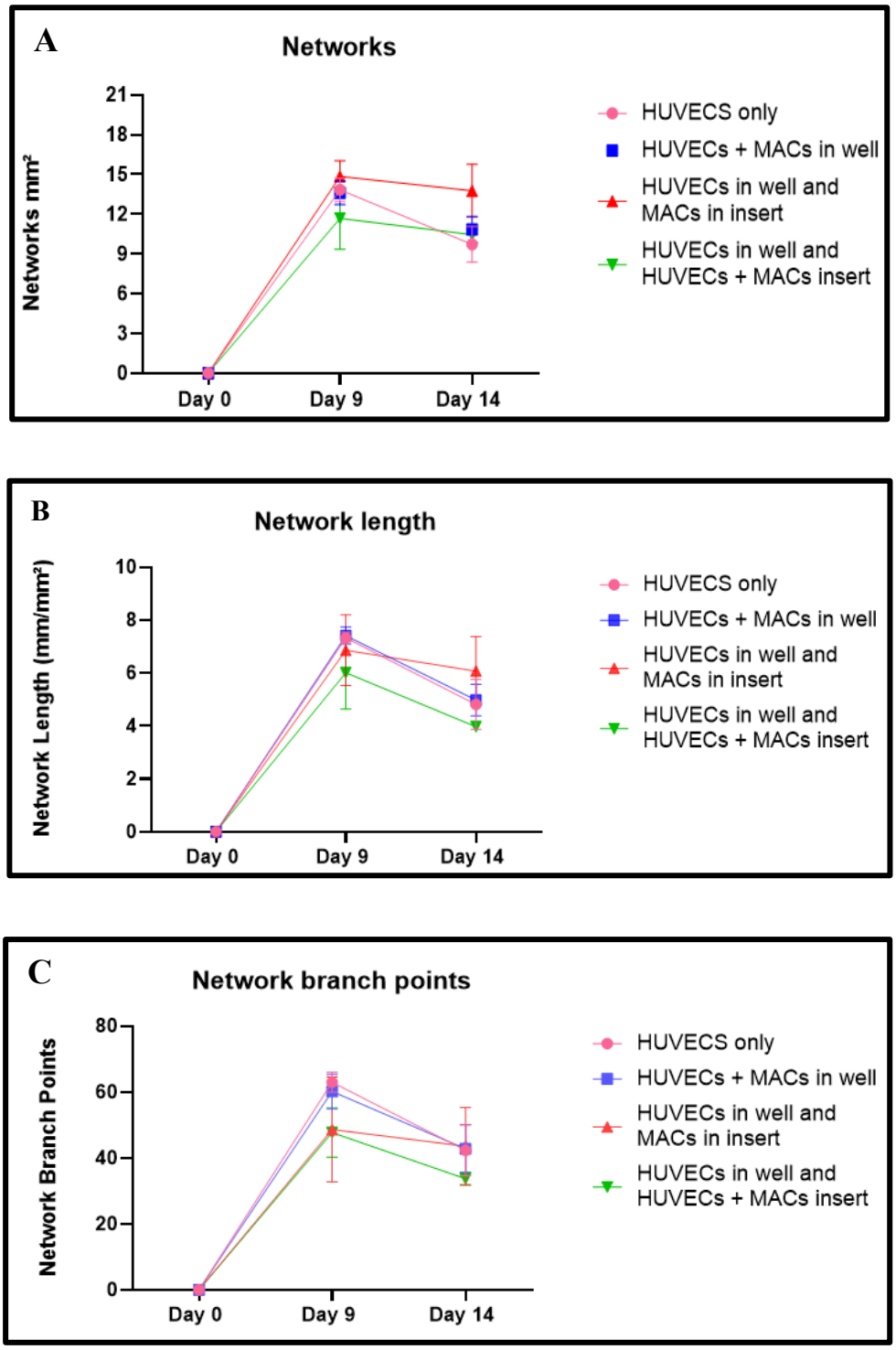


Figure 11. Quantification results from the third experiment of MACs and HUVECs co-cultured in Transwell®. Increased networks per mm^2 and increased network lengths per mm/mm^2 at all time points are highest when HUVECs were co-cultured with only MACs in the insert. No significant difference is seen between the groups when network branch points are considered.

3 DISCUSSION

3.1 Culture conditions influence cell growth and formation of tube-like structures

In this project, culture conditions for co-cultures of HUVECs and MSCs were optimised using EGM or EGM 2 media and cells were grown on either non-coated wells or on wells coated with fibronectin. Cell adhesion, proliferation and formation of tube-like structures were used as parameters to assess cell viability and survival in the different conditions tested.

Fibronectin is an ubiquitous high molecular weight glycoprotein commonly found in ECM. It plays a major role in attaching cells to the ECM and mobilising them during embryonic development and wound healing and is pertinent for the development of vascular structures. Researchers have shown that coating of culture plates with connective proteins increases the potential of MSCs to adhere, expand and spread in *in vitro* cultures (Yeo & Weiss, 2019). Relou et al., have also shown that HUVECs show better EC proliferation when cultured on plates coated with fibronectin, collagen, gelatin and hyaluronan compared to cultures on non-coated surfaces. Our results are in alignment with this theory and a clear trend of better cell survival and tube-like structure formation was seen when MSCs and HUVECs were co-cultured on fibronectin coated plates.

Endothelial growth media (EGM) and endothelial growth media 2 (EGM 2) are both low serum culture media with endothelial growth supplement factors. The major difference between the two is that EGM 2 contains VEGF and insulin-like growth factor while EGM lacks these but contains endothelial cell growth supplement extracted from bovine hypothalamus. Researchers have shown that VEGF secreted by MSCs increases angiogenic sprouting of HUVECs (Beckermann et al., 2008). The results from our optimisation experiments however show that the cells grow better and HUVECs have better tube formation capacity, when cultured in EGM alone with fibronectin coating. This observation is a little perplexing, since it is a well known fact that VEGF promotes angiogenesis, while, in our experiments, when the cells were co-cultured in EGM 2 containing VEGF, no significant tubes were formed nor was the cell proliferation high. These results were extrapolated for the other experiments in this project and good cellular viability was seen when primary cells of our interest were co-cultured with HUVECs in EGM on fibronectin coated wells. However, no clear evidence behind why the cells prefer EGM to EGM 2 was found.

Furthermore, no statistically significant differences between the groups was seen when tube formation and network parameters were quantified, despite the fact that we observed clear morphological evidence that cells cultured in EGM with fibronectin coated plates survived better. This indicates that although these culture conditions promote cell viability, they may perhaps not have that much effect on the proangiogenic capacity of ECs and on the capacity of MSCs to support angiogenesis. Going forward, culture conditions could be slightly modified by the addition of exogenous cytokines or growth factors to see if these affect or promote better tubulogenesis when MSCs and HUVECs are co-cultured.

3.2 MSCs and HUVECs cultured in Transwell® setup give rise to tube like structures

During this project, we aimed to see if MSCs and HUVECs interact with each other in a paracrine manner to induce formation of tubular structures as seen in *de novo* vascularisation. In order to assess this, we cultured MSCs in a Transwell® cell culture insert. These inserts are porous membranes that facilitate cell-cell interaction and exchange of soluble bioactive molecules between the cells.

Researchers have shown that MSCs can act as a source of VEGF and the presence of VEGF, a positive regulator of angiogenesis, has been observed in cultures of human MSCs (Kagiwada et al., 2008). Others have shown that when MSCs were co-cultured with HUVECs without the presence of any ECM proteins, expression of the VEGF-A gene was greatly enhanced compared to monoculture groups (Oki et al., 2018). They further also showed that when HUVECs and MSCs were co-cultured without any ECM proteins, lumen-like structures were observed after just 72 hours of culturing. These studies indicate that MSCs play a profound role in enhancing the angiogenic capacity of HUVECs to form tubular structures. Based on this existing evidence, we wanted to see if MSCs can still influence HUVECs to form these lumen like structures, when they were not grown in direct contact with each other.

In our experiments, we plated HUVECs with the MSCs in a Transwell® setup. Here, we tried to see if MSCs can still influence HUVECs to form tubular structures by releasing any soluble, paracrine pro-angiogenic factors. Results show that MSCs are capable of influencing HUVECs to form tube like structures, even if they are cultured in a Transwell® setup where they are not in direct contact with the endothelial cells.

Unfortunately, no statistically significant differences were found between the groups when the number of networks, network length and network branch point parameters were quantified. Further, our HUVECs were also plated onto culture wells coated with fibronectin. When MSCs were co-cultured with HUVECs in the cell culture insert and HUVECs at the bottom of the well, slight increases in network length and network branch points were noted. The reason behind this is not fully known, but we can at least conclude that MSCs do secrete certain paracrine factors that facilitate this tube formation. Whether this certain factor is VEGF or something else was not elucidated in this project and further assays like RT-PCR and ELISA need to be conducted to investigate the molecular mechanisms behind this phenomenon.

3.3 Functionality of MACs using *in vitro* Transwell® co-culture models

The central aim of this project was to test the functionality of MACs when co-cultured with HUVECs in a Transwell® setup. To achieve this, PB-MNCs were first co-cultured with MSCs and later our cells of interest, i.e., MACs expressing the CD14 and CD31 markers were isolated by means of magnetic-activated cell sorting. These MACs were then placed in cell culture inserts and cultured with HUVECs. Our lab has previously observed that when MNCs and MSCs are co-cultured, EC-like, spindle shaped cells positive for CD31, CD14 and CD45 surface markers are formed. These monocytic cells were assumed to be the MAC population and both 2D and 3D co-cultures with HUVECs were done to check if they can support angiogenesis *in vitro*. The results of these experiments showed that MACs are indeed proangiogenic in nature and induce looping and branching structures in HUVECs when co-cultured (Uusitalo-Kylmälä et al., 2021). Based on these previous observations, we hypothesized that MACs with HUVECs in a Transwell® setup interact to form tube-like angiogenic structures and that this tube formation is either affected by the cell-cell contact and secreted paracrine growth factors or both.

Results from the experiments show quite conflicting data. In the first set of experiments, the most number of networks per mm^2 and network length per mm/mm^2 were seen when HUVECs were co-cultured with MACs and HUVECs in a Transwell® insert. Data from the first set of experiments also showed that network formation peaked around day 4 for all groups. This raises the question whether secretion of paracrine factors peaks during the early stages of co-culture and declines in the following days.

During the second attempt, extremely inconsistent results were seen. The highest number of networks per mm^2 were seen when HUVECs were co-cultured with MACs and HUVECs in a Transwell® insert. However, increased number of network branch points and network length was seen in the control group where HUVECs and MACs were co-cultured with no cell culture inserts. In the final attempt, maximal network formation per mm^2 and network length per mm/mm^2 were seen when HUVECs were co-cultured with only MACs in the insert. When network branch points were considered, no real difference between the control groups and experimental groups were observed in any of the three experiments.

Furthermore, no statistical difference was found between any of the groups despite the fact that clear morphological evidence of looping and branching structures were seen when these cells were microscopically evaluated. In conclusion, our hypotheses that MACs interact with HUVECs in a Transwell® setup to produce tubular structures as seen in *de novo* vascularisation was proved right to a certain extent. However, the exact mechanisms by which these cells interact still remains unknown. These results also do not help to determine if culturing these cells in a Transwell® setup boosts their proangiogenic capabalities compared to when they're grown in a conventional direct co-culture model.

3.4 Limitations of this study

This project has quite a few limitations and falls short on several fronts. Firstly, the cells used for the entirety of this study are primary cells. Primary cells by nature are quite heterogenous and function a little different from culture to culture. Variations are also due to methodological differences that occur during isolation of MACs from MSC-MNC co-cultures. Also personnel inexperience and technical variations can further amplify these differences. The number of MACs that were isolated also varied from experiment to experiment. For instance, during the second isolation procedure, quite low number of MACs were obtained despite following the exact protocol. This in turn gave very poor and confusing results, when these MACs were plated with HUVECs in Transwell® setup. This issue could probably be sorted if the procedures were conducted by a more experienced researcher. Next, cells were cultured for upto 14 days in the IncuCyte®. This is quite a long time period and although cells did not die, the number of viable cells decreased over time in all the groups. However, reducing the number of culture days is not helpful, as the formation of tubular structures only takes places around day 10.

Maybe adding exogenous growth factors could help in improving cell viability; however, this was not ideal or recommended here, since the main aim of this project was to see if MACs release any proangiogenic factors of their own to influence the formation of tubes. Nevertheless, it is wise to consider if better tube formation could have been observed if the cells remained viable for a prolonged period of time. The first time these cells were placed in the IncuCyte®, we noticed cells dying at quite an alarming rate. We later found out that this was because the IncuCyte® is a shared imaging instrument and increased number of people kept on opening and closing the apparatus, thus causing the media to evaporate and altering the required cell culture conditions. This issue was resolved by adding PBS to the empty wells and normal cell growth was observed in the future experiments.

In this project, we placed the culture plates in the IncuCyte® for the entire 14 day period. Endothelial cells do not form any visible tubular structures for the first one week of culturing and thus Incucyte® could be utilized only during the last week of culture. Nevertheless, by this approach any potential changes happening prior to this timeframe, would be missed. Although numerical data does show that network formation and network length peaks around day 4, this was not visibly seen when the IncuCyte® acquired automated images of the plate. Placing the plate in the IncuCyte® for so long also deteriorates the cell health since constant opening and closing of the IncuCyte® door affects cells in a negative manner.

Futhermore, primary cells are very sensitive cells and ensuring their survival is quite difficult in a monoculture let alone in a co-culture model. MACs isolation from the MSC-MNC co-culture is also quite a time consuming process and these cells need to be freshly isolated before every experiment. These cells are non-proliferative and do not survive freezing. These properties make them not very easy to work with. Obtaining consistent *in vitro* results with experiments conducted using these cells is quite a challenge and requires careful planning and execution. Finally, in this study we did not investigate what proangiogenic factors were released by the MACs to induce tube formation in co-culture models, but it will be the topic of future studies. Pinpointing these factors will provide more substantial insights into the cellular crosstalk that takes place during the angiogenesis process.

3.5 Future directions for studying angiogenesis using MACs and co-culture models

Going forward, a few changes can be done when using this Transwell set up for co-culture models. Firstly, finding an alternative way to image the co-cultured cells instead of using the IncuCyte® real time imaging system could help in obtaining more consistent results. In case the, image acquistion and tube formation parameters are done using the IncuCyte®, the cells could be cultured in a normal incubator for the first week and then transferred to the IncuCyte®. Second, in this project, we never tested if MACs cultured alone are capable of forming tube-like structures on their own. Future studies should at least include this as a control group to assess the true tube forming capacity of these monocytic cells. Finally, media in which the cells were cultured was collected during this project at various time points. However, due to time constraints we were not able to run ELISA assays using this conditioned media. Kwon et al., (2014) have already been able to show that conditioned media obtained from human MSC cultures contain several growth factors such as VEGF, hepatocyte growth factor (HGF), monocyte chemotactic protein-1 (MCP-1), interleukin-6 (IL-6), and transforming growth factor-beta1 (TGF-β1), which augment the angiogenic potential of endothelial cells *in vitro*.

In the future, conditioned media used to culture the cells should be tested to elucidate, which proangiogenic factors are secreted by the cells when they are cultured in a Transwell® setup with no direct cell-cell contact. Understanding which molecules play a key role in the angiogenesis process can help in pinpointing the key regulators of neovascularisation. Furthermore, once the cytokines and growth factors are known, cells could be modified by gene expression technologies and upregulation of the corresponding gene may lead to enhanced vascularisation or promote better tubule formation.

4 MATERIALS AND METHODS

4.1 Cell culture

4.1.1 Human mesenchymal stem cells

Human mesenchymal stem cells (MSCs) were harvested from the iliac bone marrow of a healthy 21-year-old female donor after an informed consent form under the protocol approved by the Ethics committee of the Helsinki University Central Hospital, Finland, was signed. Cells were then isolated, expanded, and cryopreserved in liquid nitrogen using previously optimized methods.

In this project, cells were thawed prior to use and cultured in α- minimal essential medium (α- MEM; Gibco, Grand Island, USA, Ref no. 41061-029) containing 10% foetal bovine serum (FBS; U.S. origin, Invitrogen, cat#16000-044) and 1% penicillin streptomycin (PS, Gibco) henceforth referred to as basal media. Cells at passages 6 to 8 were used for the experiments.

4.1.2 Human umbilical vein endothelial cells

Commercially available GFP-expressing human umbilical vein endothelial cells (GFP-HUVECs) (IncuCyte® Cytolight Green HUVECs, Essen Bioscience, Sartorius, cat#4453) were expanded and cultured in Endothelial Growth Medium 2 (EGM 2, PromoCell, C-22011) with the Supplemental Mix (PromoCell GmbH, Heidelberg, Germany, cat# 39216) and 1% penicillin streptomycin (PS, Gibco). Cells were cryopreserved in liquid nitrogen and thawed prior to use in experiments. Cells at passages 7-8 were used for the experiments.

4.1.3 Peripheral blood-derived mononuclear cells

Peripheral blood sample (average volume 30 mL) was collected from one healthy 25-year-old male donor. The local Ethical Committee of University of Turku approved the protocol, and the donor signed an informed consent. Blood was always drawn on the day of the experiment and mononuclear cells were isolated from the freshly drawn blood samples by Ficoll density gradient centrifugation method and cultured with MSCs as described below (see 4.3).

4.2 Optimization of culture conditions

Culture conditions for optimal tube formation when MSCs and HUVECs were grown together was checked using four different parameters i.e., Endothelial Growth Media (EGM, PromoCell, C-22010) with Supplemental Mix (PromoCell GmbH, Heidelberg, Germany, cat# 39215) and 1% PS, EGM 2, fibronectin coating and no fibronectin coating. The cells were plated in a 96 well plate (96-Well CytoOne® Plate, TC-Treated, cat#CC7682-7596) and wells of the fibronectin groups were coated with 1 ng/mL fibronectin prior to plating cells. Experimental groups were as follows: MSCs and HUVECs in EGM media with fibronectin coating, MSCs and HUVECs in EGM media without fibronectin coating, MSCs and HUVECs in EGM 2 media with fibronectin coating, and MSCs and HUVECs in EGM 2 media without fibronectin coating. Control groups were as follows: MSCs alone in EGM without fibronectin, MSCs alone in EGM

with fibronectin, HUVECs alone in EGM without fibronectin and HUVECs alone in EGM with fibronectin. Similar control groups with EGM 2 were included as well. Cells were seeded at a density of 500 cells/well for MSCs and 1000 cells/well in the case of HUVECs. Cells were observed using real time cell imaging software IncuCyte® S3. Half of the media was changed every alternate day.

4.3 MSC-MNC co-culture for MACs isolation

The co-culture set up was prepared in such a way that MSCs were thawed and cultured first in one or two T75 flasks in basal medium (1000 cells/cm^2). Half of the medium was changed every 3-4 days. Cells were allowed to reach confluency and then harvested using trypsin/EDTA (Gibco) before re-plating into three T75 flasks (1000 cells/cm^2). After one week of expansion, cells were harvested again, re-plated onto three T75 flasks (2500 cells/cm^2) for experiments, and allowed to grow for 3 days. Mononuclear cells (MNCs) were isolated from the peripheral blood sample using Ficoll density gradient centrifugation method (Ficoll-Paque PLUS, GE Healthcare Bio-Sciences AB, Sweden). The MNCs were added to the flasks containing MSCs at a cell density of 50000 cells/cm^2. Cells were cultured in basal media for one week and half of the medium was changed every 3-4 days.

4.4 Magnetic-activated cell sorting for isolation of MACs from PB source

Endothelial progenitor cells of our interest i.e., $CD14^+CD31^+$ cells, hereafter referred to as Myeloid Angiogenic Cells (MACs) were isolated from the MSC-MNC co-culture by means of magnetic activated cell sorting (MACS®). Cell sorting was performed with magnetic nanoparticles coated with antibodies against $CD14^+$ and $CD31^+$ according to the manufacturer's instructions (Miltenyi Biotec). Cells in the co-culture were trypsinised and counted. After counting, the cell suspension was subjected to centrifugation at 300 g for 10 minutes, supernatant was discarded, and the pellet was dissolved in 80 µL of cold buffer (1x PBS + 1% FBS), henceforth known as MACS buffer. 20 µL of CD14 microbeads (Miltenyi Biotec, 130-050-201) were added per 10^7 cells. The mixture was incubated at 4°C in a cold room rotator for 15 mins. The cell suspension was then subjected to a strong magnetic field (Miltenyi Biotec) with a LS column (Miltenyi Biotec, 130-042-401). The cells expressing the surface markers CD14 and CD31 remain in the column while the remaining non-specific cells were collected separately. The LS column is then removed from the magnetic field, and cells positive for CD14 were eluted by washing the column with 5 mL of cold MACS buffer. Cells were centrifuged for 5

minutes at 300 g. The supernatant was discarded, pellet was dissolved in 1 mL EGM and cells were counted. After counting, cells were again centrifuged for 3 minutes at 300 g. Supernatant was aspirated and cells were resuspended in 60 µL of EGM. 20 µL of FcR blocking reagent (Miltenyi Biotec, 130-059-901) was added to this suspension followed by 20 µL of CD31 microbeads (Miltenyi Biotec, 130-046-702) per 10^7 cells. The mixture was incubated at 4°C in a cold room rotator for 15 mins. 1 mL of EGM was added to the cell suspension and cells were centrifuged at 300 g for 3 minutes. Cell pellet was resuspended in EGM medium and subjected to magnetic field as mentioned above. Unlabelled cells were collected as flow through and $CD14^+CD31^+$ cells were eluted with 5 mL of EGM medium as aforementioned. Cell suspension was then centrifuged again at 300 g for 5 minutes. Supernatant was discarded and pellet was resuspended in 1 ml EGM. Cells were then counted. The cell sorting success by this protocol was previously checked by our lab using flow cytometry analyses.

4.5 Transwell® cell culture set up

4.5.1 Transwell® co-culture with HUVECs and MSCs

Cells were cultured in a 24-well plate (Corning cat# 356008) coated with fibronectin (1 µg/ml) with 0.4 µm inserts (Falcon® Permeable Support Transparent PET Membrane, cat# 353095) to see if HUVECs in the presence of MSCs give rise to tube-like structures. HUVECs were plated a day before to allow cells to attach to the plate and the following day MSCs were harvested as described above and added to the inserts and wells. HUVECs were seeded at a density of 6000 cells/well and 1000 cells/insert. MSCs were seeded at a density of 3000 cells/well and 500 cells/insert. Control groups included HUVECs alone and HUVECs with MSCs without inserts. Experimental groups included HUVECs in the well with MSCs alone in insert and HUVECs in the well with MSCs as well as HUVECs in the insert (Figure 12). Cells were cultured in EGM for at least 14 days. Three biological replicates were performed, and each experimental sample group consisted of three parallel technical replicates.

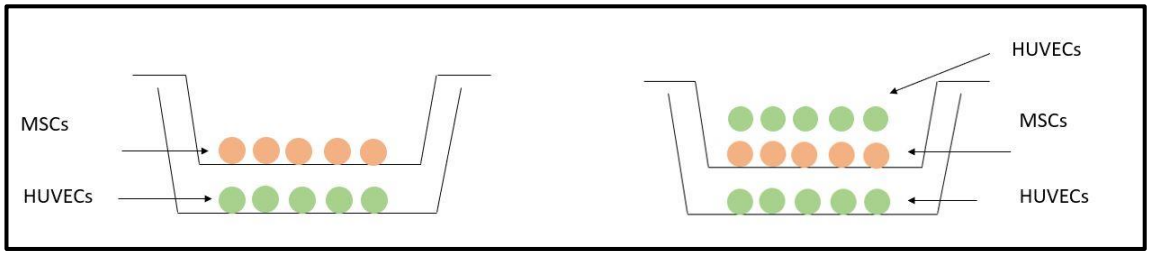


Figure 12. Diagrammatic representation of experimental groups in the Transwell® co-culture model with HUVECs and MSCs.

4.5.2 Transwell® co-culture with HUVECs and MACs

Cells were cultured in a 24-well plate (Corning cat# 356008) coated with fibronectin (1 µg/ml) with 0.4 µm inserts (Falcon® Permeable Support Transparent PET Membrane, cat# 353095). HUVECs were plated a day before to allow cells to adhere to the plate and the following day MACs were isolated from MSC-MNC co-culture as described above and added to the wells and inserts. Cells were seeded at a density of 6000 HUVECs/well and 12,000 MACs/well. Cells in the insert were plated at a density of 1000 cells/insert for HUVECs and 2000 cells/insert for MACs. Control groups included HUVECs alone and HUVECs with MACs without inserts. Experimental groups included HUVECs in the well with MACs alone in insert and HUVECs in the well with MACs as well as HUVECs in the insert. Cells were cultured in EGM for at least 14 days. Half of the media used to culture the cells in was changed every alternate day. Three biological replicates were performed, and each experimental sample group consisted of three parallel technical replicates.

4.6 Real time cell imaging *(*IncuCyte® S3 live-cell analysis*)*

HUVECs and MACs were co-cultured in a 24-well plate setup with Transwell® inserts as described above. Cell proliferation, morphological and structural changes were observed using the real-time cell imaging system (IncuCyte® S3, Sartorius).

Cells were imaged and images were stored automatically by the system at regular 2- or 4-hour intervals. Images were taken with the 10x objective. IncuCyte® Angiogenesis Analysis Software Module (Cat. No. 9600-0011) was used to analyze the number and length of networks, as well as the number of network branch points.

4.7 Microscopy imaging

Cells were fixed with paraformaldehyde after 14 days of culturing and cell morphology and tube-like structures were imaged using both a standard light microscope and multichannel fluorescence microscope (EVOS M5000). Images were acquired using a 10x objective lens for both microscopes.

4.8 Statistical methods

Statistical analyses and quantification graphs were made using Graph Pad Prism 9. Statistical significances between the experimental groups were assessed using the One-Way-ANOVA followed by Bonferroni multiple comparison test. All experiments were repeated three times and conducted as triplicates to reduce experimental errors.

5 ACKNOWLEGEMENTS

I would like to take the opportunity to thank PhD, Adjunct professor Terhi Heino for giving me constant support and guidance throughout this project. Her guidance has greatly aided me in learning new skills both inside and outside the lab and I am very thankful to her for giving me the opportunity to be a part of her research group. I also would like to thank doctoral candidate Liina Uusitalo-Kylmälä for her constant support and help with my experimental methods. She has gone above and beyond in her efforts and I am extremely grateful for her help. I would like to thank MSc Karoliina Kajander for her insightful inputs and fellow Master's student Nicko Widjaja for his kind assistance in being a willing blood donor for my experiments. Finally, I would also like to thank all my friends and family who have been a great support system for me.

6 ABBREVIATIONS

2D – two-dimensional

3D – three-dimensional

CAC – Circulating angiogenic cell

CAD – Coronary Artery Disease

CD14 – Cell differentiation marker 14

CD31 - Cell differentiation marker 31

CD45 - Cell differentiation marker 45

CD146 – Cell differentiation marker 146

EC - Endothelial cell

ECFC - Endothelial colony forming cell

ECM – Extracellular matrix

EGM – Endothelial Growth Media

EGM 2 – Endothelial Growth Media 2

EPC - Endothelial progenitor cell

HUVEC - Human umbilical vein endothelial cell

MAC - Myeloid angiogenic cell

MMP – Matrix metalloprotease

MNC - Mononuclear cell

MSC - Mesenchymal stromal cell

PB-MNC – Peripheral blood-derived mononuclear cell

VEGF - Vascular endothelial growth factor

VEGFR2 – Vascular endothelial growth factor receptor 2

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