Establishment of 3D co-culture model of mesenchymal stem cells and mononuclear cells for studying vasculogenesis/angiogenesis, and the magnetic isolation of endothelial progenitor cells from mononuclear cells

Master's Thesis

University of Turku

MSc Degree Programme in

Drug Discovery and Development

04.02.2019

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MÄKELÄ, MATTI: Establishment of 3D co-culture model of mesenchymal stem cells and mononuclear cells for studying vasculogenesis/angiogenesis, and the magnetic isolation of endothelial progenitor cells from mononuclear cells

Master's Thesis, 49 pages, 5 appendices

Drug Discovery and Development

February 2019

Bone tissue engineering that stimulates bone formation has improved normal fracture healing, but its true clinical impact is still lacking. One of the biggest problems with bone tissue engineered grafts is an inadequate blood supply to the site of implantation.

Our research group has previously shown that tubular, vessel-like structures are formed in the regular co-cultures of bone marrow derived mesenchymal stem cells (MSCs) and peripheral blood derived mononuclear cells (PB-MNCs). In addition, enhanced differentiation of both bone-forming osteoblasts and pericytes supporting vessel structures was observed. This offers an interesting possibility for a new cell-based therapy and could possibly solve the problem with inadequate blood supply.

In this project, a three-dimensional co-culture model was established to study angiogenesis/vasculogenesis by mixing type I collagen with Matrigel in four different ratios and culturing the cells within these gels. Both human umbilical vein endothelial cells (HUVECs) as well as MSCs and PB-MNCs were used. Cell proliferation and viability was analysed with AlamarBlue assay and live cell monitoring was done by IncuCyte ZOOM. Endothelial cell precursors and monocytes were isolated from peripheral blood by magnet activated cell sorting (MACS) with CD34 and CD14 markers, respectively. Isolated cells were grown with MSCs in a regular monolayer culture. Numbers of rounded and elongated cells were counted from microscope images with ImageJ program. Immunofluorescence staining for endothelial marker CD31 and pericyte marker PDGFRB was done on the co-cultures.

Results indicate that the morphology of the cells changed inside the gels. Cells started to reach out to neighbouring cells and formed networks instead of a single confluent layer. Primary cells stayed viable and started to proliferate in each gel. In the co-cultures with CD34 and CD14 selected cells, the number of rounded cells decreased, while the number of elongated cells increased, indicating a possible endothelial cell differentiation.

In conclusion, elongated, endothelial cell-like morphology and the formation of cellular networks appeared promising for the formation of vessel-like structures. Further studies are still needed both to confirm these results and to study the endothelial cell and pericyte differentiation in more detail.

Keywords: Cell cultures, Three-dimensional, Mesenchymal stem cells, Mononuclear cells

TURUN YLIOPISTO

Biolääketieteen laitos, Lääketieteellinen tiedekunta

MÄKELÄ, MATTI: Kolmiulotteisen mesenkymaalisten kantasolujen ja mononukleaarisolujen yhteisviljelmän perustaminen angiogeneesin/vaskulogeneesin tutkimista varten, ja endoteelisolujen esiasteiden magneettinen eristäminen mononukleaarisoluista

Pro gradu tutkielma, 49 sivua, 5 liitettä

Drug Discovery and Development

Helmikuu 2019

Luukudosteknologia pyrkii lisäämään luun luonnollista parantumiskykyä käyttäen erilaisia biomateriaaleja ja soluterapiaa. Se on osoittanut hyötynsä luumurtumien paranemisessa, mutta ongelmiakin vielä on. Yksi suurimmista haasteista on riittävän verenkierron aikaansaaminen, mikä aiheuttaa siirteiden epäonnistumisen.

Tutkimusryhmämme on aikaisemmin osoittanut, että verisuonten kaltaisia rakenteita muodostuu, kun luuytimen mesenkymaalisia kantasoluja (MSC) ja perifeerisen veren mononukleaarisoluja (PB-MNC) viljellään yhdessä. Lisäksi sekä luuta muodostavien osteoblastien että suonirakenteita tukevien perisyyttien erilaistuminen oli lisääntynyt. Tämä voisi mahdollistaa uuden mallin soluterapiaan ja tarjota ratkaisun ongelmaan riittävän verenkierron suhteen.

Tässä projektissa kehitettiin kolmiulotteinen yhteissoluviljelmä angiogeneesin tutkimiseen sekoittamalla tyypin 1 kollageenä ja Matrigel:iä eri pitoisuuksissa. Soluja viljeltiin kahden tällaisen matriksin välissä. Kolmea eri solutyyppiä käytettiin projektin aikana: ihmisen napanuoran laskimon endoteelisoluja (HUVEC), sekä MSC- ja PB-MNC-soluja. Solujen jakautumista ja elinkykyä tutkittiin AlamarBlue -metodilla. IncuCyte ZOOM:illa seurattiin solujen kasvua ja morfologiaa. Lisäksi tässä projektissa pyrittiin eristämään perifeerisestä verestä endoteelisolujen esiasteita ja monosyyttejä käyttämällä MACS-menetelmää ja CD34/CD14 markkereita. Näin eristettyjä solufraktioita viljeltiin yhdessä MSC:jen kanssa. Pyöreiden ja pitkulaisten solujen määrät analysoitiin ImageJ -ohjelman avulla. Immunofluoresenssivärjäyksiä tehtiin käyttäen CD31:tä endoteelimarkkerina ja PDGFRB:tä perisyyttimarkkerina.

Tulokset osoittavat, että solujen morfologia muuttui kolmiulotteisissa viljelmissä. Solut alkoivat kurottautua kohti viereisiä soluja ja muodostaa soluverkostoja yhden ainoan yhtenäisen solumaton sijaan. Lisäksi CD34- ja CD14- markkereita ilmentävien solujen ja MSC:jen yhteisviljelmissä pyöreiden solujen lukumäärä pieneni ja pitkulaisten solujen lukumäärä kasvoi, mikä viittaisi solujen differentiaatioon endoteelisoluiksi.

Endoteelisolujen kaltainen morfologia ja soluverkostojen muodostuminen yhteisviljelmässä vaikuttaa lupaavalta, mutta lisätutkimuksia tarvitaan varmistamaan nämä tulokset.

Avainsanat: soluviljelmä, kolmiulotteinen, mesenkymaalinen kantasolu, mononukleaarisolu

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1 INTRODUCTION

1.1 BONES AND BONE TISSUE

Bone tissue is a living component of the human body. It is a dense, mineralized connective tissue type, which is under constant change, when old bone tissue is broken down and replaced by a new tissue (Florencio-Silva et al., 2015). Bone cells play an important role in this event. Osteoclasts are the ones responsible for breaking down the old bone, and osteoblasts continue to form the new bone as this happens. The balance between these two is important to the functional bone. However, this balance can be disturbed in various pathological conditions, such as osteoporosis (Raiz, 2005). In addition to bone cells, bone tissue also needs nutrients, growth factors, and oxygen delivered by an adequate blood supply to function properly. However, unlike other tissues in the body, bone has a special ability to heal without forming any scars (Marsell and Einhorn, 2011). If these scars did form, it would compromise the sturdiness of bones over time and hence the function of bones.

Bones allow the movement of the body and the upright position in which we stand. In addition, bones offer protection to the important inner organs, contribute to the mineral homeostasis, and to the differentiation of red and white blood cells in the bone marrow (BM). Bones are composed of two different types of bone tissue. Spongy bone is a porous, relatively light part of the bone which contains the bone marrow. It is surrounded by harder and denser compact bone that is capable of handling mechanical stress. This unique composition gives bone its properties. It is light enough to allow efficient movement but tough enough to handle constant mechanical stress placed upon it.

Despite of this, a high impact, like a collision in the car accident or jumping from the high altitudes, can cause bones to break. In addition, a constant, high-impact mechanical stress (for example strenuous running or other sports) can cause bones to weaken by accumulation of microfractures, which (if not repaired) will finally cause so called stress fractures (Goolsby and Boniquit, 2017). If bone breaks, an event called fracture healing happens. Fracture healing is a highly

regulated process that requires assistance of numerous cell types, and an establishment of an adequate blood supply. It happens with several recognizable steps and takes usually a long time before complete recovery. In some cases, fracture healing can be imperfect which leads to delayed unions (meaning it takes longer time for fracture to heal) or non-unions (meaning that fracture fails to heal at all). This imperfect healing especially happens in the cases of inadequate blood supply (Lu et al., 2007) to the site of fracture, and in the other possible cases like infection (Mills et al., 2016), and a gap between the two ends of fractured necrotic bone (Mills et al., 2016) for example.

1.2 BONE TISSUE ENGINEERING

Bone tissue engineering (BTE) is a field of study that tries to enhance the natural bone fracture healing or *de novo* synthesis of bone tissue by utilizing several different methods (Black et al., 2015). These methods include different scaffolds and biomaterials, utilization of various cell types, establishment of *in vivo* models, and development of vascularized bone grafts and much more (Black et al., 2015) (Amini et al., 2012). BTE has become an important research field due to the increased life expectancy of individuals, which has been achieved because of major improvements to the general quality of life, as well as medical strategies and new medical innovations. However, this means that in an individual's life there exists more chances to suffer from the problems and diseases associated with high age. For example, more people might suffer from one or several bone fractures in their life time, which will become a major socio-economic burden. Such problems also lower the quality of life of an affected individual. For these reasons improvements in the strategies to address fracture healing and other musculoskeletal diseases are still needed. There have already been major advances in the field of BTE during the last decade, but several problems still exist. One of the biggest current limitations is the lack of an adequate blood supply to the site of implantation which causes engineered grafts to fail (Amini et al., 2012). Other notable problems include the cost of these grafts (Amini et al., 2012), the lack of information behind the stem cell action in bone defects (Ma et al., 2014), and difficulty to choose appropriate biomaterials for each patient case (Amini et al., 2012).

1.2.1 BIOMATERIALS AND SCAFFOLDS

There are several prerequisites for biomaterials to be appropriate for BTE. Biomaterials should be biocompatible (Perez et al., 2018), meaning that implantation to the host will not cause a severe immune response against the graft. The biomaterials should also be biodegradable (Perez et al., 2018), as the implanted biomaterials should only be temporary, and a newly formed bone should finally replace the biomaterial. It should also provide enough mechanical stability and support to the healing bone (Perez et al., 2018). In addition to these properties, biomaterials should have abilities to affect neighbouring cells (e.g. on cell proliferation and differentiation) and have osteoinductive and osteoconductive properties (Perez et al., 2018). These prerequisites make finding suitable biomaterials a challenging matter. Not a single biomaterial currently used in BTE fulfils all these properties.

In BTE the following biomaterials have already been developed: ceramics, polymers, composites, advanced hydrogels, and immune-modulatory biomaterials (Amini et al., 2012). Ceramics are highly biocompatible, have good bioactivity, and are slow wearing materials. The most commonly used ones are calcium phosphate and tricalcium phosphate (Perez et al., 2018). However, their use is limited because they are too brittle and do not offer enough mechanical strength for BTE. Polymers can either be synthetic or natural (e.g. polylactides and collagen). From these polymers, poly(ε-caprolactone) (PCL) is the most studied one and it is considered as a good biomaterial for BTE purposes (Woodruff and Hutmacher, 2010). PCL's mechanical properties are maintained for several months and it is fairly biocompatible and easy to manufacture (Perez et al., 2018). Otherwise, polymers fall short in the categories of load-bearing capabilities and biocompatibility. Composites are combination of polymers and ceramics. The aim is to take advantage of the properties of each component, while avoiding the limitations of each individual biomaterial. In addition to this, composites can be enhanced by adding metals to them (Perez et al., 2018). By doing this, benefits can be seen for example with osteogenesis (Perez et al., 2018). The usage of advanced hydrogels is an intriguing possibility, since hydrogels are capable of functioning as a matrix for tissue engineering and offer the required biocompatibility (Amini et al., 2012). In addition, advanced hydrogels can mimic the structure of surrounding extracellular matrix which makes them

unique compared to other biomaterials (Amini et al., 2012). Immune-modulatory biomaterials have an ability to affect the host's immune response in a manner that favours bone regeneration (Amini et al., 2012).

Scaffolds are structures that are built from the biomaterials, the choice of which completely depends on the nature of the fracture site, economical cost, and the effectiveness of the biomaterial in each patient case (Roseti et al., 2017). Scaffolds mirror the properties of biomaterials such as biocompatibility and biodegradability. In addition, engineered scaffolds should have similar mechanical properties as the site of implantation (O'Brien, 2011). However, even more important is an adequate porous structure in these scaffolds. This allows cells to migrate through the scaffolds, as well as waste and nutrients to pass the scaffold, and furthermore helps to achieve a proper vascularization, which is important for a successful graft (O'Brien, 2011). Without these properties, grafts tend to fail *in vivo*.

1.2.2 CELL-BASED STRATEGIES

The effectiveness of biomaterials and scaffolds can be enhanced by utilizing different cell types. The cells are added to the scaffolds before the implantation to the host and they can then affect the bone regeneration, vascularization, or other important phenomena in the fracture healing. There are many things to take into consideration when deciding which cell type to choose. One can choose to use primary osteogenic cells with limited proliferation capabilities, stem cells capable of self-renewing, cells with osteogenic potential, cells with vasculogenic potential, or cell types with potential for both vasculogenesis and osteogenesis (Marot et al., 2010). In addition to differences in cell types and their potential for BTE, things like the source of cells (autologous or allogeneic), the ease of harvesting from donor, how homogenic the harvested cell population is, how easy it is to induce the wanted cell phenotype, the ease of expanding of cell cultures *in vitro*, the stability of graft after implantation, and many more should be taken into consideration (Marot et al., 2010).

Stem cells used the most commonly in BTE can be divided into adult stem cells and pluripotent embryonic stem cells. Bone formation consistency has not been reached with pluripotent embryonic stem cells which makes using of adult stem

cells more intriguing (Ma et al., 2014). In addition, ethical issues must be considered when embryonic stem cells are being used. Adult stem cells can be derived from the bone marrow, adipose tissue, or the dental pulp (Ma et al., 2014). The use of adult stem cells is intriguing possibility because of the availability of the cells and the harvesting can be done in the conjunction with other medical procedures. However, primary osteogenic cells are also readily available and can be collected from bone and periosteum of the donor. The ideal choice of the cell types to use is still in the progress (Marot et al., 2010). The application of different cells with bioengineered scaffolds is a strategy that possibly can increase the success rates of the grafts. However, as a cell-based therapy the regulations concerning this kind of approach are stricter than with just scaffolds and much more research is needed before these strategies can become clinically relevant (Marot et al., 2010).

1.3 ANGIOGENESIS AND VASCULOGENESIS

Angiogenesis and vasculogenesis are both phenomena related to the formation of blood vessels. In angiogenesis new vessels are being formed from pre-existing blood vessels by sprouting. Whereas in vasculogenesis, new blood vessels are being formed from nothing, *de novo*. Both phenomena are normal during human development. However, they also take place in an individual that has reached adulthood, since angiogenesis and vasculogenesis can also happen in some physiological (e.g. pregnancy) and pathological (e.g. tumorigenesis) conditions (Stegen et al., 2015). In addition, formation of new blood vessels is especially needed after tissue trauma, where the establishment of an adequate blood supply to the site of injury is important, for example in the bone fractures (Hankenson et al., 2011). Blood supply is responsible for transferring cells like osteoblast progenitors which have a role in new bone formation to the site of the fracture (Hankenson et al., 2011). In addition, blood supply is important for gas, nutrient, and waste exchange in bone (Hankenson et al., 2011). Depending on the situation, angiogenesis and vasculogenesis can also be harmful to the individual. In the case of malignant tumours, angiogenesis and vasculogenesis are considered universally as undesired events because thereby tumour is becoming self-sustaining, metastases more easily, and becomes more difficult to treat.

Angiogenesis starts with the sprouting of endothelial cells (ECs). This is initiated by the increased levels of angiogenic growth factors, which cause ECs to degrade the basement membrane, which normally keeps ECs from migrating. This is done with help of metalloproteinases. After this process, some ECs form tip cells that express filopodia and start to move towards attractive cues. The tip cells are then followed by stalk cells (also formed from ECs) which are highly proliferative and capable of forming branches and lumen of the newly formed vessels. New vessel connection is established by interactions between two tip cells and finally, the newly formed connection is stabilized by different events, such as the recruitment of pericytes and the deposition of extracellular matrix (Stegen et al., 2015).

Vasculogenesis is mediated by endothelial progenitor cells (EPCs), which are derived either from the bone marrow (Madeddu, 2005) or from the blood circulation (Hankenson et al., 2011). These progenitors are recruited to the site of injury where they start to differentiate to mature ECs. This mobilization seems to be regulated by ischemic conditions in the tissue (Hankenson et al., 2011). In addition to hypoxia, vasculogenesis is regulated by different angiogenic growth factors from which vascular endothelial growth factor (VEGF) seems to hold a major role (Madeddu, 2005). There are also many other important growth factors for both vasculogenesis and angiogenesis. If angiogenesis is somehow disturbed or simply inadequate, problems may arise. For example, in bone fracture healing, non-unions and delayed unions happen more frequently without an adequate blood supply (Stegen et al., 2015). Medical strategies which are trying to regulate angiogenesis and vasculogenesis in some way are an interesting option when trying to improve the fracture healing. This would, for example, increase the success rate of engineered grafts which are currently being used for bone healing.

1.4 MESENCHYMAL STEM CELLS AND PERICYTES

1.4.1 MESENCHYMAL STEM CELLS

Mesenchymal stem or stromal cells (MSCs) are multipotent cells that can be derived from different sources in an adult human (Murray et al., 2015). These

sources include bone marrow, dental pulp, and adipose tissue (Main et al., 2014). Being multipotent cells, MSCs can differentiate into various cell lineages. For example, MSCs can differentiate into chondrocytes, osteoblasts, and adipocytes. This differentiation is dependent on environmental factors, like growth factors and cell to cell connections (Murray et al., 2015). This aspect makes MSCs an interesting research subject and possibly relevant in clinical applications. However, mechanisms behind the differentiation process into different lineages is not yet completely established and more studies are needed (Ullah et al., 2015). In addition, studies on cell homing to the specific tissue or site of injury after systemic administration is still needed (Kumar et al., 2010). This information would help to establish effective treatments for different pathological conditions, such as bone fracture healing, cardiac diseases, and neurological disorders like Alzheimer's (Sudulaguntla et al., 2017).

The interest of MSCs in tissue engineering has recently increased (Kumar et al., 2010). The aspect of regenerating tissues and organs is an intriguing opportunity (Wong et al., 2015), which in turn has increased research conducted on MSCs. Because of this, the need for defining specific criteria for MSCs has arisen. In 2006, the International Society of Cellular Therapy agreed on a minimum criteria for the definition of MSCs (Dominici et al., 2006). According to this definition, MSCs (*in vitro*) must be capable of adhering to plastic surfaces and express the following surface antigens: CD105, CD73 and CD90 (over 95% of cell population). In addition, the following antigens must be absent: CD45, CD34, CD14, CD11b, CD79a, CD19 and HLA-DR. Finally, true MSCs should be able to differentiate into three separate cell lineages (osteoblasts, chondrocytes and adipocytes) *in vitro* (Murray et al., 2014).

1.4.2 PERICYTES

Pericytes are cells that can be found in different locations in the body such as vasculature, liver, and kidney (Wong et al., 2015). The pericyte function and markers differ depending on the location (Wong et al., 2015). For example, in the vasculature pericytes are supporting cells that help to maintain vessel stability and have a role in angiogenesis (Wong et al., 2015). There can also be differences between pericytes in the same tissue.

One study has suggested that pericytes could have different functions depending on the location on a single capillary (middle, closer to the arteriole end etc.)

(Attwell et al., 2016). The possibility of different subtypes is important to be kept in mind when targeting or studying pericytes. Recently, it has been found out that pericytes and MSCs are somehow connected. There exist at least two different hypotheses currently. According to them, either pericytes are precursors of MSCs or *in vivo* counterparts of MSCs (Wong et al., 2015). There are studies indicating that pericytes isolated from different tissues have similar abilities as MSCs when cultured, expressing the same surface antigens and having an ability to differentiate into the same cell lineages (Crisan et al., 2008). This could suggest that MSCs are derived from pericytes. However, this is not yet universally accepted.

1.5 PERIPHERAL BLOOD MONONUCLEAR CELLS

Peripheral blood mononuclear cells (PB-MNCs) is a heterogenous cell fraction, containing e.g. lymphocytes, monocytes, and dendritic cells. This cell population can differentiate into several different cells when in association with the proper inducing environment. The reactivity of immune cells is influenced by different factors like nutritional status, hormone levels, infections, and inflammation, which in turn has an effect on the PB-MNC population of an individual (Kleiveland., 2015). An interesting cell population in this fraction are the endothelial progenitor cells (EPCs), which can be isolated with several different surface markers, for example CD34 (Kleiveland., 2015)

1.5.1 ENDOTHELIAL PROGENITOR CELLS

EPCs are a part of mononuclear cell population, which gives rise to endothelial cells and hence have an important role in the postnatal angiogenesis and vasculogenesis (Eguchi et al., 2007). In peripheral blood, the number of EPCs is low in normal conditions whereas they are more abundant in bone marrow. However, the number of circulating EPCs can increase in the ischemic conditions (Tongers et al., 2010) and by the effect of different cytokines (Matsumoto et al., 2008). A widely accepted characterization of EPCs is the expression of CD34 marker and vascular endothelial growth factor receptor-2 (Hu et al., 2008). However, there is increasing evidence that EPCs express also several other cell surface markers, including for example ones for the monocytic cell lineage, such as CD14 (Hu et al., 2008). EPCs have an interesting therapeutic potential in the vascular diseases (atherosclerosis) and other conditions (fracture healing).

1.5.2 CD34 POSITIVE FRACTION

CD34 is a transmembrane glycoprotein expressed on the surface of different cell types (Sidney et al., 2014). It has been historically associated with haematopoietic stem/progenitor cells. However, currently CD34 is also associated with several non-haematopoietic cell types, such as vascular endothelial progenitors, interstitial cells, and epithelial progenitors etc. (Sidney et al., 2014). In the bone fracture healing, CD34 positive MNC fraction has been suggested to offer unique therapeutic potential because it contains cells capable of differentiating to both ECs and osteoblasts (Kuroda et al., 2014) (Fukui et al., 2015). This means that this fraction contributes to the suitable environment for fracture healing via both angiogenesis/vasculogenesis and osteogenesis. In addition, the isolation of CD34 -positive PB-MNCs is fairly non-invasive and does not require much from donor in comparison to cells isolated, for example, from BM aspirate. There have already been successes when systemically injecting these cells into nude rats with non-union femur fracture (Matsumoto et al., 2006). Enhanced angiogenesis and osteogenesis were observed in this study in the rats that obtained CD34+ cells, and a bridging callus was formed by week 8, while this did not happen in control animals (Matsumoto et al., 2006). However, the effectiveness of this method correlates with the available quantity of CD34+ PB-MNCs in the site of injury (Kuroda et al., 2014). In these studies, the localization in other tissues such as lungs and brain was also observed (Kuroda et al., 2014). A couple of clinical trials have also been initiated with these strategies (Matsumoto et al., 2008). Altogether, CD34+ PB-MNCs appear as cells with an interesting potential in tissue engineering and cell-based therapies.

1.5.3 CD14 POSITIVE FRACTION

CD14 is a receptor for lipopolysaccharide and there exist two isoforms: one expressed on cell surface and a soluble form (Ziegler-Heitbrock et al., 1993). CD14 is considered as a haematopoietic cell marker (Zigdon-Giladi et al., 2014) but, there is evidence that CD14 could be used to isolate other cells too, especially circulating EPCs. There is data suggesting that two subpopulations of EPCs exist in the circulating blood; one CD34 positive and the other CD14 positive subpopulation (Hu et al., 2008). It is well established that CD34 fraction contains EPCs but also CD14+ fraction can give rise to the endothelial-like cells (Pujol et al., 2000). These cells express common endothelial markers, such as

von Willebrand factor and CD34 (although weakly), and their morphology changes during culturing from MNCs with cytoplasm starting to extend to neighbouring cells to either large oval cells or spindle-shaped granulated cells (Pujol et al., 2000). This could mean that EPCs are present in this fraction too.

1.6 PREVIOUS STUDIES UTILIZING CO-CULTURES OF MSCs AND PB-MNCs

There are previous studies on co-culturing of PB-MNCs and BM derived MSCs (Joensuu et al., 2011). In these studies, cell differentiation, morphological changes, and differences in the gene expression were evaluated. During the time of one week in culture, non-adherent MSCs and MNCs started to attach to the plastic. At the same time their morphology changed, and they began migrating in the cultures. Cells became elongated instead of rounded and finally, after four weeks in culture the formation of tube-like structures was observed. In addition to the morphological changes, the cell differentiation was confirmed with immunohistochemistry for CD-31 (PECAM-1) and endoglin. CD-31 staining was observed in the co-cultures, but not in the cultures of MSCs without any MNCs. Endoglin expression was also observed, but it was definitely weaker. In addition, mRNA levels of VEGFR1 and VEGFR2 were observed by RT-PCR. VEGFR1 expression also increased over time, indicating endothelial cell differentiation.

These cultures were performed in normal medium without any exogenously added growth factors. It has also been shown that exogenous VEGF enhances the EC differentiation in MNC cultures. In another study done with similar co-cultures in basal and osteoblastic conditions, osteoblastic differentiation and enhanced bone formation were seen when cells were cultured in the osteogenic medium with exogenous VEGF (Joensuu et al., 2015).

The results of these studies suggest that the co-culture of BM-MSCs and PB-MNCs has a potential for tissue engineering. The formation of vessel-like structures and the observed endothelial cell differentiation could possibly help to overcome the major current problem with BTE: the lack of proper blood supply. In addition, enhanced osteoblastic differentiation correlates with the bone formation and thus the implantation of PB-MNCs and BM-MSCs with biomaterial-based scaffolds could promote the healing of non-union and delayed union

fractures. For these reasons, co-culture of BM-MSCs and PB-MNCs is an interesting research subject.

1.7 3D CELL CULTURES

The interest in three-dimensional (3D) cell culture models has peaked during the last few decades. The advances in the imaging methods and the development of several different matrices (such as collagen, Matrigel, gelatin, laminin, vitronectin, and agarose) has increased the usage of 3D models (Ravi et al., 2015). Various matrices have already been used with several different cell lines and primary cells (Ravi et al., 2015). Three dimensional models can be used to study drug response, cell morphology, tissue architecture, microenvironment, cell motility, cell adhesion, cell differentiation, and signalling among the other things (Ravi et al., 2015). 3D models can be considered as an extension of *in vitro* experiment that are trying to bridge the gap between *in vitro* and *in vivo*.

The data obtained from 3D models is more relevant when compared to the information from regular monolayer (2D) cultures (Edmondson et al., 2014). In 2D cultures, cells are growing as a single, adherent layer on a plastic or glass surface and they are provided with an appropriate amount of nutrients, growth factors, and oxygen depending on a study. The cell proliferation is controlled when the non-adherent, necrotic cells are removed during the medium change. Usually, the morphology of cells cultured in these conditions differs from their *in vivo* counterparts, since the cells are commonly flatter and stretch out more (Edmondson et al., 2014). It is important to notice that cell morphology is an important factor in the normal cell function, meaning that the cells cultured in 2D could act differently from the ones *in vivo*. In addition to this limitation, 2D cultures do not take into account interactions between cell and extracellular matrix, possible changes in cell to cell interactions, cell populations, and different cellular structures (Edmondson et al., 2014). Three-dimensional cultures address these limitations which can then be considered before *in vivo* experimentation, for example.

The benefits of three-dimensional culturing are clear, but also problems do exist. The choice of matrices is dependent on the cell type (Ravi et al., 2015) and the potential combinations of various matrices and cell types offers a vast amount of choices to choose from. This means that research conducted in specific

conditions might not be relevant for the other studies, which increases the amount of research needed. 3D models are also suffering from problems such as poor reproducibility because of biometric scaffolds, high workload before the culturing, and they are more expensive when doing the large-scale experiments (Antoni et al., 2015). In addition, there might be problems when trying to analyse (sensitivity and performance with high-throughput screening instruments) or image (transparency of the material and the size of scaffold) these cultures (Antoni et al., 2015). Despite of this, the need 3D cultures is well recognized in order to study cell functionality in the environment closer to *in vivo*, and to minimize the cost of animal experiments and failures in the clinical phases.

1.8 AIM OF THE PROJECT

This master's thesis project has two aims: the first was to establish a 3D co-culture model of BM-MSCs and PB-MNCs, and the second was establishment and optimization of magnet activated cell sorting (MACS) -method that could be used for MNCs. In 3D experiments, we aimed to establish a model that can be used to study angiogenesis and vasculogenesis *in vitro*. We focused on the optimization of 3D cell culture conditions to ensure cell viability and *de novo* formation of vessel-like structures *in vitro* which have been seen in the previous studies utilizing 2D cultures of BM-MSCs and PB-MNCs (Joensuu et al., 2011). The matrices for 3D-cultures were Matrigel and collagen type 1 and they were selected based on the natural environment of each cell type. Our hypothesis was that MSCs would prefer collagen and MNCs would prefer Matrigel. We also aimed for establishing immunostaining protocols for 3D-cultures and the purpose was to see if the same antibodies and procedure can be used as in regular monolayer cultures.

The aim of MACS experiments was to specifically isolate endothelial progenitor cells from the PB-MNC fraction and compare this isolated fraction to the MNC fraction in MSC-MNC co-cultures. The isolation was done by utilizing two different surface antigens (CD34 and CD14) and the choice of surface antigens was done on the basis of literature. Both of these cell surface markers have been identified on EPCs (Hu et al., 2008). In co-cultures with MACS-isolated cells we used immunostaining to evaluate the origin of ECs and pericytes in our cultures. Our

hypothesis was that MSCs would differentiate into pericytes and MNCs or the isolated fractions would differentiate into ECs.

2 RESULTS

2.1 OPTIMIZATION OF 3D CULTURES WITH HUVECS

During this project, two different optimization rounds were done with human umbilical vein endothelial cells, also known as HUVECs. We found out that HUVECs stayed viable and started to proliferate in every matrix composition (pure collagen, or 1:1, 1:2, and 1:3 Matrigel to collagen ratios). Cell number had effect on the appearance of the cells, but not on their viability inside the gel (3 000, 7 000, 15 000, and 75 000 cells/cm2). Cell culture wells with a higher cell number were almost full immediately after the cell attachment and we found out that 75 000 cells/cm2 was too large to acquire reliable data with live cell monitoring (Fig. 1A). HUVEC cell morphology started to change with cell numbers 3 000/ cm2, 7 000/ cm2 and 15 000/ cm2. HUVEC cells started to reach out to the neighbouring cells and formed networks inside the wells (Fig 1C and D).

In the regular monolayer cultures HUVECs occupied a single confluent layer (Fig. 1B). There seemed not to be any relevant differences between the different matrix compositions when cell proliferation was considered during the first optimization round except in the group of 75 000 cells/cm2 (Fig. 2 A, B, C). However, data from this group is not reliable because of immediate maximum confluence of the wells. Because there was not differences in other groups, it was safe to leave one matrix composition (1:2) out from the second optimization round. No differences in the cell proliferation were observed during the second optimization round either (Fig. 3 A, B, C). 2D cultures of each group were used as controls. Cell proliferation was the highest in these controls.

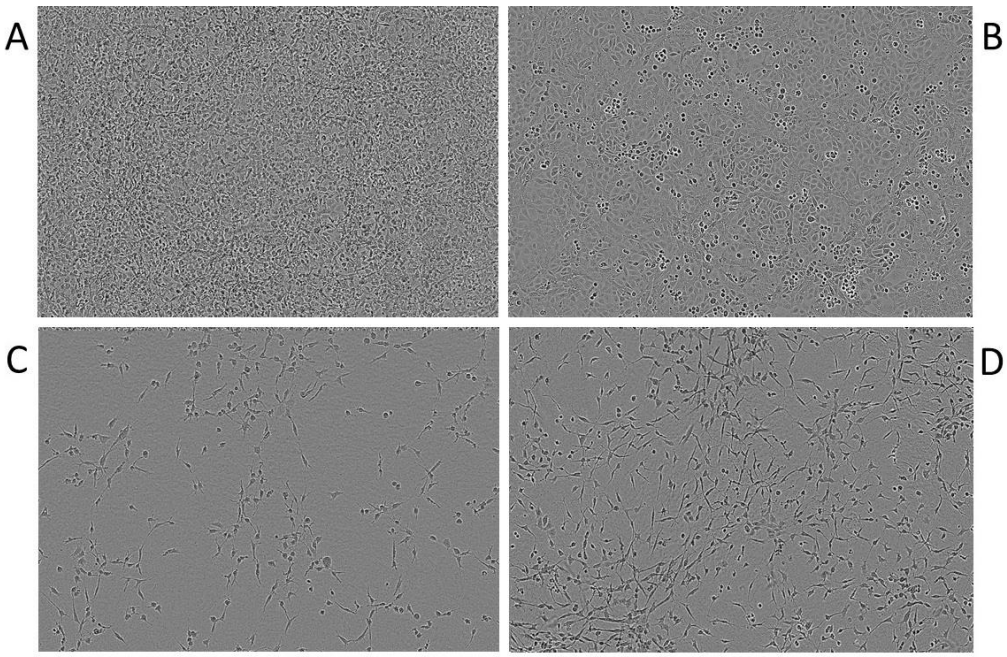

Figure 1. Images acquired by IncuCyte ZOOM of a cell culture well containing HUVECs in the different numbers of cells. A: 75 000 cells/cm2, day 1, and matrix composition of 1:1 (collagen: Matrigel); HUVECs formed a single confluent layer right after the initiation of the culture. B: 3 000 cells/cm2, day 9 as a regular 2D culture; HUVECs occupied the well as a single confluent layer after several days of culture. C: 3 000 cells/cm2, day 1, and matrix composition of 2:1 (collagen: Matrigel); HUVECs can be seen all around the well but their morphology has not started to change yet. D: the same well as in the image C, day 9; HUVECs are starting to reach out to the neighbouring cells and form cellular networks within the gel.

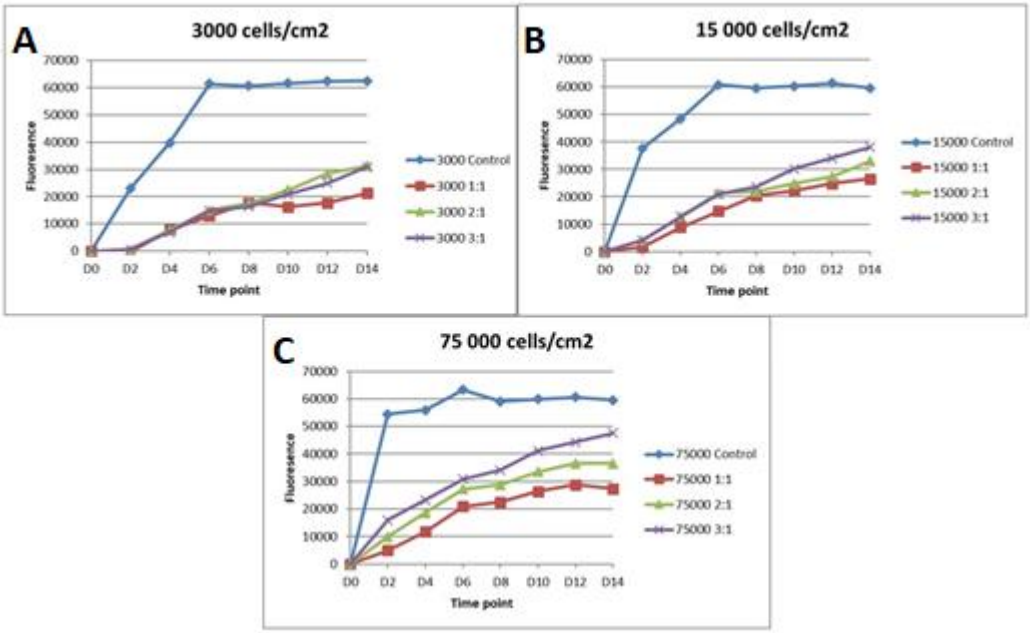

Figure 2. The results from AlamarBlue assay with HUVECs in the first optimization round with cell numbers of 3 000 (A), 15 000 (B), and 75 000 cells/cm2 (C) and matrix compositions of only collagen, 1:1, 2:1, and 3:1 (collagen:Matrigel). Differences in cell proliferation seem to be minimal between each cell number in different matrix compositions. Regular 2D monolayer culture was used as control.

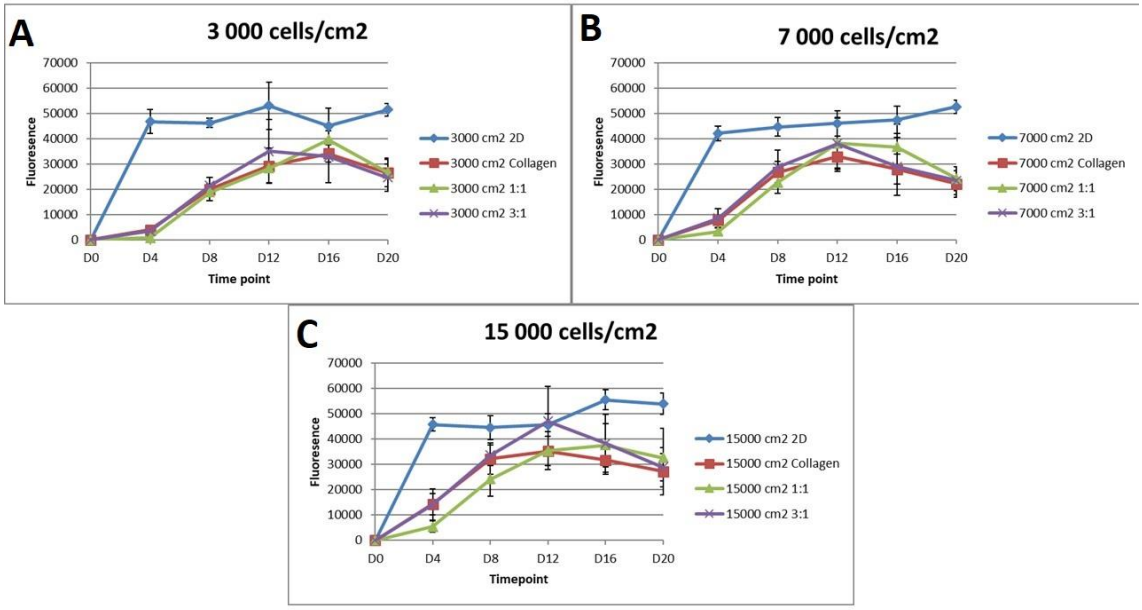

Figure 3. The results from AlamarBlue assay with HUVECs in the second optimization round with cell numbers of 3 000 (A), 7 000 (B), and 15 000 cells/cm2 (C) and matrix compositions of only collagen, 1:1, and 3:1

(collagen:Matrigel). Differences in cell proliferation between matrix compositions seem to be minimal within each cell number. Regular 2D monolayer culture is used as control.

2.2 CELLS STAY FUNCTIONAL AND START FORMING CELLULAR NETWORKS IN 3D CO-CULTURES

Results from 3D co-cultures of MSCs and MNCs were quite similar to the results acquired with HUVECs. We found out that there were not any significant differences in the cell proliferation between different matrix compositions (collagen, 1:1, and 1:3 Matrigel:collagen) in the MNC and MSC+MNC groups. However, there was a statistically significant difference (Wilcoxon signed-rank test; $p<0.05$) between only collagen and the matrix composition of 1:1 in three different timepoints (day 8, 12, and 20) in the MSC group (Fig. 4A). It seems that collagen is the best choice for cell proliferation if only MSCs are considered. No networks were observed in 2D cultures (Fig. 5A) but the morphology of the cells changed inside 3D cultures in a similar way as with HUVECs as MSCs started to reach out to the neighbouring cells and formed cellular networks (Fig. 5B). Despite of morphological changes, we did not see any tube-like structures during these experiments. MNCs however seemed to locate around aforementioned networks (Fig. 5B). We also noticed that 3D gels started to detach from the cell culture plastic surfaces after three weeks of culturing, which might impair the formation of tube-like structures.

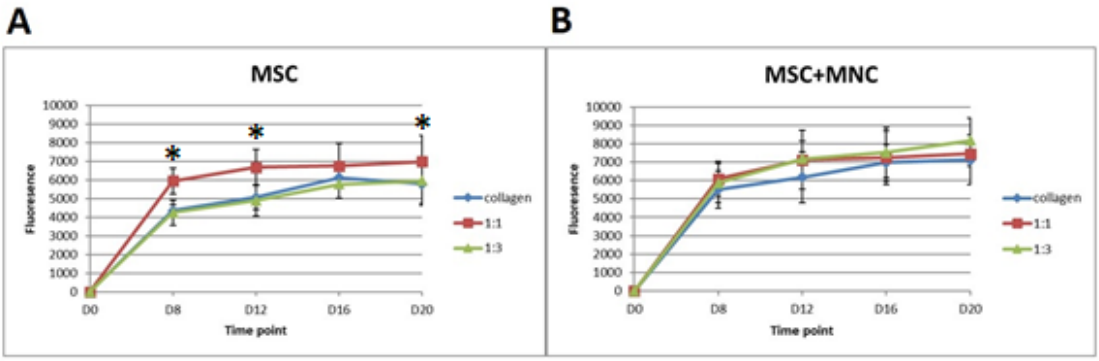

Figure 4. The results from AlamarBlue assay with MSCs alone (A) and MSC-MNC co-cultures (B) with 2 500 cells/cm2 for MSCs and 50 000 cells/cm2 for MNCs. The used matrix compositions were only collagen, as well as 1:1 and 1:3 (Matrigel:collagen). Time points of days 8, 12, and 20 were statistically different

in the group of MSCs when comparing only collagen to 1:1 Matrigel:collagen gel ratio (Wilcoxon signed-rank test; $p<0.05$).

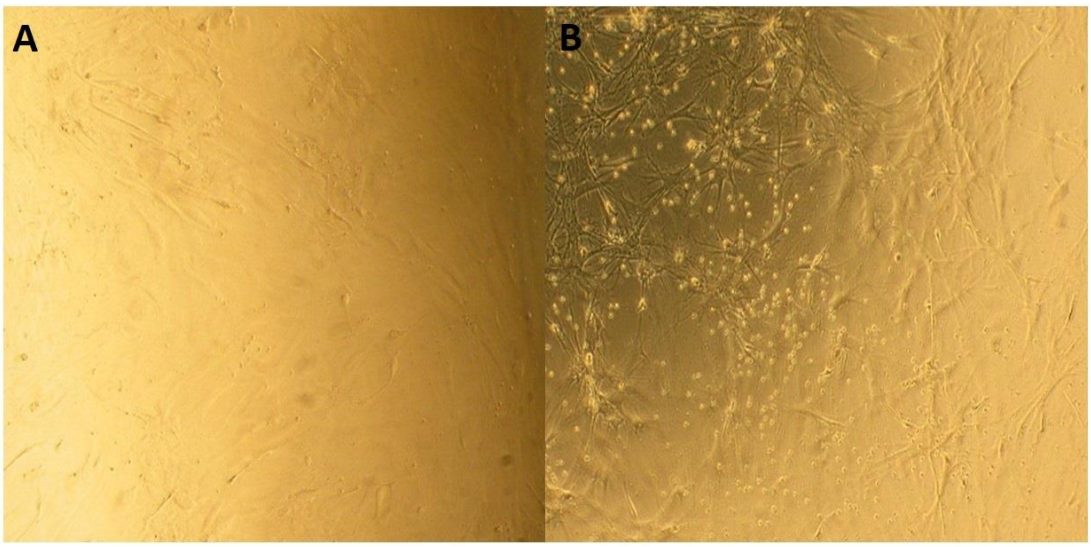

Figure 5. Images acquired by a regular digital camera from normal light microscope. Both wells are containing MSCs and MNCs in either 2D culture (A) or 3D culture (B). Time point for both images is day 12 and the initial cell numbers were 50 000 cells/cm2 for MNCs and 2 500 cells/cm2 for MSCs. Morphological changes can be seen in 3D culture where MSCs start to form networks, and MNCs start to locate around these networks.

2.3 THE NUMBER OF CD34 ISOLATED CELLS WITH DIFFERENT MORPHOLOGY CHANGED IN CO-CULTURES

In the MACS experiment with CD34 binding magnetic beads, we noticed that the isolation protocol is working. Isolation of MNC population yielded two different cell fractions but the number of isolated cells, however, was low. Only 500 000 out of 68 x 10^6 cells (0.74 %) were CD34 positive, while the number of CD34 negative cells was 61 x 10^6. Individual control cultures for each cell type used (MSC, MNC, CD34+ and CD34-) were made, and cells stayed viable during the culture of up to 21 days. In the co-culture groups of MSC+MNC/CD34-/CD34+, we noticed that the number of elongated cells increased (Fig. 6) and at the same time the number of rounded cells decreased. However, there were no statistically significant

differences when comparing the number of elongated cells between MSC+MNC and MSC+CD34- or MSC+CD34+ groups at any of the time points.

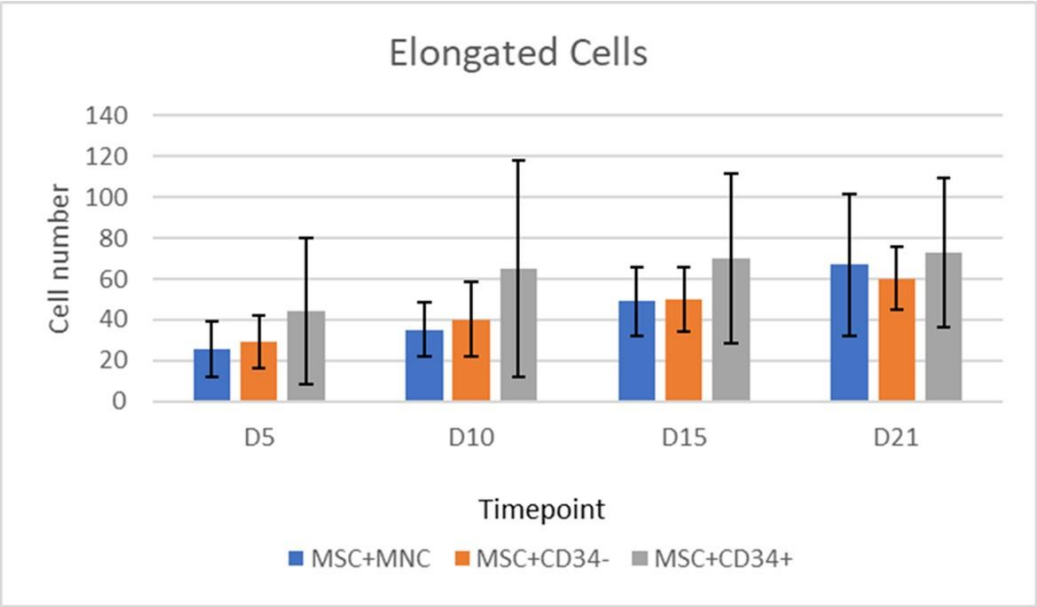

Figure 6. Analysis of numbers of elongated cells in the different co-cultures done with ImageJ. Results are showing the next groups: MSC+MNC, MSC+CD34-, and MSC+CD34+ at four different timepoints (days 5, 10, 15, and 21). The number of elongated cells keeps increasing in each group over time, however, there are not statistically significant differences between the groups (Wilcoxon signed-rank test; $p>0.05$).

2.4 CD14+ CELLS STAY MORE VIABLE THAN CD34+ CELLS IN THE CO-CULTURES

In the MACS experiment with CD14 binding magnetic beads, we found out that the isolation protocol is working as intended. The cell yield was also larger than with CD34 magnetic beads. 6.8×10^6 out of 76.5×10^6 cells were CD14 positive (10.41%), while the number of negative cells was 65.3×10^6. Individual control cultures for each cell type (MSC, MNC, CD14+, and CD14-) were also used in this experiment. During the co-culture with MSCs, we observed that CD14 positive cell fraction stayed viable better than the CD14 negative cell fraction (Fig. 7). We also observed similar change in the cell morphology as with cells isolated with CD34 magnetic beads; unfortunately, there is no numerical data to support

this observation because of the lack of access to the IncuCyte equipment at the time. However, we observed that the number of elongated cells increased as the number of rounded cells decreased. Neither in this experiment, were tube-like structures observed even if the cultures were continued up to three weeks.

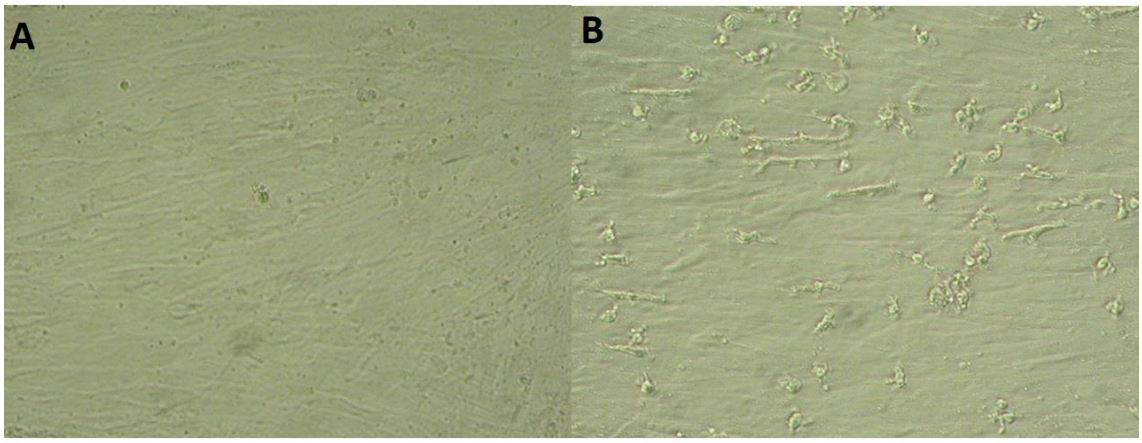

Figure 7. Images acquired by a digital camera under normal light microscope. Both wells contain MSCs and either CD14- (A) or CD14+ (B) isolated cell fraction in a regular 2D culture. Time point for both images is day 21. Figure shows that CD14+ fraction stayed viable better than the CD14- fraction in the co-cultures with MSCs. In image A basically only MSCs can be observed.

2.5 IMMUNOSTAINING OF 2D AND 3D CULTURES

Primary antibodies against CD31 (used as an endothelial marker) and PDGFRB (used as a pericyte marker) worked as intended in the regular monolayer co-/cultures. MNCs stained more prominently with the CD31 antibody, which is in agreement with our hypothesis. However, no distinction which cells differentiated into pericytes could be confirmed because MSCs stained for both CD31 and PDGFRB (Fig. 8). In addition, we found out that our protocol for 3D immunostaining is working but it is highly dependent on the condition of the gel.

(Results are not shown here because the conditions of gels in 3D cultures were suboptimal and the staining was done only once).

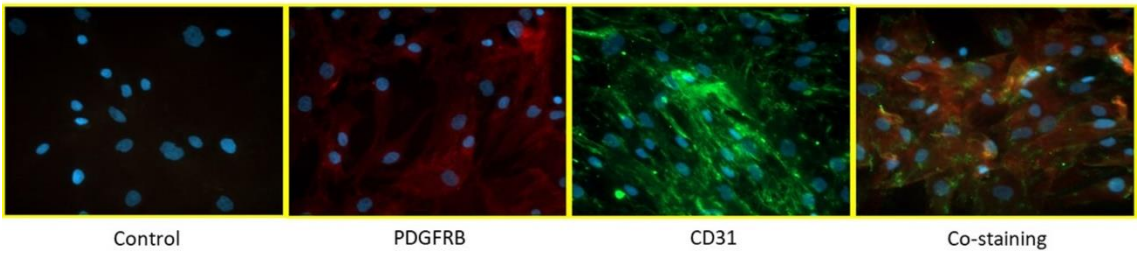

Figure 8. Images showing staining of MSC+MNC co-culture with CD31 (green) and PDGFRB (red) markers. The large round/oval nuclei (blue) are those of MSCs and the small round nuclei those of MNCs. MNCs stained more prominently with CD31 as can be seen from the co-staining, while MSCs were positive for both markers.

3 DISCUSSION

3.1 ESTABLISHMENT OF 3D CULTURES

Results from 3D experiments showed that HUVECs can be used to optimize conditions for MSCs and MNCs. HUVECs are easy to handle and grow, they are commercially available and an inexpensive choice. They can handle better the mechanical and chemical stress, and differences in culture conditions. Although the choice was primarily done because of these reasons, HUVECs are still endothelial cells that should give us enough information about the planned experiments with other, more sensitive primary cells. Indeed, both HUVECs and MSCs/MNCs stayed viable and kept proliferating in our culture conditions. However, results with HUVECs were not necessarily directly applicable to other cells, and in the case of problems optimization should be done again with MSCs and MNCs specifically.

Many different matrix compositions (only collagen, as well as 1:1, 1:2, and 1:3 Matrigel to collagen ratios) and cell numbers (HUVECs: 3 000, 7 000, 15 000, and 75 000 cell/cm2; MNCs: 50 000 cells/cm2; MSCs 2 500 cells/cm2) were

tested in this project. The purpose was to find the most suitable conditions for the optimal vasculogenesis/angiogenesis in the 3D MSC+MNC co-culture. The idea behind the choosing of the mixture of Matrigel and collagen was an assumption that MSCs would prefer collagen and MNCs would, instead, prefer Matrigel because of their natural environments. MSCs are in close contact with collagen in the bone environment, while the extracellular matrix for MNCs is more complex. Our results indicate that MNCs differentiate into endothelial cells that form the wall of the tube-like structures *in vitro* and because of this, the mixture of extracellular matrix proteins like Matrigel could be more suitable for MNCs. The other part of this assumption is that MSCs differentiate into the pericytes that offer support for forming vessels.

However, results from 3D experiments indicated that there were no differences in cell proliferation and viability between the gels. at least, not in the beginning of these cultures. This indicated that this kind of model can be at least used to study these specific primary cells and HUVECs. In addition, it seems that this model could be used to study vasculogenesis and angiogenesis. This conclusion is based on the observation that both primary cells and HUVECs started to reach out for the neighbouring cells and formed networks. However, changes to the medium used might be necessary to see tube-like structures with these culture conditions. The gels with Matrigel started to detach from the bottom of wells after three weeks of culturing. After detachment, there is a chance that gels start to fold on top of themselves which creates several layers with cells inside the gel. If this happens, it is impossible to say if cells have proliferated and moved into different layers or not. This in turn could have an effect on data collected. In addition, folding of gels makes imaging of samples really challenging. It is possible that the tube-like structures cannot be obtained with this kind of model and time frame without any exogenously added growth factors. However, gels containing only collagen seemed to stay better and longer attached to the bottom of wells. According to these experiments, using only collagen would be a better choice both economically (cheaper than Matrigel) and experimentally (easier to handle, no statistically significant differences).

As for the cell numbers used, after the initial rounds with HUVECs and several different cell numbers there were not any statistically significant differences to be seen in the cell proliferation. This made the potential testing of different cell

numbers for primary cells little redundant and we decided to use the same MSC and MNC numbers as in the previous studies (Joensuu et al., 2011 and 2015). However, this means that we do not know if different primary cell numbers would have some effect on cell proliferation or the formation of tube-like structures. There is a chance that MSCs and MNCs were not able to come into the contact with each other fast enough to form clear vessel-like structures. Again, currently it seems that three weeks culture time is a complete maximum for these cultures. However, despite of difficulties it seems that the vessel-like structures could be formed in this 3D model with a little additional optimization.

3.2 LIMITATIONS OF 3D CULTURES

We did two different experiments with primary cells in the 3D setup during this project. Our data, however, is mainly from the second experiment because of practical difficulties in the first experiment. The first experiment was an initial touch to this kind of research and there were a couple of problems. For example, the start of experiment took an additional day because of the two different cell types needing to attach to the wells. Something that did not come to mind when working only with just HUVECs. Data collected from this first experiment suggested that only collagen would be the worst possible choice for co-cultures of MSCs and MNCs when the cell proliferation was considered. Then again, the same experiment suggested that only collagen would the best possible choice for these cell types when cultured separately. In addition, we ran out of collagen during the starting of cultures which made the number of parallel wells low, which probably also affected our initial results. The experiment was repeated, and the results shown and discussed in paragraphs above are from the second experiment. These practical issues highlighted the time-consuming nature of 3D cultures and brings up the need for additional research on the matter.

Additional challenges with 3D cultures during this project were related to imaging. Both fluorescence imaging and imaging with IncuCyte ZOOM proved to be difficult, at least occasionally. The auto focusing function in IncuCyte ZOOM was the main problem with live-monitoring. This function searches for a layer that contains cells in the culture and automatically focuses on this layer. However, in a 3D culture there is a high chance that there exists more than just one layer with

cells. Because of these issues, the images collected were occasionally unusable. However, this did not prevent the data collection because of several parallel wells in our experiments. The other problem was with fluorescence imaging. The imaging protocol itself was working but the condition of samples was suboptimal. The samples had folded several times on themselves because of early detachment from the bottom of wells, which made the distinction of different layers difficult. This is likely due to the combination of inexperienced researcher, too long culture times before fixation, and too many procedures causing mechanical stress to the matrices during the experiments. This problem could possibly be solved with increased experience and additional optimization of culture conditions in the future.

3.3 3D EXPERIMENTS IN THE FUTURE

There are a couple possibilities that could be done in the future 3D experiments based on the results from this project. First of all, experiments could be conducted only with the one matrix composition. From all of the tested 3D gels, collagen type 1 is mechanically the strongest and also economically the cheapest option. It is easier to handle and can persist a couple of mistakes. Collagen survived longer in cultures without starting to detach from the bottom of well which could help with the quality of samples for imaging, for example. However, if tube-like structures are not seen in the future 3D cultures, the choice for collagen type 1 should be questioned.

In this project, we did not see any tube-like structures although they were previously observed in the similar kind of experiments with regular monolayer cultures (Joensuu et al., 2011). The angiogenic potential could maybe be improved by using a higher cell number for MSCs and MNCs or adding some exogenous factor, such as VEGF, which is known to support the formation of blood vessels. Another option is to aim for longer cultures longer than three weeks, while avoiding unnecessary mechanical stress, such as methods that require pipetting the medium out from the wells. It is possible that the cells inside the gel could stay viable even with fewer medium changes. This is based on the observation that it takes much longer time for medium to reach the cells in the 3D culture than in a monolayer culture. However, there is no data from this project to support this. It is possible that this kind of approach could also compromise cell

viability, which should be considered if the cells are difficult to obtain, isolate or if they are expensive.

Imaging methods should be also optimized for the future experiments. IncuCyte ZOOM might not be the best choice for daily monitoring and maybe just a regular digital camera could be utilized for this purpose. However, this makes monitoring of cultures in turn significantly more time-consuming for the researcher. Pros and cons should be weighed if the daily monitoring is a necessity. Of course, lengthening the time between the monitoring days is a possibility that could offer the best of the both choices. In this project, we saw some success with the immunostaining. Our results show that designated markers were working as intended but we did not gain any additional information when compared to monolayer cultures. Unfortunately, only a regular fluorescence microscope was used for the imaging of the 3D cultures, instead of e.g. a confocal microscope. This approach would be a good choice for future experiments, since a confocal microscope can be used to imagine several different layers from the same spot to create a 3D model of the cultured cells. This data could then be used to define the number of branches, the length and the diameter of tube-like structures, and even to define the percentage of the area covered by these structures.

3.4 ISOLATION OF CELLS WITH THE MACS METHOD

During this project, in total three experiments (two of which were partially unsuccessful) with CD34 specific magnetic beads and only one experiment with CD14 specific magnetic beads were performed. CD34 is considered as a common stem cell marker and CD14 is located on the surface of endothelial cells. Our hypothesis was that the fraction containing endothelial progenitor cells could be isolated with these magnetic beads. The isolations with both markers were successful, but the amount isolated with CD34 was significantly lower than the number isolated with CD14. The percentage of CD34 positive cells was only 0.74% of the whole MNC population, whereas with the isolation for CD14 marker 10.41% positive cells were obtained. It is clear that the number of CD34 positive cells in the peripheral blood is very low, which will lead to too few replicates in the experiments, which in turn might affect the results. This makes CD14 a better choice, when only cell numbers are considered. When co-culturing the CD34+ or CD14+ cells together with MSCs, we got data on cell morphology and the number

of cells with different morphologies in the several time points. However, we did not see any tube-like structures; not even with just MNC population used as a control.

In the population isolated with CD34+ magnetic beads the cell morphology changed as the number of elongated cells increased and the number of round cells decreased. MNCs were rounded cells in the beginning of the culture and thus the change in their morphology would indicate that differentiation towards endothelial cells, which was the desired outcome, happens in CD34 positive fraction. However, we did not observe any statistically significant differences in the number of elongated cells when comparing different isolated fractions (CD34+ and CD34-) to the whole MNC fraction. This instead could mean that the central cell population can't be isolated by targeting the CD34 surface antigen. However, because of lack of time and therefore the possibility to repeat the experiments during this project more studies are needed to confirm these results with CD34+ and CD34- cells.

We saw similar kind of results with the cell populations isolated with CD14 specific magnetic beads. The number of elongated cells increased, while the number of round cells kept decreasing in co-cultures. In addition to these results, we noticed that the CD14+ fraction stayed longer viable in the co-cultures than the CD14- fraction. There is no numerical data about this matter, but the differences could be clearly observed from the microscopic images. In the CD14- fraction there were hardly any cells after 21 days in culture, while the CD14+ fraction resembled the cultures containing MSCs and MNCs. This could mean that EPCs are within the CD14 positive cell fraction and CD14 might be a better choice than CD34 for the isolation of EPCs. This however raises the question what causes the problems with cell viability in CD14 negative cell fraction. It is possible that cell to cell contact with MSCs triggers differentiation towards a certain cell type and precursors for this exact differentiation are not within the CD14 negative cell fraction. The lack of specific growth factors could then affect the cell viability in these populations, but more studies are needed to confirm these speculations.

3.5 LIMITATIONS OF MACS EXPERIMENTS

There were a couple of challenges during the MACS-based cell isolations, which can have an effect on the obtained results. The cell number of the whole isolated

MNC population from donor's blood varied on different days. This might be due to the natural variability of donor's blood or the efficiency of collecting the MNC fraction after the Ficoll gradient centrifugation. In addition, during the first experiments it was noticed that the cell pellet obtained from gradient centrifugation was dispersing on its own during the washing of the pellet. This problem was corrected by adding an extra centrifugation step to the next experiments. Dispersing pellet, in turn, might have caused problems of an adequate yield of isolated cells with CD34 specific magnetic beads because the lack of MNCs from the previous steps. The origin of initial blood sample likely also had an effect on the yield of isolated CD34 cell population. Our blood sample was from a major vein (systemic circulation in arm) which is known to contain less of stem cells than, for example, umbilical vein blood. The low numbers of isolated CD34+ cells in turn caused the number of parallel samples for different time points to be relatively low, which could affect the results. With CD14 magnetic beads, the MNC isolation was successful and there was an adequate yield of isolated cells. Blood sample being from peripheral blood seemed not to have similar effect with CD14 specific magnetic beads than it did with CD34 magnetic beads, at least what can be told according to this one experiment with these CD14 specific beads.

In none of these experiments, the formation of tube-like structures was seen. The lack of these structures could mean that MSCs used have been divided too many times (the cells were in passages 5-6) to support endothelial cell differentiation or that the medium used might not have been optimal for endothelial cell differentiation. In addition, the culture time might have been too short or the inexperience in working with primary cells could have had an effect on the results. In addition, the condition of donor's blood, and hence the number of MNCs, could have differed from day to day. These lower numbers of MNCs in peripheral blood did not help. This led to the lower number of parallel wells, which means the less mistakes, ones leading to disqualification of the well, can be made without affecting results. This is specifically strange because these structures were seen in the previous studies (2D cultures with MSCs and MNCs) conducted by our research group.

3.6 MACS EXPERIMENTS IN THE FUTURE

In the future, the experiments that were performed during this project should be repeated a couple of times to confirm these results. If the results are similar, CD34 surface antigen can be left out from the next experiments, because CD14 surface antigen seems to be a better candidate for EPC isolation. The other possible choice could be to test CD34 magnetic beads with umbilical cord blood instead of peripheral blood and see if results are different. At least this should yield to a larger number of isolated cells to be able to have more parallel wells in the experiments which in turn would give more reliable data. The similar counting of elongated and round cells should be made in the experiment with cells isolated for CD14 surface marker. This data should then be compared with data from CD34 experiments and used to decide the best surface marker choice for EPC isolation. To confirm that the isolated cells really are EPCs, gene expression studies are furthermore needed. In addition to these steps, methodological improvements should be made to confirm the formation of tube-like structures in co-cultures.

4 MATERIALS AND METHODS

4.1 CELLS

In this project three different cell types were used. Human umbilical vein endothelial cells (HUVECs) were from commercial sources (ATCC), and not isolated by our research group. Mesenchymal stem cells (MSCs) were isolated from a BM aspirate of a single volunteer donor and the cells were stored in the liquid nitrogen and thawn right before the initial cultures were started. MSCs were plated for experiments one day before PB-MNCs, which were always isolated from the fresh peripheral blood sample of a healthy volunteer in the morning of the first day of experiments. Isolation was done by density gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare Bio-Sciences AB, Sweden). For exact protocol see appendix 1. In addition, CD34+, CD34-, CD14+, and CD14- cell fractions isolated with MACS (see below) were used in this project. Isolation was done from the whole MNC cell population. There are appropriate ethical permissions available for collecting the BM and PB samples.

4.2 3D CULTURES AND CO-CULTURES

The establishment of 3D cultures and co-cultures was started by preparing the appropriate solutions for different matrices. Protocol by Harvard Medical School's Department of Cell Biology (https://brugge.med.harvard.edu/protocols) was used as a basis. The gels were composed of the mixture of rat tail collagen type 1 (Corning) and growth factor reduced Matrigel (Corning) in different ratios. During this project the following ratios were used: only collagen, 1:1, 1:2, and 1:3 (Matrigel:collagen). The concentration of collagen was in all cases 1.6 mg/ml. For exact protocol see appendix 2. 3D co-cultures were done by utilizing a "sandwich" model (Fig. 9). In this model, a base layer of gel was layered first and allowed to stiffen for the incubation period of at least 30 min and up to 1h. Thereafter, a solution containing cells and basal medium was added on top of the base layer. HUVECs were cultured at 3 000, 7 000, 15 000, and 75 000 cells/cm2. MSCs were cultured at 2 500 cells/cm2 and PB-MNCs at 50 000 cells/cm2. Basal medium was alpha-MEM (Gibco), supplemented with 10% fetal bovine serum (inactivated, USA), and 1% penicillin-streptomycin (Gibco). Cells were allowed to attach to the base layer overnight. Cultures were done on 96-well plates and 8-well chamber slides (Nunc Lab-Tek, glass).

The next morning, after the cell attachment was confirmed with a light microscope, a top layer of appropriate gel was added to the wells. After an incubation period of 30 min, 100 µl basal medium was added to the wells. For regular monolayer co-cultures, an appropriate amount of the solution consisting of cells and medium was immediately added to the wells, and cells were allowed to attach overnight. Endothelial cell growth medium (EGM) was used for cultures with HUVECs instead of basal medium. EGM contained the supplement mix (C-39215) provided by the manufacturer (PromoCell GmbH, Germany). Half of the medium was changed every four days for every culture. The number of parallel wells was 8 for each group, except when culturing with chamber slides which were used only for imaging (4 parallel wells for each group).

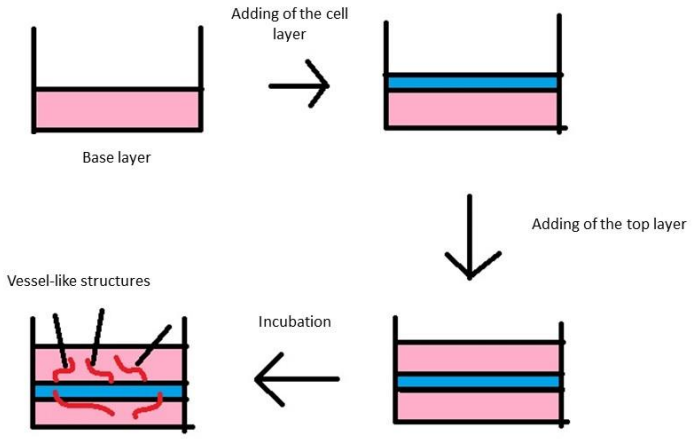

Figure 9. Theoretical image of established 3D culture model. The base layer of a wanted matrix composition is done first. Following this step, the used cells are seeded on top of the base layer after solidification. An adequate incubation time is ensured for cell attachment. In the final step the top layer is layered on top of the cell layer.

4.3 ALAMARBLUE ASSAY

AlamarBlue assay (Invitrogen, USA) was used to study cell viability and proliferation during the cultures. The assay was conducted on 3D and regular monolayer co-/cultures every four days. 10% AlamarBlue -solution was used in the assays and the incubation time for 3D cultures was 3-4 h depending on the experiment. For monolayer cultures, incubation time was 30 min. Two control wells with only AlamarBlue -solution were used as a blank in the assay. Fluorescence was measured at the wavelength of 550-580 nm with software MikroWin 2000 with microplate multimethod reader (Chameleon, Hidex).

4.4 LIVE MONITORING OF THE CELLS

Cell morphology and proliferation was studied by using IncuCyte ZOOM, regular IncuCyte and a light microscope. Because of the overlapping reservations and long monitoring periods, the constant use of IncuCyte ZOOM was not possible

during the project. IncuCyte ZOOM is a machine that can be programmed to take focused set of pictures after the certain time periods. In this case, time period was two hours. Certain cultures were observed once a day with regular light microscope when the use of IncuCyte ZOOM was not possible. Cultures were monitored from two to three weeks depending on the experiment. Causing stress, mechanical or otherwise, to the cultures was avoided during the project.

4.5 IMMUNOFLUORESCENCE STAINING OF CELLS

Depending on the experiment and the cell types in the culture, the cultures were finished at several different time points. Cells were fixed with 2% paraformaldehyde for 15 min (monolayers) or 3 h (3D cultures) at room temperature. The samples were then preserved in PBS at +4°C until the start of immunofluorescence staining. First, 3% bovine serum albumin (BSA) in PBS was used for blocking the unspecific binding of antibodies (incubation time of 1 hour at room temperature). Antibodies against CD31and PDGFRB were used as during this project (see details in appendices 3 and 4) and incubated overnight with the fixed cells. Alexa Fluor 594- and Alexa Fluor 480-labelled secondary antibodies were used for detection. Incubation time for the secondary antibodies was 1 h and the cultures were kept in the dark during incubation. 0,5% Triton-PBS was used for permeabilization of the 3D gels. For exact protocols, see appendices 2 and 3. Samples were observed on the same day with fluorescence microscope (ZEISS Axioimager) and images were recorded and analysed with ZEN lite software (ZEISS).

4.6 MACS

Magnet activated cell sorting or MACS was used to isolate specific cell fractions from the whole PB-MNC population. Magnetic beads targeting CD14 and CD34 surface antigens (Miltenyi Biotec) were used during this project. MNC population isolated with a density gradient centrifugation was mixed with a solution containing these aforementioned magnetic beads. 100 µl of magnetic beads were used for every 100 x 10^6 cells. Cells were incubated for 1 hour in the contact with beads (+4°C). After incubation cells were placed inside a commercial LS column (Miltenyi Biotec) and a powerful midiMACS magnet (Miltenyi Biotec) which caused the cells labelled with magnetic beads to stick to the side of the column.

This step was done on a magnetic standing surface called multi-stand (Miltenyi Biotec). For an informative picture of a setup, see Fig. 10. Non-labelled cells passed through these columns and labelled cells were collected into a different tube. Manufacturer's recommended protocol was used with minor changes during this project. For exact protocol, see appendix 5.

Figure 10. Picture taken during the laboratory work in this project. Image shows a multi-stand (black object) on which midiMACS magnet (purple object) is placed. Finally, the LS column is placed into midiMACS magnet. The setup was used to isolate the CD34+ or CD14+ cell fraction from PB-MNCs with specific magnetic beads.

4.7 STATISTICAL ANALYSES

Statistical analysis was done by using a couple of different softwares for different purposes. Microsoft Excel 2016 was used to make graphs and charts of the results. R studio (3.4.4) was used to make correlations between different groups and to determine if there are any statistically significant differences. Wilcoxon

signed-rank test was used in all cases because collected data was not normally distributed. ImageJ (version 1.51w) was used to count the number of elongated (0-15% round) and round cells (85-100% round) in the regular monolayer cultures.

5 ACKNOWLEDGEMENTS

I want to thank PhD, Adjunct professor Terhi Heino for giving me this opportunity and for constant help during the experimental part and during the writing. In addition, I want to thank MSc Liina Uusitalo-Kylmälä for constant help with laboratory methods and experiments. Finally, I want to thank my friend, Sami Oksanen, for being a willing blood donor for this project.

6 ABBREVATION LIST

- 2D: two-dimensional
- 3D: three-dimensional
- BM: bone marrow
- BTE: bone tissue engineering
- EC: endothelial cell
- EGM: endothelial cell growth medium
- EPC: endothelial progenitor cell
- HUVEC: human umbilical vein endothelial cell
- MACS: magnet assisted cell sorting
- MNC: mononuclear cell
- MSC: mesenchymal stem cell
- PB: peripheral blood
- PCL: poly(ε-caprolactone)
- PDGFRB: platelet derived growth factor receptor beta
- VEGF: vascular endothelial growth factor
- VEGFR: vascular endothelial growth factor receptor

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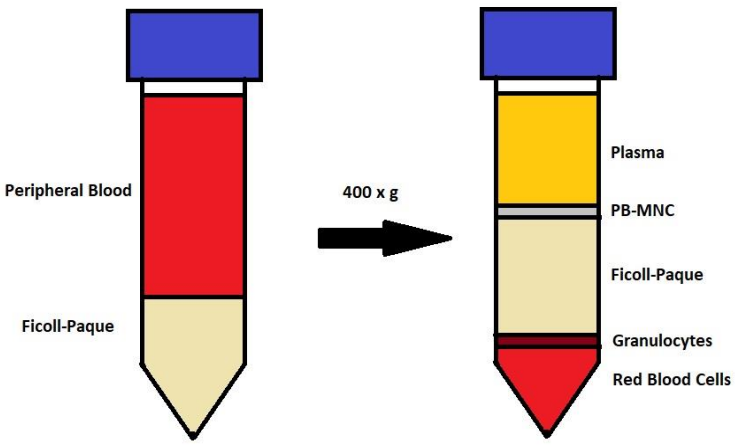
8. APPENDICES

8.1 APPENDIX 1: FICOLL-PAQUE DENSITY GRADIENT CENTRIFUGATION

1. Collection of the blood sample
    a. write down the volume of the sample
    b. write down time when taken and time when isolation started
2. Mix blood with 1xPBS (same volume as retrieved blood)
3. 20 ml Ficoll in a 50 ml falcon tube
4. Add carefully the sample (~20 ml) on top of the Ficoll
5. Centrifugation 400 g, 30 min, brake 1
6. Collect the serum fraction in a 50 ml Falcon tube
7. Collect the mononuclear cells (MNCs) carefully with sterile Pasteur pipette in a 50 ml Falcon tube
8. Fill the tube halfway with sterile 1xPBS and suspend to brake cell aggregates
9. Fill to 50 ml with PBS
10. Centrifugation 400 g, 15 min, brake 9
11. Discard the PBS
12. Wash the pellet with 1xPBS as described above, 400 g, 10 min, brake 9
13. Discard the PBS and dilute the cell pellet in 1 ml basal media
14. Count the cells with 2% acetic acid (390 µl acetic acid + 10 µl cells)



8.2 APPENDIX 2: PROTOCOL: COLLAGEN-MATRIGEL MIXTURE AND 3D CULTURE PLATING

Preparation:

1. Thaw Matrigel on ice overnight at +4°C. Make sure there is not any ice on the cap ring. Once thawed Matrigel can be stored s 1.0 ml aliquots at -20°C.
2. Chill pipette tips, all vials, and plates used during the work.
3. Always work on ice.

Materials:

1. Collagen type 1, rat tail
2. Sterile 10X PBS
3. Sterile 1 N NaOH
4. Matrigel

Mixing:

1. Add 10X PBS to the tube. (Formula: Solution's Final Volume/10 in mL)
2. Calculate wanted collagen concentration. Do not add to the tube yet. (Formula Final volume X Final collagen concentration in mg/mL/ Concentration in bottle)
3. Add to the tube the wanted volume of 1N NaOH. (Formula: (volume collagen to be added) x 0.023 mL)
4. Add Matrigel with the following formula: (final volume) – (volume collagen) – (volume 10X PBS) – (Volume 1 N NaOH) = volume Matrigel to add. Or the same volume with collagen and the rest will be sterile dH_2O.
5. Immediately add collagen, leave the tube on ice, and proceed with plating.

Plating:

1. Add the mixture to the wells to create the base layer.
2. Spread evenly.
3. Let the base layer to solidify for the time it takes to collect and calculate cells or at least 30 minutes in the incubator (+37°C, CO_2 5%)
4. Add the wanted number of cells on the base layer.

5. Incubate for 30 minutes in the incubator (+37°C, CO_2 5%) to allow cell attachment.
6. Add the top layer. Utilize well walls when pipetting to avoid disturbing the cells.
7. Incubate for 60 minutes.
8. Add cell medium to the top and change every four days.

8.3 APPENDIX 3: IMMUNOSTAINING CD31 AND PDGFRB ON CULTURES (2D)

Fixation:

1. Remove medium from wells
2. Add 200 µl of PFA to wells
3. Incubate 15 min RT
4. Remove PFA from wells
5. Wash with 1xPBS two times
6. Add 1xPBS to wells and store plates at +4°C

Blocking:

1. Do the solution 3% BSA in PBS if needed
2. Add 3%BSA in PBS to wells 200 µl
3. Incubate 60 min RT

Immunostaining the first day:

1. Dilute primary antibodies to 1% BSA in PBS
    - Anti-CD31 dilution (ab24590) 1:100
    - Anti-PDGFRB dilution (ab32570) 1:100
2. Remove blocking solution
3. Add primary antibody to wells 150 µl per well (**REMEMBER NEGATIVE CONTROL FOR EACH GROUP**)
4. Incubate overnight at +4°C

Immunostaining the second day:

1. Wash with PBS three times 5min
2. Dilute secondary antibodies to 1% BSA in PBS
    - Goat Anti-Mouse dilution 1:1000
3. Add secondary antibody to wells 150 µl per well
4. Incubate 1h RT in the dark
5. Wash with PBS three times 5min in the dark
6. Add DAPI 30 µl (DNA stain) and place the cover slide over the sample

8.4 APPENDIX 4: IMMUNOSTAINING ON 3D CULTURES

Fixation:

7. Remove medium from wells
8. Add 200 µl of PFA to wells
9. Incubate 3h RT
10. Remove PFA from wells
11. Wash with 1xPBS two times
12. Add 1xPBS to wells and store plates at +4°C

Immunostaining the first day:

5. Permeabilize with 0.5% Triton in PBS 10 min +4°C
6. Wash 3xPBS 10 min each
7. Blocking with 3% BSA in PBS 60 min RT
8. Dilute primary antibodies to 1% BSA in PBS
    - Anti-CD31 dilution 1:100
9. Remove blocking solution
10. Add primary antibody to wells 150 µl per well (**REMEMBER NEGATIVE CONTROL FOR EACH GROUP**)
11. Incubate overnight at +4°C

Immunostaining the second day:

7. Wash with PBS three times 20 min
8. Dilute secondary antibodies to 1% BSA in PBS
    - AF488 dilution 1:1000
9. Add secondary antibody to wells 150 µl per well
10. Incubate 1h RT in the dark
11. Wash with PBS three times 10 min in the dark
12. Do Hoechst solution 1 microgram per ml
13. Add 200 microliters per well, incubate 3 min
14. Rinse once with PBS
15. Using scalpel blade no. 11 cut through the holding plate and lift gently
16. Add cover glass

8.5 APPENDIX 5: MACS ISOLATION WITH CD34 AND CD14 MAGNETIC BEADS

First step: Ficoll-Plaque density gradient centrifugation

1. Collection of the blood sample
    a. write down the volume of the sample
    b. write down time when taken and time when isolation started
2. Mix blood with 1xPBS (same volume as retrieved blood)
3. 20 ml Ficoll in a 50 ml falcon tube
4. Add carefully the sample (~20 ml) on top of the Ficoll
5. Centrifugation 400 g, 30 min, brake 1
6. Collect the serum fraction in a 50 ml Falcon tube
7. Collect the mononuclear cells (MNCs) carefully with sterile Pasteur pipette in a 50 ml Falcon tube
8. Fill the tube halfway with sterile 1xPBS and suspend to brake cell aggregates
9. Fill to 50 ml with PBS
10. Centrifugation 400 g, 15 min, brake 9
11. Discard the PBS
12. Wash the pellet with 1xPBS as described above, 400 g, 10 min, brake 9
13. Discard the PBS and dilute the cell pellet in 1 ml basal media
14. Count the cells with 2% acetic acid (390 µl acetic acid + 10 µl cells)

Second step: MACS

1. Centrifugation, 400 g, 10 min, brake 9
2. Adjust to 10 x 10^6 in 80 µl of cold PBS+1%FBS in Eppendorf
3. Add the magnetic beads CD34 (130-046-702) or CD14 (130-050-201) 100 µl per 100 x 10^6 cells.
4. Incubation 1h, shaking, +4°C
5. Washing of MACS columns with 3ml cold PBS
6. Add the cells into the column, rinse Eppendorf with 1 ml cold PBS
7. Wash the column with 3 ml of cold PBS three times to get rid of unbound cells
8. Take the column out of the magnet and rinse with 5 ml of cold PBS to collect bound cells. Use the provided plunger (fast).
9. Count the isolated cells with trypan blue