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Ana Carolina Santo Mendes

MESENCHYMAL STROMAL CELL AND ENDOTHELIAL PROGENITOR CELL CO-CULTURES TO INDUCE VASCULARIZATION *IN VITRO*

Dissertação no âmbito do Mestrado em Bioquímica, orientada pela Professora Doutora Terhi Johanna Heino, co-orientada pela Professora Doutora Maria de Melim Vasconcelos de Vitorino Morais e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia.

Agosto de 2019

Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia
da Universidade de Coimbra

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The present study was performed in the Institute of Biomedicine of University of Turku, under the supervision of Professor Doctor Terhi Johanna Heino and the co-supervision of Professor Doctor Paula Maria de Melim Vasconcelos de Vitorino Morais from the Department of Life Science of University of Coimbra.

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Abbreviations

Ang-1	angiopoietin-1
Ang-2	angiopoietin-2
BM	bone marrow
BMP	bone morphogenic protein
BSA	bovine serum albumin
$CD14^-$ cell	CD14-negative cell
$CD14^+$ cell	CD14-positive cell
$CD14^+CD34^-$ cell	CD14-positiveCD34-negative cell
$CD14^-CD34^-$ cell	CD14-negativeCD34-negative cell
$CD34^-$ cell	CD34-negative cell
$CD34^+$ cell	CD34-positive cell
CNS	central nervous system
EC	endothelial cell
EPC	endothelial progenitor cell
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FGF	fibroblast growth factor
HC	hematopoietic cell
HSC	hematopoietic stem cell
HUVEC	human umbilical vein endothelial cell
iPSC	induced pluripotent stem cell
MACS	magnetic-activated cell sorting
MNC	mononuclear cell
MSC	mesenchymal stromal cells
NG2	neural/glial antigen 2
PB	peripheral blood

PB-MNC	peripheral blood- mononuclear cell
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PDGF-BB	platelet-derived growth factor-BB
PDGFRβ	platelet-derived growth factor receptor-β
PECAM	platelet endothelial cell adhesion molecule
PFA	paraformaldehyde
RT	room temperature
SMC	smooth muscle cell
SD	standard deviation
TGF-β	transforming growth factor β
VEGF	vascular endothelial growth factor
VEGFR1	vascular endothelial growth factor receptor 1
α-MEM	α-minimal essential medium
α-SMA	α-smooth muscle actin

Resumo

Ao longo das últimas décadas, as células estromais mesenquimais (MSCs) têm vindo a ser estudadas na tentativa de se tornarem numa nova ferramenta de regeneração de tecidos, devido à sua capacidade imuno-modulatória e à habilidade para reparar diferentes tipos de tecidos. No entanto, tem sido observado que em algumas situações de doença, o uso isolado de MSCs pode não ser suficiente devido à falta de vascularização nos tecidos. Neste sentido, a co-cultura de MSCs com células endoteliais (ECs) tem sido sugerida como uma nova abordagem para o aumento da vascularização. Contudo, para isolar ECs são necessárias cirurgias bastante invasivas. Como alternativa às ECs, tem sido proposto o uso de células progenitores endoteliais (EPCs), que facilmente são isoladas a partir de células mononucleares (MNCs), presentes em sangue periférico (PB). Em estudos anteriores, realizados neste grupo de investigação, foi demonstrado que a co-cultura de MSCs com MNCs promoveu a formação de estruturas tubulares, semelhantes a vasos sanguíneos, e que ocorreu diferenciação espontânea em ECs e pericitos, essenciais à angiogénese e à vascularização.

Este projeto de investigação teve como objetivos analisar o potencial de diferentes células progenitoras na indução da vascularização, quando em co-cultura com MSCs, e avaliar a origem de ECs e pericitos anteriormente encontrados nas nossas co-culturas. Para tal, recorrendo à técnica *magnetic-activated cell sorting* (MACS) e ao uso dos marcadores CD14 e CD34, isolou-se as EPCs a partir de PB-MNCs. Em seguida, com recurso ao sistema IncuCyte e ao ImageJ, estudou-se as variações na morfologia celular e recorrendo a imunocitoquímica e a qPCR, avaliou-se ainda a origem das ECs e dos pericitos.

Os resultados obtidos, mostraram que as células $CD14^+$, $CD34^+$ e $CD34^-$, quando em co-cultura com MSCs, adquirem uma morfologia semelhante às ECs (em forma de fuso), indicando que as MSCs são responsáveis por providenciar os sinais essenciais para o comprometimento das células neste tipo de morfologia. Observou-se que, de entre todas as co-culturas avaliadas, as co-culturas MSC-$CD14^+$ e MSC-$CD34^+$ foram as que apresentaram uma maior capacidade de vascularização. Provou-se ainda que a diferenciação em ECs ocorre apenas a partir de MNCs. Enquanto, por outro lado, a diferenciação em pericitos tanto ocorre a partir de MSCs como de MNCs. Foi ainda interessante verificar, que quando em co-cultura com MSCs, ambas as células $CD14^+CD34^-$ e $CD14^-CD34^-$ se diferenciam em pericitos, sugerindo que nas MNCs existem pelo menos duas populações diferentes de células capazes de se diferenciarem em pericitos.

Em suma, os resultados mostraram que a co-cultura de PB-MNCs com MSCs apresenta potencial angiogénico, comprovado pela diferenciação em ECs e pela presença de diferentes populações de pericitos. Salientando assim, o potencial do uso de células derivadas de sangue em engenharia de tecidos e terapias de regeneração.

PALAVRAS-CHAVE: células estromais mesenquimais, células progenitoras endoteliais, pericitos, co-culturas, vascularização

Abstract

Mesenchymal stromal cells (MSCs) have been explored as a new tissue regeneration tool due to their immunomodulatory potential and the ability to repair several tissues. However, in some situations of disease and injury, using only MSCs alone is may not enough due to the lack of vascularization in the tissues. Therefore, co-cultures of MSCs with endothelial cells (ECs) or they progenitors have been suggested as a novel approach to improve vascularization. Nevertheless, isolating primary ECs from blood vessels requires invasive surgeries. As an alternative to ECs, endothelial progenitor cells (EPCs), which can be found in peripheral blood mononuclear cell (PB-MNC) fraction, can be used.

In previous studies, our research group has demonstrated that human MSCs co-cultured with MNCs promotes the formation of vessel-like tubular structures and that there is spontaneous EC and pericyte differentiation, which is essential for angiogenesis and vascularization.

In this research project, we aimed to analyze the potential of different progenitor cells in peripheral blood to induce vascularization in co-cultures with MSCs and to evaluate the origin of ECs and pericytes previously found in our co-cultures. For that, we isolated EPCs from PB-MNCs by magnetic-activated cell sorting (MACS) using CD14 and CD34 surface markers, studied cell morphology changes by IncuCyte system and ImageJ and evaluated EC and pericyte differentiation by immunocytochemistry and qPCR.

Our results demonstrated that $CD14^+$, $CD34^+$ and $CD34^-$ cells in co-culture with MSCs acquired an EC-like morphology (spindle-shaped), indicating that MSCs provide the essential signals to commit the cells into spindle-shaped morphology. We showed that among all the evaluated co-cultures, MSC-$CD14^+$ and MSC-$CD34^+$ co-cultures presented the highest vasculogenic capacity. We also proved that MNCs are the source of ECs in our co-cultures, while the pericytes originate both from MSCs and MNCs. Furthermore, $CD14^+CD34^-$ and $CD14^-CD34^-$ cells in co-culture with MSCs showed pericyte differentiation, which interestingly suggests that within MNCs there are at least two different populations of pericytes.

In conclusion, our results demonstrate that co-cultures of PB-MNCs and MSCs possess angiogenic capacity, shown by the EC differentiation, as well as the presence of different subpopulations of pericytes. This highlights the potential of the use of blood-derived cells in tissue engineering and tissue regeneration therapies.

***KEYWORDS**:* mesenchymal stromal cells, endothelial progenitor cells, pericytes, co-cultures, vascularization

1. Introduction

1.1. Tissue Regeneration

Regeneration is the ability to replace tissue structure and function after disease or injury and it is based on our self-healing potential (Sandoval-Guzmán and Currie, 2018). The regenerative potential is different between different organs and tissues (Ferreira et al., 2018). For instance, bone, cartilage, nerves, muscle, skin, pancreas, blood and liver are highly regenerative tissues, while central nervous system (CNS), kidney and heart present limited regenerative potential (Tal et al., 2010; Mokalled and Poss, 2018; Giannoudis and Pountos, 2005).

Mesenchymal stromal cells (MSCs) have been explored as a new tissue regeneration tool due to their immunomodulatory potential and the ability to repair several tissues (Ferreira et al., 2018) (Fig.1). Initially, it was thought that MSCs could only be used in bone and cartilage regeneration (Caplan, 1991). Nevertheless, MSCs have recently been tested in the treatment of stroke, myocardial infarction, multiple sclerosis, diabetes, lung injuries, liver cirrhosis and auto-immune diseases, such as lupus and Crohn's disease (Ferreira et al., 2018).

MSCs can secrete various soluble factors, cytokines, chemokines and growth factors, which contribute to their immunomodulatory potential (Madrigal et al., 2014) (Fig. 1). These different factors in different microenvironments can stimulate vascularization, promote tissue remodelling, modulate the immune system, recruit other cells types and inhibit cell death and fibrosis (Ferreira et al., 2018).

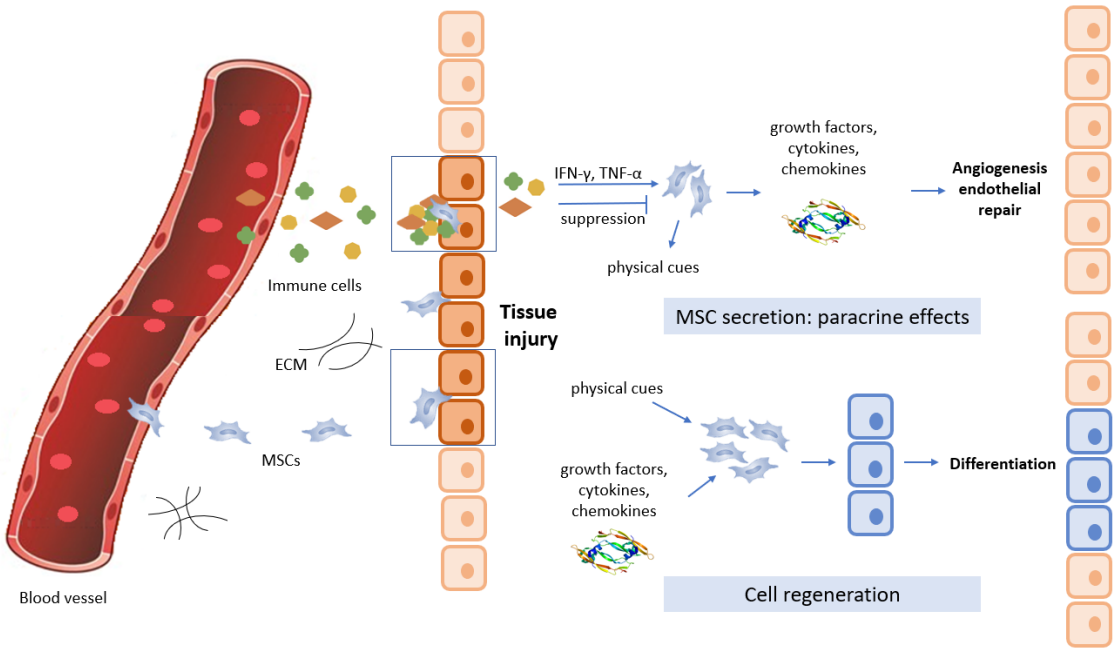

Figure 1. Representation of the immunomodulatory and regenerative potential of MSCs. Modified from Bunpetch et al., 2019.

To mimic the physiological *in vivo* conditions, MSCs can be cultured in three-dimensional cell cultures. It has been observed that three-dimensional cultures of MSCs induce the production of factors associated with cell proliferation and survival as well as vascularization, which in turn leads to increased immunomodulatory, anti-apoptotic, anti-fibrotic and angiogenic activities of MSCs (Ferreira et al., 2018).

The therapeutic potential of MSCs is incontestable. However, in some situations of disease and injury, using MSCs alone may not be enough. For example, porous biomaterials can be seeded with MSCs *in vitro*; nonetheless, implantation often fails *in vivo* due to the lack of nutrients and oxygen in the implant parts which are non-vascularized (Muschler et al., 2004). Vascularization is the key to success for tissue regeneration and engineering. New evidence has suggested that vascularization can be improved by co-culturing MSCs with endothelial cells (ECs) or they progenitors (Joensuu et al., 2018).

1.2. Vascularization

Blood vessels provide nutrients and oxygen to cells and are responsible to collect waste products from tissues (Risau and Flamme, 1995). The inner layer of blood vessels is composed of ECs, while the outer layer is composed of pericytes.

Blood vessels form an extensive and dense network, being essential for all tissues. The lack of functional or structural blood vessels promotes several diseases, such as stroke, myocardial infarction and neurodegenerative disorders (Potente et al., 2011). On the other hand, abnormal remodelling of blood vessels or excessive vascular growth contributes to ocular diseases, inflammatory disorders and cancer (Carmeliet, 2003; Folkman, 2007).

There are different mechanisms of blood vessel formation and regeneration, known as vasculogenesis, angiogenesis and arteriogenesis (Fig. 2).

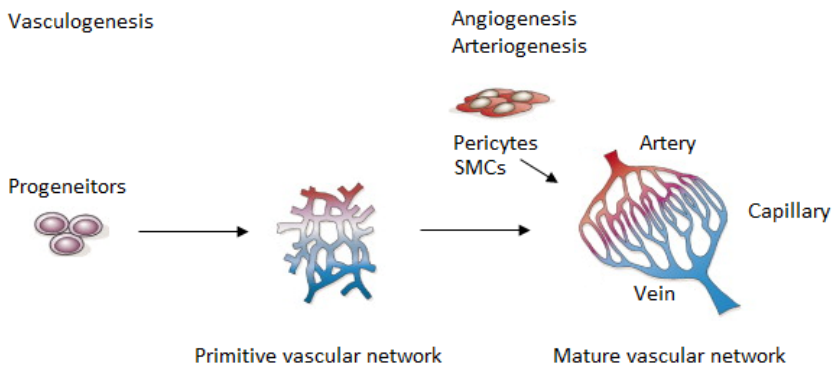

Figure 2. Vascular development. Modified from Carmeliet, 2005.

1.2.1. Vasculogenesis

Vasculogenesis is a mechanism of de novo formation of blood vessels. This process occurs both in the embryo and adult. During the embryonal development, after mesoderm formation, endothelial progenitor cells (EPCs), called angioblasts, and hematopoietic progenitor cells form solid cellular clumps called blood islands (Risau and Flamme, 1995) (Fig. 3). The outer cells of theses solid clumps are subsequently differentiated into ECs, while the inner cells turn into hematopoietic cells (HCs) (Gonzalez-Crussi, 1971). After blood island differentiation and fusion, occurs the differentiation and organization of ECs into a primary capillary plexus and later into a primitive vascular network (Risau and Flamme, 1995). A close association of ECs and HCs in the blood islands has lead into the assumption that both cell types are derived from a common progenitor, known as hemangioblast (Sabin, 1917; Risau and Flamme, 1995). This assumption is further supported by the common expression of the same genes, such as CD34, platelet endothelial cell adhesion molecule (PECAM), which is more commonly known as CD31, and CD133 in both angiogenic and hematopoietic lineages (Fina et al., 1990; Newman et al., 1990; Fischer et al., 2006).

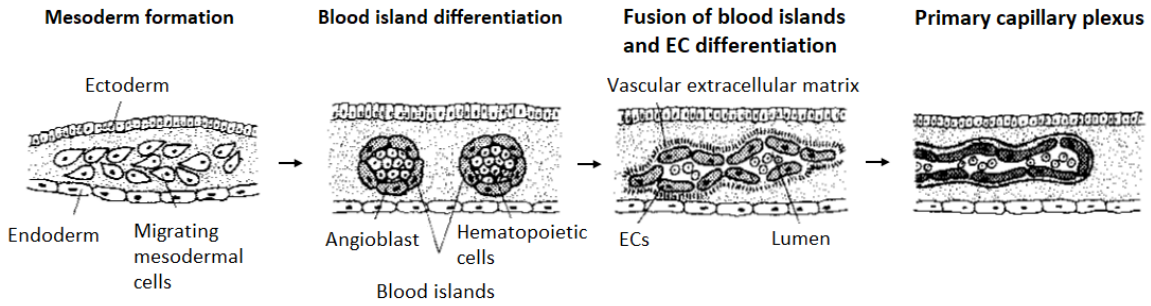

Figure 3. Vasculogenesis in the embryo. Modified from Risau and Flamme, 1995.

As previously mentioned, vasculogenesis can also occurs in the adult, in ischemic, malignant or inflamed situations (Carmeliet, 2003). In this case, bone marrow-derived EPCs are recruited into the circulation and migrate to sites of neovascularization to stimulate new blood vessel growth by releasing angiogenic factors (Fischer et al., 2006) (Fig 4).

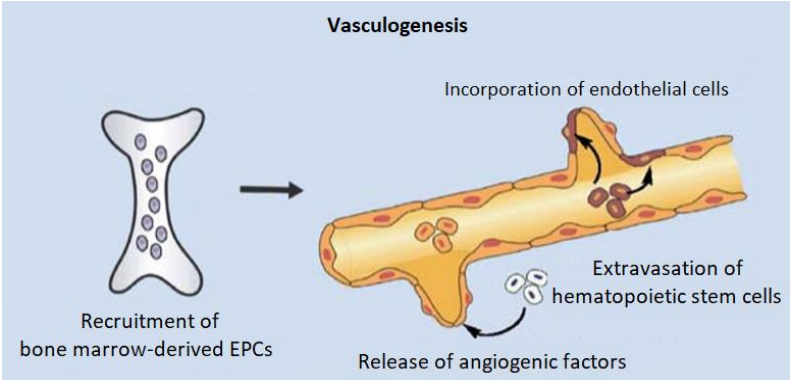

Figure 4. Vasculogenesis in adult. Modified from Fischer et al., 2006.

1.2.2. Angiogenesis

Angiogenesis is the formation of new blood vessels from pre-existing ones. Normally it occurs in the cycling ovary and during pregnancy. Nevertheless, angiogenesis is also reactivated in situations of hypoxia, inflammation, wound healing and tissue repair (Carmeliet, 2003). This blood vessel regeneration mechanism takes place by the detachment of pericytes from vessels and thereafter ECs break from their original position and stimulate the sprouting, branching and formation of new blood vessels (Phng and Gerhardt, 2009) (Fig. 5).

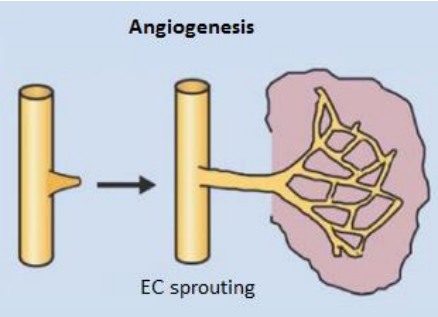

Figure 5. Angiogenesis. Modified from Fischer et al., 2006.

Angiogenesis is a well-orchestrated process dependent of signalling factors and various pathways. Vascular endothelial growth factor (VEGF) is one of the essential growth factors involved in angiogenesis, being responsible for stimulating the survival, proliferation, migration and differentiation of ECs (Ferrara et al., 2001). Fibroblast growth factor (FGF) is another key growth factor in angiogenesis, where it acts by promoting EC proliferation, migration and differentiation (Iruela-Arispe and Davis, 2009). Angiopoietins, angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2), are responsible for stabilizing and destabilizing the vascular networks (Rouwkema and Rademhosseini, 2016). Ang-1 mediates EC junctions, promotes EC survival and endothelial-mural cell interactions (Rouwkema and Rademhosseini, 2016). Ang-2, on the other hand, is produced during vascular remodeling by ECs and its effect is VEGF-dependent (Rouwkema and Rademhosseini, 2016). In the presence of VEGF, Ang-2 promotes vascular remodeling, while in the absence of VEGF, Ang-2 is responsible for vessel regression and EC death (Rouwkema and Rademhosseini, 2016). Platelet-derived growth factor (PDGF) plays an important role in vascular maturation and remodelling (Rouwkema and Rademhosseini, 2016). All these growth factors are also important for vasculogenesis and arteriogenesis, but they have mostly been studied with respect to angiogenesis.

In some cases, the balance between pro and anti-angiogenic molecules is disturbed, causing an angiogenic switch, which lead to more than 70 disorders, such as malignant, inflammatory and ocular disorders and affect many processes, as for example in obesity, multiple sclerosis, arthritis, asthma, cirrhosis, endometriosis, bacterial infections and autoimmune diseases (Carmeliet, 2003). However, in other diseases, such as amyotrophic lateral sclerosis, Alzheimer's diseases, diabetes,

hypertension, stroke, Crohn's disease, atherosclerosis and cardiac failure angiogenesis is insufficient and occurs vessel regression (Carmeliet, 2003).

1.2.3. Arteriogenesis

After the establishment of vascular networks by vasculogenesis and angiogenesis, it is necessary to ensure that vascular networks are functional, stable, durable and non-leaky. For that, the recruitment of pericytes and smooth muscle cells (SMCs) is required for a process known as arteriogenesis (Fig. 6). Besides that, pericytes and SMCs can regulate EC survival, proliferation and differentiation as well as assist ECs in obtaining their specialized functions (Fischer et al., 2006).

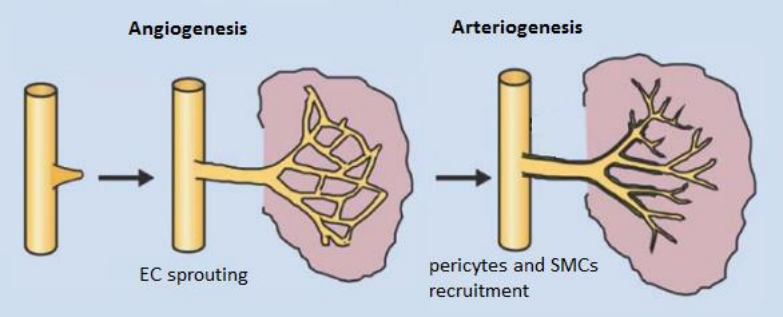

Figure 6. Arteriogenesis. Modified from Fischer et al., 2006.

1.2.4. Vascularization in tissue regeneration

Apart from cartilage, cornea and epidermis, that are non-vascular, all tissues communicate with blood vessels that provide them oxygen and nutrients (Fischer et al., 2006). Thus, promoting vascularization is an essential goal in tissue regeneration and engineering (Fu and Wang, 2018).

In the last decade, it was shown that co-culturing cells, such as ECs, which secrete angiogenic factors with mural precursors, as for example embryonic fibroblasts or MSCs, can induce vascular networks (Levenberg et al., 2005; Fuchs et al., 2007; Evensen et al., 2009). However, the isolation of primary ECs requires invasive procedures from large diameter vessels (Peters, 2018). As an alternative to ECs, human umbilical vein endothelial cells (HUVECs), EPCs and induced pluripotent stem cells (iPSCs) can be used (Pellegata et al., 2018).

HUVECs are vasculature-derived ECs and very popular due to their simple isolation, low cost and high angiogenic potential (Staton et al., 2009). Nevertheless, HUVECs, when translated into several animal models, have shown poor engraftment and anastomosis (Pellegata et al., 2018). Besides that, they are fully differentiated ECs and have no potential to differentiate into other endothelial phenotypes (Fu and Wang, 2018).

EPCs, on the other hand, are a recent subject of interest due to the ability of organ-specific commitment into several endothelial phenotypes (Fu and Wang, 2018). They can be found in adult peripheral blood (PB) (Asahara et al., 1997), umbilical cord blood (Murga et al., 2004) and bone marrow (BM) (Hamilton et al., 2004).

More recently, iPSCs have been studied as a promising source of ECs. For example, Orlova et al. showed that human iPSCs-derived ECs, when injected into the zebrafish model, were able to form blood vessels (Orlova et al., 2014). However, although promising, the clinical use of iPSC-derived ECs is still a concern given their tumorigenic potential (Cossu et al., 2018).

1.3. Important cells for vascularization

1.3.1. Mesenchymal stromal cells

Mesenchymal stromal cells were identified by Friedenstein et al. as non-hematopoietic, spindle-shaped, plastic-adherent and colony forming cells in BM (Friedenstein et al., 1968). Afterwards, it was discovered that, in addition to BM, MSCs exist in other tissues, such as skeletal and smooth muscle, PB, umbilical cord blood, adipose tissue, synovia and in perivascular niches of several postnatal tissues and organs, contributing to normal tissue turnover (Galmiche et al., 1993; Bosch et al., 2000; Erices et al., 2000; Zuk et al., 2001; Kuznetsov et al., 2001; Crisan et al., 2008; Karystinou et al., 2009; Chapurlat and Confavreux, 2016).

Over the years, several studies have demonstrated the potential of MSCs to generate cartilage and bone and to differentiate into adipogenic, chondrogenic and osteogenic lineages (Friedenstein et al., 1968; Long et al., 1990; Caplan, 1991; Pittenger et al., 1999; Adams and Scadden, 2006) (Fig. 7).

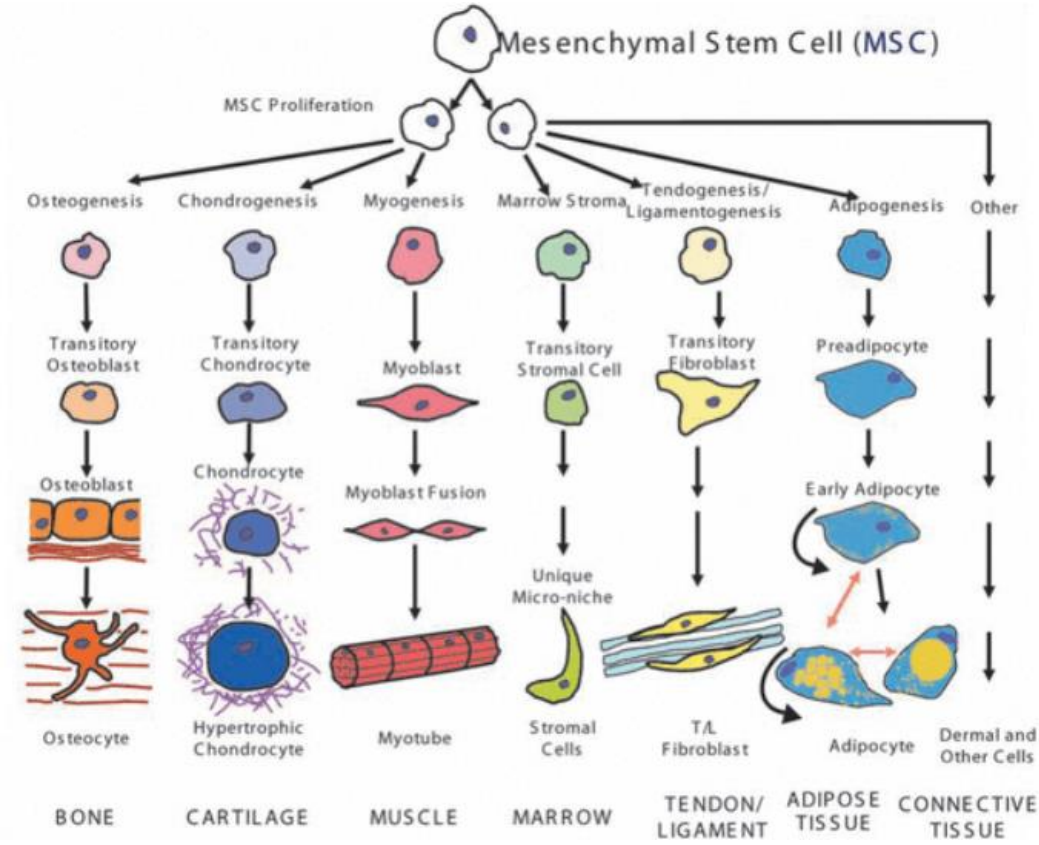

Figure 7. Multi-potentiality of MSCs to generate different cells types. Modified from Caplan, 2010.

More recently, it has also been demonstrated that MSCs can promote tissue repair in other tissues associated with cardiac, neurological, auto-immune, liver and lung diseases (Ferreira et al., 2018). Besides that, MSCs regulate the differentiation, cell senescence and function of other cells, and it has been suggested that they have potential to enhance the vascularization of several tissues, due to their role in hematopoietic stem cell (HSC) niches and the ability to enhance angiogenesis (Méndez-Ferrer et al., 2010; Nombela-Arrieta et al., 2011; Guo et al., 2012). In addition, several studies have documented that MSCs have immunomodulatory properties (Horwitz et al., 1999; Le Blanc and Mougiakakos, 2012; De Miguel et al., 2012; Ma et al., 2014).

MSCs secrete many different growth factors, such as bone morphogenic proteins (BMPs), VEGF, transforming growth factor β (TGF-β) and platelet-derived growth factor-BB (PDGF-BB). BMPs regulate osteogenesis (Gaur et al., 2005), while VEGF can both accelerate bone regeneration and promote angiogenesis (Kleinheinz et al., 2005). TGF-β expressed by MSCs is responsible for bone regeneration (Ferreira et al., 2018), while when expressed by pericytes and ECs, it controls maturation, proliferation and contributes for vessel stabilization (Gaceb et al., 2018). PDGF-BB when secreted by MSCs is involved in bone regeneration (Ferreira et al., 2018) and when secreted by ECs, through the binding to platelet-derived growth factor receptor-β (PDGFRβ) on pericytes, causes their proliferation, recruitment and migration to sites of angiogenesis and neovascularization (Gaceb et al., 2018)

Extensive attempts have been made to identify a specific marker profile for MSCs. Because of this, the International Society for Cellular Therapy has proposed the minimal phenotypic and functional criteria to identify MSCs (Dominici et al., 2006). This definition states that MSCs must differentiate into osteoblasts, adipocytes and chondrocytes *in vitro*, express the cell surface markers CD105, CD73, and CD90 and be plastic adherent (Dominici et al., 2006). However, MSCs should not express the hematopoietic progenitor and endothelial marker CD34, the monocyte and macrophage markers CD11b and CD14, the leukocyte marker CD45, the B-cell markers CD19 and CD79a and HLA-DR (Dominici et al., 2006). Years later, various new markers have been suggested to define MSCs, such as CD271, CD200, CD49a, CD24, SSEA4, GD2, Stro-1, CD146, Sca1, PDGFRα and PDGFRβ (Pontikoglou et al., 2011).

More than 25 years ago, MSCs were named mesenchymal stem cells by Caplan (Caplan, 1991). Nevertheless, many researchers, including Caplan, agree that there is an issue with “stem cell” part of the name, due to fact that MSCs never differentiate into all tissues, but only secrete different factors and biomolecules (Caplan, 1991, 2010, 2017). To solve this discrepancy of nomenclature, other names have been suggested, such as mesenchymal stromal cells and medicinal signaling cells (Horwitz et al., 2005; Caplan, 2010, 2017). However, no consensus has yet been reached and e.g. marrow stromal cells, multipotent stromal cells, mesodermal stem cells, mesenchymal stromal cells and medicinal signaling cells have been the names used to define the same cell type (Caplan, 2017).

MSCs can be found in several tissues, but mainly due to the higher amount in BM, umbilical cord and adipose tissue, these are the most common sources (Kern et al., 2006; Hass et al., 2011). There are several protocols available for MSC isolation and culture. Usually, these protocols include density gradient centrifugations of the sample in Ficoll or Percoll. This allows the separation of mononuclear cell (MNC) fraction, which contains MSCs, HSCs, monocytes, T cells and B cells, and separate them from the other marrow constituents, such as lipids, plasma and red blood cells.

In MSC cultures, there is an initial phase of high proliferation, followed by a plateau, where the proliferation is maintained until a limit is reached (Banfi et al., 2000). This occurs because the growth of cells is limited due to cellular senescence (Hayflick, 1965). In addition, increasing passages and the expansion of MSCs causes loss of spindle-shaped morphology (Hayflick, 1965). It has also been shown that after 4 or 5 passages random chromosomes alterations can take place and that with higher the passages, there is also increase in karyotype alterations and decrease in the proliferation and multi-potency capacity (Banfi et al., 2000; Satija et al., 2007). However, the adipogenic and osteoblastic differentiation capacity seem unaffected (Binato et al., 2013).

1.3.2. Pericytes

Pericytes are contractile cells located on the basement of blood vessels, separated from ECs by a basement membrane synthesized by both cell types (Bergers and Song, 2005; Díaz-Flores er al., 2009). Pericytes are ubiquitously present in all tissues (Shepro and Morel, 1993); however, they play an essential role in the CNS, being responsible for coordinating the neovascular functions, such as blood-brain barrier formation and maintenance, angiogenesis, vascular stability and clearance of toxic cellular products and thereby promoting CNS homeostasis and neuronal function (Winkler et al., 2011). They are a heterogeneous cell population and lack specific markers, which makes it difficult to identify them (Gaceb et al., 2018). Alpha-smooth muscle actin (α-SMA) has been used as a pericyte marker. However, it has been observed that is not an optimal marker to recognize early stage pericytes in neovascularization, and thus PDGFRβ and neural/glial antigen 2 (NG2) have emerged as appropriate pericyte markers for initial states of vessel formation (Stallcup, 2018). PDGFRβ is a transmembrane receptor, responsible to recruit pericytes in response to PDGF-BB by ECs in angiogenesis (Hellström et al., 1999). NG2, on the other hand, is a cell surface component responsible for pericyte proliferation, motility and also for maturation and formation of EC junctions (Stallcup, 2018).

It has been demonstrated that pericytes are responsible to support ECs, to maintain blood vessels, to regulate angiogenesis, blood flow and vascular permeability as well as to support tissue repair and regeneration (Hellström et al., 2001; Pallone and Stilldorff, 2001; Enge et al., 2002; Chen et al., 2014; Bodnar et al., 2016) (Fig. 8). Pericytes perform their functions by secreting growth factors, angiogenic molecules, inflammatory molecules and extracellular vesicles (Gaceb et al., 2018)

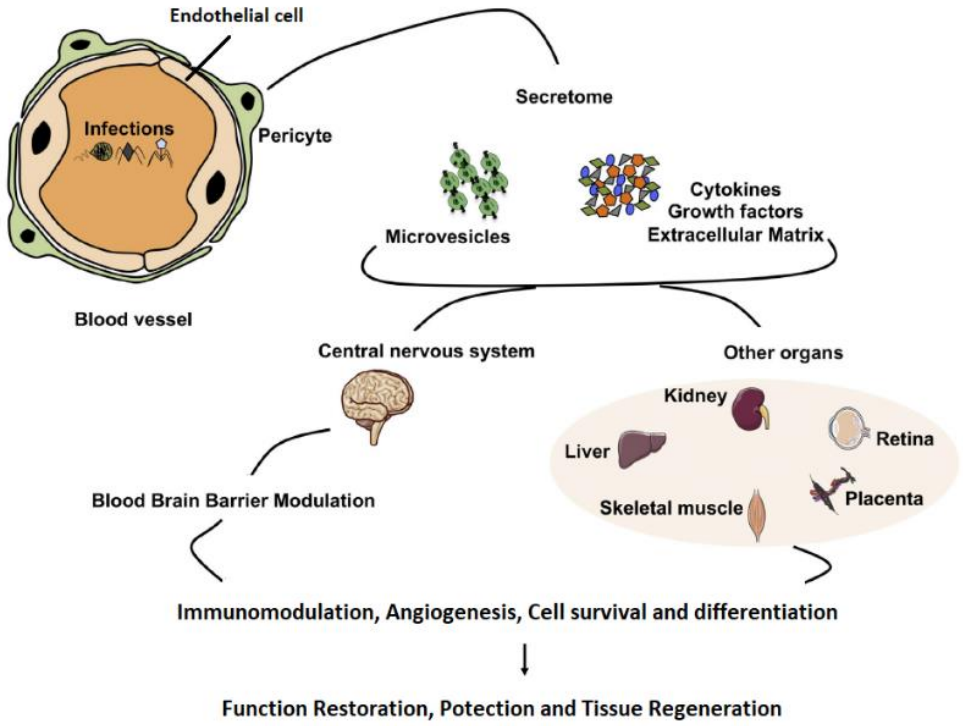

Figure 8. Pericytes. Modified from Gaceb et al., 2018.

Some of the growth factors and angiogenic molecules expressed by pericytes are Ang-1, VEGF, TGF-β and PDGF-BB (Gaceb et al., 2018). Ang-1 is essential to promote stabilization and maturation of blood vessels and regulate vascular remodeling contributing to tissue repair (Sundberg et al., 2002). The other growth factors were discussed in more detail in Chapter 1.3.1. Since angiogenesis plays an essential role in tissue regeneration (Armulik et al., 2010) it is possible that pericytes have potential in cell therapy for tissue regeneration and engineering.

Over the years, the relation between MSCs and pericytes has been discussed. Some studies have suggested that MSCs could differentiate into pericytes (Loibl et al., 2014), while other studies suggest that pericytes isolated from different tissues can give rise to MSCs (Caplan, 2008; Crisan et al., 2008). However, no comprehensive and detailed comparison of MSC and pericyte properties has been performed, neither *in vivo* nor *in vitro*. Thus, the final answer to the interrelationship between these two cell types remains unsolved.

1.3.3. Endothelial progenitor cells

In 1997, Asahara et al. identified and described EPCs present in adult PB (Asahara et al., 1997). Nowadays it is known that umbilical cord blood and BM are additional sources of EPCs (Murga et al., 2004; Hamilton et al., 2004).

EPCs have a key role in neovascularization (Takahashi et al., 1999) and in postnatal vasculogenesis and angiogenesis (Asahara et al., 1997; Eguchi et al., 2007). They secrete cytokines and growth factors in damaged tissues (Wang et al., 2018), which leads to proliferation, migration and differentiation into ECs (Luttun et al., 2002). However, EPCs have not yet the characteristic mature endothelial cell markers (Luttun et al., 2002).

EPCs express CD34, CD14 and CD133. CD34 is a cell surface glycoprotein expressed on HSCs and ECs. Nevertheless, ECs remain positive for CD34, while HSCs cease to express CD34, when differentiating into mature HCs (Asahara et al., 1997). Thus, CD34 antigen is a widely accepted cell marker to identify ECs and their progenitors (Murga et al., 2004). Despite these evidences, Harraz et al. have suggested the existence of a CD34-negative ($CD34^-$), CD14-positive ($CD14^+$) cell population, which is able to differentiate into endothelial-like cells (Harraz et al., 2001). CD34 inhibits the differentiation of progenitor cells and it is responsible for enhancing proliferation, angiogenesis, osteogenesis and fracture healing (Matsumoto et al., 2006; Kuroda et al., 2014). CD14 is a co-receptor for a lipopolysaccharide and considered as a monocyte and macrophage marker (Ziegler-Heitbrock and Ulevitch, 1993). However, it has also been observed that $CD14^+$ cells can, in the presence of angiogenic growth factors, obtain spindle-shaped morphology and differentiate into endothelial-like cells, which suggests the possibility of using CD14 marker to isolate EPCs (Pujol et al., 2000; Zhang et al., 2005).

There are several important endothelial markers that can be used to distinguish EPCs from more mature ECs, such as CD31 and vascular endothelial growth factor receptor 1 (VEGFR1). CD31 is an integral membrane glycoprotein, essential for endothelial cell-cell adhesion (Albelda et al., 1991). The expression of CD31 reflects the adhesion and differentiation of ECs during their migration. VEGFR1 is a member of the VEGFR family, strongly expressed in vascular ECs (Dumont et al., 1995), responsible for regulate angiogenesis and vasculogenesis during development (Shibuya, 2011).

Additionally, it has been observed that EPCs co-cultured with MSCs or fibroblasts, can generate a durable and stable vascular network (Chen et al., 2012). Thus, EPCs and they co-cultures show great potential for therapeutic vascularization and tissue regeneration (Peters, 2018).

Blood-derived EPCs can be found within the peripheral blood-mononuclear cell (PB-MNC) fraction. To isolate EPCs from PB it is necessary to first isolate the PB-MNC fraction using a density gradient centrifugation protocol, which allows the separation of MNCs from the other blood constituents. After collection of the PB-MNCs, two different techniques can be used: fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS).

FACS is a technique that allows separate live and functional cell populations into sub-populations based on fluorescent labelling of certain surface markers (Picot et al., 2012) (Fig.9). In this technique, after staining the cells using fluorophore-conjugated antibodies, they can be separated depending on which fluorophore they have been stained with (Picot et al., 2012).

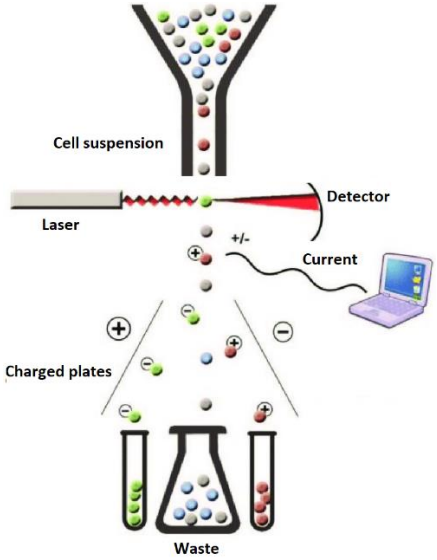

Figure 9. Fluorescence-activated cell sorting. Modified from Mehanna, 2017.

MACS, on the other hand, allows the separation of live and functional cell populations into subpopulations based on magnetic labeling. For that, magnetic microbeads which are conjugated with specific antibodies against a precise cell surface antigen, are used. They do not activate cells, are of small size and do not have to be removed for downstream applications. MACS technology is based on three steps (Fig. 10). These three steps are magnetic labelling, magnetic separation, and elution of labelled cells. Magnetic labelling consists of the detection of specific antigens using magnetic microbeads. The detection of this specific antigens allows the isolation of the specific cell populations. Magnetic separation, the second step, involves the separation of the labelled cells, known also as a positive fraction, from the other cells present in the sample, known as a negative fraction. This separation takes place with a magnetic separator present in the column, where the positive fraction is being held. The last step of MACS, called elution of labelled cells, consists of the removal of the column from the magnetic separator, elution of the positive fraction and collecting it.

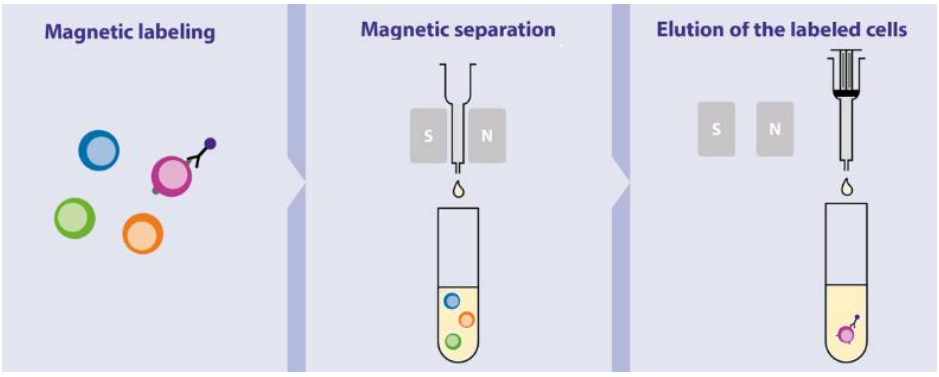

Figure 10. Magnetic-activated cell sorting. Modified from Miltenyibiotec.com, 2019.

FACS has been widely used to isolate EPCs and ECs from PB-MNCs (Asahara et al., 1997; Pujol et al., 2000; Harraz et al., 2001). Nevertheless, several studies have shown that when FACS and MACS are compared, the isolation with MACS- is quicker, easier and leads to higher viability and higher yield compared to FACS (De Wynter et al., 1995; Gomes at al., 2001; Willasch et al., 2010; Sutermaster and Darling, 2019). This suggests that MACS method is more attractive when selecting a low-abundance cell population or having a sensitive sample (Sutermaster and Darling, 2019).

1.4. Aims

In previous studies, it has been demonstrated that human MSCs co-cultured with MNCs promote the formation of vessel-like tubular structures and that there is spontaneous EC and pericyte differentiation, which could induce and enhance angiogenesis (Joensuu et al., 2011; Joensuu et al., 2018).

We hypothesize that the cellular and molecular interactions between MSCs and EPCs are important for vascularization and by studying them *in vitro* we will be able to improve future cell-based tissue engineering applications. Therefore, the aims of this research project were to analyse the potential of different progenitor cells in PB to induce vascularization in co-cultures with MSCs and to evaluate the origin of ECs and pericytes previously found in our co-cultures. For that, several experiments were performed to evaluate cell morphology and expression of EC and pericyte markers in co-cultures.

2. Materials and methods

2.1. Culture of MSCs

Human MSCs used in these experiments were from a 21-years-old healthy female donor and were previously isolated from iliac BM, stored in liquid nitrogen and culture expanded. The cells were cultured in cell density of 1 000 cells/cm^2 in 75 cm^2 cell culture flasks using basal medium, containing α-minimum essential medium (α-MEM; Gibco, Paisley, UK), 100 µg/mL streptomycin, 100 IU/mL penicillin (PS, Gibco, Paisley, UK) and 9% fetal bovine serum (FBS, US-origin, 16000-044, Gibco). Half of the medium was changed every 3 or 4 days. When cell culture flasks became confluent, 0,05% Trypsin/EDTA (Gibco, Paisley, UK) was used to detach the cells from the flask. The cells were counted and re-plated at 1 000 cells/cm^2. Passages between 5 and 8 were used for the experiments.

2.2. Co-culture of MSCs and MNC fractions

2.2.1. Isolation of MNCs and co-culture with MSCs

To co-culture MSCs and MNCs, MSCs were collected from cell culture flasks using 0,05% Trypsin/EDTA (Gibco, Paisley, UK) and plated into chamber slides (2 500 cells/cm2), 24-well cell culture plates (2 500 cells/cm^2) and T25-cell culture flasks (1 000 cells/cm^2). After 3-4 days of culturing, MNCs were isolated from PB sample (average of 41,5 mL) of a 25-years-old healthy male donor using Ficoll density gradient centrifugation (Ficoll-Paque Plus). MNCs were counted and part of them were used directly for co-cultures with MSCs (50 000 cells/cm^2), while other MNCs were used for the MACS cell separation method (Miltenyi Biotec) to magnetically separate $CD14^+$ and CD34-positive ($CD34^+$) cell fractions.

2.2.2. Isolation of $CD14^+$ cells and co-culture with MSCs

MACS was used to isolate $CD14^+$ cells from the whole MNC population. After isolating the whole MNC fraction, 80 µL of cold buffer (1x PBS + 1% FBS) and 20 µL of CD14 microbeads (CD14 MicroBead Kit, Miltenyi Biotec, cat., 130-050-201) were added per 10^7 total cells and incubated in shaking for 15 minutes at +4°C. This first step called magnetic labelling, was followed by the magnetic separation step. A LS column (Miltenyi Biotec, cat., 130-042-401) was placed in the magnetic field (MidiMACS Separator, Miltenyi Biotec, cat., 130-042-302) and a 50 mL falcon was placed underneath the column. Three mL of cold buffer and the solution containing the MNCs were added into the column. Finally, the column was rinsed three times with 3 mL of cold buffer. This magnetic separation step allowed the collection of CD14-negative ($CD14^-$) fraction. Then, the column was removed from the magnetic field and 5 mL of cold buffer was pipetted into the column and the magnetically labelled cells were flush out to a 15 mL falcon by firmly pushing the plunger into the column. This final step, called elution of labelled cells, allowed the collection of the $CD14^+$ fraction. After obtaining the $CD14^+$ and $CD14^-$ fractions, the cells were counted and plated in co-

culture with MSCs (50 000 cells/cm^2). Co-culture with whole MNC fraction was used as control. Basal medium was used to culture the cells and half of medium was changed every 3 or 4 days.

2.2.3. Isolation of CD34$^+$ cells and co-culture with MSCs

MACS was also used to isolate the CD34$^+$ cells from the whole MNC fraction. After isolating the whole MNC population, 300 µL of cold buffer (1x PBS + 1% FBS), 100 µL of FcR Bloking Reagent (Miltenyi Biotec, cat., 130-046-702) and 100 µL of CD34 microbeads (CD34 MicroBead Kit, Miltenyi Biotec, cat., 130-046-702) were added per 10^8 total cells and incubated in shaking for 30 minutes at +4°C. This magnetic labelling step was followed by the magnetic separation (see Chapter 2.2.2 above). This step allowed the collection of CD34$^+$ and CD34$^-$ fractions, which were counted and the cells were plated in co-culture with MSCs (50 000 cells/cm^2). Co-culture with whole MNC fraction was used as control. Basal medium was used to culture the cells and half of medium was changed every 3 or 4 days.

2.2.4. Isolation of CD14$^+$CD34$^-$ cells and co-culture with MSCs

MACS was also used to isolate the CD14$^+$ and CD14$^-$ population from the CD34$^-$ cells. After collecting the CD34$^-$ cells, 80 µL of cold buffer (1x PBS + 1% FBS) and 20 µL of CD14 microbeads (CD14 MicroBead Kit, Miltenyi Biotec, cat., 130-050-201) were added per 10^7 total cells and incubated in shaking for 15 minutes at +4°C. This magnetic labelling step was followed by the magnetic separation (see Chapter 2.2.2 above). This step allowed the collection of CD14-positive-CD34-negative (CD14$^+$CD34$^-$) and CD14-negative-CD34-negative (CD14$^-$CD34$^-$) fractions, which were counted and the cells were plated in co-culture with MSCs (50 000 cells/cm^2). MSC-MNC and MSC-CD34$^-$ co-cultures were used as control. Basal medium was used to culture the cells and half of medium was changed every 3 or 4 days.

2.3. IncuCyte real-time cell imaging system

To study cell morphology an IncuCyte system (IncuCyte ZOOM or IncuCyte S3) was used. The cells were co-cultured in 24-well plates (Falcon) for up to 21 days and images were acquired every two hours with a 10x objective by IncuCyte. Each experimental group consisted in four parallel samples (wells) and from each well four images with an area of 2,3mm^2 each were recorded. Spindle-shaped cells were quantified using the Cell Counter plugin of Image Processing and Analysis Java (ImageJ) and the program settings were configured to take into account only the spindle-shaped cells. After quantifying the number of spindle-shaped cells in each IncuCyte image, the average number of cells was calculated and considered as the number of spindle-shaped cells per 2,3 mm^2 of each sample.

2.4. Immunocytochemistry

After the cells were cultured for 7, 14 or 21 days in 8-well chamber slides (Millicell), they were fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature (RT), washed with phosphate-buffered saline (PBS) and stored at 4°C until used. Cultures for the immunocytochemistry of CD31 were blocked with 3% bovine serum albumin (BSA) in PBS for 1 hour at RT, and incubated overnight at 4°C, with a mouse monoclonal antibody against CD31 (1:20) (Abcam, ab9498) in 1% BSA in PBS. The next day, after several washes with PBS, the cells were incubated in the dark, with an Alexa488-labelled goat anti-mouse antibody (1:1000) (Abcam, ab150113) in 1% BSA in PBS. Finally, the cultures were washed in the dark with PBS and mounting medium with DAPI (Vector, cat. H-1200) was used to stain the nuclei.

Cultures stained for VEGFR1 were blocked with 3% BSA in PBS for 1 hour at RT and incubated with a rabbit monoclonal antibody against VEGFR1 (1:100) (Abcam, ab32152) in 1% BSA in PBS overnight at 4°C. The next day, after several washes with PBS, the cells were incubated in the dark with an Alexa594-labelled goat anti-rabbit antibody (1:1000) (Abcam, ab150080) in 1% BSA in PBS. Finally, the cultures were washed in the dark with PBS and mounting medium with DAPI (Vector, cat. H-1200) was used to stain the nuclei. To stain the cultures for PDGFRβ and NG2, the same procedures as for VEGFR1 were used, except for the primary antibodies, which in this case were a rabbit monoclonal antibody against PDGFRβ (1:100) (Abcam, ab32570) and a rabbit monoclonal antibody against NG2 (1:100) (Abcam, ab183929), respectively. CD31 and VEGFR1 were used to detect ECs, while PDGFRβ and NG2 were used to detect pericytes. Samples incubated with only 1% BSA in PBS, instead of the respective primary antibody, were used as a negative control. Images were acquired at RT with a Zeiss Axio Imager M1 microscope using Plan-neofluar 20x air objective.

2.5. Real-time qPCR

For the analysis of mRNA expression levels of EC markers CD31 and VEGFR1 and pericyte markers NG2 and PDGFRβ, total RNA was isolated on days 7 and 14 of co-cultures according to manufacturer's instructions (GenEluteT Mammalian Genomic DNA Miniprep Kits; Merck). RNA samples were purified before reserve transcription using DNase I (Merck) and RNA quality was checked by Nanodrop Spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized from equal amounts of mRNA using SensiFAST cDNA Synthesis Kit (Bioline). Specific primers were used at a concentration of 25nM (Table 1). SensiFAST™ SYBR No-ROX Kit was used for real-time qPCR (qPCR) according to manufacturer's instructions and β-actin was used as a housekeeping reference gene. Results were analyzed by using MSCs alone as calibrator.

Table 1. Primers.

Gene	Forward primer	Reverse primer	Tm
β-actin	AGATCAAGATCATTGCTCCTCCTG	AGCTCAGTAACAGTCCGCCT	60°C
CD31	TGCCGTGGAAAGCAGATACTC	AGCCTGAGGAATTGCTGTGTT	60°C
VEGFR1	GAAATCACCTACGTGCCGGA	ACGTTCAGATGGTGGCCAAT	60°C
NG2	ACACGGATGCCACCCTACAAG	GGGCTCTTCACTGAGAATACGA	60°C
PDGFRβ	CAAGGACACCATGCGGCTTC	AGCAGGTCAGAACGAAGGTG	61°C

2.6. Statistical analysis

To evaluate the statistical differences between groups, paired t-test or one-way ANOVA followed by Bonferroni multiple comparison test were performed. All the experiments were repeated at least three times and in order to reduce the experiment-to-experiment variation in qPCR measurements, data from each experiment was normalized by dividing all values from each experiment with the highest value of the experiment. Data were presented as mean ± standard deviation (SD) and p-value ≤ 0.05 was considered statistically significant. The statistical analyses were performed using GraphPad Prism 8.1.2 (GraphPad Software, San Diego, CA, USA).

3. Results

During this research project ten experiments were performed. The seven first experiments were performed to evaluate cell morphology by IncuCyte for 21 days and expression of EC and pericyte markers by immunocytochemistry on days 7, 14 and 21. The last three experiments were performed to evaluate the expression of EC and pericyte markers by qPCR on days 7 and 14.

3.1. Abundance of MNCs and EPCs in peripheral blood

Blood samples were collected from the same donor for all the experiments to avoid the effects of potential inter-individual variations in the quality and composition of cells. Despite of this, the blood sample volumes varied between 40,5 and 50 mL in different experiments and there were also differences in the total numbers of isolated cells, thereby affecting the number of cells available for co-cultures (Tables 2 and 3).

After the PB-MNCs were isolated by density gradient centrifugation, the cells were fractionated using CD14 and CD34 microbeads. When MNCs were incubated with CD14 microbeads, 10% of the cells were $CD14^+$ and 90% were $CD14^-$, showing that there are more $CD14^-$ than $CD14^+$ cells within the PB-MNCs. While, when MNCs were incubated with CD34 microbeads, 1% of the cells were $CD34^+$ and 99% were $CD34^-$, demonstrating the very low number of $CD34^+$ cells within the PB-MNCs. The double isolations, on the other hand, showed that 9% of the cells were $CD14^+CD34^-$ and 91% were $CD14^-CD34^-$.

Table 2. Volume of blood, abundance of isolated MNCs, $CD14^+$, $CD14^-$, $CD34^+$, $CD34^-$, $CD14^+CD34^-$ and $CD14^-CD34^-$ cells and percentage of lost cells in the seven first experiments (Part I).

	1st exp	2nd exp	3rd exp		4th exp	5th exp	6th exp	7th exp
Blood (mL)	40,5	35,5	38		50	41	41,5	44,5
MNCs	121,2 x10^6	114 x10^6	121,4 x10^6		50 x10^6	57 x10^6	65 x10^6	130 x10^6
MNCs -co-culture	1,2 x10^6	2,4 x10^6	1,4 x10^6		9,6 x10^5	8,2 x10^5	1 x10^6	2 x10^6
MNCs – MACS	120 x10^6	111 x10^6	60 x10^6	60 x10^6	49 x10^6	56 x10^6	64 x10^6	128 x10^6
$CD14^+$ cells	2,5 x10^6	5 x10^6	4 x10^6	___	___	___	___	___
$CD14^-$ cells	42 x10^6	52 x10^6	33 x10^6	___	___	___	___	___
$CD34^+$ cells	___	___	___	4,7 x10^5	4,6 x10^5	3,4 x10^5	4,9 x10^5	1,1 x10^6
$CD34^-$ cells	___	___	___	35 x10^6	46 x10^6	48 x10^6	63 x10^6	121 x10^6
$CD34^-$ cell – co-culture	___	___	___	___	___	8,2 x10^5	1,2 x10^6	1,4 x10^6
$CD34^-$ cells-MACS	___	___	___	___	___	47,5 x10^6	62 x10^6	119,6 x10^6
$CD14^+$ $CD34^-$ cells	___	___	___	___	___	2,5 x10^6	6,3 x10^6	11 x10^6
$CD14^-$ $CD34^-$ cells	___	___	___	___	___	38 x10^6	53 x10^6	88,4 x10^6
Lost cells (%)	63	49	38	41	5	14-15	1-4	5-17

Table 3. Volume of blood, abundance of isolated MNCs, $CD14^+$, $CD14^-$, $CD34^+$ and $CD34^-$ cells and percentage of lost cells in the last three experiments (Part II).

	8th exp		**9th exp**		**10th exp**	
Blood (mL)	42		41		41	
MNCs	146 x10^6		70 x10^6		65 x10^6	
MNCs - co-culture	3 x10^6		3 x10^6		3 x10^6	
MNCs - MACS	71,5 x10^6	71,5 x10^6	33,5 x10^6	33,5 x10^6	31 x10^6	31 x10^6
$CD14^+$ cells	8,2 x10^6	___	4 x10^6	___	3,6 x10^6	___
$CD14^-$ cells	50 x10^6	___	26 x10^6	___	27 x10^6	___
$CD34^+$ cells	___	3,2 x10^5	___	3,9 x10^5	___	2,8 x10^5
$CD34^-$ cells	___	50,4 x10^6	___	32,5 x10^6	___	30 x10^6
Lost cells (%)	19	29	10	2	1	2

As the project proceeded, it was noticed that the number of lost cells was decreasing throughout the ten experiments, particularly when the last experiments were compared with the three first experiments where 38-63% of the cells were lost (Tables 2 and 3).

As mentioned above, the number of isolated $CD34^+$ cells was low and because of this, the immunocytochemical stainings for EC and pericyte markers could only be performed on days 7, 14 and 21 in the 5th and 7th experiment and the qPCR analysis only on day 7 in the last three experiments. Additionally, because of the low number of $CD34^+$ cells, it was not possible to perform IncuCyte analysis in culture alone.

3.2. Morphology of cultured cells

In order to study cell morphology, images of human MSCs, MNCs as well as, $CD14^+$, $CD14^-$ and $CD34^-$ cells were constantly recorded using IncuCyte for up to 21 days. After 1-2 days of culture, MSCs started to adhere to the plastic surface as single cells. As they grew, their fibroblast-like morphology became more evident and they began to form colonies (Fig. 11A). On day 14 the cells exhibited long fibroblast-like morphology (Fig.11B) and on day 21 60-70% of confluence was observed (Fig. 11C).

MNCs are a heterogeneous cell population. After seven days of culture, some cells started to change either into a spindle-shaped or monocyte-like cell morphology (round and plastic-adherent cells), while the other cells maintained the rounded morphology (Fig. 11D). After 14 and 21 days of culture, both rounded, monocyte-like, as well as spindle-shaped cells were observed (Fig. 11E, 11F).

Some CD14$^+$ cells started to change their morphology from a rounded to a spindle-shaped morphology after seven days of culture (Fig. 11G). However, on days 14 and 21 some of the CD14$^+$ cells presented a monocyte-like cell morphology (Fig. 11H, 11I). CD14$^-$ cells, on the other hand, maintained the round cell morphology over time (Fig. 11J-L).

Some of CD34$^-$ cells started to change into spindle-shaped or monocyte-like cells after seven days of culture (Fig. 11M). Nevertheless, at all time points, in addition to spindle-shaped and monocyte-like cell morphology, a considerable number of rounded cells was also observed (Fig. 11M-O).

In all cultures, expect for MSCs, no confluence above 50% was observed (Fig. 11). Relatively low cell numbers were noticed especially in CD14$^-$ and CD34$^-$ cultures, indicating that these cells do not survive well alone in prolonged cultures (Fig. 11J-O). Additionally, due to the low number of CD34$^+$ cells that were obtained from PB with MACS, it was not possible to evaluate them in culture alone.

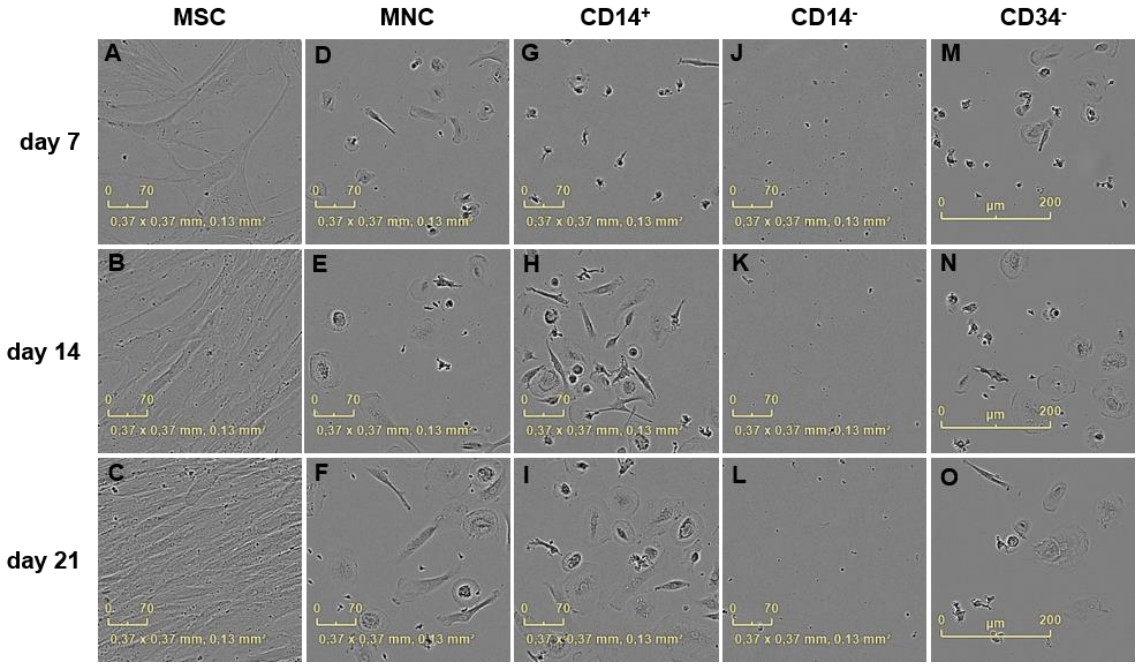

Figure 11. Morphological changes in cultures of MSCs, MNCs, CD14$^+$, CD14$^-$ and CD34$^-$ cells. Representative images of MSCs (A-C), MNCs (D-F), CD14$^+$ (G-I), CD14$^-$ (J-L) and CD34$^-$ (M-O) cells obtained by IncuCyte on days 7, 14 and 21.

To quantify the number of cultured cells that acquired a spindle-shaped morphology, ImageJ was used for IncuCyte images obtained on days 7, 14 and 21. As evident from the IncuCyte images (Fig. 11), it was observed that on days 14 and 21 CD14$^+$ cell cultures contained significantly more spindle-shaped cells than MNC cultures ($p = 0{,}0021$ on day 14 and $p = 0{,}0007$ on day 21), while CD14$^-$ cultures contained significantly less spindle-shaped cells than MNC cultures ($p = 0{,}0488$ on day 14 and $p = 0{,}0269$ on day 21) (Fig. 12). CD34$^-$ cells, on the other hand, were the cell population that presented the most spindle-shaped cells, compared with MNCs ($p = 0{,}0003$ on day 7, $p < 0.0001$ on day 14 and $p = 0.0002$ on day 21) (Fig. 12).

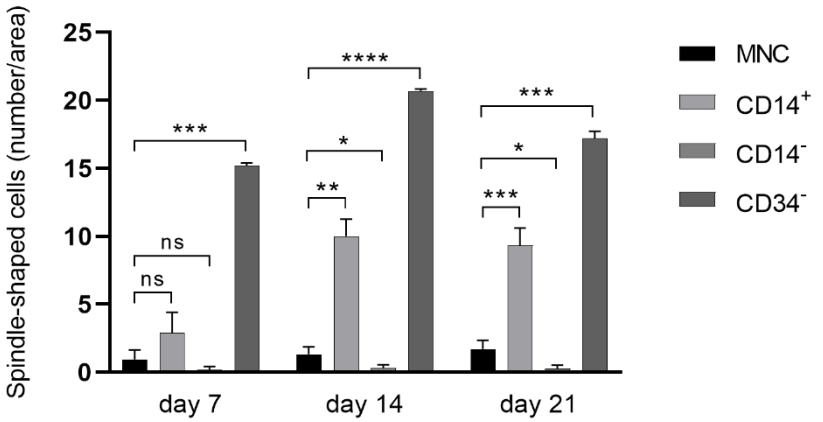

Figure 12. Number of spindle-shaped cells in cultures of MNCs, $CD14^+$, $CD14^-$ and $CD34^-$ cells. Spindle-shaped cells were quantified on days 7, 14 and 21 by ImageJ, using IncuCyte images with an area of 2,3 mm^2. Data is expressed as mean ± SD; ns, non-significant; * p≤0,05; ** p≤0,01; *** p≤0,001; **** p≤0.0001. One-way ANOVA followed by Bonferroni multiple comparison test was used. The results are representative of four independent experiments and each experiment included four replicate samples.

3.3. Morphology of co-cultured cells

To study cell morphology in co-cultures, MNCs, $CD14^+$, $CD14^-$, $CD34^+$, $CD34^-$, $CD14^+CD34^-$ and $CD14^-CD34^-$ cells were co-cultured with MSCs for up to 21 days and imaged using IncuCyte. Then, to determine the number of cells that acquired a spindle-shaped morphology, ImageJ was used to quantify cell number on days 7, 14 and 21.

3.3.1. Morphology of $CD14^+$ and $CD14^-$ cells when co-cultured with MSCs

MNCs attached over the MSCs after 2-3 days of co-culture. After seven days, some of MNCs become spindle-shaped (Fig. 13A) and over the time the number of rounded cells that turned spindle-shaped increased (Fig 13B, 13C). As MNCs, also $CD14^+$ cells were observed to attach over the MSCs after 2-3 days of co-culture. However, it was also visually observed that the number of $CD14^+$ cells that attached over the MSCs was higher than the number of MNCs that attached over the MSCs. After seven days of co-culture, $CD14^+$ cells started to sprout and form tube-like structures (Fig. 13D) and after 21 days, almost all the cells presented a spindle-shaped morphology (Fig. 13F). Suggesting that MSC-$CD14^+$ co-cultures have a higher and better angiogenic potential than MSC-MNC co-cultures. $CD14^-$ cells, on the other hand, did not attach over the MSCs and maintained the rounded morphology during the 21 days of co-culture, showing no induction of endothelial-like cell differentiation (Fig. 13G-I).

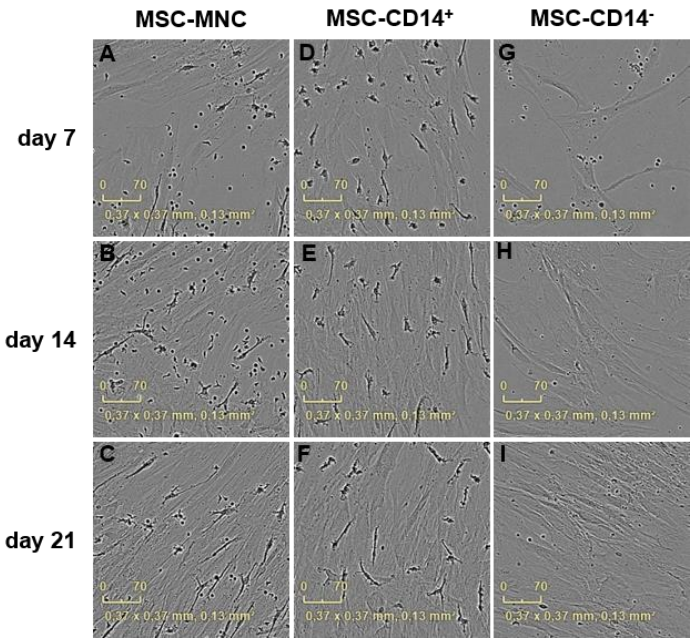

Figure 13. Morphological changes in co-cultures of MNCs, CD14$^+$ and CD14$^-$ cells with MSCs. Representative images of MSC-MNC (A-C), MSC-CD14$^+$ (D-F) and MSC-CD14$^-$ (G-I) co-cultures obtained by IncuCyte on days 7, 14 and 21.

As mentioned above, after IncuCyte images acquisition and visual evaluation of morphological changes, spindle-shaped cells were quantified with ImageJ, using IncuCyte images of days 7, 14 and 21. The quantification allowed to confirm what the IncuCyte images indicated: MSC-CD14$^+$ co-cultures contained more sprouting, tube-forming cells than MSC-MNC co-cultures at all time points (Fig. 14). However, the differences between the two groups were significant only on days 14 and 21 ($p = 0.0076$ on day 14 and $p = 0.0061$ on day 21) (Fig. 14). Furthermore, the quantification showed that MSC-CD14$^-$ co-cultures contained a very few spindle-shaped cells (Fig. 14). Nevertheless, statistically significant differences were observed between MSC-CD14$^-$ and MSC-MNC co-cultures at all time points, demonstrating that co-cultures of MSC-CD14$^-$ presented less spindle-shaped cells than the other co-cultures ($p = 0.0039$ on day 7, $p = 0.0209$ on day 14 and $p = 0.0130$ on day 21) (Fig.14).

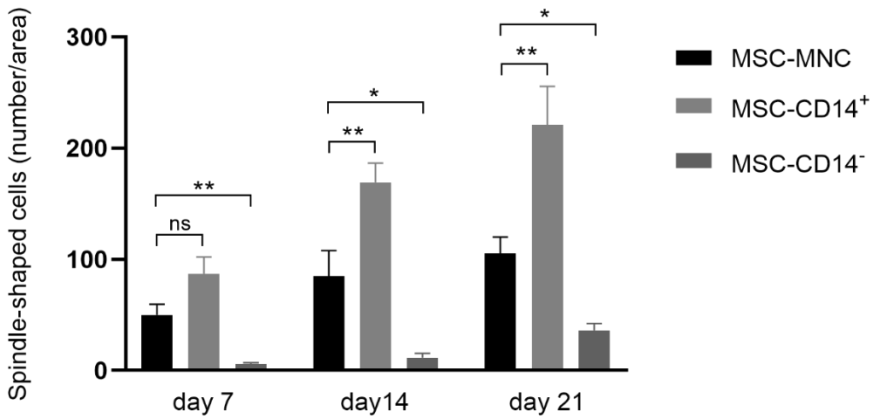

Figure 14. Number of spindle-shaped cells in co-cultures of MNCs, CD14$^+$ and CD14$^-$ cells with MSCs. Spindle-shaped cells of MSC-MNC, MSC-CD14$^+$ and MSC-CD14$^-$ co-cultures were quantified on days 7, 14 and 21 by ImageJ, using IncuCyte images with an area of 2,3 mm^2. Data is expressed as mean ± SD; ns, non-significant; * p≤0,05; ** p≤0,01. One-way ANOVA followed by Bonferroni multiple comparison test was used. The results are representative of three independent experiments and each experiment included four replicate samples.

3.3.2. Morphology of CD34$^+$ and CD34$^-$ cells when co-cultured with MSCs

When morphological changes in CD34$^+$ and CD34$^-$ cells were evaluated, it was observed that both cell types attached over the MSCs after 2-3 days of co-culture. After seven days, some of CD34$^+$ cells started to change from a rounded to a spindle-shaped morphology (Fig. 15D) and after 21 days of co-culture both rounded and spindle-shaped cells were observed (Fig. 15F). Similarly to CD34$^+$ cells, also some CD34$^-$ cells obtained spindle-shaped morphology, after seven days of co-culture (Fig. 15G) and after 21 days some of the CD34$^+$ cells were spindle-shaped, while other cells maintained the rounded morphology (Fig. 15I).

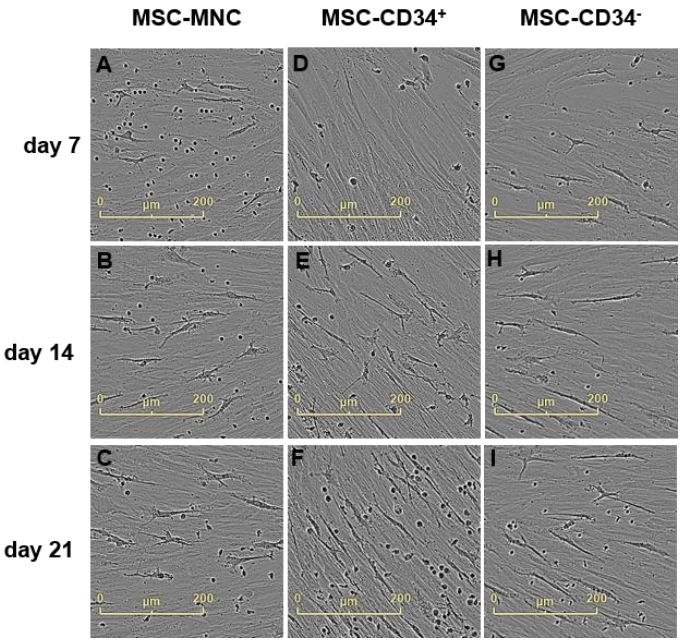

Figure 15. Morphological changes in co-cultures of MNCs, CD34$^+$ and CD34$^-$ cells with MSCs. Representative images of MSC-MNC (A-C), MSC-CD34$^+$ (D-F) and MSC-CD34$^-$ (G-I) co-cultures obtained by IncuCyte on days 7, 14 and 21.

The quantification of spindle-shaped cells by ImageJ showed that MSC-CD34$^+$ co-cultures presented more tube-like structures than MSC-MNC co-cultures after 14 and 21 days of co-culture ($p = 0{,}0199$ on day 14 and $p = 0{,}0241$ on day 21) (Fig. 16). In contrast, statistically significant differences between MSC-CD34$^-$ and MSC-MNC co-cultures were observed just on day 7 ($p = 0{,}0110$), suggesting that both CD34$^-$ cells and MNCs have a similar capacity to differentiate into spindle-shaped cells when co-cultured with MSCs (Fig. 16).

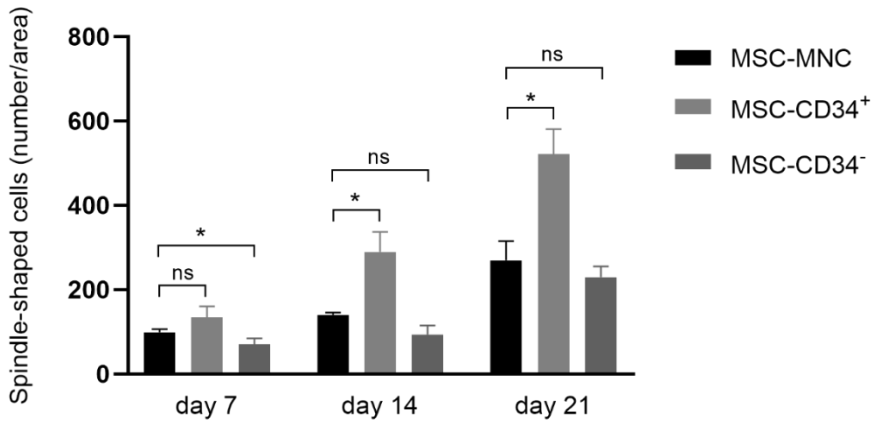

Figure 16. Number of spindle-shaped cells in co-cultures of MNCs, CD34$^+$ and CD34$^-$ cells with MSCs. Spindle-shaped cells of MSC-MNC, MSC-CD34$^+$ and MSC-CD34$^-$ co-cultures were quantified on days 7, 14 and 21 by ImageJ, using IncuCyte images with an area of 2,3 mm^2. Data is expressed as mean ± SD; ns, non-significant; * $p \leq 0{,}05$. One-way ANOVA followed by Bonferroni multiple comparison test was used. The results are representative of four independent experiments and each experiment included four replicate samples.

3.3.3. Morphology of $CD14^+CD34^-$ and $CD14^-CD34^-$ cells when co-cultured with MSCs

After observing that in co-culture with MSCs almost all $CD14^+$ cells formed tube-like structures and just a few $CD14^-$ cells presented spindle-shaped morphology, we tried to observe if the heterogeneous populations of MSC-$CD34^+$ and MSC-$CD34^-$ co-cultures contained $CD14^+$ cells. However, only the number of isolated $CD34^-$ cells was high enough to perform such double isolation experiments. Thus, after $CD34^-$ cells were isolated from PB-MNCs, some of these cells were incubated with CD14 microbeads and thereby $CD14^+CD34^-$ and $CD14^-CD34^-$ cell populations were obtained and cultured with MSCs for 21 days. After 2-3 days of co-culture, $CD14^+CD34^-$ cells attached over the MSCs. Nevertheless, the same did not happen with $CD14^-CD34^-$ cells in co-culture. After seven days of co-culture, some of the $CD14^+CD34^-$ cells started to become spindle-shaped and their number increased over the 21 days (Fig. 17D-F). However, a substantial number of cells did not reach that morphology (Fig. 17D-F). In MSC-$CD14^-CD34^-$ co-cultures, on the other hand, although almost all the cells maintain the rounded cell morphology, a few cells developed well-defined tube like-structures on day 21 (Fig. 17G-I).

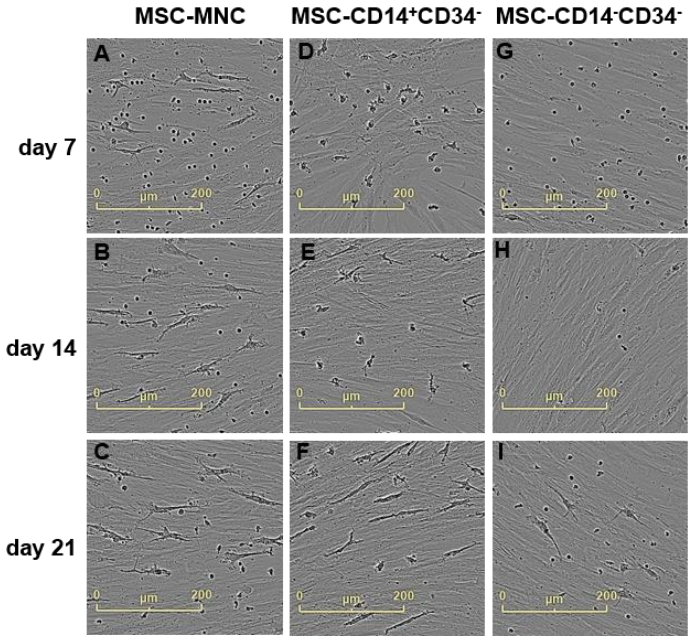

Figure 17. Morphological changes in co-cultures of MNCs, $CD14^+CD34^-$ and $CD14^-CD34^-$ cells with MSCs. Representative images of MSC-MNC (A-C), MSC-$CD14^+CD34^-$ (D-F) and MSC-$CD14^-CD34^-$ (G-I) co-cultures obtained by IncuCyte on days 7, 14 and 21.

When the number of spindle-shaped cells was quantified, a statistically significant difference was observed between MSC-$CD14^+CD34^-$ and MSC-MNC co-cultures only on day 14 ($p = 0.0121$) (Fig. 18). Nevertheless, significant differences were observed between MSC-$CD14^-CD34^-$ and MSC-MNC co-cultures at all time points ($p = 0{,}004$ on day 7, $p < 0{,}0001$ on day 14 and $p = 0{,}0106$ on day 21), showing that MSC-$CD14^-CD34^-$ co-cultures had a poorer capacity for EC differentiation than MSC-MNC co-cultures (Fig. 18).

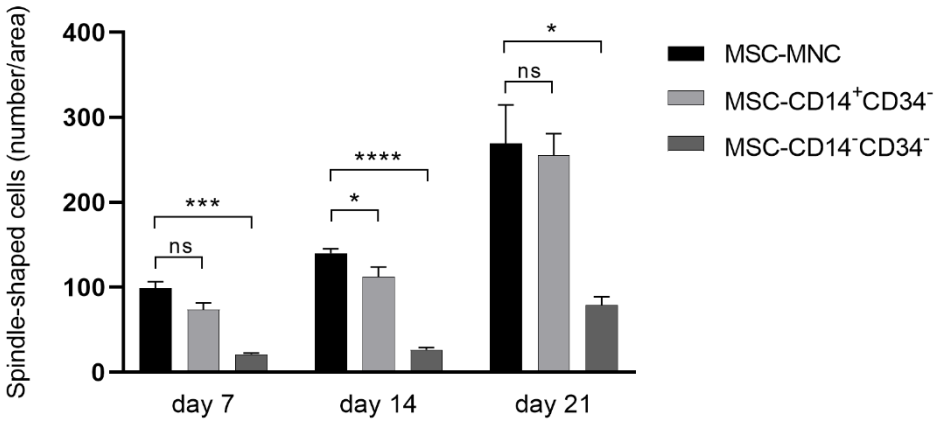

Figure 18. Number of spindle-shaped cells in co-cultures of MNCs, $CD14^+CD34^-$ and $CD14^-CD34^-$ cells with MSCs. Spindle-shaped cells of MSC-MNC, MSC-$CD14^+CD34^-$ and MSC-$CD14^-CD34^-$ co-cultures were quantified on days 7, 14 and 21 by ImageJ, using IncuCyte images with an area of 2,3 mm^2. Data is expressed as mean ± SD; ns, non-significant; * p≤0,05; ** p≤0,01 and *** p≤0,001. One-way ANOVA followed by Bonferroni multiple comparison test was used. The results are representative of three independent experiments and each experiment included four replicate samples.

3.4. Morphology of cultured cells vs Morphology of co-cultured cells

To evaluate the vasculogenic potential of co-culturing MNCs, $CD14^+$, $CD14^-$ and $CD34^-$ cells with MSCs, the numbers of spindle-shaped cells in culture and in co-culture were compared. The results demonstrate that co-culturing all the different types of cells with MSCs significantly increased the number of spindle-shaped cells at all time points (Fig. 19).

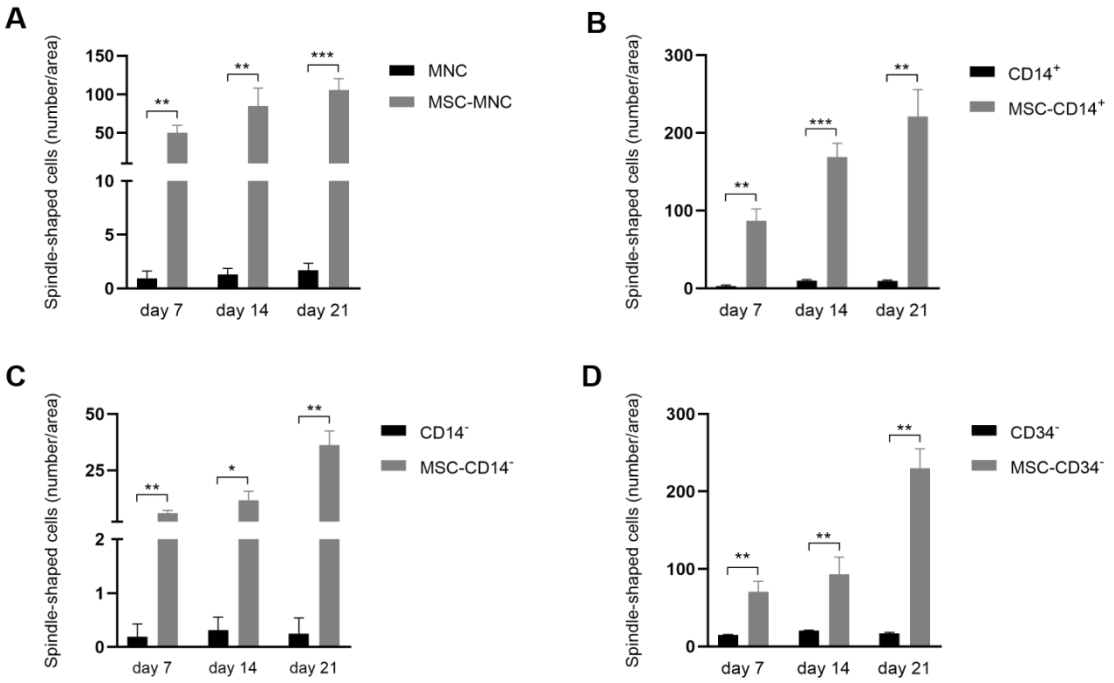

Figure 19. Number of spindle-shaped cells in cultures vs in co-cultures. Number of spindle-shaped cells was analysed in MNC cultures vs MSC-MNC co-cultures (A), $CD14^+$ cultures vs MSC-$CD14^+$ co-cultures (B), $CD14^-$ cultures vs MSC-$CD14^-$ co-cultures (C) and $CD34^-$ cultures vs MSC-$CD34^-$ co-cultures (D). Data is expressed as mean ± SD; * p≤0,05; ** p≤0,01 and *** p≤0,001. Paired t-test was used. The results are representative of seven independent experiments and each experiment included four replicate samples.

3.5. EC and pericyte marker expression in cultured cells by immunocytochemistry

In order to evaluate whether MSCs, MNCs, $CD14^+$, $CD14^-$ or $CD34^-$ cells express EC and pericyte markers, immunocytochemical stainings were performed. For this, the EC markers, CD31 and VEGFR1 and the pericyte markers, NG2 and PDGFRβ were used. The expression of the different markers was evaluated in three different time points: on day 7, 14 and 21. As previously mentioned, the expression of EC and pericyte markers was not evaluated in cultures of $CD34^+$ cells due to the low number of isolated cells.

MSCs did not express CD31 (Fig. 20A, 20E, 20I) or VEGFR1 (Fig. 20B, 20F, 20J) at any time point, but some MSCs expressed both pericyte markers, NG2 (Fig. 20C, 20G, 20K) and PDGFRβ (Fig. 20D, 20H, 20L) at all time points. Additionally, no background staining was observed in the negative controls (Fig. 20M-P).

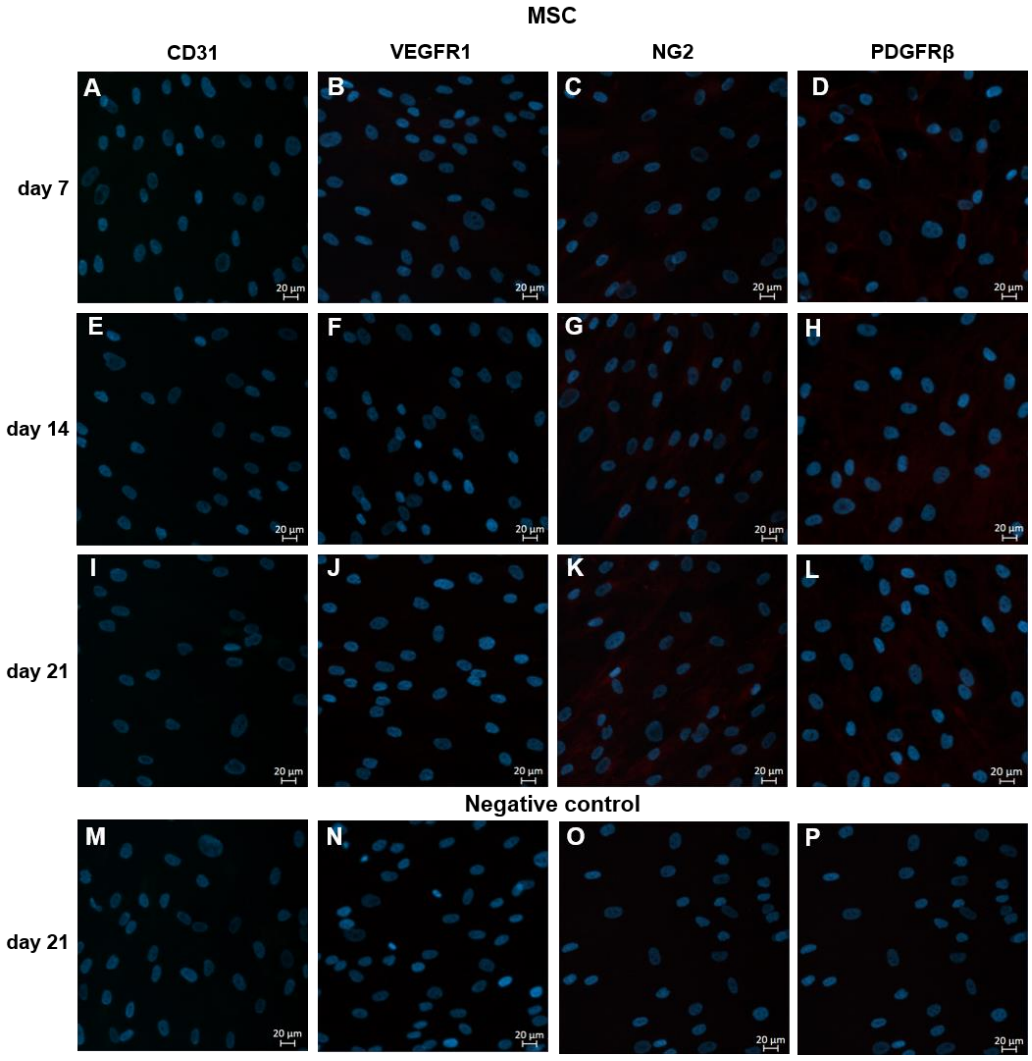

Figure 20. Cultures of MSCs stained for EC and pericyte markers. Representative images of MSCs in culture stained for EC markers, CD31 (green) and VEGFR1 (red) on day 7 (A, B), day 14 (E, F) and day 21 (I, J) and for pericyte markers, NG2 (red) and PDGFRβ (red) on day 7 (C, D), day 14 (G, H) and day 21 (K, L) and corresponding negative controls for each marker on day 21 (M-P). The results are representative of seven independent experiments.

On the other hand, MNCs were shown to express both EC markers, CD31 and VEGFR1 on days 7 (results not shown), 14 (Fig. 21A, 21B) and 21 (Fig. 21E, 21F). Interestingly, MNCs expressed the pericyte markers NG2 and PDGFRβ on days 14 (Fig. 21C, 21D) and 21 (Fig. 21G, 21H). However, the expression of PDGFRβ was very weak in both time points (Fig. 21D, 21H). No background staining was observed in the negative controls (Fig. 21I-L).

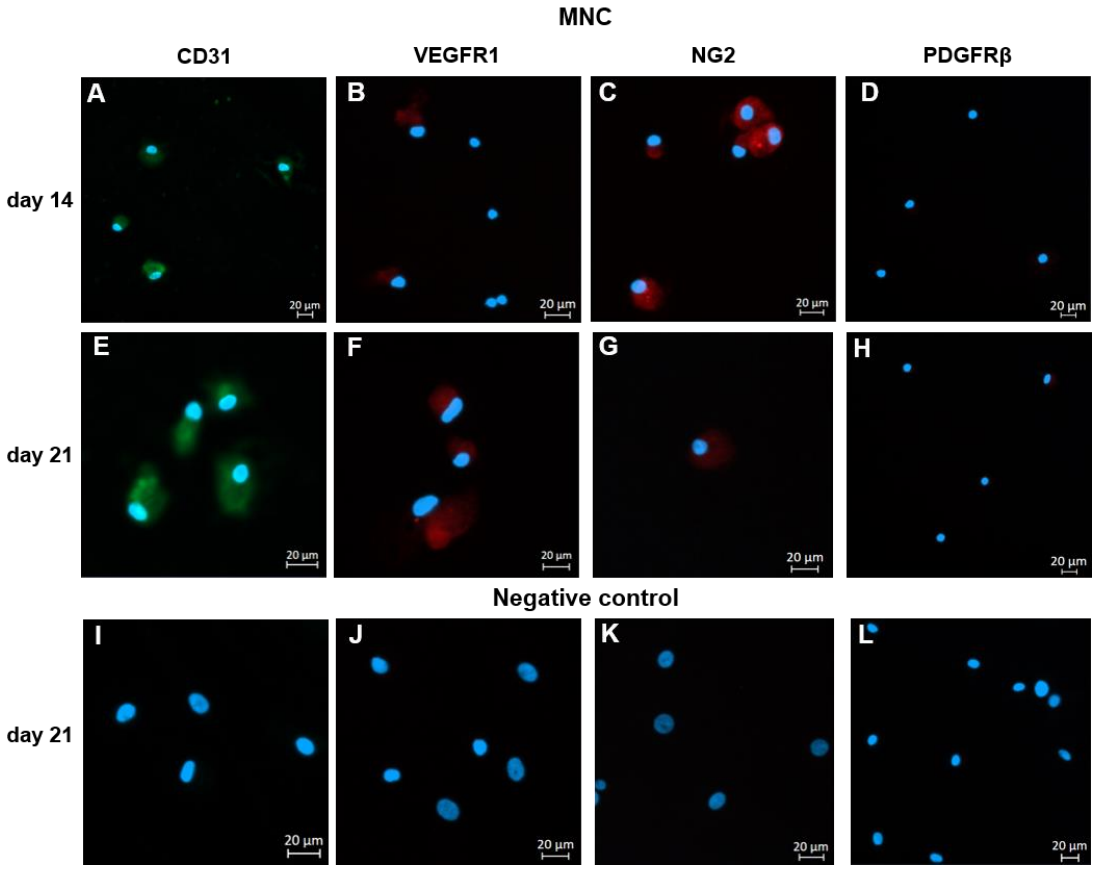

Figure 21. Cultures of MNCs stained for EC and pericyte markers. Representative images of MNCs in culture stained for EC markers, CD31 (green) and VEGFR1 (red) on day 14 (A, B) and day 21 (E, F) and for pericyte markers, NG2 (red) and PDGFRβ (red) on day 14 (C, D) and day 21 (G, H) and corresponding negative controls for each marker on day 21 (I-L). The results are representative of four independent experiments.

Immunocytochemical staining of $CD14^+$ cells, showed that these cells express EC markers, CD31 and VEGFR1 on days 7 (results not shown), 14 (Fig. 22A, 22B) and 21 (Fig. 22E, 22F). These cells also expressed the pericyte marker NG2 on days 14 (Fig. 22C) and 21 (Fig. 22G), with a stronger staining on day 14. In addition, a weak expression of PDGFRβ in some $CD14^+$ cells was observed on day 14 (Fig. 22D), but not on day 21 (Fig. 22H). $CD14^-$ cells, on the other hand, did not express any EC or pericyte marker at any time point (Fig. 23A-H). Additionally, also on these cultures no background staining was observed (Fig. 22I-L, 23I-L).

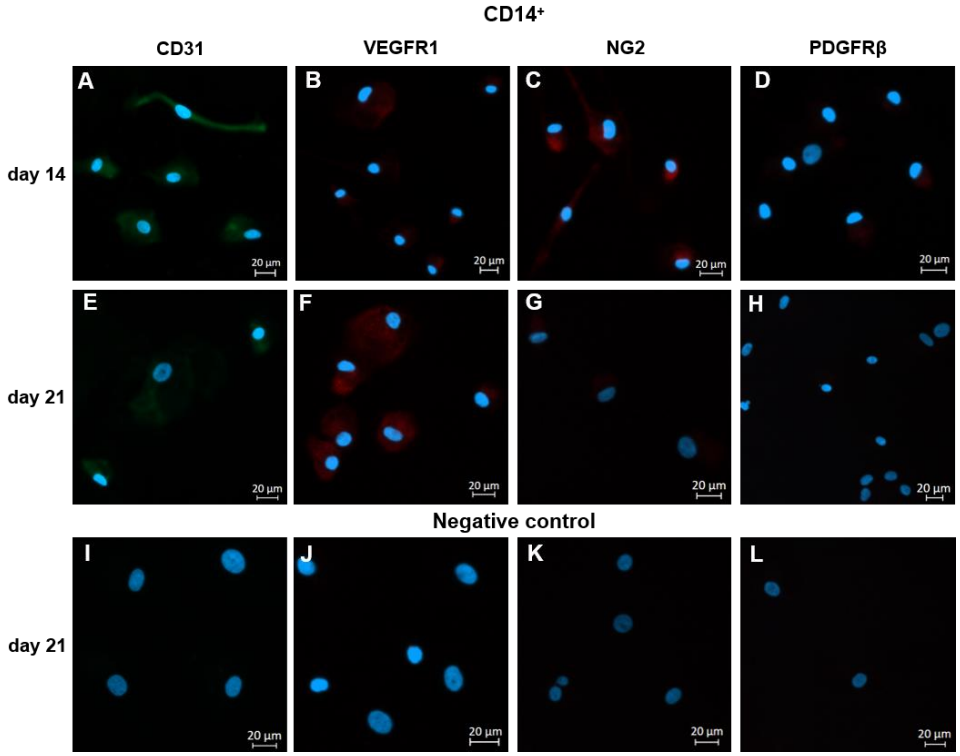

Figure 22. Cultures of CD14$^+$ cells stained for EC and pericyte markers. Representative images of CD14$^+$ cells in culture stained for EC markers, CD31 (green) and VEGFR1 (red) on day 14 (A, B) and day 21 (E, F) and for pericyte markers, NG2 (red) and PDGFRβ (red) on day 14 (C, D) and day 21 (G, H) and corresponding negative controls for each marker on day 21 (I-L). The results are representative of three independent experiments.

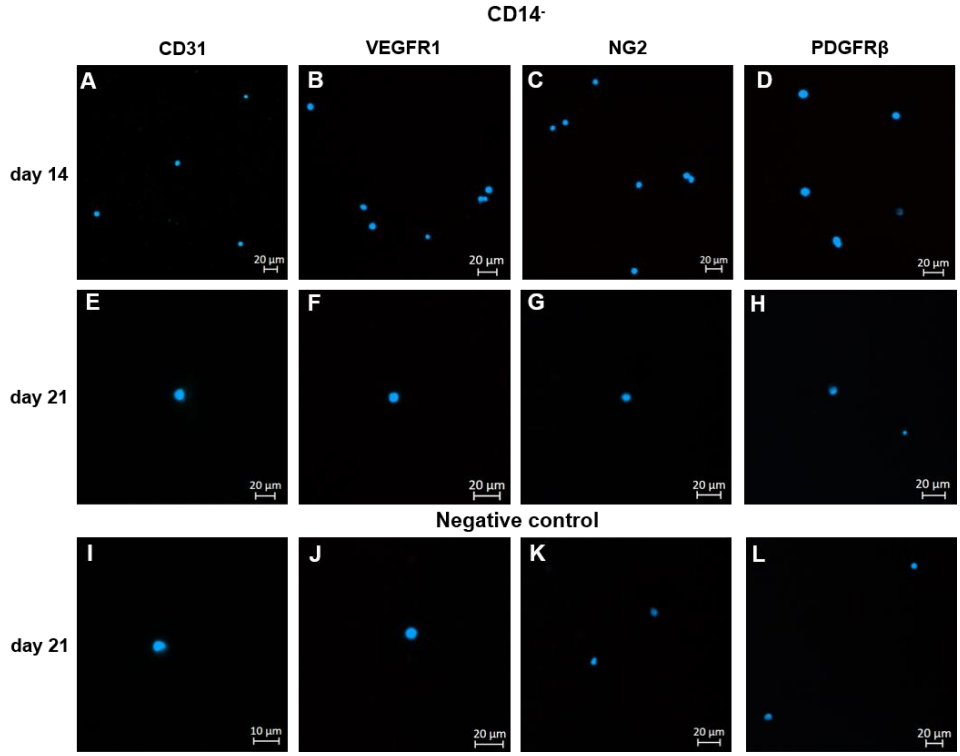

Figure 23. Cultures of CD14$^-$ cells stained for EC and pericyte markers. Representative images of CD14$^-$ cells in culture stained for EC markers, CD31 (green) and VEGFR1 (red) on day 14 (A, B) and day 21 (E, F) and for pericyte markers, NG2 (red) and PDGFRβ (red) on day 14 (C, D) and day 21 (G, H) and corresponding negative controls for each marker on day 21 (I-L). The results are representative of three independent experiments.

The immunocytochemistry stainings of CD34$^-$ cells show that some of these cells express both EC markers, CD31 (Fig. 24A, 24D) and VEGFR1 (Fig. 24B, 24E) as well as the pericyte marker NG2 (Fig. 24C, 24F) on days 14 and 21. PDGFRβ staining on these samples could not be performed due to technical limitations. No background staining was observed in the negative controls (Fig. 24G-I).

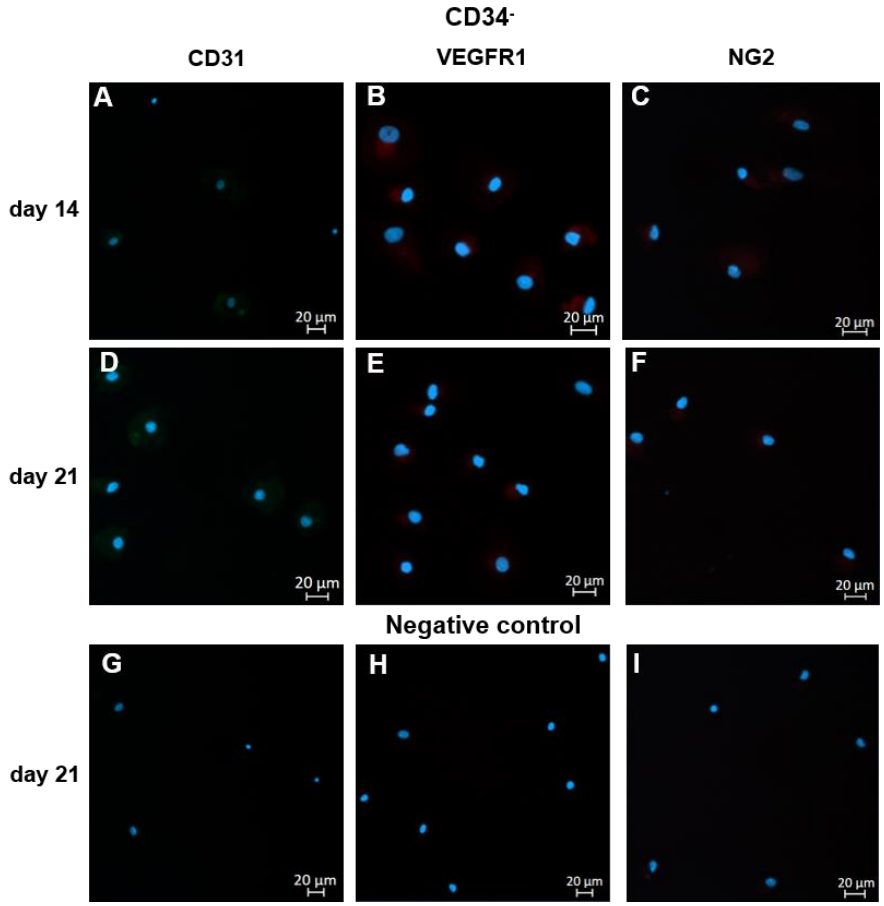

Figure 24. Cultures of CD34$^-$ cells stained for EC and pericyte markers. Representative images of CD34$^-$ cells in culture stained for EC markers, CD31 (green) and VEGFR1 (red) on day 14 (A, B) and day 21 (D, E) and for pericyte marker NG2 (red) on day 14 (C) and day 21 (F) and corresponding negative controls for each marker on day 21 (G-I). The results are representative of two independent experiments.

Table 4. Summary of immunocytochemical stainings of cultured MSCs, MNCs, CD14$^+$, CD14$^-$ and CD34$^-$ cells. The cells were grown and after 7, 14 or 21 days were stained for EC (CD31 and VEGFR1) and pericyte (NG2 and PDGFRβ) markers. Data was analyzed with a fluorescence microscope and the intensity of staining is expressed as -, negative staining; (+), weak staining; + moderate staining; ++, strong staining; +++ very strong staining; n.a., data not available.

	CD31			VEGFR1			NG2			PDGFRβ		
	7	14	21	7	14	21	7	14	21	7	14	21
MSC	-	-	-	-	-	-	(+)	+	++	+	++	++
MNC	n.a.	+++	+++	n.a.	++	+++	n.a.	+++	++	n.a.	(+)	(+)
CD14$^+$	n.a.	++	+	n.a.	++	+++	n.a.	++	(+)	n.a.	(+)	-
CD14$^-$	n.a.	-	-	n.a.	-	-	n.a.	-	-	n.a.	-	-
CD34$^-$	n.a.	(+)	+	n.a.	++	+	n.a.	+	+	n.a.	n.a.	n.a.

3.6. EC and pericyte marker expression in co-cultured cells by immunocytochemistry and qPCR

To evaluate the expression of EC and pericyte markers in co-cultures of MSC-MNC, MSC-CD14$^+$, MSC-CD14$^-$, MSC-CD34$^+$ and MSC-CD34$^-$, immunocytochemical stainings for CD31, VEGFR1, NG2 and PDGFRβ were performed on samples from days 7, 14 and 21 of culture.

The immunocytochemistry results showed that MNCs in co-culture with MSCs express both CD31 (Fig. 25A, 25E, 25I,) and VEGFR1 (Fig. 25B, 25F, 25J) at all time points. When in co-culture, some MSCs and some MNCs were observed to express NG2 at all time points (Fig. 25C, 25G, 25K). Additionally, it was observed that MNCs in co-culture with MSCs did not express PDGFRβ at any time point, while some MSCs expressed PDGFRβ at all time points (Fig. 25D, 25H, 25L). No background staining was observed in any of the negative controls (Fig. 25M-P).

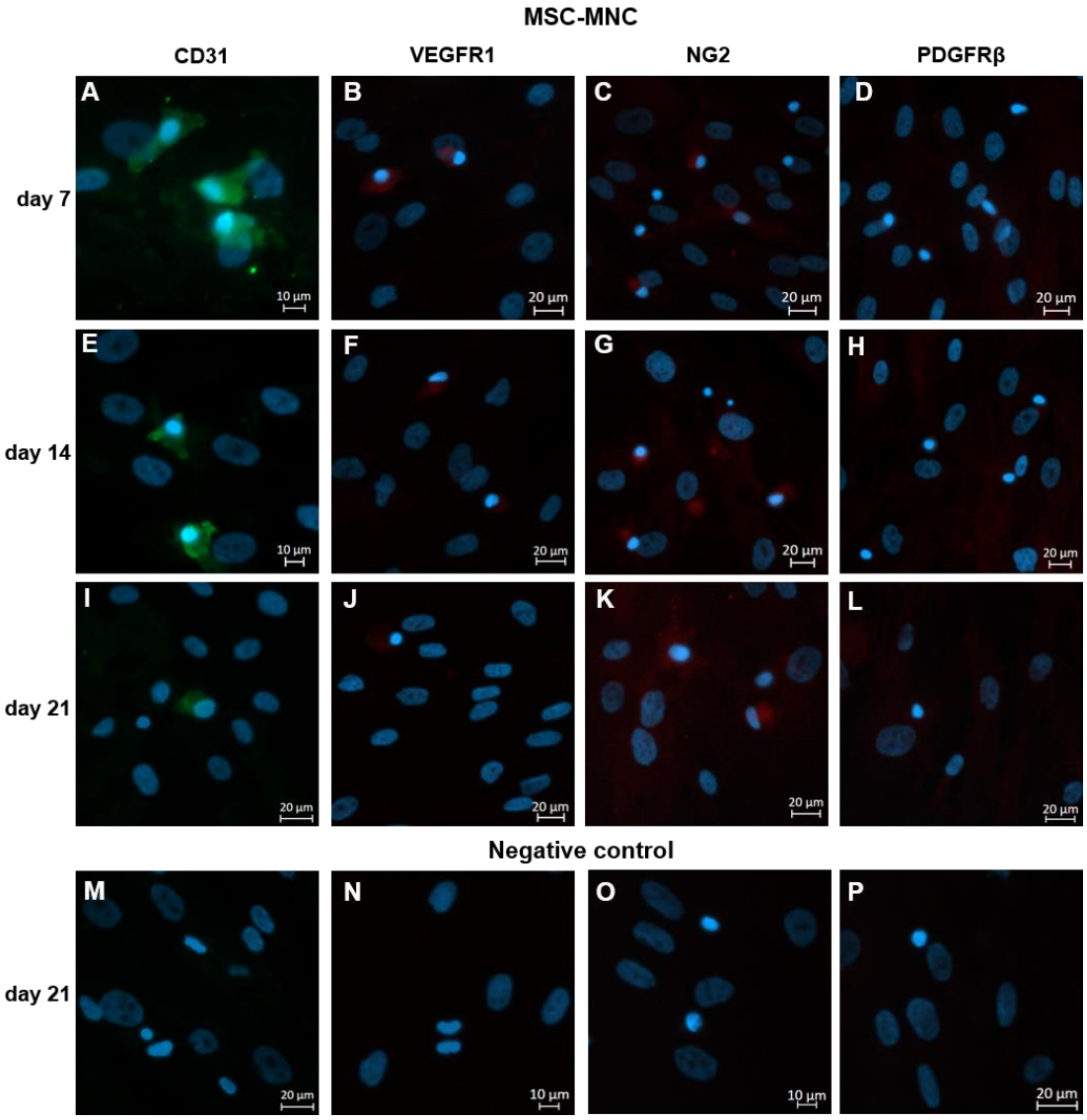

Figure 25. Co-cultures of MSC-MNC stained for EC and pericyte markers. Representative images of MSC-MNC co-cultures stained for EC markers, CD31 (green) and VEGFR1 (red) on day 7 (A, B), day 14 (E, F) and day 21 (I, J) and for pericyte markers, NG2 (red) and PDGFRβ (red) on day 7 (C, D), day 14 (G, H) and day 21 (K, L) and corresponding negative controls for each marker on day 21 (M-P). The results are representative of seven independent experiments.

Immunostaining of MSC-CD14$^+$ co-cultures showed that CD14$^+$ cells expressed both EC markers, CD31 (Fig. 26A, 26E, 26I) and VEGFR1 (Fig. 26B, 26F, 26J) at all time points. Furthermore, some MSCs and CD14$^+$ cells expressed both NG2 and PDGFRβ. The expression of NG2 was observed at all time points (Fig. 26C, 26G, 26K), while PDGFRβ was expressed only on days 14 and 21 (Fig. 26H, 26L). Again, no background staining was observed (Fig. 26M-P).

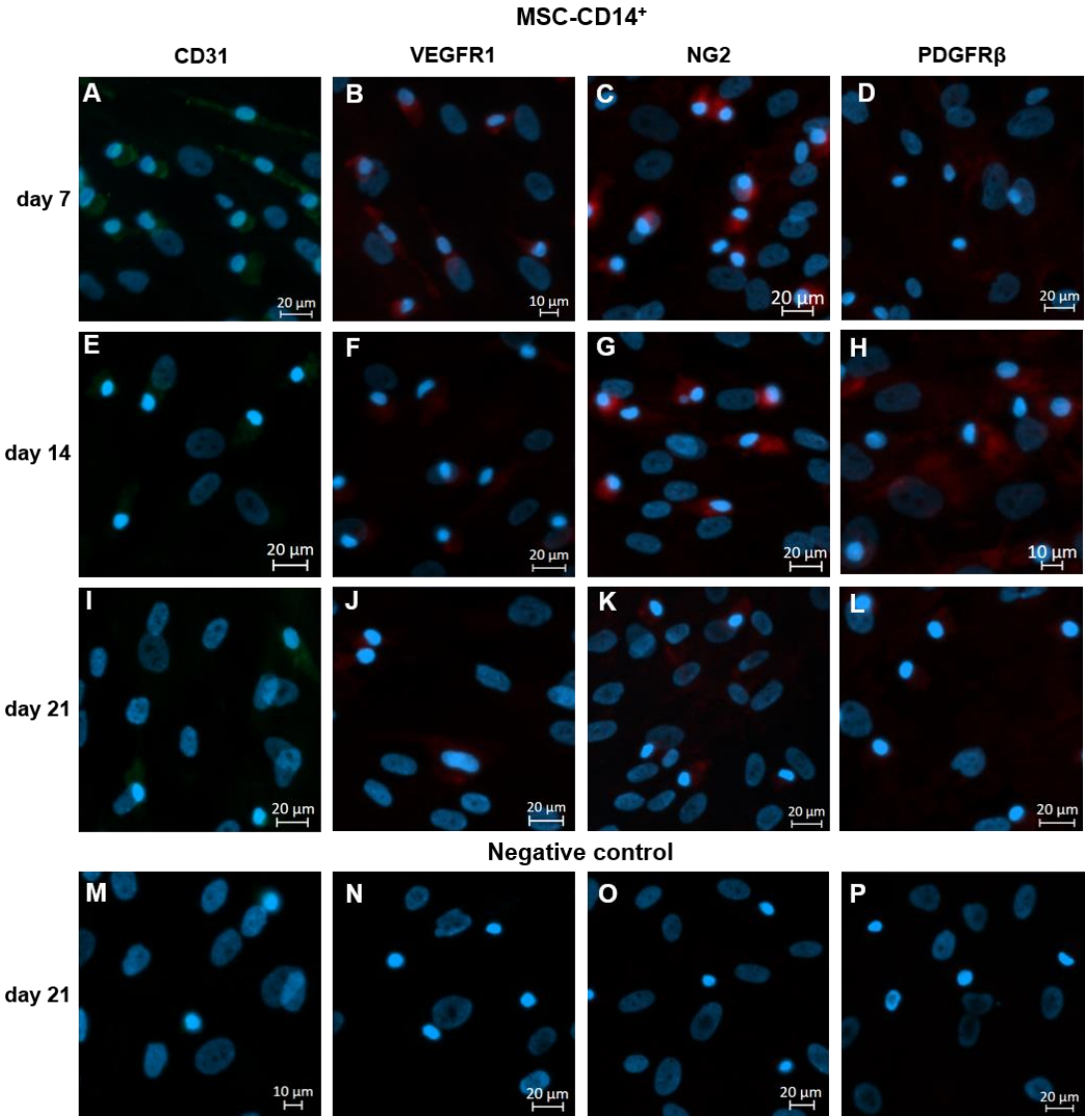

Figure 26. Co-cultures of MSC-CD14$^+$ stained for EC and pericyte markers. Representative images of MSC-CD14$^+$ co-cultures stained for EC markers, CD31 (green) and VEGFR1 (red) on day 7 (A, B), day 14 (E, F) and day 21 (I, J) and for pericyte markers, NG2 (red) and PDGFRβ (red) on day 7 (C, D), day 14 (G, H) and day 21 (K, L) and corresponding negative controls for each marker on day 21 (M-P). The results are representative of three independent experiments.

In MSC-CD14$^-$ co-cultures neither MSCs nor the CD14$^-$ cells expressed CD31 at any time point (Fig. 27A, 27E, 27I). However, the expression of VEGFR1 was observed in CD14$^-$ cells on day 7 (Fig. 27B) and PDGFRβ on day 14 (Fig. 27H) when these cells were co-cultured with MSCs. Furthermore, in these co-cultures MSCs expressed NG2 (Fig. 27C, 27G, 27K) and PDGFRβ (Fig. 27D, 27H, 27L) at all time points. Also here, no background staining was observed (Fig. 27M-P).

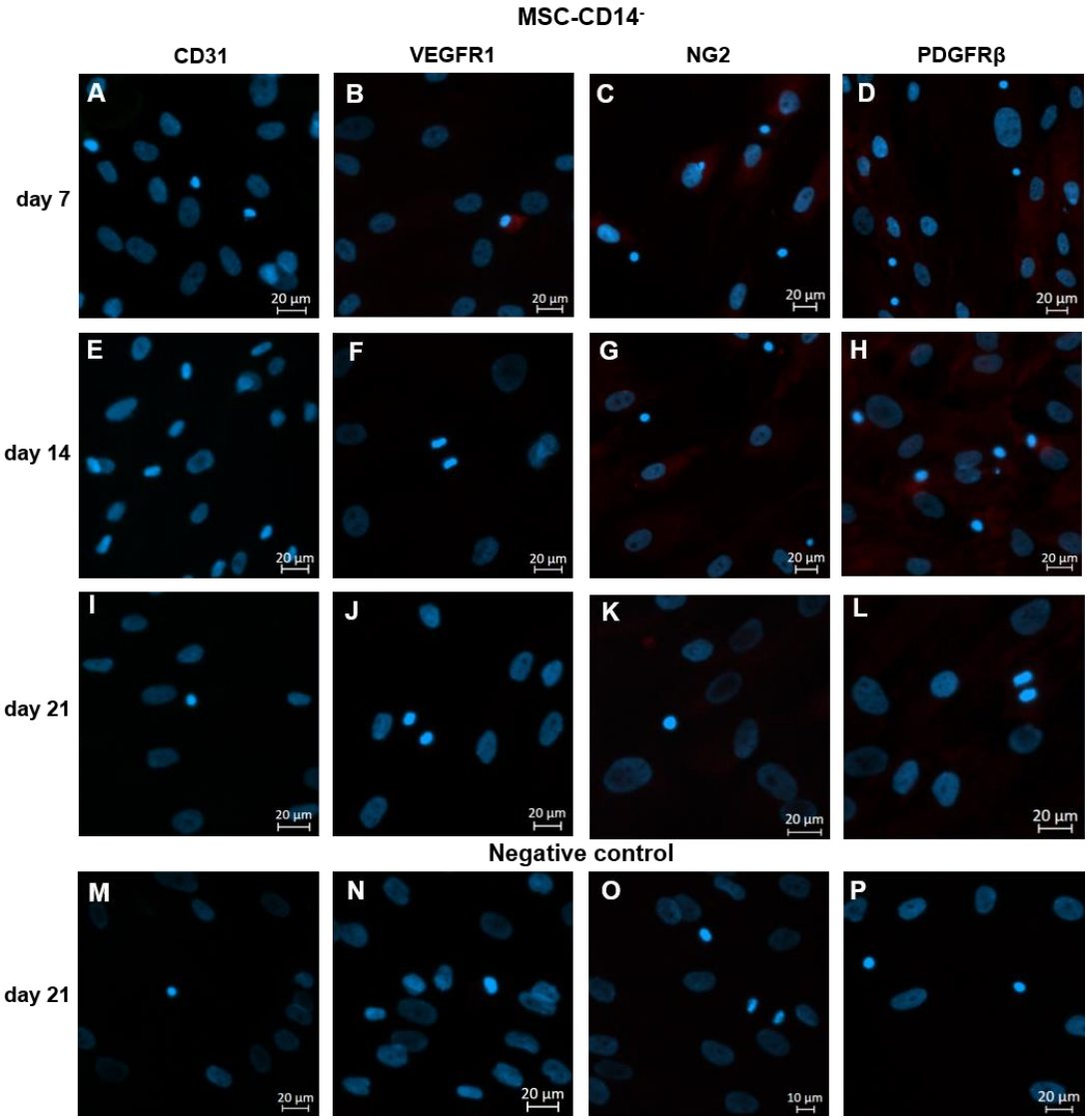

Figure 27. Co-cultures of MSC-CD14$^-$ stained for EC and pericyte markers. Representative images of MSC-CD14$^-$ co-cultures stained for EC markers, CD31 (green) and VEGFR1 (red) on day 7 (A, B), day 14 (E, F) and day 21 (I, J) and for pericyte markers, NG2 (red) and PDGFRβ (red) on day 7 (C, D), day 14 (G, H) and day 21 (K, L) and corresponding negative controls for each marker on day 21 (M-P). The results are representative of three independent experiments.

In MSC-CD34$^+$ co-cultures the immunostaining results showed that some CD34$^+$ cells expressed CD31 (Fig. 28A, 28D, 28G), VEGFR1 (Fig. 28B, 28E, 28H) and the pericyte marker NG2 (Fig. 28C, 28F, 28I) at all time points. No background staining was observed (Fig. 28J-L). Additionally, PDGFRβ staining could not be performed due to technical limitations.

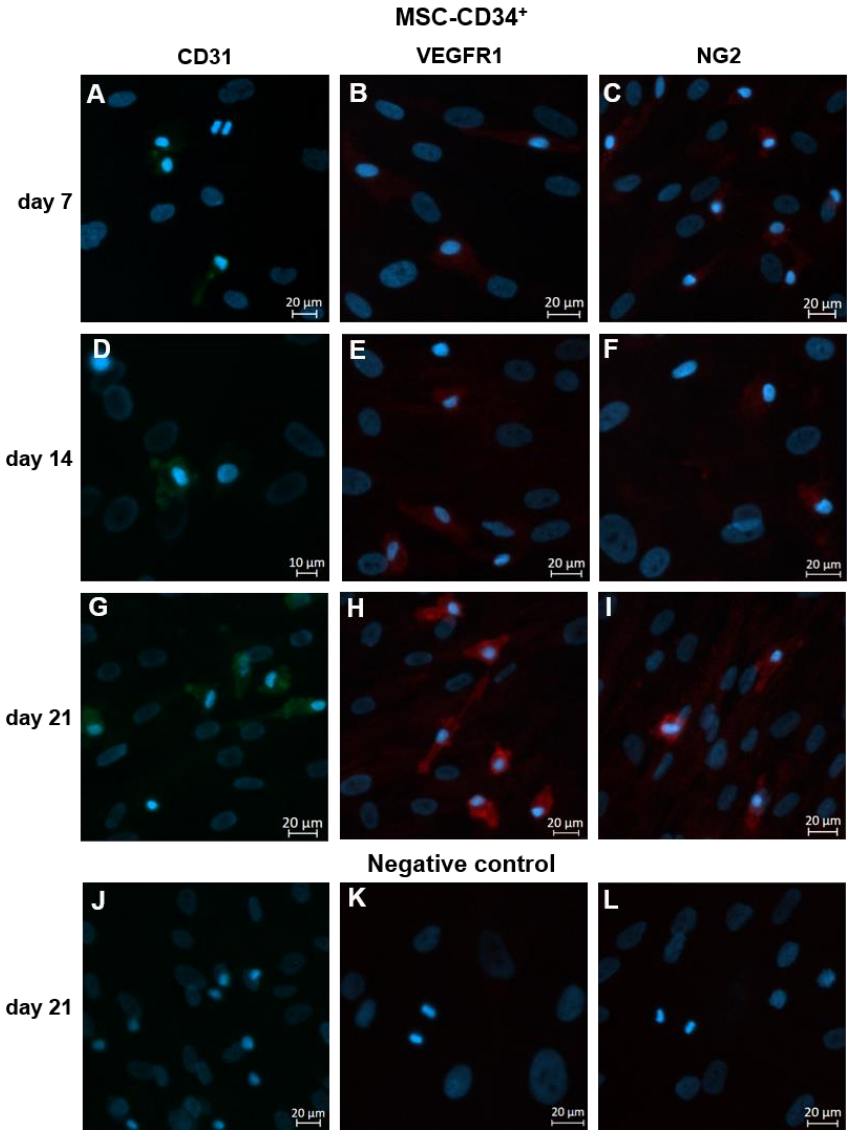

Figure 28. Co-cultures of MSC-CD34$^+$ stained for EC and pericyte markers. Representative images of MSC-CD34$^+$ co-cultures stained for EC markers, CD31 (green) and VEGFR1 (red) on day 7 (A, B), day 14 (D, E) and day 21 (G, H) and for pericyte marker NG2 (red) on day 7 (C), day 14 (F) and day 21 (I) and corresponding negative controls for each marker on day 21 (J-L). The results are representative of two independent experiments.

MSC-CD34$^-$ co-cultures showed the same expression pattern as the MSC-CD34$^+$ co-cultures. That is, some of CD34$^-$ cells expressed the EC markers CD31 (Fig. 29A, 29D, 29G) and VEGFR1 (Fig. 29B, 29E, 29H) and both MSCs and CD34$^-$ cells expressed the pericyte marker NG2 (Fig. 29C, 29F, 29I) at all time points. Again, no background staining was observed (Fig. 29J-L) and PDGFRβ staining could not be performed due to technical limitations.

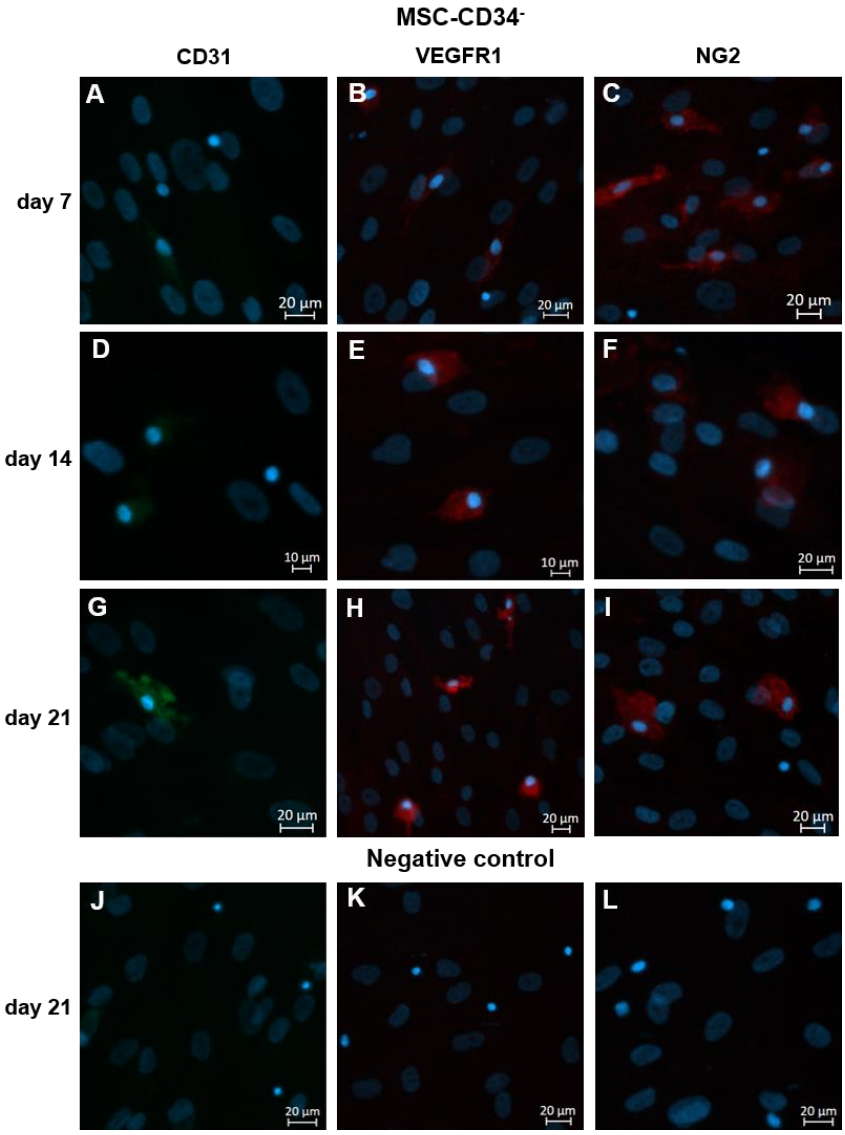

Figure 29. Co-cultures of MSC-CD34$^-$ stained for EC and pericyte markers. Representative images of MSC-CD34$^-$ co-cultures stained for EC markers, CD31 (green) and VEGFR1 (red) on day 7 (A, B), day 14 (D, E) and day 21 (G, H) and for pericyte marker NG2 (red) on day 7 (C), day 14 (F) and day 21 (I) and corresponding negative controls for each marker on day 21 (J-L). The results are representative of three independent experiments.

MSC-CD14$^+$CD34$^-$ co-cultures showed positive staining for both EC and pericyte markers at all time points (Fig. 30). Furthermore, it was observed that VEGFR1 (Fig. 30B, 30F, 30J), NG2 (Fig. 30C, 30G, 30K) and PDGFRβ (Fig. 30D, 30H, 30L) were expressed in both CD14$^+$CD34$^-$ cells and MSCs, while the EC marker CD31 was just expressed in CD14$^+$CD34$^-$ cells (Fig. 30A, 30E, 30I). Additionally, no background staining was observed (Fig. 30M-P).

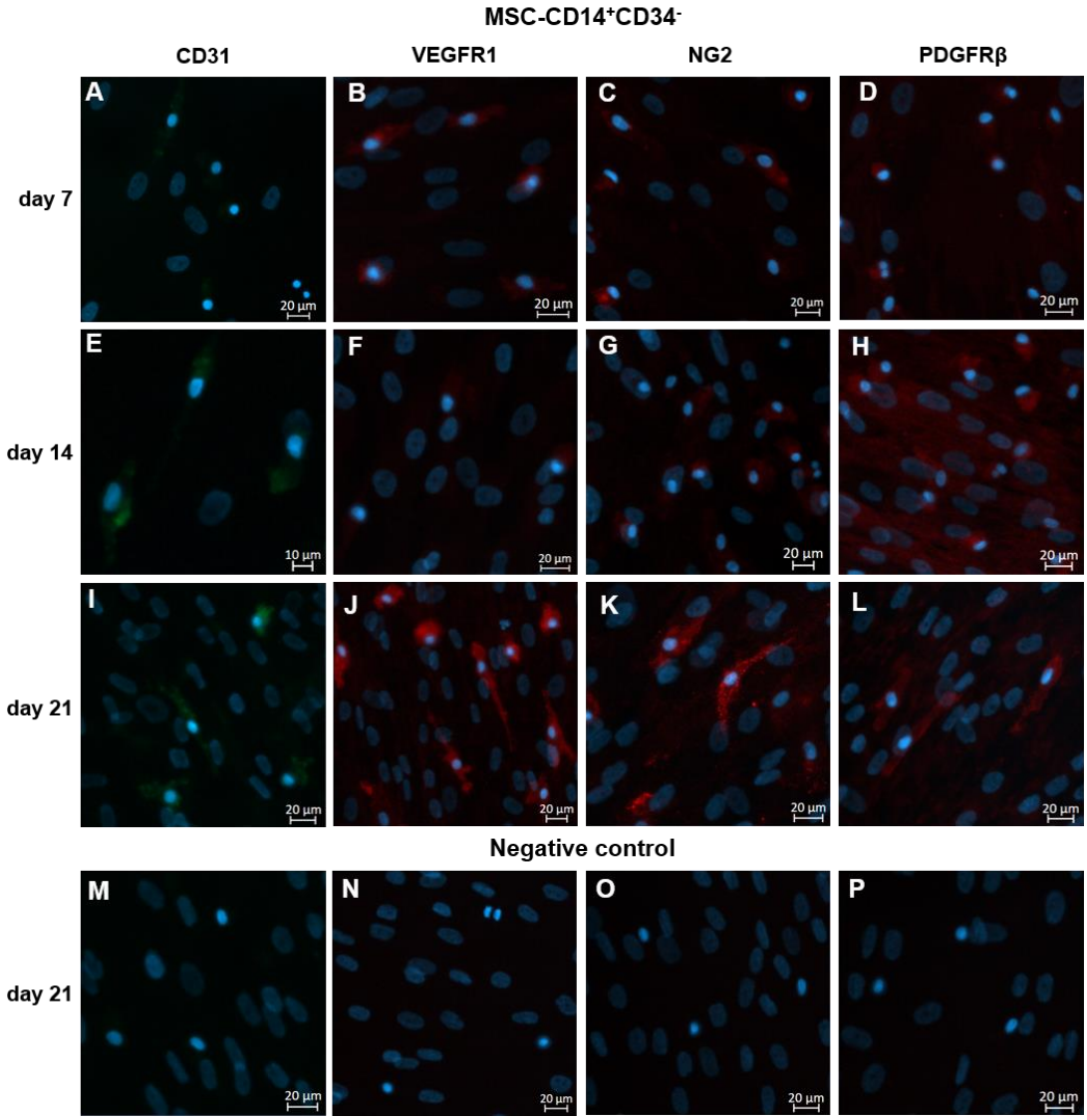

Figure 30. Co-cultures of MSC-CD14$^+$CD34$^-$ stained for EC and pericyte markers. Representative images of MSC-CD14$^+$CD34$^-$ co-cultures stained for EC markers, CD31 (green) and VEGFR1 (red) on day 7 (A, B), day 14 (E, F) and day 21 (I, J) and for pericyte markers, NG2 (red) and PDGFRβ (red) on day 7 (C, D), day 14 (G, H) and day 21 (K, L) and corresponding negative controls for each marker on day 21 (M-P). The results are representative of three independent experiments.

The evaluation of EC and pericyte marker expression in MSC-CD14$^-$CD34$^-$ co-cultures showed that CD31 was not expressed at any time point (Fig. 31A, 31E, 31I). However, VEGFR1 was expressed on day 14 in MSCs (Fig. 31F) and on day 21 in both MSCs and CD14$^-$CD34$^-$ cells (Fig. 31J). Expression of NG2 was observed in both MSCs and CD14$^-$CD34$^-$ cells at all time points (Fig. 31C, 31G, 31K). Nevertheless, expression of PDGFRβ was just observed on day 21 (Fig. 31L). Again, no background staining was observed (Fig. 31M-P).

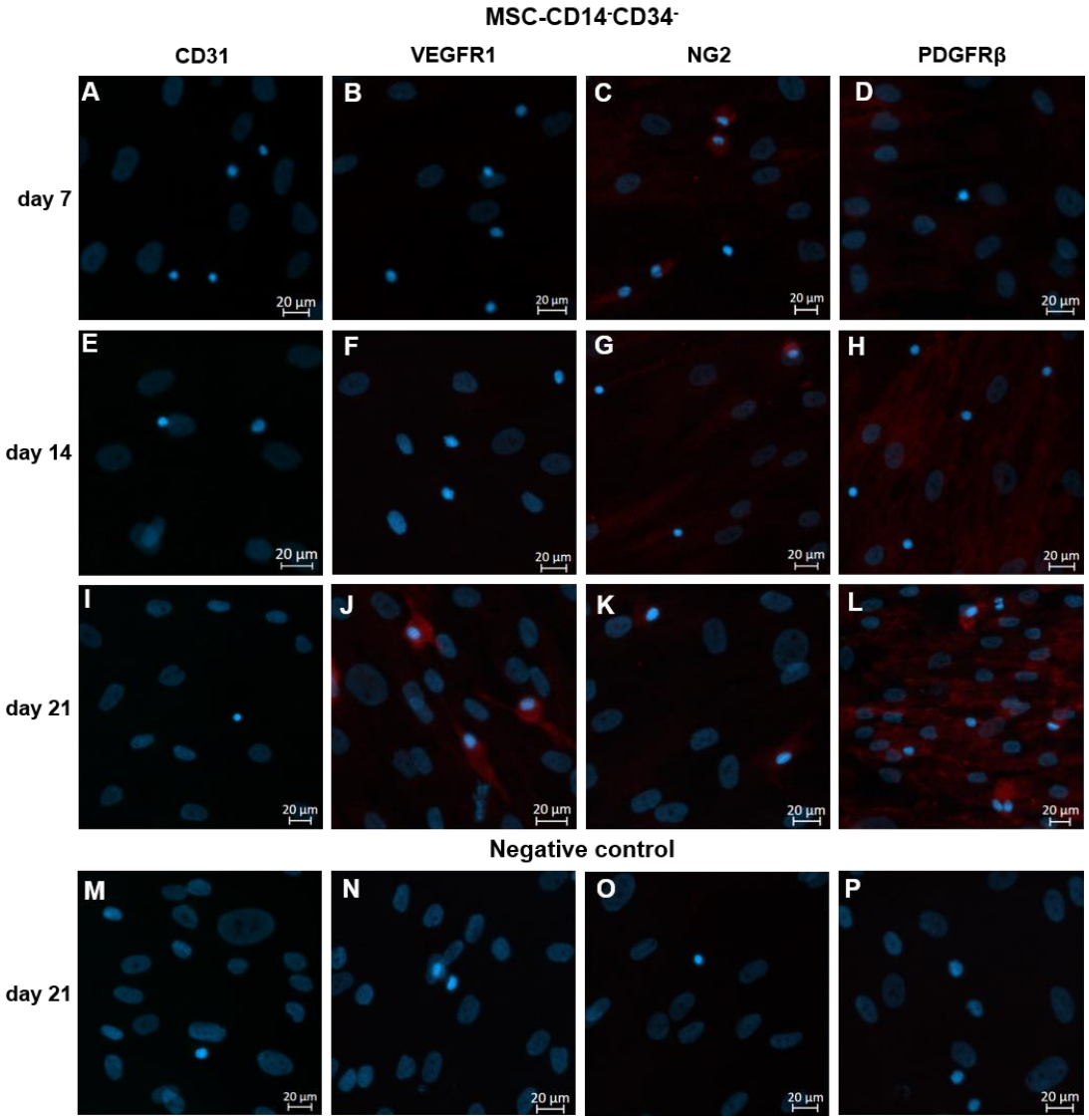

Figure 31. Co-cultures of MSC-CD14$^-$CD34$^-$ stained for EC and pericyte markers. Representative images of MSC-CD14$^-$CD34$^-$ co-cultures stained for EC markers, CD31 (green) and VEGFR1 (red) on day 7 (A, B), day 14 (E, F) and day 21 (I, J) and for pericyte markers, NG2 (red) and PDGFRβ (red) on day 7 (C, D), day 14 (G, H) and day 21 (K, L) and corresponding negative controls for each marker on day 21 (M-P). The results are representative of three independent experiments.

Table 5. Summary of immunocytochemical stainings of MSC-MNC, MSC-CD14$^+$, MSC-CD14$^-$, MSC-CD34$^+$, MSC-CD34$^-$, MSC-CD14$^+$CD34$^-$ and MSC-CD14$^-$CD34$^-$ co-cultures. The cells were grown and after 7, 14 or 21 days were stained for EC (CD31 and VEGFR1) and pericyte (NG2 and PDGFRβ) markers. Data was analyzed with a fluorescence microscope and the intensity of staining is expressed as -, negative staining; (+), weak staining; + moderate staining; ++, strong staining; +++ very strong staining; n.a., data not available.

	CD31			VEGFR1			NG2			PDGFRβ		
	7	14	21	7	14	21	7	14	21	7	14	21
MSC - MNC	+++	+++	++	++	++	++	++	+++	+++	+	++	++
MSC - CD14$^+$	++	++	++	++	++	++	+++	+++	++	+	+++	++
MSC - CD14$^-$	-	-	-	++	-	-	++	++	+	++	+++	++
MSC - CD34$^+$	++	++	++	++	++	+++	++	++	+++	n.a.	n.a.	n.a.
MSC - CD34$^-$	+	+	+++	++	+++	+++	+++	+++	+++	n.a.	n.a.	n.a.
MSC - CD14$^+$CD34$^-$	(+)	++	++	++	+	+++	++	++	+++	++	+++	+++
MSC - CD14$^-$CD34$^-$	-	-	-	-	(+)	+++	++	++	++	++	+++	+++

In order to confirm the results obtained by immunocytochemistry, the CD31, VEGFR1, NG2 and PDGFRβ marker gene expression on days 7 and 14 was analyzed by qPCR. As previously mentioned, mRNA expression was evaluated in all the groups both on days 7 and 14, except for the MSC-CD34$^+$ co-cultures, which were just analyzed on day 7 due to the limited number of isolated CD34$^+$ cells.

The results showed that CD31 was upregulated in MSC-CD14$^+$ co-cultures ($p < 0{,}0001$ on days 7 and 14) and downregulated in MSC-CD14$^-$ co-cultures when compared with MSC-MNC co-cultures ($p = 0{,}0001$ on days 7 and $p < 0{,}0001$ on day 14) (Fig. 32A). These results confirmed what had already been suggested by our immunocytochemistry results: expression level of CD31 was higher in MSC-CD14$^+$ co-cultures compared to MSC-CD14$^-$ co-cultures in both time points, confirming the higher capacity of CD14$^+$ cells to differentiate into ECs. Nevertheless, VEGFR1 was more highly expressed in MSC-CD14$^-$ co-cultures when compared with MSC-MNC co-cultures on day 7 ($p = 0{,}0103$), while no significant differences were observed on day 14 (Fig. 32B).

MSC-CD14$^+$ co-cultures, on the other hand, showed no significant differences in VEGFR1 expression at either time point when compared with MSC-MNC co-cultures (Fig. 32B).

When the expression levels of NG2 and PDGFRβ were compared in MSC-CD14$^+$ and MSC-CD14$^-$ co-cultures with MSC-MNC co-cultures, no significant differences were observed for either pericyte gene (Fig. 32C, 32D), except for MSC-CD14$^+$ co-cultures that showed a slight downregulation of PDGFRβ on day 7 (p = 0,0304) (Fig. 32D).

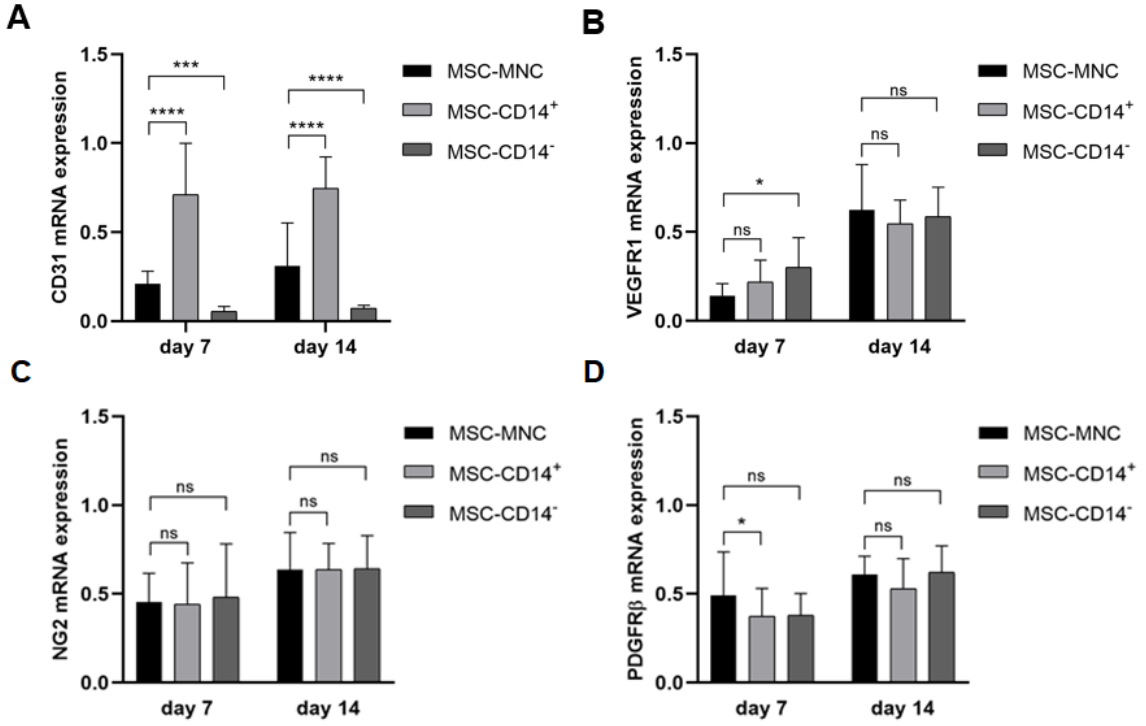

Figure 32. Expression levels of EC and pericyte markers in MSC-CD14$^+$ and MSC-CD14$^-$ co-cultures. mRNA expression levels of CD31 (A), VEGFR1(B), NG2 (C) and PDGFRβ (D) were analysed in MSC-MNC, MSC-CD14$^+$ and MSC-CD14$^-$ co-cultures on days 7 and 14. Data is expressed as mean ± SD; ns, non-significant; * p≤0,05; *** p≤0,001 and **** p≤0,0001. One-way ANOVA followed by Bonferroni multiple comparison test was used. The results are from three independent experiments and in each experiment three samples were analysed in duplicate.

Expression of CD31 in MSC-CD34$^+$ co-cultures was not significantly different to MSC-MNC co-cultures (Fig. 33A). Nevertheless, VEGFR1 was upregulated in these co-cultures when compared with control co-cultures (p = 0,0008) (Fig. 33B). In MSC-CD34$^-$ co-cultures, the expression of CD31 and VEGFR1 was slightly higher than in control co-cultures on day 7 (p = 0,0301 and p = 0,0188, respectively) (Fig. 33A, 33B). However, on day 14 no significant differences were observed between MSC-CD34$^-$ and MSC-MNC co-cultures for either EC marker (Fig. 33A, 33B). Taken together, these results and those mentioned above suggest that both EC markers may not be expressed in all the co-cultures in a temporally similar manner.

The expression of NG2 in MSC-CD34$^+$ co-cultures was not significantly different from MSC-MNC co-cultures (Fig. 33C). On the other hand, NG2 was downregulated in MSC-CD34$^-$ co-cultures, both on days 7 and 14 (p = 0,0014 and 0,0123, respectively) (Fig. 33C). Additionally, the results showed no significant differences in PDGFRβ expression when MSC-CD34$^+$ and MSC-CD34$^-$ co-cultures were compared with MSC-MNC co-cultures (Fig. 33D).

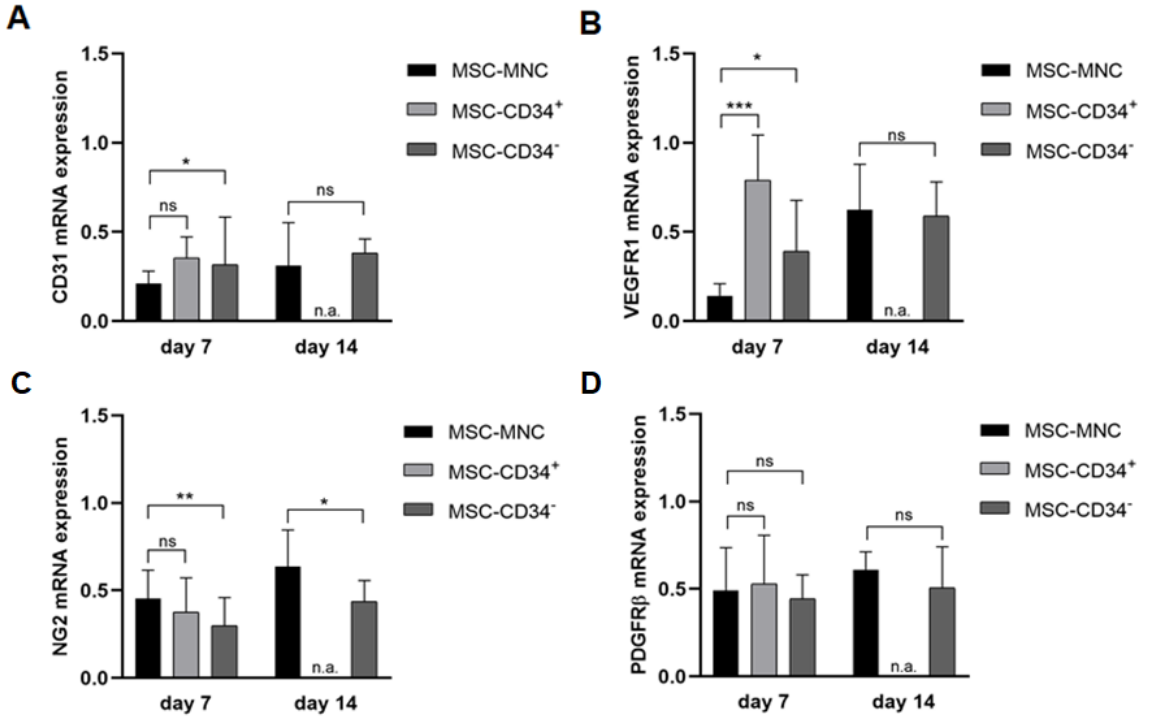

Figure 33. Expression levels of EC and pericyte markers in MSC-CD34$^+$ and MSC-CD34$^-$ co-cultures. mRNA expression levels of CD31 (A), VEGFR1(B), NG2 (C) and PDGFRβ (D) were analysed in MSC-MNC, MSC-CD34$^+$ and MSC-CD34$^-$ co-cultures on day 7 and in MSC-MNC and MSC-CD34$^-$ co-cultures on day 14. Data is expressed as mean ± SD; n.a., data not available; ns, non-significant; * p≤0,05; ** p≤0,01 and *** p≤0,001. One-way ANOVA followed by Bonferroni multiple comparison test was used. The results are from three independent experiments and in each experiment three samples were analysed in duplicate, except for MSC-CD34$^+$ co-cultures where only one sample was obtained for analysis.

4. Discussion

Neovascularization is an important issue for the success of tissue regeneration therapies and tissue engineered constructs. To solve this issue, MSCs and EPCs have been studied due to the angiogenic and vasculogenic potential that both these cell types might have. MSCs have been described to be able to differentiate into pericytes (Blocki et al., 2013), ECs (Oswald et al. 2014), and SMCs (Au et al., 2008), while EPCs derived from PB-MNCs have been reported to differentiate into ECs (Asahara et al., 1997; Boyer et al., 2000; Pujol et al., 2000; Harraz et al., 2001). Besides that, it has been hypothesized that pericytes, which are the cells responsible for maintaining blood vessels, supporting ECs and regulating angiogenesis, could contain different populations: one which is a MSCs subpopulation and another which has a monocyte origin (Blocki et al., 2018). This suggests that pericyte differentiation might also be an important aspect in developing tissue regenerative therapies. Additionally, recently, co-culture systems of MSCs and MNCs have been established and their angiogenic potential was demonstrated by EC and pericyte differentiation (Joensuu et al., 2011; Joensuu et al., 2018). Nevertheless, the contribution of specific cell populations that induced the vasculogenic potential remained unknown.

To address this question, different cell populations were cultured and co-cultured with MSCs to evaluate the vasculogenic capacity by IncuCyte and ImageJ, as well as to determine the origin of ECs and pericytes by immunocytochemistry and qPCR in this research project.

Immunocytochemical staining results demonstrated that cultured MSCs express the pericyte markers NG2 and PDGFRβ. However, not all the MSCs were positive for these markers confirming the well-known heterogeneity of this cell population (Horwitz et al., 2005). Furthermore, we demonstrated that our MSCs were negative for both EC markers, CD31 and VEGFR1, while MNCs were positive for these markers, both in culture and in co-culture. This indicates that EPCs in our co-culture setup originate from MNCs. Additionally, it was observed that when MNCs were alone in culture they had a rounded morphology or changed their morphology into monocyte-like or spindle-shaped, while in co-culture with MSCs only changes into a spindle-shaped morphology were observed. This suggests that MSCs produce the essential signals for MNCs differentiate into spindle-shaped cells.

It has been described that EPCs can be found within the PB-MNC population. According to some studies, EPCs are positive for CD14 and CD34 (Pujol et al., 2000; Asahara et al., 1997), while according to others EPCs would be negative for CD34 (Harraz et al., 2001; Murga et al., 2004). In the current study, the results of EPC isolation from PB-MNCs showed that $CD14^+$ cells are on average ten times more abundant than $CD34^+$ cells and that just 1% of PB-MNCs are $CD34^+$ cells, confirming the low number of $CD34^+$ cells in the PB-MNC fraction, as previously described by Asahara et al. (Asahara et al., 1997). We also demonstrated that in co-culture only cells with a spindle-shaped morphology were observed, while when cultured alone the $CD14^+$ cells changed also for monocyte-like morphology. This indicates that MSCs provide the essential signals for $CD14^+$ cells to differentiate into spindle-shaped cells. Furthermore, we showed that the number of

spindle-shaped cells was approximately doubled after 21 days of MSC-CD14$^+$ co-culture when compared to MSC-MNC co-cultures, suggesting a higher capacity of MSC-CD14$^+$ co-cultures for vascularization. Immunocytochemical staining results demonstrated that CD14$^+$ cells expressed CD31, VEGFR1, NG2 and PDGFRβ both alone in culture and in co-culture with MSCs. Then, by qPCR we showed that CD31 was upregulated in these co-cultures both on days 7 and 14, confirming the increased vasculogenic capacity by EC differentiation.

CD14$^-$ cells were also studied alone in culture and in co-culture. The results demonstrated that just a few cells acquired a spindle-shaped morphology both alone in culture and in co-culture, suggesting that MSC-CD14$^-$ co-cultures do not possess a good capacity for vascularization. Moreover, the immunocytochemical staining results showed that when in culture alone, none of CD14$^-$ cells were positive for EC or pericyte markers, while in co-culture with MSCs, CD14$^-$ cells expressed VEGFR1 and PDGFRβ. Additionally, the qPCR results demonstrated that in these co-cultures the CD31 marker gene was downregulated on days 7 and 14, while VEGFR1 was slightly upregulated on day 7 compared to the control co-cultures. Taken together these results and the study where Eilken et al. pointed out pericytes as VEGFR1 expressing cells that may contribute for regulating the EC sprouting, we hypothesize that CD14$^-$ cells expressing VEGFR1 are the same cells that express PDGFRβ and that this indicates pericyte differentiation (Eilken et al., 2017). However, to confirm such hypothesis we should have made a double immunostaining for both markers.

From the analysis of the potential of CD34$^+$ cells in co-culture with MSCs, we demonstrated what had already been shown with MSC-CD14$^+$ co-cultures. That is, the number of spindle-shaped cells was approximately two times higher than in the control co-cultures after 21 days of co-culture. This shows that MSC-CD34$^+$ co-cultures have a high vasculogenic potential and both MSC-CD34$^+$ and MSC-CD14$^+$ co-cultures present the same capacity for vascularization. Furthermore, by immunocytochemistry we observed that MSC-CD34$^+$ co-cultures expressed both EC markers, CD31 and VEGFR1 and the pericyte marker NG2, while by qPCR no significant differences were observed in the expression of CD31, NG2 or PDGFRβ in either time point, when compared with MSC-MNC co-cultures. Nevertheless, VEGFR1 was upregulated in these co-cultures on day 7, demonstrating that as in MSC-CD14$^+$ and MSC-CD14$^-$ co-cultures the expression of both EC markers CD31 and VEGFR1 may not occur in a temporally similar manner.

The experiments with CD34$^-$ cells alone in culture and in co-culture demonstrated that when in culture, these cells acquired both spindle-shaped and monocyte-like morphology, while in co-culture only spindle-shaped morphology was observed. This is in line with our previous observations in other co-cultures and indicates that MSCs produce the crucial signals for CD34$^-$ cells to differentiate into spindle-shaped cells. Immunocytochemical stainings demonstrated that CD34$^-$ cells expressed CD31, VEGFR1 and NG2 both alone in culture and in co-culture with MSCs. Moreover, the qPCR results showed upregulation for both CD31 and VEGFR1 on day 7,

corroborating with immunocytochemical results and indicating EC differentiation. However, the pericyte marker NG2 was downregulated on days 7 and 14, suggesting less pericyte differentiation in these co-cultures than in MSC-MNC control co-cultures.

Finally, we analyzed the potential of double-isolated $CD14^+CD34^-$ and $CD14^-CD34^-$ cells to induce vascularization when in co-culture with MSCs. Our results demonstrated that MSC-$CD14^+CD34^-$ co-cultures present approximately the same vasculogenic potential as MSC-MNC control co-cultures, while MSC-$CD14^-CD34^-$ co-cultures presented less than half of the vasculogenic capacity of control co-cultures. Interestingly, both $CD14^+CD34^-$ and $CD14^-CD34^-$ cells expressed the pericyte markers, NG2 and PDGFRβ, suggesting that there are at least two different populations of pericyte-like cells within MNCs. According to Stapor et al., different pericyte populations could have different specialized functions, such as recruitment, paracrine signaling, extracellular matrix modulation, as well as direct interactions with ECs (Stapor et al., 2014). This, together with our observations, suggests that the two different populations of pericyte-like cells we found within MNCs could play different functions during angiogenesis.

To avoid potential inter-individual variations, all ten experiments were performed using blood samples from the same donor. Despite of this, there was a clear variation in the number of isolated cells between experiments, which is most likely caused by individual variation and the quality of the samples. Additionally, many cells were lost during the isolations, especially in the first three experiments, where 38-63% of the cells were lost. These losses were most likely due to the wrong execution of step three of the MACS protocol: i.e., the column was not removed from the magnetic separator as it should have and therefore many of the cells that were attached to the magnetic microbeads must have remained in the column. Furthermore, during this research project we faced two main limitations: one related to the fact that we needed to expand MSC cultures up to 16-21 days before we had enough cells for co-cultures with MNCs and another associated to the restricted execution of different experiments simultaneously, which was caused by the time frame for collection of blood samples from the same donor.

One of the aims in this study was to evaluate the pericyte origin in our co-cultures. As mentioned above, pericytes are responsible to maintain blood vessels, support ECs and regulate angiogenesis - the functions that will be performed after the blood vessels are formed. Because of that and because we did not find any significant upregulation of pericyte markers in most of our co-cultures by qPCR, it is possible that an extended culturing time could have improved the pericyte differentiation in our co-cultures.

The experiments in this research project were performed using basal medium which contains α-MEM, FBS, streptomycin and penicillin. We could have added growth factors or even used a richer and more appropriate medium for EC differentiation, such as endothelial cell growth medium. However, we tried to keep our study as natural and simple as possible for three reasons. First, because in co-culture it would have been very difficult to predict what effects some additional

growth factors or medium components could have had on each cells type. Second, because by keeping it simple and still observing vasculogenic potential, we are able to hypothesize that even better results could be obtained with the addition of growth factors or using a more enriched medium. Third, this way we were able to get closer to the applicability in tissue engineering and regeneration therapies. If one day such co-cultures would be implanted in an animal model or in the human body, the tissue itself around the implant may not have all the favorable factors for success and thus we can rely on the fact that already at a more basic level our implant has the vasculogenic capacity. Nevertheless, it would be interesting to carry out a study in which growth factors were added or a more enriched medium was used in the same setup. Furthermore, for future applications, it would be important to use the patient's own cells to avoid rejection.

5. Conclusions and future perspectives

In this research project, we aimed to find the specific cell populations, which are present in PB-MNCs and that in co-culture with MSCs are able to induce the greatest vasculogenic potential. Another aim was to identify the origin of ECs and pericytes previous observed in MSC-MNC co-cultures.

Here we showed that when cultured alone, $CD14^+$, $CD34^+$ and $CD34^-$ cells changed into a monocyte-like or spindle-shaped morphology, while in co-culture these cells just acquired a spindle-shaped morphology. This indicates that MSCs provide the essential signals for these cells to differentiate into spindle-shaped cells and prevents their commitment into monocyte-like morphology.

We identified MSC-$CD14^+$ and MSC-$CD34^+$ co-cultures as the setup with greatest vasculogenic potential, which demonstrates that $CD14^+$ and $CD34^+$ cell populations contain more EPCs than the other evaluated fractions. We also showed that MSC-$CD14^+CD34^-$ co-cultures presented higher vasculogenic capacity than MSC-$CD14^-CD34^-$ co-cultures. However, in the future, it would also be very important to compare MSC-$CD14^+CD34^-$ and MSC-$CD14^+CD34^+$ co-cultures for a better evaluation and understanding of the vasculogenic potential of these cell populations.

We demonstrated that EC markers were expressed by $CD14^+$, $CD34^+$ and $CD34^-$ cells, thus showing that ECs differentiate from MNCs and not from MSCs. We furthermore showed that pericyte markers were express both by some MSCs, as well as by $CD14^+$, $CD14^-$, $CD34^+$ and $CD34^-$ cells and interestingly, both pericyte markers were expressed also in MSC-$CD14^+CD34^-$ and MSC-$CD14^-CD34^-$ co-cultures, proving that pericyte-like cells originate from both MSCs and MNCs and thus raising the hypothesis that there are at least two populations of pericyte-like cells with different angiogenic functions within the PB-MNCs. The existence of pericytes and their progenitors in PB thus opens up the possibility for the use of these blood-derived cells as autogenous sources to improve vascularization in tissue regeneration therapies and tissue engineering constructs.

Despite these promising results, further investigations are needed. Next steps should include: the use of other surface markers, such as the early EPC marker CD133 and the typical EC marker CD31 to even more specifically isolate different PB-MNC subpopulations and to evaluate their angiogenic potential in co-culture with MSCs; the use of double immunocytochemical stainings to clarify, which cells are true pericytes and which are ECs; the characterization of MSC secretome and addition of various biomolecules into the cultures of different MNC fractions to reveal the key molecular regulators responsible for the vasculogenic capacity; as well as the establishment of three-dimensional co-cultures, which would better mimic the *in vivo* environment for evaluating the formation of vascular structures. Furthermore, it would be interesting to use the chick embryo chorioallantoic membrane (CAM) model to study the *in vivo* potential of our co-cultures. This model would be performed by implantation of our co-cultures on the CAM through a hole cut in the egg shell. Then, after 1-3 days, neovascularization and angiogenesis could be quantified via image analysis or colorimetric detection methods.

6. References

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