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MESENCHYMAL STROMAL CELLS AND CIRCULATING ANGIOGENIC CELLS IN VASCULARIZATION

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TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA – SER. D OSA – TOM. 1659 | MEDICA – ODONTOLOGICA | TURKU 2022

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ISBN 978-951-29-8971-3 (PRINT)
ISBN 978-951-29-8972-0 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama, Turku, Finland 2022

To my family

UNIVERSITY OF TURKU
Faculty of Medicine
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Cell Biology and Anatomy
LIINA UUSITALO-KYLMÄLÄ: Mesenchymal stromal cells and circulating angiogenic cells in vascularization
Doctoral Dissertation, 121 pp.
Turku Doctoral Programme of Molecular Medicine (TuDMM)
October 2022

ABSTRACT

Vascularization is essential for tissue healing and reduced ability to regrow blood vessels can contribute, for example, to impaired wound healing and bone formation after injury. Generation of functional blood vessels is the key challenge for regenerative medicine and further development of vascular tissue engineering methods are still needed.

Mesenchymal stromal/stem cells (MSCs) contribute to tissue healing through their differentiation capacity and by promoting new blood vessel formation. Endothelial progenitor cells (EPCs), which can be isolated from peripheral blood (PB) and cultured in vitro are consistently reported to produce two different types of EPCs. Endothelial colony forming cells (ECFCs) can differentiate into mature endothelial cells and themselves form capillary-like structures, while myeloid angiogenic cells (MACs) increase vascularization in a paracrine manner. Thus, co-cultures of MSCs and PB derived cells may present a potential cell-based tool to enhance angiogenesis in tissue transplants and to promote tissue healing.

In this thesis, increased expression of proangiogenic factors was demonstrated in co-cultures of bone marrow (BM) derived MSCs and PB derived mononuclear cells (MNCs) in both basal and osteoblastic culture conditions, while the expression profiles differed between the two conditions. Furthermore, differentiation of functional MACs was observed in the MSC-MNC co-cultures and pericyte-like cells with different marker profiles were discovered within both MSCs and MNCs. In addition, we showed, for the first time, that circulating MACs and hematopoietic pericyte-like cells are found in both patients with traumatic skin defect and in healthy controls. These results suggest that MACs are not only generated in vitro but they do exist in circulation in vivo.

To improve the therapeutic competency of MSCs and MNCs, it is important to understand their contribution to neovascularization. Results presented here provide important knowledge on the functions and interactions between MSCs and MNCs and thus improve their potential for future clinical use in regenerative medicine.

KEYWORDS: mesenchymal stromal cell, mononuclear cell, myeloid angiogenic cell, pericyte, co-culture, vascularization

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
Biolääketieteen laitos
Solubiologia ja anatomia
LIINA UUSITALO-KYLMÄLÄ: Mesenkymaaliset kantasolut ja verenkierron angiogeeniset solut verisuonten uudismuodostuksessa.
Väitöskirja, 121 s.
Molekyylilääketieteen tohtoriohjelma (TuDMM)
Lokakuu 2022

TIIVISTELMÄ

Verisuonten uudismuodostus on merkittävässä roolissa kudosvaurioiden korjaamisessa ja heikentyneen verisuonituksen seurauksena voikin olla muun muassa haavan tai luunmurtuman paranemisen hidastuminen. Regeneratiivisen lääketieteen yksi suurimmista haasteista on saada aikaan tehokas ja toimiva verisuonitus, minkä vuoksi tarvitaan menetelmiä, joilla esimerkiksi kudossiirteiden verisuonten uudismuodostusta voitaisiin tehostaa.

Mesenkymaaliset kantasolut (eng. MSCs) edistävät kudosten paranemista niin erilaistumalla mesenkymaalisten kudosten eri soluiksi kuin erittämällä esimerkiksi tulehdusreaktiota estäviä sekä verisuonten muodostukseen tarvittavia tekijöitä. Verenkierrosta eristettävä mononukleaarisolupopulaatio (eng. MNCs) puolestaan sisältää kahdenlaisia verisuonten uudismuodostukseen osallistuvia endoteelisolujen esiasteita. Todelliset endoteelisolujen esiastesolut voivat itse muodostaa verisuonimaisia rakenteita, kun taas myeloidisen linjan angiogeeniset esiastesolut (eng. MACs) lisäävät verisuonten muodostusta parakriinisesti. Mesenkymaalisten kantasolujen ja verenkierron solujen yhteisviljelmiä voidaankin mahdollisesti hyödyntää kudosteknologian alalla uudisverisuonituksen tehostamiseksi kudossiirteissä.

Tässä väitöskirjassa osoitettiin, että kun MSC-soluja ja verenkierron MNC-soluja viljeltiin yhdessä sekä perusolosuhteissa, että luun muodostusta lisäävissä olosuhteissa, verisuonten muodostukseen tarvittavien tekijöiden ilmentyminen lisääntyi. Lisäksi osoitettiin, että yhteisviljelmässä erilaistui sekä verisuonten muodostukseen tarvittavia perisyyttejä että toiminnallisia MAC-soluja. Näiden tulosten lisäksi MAC-soluja ja perisyyttimäisiä soluja löydettiin traumaattisista ihovaurioista kärsivien potilaiden sekä terveiden luovuttajien verenkierrosta. Nämä tulokset viittaavat siihen, että MAC-soluja ei muodostu vain viljelyolosuhteissa, vaan niitä esiintyy myös verenkierrossa.

MSC:jen ja MNC:jen välisten vuorovaikutusten ja niiden merkityksen ymmärtäminen kudosten korjaamisessa ja uusiutumisessa onkin keskeistä, jotta niitä voitaisiin tulevaisuudessa paremmin hyödyntää kliinisessä käytössä esimerkiksi tehostamassa uudisverisuonitusta kudossiirteessä.

AVAINSANAT: Mesenkymaalinen kantasolu, mononukleaarisolu, myeloidisen linjan angiogeeninen esiastesolu, perisyytti, yhteisviljelmä, verisuonitus

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Abbreviations

2D	two-dimensional
3D	three-dimensional
αMEM	alpha minimum essential medium
αSMA	alpha smooth muscle actin
ALP	alkaline phosphatase
ANG1	angiopoietin-1
ASC	adipose tissue-derived mesenchymal stromal cell
BM	bone marrow
BM-MSC	bone marrow derived mesenchymal stromal cell
BMP2	bone morphogenic protein-2
BSA	bovine serum albumin
BTE	bone tissue engineering
CAM	chick chorioallantoic membrane
DAB	diaminobenzene
Dll4	delta like canonical Notch ligand 4
EC	endothelial cell
ECFC	endothelial colony forming cell
EGM	endothelial growth medium
EGM-2	endothelial growth medium-2
EPC	endothelial progenitor cell
ELISA	enzyme-linked immunosorbent assay
EV	extracellular vesicle
FBS	fetal bovine serum
FGF2	fibroblast growth factor-2
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP-HUVEC	green fluorescent protein expressing human umbilical vein endothelial cell
HRP	horseradish peroxidase
HSC	hematopoietic stem cell
HUVEC	human umbilical vein endothelial cell
ICC	immunocytochemistry

IHC	immunohistochemistry
IF	immunofluorescense
IL-8	interleukin 8
iPSC	induced pluripotent stem cell
MAC	myeloid angiogenic cell
mRNA	messenger RNA
miRNA	micro RNA
MSC	mesenchymal stromal cell
MNC	mononuclear cell
NG2	neural glial antigen 2
OB-medium	osteoblastic differentiation medium
PB	peripheral blood
PB-MNC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PDGF	platelet derived growth factor
PDGFRβ	platelet-derived growth factor receptor beta
PDGFB	platelet-derived growth factor subunit B
PIGF	placental-like growth factor
PTN	pleiotrophin
qPCR	real-time quantitative polymerase chain reaction
RT	room temperature
scRNA-seq	single cell RNA sequencing
SDF1	stromal derived growth factor-1
STSG	split-thickness skin graft
TBS	tris-buffered saline
TGFβ	transforming growth factor-β
VEGF	vascular endothelial growth factor
VEGFR1	vascular endothelial growth factor receptor-1
VEGFR2	vascular endothelial growth factor receptor-2
VEGFR3	vascular endothelial growth factor receptor-3
VSMC	vascular smooth muscle cell
vWF	von Willebrand factor

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Katriina Joensuu, **Liina Uusitalo-Kylmälä**, Teuvo A Hentunen, Terhi J Heino. Angiogenic potential of human mesenchymal stromal cell and circulating mononuclear cell co-cultures is reflected in the expression profiles of proangiogenic factors leading to endothelial cell and pericyte differentiation. *J Tissue Eng Regen Med.* 2018 Mar;12(3):775–783.

II **Liina Uusitalo-Kylmälä**, Ana Carolina Santo Mendes, Lauri Polari, Katriina Joensuu and Terhi J. Heino. An in vitro co-culture model of bone marrow mesenchymal stromal cells and peripheral blood mononuclear cells promotes the differentiation of myeloid angiogenic cells and pericyte-like cells. *Stem Cells Dev*. 2021 Mar;30(6):309–324.

III **Liina Uusitalo-Kylmälä**, Katriina Joensuu, Kristiina Hietanen, Juha Paloneva, Terhi J Heino. Evidence for the in vivo existence and mobilization of myeloid angiogenic cells and pericyte-like cells in wound patients after skin grafting. *Wound Repair Regen*. 2022 Aug 25. doi: 10.1111/wrr.13047.

1 Introduction

Vascularization has an essential role during tissue healing, since vascular networks supply cells with nutrients and oxygen. Reduced ability to regrow blood vessels contributes, for example, to impaired wound healing and bone formation. In addition, poor vascularization remains a major obstacle in regenerative medicine and tissue engineering (Novosel et al., 2011). Endothelial cells (EC) and pericytes are the essential cellular components of microvessels, as ECs form the endothelium that lines the inner surface of vessels, and pericytes further support the vascular structure.

Mesenchymal stromal cells (MSCs) are multipotent stem cells of mesodermal origin and because of their ability to promote tissue healing and regulate immune response they have gained great interest in the fields of regenerative medicine and tissue engineering (Caplan et al., 2011). MSCs reside in stromal tissues of the body and they can be differentiated in vitro, for example, into bone forming osteoblasts (Dominici et al., 2006). In addition, MSCs include a specialized subpopulation of pericytes (Blocki et al., 2013). Interestingly, pericytes with a monocyte origin have also been observed (Blocki et al., 2018). Besides such pericyte-like cells, peripheral blood derived mononuclear cells (PB-MNCs) consist of immune cells, which are needed for the inflammatory phase of tissue healing, and two types of endothelial progenitor cells (EPCs) (Medina et al., 2017). Endothelial colony forming cells (ECFCs) can differentiate into mature ECs and have tube forming capacity in vitro and in vivo, while myeloid angiogenic cells (MACs) can enhance vascularization in a paracrine manner (Medina, 2017). All this indicates that both MSCs and PB-MNCs can have potential to enhance angiogenesis in tissue transplants and thus to promote tissue healing.

The aim of this thesis was to study whether co-cultures of bone marrow derived MSCs (BM-MSCs) with PB-MNCs can form an in vitro environment promoting angiogenic cell differentiation. To achieve this, we measured the expression of proangiogenic factors and analysed the cells differentiating in the MSC-MNC co-cultures by multiple different methods. In addition, we evaluated the presence of angiogenic cells in the circulation of human patients with traumatic skin defect before and after skin graft surgery and in healthy controls.

2 Review of the Literature

2.1 Formation of blood vessels

Vascular system includes arteries, veins, and capillaries and each of these has a specific role in the circulation. Arteries are blood vessels that deliver oxygenated blood from the heart to the tissues, while veins carry blood back into the heart. Capillaries are smaller blood vessels that form the connection between arteries and veins and distribute oxygen and nutrients to the body (Krüger-Genge et al., 2019). Capillaries are often referred as microcirculation and they can be divided into three types: continuous, fenestrated, and discontinuous which can be found in different regions of the body. Differences between these capillary types are discussed in more detail in chapter 2.2.1. Larger blood vessel, namely arteries and veins consist of three layers: adventitia, media, and intima. In intima ECs form the inner lining of vessel wall, which regulates the exchange of nutrients and cells between bloodstream and surrounding tissues, whereas mural cells including pericytes and vascular smooth muscle cells (VSMC), in the media, envelop endothelial cells providing support to the vascular structure (Bergers et al., 2005) (Figure 1). Pericytes are embedded specifically within the vascular basement membrane of microvessels, while VSMCs are found around large arteries and veins. Both endothelial cells and pericytes adjust their number and arrangements depending on the function of the vessels and organs in which they are present (Armulik et al., 2005; Eelen et al., 2020).

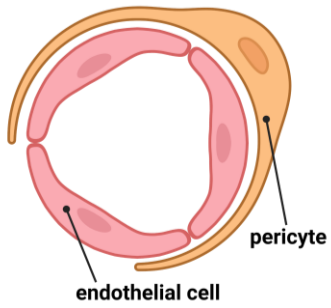

Figure 1. Endothelial cells form a thin cell layer called endothelium that lines all blood vessels and controls the passage of materials into and out of the bloodstream. Pericytes wrap around the endothelial cells thus supporting the vascular structure. Created in Biorender.com.

Adventitia is the outer layer of vessels, and it is considered as a key regulator of vascular wall function and structure. It consists of a variety of cells including fibroblasts, immune cells, progenitor cells, nutrient vessel (vasa vasorum) endothelial cells and pericytes, and adrenergic nerves and thus making it the most complex layer of the vessel wall (Stenmark et al., 2013).

Even though blood vessels consist of multiple interacting cell types, VSMCs and pericytes are the most well-studied blood vessel associated cells in addition to ECs. They can regulate the vessel blood flow by producing vasoconstriction and vasodilation. In addition, the interactions between endothelial cells and mural cells are important in the regulation of vessel formation (Bergers et al. 2005), which occurs through vasculogenesis and angiogenesis. Vasculogenesis is defined as the differentiation of endothelial progenitor cells (angioblasts) into endothelial cells and formation of a primitive vascular network in the embryo while angiogenesis refers to the growth of new capillaries from pre-existing blood vessels either via sprouting or intussusception (Naito et al., 2020). However, it has been shown that angiogenesis also occurs in developing embryo and vasculogenesis take place in adult tissues, for example during wound healing (Ratajska et al., 2017; Velazquez, 2007). Angiogenesis usually occurs in nutrient-deprived and hypoxic environments when restoration of blood flow is required to support the growth and function of a tissue, for example, during bone fracture repair and wound healing (Eelen, 2020).

2.1.1 Vasculogenesis

Vasculogenesis was originally defined as the de novo formation of blood vessel from endothelial progenitor cells, also known as angioblasts (Risau, 1997). The term angioblast has commonly been used to denote the progenitor cells that gives rise to embryonic vasculogenesis, while term endothelial progenitor cell (EPC) is used to describe the vascular formation in the adults (Kovacic et al., 2008). However, vasculogenesis has more recently been reported to occur also after birth in adult tissues (Ratajska, 2017).

Embryonic vasculogenesis

Formation of blood vessels during embryonal development initiates when mesodermal stem cells differentiate into hemangioblasts, which continue to differentiate into hematopoietic stem cells and angioblasts. Angioblasts can differentiate into endothelial cells, which in turn form early vascular networks (Patel-Hett et al., 2011). Hemangioblasts are multipotent cells capable of generating endothelial or hematopoietic cells depending on the local environment (Amaya, 2013). The existence of hemangioblasts has been demonstrated in vitro and in early

mouse embryo (Huber et al., 2004), even though the isolation of these cells and demonstrating their precise location within the developing embryo is still a challenge (Ratajska, 2017). However, single-cell RNA sequencing technique has shed some light on the emergence of the first hematopoietic stem cells (HSCs) and endothelial evolutions (Hou et al., 2020; Zeng et al., 2019).

Postnatal vasculogenesis

One of the most interesting discoveries in the field of vascular biology has been the detection of endothelial progenitor cells in adult circulation (Käßmeyer et al., 2009). EPCs have been identified in the peripheral blood (PB), bone marrow (BM), cord blood and solid tissues but not all of them represent true EPCs capable of forming blood vessels (Ratajska, 2017). There has been inconsistency in naming these cells (Medina, 2017), which will be discussed later in more detail. BM derived EPCs have been shown to be involved, for example, in blood vessel formation during wound healing (Bauer et al., 2006) and in tumour growth (Lyden et al., 2001) in mice. Postnatal vasculogenesis is an emerging field of research and the potential use of EPCs as a therapeutic treatment to increase postnatal vasculogenesis and angiogenesis in ischemic diseases might be of value in the future.

2.1.2 Sprouting angiogenesis

Growth of new blood vessels from pre-existing ones, also commonly known as sprouting angiogenesis, is initiated under hypoxic conditions by proangiogenic growth factors, such as vascular endothelial growth factor (VEGF) (Ribatti et al., 2012). VEGF activates ECs, which then start to degrade the basement membrane thus allowing tip endothelial cells to protrude and migrate towards the hypoxic area (Arroyo et al., 2010). Tip ECs are followed by stalk ECs, which are highly proliferative and therefore ensure sprout elongation and lumen formation. Lumens are formed by ECs and blood flow together with multiple signalling pathways and thus regulate the final size of inner diameter of the vessels (Iruela-Arispe et al., 2009). A new blood vessel is formed when two tip cells from adjacent sprouts meet and anastomose (Eelen, 2020).

The term angiogenesis was first introduced already in 1794 by the British surgeon John Hunter, and angiogenesis has been widely studied since then (Ribatti, 2014). The most used in vivo angiogenesis models include rabbit cornea, developing mouse retina and the intersegmental vessel growth in zebrafish (Ribatti, 2014). In vitro models with ECs have allowed studying endothelial migration and proliferation, as well as proteolytic digestion of the extracellular matrix, which is needed for the invasion of vasculature into surrounding connective tissue (Ribatti,

2012). Fibrin bead assay is one of the in vitro assays, which can be used to study early stages of angiogenesis, specifically sprouting and lumen formation. In this model, EC-coated beads are embedded in a fibrin gel and allowed to sprout and form vessels, which can then be observed under microscopy (Nakatsu et al., 2007).

VEGF and Notch signalling pathways are the key regulators of sprouting angiogenesis (Melincovici et al., 2018). VEGF activates vascular endothelial growth factor receptor 2 (VEGFR2) in tip ECs, which in turn increases the expression of notch ligand delta-like protein 4 (Dll4) (Hellström et al., 2007). Dll4 activates Notch signalling in the stalk ECs, which inhibits VEGFR2 signalling and activates VEGFR1 signalling in the stalk ECs. VEGFR1 in turn is involved in guiding and limiting tip cell formation and thus controls the cell proliferation and formation of blood vessels (Naito, 2020) (Figure 2A). In addition to ECs and pericytes, crosstalk between different cell types has an essential role during angiogenesis and it has been studied specifically in cancer models. Macrophages, neutrophils, B-cells and T-cells, as well as fibroblasts and adipocytes have been shown to play a part during tumour angiogenesis, either by promoting or limiting angiogenesis (De Palma et al., 2017).

2.1.3 Intussusceptive angiogenesis

The process of angiogenesis was considered to occur only through sprouting, but generation of blood vessels through another mechanism, called intussusceptive angiogenesis is also recognized today. Intussusceptive angiogenesis involves formation of blood vessels by a splitting process, where the pre-existing vessels are split in two (Figure 2B). The process begins when endothelial cells of opposing inner vessel walls migrate into the capillary lumen forming an intraluminal pillar (Burri et al., 2004). Cellular junctions of the ECs are reorganized, and the endothelial bilayer and basement membranes are perforated to allow growth factors, myofibroblasts and pericytes to enter the lumen, where they produce components of extracellular matrix forming a tissue pillar (Djonov et al., 2003). Finally, several pillars grow and fuse with each other leading to splitting of the initial blood vessel into two new vessels (De Spiegelaere et al., 2012).

Mechanism of intussusception has been studied e.g. in mice, zebrafish and by chick chorioallantoic membrane (CAM) assay. It has been shown that blood flow rate and changes in the blood pressure have critical effects on vascular adaptation and formation of intravascular pillars (Díaz-Flores et al., 2020). Molecular signals participating in intussusceptive angiogenesis are less well known compared to sprouting angiogenesis, however, both VEGF (Baum et al., 2010) and Notch signalling (Dimova et al., 2013) has been shown to take part in intussusception. It was recently demonstrated by Arpino and others that, contrary to the general view, angiogenesis after ischemic skeletal muscle injury in mice was dominated by the intussusception

and not by endothelial sprouting. In addition, they showed that the intussusception was accelerated by inhibiting VEGFR2 signalling (Arpino et al., 2021).

Both sprouting angiogenesis and intussusception occur in growing tissues, as well as in cancer. Sprouting is invasive process relying largely on cell proliferation, while in intussusceptive angiogenesis, cell proliferation is kept to a minimum as the ECs grow in size and flatten rather than proliferate (Burri, 2004). In most of the developing vascular beds, the vascular network is first formed through sprouting angiogenesis and intussusceptive angiogenesis takes over at a later stage (De Spiegelaere, 2012). Since intussusceptive angiogenesis was described more than 100 year later than sprouting angiogenesis, understanding its molecular mechanisms and functional implications still requires more research. It is essential that future angiogenic research considers both phenomena, sprouting and intussusception.

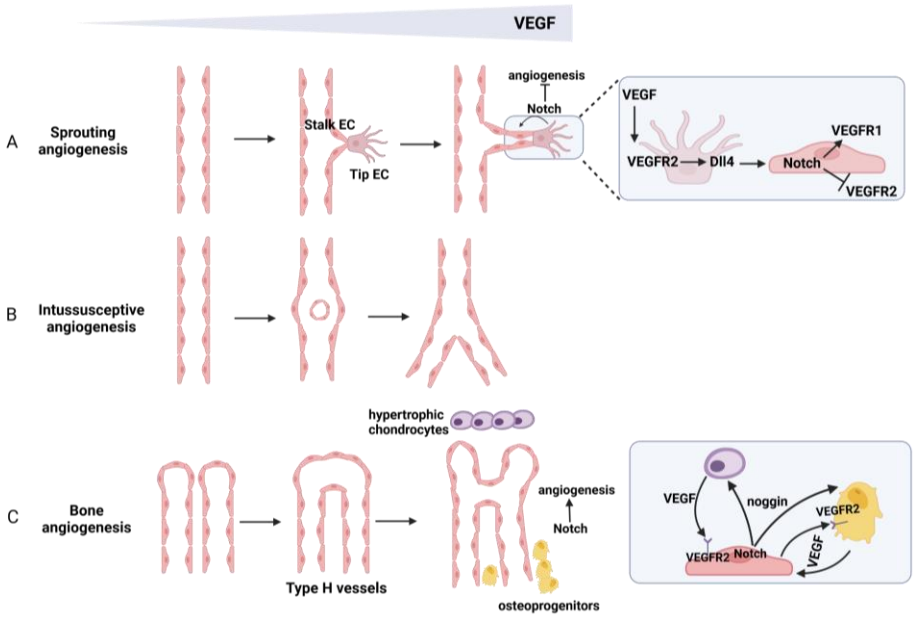

Figure 2. Simplified diagram of different angiogenic processes. Sprouting angiogenesis occurs in response to proangiogenic signals, e.g. VEGF, which activates endothelial cells (ECs) and these activated tip ECs begin to sprout and guide the following stalk ECs towards the chemical stimulus. Tip cells express Dll4 that activate Notch signalling, which coordinates tip versus stalk phenotypes within the growing sprouts. Intussusceptive angiogenesis requires endothelial pillar formation, which leads to splitting of the vessel in two. Bone angiogenesis is mediated by bud-shaped protrusions and EC proliferation is controlled by chondrocyte secreted VEGF. In contrast to sprouting angiogenesis, Notch signalling increases angiogenesis in bone. Notch signalling increases also the differentiation of osteoprogenitors and promotes chondrocyte maturation through noggin protein. The figure is based on the following publications: Hendriks & Ramasamy., 2020 and Stegen & Carmeliet., 2016. Created in BioRender.com.

2.1.4 Bone angiogenesis

Bone can be formed through endochondral ossification and intramembranous ossification. During intramembranous ossification MSCs differentiate directly into bone forming osteoblasts, while during endochondral ossification MSCs differentiate initially into chondrocytes and the process involves the replacement of hyaline cartilage with bone tissue. Vascularization is important in both ossification processes, as well as in bone remodelling for the supply of oxygen and nutrients and removal of waste products.

Bone is a highly vascularized tissue and suggestion that the vascular system is essential for osteogenesis was made already in 1763 by Albrecht von Haller in his book *Experimentorum de ossium formatione* (Trueta, 1963). Later research supported this, but despite the evidence indicating that angiogenesis and bone formation are tightly coupled, relatively little has been known about the underlying cellular and molecular mechanisms. The existence of bone specific vessel subtype with distinct morphological, molecular, and functional properties was discovered only recently in 2014 by Kusumbe, Ramasamy and Adams by using genetically modified mice (Kusumbe et al., 2014). They showed that bone capillaries could be divided into H and L subtypes based on their marker expression and functional characteristics. Type H capillaries are defined by high expression levels of CD31 and endomucin, whereas type L vessels are characterized by low expression of these markers (Kusumbe, 2014). Type H vessels are found in both periosteum and endosteum of the diaphysis and near the growth plate in the metaphysis, where they are organized as columns connected to each other at their distal end (Figure 3). In contrast, type L vessels form dense, highly branched capillary network in the bone marrow cavity of the diaphysis (Kusumbe, 2014). Type H capillaries are associated with osteogenesis since they produce factors that stimulate proliferation and differentiation of osteoprogenitors in the BM, thus directing bone formation (Peng et al., 2020).

Today, knowledge on coupling of osteogenesis and angiogenesis is better established, since endothelial cells are known to provide oxygen and nutrients to bone cells, which in turn secrete proangiogenic factors, such as VEGF. In addition, endothelial cells produce factors, such as bone morphogenic protein-2 (BMP2) and noggin that further regulate chondrocyte maturation and bone cell behaviour (Stegen et al., 2018). Bone angiogenesis differs from the typical sprouting angiogenesis in several ways (Hendriks et al., 2020) (Figure 2C). There are no tip endothelium cells in the bone. Instead, the vessel growth is mediated by bud-shaped protrusions. Furthermore, Notch signalling increases angiogenesis in postnatal long bones, while restricting sprouting in other organs (Ramasamy et al., 2014). On the other hand, VEGF is one the most important factors influencing also bone angiogenesis (Hu et al., 2016; Ramasamy et al., 2016). Endothelial cell proliferation and angiogenesis are controlled by hypertrophic chondrocyte secreted VEGF, as well as Notch signalling, which is activated by blood flow (Maes et al., 2012).

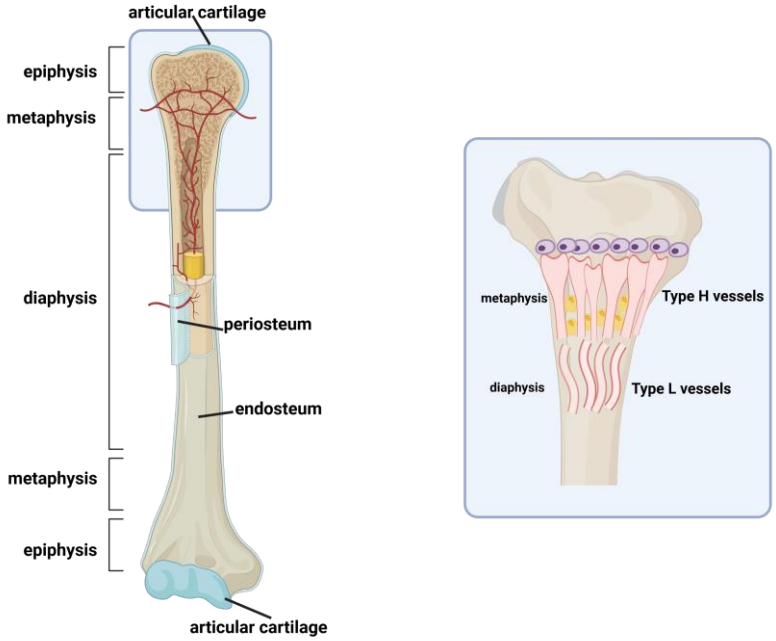

Figure 3. Long bone consists of the diaphysis, the epiphyses at the proximal and distal ends of the bone, and the metaphyses which, in a growing bone, contain the growth plate. A long bone is covered by articular cartilage at its epiphyses and by periosteum around the diaphysis. Endosteum is a thin membrane that covers the internal bone surface. Angiogenesis and bone formation are tightly coupled by type H vessels, which are located near the growth plate and in the periosteum and the endosteum of the diaphysis. Type H vessels are organized as straight columns interconnected at their distal end, surrounded by osteoprogenitors. These vessels can direct bone formation by producing factors that enhance proliferation and differentiation of osteoprogenitors. In contrast, type L vessels form dense, highly branched capillary network in the diaphysis. Modified from Ramasamy et al., 2016. Created in BioRender.com.

2.1.5 Proangiogenic factors

As previously mentioned, VEGF is one of the key regulators of angiogenesis and skeletal growth (Hu, 2016; Hu et al., 2017; Melincovici, 2018). VEGF family includes several members: VEGFA, also referred to as VEGF, as well as VEGFB, VEGFC, VEGFD and placental-like growth factor (PIGF), and they function through their receptors VEGFR1, VEGFR2, and VEGFR3. Both VEGFR1 and VEGFR2 are expressed as soluble and membrane-bound forms. Especially VEGFR2 is expressed in ECs, and it is considered to be the main mediator of EC proliferation, migration and activation in response to VEGF (Eelen, 2020). VEGFR1 is considered as a decoy receptor for VEGF, thereby preventing VEGF from binding to VEGFR2 (Koch et al., 2012; Melincovici, 2018). However, VEGFRB and PIGF can compete with VEGF in binding to VEGFR1 thus releasing VEGF. Along with ECs, VEGFR1 is

expressed by inflammatory cells, monocytes, macrophages and bone marrow progenitor cells, and activation of VEGFR1 is also shown to be involved in pathological angiogenesis (Cao, 2009).

In addition to VEGF, other proangiogenic factors associated with angiogenesis include stromal derived growth factor-1 (SDF1) (Strasser et al., 2010; M. Zhang et al., 2017), pleiotrophin (PTN) (Lamprou et al., 2020; Papadimitriou et al., 2016; Perez-Pinera et al., 2008), and fibroblast growth factor-2 (FGF2) (Jia et al., 2021). They are all recognized as important factors in endothelial tube formation. It has also been suggested that FGF2 stimulates angiogenesis and bone formation during bone regeneration (Kigami et al., 2014). Bone morphogenetic proteins has been reported to regulate blood vessel formation (García de Vinuesa et al., 2016), and particularly BMP2, -4 and -6 are suggested to be proangiogenic (Pulkkinen et al., 2021) and BMP2 and BMP6 can induce endothelial sprouting in vitro (Benn et al., 2017). BMP6 has also shown to induce angiogenesis in vivo in a matrigel plugin assay in nude mice (Pulkkinen, 2021).

Transforming growth factor beta (TGFβ) contributes to the recruitment of pericytes into the neovascularization site (Kemp et al., 2020). Other signalling pathways regulating pericyte-endothelial cell communication include angiopoietin1 (ANG1)/TIE2 and platelet derived growth factor (PDGF/PDGFRβ) signalling pathways. Pericytes produce ANG1, while TIE2 is expressed by ECs. By binding to TIE2 receptor, ANG1 stimulates EC survival and angiogenesis (Caporarello et al., 2019; Jeansson et al., 2011). PDGFRβ-expressing pericytes are recruited during angiogenesis by the tip-endothelial cell secreted platelet-derived growth factor subunit B (PDGFB) (Armulik, 2005; Gaengel et al., 2009).

2.2 Angiogenic cells

2.2.1 Endothelial cells

As stated previously, endothelial cells form the inner cellular lining of all blood vessels, called endothelium. It forms the barrier between vessels and tissues controlling the transport of circulating molecules, cells, and pathogens between the tissue and the bloodstream. ECs are a heterogeneous cell type, and they differ depending on the vessel type and the organ they exist (Marcu et al., 2018). The endothelium of arteries and veins forms a continuous monolayer of cells held together by tight junctions, whereas the endothelium of capillaries can be continuous, fenestrated, or discontinuous depending on their tissue-specific function (Figure 4). Organs involved in filtration or secretion, for example kidney, have fenestrated capillaries containing small pores in their endothelium allowing the fluid flow and exchange of small molecules (Aird, 2012; Hennigs et al., 2021).

Discontinuous endothelium, which is found e.g., in liver and BM, allows large molecules and circulating cells to travel through gaps in both the endothelial layer and the basement membrane. The most common EC markers are CD31 and von Willebrand Factor (vWF) (Table 1), but since ECs differ phenotypically, these markers are expressed in a heterogeneous pattern along different EC populations (Pusztaszeri et al., 2006).

Determining the heterogeneity of endothelial cells still requires further investigation as it is not completely clear, whether ECs within a single tissue are phenotypically different at the single cell level and whether ECs in a particular vascular bed (arteries, capillaries, veins) are heterogeneous across different tissues. A recent murine study shows that ECs from different vascular beds showed similar transcriptome in different tissues, and the heterogeneity of endothelial cells was contributed by the tissue rather than the vessel type. They also demonstrated that capillary ECs are the most heterogeneous compared to arterial and venous ECs which shared more markers in more tissues (Kalucka et al., 2020).

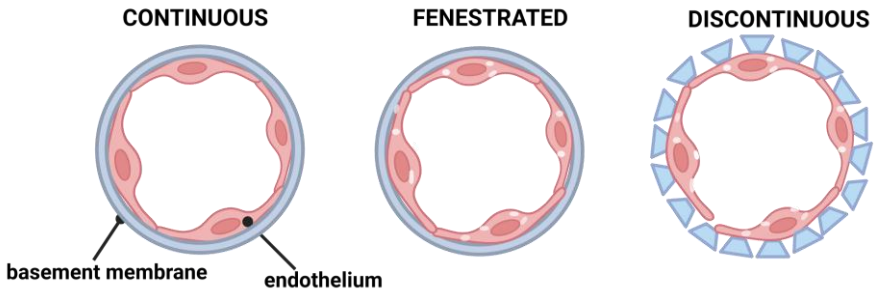

Figure 4. Endothelium formed by ECs vary between the vessel type and function of the organ they exist. ECs are coupled by tight junctions in continuous endothelium, whereas fenestrated endothelium contains small pores. Discontinuous endothelium contains gaps in both the endothelial layer and the basement membrane. Based on the original publication: Dellaquila et al., 2021. Created in BioRender.com.

2.2.2 Endothelial progenitors

As mentioned previously, the development of vascular system in vertebrates has been explained by mesodermal angioblasts differentiating into endothelial cells followed by formation of vascular network. However, the discovery of adult EPCs among the PB-MNC fraction has challenged this view (Asahara et al., 1997). EPCs have been typically defined as the cells capable of differentiating into ECs and forming new blood vessels. However, as the research related to EPCs has expanded, there has been controversy around the definition of EPCs, thus creating confusion in the field.

Currently, it is recognized that EPCs isolated from PB or umbilical cord blood and then cultured in vitro, consistently produce two different types of EPCs. These cells are described as early EPCs and late EPCs according to their time-dependent appearance in culture (Hur et al., 2004). These cells have been given multiple different names based on their phenotype or function (Medina, 2017), and there are still some inconsistencies in the nomenclature of these cells. Thus, it has recently been suggested to use terms endothelial colony forming cells (ECFCs) and myeloid angiogenic cells (MACs) to clarify both their phenotypic lineage and function (Medina, 2017).

Applying EPCs to vascularize engineered tissues is of great interest because of their potential to enhance vascular formation and because they are relatively easy to isolate from peripheral blood or umbilical cord blood (Ingram et al., 2004). Another benefit is the possibility to use of autologous cells, which makes it possible to avoid potential immunogenic concerns (Faris et al., 2020).

2.2.2.1 Endothelial colony forming cells

ECFCs, also known as late EPCs, represent the true endothelial progenitors, since they can differentiate into mature endothelial cells and have tube forming capacity in vitro (Medina, 2017). These cultured ECFCs are characterized as being positive for CD31, CD105 and CD146 surface markers (Table 1) and negative for hematopoietic marker CD45 and monocyte-associated marker CD14 (Medina, 2017). ECFCs can be isolated by culturing peripheral blood or umbilical cord blood MNC fraction for 2-3 weeks in endothelial differentiating medium (Faris, 2020). Bischoff lab has been studying the vasculogenic potential of human blood-derived endothelial progenitors and they have shown that ECFCs together with BM-MSCs can form functional microvascular networks in vivo when co-implanted into immunodeficient mice and thus support the aim of using human EPCs to form vascular networks in engineered organs and tissues (Melero-Martin et al., 2008).

On the other hand, ECFCs have also been isolated directly from blood and these circulating ECFCs have been identified as positive for CD34 and VEGFR2 surface markers by flow cytometry (Asahara, 1997). However, there are some disagreements surrounding this definition, since CD34 and VEGFR2 are also used to identify mature circulating ECs. Thus, it has been suggested to include an additional stem cell marker CD133 to better identify the progenitor cells (Peichev et al., 2000). However, there are still some contradictory results, whether these $CD34^{+}VEGFR2^{+}CD133^{+}$ cells represent true ECFCs (Medina, 2017). There is also debate about the origin of circulating ECFC. It has been suggested that ECFCs are of BM origin (Lin et al., 2000), but this was later challenged by other studies proposing that ECFCs more likely originate from tissue vascular niche,

which is the location of resident EPCs (Tura et al., 2013) and not from BM (Fujisawa et al., 2019).

The therapeutic potential of ECFCs as an autologous treatment to induce neovascularization in patients suffering ischemic diseases is under investigation (Faris, 2020). However, ECFCs do not survive in the harsh microenvironment of hypoxic tissues as they attend to lose their capacity to proliferate, migrate and engraft within vessels (Faris, 2020). Therefore, there is a need to improve their regenerative potential. Future work should thus focus towards understanding the possible phenotypic and functional differences between organ-specific origin of ECFCs (Dight et al., 2021) and to explore the possibility to directly target the tissue-resident ECFCs and to promote their expansion, thus enhancing their regenerative potential directly in patient (Faris, 2020).

2.2.2.2 Myeloid angiogenic cells

MACs, also known as early EPCs, display a hematopoietic phenotype and, unlike ECFCs, cannot differentiate into ECs. Early EPCs have been shown to appear in culture as spindle-shaped cells that demonstrate a low proliferative potential and no tendency to form colonies (Medina et al., 2010). These in vitro generated MACs are characterized as positive for hematopoietic marker CD45 and monocyte marker CD14, as well as for endothelial marker CD31, and as being negative for CD146, and CD34 surface marker (Table 1).

MACs have been shown to enhance retinal microvascular repair in mice in a paracrine manner by interleukin 8 (IL-8) –mediated transactivation of VEGFR2 (Medina et al., 2011). MACs also were shown to share similarities with M2 macrophages, indicating that MACs might represent an alternative macrophage subpopulation, and as such, promote repair and limit tissue injury (Medina, 2011). On the other hand, the same research group more recently demonstrated that MACs are similar but still separate from M2-activated macrophages (Chambers et al., 2018).

In vitro generated MACs have shown promising outcomes in preclinical models, for example, for critical limb ischemia (Chambers, 2018), and potentially offer a new tool for cell-based therapies to treat ischemic diseases because of their proangiogenic properties. However, the in vivo existence of circulating MACs has not yet been recognised.

Table 1. Common markers used to define endothelial cells (ECs), endothelial colony forming cells (ECFCs), myeloid angiogenic cells (MACs), pericytes, monocytes, mesenchymal stromal cells (MSCs) and osteoblasts.

MARKER	DEFINITION	EXPRESSION	REFERENCE
ALP	Alkaline phosphatase	osteoblasts	Grellier et al. 2009
αSMA	Actin Alpha 1, Skeletal Muscle (ACTA1)	pericytes	Wong et al., 2015
CD14	Myeloid Cell-Specific Leucine-Rich Glycoprotein	monocytes, MACs	Zhang et al. 2005, Medina et al., 2017
CD31	Platelet And Endothelial Cell Adhesion Molecule 1 (PECAM1)	ECs, ECFCs, MACs	Pusztaszeri et al. 2006, Medina et al. 2017, Asahara et al., 1997,
CD34	Hematopoietic Progenitor Cell Antigen	ECFCs, ECs	Asahara et al., 1997, Boyer et al., 2000
CD45	Protein Tyrosine Phosphatase Receptor Type C (PTPRC)	hematopoietic cells, MACs	Medina et al., 2017
CD133	Prominin 1	ECFCs	Peichev et al., 2000
CD146	Melanoma Cell Adhesion Molecule	ECFCs, MSCs, pericytes	Medina et al., 2017, Peichev et al., 2000, Wong et al., 2015, Crisan et al., 2008,
NG2	Chondroitin Sulfate Proteoglycan 4 (CSPG4)	pericytes	Wong et al., 2015
PDGFRβ	Platelet Derived Growth Factor Receptor Beta	MSCs, pericytes	Wong et al. 2015, Crisan et al., 2008
VEGFR1	Fms Related Receptor Tyrosine Kinase 1 (FLT1)	monocytes, ECs	Zhang et al. 2005, Pusztaszeri et al., 2006
VEGFR2	Kinase Insert Domain Receptor (KDR)	ECs, ECFCs	Pusztaszeri et al., 2006, Asahara et al., 1997,
vWF	von Willebrand Factor	ECs	Pusztaszeri et al., 2006

2.2.3 Peripheral blood derived mononuclear cells

ECFCs and MACs can be generated in vitro by culturing PB-MNCs, which can be isolated from blood by several methods. One routine technique used to isolate MNCs is gradient density centrifugation, which take advantage of the density differences between mononuclear cells and other components in the blood. The buffy coat, i.e. the MNC fraction, obtained by this method, contains several different cell types, such as, T-cells, B-cells, natural killer cells, as well as granulocytes (neutrophils, eosinophils, and basophils) and monocytes, which can further differentiate into macrophages and dendritic cells (Figure 5). All blood cells derive from hematopoietic stem cells (HSCs), which reside in the BM. In addition to HSCs, bone

marrow microenvironment contains a variety of cell types at different stages of maturation. These cells include for example mesenchymal stromal cells, perivascular cells, and adipocytes (Morrison et al., 2014). BM adipocytes are suggested to have supporting role in the hematopoietic niche and directly sustain the survival of hematopoietic stem cells (Mattiucci et al., 2018).

Myeloid cells

Platelets, erythrocytes, granulocytes, monocytes, macrophages, and dendritic cells are collectively called myeloid cells. However, platelets and mature erythrocytes do not have nucleus and are thus not found within the MNCs, so they are not discussed here any further. Different cell types within the MNCs can be isolated and studied based on their surface marker expression. CD14 expressing monocytes are the most common cell type in the MNC fraction and CD45 along with CD34 are commonly used markers of hematopoietic stem cells. However, today CD34 is an established marker of several cell types (Sidney et al., 2014) and for instance, circulating EPCs were originally identified among the $CD14^+$ (Zhang et al., 2005) and $CD34^+$ cells (Boyer et al., 2000) (Table 1).

Distinct tissue-resident macrophage populations are mostly derived from the yolk sac during embryogenesis (Wynn et al., 2016). These tissue-resident macrophages are important during development and have critical role in normal tissue homeostasis. However, following tissue injury, inflammatory monocytes are recruited from the BM to the wound site, where they differentiate into macrophages (Wynn, 2016). Monocyte derived macrophages are a heterogeneous cell population (Gordon et al., 2005) present in almost all tissues (Moore et al., 2019). Both tissue-resident and recruited macrophages are important to the inflammatory phase of the wound healing, as they can phagocytose debris at the wound site thus protect the host against infection (Yousuf et al., 2017). In addition, the role of macrophages in promoting vessel development has also been shown (Moore, 2019).

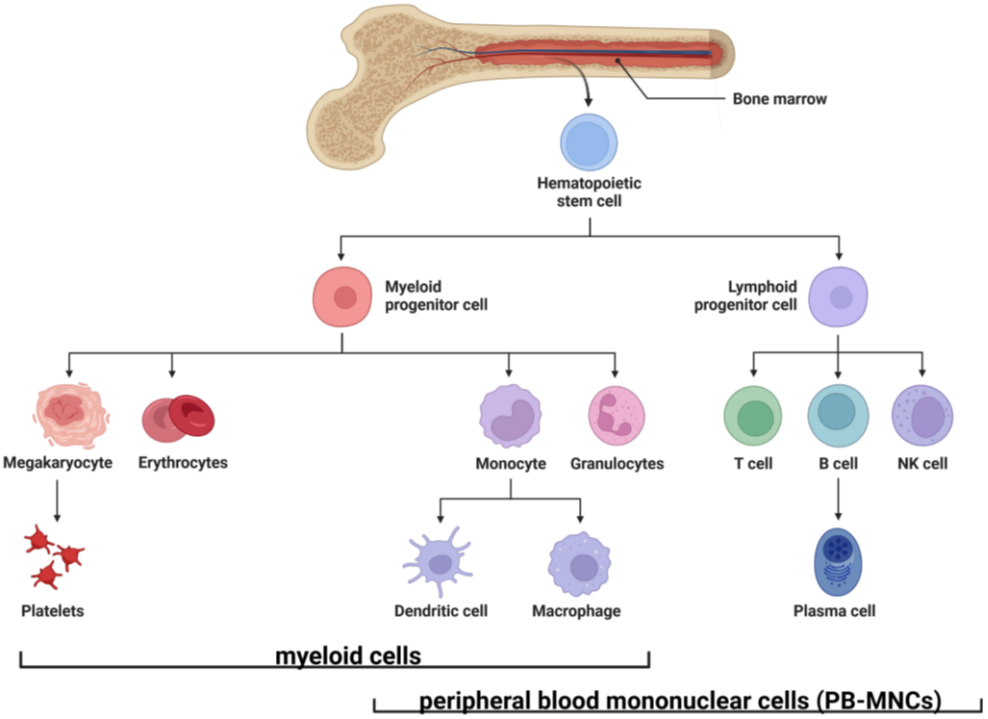

Figure 5. Bone marrow derived hematopoietic stem cells give rise to different types of blood cells. The myeloid cells within the mononuclear cell fraction isolated from peripheral blood include monocytes, macrophages, granulocytes, and dendritic cells. Created in BioRender.com.

2.2.4 Mesenchymal stromal cells

In addition to PB-MNCs, MSCs represent another cell population with a great potential for regenerative medicine. However, MSCs are a group of heterogeneous cells and there are still some uncertainties about their identification and classification, which limits their clinical use. Traditionally, MSCs have been characterized as stromal precursor cells, with multipotent differentiation capacity in vitro and they have been shown to have also immunomodulatory abilities. In recent years, however, lineage tracing and single-cell sequencing has clarified several subgroups of MSCs and their roles in both normal physiological and pathological conditions (Gao et al., 2021).

History and nomenclature

Friedenstein and colleagues discovered MSCs in the 1970s as fibroblast colony forming cells isolated from the BM of a guinea pig (Friedenstein et al., 1970). Later in the 1990s, Arnold Caplan officially named these cells as mesenchymal stem cells

(Caplan, 1991). However, as the knowledge of MSCs has since then expanded, this led Caplan to later suggest that MSCs should rather be referred as medicinal signalling cells to better describe their immunomodulatory and regenerative potential (Caplan, 2017). Other names for MSC populations in the literature include mesenchymal progenitor cells, multipotent stromal cells, and mesenchymal precursor cells (Samsonraj et al., 2017). It has also been shown that, in addition to the bone marrow, MSCs can be harvested from other tissues as well (Samsonraj, 2017), such as peripheral blood (Alm et al., 2010), placenta (Fukuchi et al., 2004), umbilical cord (O. K. Lee et al., 2004), dental pulp (Gronthos et al., 2000) and adipose tissue (Zuk et al., 2002). These cells can differentiate into cells of different connective tissue lineages including osteoblasts, chondrocytes, and adipocytes (Caplan, 1991; Pittenger et al., 1999) (Figure 6). MSCs have been reported to differentiate also into myocytes (Gang et al., 2004), fibroblasts (Lee et al., 2006) and endothelial cells (Oswald et al., 2004).

Tissue specificity of MSCs

Adipose tissue-derived MSCs (ASCs) and bone marrow-derived MSCs (BM-MSCs) are probably the most used MSC-like populations, and they share many biological characteristics. Their immunophenotypes are more than 90% identical, although some differences do exist (Strioga et al., 2012). There are variations in their differentiation potential, transcriptome, proteome, and immunomodulatory activities. For example, ASCs are more genetically and morphologically stable when cells are cultured for a long time (Strioga, 2012). It has also been suggested that ASCs have better proangiogenic action compared to BM-MSCs (Kim et al., 2007). Some of the differences are related to different isolation and culture protocols. The isolation of ASCs is safer, and significantly larger amounts of ASCs can be obtained compared to BM (Strioga, 2012). In addition, human placenta-derived cells have also been suggested to have similar multilineage differentiation potential like BM-MSCs (Miao et al., 2006).

Classification into subgroups

Because of the heterogeneous mixture of cells within the MSC population, there has been inconsistency related to the characterization of MSCs. Therefore, the International Society for Cell and Gene Therapy (ISCT) provided the following minimum criteria to define mesenchymal stromal cells, regardless of their source (Dominici, 2006):

1. plastic adherence in standard culture conditions
2. expression of CD73, CD90 and CD105 surface markers and lack of CD14, CD34, CD45 or CD11b, CD79a or CD19 and HLA-DR expression
3. differentiation capacity into osteoblasts, adipocytes, and chondrocytes under specific culture conditions in vitro

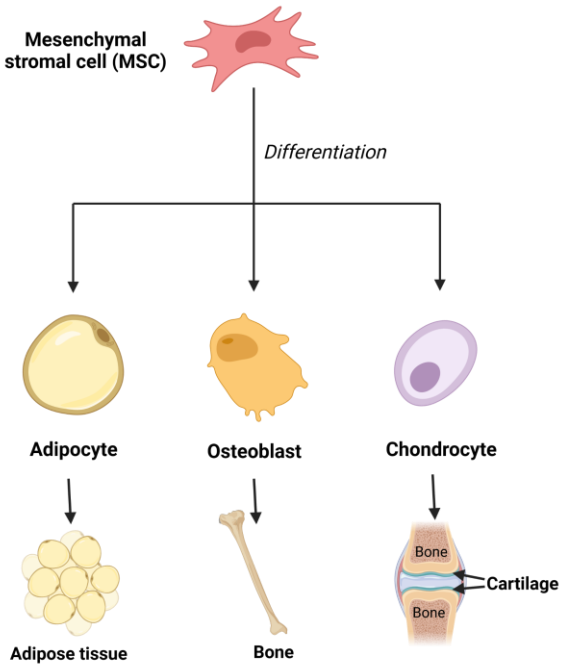

Figure 6. One of the criteria when characterizing mesenchymal stromal cells, is the tri-lineage in vitro differentiation capacity into adipose tissue composing adipocytes, bone forming osteoblasts and cartilage producing chondrocytes. Created in BioRender.com

More recently, BM-MSCs have been classified either as multipotent stem cells, as skeletal stem cells or as adipocyte lineage cells (Gao, 2021). Both mice and human studies have demonstrated evidence about the existence of a more uniform purified skeletal stem cell population in the bone. These cells give rise to bone and cartilage, but do not differentiate into adipocytes, suggesting that adipocytes originate from other sources of resident bone marrow stem cells (Chan et al., 2015, 2018). Furthermore, large-scale single cell transcriptome profiling of mice bone marrow mesenchymal lineage cells identified adipogenic lineage precursors in the BM (Zhong et al., 2020). The regulation of bone marrow stem cell differentiation fate is a complex network involving an increase in intracellular transcription factors, as well as signalling pathways, microRNAs, and extracellular elements such as hypoxia and mechanical stimulation (Gao, 2021).

Clinical applications of MSCs to promote vascularization

Studies in animal models of ischemic diseases have shown that treatment with BM-MSCs induces angiogenesis and thus improves the ischemic conditions (Al-Khaldi et al., 2003; Iwase et al., 2005). It has been suggested that MSCs could enhance

angiogenesis by differentiating into ECs (Liu et al., 2017; Oswald, 2004), but today the focus is more on their paracrine action (Maacha et al., 2020). Culture expanded BM-MSCs are known to secrete various angiogenic factors, such as monocyte chemoattractant protein 1, interleukin 6, and VEGF (Kwon et al., 2014). Another paracrine mechanism of MSCs involves the secretion extracellular vesicles (EVs) (Maacha, 2020). Exosomes are one type of EVs involved in intercellular communication and BM-MSCs derived exosomes have been shown to promote angiogenesis in vitro (Shabbir et al., 2015) and to accelerate wound healing in diabetic rats (Ding et al., 2019).

In addition to BM-MSCs, ASCs promote angiogenesis in vitro (Kachgal et al., 2011) and in vivo (Matsuda et al., 2013). As discussed previously there are differences between MSCs isolated from different tissues. While both BM-MSC and ASCs support the formation of microvascular networks when co-cultured with ECs, they promote the angiogenesis via different mechanisms (Kachgal, 2011) and there are differences also in the network phenotypes (Mykuliak et al., 2022). MSC-derived exosomes have also been shown to differ by their wound healing capacity depending on the tissue they were isolated from (Hoang et al., 2020). Whereas MSCs of diverse tissue types share some common features, it has become evident that MSCs of each tissue type also possess unique properties. This highlights the importance of choosing the application-specific stromal cell source for designing efficient stem cell-based therapies.

In conclusion, MSCs have been shown to play an important role in accelerating tissue repair by promoting the formation of blood vessels. However, inconsistent characteristics of the MSCs limit the clinical development of MSC-based therapies (Zhou et al., 2021). Further studies are still needed to fully understand both the heterogeneity and function of the MSCs in vivo (Arthur et al., 2020).

2.2.5 Pericytes

Pericytes are multipotent cells important for angiogenesis, and similarly to MSCs, their identification has been challenging due to their heterogeneity in terms of definition, tissue distribution, origin, phenotype, and multifunctional properties (Harrell et al., 2018). Charles Rouget was the first to describe pericytes (also called Rouget cells) already in 1873, but their function is still not completely elucidated (Armulik et al., 2011). Pericytes surround the endothelial cells of small vessels thus supporting the vascular structure in various organs, but they have also been suggested to have a wider role in tissue repair (Wong et al., 2015).

Origin and identification of pericytes

Identifying pericytes has been a major challenge due to the lack of a specific pericyte marker. Human placenta-derived pericytes possess similar cell marker profile as

BM-MSCs and they can also differentiate into adipocytes, osteoblasts, and chondrocytes in vitro, indicating that pericytes could represent a subpopulation of BM-MSCs. However, not all BM-MSCs can behave as pericytes in vitro and maintain endothelial tube networks (Blocki, 2013). Pericytes are usually isolated as $CD146^{+}CD34^{-}$ cells from various tissues (Crisan et al., 2008) and they have been reported to express e.g. markers such as platelet-derived growth factor receptor beta (PDGFRβ), alpha smooth muscle actin (αSMA), and neural glial antigen 2 (NG2) (Table 1) (Wong, 2015).

MSCs have been suggested to reside in a perivascular niche in vivo (Crisan et al., 2012) and since pericytes exhibit similar features as MSCs, it has also been proposed that pericytes and MSCs could have the same origin (Crisan, 2008) and that pericytes could behave as MSCs in vivo (W. C. W. Chen et al., 2015). However, this hypothesis was later challenged by a study indicating that the MSC-like behaviour of pericytes arises from artificial cell manipulations ex vivo and does not exist in vivo (Guimarães-Camboa et al., 2017). MSCs and pericytes share similarities in terms of their marker expression such as PDGFRβ and CD146. However, there are also unique markers to identify them: for example, NG2 and αSMA are commonly expressed by pericytes but not by the MSCs (Wong, 2015).

It is clear that pericytes represent a heterogeneous cell type and the existence of hematopoietic pericytes having a monocytic origin has moreover been hypothesized (Blocki et al., 2015). Hematopoietic pericytes are suggested to derive from hematopoietic stem cells via monocytic lineage and contribute at the early stages of angiogenesis for instance during vessel sprouting, whereas mesenchymal pericytes would be present later in more mature vessels thus inducing vessel maturation and stabilisation (Blocki, 2018).

Pericytes in tissue repair

The interactions between pericytes and ECs are important for vascular regeneration via angiogenesis. Pericytes are known to communicate with endothelial cells directly via gap junctions, tight junctions, focal adhesions, and soluble factors (Zhu et al., 2022).The signalling pathways involved in the crosstalk between ECs and pericytes were described earlier in the chapter 2.1.5.

In addition to contributing to angiogenesis, pericytes have been identified as a cell source with osteogenic properties. Pericytes expressing CD146 and NG2 were isolated from mouse embryos and were shown to contribute to bone fracture healing (Supakul et al., 2019). $CD146^{+}CD34^{-}CD45^{-}$ pericytes isolated from human adipose tissue can differentiate into osteoblasts and form bone after intramuscular implantation into a mouse (James et al., 2012). Thus, pericytes might be a promising therapeutic candidate to enhance bone healing (Zhu, 2022).

Wound healing is a complex process involving several overlapping phases, which are discussed later in more detail, and pericytes are involved in many of these events (Bodnar et al., 2016; Thomas et al., 2017). Inflammation is one of the key phases of wound healing and pericytes have shown to regulate the extravasation of neutrophils in mice (Proebstl et al., 2012; S. Wang et al., 2012). Inhibition of PDGFRβ impairs cutaneous wound healing in mice and inhibits the proliferation and migration of pericytes and fibroblasts in vitro, implying that PDGFRβ signalling is important during the early phases of wound healing (Rajkumar et al., 2006). It seems that pericytes have an important role in chronic and acute healing processes, but their role during wound healing is not yet completely elucidated and requires further research.

2.3 In vitro vascular models

A major limitation in current tissue engineering applications is the lack of a sufficient vascularization. To approach this challenge, appropriate in vitro methods to study neovascularization are needed. One strategy is based on the ECs and their ability to form new blood vessels (Novosel, 2011) but the angiogenic potential of different factors and cells, for example MSCs and pericytes, can be studied by various assays (Figure 7). Most of the in vitro models use two-dimensional (2D) cultures due to their ease of use, although three-dimensional (3D) culture models represent the real in vivo situation better. Today, several different 3D models have been established to study the cell-cell interactions, microenvironmental cues and effect of biomaterials and growth factors that direct angiogenesis (Zucchelli et al., 2019). When establishing in vitro vascular models, one should take into consideration where and how the cells work in the body and try to mimic that.

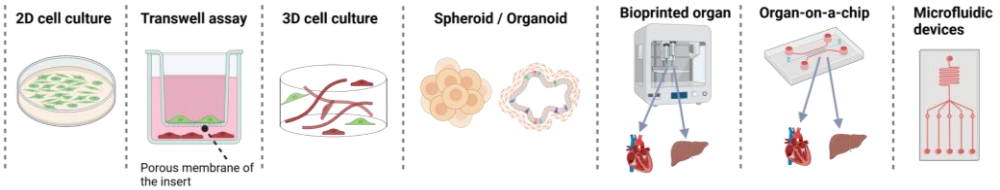

Figure 7. Two-dimensional (2D) cell culture methods are the most used type of in vitro assays. Transwell assay is used to study the cell migration or effects of soluble factors. Three-dimensional (3D) cell cultures are artificially created environments to better model the in vivo situation. Spheroids are 3D cell cultures that arrange themselves during proliferation into sphere-like formation while organoids can be described as cells grown in 3D to form structural units that partly resemble the organ. Bioprinted organs or organ-on-a-chip models can also be used to imitate structures and functions of an organ. Microfluidic devices can be used to study e.g. normal and disrupted flow. Modified from Dellaquila et al., 2021. Created in BioRender.com.

Cell source selection

Many of the angiogenic assays employ ECs, such as human umbilical vein endothelial cells (HUVECs) or human dermal microvascular endothelial cells, due to the ease of obtaining these cells (Stryker et al., 2019). However, ECs are not homogenous and there is variation between different donors, which can cause inconsistency in functional outcomes of the formed vascular networks. Furthermore, primary cells have also low proliferation rate and limited lifespan (Masson-Meyers et al., 2021). To overcome some of these challenges, the use of pluripotent stem cells is also being investigated. They are capable of self-renewing indefinitely in culture and differentiate into different types of cells. The use of EPCs in enhancing vascularization is also under investigation (Ratajska, 2017).

Co-culture systems

Although ECs are the main cell type used to study angiogenesis in vitro, monocultures are not adequate in mimicking physiological angiogenesis. Instead, angiogenesis in vivo involves also supporting stromal cells, e.g. MSCs and/or pericytes. Such complex physiological conditions can be mimicked by using different approaches. Co-culture assays are commonly used to investigate the communication between different cells and e.g. the effects of MSCs on EC behaviour and tube formation capacity (Masson-Meyers, 2021). Co-cultures can be performed in 2D, but the 3D-models are better representatives of the in vivo physiological environment (Zucchelli, 2019). In vivo microenvironment can moreover be replicated by using different scaffolds and matrices resembling the natural extracellular matrix. Scaffolds such as collagen I, fibrin or Matrigel have an important effect on the structural and mechanical properties of the vessel formation and can also be used both as 2D and 3D models (Stryker, 2019).

Three-dimensional (3D) culture models

While in 2D cell culture cells usually grow in a monolayer, 3D model allows cells to grow in more than two directions. 3D models include e.g. spheroids, which are multicellular 3D models that form due to the tendency of adherent cells to aggregate. The spheroid-based sprouting assay enables to study the effects of the pro- and anti-angiogenic determinants to endothelial cell function (Heiss et al., 2015). More complex organoids are miniaturized and simplified versions of an organ produced in 3D. Organoids contain multiple organ-specific cell types and can recapitulate some specific function of the respective organ (Dellaquila et al., 2021). For example, skin organoids were developed by differentiating and guiding human induced pluripotent stem cells (iPSCs) over a month's long process to generate human skin tissue (Lee

& Koehler, 2021). Although organoids can stimulate the structures and functions of organs in vitro, the 3D models still have a limited capacity to form functional vascular networks (Zhao et al., 2021). To overcome these limitations, latest advancements in vascularization strategies in tissue engineering are bioprinted tissues and organ-on-a-chip models (Dellaquila, 2021). Organ-on-a-chip models try to imitate the structure of human organs and to model the function of human tissues and diseased states. Microfluidic devices for in vitro 3D assays are used to replicate the pressure, strain, and shear stress waveforms associated with both normal and disrupted flow and thus better mimicking the physiological and pathological conditions of the in vivo systems (Zucchelli, 2019).

Culture conditions

In addition to co-cultures of ECs and supporting cells and the use of suitable scaffold, choosing the appropriate culture conditions are important for cell-based vascularization strategies. These conditions include cell seeding density and ratio between different cell types. Choice of culture medium is also critical for cell culture models, and in a co-culture system, the more sensitive cell type will usually determine the final formulation of culture medium.

Cell differentiation is a highly significant process for living organisms, and culture conditions also determine the differentiation fate of the progenitor cells. For example, MSCs can be differentiated into osteoblasts by adding ascorbic acid, natrium-beta-glycerophosphate and dexamethasone to the culture medium.

Methods to assess vascularization

To investigate how cells are affected by different culture conditions, various methods can be used to analyse cell proliferation, viability, and migration. Migration of ECs, in response to the chemical stimuli can be studied with various methods. These include wound healing assay, in which a scratch is generated on a confluent cell monolayer or Boyden chamber also known as Transwell assay, which can be used to measure both the migration of ECs and the chemotactic capability of the test substances (Stryker, 2019). Depending on the membrane pore size, Transwell assay can also be used as a co-culture method to examine the interactions via soluble, paracrine factors between different cell types. Proliferation assays can be used to evaluate whether the factor to be tested is proangiogenic or antiangiogenic (Goodwin, 2007).

Vascularisation can be analysed by different tube forming assays and by quantifying the sprout formation, analysing the lumen width and volume, as well as assessing the number, and branching of the tube-like structures formed in vitro.

Histological staining and immunolabeling by immunohistochemical (IHC) or immunofluorescence (IF) methods can be used to visualize the structure and morphology of the blood vessels and to quantify e.g. the number and diameter of the vessels in the tissue sample. Proangiogenic growth factors involved in the vessel formation can be analysed by enzyme-linked immunosorbent assay (ELISA), Western blot and real-time quantitative polymerase chain reaction (qPCR) methods (Masson-Meyers, 2021). ELISA is used to measure the soluble factors secreted by the cells. Western blot provides information about the protein expression, while qPCR is used to measure the expression of factors at an mRNA level.

As already previously discussed, defining cell heterogeneity and phenotype still requires further investigation. Single cell analysis has made it possible to quantify DNA, RNA and protein variations at a single cell level and can be used to identify cell subpopulations within a given organ. One of the most used single cell analysis techniques is single cell RNA sequencing (scRNA-seq), which provides information about RNA molecules in individual cells. Other technique is spatial transcriptomics, which is a molecular profiling method that can be used to specify the spatial distribution of mRNA molecules (Longo et al., 2021). These methods have revolutionized the studies on tissue development, cellular heterogeneity within tissues and cellular response to injury (Chavkin et al., 2020). Obtaining this new information is important for the vascular biology field, as vascular cells are abundantly distributed throughout all tissues and must maintain various functions to ensure tissue homeostasis.

2.4 Wound healing and skin regeneration

Skin is composed of different layers, but the two main layers are called epidermis and dermis (Figure 8). Epidermis consists of different cell types, i.e., keratinocytes, melanocytes, Merkel's cells, and Langerhans cells, while dermis is a connective tissue composed of sebaceous glands, sweat glands, hair follicles and fibroblasts, macrophages, and mast cells. Dermis also contains blood vessel, which provide nutrients to the skin and are crucial during wound healing (Pasparakis et al., 2014). Different skin stem cells are capable of extensive proliferation and differentiation thus providing the excessive regeneration capacity of the skin (Díaz-García et al., 2021).

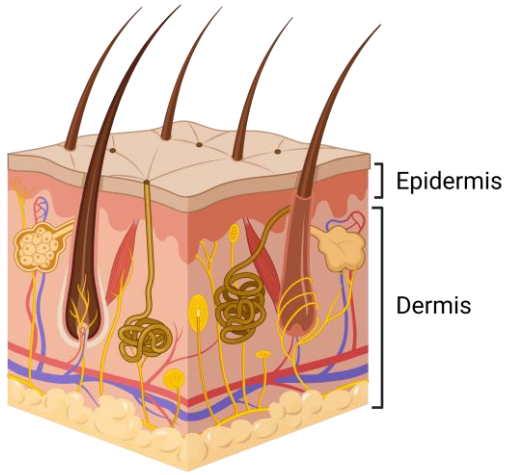

Figure 8. Skin consists of two main layers, outermost layer called epidermis and the inner layer known as dermis. Created in BioRender.com.

Wound healing process can be divided into four overlapping phases that include haemostasis, inflammation, proliferation, and remodelling also known as maturation (Díaz-García, 2021). Wound healing involves interactions between various cell types, growth factors and cytokines. Immediately after the injury, blood vessels are damaged leading to inflammation and development of hypoxia. Hypoxia and inflammatory cytokines activate platelets, which are central in the recruitment of immune cells, such as neutrophils, and macrophages and their circulating monocyte progenitors, to the injury site (Gonzalez et al., 2016). The inflammation is followed by the proliferative phase, where keratinocytes, fibroblasts, macrophages, and endothelial cells are activated to coordinate wound closure, matrix deposition and angiogenesis (Wilkinson et al., 2020). Angiogenesis is triggered by hypoxia-inducible factor and release of VEGF, as well as other growth factors (Hashimoto et al., 2015). Final phases include remodelling of collagen and maturation of the newly formed blood vessel networks (Yousuf, 2017). The wound healing time is affected by several different factors such as wound size, depth, and location. However, the inflammatory phase usually lasts several days, while the proliferative phase can last several weeks and the remodelling/maturation begins about 21 days after an injury and can continue for a year or more in case of delayed healing (Wilkinson, 2020) (Figure 9).

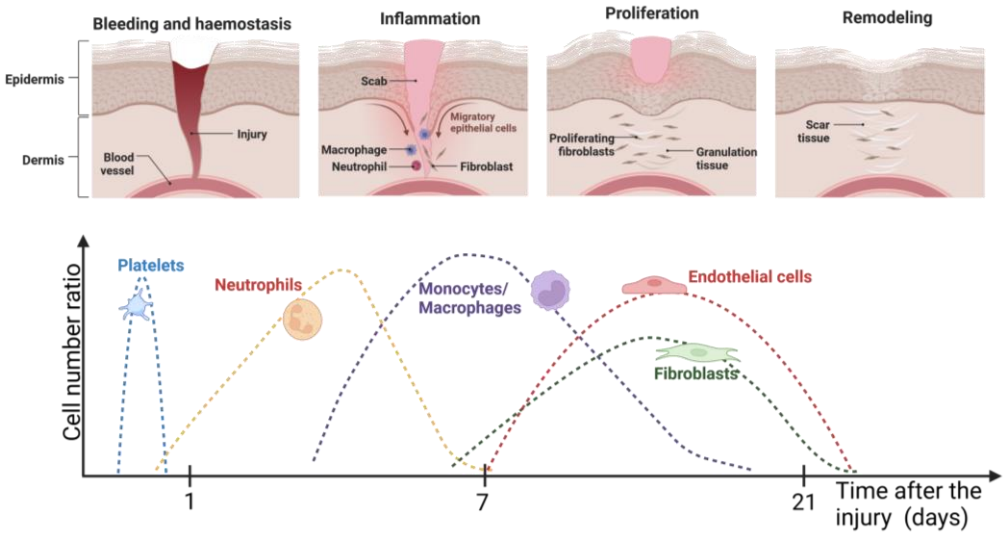

Figure 9. Wound healing process can be divided into four overlapping stages: haemostasis, inflammation, proliferation, and remodeling. Haemostasis happens immediately after the injury when activated platelets release factors to initiate the formation of a fibrin clot to prevent blood loss. Inflammation phase lasts for few days and it begins with neutrophil influx to the wound site to prevent infection. Monocytes arrive later and differentiate into macrophages to further eliminate the damaged cells and pathogens from the wound area. Migration of fibroblasts into the site of injury initiates the proliferation stage. This stage is characterized by the formation of granulation tissue, angiogenesis, and re-epithelialization of the epidermis. Remodeling phase begins approximately 21 days after the injury and can last for a year or more. Figure is based on the following publications: Wilkinson & Hardman. 2020., and Guillamat-Prats. 2021. Created in BioRender.com

The process of wound healing can sometimes be impaired or compromised, as in the case of diabetic ulcers, because of the reduced ability to regrow blood vessels through angiogenesis (Veith et al., 2019). Non-healing cutaneous wounds can be caused by e.g. infections, poor circulation, or aging (Guo et al., 2010). In addition, extensive skin defects can occur after severe burns or trauma.

Split-thickness skin grafts (STSG) are still a common treatment for skin defects, when full thickness autologous skin grafts or skin flap transplants are inapplicable (Chen et al., 2017). Skin grafting involves the transfer of cutaneous tissue from a donor site to a recipient site. STSG refers to a graft that contains the epidermis and a portion of the dermis, while full-thickness skin graft consists of the epidermis and entire dermis. Effective vascularization of the graft is crucially important for the successful engraftment of these skin grafts (Frueh et al., 2018) and insufficient blood supply at the early stages can cause graft necrosis and fibrosis (Chen et al., 2017). Inosculation, where vascular network is established between the graft and recipient site, occurs within 48-72 hours after grafting and the graft typically integrate into the wound bed after 5 to 7 post-operative days. As angiogenesis is necessary for the

successful engraftment of skin grafts, understanding the biological processes behind revascularization of skin substitutes and applying this knowledge to clinical practice is a prerequisite for the successful treatment of skin defects (Frueh, 2018).

2.5 Clinical implications of vascularization

Vascularization is an important aspect both in the progress and treatment of multiple diseases. In the case of ischemic diseases and impaired tissue healing, angiogenesis is reduced and can be treated by stimulating the blood vessel growth with e.g. drugs or gene therapy vectors (Carmeliet et al., 2011). On the other hand, cancer or blinding eye disease, such as interstitial keratitis or retinopathy of prematurity, often cause excessive blood vessel formation and antiangiogenic therapies can be used to attenuate the vessel growth (Yoo et al., 2013). As VEGF signalling is considered the main angiogenesis promoter, most of the approved anti-angiogenic treatments target VEGF (Lopes-Coelho et al., 2021). However, in case of cancer, targeting only one growth factor is not considered the best option as cancer usually just circumvents this pathway and then uses another for growth (Lopes-Coelho, 2021).

In regenerative medicine, tissue engineering aims to restore or replace injured tissues or to maintain and improve tissue functionality. However, insufficient vascularization is the main challenge in tissue engineering (Novosel, 2011). Cell-based vascularization strategies aim to apply cells and growth factors to generate new blood vessels, while scaffold-based strategy also includes the use of biomaterials (Novosel, 2011). In classic tissue engineering, cells are introduced into a suitable scaffold and implanted to treat the tissue defects. However, scaffolds lack vascularization, which is crucial for proper adhesion of the implanted scaffolds and is required for the construct to be functional (Masson-Meyers, 2021). Thus, better methods to enhance vascularization are clearly needed.

Vascularization strategies in skin tissue engineering

Non-healing, cutaneous wounds, as well as chronic, non-healing ulcers have become a major burden to the healthcare worldwide and several attempts have been made to develop treatments to enhance wound healing by inducing vascularization (Veith, 2019). Autologous skin grafts, used in wound healing, are fragile and have also other disadvantages, such as the unavoidable damage caused to the donor site. Thus, engineered constructs could serve as an alternative to autologous grafts (Chen et al., 2017). When a construct is implanted to the recipient area to restore the tissue defect, it should be slowly vascularized and start to adhere. Biomaterials used for tissue engineering can usually be divided into natural and synthetic biomaterials, and collagen and fibrin are examples of natural biomaterials used as scaffolds for skin

regeneration (Lukomskyj et al., 2022). Even though there are several commercially available tissue-engineered skin substitutes, there are also challenges associated with their use, such as appropriate vascularization (Chen et al., 2017).

One approach is to promote the ingrowth of blood vessels from the host by delivering different compounds to the wound site, thus aiming to induce therapeutic vascularization (Veith, 2019). Supplying exogenous growth factors, such as PDGFs and VEGF to the wound site have demonstrated great promise in enhancing angiogenesis in animal models (Veith, 2019; Xie et al., 2013). However, because of the limited time of biological activity at the wound site, these treatments often require repeated administration, which then increases cost and may cause adverse side effects (Nurkesh et al., 2020). Thus, almost all the growth factor-based treatments have failed to show clear benefits for enhancing wound healing in clinical trials (Veith, 2019). These limitations have been approached by developing biocompatible materials, such as hydrogels, which would serve as a drug delivery system by protecting growth factors from protease degradation and allowing a stable long-term release of the drug, thus prolonging drug bioactivity and providing sustainable drug delivery, and therefore eliminating the need for repeated administration (Nurkesh, 2020). However, scaffold-based approaches also involve the optimization of scaffold properties (Masson-Meyers, 2021). One opportunity to be studied is the stem cell-based scaffold-free technique, where cells are grown in a sheet producing extracellular matrix in culture and are then implanted as a cell-matrix construct, providing a good, more natural microenvironment for vascularization (Masson-Meyers, 2021).

Another approach to speed the adhesion process and thus enhancing wound healing, is to prevascularize the constructs in vitro before implantation (Masson-Meyers, 2021). Cell-based vascularization strategies of the constructs are currently under investigation. Because of their ability to form new vessels, mature endothelial cell types, such as HUVECs, have been used in many vascularization studies (Hendrickx et al., 2011). However, mature ECs possess some disadvantages, such as low proliferation rate, and heterogeneity (Novosel, 2011). As an alternative for mature ECs, the use progenitor cells, such as ECFCs and MACs, are being studied (Hendrickx, 2011; Masson-Meyers, 2021). In addition to endothelial cells, supporting cells are also needed to mimic the physiological angiogenesis in tissue engineering applications. For instance, ASCs, BM-MSCs and iPSCs have been investigated because of their paracrine effects, alteration of the immune response, and for their ability to differentiate into vascular lineage cells (Veith, 2019).

Choosing the right cell type is important and one criterion is the practicability for clinical applications. For example, in acute situation such as trauma, the cells should be readily available with sufficient amounts for therapy (Hendrickx, 2011). Cell-based therapies to improve the survival and therapeutic effects of skin grafts

have been developed as a new approach during the last years. For example, it has been demonstrated that in vitro prevascularized human MSC cell sheets implanted in a rat full thickness skin wound model and covered with autologous STSG showed less haemorrhage and necrosis, and lower inflammatory cell infiltration compared to STSG alone (Chen et al., 2017). In addition, cell sheet implantation significantly improved cosmetic appearance relative to the STSG control group (Chen et al., 2017). Thus, the strategy of combining split thickness skin grafts with pre-vascularized implant might to be a promising approach in regenerative treatment of full thickness skin wounds in the future. However, currently there are no cell-based proangiogenic treatments in clinical use, which demonstrates the need for further investigations (Domaszewska-Szostek et al., 2019).

Vascularization strategies in bone tissue engineering

Bone is a highly vascularized tissue and there is an important connection between bone formation and angiogenesis, since bone development, maturation, remodelling, and regeneration are dependent on the of blood vessel supply (Simunovic et al., 2021). Thus, vascularization is an essential element to be considered in bone tissue engineering (BTE).

Vascularization strategies in BTE include the use of angiogenic growth factors and cells, the same as in the skin tissue engineering. As discussed earlier, the angiogenic factor VEGF has a central role in the coupling of angiogenesis and osteogenesis, and it is important factor in BTE and bone regeneration (Diomede et al., 2020; Dreyer et al., 2020). For example, Chen and colleagues established a system, where VEGF was loaded to the heparin cross-linked demineralized bone matrices and it was shown to improve the vascularization of the scaffold when implanted subcutaneously into rats (Chen et al., 2010).

As a cell-based strategy, co-culturing vascular and osteogenic lineage cells has become a central research area in BTE (Simunovic, 2021). In addition, the use of bioactive glasses, that release bioactive ions (Qazi et al., 2018), and 3D-printed scaffolds (Wang et al., 2020) are under investigation. It has been shown that, at a proper dosage, copper has proangiogenic functions and can activate proangiogenic growth factors, such as VEGF (Sen et al., 2002). Thus, Wang and colleagues used copper-doped bioactive glass in combination with 3D printed scaffold in their studies and demonstrated that Cu^{2+} released from 3D scaffold stimulated the angiogenesis in a co-culture of BM-MSCs and HUVECs. Osteoblastic differentiation of BM-MSCs was also supported by the scaffold, although copper suppressed the differentiation of osteoblasts (Wang et al., 2019).

Regenerative medicine is a quickly evolving field that combines the use of tissue engineering with materials science, stem cell biology, and developmental biology.

Strategies to support and enhance vascularization have an important role in tissue engineering and especially in BTE. However, all the strategies still have disadvantages, such as short half-life of some angiogenic growth factors, limited survival of implanted cells, and other technical challenges and thus more studies are still needed. However, despite all the challenges, regenerative medicine is progressing, and tissue engineering applications are likely to increase in clinical settings in the future (Simunovic, 2021).

3 Aims

Developing functional and efficient vascular networks, and finding an optimal endothelial cell source remain challenges in regenerative medicine and tissue engineering and therefore improved strategies to generate vascular system in tissue transplants are needed. However, the cellular interactions in angio- and vasculogenesis are not yet completely understood. Especially the role of endothelial progenitor cells (EPCs) and pericytes in tissue repair requires further investigation. EPCs have been identified among the mononuclear cells (MNCs) of peripheral blood (PB) and umbilical cord blood. In addition, mesenchymal stromal cells (MSCs) have been shown to induce angiogenesis by producing paracrine signals and by differentiating into pericytes. Thus, the aim of this PhD project was to study the angiogenic potential of bone marrow derived MSCs and peripheral blood derived MNCs.

The specific aims were:

1. To evaluate the in vitro angiogenic potential of human MSC-MNC co-cultures both in basal conditions and during osteoblastic differentiation.
2. To study the cellular phenotypes and functionality of cells formed in MSC-MNC co-culture set-up and to analyse the cellular components of the PB-MNC fraction
3. To investigate, whether myeloid angiogenic cells (MACs) exist in vivo and to determine if MACs and pericyte-like cells are present in human circulation during wound healing.

4 Materials and Methods

4.1 Study subjects and cell isolation

4.1.1 Skin graft patients and surgical procedure (**III**)

Patient with a traumatic full thickness skin defect in the lower limb were recruited to the study. Patients suffered of atherosclerosis, type I diabetes mellitus, hypertensio arterialis, tetraplegia, or Marfan´s syndrome, all of which are syndromes causing impaired circulatory functions and thus impaired wound healing. The wound areas were approximately 5x5 cm to 7x8 cm and patients underwent reconstruction with split thickness skin graft (STSG) harvested from patient's thigh by using a Zimmer dermatome. Thickness of the STSGs were 10/1000 inch each and the grafts were meshed in 1:1.5 manner. The wound beds of recipient areas were revised and covered with meshed STSGs and attached with surgical staples. After transplantation, the wounds were covered with traditional wound dressings and the affected limbs were immobilized for 6 days. Blood samples were drawn before the surgery and on the first and sixth postoperative days for further analysis (Table 2). Furthermore, skin biopsies for histological analyses were taken from the wound bed in local anaesthesia on 6th and 14th postoperative days.

4.1.2 Peripheral blood mononuclear cells (**I, II, III**)

Peripheral blood samples (volume range 18 - 40 ml) were drawn from healthy donors and skin graft patients (Table 2) and mononuclear cells were isolated by gradient centrifugation (Ficoll-Paque Plus, Cytiva).

Table 2. Demographic data of MNC donors.

GENDER	AGE	STATUS	PUBLICATION
M	25	healthy	**I, II**
M	29	healthy	**II**
F	33	healthy	**II**
M	75	healthy	**III**
M	39	healthy	**III**
M	42	healthy	**III**
F	59	healthy	**III**
M	77	skin graft patient	**III**
M	40	skin graft patient	**III**
M	45	skin graft patient	**III**
F	60	skin graft patient	**III**

Each blood sample was mixed in 1:1 ratio with 1x phosphate-buffered saline (PBS) and slowly layered on top of an equal volume of Ficoll-Paque Plus solution in a 50 ml tube. Samples were centrifuged 400g for 30 minutes. Mononuclear cell fraction (PB-MNCs) was carefully collected and washed twice with PBS (Figure 10). Thereafter, PB-MNCs were counted and either plated in co-cultures or subjected to further sorting or analyses.

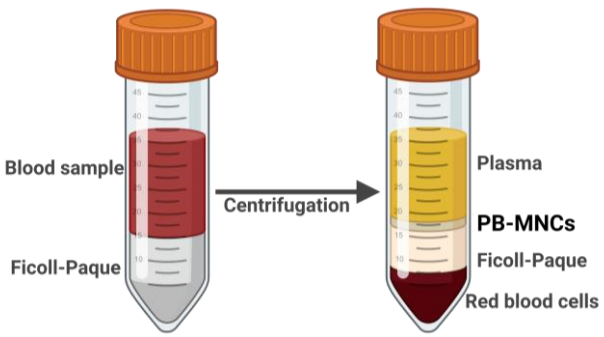

Figure 10. Isolation of MNCs from PB by gradient centrifugation (Ficoll-Paque plus). Created in BioRender.com.

4.1.3 Bone marrow mesenchymal stromal cells (**I, II**)

Human MSCs were isolated from iliac bone marrow of a healthy 21-year-old female donor. After isolation, the cells were expanded and stored in liquid nitrogen, as

previously described (Alm, 2010). In brief, the BM aspirate was mixed with alpha minimum essential medium (αMEM; Gibco), which contained 100 IU/ml of penicillin and 100 µg/ml of streptomycin (Gibco) and 20 IU/ml of heparin (Heparin Leo; LEO Pharma A/S) and cells were isolated by density gradient centrifugation (Ficoll Paque Plus; Amersham Pharmacia). Cells were seeded at 1×10^6 cells in a 25 cm^2 tissue culture flasks (Gibco) and cultured in αMEM containing 10% fetal bovine serum (FBS) and antibiotics. After 48 hours of culture, non-adherent cells were discarded, and medium was changed every 3–4 days. Upon confluency, cells were harvested using trypsin/EDTA (Gibco), counted and replated at 1000 cells/cm^2. BM-MSCs were identified according to internationally verified criteria (Dominici et al., 2006), described in chapter 2.2.4 (Table 3). Cells at passages 2 to 6 were used for the experiments.

4.1.4 Human umbilical vein endothelial cells (**II**)

Green fluorescent protein expressing human umbilical vein endothelial cells (GFP-HUVECs) (IncuCyte Cytolight Green HUVECs, Sartorius, cat#4453) were cultured in Endothelial Cell Growth Medium 2 (EGM-2, PromoCell, C-22011) and cells at passages 4 to 8 were used for the experiments.

Table 3. Cells used in the study and the markers used to define the cell types.

CELLS	PHENOTYPE	PUBLICATION
BM-MSC	$CD105^+CD73^+CD90^+CD14^-CD45^-$	I, II
PB-MNC	$CD14^+CD45^+$	I, II, III
HUVEC	$CD31^+$	II
MAC	$CD14^+CD45^+CD31^+CD34^-$	II, III
BM-MSC derived pericyte	$\alpha SMA^+PDGFR\beta^+NG2^+CD146^+$	II
PB-MNC derived pericyte	$CD14^+CD45^+NG2^+PDGFR\beta^+$	II, III
Osteoblast	ALP	I

4.2 Ethical approvals (**I, II, III**)

All donors signed an informed consent before participating in the study and collection of samples followed the Declaration of Helsinki ethical principles. The Ethical committee of the Helsinki University Central Hospital, Finland approved the study protocol for MSC isolation. The local Ethical Committee of University of Turku approved the protocol for MNC isolation of healthy donors, while the

protocols for skin graft surgery and blood and skin biopsy sampling of patients were approved by Tampere University Central Hospital.

4.3 Cell cultures

4.3.1 Two-dimensional MSC-MNC co-cultures (**I, II**)

BM-MSCs were first cultured in T75 flasks (1000 cells/cm^2) in basal medium (Table 4). After the cells were approximately 50% confluent, they were detached, counted, and plated into 96-well or 24-well culture plates (2500 cells/cm^2), chamber slides (2500 cells/cm^2) or T25 cell culture flasks (1000 cells/cm^2). BM-MSCs were first cultured in basal medium and after 3 to 4 days, PB-MNCs were isolated and added to the co-cultures in the ratio of 1:20 (MSC:MNC). Half of the media was changed every 3 to 4 days and the cells were cultured for a total of 5, 7, 10 or 14 days.

Osteoblastic differentiation of BM-MSCs was performed as previously described, except for adding the osteogenic medium to the cells on the same day as co-culturing with PB-MNCs was started. Osteoblastic differentiation medium (OB-medium) was basal medium supplemented with 10 mM Na-β-glycerophosphate (Merck) and 0.05 mM ascorbic acid-2-phosphate (Sigma–Aldrich). Dexamethasone (100 nM) was included in the OB medium for the first week of culture (Table 5). Recombinant human VEGF at 5 ng/ml (R&D Systems) was used to study the role of exogenously added VEGF.

Table 4. Contents of the basal medium.

REAGENT	RATIO	MANUFACTURER	CAT.NO.
Mem αlpha	90 %	Thermo Fisher Scientific (Gibco)	41061037
Fetal bovine serum, certified, USA	9 %	Thermo Fisher Scientific (Gibco)	16000044
Penicillin-streptomycin	1 %	Thermo Fisher Scientific (Gibco)	15140122

Table 5: OB-medium was basal medium (Table 4) supplemented with β-glycerol phosphate disodium salt pentahydrate, ascorbic acid-2-phosphate, and dexamethasone.

REAGENT	RATIO	MANUFACTURER	CAT.NO
Beta glycerol phosphate disodium salt pentahydrate	10 mM	Merck (Sigma–Aldrich)	50020
Ascorbic acid-2-phosphate	0.05 mM	Merck (Sigma–Aldrich)	A8960
Dexamethasone	100 nM	Sigma–Aldrich	D4902

4.3.2 Three-dimensional HUVEC-MAC co-cultures (**II**)

MACs were generated in vitro by culturing MSCs with MNCs in basal medium as described in the previous chapter, and isolated by magnetic activated cell sorting as described below (see chapter 4.4). Fibrin Gel In Vitro Angiogenesis Assay Kit (ECM630; Sigma-Aldrich) was used to study the capacity of MACs to enhance the tube formation of HUVECs in a 3D-model. Fibrin gel was prepared by mixing fibrinogen solution (30 µl/well) with thrombin solution (20 µl/well) in a 96-well plate, after which the plate was placed in 37°C for 45–60 min for the gel to polymerize. GFP-HUVECs (5000 cells/well) were added to the plate and allowed to adhere overnight. $CD14^+$ $CD31^+$ MACs (5000 cells/well) were then added to the wells and allowed to adhere for 2 h before adding another layer of fibrin gel (50 µl/well). Cells were cultured in EGM (not supplemented with VEGF) (C-22010, Promocell) for 4 to 7 days.

4.4 Magnetic activated cell sorting (**II**)

Cell sorting was performed with magnetic beads (Figure 11). After isolating the whole MNC fraction (see chapter 4.1.2), the cells were labelled with magnetic microbeads coated with antibodies against CD14, CD34 or CD31 (Table 6).

Table 6. Microbeads used in magnetic activated cell sorting.

MICROBEADS	VOLUME	MANUFACTURER	CAT.NO.
CD14	20 µl / 10^7 cells	Miltenyi Biotec	130-050-201
CD34	100 µl / 10^8 cells	Miltenyi Biotec	130-046-702
CD31	20 µl / 10^7 cells	Miltenyi Biotec	130-091-935

To isolate $CD14^+CD31^+$ MACs for 3D functional assays, MSCs and MNCs were co-cultured in basal medium for 7 days, after which cells were detached by Accutase (Life Technologies). The cell suspension was then loaded onto a LS column (Miltenyi Biotec, 130-042-401), which was placed in a separator with a strong magnetic field (Miltenyi Biotec). The cells expressing the specific antigen are retained within the column, while the unlabelled cells pass through. After washing, the column is removed from the magnetic separation and the target cells are eluted (Figure 11). In addition to $CD14^+CD31^+$ MACs, cells with $CD14^+$, $CD14^-$, $CD34^+$, $CD34^-$, $CD34^-CD14^+$ and $CD34^-CD14^-$ phenotypes were isolated with this method and used in the study. The sorting success was confirmed by flow cytometry as described below (chapter 4.5).

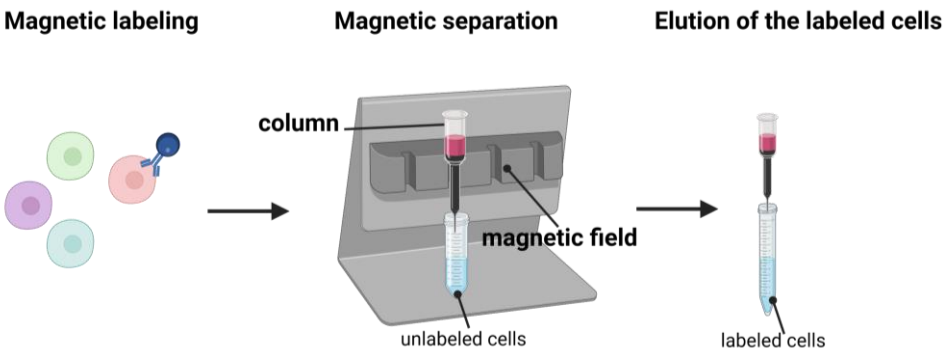

Figure 11. Simplified illustration about magnetic cell separation protocol. Created in BioRender.com.

4.5 Flow cytometry (**II, III**)

Flow cytometry was used to confirm the success of magnetic activated cell sorting and to determine the presence of MACs and pericyte-like cells within the MSC-MNC co-cultures or in the plain MNC population. Cells were filtered through 35-µm pore cell-strainer snap caps (Corning Incorporation), transferred to a 96-well plate (100 000 cells / well) and Fc receptors were blocked with Human BD Fc Block reagent (BD Biosciences). Cells were stained with fluorochrome-labelled anti-human monoclonal antibodies (Table 7). After staining, cells were washed with PBS, after which flow cytometric analysis was performed using BD LSR Fortessa (BD Biosciences) and results were analysed with Flowing Software (Turku Centre of Biotechnology, Turku, Finland). The percentage of cells expressing a specific phenotype was calculated according to the number of cells in the specific dot plot quadrants.

Table 7. Antibodies used in flow cytometry.

ANTIBODY	FLUOROCHROME	MANUFACTURER	CAT.NO.
anti-CD14	Steptavidin-Allophycocyanin/Cyanine7 (APC/Cyanide7)	BioLegend	367107
anti-CD45	R-Phycoerythrin (PE)	BioLegend	368509
anti-CD31	Brilliant Violet 711	BioLegend	303135
anti-CD34	Brilliant Violent 421	BioLegend	343609
anti-CD146	Steptavidin-Allophycocyanin (APC)	BioLegend	361015
anti-NG2	Alexa Fluor 488	Invitrogen	53-6504-80

4.6 Cytochemistry and histology (**I, II, III**)

4.6.1 Immunocytochemistry (**I, II**)

Cells were fixed with 3% paraformaldehyde, after which these fixed cell samples were blocked with 3% bovine serum albumin (BSA) in PBS for 1 hour at room temperature (RT) and incubated overnight at +4°C with a monoclonal primary antibody diluted in 1% BSA in PBS (Table 8). For the detection of intracellular marker αSMA, the cells were initially permeabilized with 0.2% Triton X-100 in PBS for 10 min on ice and then washed 3 times for 5 min with PBS before blocking the unspecific binding of the antibody. Samples in which primary antibody was absent but secondary antibody was included, were used as negative controls. After the incubation with primary antibodies, cells were washed 3 times for 5 min with PBS and incubated with a biotin-conjugated goat anti-rabbit (Vector Laboratories) or goat anti-mouse secondary antibodies (DakoCytomation, 1:200 for both). Bound antibodies were detected by Vectastain ABC kit and diaminobenzene (DAB) (Vector Laboratories). For fluorescent detection goat anti-mouse (ab150113, Abcam) or goat anti-rabbit (ab150080, Abcam) secondary antibodies were diluted in 1% BSA in PBS (1:1000) for 1 h at RT in the dark. Mounting medium with DAPI (Vector) was used to mount the samples and to stain the nucleus.

Table 8. Primary antibodies used in immunocytochemistry.

ANTIBODY	DILUTION (PUBLICATION)	MANUFACTURER	CAT.NO.
Mouse monoclonal to CD31	1:200 (**II**)	Abcam	ab9498
Rabbit monoclonal to VEGFR1	1:200 (**I**, **II**)	Abcam	ab32152
Mouse monoclonal to αSMA	1:100 (**I**), 1:200 (**II**)	Abcam	ab7817
Rabbit monoclonal to PDGFRβ	1:100 (**II**)	Abcam	ab32570
Rabbit monoclonal to NG2	1:100 (**II**)	Abcam	ab183929

4.6.2 Immunohistochemistry (**III**)

Paraffin embedded skin biopsies were cut into 5 µm sections and slides were deparaffinised and rehydrated before immunohistochemical (IHC) staining. Antigen retrieval was performed using either sodium citrate pH 6 or Tris-EDTA buffer pH 9 depending on the antibody. Slides were washed with Tris-Buffered Saline (TBS) plus 0,025% Triton X-100 and blocked with 10% normal goat serum diluted in TBS for 1 hour at RT. All antibodies were diluted in TBS plus 1% BSA. Primary antibodies (Table 9) were incubated overnight at +4°C and washed with TBS plus 0,025% Triton X-100.

Blocking of endogenous peroxidases was performed with 0,3% H_2O_2 in TBS for 15 min at RT. Horseradish peroxidase (HRP) conjugated goat anti-mouse (ab205719) or goat anti-rabbit (ab205718) secondary antibodies were used as 1:2000 dilution and incubated for 1 hour at RT and washed with TBS. DAB was used to develop the staining and Papanicolau's hematoxylin (Millipore) in 1:5 dilution was used for counterstaining. Samples with no primary antibody was used as negative controls.

Immunofluorescence (IF) detection was done in the same manner, except for no blocking of endogenous peroxidase was performed and dilutions of the antibodies were different (Table 9). Goat anti-mouse Alexa Fluor 488 (ab150113, 1:1000) and goat anti-rabbit Alexa Fluor 594 (ab150080, 1:1000) were used as secondary antibodies, and mounting medium with DAPI (Vector) was used to mount the samples and stain the nucleus.

Table 9. Primary antibodies used in immunohistochemistry.

ANTIBODY	DILUTION (IHC)	DILUTION (IF)	MANUFACTURER	CAT.NO.
Mouse monoclonal to CD14	1:200	-	Abcam	ab181470
Mouse monoclonal to CD45	1:500	-	Abcam	ab8216
Mouse monoclonal to CD31	1:500	1:100	Abcam	ab9498
Rabbit monoclonal to PDGFRβ	1:100	1:100	Abcam	ab32570
Rabbit polyclonal to NG2	1:500	1:100	Abcam	ab129051

4.7 Image analysis (**I, II, III**)

Morphological changes of the cells were evaluated by using light microscopy and real-time cell imaging system (IncuCyte ZOOM or IncuCyte S3, Sartorius). The IncuCyte software system automatically acquired and recorded images every 2 hour intervals with a 10x objective. Specific Incucyte Angiogenesis module (Cat. No. 9600-0011) was used to analyse the effect of MACs on the HUVECs ability to form networks. Images were taken every 6 hours with a 4x objective. Skin sample slides were scanned with Pannoramic P1000 or Pannoramic midi slide scanner. Blood vessels and cell morphologies and phenotypes were analysed by ImageJ or Fiji.

4.8 Real-time-quantitative PCR (**I, II, III**)

Total RNA was isolated according to manufacturer's instructions (GenElute Mammalian Total RNA, Merck). RNA quality was checked by Nanodrop Spectrophotometer (Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized from mRNA by High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher

Scientific) or SensiFAST cDNA Synthesis kit (Bioline). Specific primers and probes were purchased from Integrated DNA Technologies Inc or Metabion (Table 10). Primer concentrations in each PCR-reaction were 25 nM. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) or beta-actin (*ACTB*) was used as a housekeeping reference gene and results from co-cultures were analysed by using MSCs alone as calibrator, while with the patients' PB-MNC samples, results from day 0 were used as a calibrator. SensiFAST SYBR No-ROX Kit was used for qPCR reaction according to the manufacturer's protocol. Relative gene expression was quantified by using the ΔΔCt method where the fold change was calculated with the $2^{-\Delta\Delta Ct}$ formula.

Table 10. qPCR primers used in the study.

GENE (PUBLICATION)	FORWARD PRIMER	REVERSE PRIMER
ACTA2 (**I**)	GCTGATAATGACAACTGTATGTGC	ATGGTTTTGTCCCGCAGTA
ACTA2 (**II, III**)	CTCAAGTCTGTCTTTGCTCCTT	GTGCTGTCCTCTTCTTCACACA
ALP (**I**)	CCGACTACTACGCCAAGGA	GTTCAGGTACCGCTTCTCG
ANGPT1 (**I**)	TTTGACCAGAGTTTTTCCATGTG	GAAGCAGCAACGCTAGAAGA
BMP2 (**I**)	AGAAGAGCGACCCTCACA	CTTCATAGCCAGGTAACGGTT
PECAM1 (**II, III**)	TGCCGTGGAAAGCAGATACTC	AGCCTGAGGAATTGCTGTGTT
FGF2 (**I**)	CATGAACGCCAAGGTCGT	CATCTGTAGCTCAGGCTGAC
CSPG4 (**II, III**)	ACACGGATGCCACCCTACAAG	GGGCTCTTCACTGAGAATACGA
PDGFRB (**I**)	GGACTTTATACTTGTCGTGTAAGGA	CCTGCTGTTTTCGATGTTTCAC
PDGFRB (**II, III**)	CAAGGACACCATGCGGCTTC	AGCAGGTCAGAACGAAGGTG
PIGF (**I**)	CCAAATACATCCTCTAAAAGAAGTTCA	CCAACTCTATCAGTGGTGCTC
PTN (**I**)	GAAGCAGGGAAGAAAGAGAAAC	CTCAGCTCCAGTCCGAGT
SDF1 (**I**)	GAGTATGAGAGTGACGAGAAAGC	GGTCAAGGGTCAGGAGTTC
TGFB (**I**)	CTGAACGTGGTCAACCTGTT	GTCCACCAGGTCTCCGTA
VEGFA (**I**)	CCATGAACTTTCTGCTGTCTTG	GCGCTGATAGACATCCATGA
VEGFA (**II**, **III**)	GCGGATCAAACCTCACCAAG	ACAAATGCTTTCTCCGCTCTG
FLT1 (**I**)	CCAGAGCCTGCACATCA	TGCTCTCCACGCAGAGG
FLT1 (**II, III**)	GAAATCACCTACGTGCCGGA	ACGTTCAGATGGTGGCCAAT
KDR (**II**, **III**)	GAGCCATGTGGTCTCTCTGG	GAGTGGTGCCGTACTGGTAG
VWF (**I**)	CACTGACTACCTCATGAAGATCC	AAGTCCAGAGCTACATAACACAG
ACTB (**II**, **III**)	AGATCAAGATCATTGCTCCTCCTG	AGCTCAGTAACAGTCCGCCT
GAPDH (**I**)	ACATCGCTCAGACACCATG	TGTAGTTGAGGTCAATGAAGGG

4.9 Analysis of osteoblastic differentiation (**I**)

Cells were fixed with 3% paraformaldehyde and osteoblast differentiation was analysed by alkaline phosphatase (ALP) staining kit according to manufacturer's instructions (Alkaline phosphatase kit, 86-R, Sigma-Aldrich).

4.10 ELISA assay (**II**)

ELISA assay was used to measure the presence and levels of VEGF in cell cultures (human VEGF ELISA kit, KHG0111, Invitrogen, Thermo Fisher Scientific). Mediums were collected from PB-MNCs, BM-MSCs and their co-cultures as well as from HUVEC-MAC co-cultures. Basal medium and EGM medium without cells were used as a background control. Absorbance was measured by an ELISA plate reader at 450 nm and VEGF concentration in samples and controls was quantified based on the assay standard curve.

4.11 Statistical analysis (**I, II, III**)

Statistical analyses were conducted by using Microsoft Excel (I) or GraphPad Prism 8 software (II, III). Student's t-test was used for pairwise comparison and one-way-ANOVA followed by Dunnett's test or Bonferroni correction were used for multiple comparison tests. Kruskal-Wallis test followed by Dunn's multiple comparison was used for a non-parametric analysis depending on the normality of the data.

5 Results

5.1 MSC-MNC co-culture show differentiation of angiogenic cells in both basal and osteoblastic conditions

Our group had previously discovered the formation of vessel-like structures when PB-MNCs were co-cultured with BM-MSCs (Joensuu et al., 2011), as well as the enhanced osteoblastic differentiation in MSC-MNC co-cultures (Joensuu et al., 2015). Thus, in this PhD study, the first aim was to compare the angiogenic potential of MSC-MNC co-cultures in both basal and osteoblastic conditions. Differentiation of osteoblasts, and therefore the success of the osteoblastic induction, was demonstrated via positive ALP mRNA expression and ALP staining. Interestingly, most prominent, although not statistically significant, osteoblastic differentiation was observed in the MSC-MNC co-cultures in OB-medium (**I**, Figure 2). This was in line with our previous observations (Joensuu, 2015) and suggested that PB-MNCs support osteogenesis.

To analyse the formation of EC-like cells in the co-cultures, we first evaluated the morphology of the cells. In co-culture with BM-MSCs, PB-MNCs transformed into spindle-shaped cells more efficiently, compared to monocultures (**II**, Figure 1A-B), and the number of these cells increased over time (**II**, Figure 1C). However, this morphological change was more significant in basal compared to osteoblastic conditions, in which PB-MNCs remained round-shaped (**I**, Figure 1).

To study further the angiogenic potential of MSC-MNC co-cultures, we analysed the gene expression of various angiogenic factors. The observations on different cellular responses between basal and osteoblastic conditions were reflected in the expression patterns of proangiogenic factors on days 5 and 10 (**I**, Figure 4). Higher expression of *SDF1* was observed in the co-cultures in basal condition on both days. However, the expression of *SDF1* was downregulated on day 10 compared to day 5. Both *ANGPT1* (ANG1) and *BMP2* were upregulated in osteoblastic condition and the expression of *BMP2* was further upregulated in co-culture compared to monoculture on day 5. The expression levels of *PTN* and *PIGF* were also higher in the osteoblastic differentiation medium compared to basal medium on day 10 (**I**, Figure 4).

Gene expression of endothelial markers *FLT1* (VEGFR1), and *vWF* as well as pericyte recruiter *PDGFRB* and pericyte inducer *TGFB* were upregulated after 5 days of co-culture in both basal and OB-medium (**I**, Figures 3 and 5). On the other hand, gene expression of pericyte markers *ACTA2* (αSMA) and *PDGFRB* were downregulated in the co-culture on day 7 in basal medium and no significant differences were observed on day 14 (**II**, Figure 8). Interestingly, the expression level of *CSPG4* (NG2) was approximately the same in monocultures and in co-culture on day 7, while on day 14 the expression was significantly downregulated in co-culture compared to MSCs alone (**II**, Figure 8). Nevertheless, the expression of EC-markers *FLT1* (VEGFR1), *PECAM1* (CD31), and *KDR* (VEGFR2) were upregulated in the MSC-MNC co-cultures compared to MSCs alone in basal conditions on days 7 and 14 (**II**, Figure 2). Differentiation of CD31, and VEGFR1 expressing EC-like cells as well as αSMA, PDGFRβ, and NG2 expressing pericyte-like cells in the co-cultures was further demonstrated by immunocytochemical stainings on day 5 (**I**, Figures 3 and 5) and on day 7 (**II**, Figures 1 and 8). This verified that despite the differences, both conditions supported the differentiation of cells towards angiogenic cell phenotype in co-cultures.

5.2 Both BM-MSC and PB-MNC populations contain pericyte-like cells

Since the cells with a pericyte-like phenotype were shown to differentiate in MSC-MNC co-cultures, the origin of these cells was further analysed by flow cytometry, immunochemistry, and mRNA analysis. Results showed the existence of pericyte-like cells within both BM-MSC and PB-MNC populations (**I**, **II**, **III**). However, the marker profile of these cells was different depending on which cell population they originated. αSMA expressing cells were identified among the BM-MSCs, but not among the PB-MNCs, when either of these cell types was cultured alone in monocultures (**I**, Figure 5 and **II**, Figure 8). However, since MSCs are known to be a heterogeneous cell population, not all BM-MSCs expressed αSMA. In addition, PDGFRβ$^+$ cells and NG2$^+$ cells were observed among BM-MSCs (**II**, Figure 8) and flow cytometry analysis further verified the existence of NG2 expressing cells among the MSCs and that MSCs expressed also CD146 marker (**II**, Figure 9). This indicated the existence of cells with pericyte-like phenotype among the BM-MSCs.

PB-MNCs differentiated into PDGFRβ expressing cells in MSC-MNC co-cultures, suggesting that BM-MSCs support the differentiation of PB-MNCs into pericyte-like cells (**II**, Figure 8). In addition, PB-MNCs expressed NG2 at both mRNA and protein level (**II**, Figure 8). Flow cytometry analysis further confirmed the presence of NG2$^+$ pericyte-like cells among the MNCs isolated from healthy donor and in skin graft patients (**II**, Figure 9 and **III**, Figure 1). Surprisingly, the

number of circulating $CD14^+CD45^+NG2^+$ hematopoietic pericyte-like cell was significantly higher in healthy donors compared to patients with traumatic skin defect before surgery. However, the number of these pericyte-like cells increased slightly in the circulation after skin grafting (**III**, Figure 1). Furthermore, $NG2^+$ and $PDGFR\beta^+$ cells were observed around small vessels at the graft site during wound healing, suggesting that these cells represent true pericytes (**III**, Figure 3). These intriguing results show that pericyte-like cells of PB-MNC-origin are not only formed in vitro in co-cultures with BM-MSCs but can also exist in circulation in vivo. However, these results need to be verified with more patients.

5.3 Spindle-shaped cells formed in MSC-MNC co-cultures are myeloid angiogenic cells

The spindle-shaped cells formed in the MSC-MNC co-cultures were further characterized by magnetic activated cell sorting, which demonstrated that both $CD14^+$ and $CD34^+$ MNCs had high differentiation potential into elongated EPC-like cells (**II**, Figure 3). However, mRNA analysis and immunocytochemistry (ICC) demonstrated that most of the CD31 expressing cells were among the $CD14^+$ MNCs (**II**, Figure 4). To elucidate the phenotype of these cells more specifically, magnetic activated cells sorting was used to isolate $CD14^+CD31^+$ double positive cells from the MSC-MNC co-cultures. The isolated cells were further analysed by flow cytometry and these cells were shown to be $CD14^+CD45^+CD31^+CD34^-$ thus confirming their MAC-phenotype (**II**, Figure 5).

5.4 MACs support angiogenesis in vitro and exist in circulation in vivo

Functionality of the MACs formed in the MSC-MNC co-cultures was demonstrated by 3D tube formation assay and IncuCyte angiogenesis analysis. HUVECs represent true endothelial cells with a tube forming ability, and when co-cultured with MACs, HUVECs formed more loops and branching points, which was further confirmed by the quantification of branching points and networks (**II**, Figure 6). These results show that MACs support the tube forming capacity of HUVECs in vitro.

To study whether MACs exist in vivo, cells with $CD14^+CD45^+CD31^+CD34^-$ phenotype were analysed from the circulation of patients with a traumatic tissue defect and from healthy controls. When compared to patients before surgery, the number of MACs was significantly lower in older controls (age 75 and 59 years), while in younger controls (age 39 and 42 years) the number was higher or similar compared to corresponding patients. However, in all patients, the number of MACs increased during the early phases of wound healing (**III**, Figure 1). In addition,

successful vascularization of the skin grafts was demonstrated by analysing the formation of blood vessels in patient's skin biopsies post-surgery (**III**, Figure 3). CD14 and CD45 expressing myeloid cells were also shown to participate in wound healing and CD14, CD45 and CD31 expressing cells were found to be co-localized within the same regions in the regenerating skin (**III**, Figure 4). Taken together, this data suggests that MACs could contribute to angiogenesis also in vivo. However, due to the limited patient number, further studies are needed to confirm these results.

5.5 VEGF is secreted by BM-MSCs but not MACs

As VEGF is one of the key factors regulating angiogenesis, its effect on the angiogenic potential of BM-MSCs and PB-MNCs was studied by adding exogenous VEGF into co-cultures performed either in basal or in osteoblastic conditions. As a result, the formation of spindle-shaped cells in osteoblastic medium was enhanced after 14 days of co-culture, even though this difference did not reach statistical significance (**I**, Figure 6). However, this suggests that addition of exogenous VEGF promotes the differentiation of EC-like cells in osteogenesis-inducing conditions over time.

To elucidate weather VEGF is the key signal for inducing angiogenic differentiation in co-cultures, an ELISA assay was used to measure the presence and levels of VEGF in mono vs. co-cultures performed in basal medium. BM-MSCs produced VEGF with increasing concentration over time and the concentration was further increased in MSC-MNC co-cultures, even though PB-MNCs alone did not secrete VEGF (**II**, Figure 7). No detectable levels of VEGF secreted by MACs was found either, indicating that they stimulate angiogenesis through some other molecular mechanism.

Expression levels of *VEGF* were also analysed from the total MNC fractions of skin graft patients both before the surgery and on day 1 and 6 post surgery. Gene expression differed between the patients, since in two patients the expression was upregulated post-surgery, while in two patients it was downregulated (**III**, Figure 2). These differing results might be explained by the diverse roles of VEGF during different conditions (Apte et al., 2019) and the fact that all patients used in this study suffered from different underlying diseases, as described above (chapter 4.1.1).

6 Discussion

Angiogenic potential of MSC-MNC co-cultures

Vascularization is an important biological phenomenon in tissue healing. As many diseases with defective tissue repair are characterized by insufficient vascularization, effective methods to enhance postnatal vasculogenesis and angiogenesis, as well as finding an optimal endothelial cell source, are needed for clinical applications. The aim of this research project was to study the angiogenic potential of bone marrow derived mesenchymal stromal cells (BM-MSCs) and peripheral blood derived mononuclear cells (PB-MNCs). We established an easy-to-implement 2D in vitro co-culture model to study the interactions of BM-MSCs and PB-MNCs and their contribution to neo-vessel formation. We demonstrated increased expression of proangiogenic factors and differentiation of both pericyte-like cells and myeloid angiogenic cells (MACs) in MSC-MNC co-cultures in basal medium without any added supplements. Furthermore, for the first time, we found that MACs and pericyte-like cells exist in human circulation and showed that the number of these cells was increased in the circulation of traumatic wound patients after skin grafting, suggesting that they play a role in tissue healing in vivo.

MACs have previously been obtained in short-term cultures of PB-MNCs on fibronectin-coated plates in endothelial cell culture medium containing different proangiogenic growth factors, such as VEGF (Hur, 2004). We were able to obtain these cells by co-culturing PB-MNCs with BM-MSCs without any coating or exogenous growth factors, suggesting that BM-MSCs could directly promote the differentiation of MACs. Mesenchymal stromal cells are known to contribute to tissue healing by attenuating local inflammatory process through their immunomodulatory properties and by mediating angiogenesis by secreting VEGF (Caplan, 2011; Mayer et al., 2005). In the current study, we showed that the secretion of VEGF by BM-MSCs was increased when they had a direct contact with PB-MNCs, indicating that PB-MNCs could guide BM-MSCs to secrete more VEGF. These results would further suggest that in our co-culture model BM-MSCs regulate the formation of MACs by secreting VEGF. This hypothesis is supported by other studies, which have also shown increased secretion of VEGF by MSCs and differentiation of proposed ECFCs when co-cultured with BM-MSCs in 3D fibrin

matrix (Rüger et al., 2018, 2020). Furthermore, Rüger and colleagues showed formation of vascular structures when they co-cultured BM-MSCs with PB-MNCs in a fibrin-based 3D model. We did not observe formation of tube-like structures nor detect ECFCs in our co-cultures, which could be because our MSC-MNC co-cultures were performed in 2D. Thus, fibrin-based 3D culture conditions might support the formation of ECFCs instead of MACs.

Identification of functional MACs in vitro and in vivo

As we observed the formation of spindle-shaped cells, that are supposed to reflect the differentiation of ECs or EC-like cells, in our MSC-MNC co-culture model, we began to investigate these cells and their angiogenic potential in more detail. The proposed angiogenic potential of co-cultures was further supported by qPCR analysis, which showed increased levels of endothelial markers *FLT1* (VEGFR1) and *vWF* in both basal and osteoblastic culture conditions. However, since the formation of elongated EC-like cells was more prominent in the basal medium compared to osteoblastic conditions, we continued the investigations in basal conditions.

Since both PB-MNCs and BM-MSCs have been shown to differentiate into EC-like cells (Asahara, 1997; Oswald, 2004), we wanted to determine from which cell type the spindle-shaped cells in our co-culture system originated. We demonstrated by immunocytochemical analysis that the elongated cells of PB-MNC origin expressed endothelial markers VEGFR1 and CD31, but BM-MSCs did not. Furthermore, we showed that the CD31 expressing spindle-shaped cells were derived from $CD14^+CD45^+$ myeloid cells, which indicated that they could represent MACs, which have been reported to be, as the name implies, of myeloid origin and to modulate angiogenesis in a paracrine manner (Medina, 2011). In addition, the cells formed in our co-cultures lacked the expression of CD34, which is considered as a marker for mature circulating ECs (Peichev, 2000). We further analysed the proangiogenic functionality of the EC-like cells formed in the MSC-MNC co-cultures and demonstrated that these cells indeed stimulated the formation of tube-like structures and networks when co-cultured with HUVECs. This further confirmed that even though they themselves do not have the tube forming capacity, they still could stimulate angiogenesis in vitro.

There are previous studies, which have suggested that MACs are generated only by in vitro culturing of PB-MNCs (Medina, 2017). We also observed these cells to be formed in MSC-MNC co-cultures but wanted also to further investigate the existence of MACs in vivo by analysing the number of $CD14^+CD45^+CD31^+CD34^-$ cells in human circulation. To our knowledge, this was the first study to show that MACs exist in vivo. Furthermore, by comparing the number of MACs in the

circulation of healthy controls to traumatic wound patients, we showed similar or higher levels of MACs in younger controls, while in older controls the number was significantly lower compared to patients at pre-operation. This suggests that MACs might have age-related functions. However, in all patients, the levels of circulating MACs were increased after skin grafting indicating that these cells could be associated with tissue trauma and potentially also be mobilized in circulation in response to trauma. This is supported by another study showing a rapid rise of ECFCs in the circulation of burn patients (Fox et al., 2008), indicating that circulating cells, with the ability to enhance angiogenesis, are mobilized quickly in response to trauma. Furthermore, since in vitro generated MACs have been shown to enhance angiogenesis in vivo when injected into a mouse (Medina, 2011), and formation of blood vessels has important role during wound healing, it can be hypothesized that these circulating MACs could contribute to wound healing by enhancing vascularization. For further support, our quantitative analysis of blood vessels at the graft site demonstrated effective vascularization in the traumatic wound patients after skin grafting.

Besides MACs, macrophages are another cell type with a myeloid origin. Macrophages have been shown to have a critical role during tissue repair and specifically the macrophages polarized towards the anti-inflammatory M2 phenotype, have been indicated to promote angiogenesis in vitro and in vivo in mice (Jetten et al., 2014). It has been suggested that MACs could represent an alternative M2 macrophage subtype, because they were shown to resemble M2 macrophages based on molecular profiling (Medina, 2011). However, it has been shown that MACs differ from M2 macrophages at least by high CD163 expression (Chambers, 2018). Even though we did not analyse CD163 expression in the $CD14^{+}CD45^{+}CD31^{+}CD34^{-}$ cell population and thus cannot conclude whether they are M2 cells or not, we nevertheless showed that cells with angiogenic capacity are formed in the MNC-MSC co-cultures and similar kinds of cells can be found in human circulation as a result of tissue trauma.

Identification of pericyte-like cells within BM-MSC and PB-MNC populations

In our MSC-MNC co-cultures, pericyte-like cells were identified within both BM-MSC and PB-MNC populations, but cells had different marker profiles depending on their origin. First, we demonstrated by qPCR that the gene expression of pericyte induction factor *TGFB* and pericyte marker *PDGFRB* was upregulated in the MSC-MNC co-culture, compared to BM-MSCs alone, in both basal and osteoblastic conditions. This indicated differentiation of pericyte-like cells in the co-cultures. However, since PB-MNCs alone were not included as a control, we could not be certain from which cell population these cells originated.

Pericytes are indeed challenging to identify due to their heterogeneity, and lack of a specific pericyte marker (Armulik, 2011). Therefore, several markers are usually used for their identification. Pericytes also display similarities with BM-MSCs in terms of their marker profile, ability to self-renew, and potential to differentiate into multiple mesenchymal cell types in vitro, which makes the identification even more complex (Wong, 2015). We identified αSMA, PDGFRβ and NG2 expressing cells among the BM-MSCs by immunocytochemical stainings and by assessing the mRNA expression levels. Flow cytometry analysis further showed that BM-MSCs both alone and in co-cultures expressed CD146 surface marker, which is a marker associated with the commitment of a BM-MSC subpopulation to a vascular smooth muscle cell lineage (Espagnolle et al., 2014). Nevertheless, not all of our BM-MSCs expressed the pericyte markers studied, reflecting the heterogeneity of BM-MSCs and distinguishing them as non-pericytic MSCs and pericyte-like MSCs. This is consistent with the study by Blocki et al. who reported that pericytes represent a subpopulation of BM-MSCs, but not all BM-MSCs can act as pericytes (Blocki, 2013). Despite the shared phenotype between BM-MSCs and pericytes, NG2 and αSMA markers have been shown to be expressed by only pericytes but not by BM-MSCs (Wong, 2015), thus allowing to distinguish these cells from each other. We observed NG2 and αSMA expressing cells within our BM-MSCs, thus verifying the existence of pericyte-like cells.

Even though cells with pericyte-like phenotype were observed among BM-MSCs, to our surprise also part of the cells within PB-MNC population started to express PDGFRβ in co-culture with BM-MSCs. Flow cytometry analysis further supported this observation, since $CD14^{+}CD45^{+}$ PB-MNCs, which were separated by magnetic beads, started to express CD146 and NG2 in co-culture with BM-MSCs over time. These results support the previous hypothesis of an alternative pericyte-like cell population of PB-MNC origin (Blocki, 2015). These hematopoietic pericytes are suggested to be involved in the early stages of angiogenesis, such as during vessel sprouting, whereas BM-MSC derived pericytes are present on mature vessels and induce vessel maturation and stabilization (Blocki, 2018). Our results are in line with this hypothesis, since the presence of αSMA, in subsets of pericytes, is suggested to be correlated with the ability of pericytes to contract and control blood pressure (Crisan, 2012) and we observed αSMA expressing pericyte-like cells only among BM-MSCs and not among PB-MNC. In addition, our results further showed increased differentiation of pericyte-like cells among PB-MNCs in co-cultures, suggesting that BM-MSCs could stimulate the differentiation of these hematopoietic pericytes.

In our study, pericyte-like cells of hematopoietic origin were demonstrated within the cultured PB-MNCs, but the origin of pericytes across all tissues in vivo is not fully known. Thus, to further analyse the relevance of these cells in vivo, we

evaluated their number in the circulation of healthy donors and human skin graft patients. NG2 has been shown to label pericyte-like cells on multiple types of vasculatures in different organs (Stallcup, 2018), and thus we analysed the number the $CD14^{+}CD45^{+}NG2^{+}$ cells among the PB-MNCs by flow cytometry. We found a small population of these hematopoietic pericyte-like cells in the circulation of both healthy subjects and skin graft patients. This was an interesting finding because, pericytes mostly develop from the mesoderm during embryogenesis (Thomas, 2017), and their origin is diverse in different tissues. For example, it is not entirely known what the origin of pericytes is in the organs that arise from the ectoderm, such as skin. Mouse studies have suggested that pericytes would originate from skin fibroblasts during wound healing (Goss et al., 2021) and that myeloid progenitors could also differentiate into pericytes during the embryonic development of skin vasculature (Yamazaki et al., 2017). Our data suggests that, in addition to tissue-resident pericytes, pericyte-like cells also exist in the circulation.

Limitations of the study

When investigating the angiogenic potential of MSC-MNC co-cultures, we used BM-MSCs from only one donor and a small number of PB-MNC donors. This was justified by the fact that high intra-donor variation is a significant issue when working with human primary cells and it is challenging to get repeatable results if one uses cells from many donors of different ages and genders. In addition, long-term co-cultures are time-consuming and laborious, which would have significantly increased the workload. However, in order to conclude that the observations we made are universal, our experiments should be repeated, and the data confirmed by using cells from multiple donors. A small patient sample size was also a limitation, when studying the number of MACs and pericyte-like cells in the circulation, and thus further studies with more patients are needed to confirm our results. In addition, more comprehensive cellular and molecular analyses would be needed to confirm our hypothesis about the role of circulating MACs and pericyte-like cells during tissue healing.

Future aspects

We have shown that cells contributing to vascularization differentiate in our MSC-MNC co-cultures, and thus it would be noteworthy to investigate the mechanisms between BM-MSCs and PB-MNCs in both osteoblastic and basal conditions in more detail. We have previously shown that these cells communicate through paracrine signalling as well as through a direct cell-cell contact, and that PB-MNCs enhance the differentiation of BM-MSCs into osteoblasts in vitro (Joensuu, 2015). As Notch

signalling is known to be important in the communication between endothelial cells and osteoprogenitors, it would be of great interest to study the effect of Notch signalling in the formation of osteoblasts and MACs in the MSC-MNC co-cultures. Another possible mechanism for cell-cell communication are small channels called gap junctions, which physically connect adjacent cells and allow the transport of small molecules directly from one cell to another. The role of both Notch signalling and gap junctions could be studied by inhibiting their function in MSC-MNC co-cultures and analysing the effects on morphology, proliferation, and differentiation of BM-MSCs and PB-MNCs.

There is also an increasing interest in the role of extracellular vesicles (EVs), such as exosomes in cell-to-cell communication. Exosomes are known to contain messenger RNA (mRNA) and microRNA (miRNA) as well as various proteins, and it has been shown that functional RNAs can be transferred from one cell to another by exosomes (Valadi et al., 2007). MSC derived EVs have been shown to have important effect on the immune effector cells (Bazzoni et al., 2020) and thus it is possible that the exosome-mediated miRNA-signalling could also contribute to the interactions between BM-MSCs and PB-MNCs. The role of exosomes in MSC-MNC interaction could be studied by isolating EVs from BM-MSCs by ultracentrifugation method (Théry et al., 2006) and since cells secrete a variety of different vesicles, they must be characterized as exosomes by using electron microscopy and Western blotting methods (Lötvall et al., 2014). The role of exosomes on differentiation of PB-MNCs could be evaluated by ICC, imaging methods and qPCR. The presence of miRNAs in exosomes during angiogenic differentiation could be measured using qPCR and next-generation sequencing, which is not only suitable for profiling of known miRNAs as qPCR, but it is also able to detect unknown miRNAs.

Since we demonstrated the differentiation of pericyte-like cells and MACs in MSC-MNC co-cultures and since MACs were capable of stimulating tube formation of HUVECs in vitro, it would also be interesting to investigate the molecular mechanisms through which MACs enhance angiogenesis. MACs have been suggested to modulate angiogenesis through IL-8, which is one of the major mediators of the inflammatory response (Medina, 2011), suggesting that IL-8 might be one of the contributing factors also on our experimental setup. Another possible factor might be SDF1 as it is important factor in vascularization (Zhang et al., 2017) and we have previously shown higher expression of SDF1 in our MSC-MNC co-culture compared to BM-MSCs alone (Joensuu et al., 2018). Furthermore, we discovered that MACs and pericyte-like cells exist in the circulation and that their number increases in the early phases of wound healing, suggesting that they could contribute to wound repair and regeneration. Next step would be to isolate MACs and pericyte-like cells from the circulation and to perform comprehensive cellular

and molecular analyses to these cells by using for example scRNA-seq. Their functionality should also be analysed by 3D angiogenesis assays such as fibrin bead assay, so that their role in tissue repair would be truly understood.

Cell therapy and using endothelial progenitor cells has been considered promising tool e.g. for ischemic diseases. However, clinical trials have failed mostly due to the poor characterization of these cells and thus their behaviour in disease-related microenvironment needs further investigation. Hypoxia is one of the main factors associated with vascular insufficiency and tissue ischemia and low oxygen has been shown to alter the ECFCs shape, proliferation, and size in vitro (Hookham et al., 2016) and thus the response of MACs to hypoxia would also be important to investigate in the future. In addition, diabetes mellitus is a metabolic disease that can lead to vascular complications and high glucose conditions has been reported to cause dysfunction of ECFCs (Lyons et al., 2020). Number of the circulating ECFCs has been shown to decrease in diabetic patients (Fadini et al., 2005) and impaired proliferation of diabetic ECFCs have also been demonstrated (Tepper et al., 2002). In addition, the proangiogenic properties of in vitro generated MACs have been shown to be impaired under diabetic conditions (Chambers, 2018). In the future it would be of interest to study the effects of diabetes on the number and function of circulating MACs and pericytes and the potential of MACs to resolve diabetic vascular complications.

7 Conclusions

Based on the results and discussion presented in this thesis, the following conclusion can be made:

1. There is angiogenic potential in the MSC-MNC co-cultures in both basal and osteoblastic conditions without any added supplements, even though the expression profiles of proangiogenic growth factors differed between the culture conditions.
2. Spindle-shaped cells expressing endothelial markers in the MSC-MNC co-culture are myeloid angiogenic cells of PB-MNC origin.
3. MACs support the tube forming capacity of endothelial cells in vitro.
4. Pericyte-like cells with different marker profiles are found within both BM-MSC and PB-MNC fractions.
5. MACs and pericyte-like cells exist in the human circulation and their number was increased in traumatic wound patients after skin grafting.

Acknowledgements

This study was carried out in the Institute of Biomedicine within the former Department of Cell Biology and Anatomy, Faculty of Medicine, University of Turku between the years 2015 and 2022. I want to thank the head of the former Department of Cell Biology and Anatomy, Professor Juha Peltonen and the head of the Institute of Biomedicine, Professor Sari Mäkelä for providing the research facilities to conduct this research. This study was supported by the Turku Doctoral Programme of Molecular Medicine (TuDMM). In addition, I was fortunate to receive funding from several foundations, which enabled me to complete this work: Turku University Foundation, Björkqvist Fund, University of Turku Joint Research Grant Fund, Otto A. Malm Donation Fund and Orion Research Foundation.

I am forever grateful to my supervisor Adjunct Professor Terhi Heino for believing in me and giving me the opportunity to become a doctoral researcher. She always found the time to advice and help me and I feel privileged to have her as my mentor.

I wish to express my warmest gratitude to Professor Mikko Niemi and Adjunct Professor Johanna Laakkonen for reviewing this thesis. Their thorough evaluation and excellent comments significantly improved the quality of this thesis. I also want to thank my follow-up committee members, Professor Juha Tuukkanen, Adjunct Professor Riku Kiviranta, and Adjunct Professor Anna-Marja Säämänen for the annual meetings and valuable comments during these years. In addition, I am sincerely grateful to Annukka for allowing me to participate in one her research projects and for teaching me new skills.

I want to express my sincerest gratitude to all my co-authors. In particular, I wish to acknowledge MD, PhD Katriina Joensuu, whose contribution and support has been most important. Adjunct Professor Teuvo Hentunen, PhD Lauri Polari and MSc Ana Carolina Santo Mendes are thanked for their valuable contribution to the original publications of this study.

I am grateful to Adjunct Professor Kaisa Ivaska-Papaioannou and Adjunct Professor Jorma Määttä for creating a supportive work environment and for sharing their scientific knowledge. In addition, I owe my deepest gratitude to my colleagues MSc Milja Arponen, MSc Anja Hjelt and MSc Karoliina Kajander. I have enjoyed

our discussions of science and life and it has always been nice to come to work because of you. It has also been a pleasure to get to know you outside the office. In addition, I want to thank the current and former members of the BoneLab Turku, MSc Niki Jalava, MSc Nicko Widjaja, MSc Nataliia Petruk, MSc Petri Rummukainen, PhD Fan Wang and PhD Vappu Nieminen-Pihala. I also want to acknowledge latest member of our group, Veera Valtonen.

I want to thank Miso Immonen, Merja Lakkisto, and Pia Tuominen for their technical support and for the pleasant company during lunch and coffee breaks. I am extremely grateful to Johanna Järvi for her help with collecting the blood samples.

Special thanks belong to my dear friend and colleague PhD Tiina Kähkönen for listening, guiding, and supporting me all these years. In addition, I wish to acknowledge my dear friends outside the academia. Nita Salo, we have been friends forever and you have always been like a sister to me. Your support is irreplaceable. I want to thank Sonja Varvas for listening and being there for me. You always know the right words what to say. I also want to thank Nina Lehtonen, Sini Mäkinen, Noora Kajander, and Hanni Toivanen. Life is more than just work and research and I am grateful for having all of you in my life.

Finally, I wish to thank my family. My brother Sakari and his family Teija, Hilla and Eemeli. I am sincerely grateful to my parents Matti and Pirkko for their endless love and support. I could not have achieved this without them. I want to thank my husband Jarno for his love and support throughout this journey and my beloved son Joonatan, you are the most important part of my life.

Turku, September 2022
Liina Uusitalo-Kylmälä

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Joensuu K, Uusitalo-Kylmälä L, Hentunen TA & Heino TJ (2018) Angiogenic potential of human mesenchymal stromal cell and circulating mononuclear cell co-cultures is reflected in the expression profiles of proangiogenic factors leading to endothelial cell and pericyte differentiation.
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I

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II

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