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INTERACTIONS BETWEEN HUMAN MESENCHYMAL STEM CELLS AND CIRCULATING MONONUCLEAR CELLS IN BONE REGENERATION

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Interactions between human mesenchymal stem cells and circulating mononuclear cells in bone regeneration

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ABSTRACT

Despite the regenerative actions of bone marrow mesenchymal stem cells (BM-MSCs) and optimal reduction, ossification is delayed in almost 10% of all fractures, requiring surgical intervention. The gold standard for treating non-unions and delayed unions is a tissue auto- or allograft, which, nevertheless, can cause several specific problems, such as donor site morbidity, disease transmission and immunological rejection. To overcome these problems, tissue-engineered bone grafts are being developed. They are sensitive to hypoxia, however, and can quickly undergo apoptosis in the host tissue. Therefore, improved methods for inducing proper angiogenesis and tissue endothelialisation are vital. It is known that peripheral blood mononuclear cells (PB-MNCs) include endothelial progenitor cells (EPCs), which are responsible for neoangiogenesis in adult tissues, and these cells have been suggested as a potential source of bone graft vascularization. The aims of this study were to utilize the interactions between human BM-MSCs and PB-MNCs: Firstly, to develop a method of endothelialising an MSC-culture; secondly, to optimize the differentiation capacity of MSCs into bone-forming osteoblasts; and, thirdly, to characterize the cellular and molecular factors essential in these processes. The results showed that a co-culture of human MSCs and MNCs led to a powerful endothelial cell differentiation and tubular structure formation, even without any exogenous growth factors. Furthermore, the osteoblastic differentiation and bone formation in the co-culture setting was more efficient than in monocultures and was further potentiated when cultures were supplemented with exogenous VEGF. Finally, it was shown that both endothelial and pericyte differentiation were induced in MSC-MNC co-cultures and that the expression profile of various proangiogenic factors was dependent on culture conditions. In conclusion, this study demonstrates that the key events that stimulate successful bone healing can be induced in co-cultures of human MSCs and MNCs. This co-culture method can be used to enhance osteoblast, endothelial cell and pericyte differentiation and could therefore have potential in the further development of tissue-engineered bone grafts.

Keywords: Angiogenesis, bone formation, endothelial cells, mesenchymal stem/stromal cells, mononuclear cells, proangiogenic factors, tissue engineering, vascular endothelial growth factor

Katriina Joensuu

Ihmisen mesenkymaalisten kantasolujen ja verenkierron mononukleaaristen solujen vuorovaikutukset luunmurtuman paranemisessa

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TIIVISTELMÄ

Luuytimen mesenkymaaliset kantasolut erilaistuvat luuta muodostaviksi osteoblasteiksi eri signaalien ohjaamina. Ne toimivat kantasolureservinä, mutta myös tärkeinä tekijöinä kudosten uusiutumisessa, kuten murtuman paranemisessa. Solujen toiminnasta ja murtuman optimaalisesta reduktiosta huolimatta n. 10 %:ssa murtumia luutuminen viivästyy, jolloin tarvitaan luusiirrettä. Tähän liittyy kuitenkin erityisiä ongelmia, kuten luovuttajakudoksen vaurioita ja immunologisia hyljintäreaktioita, minkä vuoksi onkin pyritty kehittämään kudosteknologisesti valmistettuja siirteitä. Nämä ovat kuitenkin herkkiä hapenpuutteelle, minkä vuoksi tarvitaan soluviljelymenetelmiä, joilla voitaisiin tehostaa verisuonten uudismuodostusta kudossiirteessä.

Verenkierron mononukleaaristen solujen joukossa on verisuonten uudismuodostukseen osallistuvia endoteelisolujen esiasteita. Tämän väitöskirjatyön tavoitteena olikin edellä mainittujen solujen vuorovaikutuksia hyödyntämällä tehostaa mesenkymaalisten kantasolujen erilaistumiskykyä luuta muodostaviksi soluiksi ja saada aikaan hapensaannin kannalta keskeinen soluviljelmän endotelisaatio. Lisäksi tavoitteena oli karakterisoida näissä prosesseissa keskeisiä molekulaarisia signaalitekijöitä.

Tässä työssä osoitettiin, että ihmisen mesenkymaaliset kantasolut saivat aikaan mononukleaaristen solujen erilaistumisen juostemaisia rakenteita muodostaviksi endoteelisoluiksi ilman lisättyjä kasvutekijöitä. Työssä todettiin myös, että mesenkymaalisten kantasolujen ja mononukleaaristen solujen yhteisviljelmissä luun muodostus on tehokkaampaa kuin yksittäisviljelmissä. Lisäksi osoitettiin, että luunmuodostus tehostui edelleen, kun yhteisviljelmään lisättiin verisuonen endoteelisolukasvutekijää (VEGF). Yhteisviljelmässä tapahtui myös sekä endoteelisolujen että perisyyttien erilaistumista. Molekulaarisia mekanismeja ja eri signaalitekijöitä tutkittiin tarkemmin Transwell-kalvomenetelmää sekä kvantitatiivista RT-PCR:ää käyttäen ja todettiin, että proangiogeeniset tekijät ilmentyvät eri tavoin eri viljelyolosuhteissa. Tässä työssä kehitetyssä soluviljelymallissa pystyttiin siis saamaan aikaan monet murtuman paranemisen kannalta keskeiset solutapahtumat, ja se avaakin uusia näkökulmia tulevaisuuden kudosteknologisesti valmistettujen luusiirteiden kehitystyölle.

Avainsanat: Angiogeneesi, endoteelisolu, luun muodostus, mesenkymaalinen kantasolu, mononukleaarinen solu, verisuonen endoteelisolukasvutekijä

TABLE OF CONTENTS

ABBREVIATIONS

ALP	alkaline phosphatase
Ang-1	angiopoietin-1
Ang-2	angiopoietin-2
BM	bone marrow
BMP	bone morphogenetic protein
BMU	basic multicellular unit
BRC	Bone remodelling compartment
BSA	bovine serum albumin
EC	endothelial cell
ECM	extracellular matrix
EPC	endothelial progenitor cell
ET-1	endothelin 1
ERK	extracellular-signal-regulated kinase
FGF	fibroblast growth factor
HIF	hypoxia-inducible factor
HSC	haematopoietic stem cell
hUVEC	human umbilical vein endothelial cell
IGF	insulin-like growth factor
MAPK	mitogen-activated protein kinase
M-CSF	macrophage-colony stimulating factor
MNC	mononuclear cell
MSC	mesenchymal stem/stromal cell
OC	osteocalcin
ON	osteonectin
OP	osteoponti
OPG	osteoprotegerin
PB	peripheral blood
PDGF	platelet-derived growth factor
PDGFRβ	platelet-derived growth factor receptor β
PECAM-1	platelet endothelial cell adhesion molecule 1

PIGF	placental-like growth factor
PTH	parathyroid hormone
PTN	pleiotrophin
RANKL	receptor activator of nuclear factor kappa B ligand
RT	room temperature
Runx2	Runt-related transcription factor 2
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
vWF	von Willebrand factor
SDF-1	stromal-cell-derived growth factor 1
α-SMA	α smooth muscle actin
TGF-β	transforming growth factor β
TRACP	tartrate-resistant acid phosphatase
3DP	three-dimensional printing

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I–III. The original publications have been reproduced with the permission of the copyright holders.

I **Joensuu K**, Paatero I, Alm JJ, Elenius K, Aro HT, Heino TJ, Hentunen TA. Interaction between marrow-derived human mesenchymal stem cells and peripheral blood mononuclear cells in endothelial cell differentiation. *Scand J Surg,* 2011;100:216-222.

II **Joensuu K**, Uusitalo L, Alm JJ, Aro HT, Hentunen TA, Heino TJ. Enhanced osteoblastic differentiation and bone formation in co-culture of human bone marrow mesenchymal stromal cells and peripheral blood mononuclear cells with exogenous VEGF. *Orthop Traumatol Surg Res,* 2015;101:381-386.

III **Joensuu K***, Uusitalo L*, Hentunen TA, Heino TJ. 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 *Equal contribution. Manuscript.

1. INTRODUCTION

Mesenchymal stem cells (MSCs) reside in most adult tissues where they contribute to normal tissue turnover and repair. In addition to direct differentiation into several cell types, e.g. osteoblasts, chondrocytes and adipocytes (Pittenger et al. 1999, Jiang et al. 2002), MSCs also have trophic and regulatory functions. Therapeutic use of MSCs has been reported in the treatment of various conditions, including bone fracture, nerve injury and myocardial infarction (Berry et al. 2006), and it is an area of active research and development.

In all types of tissue healing and regeneration, angiogenesis is decisive for a successful outcome. Traditionally, the concept of tissue engineering has constituted three main components: cells, growth factors and a scaffold material (Muschler et al. 2004). However, the cells will consume all available oxygen within a few hours, while it will take several days for new blood vessels to grow and deliver oxygen and nutrients into the implant (Service 2000). Therefore, angiogenesis has been presented as the fourth essentiality of tissue engineering (Giannoudis et al. 2008).

Peripheral blood (PB) mononuclear cells (MNCs) include endothelial progenitor cells (EPCs), which are able to differentiate into blood vessel endothelial cells (ECs) (Asahara et al. 1997). EPCs have been identified among CD34+ (Asahara et al. 1997, Shi et al. 1998, Zhang et al. 2005) and CD14+ populations of MNCs (Asahara et al. 1999, Takahashi et al. 1999, Guillotin et al. 2004). For tissue engineering, it is vital to find an optimal EC source for angiogenesis without triggering any immunoreactions in the body. Therefore, PB-MNCs seem ideal for this purpose.

MSCs and osteoblasts regulate the survival and differentiation of haematopoietic cells (HSCs). It has also been shown that MSCs and osteoblasts are able to produce a variety of growth factors needed in EC differentiation, survival and growth (Kasper et al. 2007, Grellier et al. 2009, Guo et al. 2012). Furthermore, MSCs support *in vivo* blood vessel formation by differentiating into perivascular cells (Au et al. 2008). Interestingly, the interactions also seem to work the other way around: endothelial cells have been shown to increase the osteogenic gene expression as well as mineralization of MSCs *in vitro* and *in vivo* (Griffith et al. 2002, Thébaud et al. 2012). In addition, CD34+ fraction of PB-MNCs has recently been shown to be important for angiogenesis and bone formation during clinical fracture healing (Kuroda et al. 2014), thereby having further potential in bone tissue engineering.

In most studies aiming at pre-vascularization of implants, human umbilical vein endothelial cells (hUVECs) or other mature ECs have been used, although these cells are difficult to access and cannot be obtained in large quantities (Grellier et al. 2009). Therefore, there is increasing interest in applying EPCs and HSCs to the vascularization of tissue engineered implants.

Interactions between MNCs and MSC-derived cells, such as direct cell-to-cell contacts and paracrine signalling leading to osteoblastic differentiation, osteoclastogenesis and angiogenesis, are critical in bone formation and turnover. The cell communication mechanisms in bone remodelling and angiogenesis are coupled by several various growth factors, including vascular endothelial growth factor (VEGF) (Clarkin and Gerstenfeld 2013). The aim of this study was to utilize these interactions to establish better *in vitro* methods for enhanced EC and osteoblastic differentiation and to further characterize the molecular and cellular mechanisms behind the processes.

2. REVIEW OF THE LITERATURE

2.1. Bone structure and function

The adult human skeleton has a total of 213 bones, excluding the sesamoids. The skeleton serves a variety of functions: it acts as a structural support, permits movement, protects vital organs, maintains mineral homeostasis and acid-base balance, is a reservoir of growth factors and cytokines, and provides an environment for stem cell niches in the bone marrow (BM). Bone is one of the main connective tissues in the human body. It is characterized by extracellular matrix (ECM) of mainly type I collagen and a high mineral deposition of hydroxyapatite (Ca10[PO4]6[OH]2), which contributes to the high tissue density and strength.

The adult human skeleton is composed of 80% of cortical and of 20% of trabecular bone (Eriksen et al. 1994) (Fig. 1). Cortical bone is solid and dense and surrounds the marrow space. It provides the skeleton with mechanical and structural support. Trabecular bone is the cancellous filling the bone marrow compartment and responsible for the metabolic functions of bone. Both of these bone structures are composed of osteons called Haversian systems. In cortical bone, the osteons are structured as parallel and composed of dense layers called lamellae. Trabecular bone is composed of a network of thin calcified tissue, which is occupied by highly vascular bone marrow, including haematopoietic stem cells (HSCs), MSCs and the cells of the immune system. The main function of bone marrow is to maintain haematopoiesis and B-cell maturation. The outer surface of cortical bone is lined by periosteum, which provides attachment for tendon and muscles. It consists of fibroblasts and osteoprecursor cells embedded in fibrous ECM. The inner surface of bone is covered by membranous endosteum, containing vessels, osteoblasts and osteoclasts.

There are four primary cell types in bone tissue: osteoblasts, osteocytes, bone lining cells and osteoclasts. Osteoprogenitors reside in BM, the periosteum and bone canals and migrate and differentiate into bone-forming osteoblasts. Osteoblasts secrete the mineralized bone matrix, and some of them become osteocytes, as they become trapped within the bone tissue. Osteoclasts, on the other hand, are MNC-derived, giant, multinucleated bone-resorbing cells, whose main function is to digest bone. In bone tissue reside also HSCs, ECs and their progenitors, as well as osteal macrophages, which can interact with each other and thereby have a special role in the orchestrated action in bone homeostasis.

2.2. Bone remodelling

At a microscopic level, bone is a dynamic tissue, although, macroscopically, it may seem static. Bone remodelling is a strictly regulated lifelong process, in which bone maintains the mineral metabolism, replies to mechanical loading and repairs the constantly developed microdamage. Bone remodelling is based on the carefully regulated actions of bone-resorbing osteoclasts and bone-forming osteoblasts. The main unit where remodelling takes place was named the Basic Multicellular Unit (BMU) by Harald Frost (1969). It includes osteoclasts and osteoblasts located in the bone remodelling cavity. Remodelling in cancellous bone near the BM is initiated by resorption: an osteoclast erodes a resorption lacuna in 30–40 days, followed by the formation of new bone by osteoblasts over a period of 150 days (Eriksen 1994). Hattner and co-workers (1965) showed that bone formation in the adult spongy bone occur after bone resorption in at least 96.7% of cases. Only 3% of bone formation occurs without previous resorption and is called bone modelling (Hattner et al. 1965).

The cellular activities of BMU are divided into four distinct phases: the activation of the bone surface, the recruitment of osteoclasts and bone resorption, coupling from resorption to formation (or a reversal), and the recruitment of osteoblasts and bone formation (Ikeda and Takeshita 2014). In cortical bone (distant from bone marrow), remodelling takes place in tunnels where osteoclasts form "cutting cones" to remove damaged bone, followed by bone formation of osteoblasts (Agerbaek et al. 1991). This remodelling cycle of cortical bone continues for a median of 120 days (Agerbaek et al. 1991). On average, 2%–10% of the adult human skeleton is remodelled annually. Most of the bone turnover takes place at the endosteal compartment of trabecular bone.

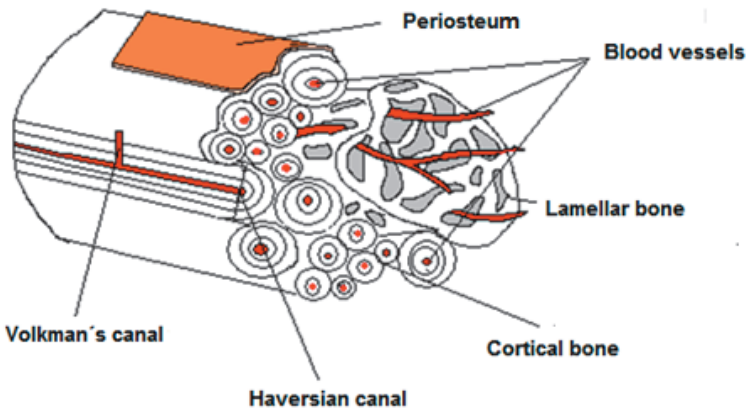

Figure 1. Illustration of bone structure. Modified from Stevens and Lowe 1997.

During the bone remodelling process, OCs and osteoblasts interact in the bone remodelling compartments (BRC), which are spaces separated from the bone marrow cavity by canopy structures formed by osteoblastic cells (Hauge et al. 2001). BRCs have been shown to be in immediate contact with the capillaries, which allows the communication between BRCs, BM and systemic circulation (Andersen et al. 2009).

2.3. Bone cells and their neighbours

2.3.1. Cells of mesenchymal lineage

Mesenchymal stem cells

MSCs were originally identified nearly five decades ago by Friedenstein and co-workers (1968) as non-haematopoietic, plastic-adherent, spindle-shaped, colony-forming cells in the BM. Later, it has been discovered that, in addition to BM, these multipotent cells reside in most adult tissues contributing to normal tissue turnover. Early studies demonstrated the osteogenic capacity of MSCs (Friedenstein et al. 1968). Caplan (1991) described their ability to generate cartilage and bone and named the cells "mesenchymal stem cells". Finally, Pittenger et al. (1999) demonstrated the multi-potentiality of MSCs by revealing their ability to differentiate into adipogenic, chondrogenic and osteogenic lineage.

Characteristics and origin of mesenchymal stem cells

MSC-like cells have also been isolated from PB (Kuznetsov et al. 2001), umbilical cord blood (Erices et al. 2000), adipose tissue (Zuk et al. 2001), synovia (Karystinou et al. 2009) and skeletal muscle (Bosch et al. 2000), and from the perivascular niches of various other postnatal tissues and organs (Crisan et al. 2008). MSCs are often referred to as a single cell type, but it has been suggested that they comprise a heterogeneous population of cells with a diverse differentiation capacity (Nombela-Arrieta et al. 2011). MSCs indeed have source-dependent characteristics, but the common criteria include the expression of certain cell surface markers (CD105, CD73 and CD90) and a lack of expression of certain markers (CD45, CD34, CD14/CD11b and CD19/CD79) as well as the capacity to adhere to plastic and to differentiate into bone, cartilage and adipose lineages (Dominici et al. 2006).

In addition to the direct capacity to differentiate into multiple cell types (Fig. 2), MSCs regulate differentiation, cell senescence and functions of other cell types. Recently, the regulative functions of MSCs have been considered to be more and more important from the therapeutic point of view. It has been suggested that MSCs could have potential in enhancing the vascularization of various tissues, since they enhance angiogenesis and endothelial differentiation of HSC-derived progenitor cells (Nombela-Arrieta et al. 2011, Guo et al. 2012), in addition to

enhancing the arteriogenesis of ischaemic heart muscle (Guven et al. 2006) as well as the angiogenesis of an ischaemic cortex after stroke (Guo et al. 2012).

Several studies have demonstrated that MSCs possess an immunoregulatory function *in vitro* and *in vivo* (Ma et al. 2014). *In vitro*, MSCs inhibit the proliferation of immune cells such as T and B cells as well as dendritic cells (De Miguel et al. 2012). Moreover, MSCs can stop a variety of immune cell functions such as cytokine secretion and cytotoxicity. *In vivo,* MSCs have been successfully used to treat graft versus host disease in HSC transplantations (Ringdén et al. 2006) and autoimmune diseases such as systemic lupus erythematosus (Castro-Manrreza and Montesinos 2015).

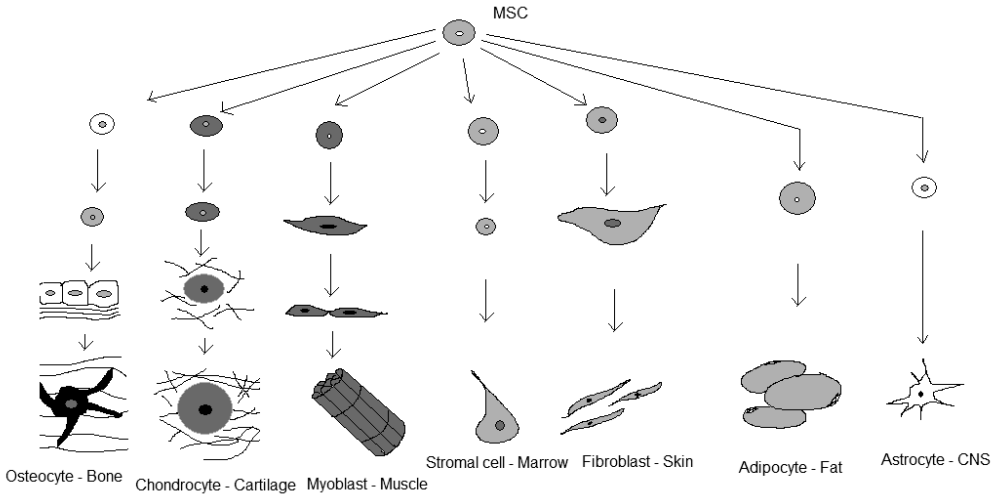

Figure 2. The multilineage potential of adult mesenchymal stromal/stem cells. CNS=Central nervous system. Modified from Säämänen et al. 2010.

Isolation and culture of bone-marrow-derived mesenchymal stem cells

There are no standardized protocols for the isolation and culture expansion of BM-MSCs, and the applied culture conditions vary greatly between different laboratories and research groups. Isolation procedures usually include density gradient centrifugation (such as Ficoll or Percoll) to separate the MNC fraction from other marrow constituents, such as plasma, lipids and red blood cells. The MNC fraction contains T cells, B cells, monocytes, HSCs and MSCs. MSCs represent the adherent colony-forming cell population. The density gradient centrifugation process has, however, been reported to reduce the total yield of MNCs from BM, but when isolation with Ficoll was compared to Percoll, no difference was observed in MNC yields (Pösel et al. 2012). Nevertheless, cells isolated with density gradient centrifugation have been shown to possess shorter telomere length and lower CFU efficiency than plastic-plated whole BM cultures (Mareschi et al. 2012).

MSC populations tend to become more homogenous after *in vitro* cultivation (Bara et al. 2014), and, after extended *in vitro* expansion, they undergo replicative senescence, which is subject to significant donor variation (Bruder et al. 1997). It seems that the proliferation rate does not correlate with donor age alone, but is also attributed to sampling variation during aspiration and to the proportion of highly proliferative cells in the cell isolate (Fennema et al. 2009, Bara et al. 2014). Even though MSCs are found near vascular structures, their surroundings are relatively hypoxic, which may be necessary in order to maintain the MSCs in an undifferentiated state and should therefore be taken into account in culture conditions (Mohyeldin et al. 2010).

Osteogenic differentiation of mesenchymal stem cells in vitro

MSCs are considered an attractive cell source for osteoprogenitors in regenerative medicine. They can be expanded in culture for several passages, and their osteoblastic differentiation capacity has been optimized in quite a few studies (Service 2000, Muschler et al. 2004). Culture conditions usually contain ascorbic acid, β-glyserophosphate and dexamethasone. Osteogenic differentiation of MSCs is defined by four stages: cellular commitment, proliferation, matrix maturation and mineralization. Osteoblastic cells change their morphology and start to express alkaline phosphatase (ALP), type I collagen, osteocalcin (OC) and osteopontin (OP) during the culture period. The mechanisms will be discussed in more detail in the next section. However, the *in vitro* differentiation of human MSCs into true bone forming osteoblasts is not yet efficient enough for cell-based therapies to reach the clinic (Service 2000, Muschler et al. 2004). The final breakthrough in clinical use is still missing, mainly due to obstacles in the large-scale commercial implementation of MSCs (Wagner et al. 2009).

Osteoblasts

Osteoblasts are cells of mesenchymal origin. Their differentiation involves a series of events (during which the cells turn/evolve) from MSCs into proliferating preosteoblasts, bone matrix-producing osteoblasts and, finally, into osteocytes or bone lining cells. The primary function of osteoblasts is to secrete and mineralize ECM, and they are found on bone surfaces (Rao and Stegemann 2013). ALP activity serves as a marker of their differentiation. As bone is formed, some osteoblasts become trapped in the matrix and undergo morphological and other phenotypic changes to become osteocytes, which account for the majority of the bone cells (Rao and Stegeman 2013).

The earliest osteoblastic marker is Runt-related transcription factor 2 (Runx2), which is essential for osteoblastic differentiation during the whole differentiation sequence (Komori et al. 1997). Runx2 regulates the expression of, e.g., OC, vascular endothelial growth factor (VEGF) and receptor activator of nuclear factor kappa B ligand (RANKL) (Lian et al. 2006), as well as collagen type I and ALP

(Ducy et al. 1999). Osterix is another important transcription factor required in the differentiation of MSCs into mature osteoblasts (Nakashima et al. 2002). It acts downstream from Runx2, and its absence leads to perinatal lethality due to serious defects in bone formation (Nakashima et al. 2002). Runx2 is known to positively regulate the expression of osterix (Nishio et al. 2006).

A wide variety of growth factors and hormones are known to have an impact on osteoblastic differentiation, including autocrine, paracrine or endocrine actions. These factors include, for example, bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), sex hormones and parathyroid hormone (PTH) (Qin et al. 2003).

Central endrocrine factors in osteoblastic differentiation

Oestrogen is an important factor in bone homeostasis in both sexes. Low oestrogen levels as well as the mutation of oestrogen receptor (ER) leads to osteoporosis. However, the exact mechanism of how oestrogen influences on bone cells is still unclear in detail. Osteoblasts are known to express ERs (Eriksen et al. 1994). In addition to the direct action on ERs, oestrogen acts via second messengers such as interleukins, prostaglandins and transforming growth factor β (TGF-β). Androgens have also been shown to have a positive influence on bone metabolism and osteoblastic differentiation (Kasperk et al. 1989). PTH is an essential factor in mineral homeostasis. Osteoblasts are known to express high levels of PTH receptors. PTH inhibits the apoptosis of osteoblasts, but when secreted at high levels, PTH stimulates bone resorption. BMPs and PTH are known to act through Wnt/β-catenin signalling pathways (Westendorf et al. 2004). Wnt/β-catenin signalling regulates osteogenesis by multiple mechanisms. Its activation is needed in osteoblastic lineage commitment, and it directly stimulates the Runx2 gene expression (Gaur et al. 2005) and promotes the proliferation and mineralization of osteoblasts (Kato et al. 2002). Mature osteoblasts are characterized by the expression of ALP and type I collagen (Murshed et al. 2005) as well as regulators of matrix mineralization such as OC, ON and osteopontin (OP). Vitamin D has also been shown to increase the expression of ALP, collagen type I, OC and OP, possibly via Runx2 transcription factor (Ducy et al. 1997).

Osteocytes and bone lining cells

Most of the osteoblasts undergo apoptosis after bone formation. However, at the end of their lifespan, some osteoblasts transform into osteocytes which form spaces called lacunae in the osteons, while others differentiate into bone lining cells. Osteocytes and bone lining cells are defined by their location. Bone lining cells are found on quiescent bone surfaces and are in contact with osteocytes (Rubinacchi et al. 1998). Mature osteocytes are stellate-shaped cells forming the lacuno-canalicular network inside the bone. Osteocytes communicate through these small channels, maintaining homeostasis. They are thought to guide bone

remodelling by activating osteoclastogenesis via their connections to bone lining cells (Seeman, 2008). Furthermore, viable osteocytes inhibit osteoclastogenesis (Heino et al. 2002, Kurata et al. 2006), while apoptotic osteocytes activate osteoclasts and thereby initiate the bone remodelling cycle after microdamage (Kurata et al. 2006, Kogianni et al. 2008, Kennedy et al. 2014). Osteocytes also have effects on osteoblasts – e.g., by secreting sclerostin, known as an osteocyte-specific cysteine knot-secreted glycoprotein. Sclerostin is thought to depress bone formation, as sclerostin-deficient mice exhibit dramatically increased bone mass by increased bone formation (Li et al. 2008). In contrast, overexpression of sclerostin decreases bone formation by inhibiting Wnt signalling and osteoblastogenesis (Li et al. 2008). Sclerostin antibody treatment has been developed as a new method to treat postmenopausal osteoporosis (Padhu et al. 2011).

Some of the osteocytic cellular processes are also in direct contact with BM (Kamioka et al. 2001), suggesting a central role for osteocytes in mineral homeostasis. Osteocytes have been described to secrete FGF23, a growth factor that is a central regulator in phosphate and vitamin D metabolism (Liu et al. 2003). FGF23 participates in several endocrine feedback loops (Quarles, 2011). The principal biological action of FGF23 in the kidney is to inhibit phosphate reabsorption by decreasing Na-dependent Pi co-transporters and to suppress $1,25(OH)_2D$ levels (Shimada et al. 2005). FGF23 also stimulates the catabolism of $1,25(OH)_2D$ by activating the 24-hydroxylase (Cyp24) (Shimada et al. 2005) and inhibiting Cyp27b1 (Shimada et al. 2005). In the parathyroid gland, FGF23 directly suppresses PTH mRNA expression *in vitro* and decreases serum PTH *in vivo* (Ben-Dov et al. 2007).

Pericytes

Pericytes are contractile cells located on the basement membrane of most vessels. Capillaries, arterioles and venules are covered by pericytes at a ratio of 10 ECs for each pericyte, depending on the tissue (Armulik et al. 2011). The exact function of pericytes is not completely understood (Gomez-Gaviro et al. 2012), but they are reported to support ECs as well as differentiate into a variety of mesodermal cell types such as osteoblasts, myocytes and adipocytes (Crisan et al. 2008, Armulik et al. 2011). Therefore, mural pericytes are suggested as an MSC subpopulation, although not all MSCs possess pericyte characteristics (Blocki et al. 2013).

TGF-β is the main inducer of pericyte differentiation and proliferation (Melchiorri et al. 2014). It acts via two receptors, Alk-1 and Alk-5, the first inducing proliferation and migration and the latter mainly cell differentiation and maturation (Armulik et al. 2011). Platelet-derived growth factor receptor β (PDGFRβ) is another pericyte marker and important in the recruitment of pericytes by ECs in angiogenesis (Melchiorri et al. 2014). Notch-signalling is

central in PDGFRβ-expression and crucial to angiogenic sprouting and tubule-formation, also having an important role in pericyte–EC interactions (Domenga et al. 2004, Liu et al. 2010). Stromal-cell-derived growth factor 1 (SDF-1) has a role in pericyte recruitment and endothelial tube formation and maturation (Stratman et al. 2010), and its actions are suggested to associate with the PDGF pathway (Song et al. 2009).

2.3.2. Cells of haematopoietic lineage

Haematopoietic stem cells

HSCs give rise to lymphoid and myeloid progenitor cells, which produce all the mature blood and immune cells of the body (Dunn, 1971), as well as, for instance, monocytes and CD34+ endothelial progenitor cells. HSCs are located around blood vessels in specific microenvironments in the BM. Human HSCs are lineage negative (Lin^-) and positive for CD34 (Notta et al. 2011), and they may be further selected by using various surface markers, including CD45 and CD41 (Lim et al, 2013).

Many different cell types, such as osteoblasts, endothelial cells and MSCs, have been characterized as contributors to HSC niche formation (Mendez-Ferrer et al. 2010, Gomez-Gaviro et al. 2012). Anatomically, HSC niches are distinguished into osteoblastic and vascular niches (Yin and Lin 2006), located either in the immediate proximity of trabecular bone (Nilsson et al. 2001) or in the sinusoidal vascular endothelium (Kiel et al. 2008). These two niches play different roles in regulating the quiescence and proliferative status of HSCs (Balduino et al. 2012). Quiescent/slow-cycling HSCs are typically concentrated in the endosteal niche and in close contact with osteoblasts, which affects HSC function and expansion (Calvi et al. 2003). Conversely, proliferative/fast-cycling HSCs prefer to localize into the perivascular niche, which supports stem cell expansion and development (Calvi et al. 2003, Kiel et al. 2007).

In addition to cellular components, there are also acellular ECM components in the osteoblastic niche, which regulate HSC function (Bodo et al. 2009). Bone-resorbing osteoclasts also have a role in HSC regulation, since the inhibition of osteoclast function has been shown to reduce the HSC number and to impair their quiescence (Lymperi et al. 2011). It has been discovered that nestin+ MSCs have a high expression level of genes related to HSC maintenance and that they are spatially associated with HSCs and adrenergic nerve fibres (Shen et al. 2004). There is also evidence that HSCs may induce MSCs to differentiate into osteoblasts (Jung et al. 2008).

Bone and BM share a close developmental relationship, and physiological changes are often reflected in both compartments, such as in increased bone remodelling, when haematopoietic (red) marrow is dominating the marrow space instead of

the inactive fatty (yellow) marrow (Krempien et al. 1978, Eventov et al. 1991). In addition, bone and BM are anatomically connected, indicating that interactions are likely to exist between them. It has been suggested that both the BM and the HSC endosteal niche cannot be generated or sustained independently of bone formation (Chan et al. 2009).

Mononuclear cells

MNC fraction in the PB contains, for example, T-cells, B-cells, monocytes and CD34+ EPCs. The PB-MNC-fraction is always a mixture of these different cell types and, therefore, the numbers of different cells and cytokines vary between donors and sampling time (Maccarrone et al. 2013). PB-MNCs are usually isolated from blood with density gradient centrifugation (such as Ficoll or Percoll) protocols similar to those used for BM-MNCs, as discussed earlier.

Different cell and progenitor pools within the PB-MNC fraction can be studied and isolated based on the expression of different surface markers. CD34 is a cell surface glycoprotein that is considered an early haematopoietic marker of monocyte-lineage cells. It promotes proliferation and blocks the differentiation of progenitor cells as well as functions as a cell–cell adhesion factor. It may also mediate the attachment of HSCs to BM ECM or directly to MSCs and enhance the migration of HSCs (Nielsen and McNaghny 2008). CD14, in turn, acts as a co-receptor for the detection of bacterial lipopolysaccharide. HSCs give rise also to circulating CD14+ monocytes in the BM. In adult humans, monocytes consist of 5%–10% of circulating white blood cells. They are a heterogeneous population as regards their surface markers, phagocytic capacity and differentiation potential. Furthermore, circulating monocytes have the capacity to differentiate into a variety of phagocytes, including macrophages, dendritic cells, osteoclasts, microglia and Kupffer cells.

Strategies to improve angiogenesis together with bone formation are essential for tissue-engineered implants. ECs are inappropriate for clinical applications as they are not easily accessible and cannot be attained in sufficient quantities. PB-MNCs seem an interesting endothelial progenitor cell source for tissue engineering. Several distinct progenitor cell populations have been reported in the PB-MNC fraction, including HSCs (Damon and Damon 2009), EPCs (Asahara et al. 1997), MSCs (Zvaifler et al. 2000), osteoclast precursor cells (Costa-Rodrigues et al. 2010, Hu et al. 2011) and circulating fibrocytes (Bucala et al. 1994), suggesting that PB-MNCs may possess the potential to differentiate into a multitude of mature functional cell types in specific microenvironments (Zhang and Huang 2012).

CD34+ and CD14+ MNC populations are known for their pre-angiogenic properties (Takahashi et al. 1999, Zhang et al. 2005), and the use of PB-MNCs as a cell source for tissue-engineered bone constructs is further supported by evidence of a pre-osteoblastic cell population (Eghbali-Fatourechi et al. 2005,

Kuroda et al. 2014), which appears to be comprised of two populations: one related to haematopoietic stem cells/endothelial progenitor cells and the other to plastic-adherent MSCs. It has been suggested that these CD34+ preosteoblastic cells of haematopoietic origin are clinically relevant in bone regeneration and fracture healing (Kuroda et al. 2014). Kuznetsov et al. (2001) identified circulating adherent, clonogenic, fibroblast-like cells with osteogenic and adipogenic potential from the blood of four mammalian species (mouse, rabbit, guinea pig and human). These cells were positive for ON, OP, collagen 1 and α-smooth muscle actin (α-SMA), and negative for the expression of haematopoietic and endothelial markers.

Osteoclasts

Osteoclasts are giant multinucleated cells responsible for bone resorption, thereby having a central role in the bone remodelling process and skeletal development. Osteoclasts are usually located on the endosteal calcified bone surface in a resorption lacuna. There are several characteristic features that can be used for their identification: multiple nuclei (usually 4–20), abundant Golgi complexes, transportation of lysosomal vesicles across the cell and the cytoplasmic expression of tartrate-resistant acid phosphatase (TRACP). In addition, osteoclasts are characterized by the formation of actin ring when attaching to the bone surface. The osteoclasts' ruffled border membrane domain faces the bone surface, and they secrete protons to the resorption lacuna, which becomes acidic and therefore ensures the degradation of the mineralized matrix during bone resorption. They also secrete proteolytic enzymes, such as cathepsin K, to break down the collagen matrix. The digested matrix is then transported across the cell in lysosomal vesicles and released to the surroundings from the functional secretory domain of the basal membrane (Väänänen and Zhao, 2008).

A central mechanism in the regulation of osteoclast differentiation is the RANKL/RANK/OPG pathway. Osteoblasts express and secrete RANKL, which binds to the membrane receptor RANK expressed on mononuclear osteoclast precursor cells, causing the cells to proliferate and differentiate into mononuclear pre-osteoclasts. Osteoprotegerin (OPG) is a soluble decoy receptor for RANKL, also produced by osteoblasts, which inhibits osteoclastogenesis (Khosla 2001). The majority of endocrine, paracrine and autocrine factors affecting bone resorption, such as oestrogen and PTH, act through the RANKL/RANK/OPG pathway (Khosla 2001). Macrophage-colony stimulating factor (M-CSF) is another central factor in osteoclast differentiation. Damaged osteocytes have been shown to produce M-CSF and RANKL to stimulate osteoclastic differentiation and activation, which is needed for microdamage repair (Kurata et al. 2006).

Endothelial cells and their progenitors

EPCs, which arise from HSCs, are found among the CD34+ (Shi et al. 1998, Boyer et al. 2000) and CD14+ (Zhang et al. 2005) population of MNCs. They are important cells in postnatal vasculogenesis (Asahara et al. 1999) and neovascularization (Takahashi et al. 1999, Asahara et al. 2004). Even though EPCs are normally rare in the peripheral circulation, they have been shown to be mobilized from BM in different stress situations, such as myocardial or hind limb ischaemia (Takahashi et al. 1999, Guven 2006).

ECs provide the inner surface of the vessel and are in direct contact with blood. In vascular ECs, endothelial junctions have to maintain blood vessel homeostasis while retaining their ability to rearrange during angiogenesis. Both adherens and tight junctions join neighbouring cells together and can adapt quickly to changes in the perivascular microenvironment, such as angiogenic/antiangiogenic signals, blood flow, shear stress and inflammatory conditions. The adherens junction protein, vascular endothelial cadherin (VE-cadherin), is specifically responsible for these junctions. Other endothelial markers are VEGF receptors 1, 2 and 3 that are discussed later. The von Willebrand factor (vWF) is a glycoprotein involved in arterial thrombus formation, which is expressed by mature ECs and widely used as an endothelial cell marker. Platelet Endothelial Cell Adhesion Molecule 1 (PECAM-1) is an integral membrane glycoprotein belonging to the immunoglobulin superfamily and an important vascular molecule, especially involved in endothelial cell–cell adhesion (Albelda 1991). The expression profile of PECAM-1 indicates the progress of EC differentiation and adhesion during EC migration. Endoglin, in turn, is a receptor for TGF-β, which induces EC proliferation in angiogenesis (Mercado-Pimentel 2007). It is highly expressed on human vascular ECs, especially in proliferating ECs.

Endothelial cells are reported to stimulate the osteoblastic differentiation of MSCs and bone formation (Guillotin et al. 2009) as well as regulate HSCs in the bone marrow (Shi et al. 1998). Furthermore, they form the perivascular niche for precursor cells in various organs and tissues, such as muscle and testis (Christov et al. 2007, Yoshida et al. 2007, Yin et al. 2006). It has also been shown that neurogenesis is associated with angiogenesis (Tavazoie et al. 2008, Kiel et al. 2005) and that ECs can stimulate neurogenesis and self-renewal of neural stem cells (Shen et al. 2004)

Osteal macrophages

Macrophages are heterogeneous MNCs derived from HSCs that migrate into a variety of tissues to differentiate into resident tissue macrophages (Pettit et al. 2008). They are closely related to osteoclasts but differ morphologically as well as in terms of their protein expression profiles (Gordon et al. 2005). It has been shown that tissue macrophages such as microglia in the brain and Kupffer cells in

the liver play important roles in tissue homeostasis and development (Gordon et al. 2005, Pettit et al. 2009). A special cell population in bone tissue has been discovered at the site of bone modelling: osteal macrophages or osteomacs (Chang et al. 2008). These cells form a canopy structure above mature osteoblasts at bone formation sites and constitute approximately one sixth of the total cells in osteal tissues. In contrast, the canopy structures of bone remodelling compartments are formed by cells of osteoblast lineage in immediate contact with the capillaries (Andersen et al. 2009).

Osteomacs are not considered as an osteoclastic subpopulation as they do not express common osteoclastic markers and lack multinucleation, but they do have the ability to differentiate toward multinucleated cell phenotypes through cell plasticity (Takeshita et al. 2000). However, osteomacs are not considered as the most preferred osteoclastic precursor cell *in vivo* (Pettit et al. 2008).

The function of osteomacs is to enhance bone mineralization by osteoblasts. It appears that osteomacs are essential at the bone remodelling site, because depleting them caused a major loss of osteoblast bone-forming surface (Andersen et al. 2009). Osteomacs also have an important role in bone healing. In a mouse tibial bone injury model, immunohistochemical stainings revealed that macrophages were present within the bone injury site, promoting bone healing (Alexander et al. 2011). Macrophages are indeed demonstrated to produce various potent growth factors important in bone remodelling, such as TGF-β and BMP-2 (Assoian et al. 1987, Champagne et al. 2002). Due to their close proximity to bone surfaces, osteomacs are suggested to have an important role in detecting and responding to bone damage (Miron and Bosshardt 2016).

Osteomacs and other macrophage-lineage cells play a special role in biomaterial science. Hydrophilic and anionic surfaces have been demonstrated to decrease macrophage adherence, thus reducing foreign body reaction (Brodbeck et al. 2002). In bone, osteomacs are responsible for osteoconductive and -inductive actions in the surfaces of implants (Miron and Bosshardt 2016).

2.4. Blood vessel (re)generation mechanisms

ECs that form the inner layer of blood vessels are the major regulators of vascular physiology as they control blood flow and vessel tone and provide thrombo-resistance and permeability. The outer surface of blood vessels is composed of mural cells: pericytes and smooth muscle cells in larger vessels. Blood vessels are found in most tissues to deliver essential oxygen as well as metabolic and nutritional factors, but also serving as a reservoir for tissue-specific stem cell niches in the form of pericytes. Therefore, it can be suggested that ECs and pericytes have several regulative and interactive roles in various tissues.

Vasculogenesis takes place during embryonal development, when mesodermal cells form new blood vessels (Fig. 3A). It involves three stages: 1) the differentiation of mesodermal cells into angioblasts/haemangioblasts, 2) the differentiation of angioblasts/haemangioblasts into ECs, and 3) the organization of ECs into primary capillary plexus (Moon and West 2008). Capillaries are composed of ECs, basal membrane and pericytes. In angiogenesis, blood vessels sprout from pre-existing vessels or are formed by EC precursors (Pepper et al. 2002) (Fig. 3B).

Angiogenesis occurs in response to ishaemia and hypoxia, and it is characterized by ECM degradation and the detachment of pericytes from capillaries and microvessels (<100 μm in diameter), allowing the endothelial tip cells to become invasive and form filopodia and lamellipodia (Watt et al. 2013). Tip cells proliferate, extend the vessels and form ECM, junctions and lumens, and finally anastomose or inosculate with other tip cells (Fig. 3B) (Watt et al. 2013). Hypoxic tissues secrete VEGF to induce angiogenesis, thereby expanding the capillary network and increasing nutrient and oxygen supply (Shweiki et al. 1992).

Arteriogenesis, on the other hand, means the growth of collateral vessels and is one of the most powerful revascularization mechanisms in adults (Fig. 3C). Arteriogenesis is thought to be initiated by shear-stress-induced activation of ECs in the vascular wall, macrophage and lymphocyte recruitment and adhesion, remodelling of the vascular wall by released proteases and cell proliferation (Watt et al. 2013). MSCs have been reported to play a central role in this process by releasing angiogenic factors and proteases (Kim et al. 2006).

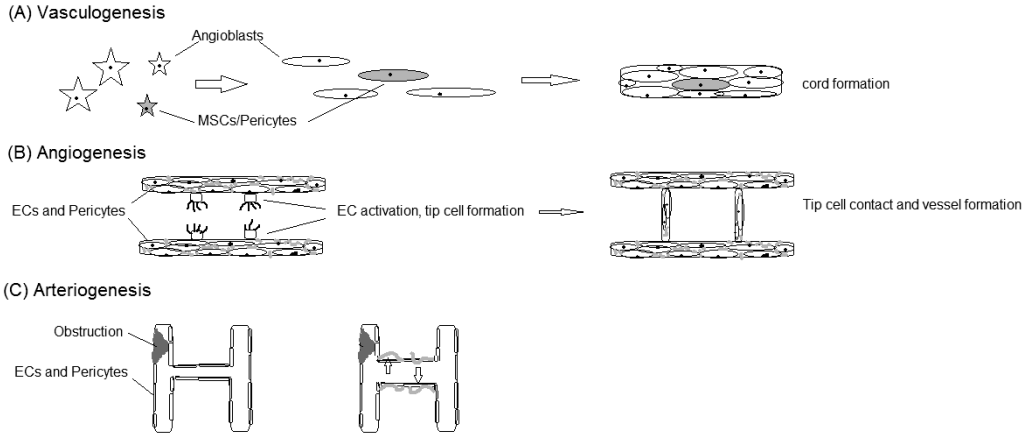

Figure 3. New blood vessel formation through vasculogenesis for the formation of vessels *de novo* from progenitor cells (A), or through angiogenesis where ECs respond to hypoxia first by movement of MSCs/pericytes away from ECs and forming tip cells (B). ECs then proliferate, extending the tip cells and forming lumen. These vessels are then stabilized with MSCs/pericytes. Arteriogenesis can occur in the absence of hypoxia with an increase in luminal diameter as a result in paracrine signalling and regulating interactions between ECs and MSCs (C). The figure has been modified from Watt et al. 2013.

2.4.1. Factors mediating angiogenesis

Vascular endothelial growth factor

VEGF is a powerful and the best known factor in angiogenesis. ECs proliferate in response to VEGF, and the vascular sprouts are guided by VEGF (Gerhardt et al. 2003). VEGFs induce EC differentiation, migration, survival and proliferation as well as promote vascular permeability. The VEGF protein family consists of VEGF-A, B, C and D (Ferrara et al. 2003). VEGFs mediate their effects on ECs by activating the cell surface VEGF receptor tyrosine kinases, VEGFR-1, 2 and 3 (Koch et al. 2011). Certain VEGF ligands are selectively bound by VEGFRs – VEGF-A binds VEGFR1 and VEGFR2, and VEGF-C binds VEGFR2 and VEGFR3. VEGFR3 activation by VEGF-C regulates the lymphatic vasculature, and the VEGF-A activation of VEGFR2 is the main regulator of EC function (Koch et al. 2011). VEGF receptors 1 and 2 are expressed on the cell surface of most blood ECs. The role of VEGFR1 on the surface of ECs is considered to be a "decoy" for not having an effective mitogenic signal to ECs (Ferrara et al. 2003). Instead, it is needed in the migration of the monocytes in response to VEGF (Ferrara et al. 2003). VEGFR2 is considered to be a major mediator of EC mitogenesis and survival (Ferrara et al. 2003).

The critical role of VEGF-A has been demonstrated by, for example, Shalaby et al. (1995), while mice heterozygous for VEGF-A or homozygous null for VEGFR2 died embryonically and displayed severe vascular defects. In addition, Sakurai and co-workers demonstrated that mice carrying a single amino acid mutation preventing the VEGFR2-induced activation of the extracellular-signal-regulated kinase1/2 (ERK1/2) die *in utero* because of vascular defects. Furthermore, activation of VEGFR2 triggers multiple signalling pathways regulating EC proliferation, migration, adhesion, survival and lumenisation (Koch et al. 2011). Activation of the phosphatidylinositol 3-kinase/Akt signalling pathway promotes EC survival by inhibiting apoptosis (Gerber et al. 1998). Phosphorylation of VEGFR2 activates ERK signalling cascade, where ERK1/2 phosphorylation induces EC proliferation and network formation, and increases vessel lumen size (Mavria et al. 2006).

Other proangiogenic factors

Besides VEGF, there are various other proangiogenic factors. For example, the sprout formation is facilitated by VEGF, FGF, VE-cadherin and angiopoietin-2 (Ang-2), which are released from hypoxic tissues (Bae et al. 2012). Platelet-derived growth factor-BB (PDGF-BB) and angiopoietin-1 (Ang-1) recruit MSCs to the site of neovascularization, and TGF-β guides the differentiation of MSCs into mural cells (Hirschi et al. 1998). Even the ECM is crucial, since type I collagen fibres have been shown to be essential for angiogenesis when ECs form capillary structures (Moon and West 2008). Matrix metalloproteinases, in turn, degrade the

basement membrane, followed by the migration of ECs, which is mediated by signalling through EC surface proteins such as Notch, VEGFR and neuropilins (Bae et al. 2012).

2.4.2. Communication between haematopoietic and mesenchymal cells

Cell-to-cell communication takes place via two main mechanisms: via paracrine, soluble signals or via direct physical cell contacts (such as gap junctions). Gap junctions link the cytoplasm of adjacent cells, allowing a free diffusion of particles less than 100 daltons between the cells. Gap junctions are intercellular channels, which are homo- and hetero-hexamers of connexin proteins. They facilitate the passage of secondary messengers (such as calcium and cAMP) between various cells. The permeability of gap junctions is controlled by external stimuli, such as mechanical, shear, ionic and ischaemic stresses, as well as by intracellular signals, such as membrane potential and phosphorylation status (Giepmans 2004). The existence of the multifunctional connexin-43 (Cx43) and connexin-45 (Cx45) gap-junction proteins in the BM compartments suggests a possibility for a direct intercellular communication between MSC- and HSC-derived cells (Rosendaal et al. 1994). Nestin-positive MSCs have an abundant CXC12 production and a high expression of Cx43 and Cx45 (Mendez-Ferrer et al. 2010). It has been shown that intercellular communication via the Cx43 and Cx45 gap-junction channels between MSCs coordinate the ability of MSCs to support the regulation of stromal-derived factor-1 (SDF-1, also known as CXCL12) production and secretion. SDF-1 is an essential factor in maintaining undifferentiated HSCs, in HSC homing and survival, and in controlling HSC proliferation and differentiation (Ding and Morrison 2013).

Endothelial cell–osteoblast crosstalk

Aguirre and co-workers (2010) have demonstrated that EPCs and MSCs interact both directly through gap junctions and indirectly through paracrine signalling. An increased expression of proangiogenic factors, such as VEGF, IGF1 and Ang-1, has recently been shown in co-cultures of MSCs and EPCs (Aguirre et al. 2010, Rahbarghazi et al. 2013, Li and Wang 2013). EPCs and MSCs have both been demonstrated to differentiate into endothelial phenotypes in the co-cultures (Aguirre et al. 2010, Rahbarghazi et al. 2013). It has also been shown that MSCs differentiate into smooth muscle cell/pericyte lineage when co-cultured with EPCs or HUVECs, serving as a mural, stabilizing cells in the tubular structures of ECs, and that the signalling path is strictly cell-contact- and ERK-dependent and does not involve gap junctions (Goerke et al. 2012).

ECs and their progenitors produce various factors affecting the migration, proliferation and differentiation of osteoblasts and MSCs (Table 1). BMP-2 and VEGF expression is up-regulated in ECs by hypoxia (Bouletreau et al. 2002), thus stimulating osteoblastic differentiation of MSCs and bone formation. On the other

hand, vasoconstrictor endothelin-1 (ET1) produced by ECs downregulates the expression of VEGF-A associated with the proliferation and differentiation of MSCs (Veillette et al. 2004), while IGF stimulates the migration of MSCs (Fiedler et al. 2006). In a co-culture of human BM-derived MSCs and human dermal microvascular ECs, osteoblastic markers ALP and OC were strongly upregulated due to the high production of BMP-2 (Kaigler et al. 2005).

VEGF is a key factor in the crosstalk between ECs and MSCs/osteoblasts (Table 2). It is produced by osteoblast lineage cells, such as osteoblasts and MSCs, and stimulates EC proliferation and migration as well as the formation of capillary-like structures (Grellier et al. 2009). The secretion of VEGF is stimulated by BMPs, FGF-2, IGF and TGF-β (Carano et al. 2003, Keramaris et al. 2008). These proangiogenic factors secreted by MSCs and osteoblasts cause upregulation of VEGFRs by ECs, which, in turn, increases the ALP expression of MSCs and osteoblasts (Grellier et al. 2009). Hoch and co-workers (2012) demonstrated the differentiation-dependent secretion of proangiogenic factors by human MSCs, but when differentiating towards osteoblastic lineage, the secretion of VEGF decreased. Furthermore, Thèbaud et al. (2012) showed that EPCs stimulate osteogenic differentiation of MSCs despite the differentiation status of ECs (progenitor cells vs. mature ECs).

FGF-2 is another important link between osteo- and angiogenesis (Table 1, 2). It activates the proliferation of ECs (Distler et al. 2003) and osteoblastic differentiation of MSCs (Tokuda et al. 2000). TGF-β is expressed by ECs and osteoblasts and stimulates the migration of both of these cell types (Kanaan and Kanaan, 2006). It also stimulates the differentiation of MSCs into pericytes (Lebrin et al. 2005).

Pericytes are known to express Ang-1, and gene knockdown of Ang-1 in these cells limited their ability to regulate microvessel assembly (Sacchetti et al. 2007). Ang-1 is known to recruit MSCs to the site of neovascularization (Hirschi et al. 1998). SDF-1 has a role in pericyte recruitment and endothelial tube formation and maturation (Stratman et al. 2010), and it is also an important factor in the regulation of stem cell niche by MSC- and HSC-derived cells (Ding and Morrison 2013).

Pleiotrophin (PTN) is another angiogenic growth factor that might also have a role in bone remodelling (Lamprou et al. 2014). PTN is shown to promote endothelial cell proliferation and differentiation, in addition to inducing the proliferation of human PB-MNCs (Achour et al. 2001). It also increases the mRNA expression of the VEGFR1 in EC cultures (Kokolakis et al. 2006).

Table 1. Cell types and key growth factors in the angiogenesis of bone tissue

Cell type	Function in angiogenesis	Markers	Growth factors expressed	References
MSC	Production of proangiogenic factors Differentiation into pericytes Differentiation into ECs Homing ability	CD166, CD106, CD146, SH2, Stro-1	VEGF, BMPs, FGF-2, IGF, Ang-1, TGF-β	Melchiorri et al. 2014, Li et al. 2013, Carano et al. 2003, Keramaris et al. 2008
Osteoblast	Production of VEGF in hypoxia	ALP, Collagen I, Osteonectin, Osteocalcin	VEGF, PTH	Grellier et al. 2009
EC	Innermost layer of blood vessels Regulatory role in angiogenesis	VEGFR1, CD31, VE-cadherin, Endoglin	VEGF, IGF, FGF-2	Ferrara et al. 2003, Albelda 1991, Mercado-Pimentel 2007, Clarkin and Gerstenfield 2013
EPC	Differentiation into ECs Expression of VEGF Support new vessel formation	CD34, CD14	BMP-2, BMP-4, VEGF, FGF-2	Asahara et al. 1997, Shi et al. 1998, Zhang et al. 2005, Asahara et al. 1999, Takahashi et al. 1999, Guillotin et al. 2004, Smadja et al. 2008, Clarkin and Gerstenfeld 2013
Pericyte	Wrap around EC layer Initiate vessel maturation Regulation of microvessel integrity, structure and function Origin of perivascular progenitor cells?	α-SMA, PDGFRβ, NG2-proteoglycan	TGF-β, Ang-1, MMPs	Lebrin et al. 2005, Goerke et al. 2012, Melchiorri et al. 2014, van Dijk et al. 2015

Table 2. Central growth factors in coupling bone repair and angiogenesis.

Growth factor	Influence	Function	Reference
VEGF	Angiogenic and osteogenic	Central mediator for other growth factors	Ferrara 2003, Clarkin 2008, Saran et al. 2014
TGF-β	Angiogenic and osteogenic	Migration of osteoblasts and ECs increases Differentiation of MSCs into pericytes and osteoblasts increases	Kanaan and Kanaan 2006 Lebrin et al. 2005
IGF	Angiogenic and osteogenic	Migration of MSCs increases Increases production of VEGF	Fiedler et al. 2006
FGF-2	Angiogenic and osteogenic	Proliferation of ECs increases Osteoblastic differentiation of MSCs increases	Distler et al. 2003 Tokuda et al. 2000
BMP-2, 4, 7	Osteogenic and indirectly angiogenic	Differentiation of osteoblasts increases Production of VEGF by osteoblasts increases	Saran et al. 2014 Clarkin and Gerstenfeld 2012
PDGF	Angiogenic and osteogenic	Chemoattractant and mitogenic for osteoblasts Recruitment of pericytes increases	Saran et al. 2014 Melchiorri et al. 2014

2.4.3. VEGF in coupling bone remodelling and angiogenesis

Communication between vascular and bone cells is central in bone biology throughout the whole lifespan from embryonal development to adulthood as well as in fracture healing and tissue-engineered bone grafts. Osteogenic precursor cells are found in vascular supplies in a pericyte-like fashion during embryogenesis as well as in fracture healing (Maes et al. 2010). Although VEGF is a central mediator between bone remodelling and angiogenesis (Table 1 and 2), the crosstalk between bone cells and ECs in the control of bone angiogenesis seems undefined (Clarkin et al. 2008, Clarkin and Gerstenfeld 2013).

Osteoblasts produce VEGF in response to different stimuli: hypoxia is one of the most potent inducers, and it acts via hypoxia-inducible factor (HIF-1) (Steinbrech et al. 2000). Overexpression of HIF-1 increases both angiogenesis and osteogenesis. Bone cell mechanotransduction stimulates also angiogenesis by the production of proangiogenic factors including VEGF by osteoblasts (Yao et al. 2004, Jaasma et al. 2007). In addition, a wide range of anabolic factors including oestrogens, IGF-1, FGF-2, TGF-β and BMPs 2, 4, 6 and 7 stimulate the osteoblastic production of VEGF and most of these factors act via mitogen-activated protein kinase (MAPK)-pathway (Clarkin and Gerstenfeld 2013). Transcription factor Osterix regulates VEGF expression while inducing osteoblast differentiation (Tang et al. 2012). The sonic hedgehog pathway is implicated as one of the main signalling pathways controlling angiogenesis and osteogenesis (Dohle et al. 2010).

VEGF also has a role in osteoclast function. Hypoxia is a potent inducer of VEGF production in osteoclasts (Trabec-Reynolds et al. 2010). One study has suggested that VEGF upregulates RANK on endothelial cells, increasing cell-sensitivity to RANKL and thus promoting angiogenesis, whereas the up-regulation of RANK by ECs after VEGF treatment indirectly enhanced the adhesion of MNCs (Min et al. 2003). VEGF has also been shown to upregulate M-CSF (Niida et al. 2005).

2.5. Tissue engineering

Tissue engineering, the combining of cells and biomaterials to build tissues and organs *in vitro*, is one of the most promising fields to address the limited supply of organs for transplantation (Khamdemhosseini et al. 2009). The use of either autologous or allogenic grafts in traditional transplantation surgery has serious problems, such as donor site morbidity, the risk of disease transmission and extended immunosuppression as well as a lack of appropriate donor tissue (Moon and West 2008). *In vivo* implantation of tissue-engineered tissues could reduce these problems.

Traditionally, tissue engineering is thought to involve the isolation and seeding of stem cells or organo-specific cells on different scaffold biomaterials. The scaffolds

will then serve as an artificial ECM for the cells to attach to, proliferate and form tissue constructs prior to implantation (Chen et al. 2010). Although there have been tremendous efforts, success in tissue engineering has mostly been limited to avascular tissues such as the epidermis of the skin, cartilage and bladder (Bae et al. 2012). Proximity to a vascular network is necessary for adequate delivery of nutrients and oxygen as well as removal of waste products, thus having a significant impact on cell survival. Vascular growth and tissue remodelling are coupled processes (Bae et al. 2012), which further highlights the importance of vasculature in regenerative medicine. The development of efficient vascularization strategies is a key challenge (Novosel et al. 2011).

2.5.1. Bone tissue engineering

Tissue engineering has great promise in bone repair (Muschler et al. 2004, Giannoudis et al. 2007). Bone tissue has a lifelong renewal capacity through its stem cells and is one of those rare organs that can heal without developing any scar tissue. Still, large bone defects are a significant clinical problem worldwide. Traditionally, the concept of tissue engineering has constituted three main components: cells, growth factors and a scaffold material (Giannoudis et al. 2007). Currently, angiogenesis has been presented as the fourth essentiality of the bone tissue engineering/reconstruction concept, and techniques for the endothelialisation of tissue engineered grafts are crucial.

The main causes of fracture non-unions and delayed unions are tissue instability and the lack of vasculature around the defect (Rao and Stegemann 2013). Although the healing response can lead to physiological remodelling, non-unions and large bone defects require surgical intervention. Autografts, allografts and xenografts are used as treatment options, but they are associated with complications such as donor site morbidity, disease transmission and immunological rejection (Rao and Stagemann 2013). The gold standard for the treatment of a large bone defect is a tissue autograft from the patient (Khan et al. 2008). Allografts entail the risk of disease transmission and failure over long-term use (Wheeler and Enneking 2005). Xenografts involve the transplantation of bone tissue across species and carry the risk of disease transmission. In addition, xenografts must undergo sterilization processes that cause a loss of osteoinductive factors within the grafting material (Schroeder and Mosheiff 2011).

2.5.2. Biomaterials in bone tissue engineering

Biomaterials are industrially manufactured or natural non-toxic materials, which are developed to heal, replace or support any tissue or organ in the body. Biomaterials in bone tissue engineering have to be biocompatible, i.e. anti-immunogenic and non-toxic, the degradation time has to be ideal for bone growth, the material has to be osteoconductive (allowing bone growth), and the material

has to tolerate sterilization procedures. The pore size of the material has to be optimal for tissue growth and angiogenesis and the surface roughness ideal for cell adhesion, proliferation and differentiation (Logeart-Avramoglou et al. 2005). A high variety of materials have been used in bone tissue engineering, such as metals, ceramics, synthetic and natural polymers as well as composites. Material selection is a difficult and critical factor in bone tissue engineering as the properties of a scaffold mainly depend on the nature of the biomaterial.

Metals are biocompatible, strong and inexpensive materials for bone repair, but they are not biodegradable and have higher moduli than bone, inducing stress shielding (Zhang et al. 2008). They are the main material type for prosthetic surgery in large joints, such as the hip and knee, but are not suitable for proper tissue engineering applications.

Bioceramics such as β-tricalcium phosphate are bioactive and have structural and compositional similarity with the mineral phase of bone and, therefore, have been widely used in bone repair (El-Ghannam 2009). Bioactive glass forms strong chemical bonds with bone, as demonstrated by Hench (1973). When bioactive glass is in touch with physiological fluids, calcium phosphate precipitates on the glass surface. The process depends on silica concentration. Osteoprogenitors and bone proteins attach to the bioactive surface of ceramics while osteoblasts produce bone ECM, integrating the biomaterial to the bone ECM (Cordonnier et al. 2011). However, the low mechanical properties and brittleness of bioactive glass and other ceramics make them unsuitable for large bone defect repair (Rezwan et al. 2006). In addition, despite the fabrication of composite ceramics, the degradation rate of ceramics is low compared to the bone healing time (Cordonnier et al. 2011).

Biodegradable synthetic polymers such as poly lactic acid and poly lactic-co-glycolic acid seem ideal material for bone tissue engineering. They are biocompatible and easily processed, and the degradation rate can be controlled (Cordonnier et al. 2011). In addition, the mechanical properties and pore characteristics can be tailored to be ideal for bone regeneration. However, the lack of cell recognition sites obstructs cellular penetration and adhesion, unlike with natural polymers such as collagen and chitosan (Nguyen et al. 2012).

Hydrogels are polymeric networks made of synthetic or natural polymers that have the ability to absorb large volumes of water (Peppas et al. 2006). They have remarkably similar properties to bone ECM, making them the leading candidates for an engineered tissue scaffold (Nguyen et al. 2012). However, the mechanical properties are too low for bone tissue *in vivo*. The limited properties of each biomaterial have led the focus to combining synthetic and natural polymers to create a balance between mechanical properties and biological signals (Nguyen et al. 2012).

Recently, three-dimensional printing (3DP) has held the spotlight in bone tissue engineering. 3DP is a potential technology in scaffold manufacture due to the ability to directly print porous scaffolds with a designed shape, controlled chemistry and interconnected porosity (Bose et al. 213). Biodegradable synthetic polymers as well as composites of synthetic and natural polymers have successfully been used in 3DP, sometimes even with site-specific growth factor/drug delivery abilities (Bose et al. 2013).

2.6. Approaches to the prevascularization of bioengineered tissues

The development of new blood vessels into an implanted tissue construct is a challenging and time-consuming process. Even highly successful proangiogenic strategies will not be able to prevent apoptosis of the cells in the centre of three-dimensional tissue constructs. This has led to the development of prevascularization techniques in tissue engineering. The central idea is to generate microvascular networks within the tissue constructs before implantation. The network has to develop interconnections to the blood vessels of the host tissue, which is also called inosculation (Laschke et al. 2006).

The perfusion could theoretically be instantaneous if the pre-engineered network in the implant is sufficiently organized, and the vascular network can be microsurgically connected to the patient during the implantation procedure. An optimal vascular network for engineered tissue needs to possess several characteristics: All of the cells need to be within a distance of 200 µm from a vessel. Therefore, the vascular network should be organized as a vascular tree, where larger vessels branch into smaller vessels, which ultimately branch into capillaries that are distributed throughout the tissue volume. The vascular network should act as a barrier and selectively control the passage of materials from the vessels to the surrounding tissue. Finally, the network should be easily connected to the vasculature of the patient, or it should be microsurgically connected to it. For the microsurgical approach, vascular structures with a diameter of several hundred micrometres are needed. (Rouwkema and Khademhosseini 2016)

Co-cultures seem to be one possibility for the prevascularization of tissue constructs. Scaffolds can be seeded *in vitro* with ECs or EPCs, which have the capacity to spontaneously self-assemble into capillary-like structures (Tremblay et al. 2005, Shepherd et al. 2006). For more stable results, it is possible to co-seed ECs/EPCs with stabilizing cells, such as MSCs as Koike et al. have suggested (2004). They seeded HUVECs and 10T1/2 mesenchymal precursor cells into fibronectin type I collagen gels and implanted them into mice. The blood-perfused vessels were covered with 10T1/2 mural cells and were stable and functional for over a year.

EC patterning to achieve a vascular tree is an important area of focus in many studies. By using novel fabrication methods of different biomaterials, the organization of vascular cells can be controlled and designed by keeping in mind the fact that all cells in the tissues are organized within 200 μm from a vessel (Rouwkema and Khademhosseini 2016). Several combinations of biomaterials (mainly hydrogels) have been studied to attain complex channel networks, such as gelatin (Golden et al. 2006) and polydimethylsiloxane molds (Zheng et al. 2012). Linville and co-workers (2016) recently showed that channels as narrow as 20 μm can be seeded successfully with ECs, resulting in millimetre-long perfusable capillaries. Vascular organization can also be guided by using chemical signals, such as VEGF, PDGF and Ang-1, after the channels have first been microfabricated in biomaterials (collagen hydrogels) (Rouwkema and Khademhosseini 2016).

3. AIMS

Sufficient angiogenesis is the key factor in bone fracture healing as well as in tissue engineering. The main aim of this study was to develop an optimal co-culture assay for enhanced endothelialisation and osteoblastic differentiation and to characterize the key cellular and molecular factors contributing to the cellular interactions.

The PhD project has three specific aims:

1) **To investigate the interactions between human BM-MSCs and PB-MNCs in endothelial cell differentiation** by co-culturing human bone-marrow-derived MSCs and peripheral blood MNCs in a basal medium and studying endothelial cell differentiation and assembly in the co-culture.

2) **To study the interactions between human MSCs and PB-MNCs in osteoblastic differentiation and bone formation** and to develop an efficient *in vitro* bone formation model.

3) **To characterize the cellular and molecular interaction mechanisms between MSCs and MNCs** by identifying the specific factors leading to enhanced bone formation and EC differentiation in MSC-MNC co-cultures.

4. MATERIALS AND METHODS

4.1. Cell isolation and culture (I–III)

Human MSCs were isolated from 5–6-ml iliac BM aspirates under spinal anaesthesia from eight donors (Table 3). BM aspirates were mixed with α-minimum essential medium (α-MEM; Gibco, Paisley, UK) containing 100 IU/ml of penicillin and 100 µg/ml of streptomycin (PS, Gibco) and 20 IU/ml of heparin (Heparin Leo; LEO Pharma A/S, Ballerup, Denmark). Cells were isolated by density gradient centrifugation (Ficoll Paque Plus, Amersham Pharmacia, UK) and seeded at 1×10^6 cells/cm^2 in 25 cm^2 tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ, USA), then cultured in α-MEM containing 10% foetal bovine serum (FBS, U.S. origin, Invitrogen, cat#16000-044) and PS, hereafter referred to as basal medium. After culturing for 48 hours, non-adherent cells were discarded and the medium was thereafter changed every four days. Upon confluency, cells were harvested using trypsin/EDTA (Gibco), counted and re-plated at 1000 cells/cm^2. Passages 2–6 were used for the experiments.

Table 3. Demographic data of MSC donors used in the study.

Donor	Sex	Age (y)	Status
#1	F	70	hip fracture
#2	F	21	healthy volunteer
#3	F	22	healthy volunteer
#4	F	19	ankle fracture
#5	F	60	ankle fracture
#6	F	72	hip osteoarthritis
#7	M	51	ankle fracture
#8	F	23	tibia fracture

4.2. MSC-MNC co-cultures for EC differentiation (I, III)

To prepare the MSC-MNC co-cultures, MSCs were harvested as described above and plated into 24-well tissue culture plates (2500 cells/cm^2). After 5 days, PB samples (average volume 40 ml) were drawn from healthy donors, and MNCs were isolated by means of Ficoll density gradient centrifugation. The MNCs were added to the MSC cultures at day 5 at the cell density of 5×10^4 MNCs/cm^2, except for the controls including plain MSCs. Three to six parallel samples were cultured from each MSC donor (I, n = 3; III, n = 1) for each time point investigated (I: 5, 10, 15, 20 days; III: 5, 10 and 14 days). Cells were cultured in the basal medium, and half of the medium was changed every 4–5 days.

4.3. MSC-MNC co-cultures for osteoblastic differentiation (II, III)

The experimental setup for study II included four different groups that were all cultured in an osteogenic (OB) medium: 1) MSCs, 2) MSCs + VEGF, 3) MSCs and MNCs, and 4) MSCs and MNCs + VEGF. For study III, the experimental setup included four different groups: 5) MSCs in basal medium, 6) MSCs and MNCs in basal medium, 7) MSCs in OB medium, and 8) MSCs and MNCs in OB medium. Each group consisted of three to four parallel samples. MNCs ($1 \times 10^5/cm^2$) were added to groups 3, 4, 6 and 8.

The OB medium was basal medium supplemented with 10 mM Na-β-glycerophosphate (Merck) and 0.05 mM ascorbic acid-2-phosphate (Sigma-Aldrich). Dexamethasone (10^{-7} M) was included for the first week. Recombinant hVEGF-A (R&D Systems, Minneapolis, USA) was added at 5 ng/ml to groups 2 and 4. Half of the medium was changed every 4–5 days, and fresh VEGF-A was simultaneously added. For some experiments, MSCs and MNCs were first co-cultured in basal medium for 2 weeks before switching to OB medium with VEGF. MSCs from donors #1 and #2 were used here in 4 parallel samples, and the cultures were otherwise performed as described above.

4.4. Real-Time Cell Imaging System IncuCyte (III)

For study III, groups 5–8 from donor #2 were visualised using a real-time cell imaging system (IncuCyte live-cell ESSEN BioScience Inc, Ann Arbor, Michigan, USA) to study cell proliferation, differentiation, migration and tube formation. Cells were cultured in 24- and 48-well plates for 14 days. Images were automatically acquired and registered by the IncuCyte software system. Updates were recorded at 4-h intervals for the duration of the experiment. Sprouting spindle-shaped cells were quantified with the Cell Counter plugin of Image Processing and Analysis Java (ImageJ) after 3, 5, 7, 10 and 14 days of co-culture in 48-well plates.

To study the effect of exogenous VEGF in cell sprouting in MSC-MNC cultures in OB medium, MSC-MNC co-cultures in OB medium were prepared as described earlier, and recombinant hVEGF-A (R&D Systems, Minneapolis, USA) was added at 5 ng/ml to the co-cultures. Half of the medium was changed every 4–5 days, and fresh VEGF-A was simultaneously added. Cells were otherwise co-cultured and visualised with IncuCyte as described.

4.5. Transwell® cell cultures (II)

The Transwell® system with 0.4 μm inserts (Translucent, High Density PET Membrane, BD Falcon) was used to study whether VEGF-supplemented co-

cultures require cell–cell contact between MSCs and MNCs, and whether the osteogenic factors produced by these cells are soluble. MSCs (donor #2) alone or MSC-MNC co-cultures in OB medium supplemented with VEGF were seeded in the lower well as four parallel samples. MNCs were seeded either alone or as co-cultures with MSCs in the upper cell culture inserts. As a control, an MSC-MNC co-culture with an empty insert was used.

4.6. Immunocytochemistry (I, III)

After 5, 10, 15 and 20 days (study I) or 10 and 14 days (study III) of culture, cells were fixed in 2% paraformaldehyde (PFA). Cultures for PECAM-1 and Endoglin were blocked with 3% bovine serum albumin (BSA) for 1 h at room temperature (RT), washed with PBS and incubated for 1 h at RT with rabbit anti-human PECAM-1 (CD31) antibody (1:50) (Santa Cruz Biotechnology, Santa Cruz, California, USA) and mouse anti-human endoglin (CD105) antibody (1:20) (DakoCytomation Inc, Denmark).

Cultures that were stained for anti-human α-SMA were permeabilised with 0.2% Triton X-100 and blocked with 3% BSA for 1 h at RT then incubated for overnight at +4 °C with mouse anti-human α-SMA antibody (1:100) (Abcam, #ab7817).

Cultures for the immunocytochemistry of VEGFR1 were first treated with 3% H_2O_2 in methanol for 10 min. RT, washed three times with dH_2O and once with 0.05% Tween-20 in PBS, then blocked with 3% BSA for 30 min. at RT, washed with PBS and incubated overnight at 4 °C with rabbit anti-human VEGFR1 antibody (1:200) (Abcam, #32152).

Biotin-conjugated secondary antibodies included goat anti-rabbit antibody (Vector Laboratories) and polyclonal rabbit anti-mouse antibody (DakoCytomation) (1:200 for both). Samples with omitted primary antibodies were used as negative controls. Bound antibodies were detected with the Vectastain ABC kit and diaminobenzidine (0.52 mg/ml) (both from Vector Laboratories). The samples were examined and photographed under a light microscope (Leitz Aristoplan). Positively stained areas for α-SMA and VEGFR1 were quantified from the images using an automated image analysis, and the stained areas (cm^2) were converted to a percentage of the total area.

4.7. cDNA synthesis and quantitative real-time RT-PCR

4.7.1. Expression of VEGFR1 and VEGFR2 (I)

For the analysis of the mRNA expression levels of VEGFR1 and VEGFR2, cells from three donors (donors #5, #6 and #8) were cultured in 10 cm^2 culture dishes. Total RNA was isolated at 5, 10, 15 and 20 days of culture using a kit (GenElute™

Mammalian Total RNA, Sigma-Aldrich, St. Louis, USA) according to the manufacturer's instructions. One microgram of total RNA was treated with 5 units of DNase I (Roche, Indianapolis, USA) and subjected to first-strand cDNA synthesis using MuMLV-H(-) reverse transcriptase (Promega, UK; 200 units) and random hexamer primers (Promega, 0,5 μg).

PCR reaction was performed in duplicates. PCR was carried out in a solution containing 100 nmol of primers, 200 nm of 5′ 6-FAM- or VIC-labelled probe, 12.5 μl of TaqMan universal PCR Master Mix (PE Biosystems), and 0.5 μl of template cDNA in a final volume of 25 μl. Thermal cycling was performed with an ABI PRISM 7700 Sequence Detector (PE Biosystems). Cycling was initiated with 2 min. at 50°C and 10 min. at 95°C, followed by 40 15-second cycles at 95°C and 1 min. at 60°C.

The VEGFR1 and VEGFR2 probes were from Universal Probelibrary (Roche). The following primers and probes were used: VEGFR1-left ATG CCA GCA AGT GGG AGTT, VEGFR1-right CAA AAG CCC CTC TTC CAA GT, VEGFR1-probe CTG GGC AA (Probe #61), VEGFR2-left TGA ACT AAA TGT GGG GA TTG ACT, VEGFR2-right CGG TTT ACA AGT TTC TTA TGC TGA, VEGFR2-probe ACT GGG AA (Probe #48), GAPDH-left ACC AGG CGC CCA ATA CGA CCA A, GAPDH-right GTT CGA ACA GTC AGC CGC ATC, and GAPDH-probe GGA ATT TGC CAT GGG TGG A.

4.7.2. Expression of proangiogenic factors, EC and pericyte markers (III)

For the analysis of the mRNA expression levels of proangiogenic factors, as well as inductive factors in pericyte differentiation and markers of osteoblastic, EC and pericyte differentiation, total RNA was isolated at 5 and 10 of culture using a kit (GenElute™ Mammalian Total RNA, Sigma-Aldrich, St. Louis, USA) as described above.

RNA quality check was performed by using the Nanodrop Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) to measure absorbance in 260/280 wave lengths. 200 ng of total RNA was subjected to first-strand cDNA synthesis using MuMLV-H(-) reverse transcriptase and random octamers and oligo dT-16 primers (High-Capacity RNA-to-cDNA™ Kit, Thermo Fisher Scientific, Wilmington, USA).

PCR reaction was performed in four parallel samples. Specific primers and probes were purchased from ITD (Integrated DNA Technologies, Inc., California, USA) (Table 3). The concentrations of the primers in each PCR reaction were 0,5 μM and of the probes 0,25 μM. PCR was carried out in a solution containing a 5′ 6-FAM-labelled probe in 7 μl of Kaba Probe Fast qPCR master mix (Kaba Biosystems, CA, USA), and 200 ng of template cDNA in a final volume of 10 μl. Thermal cycling was performed with Master Mix QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific, Wilmington, USA). Cycling was initiated with 3 min. at 95°C, followed by 40 3-second cycles at 95°C and 30 s at 60°C.

Table 4. qPCR primers and probes in study III.

α-SMA		
	PrimeTime Probe	/56-FAM/AAC AGG AGG /ZEN/ATG GTG GTT TGA TGC T/3IABkFQ/
	PrimeTime Primer 2	GCT GAT AAT GAC AAC TGT ATG TGC
	PrimeTime Primer 1	ATG GTT TTG TCC CGC AGT A
ANG-1		
	PrimeTime Probe	/56-FAM/CCG CAG TCC /ZEN/GTC TAA GAA GCA CG/3IABkFQ/
	PrimeTime Primer 2	TTT GAC CAG AGT TTT TCC ATG TG
	PrimeTime Primer 1	GAA GCA GCA ACG CTA GAA GA
BMP-2		
	PrimeTime Probe	/56-FAM/TCA AGC AGA /ZEN/AGA GAG AGG AGT TGT GTC T/3IABkFQ/
	PrimeTime Primer 2	AGA AGA GCG ACC CTC ACA
	PrimeTime Primer 1	CTT CAT AGC CAG GTA ACG GTT
FGF-2		
	PrimeTime Probe	/56-FAM/CGT GCT GGT /ZEN/CCT CGT GCT GA/3IABkFQ/
	PrimeTime Primer 2	CAT GAA CGC CAA GGT CGT
	PrimeTime Primer 1	CAT CTG TAG CTC AGG CTG AC
SDF-1		
	PrimeTime Probe	/56-FAM/ACA AGC ACT /ZEN/CCC ACT TCA TCT GGA AC/3IABkFQ/
	PrimeTime Primer 2	GAG TAT GAG AGT GAC GAG AAA GC
	PrimeTime Primer 1	GGT CAA GGG TCA GGA GTT C
ALP		
	PrimeTime Probe	/56-FAM/ACC CGC GTG /ZEN/CTA ATG GTG GAA /3IABkFQ/
	PrimeTime Primer 2	CCG ACT ACT ACG CCA AGG A
	PrimeTime Primer 1	GTT CAG GTA CCG CTT CTC G
TGF-β		
	PrimeTime Probe	/56-FAM/TAG ATG GGT /ZEN/CCT CCT TTG GTG CAG /3IABkFQ/
	PrimeTime Primer 2	CTG AAC GTG GTC AAC CTG TT
	PrimeTime Primer 1	GTC CAC CAG GTC TCC GTA
PDGFR		
	PrimeTime Probe	/56-FAM/CCA TCA TTC /ZEN/AAA TCT GTT AAC ACC TCA GTG C/3IABkFQ/
	PrimeTime Primer 2	GGA CTT TAT ACT TGT CGT GTA AGG A
	PrimeTime Primer 1	CCT GCT GTT TTC GAT GTT TCA C
VEGFR1		
	PrimeTime Probe	/56-FAM/TCA TCC AGG /ZEN/AGC TGT CCC TCA GG/3IABkFQ/
	PrimeTime Primer 2	CCA GAG CCT GCA CAT CA
	PrimeTime Primer 1	TGC TCT CCA CGC AGA GG
VWF		
	PrimeTime Probe	/56-FAM/TCC CGG ACA /ZEN/ATC TCA CGC TCA G/3IABkFQ/
	PrimeTime Primer 2	CAC TGA CTA CCT CAT GAA GAT CC
	PrimeTime Primer 1	AAG TCC AGA GCT ACA TAA CAC AG
VEGFA		
	PrimeTime Probe	/56-FAM/TGC TCT ACC /ZEN/TCC ACC ATG CCA AG/3IABkFQ/
	PrimeTime Primer 2	CCA TGA ACT TTC TGC TGT CTT G
	PrimeTime Primer 1	GCG CTG ATA GAC ATC CAT GA
PTN		
	PrimeTime Probe	/56-FAM/TGT GGA GAA /ZEN/TGG CAG TGG AGT GTG /3IABkFQ/
	PrimeTime Primer 2	GAA GCA GGG AAG AAA GAG AAA C
	PrimeTime Primer 1	CTC AGC TCC AGT CCG AGT
PIGF		
	PrimeTime Probe	/56-FAM/TGT CTT GTT /ZEN/TCT CCT TTC ATG TAA TTT TTG TTC TGT /3IABkFQ/
	PrimeTime Primer 2	CCA AAT ACA TCC TCT AAA AGA AGT TCA
	PrimeTime Primer 1	CCA ACT CTA TCA GTG GTG CTC
GAPDH		
	PrimeTime Probe	/56-FAM/AAG GTC GGA /ZEN/GTC AAC GGA TTT GGT C/3IABkFQ/
	PrimeTime Primer 2	ACA TCG CTC AGA CAC CAT G
	PrimeTime Primer 1	TGT AGT TGA GGT CAA TGA AGG G

The accumulation of the specific PCR products was detected in real time as an increase in fluorescence. The observed fluorescence was plotted against cycle number to generate amplification plots and to determine CT values, i.e. the cycle numbers at which the fluorescence signal exceeded a CT value of 0.05 relative fluorescence units. Each determination of a CT value was made in duplicate and normalized with the CT values of simultaneous duplicate GAPDH expression measurements from the same samples. The range between two parallel CT values was <5% of the mean in all of the measurements. The relative expression of the gene analysed (target gene) was estimated using the formula: relative expression = $2-\Delta CT$, where ΔCT = CT(target gene) − CT(GAPDH). The quantity of a specific gene was expressed as a percentage of the quantity of GAPDH mRNA after multiplying the relative target gene expression by a factor of 100.

4.8. Three-dimensional MSC-MNC co-cultures and demonstration of the tartrate-resistant acid phosphatase expression of endothelial-like cells (I)

Type I collagen sponge (Spongostan, Johnson & Johnson, UK) was cut in 5 mm × 5 mm pieces and incubated in basal medium in 24-well plates for 30 min. at 37 °C. Ten thousand passage 6 MSCs from donor #8 in 40 µl medium were added to the centre of the sponge pieces and cultured in basal medium, and, after 3 days, 100 000 MNCs were added, with the exception of the controls. After 15 and 20 days, the 3D co-cultures were fixed with 2% PFA. Co-cultures were stained for tartrate-resistant acid phosphate (TRACP) using a Leukocyte Acid Phosphatase kit (Sigma-Aldrich) to identify MNC-derived cells.

4.9. Analysis of osteoblastic differentiation (II–III)

Osteoblastic differentiation was analysed after 2 weeks by staining the cells for ALP using an Alkaline phosphatase kit (86-R, Sigma-Aldrich) according to the manufacturer´s instructions. Bone nodules were detected by von Kossa staining for deposited calcium after 4 weeks.

The ALP- and von-Kossa-stained areas were quantified using an automated image analysis. In brief, the plates were scanned using a flatbed scanner with a transparency adaptor (HP ScanJet 5370C) at 600 dpi resolution and saved as 24-bit colour images in TIFF format. Transparency exposure adjustments were maintained constant to create images of equal intensity. Positively stained areas were histomorphometrically quantified from the images using an automated image analysis, and the stained areas (cm^2) were converted to a percentage of the total area.

ALP activity was determined from cell lysates from four replicate wells after two weeks (II). The cells were extracted into 100 µl/well of Triton X-100 buffer (50 mM Tris–HCl, 0.1% Triton X-100, 0.9% NaCl, pH 7.6) and frozen and thawed three times. Enzyme activity was determined colorimetrically using *p*-nitrophenylphosphate as a substrate at pH 10. The absorbance at 405 nm was measured with a 96-well plate reader (Wallac 1420 Victor^2, PerkinElmer Life Sciences, Turku, Finland). The total protein content was analysed from the same samples with the protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA, USA) and BSA as a standard.

Calcium concentrations were determined from four replicate wells for study II at four weeks. The cultures were washed three times with Ca^{2+}- and Mg^{2+}-free PBS and incubated in 100 µl/well of 0.6 M HCl overnight at RT. The assay was performed with a Calcium kit (Roche Diagnostics, Boehringer-Mannheim, Germany). Chromogen solution (containing *o*-cresol-phtalein complexone) was mixed with ethanolamine buffer in a ratio of 1:4, and this reagent mixture was then further mixed with cell extracts (250 µl of mixture + 25 µl of extract). The absorbance of each sample was measured at 570 nm as two replicates. Absolute calcium concentration was determined by a standard curve made of 2 mM calcium solution provided by the manufacturer.

4.10. Measures of cell proliferation (II)

Cell proliferation was measured by assessing the metabolic activity of growing cells at 7, 14, 21 and 28 days of culture by using the AlamarBlue® assay (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions in four replicates/group. Furthermore, fluorescence counts were used to quantify the specific ALP activities.

4.11. Statistical procedures (I–III)

In the statistical analyses, paired Student´s t-test was used for pairwise comparisons between different groups. Each culture condition (group) consisted of four replicate samples (wells). Data is presented as mean ± SD, and a p-value ≤0.05 is considered statistically significant.

5. RESULTS

5.1. MSC-MNC co-cultures in basal medium demonstrating endothelial cell differentiation and tube-like structure formation (I, III)

Human MSC and MNC co-cultures were observed by using a light microscope or IncuCyte during a co-culture period of 14–20 days. An MNC-derived cell population was observed to attach over the MSC layer after 2–3 days of co-culture. Cells became spindle-shaped and started to migrate and form tube-like structures after one week of culture. After two weeks, cells in the middle of the cord organized into cobblestone structures, while cells in the periphery of the cord were arranged in a capillary-like pattern (Fig. 4).

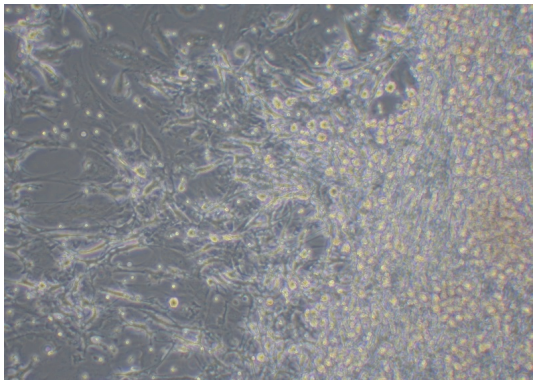

Figure 4. MSC-MNC co-culture in basal medium after 14 days of culture observed using a light microscope (magnification 1:20). Cells in the periphery appeared spindle-shaped and cells in the middle of the cord formed cobblestone-like structures, characteristic of ECs.

Vessel-like formations were observed, and after 20 days of co-culture, 3D structures of approximately 1 cm in length could be seen with the naked eye (Fig. 5).

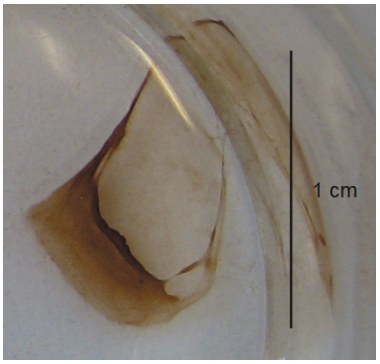

Figure 5. MSC-MNC co-culture was immunostained for PECAM-1 after 20 days of culture, and tubular PECAM-1-positive tubular structures were observed.

5.2. MSC-MNC co-cultures in OB-medium show enhanced osteoblastic differentiation (II, III)

5.2.1. MSCs are the only proliferating cells in co-cultures (II)

Human MSCs and MNCs were co-cultured in either OB or basal medium with or without exogenous VEGF. No differences were observed in cell proliferation (assayed with Alamar blue) between the groups during the first 2 weeks of culture in any of the three MSC donors used in these experiments, suggesting that MSCs were the only proliferating cells in these cultures.

5.2.2. ALP staining, Ca measurement and qPCR for ALP demonstrated enhanced osteogenesis in co-cultures (II, III)

The strongest ALP staining was observed in the MSC-MNC co-cultures supplemented with exogenous VEGF, which was confirmed by quantifying the positively-stained surface areas. No staining was observed when MSCs were cultured in basal medium. The highest Ca concentrations were observed in MSC-MNC co-cultures supplemented with exogenous VEGF.

A strong osteoblastic differentiation of MSCs was observed at mRNA level on days 5 and 10 ($p \leq 0.01$) and at protein level on day 14 ($p \leq 0.001$) (III). The most efficient osteoblastic differentiation was demonstrated in the MSC-MNC co-cultures performed in OB medium.

5.2.3. ALP activities further supported the data of enhanced osteogenesis in co-cultures (II)

ALP activity was significantly higher in MSC-MNC co-cultures supplemented with exogenous VEGF when compared to the control (MSCs in OB medium). Enzymatic ALP activities were not normalized against protein or DNA content as usual, since those parameters are skewed by the unequal cell numbers in the co-cultures vs. plain MSC cultures. Therefore, specific ALP activity was calculated in a single MSC donor (#2) in relation to cell proliferation (Alamar blue fluorescence counts at 2 weeks), confirming that enzymatic ALP activity (as counts at 405 nm) and specific ALP activity showed a similar response.

ALP activities (counts at 405 nm) as % of control were calculated separately for each donor due to the high variation in ALP levels between different donors. ALP activities were significantly highest in the MSC-MNC co-cultures supplemented with exogenous VEGF, verified by the pooled data. No ALP activity was detected when PB-MNCs were cultured alone in OB medium either with or without VEGF.

The highest ALP activity was observed in those MSC-MNC co-cultures where OB medium was added from the beginning of the co-culture period. ALP activities were significantly lower when co-cultured in basal medium for two weeks before

osteoblastic induction. This indicates that, in addition to cell–cell interactions between MSCs and MNCs, the components of OB medium are crucial in mediating the positive effects on differentiation.

5.2.4. Transwell® cell culture experiments demonstrated that physical contact between MSCs and MNCs is crucial in enhanced osteogenesis (II)

Physical contact was shown to be needed between MSCs and MNCs to enhance osteoblastic differentiation in VEGF-supplemented co-cultures. MSCs and MNCs in VEGF-supplemented OB medium separated with the permeable membrane showed significantly lower ALP activities after two weeks when compared to the co-cultures. In addition, factors produced after cell–cell contact appear to be soluble, as MSCs and MNCs co-cultured in the upper compartment of the transwell system enhanced the osteoblastic differentiation of the monocultured MSCs isolated by the membrane in the lower wells.

5.3. Immunocytochemistry demonstrated endothelial cell and pericyte differentiation in the co-cultures (I, III)

5.3.1. Endothelial cell differentiation was demonstrated by the immunocytochemistry of PECAM-1 (I), endoglin (I) and VEGFR1 (III)

The expression of PECAM-1 became stronger with longer culture periods in the MSC-MNC co-cultures in basal medium. Spindle-shaped PECAM-1-positive cells were observed at day 10. Finally, at day 20, PECAM-1-positive cord-like structures were seen with the naked eye (Fig. 5). No PECAM-1-positive cells were discovered in the MSC cultures without MNCs. Endoglin and VEGFR1 stainings showed very similar results (Fig. 6). MSCs cultured without MNCs showed a qualitatively much weaker staining for endoglin compared to co-cultures.

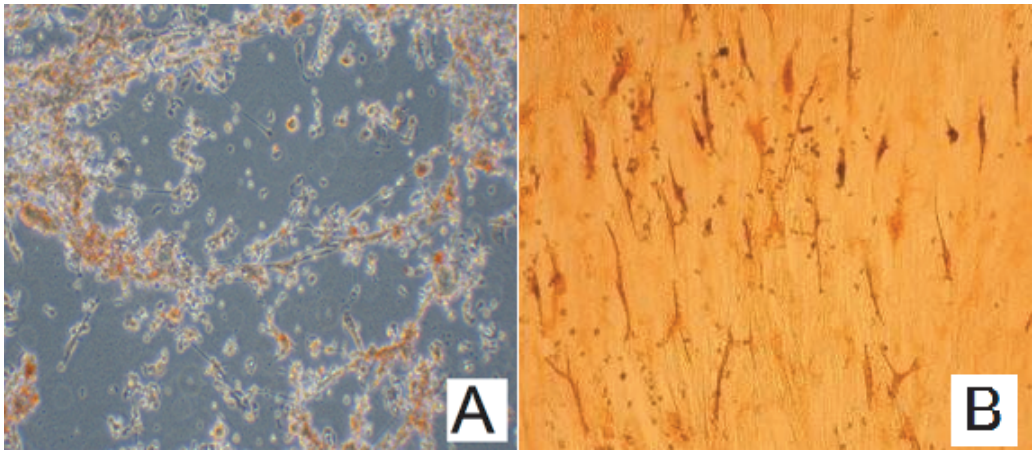

Figure 6. MSC-MNC co-cultures in basal medium were stained for Endoglin (A) and VEGFR1 (B) after 14 days of culture. Magnification 1:20.

Expression of VEGFR1 in MSC-MNC co-cultures was also observed in OB medium in a similar manner as with basal medium. However, no statistically significant differences could be observed in the quantification of VEGFR1-positively stained cells either in basal or OB medium, probably due to background staining of MSCs and differentiated osteoblasts.

5.3.2. Pericyte differentiation was demonstrated by immunocytochemistry of α-SMA

Immunostainings for pericyte marker α-SMA were performed after 14 days of MSC-MNC co-culture in basal and OB medium. The stainings demonstrated a pericyte differentiation in the cultures (Fig. 7), which was further studied as described in section 5.3.

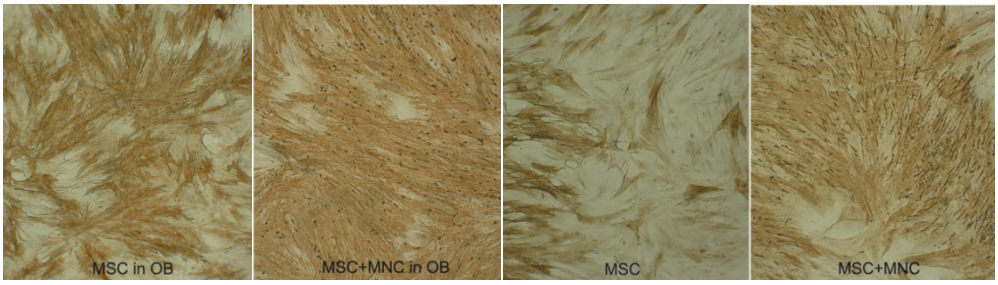

Figure 7. MSC-MNC co-cultures in basal and OB medium were stained for α-SMA after 14 days of culture.

5.4. Quantitative real-time PCR for studying endothelial cell (I, III) and pericyte differentiation (III) as well as the expression of proangiogenic factors (III)

5.4.1. Endothelial cell differentiation was further demonstrated by the expression of VEGFR1 (I, III) and VEGFR2 (I) as well as the von WIllebrand factor (III)

Quantitative RT-PCR was used to measure the expression levels of VEGFR1and VEGFR2 from MSC-MNC co-cultures in basal medium, as well as that of various proangiogenic factors, endothelial cell markers and pericyte inducers and markers from the co-cultures in basal and OB medium

VEGFR1 was up-regulated at 10 days, and the expression levels increased over time in the MSC-MNC co-cultures in basal medium as well as in OB medium, while for vWF the increase did not reach statistical significance in basal medium. However, in OB medium, the expression levels for both VEGFR1 and vWF were significantly higher in the co-cultures than in (plain) MSC cultures ($p \leq 0.01$ for VEGFR1 and $p \leq 0.05$ for vWF). The expression level of VEGFR2 mRNA was less than 0.5% of the control (GAPDH) in both co-cultures and control cultures at all time points.

5.4.2. Expression profiles of proangiogenic factors differ between culture conditions (III)

The mRNA expressions of VEGF, Ang-1, SDF-1, placental-like growth factor (PIGF), PTN, FGF-2 and BMP-2 were studied by means of qPCR after 5 and 10 days of culture from MSC-MNC co-cultures in basal and OB medium. The expression profiles of individual proangiogenic factors seemed to be different between the two culture conditions, although EC differentiation was demonstrated in the co-cultures of both of the studied culture conditions.

Co-cultures in basal vs. OB medium were compared. A significantly higher expression of SDF-1 ($p \leq 0.01$ at day 5 and $p \leq 0.001$ at day 10) and VEGF ($p \leq 0.01$ at day 5) was observed in the basal medium. However, Ang-1 was expressed at significantly higher levels in the osteogenic induction of MSC-MNC co-cultures than in basal medium ($p \leq 0.01$ at day 5 and $p \leq 0.001$ at day 10). PTN expression was significantly higher in basal medium than in OB medium at day 5 ($p \leq 0.05$), while at day 10, the expression level of PTN was higher in the co-cultures in OB medium compared to the co-cultures in basal medium ($p \leq 0.05$). BMP-2 was highly expressed in both co-culture setups, although the expression level was significantly higher in OB medium compared to basal medium at both time points ($p \leq 0.01$ at day 5 and $p \leq 0.001$ at day 10).

5.4.3. Differentiation of pericytes in the MSC-MNC co-cultures in both culture conditions was further demonstrated by qRT-PCR (III)

Pericyte inducer TGF-β was expressed in both co-culture setups at day 5, but not in the plain MSC cultures. Pericyte marker PDGFRβ was expressed in both MSC-MNC co-culture groups but not in plain MSCs, demonstrating a pericyte differentiation of MSCs in the presence of PB-derived MNCs.

5.5. 3D MSC-MNC co-cultures and a demonstration of the TRACP expression of endothelial-like cells (I)

The 3D co-cultures of MSCs and MNCs in a bovine collagen sponge showed that tube-forming cells were able to attach to collagen fibres. After 15 and 20 days, the cultures were stained for TRACP to locate the monocyte-derived cells. TRACP-positive longitudinal cells were detected evenly spread throughout the sponge.

5.6. The effect of exogenous VEGF in cell sprouting capacity (III)

Finally, we evaluated whether exogenous VEGF increases cell sprouting in MSC-MNC co-cultures in osteoblastic conditions. MSCs and MNCs were co-cultured in OB medium with and without exogenous VEGF, monitored with IncuCyte, and the quantities of sprouting cells were quantified. As a result, after 14 days of co-culture, there were more sprouting cells in the co-cultures in OB medium with exogenous VEGF.

6. DISCUSSION

Insufficient angiogenesis is the major reason for failure in bone grafting and bone tissue engineering. Therefore, the development of efficient methods to endothelialise bone tissue grafts is vital. In this study, we demonstrated a spontaneous EC and pericyte differentiation as well as vessel-like structure formation in human MSC-MNC co-cultures. We observed the expression of multiple proangiogenic factors during the co-culture. We also demonstrated EC and pericyte differentiation in the MSC-MNC co-cultures in OB medium, which was reflected by the different expression profiles of proangiogenic factors. In addition, we showed that osteoblastic differentiation and bone formation was enhanced in MSC-MNC co-cultures and was further potentiated by exogenous VEGF. These findings suggest that interactions between MSCs and MNCs could be utilised to induce and enhance angiogenesis and osteogenesis in future bone tissue engineering applications.

6.1. MSC-MNC co-cultures

We established a novel co-culture method of human BM-MSCs and PB-MNCs in which cells were cultured either in basal or in OB medium, and morphological changes were observed by means of light microscopy and the IncuCyte imaging system. Cell sprouting and tube-like structure formation was discovered in the co-cultures in basal medium and only later in OB medium, suggesting that more efficient angiogenesis takes place in basal medium. The EC, pericyte and osteoblastic differentiation were further studied by evaluating the expression of different proangiogenic factors and inducers.

We used BM as an MSC source throughout the study (I–III). BM is still considered to be the most common source of MSCs, even though alternative and easily accessible sources of MSCs have been introduced. Adipose-derived stem cells have been demonstrated to be genetically and morphologically more stable in culture when compared to BM-MSCs (Izadpanah et al. 2006). They also have a lower senescence ratio (Kern et al. 2006) and a higher proliferative capacity, in addition to retaining higher differentiation potential for a longer period in culture (Izadpanah et al. 2006). Adipose-derived MSCs have also been demonstrated to be a more efficient support to haematopoiesis (both *in vitro* and *in vivo*) (Kern et al. 2006) and to have fewer immunophenotypical differences (Strioga et al. 2012). These properties also make adipose-derived MSCs an attractive cell source to be tested for clinical use. They have indeed been used in clinical studies for the treatment of a variety of clinical conditions, including coronary disease, acute

myocardial ischaemia, femoral head osteonecrosis, calvarial defects, breast reconstruction, and facial lipoatrophy (Sousa et al. 2014).

The gradient density centrifugation method is a routine isolation method for BM-MSCs in our laboratory. There is evidence that density centrifugation might not be the most optimal isolation method for BM-MSCs. Mareschi and co-workers (2012) plated the whole bone marrow at a low cell density, resulting in MSCs with a longer telemore length and increased CFU efficiency compared to density-gradient-centrifuged MSCs. However, no significant differences between the Ficoll, Percoll or whole-BM separation methods have been demonstrated in regard to cell morphology, growth rate at the first passage, immunophenotype, or differentiation potential (Torre et al. 2015). However, Mareschi and co-workers further demonstrated that a direct selection of MSCs from BM cells by means of adhesion to culture plastic could be a more advantageous method when compared to MSCs obtained by means of gradient separation. Furthermore, the presence of a non-adherent MSC population with a high differentiation and proliferation potential has been suggested (Di Maggio et al. 2012), and this population might be lost when density centrifugation and cell adherence are used as an isolation method.

We cultured MSCs in α-MEM supplied with 10% foetal bovine serum. Using FBS is problematic when aiming for clinical applications, since it contains xenogenic proteins. Alternatively, MSCs may be cultured using autologous serum or platelet-rich plasma, or under serum-free conditions with growth factor supplementation (Tonti et al. 2009). MSCs expanded in FBS are reported to be less proliferative and subject to extensive gene expression changes compared to MSCs expanded in autologous serum (Shahdadfar et al. 2005). An upregulated expression of genes associated with cell-cycle inhibition and differentiation has been observed in FBS-supplemented MSCs, whereas the transcriptome of MSCs cultured with autologous serum was comparably stable, suggesting that FSB may maintain MSCs in a more primitive state (Shahdadfar et al. 2005). Interestingly, both proliferation rate and osteogenic differentiation appear to be enhanced by platelet-rich plasma from younger (<35 years) versus older (>45 years) donors (Lohmann et al. 2012). However, this result was not attributed to individual or combinatorial levels of growth factors or hormones. There are also significant differences in composition and quality between commercial FBSs. Some products have higher amounts of growth factors than others, influencing cell proliferation and differentiation, for example, and this should be taken into account. In the present experiments, we used FBS of U.S. origin (Invitrogen), which has been demonstrated to be rich in growth factors and is the gold standard serum for MSC and OB cultures in our laboratory.

In the co-cultures, we used MSCs together with allogenic MNCs without typing HLA molecules from the donors. Possibly due to the immunomodulative and suppressive functions of MSCs (Ma et al. 2014), we did not observe any cell death

or macrophage, nor dendritic cell activation in the co-cultures. However, it would be interesting to study the endothelial cell differentiation mechanisms and osteoblastic differentiation in autologous MSC-MNC cultures as well.

6.2. Endothelial cell differentiation

In the MSC-MNC co-cultures cultured in basal medium, the expression of PECAM-1 and endoglin increased over the culture periods (I), indicating EC differentiation. This observation was supported by the morphological changes, when the cells migrated and formed vessel-like structures in the co-cultures. The mRNA expression of VEGFR1 was significantly up-regulated in the co-cultures in both basal and osteoblastic medium when compared to MSC control cultures, while the mRNA expression of VEGFR2 was very low (<0.5%). VEGFR1 on the EC surface is needed for the migration of the monocytes in response to VEGF (Mercado-Pimentel et al. 2007), which probably explains the earlier expression of VEGFR1 in the co-cultures. The expression profile of vWF was similar to that of VEGFR1 in the co-cultures in both basal and OB medium, although the increase was not statistically significant in basal medium.

Some studies have implicated that MSCs could differentiate along an endothelial lineage (Oswald et al. 2004, Wang et al. 2006, Pankajakshan et al. 2013) in the presence of VEGF and 2% FBS (Oswald et al. 2004), or in combination with VEGF and epidermal growth factor (EGF) or FGF-2 (Wang et al. 2006). However, we observed no EC differentiation (I) in the MSC cultures supplemented with exogenous VEGF, suggesting that MNC-derived cells are the source of differentiated ECs in the co-cultures. We further demonstrated that the spindle-shaped tubule forming cells are from a monocyte lineage by staining them for TRACP (I).

6.3. Osteoblastic differentiation

In addition to endothelialisation and angiogenesis, more efficient techniques in the osteoblastic differentiation and bone formation of MSCs are needed for cell-based applications to reach the clinic. In the second part of the study, we aimed to enhance the osteogenesis of MSCs in the co-culture setup and demonstrated that, indeed, in a co-culture of human BM-MSCs and PB-MNCs in osteogenic medium supplemented with VEGF, osteoblastic differentiation was significantly increased (II). VEGF was shown to potentiate the osteoblastic differentiation assessed by ALP expression and Ca concentrations in the MSC-MNC co-cultures, while it had no effect on MSCs cultured alone in osteogenic medium.

CD34+ and CD14+ MNC populations are known for their pre-angiogenic properties (Takahashi et al. 1999), and the use of PB-MNCs in tissue engineered

bone constructs is further supported by the presence of a pre-osteoblastic cell population (Kuroda et al. 2014). However, we observed no ALP activity or mineralization of the plain MNCs cultured with osteogenic supplements, indicating that MSCs, not MNCs, are the main pre-osteoblastic cells contributing to osteogenesis in this co-culture setup. Since we used the whole PB-MNC fraction instead of purified CD14+/CD34+ progenitor cells in the co-cultures, we cannot fully conclude which cell type(s) in the MNC-fraction were responsible for EC differentiation and inducing the stimulatory effect on osteoblastic differentiation (II). Nevertheless, there is data showing that the CD14+ cells would be the central players inducing the osteogenic differentiation of BM-MSCs via a cell-contact dependent production of soluble factors (Guihard et al. 2012, Nicolaidou et al. 2012). This is supported by our Transwell experiments demonstrating that cell contact is necessary for optimal osteogenesis (II).

Kuwana and co-workers (2003) have discovered a CD14+/CD34+ cell population in PB that formed a fibroblastic phenotype on plastic and expressed collagen type I. These cells were able to differentiate towards endothelial lineage, expressing PECAM, endoglin, VEGFR1 and vWF after culture in an endothelial cell basal medium. These cells were suggested to participate in angiogenesis and were termed monocyte-derived multipotential cells. Romagnani et al. (2005) demonstrated that most CD14+ cells present in the BM were found to be CD14/CD34 double-positive cells. These circulating CD14+CD34+ cells, but not CD14+CD34− cells, proliferated in response to different stem cell growth factors and exhibited multipotency, as shown by differentiating into ECs, osteoblasts, adipocytes and neural cells.

Liu and co-workers (2012) have suggested that VEGF stimulates osteoblastic differentiation of MSCs through an intracellular mechanism, which supports our findings. Behr et al. (2011) showed that locally applied VEGF increased the osteogenic healing capacity of human MSCs by promoting osteoblastic and EC differentiation. However, we did not observe any differences between the plain MSCs cultured in OB medium or OB medium supplemented with VEGF, indicating that the mechanism requires the presence of cells from the MNC fraction. MSCs have indeed been described to have a CD14-crossreactive epitope (Pilz et al. 2011), demonstrating a cell–cell contact-dependent interaction mechanism.

We observed that, after co-culturing MSCs and MNCs in basal medium for two weeks, the osteoblastic differentiation was not as efficient as in co-cultures where osteoblastic induction was initiated immediately after adding the MNCs. This result indicates that osteoinductive components and monocyte/macrophage lineage cells, rather than the ECs, are needed for the strong osteoblastic differentiation in the co-cultures (II).

Our Transwell experiments showed that the physical contact between MSCs and MNCs is essential for the strong induction of bone formation in the co-culture settings but that simultaneously produced soluble factors are also needed for osteogenesis. MSCs produce various pro-angiogenic and osteogenic factors, such as FGF-2 and BMP-2, in co-cultures of MSCs and EPCs (Li et al. 2013), indicating that these factors could also be relevant in our experimental setup. The production of these factors has been shown to decrease during the osteoblastic differentiation process (Hoch et al. 2012), which is not in line with our results from the gene expression analysis where we showed that the expression of BMP-2 was highly expressed in the co-cultures in OB medium and the expression levels of FGF-2 were lower in both culture conditions (III). However, exogenous VEGF stimulated the osteoblastic differentiation, possibly also contributing to the expression profiles of pro-angiogenic factors.

6.4. The expression of proangiogenic factors and pericyte differentiation

We further aimed to elucidate the molecular mechanisms by studying the expression profiles of various proangiogenic factors and inducers in co-cultures (III). In the MSC-MNC co-cultures in basal medium, we observed that VEGF and SDF-1 were clearly expressed, whereas in MSC-MNC co-cultures in OB medium, in contrast, the expression of BMP-2 and Ang-1 were clearly the highest. Therefore, we concluded that the observed difference between tube-forming cells in different culture conditions was most likely due to a different pattern of proangiogenic factor expression. VEGF has been shown to promote EC proliferation, migration and the formation of capillary-like structures (Gerhardt et al. 2003), and SDF-1 is another important factor in endothelial tube formation and maturation (Stratman et al. 2010). Both of these factors were expressed in the co-cultures in basal medium, but not in co-cultures in OB medium, probably explaining the lower tube-forming capacity of OB-supplemented co-cultures.

Pericytes that are important cells in stabilizing the tubular structures were also observed in the co-cultures in basal and OB medium. Immunostaining for α-SMA demonstrated that MSC monocultures also expressed this pericyte marker characteristic to mural cells, indicating the heterogeneity of BM-MSCs. Interestingly, pericyte inducer TGF-β and another pericyte marker, PDGFRβ, were strongly expressed in both co-culture setups, but not in the plain MSC cultures. It has been shown that MSCs differentiate into smooth muscle cell/pericyte lineage when co-cultured with EPCs or HUVECs, serving as mural, stabilizing cells in the tubular structures of ECs (Goerke et al. 2012), and thereby supporting our data.

The expression of VEGF was strongly decreased in the co-cultures in OB medium (III). According to the literature, the secretion of VEGF is stimulated by BMPs, FGF-2 and TGF-β (Keramaris et al. 2008). These proangiogenic factors secreted by MSCs and osteoblasts cause upregulation of VEGFRs by ECs, which, in turn, increases ALP expression in osteoblasts (Grellier et al. 2009). Hoch and co-workers (2012) have demonstrated a differentiation-dependent secretion of proangiogenic factors by human MSCs, but when differentiating towards osteoblastic lineage, the secretion of VEGF decreased. Our results demonstrate similar decreased expression of VEGF during osteoblastic differentiation, although angiogenic potential still remained in the osteoblastic co-culture conditions probably due to the expression of other proangiogenic factors such as Ang-1, BMP-2 and PTN.

The expression of both VEGF and SDF-1 was lower in osteoblastic conditions than in basal medium, probably leading to poorer sprouting capacity, as these factors (alongside with pericytes) are known to be important in endothelial tube formation and maturation (Grellier et al. 2009, Stratman et al. 2010). Therefore, we decided to study whether exogenous VEGF increases cell sprouting in MSC-MNC co-cultures cultured in OB medium. After 14 days of co-culture, significantly more sprouting cells were observed in the osteoblastic co-cultures with exogenous VEGF. It can be suggested that the sprouting cells are also needed in enhanced bone formation capacity.

The interaction mechanisms behind EC, osteoblastic and pericyte differentiation in basal and OB medium are summarised below in Figures 8 and 9. When co-cultured in basal medium, MNCs produce TGF- β, PDGF and SDF-1. Due to cell–cell contact, MSCs begin to produce SDF-1, VEGF-A and BMP-2, thereby inducing EC differentiation of MNCs, as well as starting to differentiate into pericytes. Due to the actions of VEGF and SDF-1, ECs start to sprout and form tube-like structures (Fig. 8). Instead, when co-cultured in OB medium, MSCs begin to produce Ang-1, PTN and BMP-2, inducing EC differentiation of MNCs and initiating differentiation into pericytes. Although the sprouting capacity of ECs is poorer in OB medium compared to co-cultures in basal medium. When exogenic VEGF is added to the osteoblastic cultures, ECs starts to sprout and form tube-like structures, possibly further enhancing the osteoblastic differentiation (Fig. 9).

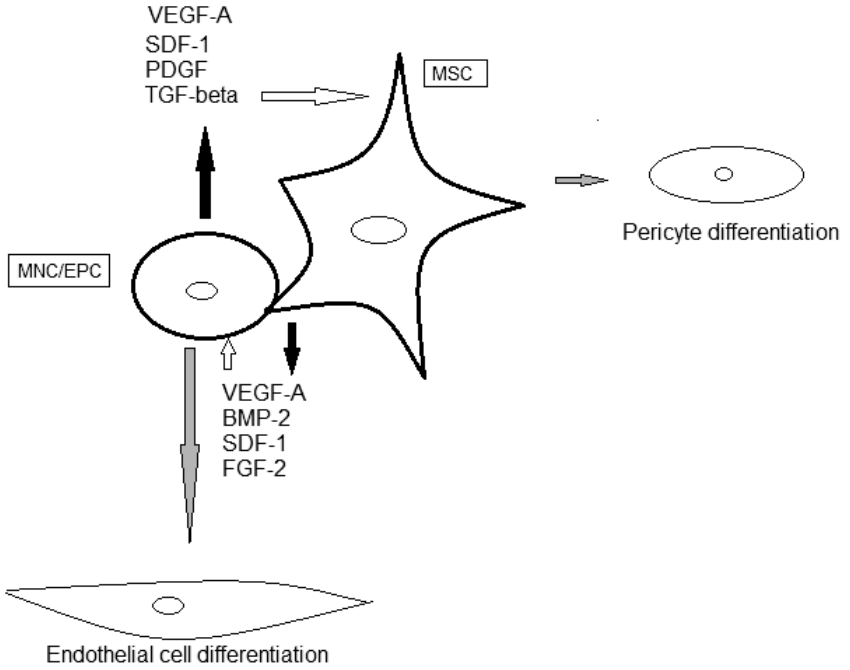

Figure 8. Summary of interactions on MSC and MNC co-cultures in basal medium.

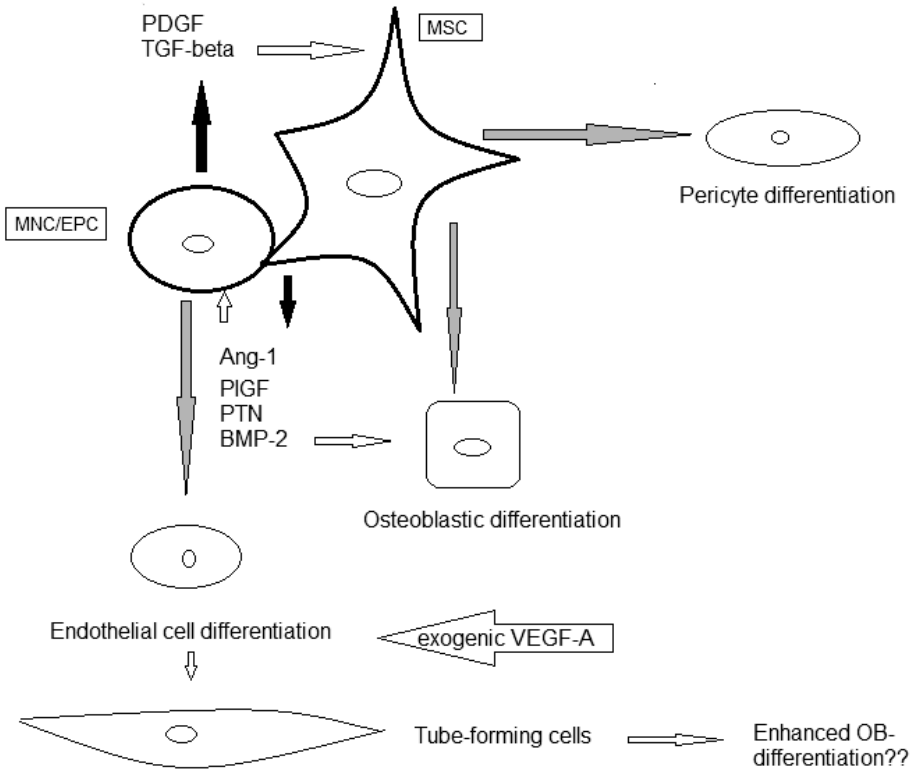

Figure 9. Summary of interactions in MSC and MNC co-cultures in osteoblastic medium.

7. FUTURE ASPECTS

In this study, we demonstrated EC and pericyte differentiation in MSC-MNC co-cultures performed in either basal or OB medium. In addition, we showed that osteoblastic differentiation was enhanced in the co-cultures and was further potentiated with exogenous VEGF. Our results indicate that this co-culture assay could have potential when aiming towards enhanced angiogenesis and osteogenesis in tissue engineering.

In the future, it is important to more closely study the cellular components of MNC fraction used in our cultures. According to the literature, it is likely that the active cell populations in the co-cultures are CD34+ and CD14+ cells. It would be of interest to repeat the experiments with immunoseparated cell fractions. In addition, more detailed data on the interaction mechanisms of MNCs and MSCs is needed. Since we showed that physical contact between the cells is required for the enhanced osteoblastic differentiation in the co-cultures, it would be interesting to inhibit gap junctions and cell–cell adhesion molecules to validate this finding. For soluble factors, the approach would be the inhibition of the key growth factors like TGF-β, Ang-1 and SDF-1 by, e.g., neutralizing antibodies.

We showed that the osteoblastic differentiation of MSCs and the tube-forming capacity of ECs was enhanced when exogenous VEGF was added to the co-cultures in OB medium. It can be hypothesized that the sprouting of ECs potentiates the osteoblastic differentiation. This could be studied by evaluating the expression profiles of proangiogenic and osteogenic factors of tube-forming ECs and then comparing the profiles against inactive ECs to identify the critical factors.

Co-cultures are considered one possibility for prevascularization of tissue-engineered constructs (Rouwkema and Khademhosseini 2016) and, accordingly, the next step would be to test our co-culture model on biomaterials. Scaffolds such as hydrogels or porous hydroxyapatite could be seeded with MNCs and MSCs *in vitro*. EPCs have been shown to possess the capacity to spontaneously self-assemble into capillary-like structures on biomaterials (Tremblay et al. 2005, Shepherd et al. 2006), indicating that our co-culture model could also have similar angiogenic potential when seeded on scaffold materials. In addition, we have MSCs as stabilizing cells that can differentiate into pericytes and thus support capillary-like structure formation. MSCs and MNCs could be seeded into different scaffold materials with optimal pore size, after which the capillary-like structure formation, as well as EC and pericyte differentiation, could be studied. Furthermore, osteoblastic differentiation and vascular organization with exogenous VEGF could be analysed on micro-fabricated biomaterials such as collagen hydrogels. Finally, *in vivo* studies with animal models would be needed to verify the functionality of microvessels and enhanced bone formation and fracture healing capacity.

8. CONCLUSIONS

Improved angiogenesis and bone formation in tissue-engineered implants are necessary for successful clinical applications. PB-MNCs seem an interesting endothelial progenitor cell source for tissue engineering due to their angiogenic properties as well as their availability. MSCs, in turn, are described to produce proangiogenic factors as well as differentiate into mural pericytes, making MSCs and MNCs as an attractive co-culture setup for regenerative medicine.

Based on the results and discussion presented in this thesis, the following conclusions can be drawn:

1. EC differentiation and tube-like structure formation occur in co-cultures of human BM-MSCs and PB-MNCs without exogenously supplied growth factors.

2. Osteoblastic differentiation and culture mineralization are enhanced in co-cultures of human BM-MSCs and PB-MNCs in the presence of routine osteogenic supplements and are further enhanced with exogenous VEGF. The mechanisms leading to enhanced osteoblastic differentiation require cell–cell contact between MSCs and MNCs, and the central factors produced by cell-contact stimuli are soluble.

3. Pericyte differentiation takes place in MSC-MNC co-cultures in both basal and osteoblastic medium. In addition, EC differentiation also occurs in MSC-MNC co-cultures in osteoblastic medium.

4. The mRNA expression levels of multiple proangiogenic factors differ between MSC-MNC co-cultures in basal vs. OB medium. Despite of the differences in the expression profiles of proangiogenic factors, EC and pericyte differentiation takes place in both culture conditions. Nevertheless, the tube-formation capacity is poorer in an osteoblastic environment.

5. When exogenous VEGF is added to MSC-MNC co-cultures in OB medium, the tube-formation as well as bone formation capacity is enhanced, indicating that this co-culture assay could have potential in bone tissue engineering.

6. According to our results, the most optimal co-culture setup for bone tissue engineering would be human MSCs and MNCs co-cultured in OB medium with exogenous VEGF to enhance osteoblastic differentiation and bone formation as well as EC differentiation and tube formation. In addition to osteoblasts and ECs, this co-culture assay includes stabilizing mural pericytes, which are important for angiogenesis.

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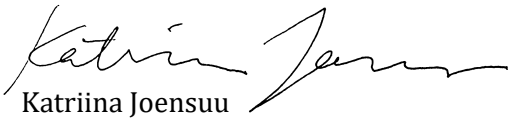

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