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**Interactions of early endothelial progenitor cells (EPC) and
outgrowth endothelial cells (OEC) to improve the vascularization
of bone tissue constructs**

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Abbreviations

Ang-1	Angiopoetin-1
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CFU-f	Colony-forming unit-fibroblasts
CLSM	Confocal laser scanning microscopy
CO ₂	Carbon dioxide
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleotide acide
ECM	Extracellular matrix
EC	Endothelial cells
EGM-2	Endothelial cell growth medium-2
ELISA	Enzyme-linked immunosorbent assay
EPC	Endothelial progenitor cells
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
HA	Hydroxyapatite
HIF	Hypoxia-inducible factor
MCP-1	Monocyte chemotactic protein-1
MMP9	Matrix metallopeptidase 9
MMR	Macrophage mannose receptor
MSC	Mesenchymal stem cells
O ₂	Oxygen
ODM	Osteogenic differentiation medium
OEC	Outgrowth endothelial cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PU	Polyurethane
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RNA	Ribonucleic acid
SM-Actin	Smooth muscle actin
TGF- β	Transforming growth factors- β
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor
2D	Two dimensional
3D	Three dimensional

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1. Introduction

1.1 Tissue engineering

Each year, millions of people suffer from tissue loss or end-stage organ failure by cancer, congenital anomaly, or trauma that could be treated with artificial organs (including tissues) or organ transplantation that are the first choice to reconstruct the devastated tissues or organs [1]. Although those therapies have much advanced, many patients die while waiting for the transplantation of tissues or organs due to the limitations in suitable donor tissues [2, 3]. After transplantation of xenogenic or allogenic tissue patients might have to accept a life-long immunosuppressive therapy with its associated effects such as renal failure, liver toxicity, hypertension, and diabetes mellitus for organ transplantation and also are at risk of infection, acute and chronic rejection [4].

Tissue engineering as a new potential treatment provides solutions to many kinds of disease. In 1993, the term tissue engineering was described by Langer and Vacanti as “an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function”[5]. In 2005, MacArthur and Oreffo defined tissue engineering as the “understanding the principles of tissue growth, and applying this to produce functional replacement tissue for clinical use”[6].

Tissue engineering can be used to enhance tissues and organs functions. In general, there are three main approaches in tissue engineering and regenerative medicine: (i) to use freshly isolated or cultured cells for cellular therapy; (ii) to use biomaterials for tissue regeneration in

situ; and (iii) to use a combination of cells and scaffolds [7] (Figure 1.1) according to the definition of tissue engineering as described above. The major goal of tissue engineering is to replace or to repair damaged tissues or organs and to achieve complete tissue regeneration. In order to create bioartificial tissues, three main components are combined as described in figure 1: cells, matrices, and growth factors [8].

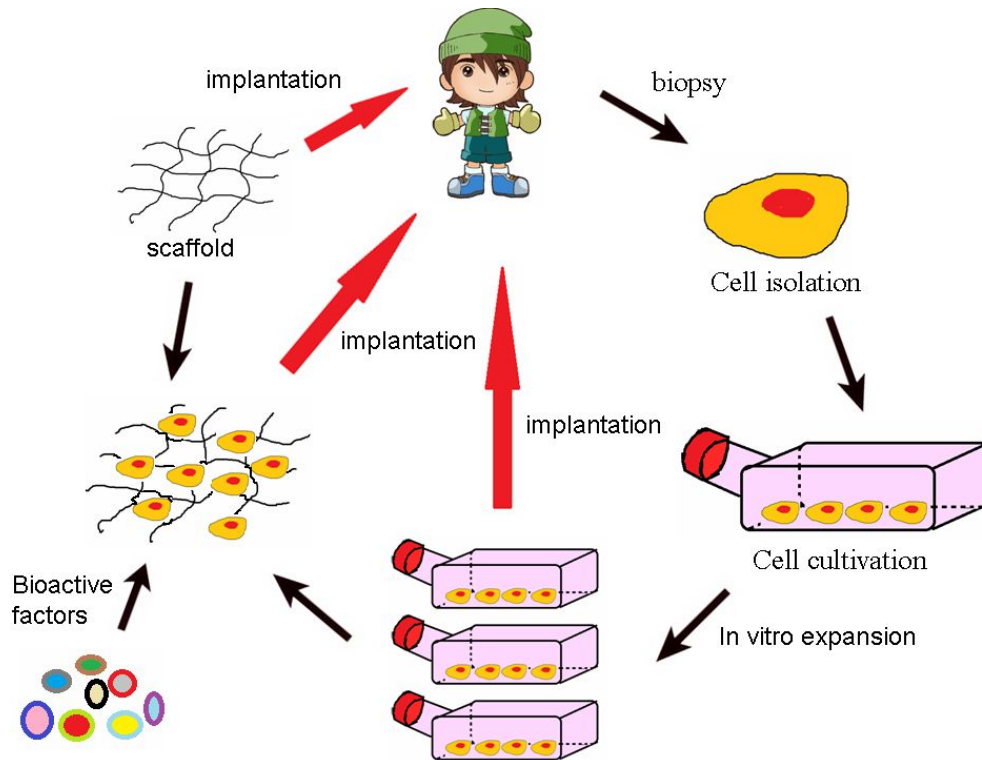


Figure 1.1 General strategies for tissue engineering: harvested cells, bioactive factors, scaffold.

The cells are the elements for functional tissues and thus play a significant role in tissue engineering. There are two main types of cells that can be used for tissue engineering [9]. One option would be to use mature autologous organ-specific cells from the patient itself. Nevertheless, it is a problem that mature cells are difficult to harvest in a sufficient number and in an adequate quality of cells, especially in the case of older patients. For example, it is exceedingly hard to harvest cardiac cells and spinal cord or peripheral nerve cells from patients [1]. The other type of cells which can be harvested from patients, stem cells, might

solve this problem. Adult stem cells such as mesenchymal stem cells for instance are more proliferative and have a higher differentiation potential .

Scaffolds consisting of different types of biomaterials are designed to give a certain shape to the bioengineered tissue and to guarantee the mechanical properties. In addition, they serve as carriers for implanted cells or delivery systems for growth factors involved in tissue regeneration. As such they are also important subjects for tissue engineering and regenerative medicine. Multiple types of scaffolds have been developed for tissue engineering, but the aim of a clinically useful tissue scaffold is still problematic. A good scaffolding material for tissue engineering should be stable, biocompatible and should support the tissue regeneration at site of implantation [11]. The interaction of the material with the tissue and specific cell types is an important criterion to select the biomaterial but also includes a suitable scaffold shape. Scaffolds should be designed to perform some biological functions. Firstly, scaffold should promote cell adhesion and spreading of cultured cells on the artificial extracellular matrix which can be supported by including sequences derived from elastin and fibronectin [12]. In the second place, scaffolds should allow sufficient transport of O₂, CO₂, nutrients, and regulatory factors to support cell proliferation and differentiation [13]. This can be guaranteed by the physio-chemical properties and the scaffolding architecture including factors such as pore size and interconnectivity. Finally, scaffolds would have to keep a balance between biodegradation resulting in products causing no inflammation or toxicity [14, 15] and the rate of tissue regeneration. In the future, the biomaterials should be useful to control cellular signaling processes and cell-matrix interactions involved in tissue repair.

There are so many proteins that play an important role in proliferation and differentiation of cells during tissue repair and which be used in tissue engineering and regenerative medicine. These proteins, such as bone morphogenetic proteins (BMPs), basic fibroblast growth factor (bFGF or FGF-2), vascular endothelial growth factor (VEGF), and transforming growth factors- β (TGF- β), are secreted by cells themselves or as a result of communication with surrounding cells [16]. These proteins are called cell growth factors that start their biological

action by binding to specific receptors on the surface of target cells. Understanding the biological functions and performances of extracellular matrix proteins and growth factors is necessary for the strategic delivery of growth factors. This is due to the fact that extracellular matrix contains many components such as adhesive molecules, and proteins regulating the activity of a number of growth factors [17]. Thus, it is essential to know how to deliver growth factors to the site of action. Three methods have been used for the growth factor delivery [1]: (1) DNA plasmids that include the gene encoding for the desired growth factors. (2) Genetic modified cells secreting growth factors at implantation side. (3) Growth factor protein added to a carrier in the sense of drug delivery. The science and engineering behind growth factor delivery providing a high impact on regenerative medicine will progress in the future.

1.2 Bone tissue engineering

Bone is a dynamic, highly vascularised and innervated tissue, subjected to continuous healing and remodeling without leaving a scar [18]. Fracture healing is the result of bone regeneration through intramembranous and/or endochondral ossification and the healing process results in the new formed bone which is not different from the adjacent healthy bone [19]. However, bone regeneration fails in some cases because there are so many risk factors such as gender, age, diet, diabetes, smoking, alcohol, type of fracture and infection in fracture healing [20]. Considering the growing number of patients who suffer from large bone defects occurring after trauma, infection, tumor resection, it is critical for scientists and doctors to enhance the bone healing process [21].

1.2.1 Scaffolds for bone tissue engineering

Bone defects and injuries represent a common clinical problem in our society. Most therapies

in the clinical practice are based on autografts for bone defects [22]. The current gold standard for bone transplantation are autografts including all the three elements for new bone growth: osteoconductivity, osteogenicity, and osteoinductivity [23]. However, autografts have numerous drawbacks that can induce additional surgical procedures, post-operative pain, blood loss, hematoma formation, nerve injury, infection and cosmetic defects [24]. Considering the complications associated with bone graftings, suitable scaffolds can support the bone repair by guiding the proliferation and differentiation of cells and acting as mechanical stabilizer.

Several scaffolds are available and they may be classified as natural or synthetic biomaterials that are to provide a 3D environment for cells and tissue to grow on. Natural polymers usually have organized structures which induce cellular growth at various stages of development [25]. Biodegradable polyurethane (PU) is a very useful biocompatible material that has been used as a temporary extracellular matrix for bone tissue engineering, but biocompatible polyurethane is lacking of bioactive groups and thus has limitations in osteoinductivity and osteoconductivity [26]. It is possible that polyurethane coated with bioactive ceramic particles such as hydroxyapatite would enhance the bioactivity and improve the mechanical properties of the scaffold for bone tissue engineering [27] (Figure 1.2).



Figure 1.2 Photo of PU-HA scaffold [28].

Hydroxyapatite (HA) is the major inorganic component of natural bone and has been made from calcium phosphate for the use as an orthopaedic and dental material because of its excellent bioactive and biocompatibility properties [29]. HA is an important component of medical relevance in terms of its strong osteoconductive but minimally osteogenic properties [30]. The synthesis of HA powder has been performed over the past years using different techniques, such as precipitation, hydrothermal, and sol-gel methods [31]. The commonly used scaffold architectures reported in the literature for proper cellular seeding and vascularization is a pore size of at least 100 μm [32]. The inclusion of HA into the PU will improve the mechanical properties as well as the cell differentiation and proliferation for bone regeneration. Many advantages for this type of scaffold derive as well from the economical effective and technically simple production procedure which will have an impact on bone tissue engineering in the future.

1.2.2 Bone tissue engineering with mesenchymal stem cells

Bone marrow contains mesenchymal stem cells (MSC) which are a widely used cell type for bone tissue engineering strategies as described in the scientific and clinical literature (Figure 1.3). MSC support hematopoietic stem cells and are able to differentiate into multiple cell lineages, including bone, cartilage, muscle, marrow stroma, tendon, fat, dermis, and connective tissues [33] (Figure 1.4). In 1970, bone marrow stromal cells were reported by Friedenstein and colleagues who demonstrated that stromal cells can be described as colony-forming unit-fibroblasts (CFU-f). Actually, MSC are isolated from low-density mononuclear fraction, and resemble 0.001-0.01%, of the total population in bone marrow. By their adherence to plastic surfaces they can be distinguished from hematopoietic cells [35, 36]. It is considered to be easy and effective to isolate and to expand MSC as well as to differentiate them into multiple lineages under specific culture conditions. They can be

isolated from various kinds of biological sources such as bone marrow, peripheral blood, umbilical cord blood, and placenta [37]. Considering the ability of MSC to form osteoblasts, MSC are very important for enhancing bone regeneration, and thus seem to be of great potential for bone tissue engineering.

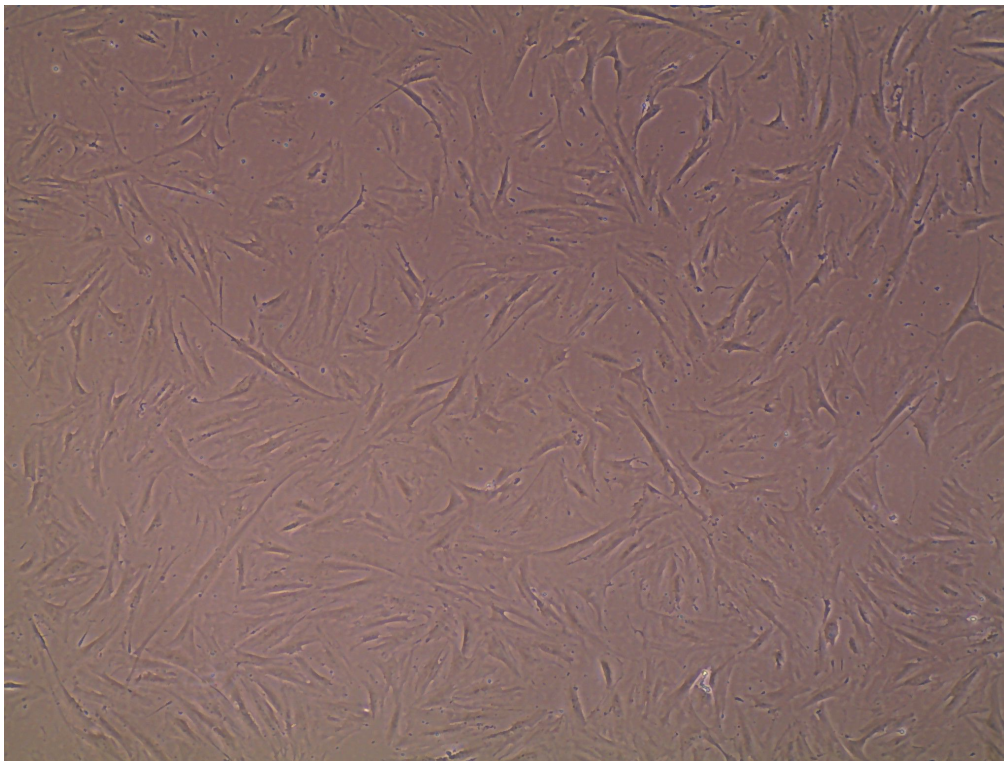


Figure 1.3 Overview of morphology of mesenchymal stem cell.

In bone repair and regenerative medicine, it has been shown that it is possible to use MSC for clinical applications because they are not only to harvest easily and in high cell numbers, but also easily to culture and keep their differentiation potential for bone tissue engineering [38]. Furthermore, MSC can be differentiated very effectively into osteoblastic cells, compared to their lower potential to develop chondrocytes or adipose cells [39]. The differentiation of MSC into osteoblastic phenotype was achieved by osteogenic supplements dexamethasone, beta-glycerolphosphate, and L-ascorbic acid added to the cell culture medium.

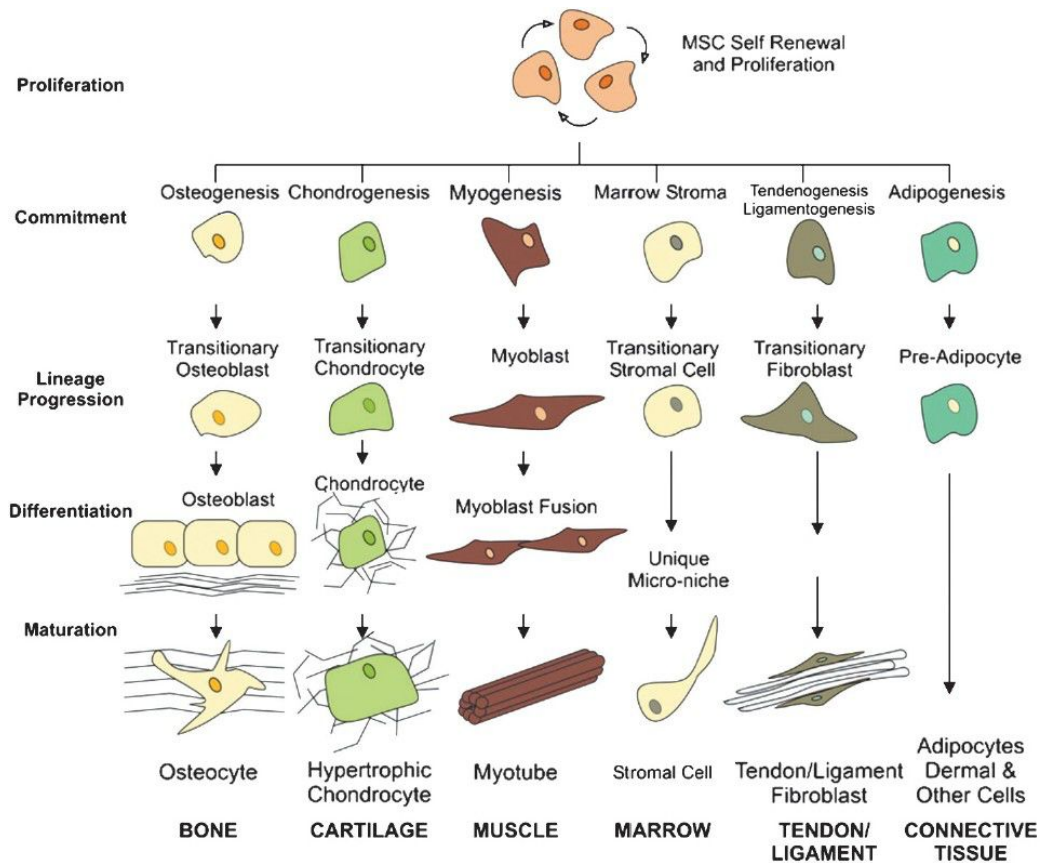


Figure 1.4 Overview of mesenchymal stem cell differentiation [41].

In some clinical case reports, ex vivo expanded autologous MSC were successfully injected into large bone defects of patients to treat constricted fracture healing [42]. In addition, it has been shown that autologous bone marrow mononuclear cells implanted in the necrotic lesion of the femoral head result in the effective treatment of early stages of osteonecrosis [43]. When MSC proliferate and differentiate into osteogenic lineages, different growth factors are released from their neighbor cells during the formation of new bone [44, 45]. Further, MSC are also able to assist the neovascularization process by releasing of proangiogenic factors containing vascular endothelial growth factor (VEGF), angiopoetin-1 (Ang-1) and basic fibroblast growth factor (bFGF) [46]. In summary, MSC are a promising candidate cell type for the generation of new bone and might be also supportive in the formation of new blood vessels.

1.2.3 Bone blood vessel in bone regeneration

The development of a functional vascular system is a prerequisite for successful fracture healing and is therefore a highly important task in bone tissue engineering. Therefore the mechanism of neovascularization have to be understood in order to create new therapeutical tools to support this regeneration process. During embryonic development mesoderm derived precursor cells such as hemangioblasts are stimulated by VEGF and FGF signaling to undergo their first critical steps of differentiation to both hematopoietic and endothelial cells (EC) [47] (figure 1.5). The sufficient production of blood cells is supported by hematopoietic stem cells in bone marrow. In the early embryogenesis, a primary capillary network develops by a process called vasculogenesis in which angioblasts, or endothelial progenitors, differentiate to form primitive blood vessels. VEGF and its the signaling through VEGF-R2/Flk-1, plays an important role in this developmental process [48] (figure 1.5).

In contrast to these processes in the development, it was thought that the blood vessel formation in an adult organism is purely based on the sprouting or splitting of existing capillaries. This process defined as angiogenesis is dependent on the dominant proangiogenic action of VEGF and angiopoietin 2 (figure 1.5). Subsequently, the vessel stabilization is the related to the recruitment of pericytes and smooth muscle cells to support a mature vasculature.

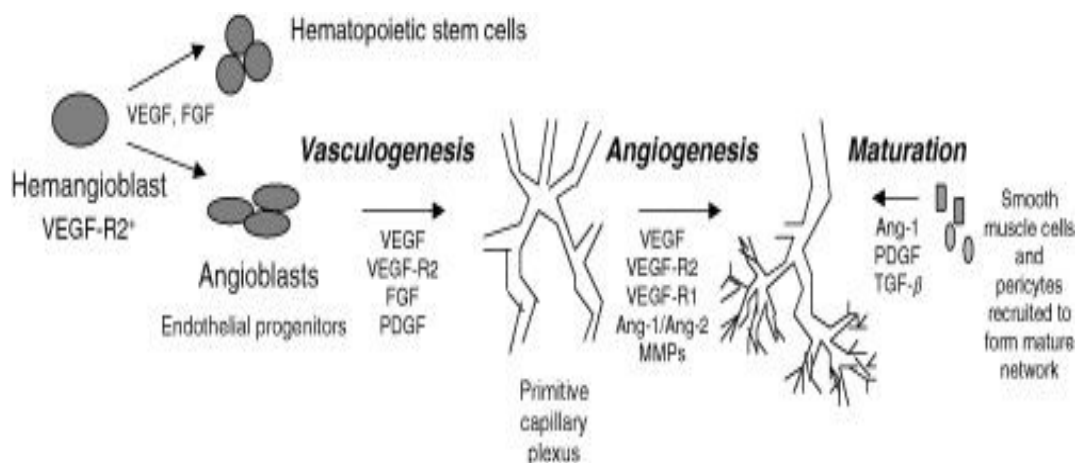


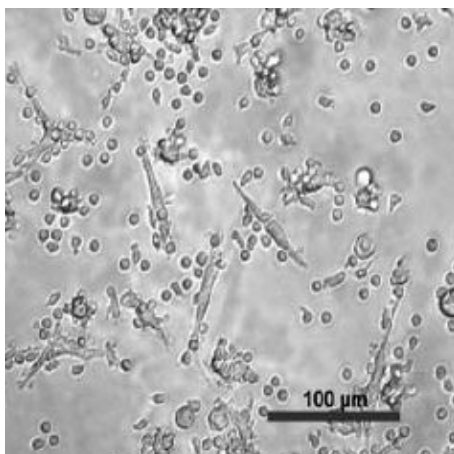
Figure 1.5 Overview of vascular development [49].

Bone is one of a well-vascularized tissues in the body. The periosteum, the surrounding muscles and the outer 1/3-rd of the underlying cortex are supplied by the periosteal circulation and the medullary circulation supplies the inner 2/3-rds of the cortex and bone marrow [50]. The bone vasculature consists of an arteriole, which divides into a complex anastomosed network of capillaries [15]. The vascular network supplies nutrients for mesenchymal cells which differentiate into chondrocytes followed up by endochondrial bone formation in a secondary ossification centre [51]. Thus, the development of vessel network is essential to the homeostasis of bone tissue.

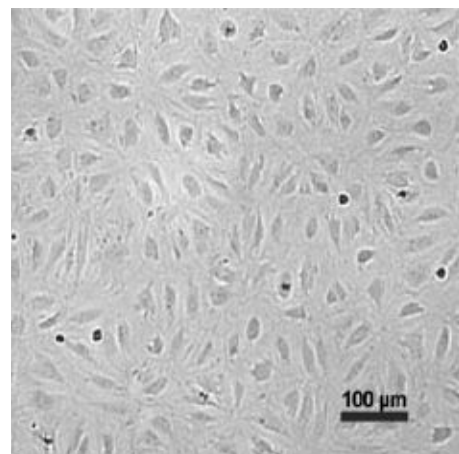
It is a common conception that atrophic non-unions are usually more difficult to treat, as they suffer from poor blood supply at the fracture site [52]. Thus, the concept has been developed that enhancing vascularization in the field of non-union fracture is essential for advancing the orthopedic care. This concept is also supported by new findings regarding the types of mechanisms involved in new blood vessel formation, which identified the contribution of hemangioblasts or endothelial progenitor cells in adult blood vessel formation and repair. In 1997, endothelial progenitor cells were initially identified by Isner and Asahara who demonstrated that progenitor cells were able to initiate neovascularization in vitro and vivo [53]. Since then endothelial progenitor cells are thought to make a significant contribution to vessel growth, both in the embryo and in ischemic, malignant or inflamed tissue in the adult [54]. Adult vasculogenesis occurs through differentiation of local endothelial progenitor cells, or more likely, through circulating endothelial progenitor cells (EPCs) [55] recruited to the sites of blood vessel reorganization. Endothelial progenitor cells are suggested to release angiogenic growth factors that promote blood-vessel development [56]. Nevertheless, until now no consensus on the definition of endothelial progenitor cells and their method of action exists. Therefore, it is important that isolation methods, the identification of EPC, as well as the knowledge about their methods of action further develops in order to apply such cells for the bone vascularization and regeneration.

According to the literature, there are two main populations of cells which can be isolated from the mononuclear cell fraction from the peripheral blood which are defined as early endothelial progenitor cells and colony forming endothelial cells or outgrowth endothelial cell according to their time-dependent appearance [57] (figure 1.6). These cell types develop from mixed populations of mononuclear cells which are cultured in an endothelial cell culture medium with endothelial growth factors on collagen or fibronectin coated cell culture plates. After several days, the adherent cells with spindle shaped morphology express cell surface markers such as CD34, CD31 and CD45 [58]. Those cells which show some endothelial markers but also hematopoietic characteristics such as the expression of CD45 are termed early endothelial progenitor cells. In contrast, late EPC, named also outgrowth endothelial cells (OEC) or endothelial colony forming cells appear as endothelial colonies after 3 weeks in culture. These cells are considered as mature endothelial cells according to their endothelial phenotype and their high proliferative, migratory, and tube forming capabilities [59]. OEC exhibit a typical cobblestone-like morphology [60] and show key characteristics of mature endothelial cells such as the expression of CD31, CD34, and CD146 [58]. Despite differences of EPC and OEC in gene expression, they both have been shown to contribute to neovasculogenesis in vivo. It has been shown that early EPC secrete angiogenic cytokines which trigger angiogenesis, whereas OEC act as mature endothelial cells and are able to provide a sufficient number of endothelial cells [57].

EPC



OEC



EPC	OEC
Spindle shaped morphology	Cobblestone-like morphology
After 7 days in culture	After 2-3 weeks in culture
CD31+, vWF (+)	CD31+, vWF (+++)
CD45+, CD14+, CD146-	CD45-, CD14-, CD146+
Low proliferative potential	High proliferative potential
No tube formation	Tube formation
Secretion of angiogenic factors	High vasculogenic potential

Figure 1.6 Overview of morphology of endothelial progenitor cells and outgrowth endothelial cells [56, 57, 59-62].

Signaling processes by angiogenic growth factors make a significant contribution to new blood vessel formation in the bone. In this context also VEGF plays a critical role in angiogenesis. Studies by Asahara have demonstrated that vascular endothelial growth factors (VEGF) is not only essential for increasing endothelial cell differentiation and proliferation, but also enhances tube formation and the mobilization as well as the recruitment of endothelial progenitor cells [63]. VEGF is highly increased in the early phases post-fracture and has been shown to promote the bone formation during distraction osteogenesis in fracture healing [64]. This important role for VEGF signaling in bone repair is supported for instance by studies using VEGF receptor-1 and VEGF receptor-2 blocking antibodies [65]. Hypoxia-inducible factor (HIF) pathway plays a central role in activating angiogenesis in areas of low oxygen tension, including the avascular portion of the cartilaginous callus [66]. As such VEGF gene expression is enhanced directly by the transcription factor HIF. Lastly, it is well-accepted that signaling molecules play a role in vascularization at the fracture site.

1.3 Myeloid cells and macrophage-like cells in vascularization

Hematopoietic stem cells are multipotential cells residing in the bone marrow which give rise to all blood cell types including myeloid (monocyte, macrophage, neutrophil, eosinophil, basophil, megakaryocyte, platelets, and red blood cells) and lymphoid (B cells, T cells, and natural killer cells) cell lineages [67] (figure 1.7).

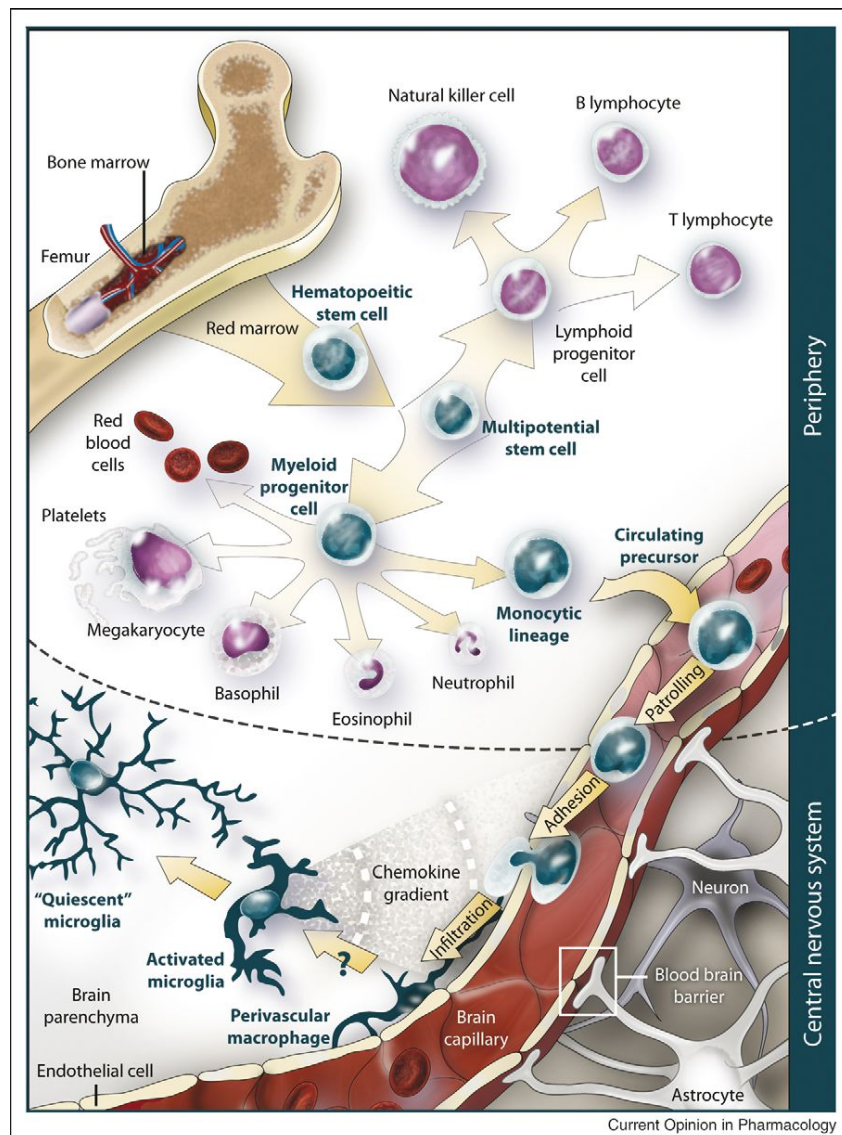


Figure 1.7 Hematopoietic stem cells give rise to myeloid progenitor cell lineages and lymphoid progenitor cell lineages [68].

Cells of monocytic lineages are related to circulating blood progenitors with partly macrophage-like characteristics which are involved in the repair and neovascularization in a series of different tissues [69]. Myeloid angiogenic cells and endothelial progenitor cells share a series of common characteristics in terms of the secretion of proangiogenic molecules and the active contribution to blood vessel reorganization. For instance myeloid cell derived interleukin-8 (IL-8) is able to transactivate vascular endothelial growth factor receptor-2 (VEGFR2) independently of extracellular VEGF [70]. Myeloid cells are known to improve angiogenesis in experimental set up [71]. Macrophages belong to the myeloid cell lineages that arise from monocytic progenitor cells that differentiate into tissue macrophages. Macrophages have a range of different phenotypes depending on the environmental stimuli [72]. Activated macrophages include classical activated M1 phenotypes, associated with active microbial killing and proinflammatory characteristics, and alternative activated M2 phenotypes, associated with tissue remodeling and proangiogenic function [71] (figure 1.8). M2 are supposed to play a key role in angiogenesis by releasing a number of potent proangiogenic growth factors such as VEGF and bFGF [73]. Accordingly, myeloid cells and macrophage cells have major therapeutic value in the clinical setting.

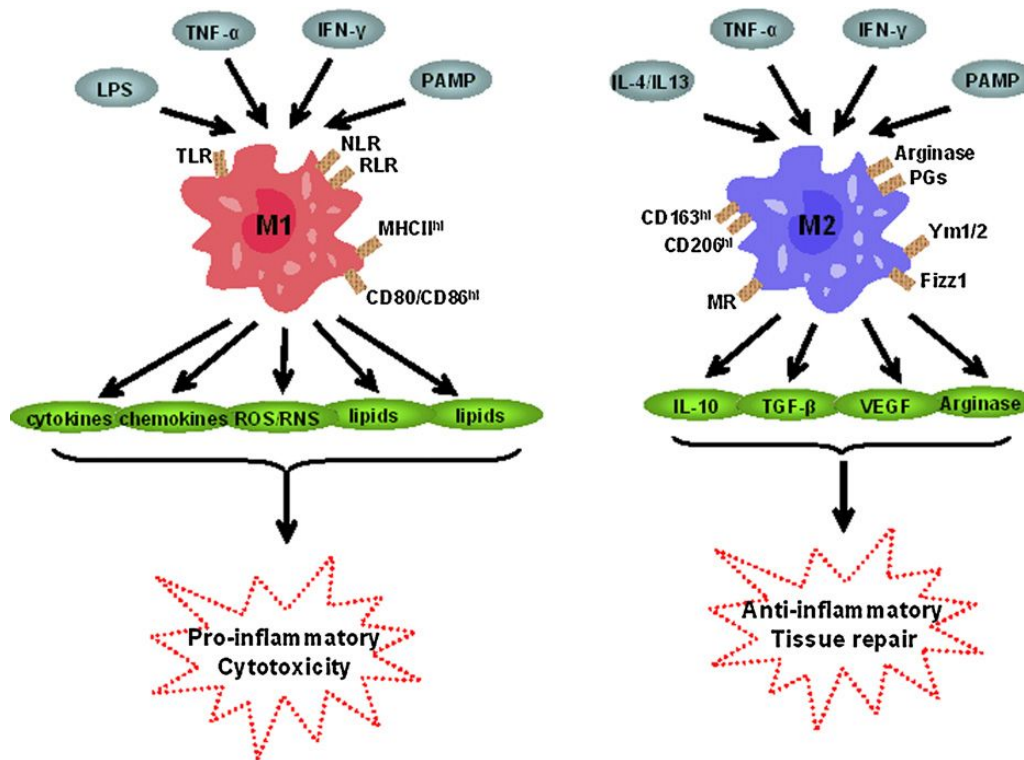


Figure 1.8 Macrophages with distinct phenotypes [74].

1.4 Co-culture system in bone tissue engineering

Bone regenerative medicine in general as well as the still unsolved problem of the neovascularization in larger bone defects remains a significant clinical challenge. Co-culture models were used for analysis of cell-to-cell communication through three mechanisms: (i) direct membrane-membrane interactions between two adjacent cells, (ii) adjacent cells communicating using gap junctions form direct connectivity between two cytoplasm, and (iii) factors released from extracellular matrix that activate receptors on the target cells [75]. In this context co-culture approaches have been established due to the findings by several groups aiming at the prevascularization of tissue engineered constructs. The vasculature is essential for oxygen delivery, nutrients exchange, and soluble factors transport to support new bone formation in the body [76]. In co-culture models, VEGF secreted by bone-forming cells containing mesenchymal stem cells, osteoprogenitors, and osteoblasts plays a major role in

osteo-endothelial communication [77, 78]. On the other hand, VEGF also can support the recruitment of progenitor cells and lead to their differentiation into osteoblasts through activation of receptors [79]. In addition, it is well-accepted that osteoblasts seem to be a considerable cellular source of angiopoietin-1 (Ang-1) [80] and that VEGF and Ang-1 promote angiogenic response and vascular stabilization [81]. The cellular crosstalk phenomena in co-culture models guiding angiogenesis and osteogenesis during bone repair show a high degree of complexity [82], but this knowledge is essential for the translation of bone tissue engineering. In addition to use these models as tools to study bone repair processes co-culture approaches or co-implantations strategies seem to be a therapeutical option to enhance bone and vascularization.

1.5 Aim of the study

The development in science and in new technologies, has resulted in clear improvements in the field of vascularized bone tissue, but large bone defects/injuries still represent a major challenge in the field of bone tissue engineering and regenerative medicine. An understanding of the interaction of osteogenesis and angiogenesis will enhance the process of bone development and repair. In vivo, EPC have proved their therapeutic potential ability to form vascular networks that will allow suitable vascularization of engineered organs and tissues [83]. Endothelial progenitor cells isolated from human peripheral blood seem to be a potential autologous cell source for neovascularization in bone tissue engineering. The present study is focused on the role of endothelial progenitor cells in the formation of angiogenic structures in different co-culture models. Using this co-culture model to mimick the bone regeneration process the effect of EPC on angiogenic structures was evaluated. According to present knowledge endothelial progenitor cells might act myeloid cells, Therefore endothelial progenitor cells were added to co-cultures of MSC and OEC and their properties to support new blood vessel formation were investigated in vitro. The co-localization of myeloid cells and angiogenic structures are analyzed by CLSM, and the formation of angiogenic structures

were analysed quantitatively in the vitro model system. Analysis of the molecular mechanisms in the angiogenic process and angiogenic activation including vascular growth factors and factors involved in vessel stabilization was performed to understand the interaction of different cell types. This also included the evaluation of myeloid marker to assess a potential contribution of myeloid cells contained within the EPC cultures.

2. Materials and Methods

2.1 Materials

2.1.1 Instruments

Instrument	Model	Manufacturer
Centrifuge	Biofuge primo R	Thermo Scientific, Langenselbold, Germany
Centrifuge	Multifuge 3 S-R	Thermo Scientific, Langenselbold, Germany
Cryo-microtome	HM550	Thermo scientific, Dreieich, Germany
Analytical balance	BP211 D	Sartorius, Göttingen, Germany
Fluorescence microscope	Axioskop 40	Zeiss, Germany
Fluorescence microplate reader	Spectra fluor plus	Tecan, Crailsheim, Germany
Fridge (4 °C)	Uks 3602 index 12/001	Liebherr, Austria
Freezer (-20 °C)	Premium no frost	Liebherr, Austria
Freezer (-80 °C)	Hera freeze	Heraeus, Hanau, Germany
FUSION imaging system	Fusion SL lourmat	vilber PEQLAB, Erlangen, Germany
Incubator	Hera cell 240	Heraeus, Hanau, Germany
Laminar flow	Hera safe	Heraeus, Hanau, Germany
Light microscope	Axiovert 25	Zeiss, Göttingen, Germany
Liquid nitrogen tank	Locator 4 plus	Thermo Scientific, Dreieich, Germany

Micro plate reader	Apollo LB 911 TECAN	Berthold Technologies, Austria
Nano Drop	Nano Drop 2000	Peqlab, Erlangen, Germany
Orbital Incubator	SI50	Stuart, ST15 OSA, UK
pH meter	HI 991001	HANNA, Kehl am Rhein, Germany
Pipette	10 µl, 100 µl, 1000µl	Eppendorf, Hamburg, Germany
Pipetus	100-240 volt	Hirschmann Laborgeräte, Eberstadt Germany
Real Time PCR cyclers	iCycler iQ	Bio-Rad, Hercules, CA, USA
Vacuum pump	Vacu boy	Integra Biosciences, Fernwald, Germany
Vortex	Vortex-Genie2™	Scientific Industries, New York, USA

2.1.2 Consumables

Consumable	Manufacturer
Cell strainer (40 µm, 100 µm)	BD Falcon, Franklin Lakes, NJ, USA
Centrifuge tubes (15 ml, 50 ml)	Sarstedt, Nümbrecht, Germany
Cover glasses (18x18 mm)	Menzel-Gläser, Braunschweig, Germany
Cryovials (2 ml, 5 ml)	Greiner, Frickenhausen, Germany
ELISA Microplate (96 well)	Nunc, Langenselbold, Germany
Lab-Tek Chamber slide (2 well, 8 well)	Nunc, Langenselbold, Germany
Laboratory bottle (100 ml, 250 ml, 500 ml, 1 l)	Schott, Mainz, Germany
Microscope slides	Menzel-Gläser, Braunschweig, Germany
Micro tubes (1,5 ml, 2 ml)	Sarstedt, Nümbrecht, Germany
Parafilm	Pechiney Plastic Packaging, Menasha, USA
Pasteur pipettes	Hecht-Assistant, Sondheim, Germany
Pipette tips (10 µl, 100 µl, 1000 µl)	Greiner, Frickenhausen, Germany
Pipette tips (1000 µl)	Sarstedt, Nümbrecht, Germany
Pipette filter tips (10 µl, 100 µl, 1000 µl)	Sarstedt, Nümbrecht, Germany

Ritip (1,25 ml, 2,5 ml, 5 ml, 12,5 ml)	Th. Geyer, Hamburg, Germany
Save seal micro tubes (0,5 ml)	Sarstedt, Nümbrecht, Germany
Serological pipettes (5 ml, 10 ml, 25 ml)	Sarstedt, Nümbrecht, Germany
Tissue culture flasks (25 cm ² , 75 cm ²)	Sarstedt, Nümbrecht, Germany
Tissue culture plate, 24-well	TPP, Trasadingen, Switzerland
Tissue culture plate, 96-well	Sarstedt, Nümbrecht, Germany

2.1.3 Chemicals

Name	Manufacturer
β -Mercaptoethanol	Sigma-Aldrich, Steinheim, Germany
β -glycerol phosphate	Sigma, St. Louis, MO, USA
Ascorbate-2-phosphate	Sigma, St. Louis, MO, USA
Biocoll Separating Solution	Biochrome, Berlin, Germany
BSA (albumin from bovine serum)	Sigma, Steinheim, Germany
Collagen Type I Rat Tail (3,66 mg/ml)	BD Biosciences, Bedford, MA
DAPI (4',6-diamidino-2-phenylindole)	Molecular Probes, Eugene, Oregon, USA
Dexamethasone	Sigma, St. Louis, MO, USA
DMEM, high Glucose (4,5 g/l)	PAA Laboratories, Pasching, Austria
DMEM/Ham's F-12 (1:1), with stabile glutamine	Biochrom, Berlin, Germany
DMSO (dimethyl sulfoxide)	Sigma-Aldrich, Steinheim, Germany
EGM-2 BulletKit® (endothelial growth medium 2)	Lonza Walkersville, MD, USA
Elite mouse IgG	Vectastain, Germany
Fluoromount	Sigma, St. Louis, MO, USA
FBS (fetal bovine serum)	PAA Laboratories, Pasching, Austria
Fibronectin (human plasma fibronectin purified protein) (1 mg/ml)	Millipore, Temecula, CA, USA
Freezing medium	Leica, Germany

Impact DAB	Vectastain, Germany
Paraformaldehyde (4 %)	USB, Cleveland, USA
Penicillin/Streptomycin (100x) (10.000 U/ml/10 mg/ml)	PAA Laboratories, Pasching, Austria
PBS Dulbeco's (1x) (phosphate-buffered saline)	PAA Laboratories, Pasching, Austria
PBS Dulbeco's (10x) (phosphate-buffered saline)	PAA Laboratories, Pasching, Austria
QuantiTect™ SYBR® Green PCR Master Mix	Qiagen, Hilden, Germany
QuantiTect™ SYBR® Green primer assay	Qiagen, Hilden, Germany
RNase free water	Qiagen, Hilden, Germany
Substrate Reagent Pack	R&D Systems, Wiesbaden, Germany
Triton™ X-100	Sigma, Taufkirchen, Germany
Trypsin/EDTA (10x)	PAA Laboratories, Pasching, Austria
Tween® 20	Roth, Germany

2.1.4 Antibodies

Primary antibody	Source	Dilution	Manufacturer
anti-human CD31	mouse	1:50	Dako, Glostrup, Denmark
anti-human CD11b	rabbit	1:50	Epitomics, Burlingame, CA, USA
Secondary antibody	Source	Dilution	Manufacturer
Alexa Fluor 488 anti mouse	goat	1:1000	Invitrogen-Molecular Probes, Eugene, Oregon, USA
Alexa Fluor 555 anti rabbit	donkey	1:1000	Invitrogen-Molecular Probes, Eugene, Oregon, USA

2.1.5 Primers

Gene name	Primer assay name	Catalogue number
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vWF	Hs_VWF_1_SG QuantiTect Primer Assay	Quiagen/QT00051975
CD146	Hs_MCAM_1_SG QuantiTect Primer Assay	Quiagen/QT00079842
sm-Actin	Hs_ACTA_1_SG QuantiTect Primer Assay	Quiagen/QT00088102
CD11b	Hs_ITGAM_1_SG QuantiTect Primer Assay	Quiagen/QT00031500
IL-8	Hs_IL8_1_SG QuantiTect Primer Assay	Quiagen/QT00000322
CD68	Hs_CD68_1_SG QuantiTect Primer Assay	Quiagen/QT00037184
CD163	Hs_CD163_1_SG QuantiTect Primer Assay	Quiagen/QT00074641
Integrin α 5	Hs_ITGA5_1_SG QuantiTect Primer Assay	Quiagen/QT00080871
GAPDH	Hs_GAPDH_1_SG QuantiTect Primer Assay	Quiagen/QT00079247

Gene name	Sequence	Annealing temperature
	For	5'-
	CCGGATCTATGACTCAGGGACCAT-3'	
CD 31	rev 5'-GGATGGCCTCTTTCTTGTCCAG-3'	55°C
	(Eurofins)	
	for 5'-AAATGTGACAAGCCAAGGCGG-3'	
	rev	
VEGF	5'-AAAGTGCTCCTCGAAGAGTCTCC-3'	55°C
	(Eurofins)	

2.1.6 Kits

Name	Manufacturer
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DuoSet® human CXCL8/IL-8	R&D Systems, Wiesbaden, Germany
DuoSet® human VEGF	R&D Systems, Wiesbaden, Germany
Quant-iT Pico Green dsDNA Assay Kit	Invitrogen, Germany
RNeasy Micro kit	Qiagen, Hilden, Germany
RNeasy Kit	Qiagen, Hilden, Germany
RNA-to cDNA Kit	Applied Biosystems, Carlsbad, USA

2.2 Isolation and expansion of human endothelial progenitor cells (EPC) and outgrowth endothelial cells (OEC)

Endothelial progenitor cells and outgrowth endothelial cells were isolated and cultured according to protocols as described previously . The mononuclear cells were isolated from peripheral blood buffy coats, 25ml peripheral blood was diluted 1:2 with 25ml phosphate buffered saline in 50ml tube and then 35ml of this mixture that was carefully added to 15 ml of Bicolll were centrifugated for 35 minutes at 400 x g without break, by Biocoll gradient centrifugation (figure 2.1). The mononuclear cells were cultured in endothelial cell growth medium-2 (EGM-2) with full supplements from the kit (10 ml FBS, 0.2 ml Hydrocortisone, 2 ml hFGF, 0.5 ml VEGF, 0.5 ml IFG-1, 0.5 ml ascorbic acid, 0.5 ml hEGF, 0.5 ml Heparin), 5% FBS, and 1% penicillin/streptomycin, on collagen coated well plates. 5×10^6 mononuclear cells/well were seeded on 24 culture-well plates and then fed with fresh medium every second day. After 1 week of culture, adherent cells were collected by trypsinization and used as EPC or reseeded in a density of 0.5×10^6 cells/well to collect OEC. Then colonies of OEC with cobble-stone-like morphology appearing after 2-3 weeks in culture were trypsinized and expanded over several passages as described before.

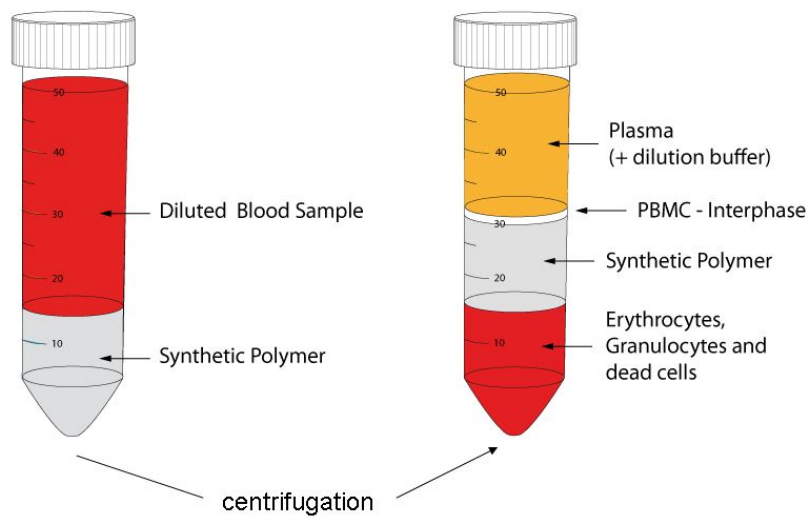


Figure 2.1 Isolation of mononuclear cells from peripheral blood buffy coat. According <http://pluriselect.com/buffy-coat.html>

2.3 Isolation and expansion of mesenchymal stem cells (MSC)

Human MSC isolated from bone marrow of human cancellous bone fragments of adult donors were cultured and expanded as previously described [87]. Bone fragments were washed several times with phosphate buffered saline to collect the loosely associated cell fractions. The washing solution containing bone marrow residues was filtered by cell strainer and centrifuged for 10 minutes at 400 x g, and the cell pellet was resuspended in DMEM/Ham F-12 supplemented with 20% FBS and 1% Penicillin/Streptomycin. Cells were seeded at a density of 2×10^6 cells/cm² on collagen-coated flasks (T75). MSC-like colonies appearing after 7-10 days were expanded using a splitting ratio of 1:3 and were cultivated in DMEM-Ham F12 supplemented with 20% FBS and 1% Penicillin/Streptomycin. Then MSC were cultivated in osteogenic differentiation medium (ODM) in passage two for 2 weeks to induce

osteogenic differentiation before starting experiments. ODM consists of DMEM-Ham F-12 supplemented with 0.1 μ M dexamethasone, 10 μ M β -glycerol phosphate, 50 mM ascorbate-2-phosphate, 10%FBS, and 1% Penicillin/Streptomycin.

2.4 Coculture and triple culture of MSC, OEC and EPC

To study the influence of EPC-derived myeloid cells on angiogenesis we used co-cultures based on 1) MSC and EPC, 2) MSC and OEC and 3) triple cultures of MSC and OEC to which we added 5% and 10% EPC per total cells. In a first step MSC were seeded onto the fibronectin coated 24-culture well plates or on fibronectin-coated Thermanox coverslips at a density of 200.000 cells/well and cultivated in ODM. After 24 h, 200.000 OEC were added for co-cultures of MSC/OEC, and 200.000 OEC plus 5% or 10% of EPC for triple cultures. In addition 10% EPC per well were added to MSC for the MSC/EPC set up. Co-culture and triple-culture were maintained in EGM-2 for 4 or 14 days.

2.5 Immunofluorescence staining

After 4 or 14 days co-cultures and triple-cultures seeded on Thermanox coverslips as described above were prepared for immunofluorescence staining. After fixation with 4% paraformaldehyde, cells were washed three times with PBS and permeabilized using 0.1% Triton[®]X-100. Cells were incubated with the following primary antibodies diluted in 1% bovine serum albumin (BSA) in PBS for 1 hour at room temperature: CD31, CD11b. After washing 3 times with PBS, cells were incubated with the corresponding fluorescently labeled secondary antibodies diluted 1:1000 in 1% BSA in PBS for 1 hour at room temperature. For nuclear counterstaining, cells were treated with 4',6-diamidino-2-phenylindole (DAPI). Samples were then observed using Zeiss LSM 510 Meta confocal laser scanning microscope.

2.6 Image quantification and colocalization studies

Microscopic images of immunofluorescence-stained co-cultures and triple-cultures at different time points were analysed using ImageJ 1.43 was preformed by MetaPhysiol, Dr. Harald Schmidt [88] as introduced previously [89]. In brief, the tube-like structures were extracted from the immunofluorescent images stained for CD31 [90] and analyzed for the area, length, branching points and endpoints. According to these results, three regions of interest were established, the total area of tube-like structures and circular regions ($r = 50$ pixel) around the branching- and endpoints. Finally, the immunofluorescent images stained for CD11b were analyzed for co-localization of positive cells by determination of the integrated density of CD11b fluorescence at the regions of interest. The relative intensity (relative intensity = integrated density per area) for each region was calculated and the relative intensity of the whole image was used as a reference. The results of image analysis experiments are given as means \pm s.d., and significant differences between means were calculated with MS-Excel. According to the results of a variance ratio analysis (F-test, $p < 0.05$) an unpaired t-test for either homoscedastic or heteroscedastic variances was performed (* $p < 0.05$ and ** $p < 0.01$).

2.7 Gene expression analysis

2.7.1 RNA isolation

RNA isolation was performed using RNeasy Micro kit. The medium was taken away from the 24-well-plates, the cells from different experiments were lysed in 500 μ l lysis buffer

containing 1 % -Mercaptoethanol in RLT-buffer to stabilize the lysates. After an incubation period of 5 minutes, the cell lysates were directly taken into a QIA shredder mini spin column and centrifugated at 13.000 rpm for 3 minutes. The column was thrown away, 500 µl of 70% ethanol was added to the cell lysates and mixed till the fluid was clear. Then, maximum 500 µl of the solution was added to the RNeasy Mini away and centrifugated at 10.000 rpm for 30 seconds. After the flow through, the passage was thrown away and this step needed to be repeated again. To wash the filter, 350 µl RW1 buffer was put into the column and incubated for 10 minutes. After this, the column was centrifugated at 10.000 rpm for 30 seconds. Then, 78 µl DNase mix (70 µl RDD buffer and 10 µl DNase for one sample) was given into the column and incubated for at least 30 minutes at room temperature to destroy DNA. After this incubation time, 350 µl RW1 buffer was added to the column which was centrifugated at 10.000 rpm for 30 seconds. 350 µl of RW1 buffer was given into the column and incubated for 5 minutes at room temperature for washing the filter, then the column was centrifugated at 10.000 rpm for 30 seconds. The column was transferred into a new 2 ml tube and filtered with 500 µl RPE buffer, before this column was centrifugated at 13.000 rpm for 30 seconds. This step was repeated again. Therefore, 30 µl of nuclease free water was added to the column and incubated for 10 minutes. Then the column was centrifugated at 13.000 rpm for 3 min. The concentration of RNA can be measured by a Nano Drop 2000c spectrophotometer. 2 µl of the RNA solution was added onto the lower optical surface for measuring. NanoDrop software was opened to measure the concentration of RNA using a microvolume spectrophotometer.

2.7.2 Reverse transcription

For the transcription of RNA into DNA, 1000 ng RNA was taken into a solution containing 10 µl RT buffer and 1µl RT Enzyme Mix after the was denatured at 70 ° C for 10 minutes. The solution was filled up to 20 µl with RNase free water and mixed as well as centrifugated. The samples were incubated in a water bath at 37 ° C for 1 hour. Finally, the solution was incubated at 95 ° C for 5 minutes and filled up to 100 µl with RNase free water.

2.7.3 RT-PCR

To assess the expression of endothelial and myeloid markers, as well as the expression of angiogenic factors, semi-quantitative real-time polymerase chain reaction (RT-PCR) was performed for CD146, von Willebrand factor (vWF), CD31, sm-actin, CD11b, IL-8, VEGF, and Glycerin-Aldehyde-3-phosphate (GAPDH) as internal control. For detailed information of used primers please refer to table 1. For gene expression analysis, total RNA was extracted from different culture set ups using RNeasy Kit according to the manufacturer's protocol. A total amount of 1µg RNA was transcribed into cDNA using High capacity RNA-to cDNA Kit. Quantitative real-time PCR was performed using iCycler iQ Real-Time Detection System. 12.5 µL of QuantiTect™ SYBR® Green PCR Master Mix, 2.5 µL of QuantiTect™ SYBR® Green primer assay, 6 µL of RNase free water, and 4 µL of cDNA were used for one reaction. For amplification, the following thermocycler program was performed. 1 cycle: 2 min at 50 °C; 1 cycle: 8 min at 95 °C; 40 cycles: 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C; 80 cycles: 30 s at 55 °C; 1 cycle: hold at 20 °C. Glycerin-Aldehyde-3-phosphate (GAPDH) was used as internal control and relative gene expression was determined using the $\Delta\Delta C_t$ method. Gene expression was compared setting control cultures to 1 (reference value) as indicated in the relevant figures.

2.8 Quantification of different growth factors using enzyme-linked immunosorbent assay (ELISA)

The concentration of different growth factors in cell culture supernatants from different culture set ups of MSC, OEC and EPC in EGM-2 collected after 4 and 14 days were measured using ELISA DuoSet® for IL-8 and VEGF. ELISA was performed according to the manufacturer's instructions. Results for each sample were normalized to the DNA content of

each sample to compensate for the different cell numbers in the set up

2.8.1 VEGF (ELISA)

Cell culture supernatants were centrifuged and stored at $\leq -20^{\circ}\text{C}$. VEGF standard was prepared with 2000 pg/mL of calibrator diluent RD5K (figure 2.2). 500 μL of calibrator diluent RD5K was added into each tube and mixed each tube thoroughly before the next transfer. 50 μL of Assay Diluent RD1W was added to each well. Then 200 μL of standard, control, or sample were added to each well. The plate was covered with the adhesive strip and incubated for 2 hours at room temperature. Each well were washed three times with wash buffer (400 μL) per well. 200 μL of VEGF conjugate was added to each well and incubated for 2 hours at room temperature. Each well were washed three times with wash buffer (400 μL) per well. 200 μL of substrate solution was added to each well and protected it from light as well as incubated for 20 minutes at room temperature. 50 μL of stop solution was added to each well. The optical density of each well was determined within 30 minutes. A streptavidin-HRP (horseradish-peroxidase) colorimetric reaction was used to determine protein concentrations. The optical density of each well was measured using an automatic plate reader capable of measuring absorbance at 450 nm. After background subtraction concentrations were determined according to a standard calibration curve.

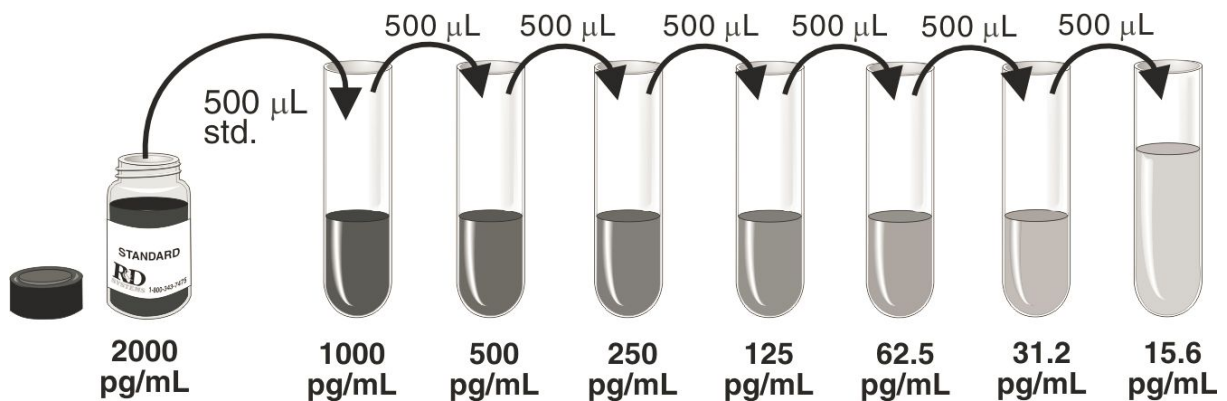


Figure 2.2 Prepare VEGF standard. According R&D systems.

2.8.2 IL-8 (ELISA)

The 96-well microplate were coated with 100 μL per well of the diluted Capture Antibody and incubated overnight at room temperature. Each well were washed three times with wash buffer (400 μL) per well. The plate was blocked by adding 300 μL of Block Buffer to each well and incubated at room temperature for a minimum of 1 hour. Each well were washed three times with wash buffer (400 μL) per well. 100 μL of standard or sample was added to each well and covered with an adhesive strip as well as incubate for 2 hours at room temperature. Each well were washed three times with wash buffer (400 μL) per well. 100 μL of the detection antibody diluted in reagent diluent was added to each well and incubated 2 hours at room temperature. Each well were washed three times with wash buffer (400 μL) per well. 100 μL of the working dilution of Streptavidin-HRP was added to each well and incubated for 20 minutes at room temperature as well as avoided placing the plate in direct light. Each well were washed three times with wash buffer (400 μL) per well. 100 μL of substrate solution was added to each well and incubated for 20 minutes at room temperature as well as avoided placing the plate in direct light. 50 μL of stop solution was added to each well and mixed them. The optical density of each well was measured using an automatic plate reader capable of measuring absorbance at 450 nm. After background subtraction concentrations were determined according to a standard calibration curve.

2.8.3 Quantitative DNA

The DNA content of co-cultures and triple cultures was determined by Quant-iT Pico Green dsDNA Assay Kit according to the manufacturer's protocol. Trypsinize the cells with 250 μL Trysin/EDTA per each well and take the cells into Eppi as well as centrifuge at 14000 rmp for 5 min. Then destroy the cells in an ultrasound bath to release the DNA into the supernant. Make a range standard:

Stock-std: 100 µg/mL

Working solution: 2µg/mL (20µL stock solution + 980 µL TE buffer)

Table 1. Protocol for preparing a stand curve.

standard	Working solution	TE-buffer
1ng/mL	1 µL	1999 µL
10 ng/mL	5 µL	995 µL
100 ng/mL	50 µL	950 µL
1000 ng/mL	500µL	500 µL

Then add 100 µL TE buffer into each well. Picogreen solution is diluted 1:200 and 72 µL is added to each well. For the quantification, 28 µL of the sample and of the standards are added to wells in a 96 well plate and incubated for 10 minutes in the dark at room temperature.

The DNA content of co-cultures and triple cultures was analysed with a fluorescence microplate reader at an excitation/emission wavelength of 485/535 nm. In co-cultures and triple cultures ELISA data were normalized to the DNA content of each sample to compensate for the different cell numbers in the set up.

2.9 Protein arrays for angiogenesis- and hematopoiesis-related proteins

In a first step MSC were seeded onto the fibronectin coated 6-culture well plates at a density of 2.14×10^5 cells/cm² for MSC monocultures and 1.07×10^5 cells/cm² for MSC/OEC co-cultures and triple-cultures (OEC/MS/10% EPC) and cultivated in ODM. After 24 h, OEC in a density of 1.07×10^5 cells/cm² were added for MSC/OEC co-cultures and for triple-cultures (OEC/MS/10% EPC). 10% per total cells of EPC were added to triple

cultures. After 4 days, cells were lysed in buffers according to the recommendations for the protein arrays. In addition, proteins were harvested from the corresponding cell culture supernatants after centrifugation. The total protein concentration in individual samples was determined by the Bradford assay and measured at 562 nm using an automatic plate reader. Protein-extracts derived from 3 different donors were subsequently pooled group wise at equal ratios to assess an average protein profile for each sample group. Proteome Profiler™ Human Angiogenesis Array and Proteome Profiler™ Human Hematopoietic Array were performed according to the manufacturer's protocol. Proteins were detected by chemiluminescence and analyzed quantitatively using FUSION imaging system equipped with fusion software.

2.9.1 Angiogenesis – related proteins

Cell culture supernatants were centrifuged and stored at $\leq -20^{\circ}$ C. Cells were washed with PBS and added lysis buffer. The lysates were rocked gently at 2-8 °C for 30 minutes. Quantitation of sample protein concentrations using a total protein assay was recommended. 2.0 mL of array buffer 7 as a block buffer was pipetted into each well of the 4-well multi-dish. Each membrane was placed in a well of the 4-well multi-dish. 4-well multi-dish was on a rocking platform shaker for one hour. Each sample was added to 0.5 mL of array buffer 4 and adjusted to a final volume of 1.5 mL with array buffer 5 as necessary. 15 μ L of reconstituted detection antibody cocktail was added to each sample and incubated at room temperature for one hour. Array buffer 7 was aspirated from the wells of the 4-Well Multi-dish and samples were added to the wells as well as incubated overnight at 2-8 °C on a rocking platform shaker. Membranes were washed with wash buffer three times. Then 2.0 mL of diluted

Streptavidin-HRP was pipetted into each well and incubated for 30 minutes at room temperature on a rocking platform shaker. Membranes were washed with wash buffer three times. 1 mL of Chemi Reagent Mix was pipetted onto each membrane and waited for testing.

2.9.2 Hematopoiesis – related proteins

Hematopoietic panel was divided into two parts (H and C). 2.0 mL of 1x array buffer 8/1 as a block buffer was pipetted into each well of the 4-well multi-dish. Each membrane was placed in a well of the 4-well multi-dish and was on a rocking platform shaker for one hour. 0.3 mL of Array buffer 8 was added to each sample and adjusted to a final volume of 3 mL with array buffer 1 as necessary. 1x array buffer 8/1 was aspirated from the wells of the 4-well multi-dish and 1.5 mL of the sample was added to each well as well as incubated overnight at 2-8 °C on a rocking platform shaker. Membranes were washed with wash buffer three times. For part C and part H array, 30 µL of reconstituted detection antibody cocktail C and H was diluted to 1.5 mL with 1X Array Buffer 8/1. 1.5 mL of the dilution was pipetted into each well of the 4-well multi-dish and incubated for 2 hours at room temperature on a rocking platform shaker. Membranes were washed with wash buffer three times. 1 mL of chemi reagent mix were pipetted onto each membrane and waited for testing.

2.10 Prevascularization of polyurethane-hydroxyapatite (PU-HA) scaffolds

Before cell seeding scaffolds were placed in 96-well plates and coated with 10 µg/mL fibronectin in PBS for 1 h at 37 °C. PU-HA were seeded with 1) 350.000 MSC and 350.000 OEC or 2) 350.000 MSC and 350.000 OEC plus 5% of total cells EPC (35.000) 3) 350.000 MSC and 350.000 OEC plus 10% of total cells (70.000) EPC and 4) 700.000 MSC and 10%

(70.000) EPC per scaffold. After seeding MSC in a first step samples were placed in the incubator for 4 h before transferring them to a new 24-well plate with 1.5 mL of fresh ODM medium. After 24 h, OEC and EPC were seeded into the MSC-seeded PU-HA scaffolds and the scaffolds were incubated at 37 °C for 4 and 14 days. Then samples were prepared a) for CLSM as described previously or b) For cryosections of scaffolds were performed according to standard procedures after fixing in ice cold methanol. After embedding in freezing medium scaffolds were sectioned on a cyro-microtome into sections of 9 or 12 µm in thickness. Sections were postfixed with 4 % paraformaldehyde and stained by immunofluorescence as described in earlier sections (section 2.5) or by immunohistochemistry using CD31 as primary antibody followed by the detection using the elite kit and DAB for the colorimetric reaction. Nuclear counterstaining was performed by hemalaun.

2.11 Statistical analyses

Data are presented as mean values \pm standard deviation of the mean. Statistical significance was assessed using the unpaired students t-test (p-value *p<0.05 and **p<0.01). This test was chosen to compare different set ups of co-cultures and triple-cultures. Statistical analysis was performed with Excel. Statistical significance was demonstrated when the p-values were less than 0.05.

3. Results

3.1 Colocalization of endogenous CD11b-positive cells in triple-cultures system

Immunofluorescent labeling of CD11b-positive cells was performed in triple-cultures of MSC with OEC and EPC after 14 days by CLSM (figure 3.1). In addition, vascular structures have been visualized by staining for the endothelial marker CD31 (depicted in green) and indicated the co-localization of CD11b-positive cells (depicted in red) and vascular structures. The CD11b-positive cells were located near to the vascular structures as indicated by CLSM. Additional results from the group and corresponding image analysis (not part of the present thesis) also conformed this morphological observation in statistical evaluations.

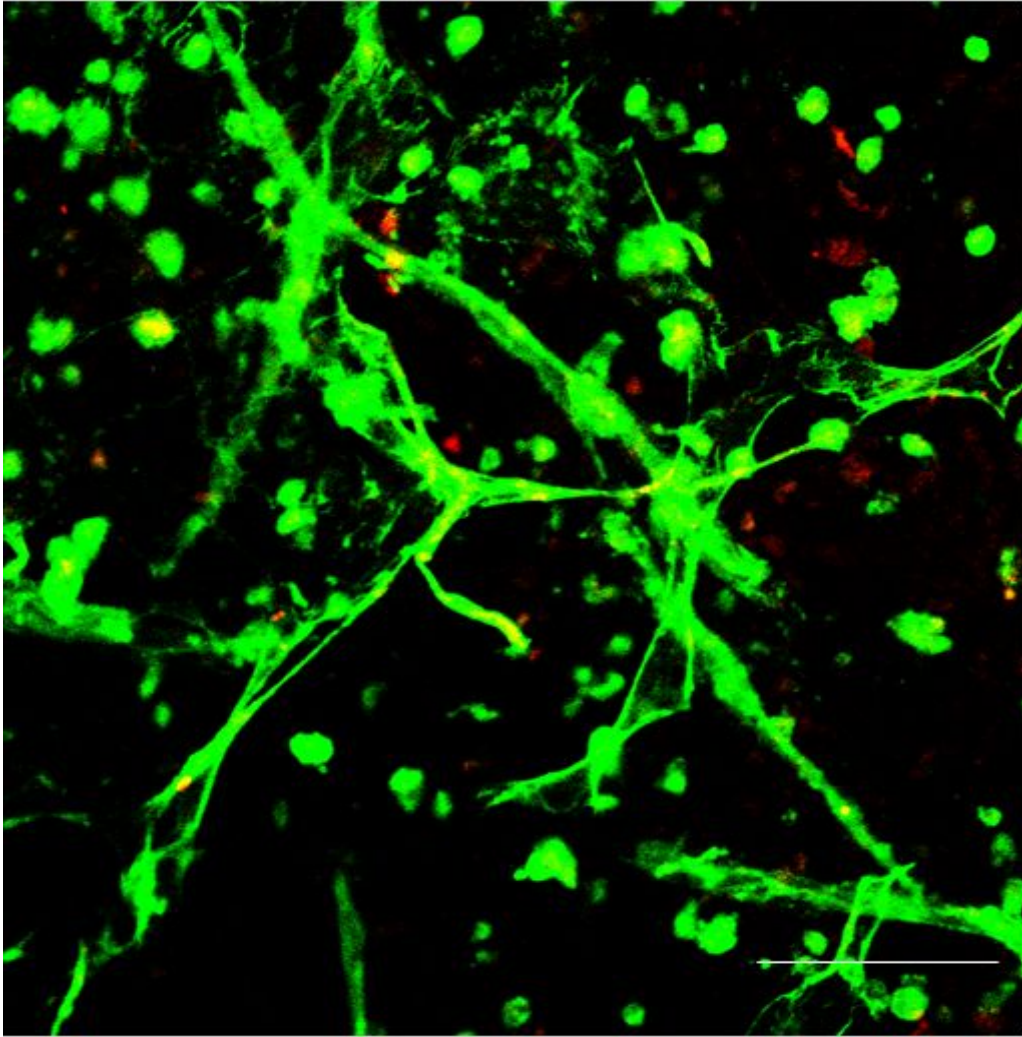


Figure 3.1 Co-localization of myeloid cells with angiogenic structures in triple-cultures (MSC+OEC+5%EPC) after 14 days. The triple-cultures are stained for CD31 (green) and CD11b (red), bar=100 μ m.

3.2 Effects of myeloid cells on angiogenic structures determined by confocal microscopy and quantitative picture analysis

To further define the role of the CD11b-positive or myeloid cells during angiogenesis and to see whether EPC act as a source of CD11b-positive or myeloid cells, we actively added early EPC to the co-culture system and evaluated their effects on the formation of vascular

structures.

The formation of angiogenic structures and the associated myeloid cells after 4 (figure 3.2 A, B) and 14 days (figure 3.2 C, D) were investigated by immunofluorescent staining for endothelial markers such as CD31 (A, C; depicted in green). In addition, CD11b (depicted in red, figure 3.2 A-D) was used as a marker for myeloid cells. During the time course of the co-cultures OEC formed angiogenic structures characterized by elongated endothelial morphology. The addition of early EPC to the co-cultures of MSC and OEC seemed to increase the number of vascular structures present in the co-cultures, whereas in additional controls of MSC/EPC co-cultures no vascular structures were detected at the investigated time points. Myeloid cells were characterized by a more rounded morphology and CD11b expression depicted in red. In addition, rounded CD11b-positive cells partly expressed also the endothelial marker CD31. CD11b-positive myeloid cells were closely associated with the angiogenic structures, indicating an active role in the formation of vascular structures to be further defined. A potential integration of myeloid cells into vascular structures might occur but could not be proven at the present stage of investigation.

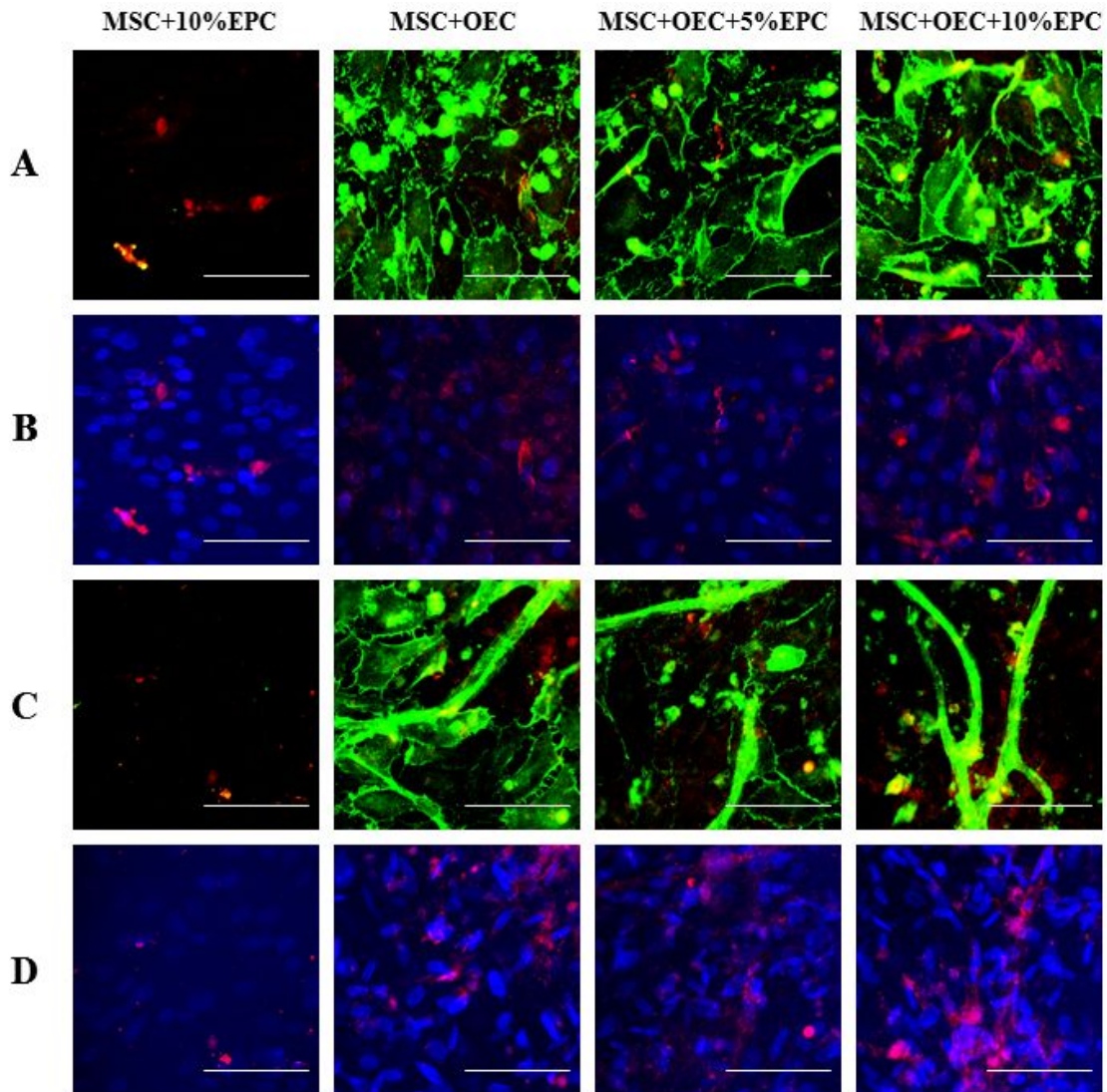
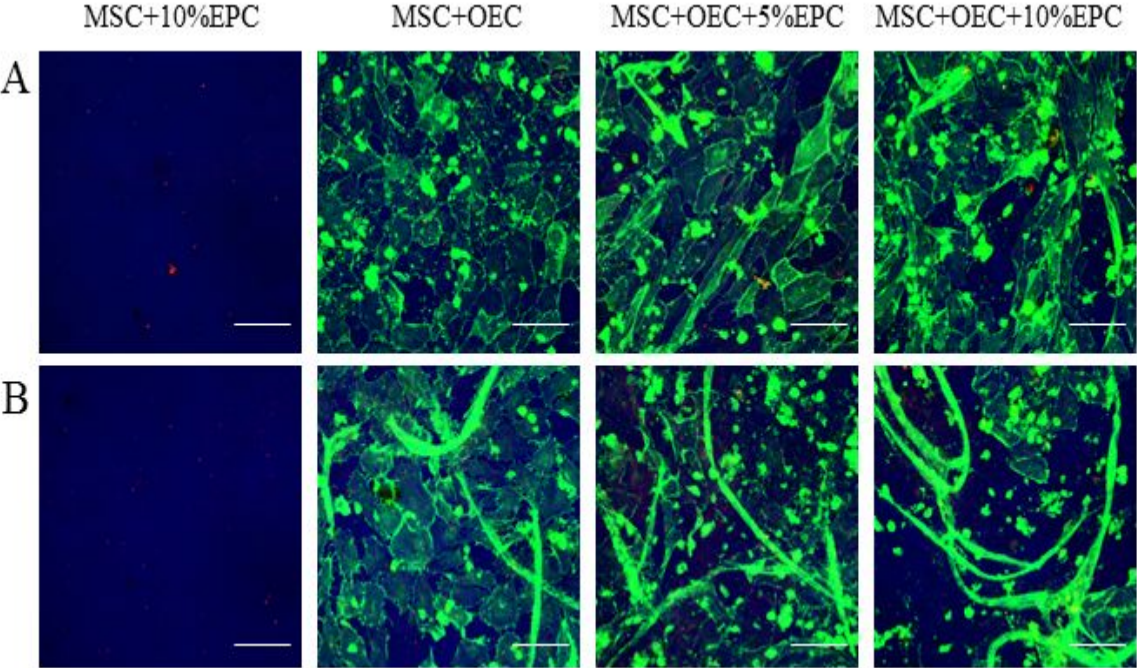


Figure 3.2 Immunofluorescent staining of co-cultures (MSC+10%EPC, MSC+OEC) or triple-cultures (MSC+OEC+5%EPC or MSC+OEC+10%EPC). Co-cultures and triple-cultures as indicated in the individual columns were analyzed for the endothelial marker CD31 depicted in green (A, C) and myeloid marker CD11b depicted in red (A,B,C,D) after 4 days (A,B) and 14 days (C,D). nuclei (blue), bar=100 μ m.

The effect of the addition of EPC was quantified by image analysis in cultures of at least three different donors as depicted in figure 3. Representative overview pictures underlying the quantification process are depicted for day 4 (figure 3.3 A) and for day 14 (figure 3.3 B). In

comparison to the control group, represented by the co-culture of MSC and OEC, EPC added in a ratio of 10% per total cells to the co-culture resulted in a significant increase in the area of vascular structures (figure 3.3 C) at both investigated time points and with regard to the skeleton of the vascular structures (figure 3.3 D) after 14 days of the culture process.



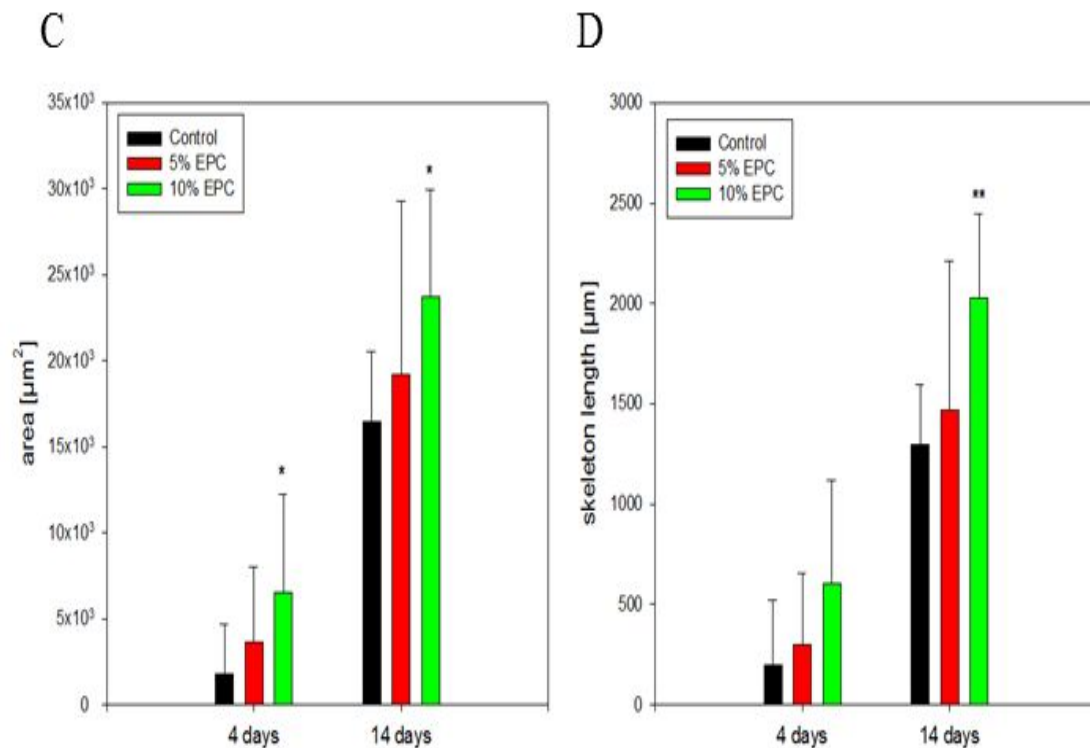
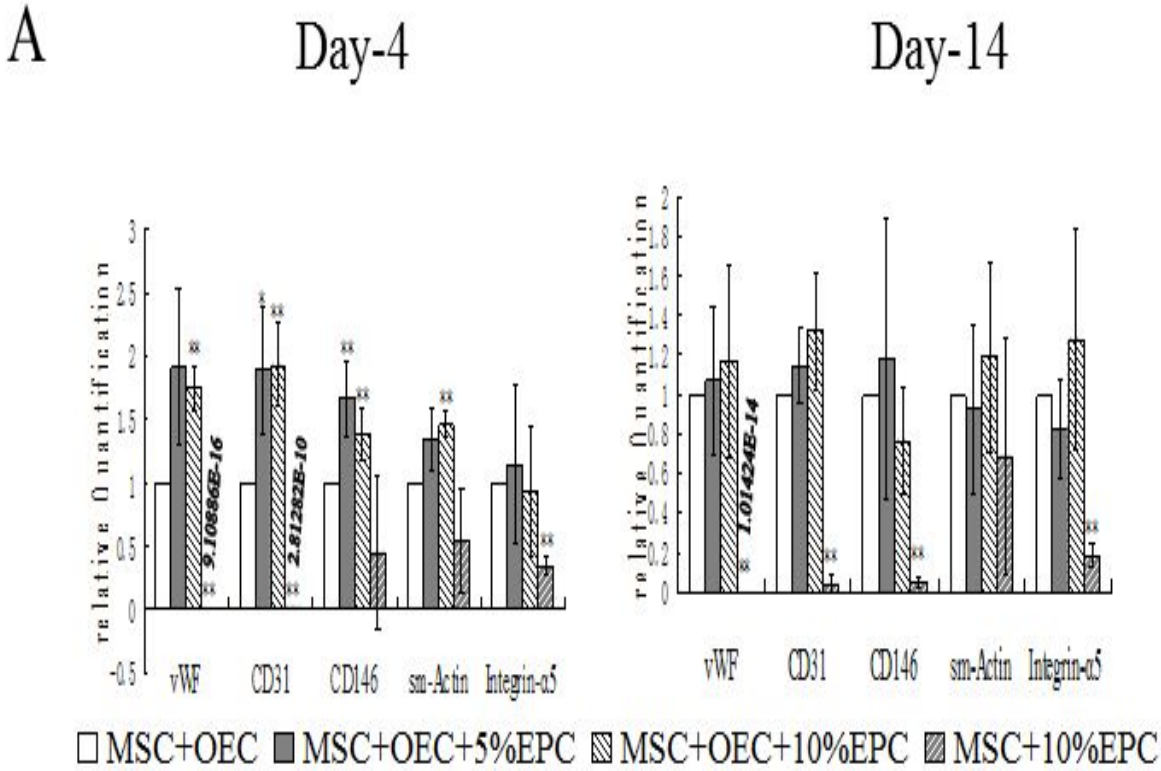


Figure 3.3 Quantitative assessment of vascular structures influenced by the addition of EPC. Co-cultures (MSC, OEC and MSC, EPC) and triple-cultures (MSC+OEC+5%EPC, MSC+OEC+10%EPC) were stained for the endothelial marker CD31 after 4 days (A) and 14 days (B). The area (C) and the total skeleton length (D) of vascular structures formed by co-cultures and triple-cultures after 4 days and 14 days were assessed quantitatively. Statistical significance was defined as a P-value of less than 0.05 ($p < 0.05$ and ** $p < 0.01$) $n=9$ samples. bar: $100\mu\text{m}$.*

3.3 Effects of myeloid cells on endothelial, myeloid and vessel-stabilizing markers and genes

To assess the effects exerted by myeloid cells added to the culture system in more detail, we further evaluated endothelial markers such as vWF, CD31, CD146, as well as smooth muscle actin involved in vascular stabilization and integrin- $\alpha 5$ by quantitative real time PCR (figure 3.4 A). Compared to the co-cultures of OEC and MSC used as reference group, the expression of endothelial markers at day 4 was significantly higher in groups to which EPC were added (figure 3.4 A), although the effects comparing 5% and 10% of EPC were not consistent. The same trend was observed for smooth muscle actin associated with the vascular stabilization by pericytes and integrin α -5 associated with angiogenic activation of endothelial cells.



B

Day-4

Day-14

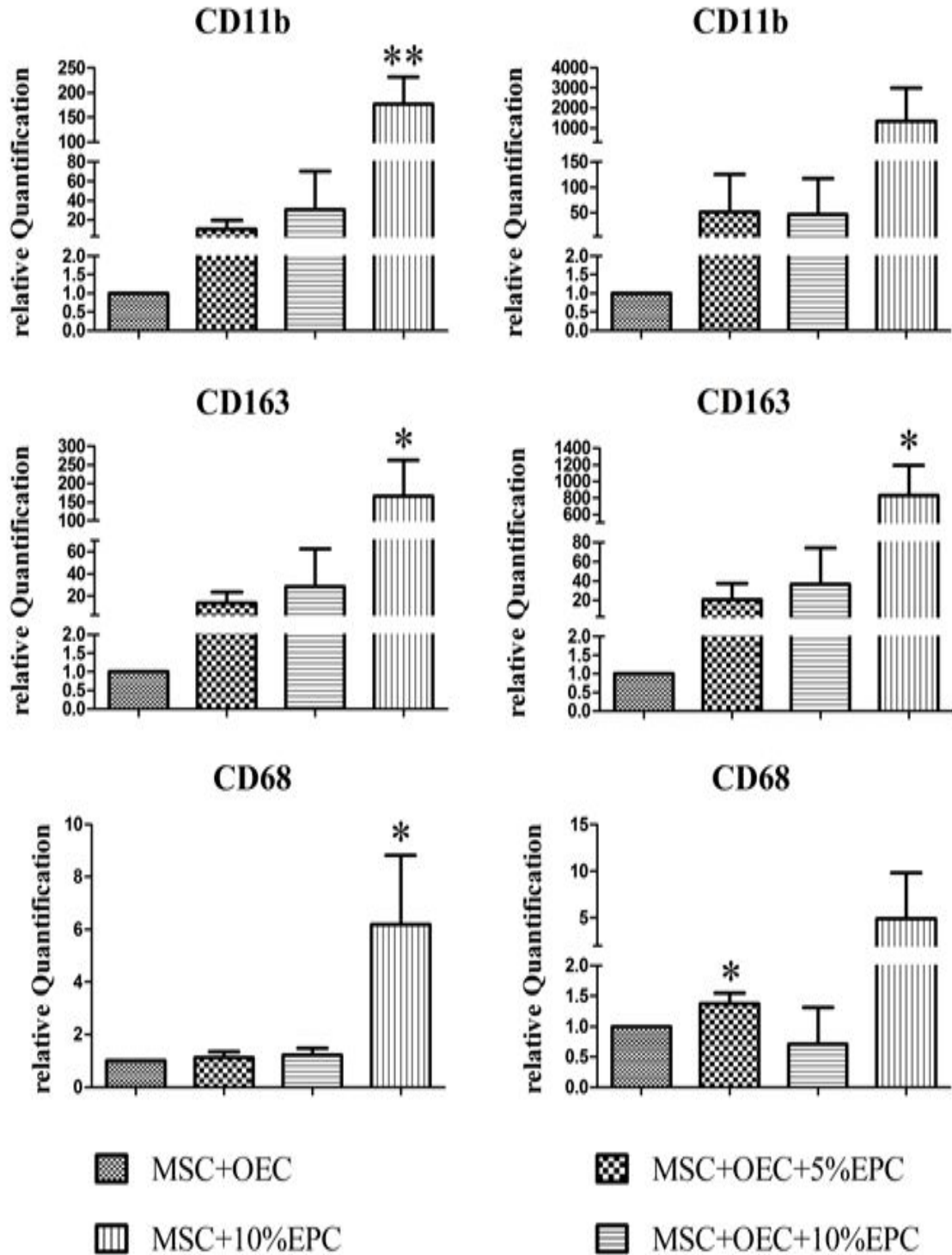


Figure 3.4 Expression of endothelial and myeloid markers. Relative expression of endothelial or angiogenesis-related markers (A) and myeloid or macrophage-associated markers (B) in co-cultures and triple-cultures was investigated by quantitative real-time PCR after 4 days and 14 days. GAPDH was used as an endogenous standard to normalize the data and control co-cultures were set to 1 ($p < 0.05$ and ** $p < 0.01$) $n=3$.*

In addition, real time PCR was performed to analyse myeloid or macrophage-related markers expressed by EPC or macrophage-like cells in the co-cultures. In these real time PCR experiments the highest expression of CD11b as a marker of myeloid cells was observed in the MSC/10% EPC co-cultures compared to the other groups, which confirms that EPC are the main source of myeloid cells in our co-culture scenarios (figure 3.4 B). Similar findings were observed for CD68 and CD163 used as macrophage markers for which we found significantly higher expression rates in MSC/EPC co-cultures than in the OEC/pOB cultures.

3.4 Expression and secretion of IL-8 and VEGF

To assess the mechanisms responsible for the positive angiogenic effect by the EPC or myeloid cell populations, we analysed the expression of IL-8 and VEGF by real time PCR, as well as the corresponding concentrations of IL-8 and VEGF in cell cultures supernatants by ELISA (figure 3.5). To cope with the different cell numbers in the co-culture set up, VEGF and IL-8 concentrations were normalized to the DNA content. The highest levels of free IL-8 and VEGF were found in the MSC/ 10%EPC group (figure 3.5 B, D), whereas in the co-cultures where mature endothelial cells were present, the concentration of free IL-8 and VEGF were lower and showed no differences between the groups at day 4 of the culture. The expression rates of VEGF and IL-8 after 14 days of cultures were slightly higher in the MSC/OEC cultures to which we added the EPC. These results might indicate that EPC or myeloid cells produce IL-8 and trigger the release of VEGF which is usually found in high concentrations in osteogenic cell culture supernatants in accordance with our previous reports

[91, 92].

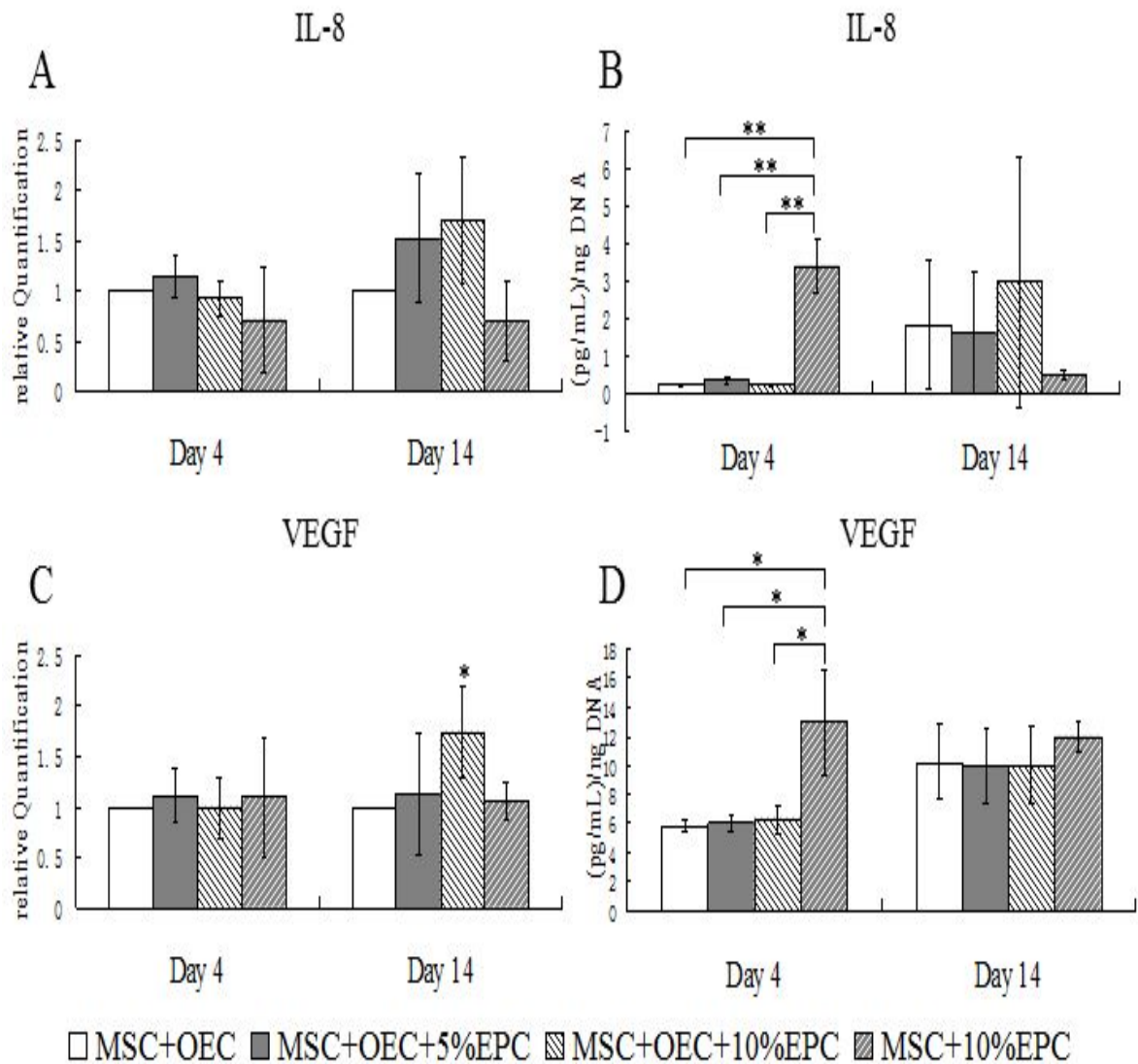


Figure 3.5 Relative quantification of gene expression of the proangiogenic factors IL-8 (A) and VEGF (C) in co-cultures and triple-cultures after 4 days and 14 days. GAPDH was used as an endogenous standard to normalize the data and control co-cultures were set to 1 (* $p < 0.05$ and ** $p < 0.01$) $n=3$. (B, D) Release of proangiogenic factors in co-cultures and triple-cultures. The concentration of IL-8 (B) and VEGF (D) in the supernatants of co-cultures and triple-cultures after 4 days and 14 days was measured using an enzyme-linked-immunosorbent-assay. Results were normalized to the DNA content of each

*sample to compensate for the different cell numbers in the set up (*p < 0.05 and **p < 0.01)*
n=3.

3.5 Protein arrays for angiogenesis- and hematopoiesis-related proteins

To assess the production of factors guiding angiogenesis in individual cell types and the effects of the co-cultures on these molecules, we performed protein arrays for angiogenesis- and hematopoiesis-related factors. Protein extracts were harvested from cell culture supernatants and cell lysates to analyse soluble and membrane- or matrix-bound fractions of the proteins. For this purpose proteins from the different fractions and culture approaches were pooled in equal ratios from three independent experiments and thus are depicted as mean values. In figure 3.6 A and figure 3.6 B results are depicted for VEGF, IL-8 and MCP-1 in cell culture supernatants (A) and cell lysates (B). Amongst the groups we assessed, MSC as monocultures were identified as the main producer of VEGF. In the co-cultures the levels of free VEGF are reduced in comparison to MSC monocultures. Moreover, the addition of EPC to the co-cultures of MSC and OEC further reduced the levels of free VEGF, whereas the cell-bound fraction of VEGF in the triple culture of MSC, OEC and EPC, was slightly higher. The highest IL-8 values in the supernatant were found in those of the co-cultures of OEC and MSC, whereas the highest levels for IL-8 in the cell lysates were found in OEC monocultures. In addition, in OEC lysates MCP-1 (monocyte-chemoattractant protein-1) was enriched, thus favoring the idea that they are the main producer of MCP-1 within the investigated cell types. In comparison, analysis of MCP-1 in the lysates indicates that the addition of EPC or myeloid cells reduces the levels of free MCP-1.

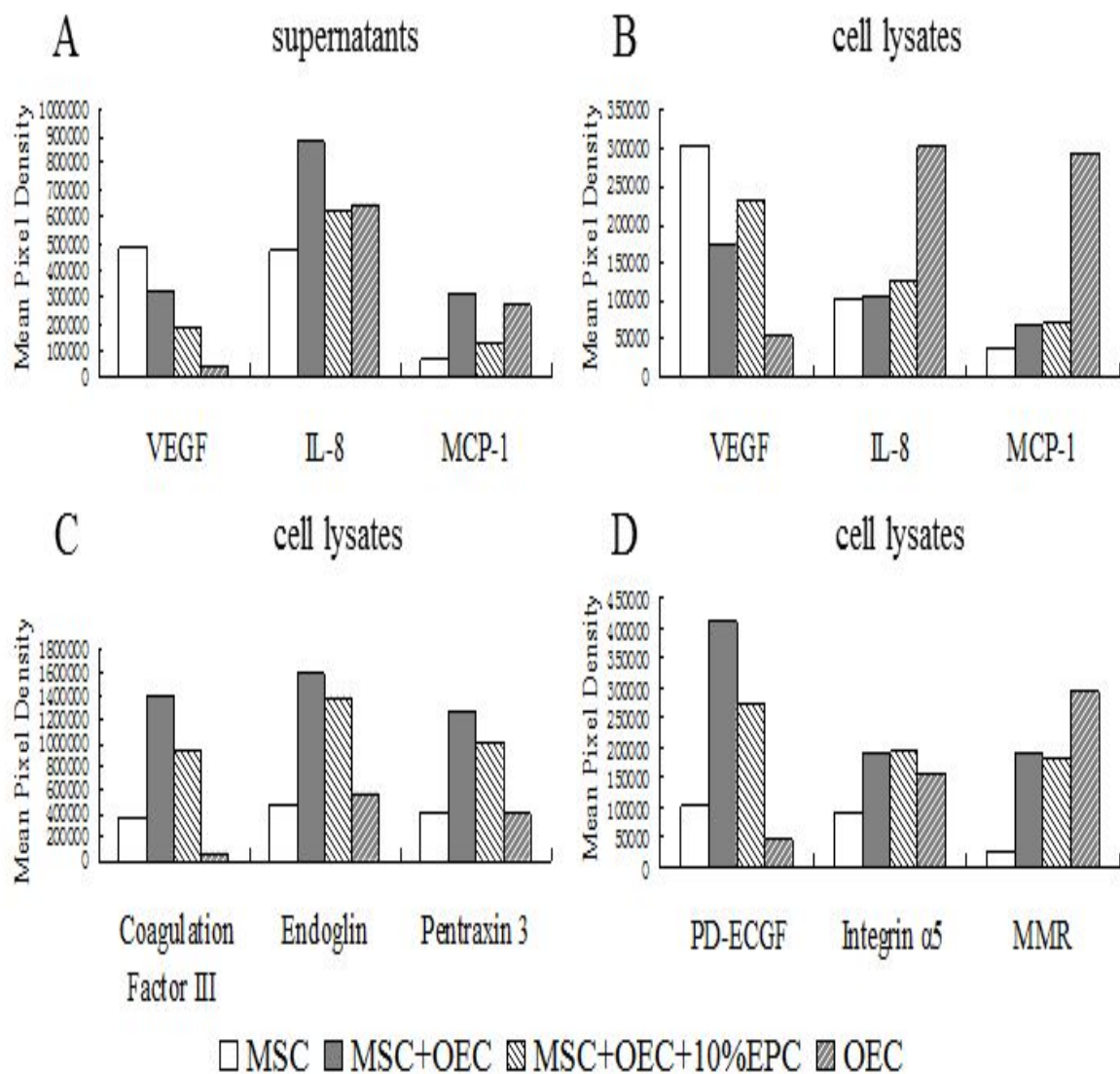


Figure 3.6 Human Proteome Profiler Array for Angiogenesis and Hematopoiesis. Quantitative read-out of protein arrays for VEGF, IL-8, MCP-1 in the cell culture supernatants (A) and cell lysates (B) in mono-, co- and triple cultures. Key molecules which drive angiogenesis found in the cell culture lysates (C,D). Results were depicted as mean pixel densities resulting from protein profiler experiments by pooling the protein extracts in equal ratios from 3 different donors.

By the protein arrays from cell lysates (figure 3.6 C,D) we identified several molecules which were upregulated in co- and triple-cultures containing angiogenic structures, compared to

MSC or OEC monocultures, in which no angiogenic structures were observed. These molecules include coagulation factor III (also known as tissue factor), endoglin, pentraxin 3, PD-ECGF and integrin- α 5. Furthermore, protein lysates of OEC showed high levels of macrophage mannose receptor (MMR).

3.6 The formation angiogenic structures and the associated myeloid cells grown on PU-HA scaffolds

We further explored the role of the CD11b-positive or myeloid cells were added to the co-culture systems which were grown on PU-HA scaffolds. The formation of angiogenic structures after 4 (figure 3.7 A, C) and 14 days (figure 3.7 B, D) was investigated by immunofluorescent staining for endothelial marker such as CD31 (depicted in green, figure 3.7) and for myeloid cells marker such as CD11b (depicted in red, figure 3.7) by CLSM. This was performed either as overlays from 3-D projection to cope with the surface topography or performed on sections derived from the scaffolds. In addition, the formation of angiogenic structures was also observed by light microscopy after immunohistological staining for the endothelial marker such as CD31 depicted in red (figure 3.8), which also enables to observe the porous structure of the scaffolding material. During the time course of the co-cultures OEC formed angiogenic structures characterized by elongated endothelial morphology on scaffolds. Nevertheless, in further studies methods have to be developed which would allow to quantify the effect of EPC or myeloid cells on the angiogenic structures in such a 3-D model.

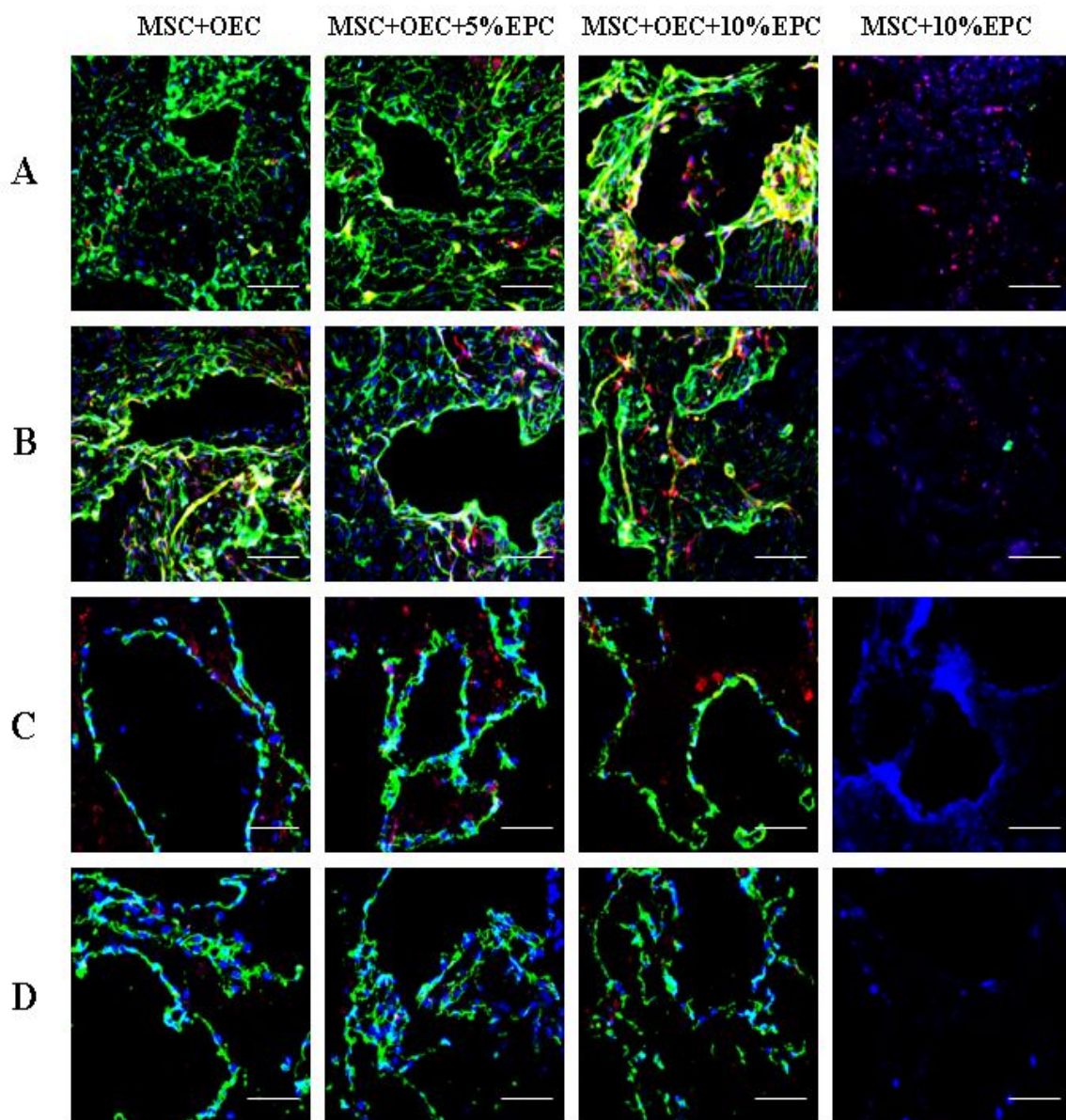


Figure 3.7 Immunofluorescent staining of co-cultures (MSC+10%EPC, MSC+OEC) or triple-cultures (MSC+OEC+5%EPC or MSC+OEC+10%EPC) on PU-HA scaffolds. Co-cultures and triple-cultures as indicated in the individual columns were analyzed for the endothelial marker CD31 depicted in green and myeloid marker CD11b depicted in red after 4 days (A, C) and 14 days (B, D). 3D pictures of PU-HA scaffolds (A, B) and the sections of PU-HA scaffolds (C,D). nuclei (blue), bar=100 μ m.

MSC+OEC

MSC+OEC+10%EPC

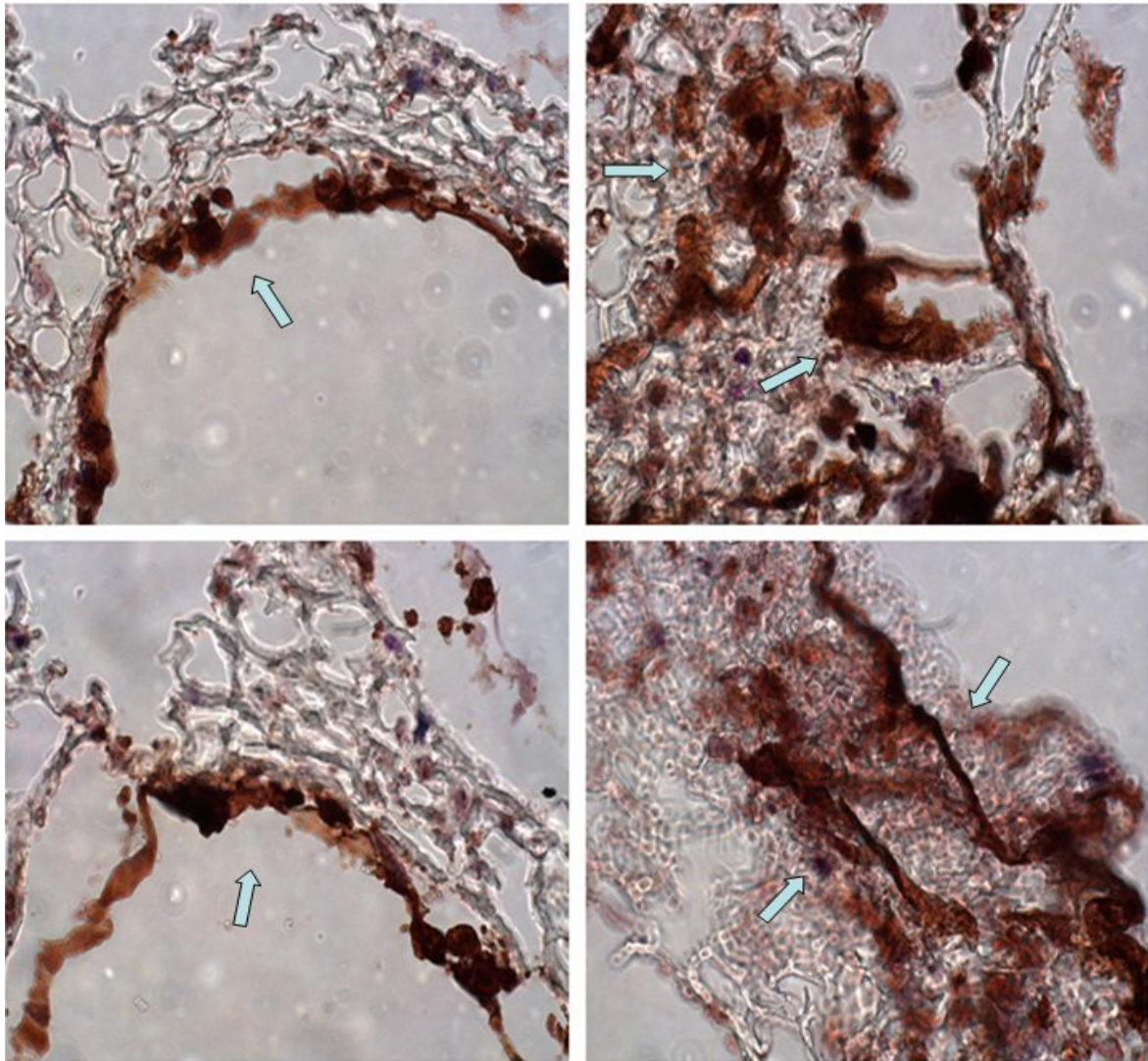


Figure 3.8 Immunohistological staining of co-cultures (MSC+OEC) or triple-cultures (MSC+OEC+10%EPC) on PU-HA scaffolds. Angiogenic structures (arrows) could be observed by light microscope. The sections of co-culture and triple-culture as indicated in the individual columns were analyzed for the endothelial marker CD31 depicted in red after 14 days.

4. Discussion

The interaction of resident endothelial cells, bone-forming cells and circulating endothelial progenitor cells is crucial for bone tissue repair and the formation of new and functional microvessels [93]. In this context it has been shown by previous data from co-culture experiments of OEC and MSC that endogenous myeloid cells are co-localized with angiogenic structures, thus implicating an important role during vascularization. Those data are part of the doctoral thesis by Gerhard Kramer (Johannes Gutenberg University, Mainz) and are also part of the manuscript by Yang Shi et al. (submitted). We hypothesized that those myeloid cells derived from EPC which are still contained as residues in OEC cultures. As a consequence in this present thesis early endothelial progenitor cells added to the co-cultures of MSC and blood-derived endothelial cells (OEC) significantly improved the formation of prevascular structures. Using several methods we have shown that early EPC act as myeloid cells and can be identified as CD11b-positive cells. The addition of myeloid cells supports the angiogenic process in different ways. This includes a beneficial effect on endothelial cell growth, as indicated by a significant increase in endothelial markers in real time PCR. In addition, EPC or myeloid cells produce angiogenic factors such as IL-8. The recruitment of myeloid cells seems to be mediated by MCP-1 expressed by OEC. Further results indicate that these myeloid cells act as M2 macrophages in co-cultures with MSC and OEC. Thus, the addition of myeloid cells or EPC to complex tissue constructs might help to support the tissue repair and neovascularization in the bone mediated by their macrophage-like properties.

4.1 The effect of co-culture system on the endothelial phenotype and angiogenic potential of outgrowth endothelial cells

It was previously shown that outgrowth endothelial cells from the peripheral blood can support the formation of vascular structures *in vitro* and *in vivo* [92, 94] and might support or accelerate blood perfusion during bone repair and in bone tissue engineered constructs. OEC form cobblestone-like colonies and express markers of mature endothelial cells. They also show a high proliferation and mature vessel-like structures under proangiogenic stimulation [60]. The results of this thesis clearly show that co-culturing of MSC and OEC influences the expression of CD146, CD31, and vWF in real time PCR as well as the formation of mature vessel-like structures *in vitro* as indicated by confocal microscopy and immunofluorescence staining for the endothelial marker CD31. In contrast there were no microvessel-like structures in co-cultures of MSC and EPC at the investigated time point in this study. Interaction between endothelial cells and osteogenic cells may be beneficial for bone repair process [95]. Endothelial cells produce growth factors such as bone morphogenic protein-2 and Endothelin-1 which effect osteoprogenitor cells differentiation and proliferation [96, 97]. In the co-culture system outgrowth endothelial cells might also support the bone repair in general by improving the vascularization and by the direct effects of endothelial cells on bone forming cells.

4.2 Effects of myeloid cells on neovascularization

In reports from the literature it has also been shown that myeloid cells from the host which are recruited to the sites of tissue repair or to implanted tissue constructs are also essential for the neovascularization process [93]. This is in accordance with a series of reports identifying myeloid cells as important mediators of neovascularization [98-100]. For example, CD11b+ cells produce high levels of MMP9 which is an important mediator in angiogenesis and plays

a critical role in vascular stability [101, 102]. However, the relative contribution of myeloid cells to vascular remodeling is still unknown.

In co-cultures of OEC with osteogenic cells the staining for the myeloid marker CD11b also indicated a co-localization of myeloid cells with angiogenic structures (Yang and Kramer et. al, submitted). In these experiments myeloid cells are characterized by their rounded morphology and also partially exhibited the expression of endothelial markers such as CD31. These characteristics are also attributes of endothelial progenitor cells. Myeloid cells are tentatively found at the tips or the nodes of the angiogenic network, which is in accordance with previous reports in the literature about the role of myeloid cells or macrophages-like cells in angiogenesis [103-105].

In this context the active addition of EPC or myeloid cells, respectively, improved the formation of vascular structures significantly. CD31 (PECAM-1) is a cell adhesion molecule (CAM) and has an important role in a number of endothelial cell functions including angiogenesis, integrin activation and cell-cell adhesion [106]. The endothelial marker CD31 was used to distinguish endothelial cells from other cells in co-culture system. The co-culture approach had three different set-ups 1) EPC were co-cultured with MSC 2) MSC were co-cultured with OEC 3) EPC were co-cultured with MSC and OEC. Comparison of different types of the co-cultures after 4 days and 14 days, showed that EPC contained cells which were able to support the formation of primitive capillary-like structures in co-cultures of MSC and OEC (Figure 3.3). In this context we evaluated a series of different markers involved in blood vessel formation. In order to specify the effects of myeloid cells in the cultures we compared the expression of endothelial markers such as vWF, CD31 and CD146, as well smooth muscle actin as an indicator for vessel stabilization by smooth muscle cells and integrin- α 5 for angiogenic activation in quantitative real time PCR. vWF (von Willebrand factor) is a glycoprotein that is synthesized in endothelial cells and essential for maintaining the normal circulation of blood after tissue injury [107]. CD146 is a cell surface molecule and is expressed in human endothelial cells as a component of the endothelial junctions [108]. After new blood vessels are being formed the blood vessels are stabilized by the association

with smooth muscle cells. It is believed that endothelial cells can induce the differentiation of mesenchymal cells into smooth muscle cells or pericytes [109]. Integrin- $\alpha 5$ is a receptor for the extracellular matrix protein fibronectin and plays a critical role in angiogenesis [110]. On the other hand Integrin- $\alpha 5$ is required for osteoblast differentiation of adult human mesenchymal stromal cells and promotes the osteogenic capacity of human mesenchymal stromal cells [111].

The increase in these markers in the groups to which we have added EPC was consistent through the evaluated endothelial markers and was statistically significant at day 4. In the group of MSC with 10% EPC the expression of endothelial markers was much lower in accordance with no obvious presence of mature endothelial cells at the investigated time point.

The mechanisms by which the EPC exert beneficial effects on endothelial cell growth could be multifactorial and might also include the transdifferentiation of subpopulations of EPC into mature endothelial cells [112]. Transdifferentiation of EPC to OEC might also occur in our experimental settings, but could not be proven at the present state. On the other hand, beneficial effects of EPC or myeloid cells on mature endothelial cells might be mediated by paracrine effects.

4.3 Interaction between EPC/myeloid cells and other cells by paracrine factors

Vascular endothelial growth factor (VEGF) is an essential regulator of physiological blood vessel growth as described already in more detail in the introduction. Besides this important role in the direct effect of VEGF in angiogenesis VEGF is essential to increase the generation of immature myeloid cells and to promote monocyte migration [114] as well as to enhance colony formation by granulocyte-macrophage progenitor cells [115]. Some groups have

suggested VEGF as a factor which is responsible for the proangiogenic effects of EPC or myeloid cells [56]. In addition, VEGF has been reported to recruit CD11b/Gr-1 and CXCR4 positive neutrophils supporting angiogenesis in mouse models [116]. In many reports from the literature, MSC were confirmed as the main producer of VEGF as it was also shown in the present study (figure 3.5). By comparing ELISA data, as well as VEGF levels by protein arrays, lower VEGF levels in supernatants and higher levels in cell lysates, reflecting the membrane- or matrix-bound fraction, characterized the triple cultures of OEC, MSC and EPC. This is in accordance with previous results investigating the ratios of VEGF in co-culture approaches. These previous studies also indicated low levels of VEGF in the supernatant but higher fractions of membrane- or cell-bound levels of VEGF in cultures with high angiogenic activity [91, 117]. ELISA data and data from the protein array gave slightly different results but this might be associated with differences in the protein harvesting procedure for the arrays or with the normalization of ELISA data to the cell number.

Another factor which has been proposed to mediate positive effects of myeloid cells on angiogenesis is IL-8 [118] triggering endothelial proliferation and angiogenesis [119, 120]. Interleukin-8 is a chemokine that was recognized as a mediator of permeability and angiogenesis. Transactivation of vascular endothelial growth factor receptor-2 by IL-8 is an important signaling pathway and leads to the increase of angiogenesis [122]. In this studies IL-8 appears to be derived from the early EPC or from mature endothelial cells. Angiogenic activation in the co-cultures of OEC and MSC leads to high levels of IL-8 in the supernatant which, in turn, are lowered by the addition of EPC to the system and a further improvement of angiogenesis. We therefore assume that this is associated with the role of IL-8 to recruit myeloid cells or endothelial progenitor cells to sites of vascular reorganization.

In addition, another molecule involved in the recruitment of EPC is MCP-1 produced by the OEC, which showed the highest rates of MCP-1 in the cell lysates. The addition of EPC leads to lower amounts of MCP-1 in the supernatants compared to the co-cultures of OEC and MSC. This seems to be in accordance with a monocyte- or macrophage-like phenotype of EPC and the role MCP-1 produced by late outgrowth endothelial cells [123] as an important regulator

of angiogenesis. MCP is closely associated with N-Cadherin [124] and induced by angiopoietin-1, leading to the recruitment of mural cells and the stabilization of angiogenic structures [125]. MCP-1 was found to be upregulated in perivascular smooth muscle cells in specimens of proliferative hemangioma but MCP-1 was not expressed in involuting hemangiomas [126]. MCP-1 is a direct mediator of angiogenesis that is able to induce endothelial cell migration and effects angiogenesis [127]. The upregulated expression of VEGF-A can be induced by MCP-1 and the subsequent angiogenesis is then induced by VEGF-A [128]. Therefore, the addition of EPC and the resulting lower MCP-1 values seem to favor a proangiogenic state of the endothelial cells.

In addition, we observed effects on several molecules involved in vascular control. Some of these molecules were much more abundant in cell lysates of co-cultures in which angiogenic structures were present than in the corresponding mono-cultures which are free of angiogenic structures. Moreover, these molecules were affected by the addition of early EPC or myeloid cells. These factors include coagulation factor III also known as tissue factor, endoglin, pentraxin 3, PD-ECGF and integrin- α 5. Tissue factor is also involved in the formation of complex and stable microvessels characterized by the stabilization of vascular structures through mesenchymal cells [129], which is mediated by the regulation of MCP-1 as described before in the literature [130] and in accordance with our observations for MCP-1, as described above. Pentraxin-3 is a negative regulator of angiogenesis due to its binding to the proangiogenic molecule fibroblast growth factor-2 [131], which is an important factor in the cellular cross talk of endothelial cells, pericytes and stromal cells. It has been shown that pentraxin-3 inhibits endothelial cell proliferation and migration [132]. Thus, the addition of EPC or myeloid cells to the system lowers the pentraxin-3 levels and contributes to the proangiogenic activity of the endothelial cells. Platelet-derived endothelial cell growth factor (PD-ECGF) is an angiogenic factor initially isolated from platelets [133] and has been detected in macrophages, stromal cells, and some epithelia [134]. Endoglin plays a multifaceted role in vascular biology and is expressed in endothelial cells in inflamed tissues. As recently shown, endoglin is involved in leucocyte adhesion and transmigration in the vascular system via the integrin α 5 β 1 receptor of leukocytes [135].

4.4 Association of macrophage with angiogenesis

Surprisingly high levels of macrophage mannose receptor (MMR) were found in the cell lysates of OEC. Although there are also reports from the literature that this marker is associated with endothelial cells [136, 137], MMR is widely accepted as a M2 macrophage marker [118, 138, 139]. Further experiments are currently underway to evaluate these findings by additional methods. Real time PCR experiments indicated that EPC contain macrophage-like cells expressing high ratios of CD11b and CD68. In co-culture with MSC they might differentiate towards cells with characteristics of M2 macrophages, as indicated by the expression of CD163 in the MSC/EPC cultures. Recently, emerging evidence has been provided that M1 macrophages in co-culture with endothelial cells [140] or mesenchymal stem cells [138, 141, 142] assume a M2-like phenotype which is responsible for anti-inflammatory and proangiogenic effects in tissue repair. In this context, the role of EPC or myeloid cells in neovascularization might be explained by their function as macrophage-like cells. Further experiments are underway to further define macrophage-like properties of EPC in the context of bone repair and neovascularization *in vitro* and *in vivo*.

4.5 Co-culture systems on PU-HA scaffolds for bone tissue engineering

Tissue engineering scaffolds should act as temporary ECM substitutes and are able to support cell and ECM interactions such as adhesion, differentiation and migration . A successful scaffold for bone repair should promote osteogenesis and the development of vascular networks in order to create a functional bone tissue. For example, mesenchymal stem cells can be seeded onto scaffolds and differentiated into chondrogenic and osteogenic cells [144]. In addition it has been shown previously that microvessel-like structures can be generated from outgrowth endothelial cells (OEC) within a scaffold by angiogenic stimuli such as the

co-culture process with osteogenic cells [86]. Thus we further explored the before mentioned the co-culture systems including also EPC or myeloid cells based on PU-HA as scaffolding materials with the aim to develop a construct which ensures endothelial and osteogenic functions.

We observed micro-vascular networks within Polyurethane-Hydroxyapatite (PU-HA) scaffolds after immunofluorescent staining and confocal laser scanning microscopy. Prevascular structures were induced by co-culturing of OEC with MSC on PU-HA scaffolds whereas no structures were observed in co-cultures of EPC with MSC on PU-HA scaffolds. Then, co-culture of OEC and MSC on PU-HA scaffolds plus the addition of EPC showed the contribution of EPC to vascular structures investigated after 4 days and 14 days. In general, the vascular structures on the scaffold increased during the culture process but an effect of the EPC on the formation of vascular structures, as well as this increase over time have to be quantified and investigated in detail in the future. Nevertheless, quantification of angiogenic structures showed the proangiogenic effect of EPC under 2-D conditions as reported in previous sections. Furthermore, an increase in vascular structures over time was already shown before in similar experiments on other types of scaffolding materials [90]. In this experimental study the growing and the maturation of the vascular network at later culture time were observed by CLSM (figure 3.7) and in immunohistochemistry of sections from the scaffolds. The endothelial cells and vascular structures staining for CD31 indicate that EPC might promote the vascular structure in triple-culture system (figure 3.8) on the scaffolds. The more vascular networks penetrate through the pores of scaffolds in the triple-cultures and reach also the inner parts of the construct (MSC+OEC+10%EPC), the better and faster the vascularization might proceed after the implantation. Here, in vivo studies would have to be performed in order to see whether this effect will be consistent in an in vivo situation.

4.6 Conclusions

Angiogenesis plays an important role in the bone repair. Endothelial progenitor cells can be isolated from the peripheral blood and expanded under specific culture conditions. In addition although endothelial progenitor cells are a heterogeneous population of cells some endothelial progenitor cells are able to give rise to outgrowth endothelial cells that have a high proliferative and vasculogenic potential. Investigation of cellular and molecular processes guiding angiogenesis may enhance our understanding of new blood formation and offer the opportunity to stimulate angiogenesis during tissue repair by suitable signaling molecules. The present study showed that the vasculogenic potential of OEC in co-culture with MSC for bone tissue engineering applications including the angiogenic activation and vessel stabilization. The angiogenic molecules such as VEGF effect the vessel growth in the co-culture system and then in a secondary effect enhance bone fracture repair and regeneration. In addition, the present study gives an insight into the pro-angiogenic action of myeloid cells or endothelial progenitor cells that seems to support the formation of microvessel-like structures. The effects of myeloid cells seem to be mediated by factors of such as IL-8, MCP-1, coagulation factor III, endoglin, pentraxin 3, PD-ECGF and integrin- α 5. Furthermore the data provide evidence that mixed application of mature endothelial cells, MSCs and endothelial progenitor cells can support the neovascularization process. Coating of Polyurethane biomaterials with Hydroxyapatite (PU-HA) combine a widely used and characterized biomaterial with the biomimetic surface of a bone substitute material. In this study PU-HA scaffolds have been shown to support the formation of a vascularized tissue with organized vascular structures. Further studies will have to reveal if the additional application of EPC leads to an improved vascularization of such constructs in vivo. For this purpose the growth of the cells types on this type of an artificial bone matrix and the formation of a complex vascular structure serves as an initial step towards an artificial bone construct on a 3-D matrix. In addition we are currently also evaluating potential effects of the triple culture of OEC, EPC and MSC on the osteogenic differentiation.

5. Summary

Repair of large skeletal defects is still a major clinical challenge in orthopaedic and reconstructive surgery. Currently investigated bone tissue engineering strategies might offer new opportunities to regenerate these defects. Bone is a well-vascularized tissue which requires an effective blood supply by nutrient artery, periosteal vessels, and epiphyseal vessels [145]. Thus a better understanding of the mechanisms and contributing cell types in blood vessel organization during bone repair might also support the bone regeneration in general. In this context the interaction of MSC and endothelial cells plays an essential role during blood vessel organization *in vivo* and can be partly mimicked in co-culture systems of endothelial cells and osteogenic cells. Recent studies have shown that myeloid cells significantly contribute to the neovascularization of the bone. Thus the present study was carried out to evaluate the role of EPC as a potential source of myeloid cells in the formation of vascular structures in a co-culture model consisting of mesenchymal stem cells (MSC) and outgrowth endothelial cells (OEC). The molecular effects of myeloid cells or EPC respectively were evaluated by quantitative real time PCR, ELISA, and protein arrays from cell culture supernatants and lysates. The active addition of EPC to co-cultures of MSC and OEC resulted in a significant increase in the formation of prevascular structures at early stages of the co-culture process. In addition, a significant increase of endothelial markers was observed indicating a beneficial effect of myeloid cells on endothelial cell growth. Furthermore, the high expression levels of CD11b and CD163 by real time PCR in co-cultures of EPC and MSC indicated that EPC act at least partly as macrophage like-cells. In initial studies this approach was then transferred to a biomaterial application based on polyurethane scaffolds coated with hydroxyapatite. First results indicate the formation of prevascular structures on this type of 3-D matrix and thus can serve as a basis for *in vivo* studies in following experiments. The relevant subpopulations with pro-angiogenic activity in EPC or myeloid

cells and their detailed function enhancing the vascularization process are currently defined in ongoing experiments.

6. References

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7. Scientific presentations and publication

Oral presentations

Y. Shi, F. Westendorf, C. Zachos, N. Steubesand, S. Lippross, A. Seekamp, S. Fuchs. Interaction of early endothelial progenitor cells and outgrowth endothelial cells to improve the formation of prevascular structures in co-cultures with mesenchymal stem cells. 16. Chirurgischen Forschungstagen 2012. Oct 04-06, 2012, Regensburg, Germany.

Schmidt H KG, **Shi Y**, Kirkpatrick CJ, Fuchs S. Object-based colocalization of angiogenic structures and myeloid cells. Proceedings of the Proceedings of ImageJ User and Developer Conference 2012. Oct 24-26, Mondorf-les-Bains, Luxembourg.

Poster presentation

Y. Shi, A. Schröder, H. Schmidt, A. Seekamp, S. Fuchs. The role of myeloid cells in neovascularization and repair of bone tissue. Interdisciplinary Symposium "Inflammation at Interfaces". Feb 22-23, 2013, Hamburg, Germany.

Publication

Yang Shi, Gerhard Kramer, Alexandra Schröder, Charles James Kirkpatrick, Andreas Seekamp, Harald Schmidt, Sabine Fuchs. Early EPC as a source of myeloid cells to improve the vascularization of bone constructs. The paper is "Under Revision for Submission to European Cells and Materials."

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9. Curriculum Vitae

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Education

Oct 2011- till date	Doctor: study at Department of Trauma Surgery, Christian-Albrechts-University, Kiel, Germany Title: Interactions of early endothelial progenitor cells (EPC) and outgrowth endothelial cells (OEC) to improve the vascularization of bone tissue constructs
Sep 2008-Jul 2011	Master: study at Department of Orthopedic Surgery, The First Affiliated Hospital of China Medical University, P.R.China Title: "Outcome of Posterolateral Fusion versus Posterior Lumbar Interbody Fusion for Lumbar degenerative disease"
Sep 2002-Jul 2007	Bachelor: study medicine at Shenyang Medical college, P.R.China
Sep 1999-Jul 2002	Highschool: Taian No.2 high school, P.R.China