Role of outgrowth endothelial cells

for applications in Tissue Engineering

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Abbrevations

ALP	Alkaline phosphatase
Ang1	Angiopoietin1
Ang2	Angiopoietin2
APS	Ammonium persulfate
BCA	Bicinchoninic acid
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BSA	Bovine serum albumine
COL	Collagen
CD	Cluster of differentiation
cDNA	Complementary DNA
DNA	Deoxyribonucleotide acide
dNTP	Deoxynucleotide phosphate
EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ethylendiamine tetraacetic acid
e.g.	Exempli gratia
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
EPC	Endothelial progenitor cells
ERK2	Extracellular signal receptor regulated kinase 2
EtOH	Ethanol
FAK	Focal adhesion kinase
FCS	Fetal bovien serum
FGF	Fibroblast growth factor
h	hour
H_2O	Water
HGF	Hepatocyte growth factor
Hh	Hedgehog
HRP	Horseradish peroxidise
Ig	Immunglobulin
IL	Interleukin
L	liter

KDR	Kinase insert domain receptor
MAPK	Mitogen activated protein kinase
MetOH	Methanol
MgCl2	Magnesium chloride
min	minute
ml	millilitre
mM	millimolar
mm	millimetre
MMP	Matrix metalloproteinases
mRNA	messenger RNA
N	Normal concentration
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
ng	nanogram
nm	nanometre
nm	nanomolar
NO	Nitric oxide
O2	Oxygen
OEC	Outgrowth endothelial cells
OSX	Osterix
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PECAM-1	Platelet endothelial cell adhesion molecule-1
Pen/Strep	Penicillin/Streptomycin mix
PFA	Paraformaldehyde
PI3K	Phosphoinositide-3-kinases
PIGF	Placental growth factor
Ptch1	Patched1
RNA	Ribonucleic acid
RT	Room temperature
RUNX2	Runt related transcription factor2
Shh	Sonic hedgehog
SMA	Alpha smooth muscle actin

Smo	Smoothened
TBS	Tris buffered saline
TE	Tris-EDTA buffer
TEMED	Tetramethylethylendiamine
TGF	Transforming growth factor
TNF-α	Tumor necrosis factor-α
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor
µg	microgram
µl	microlitre
µm	micrometre

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1. Introduction

A successful clinical application of implanted cell-constructs for bone regeneration is strongly dependent on the formation of a functional vascular network supplying the construct with oxygen and nutrients. Several approaches to improve the neovascularization of engineered tissues have been proposed during the past decade, including the use of endothelial cells or endothelial progenitor cells, strategies based on the natural interaction of the different cell types during the process of bone regeneration using co-culture systems or additional treatment of tissue engineered constructs with growth factors or morphogens.

Focus of the current study is on vascularization using outgrowth endothelial cells, especially in the context of bone tissue engineering. The study is based on an *in vitro* co-culture model consisting of outgrowth endothelial cells and primary osteoblasts which has the potential to permit further insight into underlying mechanisms of bone vascularization and could also support the identification of factors stimulating the vascularization process in therapeutic approaches. The following subchapters will give a general background, starting with fundamentals in bone tissue engineering and current vascularization strategies, bone formation and blood vessel formation, moving through endothelial progenitor cells/outgrowth endothelial cells as autologous cell source and their integration in complex engineered tissue constructs like co-culture systems and ending with the morphogen Sonic hedgehog as a potential factor stimulating the vascularization process with regard to potential applications in bone tissue engineering.

1.1 Bone tissue engineering

The repair and regeneration of large bone defects caused by trauma or diseases, such as following tumor resection or pathological degeneration and the need to transplant newly formed bone to sites of such bone defects is still a main problem in the field of regenerative medicine. The ‘golden standard’ for healing large bone defects is the transplantation of natural bone tissue from the patient (autograft) (Brown and Cruess, 1982; Gitelis and Saiz, 2002; Laurencin et al., 1999). Nevertheless, the application of autografts is restricted because of donor shortage and is associated with complications like infection, pain and donor morbidity (Gitelis and Saiz, 2002). The use of allografts, which means the transplantation of

tissue from a different individual to the patient is another commonly used application, but is also limited by a high risk of immune rejection for instance (Burg et al., 2000).

In 1993, the term Tissue Engineering was defined by Langer and Vacanti as "an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function" (Langer and Vacanti, 1993). In order to repair large bone defects and to overcome the limitations of current treatments, the field of bone tissue engineering aims to restore the natural function to a bone defect by using the natural healing response of the body, together with one or a combination of the elements cells, signalling molecules or growth factors and matrix (scaffold). The latter can be from either natural materials or from synthetic polymers and the final construct implanted into the patient (figure 1) (Albert et al., 2006; Rueger, 1998; Rueger et al., 1998; Schieker et al., 2004).

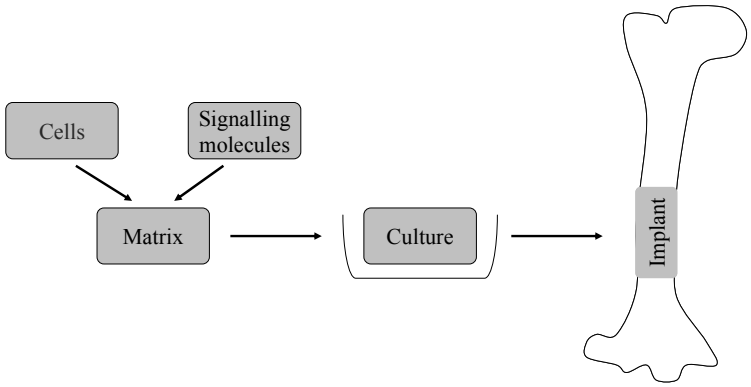


Figure 1. General strategy for bone tissue engineering: harvested cells, recombinant signalling molecules and matrices serving as a scaffold followed by the implantation of the scaffold into the patient.

Bone tissue is a highly vascularized and dynamic system with a complex construction (Caplan and Goldberg, 1999). Essential for the successful transplantation of bone constructs remains the rapid connection of the bone transplants to the hosts' vasculature. When the bone tissue engineered construct is transplanted into the patient, precultivated cells will have a limited capacity to uptake metabolic substrates due to the lack of a microvasculature. To provide sufficient supply with oxygen and nutrients via diffusion for the survival of the tissue engineered construct, metabolic active tissue must reside within 100-200 µm of a capillary (Jain et al., 2005; Kannan et al., 2005; Lovett et al., 2009). This disadvantage is known to be the major problem in regenerative bone tissue engineering strategies. Consequently, the development of strategies to overcome this problem by establishing a functional vascular

network in the tissue engineered constructs has become a major challenge in the field of bone tissue engineering and regenerative medicine.

1.1.1 Vascularization strategies in bone tissue engineering

In order to create a functional vascular network in bone engineered tissues several vascularization strategies have been developed during the past decade. These strategies include different approaches like the improvement of the scaffold architecture and functionalization (Lovett et al., 2009; Santos et al., 2008) or the use of delivery systems for proangiogenic growth factors (Geiger et al., 2005; Gu et al., 2004). In addition, prevascularization approaches using endothelial or endothelial progenitor cell based therapies (Rivron et al., 2008; Rouwkema et al., 2006) as well as complex co-culture systems consisting of heterogenous cell types are also commonly accepted strategies to enhance the vascularization process (Kaigler et al., 2003).

The scaffold architecture itself can markedly influence the vascularization properties of the graft (Lovett et al., 2009). One example of a beneficial scaffold architecture is the micro-/nano-fiber combination made from polycaprolactone which combines two important prerequisites for a bone scaffold (Tuzlakoglu et al., 2005). The microfibers of this scaffold provide mechanical support which is essential for bone regeneration. In addition, this tissue engineered scaffold mimicks the structure of the extracellular matrix through nanofibres and therefore provides a substrate for migrating endothelial cells which finally might form capillary structures under proangiogenic conditions (Santos et al., 2008). In addition, tissue engineered scaffolds may be loaded or coupled with proangiogenic factors, of which the most common factor is vascular endothelial growth factor A (VEGFA) (Zisch et al., 2003). For therapeutic approaches in terms of vascularization of regenerating tissue the focus lies on delivery systems which might allow a prolonged and constant exposure of the tissue to controlled doses of angiogenic factors (Chen et al., 2007).

Another approach to promote biomaterial vascularization is the use of endothelial cells or endothelial progenitor cells for the generation of prevascularized tissues through incorporation with endothelial cells or endothelial progenitor cells (Grellier et al., 2009; Rouwkema et al., 2006; Tabata et al., 1999). The anastomosis of a prevascularized tissue with the vasculature of the peri-implant tissue would be a beneficial therapeutic approach to

improve the sufficient vascularization of bone tissue engineered constructs. Different types of mature endothelial cells as well as endothelial progenitor cells from different sources are used for cell-based prevascularization strategies. Mature endothelial cells can be isolated from different sources like the umbilical cord, from the skin or adipose tissue. They show a low proliferation potential and limited availability. Therefore, a very promising autologues cell source for proangiogenic therapies seems to be endothelial progentitor cells (Hristov et al., 2007; Kim and von Recum, 2008; Rafii and Lyden, 2003). These cells fullfill a number of requirements that are important for a therapeutic application. They can be isolated from bone marrow, adipose tissue and peripheral blood and they possess a high proliferation capacity, reveal a high angiogenic potential in proangiogenic matrices *in vitro* and can contribute to the vascularization process *in vivo* (Gulati et al., 2003; Yoon et al., 2005).

In the context of endothelial progenitor cells for prevascularization of engineered tissue, co-culture systems consisting of endothelial progenitor cells and other cell types like osteoblasts or their precursor cells constitute a promising strategy to establish a functional vascular network in tissue engineered constructs (Finkenzeller et al., 2006; Fuchs et al., 2007; Kaigler et al., 2003). The proangiogenic effect of complex co-culture systems seems to be due to the supply of matrix components as well as angiogenic growth factors by the osteoblasts or their precursors leading to the angiogenic activation of endothelial cells (Finkenzeller et al., 2006; Mayer et al., 2005).

1.2 Bone formation

Bone exists in two architectural forms, trabecular or cancellous bone and compact, also called cortical bone. Trabecular bone, which can be found for instance in the metaphyseal end of long bones, is the most active part. This type of bone is active in growth, calcium homeostasis and hematopoiesis. On the other hand, compact bone like the shafts of the long bones is known to be more static and strong and can be found on the outer side of the bone (Yaszemski et al., 1996). Bone formation is a very complex process which occurs through two different mechanisms: intramembranous bone formation and endochondral bone formation (Campbell and Kaplan, 1992). Intramembranous bone formation means the formation of new bone directly from mesenchymal tissue and occurs during embryogenesis of the flat bones like the skull. This mechanism of intramembranous bone formation starts at the

end of the second month of gestation by condensation of mesenchymal tissue which contains osteoprogenitor cells that differentiate along a preosteoblast into an osteoblast lineage (Shapiro, 2008). The ossification process is initiated directly in the presence of adequate vascularization without a cartilage intermediate (Yaszemski et al., 1996), finally forming a trabecular bone. When sufficient bone density is formed, trabecular bone becomes remodelled to gain the optimal shape by resorption of bone by osteoclasts and new formation of bone by osteoblasts.

During endochondral bone formation, which occurs during embryogenesis of the long bones and during fracture repair, undifferentiated mesenchymal cells condense and differentiate into rapidly proliferating chondrocytes (Chung et al., 2004). The cartilage framework then becomes ossified to form the new bone. Therefore, the chondrocytes stop proliferation and become hypertrophic. This hypertrophic chondrocytes mineralize their surrounding matrix and finally undergo apoptosis. The mineralized cartilage matrix is covered with perichondrial cells which turn into osteoblasts and form a bone collar, the precursor of cortical bone. Following this, a vasculature is invading, thereby carrying osteoblasts and cartilage-degrading chondroclasts to sites of mineralized cartilage to form a primary spongiosa and finally a trabecular bone. Thus, angiogenesis, the outgrowth of new blood vessels from pre-existing vessels, seems to be a key step during ossification and highlights the close association of bone development and angiogenesis. The neovascularization during endochondral bone formation is highly controlled by the balance of positive and negative regulating factors depending on the state of chondrocyte differentiation (Carlevaro et al., 1997; Moses et al., 1990). One essential proangiogenic factor during neovascularization of the cartilage growth plate is vascular endothelial growth factor (VEGF). Carlevaro et al. have shown that VEGF is synthesized by hypertrophic chondrocytes serving as a chemoattractant for endothelial cells which finally leads to blood vessel formation during endochondral bone formation (Carlevaro et al., 2000).

1.3 Osteoblastic differentiation

30 years ago, Friedenstein and colleagues were the first group to reveal the existence of osteoprogenitor cells in the bone marrow and demonstrated that bone marrow cells were able to form osteogenic tissue *in vitro* (Friedenstein and Kuralesova, 1971). Osteoblasts as

well as chondrocytes, myoblasts and bone marrow stromal cells including adipocytes, are defined as mesenchymal cells and all these cells are hypothesized to have one common progenitor, namely the pluripotent mesenchymal stem cell (Ashton et al., 1980; Bennett et al., 1991; Bruder and Caplan, 1990; Caplan, 1991; Owen, 1988; Pittenger et al., 1999). The differentiation of mesenchymal stem cells into bone-building osteoblasts underlies complex regulatory molecular mechanisms, various hormones and factors (figure 2) (Cohen, 2006). In general, the osteoblastic differentiation process can be divided into 4 different cell stages according to Bruder and Caplan (Bruder and Caplan, 1990). Collagen type I (COL1A1) as well as alkaline phosphatase (ALP) are molecular markers of the early stages of osteoblastic differentiation, whereas osteocalcin, osteonectin, osteopontin and the mineralization of the extracellular matrix belong to the later markers of this differentiation process (Aubin, 2001; Candeliere et al., 2001). Early osteoprogenitor cells are characterized by a high proliferation potential, but these cells are negative for ALP and COL1A1. Early osteoprogenitor cells then undergo the transition into preosteoblasts which still exhibit a high proliferation capacity, but are now positive for ALP and synthesize COL1A1 and bone sialoprotein (Bruder and Caplan, 1990; Cohen, 2006). The proliferative potential decreases during further differentiation into osteoblasts. Finally, osteoblasts express necessary molecules involved in matrix mineralization like osteocalcin, osteonectin, and osteopontin (Beresford et al., 1984; Cheng et al., 1996; Nakashima and de Crombrugghe, 2003).

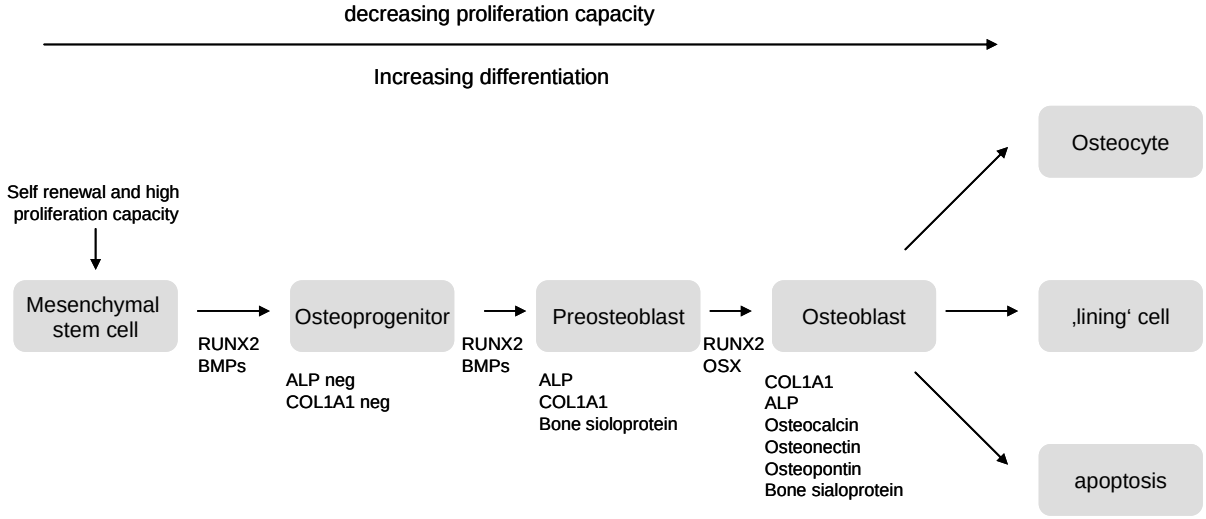


Figure 2. Schematic overview of the different stages during osteoblastic differentiation. Multipotent mesenchymal progenitors differentiate along an osteoprogenitor into preosteoblasts in the presence of BMPs and RUNX2. Preosteoblasts require osterix (OSX) to differentiate into functional osteoblasts. According to Bruder and Caplan 1990, Cohen 2006.

Of these osteoblasts, approximately 10-20 % differentiate into osteocytes, the main cells in the bone tissue. The remaining osteoblasts undergo apoptosis or become 'lining'-cells, which cover bone surfaces (Jilka et al., 1998). The differentiation process of mesenchymal stem or progenitor cells into osteoblasts is under the control of different factors including bone morphogenetic proteins (BMPs), runt-related transcription factor2 (RUNX2) and osterix (OSX) (Nakashima and de Crombrugghe, 2003) (figure2). Several groups have already shown that BMPs are potent local factors regulating osteoblastic differentiation by inducing mesenchymal stem cells to differentiate into osteoblastic lineage cells (Katagiri et al., 1993; Yamaguchi et al., 1991). At least, three different BMPs are known to be involved in osteoblastic differentiation, namely BMP2, BMP4 and BMP7 (Reddi, 2001). The transcription factor, RUNX2, also plays an essential role during osteoblastic differentiation, especially in the transition process of osteoprogenitors into preosteoblasts. Mice that lack RUNX2 have no osteoblasts (Komori et al., 1997). Under the control of OSX, another important transcription factor during osteogenic differentiation, RUNX2 expressing preosteoblasts differentiate into mature osteoblasts (Nakashima and de Crombrugghe, 2003).

Matrix mineralization, the final step during bone formation, occurs through the formation of osteoid by differentiated osteoblasts. Osteoid, the uncalcified organic matrix, is mainly composed of collagen type I (90%), osteocalcin, osteonectin, osteopontin and bone sialoprotein (Brodsky and Persikov, 2005). The mineralization of this organic osteoid matrix takes place through the deposition of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] into the collagen fibres which is mainly mediated by the activity of alkaline phosphatase (Landis, 1995; Whyte, 1994). Osteoblasts secrete ALP-containing vesicles which cleave phosphate groups. The vesicles rupture and function as centres for the hydroxyapatite crystals to grow within the collagen fibres (Ascenzi and Benvenuti, 1986; Ascenzi et al., 2008).

1.4 Blood vessel development

The first functional organ that is generated during vertebrate embryonic development is the cardiovascular system providing oxygen and nutrients necessarily required for the growth and differentiation of the developing embryo. Both angiogenesis as well as vasculogenesis are defined as processes for new vessel formation. Originally, vasculogenesis was defined as process occurring only during early embryogenesis, while angiogenesis was

defined as outgrowth of blood vessels from pre-existing vessels which takes place during postnatal life as well. Due to the discovery of endothelial progenitor cells in the adult peripheral blood by Asahara and colleagues, the understanding of blood vessel formation has changed (Asahara et al., 1997). It seems that angiogenesis and vasculogenesis are coupled and act together in the complex process of new blood vessel formation (Hristov et al., 2007).

During embryonic development the first two steps of new blood vessel formation include the differentiation of angioblasts from mesoderm, followed by the formation of a primitive blood vessel plexus (Risau and Flamme, 1995) (figure 3). The initial process of vasculogenesis constitutes mesoderm-inducing signals. These signals are essential to form angioblasts as well as hematopoietic lineage cells from the bipotential precursor of both cell types that is called a haemangioblast. During further differentiation into endothelial cells angioblasts form aggregates, also known as blood islands, which fuse with each other leading to the formation of a lumen and subsequently to the formation of a primary capillary plexus (Risau, 1997; Risau and Flamme, 1995) (figure 3).

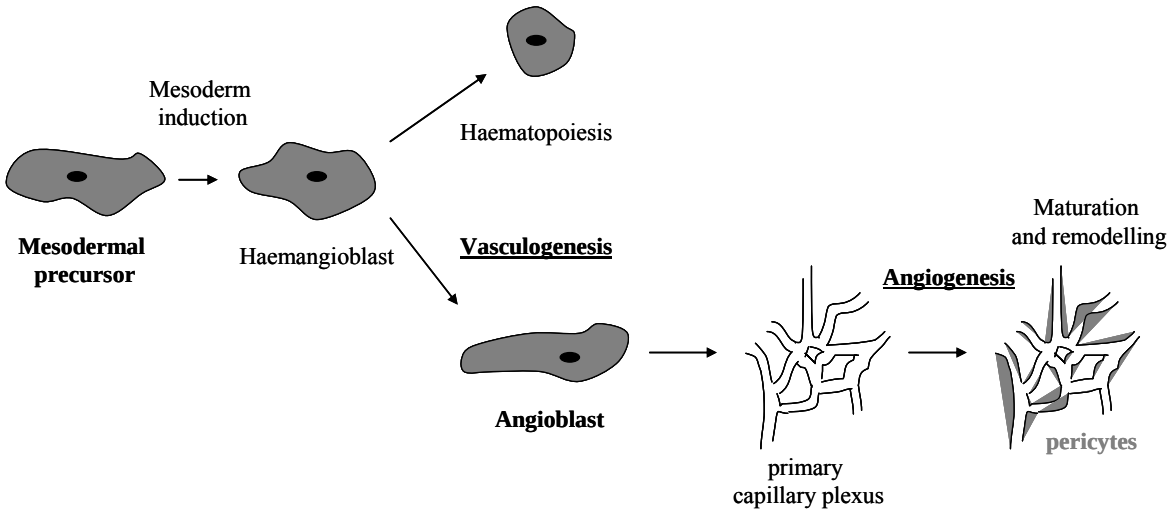


Figure 3. Schematic overview of blood vessel development including vasculogenesis, angiogenesis and blood vessel maturation and remodelling. According to Risau and Flamme 1995, Risau 1997 and Lindahl et al. 1997.

First vessels arise in the yolk sac, followed by the formation of the dorsal aorta and the cardinal veins of the developing embryo (Risau, 1997). After the process of vasculogenesis in which the primary vascular capillary plexus is built, more endothelial cells are generated and new and more complex capillaries are formed through the process of angiogenesis which can occur by sprouting or non-sprouting angiogenic mechanisms. Finally, newly formed vessels

need to be stabilized through the formation and recruitment of mural cells including pericytes and smooth muscle cells which takes place in a process called vessel maturation (Lindahl et al., 1997; Risau, 1997). The blood flow increases, the basal lamina becomes modified and the vascular wall matures through recruited pericytes. These processes finally lead to the formation of a functional and stable vascular system and the development of a microvascular endothelium, a macrovascular endothelium, arteries and veins.

1.5 Molecular regulation of blood vessel formation

The formation of new blood vessels is a highly regulated process composed of an activation phase and a resolution phase which are under the control of different growth factors, cytokines, chemokines and adhesion molecules and the complex balance between negative and positive regulating angiogenic factors. Positive angiogenic molecules include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), tumor necrosis factor α (TNF-α), angiogenin, epidermal growth factor (EGF) and angiopoietins (Folkman, 1995). On the other hand, negative angiogenic regulating molecules include for instance thrombospondin-1, prolactin, angiostatin or interferon alpha. The most important proangiogenic growth factor that controls blood vessel formation is the vascular endothelial growth factor A. VEGFA is part of a large family of angiogenic factors comprising currently 6 different members: VEGFA, VEGFB, VEGFC, VEGFD, VEGFE and placental growth factor (PIGF) (Ferrara et al., 2003). Through alternative exon splicing VEGFA can be classified in 5 different molecular variants that differ in their number of amino acids ($VEGFA_{121,145,165,189,206}$) of which $VEGFA_{165}$ is the most important splicing variant in terms of angiogenesis. VEGFA is necessarily required for the chemotaxis and differentiation of endothelial progenitor cells including angioblasts, as well as for endothelial cell proliferation, vascularization through the integration of activated endothelial cells into angiogenic structures and remodelling of vascular structures. The proliferative, mitogenic and angiogenic activities of VEGFA are mainly mediated through the receptor tyrosine kinase VEGFR2, also known as kinase insert domain receptor (KDR), the human gene encoding for VEGFR2 or fetal liver kinase-1 (Flk-1), the murine homologue for VEGFR2 (Millauer et al., 1993). Mice lacking VEGFR2 die early in embryonic development because they fail to form a vascular system (Shalaby et al., 1995). The transcription of the VEGFA gene is physiologically mediated by inflammatory processes or hypoxia. Under hypoxic conditions

hypoxia inducible factor alpha (HIF-1α) accumulates and forms heterodimers with hypoxia inducible factor beta (HIF-1ß), leading to the transcriptional activation of VEGFA. Binding of VEGFA to its receptor KDR leads to the angiogenic activation, differentiation, proliferation and sprouting of endothelial cells mediated through activation of different secondary molecular pathways (Adams and Alitalo, 2007).

1.5.1 Sprouting angiogenesis and blood vessel maturation

The process of sprouting angiogenesis consists of several steps that have been documented many years ago (Ausprunk and Folkman, 1977). As already described, the initiation of angiogenesis is activated through hypoxia-mediated upregulation of proangiogenic factors, such as VEGFA, eNOS or angiopoietin2. Within the activation phase VEGFA increases the release of nitric oxide (NO) which finally leads to the induction of vasodilation, the widening of blood vessels (Ferrara, 1999). Following the angiogenic stimulus the basal lamina and the extracellular matrix are degraded by matrixmetalloproteinases (MMPs such as MMP2 or MMP9), plasminogen activators (e.g. urokinase plasminogen activator) or by the inhibition of the corresponding protease inhibitors (tissue inhibitors of metalloproteinases) (Pepper, 2001). This proteolysis of the basement membrane can be seen as a very critical step because this allows endothelial cells to migrate and to retract. Endothelial cells migrate towards the angiogenic stimulus followed by the formation of a lumen and finally endothelial cell mitosis takes place distal to the leading tip of the sprouting vessel.

During the resolution phase, newly formed vessels become stabilized to establish a mature and functional vascular network. The maturation of vessels requires a combination of angiogenic and arteriogenic factors that lead to the covering of the newly formed vessel with mural cells. Depending on their morphology and location, mural cells can be divided into pericytes and vascular smooth muscle cells. Pericytes are associated with the small blood vessels like arterioles, venules and capillaries and they share their basement membrane with the endothelium whereas vascular smooth muscle cells are associated with arteries and veins (Armulik et al., 2005; Gaengel et al., 2009). In the literature a number of pathways have been demonstrated to be involved in mural cell differentiation, recruitment and endothelial cell covering. At least three different signalling pathways are activated during blood vessel

maturation, the PDGFB/PDGFRß pathway, the Tie/Angiopoietin system and the transforming growth factor beta (TGFß) signalling pathway (Gaengel et al., 2009; Hall, 2006; Hellstrom et al., 2001).

Platelet derived growth factor (PDGF-BB) and its receptor-beta (PDGFRß) also play a well defined role during the recruitment of pericytes to the nascent blood vessels. PDGF-BB is secreted from angiogenic sprouting endothelial cells where it serves as a chemoattractant for PDGFRß-expressing pericytes or vascular smooth muscle cells (Lindahl et al., 1997). In this way pericytes are recruited to sites of new vessel formation to stabilize the naked endothelial cells and form a durable and mature blood vessel. Additionally, platelet derived growth factor-BB also induces the differentiation of undifferentiated mesenchymal cells towards a mural cell fate (Hellstrom et al., 1999; Hirschi et al., 1999). Several *in vivo* studies have shown that insufficient recruitment of pericytes or smooth muscle cells leads to vessel enlargement and fragility in mice embryos that lack PDGF-BB (Hellstrom et al., 1999).

Another important signalling pathway involved in blood vessel maturation is the Angiopoietin/Tie system. In humans the angiopoietin family of transcription factors contains four different members, namely angiopoietin1, 2, 3 and 4 (Ang1-4). The most important angiopoietins during blood vessel formation and maturation are Ang1 and Ang2. Both are ligands for only one receptor tyrosine kinase that is called Tie-2 (Maisonpierre et al., 1997; Suri et al., 1996) but only Ang1 induces its autophosphorylation and thereby the activation of the receptor Tie-2 (Suri et al., 1996). The Tie-2 receptor is expressed in the embryonic endothelium, in the vasculature of the adult and in bone-marrow derived monocytes or mesenchymal progenitor cells (De Palma et al., 2007; Dumont et al., 1992). Ang2 competes with Ang1 and therefore acts as an inhibitor of the Ang1/Tie signalling pathway (Maisonpierre et al., 1997). Ang1 is mainly expressed by perivascular cells like pericytes or smooth muscle cells and acts in a paracrine manner on the endothelium whereas Ang2 is expressed by endothelial cells itself, suggesting an autocrine function (Fiedler et al., 2004; Scharpfenecker et al., 2005; Stratmann et al., 1998). The role of Ang1 and Ang2 in terms of angiogenesis seems to be pleiotropic and the precise balance between both molecules competing for the Tie-2 receptor is essentially required to control both the angiogenic activation of endothelial cells and the maturation of newly formed vessels. Ang1 stimulates the vessel growth in different tissues by mobilizing endothelial progenitor cells and by inducing the recruitment of pericytes (Suri et al., 1996; Visconti et al., 2002). In addition, the

angiogenic activity of Ang2 also seems to be very complex. On the one hand Ang2 is able to act as a coupled proangiogenic factor together with VEGF inducing angiogenic activity, but it can also induce endothelial cell death in the absence of VEGF (Visconti et al., 2002).

Transforming growth factor beta (TGFbeta) signalling also plays an important role during the process of vessel maturation, especially in the interaction between mural cells or pericytes and endothelial cells (Dickson et al., 1995). TGFbeta family ligands stimulate type II receptors that phosphorylate and activate type I receptors and which finally leads to the activation of the downstream signalling Smad. In terms of angiogenesis, transforming growth factor ß acts in a pleiotropic manner and dose-dependently. TGFß can contribute to the angiogenic activation by the upregulation of proangiogenic molecules and proteases at a low dose. At a high dose TGFß can also inhibit endothelial cell proliferation on the one hand and promote the stimulation of smooth muscle cell differentiation and recruitment to sites of *de novo* blood vessel formation on the other hand (Goumans et al., 2002; van den Driesche et al., 2003).

1.6 Endothelial progenitor cells

1.6.1 Endothelial progenitor cells for neovascularization

A few years ago, angiogenesis and new vessel formation in the adult were thought to involve endothelial sprouting from pre-existing vessels through proliferation and outgrowth of adult differentiated endothelial cells from the existing vessel wall that were proposed to give rise to a new vasculature. In 1997, Asahara et al. discovered a population of circulating cells found in the peripheral blood with characteristics of both endothelial cells and progenitor cells as well. This study changed the common understanding of postnatal blood vessel formation completely (Asahara et al., 1997). The newly discovered cells were termed endothelial progenitor cells (EPC) and Asahara and colleagues provided the first evidence that these bone-marrow derived cells could have regenerative properties. They reported that EPC are able to contribute to the formation of new blood vessels in the adult organism, a process called vasculogenesis. The discovery of circulating EPC that are able to differentiate into the mature vascular endothelium when needed led to a new concept of *de novo* blood vessel formation during postnatal life. Vasculogenesis, the contribution of endothelial progenitor

cells to blood vessel formation and angiogenesis, originally defined as blood vessel sprouting during postnatal life, may occur simultaneously in the adult organism (Hristov et al., 2007).

Endothelial progenitor cells can be found in the bone marrow, in adipose tissue and in the peripheral blood (Hristov et al., 2003). The majority of EPC are located in the bone marrow in close association with hematopoietic stem stells and the bone marrow stroma. In response to tissue ischemia or hypoxia EPC are recruited to sites of active neovascularization and differentiate into endothelial cells (Takakura et al., 1998). Endothelial progenitor cell recruitment is triggered through an increase of angiogenic growth factors and chemokines, such as vascular endothelial growth factor or angiopoietins (Hattori et al., 2001; Iwaguro et al., 2002). The release and mobilization of endothelial progentitor cells in the circulating blood is mediated by VEGF and granulocyte-macrophage colony stimulating factor (GM-CSF) and is dependent on the release of nitric oxide synthase expressed by stromal cells in the bone marrow (Aicher et al., 2003). Furthermore, EPC present at sites of neovascularization recruit additional EPC by releasing growth factors themselves. The released proangiogenic factors activate matrix metalloproteinases (MMPs), which in turn promote proliferation within the bone marrow and lead to EPC mobilization into the peripheral blood.

1.6.2 Characterization of endothelial progenitor cells

A number of studies on endothelial progenitor cells have been published during the past decade, but the definition, characterization and identification of endothelial progenitor cells clearly differ in the literature. There is evidence from the literature that endothelial progenitor cells isolated from the mononuclear cell fraction from peripheral blood by ficoll density gradient centrifugation are a heterogenous population of cells (Gulati et al., 2003; Hur et al., 2004; Ingram et al., 2004; Lin et al., 2000). Due to their order of appearance and their morphological characteristics, different cell types could be described (Hur et al., 2004; Prater et al., 2007). It is already known that at least two EPC cell types isolated and cultured from human peripheral blood exist, early EPC and late EPC, so-called late outgrowth endothelial cells (OEC). Late OEC are also termed as blood OEC or endothelial colony-forming cells. An overview of morphology, characteristics and functions of early endothelial progenitor cells compared to late endothelial progenitor cells (OEC) is summarized in table1.

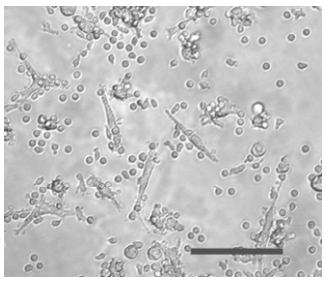
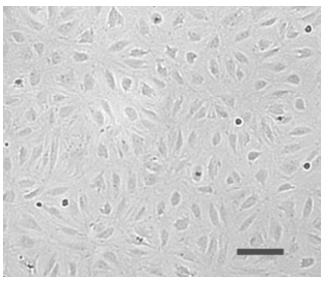
Early EPC make up the majority of the mononuclear cell fraction isolated from the peripheral blood. These cells appear after 4-7 days of cultivation and they show a spindle-shaped morphology (Lin et al., 2000; Yoon et al., 2005). Although they express a number of endothelial markers, like CD31 (PECAM1), vWF (von Willebrand factor), KDR or VE-cadherin, they do not show the typical endothelial functionality (Assmus et al., 2002; Fuchs et al., 2006). They exhibit just a low proliferative potential, a restricted expansion capacity and die after approximately 4 weeks *in vitro* (Hur et al., 2004; Rehman et al., 2003). Furthermore, they show no formation of vascular structures on proangiogenic matrices like Matrigel as shown by several groups *in vitro* (Mukai et al., 2008; Yoon et al., 2005) (table1).

Late outgrowth endothelial cells (OEC) appear as small colonies in long-term cultures after 2-3 weeks of cultivation and they possess a high proliferation capacity and a strong ability to form capillary tubes both *in vitro* and *in vivo* (Hur et al., 2004; Ingram et al., 2005; Ingram et al., 2004; Yoder et al., 2007; Yoon et al., 2005). They show a cobblestone-like morphology (Gulati et al., 2003; Yoder et al., 2007) and express a typical mature endothelial marker profile, indicating that they are more differentiated endothelial cells (table1). Previous studies already documented the angiogenic potential of OEC in different angiogenic matrices (Mukai et al., 2008). Athough only these cells show proangiogenic properties *in vitro*, both cell types, OEC as well as early EPC can contribute to neovascularization *in vivo* (Hur et al., 2004; Reyes et al., 2002). Yoon and colleagues proposed an indirect effect of early EPC on the angiogenic activation of OEC by paracrine secretion of proangiogenic factors, such as vascular endothelial growth factor (VEGF), which seem to support and improve the angiogenic capability of OEC. This highlights the complex synergy between the different endothelial progenitor cell populations during the neovascularization process (Yoon et al., 2005).

In order to isolate early EPC or OEC specifically from the mononuclear cell fraction, several surface markers have been proposed as useful (Delorme et al., 2005; Gulati et al., 2003; Timmermans et al., 2009). Gulati et al. for instance isolated OEC from the CD14-negative mononuclear cell fraction from the peripheral blood, whereas Delorme et al. separated OEC from the mononuclear cell fraction using an adhesion step in combination with a magnetic separation step for CD146 (Delorme et al., 2005). In addition, outgrowth endothelial cells seem to be negative for the endothelial progenitor cell surface marker CD133 which was

demonstrated by the group of Timmermans et al. They revealed that OEC arise out of the CD34-positive and CD45-negative mononuclear cell fraction (Timmermans et al., 2009).

Table 1. Endothelial progenitor cells. Overview of morphology, charactristics and functions. References: **1.** *Lin et al., 2000* **2.** *Yoon et al., 2005* **3.** *Rehman et al., 2003* **4.** *Gulati et al., 2003* **5.** *Ingram et al., 2004* **6.** *Ingram et al., 2005* **7.** *Yoder et al., 2007* **8.** *Hur et al., 2004* **9.** *Asahara et al., 1997* **10.** *Kalka et al., 2000* **11.** *Timmermans et al., 2007* **12.** *Delorme et al., 2005* **13.** *Gehling et al., 2000* **14.** *Peichev et al., 2000* **15.** *Urbich et al., 2004* **16.** *Assmus et al., 2002* **17.** *Fuchs et al., 2006* **18.** *Mukai et al., 2008* **19.** *Reyes et al., 2002.*

	EPC	**OEC**
Morphology	spindle shaped morphology (1-3)	Cobblestone-like morphology (4-7)
		
Order of appearance	after 7 days in culture (2,8)	after 2-3 weeks in culture (1,4,8)
Marker profile	CD31+ (3,9,10), CD45+ (3,11), CD34+ (2,9,10), CD14+ (2-4), CD146- (12), CD133+ (13-15), Flt-1 (8), eNos (8), vWF (7,8,16), VE-Cadherin (3,8,10), KDR (8,10,16), CD36+ (1), Tie2+ (4,9)	CD31+ (4,7,17), CD45- (11), CD34+ (2,11,13), CD14- (2,4), CD146+ (12,17), CD133- (11), Caveolin-1 (4,17), Flt-1 (8), eNos (4,8) ,vWF (7,8,17), VE-Cadherin (1,8,17), KDR (2,8,11), CD36+ (1), Tie-2+ (4)
Characteristics		
In vitro	low proliferative potential (2,3,8) no tube formation on Matrigel (2,18)	high proliferative potential (2,8) tube formation on Matrigel (2,18)
In vivo	vasculogenic potential through paracrine mechanisms (4), secretion of proangiogenic molecules (2)	high vasculogenic potential (4,8,19)

1.7 Co-culture of outgrowth endothelial cells and primary osteoblasts for bone tissue engineering

The rapid connection of a bone transplant to the host's vasculature ensuring the supply with oxygen and nutrients is an essential prerequisite for the successful transplantation of bone constructs. Therefore, the development of strategies to overcome this problem by establishing a functional vascular network in the tissue engineered constructs has become a major challenge in the field of bone tissue engineering and regenerative medicine. One strategy to improve vascularization of bone constructs is the use of co-culture systems consisting of heterogenous cell types creating approximately the microenvironment which can be found *in vivo.*

Several groups have shown that the bone repair process strongly depends on the functional regulation between endothelial cells and osteoblastic cells and is under the critical control of two fundamental processes, namely angiogenesis and osteogenesis (Collin-Osdoby, 1994; Decker et al., 1995; Guillotin et al., 2008). Co-culture systems consisting of endothelial cells or endothelial progenitor cells and osteoblasts or their precursors can be used from different sources and in various ways. They can be used in combination with a scaffold, co-implanted in Matrigel-plugs or in scaffold-free approaches (Fuchs et al., 2009a; Rouwkema et al., 2006; Stahl et al., 2004). Although the detailed mechanisms are currently not fully understood, it seems that co-culture systems of osteoblasts and endothelial cells induce the formation of microvessel-like structures and positively influence the angiogenic potential of endothelial cells *in vitro* and *in vivo* (Choong et al., 2006; Elbjeirami and West, 2006; Fuchs et al., 2009b; Rouwkema et al., 2006; Unger et al., 2007). Furthermore, co-culture systems of endothelial cells and osteoblasts seems to have a positive effect on the osteoblastic differentiation of the osteoblasts as well (Qu and von Schroeder, 2006; Stains and Civitelli, 2005)

The interaction of endothelial cells and osteoblasts in co-culture systems underlies complex mechanisms. Although the detailed interaction between osteoblasts and endothelial cells in the co-culture is currently under investigation, it seems that the cells communicate in two different ways (Guillotin et al., 2008; Villars et al., 2002). Co-cultures of endothelial cells and osteoblasts might interact in a direct way from cell to cell as well as in an indirect paracrine way. Direct cell to cell communication is mainly mediated via gap junctional communication.

These proteins form a transcellular channel between cells that permits the passage of ions, metabolites and other physiologically active molecules from one cell to another (Jongsma and Wilders, 2000; Rossello and Kohn, 2009). Connexin 43 is identified as the most dominant gap junction protein which mediates the intracellular transport of ions or other small molecules between osteoblasts and endothelial cells (Guillotin et al., 2004; Stains and Civitelli, 2005; Ziambaras et al., 1998). According to this, Villars et al. demonstrated an increase of alkaline phosphatase (ALP) activity in a direct co-culture system of human umbilical vein endothelial cells and primary human osteoprogenitors that was mediated by the synthesis of the gap junction protein connexion 43. Another communication mechanism in co-culture systems consisting of endothelial cells and osteoblast is the paracrine way. It has been shown that osteoblasts in co-culture with endothelial cells release higher amounts of proangiogenic molecules such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF) than in osteoblast mono-culture. These higher levels of proangiogenic factors seems to induce the angiogenic activation of endothelial cells in the co-culture system (Clarkin et al., 2008; Tokuda et al., 2003). In addition, the osteoblasts provide matrix components such as collagen I which positively influences the migratory and proliferative potential of endothelial cells (Santos et al., 2009).

Co-culture systems consisting of outgrowth endothelial cells (OEC) and primary osteoblasts (pOB) seem to be a beneficial instrument to improve the vascularization process. Outgrowth endothelial cells from the peripheral blood constitute an advantaged autologues cell source for the prevascularization of tissue engineered constructs. The isolation procedure of outgrowth endothelial cells from the peripheral blood is associated with a minimal invasive intervention in comparison with the isolation of stem cells from the bone marrow. In addition, the potential of outgrowth endothelial cells to contribute to the angiogenic process *in vitro* as well as *in vivo* has been examined and documented in several previous studies (Gulati et al., 2003; Hur et al., 2004; Mukai et al., 2008; Reyes et al., 2002; Sieminski et al., 2005; Yoon et al., 2005). In different investigations, the co-implantation of OEC together with smooth muscle cells, mesenchymal stem cells or osteoblastic cells leads to the angiogenic activation of OEC and finally to blood vessel formation by OEC (Fuchs et al., 2009a; Melero-Martin et al., 2007). The beneficial effect of osteoblasts on the cellular organization of endothelial cells into tube-like structures in the co-culture system is in accordance with reports from the literature (Fuchs et al., 2007; Rouwkema et al., 2006; Santos et al., 2009; Unger et al., 2007). Although the detailed mechanims that control the angiogenic activation of endothelial cells induced by the

co-culture are still under investigation, it seems that the co-culture of outgrowth endothelial cells with primary osteoblasts provide on the one hand proangiogenic matrix components and on the other hand angiogenic growth factors leading to the angiogenic activation of OEC.

1.8 Sonic hedgehog

Additional treatment with growth factors or proangiogenic signalling molecules to improve the vascularization of tissue engineered bone constructs is a commonly used strategy and is widely based on the treatment with classical angiogenic growth factors like vascular endothelial growth factor. Currently, one of the most promising molecular targets for a therapeutic intervention seems to be the sonic hedgehog signalling pathway due to its pivotal role in the initiation of angiogenesis and osteogenic differentiation.

1.8.1 Hedgehog proteins

Originally, hedgehog proteins were found to be morphogens, in a number of different tissues and organs during embryonic development (Chiang et al., 1996; Heine et al., 2009; Johnson et al., 1994; Nagase et al., 2007; Ruiz i Altaba, 1994). Morphogens are defined as molecules that control the pattern of tissue development during embryogenesis (Mikhailov, 1984). Three vertebrate homologues of Drosophila hedgehog exist, namely sonic hedgehog, desert Hedgehog and Indian Hedgehog (Fietz et al., 1994). Hedgehog protein is translated as a ~45kDa precursor and undergoes autocatalytic processing to produce a ~20kDa N-terminal signalling domain and a ~25kDa C-terminal domain with no known signalling role. Hedgehog signalling occurs through the interaction with its receptor patched1 (Ptch) (Cohen, 2003). In the absence of the hedgehog ligand, Ptch1 inhibits the co-receptor smoothened (Smo), a downstream protein in the Hh pathway (Nagase et al., 2007). The blocking of Smo leads to the convertion of the transcription factor Gli2/3 to its repressor form. The binding of Hh ligand to Ptch1 leads to the repression of Ptch1 which degrades Smo inhibition and finally leads to the activation of the Gli transcription factors. Activated Gli accumulates in the nucleus and controls the transcription of hedgehog target genes including Ptch1 and Gli themselves (figure 4). Sonic hedgehog (Shh) is the most studied member of the hedgehog family and is known to regulate the development of the limb bud and the central nervous

system during embryogenesis (Nagase et al., 2005; Nagase et al., 2006; Panman and Zeller, 2003). Cyclopamine, a naturally occuring teratogenic alkaloid isolated from the corn lily *Veratrum californicum* is an effective suppressor of the hedgehog signalling pathway. In 1968, Binns et al. found out that this alkaloid was responsible for several birth defects like anophtalmia and cyclopia (Binns et al., 1968) in calves that were fed with the corn lily. Cyclopamine disrupts the Hh signalling pathway by inhibiting the Hh co-receptor smoothenend and therefore this chemical is very useful to block and study the hedgehog signalling pathway (figure 4).

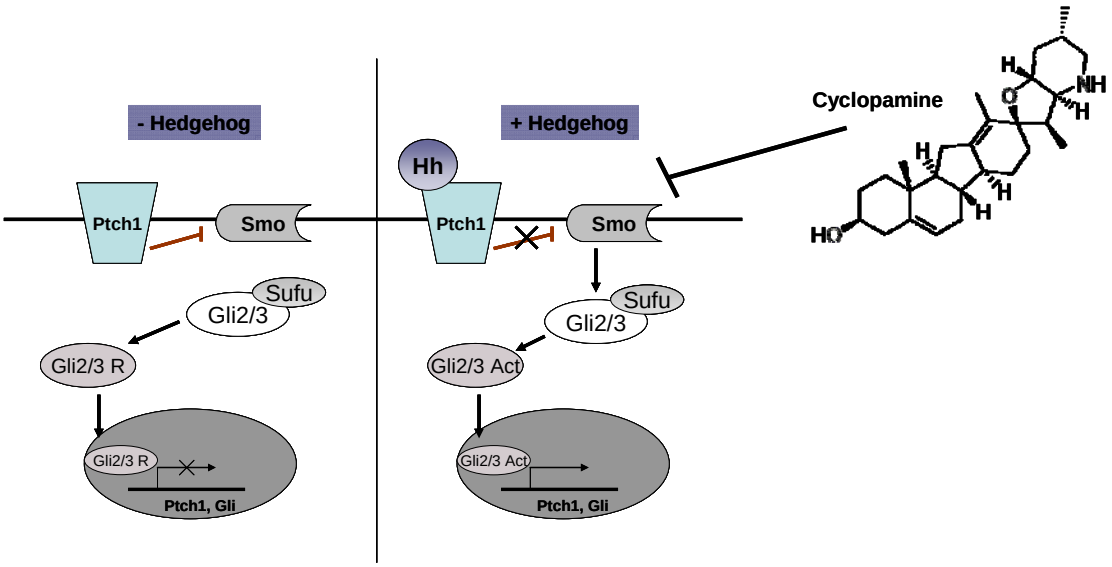


Figure 4. Schematic overview of the hedgehog pathway. Shh signaling occurs through the interaction with the patched1 (Ptch) receptor, which then activates the Gli family of transcription factors. Shh signalling can be blocked using cyclopamine. According to Cohen 2003 and Nagase et al. 2007.

1.8.2 The role of Sonic hedgehog during angiogenesis

There is increasing evidence from the literature that the morphogen Shh plays an important role in the process of embryonic angiogenesis. Lawson et al. demonstrated that Shh signalling derived from the notochords leads to aorta formation in zebrafish embryos (Lawson et al., 2002). Zebrafish embryos that lack Shh activity show practically no arterial differentiation. Another study of Vokes et al., has shown that endodermally derived Shh signalling is essential for embryonic vascular tube formation in avians (Vokes et al., 2004)

and the inhibition of this signal by treating embryos with the Hh inhibitor cyclopamine, the highly specific inhibitor of the Hh co-receptor smoothened, resulted in the disruption of vascular assembly. In addition, several recent publications pointed out the involvement of sonic hedgehog during postnatal vasculogenesis as well. Pola et al, 2001 found the presence of the Shh pathway in mice adult cardiovascular tissues and they were able to activate this pathway *in vivo,* resulting in the induction of angiogenesis through the upregulation of the proangiogenic factors VEGF and angiopoietin1 and 2 (Pola et al., 2001). Sonic hedgehog protein could induce angiogenesis in ischemic limbs and in the cornea of adult mice. Additionally, Straface et al., 2009 demonstrated a crucial function for Shh signalling in regulating angiogenesis and myogenesis during adult postnatal skeletal muscle regeneration (Straface et al., 2008).

Several *in vitro* studies regarding postnatal angiogenesis revealed indirect effects of Shh signalling as well as direct effects on endothelial cells and their angiogenic activity. Asai et al., 2006 reported an upregulation of angiogenic cytokines VEGF and angiopoietin1 in fibroblasts in response to Shh. In addition Shh treatment revealed an upregulation of stromal derived factor-1α (SDF-1) which is known to be a trafficking chemokine for hematopoietic stem cells (Asai et al., 2006). Indirect effects of Shh in terms of angiogenesis were also demonstrated by several other groups who have shown that Shh increased the expression of different proangiogenic factors in fibroblasts that might induce angiogenesis (Fujii and Kuwano, ; Lee et al., 2007; Pola et al., 2001). On the other hand, it has been shown that Shh may also act in a direct manner on endothelial cells and their angiogenic activity. Prior studies have revealed that mature endothelial cells as well as endothelial progenitor cells express the Shh receptor patched1 (Olsen et al., 2004; Yamazaki et al., 2008). Treatment of endothelial cells with microparticles harbouring sonic hedgehog protein enhanced the formation of capillary-like structures on Matrigel basement membrane matrix through the upregulation of different adhesion molecules and proangiogenic factors (Soleti et al., 2009). Asai et al., 2006 also demonstrated a direct action of Shh on the proliferation, migration, adhesion and tube formation of endothelial progenitor cells (Asai et al., 2006).

1.8.3 The role of hedgehog proteins during osteogenesis

It is generally accepted that Hh proteins play an important role during the development of the skeleton, for instance in patterning the axial of the skeleton, during craniofacial

development and in regulating endochondral ossification. During embryonic development Shh is expressed at the posterior site of the limb bud where it regulates the anterior-posterior patterning in a complex regulatory way (Riddle et al., 1993; Yang et al., 1997). An analogous mechanism of Shh action can be found in patterning the craniofacial structures as well (Helms et al., 1997).

Chiang et al, 1996 were one of the first groups to reveal a possible function for hedgehog proteins in the differentiation of osteoblastic cells. They reported that Shh-deficient mice show several defects of the distal limb skeletal elements (Chiang et al., 1996), thus indicating a regulatory function of Shh signalling during the differentiation process of skeletal or osteoblastic cells. *In vitro*, fibroblasts expressing sonic hedgehog could induce osteoblastic differentiation and ectopic bone formation (Kinto et al., 1997; Nakamura et al., 1997). Kinto et al. transfected fibroblast with sonic hedgehog and co-cultured these transfected fibroblasts with pluripotent mouse fibroblastic cell lines, as well as with a mouse osteoblastic cell line. Alkaline phosphatase activity, an early marker for osteoblastic differentiation, was clearly higher when the cell lines were co-cultivated with the Shh-transfected fibroblasts (Kinto et al., 1997). A similar experiment was performed by Nakamura et al. using a mouse mesenchymal stem cell line that was exposed to Shh-conditioned medium. Shh exposure also resulted in an increased ALP activity in the mesenchymal stem cell line (Nakamura et al., 1997). Nevertheless, the detailed mechanisms and signalling cascades, by which hedgehog proteins exactly promote osteoblastic differentiation are still poorly understood. There is increasing evidence that Shh signalling cooperates with different bone morphogenetic proteins like Bmp-2 during osteogenic differentiation (Yuasa et al., 2002).

1.9 Aim of the study

The generation of complex bioengineered tissues such as prevascularized bone constructs still represents a major challenge with respect to both the selection of suitable cell sources for cellular therapy and the identification of new molecular targets for further therapeutic intervention. Focus of the current study is on prevascularization of bone tissue engineered constructs using outgrowth endothelial cells, a subpopulation of endothelial progenitor cells isolated from peripheral blood. In this context, it should be investigated how OEC are able to integrate into complex tissue engineered constructs based on an *in vitro* co-

culture model with primary osteoblasts. This model might permit further insight into underlying mechanisms of bone vascularization. Co-cultures of outgrowth endothelial cells together with primary osteoblasts reveal a positive effect on the angiogenic activation of OEC, indicated by the formation of microvessel-like structures that increase with progressing culture time. In order to generate a stable, functional and long-lasting vasculature, newly formed vessels need to be stabilized through the recruitment of mural cells, including pericytes and vascular smooth muscle cells. Therefore, angiogenic structures induced by the co-culture system need to be investigated in terms of their maturation and stability, especially if angiogenic structures formed by OEC are associated with basement membrane proteins or mural cells. In addition, underlying molecular mechanisms associated with the proangiogenic effect must be analyzed with the aim of generating a stable and complex vascularized bioengineered tissue with regard to a possible application in bone tissue engineering. Furthermore, new concepts to stimulate and control bone regeneration or to develop constructs for bone-replacement are needed which take into consideration both osteogenesis and angiogenesis. Co-culture techniques using outgrowth endothelial cells and primary osteoblasts in combination with additional treatment with growth factors or morphogens represent a promising approach with several advantages in terms of the formation of a stable vasculature and osteogenic differentiation. Besides the more commonly used strategies to improve the vascularization of tissue engineered bone constructs which are widely based on the treatment with classical pro-angiogenic growth factors, such as VEGF, several studies have focused on the role of various morphogens or signalling factors that originally control embryonic development. One promising developmental signalling pathway with significant relevance for both angiogenesis and osteogenesis is mediated by sonic hedgehog. The aim of the present study is to analyze the effects of Shh on angiogenic activation and vessel stabilization as well as on osteogenic differentiation. Underlying mechanisms of Shh action in the co-culture system as well as molecular basics guiding angiogenesis and osteogenesis in response to Shh should also come into focus.

2. Materials and Methods

2.1 Materials

2.1.1 Instruments

Instrument	Model	Manufacturer
Analytical balance	A120S	Sartorius, Göttingen
Balance	LC420	Sartorius, Göttingen
Centrifuge	Megafuge 1.0	Kendro, Langenselbold
Centrifuge	Multifuge X3FR	Thermo Scientific, Langenselbold
CO2 incubator	C200	Labotect, Göttingen
Confocal microscope	DM RE	Leica, Wetzlar
Elektrophoresis	Mini-Protean	Bio-Rad, München
Fluorescent microplate reader	GENios plus	TECAN, Crailsheim
Fluorescent microscope	DM RX	Leica, Wetzlar
Freezing container	Cryo 1°C	Nalgene, Rochester
Heating block	Dri-Block DB-20	Techne, Burlington
Heating block	Thermomixer 5436	Eppendorf, Hamburg
Inverted fluorescent microscope	DM IRBE	Leica, Wetzlar
Laminar flow	HERAsafe KS12	Thermo Scientific
Liquid nitrogen tank	MVE Cryosystem 6000	German-Cryo, Jüchen
Magnet	MPC-1	Dynal, Hamburg
Magnetic stirrer	IKAMAG RET-GS	IKA-Werke, Stauffen
Microcentrifuge	MIKRO120	Hettich, Tuttlingen
Microscope	TS100	Nikon, Düsseldorf
Minicentrifuge	GalaxiMini	VWR, Darmstadt
Multichannel Potentiostat	VMP	Perkin Elkheimer
NanoDrop	ND-1000	NanoDrop, Wilmington
pH meter	InoLab 730	WTW, Weilheim
Plate centrifuge	Biofuge Stratos	Heraeus, Hanau
Power supply	PowerPac HC	BioRad, München
Real Time PCR cycler	7300	Applied Biosystems
Roll-mixer	Assistant RM5	Assistant, Sondheim
Shaker	Reax3	Heidolph, Schwabach
Shaker	Unimax 1010 with incubator 1000	Heidolph, Schwabach
Vacuum pump	Vacusafe Comfort	Integra Bioscience, Fernwald
Vortex	Vortex Genie2	Scientific industries
Water bath	WBT12	Reiss-Daimler Medingen

2.1.2 Consumables

Consumable	Manufacturer
6-well cell culture plates	TPP, Trasadingen
24-well cell culture plates	TPP, Trasadingen
96-well cell culture plates	TPP, Trasadingen
Thermanox coverslips (12mm)	Roth, Karlsruhe
Tubes (15ml, 50ml)	BD Falcon, San Jose
Tubes (1.5ml, 2ml)	Eppendorf, Hamburg
Cell culture flasks (25cm^2, 75cm^2)	TPP, Trasadingen
Cell strainer (40µm, 100µm)	BD Falcon, San Jose
Cell scraper	BD Falcon, San Jose
Object slides	Menzel, Braunschweig
Gelmount mounting media	Biomedia
Coverslips	Menzel, Braunschweig
Cryovials	Nalgene, Rochester
Optical adhesive film	Applied Biosystems, Foster City
Real-Time PCR optical 96-well plates	Applied Biosystems, Foster City
Protran Nitrocellullose transfer membrane	Schleicher und Schüll, Dassel
Scalpels	Braun, Tuttlingen
Parafilm	Pechiney Plastic Packaging, Chicago
Pipettes (5ml, 10ml,25ml)	Greiner, Solingen
Pipette tips (10µl, 100µl, 1000µl)	Greiner, Solingen

2.1.3 Buffers and Solutions

Buffer	composition
PBS 20X	160g NaCl 4g KCl 4g KH_2HPO_4 adjust to 1l aqua dest.
CS-buffer	30,25 g Pipes (0.1M) 0.38g EGTA (1mM) 40g Polyethylenglykol (4%) 4g NaOH adjust to 1l aqua dest.

Buffy coat buffer	2.5 ml FCS 2ml EDTA (2mM) adjust to 1l PBS
TE buffer	100mM Tris/HCl pH 8 10mM EDTA pH 8
SDS-stacking gel	3.3 ml aqua dest. 0.57 ml 1M Tris-HCl ph 6.8 22.5 µl 20% SDS 0.57 ml 40% Acrylamide 45µl 10% APS 4.5µl TEMED
SDS-separating gel (10%)	5.7 ml aqua dest. 3.0 ml 1.5M Tris-HCl ph 8.8 60µl 20% SDS 3.0 ml 40% Acrylamide 60µl 10% APS 6µl TEMED
Laemmli stock buffer	30g Tris 144g Glycine adjust to 1l aqua dest.
Running buffer	200 ml Laemmli stock 5ml 20% SDS adjust to 1l aqua dest.
Transfer buffer	100 ml Laemmli stock 250 MetOH adjust to 1l aqua dest.

2.1.4 Chemicals

Name	**Manufacturer**
Accutase	PAA Laboratories, Austria
40% Acrylamide/Bisacrylamide solution	Biorad, Hercules
APS	Biorad, Hercules
BSA	Sigma-Aldrich, St. Luis
Collagenase Type IV	Sigma-Aldrich, St. Luis
Collagen Type I	ICN Biomedicals, Eschwege
Cyclopamine	Merck, Darmstadt
DMEM F12	Gibco, Carlsbad
dNTP Mix	Quiagen, Hilden
ECL Western blotting detection reagents	Amersham Pharmacia Biotech, Freiburg

EDTA	Sigma-Aldrich, St. Luis
EGM-2 with supplements	Lonza, Verviers
EtOH	AppliChem, Darmstadt
FCS	Sigma-Aldrich, St. Luis
Fibronectin	Roche, Freiburg
Ficoll/Histopaque	Sigma-Aldrich, St. Luis
Formalin	Sigma-Aldrich, St. Luis
H_2O	Braun, Meslingen
H_2SO_4	Merck, Darmstadt
Hoechst 33342	Sigma-Aldrich, St. Luis
Isopropanol	Fluka, Basel
KCl	Calbiochem, Darmstadt
LPS	Sigma-Aldrich, St. Luis
Matrigel® basement membrane matrix	Becton Dickinson Labware, Bedford
MetOH	VWR, Darmstadt
$MgCl_2$ x 6 H_2O	Merck, Darmstadt
Milk	AppliChem, Darmstadt
NaCl	Roth, Karlsruhe
NaOH	Roth, Karlsruhe
n-butanol	Fluka, Basel
Nonfat dried milk powder	AppliChem, Darmstadt
PBS	Gibco, Carlsbad
PBS powder	AppliChem, Darmstadt
PBS tablets	Sigma-Aldrich, St. Luis
Penicillin/Streptomycin	Gibco, Carlsbad
PFA	Merck, Darmstadt
Ponceau S Solution	Sigma-Aldrich, St. Luis
Power SYBR Green Master Mix	Applied Biosystems, Foster City
SYBR Green Master Mix	Qiagen, Hilden
Precision Plus Protein Standards Dual Colour	Bio-Rad, Hercules
Precision Plus Protein Standards Western C	Bio-Rad, Hercules
Precision StrepTactivin-HRP Conjugate	Bio-Rad, Hercules
Random Primer	New England Biolabs, Frankfurt am Main
RNAse Inhibitor	Roche, Freiburg
RNAse free DNAse	Qiagen, Hilden
RotiLoad1 loading buffer	Roth, Karlsruhe
SDS	Serva, Heidelberg

Sonic Hedgehog (N-Terminal)	R&D Systems
Steptavidin HRP	R&D Systems, Wiesbaden
Streptavidin-biotinylated HRP complex	Amersham Pharmacia Biotech, Freiburg
Substrate reagent	R&D Systems, Wiesbaden
TBE	Roth, Karlsruhe
TEMED	Bio-Rad, Hercules
Tris	Roth, Karlsruhe
Tris-HCl 1M	Sigma-Aldrich, St. Luis
Triton-X 100	Sigma-Aldrich, St. Luis
Trypsin-EDTA	Gibco, Carlsbad
Tween 20	Serva, Heidelberg

2.1.5 Cell culture media

Cell culture medium (supplier)	**Supplements**
Endothelial cell basal medium-2 (Lonza)	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 (supplements from the kit) 25 ml FCS, 5 ml Penicillin/Streptomycin
Dulbeccos modified eagle medium /F12 + GlutaMAX™ (Gibco)	10% FCS 1% Penicillin/Streptomycin
Dulbeccos modified eagle medium /F12 + GlutaMAX™ (Gibco)	10% FCS 1% Penicillin/Streptomycin
VascuLifeBasal Medium (Cell systems)	VascuLife SMC Life Factor Kit

2.1.6 Kits

Kit	**Manufacturer**
BCA Protein Assay Kit	Pierce, Rockford
Osteogenesis quantitation kit	Chemicon International
Human Angiopoietin1 Duo Set	R&D Systems, Wiesbaden

Human Angiopoietin2 Duo Set	R&D Systems, Wiesbaden
Human Bmp-2 ELISA	R&D Systems, Wiesbaden
Human Bmp-4 Duo Set	R&D Systems, Wiesbaden
Human Osteocalcin ELISA	Invitrogen
Human PDGF-BB Duo Set	R&D Systems, Wiesbaden
Human Sonic Hedgehog ELISA	USCN-Life, USA
Omniscript RT PCR Kit	Qiagen, Hilden
Proteome Profiler™ Human Angiogenesis Array	R&D Systems, Wiesbaden
RNeasy micro Kit	Qiagen, Hilden
RNeasy mini Kit	Qiagen, Hilden
RT² Profiler PCR Array System Angiogenesis	SABioscience Corporation, USA
RT² Profiler PCR Array System Osteogenesis	SABioscience Corporation, USA

2.1.7 Antibodies

2.1.7.1 Primary Antibodies

Antibody	Source	Dilution	Manufacturer
Anti human CD31	mouse	1:50	Dako, Hamburg
Anti human CD31	goat	1:50	Santa Cruz, Europe
Anti human vWF	rabbit	1:8000	Dako, Hamburg
Anti human VE-Cadherin	mouse	1:100	BD Bioscience, Pharmingen
Anti human smooth muscle actin	mouse	1:100	Dako, Hamburg
Anti human Desmin	mouse	1:100	Dako, Hamburg
Anti human Desmin	mouse	1:500	Monosan, Beutelsbach
Anti human Collagen IV	mouse	1:50	Sigma-Aldrich, St. Luis
Anti human Laminin	mouse	1:50	Sigma-Aldrich, St. Luis

2.1.7.2 Secondary antibodies

Antibody	Source	Dilution	Manufacturer
Alexa Fluor 488 anti mouse	goat	1:1000	Molecular Probes, Carlsbad
Alexa Fluor 488 anti rabbit	goat	1:1000	Molecular Probes, Carlsbad
Alexa Fluor 546 anti rabbit	goat	1:1000	Molecular Probes, Carlsbad

Alexa Fluor 546 anti mouse	rabbit	1:1000	Molecular Probes, Carlsbad
Alexa Fluor 488 anti goat	donkey	1:1000	Molecular Probes, Carlsbad
Anti mouse HRP linked	sheep	1:2000	Amersham, Freiburg

2.1.8 Oligonucleotides

2.1.8.1 Primer

Primer	Sequence
α-smooth muscle actin	5' - TTC AAT GTC CCA GCC ATG TA - 3' 5' - GAA GGA ATA GCC ACG CTC AG - 3'
Calponin	5' - AGG CTC CGT GAA GAA GAT CA -3' 5' - CCA CGT TCA CCT TGT TTC CT - 3'
Smoothelin	5' - GAG TCT GCC CAA GAC CTC AG - 3' 5' - AGT CTT GGC TCG ACA CCA GT - 3'
ß-Actin	5' - GAC CTG ACT GAC TAC CTC ATG A - 3' 5' - AGC ATT TGC GGT GGA TGG AG - 3'
Patched1	5' - ACA AAC TCC TGG TGC AAA CC - 3' 5' - CTT TGT CGT GGA CCC ATT CT - 3'
CD31	5' - CAA CAG ACA TGG CAA CAA GG - 3' 5' - TTC TGG ATG GTG AAG TTG GC - 3'
PDGF-BB	5' – CTT GCA CAC TTC CCC ATC TT - 3' 5' – AGG GGG GAA GTG CAG TAG GT - 3'

2.1.8.2 Primer Assays for quantitative real-time PCR

Primer	Primer assay name	Supplier/Catalogue No.
Alkaline phosphatase	Hs_ALPL_1_SG_QuantiTectPrimerAssay	Quiagen/QT00012957
Angiopoietin-1	Hs_ANGPT1_SG_QuantiTectPrimerAssay	Quiagen/QT00046865
Angiopoietin-2	Hs_ANGPT2_SG_QuantiTectPrimerAssay	Quiagen/QT00100947
α-smooth muscle actin	Hs ACTA_1_SG QuantiTect Primer Assay	Quiagen/QT00088102
Bmp2	Hs_BMP2_1_SG_QuantiTectPrimerAssay	Quiagen/QT00012544
Bmp4	Hs_BMP4_SG_QuantiTectPrimerAssay	Quiagen/QT00012033

Collagen IV	Hs_COL4A1_1_SG QuantiTect Primer Assay	Quiagen/QT00005250
Desmin	Hs_DES_1_SGQuantiTect Primer Assay	Quiagen/QT00071778
GAPDH	Hs_GAPDH_1_SG QuantiTect Primer Assay	Quiagen/QT00079247
Gli3	Hs_Gli3_SG_QuantiTectPrimerAssay	Quiagen/QT00045682
Laminin	Hs_LAMA5_1_SG QuantiTect Primer Assay	Quiagen/QT00077819
Osteocalcin	Hs_BGLAP_1_SG_QuantiTectPrimerAssay	Quiagen/QT00232771
Osteonectin	Hs_SPARC_1_SG QuantiTect Primer Assay	Quiagen/QT00018620
Osteopontin	Hs_SPP1_1_SG_QuantiTectPrimerAssay	Quiagen/QT01008798
Patched1	Hs_PTCH_1_SG_QuantiTectPrimerAssay	Quiagen/QT00075824
PDGF-BB	Hs_PDGFB_SG_QuantiTectPrimerAssay	Quiagen/QT00001260
PI3K	Hs_PIK3CA_1_SG QuantiTect Primer Assay	Quiagen/QT00014861
RPL13A	Hs_RPL13A_1_SG_QuantiTectPrimerAssay	Quiagen/QT00089915
Runx2	Hs_RUNX2_1_SG_QuantiTectPrimerAssay	Quiagen/QT00020517
Sonic Hedgehog	Hs_Shh_3_SG_QuantiTectPrimerAssay	Quiagen/QT01156799
Tgfß	Hs_TGFB2_SG_QuantiTectPrimerAssay	Quiagen/QT00025718
VEGFA	Hs_VEGFA_2_SG_QuantiTectPrimerAssay	Quiagen/QT01036861
Von Willebrand factor	Hs_VWF_1_SG QuantiTect Primer Assay	Quiagen/QT00051975

2.2 Methods

2.2.1 Isolation and expansion of outgrowth endothelial cells (OEC) from mononuclear cells

Endothelial progenitor cells circulating in the peripheral blood are a heterogenous population of cells. According to their order of appearance, their morphology and their marker profile, endothelial progenitor cells can be divided into at least two subpopulations, namely early endothelial progenitor cells and late endothelial progenitor cells, so-called outgrowth endothelial cells (Fuchs et al., 2006). Both subpopulations can be isolated from the mononuclear cell fraction from peripheral blood buffy coats by ficoll/histopaque separation which is used to separate blood into its components. Therefore, peripheral blood was diluted 1:2 in phosphate buffered saline (PBS) containing 0.5% fetal calf serum (FCS) and 2mM ethylene diamine tetraacetic acid (EDTA) to prevent cells from clotting and subsequently centrifuged for 35 min at 400 g (without braking) with histopaque placed at the bottom of a

50ml tube. After centrifugation 3 different components become visible, from the bottom up erythrocytes, mononuclear cells and plasma. The mononuclear cell fraction was separated, washed several times in PBS and cultured in endothelial cell growth medium-2 (EGM-2) with supplements from the kit, 5% fetal calf serum and 1% penicillin/streptomycin on collagen-coated plates (35µg/ml). A total number of 5 * 10^6 cells per well were seeded on a 24-well plate. Three times a week the cells were fed with fresh medium. Early EPC appear in culture within 4-7 days with a spindle-shaped morphology and a limited expansion capacity. After 3-4 weeks, colonies with a cobblestone-like morphology appeared. These late outgrowth endothelial cells (OEC) with a typical mature endothelial marker profile and a high proliferation potential, were trypsinized and expanded on fibronectin-coated 24-well plates (10µg/ml) over several passages in a splitting ratio of 1:2. Passage numbers of OEC used for this study ranged from passage 8 to passage 20.

2.2.2 Isolation of primary osteoblasts (pOB)

Human primary osteoblasts (pOB) were isolated from human cancellous bone fragments from healthy donors according to an established protocol (Hofmann et al., 2003). Bone explants were first transferred into a sterile tube and washed several times with phosphate-buffered saline (PBS). Finally, collagenase type IV at a concentration of 1mg/ml was added for an incubation time of 1 hour at 37°C. After the enzymatic digestion bone fragments were washed several times in PBS and subsequently placed on 6-well plates cultured in Dulbecco's modified Eagle medium containing 20% FCS and 1% penicillin/streptomycin (DMEM-Ham F12). During a culture period of 2-4 weeks, cells were fed every day with DMEM-Ham F12 containing 20% FCS. When 6-well plates were approaching subconfluence, cells were transferred into T75 culture flasks cultured with DMEM-Ham F12 containing only 10% FCS and 1% penicillin/streptomycin. Cells were passaged in a ratio of 1:2 using accutase. For the present studies cells were used from several donors up to the third passage.

2.2.3 Isolation of human umbilical artery smooth muscle cells

Human umbilical artery smooth muscle cell (HUSMC) isolation was performed according to different publications (Okker-Reitsma et al., 1985; Tull et al., 2006). HUSMC

were isolated from human umbilical cords under sterile conditions. Arteries were excised from the cords, stripped off from the surrounding tissue and cut into 1mm rings in a sterile petri dish filled with PBS. Up to 10 rings were placed into one well of a 6-well plate and cultured in Dulbecco's modified eagle medium containing 20% FCS, 2ng/ml bFGF and 1% Penicillin/Streptomycin. When cells were approaching confluence after approximately 2 weeks, the amount of serum was reduced to 5% to avoid the outgrowth of fibroblasts. Cells were then transferred from the 6-well plates to T75 culture flasks and cultured in VascuLifeBasal Medium supplemented with VascuLife SMC LifeFactor Kit optimized for the culture of human smooth muscle cells.

2.2.4 Co-culture of outgrowth endothelial cells (OEC) and primary osteoblasts (pOB)

Outgrowth endothelial cells (OEC) from several donors were expanded over several passages before they were used for co-culture experiments. Passage numbers of OEC used for the co-culture experiments ranged from passage 8 to passage 20. Primary osteoblasts were used in the co-culture system in passage 3 at the latest. For each co-culture experiment at least three different donors were used.

2.2.4.1 Static co-culture

Co-cultures of outgrowth endothelial cells and primary osteoblasts were grown on fibronectin-coated (10µg/ml) Thermanox coverslips (12mm in diameter) in 24-well plates (1.766 cm^2), always seeding primary osteoblasts first (300.000/well). After one day OEC were added (200.000/well). The seeding order of OEC and pOB as well as the culture conditions in the co-culture used in this study was based on the work of Fuchs et al., 2007 (Fuchs et al., 2007). In this study the authors analysed effects of the seeding order on the co-culture system by seeding both cell types simultaneously or in different seeding steps and analysed the co-cultures after 1 week for the endothelial marker CD31 and von Willebrand factor (vWF). Microvessel-like structures could be observed when OEC were added in a second step. The co-cultures were fed 3 times per week with EGM-2 with supplements from the kit, 5% FCS and 1% Penicillin/Streptomycin. The stability of OEC at the single cell level within the co-culture with pOB was also assessed in previous work using flow cytometry and RT-PCR for endothelial markers after different cultivation time points (Fuchs et al., 2007).

The number of OEC in the co-culture stabilized around 12 % after 2 weeks of co-cultivation compared to the initial ratio of 40 % OEC which could also be confirmed at the gene expression level.

In this study, co-cultures of pOB and OEC from various cultivation time points were further processed as described in the following sections for immunofluorescence staining, gene expression analysis and protein analysis.

2.2.4.2 Co-cultures seeded on Transwells®

The use of a Transwell® filter system allows different cell types in a co-culture system to take up and secrete growth factors and signalling molecules on both sides of a transmembrane filter, their basal and their apical surface. Thereby it is possible to promote the metabolic activities of these two cell types within a co-culture system. 100.000 primary osteoblasts were seeded at the lower surface and 66.000 OECs were seeded at the upper surface of a polycarbonate transmembrane filter in a Transwell® filter system in a 24-well plate (pore size 0.4µm; 0.588 cm^2 / filter) coated with fibronectin (10µg/ml) to gain insighs into the origin of several growth factors produced by the different cell types in the co-culture. Cells were fed with EGM-2 with supplements from the kit, 5% FCS and 1% penicillin/streptomycin and cultured for different time periods. Culture supernatants as well as cell lysates of OEC and pOB were collected separately from the Transwell® filter system and then used for further experiments.

2.2.4.3 Generation of 3-D constructs of OEC and pOB

Three-dimensional (3-D) constructs of cell cultures can be generated under dynamic conditions using a rotating cell culture vessel system as already described. Therefore, cells and culture media were added in a disposable culture vessel system and were cultured under permanent rotation about a horizontal axis at a constant slow speed in order to form 3-dimensional cellular spheroids or aggregates. $2 * 10^6$ primary osteoblasts as well as $2 * 10^6$ OEC were placed together in one 10ml culture vessel system and cultured in complete EGM-2 with supplements from the kit, 5% FCS and 1% penicillin/streptomycin for 3-4 weeks and under permanent rotation according to previous studies (Fuchs et al., 2007). Fresh medium

was added every second day. The spheroids were harvested from the vessel system using a pipette tip and used for frozen sections and further for immunohistochemistry.

2.2.5 Seeding of OEC on Matrigel® Basement Membrane Matrix

Matrigel® Basement Membrane Matrix was thawed on ice at 4°C overnight and diluted 1:2 in cold EBM-2 with supplements from the kit/5% FCS and 1% P/S. 50µl of this dilution was added to each well of a 96-well plate and incubated for 30 minutes at 37°C to allow the polymerization of the Matrigel. 50.000 OEC per well of a 96-well plate were resuspended in 200µl of EBM-2 and gently pipetted on top of solidified Matrigel® Basement Membrane Matrix. OEC were treated directly after seeding with sonic hedgehog in different concentrations (2µg/ml or 5µg/ml), simultaneously with Shh and its inhibitor cyclopamine in different concentrations (5µM, 10µM, 20µM, 40µM) or with 50ng/ml vascular endothelial growth factor (VEGF) serving as a positive control for the angiogenic activation of OEC. 24 hours later, cells were imaged using an inverse microscope.

2.2.6 Sonic hedgehog stimulation of co-cultures consisting of pOB and OEC

Co-cultures were seeded as previously described on Thermanox coverslips in 24-well plates cultured in EGM-2 with supplements from the kit, 5% FCS and 1% penicillin/streptomycin. After 1 week of co-cultivation, cells were treated with 5µg/ml recombinant human sonic hedgehog (Shh) in EBM-2 with supplements from the kit, 5%FCS and 1% P/S for 24 hours and 14 days. Sonic hedgehog stimulated co-cultures were further processed for immunofluorescence staining, gene expression analysis and protein analysis. OEC and pOB mono-cultures were stimulated with recombinant Shh as well, serving as controls.

2.2.7 Cyclopamine treatment of co-cultures consisting of pOB and OEC

In control experiments co-cultures were co-cultivated for 1 week in EGM-2 with supplements from the kit, 5% FCS and 1% penicillin/streptomycin as previously described before they were treated with the sonic hedgehog inhibitor, cyclopamine at different

concentrations (5µM, 10µM, 20µM) in EGM-2 for different time points. Additionally, co-cultures were treated for 24 hours simultaneously with 5µg/ml sonic hedgehog plus cyclopamine at different concentrations (5µM, 10µM, 20µM) in EGM-2 with supplements from the kit, 5%FCS and 1% P/S. Stimulated co-cultures were further processed for immunofluorescence staining, gene expression and protein analysis.

2.2.8 VEGF treatment of co-cultures consisting of pOB and OEC

Co-cultures were seeded as previously described. After 1 week of co-cultivation, cells were treated with 50ng/ml recombinant human vascular endothelial growth factor (VEGF) in EGM-2 with supplements from the kit, 5%FCS and 1% P/S for 24 hours and 14 days. Culture supernatants were harvested and stored at -80°C until use for ELISA. Cells were fixed for immunofluorescence staining and lysed for gene expression and protein analysis.

2.2.9 Cryostat sectioning

Cell layers of static co-cultures consisting of OEC and pOB as well as 3-dimensional co-cultures of OEC and pOB generated in a rotating vessel system of different donors and different cultivation conditions and periods were snap frozen in liquid nitrogen and sectioned at a thickness of 10µm using a cryostat. Samples were stored at -20°C until use for immunohistochemical analysis. For immunofluorescent staining the slices were first thawed at room temperature before starting with the staining procedure according to 2.2.11.

2.2.10 Paraffin sectioning

Co-cultures of primary osteoblasts and outgrowth endothelial cells were seeded on the upper surface of a fibronectin-coated transmembrane filter in a Transwell® filter system and were stimulated with either 5µg/ml sonic hedgehog or 50ng/ml VEGF for 14 days as previously described. After 14 days of stimulation membranes were fixed with 3.7% PFA for 10 minutes and washed with PBS subsequently. The fixed membranes were cut into appropriate sizes and put into embedding cassettes. The samples were dehydrated for paraffin embedding in an ascending alcohol series (70%, 80%, 95%, 100%), each for 1 hour. After an

incubation step in xylene for an additional hour, membranes were embedded into paraffin blocks and cut into 4µm slices. For immunofluorescent staining of paraffin sections the sclices were rehydrated in a descending ethanol series (100%, 95%, 80%, 70%) and rinsed in distilled water before staining according to the protocol described below.

2.2.11 Immunofluorescent staining

For immunofluorescent staining cell culture medium was removed and cells were washed several times with phosphate-buffered saline (PBS). After fixation with 3.7% paraformaldehyde (PFA), cells were washed three times with PBS and permeabilized for 5 minutes using 0.1% Triton-X in PBS. Cells were washed again with PBS before being incubated with different primary antibodies diluted in 1% bovine serum albumin (BSA) in PBS for 45 minutes at room temperature. After washing 3 times with PBS, cells were incubated with fluorescently labelled secondary antibodies diluted in 1% BSA in PBS for 45 minutes in darkness at room temperature. Following this, cell nuclei were counterstained with 1µg/ml Hoechst and cells were mounted with Gelmount. The stained coverslips as well as the frozen and paraffin sections were examined using a confocal laser scanning microscope (LeicaTCS-NT).

2.2.12 Gene expression analysis

2.2.12.1 RNA isolation

RNA isolation was performed using RNeasy Mini Kit. The RNeasy Kits combine the RNA binding properties of a silica membrane with the speed of microspin technology. Cells from different experiments were trypsinized, centrifuged and lysed in RLT buffer containing high denaturing guanidine isothiocyanate which inactivates RNases and ß-mercaptoethanol (10µl/ml) to stabilize the lysates. If the cell lysates are not processed immediately they were stored at -80°C until use. For complete homogenization cell lysates were directly pipetted into a shredder spin column and centrifuged for 2 minutes at full speed. RNA was precipitated using 1 volume of 70% ethanol. After mixing gently the precipated sample was transferred to an RNeasy spin column where the total RNA binds to the silica membrane. Several washing steps with different buffers removed contaminants efficiently and are described as follows.

After washing with 350µl RW1 buffer the column was digested with DNase for 15 minutes at room temperature, followed by another washing step with 350µl RW1 buffer. After washing twice with 500µl RPE buffer RNA was eluted with 30µl RNase free water. RNA concentration was measured using a NanoDrop spectrophotometer. RNA with a concentration of at least 60ng/µl was used for complementary DNA synthesis.

2.2.12.2 Reverse transcription

One µg of extracted RNA was used to transcribe into complementary DNA (cDNA) according to a standard protocol using Omniscript Reverse Transcription Kit (Qiagen). Therefore 2µl Reverse Transcription buffer (RT buffer), 2µl Random Primer, 2µl nucleoside triphosphate (dNTPs), 1µl Reverse Transcription Omniscript enzyme, 1µl RNase inhibitor and 10µl template (1µg of RNA diluted in RNase free water) was used. The mixture was incubated at 37°C for 1 hour.

2.2.12.3 RT-PCR (reverse transcription polymerase chain reaction)

Polymerase Chain Reaction (PCR) was used to amplify different DNA products exponentially by *in vitro* enzymatic replication using a DNA polymerase. The denaturation step consists of heating the reaction to 94°C for 30 sec and causes melting of DNA template and primers by disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA. During the annealing step the reaction temperature is lowered to 50-65°C for 30 sec allowing annealing of the primers to the single-stranded DNA template. The polymerase binds to the primer-template hybrid and begins to synthesize DNA. For the elongation step a temperature of 72°C is commonly applied, depending on the type of polymerase. For PCR, a taq polymerase kit was used. Different self-designed primers were used at a concentration of 10pmol/µl. The following thermocycler program was applied:

94°C 2min, denaturation step: 94°C 30sec, annealing step : 60-65°C (depending on the primer composition) 30sec, elongation step: 72°C 30sec, 72°C 10min, 35 cycles were performed in total.

2.2.12.4 Gel electrophoresis

After amplification different PCR products were separated by their molecular weight using gel electrophoresis. By placing the molecules in wells in an agarose gel in an electric field, negatively charged DNA will move through the gel at different rates towards the positively charged anode. Therefore RT PCR products were loaded into a 1% agarose gel and electrophoresed for 45 minutes at 100 V in 1% Tris-Borat-EDTA buffer (TBE buffer) and stained with the intercalating agent ethidium bromide and the use of ultraviolet light. To determine the size of the products a common 1kb molecular weight size marker was used.

2.2.12.5 Quantitative Real-Time RT-PCR

Quantitative real-time PCR, enabling the quantification of relative gene expression, was performed using SYBR green DNA binding fluorescent dye. 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 (1ng/µL) were used for one reaction. Quantitative real-time PCR was performed in triplicates with the following cycler program:

95°C 15 min, denaturation step: 94°C 15 sec, annealing step: 55°C 30 sec, elongation step: 72°C 35 sec; dissociation: 95°C 15 sec, 60°C 1min, 95°C 15 sec, 40 cycles were performed in total.

Glycerin-aldehyde-3-phosphate (GAPDH) or ribosomal protein 13A (RPL13A) were taken as an endogenous standard and relative gene expression was determined using the ΔΔCt method. Gene expression was compared by setting control cultures to 1 (reference value) as indicated in the relevant figures.

2.2.12.6 RT^2 Profiler PCR Array System

The RT^2 Profiler PCR Array System is a pathway focused gene expression screening method using quantitative real time PCR. RT^2 Profiler PCR Arrays contain a panel of 96 primer sets per one 96 well plate for a researched set of 84 relevant pathway-focused genes plus 5 different housekeeping genes. Angiogenesis and osteogenesis RT^2 Profiler PCR Array

System were performed with 2ng cDNA of different experiments according to the manufacturers' protocol. For one reaction 12.5µl 2x SABiosciences RT^2 qPCR Master Mix, 1µl cDNA and 11.5µl RNase free water was used. The following cycler program was applied:

95°C 10 min; denaturation step: 95°C 15 sec, annealing step: 60°C 1 min; elongation step: 72°C 35sec, dissociation: 95°C 15 sec, 60°C 1min, 95°C 15 sec, 40 cycles were performed in total.

Ribosomal protein 13A (RPL13A) was taken as an endogenous standard and relative gene expression was determined using ΔΔCt method. The gene expression was compared by setting control cultures to 1 (reference value) as indicated in the relevant figures.

2.2.13 Protein expression analysis

2.2.13.1 Enzyme-linked Immunosorbent Assay (ELISA)

Culture supernatants from differently treated cells were collected and the concentrations of different growth factors were measured using ELISA DuoSets® or precoated 96-well ELISA plates. ELISA DuoSets were performed according to the manufacturers' protocol in triplicate. 96-well microplates were coated with 100µl per well of diluted capture antibody and incubated over night at room temperature while shaking (50 rounds per minute). Next day, plates were washed three times with PBS containing 0.05% Tween and blocked with 1% BSA in PBS for at least 1 hour at room temperature. After washing another three times, 100µl of the sample/standard diluted in 1% BSA in PBS was added to the plates and incubated for 2 hours at room temperature while shaking. Another washing step followed before adding 100µl of the detection antibody diluted in 1% BSA in PBS to each well for another 2 hours. A streptavidin-HRP (horseradish-peroxidase) colorimetric reaction was used to visualize protein concentrations. The optical density of each well was measured using a microplate reader (GENios plus) and a wavelength of 450 nm. Results are demonstrated in percentaged ratio to the control (control = 100%) or additionally shown as absolute values as indicated in the relevant figures. The same protocol was used for the 96-well ELISA plates, beginning with the blocking step.

2.2.13.2 Protein extraction and quantification

For cell protein extraction cells were trypsinized, centrifuged and finally lysed with 0.1% Triton-X in 0.1M Tris buffer ph 7.2. Cell lysates were mixed for 45min at 4°C, centrifuged and supernatants were transferred to new tubes and stored at -20°C until use. A BCA (bicinchoninic acid) protein Assay Reagent Kit was used to determine the protein concentration according to the manufacturers' instructions. BCA uses the reduction of Cu^{2+} to Cu^{+} by proteins in alkaline medium and the detection of these Cu^{+} in the protein lysates. Samples and standards were transferred to a 96-well plate (25µl each) and 200µl working reagent was added to each well. After an incubation time of 30 minutes at 37°C in darkness, the optical density was measured at 550nm using a microplate reader. Protein extracts were used for different protein arrays and SDS-Page (polyacrylamide electrophoresis).

2.2.13.3 SDS Polyacrylamide electrophoresis (SDS-Page)

Protein lysates were separated by their molecular weight using SDS polyacrylamide gel electrophoresis (SDS-Page) (Shapiro et al., 1967). The gel consists of 2 different components, a 5% stocking gel and a 10% separating gel, depending on the size of the proteins. The liquid separating gel (10% Polyacrylamide, 375 mM Tris-HCl pH 8.8, 0.1% SDS, 0.05% APS (ammonium persulfate), 0.005% TEMED) was poured between two glass plates and subsequently overlayed with n-butanol till gel polymerization. Afterwards n-butanol was removed and stacking gel was added (5% PAA, 125mM Tris-HCl pH 6.8, 0.1% SDS, 0.05% APS, 0.005% TEMED) on top of separating gel with a comb inserted between the two glasses. 20µg protein was mixed with RotiLoad-1 loading buffer (1:4) and incubated at 95°C for 5 min for protein denaturation before loading the samples into the wells of the stacking gel. Separation of proteins was performed at 25mA in SDS-running buffer (25mM Tris, 192mM glycine, 0.1% SDS).

2.2.13.4 Western Blot

Following SDS-Page, separated proteins were blotted from the gel onto a membrane made of nitrocellulose to make the proteins accessible for the antibody detection. Therefore, the nitrocellulose membrane was placed on top of the gel with 3 wet filters placed on both

sides of the gel/membrane. The gel/membrane with the wet filters was covered with wet sponges and put into a mini transfer chamber filled with SDS transfer buffer (25mM Tris-HCl, pH 8.0, 100mM glycine, 25% methanol) for 1 hour at 350mA. Protein transfer was controlled by Ponceau-S staining. Afterwards the membrane was blocked in 5% milk powder containing 0.2% Tween PBS (blocking solution) for 1 hour at room temperature and subsequently incubated with the primary antibody diluted in blocking solution overnight at 4°C. After washing 3 times with PBS 0.2% Tween for 5min, each membrane was incubated with the HRP-conjugated secondary antibody diluted in blocking solution for 2 hours at room temperature. To remove unbound antibody the membrane was washed again 3 times before the antibody was detected using enhanced chemiluminescent (ECL) detection reagents. Afterwards membrane was stripped with stripping buffer (100mM Glycine-HCl pH 2.8) and incubated with an antibody against ERK-2 to evaluate the protein loading per lane.

2.2.13.5 Protein arrays

Total protein concentration of different treated cultures was determined as described above and measured at 550nm using a microplate reader. Subsequently, a Proteome Profiler™ Human Angiogenesis Array study was performed according to the manufacturers' protocol to detect angiogenesis-related proteins (55 different proteins in total) within the different treated cultures. After blocking the membranes for 1 hour with blocking buffer, membranes were incubated with the detection antibody cocktail including 200µg/ml protein per sample over night at 4°C while shaking. Following several washing steps with wash buffer at room temperature, a streptavidin-HRP colorimetric reaction was used to visualize protein concentrations via a chemiluminescent detection reagent (Western lightning-ECL). Samples were analysed using Array-Pro Analyzer Version 4.5. Results are referred to a positive control and depicted as mean pixel densities, which were finally converted into percent in ratio to control. All individual values were referred to control co-cultures set to 100% (reference value).

2.2.14 Quantification of mineralization using alizarin red

To quantify the mineralization in different treated co-cultures and pOB mono-cultures, an Osteogenesis Quantitation Kit was used according to the manufacturers' protocol. Cells

were washed with PBS and fixed in 3.7 % PFA for 15 minutes at room temperature. After removing the fixative by washing three times with distilled water, 1ml/well of a 24-well plate 1x alizarin red staining solution was added and incubated at room temperature for at least 20 minutes. The excess dye was removed and cells were washed four times with deionized water. 400µl 10% acetic acid was added to each well for 30 minutes. Loosely attached cell monolayers were transferred to a 1.5 ml tube, vortexed and heated to 85°C for 10 minutes. After a centrifugation step of 15 minutes at 20.000 x g and neutralizing the pH with 10% ammonium hydroxide, 150 µl of the supernatants were used for quantification of alizarin red on a transparent 96-well plate and measured in triplicates at OD_{405} using a microplate reader. Alizarin red concentration was defined as µM alizarin red/mg protein. A BCA (bicinchoninic acid) protein Assay Reagent Kit (Pierce) was used to determine the protein concentration according to the manufacturers' instructions as already described.

2.2.15 Determination of alkaline phosphatase activity within the cell lysate

The matrix mineralization during bone formation is mainly mediated through the activity of alkaline phosphatase, an osteoblastic differentiation marker. Quantitative determination of alkaline phosphatase (ALP) activity within the cell lysates of Shh-stimulated and unstimulated co-cultures and pOB mono-cultures was performed using p-nitrophenyl phosphate (pNPP). Briefly, cells were lysed with 0.1% Triton X-100 in 0.1M Tris buffer pH 7.2, harvested with a cell scraper and incubated at room temperature for 45 min while vortexing every 15 min. 20µl of each sample were incubated with 40µl of substrate solution (0.2% pNPP in 1M diethanolamine HCl) in triplicates in a 96-well plate for 30 min at 37°C. After the incubation period 80µl of stop solution (2M NaOH/0.2 mM EDTA) was added to each well and absorbance was measured at 405 nm using a microplate reader. Absolute alkaline phosphatase activity was defined as mM pNP/mg protein

2.2.16 Image quantification

Image quantification was performed by MetaPhysiol as described in the following section. Microscropic images were analyzed using the software ImageJ 1.43 (Rasband, 1997-2007). In brief, the immunofluorescence images were corrected for the fluorescent background of the matrix by subtracting a corresponding UV-image. Tube-like structures

were extracted from the remaining background after automatic thresholding. The resulting binaries were manually corrected as overlay on the original image using Adobe Photoshop CS2. The corrected binary images reflecting the tube-like network were characterized with ImageJ. The percent area of extracted structures was calculated from black and white pixels of the binary and the total length was measured after skeletonization. The branching points (nodes) of the skeleton were determined by locating the pixels with more than two neighbours. Statistical analysis was performed with MS-Excel (Student's t test, paired, two-tailed distribution) for at least 3 different donors for each experiment and each culture condition. Per individual donor and per each culture condition, 3 images were used for quantification.

2.2.17 Statistical analysis

All experiments were performed with at least 3 different donors. The data are presented as mean values ± standard deviation. Statistical significance was evaluated using the paired student's t-test. Statistical analyses were performed with MS Excel (Microsoft Office, Microsoft) and significance was assessed by * p-value < 0.03 or * p-value < 0.05, respectively.

3. Results

3.1 Co-culture system of outgrowth endothelial cells and primary osteoblasts

3.1.1 Morphology of outgrowth endothelial cells (OEC)

Endothelial progenitor cells (EPC) isolated from the mononuclear cell fraction from peripheral blood by ficoll density gradient centrifugation are a heterogenous population of cells. They were classified on the basis of their morphological characteristics and the order of appearance in two different cell types, early EPC and late EPC, so-called late outgrowth endothelial cells (OEC). In contrast to early EPC which appear after 1 week of cultivation (figure 5A), outgrowth endothelial cells (OEC) or late endothelial progenitor cells appear as small colonies in culture after 3 or 4 weeks of cultivation (figure 5B).

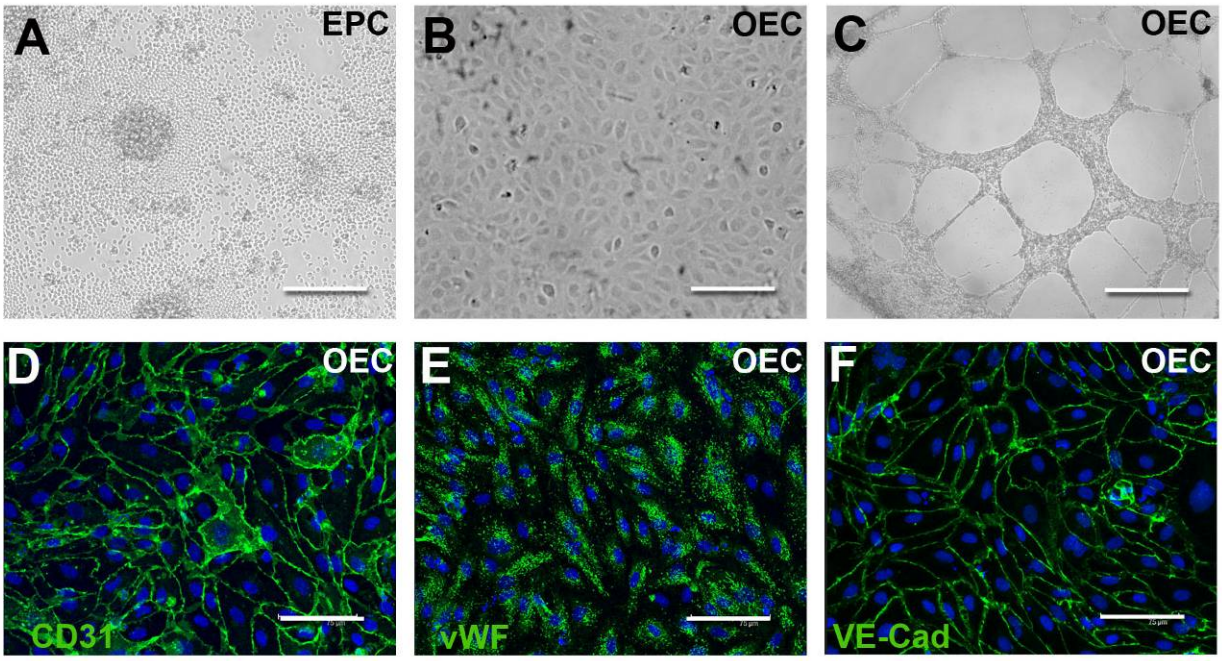


Figure 5. Characterization of outgrowth endothelial cells. In contrast to early EPC (A), OEC show a cobblestone-like morphology (B) and a high angiogenic potential when seeded on Matrigel basement membrane matrix (C) for 24 hours. D-F: OEC were stained by immunofluorescence for the endothelial marker CD31, vWF and VE-cadherin. Cell nuclei were counterstained with Hoechst. Scale bars: A/B 100µm; C 200µm; D-F 75µm.

These small colonies with a cobblestone-like morphology show, in contrast to early EPC, similarities to mature endothelial cells i.e. in terms of their phenotype, their marker profile and their angiogenic potential *in vitro* and *in vivo*. The characterization of OEC is depicted in figure 5. Outgrowth endothelial cells exhibited a typical endothelial cell morphology, have shown a high angiogenic potential when seeded on Matrigel® basement membrane matrix and a mature endothelial cell surface marker profile. OEC were positiv for the endothelial marker CD31 (also called PECAM-1 or platelet endothelial cell adhesion molecule-1), von Willebrand factor and VE-cadherin evaluated using immunohistochemical staining.

3.1.2 Co-Cultures of OEC with primary osteoblasts revealed a positive effect on the cellular organization of OEC into angiogenic structures

Previous studies already documented the angiogenic potential of OEC co-cultured with primary osteoblasts (pOB) or co-cultured with MG63, an osteoblastic cell line (Fuchs et al., 2007). In this study of Fuchs et al., OEC revealed a considerable organization into vascular structures in both types of co-cultures, whether co-cultivated with primary osteoblasts or with MG63. The formation of microvessel-like structures was achieved by co-culturing of OEC with MG63 or primary human osteoblasts in the standard culture medium for the OEC, independently of growth factor addition. In contrast to OEC in co-culture with osteoblasts, the formation of microvessel-like structures could not be observed in OEC mono-culture using the same cell culture medium.

Initial experiments in this thesis aimed at a better understanding of the interaction between OEC and primary osteoblasts in the co-culture system in terms of angiogenic activation, vessel maturation and osteogenic differentiation. Outgrowth endothelial cell were co-cultured with primary osteoblasts in endothelial cell growth medium (EGM-2) and analyzed using immunofluorescent staining for the endothelial marker PECAM (CD31) after 4 weeks of co-cultivation and compared to OEC in mono-culture (figure 6). After 4 weeks in the co-culture, OEC formed numerous microvessel-like structures, reminiscent of a prevascular network depicted in figure 6B/C. In the co-culture system with pOB, endothelial cells looked elongated and they formed intercellular contacts and luminar structures demonstrated by CD31 immunofluorescent staining of cryostatic sections (figure 6D/E). This formation of a vascular lumen by OEC in the co-culture could also be observed in paraffin sections of co-

cultures seeded on a transmembrane filter in a Transwell® filter system (figure 6F). Mono-cultures of OEC failed to organize into tube-like structures (figure6A).

According to the described study of Fuchs et al., the co-culture system consisting of primary osteoblasts and outgrowth endothelial cells revealed a remarkable effect on the angiogenic activation of OEC resulting in the formation of microvessel-like structures. This organization of OEC into angiogenic structures in the co-culture system clearly increased during the course of co-cultivation from 1 to 4 weeks of co-cultivation. Nevertheless, the molecular mechanisms that might control and support this proangiogenic effect within the co-culture system of OEC and pOB are currently not well understood.

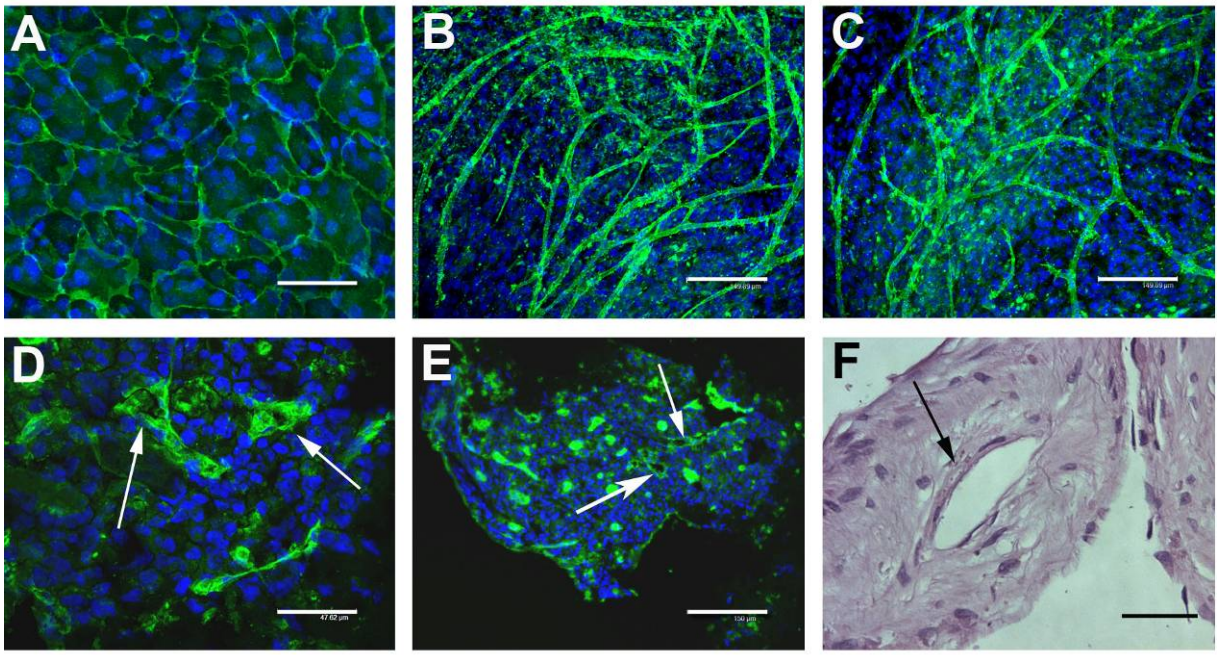
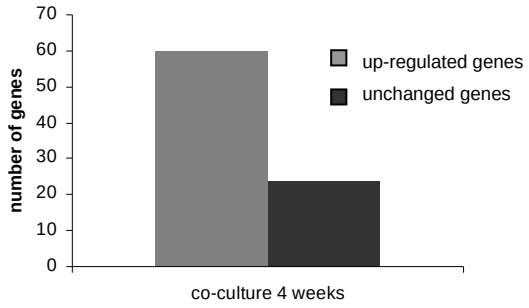


Figure 6. Co-cultures consisting of OEC and pOB. (A) OEC in mono-culture seeded on culture plastic failed to organize into angiogenic structures. (B) OEC in co-culture formed remarkable microvessel-like structures after 4 weeks of co-cultivation with pOB under static conditions (B/C) as well as under dynamic conditions generated in a rotating vessel system depicted in D/E (cryostat sections). Vessel lumen formed by OEC after 4 weeks of co-cultivation are depicted in (E/F). Cell nuclei were counterstained with Hoechst. OEC were stained using CD31 (green). Scale bars: A/D/F 50µm; B/C/E 150µm. n=6.

Due to the fact that the formation of angiogenic structures formed by OEC increased clearly during the course of co-cultivation from 1 week to 4 weeks, a human Angiogenesis RT^2 Profiler Array (PCR Array) was used to compare the expression of angiogenesis-related factors after 1 and 4 weeks of co-cultivation (figure 7). The aim was to gain insight into the molecular mechanisms and possible factors that are responsible for the proangiogenic effect of the co-culture on the OEC. The human Angiogenesis RT^2 Profiler Array profiles the expression of more than 80 genes involved in modulating the biological processes of angiogenesis and includes growth factors, their receptors, chemokines, cytokines, matrix molecules and adhesion molecules. In total, 60 genes involved in the process of angiogenesis were upregulated after 4 weeks of co-cultivation compared to 1 week, as depicted in the diagram (light grey bar) and accurately listed in the table in figure 7.



co-culture 4 weeks		co-culture 4 weeks		co-culture 4 weeks	
Akt1	1,781	FGF2	6,104	PGF	6,968
ANGPT2	3,7305	FGFR3	2,8705	PLAU	3,1175
ANGPTL4	4,051	HIF1A	4,262	PLXDC	4,0845
ANPEP	3,4175	ID1	3,9545	SERPIN	4,5425
CCL11	5,3905	ID3	2,6685	SPHK1	1,606
CCL2	1,257	IFNA1	7,2635	STAB1	2,345
CDH5	2,3585	IFNB1	2,051	TGFB1	1,4345
COL4A3	4,395	IFNG	2,2695	TGFBR	6,836
CXCL1	2,142	IL1B	5,8755	THBS1	1,746
CXCL3	6,3355	ITGAV	2,2775	THBS2	2,6205
CXCL5	8,175	ITGB3	2,084	TIMP1	2,998
CXCL6	7,2845	KDR	1,053	TIMP2	5,281
ECGF1	1,444	LAMA5	4,664	TIMP3	7,212
EDG1	7,19	LECT1	1,7375	TNF	3,6865
EFNA1	5,236	LEP	9,42	TNFAI2	1,838
EFNB2	5,4855	MDK	3,5285	VEGFA	5,411
ENG1	2,212	MMP2	5,197	VEGFC	6,892
EPHB4	3,6795	Notch4	3,54	COL18	220,803
EREG	3,5655	PDGFA	2,221	FIGF	108,783
FGF1	4,211	PECAM	1,9759	TGFB2	410,711

Figure 7. Screening of relative gene expression of angiogenesis related genes in co-cultures consisting of pOB and OEC after 1 and after 4 weeks of co-cultivation using a human Angiogenesis RT^2 Profiler Array. One week co-culture was set to 1 and served as control. Gene expression of the different genes after 4 weeks of co-cultivation relative to 1 week of co-cultivation is depicted in the table. RPL13A was taken as endogenous standard. n=3. Abbrevations of genes are explained in the appendix.

On the other hand only 20 genes exhibited no differences in gene expression depending on the co-cultivation time. The considerable upregulation of proangiogenic factors after 4 weeks of

co-cultivation is demonstrated as relative quantification of gene expression by setting 1 week of co-cultivation to 1, thus serving as reference value. The Human Angiogenesis RT^2 Profiler Array was performed with co-cultures of three different donors of OEC and pOB, respectively.

Out of these 60 upregulated genes which could be detected after 4 weeks of co-cultivation compared to 1 week of co-culture in total, collagen type 18, FIGF (VEGFD) and TGFbeta2 seemed to be the most affected genes in response to the cultivation time (red box). Collagen type 18, a nonfibrillar collagen of basement membranes (Guenther et al., 2001), was 220 times higher expressed in 4 week co-cultures compared to controls (1 week). FIGF, a member of the VEGF family (VEGF-D) exhibited a 108 times higher expression and transforming growth factor beta2 (TGFbeta2) was even 410 times higher expressed in the co-culture cultivated for 4 weeks compared to 1 week co-culture.

3.1.3 Stabilizing matrix components and mural cells were associated with microvessel-like structures in the co-culture

Blood vessels are composed of at least three cell layers, the intima, the media and the adventitia or outer layer. The wall of all vessels is composed of an inner single cell layer of endothelial cells which is surrounded by mural cells including pericytes and smooth muscle cells. In capillaries, endothelial cells and mural cells share a common basement membrane and are embedded in the extracellular matrix, which marks the outer layer of blood vessels (Jones, 1979). In order to generate stable and functional microvessels *in vitro*, it has been shown that newly formed angiogenic structures necessarily require mechanical support through the formation of stabilizing extracellular matrix proteins and mural cells including pericytes and vascular smooth muscle cells (Carmeliet, 2005).

3.1.3.1 Basement membrane proteins were associated with OEC in the co-culture system

The extracellular matrix is essential for a vascular endothelium and necessary for maintaining the organization of endothelial cells into blood vessels by providing a framework in which endothelial cells and vascular smooth muscle cells are embedded. Collagen type IV and laminin are matrix proteins associated with the basement membrane and they play

important roles during the process of angiogenesis (Herbst et al., 1988). Co-cultures of OEC and primary osteoblasts were stained using immunofluorescence for the basement membrane proteins collagen type IV and laminin to detect these proteins in the co-culture system (figure 8).

The figures revealed luminal structures formed by outgrowth endothelial cells after 4 weeks of co-cultivation in cryostat sections of 3-dimensional co-culture aggregates (figure 8B/C/E/F), generated under dynamic conditions in a rotating cell culture vessel system, as well as in frozen sections of 2-dimensional co-cultures (figure 8A/D), generated under static conditions. Luminal structures, stained positiv for the endothelial marker von Willebrand factor, were clearly surrounded by basement membrane components Laminin and Collagen IV (figure 8A/B/D/E) which demonstrated the existence of these basement membrane proteins in long term co-cultures of pOB and OEC.

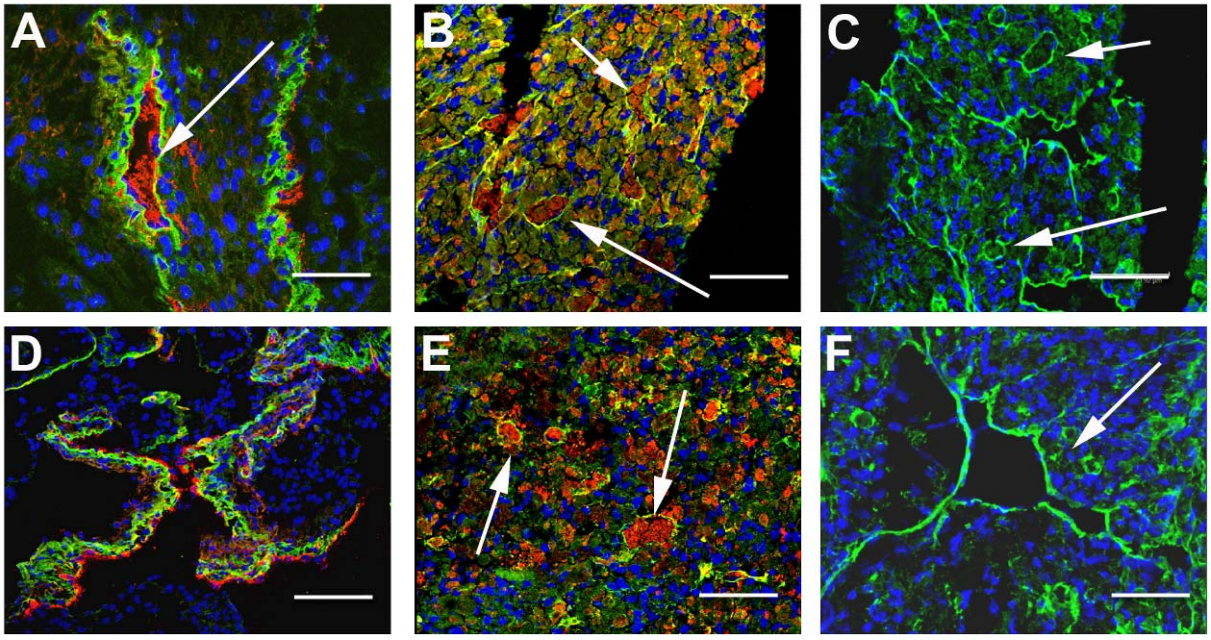


Figure 8. Cryostat sections of 2 dimensional co-cultures generated under static conditions (A/D) and cryostat sections of 3-dimensional (B/C/E/F) co-culture aggregates of pOB and OEC generated under dynamic conditions in a rotating cell culture system, both co-cultivated for 4 weeks and stained immunohistochemically for laminin (A-C, green) and collagen type IV (D-F, green). Von Willebrand factor is stained in red (A/B/D/E). Cell nuclei were counterstained with Hoechst. Arrows show the relevant expression. Scale bars 75µm. n=3.

Quantitative real time PCR evaluated the gene expression of collagen type IV and laminin in co-cultures of OEC and pOB after 4 weeks of cultivation. Both basement membrane proteins were significantly upregulated after 4 weeks of co-cultivation compared to 1 week (figure 9).

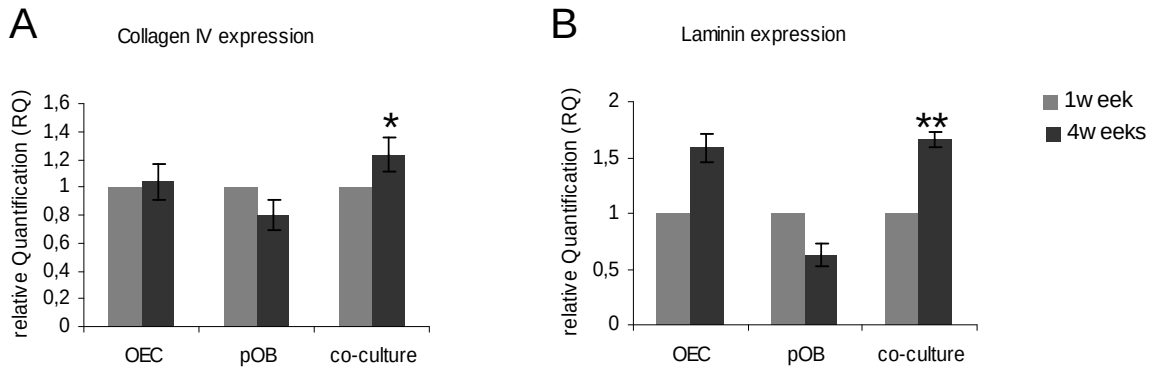


*Figure 9. Relative gene expression of collagen type IV and laminin after 1 and after 4 weeks of cultivation in pOB and OEC mono-cultures as well as in co-cultures. GAPDH was taken as an endogenous control. * significant $p < 0.05$ and ** significant $p < 0.03$. n=6.*

3.1.3.2 OEC were associated with alpha smooth muscle actin- and desmin-postitive cells in the co-culture system

The wall of all vessels is composed of a single cell layer of endothelial cells which is surrounded by mural cells (pericytes and α-smooth muscle cells) and the formation of a mature and functional vascular network necessarily requires the communication between endothelial cells and smooth muscle cells. The interactions between these two cell types in the vessel wall are involved in the control of growth and functionality of blood vessels. To investigate whether smooth muscle cells are present during the formation of microvessel-like structures in the long time co-cultures of OEC and pOB, cryostat-sections of 2- and 3-dimensional co-cultures after 4 weeks of co-cultivation were stained by immunohistochemistry for the smooth muscle cell-associated markers α-smooth muscle actin and desmin (figure 10). The existence of alpha smooth muscle actin-positive cells in the co-culture of pOB and OEC could be demonstrated in long-term co-cultures. Endothelial cells were associated with α-smooth muscle actin- and desmin-positive cells in each figure.

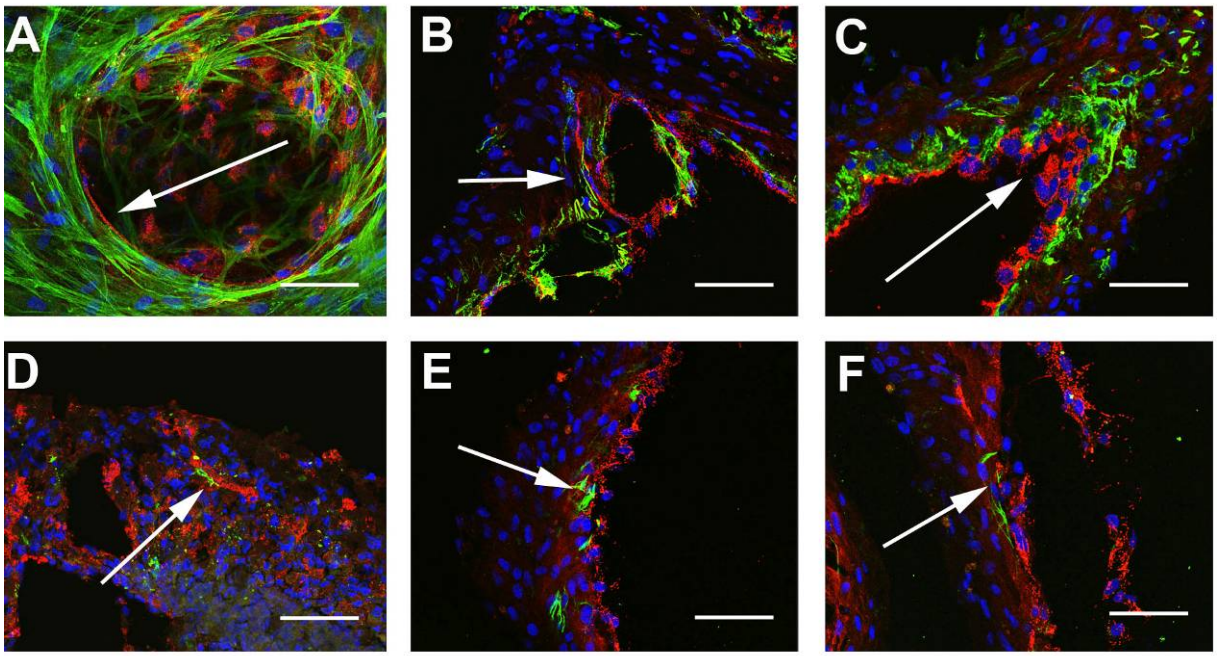


Figure 10. Co-culture of pOB and OEC (A) and cryostat sections of 2 D- (B/C/E/F) and 3-D (D) co-cultures of pOB and OEC co-cultivated for 4 weeks and stained by immunohistochemistry for pericyte-associated markers α-smooth muscle actin (A-D, green) and desmin (E/F, green). Von Willebrand factor is stained in red (A-F). Cell nuclei were counterstained with Hoechst. Scale bars 75µm. n=3.

After 4 weeks of co-cultivation luminal structures formed by endothelial cells were associated with α-smooth muscle actin-positive cells. According to this the relative gene expression of alpha-smooth muscle actin increased significantly during the course of co-cultivation evaluated using quantitative real time PCR, as depicted in figure 11.

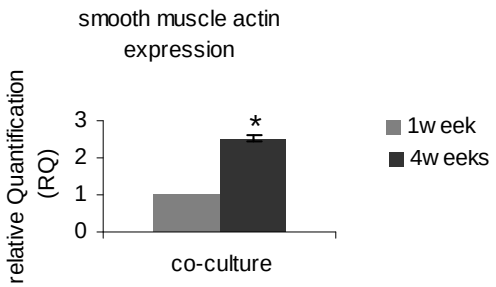


*Figure 11. Relative gene expression of α-smooth muscle actin in the co-culture of pOB and OEC after 1 week and after 4 weeks of co-cultivation. GAPDH was taken as an endogenous standard. *significant $p < 0.05$. n=6.*

The relative gene expression of alpha smooth muscle actin after 4 weeks of co-cultivation was 1.5 times higher than in the corresponding culture co-cultivated for 1 week.

Nevertheless, the origin of alpha smooth muscle actin-positive cells found in the co-culture consisting of pOB and OEC is unknown. To gain insight into the potential origin of these cells found in the co-culture system, co-cultures as well as pOB and OEC mono-cultures and human umbilical artery smooth muscle cells (HUSMC) serving as a positive control were stained immunofluorescently for the smooth muscle cell specific marker α-smooth muscle actin (figure 12).

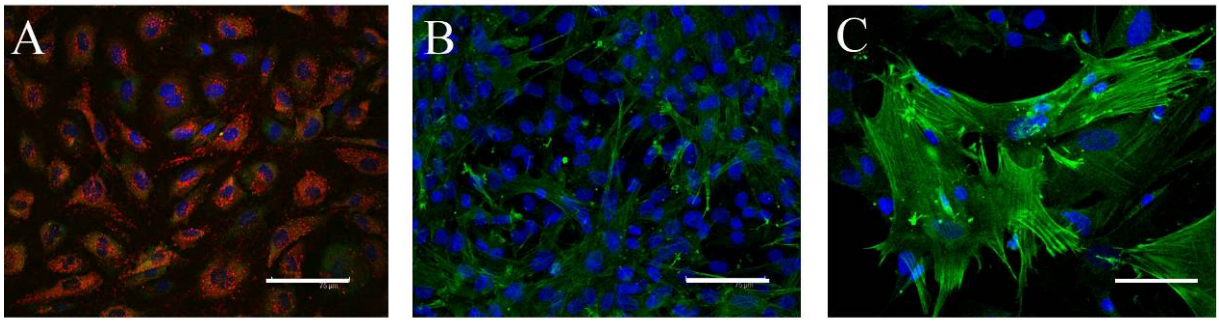


Figure 12. OEC mono-cultures (A) as well as pOB mono-cultures (B) and human umbilical artery smooth muscle cells, serving as a positive control (C), were stained using immunocytochemistry for α-smooth muscle actin (green) and vWF (red). Cell nuclei were counterstained with Hoechst (blue). Scale bars: 75µm. n=3.

Primary osteoblasts as well as HUSMC stained positively for alpha smooth muscle actin, whereas in OEC mono-cultures no smooth muscle actin expression could be detected. Additionally, RT-PCR was performed to detect smooth muscle cell-specific mRNA expression in OEC and pOB mono-cultures as well as in OEC and pOB co-cultures. HUSMC, isolated from human umbilical artery were also taken as a control. Gene expression of different smooth muscle cell-specific markers like alpha smooth muscle actin, calponin and smoothelin in HUSMC, pOB- and OEC- mono-cultures as well as in co-cultures was performed using RT-PCR (figure 13A). All smooth muscle cell-associated markers could be detected in HUSMC, pOB mono-cultures as well as in co-cultures of pOB and OEC, but not in OEC mono-cultures (figure 13A). Quantitative real time PCR detecting the expression of smooth muscle actin and desmin in pOB and OEC mono-cultures confirmed these results (figure 13B). In OEC mono-cultures the expression of alpha smooth muscle actin and desmin was almost not detectable compared to the expression of these smooth muscle cell associated markers in mono-cultures of pOB. These results supported an osteoblastic origin of alpha

smooth muscle actin positive cells in the co-culture system. This was in accordance with figure 12 in which the smooth muscle cell specific marker α-smooth muscle actin could be detected in mono-cultures of primary osteoblasts as well as in co-cultures of pOB and OEC, but not in OEC mono-cultures.

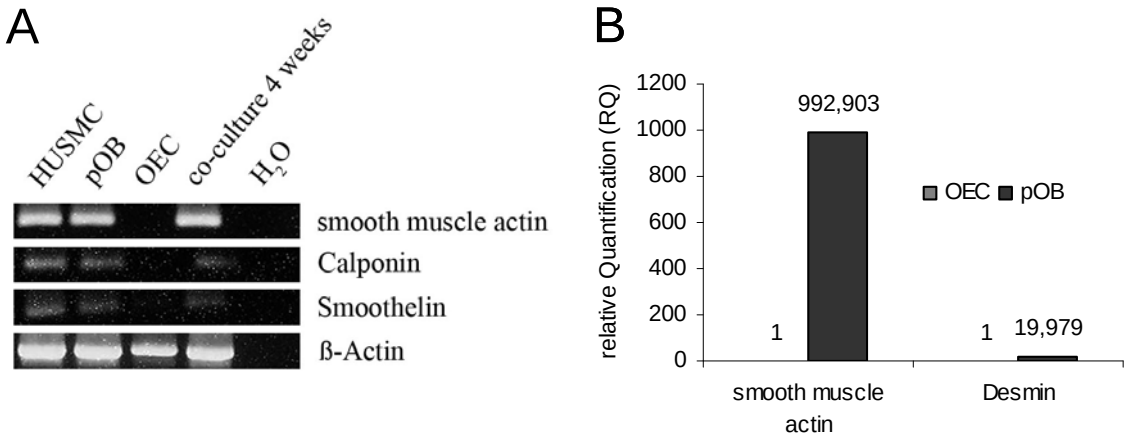
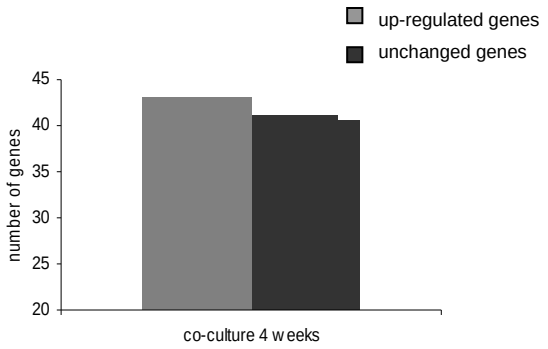


Figure 13. Gene expression analyses of smooth muscle cell specific genes alpha smooth muscle actin, calponin, smoothelin and desmin in pOB and OEC mono-cultures and co-cultures in comparison to the expression of these genes in HUSMC, serving as a positive control. Gene expression was evaluated using RT-PCR (A) as well as quantitative real time PCR (B). GAPDH was taken as endogenous standard. n=6.

3.1.4 Co-cultures of OEC and pOB seemed to promote osteogenic differentiation

The Human Osteogenesis RT^2 Profiler PCR Array profiles the expression of 84 genes involved in or related to the process of osteogenic differentiation and is therefore a beneficial instrument to screen co-cultures of primary osteoblasts and outgrowth endothelial cells during the course of co-cultivation in terms of osteogenic differentiation and matrix mineralization. The array assesses genes effecting the development of the skeletal system and the bone mineralization process including growth factors, extracellular matrix molecules and adhesion molecules. PCR Array was performed on co-cultures of 3 different donors cultivated for 1 week and 4 weeks using quantitative real time PCR (figure 14).



co-culture 4 weeks		co-culture 4 weeks		co-culture 4 weeks	
ALPL	1,213	COMP	3,414	PHEX	18,531
AMELY	84,052	CTSK	6,063	RUNX	4,102
ANXA5	2,353	EGFR	6,185	SERPI	3,12
BGN	1,226	FGF1	15,25	SMAD	7
BMP1	13,635	FGF2	15,657	SMAD	7,425
BMP4	200,071	FN1	5,165	SMAD	130,959
CALCR	2,603	ICAM1	33,7	SOX9	28,019
CD36	38,723	IGF2	6,664	STATH	20,632
CDH11	2,6	ITGA3	4,223	TGFB2	99,978
COL10A	22,794	ITGB1	1,233	TNF	11,978
COL11A	5,8	MINPP	14,156	TWIST	2,542
COL12A	1,207	MMP1	175,517	VDR	37,905
COL14A	32,383	MMP2	4,356	VEGFA	3,254
COL3A1	1,121	PDGFA	4,134	VEGFB	13,813

Figure 14. Screening of relative gene expression of osteogenesis related genes in co-cultures consisting of pOB and OEC after 1 and after 4 weeks of co-cultivation using a human Osteogenesis RT² Profiler Array. 1 week co-culture was set to 1 serving as control and gene expression of the different genes after 4 weeks of co-cultivation was evaluated relative to 1 week of co-cultivation, as depicted in the table. RPL13A was taken as endogenous standard. n=3. Abbrevations of genes are explained in the appendix.

More than 40 genes involved in osteoblastic differentiation were upregulated after 4 weeks of co-cultivation, whereas 40 genes were not affected. The upregulated genes are accurately listed in the table of which the most affected genes in response to the cultivation time are highlighted in red (figure 14). Bone morphogenic protein 4 (BMP4) for instance was expressed 200 times higher in long term co-cultures compared to 1 week of co-cultivation. AMELY, a gene that encodes for amelogenin, a member of the family of extracellular matrix proteins (Nakahori et al., 1991) was upregulated 84 times in long term co-cultures. Essential osteogenesis regulating factors like the early osteogenic transcription factor RUNX2 or the growth factor TGFß as well as the TGFbeta receptor modulating molecule SMAD were also clearly upregulated in 4 week co-cultures compared to 1 week of co-cultivation, thus indicating a positive effect on the osteogenic differentiation of the co-culture system consisting of pOB and OEC.

3.2 Influence of sonic hedgehog signalling on the co-culture system of outgrowth endothelial cells and primary osteoblasts

For a possible therapeutical intervention the identification of signalling pathways or morphogens that might affect both fundamental processes in bone regeneration and repair, namely osteogenesis and angiogenesis, could be of considerable potential. Currently, one of the most promising molecular targets that might act in this multifunctional way seems to be the sonic hedgehog signalling pathway due to its pivotal role in the initiation of angiogenesis and osteogenic differentiation. To test if sonic hedgehog might play a role in differentiation processes in the co-culture of pOB and OEC, gene expression of the Shh receptor, patched1, was analyzed in OEC and pOB mono-cultures as well as in co-cultures after 1 and after 4 weeks of cultivation using RT-PCR (figure 15).

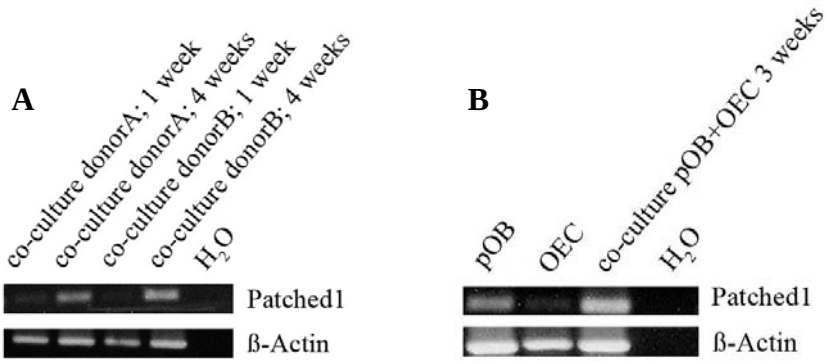


Figure 15. RT-PCR detecting the expression of the sonic hedgehog receptor, patched1 (Ptch1) in co-cultures of 1 week and 4 weeks of cultivation (A) and pOB and OEC mono-cultures compared to co-cultures (B). ß-Actin served as endogenous control. n=3.

Expression of the Shh receptor patched1 could be detected in co-cultures of 1 and 4 weeks of co-cultivation as well as in pOB and OEC mono-cultures. The expression of Ptch1 was considerable higher after 4 weeks of co-cultivation compared to 1 week (figure 15A). Mono-cultures of primary osteoblasts exhibited a significant higher expression of Ptch1 than mono-cultures of OEC (figure 15B). The existence of specific gene expression of the sonic hedgehog receptor Ptch1 in co-cultures of pOB and OEC as well as in the corresponding mono-cultures supported the idea that Shh might affect OEC as well as pOB in the co-culture

system and therefore might be a potential candidate to improve differentiation processes within the co-culture of primary osteoblasts and outgrowth endothelial cells.

Cyclopamine is a chemical that belongs to the group of steroidal alkaloids and can be isolated from the corn lily (*Veratrum californicum*) (Binns et al., 1968). Cyclopamine inhibits the hedgehog signalling pathway by targeting the smoothened protein, a patched1 co-receptor. As described before, co-cultures of outgrowth endothelial cells and primary osteoblasts under control conditions were characterized by an abundant appearance of microvessel-like structures which seemed to increase with progressing culture time. The role of Shh during angiogenesis in the co-culture system could be demonstrated in initial experiments by blocking of this vessel formation in co-cultures by incubation with the sonic hedgehog inhibitor, cyclopamine, as indicated in figure 16.

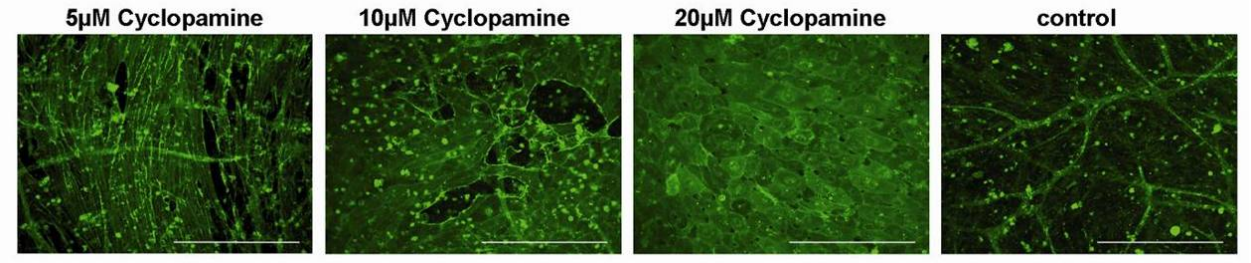


Figure 16. Immunofluorescent staining for CD31 (depicted in green) following treatment of co-cultures of OEC and pOB with cyclopamine in different concentrations (5µM, 10µM, 20µM) for 3 weeks compared to a non-treated co-culture (control). Scale bars: 300µm. n=3.

Treatment of co-cultures consisting of pOB and OEC with cyclopamine in different concentrations (5µM-20µM) revealed a dose-dependent inhibitory effect of cyclopamine on capillary morphogenesis formed by OEC in the co-culture (figure 16). Co-cultures treated with 10µM or 20µM cyclopamine revealed practically no formation of angiogenic structures.

3.2.1 Differences in the angiogenic activation dependent on sonic hedgehog or vascular endothelial growth factor stimulation in co-cultures of pOB and OEC

Based on the first results that revealed a possible role of Shh in angiogenesis in the co-culture system, effects of Shh were investigated in terms of the angiogenic activation of OEC

in the co-culture compared to treatment with a commonly used factor, VEGF, which is known to induce the formation of new vessels (Mandriota and Pepper, 1997). Co-cultures were treated either with 5µg/ml Shh or 50ng/ml VEGF for 24 hours and for 14 days and stained for the endothelial cell marker CD31 (figure 17).

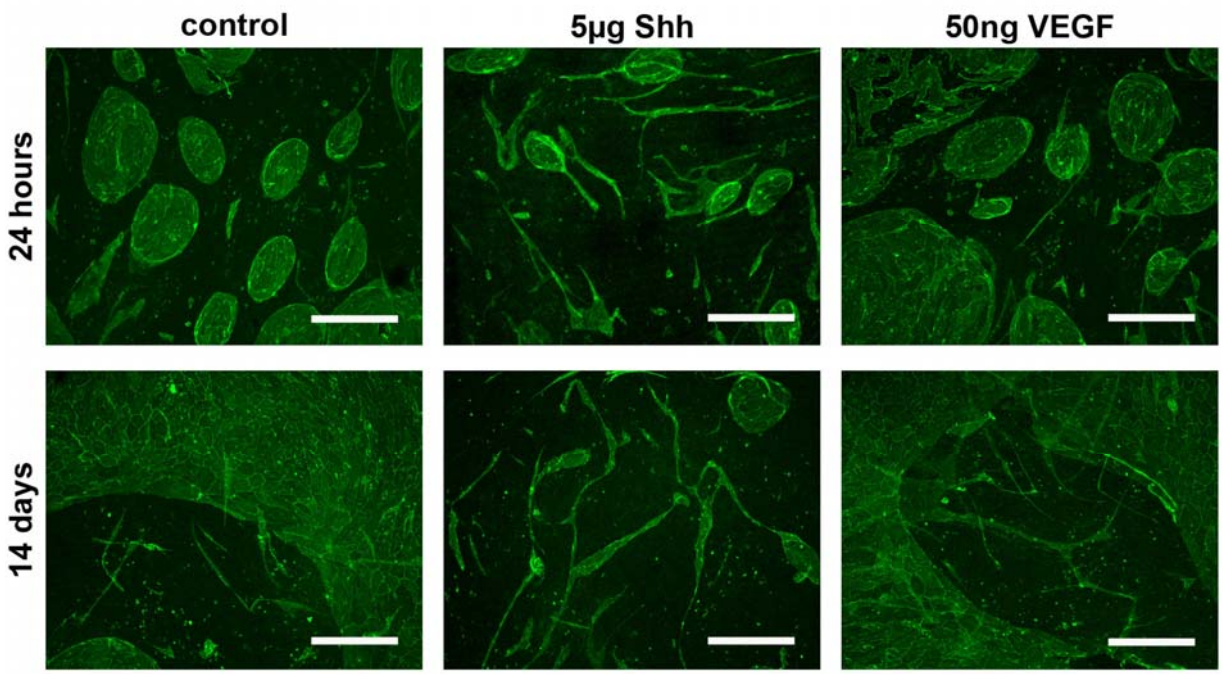


Figure 17. Differences in the angiogenic activation dependent on Shh or VEGF stimulation in co-cultures. Co-cultures were precultivated for 1 week, treated with Shh or VEGF for 24 hours and 14 days and finally stained using immunocytochemistry for the endothelial marker CD31 (green). Scale bars: 300µm. n=6.

Stimulation of co-cultures with both factors, VEGF and Shh, resulted in an increase in the formation of microvessel-like structures compared to untreated control co-cultures. In contrast to the treatment with VEGF, effects by Shh could already be observed after 24 hours (figure 17) as indicated by the formation of tube-like structures and interconnected networks. The addition of VEGF could not induce the formation of angiogenic structures after 24 hours. Nevertheless, after 14 days of treatment the amount of microvessel-like structures was comparable in VEGF and Shh stimulated co-cultures. These morphological findings could be confirmed by quantitative analyses of angiogenic structures in Shh and VEGF treated co-cultures, as depicted in figure 18.

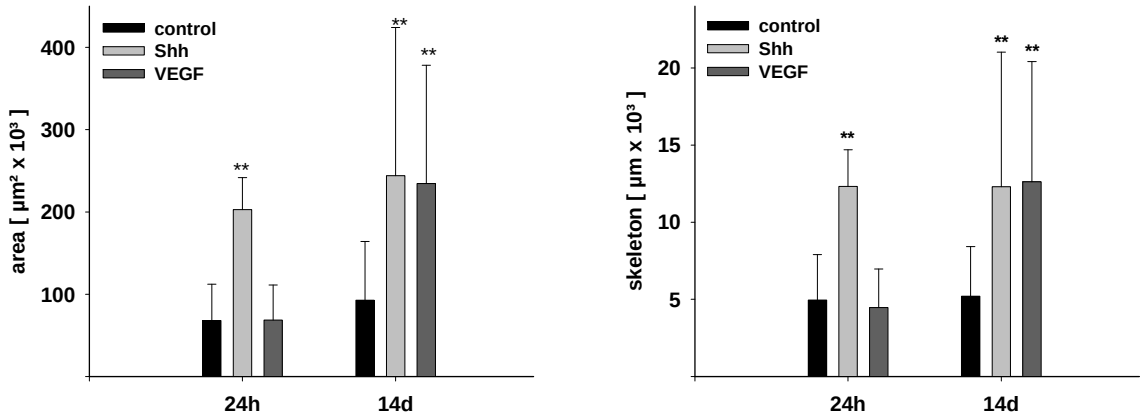


*Figure 18. Quantitative analyses of angiogenic structures in Shh- and VEGF-treated co-cultures after 24 hours and 14 days of stimulation. Area of angiogenic structures as well as the total skeleton length in Shh or VEGF treated co-cultures were analysed quantitatively after 24 hours and 14 days of stimulation. . **significant $p < 0.03$. n=3.*

The area of angiogenic structures as well as the total skeleton length was significantly higher in sonic hedgehog-stimulated co-cultures after 24 hours of stimulation compared to 24 hours VEGF treatment, whereas no significant differences could be observed between VEGF and Shh treatment after 14 days of stimulation (figure 18). Sonic hedgehog, as well as VEGF treatment, resulted in a significant increase in the area of angiogenic structures and the total skeleton length of microvessel-like structures compared to control co-cultures after 14 days of stimulation.

Angiopoietins are protein growth factors controlling the process of angiogenesis. Therefore, the concentration of angiopoietin1 and angiopoietin2 in the cell culture supernatants was measured in response to Shh and VEGF treatment using an enzyme-linked immunosorbent assay (figure 19, n=3). Angiopoietin1 concentration was significantly increased in supernatants of Shh-treated co-cultures after 24 hours and after 14 days of stimulation compared to VEGF-treated co-cultures and controls (figure 19A').

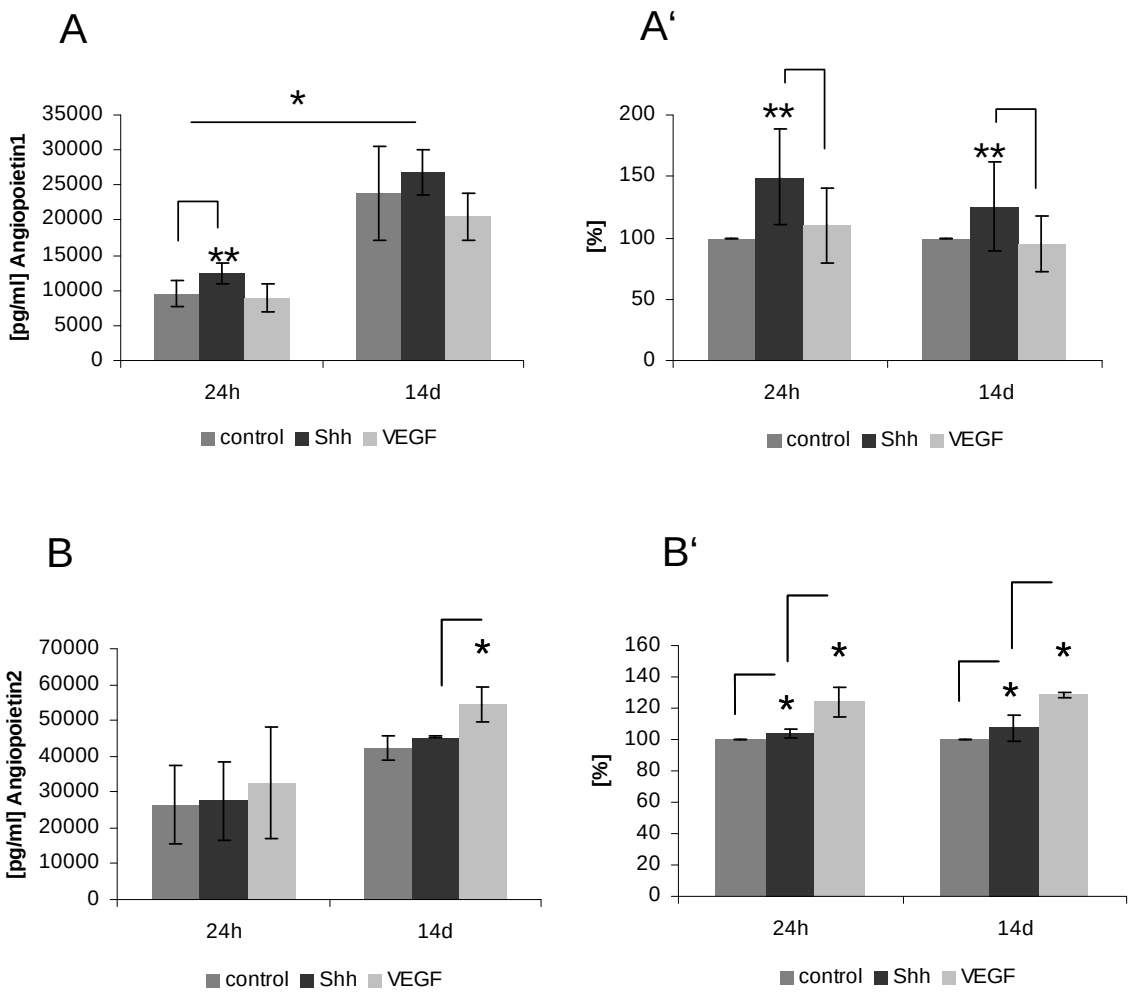


*Figure 19. Effects of Shh and VEGF treatment on the release of proangiogenic factors in co-cultures of pOB and OEC. The concentration of angiopoietin1 (A/A') and angiopoietin2 (B/B') in the supernatants of co-cultures consisting of pOB and OEC was measured in an enzyme linked immunosorbent assay after 24 hours and after 14 days of stimulation. Results are depicted as ratios (control = 100%; A'/B') and additionally shown as absolute values (A/B). [*p<0.05, **p<0.03.]*

Shh treatment of co-cultures resulted in a significant increase in the release of angiopoietin2 in the co-culture supernatants compared to controls after 24 hours and after 14 days of treatment (figure 19B') but total protein amount of angiopoietin2 was significantly higher in the supernatants of VEGF-treated co-cultures compared to Shh-treated cultures after 24 hours and after 14 days of stimulation (figure 19B'). In addition, quantification of relative gene expression of the proangiogenic factors angiopoietin1 and angiopoietin2 was assessed in response to VEGF and Shh treatment using quantitative real time PCR (figure 20). Compared to VEGF stimulation Shh treatment resulted in an upregulation of angiopoietin1 after 24

hours and 14 days of Shh stimulation compared to treatment with VEGF and controls (figure 20A; n=5). In addition, angiopoietin2 was significantly upregulated in response to 24 hours treatment with Shh whereas after 14 days of stimulation no differences in gene expression of angiopoietin2 could be detected (figure 20B; n=5).

3.2.2 Shh, but not VEGF, induced the expression of factors involved in vessel maturation and stabilization

It is generally accepted that VEGF-induced vessels lack pericytes, they are unstable, perforated and they tend to regress (Sundberg et al., 2002). To analyse a possible effect of Shh on basement membrane assembly and the maturation of newly formed vessels induced in the co-culture system gene expression levels of several basement membrane molecules, such as laminin and collagen IV, as well as smooth muscle cell/pericyte-specific marker expression, like α-smooth muscle actin, desmin, myocardin, platelet derived growth factor BB (PDGF-BB) and TGF beta in response to Shh or VEGF treatment was analysed using qReal time PCR (figure 20).

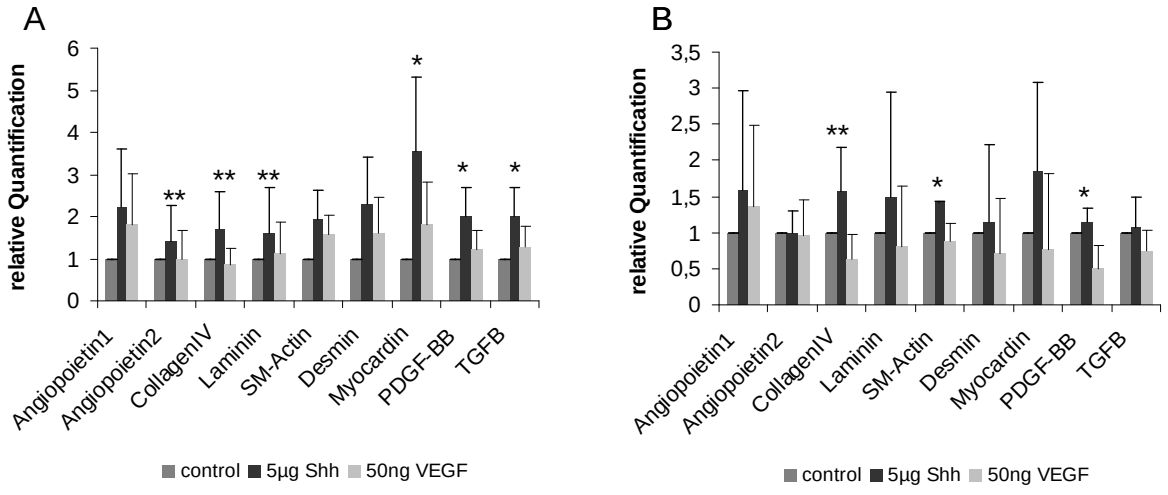


*Figure 20. Effects of Shh and VEGF on the expression of factors involved in angiogenesis. Co-cultures were treated with Shh or VEGF for 24h (A) and 14d (B). Quantitative real time PCR analysing relative gene expression of angiopoietin1, angiopoietin2, collagenIV, laminin, α-smooth muscle actin (SM-actin), desmin, myocardin, platelet-derived growth factor (PDGF-BB) and transforming growth factor ß (TGFB) was performed in response to Shh and VEGF compared to control co-cultures. GAPDH was taken as endogenous standard and control co-cultures were set to 1. n=5. [*p<0.05, **p<0.03].*

Treatment with Shh resulted in an upregulation of all tested factors compared to VEGF treatment and controls for both stimulation time points. A significant upregulation could be detected for collagen IV, laminin, myocardin, PDGF-BB and TGFß after 24 hours of Shh stimulation (figure 20A), whereas only collagen IV, α-smooth muscle actin and PDGF-BB were significantly upregulated after 14 days of Shh treatment compared to controls and VEGF-stimulated co-cultures (figure 20B).

In addition to the real time PCR experiments the levels of PDGF-BB in the supernatants of co-cultures as a growth factor associated with smooth muscle cell differentiation and vessel stabilization by mural cells were assessed using an enzyme-linked immunosorbent assay. In accordance with the increased expression of PDGF-BB at the mRNA level after 24 hours and after 14 days of Shh treatment compared to VEGF stimulation and controls, a significant increase in PDGF-BB levels in the supernatants of Shh-treated OEC mono-cultures could be detected already after 24 hours in response to Shh treatment (figure 21C, n=3).

Cryostat sections of Shh treated co-cultures (14 days treatment), stained using immunofluorescence for pericyte associated marker α-smooth muscle actin, clearly revealed the existence of alpha smooth muscle actin positive cells in the co-culture system of OEC and pOB in response to Shh treatment (figure 21A/B). Luminal structures composed of OEC were surrounded by alpha smooth muscle actin positive cells (figure 21B).

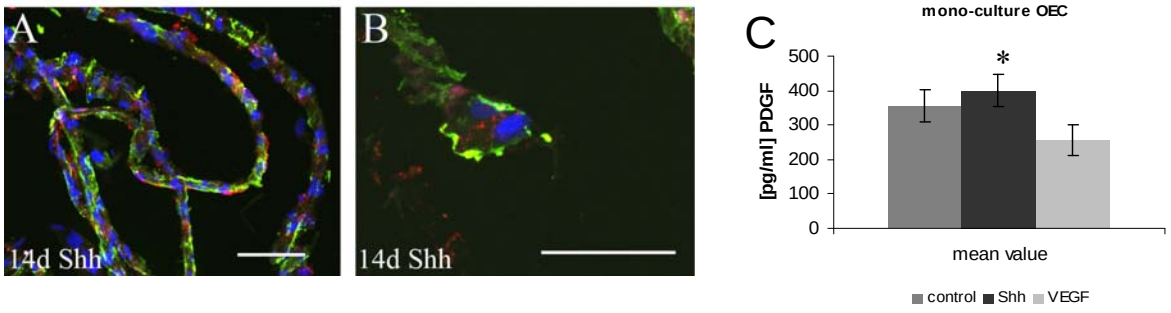


*Figure 21. Immunofluorescence staining for α-smooth muscle actin (green) in co-cultures in response to 14 days of Shh treatment (A/B). Von Willebrand factor is stained in red. Cell nuclei were counterstained with Hoechst (blue). Scale bars: 75µm. C. Concentration of PDGF-BB in supernatants of Shh- and VEGF-stimulated OEC mono-cultures measured in the supernatants of the different treated cultures using an enzyme-linked immunosorbent assay (ELISA). PDGF-BB protein concentration is demonstrated as absolute concentration. [*p<0.05, **p<0.03].*

Furthermore, the total protein amount of α-smooth muscle actin in co-culture cell lysates was assessed in response to 24 hours and 14 days of Shh and VEGF stimulation using SDS-Page and Western blot. These experiments revealed that the protein amount of alpha smooth muscle actin increased during the course of co-cultivation from 24 hours to 14 days. Nevertheless, the highest α-smooth muscle actin protein amount could be detected in sonic hedgehog-stimulated co-cultures after 14 days of Shh treatment, compared to treatment with vascular endothelial growth factor and control co-cultures, thus which supporting an ongoing process of vessel maturation and stabilization in the co-culture system of primary osteoblasts and outgrowth endothelial cells in response to sonic hedgehog (figure 22).

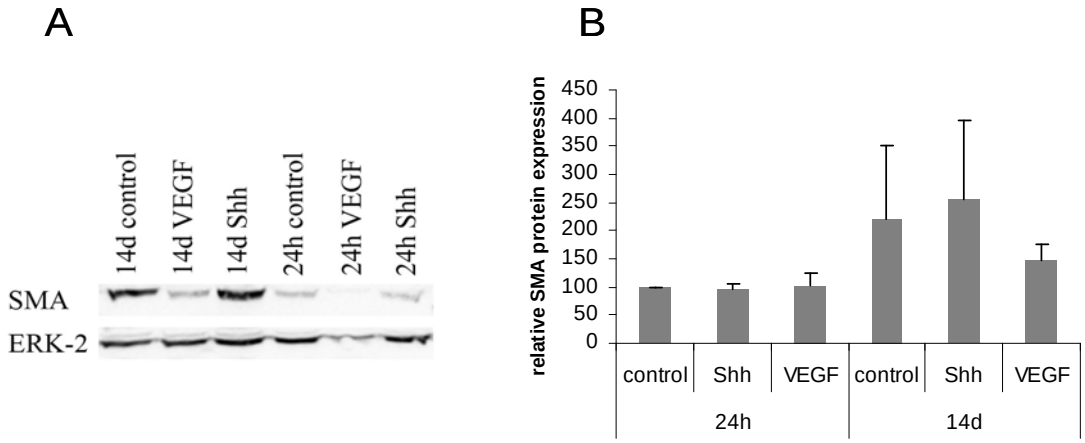


Figure 22. Effects of Shh and VEGF on the protein amount of α-smooth muscle actin in cell culture lysates in co-cultures of pOB and OEC evaluated using SDS-Page and Western blot. A. Protein amount of α-smooth muscle actin (SMA) in differently treated cultures compared to protein amount of ERK-2, used as loading control. B. Quantification of SMA protein amount. Control co-cultures cultivated for 24 hours were set to 100 percent.

3.2.3 Sonic hedgehog enhanced the formation of angiogenic structures in the co-culture system of pOB and OEC as well as in OEC mono-cultures seeded on Matrigel

As already documented, stimulation of co-cultures with 5µg recombinant sonic hedgehog revealed a considerable proangiogenic effect on OEC within the co-culture model and resulted in a significant increase in the formation of microvessel-like structures compared to control cells cultured in EGM-2 which could already be observed after 24 hours of treatment, as indicated by the formation of tube-like structures and interconnected networks (figure 23).

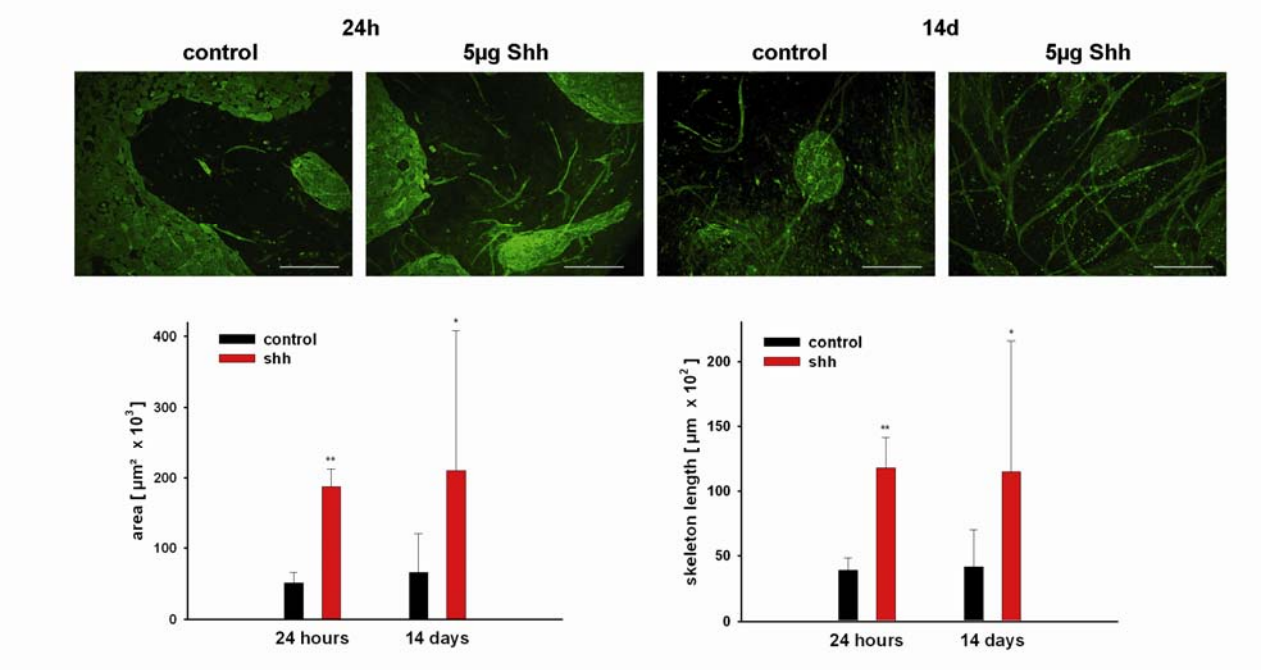


*Figure 23. Effect of Shh treatment on co-cultures consisting of pOB and OEC. Cells were co-cultured 1 week before stimulation with 5µg/ml recombinant Shh for 24 hours (n=6) and 14 days (n=3) (as described previously) and stained for the endothelial marker CD31 (green). The area as well as the total skeleton length of vascular structures formed by stimulated and non-stimulated co-cultures (n=3) after 24h and 14d were assessed quantitatively (diagrams lower row). Scale bars: 300µm. [*p<0.05, **p<0.03].*

The effect of the angiogenic stimulation by Shh was reduced when co-cultures were incubated simultaneously with Shh and cyclopamine, depending on the cyclopamine concentration as depicted in figure 24.

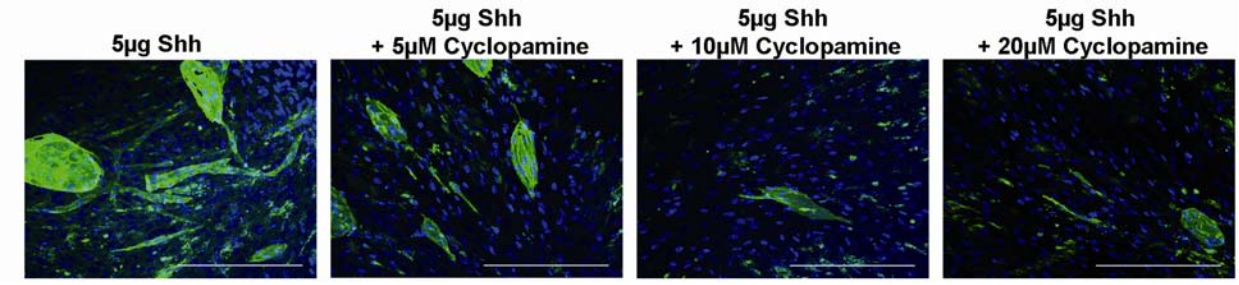


Figure 24. Simultaneous treatment of co-cultures with 5µg Shh and cyclopamine in different concentrations (5µM-20µM). Cultures were stained using immunocytochemistry for CD31 (green). Cell nuclei were counterstained with Hoechst (blue). Scale bars: 300µm. n=3.

Although the detailed underlying mechanisms are still unclear, it could be possible that Shh might act in a direct or in an indirect way on the angiogenic activation of OEC, resulting in an increased formation of angiogenic structures in the co-culture. Due to the fact that both cell types in the co-culture expressed the Shh receptor patched1 (figure 15), it is possible that Shh affects both cell types. In additional experiments using OEC mono-cultures seeded on Matrigel® basement membrane matrix Shh also stimulated the formation of angiogenic structures in OEC mono-cultures. The angiogenic-inducing effect mediated by Shh in OEC mono-cultures could be inhibited when OEC were stimulated simultaneously with Shh and the Shh inhibitor cyclopamine in a concentration dependent manner (figure 25).

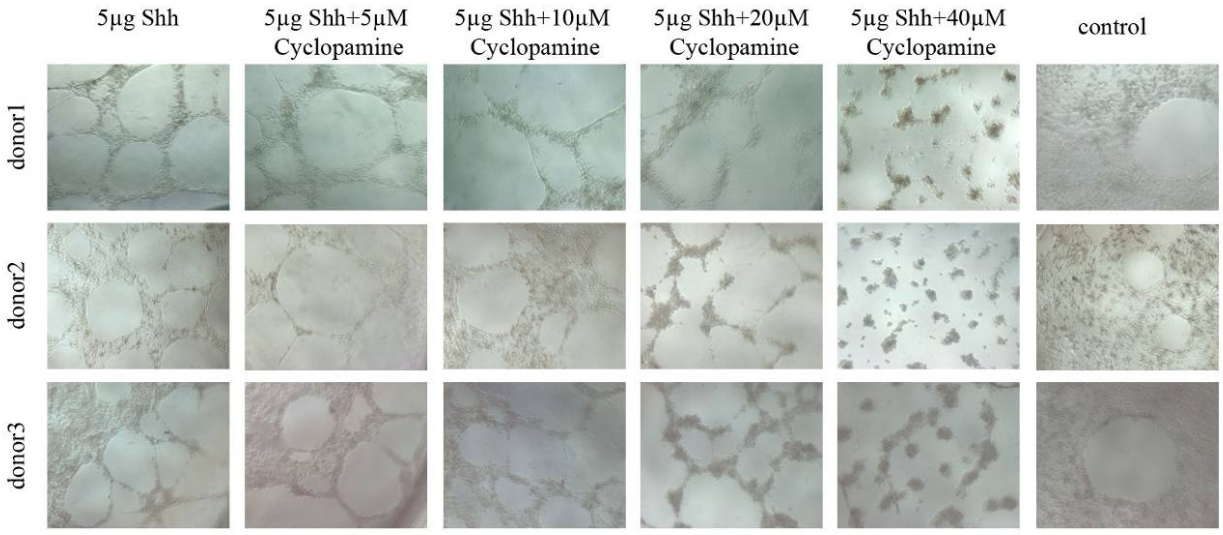


Figure 25. OEC seeded on Matrigel® Basement Membrane Matrix: Simultaneous treatment of OEC mono-cultures with recombinant human sonic hedgehog and different concentrations of the Shh inhibitor cyclopamine (5µM – 40µM) for 24 hours. n=3.

Similar to the findings in the co-culture, treatment of OEC mono-cultures with sonic hedgehog resulted in an increase in the formation of tube-like structures and capillary sprouting compared to cells cultivated in control medium (EBM-2) in the absence of Shh. Cyclopamine inhibited sonic hedgehog-mediated angiogenesis in a dose-dependent manner in co-cultures as well as in OEC mono-cultures. When OEC were treated with 5µM or 10µM cyclopamine simultaneously with 5µg/ml Shh, OEC still formed tube-like structures on Matrigel. Treatment of OEC with cyclopamine in higher concentrations (20µM and 40µM), repressed the formation of angiogenic structures by OEC, in spite of the presence of Shh (figure 25).

3.2.4 Analyses of angiogenic factors in response to sonic hedgehog: underlying mechanisms of Shh stimulation in the co-culture system

The marked positive effect of sonic hedgehog on the angiogenic activation of OEC in mono-culture as well as in co-culture with primary osteoblasts raised the question of the molecular mechanisms leading to the angiogenic activation of OEC in the co-culture. To analyse the molecular basis of Shh treatment in the co-culture, several proangiogenic molecules and growth factors, such as VEGF and angiopoietins were investigated at RNA and protein level in response to sonic hedgehog stimulation. In addition, Shh-stimulated or unstimulated mono-cultures of pOB and OEC were studied to gain insight into the potential origin of the growth factors (figure 26).

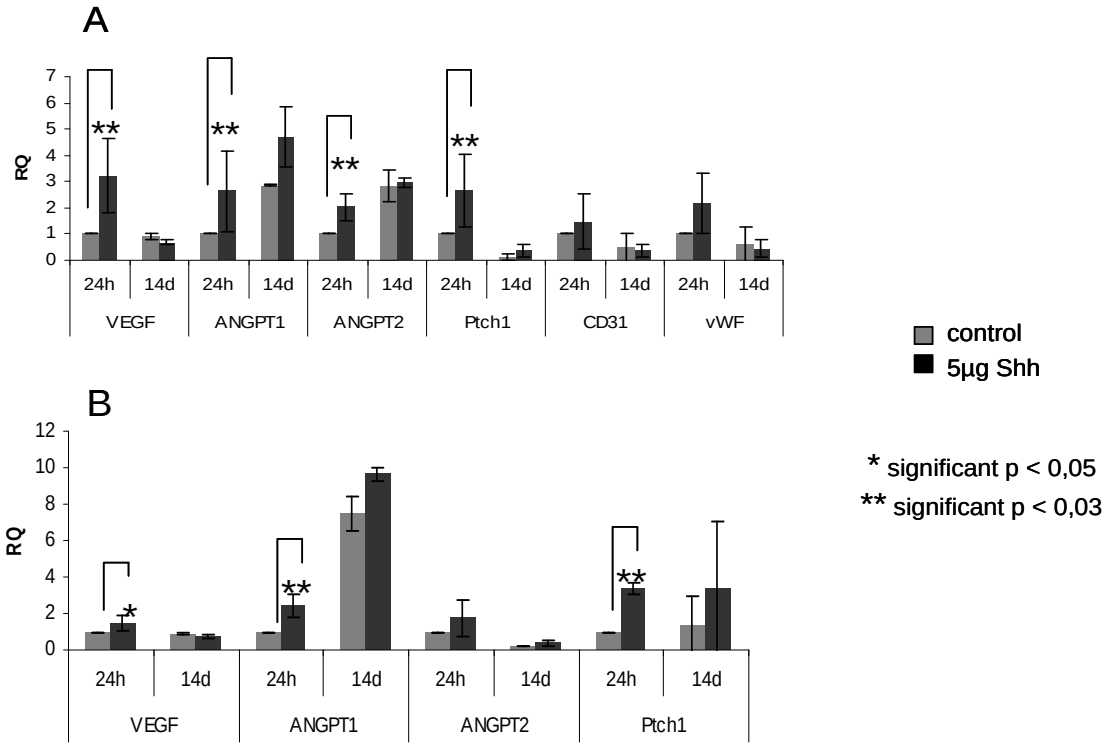


*Figure 26. Shh treatment of co-cultures (A) and pOB mono-cultures (B). A. Mean values of relative gene expression of VEGF, angiopoietin1, angiopoietin2, patched1 and the endothelial markers CD31 and vWF in Shh-treated and untreated co-cultures for 24 hours (n=6) and 14 days (n=3). B. Mean values of relative gene expression of VEGF, angiopoietin1, angiopoietin2 and patched1 in Shh-treated and untreated pOB mono-cultures for 24h (n=6) and 14d (n=3). RPL13A was taken as endogenous standard and control cultures were set to 1 (reference value). [*p<0.05, **p<0.03].*

In quantitative real-time PCR the relative gene expression of vascular endothelial growth factor (VEGF), angiopoietin1 (Ang1), angiopoietin2 (Ang2) and the hedgehog receptor patched1 (Ptch1), used as control to document hedgehog pathway activation, were significantly upregulated in sonic hedgehog-treated co-cultures after 24 hours of stimulation ($p<0.03$), as depicted in figure 26A. In addition, relative gene expression of the endothelial markers CD31 and von Willebrand factor (vWF) seemed to increase slightly after 24 hours of sonic hedgehog stimulation. During long term stimulation with Shh for 14 days, VEGF expression was downregulated compared to 24 hours of treatment, whereas angiopoietin1 and angiopoietin2 expression was not significantly changed after 14 days of treatment (figure 26B).

Similar findings for the co-cultures could be observed at protein level after determining the free VEGF levels in the cell culture supernatants by an enzyme-linked immunosorbent assay (ELISA), as depicted in figure 27. Results from ELISA for the angiogenic factors VEGF, angiopoietin1 and angiopoietin2 are shown as percentage change compared to untreated cultures (figure 27A, C, E), as well as absolute concentrations (figure 27B, D, F) to document differences in mono-cultures and co-cultures or culture time-dependent changes. The release of VEGF (A) was significantly increased in sonic hedgehog-treated co-cultures after 24 hours and after 14 days of stimulation ($p<0.05$). In addition, angiopoietin1 concentrations (C) were significantly higher in sonic hedgehog-stimulated co-cultures than in controls after 24 hours ($p<0.03$) and 14 days of stimulation ($p<0.05$).

Co-cultures of pOB and OEC as well as primary osteoblasts in mono-cultures revealed a higher concentration of angiopoietin1 in the supernatant after 14 days compared to 24 hours (D). The release of angiopoietin2 in the co-culture supernatant was higher in response to sonic hedgehog treatment compared to non-stimulated controls after 24 hours and 14 days of treatment (F). Similar effects of sonic hedgehog treatment on vascular endothelial growth factor expression in primary osteoblast mono-cultures could be detected, comparable to the results of the co-cultures. On the protein level mono-cultures of primary osteoblasts revealed a significant increase of free VEGF in the supernatant in response to sonic hedgehog treatment for 24 hours ($p<0.03$). In general, the concentration of free VEGF in the supernatants of pOB mono-cultures was higher compared to OEC mono-cultures and co-cultures of both cell types depicted in figure 27A.

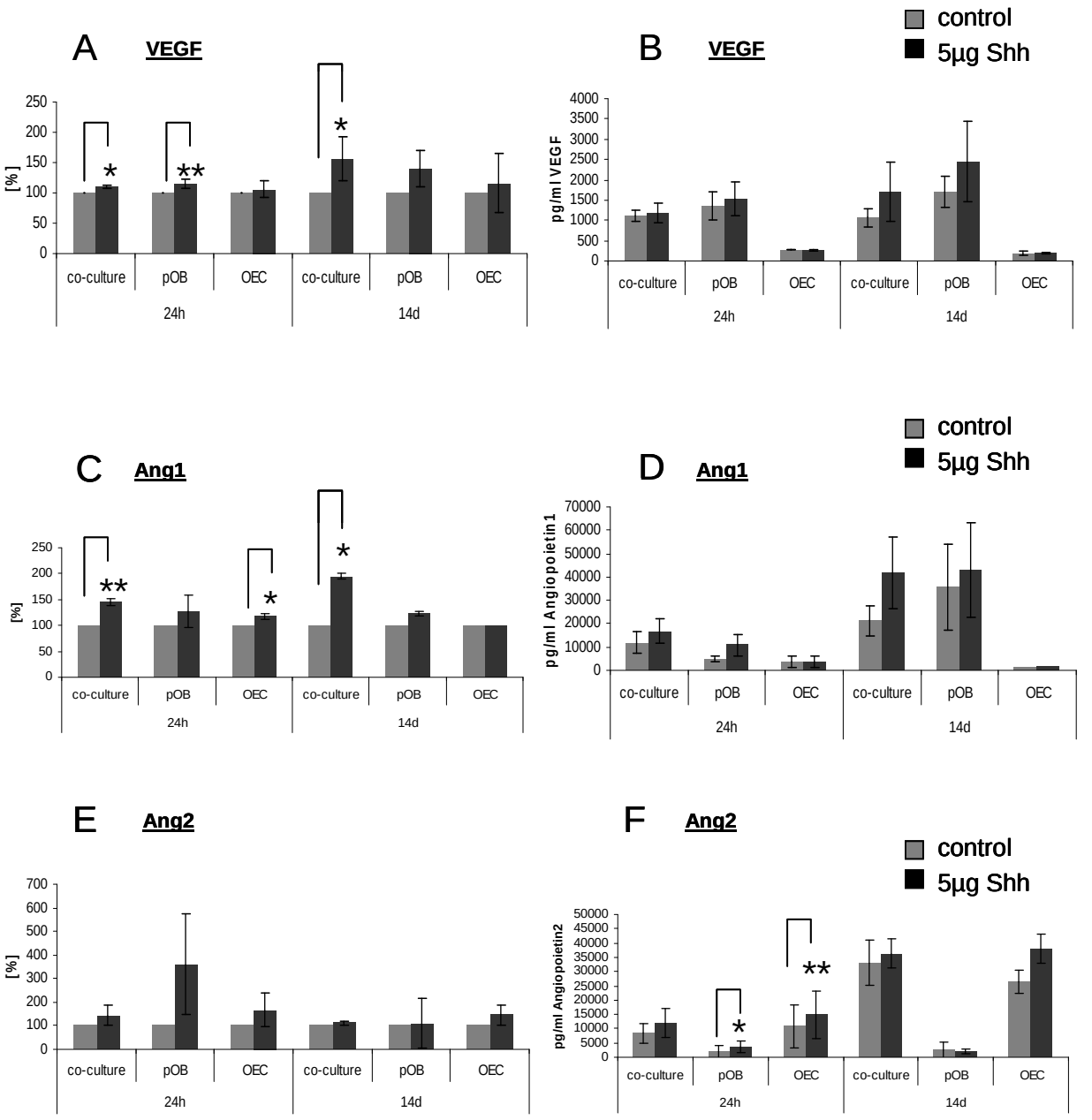


*Figure 27. Effects of Shh treatment on the release of proangiogenic factors in co-cultures, pOB mono-cultures and OEC mono-cultures. The concentration of vascular endothelial growth factor (VEGF) (A/B), angiopoietin1 (C/D) and angiopoietin2 (E/F) in the supernatants of pOB, OEC and co-cultures consisting of both cell types were measured in an enzyme-linked-immunosorbent-assay after 24 hours and after 14 days of stimulation. Results are depicted as ratios (control = 100%; A/C/E) and additionally shown as absolute values (B/D/F). [*p<0.05, **p<0.03].*

As already documented, the concentrations of VEGF and angiopoietin1 were found to be the highest in supernatants of pOB mono-cultures compared to OEC mono-cultures and co-cultures. These observations suggested that VEGF and Angiopoietin1 might originate mainly from primary osteoblasts which is in accordance with data from indirect co-cultures seeded on a transwell filter system. Indirect co-culture systems revealed a secretion of VEGF and angiopoietin1 by osteoblasts, but not by OEC (figure 28). On the other hand, angiopoietin2 concentration was higher in sonic hedgehog-stimulated OEC mono-cultures compared to co-cultures and pOB mono-cultures, which supports the hypothesis that angiopoietin2 might originate from the OEC. An indirect co-culture system was able to confirm this assumption (figure 28).

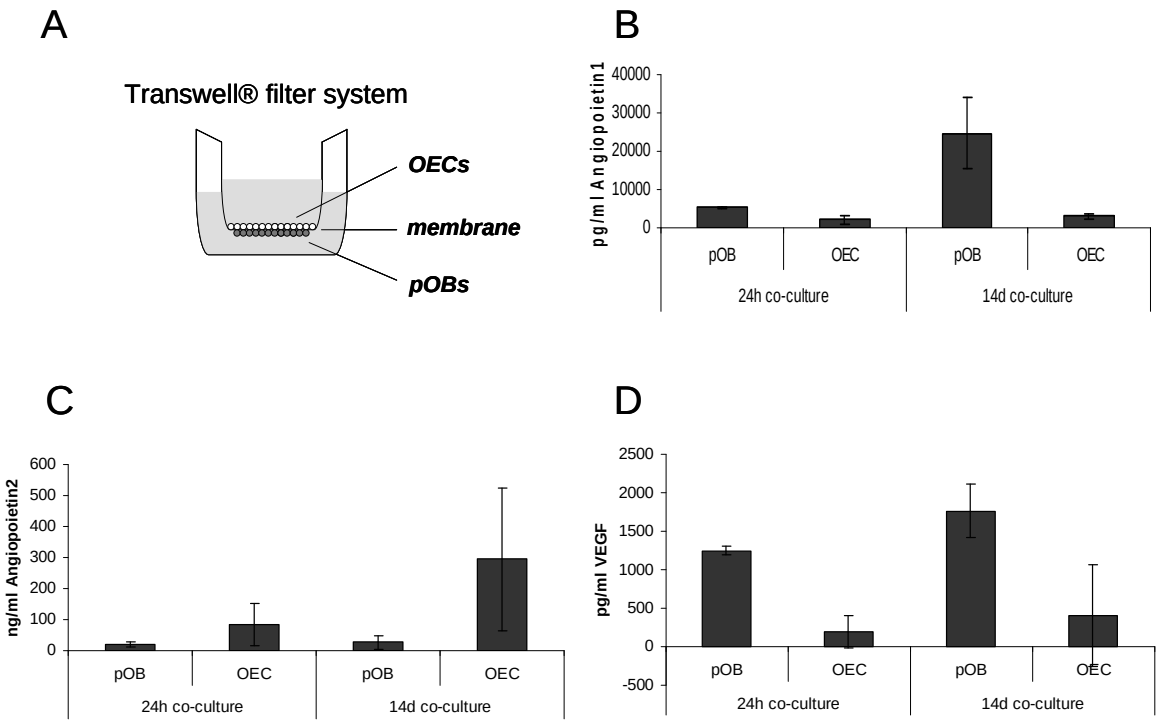


Figure 28. Indirect co-cultures on Transwell® filter systems. (A) Primary osteoblasts were seeded at the lower surface and OEC were seeded at the upper surface of a transmembrane filter in a Transwell® filter system for 24 hours and 14 days to gain insights into the origin of the growth factors found in the co-culture. Culture supernatants of OEC and pOB were collected separately from the Transwell® filter system and analysed for the angiogenic factors angiopoietin1 (B), angiopoietin2 (C) and VEGF (D) using ELISA. Results are shown as absolute values.

Results from ELISA already documented a VEGF secretion by the osteoblasts which could be enhanced by the treatment with sonic hedgehog. The free VEGF, produced by the pOB might be bound by the OEC in the co-culture. To detect surface-bound or intracellular protein levels of VEGF and other angiogenic factors, a Proteome Profiler^{TM} Human Angiogenesis protein array was performed in response to sonic hedgehog treatment. Three different donors of co-cultures of pOB and OEC as well as pOB- and OEC mono-cultures were stimulated with Shh for 24 hours (figure 29A).

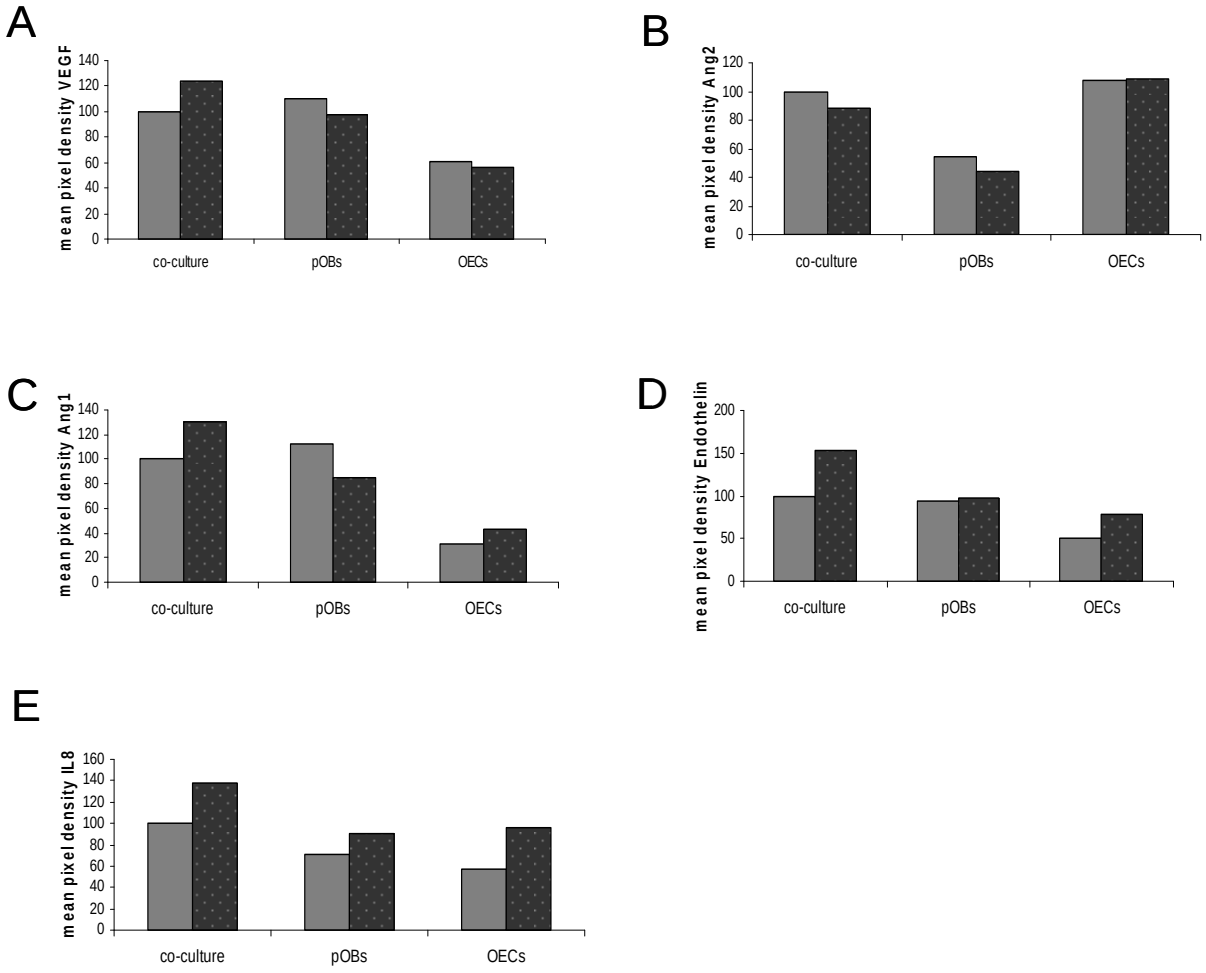


Figure 29. Determination of the intracellular protein level of proangiogenic factors in co-cultures, pOB- and OEC mono-cultures in response to Shh stimulation. The amount of VEGF (A), angiopoietin1 (B), angiopoietin2 (C), endothelin (D) and interleukin-8 (E) in the cell lysates of co-cultures, pOB mono-cultures and OEC mono-cultures was evaluated using a Proteome Profiler^{TM} Human Angiogenesis Array (n=3). Results are referred to a positive control and exhibited as mean pixel densities, which were finally converted into percentage values. All individual values were referred to control co-culture set at 100%.

The Shh-treated co-cultures exhibited an enhanced VEGF protein content (+23.7%) in cell lysates compared to non-stimulated co-cultures (figure 29A), whereas Shh-treated pOB and OEC mono-cultures revealed decreased levels of VEGF in the lysates. Shh treatment of pOB in mono-culture resulted in lower amounts of VEGF, Ang1 (figure 29B) and Ang2 (figure 29C) in the cell lysates compared to unstimulated pOB, but higher amounts of free VEGF, Ang1 and Ang2 released into the culture supernatant (figure 27). In the co-culture free VEGF concentrations might be lower compared to the corresponding mono-cultures of pOB due to binding of VEGF by OEC. Protein profiler arrays also indicated an influence of Shh on other molecules involved in the cellular crosstalk during angiogenic activation and vessel stabilization. Endothelin protein amounts in cell lysates were found to be highly enriched in response to Shh in OEC mono-cultures and in co-cultures. In addition, IL-8 protein synthesis was increased in all types of cultures by stimulation with sonic hedgehog (figure 29).

The temporal relation of the increased gene expression in response to Shh treatment and protein concentrations found in the cell culture supernatants of sonic hedgehog-treated co-cultures was also examined for the first 24 hours of treatment (figure 30, n=3).

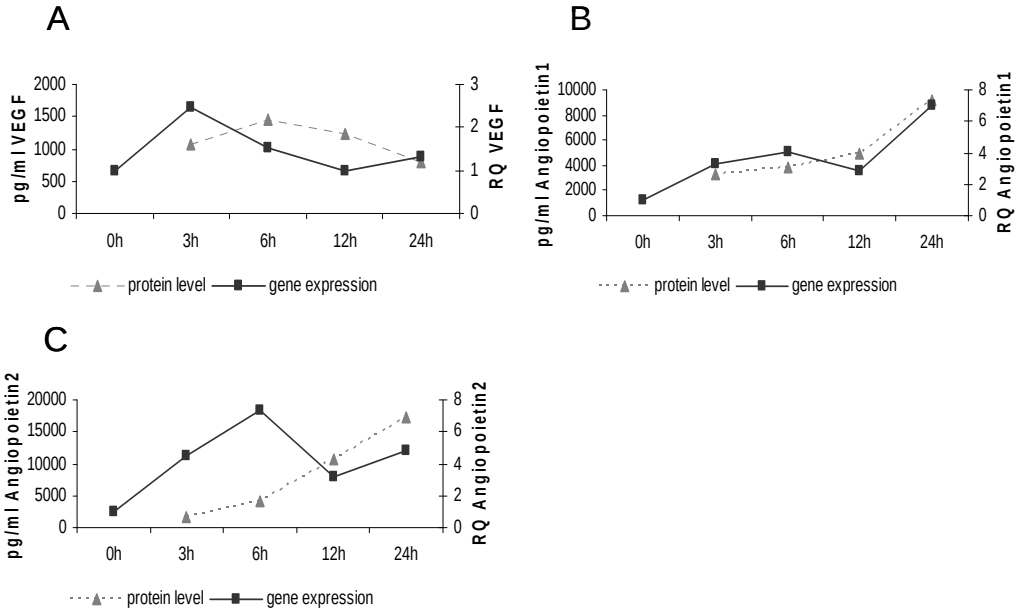


Figure 30. Time course of gene and protein expression in response to Shh. The effect of Shh on the expression of VEGF (A), Ang1 (B) and Ang2 (C) on the mRNA level using quantitative real time PCR as well as on the protein amount of these molecules in the supernatants of co-cultures evaluated using ELISA was determined after 3, 6, 12 and 24 hours of Shh stimulation. For real time experiments RPL13A was taken as endogenous control. Results from ELISA are depicted as absolute values.

The results documented the induction of VEGF, angiopoietin1 and angiopoietin2 release into the cell culture supernatants with a time delay after the increased expression of the related genes, as depicted in figure 30.

3.2.5 Sonic hedgehog treatment enhanced the osteoblastic differentiation process in co-cultures consisting of pOB and OEC

In addition to the beneficial effects of sonic hedgehog on the angiogenic potential of outgrowth endothelial cells in the co-culture system consisting of OEC and pOB, an influence of Shh on the osteoblastic differentiation in the co-culture system could also be advantageous. The stimulatory action of Shh on osteogenic differentiation was already reported in previous studies (Nakamura et al., 1997; van der Horst et al., 2003), but not in a co-culture system.

In the following subchapter, Shh signalling was investigated in co-cultures consisting of primary osteoblasts and outgrowth endothelial cells with regard to its effect on osteogenesis. Therefore, markers involved in the osteogenic differentiation process as well as functional regulating elements during osteogenesis were investigated in the co-culture system in response to sonic hedgehog treatment. In addition, the influence of Shh on matrix mineralization was evaluated using alizarin red staining and quantification.

To assess a possible effect of sonic hedgehog treatment on the osteogenic differentiation process in the co-culture system of OEC and pOB, quantitative real-time PCR to detect the expression of several molecules involved in osteogenic differentiation was performed for co-cultures (figure 31A), as well as for pOB (figure 31B) and OEC mono-cultures (data not shown) serving as controls. After 24 hours of Shh stimulation co-cultures as well as mono-cultures of primary osteoblasts exhibited a tentative upregulation of the osteogenic markers osteocalcin, osteonectin, osteopontin and alkaline phosphatase (ALP), whereas statistical significance was only documented for the upregulation of osteonectin ($p<0.05$) and ALP ($p<0.03$) in co-cultures after 24 hours of treatment (figure 31A). However, sonic hedgehog stimulation for 14 days resulted in a decrease in the expression of osteogenic markers compared to 24h treatment in co-cultures (figure 31A), without and marked changes in pOB mono-cultures (figure 31B).

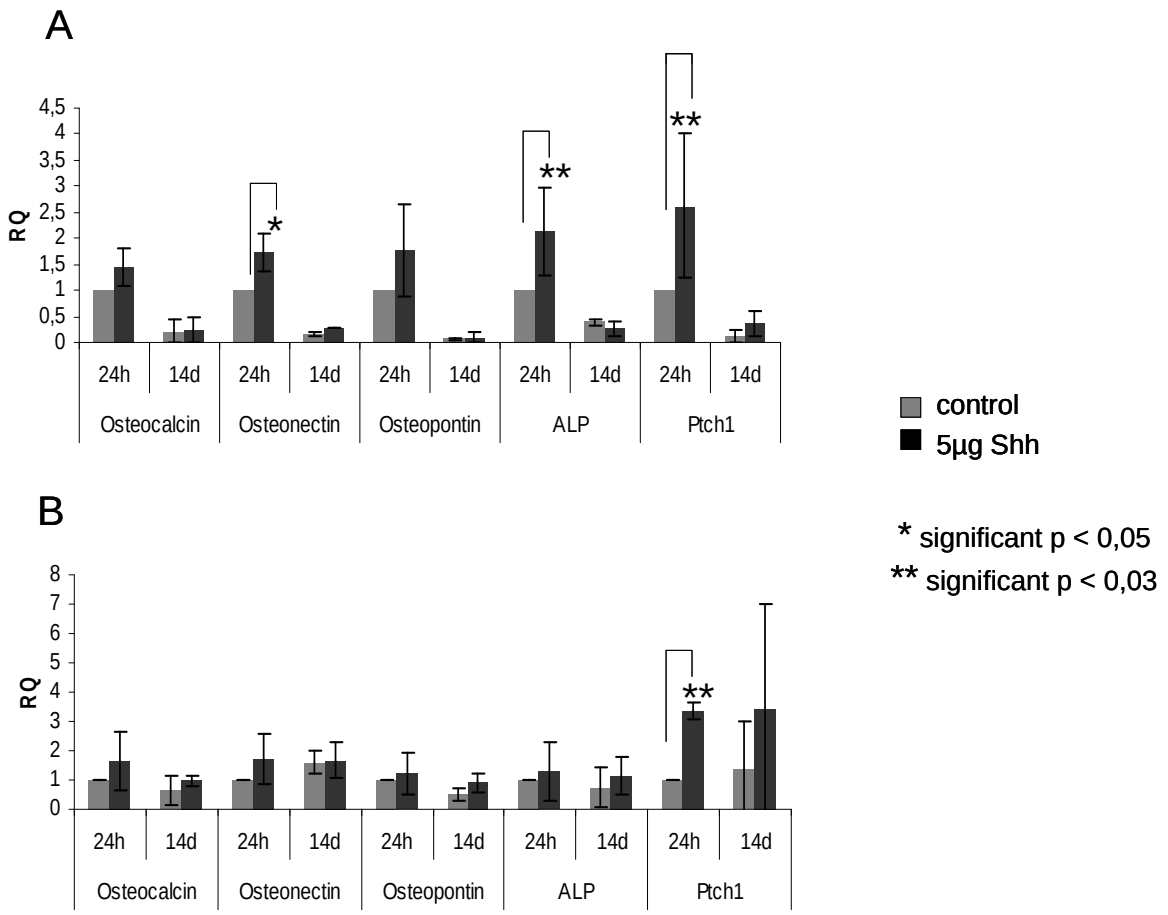


*Figure 31. Shh treatment of co-cultures (A) and pOB mono-cultures (B) resulted in an upregulation of osteogenic factors at the mRNA level. (A) Mean values of relative gene expression of different genes involved in osteogenesis in Shh treated and untreated co-cultures for 24 hours (n=6) and 14 days (n=3). (B) Mean values of relative gene expression of different genes involved in osteogenesis in Shh-treated and untreated pOB mono-cultures for 24 hours (n=6) and 14 days (n=3). RPL13A was taken as endogenous standard in both experiments (A/B). Gene expression of Shh stimulated and non-stimulated co- and mono-cultures was compared by setting control cultures to 1 (reference value). [*p<0.05, **p<0.03]*

RUNX2 (Runt-related transcription factor) is an essential transcription factor for bone formation and skeletal morphogenesis (Ducy et al., 1997) that activates the differentiation process of osteoblasts by inducing the transition of osteoprogenitor cells into preosteoblasts and finally determines the osteoblastic phenotype. Stimulation of co-cultures with sonic hedgehog resulted in an upregulation of this factor compared to non-stimulated co-cultures after 14 days of stimulation evaluated using quantitative real time PCR (figure 32).

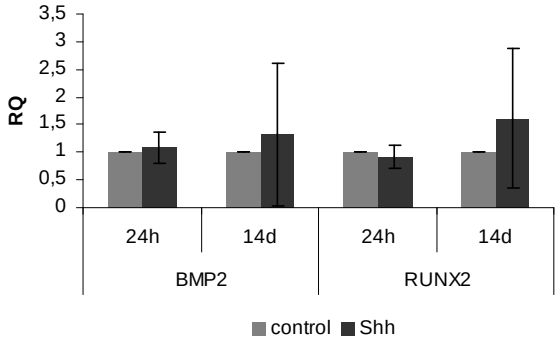


Figure 32. Expression of BMP2 and RUNX2 in co-cultures in response to Shh treatment compared to control co-cultures. Shh treatment resulted in an upregulation of osteogenic factors after 14 days of stimulation. RPL13A was taken as endogenous control. n=3

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor ß superfamily and they also play an important role in regulating osteoblastic differentiation and bone formation (Reddi, 2001). After 24 hours and after 14 days of Shh stimulation co-cultures revealed an upregulation of BMP2 compared to controls, as shown in figure 32. In cell culture supernatants the concentration of BMP2 was measured using an enzyme-linked immunosorbent assay in co-cultures of pOB and OEC, as well as in OEC and pOB mono-cultures in response to sonic hedgehog treatment. The concentration of BMP2 was increased in sonic hedgehog-treated primary osteoblasts after 24 hours and after 14 days of stimulation. In co-cultures and OEC mono-cultures Shh did not affect the BMP2 release at both time points of investigation (figure 33).

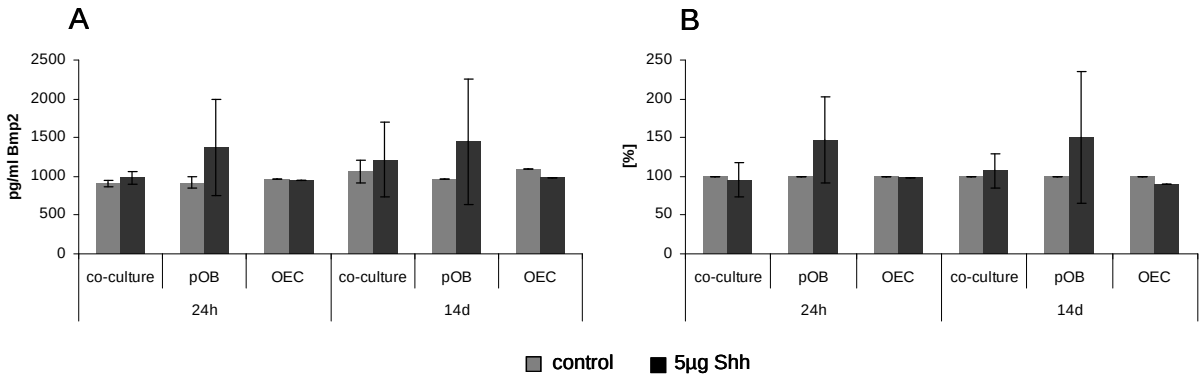


Figure 33. Effects of Shh on BMP2 release in cell culture supernatants of co-cultures as well as in OEC and pOB mono-cultures after 24 hours and 14 days of stimulation. Results are depicted as ratios (A; control=100%) and additionally shown as absolute values (B). n=3.

The potential effect of sonic hedgehog on osteoblastic differentiation as indicated by quantitative real time PCR was further investigated by analysing the effect of sonic hedgehog on the mineralization of mono-cultures and co-cultures after 24 hours and 14 days using alizarin red staining and quantification (figure 34).

In general, mineralization was consistently higher in Shh-treated co-cultures and pOB mono-cultures, compared to non-treated cultures at both time points of investigation (24 hours and 14 days), as indicated by alizarin red assays and depicted in µM alizarin red/mg protein. The mineralization of the cultures increased continuously during the course of sonic hedgehog stimulation from 24 hours, 7 days (data not shown) to 14 days in co-cultures and pOB mono-cultures and was found to be statistically significant in the co-culture after 14 days of Shh treatment ($p<0.05$) (figure 34).

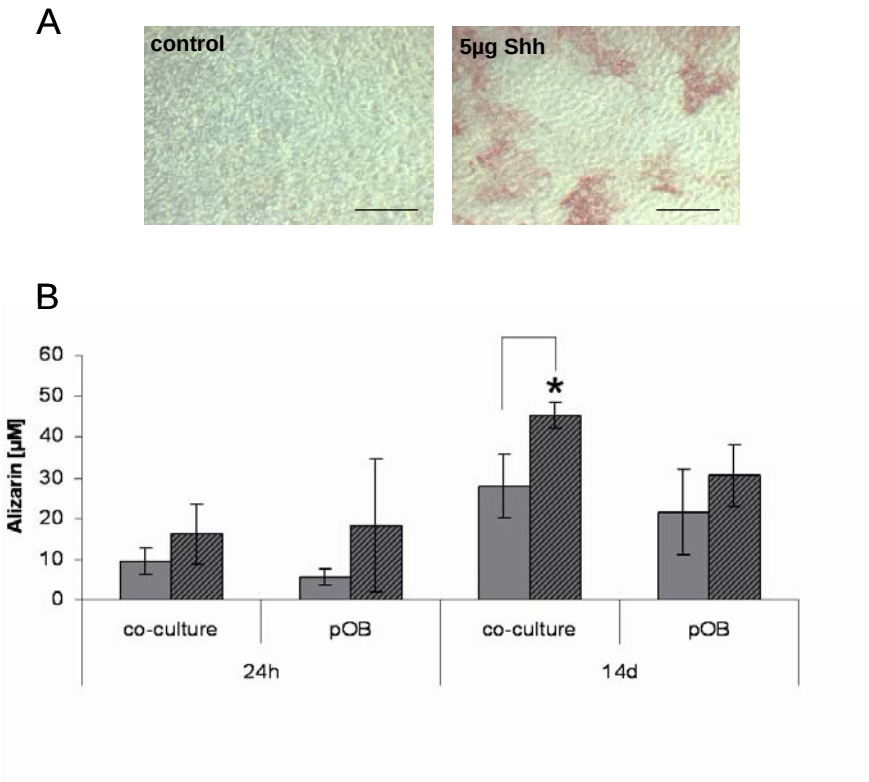


*Figure 34. Effect of Shh on osteoblastic differentiation in the co-culture system consisting of pOB and OEC. Alizarin Red staining of Shh-treated co-cultures after 24 hours (A). (B) Quantification of calcification (alizarin red) in Shh-treated co-cultures (dark bars) and pOB mono-cultures after 24 hours (n=6) and 14 days (n=3) of Shh stimulation compared to unstimulated cultures. Values represent averages of at least three independent experiments in µM alizarin red/mg Protein (B). Scale bar: 200µm. [*p<0.05, **p<0.03].*

This positive effect of Shh on the calcification could be further confirmed by comparing the activity of alkaline phosphatase, an essential enzyme during early osteoblastic differentiation, in stimulated and unstimulated co-cultures and pOB mono-cultures assessed after 24 hours and after 14 days of stimulation. Alkaline phosphatase (ALP) activity is depicted as percentage change compared to controls (figure 35A), as well as in absolute concentrations given as mM/mg protein to identify influences by mono or co-culture or culture time respectively (figure 35B).

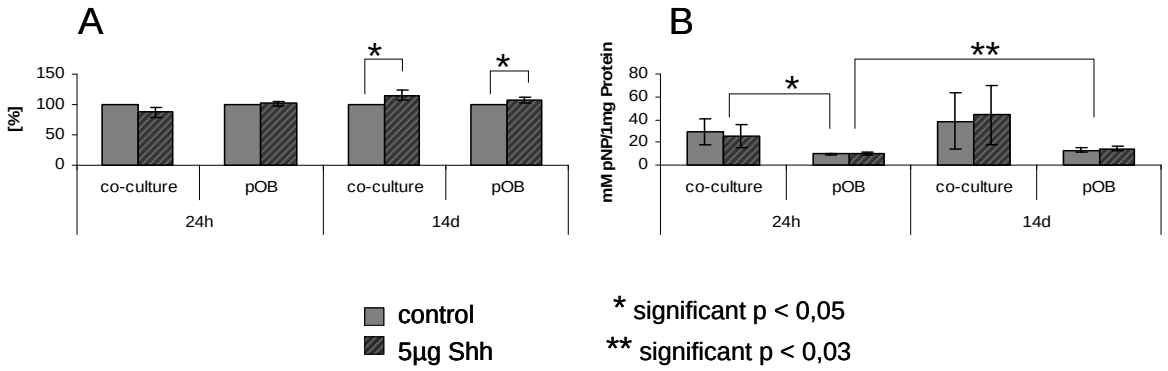


*Figure 35. Effect of Shh on osteoblastic differentiation in the co-culture system consisting of pOB and OEC. Alkaline phosphatase activity within the cell lysate in Shh-treated co-cultures and pOB mono-cultures compared to untreated cultures after 24 hours (n=3) and 14 days (n=3) of stimulation. Results are represented as averages. The absolute alkaline phosphatase activity was defined as mM pNP/mg protein and is depicted in (C). Figure (B) demonstrates the results as ratios with respect to control co-cultures (control = 100%). [*p<0.05, **p<0.03]*

Alkaline phosphatase activity was significantly increased in sonic hedgehog-treated co-cultures and mono-cultures of primary osteoblasts during 14 days of stimulation compared to the corresponding controls (figure 35A, $p<0.05$). In general, the co-cultures revealed a higher ALP activity than the pOB mono-cultures after 24 hours as well as after 14 days ($p<0.05$) (figure 35B).

4. Discussion

4.1 Co-culture system consisting of primary osteoblasts and outgrowth endothelial cells

4.1.1 Positive effect of the co-culture system on the angiogenic activation of OEC

The rapid and successful vascularization of complex tissue engineered constructs for bone regeneration is still a major problem in the field of tissue engineering. Different approaches have been developed over the past decade to provide a rapid connection of the bone construct to the host vasculature to ensure a sufficient supply of oxygen and nutrients needed for the survival of the tissue engineered construct. These vascularization strategies include different approaches like the use of delivery systems for proangiogenic growth factors (Geiger et al., 2005; Gu et al., 2004) or the generation of prevascularized tissues by incorporation of endothelial cells or endothelial progenitor cells (Rivron et al., 2008; Rouwkema et al., 2006). In addition, the use of complex co-culture systems consisting of different cell types is proposed to be another beneficial approach to enhance the vascularization process (Fuchs et al., 2007; Kaigler et al., 2003).

Outgrowth endothelial cells (OEC), a subpopulation of endothelial progenitor cells isolated and expanded from the mononuclear cell fraction from peripheral blood buffy coats, offer a very interesting autologous cell source for the prevascularization of tissue engineered constructs. In contrast to early endothelial progenitor cells (EPC), OEC reveal a more differentiated endothelial phenotype, express a typical mature endothelial marker profile with a high proliferation potential, and form vessel-like structures when seeded on Matrigel basement membrane matrix. Besides this, one major advantage of these cells is the isolation procedure, as they can be very easily collected from the peripheral blood with a minimally invasive intervention in comparison with the isolation of stem cells from the bone marrow. The potential of outgrowth endothelial cells to contribute to the angiogenic process *in vitro* as well as *in vivo* has been examined and documented in several prior studies (Gulati et al., 2003; Hur et al., 2004; Mukai et al., 2008; Reyes et al., 2002; Sieminski et al., 2005; Yoon et al., 2005). Nevertheless, the angiogenic potential of OEC depends very much on the experimental setting. Therefore, the addition of growth factors like vascular endothelial

growth factor or seeding of the cells on 3-dimensional matrices to mimick components of the extracellular matrix is often used. A beneficial strategy to obviate the need for additional growth factors and matrix molecules to promote the angiogenic activity of OEC is the use of co-culture systems. In various studies, only the co-implantation of OEC together with smooth muscle cells (Melero-Martin et al., 2007), mesenchymal stem cells (Au et al., 2008) or osteoblastic cells (Fuchs et al., 2007) has led to the angiogenic activation of OEC and finally to blood vessel formation by OEC.

Blood vessel formation is a highly regulated process which is involved in bone regeneration, and it is generally accepted that the bone repair process is under the critical control of two fundamental processes, namely angiogenesis and osteogenesis (Collin-Osdoby, 1994; Decker et al., 1995; Guillotin et al., 2008). In a co-culture system using outgrowth endothelial cells together with primary osteoblasts (pOB), OEC revealed an organization into microvessel-like structures which increased during the course of co-cultivation according to previous studies from Fuchs et al. (Fuchs et al., 2007). OEC were identified in the co-cultures using confocal microscopy and immunofluorescent staining for the endothelial marker, CD31. OEC in co-culture with pOB formed angiogenic structures, reminiscent of a vasculature, whereas OEC in mono-culture failed to form vascular-like structures. This beneficial effect of osteoblasts on the cellular organization of endothelial cells into tube-like structures in the co-culture system is in accordance with recent reports in the literature (Fuchs et al., 2007; Rouwkema et al., 2006; Santos et al., 2009; Unger et al., 2007). Although the detailed mechanisms of the "cellular crosstalk" that controls the angiogenic activation of endothelial cells induced by the co-culture are still under investigation, it seems that the co-culture with primary osteoblasts provides on the one hand proangiogenic matrix components and on the other hand angiogenic growth factors to promote angiogenic activation of OEC (Fuchs et al., 2009b; Kirkpatrick et al.).

To analyse the molecular basis for this angiogenic activation of outgrowth endothelial cells in the co-culture system of OEC and primary osteoblasts, a human Angiogenesis RT^2 Profiler Array (PCR Array) screening the expression of more than 80 genes involved in the process of angiogenesis was performed after 1 and 4 weeks of co-cultivation. In total, 60 angiogenesis-related genes were upregulated after 4 weeks of co-cultivation, which was in accordance with the increase in the formation of angiogenic structures in a time-dependenet manner, as also reported by Fuchs and Jiang (Fuchs et al., 2009b). The upregulated genes in long term co-

cultures of OEC and pOB included growth factors, their receptors, chemokines, cytokines, matrix molecules and adhesion molecules. Out of these 60 upregulated proangiogenic genes in long term co-cultures of pOB and OEC, some will be discussed exemplarily in the following sections.

The proangiogenic growth factor, vascular endothelial growth factor A (VEGFA), for instance was clearly upregulated after 4 weeks of co-cultivation compared to control co-cultures (1 week). VEGFA, known to be the most important mediator of angiogenic activation, has been proposed to be essential for the chemotaxis and differentiation of endothelial progenitor cells, including angioblasts, as well as for endothelial cell proliferation, vascularization via integration of activated endothelial cells into vessel-like structures, and also for remodelling of vascular structures (Adams and Alitalo, 2007; Leung et al., 1989; Plouet et al., 1989; Shalaby et al., 1995). The activity of VEGFA is mediated through VEGF receptor2, also called KDR or Flk-1, which is expressed on endothelial cells (Clauss et al., 1996). Gene knockout studies in mice have demonstrated that KDR is essential for the development of a vascular system. In the absence of KDR embryos fail to develop blood vessels as a result of failure of endothelial cell differentiation (Shalaby et al., 1995). Clarkin et al. showed that in a co-culture system of osteoblasts and endothelial cells VEGF is mainly expressed and released by the osteoblasts and that this osteoblast-derived VEGF leads to the activation/phosphorylation of KDR in endothelial cells and finally to angiogenic activation, thus supporting a paracrine action of osteoblast-derived VEGF on the angiogenic activation of endothelial cells (Clarkin et al., 2008). Several other groups demonstrated that bone marrow-derived mesenchymal stem cells secrete VEGF, which in turn enhances the proliferation and differentiation of endothelial cells, thus indicating a paracrine effect of VEGFA on the angiogenic activation of endothelial cells (Kaigler et al., 2003; Mayer et al., 2005). These results documented in the recent literature support an osteoblastic origin of VEGF in the co-culture system of OEC and pOB which finally leads to the angiogenic activation of OEC during the course of co-cultivation. In addition, it is generally accepted that other members of the VEGF family might be involved in the process of angiogenesis as well (Nicosia, 1998). FIGF, also called VEGFD, was also markedly upregulated in long term co-cultures of OEC and pOB as evaluated using the Angiogenesis RT^2 Profiler Array. This is in accordance with results of Marconcini et al. who described that FIGF/VEGFD also acts as a potent angiogenic factor by phophorylating VEGF receptor 2 (KDR) in primary human umbilical vein

endothelial cells *in vitro*, this finally leading to the angiogenic activation of endothelial cells (Marconcini et al., 1999).

Another upregulated proangiogenic molecule after 4 weeks of co-cultivation which was assessed using Angiogenesis PCR-Array and quantitative real-time PCR was angiopoietin2 (Ang2). The Ang-Tie system is essential for angiogenic activation of endothelial cells but is also important during later stages of vascular development, when the vessels become mature and stable as a result of pericyte coating (Augustin et al., 2009; Saharinen et al.). Angiopoietin1 and angiopoietin2 are ligands for only one receptor that is called Tie-2 or TEK (Maisonpierre et al., 1997; Suri et al., 1996), which is expressed in the embryonic endothelium, the vasculature of the adult and in bone-marrow-derived monocytes or mesenchymal progenitor cells (De Palma et al., 2007; Dumont et al., 1992). The angiogenic activity of Ang2 is very complex. Angiopoietin2 is strongly upregulated in response to hypoxia in ischaemic tissues, suggesting that this molecule might act as a potential factor in neovascularization processes at sites of injured tissue (Mandriota et al., 2000; Shyu et al., 2004). Additionally, it is generally accepted that in collaboration with VEGF, Angiopoietin2 might also act as a proangiogenic co-factor inducing the angiogenic activation of endothelial cells (Visconti et al., 2002).

Transforming growth factor beta (TGFbeta) was even 410 times more highly expressed in the co-culture cultivated for 4 weeks compared to 1 week of co-culture. TGFbeta family currently contains 33 members, critically involved in embryogenesis and in maintaining the homeostasis of adult tissues (Heldin et al., 1997). Originally, TGFbeta was discovered through its effects on fibroblasts, but during the past decade this molecule was also identified to modulate immune cells, endothelial cells and mural cells (Roberts and Sporn, 1993). Mice knockout studies revealed that TGFbeta signalling plays a critical role in angiogenesis. Several cardiovascular diseases were shown to be associated with TGFbeta mutations (Goligorsky et al., 1999). Moreover, it has been described that TGFbeta regulates the function of endothelial cells and vascular smooth muscle cells by affecting the activation state of endothelial cells, including proliferation, migration and differentiation as well as extracellular matrix production (Goumans et al., 2009).

These first results revealed a time-dependent proangiogenic effect of the co-culture of OEC and pOB, as indicated by the organization of OEC into angiogenic structures without

additional treatment with growth factors. This beneficial effect of the co-culture on the formation of microvessel-like structures by OEC clearly increased during the course of co-cultivation and is proposed to be mediated through the upregulation of different proangiogenic growth factors, e.g. vascular endothelial growth factor A, angiopoietin2 and transforming growth factor beta. The potential origin of the growth factors and the detailed mechanism of the angiogenic activation of OEC in the co-culture system will be discussed in later sections.

4.1.2 Stabilizing matrix components and mural cells associated with angiogenic structures in the co-culture system

4.1.2.1 Basement membrane proteins

Out of the 60 upregulated genes which could be detected after 4 weeks of co-cultivation as evaluated using the human Angiogenesis RT^2 Profiler Array (PCR Array), collagen type 18 seemed to be one of the most affected genes in response to the co-cultivation time. Collagen type 18, which is known to be involved in mediating the link between extra- and intracellular structural elements, was 220 times more highly expressed in 4 week co-cultures compared to controls (1 week). This molecule is a nonfibrillar collagen of basement membranes and is expressed in highly vascularised tissues such as the liver, the lung or the placenta (Guenther et al., 2001; Muragaki et al., 1994).

Blood vessels are composed of at least three cell layers, the intima, the media and the adventitia or outer layer. The wall of all vessels is composed of an inner single cell layer of endothelial cells. This inner layer is surrounded by mural cells (pericytes and smooth muscle cells). In the capillary system both endothelial cells and mural cells share a common basement membrane and are embedded in the extracellular matrix, the outer layer of blood vessels (Jones, 1979). The extracellular matrix (ECM) is essential for a vascular endothelium by providing a scaffold which is necessary for maintaining the organization of endothelial cells into blood vessels. Adhesion of endothelial cells to ECM is required for several mechanisms which are necessary for neovascularization, including endothelial cell proliferation, morphogenesis, survival and blood vessel stabilization (Davis and Senger, 2005). Numerous

extracellular matrix molecules are known to promote endothelial cell growth, migration or tube formation (Bourdoulous et al., 1998).

Several extracellular matrix glycoproteins have been identified in or closely associated with the basement membrane. Immunofluorescence staining for the basement membrane proteins laminin and collagen type IV demonstrated the existence of these proteins in long term co-culture systems of OEC and pOB. Angiogenic structures, stained positively for the endothelial marker von Willebrand factor, were clearly associated with these basement membrane proteins. According to this, relative gene expression analysis revealed a significant upregulation of laminin and collagen type IV in long term co-cultures of OEC and pOB.

Both proteins, laminin and collagen type IV, comprise the majority of proteins found in basement membranes (Herbst et al., 1988) and there is increasing evidence that these matrix proteins play important roles in the process of angiogenesis (Bell et al., 2001). The deletion of collagen IV, for instance, results in embryonic lethality (Poschl et al., 2004), which reflects the importance of this protein. Detection of these basement membrane proteins in the co-culture of pOB and OEC suggested that the co-culture system might support the angiogenic activation as well as basement membrane assembly, but the detailed mechanisms that regulate matrix assembly are yet not well understood.

4.1.2.2 Mural cells

In recent studies, the role of mural cells, i.e. pericytes and vascular smooth muscle cells (SMC), during angiogenesis has come more and more into focus. To investigate whether mural cells are present during the formation of microvessel-like structures in the co-cultures of OEC and pOB, co-cultures cultivated for 4 weeks were stained immunohistochemically for the smooth muscle cell-associated markers, α-smooth muscle actin and desmin. In the co-culture system OEC were found to be associated with α-smooth muscle actin- and desmin-positive cells. In accordance with this, relative gene expression of alpha smooth muscle actin increased significantly during the course of co-cultivation.

Vascular smooth muscle cells are found around blood vessels and are necessary for the formation of a mature and functional vascular network. During blood vessel formation newly formed vessels become enveloped by pericytes, when activated endothelial cells secrete

platelet derived growth factor (PDGF-BB) which interacts with its receptor PDGFRß on the surface of pericytes, resulting in the proliferation and recruitment of pericytes to sites of new vessels (Gerhardt and Betsholtz, 2003; von Tell et al., 2006). Absence of pericytes/smooth muscle cells during vascular development leads to endothelial hyperplasia and abnormal vascular remodelling (Xueyong et al., 2008). The existence of alpha smooth muscle actin- and desmin-positive cells associated with angiogenic structures formed by OEC in the co-culture system revealed that the co-culture did not just induce the organization of OEC into angiogenic structures, but also enhanced the maturation of newly formed microvessel-like structures through the attachment of smooth muscle cells/pericytes which finally leads to the formation of a stable and functional blood vessel (Lindahl et al., 1997; Risau, 1997).

Several experiments were performed to reveal the potential origin of α-smooth muscle actin-positive cells in the co-culture of primary osteoblasts and outgrowth endothelial cells. Immunofluorescent staining for α-smooth muscle actin demonstrated negative staining for OEC, but a positive reaction for primary osteoblasts and human umbilical artery smooth muscle cells (HUSMC), used as positive control, thus supporting an osteoblastic origin of alpha smooth muscle actin-positive cells associated with endothelial cells. Further evidence for an osteoblastic origin of smooth muscle cells within the co-culture came from an analysis of gene expression of smooth muscle cell-associated markers using quantitative real-time PCR. Expression of alpha smooth muscle actin, calponin and smoothelin could be detected in pOB mono-cultures and in long term co-cultures, as well as in control HUSMC, whereas no expression of these markers was detected in OEC mono-cultures. Primary osteoblasts used for the co-culture experiments in this study were isolated from human cancellous bone fragments in the form of small explants were cultivated for 2-4 weeks until outgrowth of primary osteoblasts occurred. These primary osteoblasts, which also originally arise from mesenchymal stem cells (Caplan, 1991; Owen, 1988; Pittenger et al., 1999), are not a homogenous cell population. Due to the isolation procedure it is possible that pericytes or pericyte progenitors also exist in the culture of primary osteoblasts. This could explain the appearance of alpha smooth muscle actin-positive cells in the co-culture system.

The ontogeny of pericytes/smooth muscle cells seems to be very complex, due to the fact that pericytes can develop from various sources. They can arise from the neuralcrest, developing into pericytes in the central nervous system but they can also originate from the cardiac system enveloping coronary vessels (Bergwerff et al., 1998; Etchevers et al., 2002). Recent

studies suggest that human smooth muscle cells may originate from the bone marrow, which indicates that there might be a circulating pericyte/smooth muscle cell progenitor in the peripheral blood. Au et al., 2008 have shown data from animal studies suggesting that human bone marrow-derived mesenchymal stem cells (hMSC) are able to differentiate into pericytes/smooth muscle cells. This supports the hypothesis that hMSC may function as progenitors which are necessary to form a functional blood vessel (Au et al., 2008). Although the results supported an osteoblastic origin of alpha smooth muscle actin-positive cells found in the co-culture of pOB and OEC, the possibility that these cells could arise from the OEC cannot be excluded. Yamashita et al. (2000) identified an embryonic vascular progenitor with the capacity to differentiate into both endothelial and smooth muscle cells in response to different growth factors, such as PDGF-BB, indicating that pericytes and endothelial cells might share a common progenitor (Yamashita et al., 2000). CD34 cell separation is commonly used to enrich endothelial progenitor cells from the mononuclear cell fraction from the peripheral blood. In 2002, Simper et al. demonstrated for the first time that CD34-positive cells can also give rise to vascular smooth muscle cells (Simper et al., 2002). In addition, it has been suggested that transplanted endothelial progenitor cells might be able to transdifferentiate into vascular smooth muscle cells as well (Iwasaki et al., 2006; Jujo et al., 2008). Isolating a population of human progenitor cells from peripheral blood with the potential to give rise to both endothelial cells and smooth muscle cells, could benefit the entire field of tissue engineering.

4.1.3 Maintained osteogenic differentiation capacity in the co-culture system

For successful bone repair a tissue engineered bone construct that combines a rapid connection to the vasculature as well as the capability to maintain an osteoblastic phenotype would be advantageous for a therapeutic intervention in bone tissue engineering. Bone is a highly vascularized tissue and bone formation is critically under the control of the interaction between the endothelium and bone cells (Kanczler and Oreffo, 2008). As explained in the previous chapters, within the co-culture systems, osteoblasts seemed to support the angiogenic activity of outgrowth endothelial cells, most likely by providing proangiogenic cues and the supply with growth factors like VEGF, angiopoietin2 and TGFbeta. These, in turn, might lead in the co-culture system to the induction of microvessel-like structures

formed by endothelial cells and vessel maturation, this being in accordance with the literature (Clarkin et al., 2008; Tokuda et al., 2003).

Besides the effect of the co-culture system on the angiogenic activation of OEC, the osteogenic potential of the co-culture system was also of interest in the scope of this thesis. In 2009, Fuchs et al. analysed the dynamic processes involved in the differentiation and functionality of both cell types in the co-culture system as a function of cultivation time (Fuchs et al., 2009b). Due to the fact that different cell types require different demands in terms of culture conditions they tested the influence of the cell culture medium on the osteogenic differentiation of pOB and found an improved calcification of the co-culture in endothelial cell growth medium (EGM-2) compared to cultivation in the osteogenic medium, DMEM F-12. In addition, they described a time-dependent upregulation of the osteogenic factors, osteocalcin and osteopontin, in co-cultures of pOB and OEC seeded on silk fibroin, thus indicating an ongoing osteoblastic differentiation in the co-culture system (Aubin, 1998; Aubin, 2001; Liu et al., 1994).

To screen for effects of the co-culture system of pOB and OEC on osteoblastic differentiation during the course of co-cultivation, a human Osteogenesis RT^2 PCR Array detecting the expression of 84 genes involved in or related to the process of osteogenic differentiation helped to profile the co-culture system in terms of osteogenesis. More that 40 osteogenesis-related genes were found to be upregulated in long term co-cultures co-cultivated for 4 weeks. Of these upregulated osteogenic genes, bone morphogenetic protein 4 (BMP4), TGFbeta2 as well as amelogenin (AMELY) and VEGF family members and their receptors were the most affected genes. These observations will be discussed in more detail below.

Bone morphogenetic protein 4 (BMP4) revealed the clearest difference in gene expression after 4 weeks of co-cultivation compared to control co-cultures (1 week). In contrast to 1 week of co-cultivation, BMP4 was upregulated 200 times after 4 weeks of co-cultivation. Bone morphogenetic proteins belong to the TGFbeta superfamily of growth factors and induce the formation of cartilage and bone, but they are also important during other developmental processes like neural crest development (Christiansen et al., 2000; Munoz-Sanjuan et al., 2002). Although the detailed molecular mechanisms that control osteoblastic differentiation are not completely understood it is generally accepted that BMPs, like BMP2, BMP4 and BMP7, are involved in the process of osteogenic differentiation during bone

formation (Reddi, 2001). The considerable upregulation of BMP4 in long term co-cultures of pOB and OEC suggested an ongoing process of osteogenic differentiation, in addition to the ongoing process of angiogenesis which has already been discussed. From the literature it is known that implantation of collagen carrier in combination with additional treatment with BMP2, BMP4 and BMP7 can heal critical sized bone defects in rodents and sheep compared to implantation of the carrier alone (Kang et al., 2004). In addition, several groups have already shown that BMPs are potent local factors regulating osteoblastic differentiation by inducing mesenchymal stem cells to differentiate into osteoblastic lineage cells (Katagiri et al., 1993; Yamaguchi et al., 1991).

One of the main growth factor acting on the formation of the skeleton is transforming growth factor beta (TGFbeta), which already came into focus in terms of the angiogenic activation of OEC in the co-culture system of pOB and OEC, as discussed in former sections. In the human Osteogenesis RT^2 PCR array TGFbeta was 100 times more highly expressed in co-cultures cultivated for 4 weeks compared to 1 week of co-cultivation. TGFbeta is expressed by endothelial cells, platelets, osteoblasts and chondrocyte and supports numerous processes, such as mesenchymal stem cell proliferation, osteoblastic and chondrocyte differentiation, bone matrix production as well as endothelial cell function, thus documenting the close association of angiogenesis and osteogenesis during the process of bone formation. Several studies reported that TGFbeta is able to induce osteoblastic differentiation during endochondral bone formation and is also crucial for regulating craniofacial development (Iwata et al., ; Ripamonti et al., 2009; Ripamonti and Roden).

Amelogenin, upregulated 84 times and in a culture time dependence, is a protein that usually plays a critical role during tooth development by regulating the growth of hydroxyapatite crystals during enamel mineralization (Nakahori et al., 1991). Amelogenins comprise a family of highly conserved extracellular matrix proteins usually strongly expressed in the dental enamel organ in the developing tooth (Brookes et al., 1994). During the past decade there is increasing evidence from the literature that amelogenins might positively influence processes of wound healing and bone formation in mesenchymal tissues (Lyngstadaas et al., 2009; Warotayanont et al., 2008). Reseland et al. revealed that amelogenin could have a positive effect on the mineralization of osteoblastic cells as demonstrated by an increase in alkaline phosphatase activity (ALP), as well as in an increased expression of the osteoblastic markers, osteocalcin and collagen type I in response to amelogenin (Reseland et al., 2006). It has been

shown that this molecule could stimulate autocrine production of bone morphogenetic proteins (BMP) and transforming growth factor beta (TGFbeta) (Suzuki et al., 2005), as well as the production of vascular endothelial growth factor (VEGF), platelet derived growth factor BB (PDGF-BB) and interleukin-6 (Lyngstadaas et al., 2001).

Another upregulated factor in long term co-cultures which reflects the close association of angiogenesis and osteogenesis was vascular endothelial growth factor (VEGF), known to have multiple activities in regulating bone formation as well. During bone development VEGF is involved in controlling the activity of endothelial cells, chondrogenic cells and osteogenic cells (Coultas et al., 2005). VEGF is highly expressed in developing bones before any blood vessels exist within the developing tissue. Finally, bone formation occurs when angiogenic activity of endothelial cells is induced by upregulation of VEGF in osteoblasts mediated through upregulation of HIF-1 alpha which is under the control of hypoxia (Zelzer et al., 2002). Besides the well established effect of VEGF on endothelial cells during bone formation, VEGF might also play a role during osteoblastic differentiation. It has already been documented that blocking VEGF inhibited bone formation in juvenile mice (Gerber et al., 1999). In addition, exogenous VEGF-A was found to stimulate the osteoblastic differentiation of a preosteoblast-like cell line (Deckers et al., 2000). A study of Street and Lenehan revealed that primary human osteoblasts express functional VEGF receptors and it was reported that VEGF induced alkaline phosphatase activity in these primary osteoblasts, suggesting a regulatory role of vascular endothelial growth factor in terms of the osteoblastic differentiation as well (Street and Lenehan, 2009).

4.1.4 Direct and indirect communication between OEC and pOB in the co-culture

The previous sections discussed the beneficial effect of the co-culture system of primary osteoblasts and outgrowth endothelial cells on angiogenic activation and vessel maturation, as well as on osteogenic differentiation processes. Although the detailed interaction between osteoblasts and endothelial cells in the co-culture is currently under investigation, it seems that the cells communicate in two different ways, namely in an indirect paracrine way as well as in a direct way (Guillotin et al., 2008; Villars et al., 2002).

One important mechanism of direct cell to cell communication is mediated by gap junctions which form a transcellular channel between cells that permits the passage of ions, metabolites

and other physiologically active molecules, including amino acids or small peptides from one cell to another (Jongsma and Wilders, 2000; Rossello and Kohn, 2009). In bone cells the most dominant gap junction protein is connexin 43 (Cx43) which participates in the process of osteoblastic differentiation by inducing a higher level of osteogenic differentiation markers when bone cells are exposed to mechanical stimuli (Stains and Civitelli, 2005; Ziambaras et al., 1998). Villars et al. demonstrated an increase of alkaline phosphatase (ALP) activity in a direct co-culture system of human umbilical vein endothelial cells (HUVEC) and primary human osteoprogenitors that was mediated by the synthesis of the gap junction protein, connexin 43. The increase of alkaline phosphatease activity did not occur when cells were seeded in an indirect co-culture system using a culture dish insert (Villars et al., 2000; Villars et al., 2002).

Additionally, several groups documented a paracrine mode of action between endothelial cells and osteoblasts in co-culture systems. It is widely accepted that within the co-culture system, the osteoblasts provide proangiogenic cues, such as matrix components like collagen, as well as growth factors, including vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF). The latter are thought to induce angiogenic activation of endothelial cells in the co-culture system (Clarkin et al., 2008; Tokuda et al., 2003). Another essential signalling factor between vascular and osteoblastic cells that acts in a paracrine way during bone formation is endothelin-1. This peptide is produced by endothelial cells and increases osteoblastic progenitor cell differentiation by increasing alkaline phosphatase activity and upregulating osteoblastic differentiation genes (von Schroeder et al., 2003). The same group has demonstrated that endothelin-1 could have a regulating function in the paracrine control of VEGF-induced angiogenesis in osteoblastic cells, thus indicating a feedback mechanism in the coupling of angiogenesis and osteogenesis during bone formation. This seems to be mediated via osterix, an early osteoblastic transcription factor (Qu and von Schroeder, 2006; Veillette and von Schroeder, 2004).

Taken together, a co-culture model consisting of outgrowth endothelial cells and primary osteoblasts appears to be a beneficial instrument to improve the angiogenic activation of OEC, as well as maintenance of osteogenic differentiation capacity of primary osteoblasts, as documented in the previous sections. In addition, this co-culture model of OEC and pOB might serve as a suitable system to study the molecular and cellular mechanisms during the

process of bone regeneration and repair and could be an advantageous *in vitro* test system to mimick the fundamental processes during bone regeneration.

4.2 Effects of Sonic Hegdehog on the co-culture system of pOB and OEC

In general, hedgehog family proteins are important morphogens mediating many embryonic developmental processes, including the development of the nervous system, limbs and skin. In addition, they play an essential role in adult morphogenesis, tissue homeostasis and repair of damaged tissue (Cohen, 2003; Heine et al., 2009; Nagase et al., 2007). In 2001, Pola et al., were the first group that pointed out the role of hedgehog proteins during angiogenesis (Pola et al., 2001). They found the presence of the hedgehog signalling pathway in adult cardiovascular tissue and were able to activate this pathway *in vivo,* resulting in the induction of angiogenesis. *In vitro*, Shh was identified as an indirect angiogenic agent mediating the upregulation of proangiogenic factors like vascular endothelial growth factor (VEGF) and angiopoietins, thus promoting the angiogenic activation of endothelial cells. Additionally, several recent publications have demonstrated that sonic hedgehog mediates capillary formation of human umbilical vein endothelial cells (HUVEC), as well as immortalized murine brain capillary endothelial cells and endothelial progenitor cells from the bone marrow, indicating direct effects of sonic hedgehog on the angiogenic activation of endothelial cells (Asai et al., 2006; Kanda et al., 2003).

In initial experiments to test a possible role of sonic hedgehog in the co-culture system of pOB and OEC, expression of the sonic hedgehog receptor, patched1 (Ptc1), could be detected in co-cultures after 1 and 4 weeks of cultivation, as well as in pOB and OEC mono-cultures. The positive effect of the co-culture system of pOB and OEC on angiogenic activation of OEC was already documented in the present and in previous studies, revealing a proangiogenic effect on OEC by the co-culture with primary osteoblasts to form luminal vascular structures *in vitro* and perfused microvessels *in vivo* which were connected to the host's vasculature (Fuchs et al., 2009a; Fuchs et al., 2007). Treatment of co-cultures with the sonic hedgehog inhibitor, cyclopamine, reduced the formation of vascular structures by OEC in the co-culture system in a dose-dependent manner, indicating that the sonic hedgehog pathway could be involved in the angiogenic activation of outgrowth endothelial cells in the co-culture system.

4.2.1 Sonic hedgehog treatment rapidly induced the angiogenic activation of OEC in co-cultures compared to VEGF treatment

To improve the process of angiogenic activation in the co-culture of pOB and OEC and to accelerate the formation of microvessel-like structures, additional treatment of co-cultures with proangiogenic growth factors or morphogens could be beneficial. Therefore, vascular endothelial growth factor A (VEGFA) is commonly used in the field of bone tissue engineering as a potent proangiogenic agent for *in vitro* and *in vivo* applications (Elcin et al., 2001; Sun et al.). Treatment of co-cultures with sonic hedgehog compared to treatment with the commonly used proangiogenic factor VEGF resulted in a marked difference in terms of angiogenic activation after 24 hours of stimulation. Stimulation of co-cultures with Shh for 24 hours revealed a significant increase in the formation of microvessel-like structures compared to 24 hours of VEGF treatment. Nevertheless, in experiments using longer periods of treatment (14 days) the angiogenic activity of OEC in Shh- or VEGF-treated cultures was comparable. In the context of currently discussed prevascularization strategies, which aim to include preformed vascular structures and result in perfused vascular structures after implantation (Fuchs et al., 2009b), Shh might be used to accelerate and improve the formation of angiogenic structures in the co-culture sytem of OEC and pOB. In addition, Shh could help reduce effectively the pre-cultivation time *in vitro*.

To analyze the molecular basis of the sonic hedgehog-mediated effect on angiogenic activation of OEC in the co-culture system, several proangiogenic factors were examined in response to 24 hours and 14 days of Shh treatment. This was performed both on the mRNA as well as on the protein level. In the co-culture system shh exerted positive effects on the expression and secretion of proangiogenic factors, such as VEGF and angiopoietins. These observations were proven on the mRNA level using quantitative real time PCR and on the protein level by ELISA and protein array studies.

By comparing the effects of Shh treatment on mono- and co-cultures utilising ELISA and protein arrays, it could be demonstrated that sonic hedgehog treatment resulted in increased secretion of VEGF by primary osteoblasts, which in turn might be responsible for the activation of endothelial cells in the co-culture. In addition, increased levels of Ang1 in the co-cultures following sonic hedgehog treatment appeared to originate from the primary osteoblasts. These observations are in accordance with reports from the literature

documenting increased levels of VEGF and Ang1 in Shh-treated mesenchymal cells (Asai et al., 2006; Pola et al., 2001) and the expression of Ang1 (Horner et al., 2001; Kasama et al., 2007; Park et al., 2006) and VEGF in osteoblasts. These observations support an indirect effect of Shh on the angiogenic activation of OEC in the co-culture system. A direct influence of Shh was also documented by the upregulation of the Shh receptor, patched1, a transcriptional target of Shh signalling, in response to Shh stimulation in OEC as well as in pOB mono-cultures and their co-cultures, indictating activation of the Shh pathway in all cell types tested. The upregulation of Ang2 gene expression or its increased release into the cell culture supernatant appears to be mediated by OEC in the co-cultures. This is in accordance with the origin of Ang2 from endothelial cells, as reported in the literature (Thomas and Augustin, 2009), and supports a direct effect of Shh on the OEC with angiogenic activation of OEC in the co-culture as well. Direct effects of sonic hedgehog on the angiogenic activity of OEC were also observed in control experiments using OEC mono-cultures seeded on Matrigel basement membrane matrix. In these controls Shh stimulation induced the formation of capillary-like structures in OEC as a result of direct effects, as previously reported for other endothelial cell types (Soleti et al., 2009) or endothelial progenitor cells (Madoka et al., 2008). Additional experiments using indirect co-cultures of pOB and OEC on transwell filter systems analysed by ELISA further confirmed the origin of VEGF and angiopoietin1 as products of the primary osteoblasts and angiopoietin2 from the OEC.

Thus, the study revealed that besides the potential to induce vascular structures after a very short incubation time, Shh also offered the advantage of stimulating at least three different angiogenic factors, including VEGF itself, but also angiopoietin1 and 2 in accordance with previous studies (Pola et al., 2001). This increase in the expression and secretion of three essential proangiogenic molecules in turn might be responsible for the promotion of the angiogenic activation of OEC in the co-culture compared to VEGF stimulation alone.

Nevertheless, in comparison to Shh treatment and untreated controls VEGF treatment of co-cultures resulted in a significant increase of angiopoietin2 in supernatants after 24 hours and after 14 days of stimulation. The roles of Ang1 and Ang2 in terms of angiogenesis seem to be pleiotropic and the precise balance between both molecules competing for the Tie-2 receptor is necessary to control angiogenic activation of endothelial cells as well as the maturation of newly formed vessels. Ang1 is thought to stimulate the growth of vessels by mobilizing endothelial progenitor cells and inducing the recruitment of pericytes (Suri et al., 1996;

Visconti et al., 2002) whereas angiopoietin2 has been proposed to act as a proangiogenic factor together with VEGF (Visconti et al., 2002). By comparing the effects of Shh and VEGF treatment on the co-culture system, it seems that VEGF treatment shifted the balance of the two angiopoietins in the system in favour of angiopoietin2. This could explain the similar angiogenic activity of outgrowth endothelial cells in co-cultures at the later time point (14 days) independent of whether the co-culture has been treated with VEGF or sonic hedgehog.

Besides the effects of sonic hedgehog on the expression and secretion of VEGF and angiopoietins, Shh also enhanced the expression of other angiogenic factors and cytokines in the co-cultures, such as endothelin-1 and interleukin-8 (IL-8), as evaluated by angiogenesis protein arrays. Interleukin-8, also named CXCL-8, was originally identified as a proinflammatory cytokine upregulated in direct proportion with progressing inflammation (Mazzucchelli et al., 1994). This cytokine is known to play an important role in endothelial cell survival (Li et al., 2003) and exerts pro-angiogenic effects on endothelial cells *in vitro* as well as *in vivo* (Heidemann et al., 2003; Koch et al., 1992; Strieter et al., 1992). Endothelin-1 is induced via hypoxia in endothelial cells (Kourembanas et al., 1991), stimulates proliferation and migration of endothelial cells and is a potent mitogen for smooth muscle cells suggesting that it might act as an angiogenesis-stimulating factor (Goligorsky et al., 1999; Morbidelli et al., 1995). Furthermore, endothelin-1 is involved in the control of the cellular cross talk of endothelial cells and osteoblasts to regulate the levels of VEGF (von Schroeder et al., 2003).

Although the co-culture process itself seems to be beneficial for the angiogenic process, leading to the formation of angiogenic structures (as demonstrated in the first chapters), sonic hedgehog might be an interesting therapeutic option to further accelerate the formation of capillary structures in co-culture systems of OEC and pOB. This was observed as a promoting effect on angiogenic activation of OEC as early as 24 hours following Shh treatment. This might be of particular importance for a fast connection to the vascular supply of the host after implantation of a tissue engineered construct.

4.2.2 Sonic hedgehog, but not VEGF, induced the expression of factors involved in vessel maturation and stabilization

Successful vascularization and formation of functional vessels also depends on stabilization of vascular structures by mural cells. Although it is generally accepted that ectopic transient VEGF expression leads to the induction of new vessels, these VEGF-induced vessels are physiologically often non-functional because they lack pericytes (Spanholtz et al., ; Sundberg et al., 2002). In order to generate a stable, functional and long-lasting vasculature, newly formed vessels need to be stabilized through the recruitment of mural cells, which include pericytes and vascular smooth muscle cells (Carmeliet, 2005; Lee et al., 1997).

In comparison to VEGF treatment, Shh treatment of co-cultures resulted in an upregulation of the smooth muscle cell-associated markers, α-smooth muscle actin, desmin and myocardin at the mRNA level at both time points of investigation (24 hours and 14 days). In accordance with these observations the total protein amount of alpha smooth muscle actin determined in western blots was higher in response to Shh stimulation compared to VEGF treatment and control co-cultures, both after 24 hours and 14 days. Nevertheless, also in untreated controls evidence of vessel stabilization in terms of smooth muscle cell expression could be detected, which, in contrast to VEGF treatment, proceeded with time of culture. This was also supported by morphological findings and was already discussed in chapter 4.1.

These findings and the associated mechanisms were further specified by the investigation of growth factors that lead to the coverage of newly formed vessels with mural cells, i.e. pericytes and smooth muscle cells. At least three different essential signalling pathways are activated during vessel stabilization, namely the angiopoietin/Tie system, the PDGFB/PDGFRß pathway and TGFß signalling system (Gaengel et al., 2009; Hellstrom et al., 2001). In the present study protein concentrations of Ang1 and Ang2 were significantly higher in Shh-treated co-cultures compared to untreated controls. As already discussed, the roles of Ang1 and Ang2 in terms of blood vessel formation are known to be very complex and the balance between these molecules is proposed to control angiogenic activation as well as vessel maturation. The higher expression and secretion of both molecules, Ang1 and Ang2 in Shh-treated co-cultures compared to controls might suggest a precise balance in Shh-stimulated co-cultures between the angiogenic activation of OEC mediated via angiopoietin2

and the commencement of the vessel maturation process mediated by angiopoietin1, secreted by the primary osteoblasts. In VEGF-treated co-cultures the balance between Ang1 and Ang2 seems to be shifted towards angiopoietin2 indicated by a significantly increased concentration of Ang2 in co-culture supernatants in response to VEGF after 14 days. This could explain the comparable number of microvessel-like structures after 14 days of stimulation in sonic hedgehog- and VEGF-treated co-cultures. In addition, this could also explain the failure in terms of vessel stabilization, e.g. smooth muscle cell assembly to sites of newly formed microvessels in the co-culture in response to VEGF which could be documented using Western Blot.

Shh treatment for 24 hours led to a significant increase of PDGF-BB in the supernatants of OEC mono-cultures, as well as a significant upregulation of PDGF-BB at the mRNA level after 24 hours and after 14 days of Shh treatment. Platelet derived growth factor (PDGF-BB) is essential for the recruitment of pericytes to naked blood vessels (Andrae et al., 2008). PDGF-BB is known to be involved in endothelial cell proliferation and angiogenesis by acting via its receptor, PDGFRbeta (Battegay et al., 1994; Thommen et al., 1997). Angiogenic sprouting endothelial cells express and secrete PDGF-BB, while pericytes or vascular smooth muscle cells express the PDGF receptor beta (PDGFRß). Therefore, PDGF-BB acts as a chemoattractant that recruits pericytes or vascular smooth muscle cells to sites of newly formed vessels (Lindahl et al., 1997; Shinbrot et al., 1994; Zerwes and Risau, 1987). Furthermore, transforming growth factor beta (TGFß)- mediated signalling pathways play an important role in the interaction between mural cells/pericytes and endothelial cells (Dickson et al., 1995). TGFß can inhibit endothelial cell proliferation and promotes the recruitment or the differentiation of smooth muscle cell at sites of *de novo* blood vessel formation (Goumans et al., 2002; van den Driesche et al., 2003). In response to Shh TGFß was also significantly upregulated after 24 hours of treatment, compared to treatment with the commonly used VEGF and untreated controls.

In conclusion, compared to VEGF Shh treatment resulted in a beneficial effect on the angiogenic activity of endothelial cells, resulting in microvessel-like structures even after short time exposure periods. This could be helpful to accelerate the vascularization process. Also contrasting with VEGF effects, Shh favoured several factors involved in vessel stabilization *in vitro*, which is one of the key factors to achieve a long-lasting and stable vascularization. The current findings are based on an *in vitro* model which might permit

further insights into underlying mechanisms of bone vascularization and also support the identification of factors to enhance the vascularization process in therapeutic approaches.

4.2.3 Role of Sonic hedgehog during the process of osteogenesis in the co-culture system

Sonic hedgehog is known to be involved in skeletal tissue formation (Chiang et al., 1996; Hammerschmidt et al., 1997; Yuasa et al., 2002). Sonic hedgehog null mice lack vertebrae and show partial defects of autopod development, documenting the importance of Shh signalling during skeletogenesis and chondroblastic and osteoblastic differentiation (Chiang et al., 1996). In addition to the effects of Shh on the angiogenic potential of endothelial cells and the effect on factors involved in vessel maturation in the co-culture, Shh further enhanced multiple key features involved in osteogenic differentiation, suggesting a synergistic effect of Shh on both processes during bone formation. Upregulation of gene expression of osteogenic differentiation markers, such as alkaline phosphatase, osteonectin, osteopontin and osteocalcin (Au et al., 2008; Aubin, 1998; Liu et al., 1994; Tepper et al., 2002) was observed in quantitative real time PCR. The assessment of calcification in the co-cultures using alizarin red and the quantification of ALP activity further supported the improvement of osteogenic differentiation as a result of Shh treatment.

The finding of an ongoing process of osteogenic differentiation in the co-culture system of pOB and OEC was(Horner et al., 2001) further analysed in terms of mechanisms and growth factors that are known to be involved in the regulation of osteoblastic differentiation. The differentiation process of mesenchymal stem or progenitor cells into osteoblasts is under the control of several molecular signalling pathways and factors, including bone morphogenetic proteins (BMPs), runt-related transcription factor2 (RUNX2) and osterix (OSX) (Nakashima and de Crombrugghe, 2003).

Several publications already reported interactive mechanisms between sonic hedgehog and BMPs in different tissues and organs during development (Fietz et al., 1994; Francis et al., 1994). Bone morphogenetic protein 2 (BMP2) was clearly upregulated in response to Shh treatment after 14 days of treatment, compared to control cultures cultivated in EGM-2 as evaluated using quantitative real time PCR. Accordingly, the protein release of BMP2 increased in pOB mono-cultures as well as in co-cultures of OEC and pOB after 14 days of Shh treatment, measured in an enzyme linked immunosorbent assay. The stimulatory action

of Shh on osteogenic differentiation was already reported in previous studies (Nakamura et al., 1997; van der Horst et al., 2003), suggesting a close interaction of Shh with BMP-2 (Yuasa et al., 2002).

Runt-related transcription factor2 (RUNX2), another early osteogenic differentiation marker, was also clearly upregulated in response to Shh treatment when treated for the longer time period (14 days). This factor is a critical transcriptional activator of osteoblastic differentiation and plays a key role during osteogenesis, especially in the transition process of osteoprogenitors to preosteoblasts and further to osteoblasts. In this context Zunich et al. reported that activation of the Shh signalling pathway in preosteoblasts led to induction of the differentiation of preosteoblasts to early osteoblasts (Zunich et al., 2009). Other groups also provided evidence that hedgehog proteins are able to promote osteoblastic differentiation in multipotent mesenchymal stem cells by upregulation of RUNX2, mediated through the transcriptional action of Gli-2 (Shimoyama et al., 2007).

In conclusion, treatment of co-cultures with the morphogen sonic hedgehog has been shown to have a promoting effect on osteoblastic as well as on angiogenic differentiation processes. Due to the multifunctionality of sonic hedgehog signalling in terms of angiogenic activation, vessel maturation and osteogenic differentiation this morphogen might be beneficial as a new therapeutic instrument in bone tissue engineering, positively influencing essential processes during bone regeneration and repair. Despite this positive effect of sonic hedgehog on the co-culture system of OEC and pOB, the use of morphogens has several limitations. Growth factors or morphogens have short half-lives and often require high doses to act in an effective way (Mistry and Mikos, 2005). Therefore, specific delivery systems should be applied to allow the release of a defined dosage of the growth factor or morphogen to achieve a high activity. Due to the fact that morphogens act in a variety of tissues and organs during embryonic development it would be important to control the range of action of sonic hedgehog to permit its effects only on specific cell types, especially in terms of potential *in vivo* applications. Gene therapy approaches could provide a controlled timing and distribution of specific growth factors or morphogens, which can be expressed in a more specific manner in different tissues using delivery vectors that incorporate e.g. inducible promoters (Rivera et al., 1999). Nevertheless, the clinical translation of such powerful strategies is associated with considerable regulatory hurdles and requires methodologies which can be shown to be safe for patients.

The present study has to be considered as fundamental research, and so additional studies are needed to analyze the underlying mechanisms and the basic control machinery during the bone repair process. In addition, cell isolation procedures have to be further optimized to achieve a suitable number of autologoes cells with a minimal expansion time for the prevascularization of engineered bone tissue. Furthermore, co-implantation strategies using outgrowth endothelial cells and primary osteoblasts have to be tested in greater detail before they can be used as therapeutic tools for a possible clinical application. In this context, especially the use of growth factors and morphogens, such as sonic hedgehog, and understanding their intervention in regenerative processes require intensive investigation.

5. Summary

Co-culture systems, consisting of outgrowth endothelial cells (OEC) and primary osteoblasts (pOB), represent a promising instrument to mimick the natural conditions in bone repair processes and provide a new concept to develop constructs for bone replacement. Furthermore, co-culture of OEC and pOB could provide new insights into the molecular and cellular mechanisms that control essential processes during bone repair. The present study described several advantages of the co-culture of pOB and OEC for bone tissue engineering applications, including beneficial effects on the angiogenic activation of OEC, as well as on the assembly of basement membrane matrix molecules and factors involved in vessel maturation and stabilization. The ongoing angiogenic process in the co-culture system proceeded during the course of co-cultivation and correlated with the upregulation of essential angiogenic factors, such as VEGF, angiopoietins, basement membrane molecules and mural cell-specific markers. Furthermore the co-culture system appeared to maintain osteogenic differentiation capacity.

Additional treatment of co-cultures with growth factors or morphogens might accelerate and improve bone formation and furthermore could be useful for potential clinical applications. In this context, the present study highlights the central role of the morphogen, sonic hedgehog, which has been shown to affect angiogenic activation as well as osteogenic differentiation in the co-culture model of OEC and pOB. Treatment of co-cultures with sonic hedgehog resulted in an increased formation of microvessel-like structures as early as after 24 hours. This proangiogenic effect was induced by the upregulation of the proangiogenic factors, VEGF, angiopoietin1 and angiopoietin 2. In contrast to treatment with a commonly used proangiogenic agent, VEGF, Shh stimulation induced an increased expression of factors associated with vessel maturation and stabilization, mediated through the upregulation of growth factors that are strongly involved in pericyte differentiation and recruitment, including PDGF-BB and TGFbeta. In addition, Shh treatment of co-cultures also resulted in an upregulation of osteogenic differentiation markers like alkaline phosphatase, osteocalcin, osteonectin and osteopontin, as well as an increased matrix calcification. This was a result of upregulation of the osteogenic differentiation regulating factors, BMP2 and RUNX2 which could be assessed in response to Shh treatment.

6. Zusammenfassung

Ko-Kultur-Modelle aus primären Osteoblasten (pOB) und sogenannten Outgrowth endothelial cells (OEC), einer Subpopulation endothelialer Progenitorzellen (EPC) aus dem peripheren Blut, bieten verschiedene Vorteile als *in vitro* Testsysteme zur Identifizierung von Faktoren, die möglicherweise zur Verbesserung der Knochenregeneration eingesetzt werden können. Darüber hinaus gelten Ko-Kulturen bzw. Ko-Implantationsstrategien auch als vorteilhaft zur Vaskularisierung von Tissue Engineering Konstrukten, da diese *in vivo* zu einem schnellen Anschluß an das Gefäßsystem des peri-implantären Gewebes führen. Es konnte gezeigt werden, dass in Ko-Kulturen aus pOB und OEC, mikrovaskuläre Strukturen mit deutlichem Lumen ausgebildet werden. Die Bildung der angiogenen Strukturen im Ko-Kultur System korrelierte mit einer höheren Expression von wichtigen proangiogenen Faktoren wie VEGF, Angiopoietine, sowie einer erhöhten Expression von Bestandteilen der extrazellulären Matrix und Markern für Perizyten bzw. glatte Muskelzellen. Auch die Fähigkeit zur osteogenen Differenzierung wird im Ko-Kultur System aufrechterhalten.

Als mögliche Faktoren, welche zur Verbesserung der Knochenregeneration verwendet werden können, gelten unter anderem Morphogene wie Sonic hedgehog (Shh). Shh spielt sowohl bei der embryonalen Vaskulogenese als auch bei der Angiogenese im adulten Organismus eine wichtige Rolle und ist darüber hinaus auch an der Knochenbildung bzw. -regeneration aktiv beteiligt. Im Rahmen dieser Dissertation wurden Ko-Kulturen bestehend aus pOB und OEC für verschiedene Zeitpunkte mit dem Morphogen Shh behandelt und es konnte bereits nach 24 Stunden Behandlung eine deutlich erhöhte Bildung von angiogenen Strukturen im System gezeigt werden. In den Experimenten war eine deutliche Hochregulation von proangiogenen Faktoren wie VEGF, Angiopoietin1 und Angiopoietin2 sowohl auf mRNA Ebene als auch auf Proteinebene zu beobachten. Im Gegensatz zu einer Behandlung mit VEGF, welches gewöhnlich als proangiogener Wachstumsfaktor eingesetzt wird, konnte durch Shh Stimulation ebenfalls eine erhöhte Expression von verschiedenen Faktoren, welche mit der Reifung und Stabilisierung von Gefäßen assoziiert sind, wie beispelsweise PDGF-BB und TGFbeta, gezeigt werden. Zudem hatte die Behandlung der Ko-Kulturen mit Shh ebenfalls eine Hochregulation von verschiedenen Markern für die osteogene Differenzierung zur Folge, wie Osteocalcin, Osteonectin und Osteopontin. Auch eine gesteigerte Kalzifizierung sowie eine erhöhte Enzymaktivität der alkalischen Phosphatase konnte in den stimulierten Ko-

Kulturen detektiert werden. Wichtige, für die osteogene Differenzierung essentielle Faktoren wie BMP2 und der Transkriptionsfaktor RUNX2 zeigten ebenfalls eine erhöhte Expression nach Behandlung mit Shh.

7. References

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8. List of figures

9. Publications and scientific presentations

9.1 Publications

PhD Thesis:

Dohle E., Fuchs S., Kolbe M., Hofmann A., Schmidt H., Kirkpatrick CJ. Comparative study assessing effcts of Sonic hedgehog and VEGF in a co-culture model for bone vascularization strategies. *European Cells and Materials*. 2011; in press.

Kolbe M., Xiang Z., **Dohle E.**, Tonak M., Kirkpatrick CJ., Fuchs S. Paracrine effects influenced by cell culture medium and consequences on microvessel-like structures in co-cultures of mesenchymal stem cells and outgrowth endothelial cells. *Tissue Engineering Part A* . 2010 submitted.

Fuchs S., **Dohle E.**, Kolbe M., Kirkpatrick CJ. Outgrowth endothelial cells: Sources, Characteristics and potential applications in Tissue Engineering and regenerative medicine. *Adv Biochem Eng Biotechnol*. 2010;123:201-17.

Kolbe M., **Dohle E.**, Katerla D., Kirkpatrick CJ. Fuchs S. Enrichment of outgrowth endothelial cells in high and low colony-forming cultures from peripheral blood progenitors. *Tissue Engineering Part C*. 2010 Oct;16(5):877-86.

Dohle E., Fuchs S., Kolbe M., Schmidt H., Kirkpatrick CJ. Sonic hedgehog promotes angiogenesis and osteogenesis in a co-culture system consisting of primary osteoblasts and outgrowth endothelial cells. *Tissue Engineering Part A*. 2010 Apr; 16 (4):1235-7.

Fuchs S., Jiang X., Schmidt H., **Dohle E.**, Ghanaati S., Orth C., Hofmann A., Migliaresi C., Kirkpatrick CJ. Dynamic processes involved in the pre-vascularization of silk fibroin constructs for bone regeneration using outgrowth endothellial cells. *Biomaterials*. 2009 Mar; 30 (7):1329-38.

Diploma thesis:

Heine P., **Dohle E.**, Schulte D. Sonic hedgehog signaling in the chick retina accelerates Meis2 downregulation simultaneously with retinal ganglien cell genesis. *Neuroreport.* 2009 Feb 18 ; 20(3):279-84

Heine P., **Dohle E.**, Bumsted-O'Brien K., Engelkamp D., Schulte D. Evidence for an evolutionary conserved role of homothorax/Meis1/2 during vertebrate retina development. *Development*. 2008 Mar; 135(5):805-11.

9.2 Scientific presentations

Oral presentations

E. Dohle, S. Fuchs, B. Pavic and CJ. Kirkpatrick. Sonic hedgehog promotes angiogenesis and osteogenesis in a co-culture system consisting of outgrowth endothelial cells and primary osteoblasts. eCM X Stem Cells for Musculoskeletal Regeneration. June 29 – July 1, 2009, Davos, Switzerland.

E. Dohle, S. Fuchs, M. Kolbe, CJ. Kirkpatrick. Effects of Sonic hedgehog (Shh) and Vascular endothelial growth factor (VEGF) on co-cultures of outgrowth endothelial cells and primary osteoblasts. TERMIS-EU Tissue Engineering and Regenerative Medicine International Society-EU. At the Crossroad: Development and Translation. June 13-17, 2010, Galway, Ireland.

Poster Presentations:

E. Dohle, S. Fuchs, B. Pavic, CJ. Kirkpatrick. Vascular basement mebrane molecules and perivascular cells in co-cultures of human outgrowth endothelial cells and primary osteoblasts. ISACB 11th Biennal Meeting. Translation of Applied Biology to the Treatment of Cardiovascular Disease. September 17-20, 2008, Bordeaux, France.

E. Dohle, S. Fuchs, B. Pavic. CJ. Kirkpatrick. Sonic hedgehog promotes both angiogenesis and osteogenesis in a co-culture system consisting of primary osteoblasts and outgrowth endothelial cells. 2nd Chinese-European Symposium on Biomaterials and Regenerative Medicine. November 17-20, 2009, Barcelona, Spain.

10. Curriculum vitae

Appendix

RT^2 Profiler^{TM} PCR Array: Human Angiogenesis

Akt1	V-akt murine thymomma viral oncogene homolog 1
ANGPT2	Angiopoietin2
ANGPTL4	Angiopoietin-like4
ANPEP	Alanyl aminopeptidase
CCL11	Chemokine ligand11
CCL2	Chemokine ligand2
CDH5	Cadherin5, VE-Cadherin
COL4A3	Collagen type4
COL18	Collagen18A
CXCL1	Chemikine ligand1
CXCL3	Chemokine ligand3
CXCL5	Chemokine ligand5
CXCL6	Chemokine ligand6
ECGF1	Endothelial cell growth factor 1 (platelet derived)
EDG1	Endothelial differentiation, sphigolipid G-protein-coupled receptor1
EFNA1	EphrinA1
EFNB2	EphrinB2
ENG1	Endoglin
EPHB4	Ephrin receptor B4
EREG	Epiregulin
FGF1	Fibroblast growth factor 1
FGF2	Fibroblast growth factor2
FGFR3	Fibroblast growth factor receptor3
FIGF	C-fos induced growth factor (vascular endothelial growth factorD)
HIF1A	Hypoxia inducible factor1, alpha subunit
ID1	Inhibitor of DNA binding1
ID3	Inhibitor of DNA binding2
IFNA1	Interferon alpha1
IFNB1	Interferon beta1
IFNG	Interferon gamma

IL1b	Interleukin1 beta
ITGAV	Integrin alpha V
ITGB3	Integrin beta3
KDR	Kinase insert domain receptor
LAMA5	Laminin alpha5
LECT1	Leukocyte cell derived chemotaxin1
LEP	Leptin
MDK	Midkine
MMP2	Matrix metallopeptidase2
Notch4	Notch homologe4
PDGFA	Platelet derived growth factorA
PECAM	Platelet endothelial cell adhesion molecule
PGF	Placental growth factor
PLAU	Plasminogen activator
STAB1	Stabilin1
TGFB1	Transforming growth factor beta1
TGFB2	Transfroming growth factor beta2
TGFBR	Transforming growth factor beta receptor
THBS1	Thrombospondin1
THBS2	Thrombospondin2
TIMP1	TIMP metallopeptidase inhibitor1
TIMP2	TIMP metallopeptidase inhibitor2
TIMP3	TIMP metallopeptidase inhibitor3
TNF	Tumor necrosis factor
TNFA12	Tumor necrosis factor alpha induced protein2
VEGFA	Vascular endothelial growth factor

RT^2 Profiler^{TM} PCR Array: Human Osteogenesis

ALPL	Alkaline phosphatase
AMELY	Amelogenin
ANXA5	Annexin A5
BGN	Biglycan
BMP4	Bone morphogenetic protein4

CALCR	Calcitocin receptor
CD36	Thrombospondin receptor
CDH11	Cadherin11
COL10A	Collagen10A
COL11A	Collagen11A
COL12A	Collagen12A
COL14A	Collagen14A
COL3A1	Collagen3A
COMP	Cartilage oligomeric matrix protein
CTSK	Cathepsin K
EGFR	Epidermal growth factor receptor
FGF1	Fibroblast growth factor1
FGF2	Fibroblast growth factor2
FN1	Fibronectin1
ICAM1	Intercellular adhesion molecule1
IGF2	Insulin like growth factor2
ITGA3	Integrin alpha3
ITGB1	Integrin beta1
MINPP	Multiple inositol polyphosphate histidine phosphatase1
MMP2	Matrix metallopeptidase2
PDGFA	Platelet derived growth factorA
PHEX	Phosphate regulating endopeptidase homolog
RUNX	Runt related transcription factor2
SERPI	Serpin peptidase inhibitor
SMAD1	Smad family member1
SMAD2	Smad family member2
SOX9	SRY sex determining region
STATH	Stetherin
TGFB2	Transforming growth factor beta2
TNF	Tumor necrosis factor
TWIST	Twist homolog1
VDR	VitaminD receptor
VEGFA	Vascular endothelial growth factorA
VEGFB	Vascular endothelial growth factorB