Interactions between endothelial and bone marrow stromal cells of relevance to bone tissue engineering

Ying Xue

Dissertation for the Degree Philosophiae Doctor (PhD)

at Faculty of Medicine and Dentistry, Department of Clinical dentistry

University of Bergen, Norway

2011

Acknowledgements

This thesis was supported by The Center for International Health (CIH) and The Center for Clinical Dental Research-Department of Clinical Dentistry, Faculty of Medicine and Dentistry, University of Bergen, Norway. My PhD scholarship was granted through the Quota program which arranged by the Norwegian Government and organized by CIH. The project was financed by The University of Bergen, The Research Council of Norway and The European Union, the 7th Frame Program. I wish to express my deep appreciation for the support.

I would like to express my gratitude to my supervisor, ***Professor Kamal Mustafa*** and my co-supervisors ***Professor Kristina Arvidson*** and ***Professor Sølve Hellem*** for excellent supervision, for guidance, and for patience and encouragement throughout the studies, especially during the most frustrating times, dealing with disappointments. I am also grateful for your support and valuable discussions and comments on the manuscripts.

Special thanks to our main collaborator ***Professor Ann-Christine Albertsson*** and ***Associate Professor Anna Finne-Wistrand,*** at The Department of Fibre and Polymer Technology, The Royal Institute of Technology, Stockholm, Sweden, for your support and for generously sharing your profound knowledge of polymers and scaffold fabrication.

I also acknowledge the contribution of ***Professor Anne Isine Bolstad,*** co-author of the fourth study, for helpful suggestions and fruitful discussions.

I would like to thank to my co-supervisor, ***Associate Professor Salah O. Ibrahim*** and PhD student ***Dr. Dipak Sapkota*** for kind help and valuable discussion of RNA isolation and PCR experiments.

I would also like to thank the staff and PhD students at The Center of Clinical Dental Research, for creating such a positive working environment: ***Rita Greiner-Simonsen, Siren Hammer, Randi Sundfjord*** for your excellent cooperation, for taking care of our laboratory and offering help whenever needed. Thanks also to my fellow doctoral students ***Shaza Idris, Kerstin Schander, Staffan Dånmark, Torbjørn Ø. Pedersen*** and ***Zhe Xing*** for your unfailing

support and sharing many funny moments inside and outside the laboratory; and ***Professor Pongsri Brudvik, Associate Professor Olav E. Bøe, Dr. Manal Mustafa, Polbhat Tripuwabhrut, Hager Zein Elabdeen, Yussra Salih, Harald Gjengedal*** and ***Torgils Lægreid*** for valuable scientific discussion and pleasant company. I have really enjoyed working together with you.

Special thanks to ***Kjell Petersen, Solveig Mjelstad Angelskår, Rita Holdhus and Anne-Kristin Stavrum*** at The Department of Informatics and The Norwegian Microarray Consortium (NMC) Core Facility Laboratory, Bergen for their assistance for your kind help with design, sample preparation and data analysis of the microarray study.

I would like also to show my sincere appreciation to the administration staff both at CIH and IKO for the endless supports: ***June-Vibecke K Indrevik***, ***Kari Nordvik***, ***Tone Hordvik***, ***Tove Russenes, Marit Stubdal*** from IKO and ***Linda Forshaw***, ***Unni Kvernhusvik Sagberg*** from CIH.

I also want to thank ***Dr. Joan Bevenius-Carrick*** for excellent linguistic revision of the manuscripts and the thesis.

Finally, I wish to acknowledge my deepest gratitude to my family: ***My father, Mother and Brother*** for all your understanding and unfailing support of my decision to study so far from home. Special thanks to my dear husband, ***Zhe Xing***, for your constant support, encouragement, patience and love.

Bergen, September, 2010

Ying Xue

Abstract

Much research effort is being directed towards the development of bone tissue engineering techniques as alternatives to grafts for the treatment of bone defects. A major challenge is that following implantation of the tissue engineered construct, inadequate vascularity limits the supply of nutrients to the proliferating osteoprogenitor cells within the scaffolding. One proposed means of addressing this issue is the addition of endothelial cells (EC), major components of vessel-formation, to bone marrow stromal cells (MSC) seeded onto a biodegradable scaffold. The aim of the present series of studies was to investigate interactions between endothelial cells and bone marrow stromal cells, with special reference to osteogenic differentiation of MSC and bone formation.

In study I, the addition of EC to MSC at a ratio of 1:5 resulted in significant increases in cell proliferation and cellular bridges between the two cell types. At the same time, increased mRNA expression of alkaline phosphatase was observed; this effect was greater than that achieved by the addition of osteogenic factors such as dexamethasone, ascorbic acid and β-glycerophosphate to the culture medium. This interesting finding suggests that under conditions of direct contact culturing, endothelial cells influence the osteogenic differentiation of MSC.

Study II was designed to select an appropriate copolymer scaffold to support the cells during the initial stages. MSC were seeded onto two porous test scaffolds, made of poly(LLA-*co*-CL) and poly(LLA-*co*-DXO). The cellular response was determined in terms of attachment, proliferation and differentiation. The results showed that poly(LLA-*co*-CL) and poly(LLA-*co*-DXO) promoted better cell attachment and growth than the control scaffolding, poly(LLA). Moreover, MSC retained their osteogenic differentiation potential on the scaffolds.

As shown in study I, EC influenced the osteogenic differentiation of MSC. In study III, this effect was investigated *in vivo*, using a rat calvarial bone defect model to compare bone regeneration by MSC grown in mono- or co-culture. Either bone marrow stromal cells alone (MSC-group) or both types of cell (CO-group) were seeded onto poly(LLA-*co*-DXO) scaffolds, cultured in spinner flasks and then implanted into symmetrical calvarial defects. After two months, new bone and vessel formation were evaluated by radiography and histology and expression of osteogenic markers by RT-PCR. Bone formation was more rapid in the CO- than in the MSC-group, but no significant differences were detected with respect to vessel formation. Expression of osteogenic markers was greater in the CO- than in the MSC-group.

Studies I & III demonstrated a significant effect of co-culture on osteogenesis. Systematic investigation continued in Study IV, namely microarray analysis with the HumanWG-6 v3.0 expression BeadChips, each array representing >48,000 probes. A global map of gene expression after communication between MSC and EC was generated in a direct-contact model. Differentially expressed genes were identified and over-represented genes were annotated by gene ontology and biological processes and pathways. The results indicated that EC had a significant impact on MSC after 5 and 15 days. Of particular interest was the disclosure of bidirectional gene regulation of angiogenesis and osteogenesis through cell signaling, cell adhesion and cellular matrix. The results suggest that the cell-matrix interaction and the TGF-beta signal pathway might play a crucial role in EC-induced gene regulation of MSC.

The results of these studies thus support bone tissue engineering concepts based on seeding of MSC and EC onto copolymer scaffolding. Together, the cell culture and animal studies indicate that cross-communication between bone marrow stromal cells and endothelial cells has influenced the osteogenic differentiation as well as enhanced the bone healing.

List of publications:

The thesis is based on the following papers:

I. Xue Y., Xing Z., Hellem S., Arvidson K., Mustafa K. Endothelial cells influence the osteogenic potential of bone marrow stromal cells. *BioMedical Engineering OnLine* 2009, **8:**34.

II. Xue Y., Dånmark S., Xing Z., Arvidson K., Albertsson AC., Hellem S., Finne-Wistrand A., Mustafa K. Growth and differentiation of bone marrow stromal cells on biodegradable polymer scaffolds: an *in vitro* study. *Journal of Biomedical Materials Research Part A* 2010, **95**: 1244-51.

III. Xing Z., Xue Y., Dånmark S., Schander K., Østvold S., Arvidson K., Hellem S., Finne-Wistrand A., Albertsson AC., Mustafa K. Effect of endothelial cells on bone regeneration using poly(L-lactide-co-1,5-dioxepan-2-one) scaffolds. *Journal of Biomedical Materials Research Part A* 2011, **96**:349-57.

IV. Xue Y., Bolstad AI., Hellem S., Arvidson K., Mustafa K. Gene expression profiles of human bone marrow stromal cells co-cultured with endothelial cells. (Manuscript).

Contents

Abbreviations

ALP	Alkaline phosphatase
BMP	Bone morphogenetic protein
cDNA	Complementary deoxyribonucleic acid
Col I	Collagen type I
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
FDR	False discovery rate
FGF	Fibroblast growth factor
HUVEC	Human umbilical vein endothelial cell
MSC	Bone marrow stromal cell
OPN	Osteopontin
PCR	Polymerase chain reaction
Poly(LLA-*co*-CL)	Poly(L-Lactide-*co*-ε-caprolactone)
Poly(LLA-*co*-DXO)	Poly(L-Lactide-*co*-1,5dioxepan-2-one)
Poly(LLA)	Poly(L-Lactide)
Runx2	Runt-related transcription factor 2
SAM	Significance analysis of microarray
SEM	Scanning electron microscopy
TGF	Transforming growth factor
VEGF	Vascular endothelial growth factor

1. Introductions

There is a widespread need for appropriate bone substitutes that allow remodeling for bone defects or voids due to disease, trauma, or ablative surgery (Frohlich et al., 2008; Kneser et al., 2006; Nakahara et al., 2009). Various limitations are associated with currently available therapies and the repair of critical-sized bone defects remains a challenge in the fields of implantology, maxillofacial surgery and orthopaedics (d'Aquino et al., 2009; Pollock et al., 2008). Much research effort is being directed towards the development of bone tissue engineering techniques as alternatives to grafts for the treatment of bone defects.

1.1 Bone Development and Remodeling

Bone is a unique organ with the ability to remodel the volume of the skeleton continuously throughout life (Bilezikian et al., 2002 ; Hill, 1998; Urist, 1965). The initial stage of bone formation is the process of cellular condensation, whereby dispersed bone marrow stromal cells proliferate and differentiate into bone cells, which could be eventually bound together by the deposit of adhesion matrix (Bilezikian et al., 2002; Laugier et al., 1998; Meyer and Wiesmann, 2006).

Subsequent bone development occurs *via* either intramembranous or endochondral ossification (Hill, 1998; Maniatopoulos et al., 1988; Urist, 1965). Intramembranous ossification with direct differentiation of mesenchymal stem cells into osteoblasts involves the replacement of connective tissue membrane sheets with bone tissue and results in the formation of flat bones (Bilezikian et al., 2002; Frohlich et al., 2008). Endochondral ossification involves the replacement of a hyaline cartilage framework with bone tissue (Meyer and Wiesmann, 2006). Both processes are closely regulated by coordinated expression and interaction of several molecules or pathways (Bilezikian et al., 2002; Frohlich

et al., 2008). The differentiation of mesenchymal cells into pre-osteoblasts is not well understood (Guillot et al., 2007; Kronenberg, 2003; Proff and Romer, 2009).

Despite the differences between the two types of ossification, vascularization is a pre-requisite for both (Bilezikian et al., 2002; Carano and Filvaroff, 2003; Hill, 1998; Santos and Reis, 2010; Towler, 2008). In intramembranous ossification there is an invasion of capillaries to transport mesenchymal stem cells. In endochondral ossification, the hypertrophic chondrocytes act as a template for bone formation, secreting angiogenic growth factors that promote the invasion of blood vessels, to replace the cartilage with bone and bone marrow (Canalis, 2009; Carano and Filvaroff, 2003). The vasculature also plays a crucial role in bone formation by the secretion of growth factors that will control the recruitment, proliferation, differentiation and function of bone-forming and bone-resorbing cells. Thus angiogenesis not only precedes osteogenesis but is also essential for its occurrence (McCarthy, 2006).

1.2 Bone defects

Bone defects or voids may result from disease, trauma, or ablative surgery. Concerning traumas, it is estimated that each year approximately 7.9 million bone fractures are sustained in the United States alone (Bishop and Einhorn, 2007; National Trauma Data Bank Report 2005). Although most fractures heal uneventfully, between 5 and 10% of patients experience complications (Kneser et al., 2006; Termaat et al., 2005). Approximately one million fractures annually, at risk of delayed union or nonunion, require hospital care. Approximately 1.5 million bone grafting operations are performed annually in the United States (Porter et al., 2009; Surgeons, 2008). Hence there is a widespread need for appropriate bone substitutes that allow remodeling of native bone tissue. Various limitations are associated with currently available therapies and the repair of critical-sized bone defects and periodontal deep intrabony defects remains a challenge in the fields of regenerative periodontal therapy, implantology,

maxillofacial surgery and orthopaedics (d'Aquino et al., 2009; Pollock et al., 2008; Zafiropoulos et al., 2007).

Current treatment options for bone defects

Several treatment strategies are currently available for management of conditions requiring bone regeneration (Bilezikian et al., 2002; Finkemeier, 2002; Meyer and Wiesmann, 2006; Vaccaro et al., 2002; Zafiropoulos et al., 2007): autologous or allergenic bone, Xenografts, demineralized bone matrix, calcium phosphate-based bone graft substitute, distraction osteogenesis, or autologous bone marrow etc.

Autologous bone grafts are regarded as the golden standard for bone regeneration. Conventional bone-grafting using autologous cortical and cancellous bone, harvested from the iliac crest, has been the standard against which all other bone-graft substitutes are assessed, but the method has some disadvantages (Frohlich et al., 2008; Kneser et al., 2006). Although there is no risk for immunogenic rejection with autogenous materials, the availability of bone for grafting is often limited (Meyer and Wiesmann, 2006). In cases of massive segmental bone loss, the autologous graft material available may be insufficient. Autogenous bone grafts may also have the potential disadvantage of a higher degree of resorption of the graft material (Zafiropoulos et al., 2007). Moreover, the harvesting procedure is associated with major complications in 8.6 % of cases and minor complications in 20.6 % (Finkemeier, 2002; Gazdag et al., 1995).

Alternatives to autologous bone grafts such as allograft bone, demineralized bone matrix, distraction osteogenesis, recombinant growth factors and synthetic implants might possibly be combined with autologous bone marrow or various growth factors, but the current treatments could not meet the demanding clinical requirements (Vaccaro et al., 2002). Therefore, it is

important to have a variety of options available to augment, expand, or substitute for autologous bone grafts.

The enormous clinical demand for treatment of bone defects has stimulated efforts to develop bone graft substitutes using the principles of bone tissue engineering (Kneser et al., 2006; Langer and Vacanti, 1993; Petite et al., 2000; Porter et al., 2009). While much work remains before this approach can be routinely applied in the clinical setting, increased understanding of bone physiology and advancements in biotechnology has led to a new surgical approach to control and modulate bone-healing (Termaat et al., 2005).

1.3 Bone tissue engineering

1.3.1 Definition

The widely accepted definition of tissue engineering was stated in 1993 by Langer and Vacanti (Langer and Vacanti, 1993). The concept is based on an understanding of tissue formation and regeneration, and aims to induce new functional tissues, rather than just to implant new spare parts (Salgado et al., 2004). As shown in Figure 1, a tissue engineering construct has three main components (Meyer and Wiesmann, 2006): a scaffold, an osteoconductive matrix capable of supporting the bone cell ingrowth and differentiation; osteogenic cells (osteoblasts or osteoblast precursors), which in the proper environment are capable of forming bone and thirdly growth factors, which can stimulate and support mitogenesis of undifferentiated bone cells to form osteoprogenitor cells.

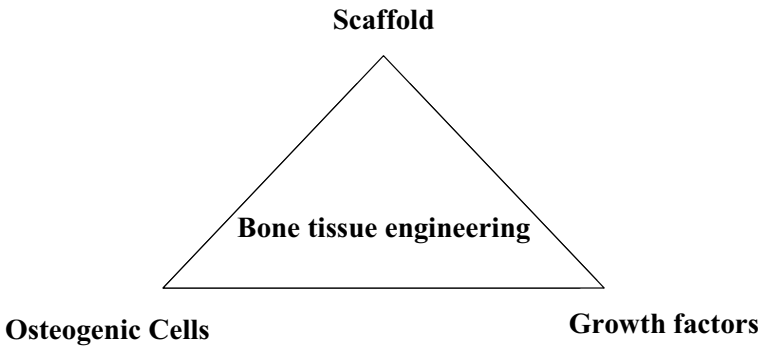

Figure 1 The three major components of bone tissue engineering

1.3.2 Scaffold

The scaffold is crucial for successful engineering of bone tissues, providing a suitable environment in which osteogenic cells can migrate, proliferate, differentiate, and promote new bone formation. It also provides structural, mechanical support during bone regeneration (Frohlich et al., 2008). The fundamental concept underlying tissue engineering is the use of the body's natural biological response to tissue damage in conjunction with engineering principles (Porter et al., 2009). In order to match the physiological needs of the bone tissues as it regenerates, an ideal scaffold must meet a number of demanding requirements: it must be biocompatible, biodegradable, promote cellular interactions and tissue development and have adequate mechanical and physical properties (Nair and Laurencin, 2007; Salgado et al., 2004).

In response to the requirements for clinical application, a variety of bone scaffolding materials has been developed in recent years, including ceramics, polymers and their combinations. Ceramics such as hydroxyapaptite and tricalcium phosphate are biomaterials with good osteoconductivity and bone-bonding ability, but with limitation of poor degradability in a biological environment (Liu and Ma, 2004; Marcacci et al., 2007). In contrast, polymers exhibit flexibility of design and composition, allowing the structure to be tailored to specific

needs. The versatility of synthetic biodegradable polymers has led to increasing interest in their potential application as scaffolding materials, enabling the fabrication of scaffolds with variations in such characteristics as form, porosity and pore size, degradation rates and mechanical properties (Karageorgiou and Kaplan, 2005; Nair and Laurencin, 2007).

Polymers

As FDA approved biomaterials, PLA and PCL have been acknowledged as potential candidates for tissue regeneration and used in a wide variety of clinical applications, such as sutures, systemic drug delivery, spinal fusion cages, coronary stents, and fixation screws (Liu and Ma, 2004; Mo et al., 2004; Rhee et al., 2004; Tang et al., 2005). *In vivo,* PLA and PCL are degraded through hydrolysis and enzymatic activity into their corresponding hydroxyl acids which are readily metabolized: thus these polymers are suitable as resorbable tissue engineering implant materials (Albertsson and Varma, 2003; Arvidson K et al., 2010; Dånmark et al., 2010; Meretoja et al., 2006; Odelius et al., 2008).

Single monomers have specific properties and limited application. Given the highly specific biochemical and biomechanical requirements of each clinical application, it is unlikely that one single scaffolding material would prove optimal for all cases of tissue-engineered bone regeneration (Lofgren and Albertsson, 1994; Ryner and Albertsson, 2002). This can be compensated for by copolymerization with other monomers to generate copolymers which have greater versatility (Albertsson and Varma, 2003; Ryner and Albertsson, 2002; Sodergard and Stolt, 2002). Properties of polymers such as degradation rates and physical and mechanical properties can be improved and adjusted over a wide range by copolymerization (Odelius et al., 2005). In order to extend the potential areas of application of polymers, research effort is directed towards controlling the architecture and tailoring the properties of the materials to suit the intended application (Albertsson and Varma, 2003; Lofgren et al., 1994; Mathisen and Albertsson, 1989).

Scaffold design

In order to optimize integration into the surrounding tissues, scaffolds for osteogenesis should simulate bone morphology, structure and function. Several requirements need to be considered in the fabrication of 3D scaffolds. Firstly, an ideal bone scaffold should have sufficient porosity to accommodate bone cells (Finkemeier, 2002; Middleton and Tipton, 2000; Srivastava and Albertsson, 2006). High interconnectivity between pores is also important for uniform cell seeding and distribution, the diffusion of nutrients to and transport of metabolites from the cell/scaffold constructs (Kyriakidou et al., 2008; Stiehler et al., 2009). Adequate mechanical stability is also important, to provide adequate support and protection before the new bone tissue has reached critical mechanical strength. In order to maintain the structural integrity, the degradation rate of the scaffolding must be tailored to match the rate of new bone formation (Edlund et al., 2008; Nair and Laurencin, 2007). The surface chemistry of the scaffold should be suitable for adhesion of bone cells. Finally, the scaffolding, while meeting all the above requirements, should also support the attachment, proliferation and differentiation of osteoblasts or osteoprogenitor cells and enhance bone regeneration (Liu and Ma, 2004; Salgado et al., 2004).

It is of fundamental importance to document the response of osteogenic cells to polymers intended for tissue engineering applications. Most studies screening new scaffolds for *in vivo* application have concerned the capacity to guide the infiltration of host cells and enhance bone regeneration (Bueno et al., 2007; Dånmark et al., 2010; Rouwkema et al., 2006). Also critical for optimization of conditions for bone tissue engineering, however, are *in vitro* studies of the effects on matrix deposition and mineralization of such variables as cell-seeding density, culture period, scaffold architecture, scaffold composition, and cell source.

1.3.3 Osteogenic cells

The goal of bone tissue engineering is the generation of new bone from osteogenic cells, supported by biocompatible, biodegradable, three-dimensional scaffolds (Guillot et al., 2007; Kneser et al., 2006). Osteogenic cells are an integral part of tissue engineering strategy, transplanted into the bone defect on the appropriate scaffolds or attracted from the host tissues by osteoinductive factors. Although the type of osteogenic cell most suitable for engineering of bone tissue has yet to be determined, bone marrow stromal cells are currently the main sources of osteogenic cells for research (Dånmark et al., 2010; Gulotta et al., 2009; Lozito et al., 2009; Oswald et al., 2004; Undale et al., 2009).

Bone marrow stromal cells

Bone marrow stromal cells (MSC), first revealed in plastic-adherence studies by Friedenstein et al (Friedenstein et al., 1968; Owen and Friedenstein, 1988), are regarded as the main source of bone progenitor cells in skeletal tissues. MSC derived from bone marrow are an obvious source of autologous osteogenic cells (Takagi and Urist, 1982). Of the cell types proposed for such application, MSC have several advantages (Undale et al., 2009). These multipotential stromal stem cells, located within the bone marrow, can differentiate into fibroblastic, osteogenic, adipogenic and reticular cells (Krebsbach et al., 1999; Lozito et al., 2009; Undale et al., 2009). Furthermore, these stem cells generate progenitors committed to one or more cell lines with an apparent degree of plasticity or interconversion. MSC are readily isolated from bone marrow and can be expanded in an *in vitro* culture system without loss of differentiation potential (Bilezikian et al., 2002). Under appropriate conditions, MSC can differentiate into osteocytes, chondrocytes, myocytes and neurons. In contrast to embryonic stem cells, there are no associated ethical issues and MSC are widely applied in bone regeneration research, both *in vitro* and *in vivo* (Drost et al., 2009; Gulotta et al., 2009; Nakahara et al., 2009).

Although engraftment of human bone marrow stromal cells has been achieved, the long-term biological effects at the implant site and the question of cell plasticity have yet to be determined. A preliminary study has shown the therapeutic effects of human bone marrow derived osteoprogenitors transplanted into children with osteogenesis imperfecta (Horwitz et al., 2002), while clinical studies by Marcacci and co-workers illustrate the potential for autologous bone marrow stromal cells (with a porous bioceramic scaffold) in the treatment of large bone defects (Marcacci et al., 2007).

Osteogenesis is a complicated process, influenced by physiological conditions, cell-to-cell interactions, extracellular matrix formation and vascularization. Previous studies have however, shown that MSC alone are unlikely to be sufficient for bone regeneration (Grellier et al., 2009; Guillotin et al., 2008; Kyriakidou et al., 2008; Yu et al., 2008).

Endothelial cells

Endothelial cells (EC) are essential to vasculogenesis: the process starts with the migration of endothelial cells or their progenitors (Jain, 2003). Endothelial cells are derived from the differentiation of mesodermal cells, which leads to the formation of the first vascular structures (Lamalice et al., 2007). These cells then give rise to the hematopoietic stem cells, which differentiate into angioblasts, the precursors of mature endothelial cells. Under the stimulation of vascular endothelial growth factor, the angioblasts and endothelial cells migrate on the extracellular matrix and remodel into tubular structures. Finally, these tubules form larger vessels (Bilezikian et al., 2002; Lamalice et al., 2007).

In contrast to vasculogenesis, angiogenesis denotes the formation of new blood vessels from preexisting ones and is essential for bone healing and growth. Recent studies have begun exploring in detail the role of vascular endothelial cells and their products in bone physiology (Unger et al., 2007; Yu et al., 2009). These studies have disclosed that bone vascular

endothelial cells may participate in a complex communication network in bone, involving endothelial cells, osteoblasts, stromal cells, and perhaps other cell types found in bone. It appears that the interaction between endothelial and bone cells is regulated by multiple systemic and local signals which may be received, transduced, and integrated by individual cells.

Interactions of MSC and EC

In the context of the intricate relationship between angiogenesis and osteogenesis (Patel et al., 2008), communication between MSC and EC is one of the most important cellular interactions for bone formation (Carano and Filvaroff, 2003; Towler, 2008). Several mechanisms are involved: interaction between membrane molecules of two adjacent cells (adherens and tight junctions); gap junction communications which form direct cytoplasmic connections; and secretion of diffusible factors from cells, or from the extracellular matrix (Grellier et al., 2009).

It has been reported that EC co-cultured with MSC are able to establish microcapillary-like structures in a three dimensional spheroids (Rouwkema et al., 2006). Cells from different endothelial and osteoblastic lineages were used. The results showed that either differentiated endothelial cells or endothelial progenitor cells could be successfully co-cultured with different bone cells, sourced from primary osteoblasts harvested from bone fragments (Clarkin et al., 2008), the osteoblastic cell line MG63 (Unger et al., 2007), and bone marrow stromal cells (Kaigler et al., 2005). Previous studies indicated that EC could effect certain levels of osteogenesis, releasing bone morphogenetic proteins (Kaigler et al., 2005) and controlling the transcription factor Osterix for bone cell differentiation (Klinkner et al., 2006). Both MSC and endothelial expressed connexion 43 (Cx43), a specific gap junction protein (Villars et al., 2000; Villars et al., 2002). These two cell types can therefore communicate via a gap junctional channel, consisting of Cx43.

In support of these *in vitro* results, *in vivo* studies also showed the beneficial effect of tissue-engineering constructs using bone cells and endothelial cells. In a rat bone defect model, co-cultured EC and bone marrow-derived osteoblasts were implanted on PCL scaffolding: the results indicated not only improved osteogenesis but also enhanced vascularization (Nakahara et al., 2009). Co-cultured osteoblasts and endothelial cells within RGD-grafted alginate microspheres in a long bone defect of mice showed significantly enhanced mineralization of the microspheres in the co-culture group (Grellier et al., 2009). Taken together, the results of these studies indicate that endothelial cells can participate not only in osteogenic differentiation *in vitro,* but also in osteogenesis *in vivo*. These studies confirmed the intrinsic relationship between angiogenesis and osteogenesis (Figure 2): intercommunication between bone marrow stromal cells and endothelial cells was identified as one of the most important cellular interactions coordinating processes involved in bone formation.

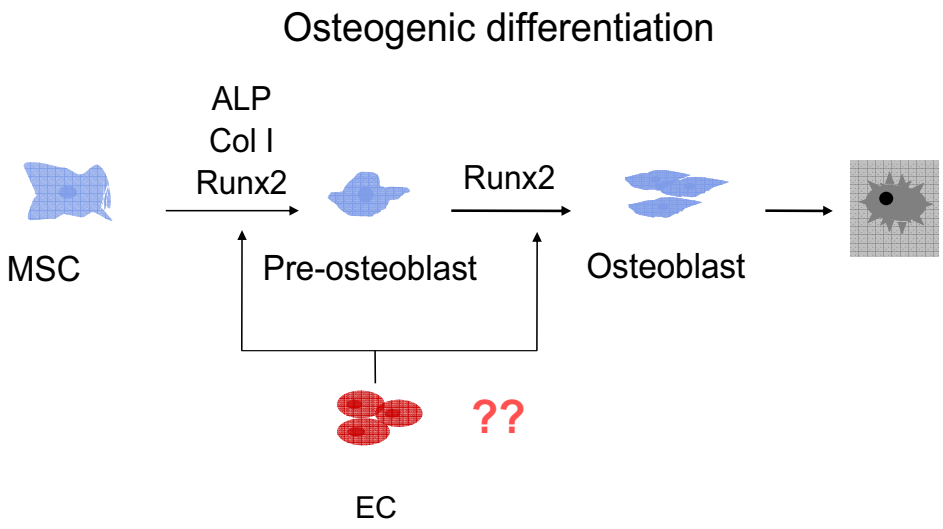

Figure 2 *The relationship between bone marrow stromal cells (MSC) and endothelial cells (EC). There are several stages of the differentiation of osteoblasts from MSC (Lian and Stein,*

1992; 1995). However, the role of EC on the commitment of osteoblast is still not yet understood.

1.3.4 Growth factors

Growth factors can regulate the replication, differentiation, and function of bone cells (Canalis et al., 2003; Deckers et al., 2002). Although no growth factors are specifically synthesized by skeletal cells, they are expressed in a variety of tissues. Some, such as PDGF and FGF, display primarily mitogenic activity for cells of the osteoblastic lineage (Canalis et al., 2007; Canalis, 2009). Bone morphogenetic proteins (BMPs) and Wnt induce the differentiation of osteoblastic cells into mature osteoblasts (Canalis et al., 2003). The most extensively researched osteoinductive bone factors are BMPs, which belong to the transforming growth factor (TGF) beta superfamily (Canalis, 2009; Nesti et al., 2007; Termaat et al., 2005). Following extensive research, in 2002 and 2003 BMP_2 and BMP_7 respectively were approved by the FDA for use as alternatives to autografts (Canalis et al., 2003; Deckers et al., 2002; Termaat et al., 2005).

Studies on growth factors also indicated that the cross-communication between EC and osteoblasts is bidirectional. EC can secrete numerous regulatory molecules that play an important role in controlling bone-forming cells (Bouletreau et al., 2002; von Schroeder et al., 2003; Wang et al., 1997), for example BMP2 and endothelian-1, which can promote osteoblastic proliferation and differentiation. On the other hand, one of the most well-researched angiogenic factors, VEGF, can be produced at the fracture site by numerous cell types, including osteoblasts (Clarkin et al., 2008; Steinbrech et al., 2000; Street et al., 2002). Osteogenic growth factors produced by EC (BMP_2, PDGF) and by osteoblasts (IGF, TGF) also have an angiogenic effect by inducing VEGF expression in osteoblasts (Deckers et al., 2002; Goad et al., 1996).

1.3.5 Current challenges in bone tissue engineering

A fully functional vascular network within bone-engineered constructs is crucial and remains a major challenge in bone tissue engineering (Grellier et al., 2009). After a bone construct is implanted *in vivo*, induction of initial vascularization is important: in particular, the survival of osteogenic cells in the interior of the scaffold is often threatened by the limited extent of initial vascularization (Kneser et al., 1999; Kneser et al., 2006). The implanted construct requires ongoing vascularization to ensure survival and integration, because it takes weeks for the host's blood circulation to establish sufficient supply to the implant (Jain, 2003; Kaigler et al., 2006; McCarthy, 2006; Meyer and Wiesmann, 2006). Several strategies have been proposed to address this problem. For example, the addition of vascular cells such as endothelial cells might offer several advantages over seeding of osteogenic cells alone (Meyer and Wiesmann, 2006; Unger et al., 2007). However, the mode of communication between the bone cells and vascular cells, at the molecular level, has yet to be fully elucidated.

In addition to addressing the limited blood supply for bone tissue engineering, the selection of an appropriate scaffolding to carry the cells and conditions at the cell-scaffold interface are also crucial determinants of successful clinical application.

2. Aims

Bone tissue engineering is a promising strategy for reconstruction/ regeneration of skeletal defects. However, insufficient blood supply after implantation remains a major obstacle to clinical success. The overall aims of the studies on which this thesis is based were to explore the interactions between bone marrow stromal cells and endothelial cells to identify possible biological pathways involved in the repair of bone defects. The studies were undertaken in order to contribute towards the development of an appropriate tissue engineered construct, comprising cells seeded onto biodegradable copolymer scaffolds.

The specific aims of the individual studies were:

- To investigate whether direct communication between bone marrow stromal cells and endothelial cells could influence the osteogenic differentiation of bone marrow stromal cells.
- To evaluate the initial cellular attachment, proliferation, and differentiation of bone marrow stromal cells onto scaffolds made of (poly(LLA-*co*-CL)), (poly(LLA-*co*-DXO)) and poly(L-Lactide).
- To determine the effect on bone regeneration of co-culturing endothelial cells and bone marrow stromal cells onto a poly(LLA-*co*-DXO) scaffold in a rat calvarial defect model.
- To identify possible biological pathways after co-culture of bone marrow stromal cells and endothelial cells and to determine the subsequent patterns of global gene expression, using microarray gene expression profiling.

3. Materials and Methods

A brief outline of the entire project is presented in Figure 3.

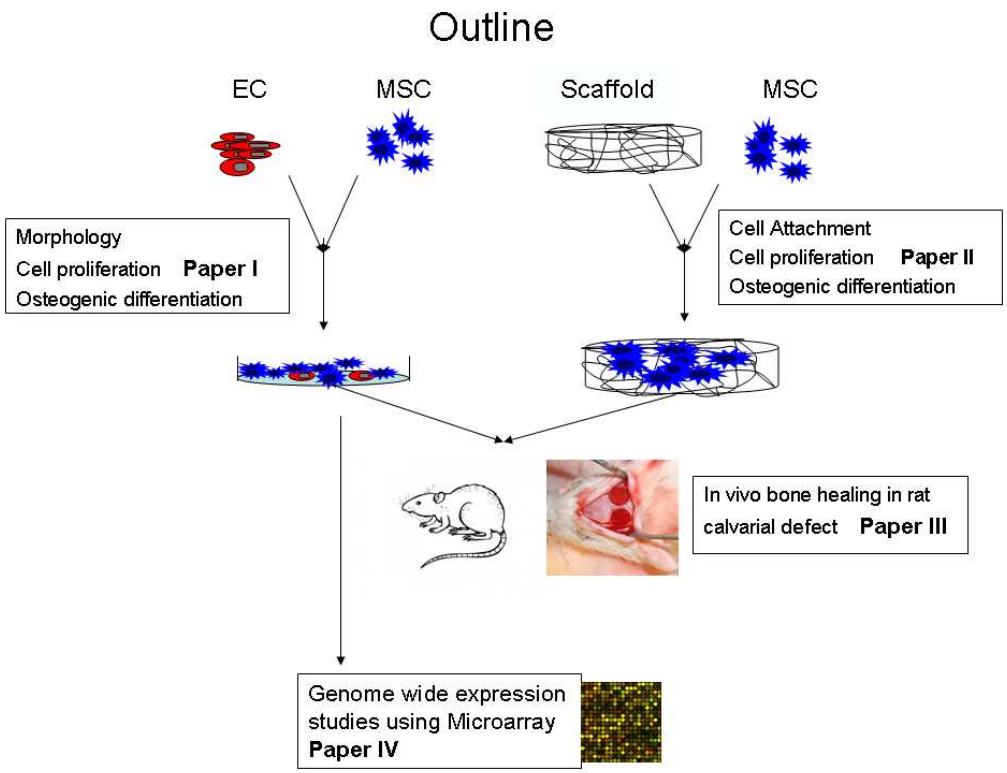

Figure 3. A brief outline of the whole study

The various experimental models, evaluations and methods are briefly summarized in Table 1. A more detailed description is provided in the individual papers (I-IV).

Table 1 *Summary of the methods used*

Experimental models	**Evaluations**	**Methods**	**Paper**
In vitro mono-culture (MSC) and co-culture (MSC/EC) on two dimensional (culture plate)	Cell Morphology Cell proliferation Gene profiling	SEM, Immunostaining SEM, WST-1 PCR, Superarray	I
MSC cultured on 3D scaffolds made of: Poly(LLA-co-CL) poly(LLA-co-DXO) poly(LLA) (control)	Cell Morphology Cell proliferation Gene profiling	SEM SEM, WST-1 PCR	II
Rat calvarial defect model	Bone regeneration Vessel Formation Gene profiling	X-ray Histological analysis PCR	III
MSC/EC and MSC on culture plate	Global gene profiling Validation	Microarray • SAM • GO Classification • pathways PCR	IV

3.1 Scaffold design and fabrication (Paper II)

Copolymer poly(LLA-*co*-CL) and poly(LLA-*co*-DXO) were synthesized as described recently (Dånmark et al., 2010). Porous scaffolds were produced from the copolymers poly(LLA-*co*-CL) and poly(LLA-*co*-DXO) by a solvent-casting-particulate-leaching method. The pore size was greater than 90 μm and the porosity 90 %. Porous scaffolds of poly(LLA) were synthesized in the same way to serve as three dimensional (3D) controls.

3.2 *In vitro* studies

3.2.1 Cell culture (Paper I, II and IV)

Primary human MSC (StemCell Technologies, Vancouver, BC, Canada) were cultured in MesenCult® complete medium according to the manufacturer's instructions. Primary human umbilical vein endothelial cells were obtained from Lonza (Clonetics®, Walkersville, MD) and were expanded in EGM® Medium containing 500 ml of Endothelial Cell Basal Medium and the following growth supplements: BBE, 2 ml; hEGF, 0.5 ml; hydrocortisone, 0.5 ml; FBS, 10 ml; GA-1000, 0.5 ml.

EC and MSC were trypsinized separately and then co-cultured at a ratio of 1:5 in a mixed, osteogenic factor-free medium. MSC were also grown alone in both osteogenic and osteogenic factor-free medium. The culture medium was changed after 3 days.

In study II, MSC were seeded at a density of 1×10^5 cells/disc onto polymer discs which fitted into the wells of a 48-well plate.

3.2.2 Morphological evaluation

Immunostaining (Paper I)

Cells grown on Ø 18 mm coverslips were rinsed in phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min. In order to distinguish the two cell types, MSC were labelled with CD90 and EC with Lectin Tritc-UEA I. The nuclei were stained with DAPI solution and the samples were examined by fluorescence microscopy.

Scanning Electron Microscopy (Paper I and II)

The samples were rinsed in 2.5% glutaraldehyde in PBS and fixed for 30 min and then fixed in 2.5% glutaraldehyde in 0.1 M Na-cacodylate PH 7.2 with 0.1 M sucrose for a further 30 min. The samples were treated with 1% osmium tetroxide in distilled water for 1h, followed by dehydration through a graded series. Critical point drying was carried out and the samples were coated with a 10 nm conducting layer of gold platinum and examined in the scanning electron microscope at a voltage of 10 kV.

3.2.3 Cell viability and proliferation tests (Paper I and II)

Cell proliferation and viability were analyzed by colorimetric assay for quantification of cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. The cells were incubated for a further 12 h at 37°C with 100 µl medium containing 10 µl WST-1 reagents. The samples were shaken for 1 min and absorbance at 450 nm was measured by a microplate spectrophotometer.

3.2.4 Gene profiling

Real-time PCR

Total RNA was isolated from cell cultures using Trizol® reagent combined with E.Z.N.A.™ Tissue RNA isolation kit. The reverse transcription reaction test was undertaken using the High Capacity cDNA Archive Kit. Quantitative RT-PCR was conducted on a StepOne system, using TaqMan gene expression assays. 6 ng of mRNA was used in each PCR reaction and mixtures were made up in 10µl triplicates for each target cDNA. The data were analysed by the relative standard curve method. GAPDH served as an endogenous control.

RT^2 Profiler PCR Array of osteogenesis (Paper I)

Contamination of genomic DNA was removed from total RNA samples by DNase I digestion prior to first-strand synthesis. cDNA synthesis was performed with the RT^2 PCR array First Strand Kit. Human Osteogenesis RT^2 Profiler PCR Array and RT^2 Real-time SyBR Green/ROX PCR Mix were purchased from SuperArray Bioscience Corporation. The ΔΔCt method was used for data analysis, and each gene fold-change was calculated as the difference in gene expression between co- and mono-cultured MSC.

3.2.5 Statistical analysis

All *in vitro* experiments were repeated at least three times using MSC from 3 separate donors and pooled human EC. The data were expressed as mean ± SD for minimum n=3. The data were tested for normal distribution and variance homogeneity, using one-way ANOVA. Differences between means were considered statistically significant when $p<0.05$. For statistical analysis, SPSS 15.0 software was used.

3.3 *In vivo* study (Paper III)

3.3.1 Isolation, induction and characterization of rat cells

MSC were isolated from the femurs of donor Lewis rats and the marrow cavity was flushed with α-MEM supplemented 15 % fetal bovine serum into a sterile falcon tube. The cells were resuspended and plated in culture flasks in α-MEM containing 10% FBS. At harvest, 1/5 of MSC were induced to EC in Endothelial Cell Growth Medium-2 using methods described previously (Yu et al., 2009). The remaining MSC were cultured in freshly prepared osteogenic stimulatory medium, containing dexamethasone, ascorbic acid and β-glycerophosphate. EC were characterized of CD31 and Flk-1 by flow cytometry.

3.3.2 Graft Preparation and surgical implantation

Poly(LLA-co-DXO) scaffolds were pre-wet with the culture media overnight. Either MSC alone (MSC-group), or both cell types (CO-group) were seeded onto the scaffold. The cell/scaffold grafts were incubated overnight and then transferred to spinner flasks for 1 week before surgery. Before implantation, CO/scaffold constructs cultured in spinner flasks were retrieved and Alexa Fluor® 568 conjugated isolectin GS-IB4 was used to reveal EC distribution and to identify any *in vitro* formation of capillary-like structures.

Symmetrical bone defects, 6 mm in diameter, were created in the parietal part of the calvaria in 12 animals. Either MSC-group or CO-group constructs were implanted on random sides of 9 animals. In three animals serving as controls, one side was implanted with unseeded scaffolds only (Scaffold-group) and the other side was left untreated (Empty-group). After 8 weeks the animals were sacrificed. The scaffolds were carefully dissected out and immediately frozen.

3.3.3 Radiographic and histological analysis

Radiographs of the retrieved parts of the skull from each rat were taken using a dental X-ray machine under exposure conditions of 10 mA, 0.08 ms and 70 KV. The exposed films were processed and the digital images were analyzed with software NIS-Elements BR.

The specimens for histological examination were fixed with 4 % paraformaldehyde and decalcified for four weeks. The specimens were embedded and then cryosectioned to 8 μm thickness. The sections were stained with Masson's Trichrome method to confirm the osteoid areas. Image analysis was performed; a common threshold was defined to include the entire osteoid area by software NIS-Elements. Isolectin GS-IB4 was applied to disclose vessel formation in the graft area.

3.3.4 Statistical Analysis

SigmaStat 3.1 was applied for statistical processing and analysis. Two-group comparison (MSC- and CO-groups) was carried out by paired t-test and significant differences were set as $p<0.05$. All values in the bar diagrams were presented as mean ± standard deviation.

3.4 Microarray study (Paper IV)

3.4.1 Experimental Design

The experiments used six different individual MSC treated with mixed EC at a ratio of 5:1 in a mixed, osteogenic factor-free medium. MSC were grown alone as a control. After 5 and 15 days' cultivation, EC were removed from co-culture by addition of 2 ml trypsin for 5 min, followed by washing with PBS. The cells were finally rinsed again with 2 ml trypsin for another 5 min at 37°C. MSC were retrieved from both co-culture and the control group.

3.4.2 Sample preparation

Total RNA from 5- and 15-day cultures was isolated using an E.Z.N.A.^{TM} Tissue RNA isolation kit. In this study, samples with a RIN value of at least 7.5 were selected. 250 ng of total RNA was reversely transcribed, amplified and labelled, using the Illumina TotalPrep RNA Amplification Kit. The amount and quality of Biotin-labelled cRNA were controlled and 1500 ng cRNA was hybridized at 58˚C for 17 hours. The iScan Reader software extracts signal intensities and saves all files for each BeadChip. The data from the scanning of arrays was investigated in GenomeStudio and J-Express 2009 as quality control measures. The SampleProbeProfile was loaded in to J-Express as two separate experiments (5 and 15 days) and further quality control of the data was undertaken.

3.4.3 Microarray Data Analysis

In the microarray data analysis, it was assumed that the majority of genes would behave similarly, irrespective of how they had been treated and that only a few genes would have been altered by treatment. These distributions need to be similar for the different samples to be comparable, as shown in Figure 4.

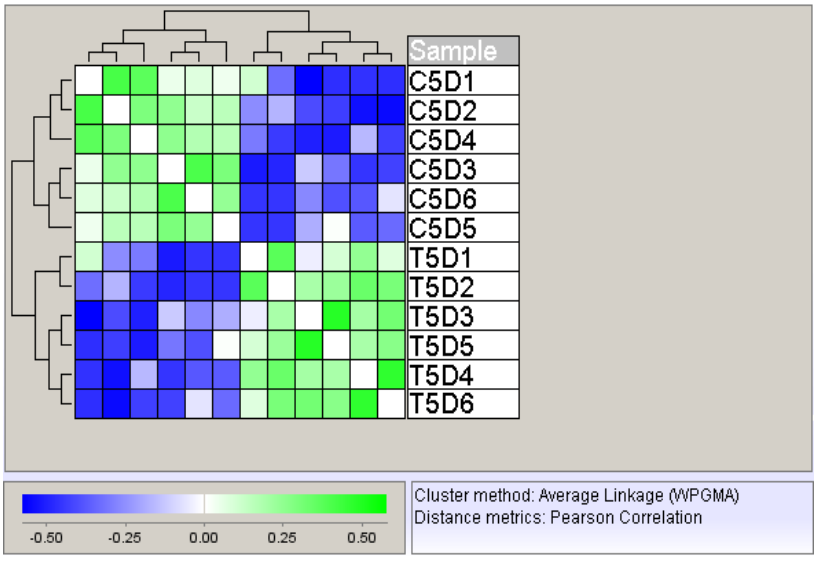

Figure 4 *Hierarchical Clustering of 5-day samples. In the 5-day experiment, hierarchical clustering of normalized data using Pearson Correlation as a measure of distance, revealed two main clusters, corresponding to the co-culture (T) and control (C) groups, indicating differential expression of genes between the two groups at the global level*

To identify genes differentially expressed between two groups, significance analysis of microarrays (SAM) method (Tusher et al., 2001) was undertaken using J-express software. Since a donor-paired group was used in this experiment, gene expression measurements were analysed by a paired SAM method. Changes in the gene-expression profile were identified at an estimated false discovery rate of less than 1%. Genes with a fold-change greater than 2 were selected. Differentially expressed genes were mapped to a gene ontology (GO) directed

acyclic graph in J-express 2.9 and compared with the total number of genes, to determine over-representation of GO terms.

To classify each cluster in more detail according to their ontological properties, lists of overrepresented genes from the SAM test were submitted to the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Dennis et al., 2003; Huang et al., 2009). The Gene Functional Classification tool in DAVID builds clusters of genes with significantly similar ontology, as tested against the whole list of genes in the Human Genome array. A similar analysis was performed with the genes against a reference list of genes through the DAVID bioinformatics database, which is a classification system (http://www.DAVID.org/) for identifying overrepresented biological processes and key pathways.

3.4.4 Validation of microarray data by RT-PCR

To validate the microarray data, real time PCR was undertaken on up- and down-regulated genes. Amplification was carried out in 96-well thermal cycle plates using a StepOne detection system. Gene expression was determined by the comparative Ct method, normalizing expression to the reference gene GADPH. For quantitative RT-PCR, the difference between groups was assessed using ANOVA, with statistical significance set at $p < 0.05$.

3.5 Ethical approvals

The study protocols of the *in vitro* studies (225.05) and the protocols for the animal experiments (project no. 2006290-31/07/2006, 20091572) were approved by Ethics Committee at Bergen University. The animal procedures were conducted in accordance with Norwegian and European Union (EU) animal safety regulations.

4. Results and Discussions

4.1 Direct Cell-to-cell communication (Paper I)

The present studies were designed using a 1:5 (EC:MSC) cell mix for 5 days of culture. Different EC:MSC ratios had been co-cultured in pilot studies and it was found that EC could grow well and exist as a continuous stimulatory factor for MSC in the 1:5 ratio, which closely simulates *in vivo* conditions (Meyer, 2006). The SEM and immunostaining results (Figure 5) indicated that there might be communication between the two cell types after 5 days of culture.

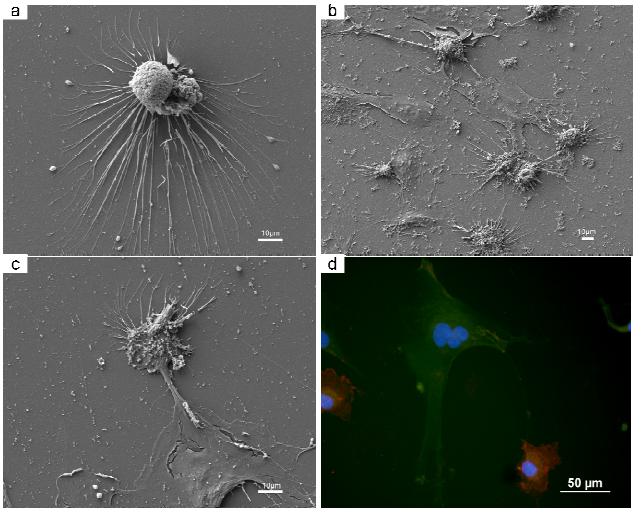

Figure 5 *Morphology of cell communications between co-cultured MSC and EC: SEM showing morphology of EC after 5 days (a); SEM of MSC and EC after 5 days (b); cellular bridge between MSC and EC disclosed by SEM (c) and by immunostaining (d).*

In the early stages of osteoblast-mediated mineralization, bone ALP is considered to have an important function in the removal of inorganic pyrophosphate, a potent inhibitor of mineralization (Sharp and Magnusson, 2008). ALP is often used as a biochemical and histochemical marker for identification and evaluation of osteogenesis. Interestingly, there was an almost 5-fold increase in ALP expression when MSC were co-cultured with EC at less

than 20% of the total cell population. This increase was confirmed by both Superarray and PCR evaluation of cells cultured in osteogenic factor-free medium. Furthermore, the PCR results showed that after 5 days of co-culture, the effect induced by EC was greater than that induced by osteogenic stimulatory medium.

However, co-culturing of MSC with EC did not influence Runx2 expression. It has been shown that Runx2 plays a role in the commitment-step to osteo-chondro progenitor cells (Komori et al., 1997; Nakashima et al., 2002; Otto et al., 1997; Phillips et al., 2006). The findings indicated that up-regulation of ALP might be independent of Runx2. However, the identification of other potential pathways will require further investigation. On the other hand, the low level of bone gamma-carboxyglutamate protein (BGLAP) expressed by MSC suggests that it is unlikely that such immature cells would have the potential to undergo terminal differentiation within 5 days of co-culture with EC. Therefore, EC could direct MSC to the early stage of osteogenic cells. The Superarray test disclosed that the genes related to chondrocyte differentiation (Sox 9, MMP2, FGF_2) (Bilezikian et al., 2002; Jin et al., 2007) were down-regulated, suggesting that EC might provide important signals for chondrocytic differentiation and have an important influence on chondrocyte commitment and maturation.

4.2 Evaluation of three different polymeric scaffolds (Paper II)

To select a copolymer scaffold appropriate for carrying cells, bone marrow stromal cells were seeded onto two porous scaffolding materials, poly(LLA-*co*-CL) and poly(LLA-*co*-DXO). The cellular responses were investigated in terms of attachment, proliferation and differentiation.

At 1 h post-seeding, SEM disclosed earlier cell-spreading on poly(LLA-co-DXO) and poly(LLA-co-CL) than on poly(LLA) scaffolds, which served as controls. At 24 h and 14 days post-seeding, MSCs in all groups showed spreading morphology and multilayers of cells

on the scaffolds. The more rapid attachment of MSC after 1 h to poly(LLA-co-DXO) and poly(LLA-co-CL) than to poly(LLA) scaffolds might be attributable to a relative improvement in hydrophilicity or other properties as a result of copolymerization. DXO is a sticky, wax-like amorphous polymer without any stability of form, but when used as a co-monomer it increases both the hydrophilicity and the degradation rate (Dånmark et al., 2010; Odelius et al., 2008). Caprolactone (CL) is tough, flexible and semicrystalline and it is permeable to low molecular weight species at body temperature. However, associated with the hydrophobicity of CL is the disadvantage of a slow degradation rate, which needs to be enhanced by copolymerization with other suitable monomers (Holmbom et al., 2005; Kim et al., 2004; Meretoja et al., 2006; Rhee et al., 2004).

Cell viability and proliferation, assessed by microscopy and WST-1 assay, indicated better proliferation on poly(LLA-co-DXO) and poly(LLA-co-CL) than on poly(LLA) after different incubation times. This might be explained by earlier attachment of MSC to poly(LLA-co-DXO) and poly(LLA-co-CL) than to poly(LLA) scaffolding. During the process of cell seeding, more rapid attachment of cells to scaffolds could enhance cell survival and accelerate tissue ingrowth (Bueno et al., 2007). Compared with a tissue culture plate, the porous scaffolds provide much greater surface areas for cell seeding and spreading. The spatial distribution of human MSC on a scaffold is quite different from that on tissue culture plates.

After the induction of osteogenic factors (dexamethasone, ascorbic acid and β-glycerophosphate), MSC in all the groups expressed the specific bone markers ALP, Runx2, Col I and OPN. This indicated that poly(LLA-co-DXO), poly(LLA-co-CL) and poly(LLA) could support the osteogenic differentiation of MSC *in vitro*; in this respect, there were no significant differences among the three scaffolding materials. However, the results showed that cells grown on tissue culture plates exhibited greater expression of ALP, Col I and Runx2 by MSC than the cells on the scaffolds.

With respect to cell attachment, proliferation and differentiation, all the results indicated that these newly developed copolymers are promising candidates as scaffolding material for bone tissue engineering.

4.3 Effect of endothelial cells on bone regeneration (Paper III)

As shown in Study I, endothelial cells might influence the differentiation of bone marrow stromal cells. Therefore, the aim of this study was to evaluate this effect *in vivo*, using a rat calvarial bone defect model. Either MSC alone (MSC-group) or co-cultured EC/MSC (CO-group) were seeded onto poly(LLA-co-DXO) scaffolds, cultured in spinner flasks and then implanted into symmetrical calvarial defects prepared in recipient rats. To investigate vessel formation, histological staining was performed with EC markers after 2 months.

After co-culture of EC and MSC in a spinner flask for one week, the EC had spontaneously organized into typical vessel-like structures. These findings suggest that under dynamic culture conditions, prevascularization could be achieved to enhance bone regenerative potential before implantation.

The bioreactor is an important factor in the formation of a functional construct (Martin et al., 2004; Stiehler et al., 2009). The cell/scaffold constructs must be cultured in an environment with sufficient nutrient and mechanical properties to induce cell phenotypes and activity. Stiehler and co-workers has shown that compared with static culture, dynamic 3-D culture in a spinner flask could achieve better proliferation, distribution and differentiation of human bone marrow stromal cells (Stiehler et al., 2009).

Gross observation of retrieved samples showed varying stages of healing. In the Empty-group, the defects were covered by a thin layer of soft tissue. The defects in the other groups were totally filled with the implanted grafts. There was no gross evidence of serious inflammation, infection or necrosis. Radiographic and histological examination showed more rapid bone

formation in the CO- than in the MSC-group (Figure 6). However, histological analysis 2 months postoperatively showed similar expression of EC markers in both groups. Furthermore, RT-PCR demonstrated that the CO-group exhibited greater expression of osteogenic markers.

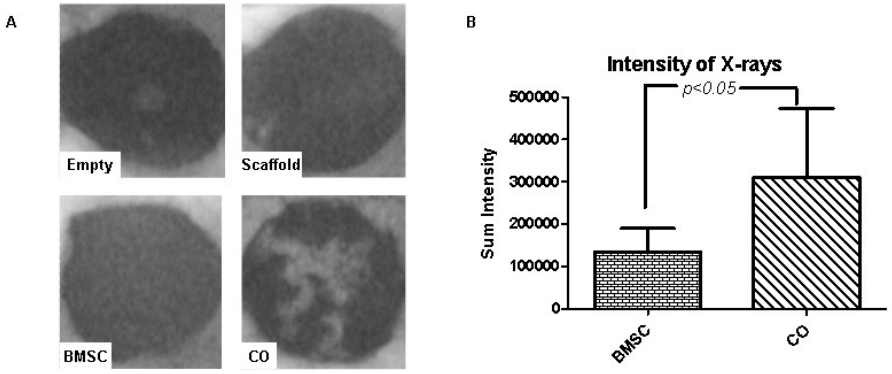

Figure 6. *X-ray results from different groups: very few high density areas were visible in the Empty, Scaffold and MSC-groups. (A) CO-group showed visibly higher density than the other groups. (B) Image analysis showed significantly higher intensity from the CO than the MSC-group.*

In vivo studies using co-culture have demonstrated improved bone regeneration, but contradictory results for vascularization (Kaigler et al., 2006; Yu et al., 2009). The present study showed no statistically significant differences between the MSC- and the CO-groups with respect to vascularization of the implanted grafts two months postoperatively. Thus the question of whether transplanted EC could have a further effect on angiogenesis in the recipient animal remains unresolved. In order to improve our understanding of angiogenesis in promoting osteogenesis in a tissue engineered bone reconstruction model, further research is needed on interactions between endothelium and osteogenic cell phenotypes.

4.4 Changes in global gene expression (Paper IV)

Based on the findings of studies I & III, demonstrating the effect on osteogenesis of co-culturing EC with MSC, further systematic investigation was carried out using microarray analysis. The HumanWG-6 v3.0 expression BeadChips, with each array representing >48,000 probes was used and a global map of gene expression following cell interactions between MSC and EC was generated in a direct-contact model.

A map was generated of differentially expressed genes of MSC after co-culture with EC for 5 and 15 days. The map disclosed complex bidirectional gene regulation mechanisms between MSC and EC. Although the top 20 gene list could only display partially differentiated genes, it could indicate the most significant changes after co-culture. Genes related to angiogenesis were upregulated by MSC after co-culture. Meanwhile, markers of osteogenesis, such as ALP, FKBP5 (Liu et al., 2007) and BMP were also up-regulated in the cocultured-MSC. Importantly, this finding might provide the basis of an alternative strategy for simultaneously improving angiogenesis and osteogenesis in engineered bone constructs: further investigation of the molecular interactions between the two cell types might provide new insights into mesenchymal stem cell biology.

Interaction between the two cell types involved not only cell-cell communication but also cell adhesion and cell-ECM communication. Among the top up-regulated genes is another important group, namely ECM related genes such as CD 93, CDH5, VWF, and MMRN1. This trend was also found in the cluster analysis. In the 5-day-experiment, GO clustering (Table 2) disclosed obvious effects on signal peptide (98 genes), cell adhesion (28 genes), extracellular matrix (27 genes), blood vessel development (18 genes), cell migration (17 genes) and ECM-receptor interaction (10 genes). The studies also indicated that EC-matrix contained certain signals and factors which could modify MSC differentiation into EC.

Table 2 *Gene Ontology annotation of overrepresented gene lists by DAVID*

5-day GO term Annotation

GO Term	Count	P Value	Fold Enrichment	Bonferroni	FDR	Enrichment Score
Signal peptide	98	1.16E-21	2.628575622	1.09E-18	1.82E-18	17.6667
Cell adhesion	28	7.02E-13	5.657907013	2.30E-10	9.51E-10	10.1803
Extracellular matrix	27	3.16E-12	5.450620082	6.35E-10	3.96E-09	9.15492
Blood vessel development	18	6.57E-08	5.183016488	1.08E-04	1.10E-04	6.70772
Cell migration	17	1.72E-06	4.362624794	0.00281331	0.002885	5.19
Response to wounding	22	9.51E-06	3.059008886	0.01546976	0.015965	3.64
Immunoglobulin domain	19	9.94E-06	3.469646478	0.00324597	0.01346	3..29
Hydroxylation	9	1.28E-06	11.21025606	4.20E-04	0.001737	3.22
EGF-like region, conserved site	17	1.35E-06	4.471613194	6.35E-04	0.001922	2.94
Chelation	4	8.97E-05	41.72706422	0.02889946	0.121339	2.83
Mesenchymal cell development	6	6.81E-04	8.439014025	0.67289333	1.137838	2.8
ECM-receptor interaction	10	1.36E-05	6.643437863	9.96E-04	0.014244	2.58
Domain:CTCK	4	0.00262892	14.25152905	0.91444934	4.039053	2.45
Response to organic substance	25	6.09E-05	2.480940351	0.09505084	0.102228	2.37
Regulation of locomotion	11	3.08E-04	4.168493974	0.39663103	0.516048	2.27
Prostaglandin receptor activity	3	0.00613438	24.53757225	0.90757121	8.186742	2.15
Urogenital system development	8	9.76E-04	5.087869325	0.79827397	1.626004	2.07
C-type lectin-like	7	0.00145361	5.6740638	0.49671796	2.05637	2.02
Tube development	10	0.00335422	3.29450998	0.99595457	5.486517	1.99
IL 17 Signaling Pathway	4	0.00518607	10.43809524	0.34712179	5.40332	1.9

It is well known that the TGF-beta pathway is important for osteogenesis differentiation and cell proliferation (Nesti et al., 2007). Bone matrix is one of the richest reservoirs of TGF-beta and osteoblasts possess several different TGF-beta receptors (Nesti et al., 2007). In the 5-day treatment group, 7 genes were recruited into this pathway (Figure 7). All theses genes are contributed most of the path of genes interaction, and the consequences of the interaction are

the osteoblast differentiation and cell cycle in Figure 7. This finding tends to support and explain the results of Study I, such as increases in bone markers (ALP, Col I) and positive effects on proliferation. The results suggest that EC could direct mesenchymal stem cells towards the osteoblastic phenotype and be considered as osteoinductive mediators in a co-culture model (Grellier et al., 2009) TGF-beta pathway suppression might explain why only early stage bone markers were detected by the microarray test.

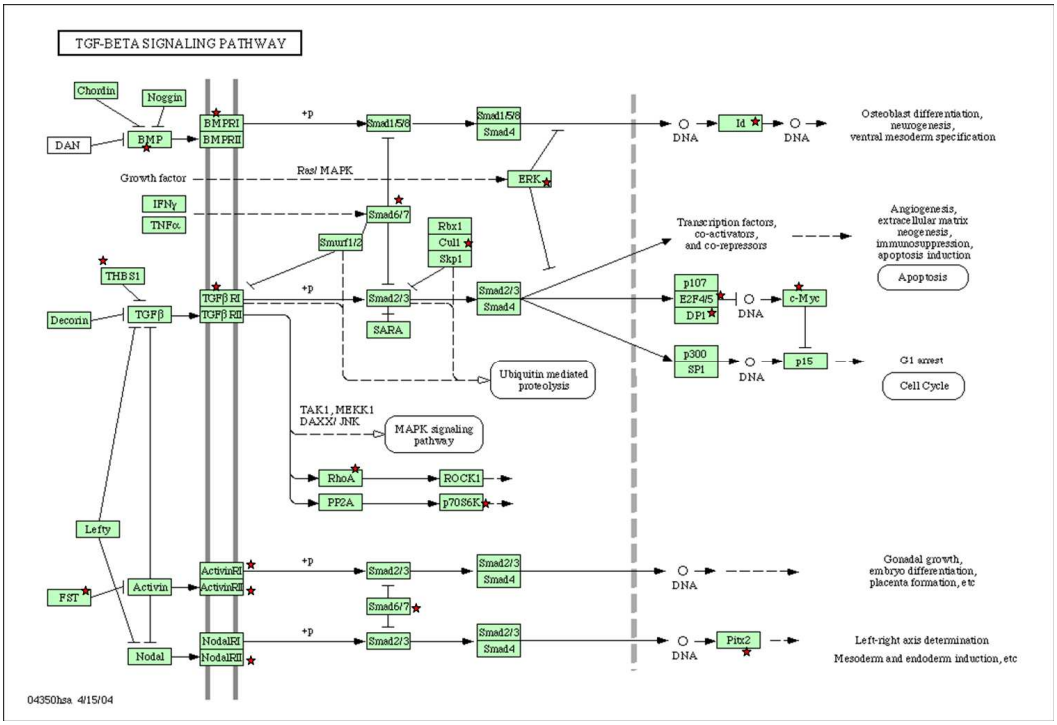

Figure 7 *TGF-beta signaling pathway:* Labeling showed 19 differentiated genes in the map

5. Conclusions

- In the absence of osteogenic factors, co-culture of bone marrow stromal cells with endothelial cells induced the expression of ALP after a short incubation time (5 days). ALP expression by bone marrow stromal cells in co-culture was even higher than that achieved by the addition of osteogenic stimulatory medium: this effect seems to be independent of Runx2.
- Co-culture of endothelial and bone marrow stromal cells on poly(LLA-co-DXO) scaffolding implanted in rat calvarial defects enhanced the osteogenic potential and induced more rapid bone formation.
- The microarray study generated a global map of gene expression of the interaction between bone marrow stromal cells and endothelial cells. Endothelial cells had a significant impact on bone marrow stromal cells after 5 and 15 days, especially with respect to cell signal, cell adhesion and cellular matrix formation.
- TGF-beta as a key biological pathway is crucial for endothelial cell-induced osteogenic gene regulation in bone marrow stromal cells.
- Human bone marrow stromal cells showed consistently better attachment and growth on scaffolds made of poly(LLA-co-DXO) and poly(LLA-co-CL) than on poly(LLA) scaffolds. This suggests that these newly developed copolymers may be promising candidates as scaffolding material for bone tissue engineering.

Future Perspectives

Many challenges remain before bone tissue engineering can be applied in the clinical setting. The co-culture model is mimicking of natural bone and results contribute to understanding of bone physiology and treatment strategies. The present study is based on only 5:1 MSC/EC,

and the addition of less than 20% endothelial cells to bone marrow stromal cells could enhance early osteogenic differentiation and increase bone regeneration. Global gene profiling indicates that several signals and biological pathways are involved in cell-to-cell communication. The finding of bidirectional gene regulation of angiogenesis and osteogenesis in co-culture offers potentially new approaches in tissue engineering by modulating bone-healing through cell-to-cell interactions. Functional studies are warranted to explore and understand the interaction between bone and endothelial cells, using *in vitro* and large animal experiments.

References

Albertsson AC, Varma IK (2003). Recent developments in ring opening polymerization of lactones for biomedical applications. *Biomacromolecules* 4*:*1466-1486.

Arvidson K, Abdallah B, Applegate L, Baldini N, Cenni E, Gomez-Barrena E *et al.* (2010). Bone regeneration and stem cells. *J Cell Mol Med* 1111*:*1582-4934.

Bilezikian J, Raisz L, Rodan G (2002). Principles of Bone Biology San Diego, CA: Elsevier Science & Technology Books.

Bishop GB, Einhorn TA (2007). Current and future clinical applications of bone morphogenetic proteins in orthopaedic trauma surgery. *Int Orthop* 31*:*721-727.

Bouletreau PJ, Warren SM, Spector JA, Peled ZM, Gerrets RP, Greenwald JA *et al.* (2002). Hypoxia and VEGF up-regulate BMP-2 mRNA and protein expression in microvascular endothelial cells: implications for fracture healing. *Plast Reconstr Surg* 109*:*2384-2397.

Bueno EM, Laevsky G, Barabino GA (2007). Enhancing cell seeding of scaffolds in tissue engineering through manipulation of hydrodynamic parameters. *J Biotechnol* 129*:*516-531.

Canalis E, Economides AN, Gazzerro E (2003). Bone morphogenetic proteins, their antagonists, and the skeleton. *Endocr Rev* 24*:*218-235.

Canalis E, Mazziotti G, Giustina A, Bilezikian JP (2007). Glucocorticoid-induced osteoporosis: pathophysiology and therapy. *Osteoporos Int* 18*:*1319-1328.

Canalis E (2009). Growth factor control of bone mass. *J Cell Biochem* 108*:*769-777.

Carano RA, Filvaroff EH (2003). Angiogenesis and bone repair. *Drug Discov Today* 8*:*980-989.

Clarkin CE, Emery RJ, Pitsillides AA, Wheeler-Jones CP (2008). Evaluation of VEGF-mediated signaling in primary human cells reveals a paracrine action for VEGF in osteoblast-mediated crosstalk to endothelial cells. *J Cell Physiol* 214*:*537-544.

d'Aquino R, De Rosa A, Lanza V, Tirino V, Laino L, Graziano A *et al.* (2009). Human mandible bone defect repair by the grafting of dental pulp stem/progenitor cells and collagen sponge biocomplexes. *Eur Cell Mater* 18*:*75-83.

Dånmark S, Finne-Wistrand A, Wendel M, Arvidson K, Albertson AC, Mustafa K (2010). Osteogenic Differentiation by Rat Bone Marrow Stromal Cells on Customized Biodegradable Polymer Scaffolds. *Jounral of Bioactive and Compatible Polymers* 25*:*207-223.

Deckers MM, van Bezooijen RL, van der Horst G, Hoogendam J, van Der Bent C, Papapoulos SE *et al.* (2002). Bone morphogenetic proteins stimulate angiogenesis through osteoblast-derived vascular endothelial growth factor A. *Endocrinology* 143*:*1545-1553.

Dennis G, Jr., Sherman BT, Hosack DA, Yang J, Gao W, Lane HC *et al.* (2003). DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* 4*:*P3.

Drost AC, Weng S, Feil G, Schafer J, Baumann S, Kanz L *et al.* (2009). *In vitro* myogenic differentiation of human bone marrow-derived mesenchymal stem cells as a potential treatment for urethral sphincter muscle repair. *Ann N Y Acad Sci* 1176*:*135-143.

Edlund U, Dånmark S, Albertsson AC (2008). A strategy for the covalent functionalization of resorbable polymers with heparin and osteoinductive growth factor. *Biomacromolecules* 9*:*901-905.

Finkemeier CG (2002). Bone-grafting and bone-graft substitutes. *J Bone Joint Surg Am* 84-A*:*454-464.

Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP (1968). Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation* 6*:*230-247.

Frohlich M, Grayson WL, Wan LQ, Marolt D, Drobnic M, Vunjak-Novakovic G (2008). Tissue engineered bone grafts: biological requirements, tissue culture and clinical relevance. *Curr Stem Cell Res Ther* 3*:*254-264.

Gazdag AR, Lane JM, Glaser D, Forster RA (1995). Alternatives to Autogenous Bone Graft: Efficacy and Indications. *J Am Acad Orthop Surg* 3*:*1-8.

Goad DL, Rubin J, Wang H, Tashjian AH, Jr., Patterson C (1996). Enhanced expression of vascular endothelial growth factor in human SaOS-2 osteoblast-like cells and murine osteoblasts induced by insulin-like growth factor I. *Endocrinology* 137*:*2262-2268.

Grellier M, Bordenave L, Amedee J (2009). Cell-to-cell communication between osteogenic and endothelial lineages: implications for tissue engineering. *Trends Biotechnol* 27*:*562-571.

Guillot PV, Cui W, Fisk NM, Polak DJ (2007). Stem cell differentiation and expansion for clinical applications of tissue engineering. *J Cell Mol Med* 11*:*935-944.

Guillotin B, Bareille R, Bourget C, Bordenave L, Amedee J (2008). Interaction between human umbilical vein endothelial cells and human osteoprogenitors triggers pleiotropic effect that may support osteoblastic, function. *Bone* 42*:*1080-1091.

Gulotta LV, Kovacevic D, Ehteshami JR, Dagher E, Packer JD, Rodeo SA (2009). Application of bone marrow-derived mesenchymal stem cells in a rotator cuff repair model. *Am J Sports Med* 37*:*2126-2133.

Hill PA (1998). Bone remodelling. *Br J Orthod* 25*:*101-107.

Holmbom J, Sodergard A, Ekholm E, Martson M, Kuusilehto A, Saukko P *et al.* (2005). Long-term evaluation of porous poly(epsilon-caprolactone-co-L-lactide) as a bone-filling material. *J Biomed Mater Res A* 75*:*308-315.

Horwitz EM, Gordon PL, Koo WK, Marx JC, Neel MD, McNall RY *et al.* (2002). Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. *Proc Natl Acad Sci U S A* 99*:*8932-8937.

Huang d, Sherman BT, Lempicki RA (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4*:*44-57.

Jain RK (2003). Molecular regulation of vessel maturation. *Nat Med* 9*:*685-693.

Jin EJ, Choi YA, Kyun Park E, Bang OS, Kang SS (2007). MMP-2 functions as a negative regulator of chondrogenic cell condensation via down-regulation of the FAK-integrin beta1 interaction. *Dev Biol* 308*:*474-484.

Kaigler D, Krebsbach PH, West ER, Horger K, Huang YC, Mooney DJ (2005). Endothelial cell modulation of bone marrow stromal cell osteogenic potential. *FASEB J* 19*:*665-667.

Kaigler D, Krebsbach PH, Wang Z, West ER, Horger K, Mooney DJ (2006). Transplanted endothelial cells enhance orthotopic bone regeneration. *J Dent Res* 85*:*633-637.

Karageorgiou V, Kaplan D (2005). Porosity of 3D biomaterial scaffolds and osteogenesis. *Biomaterials* 26*:*5474-5491.

Kim HW, Knowles JC, Kim HE (2004). Development of hydroxyapatite bone scaffold for controlled drug release via poly(epsilon-caprolactone) and hydroxyapatite hybrid coatings. *J Biomed Mater Res B Appl Biomater* 70*:*240-249.

Klinkner DB, Densmore JC, Kaul S, Noll L, Lim HJ, Weihrauch D *et al.* (2006). Endothelium-derived microparticles inhibit human cardiac valve endothelial cell function. *Shock* 25*:*575-580.

Kneser U, Kaufmann PM, Fiegel HC, Pollok JM, Kluth D, Herbst H *et al.* (1999). Long-term differentiated function of heterotopically transplanted hepatocytes on three-dimensional polymer matrices. *J Biomed Mater Res* 47*:*494-503.

Kneser U, Schaefer DJ, Polykandriotis E, Horch RE (2006). Tissue engineering of bone: the reconstructive surgeon's point of view. *J Cell Mol Med* 10*:*7-19.

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K *et al.* (1997). Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* 89*:*755-764.

Krebsbach PH, Kuznetsov SA, Bianco P, Robey PG (1999). Bone marrow stromal cells: characterization and clinical application. *Crit Rev Oral Biol Med* 10*:*165-181.

Kronenberg HM (2003). Developmental regulation of the growth plate. *Nature* 423*:*332-336.

Kyriakidou K, Lucarini G, Zizzi A, Salvolini E, Mattioli B, Mollica F *et al.* (2008). Dynamic Co-Seeding of Osteoblast and Endothelial Cells on 3D Polycaprolactone Scaffolds for

Enhanced Bone Tissue Engineering. *Journal of Bioactive and Compatible Polymers* 23*:*227-243.

Lamalice L, Le Boeuf F, Huot J (2007). Endothelial cell migration during angiogenesis. *Circ Res* 100*:*782-794.

Langer R, Vacanti JP (1993). Tissue engineering. *Science* 260*:*920-926.

Laugier P, Berger G, de Vernejoul MC (1998). [Bone remodeling: evaluation methods]. *Rev Prat* 48*:*1185-1190.

Lian JB, Stein GS (1992). Concepts of osteoblast growth and differentiation: basis for modulation of bone cell development and tissue formation. *Crit Rev Oral Biol Med* 3*:*269-305.

Lian JB, Stein GS (1995). Development of the osteoblast phenotype: molecular mechanisms mediating osteoblast growth and differentiation. *Iowa Orthop J* 15*:*118-140.

Liu TM, Martina M, Hutmacher DW, Hui JH, Lee EH, Lim B (2007). Identification of common pathways mediating differentiation of bone marrow- and adipose tissue-derived human mesenchymal stem cells into three mesenchymal lineages. *Stem Cells* 25*:*750-760.

Liu X, Ma PX (2004). Polymeric scaffolds for bone tissue engineering. *Ann Biomed Eng* 32*:*477-486.

Lofgren A, Albertsson AC (1994). Copolymers of 1,5-dioxepan-2-one and L-dilactide or D,L-dilactide - Hydrolytic Degradation Behaviour. *J Appl Polym Sci* 52*:*1327-1338.

Lofgren A, Albertsson AC, Zhang Y, Bjursten LM (1994). Copolymers of 1,5-dioxepan-2-one and L- or D,L-dilactide: *in vivo* degradation behaviour. *J Biomater Sci Polym Ed* 6*:*411-423.

Lozito TP, Taboas JM, Kuo CK, Tuan RS (2009). Mesenchymal stem cell modification of endothelial matrix regulates their vascular differentiation. *J Cell Biochem* 107*:*706-713.

Maniatopoulos C, Sodek J, Melcher AH (1988). Bone formation *in vitro* by stromal cells obtained from bone marrow of young adult rats. *Cell Tissue Res* 254*:*317-330.

Marcacci M, Kon E, Moukhachev V, Lavroukov A, Kutepov S, Quarto R *et al.* (2007). Stem cells associated with macroporous bioceramics for long bone repair: 6- to 7-year outcome of a pilot clinical study. *Tissue Eng* 13*:*947-955.

Martin I, Wendt D, Heberer M (2004). The role of bioreactors in tissue engineering. *Trends Biotechnol* 22*:*80-86.

Mathisen T, Albertsson AC (1989). Polymerization of 1,5-dioxepan-2-one. 1. Synthesis and characterization of the monomer 1,5-dioxepan-2-one and its cyclic dimer 1,5,8,12-tetraoxacyclotertradecane-2,9-one. *Macromolecules* 22*:*3838-3842.

McCarthy I (2006). The physiology of bone blood flow: a review. *J Bone Joint Surg Am* 88 Suppl 3*:*4-9.

Meretoja VV, Helminen AO, Korventausta JJ, Haapa-aho V, Seppala JV, Narhi TO (2006). Crosslinked poly(epsilon-caprolactone/D,L-lactide)/bioactive glass composite scaffolds for bone tissue engineering. *J Biomed Mater Res A* 77*:*261-268.

Meyer U, Wiesmann H (2006). Bone and Cartilage Engineering. 1 ed. Berlin: Springer-Verlag New York, LLC.

Middleton JC, Tipton AJ (2000). Synthetic biodegradable polymers as orthopedic devices. *Biomaterials* 21*:*2335-2346.

Mo XM, Xu CY, Kotaki M, Ramakrishna S (2004). Electrospun P(LLA-CL) nanofiber: a biomimetic extracellular matrix for smooth muscle cell and endothelial cell proliferation. *Biomaterials* 25*:*1883-1890.

Nair LS, Laurencin CT (2007). Biodegradable polymers as biomaterials. *Prog Polym Sci* 32*:*762-798.

Nakahara H, Misawa H, Hayashi T, Kondo E, Yuasa T, Kubota Y *et al.* (2009). Bone repair by transplantation of hTERT-immortalized human mesenchymal stem cells in mice. *Transplantation* 88*:*346-353.

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR *et al.* (2002). The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* 108*:*17-29.

National Trauma Data Bank Report (2005). The American College of Surgeons.

Nesti LJ, Caterson EJ, Li WJ, Chang R, McCann TD, Hoek JB *et al.* (2007). TGF-beta1 calcium signaling in osteoblasts. *J Cell Biochem* 101*:*348-359.

Odelius K, Plikk P, Albertsson AC (2005). Elastomeric hydrolyzable porous scaffolds: copolymers of aliphatic polyesters and a polyether-ester. *Biomacromolecules* 6*:*2718-2725.

Odelius K, Plikk P, Albertsson AC (2008). The influence of composition of porous copolyester scaffolds on reactions induced by irradiation sterilization. *Biomaterials* 29*:*129-140.

Oswald J, Boxberger S, Jorgensen B, Feldmann S, Ehninger G, Bornhauser M *et al.* (2004). Mesenchymal stem cells can be differentiated into endothelial cells *in vitro*. *Stem Cells* 22*:*377-384.

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR *et al.* (1997). Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* 89*:*765-771.

Owen M, Friedenstein AJ (1988). Stromal stem cells: marrow-derived osteogenic precursors. *Ciba Found Symp* 136*:*42-60.

Patel ZS, Young S, Tabata Y, Jansen JA, Wong ME, Mikos AG (2008). Dual delivery of an angiogenic and an osteogenic growth factor for bone regeneration in a critical size defect model. *Bone* 43*:*931-940.

Petite H, Viateau V, Bensaid W, Meunier A, de Pollak C, Bourguignon M *et al.* (2000). Tissue-engineered bone regeneration. *Nat Biotechnol* 18*:*959-963.

Phillips JE, Gersbach CA, Wojtowicz AM, Garcia AJ (2006). Glucocorticoid-induced osteogenesis is negatively regulated by Runx2/Cbfa1 serine phosphorylation. *J Cell Sci* 119*:*581-591.

Pollock R, Alcelik I, Bhatia C, Chuter G, Lingutla K, Budithi C *et al.* (2008). Donor site morbidity following iliac crest bone harvesting for cervical fusion: a comparison between minimally invasive and open techniques. *Eur Spine J* 17*:*845-852.

Porter JR, Ruckh TT, Popat KC (2009). Bone tissue engineering: a review in bone biomimetics and drug delivery strategies. *Biotechnol Prog* 25*:*1539-1560.

Proff P, Romer P (2009). The molecular mechanism behind bone remodelling: a review. *Clin Oral Investig* 13*:*355-362.

Rhee SH, Lee YK, Lim BS, Yoo JJ, Kim HJ (2004). Evaluation of a novel poly(epsilon-caprolactone)-organosiloxane hybrid material for the potential application as a bioactive and degradable bone substitute. *Biomacromolecules* 5*:*1575-1579.

Rouwkema J, de Boer J, Van Blitterswijk CA (2006). Endothelial cells assemble into a 3-dimensional prevascular network in a bone tissue engineering construct. *Tissue Eng* 12*:*2685-2693.

Ryner M, Albertsson AC (2002). Resorbable and highly elastic block copolymers from 1,5-dioxepan-2-one and L-lactide with controlled tensile properties and hydrophilicity. *Biomacromolecules* 3*:*601-608.

Salgado AJ, Coutinho OP, Reis RL (2004). Bone tissue engineering: state of the art and future trends. *Macromol Biosci* 4*:*743-765.

Santos MI, Reis RL (2010). Vascularization in bone tissue engineering: physiology, current strategies, major hurdles and future challenges. *Macromol Biosci* 10*:*12-27.

Sharp CA, Magnusson P (2008). Isoforms of bone alkaline phosphatase, stem cells, and osteoblast phenotypes. *Stem Cells Dev* 17*:*857-858.

Sodergard A, Stolt M (2002). Properties of lactic acid based polymers and their correlation with composition. *Prog Polym Sci* 27*:*1123-1163.

Srivastava RK, Albertsson AC (2006). Porous scaffolds from high molecular weight polyesters synthesized via enzyme-catalyzed ring-opening polymerization. *Biomacromolecules* 7*:*2531-2538.

Steinbrech DS, Mehrara BJ, Saadeh PB, Greenwald JA, Spector JA, Gittes GK *et al.* (2000). VEGF expression in an osteoblast-like cell line is regulated by a hypoxia response mechanism. *Am J Physiol Cell Physiol* 278*:*C853-860.

Stiehler M, Bunger C, Baatrup A, Lind M, Kassem M, Mygind T (2009). Effect of dynamic 3-D culture on proliferation, distribution, and osteogenic differentiation of human mesenchymal stem cells. *J Biomed Mater Res A* 89*:*96-107.

Street J, Bao M, deGuzman L, Bunting S, Peale FV, Jr., Ferrara N *et al.* (2002). Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. *Proc Natl Acad Sci U S A* 99*:*9656-9661.

Surgeons AAoO (2008). Facts on Orthopedic Surgeries. In: AAOS website (Available at: www.aaos.org/research/patientstats/).

Takagi K, Urist MR (1982). The role of bone marrow in bone morphogenetic protein-induced repair of femoral massive diaphyseal defects. *Clin Orthop Relat Res* 171*:*224-231.

Tang ZG, Callaghan JT, Hunt JA (2005). The physical properties and response of osteoblasts to solution cast films of PLGA doped polycaprolactone. *Biomaterials* 26*:*6618-6624.

Termaat MF, Den Boer FC, Bakker FC, Patka P, Haarman HJ (2005). Bone morphogenetic proteins. Development and clinical efficacy in the treatment of fractures and bone defects. *J Bone Joint Surg Am* 87*:*1367-1378.

Towler DA (2008). The osteogenic-angiogenic interface: novel insights into the biology of bone formation and fracture repair. *Curr Osteoporos Rep* 6*:*67-71.

Tusher VG, Tibshirani R, Chu G (2001). Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A* 98*:*5116-5121.

Undale AH, Westendorf JJ, Yaszemski MJ, Khosla S (2009). Mesenchymal stem cells for bone repair and metabolic bone diseases. *Mayo Clin Proc* 84*:*893-902.

Unger RE, Sartoris A, Peters K, Motta A, Migliaresi C, Kunkel M *et al.* (2007). Tissue-like self-assembly in cocultures of endothelial cells and osteoblasts and the formation of microcapillary-like structures on three-dimensional porous biomaterials. *Biomaterials* 28*:*3965-3976.

Urist MR (1965). Bone: formation by autoinduction. *Science* 150*:*893-899.

Vaccaro AR, Chiba K, Heller JG, Patel T, Thalgott JS, Truumees E *et al.* (2002). Bone grafting alternatives in spinal surgery. *Spine J* 2*:*206-215.

Villars F, Bordenave L, Bareille R, Amedee J (2000). Effect of human endothelial cells on human bone marrow stromal cell phenotype: role of VEGF? *J Cell Biochem* 79*:*672-685.

Villars F, Guillotin B, Amedee T, Dutoya S, Bordenave L, Bareille R *et al.* (2002). Effect of HUVEC on human osteoprogenitor cell differentiation needs heterotypic gap junction communication. *Am J Physiol Cell Physiol* 282*:*C775-785.

von Schroeder HP, Veillette CJ, Payandeh J, Qureshi A, Heersche JN (2003). Endothelin-1 promotes osteoprogenitor proliferation and differentiation in fetal rat calvarial cell cultures. *Bone* 33*:*673-684.

Wang DS, Miura M, Demura H, Sato K (1997). Anabolic effects of 1,25-dihydroxyvitamin D3 on osteoblasts are enhanced by vascular endothelial growth factor produced by osteoblasts and by growth factors produced by endothelial cells. *Endocrinology* 138*:*2953-2962.

Yu H, Vandevord PJ, Gong W, Wu B, Song Z, Matthew HW *et al.* (2008). Promotion of osteogenesis in tissue-engineered bone by pre-seeding endothelial progenitor cells-derived endothelial cells. *J Orthop Res* 26*:*1147-1152.

Yu H, VandeVord PJ, Mao L, Matthew HW, Wooley PH, Yang SY (2009). Improved tissue-engineered bone regeneration by endothelial cell mediated vascularization. *Biomaterials* 30*:*508-517.

Zafiropoulos GG, Hoffmann O, Kasaj A, Willershausen B, Weiss O, Van Dyke TE (2007). Treatment of intrabony defects using guided tissue regeneration and autogenous spongiosa alone or combined with hydroxyapatite/beta-tricalcium phosphate bone substitute or bovine-derived xenograft. *J Periodontol* 78*:*2216-2225.