

DEPARTMENT OF DENTAL MEDICINE  
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# **CRANIOFACIAL BONE TISSUE ENGINEERING WITH BIOMIMETIC CONSTRUCTS**

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*to my parents*

# ABSTRACT

The repair of bone defects and nonunions remains a significant clinical problem in orthopedic and maxillofacial reconstructive surgery. Tissue engineering offers a potential approach to overcome existing limitations related to auto- and allograft tissues. Novel biomimetic engineering strategies enable us to model the desirable physiological signaling in the bioengineered devices and to study tissue repair and regeneration under conditions resembling the human *in vivo* context. In the current thesis we aimed to better understand how a biomimetic approach in tissue engineering could be applied towards the repair and regeneration of bone tissue in the craniofacial area. In our first study we designed a biomimetic construct composed of ceramic scaffold modified with *in vitro*-derived extracellular matrix (HA-ECM), and assessed the osteogenic properties of the generated HA-ECM *in vivo*. Cell-derived matrix enhanced the osteogenic properties of ceramic scaffold, and the construct modulated the local inflammatory response in a bone repair-favorable way. We then continued to investigate the osteogenic properties of bone marrow stromal cell (BMSC)-loaded constructs and assessed the cellular components of the elicited foreign body reaction following implantation. The implications of BMSCs in the regulation of the foreign body reaction triggered by the tissue-engineered constructs were highlighted, demonstrating higher efficiency for the BMSC combination therapy. Furthermore, we investigated the effect of HA-ECM on the osteogenic differentiation of periodontal ligament progenitor cells (PDLC) and assessed the effect of PDLC-seeded HA-ECM on the bone repair. The HA-ECM enhanced the osteogenic differentiation of PDLC and the treatment with PDLC-seeded HA-ECM significantly improved calvarial bone repair. In the final study we have been able to implement a GMP-grade methodology for the biomimetic construct production under complete xeno-free conditions. The resulted tissue-engineered construct has promoted osteogenic differentiation of human MSCs *in vitro* and displayed biological safety and high biocompatibility *in vivo*. In conclusion, the work presented in this thesis highlights the functional *in vitro*-generated biomimetic tissue-engineered constructs with enhanced osteogenicity, biocompatibility and suitable handling properties, as a promising tool for craniofacial bone regeneration.

**Key words:** bone regeneration, tissue engineering, extracellular matrix, biomimetic scaffold, foreign body reaction, mesenchymal stem cells, bioceramics.

## LIST OF PUBLICATIONS

This thesis is based on the following articles and a manuscript, which are referred to in the text by their Roman numerals (I-IV).

- I. **Gregory Tour**, Mikael Wendel, Ion Tcacencu.  
Cell-derived matrix enhances osteogenic properties of hydroxyapatite.  
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- II. **Gregory Tour**, Mikael Wendel, Ion Tcacencu.  
Bone marrow stromal cells enhance osteogenic properties of hydroxyapatite scaffolds by modulating foreign body reaction.  
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- III. **Gregory Tour**, Mikael Wendel, Guido Moll, Ion Tcacencu.  
Bone repair using periodontal ligament progenitor cell-seeded constructs.  
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- IV. **Gregory Tour**, Mikael Wendel, Ion Tcacencu.  
Generation of human fibroblast-derived extracellular matrix constructs for bone tissue engineering applications: a clinical-grade approach.  
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## LIST OF ABBREVIATIONS

AA	Ascorbic acid
ACTA2	Alpha 2 smooth muscle actin
ADSC	Adipose-derived stem cells
ALP	Alkaline phosphatase
BMP	Bone morphogenetic protein
BMSC	Bone marrow stromal cell
BrdU	Bromodeoxyuridine
BSP	Bone sialoprotein
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
Col	Collagen
DPBS	Dulbecco's phosphate buffered saline
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
ESC	Embryonic stem cell
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GMP	Good manufacturing practice
HA	Hydroxyapatite
HE	Hematoxylin-eosin
HLA	Human leukocyte antigen
Hyp	Hydroxyproline
IGF	Insulin-like growth factor



IL	Interleukin
LAL	Limulus amoebocyte lysate
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MSC	Mesenchymal stem cell
MTT	Dimethyl thiazolyl diphenyl tetrazolium bromide
NFB	Newly formed bone
NK	Natural killer cell
OC	Osteocalcin
OPN	Osteopontin
PCNA	Proliferating Cell Nuclear Antigen
PDGF	Platelet-derived growth factor
PDLC	Periodontal ligament progenitor cell
PE	Phycoerythrin
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Quantitative real time polymerase chain reaction
Runx2	Runt-related transcription factor 2
S100A4	S100 calcium binding protein A4
SBC	Straumann BoneCeramic
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
TFE	TissuFleece E <sup>®</sup> equine collagen fleece
TGF- $\beta$	Transforming growth factor beta
TNF- $\alpha$	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
$\alpha$ -MEM	Minimum essential medium, alpha modification
$\beta$ -TCP	Beta-tricalcium phosphate



# 1 INTRODUCTION

## 1.1 BONE TISSUE

Bone, a mineralized mesenchymal tissue, serves at least two principal biological functions. It regulates the mineral homeostasis and provides signaling to other vital tissue systems, e.g. the parathyroid gland, kidney, vasculature, adipose tissue, hypothalamus (Fukumoto and Martin, 2009). As the essential structural connective tissue, bone protects key organs and supports locomotion. In response to mechanical forces and levels of calcium and phosphate in the blood, it is continuously turned over and remodeled through the activities of two major cell populations; bone-resorbing osteoclasts, originating from the hematopoietic lineage, and bone-forming osteoblasts of mesenchymal origin.

The mineral content exceeds 70% of the bone's dry weight, while 30% is represented by the organic component. The protein composition of the organic part is usually classified into collagenous matrix, with collagen type 1 as the major constituent comprising 90% of the organic component, and the remaining 10% of non-collagenous proteins, including cells.

### 1.1.1 Bone cell biology

Osteoblasts and osteoclasts are the two major cellular units responsible for the synthesis and remodelling of bone tissue. Osteoblasts are cuboidal bone matrix-producing cells that form a cellular layer at sites of bone formation. The layer of osteoblasts serves as a guiding template by secreting proteins towards the osteoid deposition compartment. Morphologically, they are similar to fibroblasts when cultured *in vitro* (Ducy *et al.*, 2000). Alkaline phosphatase (ALP), a cytochemical marker characteristic of osteoblast activity is expressed at osteoblast surfaces to facilitate the release of inorganic phosphate required for hydroxyapatite (HA) crystal formation. As the matrix is laid down, the osteoblasts become flattened and eventually become trapped inside the matrix where they mature into osteocytes in lacunae of the bone matrix. Osteocytes form an extensive network of long channels containing osteocytic processes extending and connecting to each other at gap junctions, serving mechanosensory role.

Osteoclasts are bone resorbing multinucleated cells of the hematopoietic lineage, formed by cellular fusion of circulating monocytes as they attach to the exposed bone surface (Chambers, 2000). Osteoclasts are dependent on the macrophage-colony stimulating factor (M-CSF) and receptor for activation of nuclear factor kappa B (NF- $\kappa$ B) RANK ligand (RANKL) expressed on osteoblasts, which is why the physical contact between the two is required for the complete formation and activation of functional osteoclasts (Teitelbaum and Ross, 2003).

### 1.1.2 Extracellular matrix of bone

Collagen fibers comprise the major portion of the organic phase and play critical roles in maintaining bone tissue structure. They are of primary importance in the processes of mineralization, providing the framework for crystal nucleation and matrix substrate for cell migration and differentiation. Another essential portion of bone extracellular matrix (ECM) is represented by the non-collagenous glycoproteins and proteoglycans that have a high ion-binding capacity necessary for the initiation of mineralization process. These include: small integrin binding ligand N-linked glycoprotein (SIBLING) family members; osteopontin (OPN), bone sialoprotein (BSP) (Hunter *et al.*, 1996), osteonectin (ON) (Termin *et al.*, 1981), proteoglycans, such as osteoadherin (Wendel *et al.*, 1998), biglycan, decorin, aggrecan, versican (Iozzo, 1998),  $\gamma$ -carboxyglutamic acid proteins like osteocalcin (OC) (Hunter *et al.*, 1996) and serum proteins.

OPN is expressed at early stages of osteoblast development prior to other matrix proteins. The early expression of OPN has been associated with its role in cell attachment to ECM, implicating its importance in linking organic and mineral phases (McKee and Nanci, 1996; Sodek *et al.*, 2000). Although, expressed in numerous tissues, OPN is abundant in mineralized bone matrix and has therefore been implicated in bone formation and remodeling (Boskey, 1995). OPN and BSP have regions enriched in Arg-Gly-Asp (RGD) cell attachment sequence and are involved in the nucleation of HA at the mineralization front. OC delays nucleation and is usually associated with the mineralized matrix of bone being expressed at later stages of osteoblast differentiation, indicating a mature osteoblast phenotype.

ECM regulates the formation of initial mineral deposition as well as orientation of the crystal growth in a defined organized fashion. Moreover, ECM components of bone have critical roles in skeletal development. This can be illustrated by a large number of skeletal dysplasia associated with mutations in matrix molecules. For instance, heterozygous mutations in *COL1A1* and *COL1A2* genes coding for the  $\alpha 1$  and  $\alpha 2$  chains of collagen type I (Col1) cause osteogenesis imperfecta (Kocher and Shapiro, 1998).

## 1.2 BONE FORMATION

### 1.2.1 Craniofacial bone development

The head, comprising the face and the skull, is probably the most anatomically sophisticated structure in all vertebrates. One remarkable and distinct feature of its development is the emergence of the neural crest and its contribution to the formation of craniofacial structures (Helms and Schneider, 2003; Trainor *et al.*, 2003).

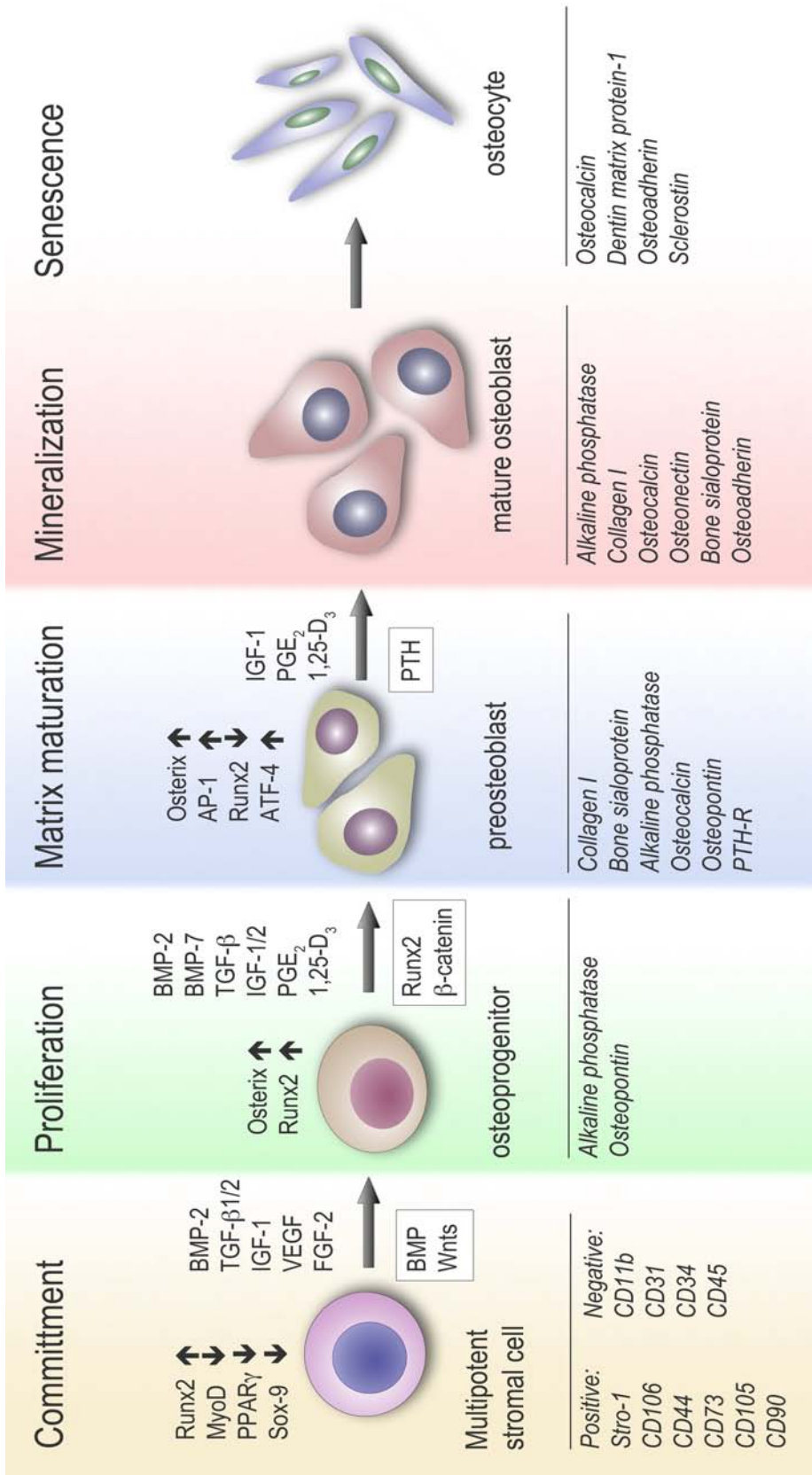
The cranial neural crest emerges from the lateral and dorsal neural crests containing cells with high potency and plasticity. These cells follow very well orchestrated migration and eventually populate the branchial arches where they start to proliferate and differentiate into specific cell types giving rise to multiple craniofacial elements through epithelial-mesenchymal interactions. These processes are strictly controlled by complex signaling pathways and involve a number of critical developmental genes (Olsen *et al.*, 2000). Craniofacial bones are mostly derived from the cranial neural crest cells through intramembranous ossification, when mesenchymal cells directly condense and differentiate into osteoblasts without endochondral model of bone formation (Nie *et al.*, 2006).

### 1.2.2 Transcriptional control of osteoblast differentiation

Numerous methodologies have been applied to identify pathways and important regulators of the lineage-specific stem cell differentiation. The genetic approach was successfully employed to demonstrate several important transcription factors that induce osteoblast-specific cell differentiation. For instance, core-binding factor  $\alpha 1$ /Runx2 (Cbfa1, Runt-related transcription factor family) (Komori *et al.*, 1997), osterix (Nakashima *et al.*, 2002) and lipoprotein related receptor 5 (Lrp5) (Qiu *et al.*, 2007) overexpression lead to osteoblast differentiation, while PPAR- $\gamma$ 2 (Lecka-Czernik *et al.*, 2002) and Sox9 (Ng *et al.*, 1997) induce adipocyte or chondrocyte lineages, correspondingly. Additionally, cells were treated with various mixtures of growth factors, hormones and extracellular matrix components to prompt differentiation. These approaches identified several factors that induced the cell toward the osteoblast phenotype, such as BMPs (Luu *et al.*, 2007), Wnt (Gaur *et al.*, 2005) or inhibiting osteoblast differentiation, e.g. Noggin (Rifas, 2007).

Runx2 (Cbfa1) is a central regulator of bone formation. It activates OC and COL1A1 genes (Komori, 2006) and may be used as a marker of the osteogenic cell lineage (Komori, 2008). Targeted disruption of Runx2 leads to complete loss of bone forming capacity by osteoblasts, indicating its importance for both endochondral and intramembranous bone formation (Komori *et al.*, 1997). Another indicator of its pleiotropic regulation of hard tissue formation can be demonstrated upon its induction in skin fibroblasts *in vitro*, where it has shown to result in osteoblast-specific gene expression (Ducy *et al.*, 1997), whereas expression of Runx2 *in vivo* leads to ectopic endochondral ossification (Takeda *et al.*, 2001). The osteoblast differentiation program is dependent on another factor, osterix, also known as Sp7, important for activation of OC, Col1A1 and BMP-2 (Nakashima *et al.*, 2002).

Several other important transcriptional regulators and signaling pathways have been identified to promote or repress the activity of the key transcriptional factors, including activating transcriptional factor 4 (Huang *et al.*, 2007), a homeobox



**Figure 1.** Transcriptional factors involved in osteoblast differentiation and corresponding cell markers expressed at the different stages.  
 $\uparrow$  = activation  $\downarrow$  = inhibition.

transcription factor *Dlx5* (Lee *et al.*, 2003), mitogen-activated protein kinase signaling pathways (Celil and Campbell, 2005).

### 1.2.3 Overview of osteoblast differentiation

As differentiation of the multipotent mesenchymal cells proceeds, the progenitor cells of each lineage acquire the specific phenotype under instruction of a number of regulatory elements. Osteoprogenitors retain patterns of the expression of marker genes that provide the characteristics to the bone phenotype. Recruitment, proliferation, differentiation and the following stages that reflect growth, proliferation, matrix synthesis and mineralization are characterized by the induction of a distinct chronologically-related set of genes (Fig. 1).

The growth and proliferation phases are associated with increased mitotic activity involving the expression of multiple cell-cycle and cell growth genes. In addition, genes associated with ECM synthesis, such as *Col1*, *OPN*, fibronectin are actively expressed. *Col1* mRNA is retained throughout the following stages of osteoblast differentiation and maturation, gradually decreasing in time.

As the committed osteoprogenitors enter the matrix secretion phase, proliferation-associated genes become downregulated, whereas *ALP* mRNA transcripts are promptly increased and the newly synthesized matrix properties and composition undergo series of modifications. The following mineralization phase is strictly coordinated by osteoblasts and further involves deposition of apatite crystals within the organic framework of ECM. The process is quite complex and it is generally accepted that the ECM components such as collagen together with several acidic macromolecules, including proteoglycans, glycoproteins and phosphoproteins regulate the mineral ions concentration and, hence, the rate of mineralization.

### 1.2.4 Factors regulating osteoblastogenesis

Circulating systemic hormones, cytokines, growth-factors or osteoblast-derived factors acting in paracrine fashion are released into the ECM and altogether affect the osteoblast differentiation process.

Bone morphogenetic proteins (BMPs) have been implicated in skeletal development and healing, being capable of initiating osteoblastogenesis of mesenchymal precursors of the adult bone marrow (Chen *et al.*, 2004). They were originally detected as proteins present in demineralized bone matrix that could induce ectopic osteogenesis (Urist, 1965). Today, over 30 members have been identified, all structurally related to transforming growth factor  $\beta$  (TGF- $\beta$ ) superfamily of signaling molecules. The BMPs are found in bone matrix and are synthesized by a number of tissues, while BMP-2, BMP-4 and BMP-7 have been implicated in ectopic osteogenesis (Sakou, 1998). Other factors such as TGF- $\beta$ 1, platelet-derived growth factor (PDGF), insulin-like growth factors (IGFs), and

members of the fibroblast growth factor (FGF) family are all able stimulate osteoblast differentiation. However, they cannot induce osteoblast differentiation from uncommitted progenitor cells in ectopic sites similarly to BMPs (Rose *et al.*, 2004). TGF- $\beta$ 1 regulates osteoblast proliferation and matrix synthesis including mineralization (Bonewald and Dallas, 1994; Janssens *et al.*, 2005). FGFs are heparin-binding growth factors regulating osteoblast differentiation in a number of pathways and cell signaling cascades (Eswarakumar *et al.*, 2005). In particular, basic (b)FGF has shown to participate in matrix mineralization by inducing ALP activity, as well as Runx2 and OC expression (Deng *et al.*, 2008).

In addition to autocrine, paracrine and endocrine signals, cell-matrix interactions are also required for osteoblast development (Grzesik and Robey, 1994). This interplay is mediated by protein ligands expressed on the cell surface responsible for facilitation of contact and anchoring to the matrix surface. Moreover, those adhesion molecules are involved in the migration of osteoblast progenitors from the bone marrow to sites of bone remodeling. More important, they play role in the control of osteoblast and osteoclast development and apoptosis (Globus *et al.*, 1998). Some of the adhesion ligands that influence osteoblast development and function include integrins, particularly  $\alpha_v\beta_3$  and  $\alpha_2\beta_1$  (Moursi *et al.*, 1997), selectins, and cadherins, as well as a family of transmembrane proteins containing disintegrin and metalloprotease domains (Rehn *et al.*, 2007). Each of these proteins recognizes distinct ligands, e.g. RGD amino acid sequence present in collagen, fibronectin, OPN, thrombospondin, BSP, and vitronectin (Ross *et al.*, 1993).

### **1.3 BONE TISSUE ENGINEERING**

It has been estimated that about one in five people reaching the age of 65 would benefit from a tissue replacement therapy during their lifetime (Lysaght and Reyes, 2001). Similar to other complex tissues, engineering bone requires cooperation of multiple disciplines including cell biology, stem cell and developmental biology, molecular biology, biomechanics, biomaterial science, immunology and transplantation science. Despite the enormous recent advances in each of the areas, the translation to the benchside and the production of functional and efficient tissue-engineered devices to replace missing tissue is limited. The complexity of the bone structure and functions requires a very well organized interactions between cells, ECM structures as well as biomechanical forces and gene and protein regulatory factors for continuous long-lasting function. Hence, engineering bone tissue requires a comprehensive approach with broad expertise (O'Keefe and Mao, 2011).



### 1.3.1 Stem cells

The drawbacks of auto-, allo- and xenograft-based bone reconstruction, such as an additional surgical procedure, infection, chronic pain, and donor dependency has generated large focus on the use of autologous cells for tissue engineering (Barry and Murphy, 2004; Bauer and Muschler, 2000; Dimitriou *et al.*, 2011; Hill *et al.*, 1999).

Stem cells are attractive candidates for tissue engineering applications due to their ability to commit to multiple cell lineages. Latest advances in stem cell biology and tissue engineering enable us to “drive” multipotent cells into the desired phenotype to generate functional tissue structures. *In vitro* and after implantation, the stem cell fate and behaviour is instructed by its intrinsic genetic and epigenetic program as well as by interactions with multiple microenvironmental elements. Cells may actively modify the parameters of their environment by synthesizing or degrading the ECM, secreting cytokines, and communicating with other cells and matrix by molecular and physical signals. In the context of tissue engineering, the “bioengineered stem cell niche” has emerged as a new paradigm comprising key factors such as regulatory molecules, ECM, physical factors and other cells (Vunjak-Novakovic and Scadden, 2011).

Adult stem cells provide tissue homeostasis throughout life and ensure regeneration following damage. Numerous types of adult stem cells have been identified, including hematopoietic, mesenchymal, endothelial, intestinal, and neuronal stem cells. Remarkably, cell populations isolated from different tissues display phenotypic similarities and share some common properties, but differ in their differentiation potential and gene expression profile in ways that reflect their tissue of origin (Noel *et al.*, 2008; Wagner *et al.*, 2005).

Mesenchymal stem cells (MSCs) isolated from a range of adult tissues have been extensively studied as a source of cells for biomedical applications, mainly due to their broad regenerative potential, paracrine regulatory effects and strong immunomodulatory activity (Pittenger *et al.*, 1999). Importantly, MSCs can be harvested and reintroduced in the same patient, therefore eliminating the immunological issues associated with allogeneic cell transplantation. However, they exhibit limited proliferation potential and an age-associated progressive decline in functionality upon *in vitro* expansion (Baxter *et al.*, 2004; Bonab *et al.*, 2006; Zhou *et al.*, 2008), which limits the generation of large quantities of functional cells for clinical applications.

On the other hand, human embryonic stem cells (ESCs) are characterized by virtually unlimited proliferation potential and ability to give rise to all tissues constituting the human body. There are also major political and ethical considerations regarding the use of human embryos, which imposes great challenges for the use of ESCs in patients. These ethical controversies have further led to intense investigations in cellular reprogramming, resulting in the generation of induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006). However,

the broader differentiation potential also represents additional challenges in directing and controlling the cell fate with higher risk of tumor formation after transplantation, currently making ESCs and iPS cells not suitable for tissue engineering applications.

The MSC phenotype has traditionally been defined through the expression of several surface markers (Foster *et al.*, 2005). The proposed minimal criteria to define human MSC by International Society for Cellular Therapy are: plastic-adherence when maintained in standard culture conditions, expression of CD105, CD73 and CD90, and negativity for CD45, CD34, CD14 or CD11b, CD79 $\alpha$  or CD19 and HLA-DR surface molecules, and ability to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro* (Dominici *et al.*, 2006).

### 1.3.2 Scaffolds as cell-delivery vehicles

Three-dimensional scaffold serves as an *in vitro* alternative for the native ECM. It is a structural and instructive template that aims at guiding and homing cells to facilitate tissue repair. Many different materials have been proposed as synthetic bone substitutes. The choice of the scaffolding material with appropriate structure, chemical composition and macro and micro-architectural parameters are of ultimate importance. Surface properties may not only significantly influence cellular adhesion and proliferation, survival, signaling and growth, but also instruct the cellular phenotype. Moreover, to allow cell in-growth and even distribution throughout the tissue-engineered device, the scaffold should have adequate pore characteristics to facilitate nutrient transport and neovascularization from the surrounding host tissue. Many of these parameters influence other scaffold properties, such as degradation rate and mechanical stability.

Hydroxyapatite (HA),  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$  of synthetic or biological origin, is considered one of the most suitable bone substitutes due to its superior osteoconductivity. Other ceramic scaffolds such as  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) and biphasic calcium phosphate are currently used for bone repair, augmentation and regeneration (LeGeros *et al.*, 2003).  $\beta$ -TCPs dissolves in the presence of osteoclasts and macrophages, whereas HA is hardly degradable (Bohner, 2000).  $\beta$ -TCP is highly biocompatible, osteoconductive, and stimulates proliferation and osteogenic differentiation of MSCs in a number of *in vivo* and *in vitro* studies (Kasten *et al.*, 2008; Takahashi *et al.*, 2005; von Doernberg *et al.*, 2006). Bone tissue engineering applications of calcium-phosphate ceramics and other bioceramics, such as bioactive glass, however have drawbacks due to their brittleness and lower mechanical strength values (Hench, 2006).

A wide variety of synthetic polymers have been investigated for tissue engineering purposes. Scaffolds composed of degradable polymers have been widely used to aid bone repair *in vivo*. The major advantages of polymer scaffolds are adjustable hydrophilicity, degradation rates and mechanical properties. These parameters can

be manipulated in different ways, most often by copolymerization or the introduction of different architecture (Holland and Mikos, 2006).

Based on our current understanding of the *in vivo* conditions, we are now able to design biomimetic scaffolds that more fully exploit the potential of cells in the dynamic *in vivo* environment by replicating the physiological milieu. Such biomimetic materials would mimic the properties of a native tissue and provide optimal signaling for seeded cells to undergo gradual replacement by the newly synthesized tissue matrix. However, new tissue formation by tissue engineering principles does not fully reflect the developmental or wound healing processes. It therefore might be unnecessary for an artificial scaffold to entirely resemble the natural ECM (Ma, 2008) since the tissue is expected to regenerate at accelerated rates compared to the slower natural development process (Chen *et al.*, 2010a).

### 1.3.3 Growth factors and gene therapy

To utilize the use of growth factors and signaling molecules in bone tissue engineering necessitates careful and thorough consideration of how these signals exert their effects in regenerating tissues. Among supposedly efficient tools are: parathyroid hormone, BMPs, Hedgehogs, IGFs, FGFs, Wnts/beta-catenin, TGF- $\beta$ , platelet-derived growth factor, prostaglandins etc (Lin and Hankenson, 2011; O'Keefe and Mao, 2011). Most of these growth factors have been effective in animal models, however, further understanding of their temporal effects on bone repair and *in vivo* kinetics will enable the eventual translation to the patient.

One example of successful translation is BMP-2, approved for clinical use in spine fusion and in tibia nonunion (Kim and Choe, 2011). BMP-2 is the leading osteoinductive growth factor used clinically in bone-related regenerative medicine today. However, supraphysiologic doses of BMP-2 are required (Walker and Wright, 2002) and concerns have emerged most often associated with the off-label use in spine surgery, including reports of inflammatory reactions (Smucker *et al.*, 2006; Vaidya *et al.*, 2007), side effects related to ectopic bone formation (Wong *et al.*, 2008) and questions about dosage and efficacy (Zara *et al.*, 2011). Together, these adverse effects along with high costs and the possibility of forming structurally abnormal and potentially mechanically unstable bone tissue may limit the overall clinical performance of BMPs.

In fact, very few biologically active agents have been approved and commercialized for therapy in humans. Unfortunately, the current growth factor delivery strategies in tissue-engineering are too complex, and still have the potential for hazardous complications in patients (Chen *et al.*, 2010b).

To date, the vastly anticipated promise of gene therapy has not been achieved, mainly due to safety concerns in clinical trials (Paez *et al.*, 2004). For clinical applications, gene therapy requires extensive information regarding the safety and efficacy of the expressed target gene as well as its delivery vehicles. BMP-2 is a

potential target gene because the recombinant protein has now been in clinical use for a decade (Jin *et al.*, 2003). Other methods to control gene expression, such as siRNA techniques, are emerging and may be more easily translated to clinical use (Cheema *et al.*, 2007).

## **1.4 INFLAMMATION AND BONE REPAIR**

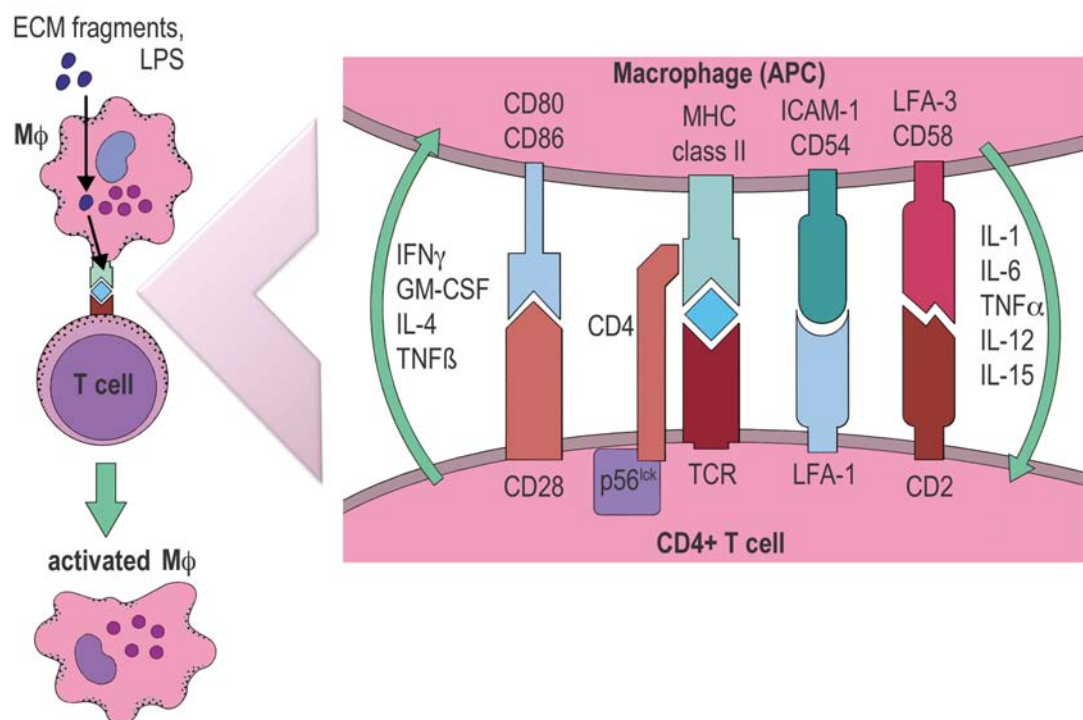
### **1.4.1 Overview of the inflammatory events following surgery**

An immune response that follows immediately after tissue injury or surgery has a large impact on the outcomes of tissue repair and regeneration. Tissue damage causes activation of complement cascades by classical or alternative mechanisms (Nilsson *et al.*, 2007) and cellular pattern recognition receptors activation as a result of cell destruction or pathogen presence (Jones, 2008). This is closely associated with inflammatory cytokine production and initiation of polymorphonuclear neutrophils (PMNs), monocytes and fibroblasts that are recruited to the wound or defect site (Lopez-Armada *et al.*, 2002). PMNs are among the first cell populations to infiltrate the wound site. They aim at removing pathogens, cell and tissue debris due to the injury and remain for only a few days (Boehler *et al.*, 2011). PMNs eliminate pathogens by phagocytosis, releasing reactive oxygen species (ROS) and inflammatory cytokines including interleukin-1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interferon- $\gamma$  (IFN- $\gamma$ ), which often leads to secondary damage in the adjacent tissues (Mountziaris and Mikos, 2008). Monocytes start to differentiate into macrophages as they enter the injury spot (Duffield, 2003). The number of macrophages normally peaks at about one week, however they can persist in the injury site for months (Beck *et al.*, 2010). Macrophages are also among the major ROS and cytokines producers additionally contributing to secondary damage. However, as will be further discussed in the following section, their presence is critical for tissue repair and regeneration as they secrete growth factors and phagocytize cell debris (Duffield, 2003).

Upon implantation of biomaterial, therapeutic device with or without foreign cells or introduction of antigens to the site of injury, the inflammatory response may become significantly intensified as a result of induced foreign body reaction. Interactions of construct device with blood or other tissue components lead to protein deposition on the biomaterial forming a provisional matrix, which affects subsequent leukocyte adhesion interactions. Therefore, the chemistry and topography of the implant surface may be principally responsible for the intensity of reactions prompted by infiltrating immune cells (Anderson *et al.*, 2008). Furthermore, cell-derived matrices can contain biological impurities or allogeneic signals resulting in increased inflammation at the implant site. Most of the synthetic scaffolds today can be produced without introducing these signals,

however some of the synthetic polymers and their degradation products can activate the complement cascade (Nilsson *et al.*, 2007).

Endotoxin is a ubiquitous contaminant that is most often associated with adverse immune reactions when present in significant amounts on implantable medical devices (Daly *et al.*, 2012). The exact amount of lipopolysaccharide (LPS) endotoxin within an implanted device that is necessary to elicit the adverse reaction is unknown. It is believed that contamination within ECM scaffolds may result in harmful effects for the host immune and remodeling responses, likely through the activation of the toll like receptor 4 signaling pathway by LPS (Brightbill *et al.*, 1999; van Putten *et al.*, 2011). The manufacturing process of biologic scaffolds from cell or tissue sources often requires decellularization, aseptic techniques and thorough quality assurance to minimize the residual LPS. However, LPS presence within these scaffolds cannot be entirely eliminated. Therefore, it is recommended that endotoxin levels in the biologic scaffolds, which do not elicit deleterious *in vivo* proinflammatory reaction, are systemically determined (Magalhaes *et al.*, 2007a).



**Figure 2.** Cell surface molecules involved in antigen presentation. Fragments of ECM, LPS molecules undergo antigen processing in APC (here, macrophage) and are presented to CD4<sup>+</sup> T-cell. Adapted from Roitt I.M., Brostoff J. and Male D.K. *Immunology*, 5th Edition, Morsby 1998.

The switch to the chronic inflammatory period is indicated by the clearance of PMNs and some macrophages from the defect site, and infiltration of lymphocytes and plasma cells responsible for the adaptive immune mechanisms. Fibroblast proliferation contributes to remodeling of the newly formed ECM to regenerate the wound. The duration and the outcomes of this constructive remodeling is to a great extent dependent on the cytokine profile of the chronic phase and formation of granulation tissue (Boruch *et al.*, 2010).

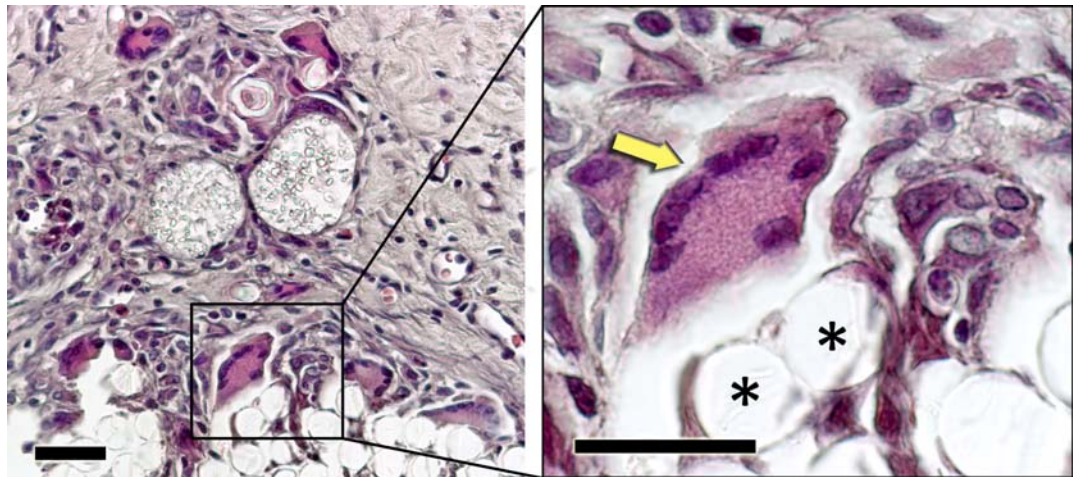
The residual macrophages produce cytokines according to their immune phenotype. The transition from an inflammatory ( $M_1$ ) phenotype toward a more regenerative or anti-inflammatory ( $M_2$ ) phenotype has shown to be well correlated with changes in the cytokine secretion profile of CD4<sup>+</sup>-helper T (Th) cells from type I (Th<sub>1</sub>) to type II (Th<sub>2</sub>) that supports resolution of inflammatory events (Martinez *et al.*, 2008). Macrophages may also attain the  $M_2$ -like phenotype after performing their primary functions related to phagocytosis (Xu *et al.*, 2006). It is generally accepted that a rapid resolution into this chronic cellular phenotype at the tissue-biomaterial interface is often associated with good implant biocompatibility, while the persistent inflammatory phenotype may indicate infection or rejection of the implant (Anderson *et al.*, 2008).

#### 1.4.2 Foreign body reaction

Host responses following implantation of biomaterials or tissue-engineered constructs include blood-material interactions, provisional matrix formation, acute inflammation, chronic inflammation, granulation tissue development, foreign body reaction and fibrous capsule development (Anderson, 2000; Anderson, 2001; Lambert, 1912).

Blood-biomaterial interactions start immediately following implantation with protein adsorption to the surface, and formation of a blood-based transient provisional matrix around the biomaterial. Injury to the connective tissue does not merely initiate the innate inflammatory response, but also leads to clot formation, involving activation of extrinsic and intrinsic coagulation mechanisms, complement, fibrinolytic, kinin-kallikrein systems and platelets. From a wound healing perspective, these blood proteins deposited on a biomaterial surface constitute the provisional matrix that launches the processes of wound healing and foreign body reaction. The newly released chemoattractants, cytokines and growth factors within the provisional matrix secures the activation and inhibition of agents capable of modulating macrophage activity, enhancing proliferation and activation of the principal cell populations in the wound healing. Chronic inflammation is histologically more diverse than acute and has been used to recognize a wide array of cellular responses. The presence of mononuclear cells, including lymphocytes and plasma cells, is classically considered as chronic inflammation and is often limited to the implant site. Another common type of chronic inflammation can be

described by the foreign body reaction where monocytes, macrophages and/or foreign body giant cells (FBGCs) are present at the tissue-biomaterial interface. As the acute and chronic inflammatory responses resolve, the newly formed granulation tissue can be identified by macrophages, fibroblast infiltration, and neovascularization. Granulation tissue is often separated from the implant or biomaterial by the cellular components of the foreign body reaction and may be described as single or several layers of monocytes, macrophages or FBGCs (Anderson *et al.*, 2008).



**Figure 3.** Foreign body giant cell (yellow arrow) adjacent to the surface of the suture material (marked with asterisks) identified at the defect site at 12 weeks after surgery. Bar = 30 $\mu$ m.

It is assumed that the formation of these giant cells enhances the defensive competence of macrophages. As expected, FBGCs are capable of delivering a more effective and focused reaction to the implant, compared with single macrophages, leading to degrading material more efficiently and sometimes even causing failure of implanted devices (Zhao *et al.*, 1991). FBGCs display membrane surface antigens similar to those found on monocytes and macrophages, explained by the fact that they are generated as the result of surface-adhered monocyte-derived macrophage fusion. In tissues removed from human implant surgeries, FBGCs have been shown *in situ* to express macrophage-associated membrane molecules as well as osteoclast markers (Al-Saffar *et al.*, 1997). Besides, they have long been implicated in wound healing mainly due to their active cytokine production and responsiveness to a wide array of cellular signals (Doussis *et al.*, 1992).

## 1.5 MODULATION OF THE INFLAMMATORY RESPONSE

Bone healing involves multiple complex cascades of molecular signaling by

inducing significant changes in the expression pattern of several thousand genes (Rundle *et al.*, 2006). In particular, the inflammatory signalling is critical for the initiation of the healing response. However, the impact of an inflammatory cytokine on the wound healing course depends on the timing and context of its expression. Therefore, a single factor may have both pro-regenerative and pro-resorptive effects on the bone tissue. For instance, proinflammatory mediators like TNF- $\alpha$ , IL-1 are mainly known for their destructive effects on bone tissue and have therefore been poorly investigated for the potential beneficial effects on the initiation of repair mechanisms. Interestingly, while carrying risks for local tissue damage and implant failure, the same pro-inflammatory factors may be among the ones initiating the healing cascade as a consequence of local changes in the tissue microenvironment following surgery (Mountziaris and Mikos, 2008).

Most of the currently available anti-inflammatory therapies intend to restrain the magnitude and diversity of inflammatory cell responses. The alternative approaches to faster and more efficiently resolve inflammation include promotion/inhibition of specific cell types by modulating the phenotype of the resident or recruited immune cell populations (Boehler *et al.*, 2011).

### 1.5.1 The impact of extracellular matrix

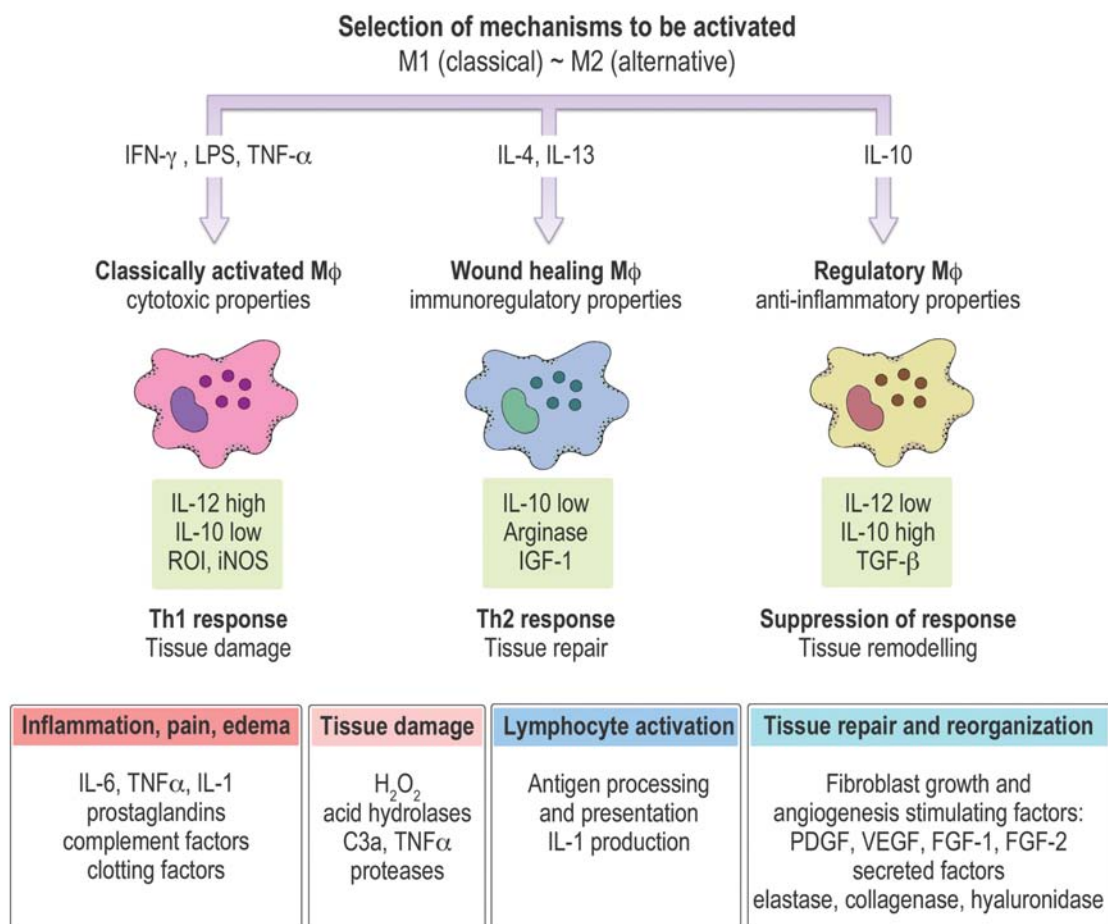
ECM comprises complexes of structural and functional proteins serving its principal role in tissue morphogenesis, maintenance of cell and tissue function and homeostasis as well as the host response to injury. Both xenogeneic and allogeneic ECMs have been utilized as scaffolds for reconstructive purposes of various tissues in both pre-clinical and clinical studies (Badylak, 2002).

The different components of the ECM and their three-dimensional arrangement and biophysical properties convey the required information to surrounding cells and modulate essential immune cell functions, such as migration into and within the defect area, immune cell activation and proliferation and cell differentiation processes, such as T-cell polarization (Sorokin, 2010). Following surgery, inflammatory cytokines and proteases, in particular matrix metalloproteinases (MMPs), are released by activated host tissue-resident cells infiltrating the wound and modify the ECM profile. In addition, cytokines such as TNF- $\alpha$ , IFN- $\gamma$  and TGF- $\beta$  start to modulate the expression of a broad range of ECM elements (Morrison *et al.*, 2009). MMPs are mostly considered as wide spectrum degraders of ECM, however by selectively cleaving ECM proteins and their receptors they may instead tune the course of inflammation following ECM implantation. This results in the generation of ECM fragments that influence the activity and function of both infiltrating and resident cells (Adair-Kirk and Senior, 2008; Davis *et al.*, 2000).



### 1.5.2 The role of macrophages

Macrophages secrete a vast array of inflammatory mediators upon activation. The transition to the activated mode occurs in response to microbial products, immune complexes, chemical mediators, certain extracellular matrix proteins, and T lymphocyte-derived cytokines (Martinez *et al.*, 2008). Alternatively, the activation of adherent macrophages is the result of phagocytosis attempt on the biomaterial surface. The activated phenotype is very efficient in secreting a range of cytokines, such as IL-1, IL-6, IL-10, IL-12, IL-18, TNF- $\alpha$ , TGF- $\beta$ , IL-8, monocyte chemoattractant protein 1, and macrophage inflammatory protein 1 $\alpha/\beta$  (Fujiwara and Kobayashi, 2005). The profile of the subsequent cytokine repertoire guides the inflammatory and wound healing response (Fig. 4).



**Figure 4.** Macrophage phenotypes. *Adapted from Mantovani et al., 2004.*

Activated macrophages exhibit several distinct phenotypes. Classical macrophage activation occurs after induction by IFN- $\gamma$  and exposure to microbial products such as LPS (Mosser, 2003). The primary function of classically activated macrophages is directed towards the elimination of intracellular pathogens, upregulation pro-

inflammatory cytokines, inhibition of anti-inflammatory cytokines and nitric oxide production. Alternative activation is initiated mainly by IL-4, IL-13 and glucocorticoids and is associated with inhibition of pro-inflammatory responses and upregulated mannose receptors (Gordon, 2003; Stein *et al.*, 1992).

In addition, alternatively activated macrophages can be involved in allergic responses and elimination of parasites (Mantovani *et al.*, 2004b), secretion of growth and angiogenic factors, upregulation of certain ECM proteins, such as fibronectin, involved in ECM remodeling during wound healing (Gratchev *et al.*, 2001; Martin and Leibovich, 2005). Alternatively activated macrophages may produce pro-fibrogenic factors that enhance fibroblast activity in contrast to classically activated macrophages (Song *et al.*, 2000).

### 1.5.3 MSCs and early modulation of immune response

To date, a significant number of studies have demonstrated an array of mechanisms allowing MSCs to suppress various immune reactions and escape immune responses, which is probably best demonstrated in an allogeneic therapy setting. The majority of studies characterize MSCs as HLA class I positive and HLA class II negative (Le Blanc *et al.*, 2003; Tse *et al.*, 2003). This expression feature is important for reduced immunogenicity of MSCs and enables the protection from natural killer (NK) cell effector mechanisms, which is nicely demonstrated by NK-mediated cytotoxicity towards cells that downregulate HLA class I (Ruggeri *et al.*, 2001). Data from *in vitro* co-culture experiments demonstrated inability of MSCs to elicit proliferative response of allogeneic lymphocytes (Le Blanc *et al.*, 2003) and escape recognition by cytotoxic T-lymphocytes and alloreactive NK cells (Rasmusson *et al.*, 2003). In addition, the low expression of HLA class II antigens and the ability to escape alloreactive lymphocytes upon IFN- $\gamma$  stimulation is another factor contributing to the reduced immunogenicity of MSC (Potian *et al.*, 2003; Tse *et al.*, 2003).

Another remarkable feature of MSCs with regard to their interplay with the immune system is absence of a number co-stimulatory surface molecules required for successful effector T-lymphocyte activation such as CD40, CD40L, CD80 and CD86 (Klyushnenkova *et al.*, 2005).

MSCs seem to mediate functions of several cellular components of immunity, however the exact mechanism of this modulation has not been elucidated. In addition to direct inhibition of T-cell proliferation, MSCs also induce regulatory CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T-cell phenotype (Treg) when present in mixed lymphocyte culture (Tang and Bluestone, 2008). Treg is a suppressor T-cell population able to limit the activation of several cell types, including CD8<sup>+</sup> T-cells, NK cells, and B-cells (Aggarwal and Pittenger, 2005; Maccario *et al.*, 2005; Selmani *et al.*, 2008). Another example of MSC-mediated immunosuppression is the alteration of dendritic cell maturation resulting in the attenuation of T-lymphocyte responses (Beyth *et al.*, 2005; Nauta *et al.*, 2006).

MSCs might mediate the functions of neutrophils and macrophages (Raffaghello *et al.*, 2008). When activated by LPS or TNF- $\alpha$ , they reprogram macrophages to produce IL-10 by releasing prostaglandin E2 (PGE2). For this reason, MSC administration to mice before or shortly after inducing sepsis by cecal ligation and puncture can reduce mortality and improve organ function (Nemeth *et al.*, 2009). IL-1 receptor antagonist, released by a subset of MSCs, can prevent activated macrophages from producing TNF- $\alpha$  (Ortiz *et al.*, 2007).

MSCs may exhibit their anti-inflammatory and immunomodulatory effects in both autocrine and paracrine fashion. Among the known soluble factors are growth factors and cytokines such TGF- $\beta$ 1 (Tse *et al.*, 2003), IFN- $\gamma$  (Krampera *et al.*, 2006), IL-1 $\beta$  (Groh *et al.*, 2005), hepatocyte growth factor (Di Nicola *et al.*, 2002), indoleamine 2,3-dioxygenase (Meisel *et al.*, 2004), IL-6 (Djouad *et al.*, 2007), IL-10 (Jiang *et al.*, 2005), PGE2 (Aggarwal and Pittenger, 2005), TNF- $\alpha$  (Beyth *et al.*, 2005), nitric oxide (Sato *et al.*, 2007), heme oxygenase-1 (Chabannes *et al.*, 2007) and HLA-G5 (Selmani *et al.*, 2008).

## **1.6 CHALLENGES IN THE CLINICAL APPLICATION OF BONE TISSUE ENGINEERING**

### **1.6.1 Legal framework and good manufacturing practice**

The translation of research-based protocols using human cells or tissues into a safe Good Manufacturing Process (GMP) requires protocols that have had careful consideration of all the risks and benefits for the patient end user. The main scope of these GMP-compliant regulations is to establish clear classification criteria for novel cell-based medicinal products (Trommelmans *et al.*, 2007). Key elements for cellular-based products include purity, sterility, stability, safety, efficacy and traceability of all involved components. In particular, the following parameters should be considered: sources and collection methods, cell seeding, proliferation rate, and culture medium (Sensebe *et al.*, 2010).

In Europe, adult MSCs are considered as Advanced Therapy Medicinal Products and are defined by the European Regulation (European Commission) 1394/2007, <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:324:0121:0137:EN:PDF>, which contains rules for “authorization, supervision, and technical requirements regarding the summary of products characteristics, labeling, and the package leaflet of Advanced Therapy Medicinal Products that are prepared following industrial methods and in academic institutions”, referring to the European GMP rules. For details, see “European Commission. EudraLex, Volume 4. Good manufacturing practice (GMP) guidelines (2011)”, available at: [http://ec.europa.eu/-health/documents/eudralex/vol-4/index\\_en.htm](http://ec.europa.eu/-health/documents/eudralex/vol-4/index_en.htm).

In the United States, human cells, tissues, or cellular and tissue-based products and their production must comply with Current Good Tissue Practice requirements,

under the Code of Federal Regulations - Title 21, part 1271, available at <http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm>. Human cells, tissues, or cellular and tissue-based products are defined as articles containing or consisting of human cells or tissues that are intended for implantation, infusion, or transfer into a human recipient. The essential Current Good Tissue Practice requirements are related to preventing the introduction, transmission, or spread of transmittable diseases by human cells, tissues, or cellular and tissue-based products.

### 1.6.2 Cell source and donor variation

A variety of cells of mesenchymal origin found in the bone marrow, trabecular bone, periosteum, cord blood, amniotic fluid, adipose tissue as well as circulating skeletal stem cells, dental pulp cells, periodontal ligament cells and cells found in almost any connective tissue – all have been shown to undergo osteoblast differentiation *in vitro*. Their ability to differentiate *in vitro* is however not always the best predictor of *in vivo* behavior of those cell populations. After collecting adequate amounts of evidence for feasibility in preclinical models, many groups have performed clinical studies with human adult stromal cells in the maxillofacial region (Schimming and Schmelzeisen, 2004; Yamada *et al.*, 2008). However, the results from these translational studies indicated a significant variation between human and animal cells such as differences in the osteogenic properties, which was not a major concern in animal models (Osyczka *et al.*, 2004; Phinney *et al.*, 1999; Siddappa *et al.*, 2007).

Numerous studies have demonstrated donor-to-donor heterogeneity in different MSC populations. These include marked disparities in growth rate, ALP levels, and osteogenic potential (Phinney, 2012). Other studies have confirmed those findings and attributed the donor-to-donor heterogeneity to several factors including sampling bias during marrow aspiration, methods used to culture and expand populations post-harvest, and the age of the donor (Banfi *et al.*, 2000; Muschler *et al.*, 2001). Indeed, cellular senescence and growth arrest are known to occur when telomeres in one or more chromosomes reach a critical length (Harley *et al.*, 1990). *In vitro*, expanded human MSCs show a replicative senescence phenotype culminating in growth arrest and loss of multipotency (Fehrer and Lepperdinger, 2005).

The use of MSCs for bone tissue engineering applications depends on their ability to expand *in vitro* while retaining their multipotency. A large variation among the donors may limit these applications. Apparently, more methods in tissue engineering will focus on complete bypass of MSCs expansion phase on plastic (Siddappa *et al.*, 2007).

### 1.6.3 Immunological aspects of allogeneic cell-based tissue engineering

The diversity of the HLA genes in the human genome, characterized by a high degree of allelic polymorphism, has developed throughout evolution to the extent that allows recognition of a very broad range of foreign proteins. The polymorphism is so great that no two individuals in an outbred population have exactly the same set of MHC genes and molecules. While beneficial to achieve the required immune response, this might represent serious issues in allogeneic cell-based tissue engineering settings. Alloreactivity, as an undesirable host response may be elicited even with minor differences between donor and recipient HLA and jeopardize the clinical outcome. This might furthermore require long-term immunosuppressive regimens with a number of drawbacks and side effects.

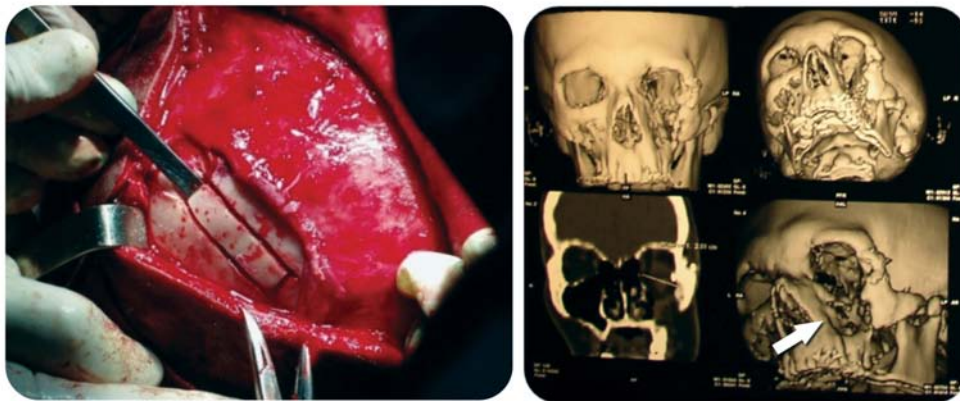
In the context of allogeneic regenerative cell-based therapy, the immunological mechanisms and allorecognition pathways of the host immune response to a cell-seeded tissue-engineered construct may have a detrimental effect on the treatment outcomes (Caballero *et al.*, 2006). In particular, the indirect allorecognition pathway mechanism might be involved in implant rejection, when alloantigens, such as the decellularized matrix, are processed and presented by the host's dendritic cells, in contrast to the direct presentation of the alloantigen, when the recipient T-cells recognize allogeneic MHC molecules on donor-derived antigen-presenting cells (Afzali *et al.*, 2007).

### 1.6.4 Tissue engineering in craniofacial area

The craniofacial area comprises structures with several tissue types where autologous grafting is the most prevalent and preferable grafting method (Fig. 5). Rib, cranium or iliac crest autograft are the “golden standards” for bony tissue replacement, but associated problems include significant bone resorption, harvesting difficulties, donor site pain, poor contouring and limited autogenous bone to fill the defect adequately (Ward *et al.*, 2010).

In areas with extended bone defects, allografts represent an alternative treatment option. However, structural bone allografts have a restricted potential to undergo remodeling with high tendency for fractures. In addition, the approach is cost-inefficient and, as mentioned earlier, carries additional risks of disease transmission and immunorejection. The ideal parameters of a bone substitute might be described as non-immunogenic, nontoxic, controllable, inexpensive, and readily available (Alsberg *et al.*, 2001). This sophisticated design of the desired scaffold challenges the field of bone tissue engineering to search for therapies that are able to perform beyond simple bone void fillers, which in turn leads to very challenging, time-consuming and costly approvals for translational products.

Some of the common conditions that require bone tissue repair are: bone defects in a number of congenital disorders, defects following removal of sinus and mandibular tumors or bony deficiencies following orthognatic corrections. The critical need for bone tissue replacement often arises from car accidents, sporting activities and gunshot wounds that result in blowout fractures of the orbital floor, orbital rim fractures, craniocerebral trauma, malunited fractures, major fractures of the maxilla or mandible, osteoradionecrosis, and dento-alveolar trauma (Rotter *et al.*, 2005).



**Figure 5.** Monocortical autograft harvest from parietal bone to replace missing orbital floor (defect indicated with arrow). *Courtesy of V. Malanchuk, 2006.*

Understanding biomechanics under physiological conditions is another factor critical for the success of craniofacial tissue engineering. Mechanical cues have shown to regulate cell and matrix biology and, hence, bone remodeling and regeneration (Mao *et al.*, 2003). One widespread illusion in the literature is that facial skeleton is not subjected to heavy mechanical stress compared to those of limb bones, possibly arising from the fact that the skull does not support body weight and from improper assumptions about *in vivo* strains exerted on craniofacial elements. Hence, the significance of mechanobiology for bone healing has been somewhat underscored. For each site intended for engineered replacement, it is critical to know how it is loaded under functional conditions, how it grows, and how its growth responds to loading (Herring and Ochareon, 2005). Several tissue-engineering approaches may currently be exploited in craniofacial area. However, the efficacy of each technique is site-dependent. For instance, the importance of the origin of MSCs used for craniofacial tissue repair has recently been discussed in the context of mesoderm- vs. neural crest-derived stem cells as more suitable and efficient candidates resulting from evidences of distinct contribution of each lineage to the regeneration of injured adult bones (Cordero *et al.*, 2011).

## 2 AIMS OF THE THESIS

The overall aim of this project was to increase our understanding of how the biomimetic approach in tissue engineering could be applied towards the repair and regeneration of bone tissue in the craniofacial area.

Specific aims:

- to design a biomimetic HA-ECM construct by exploiting the natural ability of the primary cells to secrete ECM proteins and growth factors *in vitro* (study I)
- to test the osteogenic properties of the generated construct *in vivo* (study I)
- to assess the local inflammatory response toward the construct *in vivo* (study I)
- to analyze the protein profile of the tissue-biomaterial interface (study I)
- to generate BMSC-seeded biomimetic construct and assess its osteogenic properties *in vivo* (study II)
- to assess the local tissue response toward the cell-seeded construct (study II)
- to identify cellular components of the elicited foreign body reaction (study II)
- to assess the ability of the generated construct to direct phenotype of periodontal ligament progenitor cells along the osteoblastic lineage *in vitro* (study III)
- to assess the effect of periodontal ligament progenitor cell-seeded construct on bone repair *in vivo* (study III)
- to generate constructs composed of ceramic scaffold modified with human fibroblast-derived ECM in complete xeno-free culture conditions (study IV)
- to assess the osteogenic properties of the constructs *in vitro* and the biocompatibility after implantation into rat calvaria defects (study IV)

### 3 MATERIALS AND METHODS

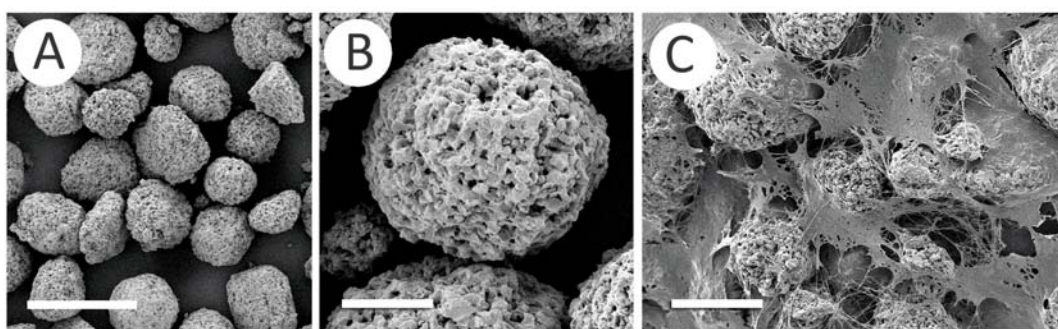
#### 3.1 ETHICAL CONSIDERATIONS

All animal care and experimental procedures were approved by the Research Ethics Committee of Karolinska University Huddinge Hospital in accordance with the policy on human care and use of laboratory animals (no. S87-06, S78-09). The experiments followed the principles for medical research according to the guidelines of the Declaration of Helsinki and the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No.85-23, revised 1996).

#### 3.2 GENERATION OF THE BIOMIMETIC CONSTRUCT

##### 3.2.1 Scaffold preparation

In the current thesis, synthetic HA microparticles (CAPTAL<sup>®</sup> Plasma Biotol Ltd, UK) were used as a platform to generate the HA-ECM constructs in studies I, II and III (Fig. 6). The HA was a kind gift from Dr. Salvador Boros, Institut Quimic de Sarrià, Universitat Ramon Llull (Spain). The sintered CAPTAL<sup>®</sup> hydroxyapatite (2 hours at 1250°C) is of high purity ( $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ,  $\geq 97.5\%$  by X-ray diffraction) and crystallinity with the surface area  $\sim 6\text{-}20 \text{ m}^2/\text{g}$ . It has a similar composition with the mineral content of the human bone, and has been the precursor material in several biomaterial developments (Wahl *et al.*, 2007). The HA microparticles were sterilized with 70% ethanol, dried out, transferred into 24-well cell culture plates



**Figure 6.** Synthetic hydroxyapatite microparticles prior to cell seeding (A) and close-up (B). Construct topography before decellularization (C); dermal fibroblasts residing on top of the HA microparticles.  
Bars = 60 $\mu\text{m}$  (A); 20 $\mu\text{m}$  (B) and 40 $\mu\text{m}$  (C).



(GIBCO, Invitrogen Inc) (10 mg HA/well), and incubated overnight in alpha-minimum essential medium ( $\alpha$ MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (50 units/ml) and streptomycin (50  $\mu$ g/ml) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. In study IV, 400-700  $\mu$ m Straumann® BoneCeramic granules (SBC) (Lot no.: Y8039; AM610; Z3475) were used as a scaffold material to generate the constructs. SBC is a fully synthetic biphasic ceramic bone substitute of medical grade purity, a combination of 60% HA and 40%  $\beta$ -TCP, 90% porous with interconnected pores of 100-500  $\mu$ m diameter.

### 3.2.2 Cell isolation and culture conditions

Primary calvarial osteoblasts (Ob) were isolated from the parietal and frontal bones, and dermal fibroblasts (DF) from the dermis of 2- to 3-day-old Sprague Dawley rat pups by sequential digestion with collagenase as previously described. (Owen and Pan, 2008; Rittié L, 2005) Briefly, the calvarial bones and dermal tissue fragments were aseptically dissected, cut into small fragments, and placed into phosphate-buffer saline solution (DPBS, GIBCO, Invitrogen Inc) containing 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Invitrogen Inc, USA). The calvarial bone fragments were incubated in 1 mg/ml collagenase A solution (Roche Diagnostics, Mannheim, Germany) for 15 min at 37°C. The supernatant was discarded and the collagenase digestion was repeated twice for 30 min. The supernatant was collected each time, mixed with  $\alpha$ MEM (GIBCO, Invitrogen Inc) containing 10% FBS (Invitrogen Inc). The fractions were pooled together. The dermal biopsies were digested once with 1 mg/ml collagenase A solution for two hours. The primary cells were dissociated from the remaining tissue fragments using a sterile 70  $\mu$ m-cell strainer (Falcon, GIBCO, Invitrogen Inc), and seeded into T-75 flasks (GIBCO, Invitrogen Inc) with  $\alpha$ MEM containing Penicillin (50 units/ml) and Streptomycin (50  $\mu$ g/ml), and 10% FBS at the density of  $2 \times 10^4$  cells/cm<sup>2</sup> at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The primary rat Ob (study I) or DF (study I, II and III) were seeded onto pre-incubated HA microparticles at the density of  $2 \times 10^5$  cells/10 mg HA/well. To increase the ECM synthesis by the rat primary cells, the culture media was supplemented with 100  $\mu$ g/ml ascorbic acid (l-AA) (Sigma-Aldrich, USA), and two thirds of the media were changed every third day. The cells were cultured for 21 days. In study IV, SBC granules were transferred into 24-well cell culture plates (GIBCO, Invitrogen Inc) (50 mg/well), and incubated overnight at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> (for details, see *Xeno-free cell culture* section below). DF were seeded onto pre-incubated granules at the density of  $2 \times 10^5$  cells/50 mg SBC/well. To enhance the ECM synthesis by the DF, the culture media was supplemented with 100  $\mu$ g/ml l-AA, and cells were cultured in the same conditions as in study I.

### 3.2.3 Cell culture in xeno-free conditions

Normal human DF (adult skin) were purchased from Lonza, USA (Clonetics™ Cat. No. CC-2511; Lot no.: 0000109944). According to the provider, the cells were derived from a 31-years old female donor after obtaining permission for their use in research applications by informed consent and legal authorization. Isolated cells were then performance assayed and tested for HIV-1, mycoplasma, Hepatitis-B, Hepatitis-C, bacteria, yeast and fungi. In addition, cell viability, morphology and proliferative capacity of the same lot were measured by the manufacturer after recovery from cryopreservation. Cells were expanded at a density of 3000 cells/cm<sup>2</sup> in sterile filtered mesenchymal stem cell serum-free basal medium (StemPro® MSC SFM Cat. No. A10332-01, Invitrogen, USA), supplemented with StemPro® MSC SFM xeno-free supplement (Cat. No. A11577-01; Lot No. 824288), 200 mM GlutaMAX™-I (Gibco®, Invitrogen), 2% human serum (Lonza, USA; Cat. No. 14-402E) and antibiotic-antimycotic solution (Gibco®, Invitrogen, USA) at 37 °C and 5% CO<sub>2</sub> with bi-weekly medium changes. According to the manufacturer, pooled and sterile filtered human serum obtained from normal human donors, tested negative for Hepatitis B surface antigen, antibodies to Hepatitis C and HIV I and II. After reaching a subconfluent state, cell passaging was performed with TrypZean animal component-free recombinant trypsin solution (Sigma-Aldrich, USA, Product Code T 3449). Cryopreservation was carried out using Synth-a-Freeze® protein-free cryopreservation medium (Catalog Number: R-005-50, Invitrogen, USA).

Human BMSCs (Poietics™ human mesenchymal stem cells, Cat. No. PT-2501; Lot no.: 0F4266) and adipose-derived stem cells (ADSC) (Cat. No. PT-5006; Lot no.: 7F4028) were purchased from Lonza, USA. The cells were harvested and cultured from normal human bone marrow of a 33-years old male donor and liposuction aspirate of a 29-years old female donor, respectively. Prior to shipping, the cells were pre-characterised for colony forming units and tested for purity by flow cytometry and for their ability to differentiate into osteogenic, chondrogenic and adipogenic lineages. Cells were positive for CD105, CD166, CD29, and CD44 and test negative for CD14, CD34 and CD45. The BMSCs and ADSCs were expanded at a density of 6000 cells/cm<sup>2</sup> in Poietics™ mesenchymal stem cell basal medium (Cat. No. PT-3238; Lot no.: 0000229448) supplemented with SingleQuots® (Cat. No. PT-4105; Lot no.: 0000223287) comprising mesenchymal cell growth supplement (Cat. No. PT-4106E), l-Glutamine (Cat no. PT-4107E) and Gentamicin-Amphotericin-B (Cat. No. PT-4504E) at 37 °C and 5% CO<sub>2</sub> with medium changed three times a week. Cultures were harvested for experimentation on attaining 80% confluence.

### 3.2.4 Cell viability and proliferation

The viability of cultured cells on different substrates in the 24-well plates was assessed by MTT colorimetric assay according to manufacturer's instructions (Roche Inc).

The MTT assay is based on the ability of mitochondrial dehydrogenases in the viable cells to cleave the tetrazolium ring of MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide yielding purple formazan crystals that are insoluble in aqueous solutions. The crystals are then dissolved in acidified isopropanol, and the resulting purple solution assessed spectrophotometrically. Briefly, 30 µl of MTT reagent was added to each well and incubated for four hours at 37°C. Then 300 µl of solubilization solution was added to each well, and the plates were incubated overnight at 37°C. Coloured formazan products were quantified by measuring absorbance at 540 nm (Labsystems Multiskan MS, Analytical Instruments, LLC). The MTT assay was performed in triplicates at day one and day 21 of culture.

### 3.2.5 Construct decellularization

HA-ECM constructs were harvested on the day 21 of static culture. Two methods were used to remove the cells from the HA-ECM constructs: Triton X-100 buffer treatment, and freeze/thaw cycles in liquid nitrogen (LN<sub>2</sub>). Briefly, at the end of the culture period all constructs were rinsed in DPBS, and either frozen in liquid nitrogen (LN<sub>2</sub>) for 10 min, undergoing freeze/thaw cycle three times, or treated with 0.5% Triton X-100 containing 20 mM NH<sub>4</sub>OH in PBS for 3 min at 37°C. The HA-ECM constructs were washed again with DPBS and double distilled (dd)H<sub>2</sub>O and stored at -80°C. To remove cells from SBC-ECM, the constructs were rinsed in DPBS and treated with sterile filtered 0.5% Triton X-100 as previously described. The SBC-ECM constructs were then washed and stored at -80°C until the day of *in vitro* experiments or surgery.

## 3.3 CONSTRUCT ANALYSIS AND CHARACTERIZATION

### 3.3.1 Macroscopic observation and light microscopy

Before decellularization the unfixed constructs were grossly observed. Then the constructs with and without cells were fixed in 4% buffered formalin for 24 hours at 4°C. The specimens were embedded in Tissue-Tek OCT compound (Sakura Finetek Europe BV, The Netherlands) and frozen in LN<sub>2</sub>. Sections of thickness 7 µm were cut from each frozen block. The cryo-sections were stained with hematoxylin and eosin (HE) (general morphology), Masson's trichrome (collagen fibers), and Alcian blue (glycosaminoglycans (GAGs)).

### 3.3.2 Scanning electron microscopy

*In vitro* generated constructs (before and after decellularization) were washed three times with DPBS, fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4 at room temperature (RT). After fixation the samples were rinsed and stored in 0.15 M cacodylate buffer supplemented with 3 mM CaCl<sub>2</sub>, pH 7.4 at 4°C until further

processing. The constructs were then centrifuged (500g) and the pellet was washed in ddH<sub>2</sub>O and the suspension was transferred to poly-l-lysine treated filters and allowed to attach for 2 min and fixed in 2% glutaraldehyde for 5 min. The filters were rinsed in ddH<sub>2</sub>O and dehydrated in 70%, 95% and absolute ethanol for 10 min each, and finally put into acetone. The filters with the pellet were then dried in a critical point dryer (Balzer, CPD 010, Liechtenstein) with carbon dioxide. After drying the filter was mounted on an aluminium stub, and coated with carbon (Bal-Tec MED 010, Liechtenstein). The specimens were analyzed in an Ultra 55 field emission scanning electron microscope (Zeiss, Oberkochen, Germany) at 5 kV.

### 3.3.3 DNA quantification

To assess the total DNA content within the decellularized HA-ECM constructs, the specimens were mechanically processed in RNase-free water by using the tissue homogenizer Ultra-Turrax T25 for 10 sec at 9500 rotation/min, and then sonicated for additional 30 min to completely loose the HA microparticles. The samples were centrifuged, and the supernatant optical density was assessed in NanoVue Plus Spectrophotometer (GE Healthcare, UK) at 260 nm, normalized to RNase-free water.

### 3.3.4 GAG assay

The samples were incubated in 1 mg/ml proteinase-K with 400mM EDTA solution at 56°C for 3 hours to solubilize the ECM, and were processed as described previously (Hoemann, 2004). To quantify the glycosaminoglycan (GAG) content, 1,9-dimethylmethylene blue (DMB) dye was added to diluted samples to form GAG-DMB aggregate, and adapted for spectrophotometer assay in a 96-microwell plate. The light absorbance was measured at 540 nm. Chondroitin sulfate sodium (Sigma-Aldrich, USA) was used as standard to quantify GAG content of the HA-ECM constructs.

### 3.3.5 Hyp-assay

The prepared samples were hydrolyzed with 6N HCl at 110°C overnight, and later dried on a heating block at 65°C. After removal of the hydrolysis byproducts the 4-Hydroxyproline (Hyp) was oxidized by *n*-propanol and Chloramine-T reagent, mixed with *p*-dimethylaminobenzaldehyde to form chromophore. The light absorbance was measured at 540 nm. Trans-4-Hydroxy-*l*-proline (Sigma-Aldrich, USA) was used as standard to quantify Hyp content of the HA-ECM constructs. The total collagen content can be calculated by multiplying amount of the total Hyp content in each sample by a factor of 8.0, assuming that Hyp represents 12.5% of the amino acid composition of collagen in most mammalian tissues (Edwards and

O'Brien Jr, 1980). Six randomly selected constructs of the same type were used for every assay, each performed in triplicate.

### 3.3.6 Lipopolysaccharide test

The chromogenic Limulus Amebocyte Lysate (LAL) QCL-1000<sup>®</sup> test (Lonza Copenhagen, Aps, Denmark) was used to assess endotoxin levels in the decellularized HA-ECM or SBC-ECM constructs. Briefly, the constructs were thoroughly vortexed and sonicated in the LAL reagent water for 30 min. Then the samples and standard dilutions of E.coli O111:B4 endotoxin were mixed with the LAL and incubated for 10 min at 37°C. The chromogenic substrate solution was added to the LAL-sample and incubated at 37°C for additional 6 min. The reaction was stopped with 25% v/v glacial acetic acid. The optical density of the samples (in triplicates) was determined spectrophotometrically at 405 nm, and the LPS concentration calculated using a standard curve.

### 3.3.7 SDS-PAGE and Western Blot analysis

SBC-ECM constructs were incubated overnight in 4M Guanidine-HCl extraction buffer followed by treatment with 0.5M EDTA-added extraction buffer for 72 hours in shaking conditions at 4°C. The precipitated protein fractions were spectrophotometrically quantified using Pierce 660 nm Protein Assay with ionic detergent compatibility reagent (Thermo Scientific, USA). 3ug of each sample in Laemmli buffer containing  $\beta$ -mercaptoethanol were electrophoresed on a SDS-PAGE 4-15% mini-gel (Bio-Rad, USA) and stained with 0.25% Coomassie Blue R-250 (Sigma, USA). Proteins were electroblotted onto nitrocellulose membrane (Hybond-ECL, GE Healthcare, UK) using Trans-Blot semi-dry transfer cell (Bio-Rad, USA) and blocked in 3% milk solution in Tris-buffered saline and 0.1% Tween-20. The membranes were probed against Col1 (1:200, Cat. No. sc-8784, goat polyclonal, Santa Cruz Biotechnology, Inc), BSP (1:2000, Cat. No. AB1854, rabbit polyclonal, Millipore, USA), BMP-2 (1:200, Cat. No. ab6285, mouse monoclonal, Abcam, UK), Col3 (1:250, Cat. No. ab82354, mouse monoclonal, Abcam, UK), OPN (1:200, Cat. No. sc-10593, goat polyclonal, Santa Cruz Biotechnology, Inc), VEGF (1:250, Cat. No. sc-7269, mouse monoclonal, Santa Cruz Biotechnology, Inc) antibodies diluted in blocking solution, followed by incubation with a corresponding horseradish peroxidase-conjugated secondary antibody (1:2000, all from DAKO, Denmark). Proteins were detected with ECL Plus Western Blotting Detection System (GE Healthcare) and visualized using ChemiDoc<sup>™</sup> XRS molecular imager system (Bio-Rad, USA).

### 3.3.8 Immunohistochemistry

Briefly, after deparaffinization in xylene and rehydration, the sections were treated with 0.2 M HCl for 10 min then with 3% H<sub>2</sub>O<sub>2</sub> to block peroxidase activity. After washing in DPBS, the cryosections of the constructs before and after decellularization were incubated for one hour at RT with the primary antibodies against rat Coll (1:500, Cat. No. AB755P, rabbit polyclonal, Millipore, USA), BSP (1:500, Cat. No. AB1854, rabbit polyclonal, Chemicon International, Inc., USA) and OPN (1:200, Cat. No. sc-10593, goat polyclonal, Santa Cruz Biotechnology, Inc., Europe). After additional washing in DPBS, the sections were incubated with the specific secondary biotinylated antibodies for one hour at RT. Peroxidase reactions were then visualized using a commercial Vectastain ABC kit (Vector Laboratories, USA). Finally, the sections were counterstained with Alcian blue or Methyl green, and mounted for light microscopy. Primary antibody was omitted from the sections used as negative controls. In study III, the cryosections were incubated for one hour at RT with the primary antibodies against OPN (1:200, Cat. No. sc-10593, goat polyclonal, Santa Cruz Biotechnology, Inc., Europe), and BMP2 (1:500, Cat. No. ab6285, mouse monoclonal, Abcam, UK).

### 3.3.9 Confocal microscopy

Confocal fluorescent images were obtained by Nikon Eclipse Ti inverted microscope system to assess the surface topography of the cell-seeded constructs in study IV. Specimens were washed with DPBS, then incubated for 40 min at RT with Alexa Fluor<sup>®</sup> 594 phalloidin solution (Cat. No. A12381, Life Technologies Co., USA) and DAPI (NucBlue<sup>™</sup> Fixed Cell Stain, Life Technologies Co., USA), and excited using respective laser wavelengths. Acquisition of z-stack images composed of 35 images (4 µm step) and three-dimensional reconstructions were produced using 20x objective in Nikon NIS elements software (Nikon, Tokyo, Japan).

## 3.4 PROGENITOR CELL ISOLATION AND IN VITRO ASSAYS

### 3.4.1 Cell harvest and culture conditions

Rat BMSC were harvested from an adult female Lewis-EGFP (enhanced green fluorescent protein) transgenic rat (strain LEW-Tg (EGFP) F455/Rrrc). The femurs and tibia were aseptically dissected followed by epiphysis resection, and the bone marrow was flushed with α-MEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. The bone marrow was dispersed by passing through a 16-gauge needle. The cells were dissociated from the remaining tissue fragments using a sterile 70 µm-cell strainer (BD Falcon, USA), and seeded into T-75 flasks (GIBCO, Invitrogen Inc) with α-MEM containing penicillin (50 units/ml) and streptomycin (50 µg/ml), and 10% FBS at 37°C in a humidified atmosphere of 5%

CO<sub>2</sub>. The non-adherent cells were washed away with DPBS after 72 h. The remaining plastic-adherent cells, representing BMSC were expanded. The BMSC at passages 3 and 4 were used for the experiments.

To harvest periodontal ligament progenitor cells (PDLC) in study III, the rat mandibles were removed and washed twice in DPBS supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg of Amphotericin B/mL (Invitrogen, USA). The molars were extracted from each mandible along with adherent periodontal ligament from the surrounding alveolar bone. To minimize contamination from gingival fibroblasts, the central one-third of each root was cut under dissecting microscope and immersed in αMEM supplemented with 10% FBS, 100 U/mL of penicillin and 100 µg/mL streptomycin sulfate (Invitrogen, USA) in a Biocoat Collagen I 12-well plate (BD Biosciences, Belgium). The cultures were incubated in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C. When the outgrowth cells derived from the tissue explants reached confluency they were detached with TrypLE (Invitrogen, USA), filtered through a 70-µm cell strainer (BD, Falcon) and sub-cultured in culture flasks until passage 3.

### 3.4.2 Phenotypic characterization by FACS analysis

In study III, the viable PDLCs at passage four were labeled with the following antibodies: Mouse IgG1/2a isotype control FITC/PE (unspecific, BD); Rat PECAM1, MCA1334A647 (CD31, Serotec); Rat VCAM1 PE, 559229 MR106, (CD106, BD); Rat ITGAM PE, MCA 275 (CD11b, Serotec); Rat Integrin B1 Chain FITC; 555005 Ha2/5, (CD29, BD); Rat Leukogate FITC/PE, DC044 (CD3/CD45, Serotec); Rat/Mouse THY1 FITC, MCA 47A488 (CD90, Serotec); Rat 5'-ecto-nucleotidase, 551123 SF/B9 (CD73, BD).

The cell suspensions were washed and analyzed on a FACScan flow cytometer (BD Biosciences, San Jose, CA) according to standard procedures (Moll et al., 2011). Cell acquisition was performed in a forward/sideward scatter (FSC/SSC) dot plot and cell debris was excluded with FSC. The fluorescence signals from 5,000 events were counted with detection of median fluorescence intensity (MFI), and analyzed with Summit v4.1 software (Dako, Fort Collins, CO).

In study IV, ADSC and BMSC at passage 4 were detached and viable cells were counted with Trypan Blue exclusion test. Cell suspensions were labeled with respective antibodies against CD45, CD14, CD31, CD34, CD44, CD46, CD55, CD59, CD73, CD90, CD105, HLA-1, HLA-2, washed and analyzed on a LSRFortessa™ Cell Analyzer (BD Biosciences, San Jose, CA). Cell acquisition was performed in a forward/sideward scatter (FSC/SSC) dot plot; cell debris was excluded with FSC. Fluorescence signals from 5,000 events were counted with detection of median fluorescence intensity (MFI) and analyzed with FACSDiva™ software (BD Biosciences, San Jose, CA).

### 3.4.3 Cell seeding

For the preparation of the cell-loaded implants (study II) the 500  $\mu$ l of the BMSC suspension containing 750 000 viable cells were mixed with 30 mg sterile HA microparticles or HA-ECM in a sterile 1.5 ml eppendorf tube. The tube was then spun down at 300 g for 1 min. The supernatant was discarded and the tube was immediately placed on ice. The average time between cell seeding and surgery did not exceed 30 min. The number of the viable cells was estimated using Trypan Blue staining.

For the ALP activity assay, MTT assay and gene expression assays (Study III), the HA microparticles or HA-ECM constructs were placed into 96-well culture plates (Costar, Corning Inc., USA; 10 mg HA/well) and PDLCs were seeded onto the scaffolds ( $1.5 \times 10^4$  cells/well) in  $\alpha$ -MEM culture media for 24 h. Culture conditions were then switched to the standard osteogenic media in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. For the *in vivo* experiments, HA-ECM constructs were placed into 24-well culture plates (30 mg HA/well); 400  $\mu$ L PDLC suspension in  $\alpha$ MEM was added onto each construct at an initial density of  $2 \times 10^5$  viable cells/well. After 30 min incubation in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C,  $\alpha$ MEM was added to a final volume of 1 ml/well. After 24 h incubation the PDLCs were cultured in standard osteogenic media for 3 or 14 days.

### 3.4.4 In vitro osteogenic differentiation/mineralization assay

PDLC, Ob and DF (study I, II and III) were seeded into 24-well plates (Costar, Corning Inc., USA;  $4 \times 10^3$  cells/cm<sup>2</sup>) and cultured in standard osteogenic media ( $\alpha$ -MEM supplemented with 10% FBS, 10 mM  $\beta$ -glycerophosphate, 50  $\mu$ g/ml ascorbic acid, and  $10^{-8}$  M dexamethasone, all from Sigma-Aldrich, USA) for 28 days. The cell monolayer was washed with DPBS, stained for 5 min with a 2% (w/v) solution of Alizarin Red S (Merck, Germany) adjusted to pH 4.2 with ammonium hydroxide, washed with water and dehydrated in series of ethanol concentrations.

To induce an osteogenic phenotype in study IV, BMSC and ADSC of passage 3 were plated at  $5 \times 10^3$  cells/cm<sup>2</sup> in a 24-well culture plate. When the cultured cells reached approximately 90% confluence, the osteoinductive medium was introduced for 21 days. Osteoblastic differentiation was induced with human MSC Osteogenic Differentiation BulletKit™ (Cat no.: PT-3002) containing osteogenic differentiation basal medium (Cat no.: PT-3924) and osteogenic SingleQuots Kit™ (Cat no.: PT-4120), all from Lonza, USA. Mineralization of the ECM was visualized by staining with Alizarin Red S as previously described.



### 3.4.5 Cellular viability within HA-ECM scaffolds

Proliferation on HA and HA-ECM constructs was evaluated with MTT colorimetric assay on 96-well plates according to manufacturer's instructions (Roche Inc.) as previously described.

### 3.4.6 Alkaline phosphatase activity

The cell-seeded HA and HA-ECM on days 1, 3, 7 and 14 were washed with DPBS, lysed with 500  $\mu$ L of 0.2% Triton X-100 (Sigma–Aldrich) *per* well and sonicated to destroy cell membranes. 100  $\mu$ L supernatant was added to 100  $\mu$ L of *p*-nitrophenylphosphate substrate buffer (Sigma–Aldrich) in a 96-well plate and incubated at 37°C for 30 min. Reaction was blocked with 30  $\mu$ L of NaOH and the absorbance was measured at 405 nm. To normalize ALP activity, total protein content was measured using Pierce 660 nm protein assay according to the manufacturer's instructions (Thermo Scientific).

### 3.4.7 Real-time qPCR

#### 3.4.7.1 RNA isolation and cDNA synthesis

Cell-seeded HA and HA-ECM constructs were rinsed twice with sterile DPBS and 100  $\mu$ L lysis buffer was added. Contents of the well were transferred to QIAshredder column (Qiagen, USA) for homogenization followed by total RNA extraction according to the manufacturer protocol (RNeasy Mini Kit, Qiagen, USA). 100 ng total RNA was reversely transcribed using iScript cDNA synthesis kit (BioRad, USA) in 20  $\mu$ L reaction on a T1 Thermocycler (Whatman Biometra, Germany) followed by spectrophotometric quantification and quality assessment with NanoVue spectrophotometer (GE Healthcare).

#### 3.4.7.2 TaqMan<sup>®</sup> gene expression assays

The difference between the mRNA levels of the key bone-related genes was analysed with 7500 Fast Real-Time PCR System (Applied Biosystems, USA). The 20  $\mu$ L reactions were performed using 40 ng of the target cDNA, TaqMan Universal PCR Master Mix (2x) and TaqMan probes (1x) (Applied Biosystems, USA). Genes and related specific assays used in study III: Alkaline phosphatase (ALP) Rn00564931\_m1, Bone gamma-carboxyglutamate protein (OC) Rn00566386\_g1, Collagen type 1 (Col1) Rn00801649\_g1, Secreted phosphoprotein 1 (OPN) Rn01449972\_m1, Runt-related transcription factor 2 (Runx2) Rn01512296\_m1, Bone morphogenetic protein 2 (BMP-2) Rn00567818\_m1, Bone morphogenetic protein 4 (BMP-4) Rn00432087\_m1, Bone morphogenetic protein 7 (BMP-7) Rn01528886\_m1, Fibroblast growth factor 1 (FGF-1) Rn00563362\_m1, Fibroblast

growth factor 2 (FGF-2) Rn00570809\_m1, Insulin-like growth factor 1 (IGF-1) Rn00710306\_m1, Platelet-derived growth factor (PDGF) Rn00709363\_m1, Vascular endothelial growth factor (VEGF) Rn00582935\_m1, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Rn01462662\_g1; and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Hs99999905\_m1, Runt-related transcription factor 2 (Runx2) Hs00231692\_m1, Alkaline phosphatase, liver/bone/kidney (ALP) Hs00758162\_m1, and bone gamma-carboxyglutamate protein (OC) Hs00609452\_g1, in study IV. The relative expression was quantified using the  $\Delta\Delta C_t$  method. Target genes were normalized against endogenous control GAPDH and calibrated to undifferentiated cells on HA (study III) or SBC alone (study IV) prior to osteogenic treatment. After an initial incubation step of 2 min at 50 °C and denaturation for 10 min at 95 °C, 40 PCR cycles (95 °C for 15 s, 60 °C for 1 min) were performed. Reactions were performed in triplicate. Three samples under each condition (substrate; time point) were used for calculating the means and standard deviations ( $n = 3$ ). The experiment was repeated on two different occasions, using same batch of cells and biomimetic constructs generated on different occasions.

### 3.4.8 Immunocytochemistry

The GFP expression of BMSCs (study II) and PDLCs (study III) was shown by fluorescent microscopy and immunocytochemistry using a primary anti-GFP antibody (Cat. No. A11122, Invitrogen, UK) as described previously. In study IV, MSCs on plastic or SBC-ECM at days 1, 7 and 14 were fixed in 4% buffered formalin for one hour at 4°C. After washing with DPBS, the specimens were incubated for one hour at RT with the primary antibodies against human OCN (1:50, Cat. No. CA-70482.16, rabbit polyclonal, Cambio Ltd, UK) or human alpha-smooth muscle actin (10 µg/ml, Cat. No. MAB1420, mouse monoclonal, R&D Systems, Europe), followed by additional washing with DPBS, and incubation with the corresponding secondary antibodies (1:50, goat anti-rabbit FITC conjugated, Ca. No. F0382, Sigma-Aldrich, Inc. or 6 µg/ml, goat anti-mouse Alexa Fluor<sup>®</sup> 488, Ca. No. A11001, Life Technologies Co., USA).

## 3.5 IMPLANTATION STUDIES

### 3.5.1 In vivo experimental design

Adult Sprague–Dawley male rats (~350 g) were used for implantation studies (I-IV). The rats were kept under uniform conditions for a period of least one week before commencement of the experiment. Free access to water and standard pelleted food was provided throughout the experiment. The rats were randomly and equally divided into four groups according to the local treatment they received.

#### 3.5.1.1 Study I

Twenty-four rats were used in the following treatment groups ( $n=6$ , each): (1) decellularized constructs generated by the rat primary calvarial Ob, *HA-OECM*, 30 mg HA plus ECM; (2) decellularized constructs generated by the rat primary dermal fibroblasts, *HA-FECM*, 30 mg HA plus ECM; (3) *HA* alone, 30 mg HA; (4) HA mixed with TissuFleece E, a mesh-like scaffold consisting of collagen type I fibrils of equine origin (Baxter AG, Austria), *HA-TFE*, 30 mg HA plus 9 mg collagen mesh, to mimic the organic/non-organic ratio in normal bone.

#### 3.5.1.2 Study II

Forty-two adult Sprague–Dawley male rats (~350 g) were randomly and equally divided into the following treatment groups: *HA alone* (30 mg HA), 2-weeks only; *HA+BMSC*, HA microparticles mixed with BMSC (30 mg HA + 750 000 viable cells); *HA-ECM*, construct alone (30 mg HA-ECM); *HA-ECM+BMSC*, construct mixed with BMSC (30 mg HA-ECM + 750 000 viable cells). Six rats from each treatment group were followed for 2 or 12 weeks after surgery.

#### 3.5.1.3 Study III

Twelve rats were randomly and equally divided into additional two groups: *HA-ECM+PDLC3* and *HA-ECM+PDLC14* (HA-ECM constructs seeded with the PDLCs and cultured in standard osteogenic media for 3 or 14 days, respectively). For cell-tracking study, additional twelve rats were randomly and equally divided into two groups: *HA-ECM+GFP<sup>+</sup>PDLC3* or *HA-ECM+GFP<sup>+</sup>PDLC14* (HA-ECM constructs seeded with GFP<sup>+</sup>PDLCs and cultured in standard osteogenic media for 3 or 14 days, respectively;  $n=6$ , each).

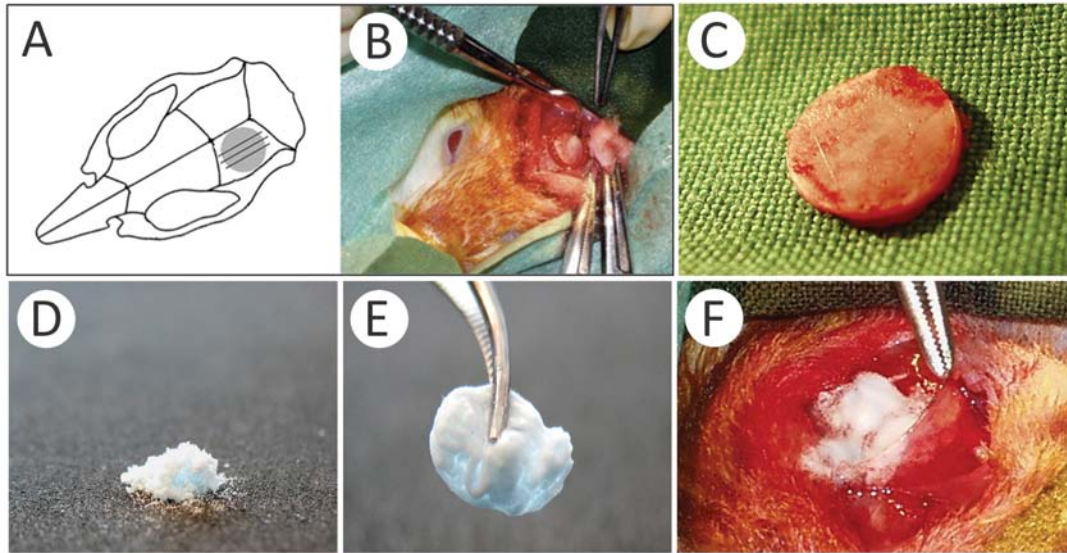
#### 3.5.1.4 Study IV

Twelve adult Sprague–Dawley male rats (~350 g) were randomly and equally divided into two groups: (1) *SBC-ECM* constructs (corresp. 50 mg SBC with ECM), and (2) *SBC* alone (50 mg SBC).

### 3.5.2 Surgical procedures

The critical-size calvarial defect model was used (Tcacencu and Wendel, 2008). The rats were anaesthetized by subcutaneous injection of Hypnorm (fentanyl/fluanisone, VetaPharma Ltd, UK) with Stesolid (Diazepam, Alpharma Aps, Denmark). During the surgery the rat was maintained on a heating pad. The ViscoTears liquid gel (Novartis) was applied on eyes for the corneal protection. The rat's head was shaved, and washed with iodine solution, and an incision was made in the sagittal plane

across the cranium. The skin and underlying tissues including the periosteum and temporal muscle were detached to expose the calvarial bone. An 8-mm full thickness circular defect was created on the left parietal region using a trephine drill with a sterile saline irrigation (Fig. 7). The defect area was evenly covered with the prepared construct or microparticles alone using periosteum elevator or dental plugger/spatula and forceps. The incisions were closed with single sutures in two layers.



**Figure 7.** Rat calvarial critical size defect model (A); bone defect is shaded grey; three parallel lines indicate section planes used for histomorphometric analysis. Creation of 8-mm circular defect using saline-cooled trephine (B). Full-thickness calvarial bone fragment after removal (C). HA alone and HA-ECM construct prior implantation (D and E, respectively); appearance of the defect site following insertion of the prepared construct (F).

### 3.5.3 BrdU (5-bromo-2-deoxyuridine) labeling

For the assessment of the cell proliferation at 2 weeks postoperatively the rats were injected subcutaneously with the BrdU labeling reagent (Invitrogen, USA, Cat.no. 00-0103) 2 h prior euthanasia.

### 3.5.4 Donor cell survival and engraftment

In study II, the host BMSCs were harvested separately from each rat euthanized at 2 weeks after surgery, and cultured as previously described for the Lewis-EGFP transgenic rat, followed by assessment for GFP expression by fluorescent microscopy, and immunocytochemistry using the primary anti-GFP antibody.

In study IV, the PDLCs were derived from the same rat using the explant method as described previously. Prior to cell seeding and implantation, GFP expression was proven by fluorescent microscopy and immunocytochemistry using a primary anti-GFP rabbit polyclonal antibody (1:400, Cat. No. A11122, Invitrogen, UK).

For cell seeding, the HA-ECM constructs were placed into 24-well culture plates (30 mg HA/well); 400  $\mu$ L of GFP<sup>+</sup> PDLC suspension in  $\alpha$ -MEM was added onto each construct at an initial density of  $2 \times 10^5$  viable cells *per* well. After 30 min incubation in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C,  $\alpha$ -MEM was added to a final volume of 1 ml *per* well. After 24 h incubation the PDLCs were cultured in standard osteogenic media for 3 or 14 days.

At 1, 4 and 10 weeks following implantation, calvaria were harvested and the tissue sections were immunostained as described elsewhere, using the same anti-GFP antibody, counterstained with Wright-Giemsa and mounted for light microscopy. Negative controls consisted of sections with omitted primary antibody as well as anti-GFP-immunostained sections of defects treated with the wild-type PDLC-loaded constructs.

### 3.5.5 Tissue retrieval and sample processing

The rats were euthanized by CO<sub>2</sub> inhalation at 2 or 12 weeks after surgery. The calvarial bone was surgically retrieved, and histologically processed. The samples were fixed in 4% neutral-buffered formaldehyde overnight at 4°C then decalcified in 12.5% EDTA, and embedded in paraffin; 5- $\mu$ m serial sections were prepared parallel to the sagittal line, and stained with hematoxylin-eosin or Masson's trichrome for the assessment of the general morphology, new bone formation, connective tissue ingrowth and the residual scaffold area. The degree of eosinophil infiltration (study II) was evaluated using Giemsa-Wright staining.

### 3.5.6 Immunohistochemistry

After deparaffinization in xylene and rehydration, the tissue sections of the calvarial bone defects were treated with 0.2 M HCl for 10 min then with 3% H<sub>2</sub>O<sub>2</sub> to block peroxidase activity. After washing in PBS, the sections were incubated for one hour at RT with the primary antibodies against rat Col1 (1:500, Cat. No. AB755P, rabbit polyclonal, Millipore, USA), BSP (1:500, Cat. No. AB1854, rabbit polyclonal, Chemicon International, Inc., USA), OPN (1:200, Cat. No. sc-10593, goat polyclonal, Santa Cruz Biotechnology, Inc., Europe), periostin (1:1000, Cat. No. RD181045050, rabbit polyclonal, BioVendor GmbH, Germany); rat macrophages (1:500, CD68 (ED1), Cat. No. MCA341GA, mouse monoclonal antibody; 1:1000, CD163 (ED2), Cat. No. MCA342GA, mouse monoclonal antibody, Serotec, UK, and 1:1000, CCR7 rabbit monoclonal antibody, Cat. No. ab32527, Abcam, UK).

In study II, the tissue sections were immunostained with the following primary antibodies: anti-GFP rabbit polyclonal antibody (1:400, Cat. No. A11122, Invitrogen, UK); anti-CD68 (ED1) mouse monoclonal antibody (1:500, Cat. No. MCA341GA, Serotec, UK); anti-PCNA mouse monoclonal antibody (1:200, Cat. No. MCA1558, Serotec, UK); anti-S100A4 rabbit polyclonal antibody (1:200, Cat. No. ab27957, Abcam, UK); anti-mieloperoxidase rabbit polyclonal antibody (1:50, Cat. No. ab9535, Abcam, UK); anti-CD45R mouse monoclonal antibody (1:100, Cat. No. 554879, BD Biosciences, Europe); anti-CD3 mouse monoclonal antibody (1:100, Cat. No. 550295, BD Biosciences, Europe).

BrdU detection was performed using anti-BrdU mouse monoclonal antibody (1:200, clone MoBU-1, Cat. No. B35128, Invitrogen). After additional washing in PBS, the sections were incubated with specific secondary biotinylated antibodies for one hour at RT. Peroxidase reactions were then visualized using a commercial Vectastain ABC kit (Vector Laboratories, USA). Finally, the sections were counterstained with Alcian blue or Methyl green, and mounted for light microscopy. Primary antibody was omitted from the sections used as negative controls. Two tissue sections from each calvarial specimen immunostained with CD68 (ED1) antibody were used to measure the macrophage (giant cell) distribution area (in pixels) using the same image-analysis software.

### 3.5.7 Histomorphometric analysis

Three central sections from each specimen were used for the measurement of the new bone areas using image-analysis software (Adobe Photoshop CS2, Adobe Systems Incorporated, San Jose, California, and ImageJ, National Institutes of Health, USA). The amount of the newly formed bone was measured twice in a blinded fashion, and expressed as the percentage of the total newly formed bone in the defect area (NFB%), or as the percentage of the total newly formed bone (NFB) to the total possible area for new bone ingrowth (study II and III) =  $NFB / (\text{total defect area} - \text{scaffold area}) \times 100\%$  (Castano-Izquierdo *et al.*, 2007).

Two tissue sections from each calvarial specimen were used to measure in a blinded manner the immunostained cell distribution profiles (study II and IV) represented in pixels per square mm (CD68, S100A4 and BrdU), or as cell number *per* square mm (PCNA-index, CD45, CD3, eosinophil and myeloperoxidase positive cells) using the same image-analysis software as for the evaluation of the new bone formation.

## 3.6 DATA ANALYSIS AND STATISTICS

The histomorphometric data were statistically analysed by the non-parametric Kruskal-Wallis and Mann-Whitney *U* test using the Statistica 8.0 software package (StatSoft). Two-tailed unpaired *t*-test was applied elsewhere for multiple comparisons of normally distributed data. The statistical significance was defined as  $p < 0.05$ . The

corresponding graphical representation was generated using Statistica 8.0, and Microsoft Office Excel (Microsoft, USA).

## 4 RESULTS AND DISCUSSION

This thesis is based on the work presented in papers I-IV (Tour *et al.*, 2011; Tour *et al.*, 2012a; Tour *et al.*, 2012b; c) and addresses the biomimetic approach to generate functional constructs for bone tissue engineering applications. The findings are presented and discussed in relation to the array of construct properties displayed *in vitro* and *in vivo* as well in the context of possible application in clinical settings.

### 4.1 CELL-DERIVED MATRIX ENHANCES OSTEOGENIC PROPERTIES OF BIOCERAMICS

Recent experimental approaches in biomimetic engineering have focused on the integration of the ECM proteins into a scaffold with the attempt to mimic the extracellular environment of the bone tissue. Several reports have previously indicated that *in vitro*-generated ECM had an impact on the osteoblastic differentiation of MSCs *in vitro* and *in vivo* (Datta *et al.*, 2006; Pham *et al.*, 2008; Pham *et al.*, 2009; Xiao *et al.*, 2004). However, to the best of our knowledge there are no studies aimed at generating ECM to improve the osteogenic properties of HA. Here we hypothesized that HA modified by ECM secreted by the primary cells possesses necessary stimuli to enhance bone tissue repair by providing a three-dimensional environment for host progenitor cells recruitment upon implantation in bone defects. The basic approach used herein was to allow cells to deposit their own ECM followed by cell removal while preserving the native constituents and topography of the generated matrix.

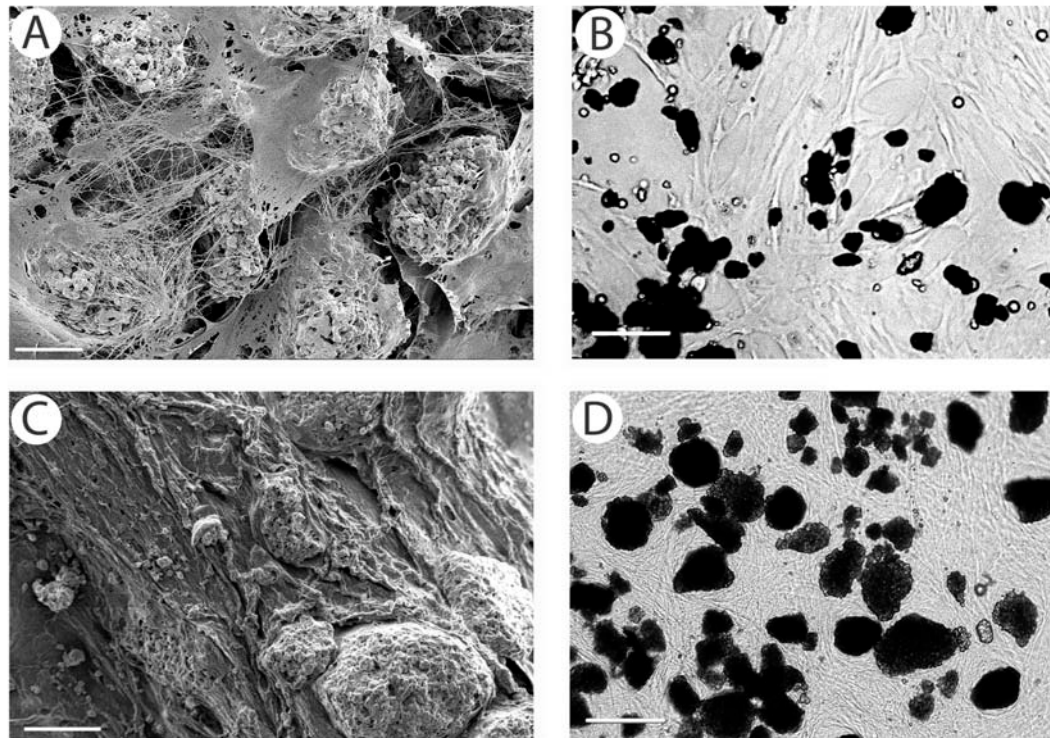
The sintered synthetic HA microparticles have previously served as a precursor material in several biomaterial developments (Wahl *et al.*, 2007) and were chosen as a primary scaffold in the current investigation due to their similar composition with the mineral content of the human bone. The primary rat DF and calvarial Ob exhibited good attachment and spreading on the microparticles, maintaining their typical flat polygonal morphology, and displaying multiple filopodia stretched out around microparticles. The cells proliferated and actively secreted ECM incorporating ceramic microparticles. This required culture at high cell density with media supplemented with ascorbic acid to facilitate the generation of three-dimensional matrices. At the end of the cultivation period, the HA microparticles were abundantly covered with a dense fibrillar matrix, resulting in a pellet-like construct on the bottom of the well.

The preference for the Triton-X extraction buffer method to obtain acellular constructs was based on the SEM observations. The ECM after Triton-X treatment



was compared with the ECM treated with other commonly used methods – freeze/thaw cycles in LN<sub>2</sub>. In the first case the ECM architecture, the fibrillar network and the porosity were better preserved, whereas after LN<sub>2</sub> freeze/thaw treatment the ECM architecture and topography were affected resulting in considerable loss of fibrillar structure, suggesting that Triton-X decellularization treatment was less harmful to the newly synthesized ECM (Fig. 8).

Removing cellular components from the cell- or tissue-derived ECM scaffolds was considered important for the concept of “off-the-shelf” availability to clinicians. In addition to be ready-to-use for graft procedures, it should be void of potential adverse immune responses triggered by the cell membrane epitopes, and allogeneic or xenogeneic DNA (Gilbert *et al.*, 2006). To our knowledge there are no official legal regulations regarding the DNA concentration limits in the cell- or tissue-derived scaffolds. The DNA remnants were detected by spectrophotometry in both osteoblast and fibroblast-derived constructs after decellularization at levels lower



**Figure 8.** Scanning electron microscopy of construct topography after decellularization with Triton-X buffer (A) and freeze-thaw LN<sub>2</sub> method (C). Light microscopy images of the construct prior (B) and after decellularization (D). Bars = 60µm (A); 20µm (B) and 40µm (C).

than 0.5 ng of DNA/mg construct dry weight. The detected DNA levels in our constructs are comparable with those in commercially available decellularized ECM scaffolds with positive clinical efficacy, suggesting that only certain higher threshold of the retained DNA may induce a detrimental host response associated with the adverse effects (Gilbert *et al.*, 2009).

The HE staining visualized the evenly distributed ECM in direct contact with the HA microparticles, which was positively stained with Alcian blue and Masson's trichrome, indicating high GAG and collagen content. The primary calvarial Ob-generated ECM had 1.5 times higher GAG content than the ECM generated by the primary DF after 21 days of culture. The HA-OECM constructs also had significantly higher collagen content compared to the HA-FECM constructs. No large variation between osteoblast- or fibroblast-derived constructs, before or after decellularization, was found with respect to the matrix morphology. As demonstrated by the immunohistochemistry, Col1 and the major non-collagenous protein components of the bone ECM (BSP and OPN) were detected in the constructs of both osteoblast and fibroblast origin. These signals were homogeneously distributed throughout the ECM, being highly expressed in the osteoblast-derived constructs, and to a lesser extent in the fibroblast-derived constructs.

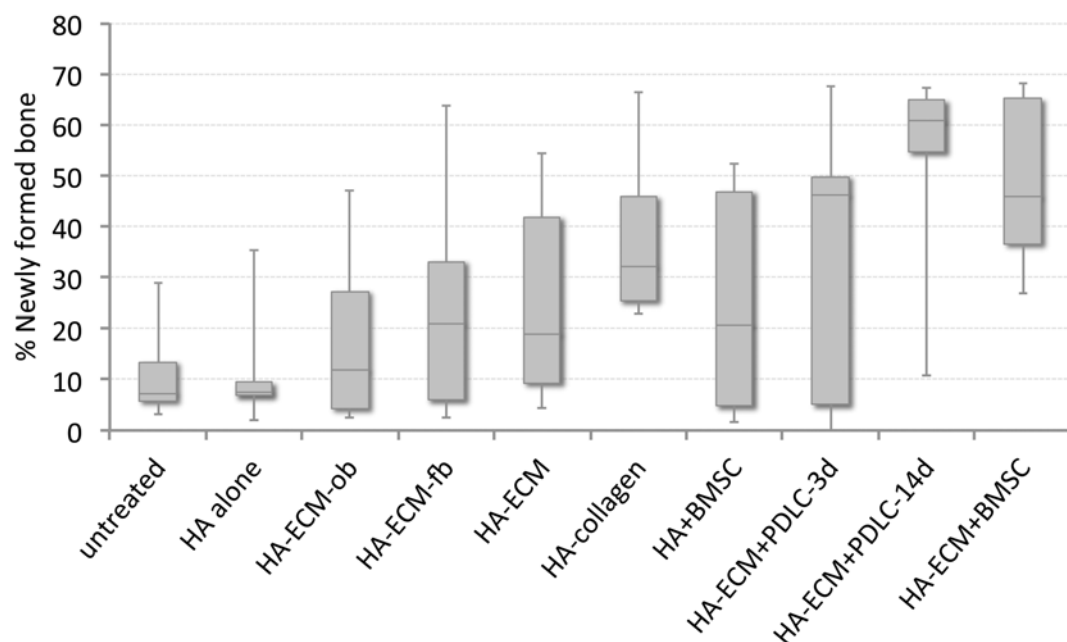
HA microparticles supported proliferation and metabolic activity of the primary cells. The proliferation of the rat primary calvarial Ob significantly increased over the cultivation period, and exceeded that of the cells grown on plastic by day 21. There was a similar increase in the proliferation of the rat primary DF cultivated on HA. No significant differences between the proliferation rates of the two cell types were observed.

To assess the LPS levels in the implanted constructs, the LAL assay was performed according to the FDA Guidelines for the end-product endotoxin testing of human and animal parenteral drugs, biological products, and medical devices (Magalhaes *et al.*, 2007b). HA-ECM constructs had very low levels of endotoxin contamination:  $0.008 \pm 0.004$  Endotoxin units (EU)/ml and  $0.005 \pm 0.002$  EU/ml for osteoblast- and fibroblast-derived constructs respectively, which corresponded to the total of 2.53 mEU/animal (for HA-OECM), and 1.4 mEU/animal (for HA-FECM), and thus can be considered as non-pyrogenic, according to the U.S. and European Pharmacopoeias. The low pyrogenicity of the HA-ECM scaffolds may allow the reconstruction of even more extended bone defects, where several grams of the HA-ECM would be required.

Collagen matrix (TFE) in combination with HA was chosen as a positive control. The TFE is widely used as a haemostatic agent, particularly in the field of thoracic and cardiovascular surgery, it is able to promote granulation tissue formation and has also been suggested as a scaffold for the tissue engineering purposes (Kofidis *et al.*, 2003). Like many other animal tissue-derived matrices, TFE has acceptable

tolerance for xenogeneic recipients since the main components of ECM are generally conserved among species. However, the *in vitro*-derived ECM offers many advantages, such as better biocompatibility, biodegradability, and the lack of the risk for the cross-species transmission of the infectious diseases (Gilbert *et al.*, 2006; Patience *et al.*, 1997).

In the present study we have demonstrated that HA-ECM constructs can induce significantly better bone repair without adding the progenitor (stem) cells or growth factors, compared to the raw HA material (Fig. 9). Significantly higher amounts of the newly formed bone were observed in the HA-ECM and HA-TFE treated defects, compared to HA alone treatment at 12 weeks after surgery, suggesting that the bioactive molecules, naturally resident in ECM, have stimulated the host cell-HA scaffold interactions.



**Figure 9.** Box-plots representing the amount of the newly formed bone at 12 weeks (median, upper and lower quartile, minimum and maximum values). HA-ECM-ob/fb = neonatal calvarial Ob/DF-derived construct, HA-ECM = adult DF-derived construct; collagen = TissuFleece E collagen matrix; BMSC = bone marrow-derived stromal cells; PDLC-3d/14d = constructs with periodontal ligament cells cultured for 3/14 days.

The data obtained from the *in vivo* experiments have indicated that the cellular origin of the ECM (DF vs Ob) was not critical for the outcomes of the bone repair induced by HA-ECM constructs. The amount of the newly formed bone was similar in the HA-FECM, HA-OECM, and HA-TFE treated defects. However, the dermal fibroblasts would be the clinically preferred source for ECM production *in vitro* as the harvesting of dermis entails much less morbidity for the patient.

A bone tissue-biomaterial interface layer rich in non-collagenous proteins has been well documented (Puleo and Nanci, 1999). In the current study, the protein profile of the biomaterial-tissue interface differed between the bone-integrated and non-integrated HA microparticles. The surface of the microparticles integrated within the newly formed bone exhibited strong immunostaining for BSP and OPN. On the contrary, surfaces of the non-integrated HA microparticles demonstrated no staining for BSP or OPN. However, the spaces between the non-integrated HA were stained for periostin. This selective accumulation of the specific proteins at the tissue-HA microparticle interface confirms the importance of the BSP and OPN for the integration of HA into the mineralizing tissue. The presence of periostin next to the non-integrated HA microparticles reported in our study, has corresponded with other observations showing the prominent periostin presence in collagen-rich tissues opposing two mineral phases, such as periodontal ligament and cranial sutures (Hamilton, 2008). High levels of periostin mRNA expression has been detected in preosteoblasts and undifferentiated mesenchymal cells at the early stage of fracture healing, further being attenuated after two weeks, and resulting in the loss of periostin mRNA expression in the mature osteoblasts as mineralization progressed (Nakazawa *et al.*, 2004). In the current study, however, the presence of the periostin at 12 weeks after surgery may represent the continuous fibrous connective tissue remodeling and may impair tissue mineralization.

An active inflammatory response was detected at the calvarial defect site at 12 weeks postoperatively demonstrated by the presence of the large number of the macrophages and FBGC. The levels of CD68<sup>+</sup> macrophage infiltration were positively correlated with the presence of ECM in the implant and with the amount of the newly formed bone (significantly larger amount of CD68<sup>+</sup> macrophages in HA-FECM, HA-OECM and HA-TFE treated bone defects compared to HA alone). The CD68<sup>+</sup> macrophages were mainly located onto the bone-non-integrated HA microparticles combined with ECM or TFE. In comparison, the CD163<sup>+</sup> macrophages were scarcely present at the defect site without direct contact with the surfaces of the HA microparticles with no difference in the cell number among the investigated groups. The lysosomal glycoprotein epitopes recognized by CD68 (ED1) antibody are expressed by most macrophage populations, as well as peripheral blood monocytes and bone marrow precursors (Pilling *et al.*, 2009), while CD163 (ED2) antibody is specific for recognizing membrane antigens of tissue-resident macrophages. Studies on functional and phenotypic diversity of the mononuclear macrophage populations in the cell-mediated immune responses have also indicated that CD68<sup>+</sup> macrophages are characterized by the production of large amounts of reactive oxygen intermediates and pro-inflammatory cytokines like IL-1, IL-12 and TNF $\alpha$  (Badylak *et al.*, 2008), whereas CD163<sup>+</sup> macrophages produce high levels of IL-10 and TGF- $\beta$ , inhibit the release of pro-inflammatory mediators, promote angiogenesis, and recruit cells for tissue remodeling (Badylak

and Gilbert, 2008; Mantovani *et al.*, 2004a; Soehnlein and Lindbom, 2010). Interestingly, in our study the macrophages and the multinucleated giant cells in contact with the HA microparticles expressed CD68 but not CD163. Obviously, the presence of CD68<sup>+</sup> cells in intimate contact with the implanted biomaterial is critical for the fate of the implant integration and, indeed, some methods have been suggested to eliminate these cells from the interface (Brodbeck *et al.*, 2002). On the other hand, we may speculate that the CD68<sup>+</sup> macrophages found at 12 weeks after surgery could have shifted their phenotype from pro-inflammatory in the early healing period to pro-regenerative as healing progressed, as has been noted for bone-tendon interface repair (Kawamura *et al.*, 2005).

The macrophages express multiple surface receptors, many of which interact with OPN, which is upregulated at the inflammatory sites such as the biomaterial implant area (Weber *et al.*, 1996). Moreover, OPN was shown to modulate macrophage adhesion, migration and cytokine release both *in vitro* and *in vivo*, and to play multiple roles in the cell-mediated immunity and wound healing (Giachelli and Steitz, 2000; Koh *et al.*, 2007). However in the current study, an increase in macrophage infiltration could not be attributed to OPN as its localization was limited merely to bone-integrated HA particles, whereas no CD68<sup>+</sup> macrophages were observed. Additionally, FBGC residing on the bone-non-integrated OPN-negative HA microparticles were most likely formed due to the lack of OPN inhibitory effect on macrophage fusion and giant cell formation, as has previously been shown *in vitro* (Tsai *et al.*, 2005).

#### **4.2 BONE MARROW STROMAL CELLS ENHANCE CONSTRUCT OSTEOGENIC PROPERTIES BY MODULATING FOREIGN BODY REACTION**

One of the critical issues for any tissue-engineered device is the interaction between the host and the cellular component of the implanted construct. At the defect site, the tissue-engineered construct is subjected to inflammatory mediators and signaling molecules such as cytokines, growth factors, and ECM proteases, which are different from the native environment of the implanted cells. Additionally, the delivered cells would be subjected to an environment specific to the foreign body reaction, such as low pH, ROS and degradative enzymes. The engineered cell-loaded construct should therefore maintain the biomimetic environment and regenerate bone tissue in the midst of this compromised milieu. In the current study we aimed to evaluate the ability of the undifferentiated BMSC delivered on the HA-based scaffolds to modulate the local cellular reaction, and to induce bone repair of the calvarial critical-sized defects in rats.

The mechanisms of BMSC-induced or supported osteogenesis are largely unknown. Some believe that the BMSC may differentiate into osteoblasts, and

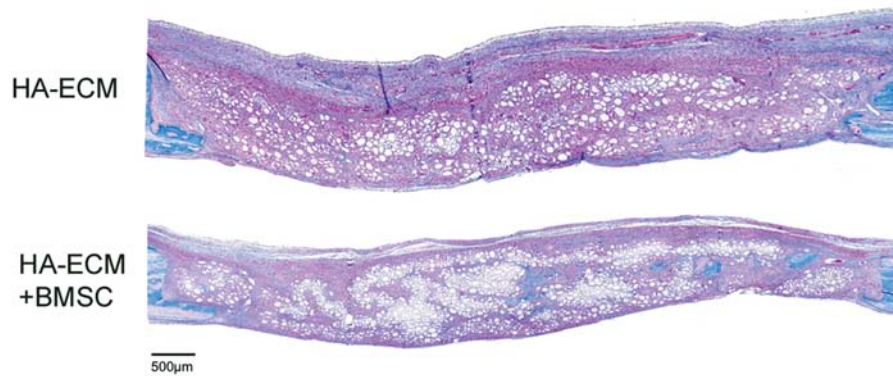
deposit bone matrix on the surface of the scaffold (Bruder et al., 1998). Alternatively, the BMSC may secrete cytokines into adjacent tissues and activate resident cell populations such as pericytes and osteoblast precursors (van Gaalen et al., 2004).

Due to the lack of a specific marker for BMSC, very little is known about their fate after implantation *in vivo*. Several studies reported the survival of BMSC up to four (Hasegawa et al., 2006), nine (De Kok et al., 2003), and even 24 weeks (Oshima et al., 2005) after implantation. In the present study the implanted BMSC expressed enhanced GFP, which is a common donor cell-tracking tool and is especially reliable when fluorescent microscopy is challenged due to the background fluorescence of bone mineral and the diminishment of signal following fixation and decalcification. The GFP expression of the cultured BMSC remained stable over the entire culture period and the harvested cells maintained high viability on scaffolds (> 98%) following *in-vitro* to *in-vivo* delivery phase. We implanted about  $7.5 \times 10^5$  viable GFP<sup>+</sup> BMSC per defect. However, no GFP signal was detected at the defect site at 2 weeks after surgery without any evidence of homing in the recipient bone marrow stroma. Our results are similar to published data (Zimmermann et al., 2011) and might be due to the hostile local environment created by acute inflammation and hypoxia. According to those data, changes in cell morphology, apoptosis and significant cell death (70% decrease) of the transplanted MSCs were evident already on day 3 after implantation. Clinically, this phenomenon might be advantageous because it limits the risk of possible complications linked to the presence of allogeneic cells. Moreover, the significant increase in new bone formation induced by the treatment with the BMSC-loaded scaffolds at 12 weeks suggests that the long-term survival and engraftment of the donor BMSC may not be required for the enhanced bone repair. It is likely that the implanted BMSC reveal their anti-inflammatory and pro-regenerative effects shortly after implantation by means of soluble factors and cell-cell interactions (Uccelli et al., 2008).

The ECM component of the construct may also affect differentiation/proliferation of the progenitor cells and may determine the recruitment of the appropriate cell types during tissue remodeling (Reing et al., 2009). While serving as a dynamic reservoir of growth factors, it might also facilitate BMSC attachment, enabling the maintenance of appropriate cell density at the moment of implantation.

The calvarial defects revealed minor new bone formation at 2 weeks postoperatively, mostly at the margins and the dural aspect (Fig. 10). The bone defects were filled with HA microparticles spread among fibrous connective tissues, comprising inflammatory cells, fibroblasts and blood vessels. No statistically significant difference in new bone formation between the groups was found at 2 weeks after surgery. The highest amount of the newly formed bone was obtained at 12 weeks with the biomimetic constructs combined with cells (HA-ECM+BMSC

group), significantly larger than with the HA-ECM, HA+BMSC ( $p < 0.05$ ) or HA treatments ( $p < 0.005$ ). This largest amount of the newly formed bone was evident despite the intense local inflammatory reaction detected at 2 weeks after surgery. The defects treated with HA-ECM or HA+BMSC revealed no statistically significant difference in the new bone formation. The HA treatment alone exhibited scarce islands of newly formed bone and the lowest repair efficiency.



**Figure 10.** Overview of rat calvaria treated with construct alone (HA-ECM) or combined with bone marrow stromal cells (HA-ECM+BMSC) reveals marked difference in inflammatory infiltrate at the defect site at 2 weeks postoperatively; Masson's trichrome staining.

We analyzed the cellular profiles of the inflammatory infiltrates at the defect sites that included eosinophils, neutrophils (PMN, myeloperoxidase-positive cells), CD68<sup>+</sup>, CD163<sup>+</sup>, CCR7<sup>+</sup>, CD45<sup>+</sup>, CD3<sup>+</sup> and S100A4<sup>+</sup> cells, as well as PCNA index and BrdU cell labeling at 2 weeks after surgery. The pattern of the cellular distribution was similar for all experimental groups. The largest accumulation of the inflammatory cells was observed predominantly along the periosteal side with fewer cells infiltrating the implants, except for the foreign body giant cells (CD68<sup>+</sup>, S100A4<sup>+</sup> and CCR7<sup>+</sup>) which were in tight contact with the bone non-integrated HA microparticles. The PCNA-positive cells were spread amongst the connective tissue between the HA microparticles.

The total CD68<sup>+</sup> cell (macrophage) number and the number of the CD68<sup>+</sup> cells in the contact with the HA microparticles was significantly lower in the HA-ECM and HA-ECM+BMSC treated defects than HA or HA+BMSC treated defects at 2 weeks after surgery. It may indicate that the ECM proteins have concealed the HA microparticles impeding the recognition by the macrophages or the foreign body giant cells (Chambers, 2000). We have also assessed the amount of the CD68<sup>+</sup> cells at 12 weeks after surgery. The levels of the CD68<sup>+</sup> cell infiltration significantly decreased by week 12 compared to week 2 only with the treatments lacking the ECM component. The amount of CD68<sup>+</sup> cells remained nearly unchanged in the

HA-ECM and HA-ECM+BMSC treated defects at 12 weeks compared to 2 weeks after surgery suggesting that the allogeneic ECM component of the biomimetic constructs supplemented chronic inflammation by maintaining a persistent macrophage infiltration as it has not been completely eliminated by 12 weeks.

We have used CCR7 and CD163 surface-markers for assessment of macrophage phenotypes in calvarial defects. The chemokine receptor CCR7 is expressed by mature dendritic cells, naïve and activated T-cells and pro-inflammatory macrophages, known as M<sub>1</sub> phenotype, whereas CD163, a membrane antigen of tissue-resident macrophages, is considered a marker for M<sub>2</sub> phenotype. In our study the CCR7<sup>+</sup> macrophages were prevalent in the calvarial defects compared to CD163<sup>+</sup> suggesting the prevalence of M<sub>1</sub>-dominant phenotype at two weeks after surgery. However, the high plasticity of macrophages makes the assignment of specific markers to different populations of macrophages very difficult.

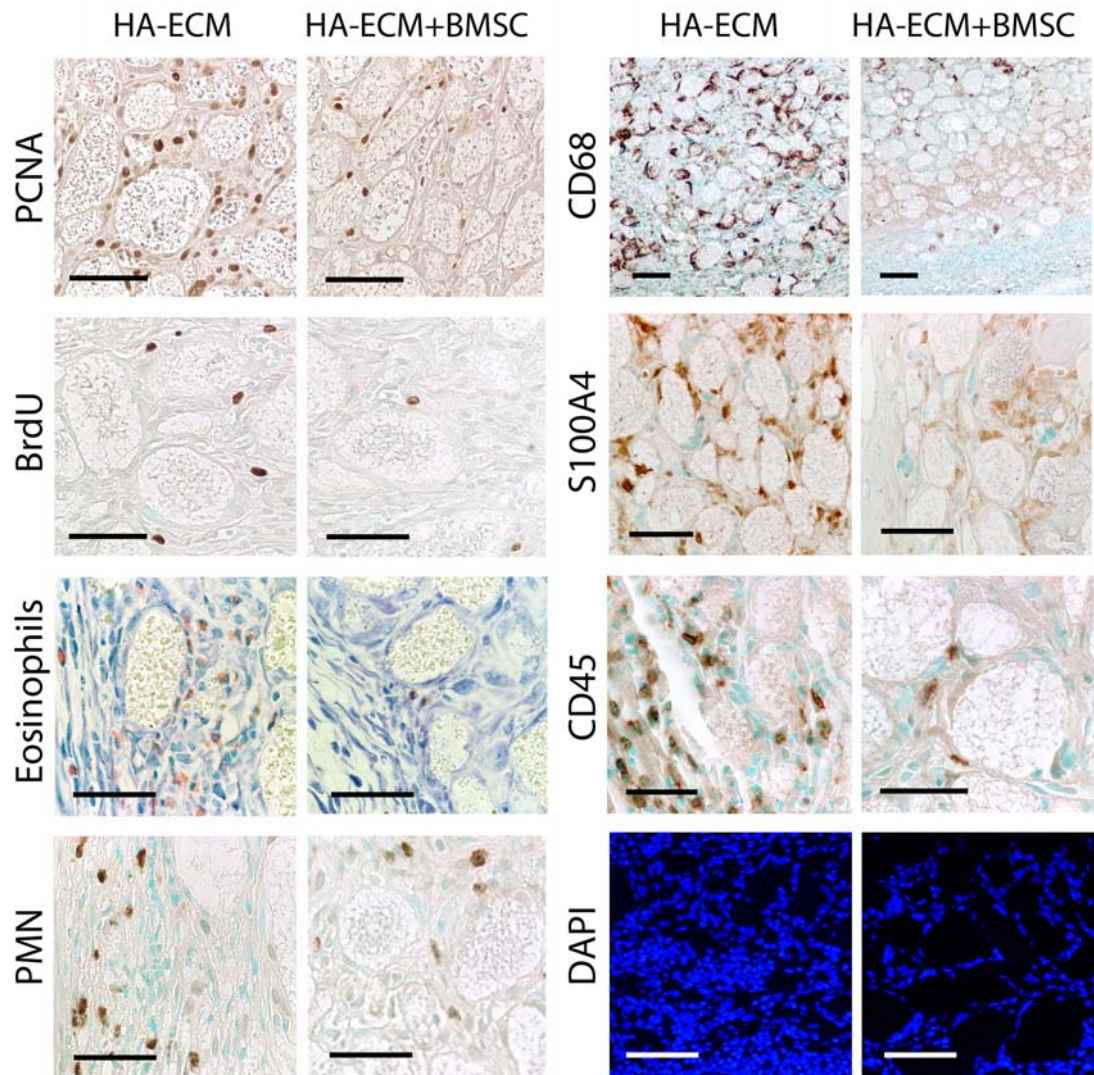
Eosinophils are traditionally considered to be the host effector cells against parasitic infections (Kariyawasam and Robinson, 2006). In addition, these cells may also contribute to both acute and chronic tissue damage caused by inflammation and are involved in transplant rejections through secretion of granule cationic proteins, the synthesis of inflammatory mediators and the production of ROS (Goldman *et al.*, 2001). The level of eosinophil infiltration was positively correlated with the presence of *in vitro* derived ECM proteins. However, the BMSC-loaded HA-ECM treatment showed a significantly lower eosinophil infiltration rate than HA-ECM treatment alone suggesting a strong anti-eosinophilic effect of the BMSC combined treatment (Fig. 11).

To further identify the abundant population of fibroblast-like cells in the tissue infiltrates we immunostained the tissue sections with the S100A4 antibody, also known as fibroblast-specific protein 1. S100A4 is considered a marker of fibroblasts in different organs undergoing tissue remodeling, fibrosis or fibroblasts derived from epithelial to mesenchymal transition (Schneider *et al.*, 2008). However, the specificity of the S100A4 for fibroblasts is controversial due to the reported overlap with macrophages and several other cell types (Inoue *et al.*, 2005). Indeed, our immunohistochemistry data revealed the S100A4<sup>+</sup> signal at both extra- and intracellular locations including fibroblast- and monocyte-like cells. We may speculate that the S100A4 could be involved in macrophage recruitment to the sites of implantation (Li *et al.*, 2010).

We have found that the vast majority of the CD45<sup>+</sup> cells are spindle-shaped. The combination of an elongated morphology, expression of CD45 and collagen production is reported to be sufficient for the identification of resident fibrocytes (Reilkoff *et al.*, 2011).



Fibrocytes have demonstrated the ability to home to areas of active tissue remodeling and wound healing, playing an active role in tissue repair via several mechanisms, such as inflammatory cytokine production, promotion of angiogenesis and secretion of ECM molecules and growth factors (Metz, 2003). In our study the fibrocyte infiltration levels at 2 weeks after surgery were positively correlated with the subsequent postoperative results of the new bone formation at 12 weeks, e.g. the HA treatment alone had the lowest rates of the CD45<sup>+</sup> cell infiltration and the new bone formation.



**Figure 11.** Representative morphology of inflammatory cell populations in HA-ECM and HA-ECM+BMSC-treated defects at 2 weeks postoperatively. Immunohistochemistry (PMN, BrdU, PCNA, CD68, S100A4, CD45), Wright-Giemsa staining (eosinophils) and pan-nuclear staining (DAPI). Bar = 50  $\mu$ m.

We may speculate that macrophages could be the major type of effector cells through which BMSC exert their immunomodulatory effects. In our case the possible mechanisms through which BMSC might modulate the course of the foreign body reaction are limited to the first few days immediately after surgery when the implanted BMSC are still viable. We believe that BMSC may interact with the host monocyte/macrophage populations, and reprogram them already at that early time point mediating the local inflammatory response. We have observed two different patterns of the cellular inflammatory reaction with the BMSC-combined treatments dependent on the presence or the absence of the matrix component. The first pattern when ECM was present showed that BMSC addition attenuated local inflammation (2-4 fold difference), which was demonstrated by a decreased PCNA-index, BrdU<sup>+</sup> cell number, significantly lower eosinophil and CD68<sup>+</sup> infiltration rates across the defect area, with the similar trend for S100A4<sup>+</sup>, CD45<sup>+</sup> and CD3<sup>+</sup> cell infiltration. The second pattern where the ECM was absent, the BMSC addition amplified the local inflammatory reaction at 2 weeks after surgery, which was demonstrated by the increased number (2-3 fold difference) of neutrophils (myeloperoxidase-positive), CD68<sup>+</sup>, CD45<sup>+</sup> and S100A4<sup>+</sup> cells. These results have demonstrated that the implanted BMSC possess the ability to subtly sense and tune the balance of the host tissue-implant interactions and the early remodeling events at the site of implantation.

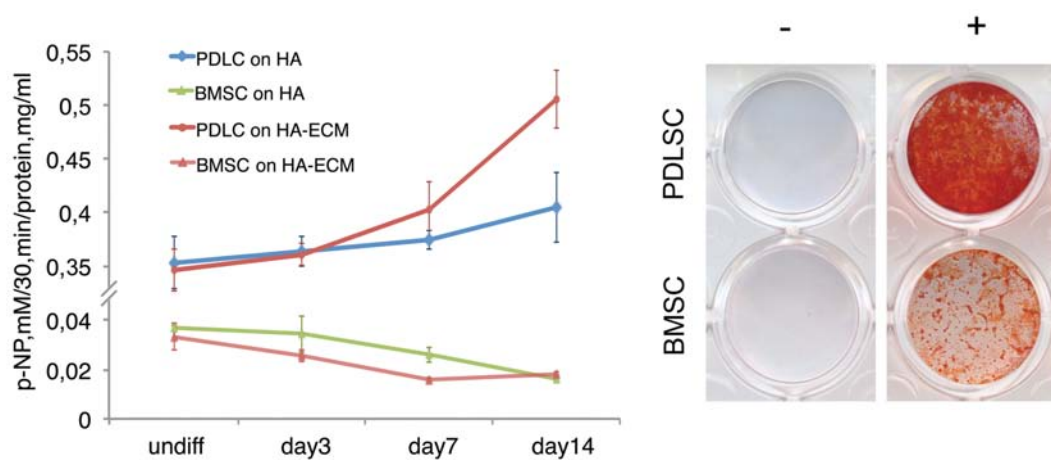
#### **4.3 PERIODONTAL LIGAMENT PROGENITOR CELL-SEEDED CONSTRUCTS ENHANCE CALVARIAL BONE REPAIR**

Mesenchymal progenitor cells found in dental tissues are capable of committing to the desired phenotype in combination with the predesigned scaffold. Despite the scant availability they may now be efficiently harvested, holding a great promise for various reconstructive therapy applications (Gronthos *et al.*, 2000; Seo *et al.*, 2004). In particular, PDLCs have excellent osteoblast-like properties and might be good candidates for both cementum/periodontal ligament complex, as well as the adjacent hard tissues repair (Hiraga *et al.*, 2009; Huang *et al.*, 2009; Kato *et al.*, 2011).

Here we demonstrated an approach using previously generated biomimetic construct seeded with PDLCs. The overall aim was to investigate the effect of the HA-ECM on the *in vitro* osteogenic differentiation of rat-derived PDLCs by evaluating the temporal gene expression and ALP activity, and to assess *in vivo* the osteogenic properties of the PDLC-seeded HA-ECM using a rat calvarial critical size defect model.

PDLCs can be easily obtained and effectively stored following common orthodontic extractions eliminating the need for more invasive procedures, such as harvesting bone chips or bone-marrow aspirations, associated with additional morbidity for the

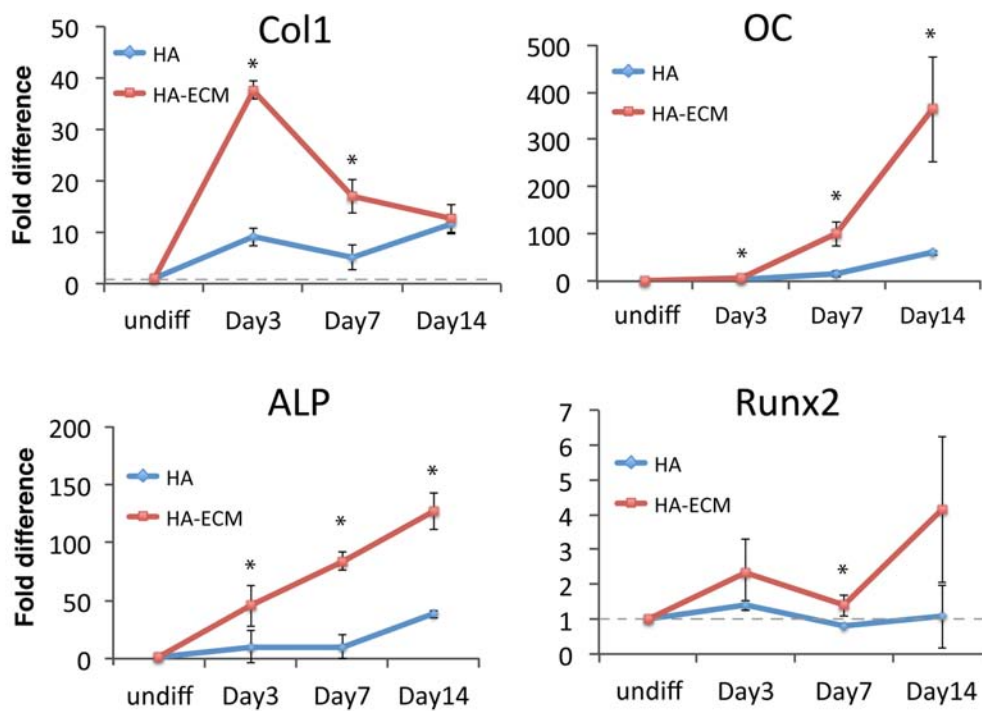
patient. Moreover, not only do they maintain stem cell characteristics, demonstrated by expression of common MSC surface molecules and ability for multipotent differentiation, but also possess superior capacity of *in vitro* osteogenic differentiation and mineralized nodule formation compared to periosteum-derived cells, bone-marrow derived cells or other dental tissue-derived progenitors (Seo *et al.*, 2004; Tsumanuma *et al.*, 2011). We have additionally studied the *in vitro* behavior of the BMSCs seeded on the biomimetic constructs and HA scaffolds in similar culture conditions and observed significantly higher ALP activity of PDLCs on both substrates compared to BMSCs during the entire culture period. Moreover, the stromal cells derived from periodontal ligament were characterized by more robust mineralization *in vitro* (Fig. 12).



**Figure 12.** Comparative osteogenic capacity of bone marrow-derived stromal cells (BMSCs) and periodontal ligament cells (PDLCs); ALP activity of cells on HA and constructs at different time intervals (on the left); mineralization assay after 28 days of treatment with regular (-) and osteogenic (+) media; note the superior osteogenic potential of cells isolated from periodontal ligament.

The isolated PDLCs maintained typical spindle-shaped fibroblast-like morphology and high proliferation rate through passages. Flow cytometry phenotyping of undifferentiated PDLCs revealed positivity for typical mesenchymal markers CD90, CD29, CD73 and negativity for representative lymphoid (CD3 and CD45), myeloid (CD11b) and endothelial markers (CD31). Upon osteogenic stimulation of the PDLCs, the mineral depositions were detected already at day 10, followed by a robust osteoblast-like differentiation by day 21 in culture. The rate of PDLCs proliferation was significantly higher on the HA-ECM constructs compared to HA or plastic already at day 3 in culture. The MTT values and the typical morphology of the seeded PDLCs demonstrated that both HA microparticles and the HA-ECM constructs supported the PDLCs attachment and exhibited high cellular compatibility.

Cell-delivery substrates capable of mimicking the extracellular environment of the bone tissue are considered to be essential for the outcome of the bone tissue-engineering therapy (Mauney et al., 2006; Pham et al., 2008). Furthermore, various experimental settings demonstrated the importance of osteogenic induction of MSCs in culture prior (Castano-Izquierdo et al., 2007; Washio et al., 2010) or after (Sikavitsas et al., 2003; Uchida et al., 2009) seeding onto the scaffolds. The PDLCs were cultured in standard osteogenic media starting directly from day 1 in culture. Our results revealed that the biomimetic construct significantly enhanced PDLCs osteogenic differentiation, as demonstrated by increased ALP production and upregulation of bone-related genes (Fig. 13).



**Figure 13.** Representative bone-related genes expressed in periodontal ligament cells at different time intervals in osteogenic culture on HA (blue) or HA-ECM construct (red) as measured by RT-qPCR; the data represent the mean fold difference in expression normalized to GAPDH and calibrated to baseline level (undifferentiated cells prior the osteogenic treatment), indicated by the dashed grey line; \* $p < 0.05$ .

Furthermore, the gene activity data facilitated the screening of the constructs for *in vivo* experiments that aimed to evaluate how the *in vitro* pre-culture period of the PDLCs on the HA-ECM constructs influenced the cells ability to regenerate bone *in vivo*. Based on our *in vitro* data, two types of the PDLC-seeded constructs (HA-ECM with PDLCs cultured for 3 days and 14 days) were selected for the

implantation in the calvarial bone defects in rats. The PDLCs were at different stages of their osteoblastic commitment: pre-osteoblast and mature osteoblast, respectively.

The histology revealed large amounts of the newly formed bone covering the defects at 12 weeks postoperatively. The interface between the biomimetic constructs and the newly formed or the host bone exhibited two distinct morphological types: the direct bone-to-bone contact or the connective tissue fibers inserted into the host bone tissue resembling the morphology of the native periodontal ligament interface. The histomorphometric analysis demonstrated that the 14-day culture period of the PDLCs on HA-ECM construct in osteogenic media was more optimal for the calvarial bone regeneration compared to 3-day culture period. We suppose that seeding the PDLCs onto the HA-ECM constructs and culturing in osteogenic media for two weeks prior implantation might facilitate the accumulation of the osteogenic growth factors within the biomimetic construct and improve the cell adaptation to the substrate (Uemura *et al.*, 2003; Yoshikawa *et al.*, 1996).

In contrast to our results, Castano-Izquierdo *et al.* showed that rat BMSCs stimulated with osteogenic media for 4 days induced the largest new bone formation (Castano-Izquierdo *et al.*, 2007). However they used a different substrate (titanium fiber mesh) and a different cell-culture setup prior implantation. Those findings may indicate that osteogenic culture protocols have to be adjusted for each type of scaffold and progenitor cell in order to achieve the best outcomes *in vivo*.

We have also evaluated the PDLC survival post-implantation using cells expressing GFP for *in vivo* tracking experiments. The GFP<sup>+</sup> PDLCs were detected at 1 and 4 weeks after surgery, located mainly within connective tissue on periosteal side with no GFP<sup>+</sup> cells present in bone tissue. The PDLC number decreased significantly over time and no PDLCs were integrated into the newly formed bone. However, the significant increase in the new bone formation induced by the treatment with the PDLC-loaded scaffolds suggests that the long-term survival and engraftment of the donor PDLCs may not be required for enhanced bone repair. It is likely that the implanted PDLCs reveal pro-regenerative effects by means of soluble factors and direct interactions with host cells without generating new bone tissue themselves.

#### **4.4 CLINICAL-GRADE APPROACH FOR GENERATION OF ECM-BASED CONSTRUCTS**

Numerous attempts with refined *in vitro* protocols have been exploited for the construct production (Salvade *et al.*, 2007; Salvade *et al.*, 2010) and MSCs expansion (Lange *et al.*, 2007) by providing stricter control of quality parameters and, therefore, a more adequate compliance to GMP-grade methodology. To date, however, no



standard clinical-grade xeno-free protocol has been established for bone tissue engineering applications.

Here we have opted for complete xeno-free culture conditions throughout the entire *in vitro* production process including cell propagation, harvesting and cryopreservation, in order to fully eliminate the side effects related to the use of FBS or similar commonly used supplements of animal origin. Furthermore, in the present study we hypothesized that SBC modified by ECM produced by normal human DF could provide a three-dimensional environment with properties favouring enhanced osteogenic differentiation of MSCs derived from bone marrow (BMSC) and adipose tissue (ADSC).

The biphasic calcium phosphate as a raw scaffold material was chosen for this study based on its ability to degrade in tissues within a physiologically optimized time frame, in contrast to essentially non-resorbable HA, or relatively fast degradable  $\beta$ -TCP (LeGeros, 2002). SBC is a clinical-grade scaffold that has previously shown to exhibit new bone formation similar to inorganic bovine bone (Cordaro *et al.*, 2008), and enhance the bone forming capacity of the autogenous bone in periodontal settings (Zafiropoulos *et al.*, 2007). However, it induces scarce mineralization when implanted alone and was reported to have little effect on osteoblastic differentiation of BMSC and PDLC when cultured *in vitro* (Mrozik *et al.*, 2012).

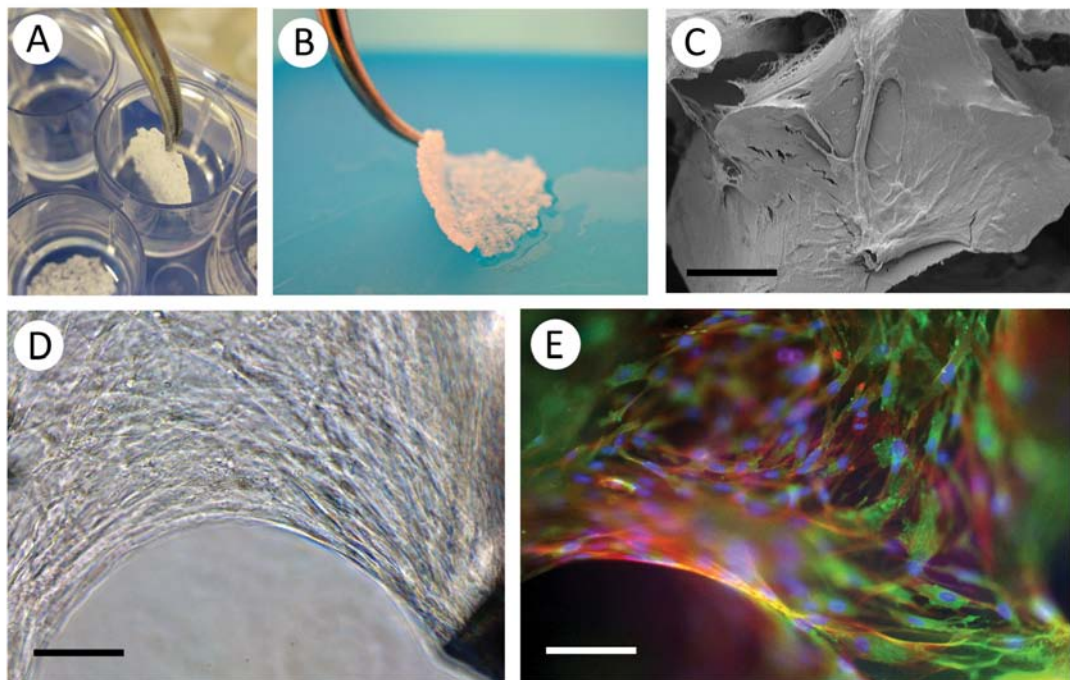
The fundamental approach in the current investigation was similar to that of our previous studies where cells were exploited to deposit proteins followed by removal of the cells while preserving the intrinsic elements and topography of the generated matrix. To produce the three-dimensional protein matrix with entrapped ceramic granules, DF were cultured at high density with media supplemented with ascorbic acid and low 2% concentration allogeneic human serum.

The SEM confirmed that SBC granules had the size of 400-700  $\mu\text{m}$  in diameter and a porous structure. DF displayed good attachment and spreading on the microparticles, maintaining their typical flat polygonal morphology and displaying several filopodia stretched out onto the granules. MTT assay demonstrated that SBC supported proliferation and metabolic activity of the normal human DF that actively secreted ECM, entrapping SBC granules and resulting in a dense membrane-like structure (Fig. 14).

Following decellularization with Triton-X at the end of the cultivation period, the SBC granules were abundantly covered with the continuous dense fibrillar matrix. The HE staining proved the presence of the evenly distributed ECM in direct contact with the SBC granules. Its fibrillar arrangement was conserved, and the matrix was positively stained with Alcian blue indicating high GAG content. Western blot analysis of the SBC-ECM revealed the presence of the typical bone matrix proteins, such as Col1, BSP, BMP-2, OPN as well as weaker signals for VEGF and Col3. The GAG and total collagen content in DF-generated ECM was  $0.36 \pm 0.08$  and  $0.72 \pm 0.07$   $\mu\text{g}/\text{mg}$  scaffold correspondingly, with levels comparable to rat fibroblast-derived

construct. The residual DNA content was detected at levels lower than 0.5 ng of DNA/mg construct dry weight, as assessed by spectrophotometry. Significantly lower LPS levels in the produced SBC-ECM constructs ( $2.84 \pm 0.9$  mEU/ml, corresponding to the total of  $0.14 \pm 0.05$  mEU/animal) can be explained by high purity medical grade scaffold and upgraded biosafety level cell culture facility used.

The multipotent capacity of the MSCs, the relatively simple way of isolation, high *ex vivo* expansion potential and immunomodulatory properties make them attractive candidates for cell therapy in skeletal tissue repair (Mosna *et al.*, 2010). BMSC and ADSC maintained typical spindle-shaped fibroblast-like morphology and high proliferation rate throughout passages 2 and 3. Flow cytometry phenotyping of



**Figure 14.** Generation of human fibroblast-derived constructs *in vitro* (A; B). Dermal fibroblasts residing on the surface of the SBC microparticle (C); SEM. Bright field view (D) and fluorescent microscopy (E) of the MSC-seeded construct after 14 days *in vitro* culture; (DAPI, Phalloidin red and anti-actin immunostaining). Bar = 100  $\mu$ m.

undifferentiated MSCs at passage 4 revealed positivity for typical mesenchymal markers HLA-I, CD90, CD29, CD73, CD44, CD105 and negativity for HLA-II, representative lymphoid (CD3 and CD45) and endothelial markers (CD31). Upon osteogenic stimulation of MSCs seeded on plastic, mineral depositions were detected as early as day 12, followed by a robust osteoblast-like differentiation by day 21 in culture. The rate of ADSC and BMSC proliferation was significantly higher on SBC-

ECM constructs compared to SBC alone already after 24 h in culture and increased over the 2-week cultivation period with higher values observed for BMSC on all substrates at day 14.

In the current study, we observed evidence of BMSC and ADSC differentiation along the osteoblast-like phenotype. An increase in ALP production, proliferation and mineralization *in vitro*, together with increased mRNA transcripts for ALP, Runx2 and OC clearly indicated hMSC osteoblast commitment on SBC-ECM. The enhanced osteogenicity of the construct was further demonstrated by an increase in mRNA transcripts of Runx2 in standard culture medium, as well as OC immunodetection, another ECM component involved in osteogenic differentiation. In fact, ECM has been shown to act as growth factor reservoir (Badylak, 2002; Badylak *et al.*, 2009) and effectively induce bone repair (Lutolf *et al.*, 2003). In addition, it may provide many active binding sites for the domains of target growth factor receptor (Reilly and Engler, 2010). It is also likely, that the topography of SBC-ECM promoted this effect as suggested by results obtained from MTT and ALP analyses when compared to SBC alone. In addition, the SBC-ECM constructs were easier to handle and exhibited better hemostatic properties than SBC, favouring clinical application in periodontal surgery and bone tissue engineering in maxillofacial area.

The majority of reports demonstrate a limited ability of ADSCs to differentiate along the osteogenic lineage compared to BMSCs (Hayashi *et al.*, 2008; Im *et al.*, 2005). In contrast, De Ugarte *et al.* found no significant difference between BMSCs and ADSCs with regard to their osteogenic capacities (De Ugarte *et al.*, 2003). In our study, samples from bone marrow and adipose tissue were collected from different donors, presumably resulting in slightly distinctive *in vitro* response to osteogenic treatment. Further, we implanted the construct or SBC scaffold alone in calvarial defects of non-immunocompromized rats. After 12 weeks, the treated calvarial bone defects were filled with the SBC granules spread among fibrous connective tissue comprising inflammatory cells, fibroblasts and blood vessels. Some granules were integrated with the islets of the newly formed bone that was mainly restricted to the dural side and areas close to the host bone margins. No defects showed a complete bone repair.

Host reactions following construct implantation often dictate the success of the treatment. To assess the biocompatibility of the generated SBC-ECM, we assessed the distribution area of CD68, a common cell-surface marker of monocyte/macrophage population and the newly formed tissue vascularization profiles. A large number of CD68<sup>+</sup> cells, including foreign body giant cells, were present in all defects at 12 weeks after surgery. However, no statistically significant differences were observed in the number of CD68<sup>+</sup> cells between the two groups. The vascularization profile at 12 weeks showed no significant differences in vessel density, size or number. The moderate inflammatory response at 12 weeks postoperatively suggested that



treatment with SBC-ECM had very little risk in relation to the elicited foreign body reaction.

## 5 CONCLUDING REMARKS

### 5.1 SUMMARY OF MAJOR FINDINGS

- The results demonstrated the successful production of functional *in vitro*-derived HA-ECM constructs for bone tissue engineering applications. ECM derived *in vitro* from the primary osteoblasts and dermal fibroblasts mimicked native bone ECM, enhanced the osteogenic properties of the HA microparticles, and modulated the local inflammatory response in a bone repair-favorable manner when implanted *in vivo*.
- The treatment of the critical-sized calvarial defects with BMSC-loaded HA constructs significantly enhanced bone repair by modulating the foreign body reaction and provided a permissive environment for new bone formation. The findings highlight the implications of BMSC in the regulation of the foreign body reaction triggered by the tissue-engineered constructs, demonstrating a higher efficiency for the BMSC combination therapy.
- *In vitro*-generated ECM deposited on HA significantly enhanced the osteogenic differentiation of PDLCs. The treatment with PDLC-seeded HA-ECM significantly improved bone repair in calvarial critical size defects
- We have been able to implement a GMP-grade methodology for the biomimetic construct production by culturing adult human dermal fibroblasts onto a clinical-grade ceramic scaffold under complete xeno-free conditions without compromising their functional properties. The resultant tissue-engineered constructs promoted osteogenic differentiation of human MSCs and displayed biological safety and high biocompatibility *in vivo*. The biomimetic design and excellent handling properties make it a promising candidate for craniofacial bone tissue engineering applications.

### 5.2 FUTURE PERSPECTIVES

In the context of present investigations, additional studies will be required to improve the osteoinductive parameters of the cell-secreted ECM, as well as to fully understand the cellular and cytokine components of the host tissue response. Elements of the host tissue response toward the implanted biomaterial are fundamental for successful biomaterial osteointegration, and understanding the

principles of the precise modulation of the local inflammatory response will become the next significant advancement in bone tissue regeneration. Furthermore, it might be relevant to study early MSCs-monocyte/macrophage interactions during the first days following implantation. Moreover, the *in vitro* assays exploring the interplay between MSCs and the inflammatory cells might help to further assess the cytokine profile behind the early remodeling events. The path forward will certainly require a deeper understanding of the exact mechanisms by which MSCs enhance bone repair and modulate the host foreign body reaction, as well as to further address the question, whether viability and survival of the transplanted cells in recipient tissues is indeed critical for the treatment outcomes.

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Mastering the driving force behind the full-spectrum performance of novel bioengineered systems in the clinical context requires communication between clinicians, biologists and engineers in ways not yet accomplished. The vast array of tools and methods of analysis in these divergent disciplines as well as the lack of interdisciplinary interaction competence constantly draw a boundary that limits the field. Today more tools are becoming available and these small changes currently happening in the field of bone regeneration enable us to study the complex biology of hard tissues with more precision. A more profound understanding of the clinical and biological requirements as well as limitations and capabilities of the advanced engineering technologies are among major barriers to overcome.

Ultimately, it is our aim as clinicians to translate those refined approaches into consistent advancements in patient care.

## 6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Kraniofaciala skelettskador förekommer vid bl.a. inflammatoriska processer, trafikolyckor, tumörkirurgi och vid strålningsterapi mot hals- och ansiktsregionen. Ofta är sådana skador allvarligt handikappande, då kroppens funktion och utseende påverkas. Vid stora bedefekter har den mänskliga kroppen svårt att själv läka ihop benet. För att få ny benvävnad att växa där det finns för lite ben eller där ben saknas helt, använder man sig idag bl.a. av mikrokirurgiska tekniker. Då flyttas kroppseget ben från ett mindre känsligt ställe, vilket innebär att kirurgen kommer att skapa en ny bedefekt. Tyvärr finns inte heller alltid rätt form, storlek eller kvalitet på bentransplantat tillgängligt. I dagsläget använder man sig även av transplantat i form av allografter eller xenografter. De tas från en annan person respektive från djur och kan medföra flera risker i form av immunologiska reaktioner och sjukdomar både hos patienten och hos donatorn. Därför skulle det vara ett stort framsteg att kunna framställa benvävnad på annat vis. En del patienter har genomgått rekonstruktiv kirurgi med hjälp av tillväxtfaktorer, men det finns fortfarande alltför många brister och frågetecken kring tekniken.

Stamcells forskning och regenerativ medicin är snabbt växande forskningsområden med stora förhoppningar om att kunna konstruera nya funktionella vävnadsdelar med hjälp av kroppsegna celler tillsammans med lämpliga biomaterial. Eftersom den benbildande processen är mycket tidskrävande har detta projekt haft för avsikt att optimera och effektivisera benläkning genom att introducera nya biomimetiska koncept för benvävnadsodling.

I det första delprojektet påvisades att det går att producera biomimetiska konstruktioner som gynnar beninväxt, genom att odla benbildande celler och bindvävsceller (fibroblaster) på materialet hydroxyapatit. Hydroxyapatit är ett syntetiskt framställt kalciumfosfat som liknar den icke-organiska komponenten i benvävnad, och har med viss framgång använts som bensubstitut, en så kallad benscaffold. Fibroblaster användes med syftet att modifiera biomaterialytor med proteiner som bygger upp så kallad extracellulär matrix för att efterlikna situationen i benvävnaden. Vidare konstaterades att konstruktionen på ett avgörande sätt bidrog till läkning av en skapad bedefekt i skalltak på råtta. Introduktion av proteiner ledde dock till en ökad risk för inflammationer i kringliggande vävnad. I den andra studien förbättrades benläkningen genom att stamceller transplanterades tillsammans med de nyodlade konstruktionerna. Ett intressant resultat av detta var att den cellbärande konstruktionen kunde dämpa inflammatoriska processer och effektivisera benbildandet efter implantationen. I det tredje delarbetet undersöktes möjligheten att ytterligare förbättra behandlingsresultat genom att använda parodontalligamentceller som är fibroblastliknande celler med ben- och stamcellsegenskaper belägna i

parodontiet. Före implantationen odlades cellerna ovanpå konstruktionerna under olika lång tid och därefter visades de kunna differentiera till osteoblastliknande celler, vilket hade positiv inverkan på defektläkning. I det sista delarbetet konstruerades ett biomimetiskt material byggt enligt samma biomimetiska metoder, men även enligt principer av god tillverkningssed (GMP).

Sammanfattningsvis har dessa studier visat lovande resultat som kan utgöra en grund för vidareutveckling av kliniskt användbara metoder och produkter för att framställa ben med hjälp av vävnadsteknik.

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