Molecular and structural patterns of guided bone regeneration (GBR)

Experimental studies on the role of GBR membrane and bone substitute materials

Ibrahim Elgali

Department of Biomaterials
Institute of Clinical Sciences
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

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ibrahim.elgali@gu.se

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To my beloved mother, my wife and my daughter

ABSTRACT

The mechanisms of guided bone regeneration (GBR) and bone healing with calcium phosphate (CaP) bone substitutes are not fully understood. The major aim of this thesis was to determine the relationship between the bone formation in bone defects and the cellular distribution and activities in association with CaP materials and/or with GBR membrane. The objectives were, firstly, to examine if the different CaP substitutes induce different cellular and molecular activities, and, secondly, to investigate the mechanisms of GBR with focus on the role of the barrier membrane in the bone healing process. A series of studies were performed in a rat trabecular bone defect model using a set of molecular (e.g. qPCR) and morphological (e.g. histology & histomorphometry) techniques.

Deproteinized bovine bone (DBB) and octa-CaP (TetraB) granules promoted bone regeneration and restitution of the defect. DBB was osteoconductive and elicited low resorption activity. TetraB induced early osteogenic and osteoclastic activities, resulting in greater bone formation than DBB. Strontium (Sr) doping of the CaP granules reduced the expression of osteoclastic resorption genes in comparison to hydroxyapatite (HA). Applying a collagen-based membrane on the defect promoted higher bone formation at all time periods. This was in parallel with upregulation of genes denoting cell recruitment and coupled bone formation and resorption (i.e. remodeling). The membrane was found to accumulate cells that expressed and released different pro-osteogenic growth factors (e.g. BMP-2). When the defect was simultaneously treated with the membrane and bone substitutes (DBB, HA, SrHA), more bone and an inhibitory effect of Sr on osteoclasts was demonstrated in the SrHA treated defect.

In conclusion, different calcium phosphate bone substitutes induce specific molecular cascades involved in the different processes of bone healing, including early inflammation, bone formation and remodeling. This promotes bone regeneration and defect restitution in comparison with the sham defect. Strontium incorporation in a synthetic CaP substitute reduces the osteoclastic resorptive activities, and promotes bone formation. Furthermore, the present results provide cellular and molecular evidence *in vivo* suggesting a novel role for the membrane during GBR, by acting as a bioactive compartment rather than as a passive barrier. The results provide new opportunities for the design of a new generation of materials to enhance bone regeneration.

Keywords: Regenerative medicine; biomaterials; bone substitute; calcium phosphate; guided bone regeneration; membrane; strontium; bone defect; bone remodeling; inflammation; cytokines; chemokines; growth factors; gene expression; histomorphometry; *in vivo*.

SAMMANFATTNING PÅ SVENSKA

Styrd vävnadsläkning bygger på principen att ett membranmaterial exkluderar mjukvävnad från att hämma benbildningen. Mekanismerna för hur membran samt benersättningmaterial kan stimulera benbildning är dock ofullständigt kända. Avhandlingens syfte är att analysera benregeneration i anslutning till kalciumfosfatberedningar och membran. I en serie experimentella studier användes en djurexperimentell model på råtta, morfologiska metoder, samt cell- och molekylärbiologiska tekniker. De kirurgiskt skapade bendefekterna lämnades tomma eller fylldes med granulat av benersättningsmaterial med eller utan ett membran som separerade den överliggande mjukvävnaden från den underliggande bendefekten.

Deproteiniserat, bovint ben (DBB) och okta-kalciumfosfat (TetraB) stimulerade benbildning och defektläkning. Analys av genutryck, morfologi och ultrastruktur visade att DBB är osteokonduktivt. TetraB stimulerade tidig ben-remodellering och kraftigare benbildning än DBB. Kalciumfosfat med strontium (SrCaP) reducerade osteoklasters genuttryck för bennedbrytning jämfört med hydroxyapatit (HA). Applikation av membran, resulterade i ökad benbildning i den underliggande bendefekten jämfört med kontroll-defekter. Dessa morfologiska fynd var kopplade till en uppreglering av gener involverade i cellrekrytering och ben-remodellering. Viktiga fynd var att membranen ackumulerade celler som uttryckte och frisatte benbildnings-stimulerande tillväxtfaktorer, samt att positiva samband påvisades mellan dessa faktorer och molekyler involverade i benremodellering i bendefekten. En kombination av membran och SrHA resulterade i mer ben i defekten. Det visades att effekten av strontium inbegriper en minskning av osteoklaster, nedreglering av osteoklasters bennedbrytande enzym samt osteoblasters genuttryck för stimulering av osteoklast-differentiering.

Sammanfattningvis så visar avhandlingen att olika benersättningsmaterial, sammansatta av kalciumfosfater, stimulerar nybildning av ben och restituerar bendefektens anatomi genom en påverkan på inflammation, benbildning och benremodellering. SrHA stimulerar benregeneration via en hämning av osteoklasters katabola effekt. Cellulära och molekylära data visar att membran, applicerad för styrd vävnadsläkning, i själva verket utgör en miljö med aktiva celler som stimulerar de benbildande processerna i den underliggande defekten. Detta fynd står i skarp kontrast till den gängse uppfattningen om hur membran för styrd vävnadsläkning fungerar. Kunskapen ger oss nya möjligheter till design och optimering av nya material i syfte att stimulera benregeneration hos patienter med skelettskador.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Elgali I, Igawa K, Palmquist A, Lennerås M, Xia W, Choi S, Chung UI, Omar O, Thomsen P. *Molecular and structural patterns of bone regeneration in surgically created defects containing bone substitutes*. Biomaterials. 2014; 35: 3229–3242.

II. Cardemil C[#], Elgali I[#], Xia W, Emanuelsson L, Norlindh B, Omar O, Thomsen P. *Strontium-doped calcium phosphate and hydroxyapatite granules promote different inflammatory and bone remodelling responses in normal and ovariectomised rats*. PLoS One. 2013; 8: e84932.

III. Turri A[#], Elgali I[#], Vazirisani F, Johansson A, Emanuelsson L, Dahlin C, Thomsen P, Omar O. *Guided bone regeneration is promoted by the molecular events in the membrane compartment*. Submitted for publication.

IV. Elgali I[#], Turri A[#], Xia W, Norlindh B, Johansson A, Dahlin C, Thomsen P, Omar O. *Guided bone regeneration using resorbable membrane and different bone substitutes: early histological and molecular events*. Submitted for publication.

[#]Equal contribution

CONTENT

ABBREVIATIONS

ALP	Alkaline phosphatase
BET	Brunauer–Emmett–Teller Technique
BMPs	Bone morphogenic proteins
BMU	Basic multicellular units
BSP	Bone sialoprotein
C5a	Complement component 5a
CaP	Calcium phosphate
CatK	Cathepsin K
Col1a1	Collagen type I alpha 1
CR	Calcitonin receptor
CT-1	Cardiotrophin-1
CXCR4	Chemokine receptor type 4
CXCL12/SDF1	Stromal cell-derived factor 1
DBB	Deproteinized bovine bone
DCPD	Dicalcium phosphate dihydrate
d-PTFE	Dense-polytetrafluoroethylene
EphB4	Ephrin type-B receptor 4
e-PTFE	Expanded polytetrafluoroethylene
ECM	Extracellular matrix
FBGCs	Foreign body giant cells

FGF-2	Fibroblast growth factor 2
GBR	Guided bone regeneration
GTR	Guided tissue regeneration
HA	Hydroxyapatite
ICP-AES	Inductively coupled plasma atomic emission spectroscopy
IGF-1	Insulin-like growth factor-1
IL-1β	Interleukin1beta
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-13	Interleukin 13
IL-17	Interleukin 17
MAPK	Mitogen-activated protein kinase
MCP	Monocalcium phosphate
M-CSF	Macrophage colony-stimulating factor
MIP	Macrophage inflammatory protein
MITF	Microphthalmia-associated transcription factor
MNGCs	Multinucleated giant cells
MSCs	Mesenchymal stem cells

NFATc1	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cells
OC	Osteocalcin
OCP	Octacalcium phosphate
OPG	Osteoprotegerin
OPN	Osteopontin
PDL	Periodontal ligaments
PMNs	Polymorphonuclear cells
PPARγ	Peroxisome proliferator-activated receptor gamma
PTH	Parathyroid hormone
PTH1R	Parathyroid hormone 1 receptor
qPCR	Quantitative-polymerase chain reaction
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RGD	Arginyl-glycyl-aspartic acid
ROI	Region of interest
Runx2	Runt-related transcription factor 2
SBF	Simulated body fluid
SrCaP	Strontium-doped calcium phosphate
SEM	Scanning electron microscope

SrHA	Strontium-doped hydroxyapatite
S1P	Sphingosine-1-phosphate
α-TCP	Alpha-tricalcium phosphate
β-TCP	Beta- tricalcium phosphate
TetraB	Tetrabone
TGF- β	Transforming growth factor-beta
TNF-α	Tumor necrosis factor alpha
TRAP	Tartrate resistant acid phosphatase
VEGF	Vascular endothelial growth factor
Wnt signaling	Wingless signaling pathway
XRD	X-ray diffraction

1 INTRODUCTION

1.1 Introductory remarks

Bone loss or insufficiency, due to local or systemic factors, remains a major challenge for bone-anchored implants. Guided bone regeneration (GBR) and bone augmentation represent two therapeutic modalities, which have been developed to restitute the bone. The first entails the application of a membrane, to cover the bone site, whereas the second includes the filling of the defect with bone substitutes.

The concept of GBR was developed based on the hypothesis that the membrane serves as a barrier, excluding non-osteogenic tissues from interfering with bone healing in the defect, thereby promoting bone formation[1]. Although the GBR concept is generally accepted, the underlying biological mechanisms and the role of the barrier membrane are yet incompletely understood.

Bone augmentation is based on implantation of biocompatible material to provide structural support to the defect site and support the intrinsic regenerative potential of the host tissue. Various forms of calcium phosphates (CaP) have been used widely as alternatives to bone autografts, the gold standard the for bone augmentation, because of their relative biocompatibility and similarity to bone mineral. A general characteristic of all CaP based materials is their osteoconductivity and ability to guide bone formation[2]. However, the ultimate outcome of bone healing is largely dependent on their specific physicochemical properties. In the context of CaP-based materials, the current knowledge of the material-cellular interactions is mainly gained from *in vivo* histological observations and *in vitro* cell culture experiments. Yet, the underlying *in vivo* mechanisms and the cellular events of the main processes of bone healing (inflammation, bone formation and remodeling) in association with CaP-based materials are incompletely understood and need further investigation.

As routine clinical procedure, GBR membrane is often applied in combination with bone grafting material. Combining e.g. the CaP-based substitutes with barrier membranes has the potential to result in a synergistic effect of both materials. While the membranes would isolate the bone defect site from non-osteogenic soft tissue, the bone substitute would maintain a three-dimensional scaffold, supporting the osteogenic cells and the promotion

of bone during healing. However, a hypothesis as such remains speculative since the mechanism of bone regeneration in conjunction with the membrane and the bone substitute is not sufficiently described.

In general, it is assumed that the design of future materials, both membranes and bone substitutes, requires an understanding of the mechanisms of tissue regeneration. Such knowledge would be beneficial for the design process, and even for tailoring of materials with specific properties for specific clinical indications.

1.2 Bone

The bony skeleton performs numerous vital functions. It shelters and supports soft tissues, and provides mechanical rigidity and stability. The skeletal surface is an attachment site and the lever arm for muscles, tendons and ligaments, which facilitate bodily movements. Bone is also a storehouse for mineral salts and fats, and the main anatomical site for hematopoiesis. The adult human skeleton consists of approximately 206 separate bones with different sizes, shapes and structure. The external surface of bone is covered by periosteum, a membrane consisting of two layers containing fibroblasts and osteoprogenitor cells. The inner surface of bone is lined by a thin layer of connective tissue called endosteum, which surrounds and walls off the inner medullary cavity of long bone[3]. The medullary cavity is occupied by the bone marrow, which is comprised of numerous blood vessels and various types of cells, e.g., adipocytes, erythrocytes, leukocytes, thrombocytes, and mesenchymal stem cells (MSCs)[4]. Bone tissue is composed of living cells embedded in a mineralized organic matrix. The organic phase consists of matrix proteins, mostly collagen type I and non-collagenous proteins e.g., bone sialoprotein (BSP), osteocalcin (OC), and proteoglycans and small amounts of lipids and osteogenic factors, e.g., bone morphogenetic proteins (BMPs). The inorganic components, primarily hydroxyapatite and other salts of calcium and phosphate represent about 70 % of the acellular part of bone[5].

1.3 Structure and composition of bone

Bone is categorized into cortical and trabecular bone. The cortical (compact) bone is the outer layer of bone, represents 80% of the skeleton, and is characterized by high density, slow turnover rate and high Young's modulus[5]. The structure of compact bone is based on osteons or Haversian systems. Each osteon consists of Haversian canal, a central channel surrounded by organized layers of bone known as concentric lamella. Between these

lamellae, osteocytes are located within lacunae, and connected with each other through cytoplasmic processes/dendrites, located in canaliculi. The Haversian canal contains one or two capillaries and nerve fibers, and is connected to the periosteum as well as the medullary cavity by transverse canals called Volkmann's canals (Figure 1). Trabecular (cancellous) bone is a porous bone enclosing numerous large spaces that give a honeycombed or spongy appearance. The bone matrix is organized into an irregular three-dimensional latticework of bony processes, called trabeculae. The spaces between the trabeculae are often filled with marrow. The trabecular bone is more elastic and has higher remodeling rate compared to the cortical bone[6].

On the microscopic level, bone tissue is classified into woven and lamellar bone. Woven bone is immature bone characterized by an irregular network of loosely packed collagen fibers that make it more flexible and mechanically weak. It forms rapidly, most notable in the fetus and during callus formation in fracture repair. It is a transitional tissue that is replaced, by the process of bone remodeling, by stronger mature tissue, i.e., lamellar bone, which is characterized by regular and parallel alignment of the collagen into concentric sheets. The lamellar pattern can be observed histologically both in compact and cancellous bone[6,7].

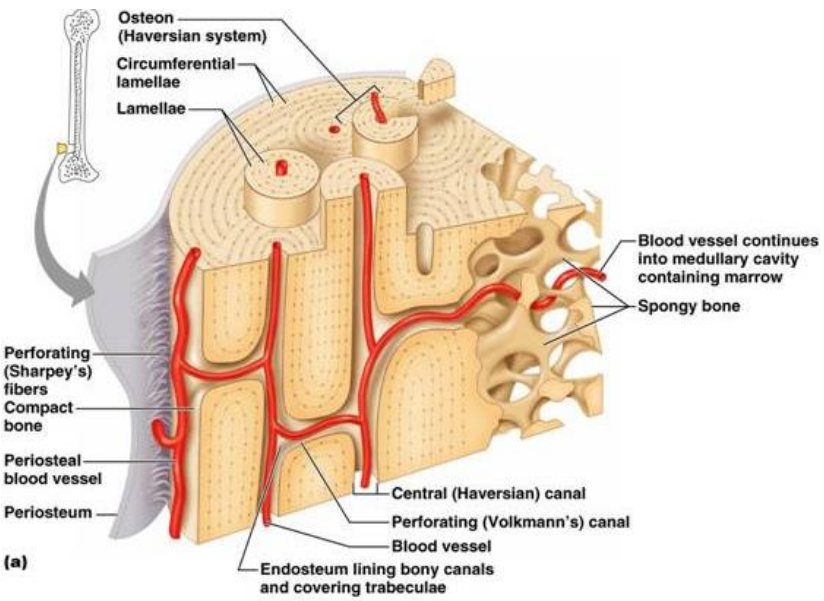


Figure 1. *The micro structure of bone showing the cortical and cancellous (spongy) bone as well as the Haversian system. MARIEB, ELAINE N.; HOEHN, KATJA, HUMAN ANATOMY AND PHYSIOLOGY, 7th Edition, © 2007. Reprinted by permission of Pearson Education, Inc., New York, New York.*

1.4 Bone cells

1.4.1 Mesenchymal stem cells (MSCs)

MSCs are multipotent stromal cells present in the bone marrow and most connective tissues, and are capable of differentiation into osteoblasts, chondrocytes, and adipocytes. Morphologically, they appear as spindle-shaped, fibroblast-like cells with a single large distinct nucleus. MSCs have a unique characteristic of selectively homing to sites of tissue injury and/or inflammation[8]. Many growth factors and chemokines secreted during bone injury regulate migration of MSCs such as insulin-like growth factor-1 (IGF-1) and stromal cell-derived factor 1 (SDF-1)[9]. It has been suggested that MSCs have an immunomodulatory function through direct cell-to-cell contact and/or release of soluble immunosuppressive/modulatory factors[10]. They can potentially interact with and inhibit proliferation and maturation of immune cells like B-lymphocytes and natural killer (NK) cells. MSCs recruited to the site of inflammation are suggested to play an important role in moderating the local inflammatory reactions via their effects on both innate and adaptive immunity[8]. During bone healing, MSCs differentiate into chondroblasts and osteoblasts to induce callus formation. In addition, they can produce trophic molecules, e.g., transforming growth factor β (TGF-β), interleukin 6 (IL-6)[11] and interleukin 10 (IL-10)[12] that can not only reduce inflammation and apoptosis in the damaged tissues, but also stimulate tissue cell regeneration. The MSC differentiation in the bone tissue is regulated by several molecules and intracellular signaling pathways. Activation of Wnt/β -catenin signaling in MSCs suppresses PPARγ, the adipogenic transcription factors, and stimulates Runx2, a transcription factor required for osteoblast differentiation. The pro-osteogenic growth factors also stimulate downstream signaling pathways (e.g., MAPK, p38, and SMAD pathways) regulating the differentiation of MSCs towards the osteochondral cell lineage[13].

1.4.2 Osteoblasts

Osteoblasts are cuboidal cells, lining a large percentage of the bone surface, and are primarily responsible for secretion of the organic matrix of bone. The osteoblasts originate from MSCs that differentiate into pre-osteoblasts, and then to osteoblasts under regulation of wide range of cytokines and growth factors[14]. The fully differentiated osteoblast appears with all characteristics of protein producing cells, e.g., large eccentric nuclei, and cytoplasm rich in secretory organelles and granules[15]. The osteoblasts deposit unmineralized matrix (osteoid) during the early phase of bone formation. Microscopically, seams of osteoblasts line the surface of newly formed matrix, where adjacent

osteoblasts are connected by gap junctions allowing the cells to function as a unit[16]. The bone matrix produced by the osteoblasts is composed of collagenous protein mainly collagen type I, and non-collagenous proteins including osteopontin (OPN), bone sialoprotein (BSP) and osteocalcin (OC)[17]. This osteoid tissue undergoes gradual mineralization by the nucleation and growth of bone apatite. Alkaline phosphatase (ALP) produced by the osteoblasts has a major role in the regulation of the bone mineralization process[18]. Osteoblasts can also express various cytokines involved in the formation of osteoclasts such as tumor necrosis factor alpha (TNF-α), receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG)[19]. The role of osteoblasts as bone forming cells is completed once they are embedded in the bone and become osteocytes. Osteoblasts can also become inactive and transform to bone-lining cells, which have a flat morphology and normally cover the surface of the quiescent bone[17].

1.4.3 Osteocytes

Osteocytes are stellate cells and constitute the main cellular component of mammalian bones, representing more than 95% of all the bone cells[15]. Once the osteoblast is embedded in the bone and becomes an osteocyte, major changes occur in the cellular morphology and the intracellular organelles such as decrease in the cell body size and increase in the cell processes[20]. Osteocytes occupy spaces (lacunae) in bone tissue, and communicate with each other by cytoplasmic extensions passing through small channels called canaliculi. At the molecular level, osteocyte differentiation is accompanied by lower production of several osteoblast markers, e.g., ALP, BSP, OC, collagen type I and Runx2[20]. Osteocytes act as mechano-sensors to control adaptive responses to mechanical loading of the skeleton. They are able to respond to the various types of stimuli and regulate skeletal hemostasis. It is believed that osteocytes can sense the need for bone remodeling[21]. Osteocytes are long-lived but not immortal cells and they die by apoptosis. The apoptosis of the osteocytes in response to bone microdamage has been suggested to initiate and increase the process of bone remodeling[22].

1.4.4 Osteoclasts

Osteoclasts are multinucleated cells that arise by the fusion of myeloid hematopoietic cells present in the bone marrow. Osteoclast precursors are either bone tissue residents or circulating monocytes[23]. Osteoclasts are characterized by a cytoplasm with a homogeneous, "foamy" appearance, due to a high concentration of vesicles and lysosomes filled with acid phosphatases[24]. Active osteoclasts exhibit a special cell membrane, known as

the ruffled border. Upon attachment to the bone surface, the osteoclast first develops the ruffled border opposing the resorption compartment and then creates an isolated microenvironment called the "sealed zone"[23,25]. Hydrochloric acid is produced by the osteoclast after mobilization of hydrogen and chlorine ions from inside the cells across the ruffled membrane. Due to the acidic environment, Howship's lacuna is formed as result of the dissolution process of the mineralized matrix. The remaining organic component is also dissolved by a collection of collagenolytic enzymes, cathepsin K (CatK) in particular[26]. Tartrate resistant acid phosphatase (TRAP) is also produced by osteoclasts and is involved in the process of bone resorption. Several chemokines regulate the recruitment, proliferation, and differentiation of osteoclast precursors at the sites of bone healing. Monocyte chemotactic protein 1 (MCP-1)[27] and stromal cell-derived factor 1 (CXCL12/SDF-1)[28] are considered as important molecules to control the migration of osteoclast precursors from the blood circulation into bone, or within a bone healing site[29]. Macrophage colony-stimulating factor (M-CSF) and RANKL expressed by osteoblasts, play a key role in osteoclast differentiation and activity. It is strongly believed that communication between the osteoprogenitors and osteoclast precursors through RANKL-RANK interaction stimulates the formation of the mature osteoclast. This interaction can be blocked by OPG, another cytokine also produced by osteoblast[30]. Furthermore, the inflammatory cytokines such as TNF-α and IL-6 are also important mediators for osteoclastogenesis during the bone remodeling process[31-33].

1.4.5 Inflammatory cells

The inflammatory infiltrate includes polymorphonuclear cells (PMNs), monocytes, and lymphocytes.

PMNs: PMN cells constitute the largest fraction of leukocytes[34]. They are the first immune cells to arrive at the site of inflammation[35]. The term polymorphonuclear leukocytes often refers specifically to neutrophil granulocytes; the most abundant PMNs. The other types of the PMNs (eosinophils and basophils) are few in numbers and are named according to staining properties of their cytoplasmic granules. In general, these cells are about 12 μm in size (about twice the size of erythrocytes) and their nuclei have a variable shape with several lobes. Neutrophils are recruited from the blood stream to the site of injury within minutes following trauma, migrate through the blood vessel wall and the extracellular matrix to the site, following the response to chemical signals such as IL-8, and C5a by a process called chemotaxis[36,37]. Neutrophils are phagocytic cells, which

interact with and may ingest foreign particles, bacteria and dead cells during the acute phase of inflammation[38]. PMNs are able to secrete pro-inflammatory cytokines, e.g., TNF-α, IL-1-β, chemokines, e.g., IL-8, and macrophage inflammatory proteins (MIPs) to allow the migration of more inflammatory cells, like monocytes/macrophages[39]. They also produce an angiogenic factor, vascular endothelial growth factor (VEGF)[40,41]. Neutrophils are short-lived, and predominate during the first several days following injury and are subsequently replaced by monocytes as the prevalent cell type[42].

Monocytes: Monocytes are phagocytic cells that circulate in the blood and constitute approximately 3 to 7% of all leukocytes in the human body[34]. They are the largest of all leukocytes (15–20 μm), identified by their large kidney shaped or notched nucleus. In normal conditions, monocytes can migrate to the connective tissue and differentiate into resident macrophages[43]. In response to inflammatory signals, monocytes migrate rapidly to sites of trauma or infection and differentiate into macrophages[44]. They express various inflammatory mediators, and also ingest and degrade microorganisms and foreign particles. During bone healing, macrophages are not only involved in the inflammatory phase, but also in bone formation and remodeling[45]. In mice, it has been shown that both systemic and local depletions of macrophages impair intramembranous ossification and delay fracture healing, whereas treatment with M-CSF increase macrophage recruitment and promote formation of woven bone[46]. Monocytes support osteogenic differentiation of MSCs via producing pro-anabolic factors such as oncostatin[47] and TNF-α[48]. Human monocytes also promote the osteogenic differentiation of MSCs via the secretion of exosomes and the up-take of exosomes in the recipient cells[49]. The monocytes/macrophages can differentiate to multinucleated cells, either osteoclasts during normal bone remodeling, or other phenotypes such as foreign body giant cells that appear in response to biomaterial implantation[50]. Although the activities of the monocytes are closely related to the immune responses and inflammation, it is believed that they play a major role in material-tissue integration[51,52]. They are also involved in cell-driven degradation of bioresorbable materials via phagocytosis and enzymatic degradation[52].

Lymphocytes: Lymphocytes (7–20 μm in size) travel in the blood, but they can normally leave blood capillaries towards the connective tissue[53]. There are three major types of lymphocyte (T cells, B cells and natural killer (NK) cells. T cells are involved in cell-mediated immunity, whereas B cells are primarily responsible for humoral immunity or antibody-driven adaptive immunity. They accumulate later during the inflammatory process. Their

presence in large numbers indicates the continuing presence of "non-self" antigens and/or infection. Nevertheless, the T cells have been suggested to play an important role of in fracture healing[54]. Several studies have shown that depletion of T-lymphocytes impairs bone healing in mice[55,56]. Furthermore, T-helper lymphocytes promote macrophage activity via secretion of cytokines such as TNF-α and IL-2[56,57]. The lymphocytes also express IL-17, which is a key mediator in the cellular immune response during osteogenesis[58]. Moreover, Th2 helper cells produce IL-4[56,57], an anti-inflammatory cytokine, which is considered as a bone resorption inhibitor[59]. Also, IL-4 and IL-13 expressed by Th2 helper lymphocytes have been shown to induce macrophage fusion and formation of giant cells at the biomaterial-tissue interface[60].

1.5 Bone healing

Bone healing is a complex, well-orchestrated process, involving interactions between different types of cells (e.g. hematopoietic and immune cells, vascular and skeletal cell precursors), and proteins as well as expression of various genes working towards restoring the function and structural integrity of bone tissue. In fact, the stages of embryonic bone development are recapitulated during bone healing[61]. Many cellular events are taking place in healing process including migration, proliferation, chemotaxis, differentiation and synthesis of extracellular proteins. It is hypothesized that all of these events are modulated when treating the healing site with calcium phosphate bone substitute and/or a GBR membrane. For proper explanation of this predictable modulation in the cellular events of bone regeneration process, understanding of the normal mechanism of bone healing is required. Bone healing is a continuous process, but can be divided into three overlapping phases (inflammation, bone formation, and remodeling).

1.5.1 Inflammation

Bone injury is associated with damage to the vasculature, bone matrix, and the surrounding soft tissues. The vascular endothelial damage results in extravasation of blood and platelet aggregation at the injury site, which initiates a cascade of blood coagulation and formation of blood clot (hematoma). A hematoma is a fibrin network that provides pathways for cellular migration whereas loss of this fibrous tissue lead to impairment of fracture healing[62]. Platelets and inflammatory cells within the hematoma release different growth factors and cytokines, which regulate the early cellular events of bone healing, such as cell migration, proliferation and synthesis of tissue matrix. The inflammatory cells including PMNs, tissue

macrophages and blood monocytes are among the earliest cells to be recruited to the injury site, releasing many pro-inflammatory cytokines and chemokines, e.g., IL-1, IL-6, TNF-α, MCP-1 and SDF-1[63]. These factors stimulate the recruitment of additional inflammatory cells, fibroblasts and MSCs. Migration and homing of MSCs to the healing site are crucial events, occurring during the early phase of bone regeneration. SDF-1 and its receptor chemokine receptor type 4 (CXCR4) are thought to have an important role in MSC recruitment. Release of SDF-1 is stimulated by the hypoxic condition in the hematoma[64]. Also owing to hypoxia, fibroblasts and endothelial cells release angiogenic factors such as VEGF to induce formation of new blood vessels. VEGF is not only considered to be an angiogenic factor, but also to act as a potent chemotactic stimulus for inflammatory cells, and a major stimulus for the migration and proliferation of MSCs and osteoblasts[65]. Furthermore, the pro-osteogenic, transforming growth factor (TGF) superfamily and BMPs are also produced during the early phase of healing, and play a significant role in the proliferation and differentiation of MSCs to fibroblasts and osteogenic lineages[66,67]. Throughout the first days of healing, fibroblasts produce collagen to form granulation tissue, which supports a variety of cell types associated with immune system and formation of extracellular matrix and blood vessels.

1.5.2 Bone formation

Bone repair can occur by different specific mechanisms primarily dependent on the biophysical environment. Bone formation takes place during the reparative stage of healing by intramembranous and/or endochondral ossification process[68]. For the intramembranous ossification, bone formation occurs directly without the formation of cartilage callus. The MSCs proliferate and condense around a profuse capillary network to form a center of ossification, where they differentiate into osteoblasts for subsequent formation of osteoid tissue[69]. On the other hand, the endochondral ossification takes place in an environment of interfragmentary space and mobility. It begins with the formation of a cartilage template, involving a cascade of recruitment, proliferation and condensation of MSCs that differentiate to chondroblasts to produce cartilagenous matrix[69,70]. The chondroblasts become chondrocytes after embedding in their own matrix and undergo a series of sequential changes, including cell proliferation, maturation and formation of hypertrophic chondrocytes, which calcify the cartilaginous matrix[71,72]. After matrix calcification, the hypertrophic chondrocytes undergo apoptosis and blood vessels penetrate the area, transporting osteoprogenitor cells to the site, which lead to replacement of the cartilaginous matrix by trabecular bone[71]. Several factors will influence the

type of ossification after bone injury, including type of injury, defect size, stability of the site, blood supply and oxygen tension. For example, the endochondral bone formation is the main process of bone repair in the bone fracture injury[73]. On the other hand, in drill-hole bone injury, which occurs in the case of creating a cylindrical bone defect, the intramembranous route is the principle process in bone formation[74]. The cellular and molecular signals that underlay these types of healing are different depending on the spectrum of the cytokines and growth factors at the site of healing.

Differentiation of osteprogenitors during bone formation

The potential sources of the MSCs that contribute to bone formation include local periosteum, bone marrow, and blood circulation[75]. Stimulation of MSCs to differentiate into the chondrocyte/osteoblast cell line is mainly regulated by TGF-β superfamily molecules, including TGF-β and BMPs. These molecules are produced by different types of cells and act on serine/threonine kinase membrane receptors on the progenitor cells. Activation of these receptors triggers intracellular signaling pathways, which stimulate the gene expression in the nucleus[76]. Many data have shown that BMPs induce a sequential cascade of events for chondro-osteogenesis, including chemotaxis, mesenchymal cell proliferation and differentiation, and controlled synthesis of extracellular matrix[77]. The regulatory effect of BMPs depends upon the type of the targeted cell, its differentiation stage, the local concentration of the ligand as well as the interaction with other circulating factors. In a comprehensive analysis of the osteogenic activity of 14 types of BMPs, BMP-2, -6, and -9 are suggested as the most potent to induce osteoblast differentiation of the MSCs[78]. Furthermore, TGF-β stimulates the recruitment of MSCs, and enhances their proliferation and differentiation toward the osteogenic lineage. In fact, binding of TGF-β/BMPs with their receptors on MSCs initiates the activation of Runx2, the osteogenic transcription factor, which in turn triggers the expression of osteogenic genes[79].

Several intracellular pathways are involved in the differentiation of the osteopogenitors, such as SMAD and p38 MAPK pathways[13]. Wnt signaling is also another important regulatory pathway for the osteogenic differentiation of MSCs. Activation of Wnt signaling pathway does not only shift the commitment of MSCs towards osteochondral lineages, but also inhibits the adipogenic differentiation[80]. Furthermore, high levels of Wnt signaling with the presence of Runx2 promote osteoblastogenesis at the expense of chondrocyte differentiation[80]. The osteogenic differentiation of MSCs is usually associated with high expression of ALP and collagen type I, the earliest markers of osteoblast phenotype. As a rule, ALP, type I collagen and the type I parathyroid receptor (PTH1R) are early markers of osteoblast

progenitors that increase as osteoblasts mature, but decline as osteoblasts become osteocytes[81]. Furthermore, in post-proliferative mature osteoblasts associated with mineralized osteoid, OC is highly up-regulated, and thus, considered as a late marker of osteoblasts[82].

1.5.3 Bone remodeling

Bone remodeling is a lifelong process of bone removal and replacement, essential for calcium homeostasis and preserving the integrity of the skeleton[83]. It also occurs during bone healing to alter the woven bone to lamellar bone structure, and restore the original shape and strength of the bone. The poorly placed trabeculae during this phase undergo bone resorption and formation at several bone sites. This process relies on the function of two principal cells of the bone tissue; the osteoclasts, that destroy the bone matrix, and the osteoblasts, the responsible cells for new bone formation[83]. The osteocytes are also another important type of cells involved in the remodeling process by their special mechano-sensory function[21,84].

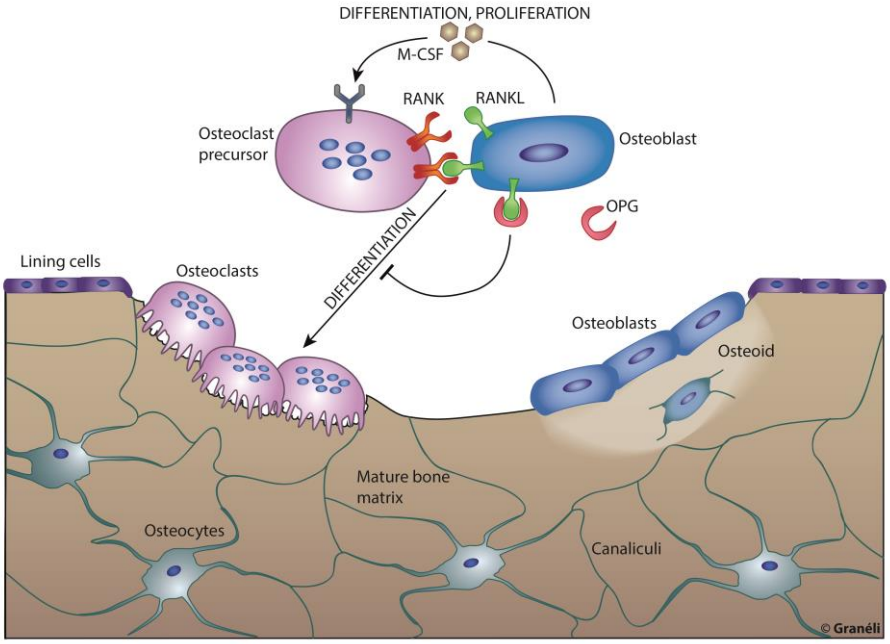


Figure 2. *Schematic illustrating of the basic multicellular unit of the bone remodeling and cells communication. Illustration: Cecilia Graneli*

The remodeling process occurs as a consequence of homeostatic demands through systemic activating signals (e.g. parathyroid hormone) and local

biomechanical cues, which initiate and sustain the process[85]. The remodeling process is initiated by the separation of the lining cells from the underlining bone in response to transmitted signals from the osteocytes. Osteoblasts and osteoclasts are then coupled within a cellular system called the basic multicellular units (BMU) (Figure 2) in which osteoclasts create a shallow resorption pit known as a Howship's lacuna to be filled later with new bone by osteoblasts[85,86]. Many cellular events and interactions are taking place at the remodeling site between the different types of bone cells, vascular and immune cells. The unique spatial and temporal arrangement of cells within the BMU ensures a coordination of the sequential phases of the bone remodeling process, which include activation, bone resorption, reversal, bone formation, and termination[83].

Communication between osteoblast and osteoclast during bone remodeling

The osteoblast and osteoclasts are coupled by a finely regulated system where the signaling take place reciprocally between the two cells. Whereas osteoclast formation is regulated by the osteoblast via RANKL and OPG[87], the differentiation and activation of the osteoblast are regulated by the osteoclast via direct[88,89] and indirect mechanisms[90-92]. In the restorative stage of bone remodeling, the osteoblast precursors produce RANKL, M-CSF, and MCP-1 (Figure 2) in response to signals generated by endocrine hormones e.g. PTH, and stimulate osteoclast formation[83]. In the context of inflammation, these cytokines are markedly increased following bone injury and, in addition to the osteoblast, are also produced by the immune cells like T cells and natural killer cells[93]. Differentiation of the osteoclast precursors to mature osteoclasts is initiated by the interaction of RANKL with RANK[94] (Figure 2). This interaction leads to activation of several transcription factors such as NF-κB, MITF, c-Fos, and NFATc[95], which are essential for osteoclast differentiation and expression of functionally relevant osteoclastic genes, including TRAP[96], CatK, and the calcitonin receptor (CR)[97]. The RANK-RANKL pathway could also be augmented by the inflammatory cytokines such TNF-α and IL-1[98,99]. The role of the osteoblast in bone resorption is also manifested by the production of matrix metalloproteinases (MMPs) in response to mechanical[100] and endocrine remodeling signals[101]. This group of enzymes degrades the unmineralized osteoid to expose the RGD adhesion sites within the mineralized bone, which allows the osteoclast attachment onto the bone surface and thereby producing bone resorption lacuna[83].

In the stage of bone formation, the osteoblasts need to produce, in the BMU, the exact amount of bone removed by osteoclasts. This balance is physiologically maintained by the locally generated cytokines that regulate bone cell communication and subsequent function.

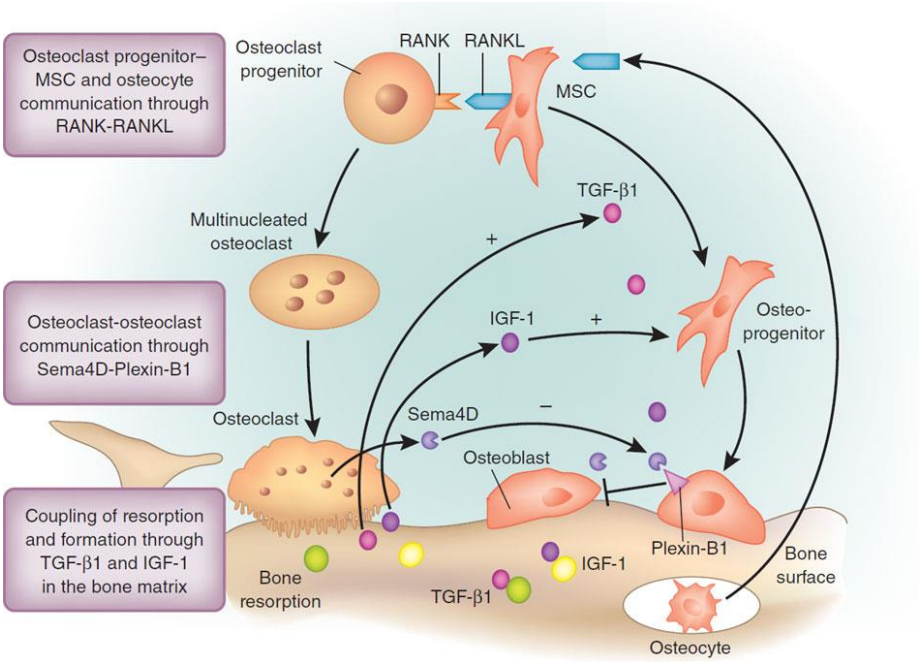


Figure 3. *Mechanisms of coupling osteoblast and osteoclast. First published by Xu Cao. 2011. Reprinted with permission from Nature Publishing Group.*

Several molecules stored in the bone matrix have been suggested to promote bone formation after their release by the osteoclastic resorption that represents the indirect effect of osteoclast on bone formation[92] (Figure 3). These molecules include different growth factors like IGF-1 and TGF-β, which stimulate osteoblast differentiation and support recruitment of MSCs to sites of bone resorption[90]. Recent studies have provided evidence that the osteoclast itself produces coupling factors that are actively involved in the interaction between the osteoclast and osteoblast. Several coupling mechanisms have been proposed; either via soluble molecules[89] or cell-cell contact[88]. For example, sphingosine-1-phosphate (S1P) is a soluble molecule secreted by osteoclasts which promotes the recruitment of osteoprogenitors and their maturation to osteoblasts[89]. Cardiotrophin-1 (CT-1) is another molecule detected in the actively resorbing osteoclasts, and has been shown to stimulate osteoblast differentiation in *vitro* and bone formation *in vivo*[102]. Furthermore, it has been reported that osteoclast-produced Sema4d has an inhibitory role on the osteoblast, equivalent to the effect of OPG on the osteoclast[103] (Figure 3). Bidirectional signaling can also be generated and transmitted between the two cells by interaction between Ephrin-B2 (ligand) on osteoclasts and EphB4 (receptor) on osteoblast precursors[104]. It is thought that the EphB4-Ephrin-B2 signaling complex simultaneously activates bone

formation and inhibits bone resorption during the transition stage of bone remodeling[105]. Direct cell-cell contact and indirect mechanisms are both required to achieve the coupling of bone formation and resorption. This is based on the assumption that the direct contact between osteoclasts and osteoblasts is not always possible, and indeed, osteoblast recruitment and matrix deposition continue for a long time after osteoclasts vacate the resorption site[83].

Table 1. Biological factors involved in bone healing and analyzed in the present thesis.

Factor	Biological process	Expressed by
TNF-α, IL1β & IL-6	• Acute inflammation and recruitment of cells • Regulation of both osteoblast and osteoclast activities	Macrophages & other inflammatory cells Osteoblasts
MCP-1	• Recruitment and activation of monocytes, leukocytes & MSCs	Monocytes, endothelial cells, fibroblasts, osteoblasts
CXCR-4	• Migration of progenitor cells (from mesenchymal and hematopoietic origins).	MSCs Endothelial cells Osteoclast precursors
VEGF & FGF-2	• Angiogenesis • Chemotaxis of monocytes • Growth and differentiation of MSCs	Monocytes MSCs Osteoblasts & chondrocytes
TGF-β & BMP-2	• Chemotaxis, proliferation and differentiation of osteoprogenitor • Differentiation of MSCs to osteoblasts • Regulation of osteoclasts	Platelets Leukocytes Fibroblast Osteoblast & chondrocytes MSCs
Col1a1, ALP & OC	• Osteogenic differentiation and bone formation	Osteoblasts & osteoprogenitors
CR, TRAP & CatK	• Osteoclastic differentiation and bone remodeling activities	Osteoclasts & pre-osteoclasts
OPG & RANKL	• Coupling of bone formation and bone remodeling	Osteoblasts

1.6 Bone augmentation

Bone augmentation is a surgical procedure performed to rebuild bone in bone deficiencies that are expected not to heal by the inherent regenerative capacity of bone tissue. It involves using natural or synthetic bone graft substitute materials to stimulate healing of bone. Bone regeneration might be accomplished through three different mechanisms: osteogenesis, osteoinduction and osteoconduction[106].

The osteogenic potential of a grafting material is governed by the presence of viable cells that are able to proliferate and differentiate to osteoblasts.

Osteoinduction is the ability of a graft material to induce the host MSCs to differentiate into bone forming cells through osteogenic growth factors.

Osteoconduction is a process whereby the bone graft supports the growth of host capillaries, vascular tissue and osteoprogenitor cells.

1.6.1 Bone grafting materials

Autogenous bone

An autogenous bone is a bone tissue transferred from one location to another within the same individual. It is the golden standard for bone augmentation and repair and has various applications in maxillofacial and orthopaedic reconstructive surgeries, e.g. spinal fusion, revision arthroplasty and repair of bone defects[107,108]. Autogenous bone can be harvested from both intraoral sites (e.g. the mandibular symphysis and ramus) and extraoral sites such as the iliac crest, distal femur and proximal tibia[107]. Due to the presence of a plethora of progenitor cells and growth factors, the iliac crest is the most common source of autograft bone. Furthermore, trabecular bone has more osteogenic potential than cortical bone due to presence of hematopoietic marrow that contains greater amount of MSCs[109]. Generally, autogenous bone is considered to have the best osteoconductive, osteogenic and osteoinductive properties among all the grafting materials currently available[110]. It provides bone matrix proteins and vital bone cells to the recipient site that enhance the overall success of the grafting procedure[111]. Despite the excellent biocompatibility of autogenous bone, the major disadvantages associated with autografting include the limited availability and donor-site morbidity. Several post-operative complications can be associated with the donor site, for example hematoma formation, nerve injury, chronic pain, bone fracture and tumor transplantation[107].

Bone graft incorporation with the host bone is a complex and incompletely understood process that involves a dynamic interplay between the bone graft and the graft environment, including host-graft mechanical interactions. This process ultimately leads to the replacement of the graft by host bone in a predictable pattern described as creeping substitution[108,112,113]. The biological response at the autograft recipient site begins with hematoma formation followed by inflammation and the subsequent formation of granulation tissue/fibrovascular tissue. The granulation tissue with the blood vessels quickly invades the graft through existing Haversian and Volkmann canals[112]. The blood vessels increase in number and size until the whole graft becomes fully vascularized and undergoes remodeling[114]. It has been reported that remodeling of the bone graft begins as soon as its vascular condition reaches the normal vasculature of bone[114]. While the vascular invasion and osteoclastic resorption of the graft progresses, the MSCs from both the graft and the recipient bed differentiate into osteoblasts and form woven bone on the surfaces of the original graft trabeculae. The hematopoietic cells also accumulate within the transplanted bone and form a viable new bone marrow. The creeping substitution continues for various periods of time depending on several factors such as the vascularity of recipient site, type of autogenous bone and the interface between the graft and host bone[113,114].

The overall incorporation mechanism is similar for cancellous and cortical bone autografts. However, they show different rates of creeping substitution and bone repair[112,115]. The autogenous cancellous bone has a highly vascularized and porous structure, and contains more viable cells than cortical bone, thereby promoting rapid revascularization and remodeling. Due to the high density of the cortical bone, a longer time for remodeling and complete revascularization is required for the cortical graft. It has been reported that the cancellous bone is typically revascularized within two weeks of implantation, whereas cortical grafts required up to two months for complete revascularization in humans[114]. For cortical grafts, the resorption process plays a much larger part in the graft incorporation. In contrast, the bone formation phase associated with cancellous bone grafts starts early, even before the restorative phase of creeping substitution[115]. Cancellous bone grafts typically become completely resorbed and replaced by new bone, whereas the cortical grafts are often incompletely remodeled for several months, and various pouches of the graft usually remain mixed with new host bone. In general, the autogenous bone eventually undergoes complete resorption and replacement by the host bone, although this may take months to years, depending on the type of autogenous grafting material and the overall healing capacity of the body[113,114].

Allogeneic bone

Allogeneic bone is a bone tissue obtained from human cadavers or living donors. It is considered as the first alternative to autologous bone graft. Bone allograft is available in various shapes, sizes, and endless quantity. The major benefit of using bone allograft is the avoidance of complications associated with the autograft harvesting procedure. Allogeneic bone has osteoinductive and osteoconductive properties, but not osteogenic potential due to the lack of viable osteogenic cells. The main disadvantages of the allografts include risk of infection transmission and host rejection[116]. To circumvent these problems, the harvested allogeneic bone undergoes disinfection and sterilization procedures using different methods including, freezing and lyophilization, radiation and ethylene oxide sterilization[117].

On implantation, the allograft demonstrates similar, but slower, sequence of biological events compared with the autograft[112]. It incorporates with host bone more slowly and incompletely than autograft. The immunological response to the allograft plays a critical role in the success of graft incorporation with the host bone[118,119] It is reported that both fresh and processed bone allografts may trigger strong immune responses, which affect the final clinical outcome. Moreover, such immune responses may delay and compromise the initial osteoinduction phase of the bone graft[118]. An allograft may also undergo rejection similar to other transplanted body organs through different mechanisms[120]. The pre-processing and sterilization of allogeneic bone reduce the risk of transmitted infection and graft rejection, but also affect the graft osteoinductivity[121]. Nevertheless, processed allografts have been shown to enhance the formation of new bone by their osteoconductive and osteoinductive properties[122]. The cross-linked collagen matrix and the available surface of the allogeneic bone support the recruitment and attachment of osteoprogenitor cells and the deposition of new bone[122]. The allograft osteoinductivity is mainly attributed to the release of the embedded growth factors in the bone matrix during active bone resorption. To enhance the osteoinductivity of allogeneic bone, decalcification has been introduced to remove the mineral phase and expose the underlying bone collagen and osteogenic growth factors such as BMPs[123,124]. The osteoinductivity of bone allograft does not depend on the mineralized or mineral deficient state only, but also on other factors such as the extent of decalcification, the donor age and size of allograft particles[123,125,126].

Xenogeneic bone

Xenogeneic bone is a tissue harvested from one species and implanted into a different species. The most commonly bone xenografts are obtained from coral, porcine, and bovine sources[106]. Bovine bone is known to be the most

used xenograft material for bone augmentation. Bovine bone was first used as freeze-dried, partially deproteinized or defatted bone. Very low success rate was reported with these materials due to the antigenicity and strong inflammatory reaction[127].

Deproteinized bovine bone (DBB) has been introduced as grafting material, containing only the mineral phase of bone after complete removal of the organic components by using different purification techniques[128]. DBB is classified amongst the calcium phosphate (CaP) group of biomaterials, and has a chemical composition nearly identical to that of human bone[128]. Although the use of DBB is limited in the orthopedic and load bearing applications[129], it is, by far, considered the most commonly used bone substitute for dental applications[127]. The DBB is widely used as grafting material in various techniques for bone augmentation and osseointegration, including maxillary sinus lifting procedure[130] and reconstruction of deficient alveolar bone[131]. Several reports have shown that DBB facilitates bone healing and implant osseointegration. However, the DBB persists for long period of time without resorption and replacement with host bone[132]. In recent years, controversy has emerged about the plausible beneficial effect of DBB when used in conjunction with the barrier membrane in guided tissue/bone regeneration. Although the use of DBB with a membrane has a good clinical documentation; some reports have shown that DBB inhibits the osseous healing and applying only a barrier membrane lead to better bone formation[133-135].

Synthetic bone substitutes

Due to the disadvantages of autogenous and allogeneic bone grafts, many efforts have been made to develop synthetic grafting materials as an alternative option for bone substitution. Various types of materials are currently available for bone repair, including metals, e.g. titanium and titanium alloys, polymers and synthetic ceramics, e.g. calcium phosphates (CaP) and bioactive glasses[136]. These materials have displayed different mechanical properties and potential of bone augmentation that explains the widespread use of certain material over the others. CaP-based biomaterials are the most common synthetic materials used in modern bone substitution, since they are claimed to be bioactive, stimulate apatite formation and bind directly to bone after implantation[137].

Advancement of calcium phosphates for bone repair

Calcium phosphates are the main component of the biological hard tissues. They are present in bone and teeth to provide stability and hardness. Structurally, natural calcium phosphate is poorly crystalline

nonstoichiometric sodium-, magnesium-, and carbonate-containing hydroxyapatite, called biological apatite[138]. The similarity with the natural apatite is the main rationale for using synthetic CaPs in different bone applications. Over the last decades, a variety of stoichiometric CaPs have been synthesized for medical purposes. The first application of CaP material for bone repair was reported in 1920 by Albee and Morrison[139]. They suggested a material called "triple calcium phosphate", able to stimulate bone growth for the treatment of bone fracture. About 50 years later, Monroe and his colleagues developed a new type of calcium phosphate (calcium-fluorapatite) to be used for dental and bone implants[140]. Nery and co-workers reported a first successful treatment of surgically created periodontal defects using a calcium phosphate that was obtained from sintering of "tricalcium phosphate reagent"[141]. The same material was further analyzed by LeGeros et al. and described as a mixture of HA and β-TCP[137].

Currently, synthetic CaP exist as different phases. For example, monocalcium phosphate (MCP), dicalcium phosphate dihydrate (DCPD), α- and β-tricalcium phosphate (α-TCP, β-TCP), octacalcium phosphate (OCP) and hydroxyapatite (HA). Both monophasic and biphasic CaP have been applied in orthopedics as bulk implant, granules, cement and as coatings on titanium implants. CaP biomaterials have a wide range of applications including repair of periodontal defects, augmentation of alveolar bone, sinus lift, tooth replacement, repair of large bone defects and spinal fusion. They are also used as scaffolds in tissue engineering for bone, cartilage and dentin regeneration[136]. Various types of CaP biomaterials have been commercialized for clinical bone augmentation. Particular attention has been given to HA due to its bioactivity, and to β-TCP due to its bioresorbability. HA, β-TCP and their mixture are the most documented calcium phosphates for bone repair[142-144]. OCP-based bone substitutes have also received great attention[145,146]. In recent years, many efforts have been made aiming to develop new CaP substitutes with high osteoinductivity by using biomimetic approaches[147] and employing principles of tissue engineering (e.g. CaP scaffolds combined with MSCs or biological cues)[148,149].

Biocompatibility of calcium phosphate materials

The CaP biomaterials have been proposed to have a superior biocompatibility due to their compositional similarity to the mineral phase of bone tissue. The bone implants and devices made of CaP induce short-term inflammation especially with less traumatic surgery and implant mobilization. The physiological nature is a major advantage for CaP ceramics. Both Ca^{2+} and PO_4^{3-} are highly acceptable to the normal physiological and cellular functions[150]. This is claimed to facilitate the integration of the material with

host tissue without strong inflammatory response and fibrous encapsulation. Moreover, CaPs are highly bioactive[151] and degrade without hindering the healing process; however, their biodegradability varies depending on the material phase composition and other physicochemical properties[136,152]. Moreover, the synthetic CaP ceramics have poor mechanical performance and different mechanism of bio-resorption[137,152].

In vitro bioactivity and cellular interaction with CaPs

The prevailing hypothesis for *in vivo* bone formation in response to CaP biomaterials is based on their bioactivity in terms of osteoconduction. CaPs stimulate bone tissue formation and accordingly directly bond with bone and form a uniquely strong biomaterial-bone interface[153]. The surface of CaP-based scaffolds sustains dissolution–re-precipitation cascades as a result of ion exchange at the solid-liquid interface in supersaturated conditions[136,153]. This process has been suggested to provide nucleation sites for the deposition of a biological apatite layer that facilitate bone bonding. It has been reported that the biological apatite layer that forms on surfaces of CaPs adsorbs circulating proteins from the biologic environment on which bone cells attach, migrate, proliferate, and differentiate, leading to matrix production[136,154-156]. In the biological systems, different types of serum proteins have been believed to be involved in the ionic exchange mechanisms of CaPs and subsequent cellular activities. However, as hundreds of proteins are present in biological fluids, their global effect on CaP reactivity is insufficiently understood[154].

One of the rationales of using CaP-based substitutes for bone augmentation is the physiological effect of Ca^{2+} and PO_4^{3-} on bone cells[157]. Ca^{2+} has been considered as one of the important mediators during bone remodeling, and to stimulate the differentiation of the osteoprogenitor cells and to produce chemotactic signals for different cell phenotypes[158]. These effects have been observed in several *in vitro* studies evaluating the osteogenic activities in response to different Ca^{2+}-functionalized biomaterials[159-161]. Also, the phosphate ion has been believed to play a critical role in the physiological mineralization of bone matrix; however, high levels of PO_4^{3-} in cell culture media have been shown to induce cell apoptosis[162]. CaP materials degrade not only by physicochemical dissolution but also with enzymatic cellular activity[163]. Multinucleated giant cells (MNGCs) have been observed in many studies in association with different types of CaP-based implants[164-166]. Although the osteoclastic phenotype of these cells is still controversial, they have been suggested to be involved in material degradation[152].

Osteoinductivity of calcium phosphate materials

CaP materials are generally known to be only osteoconductive, However, different types of CPs have been suggested to induce ectopic bone formation (osteoinductivity) like porous HA, BCP, β-TCP and OCP coating on Ti alloy[167]. Observations indicate that the geometry of bioceramics is critical for osteoinductivity[137,167], which has also been linked with the material dissolution behavior. The more soluble CaP materials have been suggested to be more osteoinductive, but a relatively stable surface is also required for bone formation to take place. To achieve this balance, biphasic calcium phosphate has been developed, based on a mixture of HA, the most stable phase, and, TCP, the more soluble phase[168]. The phase composition and the geometry are not the only determinants for osteoinductive potential of CaP ceramics. Other material properties, like microporosity, surface area and crystal shape have also been suggested to have an effect on the host cellular response and subsequently the level of bone induction[157,167].

The cellular mechanism of bone regeneration in response to CaP materials, and how the differences in their properties affect the cellular events of bone healing is still undetermined. The current knowledge on the cellular response to CaP materials is mainly based on *in vitro* data. However, the translation of these data to the *in vivo* situation is inadequate, and predictability is often inconclusive. In the highly dynamic condition *in vivo*, undefined number of biological factors is involved in the interactions between the CaP substitutes and bone tissue. The dynamic nature and the biological complexity of the *in vivo* environment is technically challenging to be translated into a simplified *in vitro* setting. Furthermore, CaP materials are reactive and their reactivity depends on their characteristics. As a consequence, the Ca^{+2} and PO_4^{3-} levels in the cell culture medium can vary substantially without being regulated and this largely affects the cellular functions and the final experimental results.

Inorganic additives to calcium phosphate

In contrast to the stoichiometric HA, bone mineral is a carbonated-HA and contains various amount of anionic and cationic substitutes such as sodium, fluoride, chloride, magnesium, strontium, zinc, copper and iron[169]. According to the biomimetic principle that a biomaterial should resemble as closely as possible the host tissue, these elements have been introduced in the preparation of CaP-based biomaterials to modulate their properties, such as crystallinity, biodegradability, and mechanical properties[170-172]. For example, the presence of magnesium and carbonate contributes to the formation of a poorly crystalline carbonated apatite that has a similarity to the bone mineral phase[150]. Moreover, ions like magnesium, silicate and strontium are

considered bioactive and doping them in the CaP lattice has been shown to improve the biological performance of CaP[173-175]

Strontium (Sr) is one of the elements that have been incorporated successfully into the calcium phosphate lattice (e.g. HA and TCP) by using different methods[176,177]. Sr has received great attention due to its natural occurrence in the bone mineral and the beneficial effect in treatment of osteoporotic bone[178]. Many *in vitro* data has indicated that strontium has a dual effect, stimulating bone formation and inhibiting bone resorption[179,180]. Although strontium-incorporated apatite may provide a promising bone substitute, the bone response to these materials and particularly the role of strontium during bone regeneration is not fully understood.

1.7 Guided tissue/bone regeneration

Guided tissue regeneration (GTR), as term, is often used synonymously and rather inappropriately with guided bone regeneration (GBR). GTR deals with the regeneration of the supporting periodontal apparatus, including cementum, periodontal ligament, and alveolar bone. GBR, on other hand, refers to the promotion of bone formation alone.

1.7.1 Guided tissue regeneration (GTR)

GTR has been introduced into clinical dental practice based on a concept that regeneration of a certain type of tissue is achieved when its progenitor cells populate the site of healing[181,182]. The procedure of GTR involves placement of a barrier membrane over the denuded lesions, to prevent migration of the epithelium and the gingival connective tissue to the root surface. This allows only the progenitor cells of adjacent periodontal ligaments (PDL) and alveolar bone to repopulate the wound healing site[181-183]. It has been suggested that cell exclusion in GTR prevents the establishment of long junctional epithelium[184] or other scenarios like ankyloses and root resorption[185]. GTR is a successful therapeutic modality for the treatment of gingival recession, and different types of periodontal defects e.g. intrabony and class II furcation defects[186,187]. It has been shown that GTR can achieve similar or even better clinical outcome compared to conventional grafting procedure[186,188].

1.7.2 Guided bone regeneration (GBR)

GBR was introduced to augment and repair bone deficiency of alveolar bone in the oral cavity. The concept of GBR was developed on the same principle as GTR, and accordingly a barrier membrane is also used, aiming to exclude

the non-osteogenic tissues from interfering with the bone regeneration process[1,181]. Bone augmentation in GBR is presumed to be achieved when the osteoprogenitors are exclusively allowed to repopulate the bone defect site by preventing the entry of soft tissue cells that may jeopardize bone formation[181]. Since establishment of its predictable intraoral approach in the late 1980s, GBR became routine surgical procedure for alveolar bone augmentation and treatment of peri-implant bone deficiencies[181]. Various non-resorbable and resorbable membranes have been used for the experimental and clinical studies in the context of GBR treatment[1]. The desirable characteristics of the membrane utilized for GBR therapy include biocompatibility, cell occlusion properties, integration by the host tissues, clinical manageability and space making ability[189].

Non-resorbable membranes

The non-resorbable barriers are available in different types, including expanded polytetrafluoroethylene (e-PTFE), or titanium mesh. e-PTFE is the earliest commercial, and clinically most popular non-resorbable membranes, and considered as standard for GBR[190]. e-PTFE is a stable material in the biological system and does not elicit an immunologic reaction. However, exposure of e-PFTE to the oral cavity results in migration of microorganisms and bacterial infection, possibly because of the highly porous structure[1]. d-PTFE with small pore size was developed to elucidate this problem. The outcome of GBR was relatively improved by using this membrane compared to the conventional e-PTFE[191]. Titanium-reinforced PTFE and titanium mesh have also been introduced to provide additional stability and space maintenance[192]. Although the use of non-resorbable membranes result a good clinical outcome, for alveolar bone augmentation, the second surgical intervention for membrane removal remains a major disadvantage. In addition, the bacterial colonization and wound infection associated with such types of membranes, can compromise the result of bone augmentation and osseointegration[193,194].

Bioresorbable membranes

The bioresorbable membranes have been developed to avoid the need for surgical removal. They are classified into synthetic and natural derived membranes. The synthetic membranes are made of polymers like polylactide (PLA) and poly-L-lactide-co-glycolide (PLGA) co-polymers[195]. The unlimited supply is the main advantage of the polymeric membranes; however, the degradation of the synthetic copolymers might elicit a strong inflammatory response leading to resorption of the regenerated bone[196,197]. In addition, it is considered that the high degradation rate of the polymeric material reduces the available function time of the barrier membrane and its

space making ability, which may affect the outcome of bone regeneration. Nevertheless, many reports have indicated a successful use of the polymeric membranes in preserving and augmenting the alveolar bone after loss of dentition[198]. Polymeric membranes have also been used with autogenous bone graft for reconstruction of mandibular and long bone defects[199,200].

The natural resorbable membranes are based on collagen, and have received great attention over the recent years. This is mainly because of the natural characteristics of the collagen material as being the principal component of connective tissue providing structural support for various tissues in the body. The interest in the collagen membranes was also because of other properties such as low immunogenicity[201]. Although some clinical reviews have indicated comparable clinical outcomes between collagen membranes and non-resorbable membranes, other studies have suggested that the collagen membranes may promote even better wound healing and bone regeneration[201]. The main disadvantage of the collagen membranes is the lack of rigidity, and thereby their use is considered limited to the types of alveolar bone defects that do not require extra fixation and stability, like bone dehiscence and fenestration and the periodontal defects[1,201]. Currently, many types of collagen membranes are commercially available for GBR in the maxillofacial surgeries. They are derived from different bovine and porcine tissues (e.g. tendon, dermis and small intestine), and their degradation vary depending on the animal source[201]. The collagen membranes are bioresorbable, and their rate of degradation might not meet the required duration for optimal tissue regeneration. Methods of cross-linking have been utilized to slow the degradation of collagen during time of implantation[202,203]. However, some reports have indicated that the prolongation of the membrane degradation is not always associated with greater periodontal/bone regeneration. During GTR, the early stage of healing has been considered as crucial period where the membrane should remain intact, after that, at the late phase of healing, the presence of the membrane might delay the maturation of connective tissue[201]. In GBR, due to the lack of rigidity, the collagen membranes are often used in conjunction with grafting material that maintains the defect space and prevent the membrane from collapsing due to the compression of the overlying soft tissue[195]. The combination of grafting materials with the barrier membrane does not only maintain the secluded space, but may also provide osteoconductive and/or osteoinductive capacities[195].

Recent advances of GBR membranes

Principles of tissue engineering have been recently suggested in the field of GBR in order to tailor make a bioactive barrier membrane, which structurally

and functionally mimics the native extracellular matrix (ECM)[204]. Electro spinning technology has been used to produce biocompatible and degradable polymers that may resemble the arrangement of the native ECM[205]. Moreover, multilayered barrier membranes, have also been proposed, with compositional and structural gradients that meet the local functional requirements[206]. Whereas bone formation would be stimulated by calcium–phosphate based nanoparticles or growth factors (e.g., BMP-2, TGF) on the hard tissue/membrane interface, bacterial colonization would be inhibited by antibacterial drugs delivered at the soft tissue/membrane interface. HA incorporated-collagen membranes have been shown to promote the functional activity of osteoblast *in vitro* and induce bone formation *in vivo*[207]. The combination of growth factors and a barrier membrane has also received great attention and suggested as a promising approach to enhance the bioactivity of the barrier membrane. Various types of resorbable membranes loaded with growth factors have suggested better bone formation and defect union compared to the corresponding unloaded membranes after implantation in different animal models[208-211].

2 AIM

The main aim of this thesis has been to determine the relationship between bone regeneration and the cell types, their distribution and activities in experimentally created bone defects augmented with calcium phosphate materials and/or with GBR membrane.

2.1 Specific aims of the included studies

- To investigate the molecular and structural events of bone healing in an experimental rat model, using surgically created defects with and without augmentation with natural deproteinized bovine bone (DBB) or synthetic tetrapod-shaped calcium phosphate (TetraB).

- To study and compare the healing process in bone defects augmented with hydroxyapatite (HA) or strontium-doped calcium phosphate (SrCaP) granules in both normal and compromized bone conditions.

- To investigate the molecular and structural pattern of GBR, with particular emphasis on the role of the barrier membrane, in bone defects covered with naturally derived resorbable membrane and compared with defects without membrane.

- To determine the relationship between the early events of bone healing and the cellular activities in response to a combination of GBR membrane and different calcium phosphate materials. Two specific aims were addressed. Firstly, to determine if the presence of bone substitutes in membrane-covered defects alters the early bone formation compared with empty, membrane-covered defects. Secondly, to explore the effect of presence of Sr ions in the HA on the early cellular and molecular activities and on the level of bone formation.

3 MATERIALS AND METHODS

3.1 Materials

In this thesis, different types of calcium phosphate granules were used as bone substitute for bone augmentation.

1. **Deproteinized bovine bone (DBB) (irregular shape):** the clinically available material was purchased as granules, 250-1000 µm (Bio-Oss®; Geistlich Pharma, Wolhusen, Switzerland) (Paper I & IV).
2. **Tetrabone (TetraB) (tetrapod shape):** the tetrapod-shaped synthetic bone granules were made of α-tricalcium phosphate and octacalcium phosphate (collaboration with University of Tokyo, Japan). (Paper I).
3. **Hydroxyapatite (HA) (column shape)**: HA powder was first prepared using precipitation methods and subsequently molded as column shape granules with a diameter of 1.5 mm and height of 1.5 mm (Paper II).
4. **Strontium-doped calcium phosphate (SrCaP) (column shape)**: SrCaP powder was prepared using surfactant-free mineralization method and subsequently molded as column shape granules with a diameter of 1.5 mm and height of 1.5 mm (Paper II).
5. **Hydroxyapatite (HA) (irregular shape):** Column shape granules were first prepared from the precipitated HA powder. The granules were then ground and sieved to smaller granules with a size of 400-600 µm (Paper IV).
6. **Strontium hydroxyapatite (SrHA) (irregular shape):** SrHA powder was synthesized also by precipitation method with adding strontium nitrate as dopant source, in the aqueous solution. SrHA was made in different ratios of Sr/Ca substitution. (SrHA005, SrHA025, and SrHA050). The granules were then ground and sieved to smaller granules with a size of 400-600 µm (Paper IV).

In addition, a membrane for guided bone regeneration was used with and without bone substitutes.

1. **Extracellular Membrane (ECM):** ECM was extracted from porcine small intestine (DynaMatrix®; Keystone Dental, Boston, USA). This membrane consists of approximately 90% collagen (mainly Type I)[212], with smaller amounts of glycosaminoglycans[213], glycoproteins and some growth factors[214] (Paper III & IV).

3.2 Material characterization

3.2.1 Morphology and surface structure

Scanning electron microscopy (SEM) was used to evaluate the morphology of the different granules and the membrane used in this thesis. The surface ultrastructure of the granules including morphology, surface texture, and crystalline orientation was also evaluated. The analysis was performed using a field-emission scanning electron microscope (FESEM, LEO 1550) working at 5 kV in secondary electron mode. Magnifications between 50 and 100,000 times were used to assess the macroscopic geometry of the materials and the micron scale surface morphology.

3.2.2 Phase composition and crystallinity

X-ray diffraction (XRD) was used to characterize and identify the crystalline structure of the materials. XRD analysis of the powder and granules was conducted on a Siemens Diffractometer 5000 with Cu (Kα) radiation at an operating condition of 40 kV and 40 mA. Crystal phase identification was determined by the database from ICDD (International Centre for Diffraction Data).

3.2.3 Elemental composition

Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was used to determine the amount of different ions in each granule type and also the ratio of strontium substitution with calcium in the strontium doped CaP materials (SrCaP, SrHA). The granules were dissolved in 1 M HCl solution and analyzed by ICP-AES (Paper II and IV).

3.2.4 Surface area and porosity

Brunauer–Emmett–Teller Technique (BET) was used to determine the surface area of the HA, SrHA and DBB granules (Paper IV). Micro computed tomography (μCT) was used to determine the porosity of the HA and SrCaP granules (Paper II).

3.2.5 *In vitro* degradation and ion release

The *in vitro* degradation of the different types of CaP granules (Paper II and IV) was evaluated in simulated body fluid (SBF). Equal amount of granules were soaked in the SBF, and put on a horizontal shaker up to 28d. The weight loss of the granules was evaluated by collecting the rest of the granules in the containers at 3, 7, 14 and 28d (Paper II) and 7 and 28d (Paper IV). The release of strontium, calcium, phosphate and magnesium was determined over time using SBF (Paper II), and Tris-HCl buffer solution (pH=7.4) (Paper IV) as dissolution media. In brief, the granules were soaked in dissolution media for different time periods (n = 3 for each granule type and time point) and the surrounding medium was collected, after 3, 7, 14 and 28d, and analyzed using ICP-AES.

3.3 Experimental designs and animal model

The experimental designs of the studies are summarized in Table 2.

3.3.1 Ethical approval

The animal experiments were approved by the University of Gothenburg Local Ethics Committee for Laboratory Animals (Paper I; dnr 301-2009, 279/2011, Paper II, III & IV; dnr 279/2011).

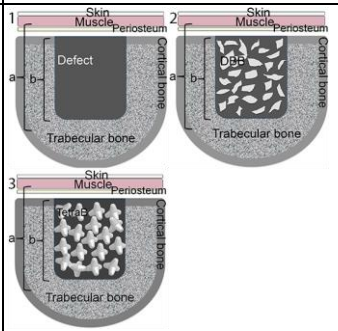
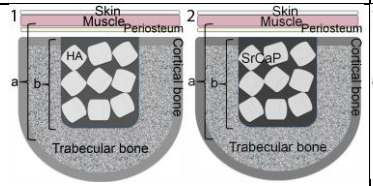
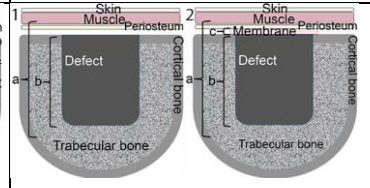
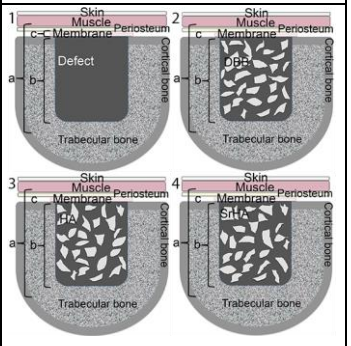
3.3.2 Pre-testing in polyurethane foam

The amount of the materials needed to fill a defect in the animal model was estimated by filling comparable sized holes created in solid rigid polyurethane foam. The foam provides consistent and uniform material with properties in the range of human bone (Sawbones®, Pacific Research Laboratories, Vashon, United States). (Paper II & IV).

3.3.3 Animal model and surgery

The Sprague Dawley rat was used as experimental animal in this thesis. Only in Paper II, ovariectomized (OVX) rats were also used in addition to the normal (non-OVX) rats. The ovariectomy was performed at 12 week of age. The rats were then received from the supplier one week later together with the normal rat. The normal and OVX rats in Paper II were allowed three weeks acclimatization period and free movement with food and water *ad libitum*, while the normal rats in the other papers were allowed 1 week. The bone regeneration was studied in trabecular femur defects augmented with different CaP substitutes with or without a barrier membrane. The defect was created bilaterally in each animal. The defect received

Table 2. Summary of the experimental design for each study in the thesis.

	Paper I	Paper II	Paper III	Paper IV
Experimental model				
Variables	1. Sham 2. Deproteinized bone (DBB) 3. Tetrapod-shaped calcium phosphate (TetraB)	1. Hydroxyapatite (HA) 2. Strontium calcium phosphate (SrCaP) Animals: Non-OVX & OVX rats	1. Sham 2. Membrane	1. Membrane, sham 2. Membrane, DBB 3. Membrane, HA 4. Membrane, SrHA
Techniques & time points	(a) Histology, histomorphometry (n=6) and ultrastructural analyses (b) Gene expression (n=8) 3, 6, 14 and 28d	(a) Histology & histomorphometry (n=8) (b) Gene expression (n=8) Baseline, 6 and 28d	(a) Histology & histomorphometry (n=8) (b) Gene expression (n=8) (c) Protein analysis (Western blot) Baseline, 3, 6 and 28d	(a) Histology, Immunohistochemistry and histomorphometry (n=6) (b) Gene expression (n=8) Baseline, 12h, 3 and 6d

either a bone substitute (DBB, TetraB, HA, SrCaP, or SrHA), ECM membrane, a combination of bone substitute and membrane or left empty (Table 2). The different sites in each study were randomized, ensuring equal distribution and rotation among the animals and between right and left locations.

The surgical procedure was similar in all papers of this thesis with some variations during the retrieval procedure depending on the subsequent analytical techniques used in each study. The surgery was performed under general anaesthesia using isoflorane inhalation (Isoba Vet, Schering-Plough, Uxbridge, UK) with a Univentor 400 anesthesia unit (Univentor, Zejtun, Malta). Anesthesia was maintained by the continuous administration of isofluorane via a mask. The distal aspect of the femur was cleaned with 5 mg/mL chlorhexidine and 70% ethanol. Subsequently, the site was shaved, anaesthetized with local anesthesia and then a longitudinal incision was made, followed by skin and periosteal reflection with a blunt instrument. A defect was made in each femoral epiphysis (trabecular bone region) using a trephine with a 2.3 mm internal diameter and 3 mm penetration depth under profuse irrigation with NaCl 0.9%. With the exception of paper I, the bone harvested from the defect site was collected from the trephine and preserved for determination of steady-state (baseline) gene expression. After augmentation of the femur defects with different materials, the subcutaneous layer of the wound was closed with resorbable polyglactin sutures (4-0, Vicryl) and the skin was closed with intracutaneous, resorbable monocryl sutures (4-0). Postoperatively, each rat received an analgesic (Temgesic 0.03 mg/kg, Reckitt & Coleman, Hull, UK) by subcutaneous injection. Animals were sacrificed using an overdose of barbiturate (Mebumal, ACO Läkemedel AB, Solna, Sweden) at different healing periods ranging from 12h up to 28d to retrieve the biological samples for different analytical techniques. In Paper II, before sample retrieval, blood was withdrawn from the jugular vein for an enzyme-linked immunosorbent assay (ELISA). After the animal sacrifice, different retrieval procedures were performed depending on the intended analysis. Sites intended for gene expression analysis (qPCR) (Papers I - IV) were retrieved using a 2.3 mm trephine and the samples were immediately preserved in tubes containing RNAlater (n = 8). The sites designated for histology, histomorphometry (Papers I - IV) and immunohistochemistry (Paper IV) were harvested en bloc using dental disc and immersed in formalin for fixation (Papers I - III; n = 8, Paper IV; n = 6). In Paper III, the membrane was gently retrieved by small tweezers and preserved for gene (qPCR) and protein analysis (Western blot) (n = 8).

3.3.4 Biological analyses

Histology and histomorphometry (Papers I-IV)

The formalin-fixated bone blocs were dehydrated in ascending series of ethanol, followed by embedding in acrylic resin (LR White) (London Resin Company Ltd, Berkshire, UK). The embedded specimens were cut along the long axis of the defect using a diamond saw. Subsequently, one half of each specimen was used to produce ground sections prepared by sawing and grinding (Exakt Apparatebau GmbH & Co, Norderstedt, Germany). The sections were made with a thickness of 10-20 mm and stained with 1% toluidine blue. The sections were then coded and evaluated blindly using light microscopy. The qualitative histological evaluation was made in a light microscope (Nikon Eclipse E600; Nikon Ltd., Tokyo, Japan). A quantitative histomorphometry analysis was performed in the same microscope using analytical software (Easy Image Measurement 2000; Bergman Labora AB, Huddinge, Sweden) to quantify the newly formed bone and the remaining bone substitute materials within the bone defect after different time periods. The analyzed histomorphometric parameters and the regions of interest in the defect in each paper are summarized in Table 3.

Histomorphometry was carried out on each section using a 10x objective. The amount of newly formed bone (Papers I-IV), the granules (Paper I and II) and bone-granule contact (Paper I) in the defect was determined using a counting procedure. A software grid consisting of multiple zones was superimposed over the tissue and the percentage area occupied by newly formed bone or granules was calculated. In order to estimate the relative proportion and distribution of the bone in the defect, the following regions were identified (Figure 4): (1) the entire defect, represented by all zones of the grid; (2) peripheral region (P), represented by zones covering the lateral and bottom borders of the defect, and central region (C), represented by zones covering the center of the defect; (3) top region (Top), represented by the upper zones adjacent to the membrane, followed by middle level (Middle) and bottom level (Bottom). The top, middle and bottom regions were only identified in Paper III and IV. The area of bone (Papers I-IV) and granules (Paper I, II) was determined separately in every zone and the area percentage was then calculated with respect to the total defect area (Papers I-IV) and to the respective region (central, peripheral; Paper II, III & IV) (top, middle, bottom; Papers III & IV). In Paper I, the bone-granule contact was determined.

Table 3. The analyzed histomorphometric parameters and the regions of interest in the defect.

Paper	Parameters	Region of interest (ROI) in the defect
I	Bone and granule area Bone-granule contact	The entire defect
II	Bone and granule area	The entire defect, peripheral & central regions
III	Bone area	The entire defect, peripheral, central, top, middle & bottom
IV	Bone area	The entire defect, peripheral, central, top, middle & bottom

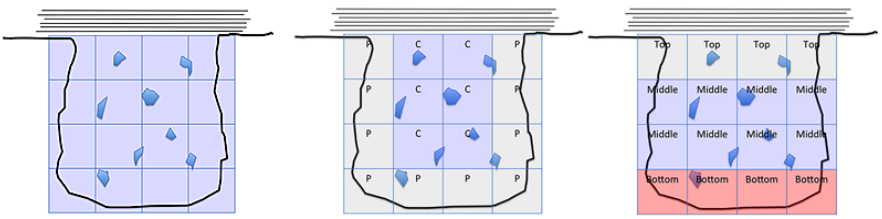


Figure 4. *Schematic diagram of the defects and the area of measurements (histomorphometry). C = Central region; P = Peripheral region.*

Immunohistochemistry (Paper IV)

Immunohistochemistry was used in Paper IV to visualize the distribution and localization of monocytes, macrophages and multinuclear cells, as well as osteoprogenitors and mesenchymal stem cells in the bone defect and the membrane at 12h, 3 and 6d of healing. After fixation, the harvested bone block was decalcified in 10% EDTA and embedded in paraffin. The paraffin-embedded block was sectioned into 3-5 µm slices with a microtome. The sections were then mounted onto glass slides and stained with hematoxylin and eosin for light microscopy. For immunostaining, 3-5 µm paraffin-embedded sections were mounted on polylysine slides (Menzel GmbH and Co KG, Braunschweig, Germany). The sections were deparaffinized, hydrated and incubated with primary antibodies CD68 (sc-58965, Santa Cruz Biotechnology, USA), and periostin (ab14041, Abcam, UK). The CD68 targets mononuclear cells (monocytes/macrophages) as well as multinucleated giant cells and osteoclasts[215-217]. The periostin was used as marker for osteoprogenitor cells and intramembranous bone formation[218].

Negative control slides were prepared by omission of the primary antibody and incubation with 1 % BSA in PBS. The immunoreactivity of CD68 and periostin was detected and visualized using Betazoid DAB Chromogen kit and horseradish peroxidase (HRP) (Biocare Medical, USA). The relative proportions of the CD68 positive cells, per mm^2 of the defect, were semi-quantitatively scored as three major subcategories: (i) mononuclear, monocyte/macrophage phenotype, (ii) multinuclear, osteoclast phenotype (associated with bone remodeling site) and (iii) multinuclear giant cell phenotype (associated with material or in soft tissue). The periostin staining was evaluated qualitatively.

Ultrastructural analysis (Paper I)

To evaluate the degree of bone mineralization in the defect and the nature of the material-bone interface, the other halves of the plastic embedded specimens were prepared for electron microscopy. The cut side was polished and the specimen was glued to a regular SEM stub and subsequently coated with a 10 nm thick conductive coating (AuPd). Backscatter SEM (BS-SEM) was performed in a Leo Ultra 55 FEG SEM (Leo Electron Microscopy Ltd., Cambridge, UK) operating at 20 kV. Magnifications between 50 and 50,000 were used to evaluate the bone response and resolve the interfacial reactions over time. For the 28d time point, transmission electron microscopy (TEM) samples across the material-bone interface were prepared using focused ion beam (FIB, FEI StrataDB235) in-situ lift out[219]. The samples were thinned to a thickness of approximately 100 nm using a decreasing ion beam current. TEM analyses were performed in a FEI Tecnai TEM/STEM, using both bright-field TEM and high-angle annular dark-field STEM (HAADF-STEM).

Gene expression (qPCR) (Papers I-IV)

qPCR is based on the polymerase chain reaction (PCR), which is used to amplify and simultaneously quantify a targeted DNA molecule. In order to identify the amount of specific mRNA in the biological sample, cDNA is first produced from the target RNA by reverse transcription to be a template in qPCR reactions. The quantity can be either an absolute number of copies or a relative amount when normalized to additional normalizing genes. In this thesis, selected panels of genes involved in inflammation, cell recruitment, bone formation and bone remodeling and coupling were analyzed in the defect sites samples (Table 4). Furthermore, genes of selected growth factors were targeted in the membrane samples (Paper III). To quantify the relative expression level of these genes in the retrieved samples. qPCR assay was performed as following:

1. Sample homogenization**:** samples were homogenized in phenol/guanidine-based Qiazol lysis reagent using 5 mm stainless steel beads (QIAGEN GmbH, Hilden, Germany) and TissueLyser (QIAGEN GmbH,Hilden, Germany). The homogenization was performed in phenol/guanidine-based trizol lysis (QIAGEN GmbH, Hilden, Germany) for defect site samples, and in Guanidinium thiocyanate lysis buffer (Macherey-Nagel, Germany) for membrane samples. For the defect site samples, the aqueous phase was used for subsequent RNA extraction. The phase separation was performed by addition of chloroform, followed by centrifugation at 12000g for 15 min.
2. RNA extraction: Total RNA from the defect site samples was extracted using RNeasy® Mini kit (QIAGEN GmbH, Hilden, Germany). The NucleoSpin RNA/protein isolation kit (Macherey-Nagel, Germany) was used to extract the total RNA from the membrane samples.
3. RNA purification: To reduce genomic DNA contamination, all samples were DNase treated with an RNase-free DNase Set (Qiagen GmbH, Hilden, Germany).
4. Reverse transcription and cDNA synthesis: Before the reverse transcription, the samples were normalized to 25 ng/µl; Reverse transcription was carried out using an iScript cDNA synthesis Kit (Bio-Rad, Hercules, USA) (Paper I, II), and GrandScript cDNA Synthesis Kit (TATAA Biocenter, Sweden) (Paper III, IV).
5. Real time polymerase chain reaction: Before the analysis, primers for the targeted genes were designed using Primer3-based software[220]. The panel of the targeted genes in each individual paper is summarized in Table 4. The expression profiles of reference genes were evaluated by geNorm[221] and Normfinder[222]software, in order to determine the best reference gene for normalization. Real-time PCR was performed on all samples in duplicates with the assays targeting the different mRNA transcripts and the best two or three selected reference genes in 10 ml reactions. The cycling conditions were 95 °C for 10 min, followed by 45 cycles of 95 °C for 20 s, 60 °C for 20 s and 72 °C for 20 s. The fluorescence was read at the end of the 72 °C step. Melting curves were recorded after the run by a stepwise temperature increase (1 °C /5 s) from 65 to 95 °C.

6. Quantification: Quantities of the target genes were normalized using the mean of Cq values of the selected reference genes. The normalized relative quantities were calculated using the delta-delta Ct method and 90% PCR efficiency ($k*1.9^{\Delta\Delta ct}$).

Table 4. The genes of interest in each paper.

Paper	site	Targeted genes	Reference genes
I	Defect	TNF-α , IL-1β, ALP, OC, TRAP and CatK	HPRT1 & YWHAZ
II	Defect	TNF-α, IL-6, caspase 3, Col1a1, ALP, OC, OPG, RANKL, CR, CatK, VEGF	HPRT1 & YWHAZ
III	Defect	TNF-α, IL-6, CXCR4, MCP-1, ALP, OC, CR, CatK, RANKL, OPG, RANK	HPRT1, GAPDH & ACTB
	Membrane	BMP-2, FGF-2, TGF-β1, VEGF	
IV	Defect	TNF-α, IL-6, MCP-1, CXCR-4, ALP, OC, RANKL, CR, CatK	HPRT1

Western blots (Paper III)

Western blot was performed to determine the presence of selected growth factors proteins (FGF-2 and BMP-2) in the native as well as in the retrieved membranes at 3, 6 and 28d.

The membrane samples were homogenized, using 5 mm stainless steel bead and TissueLyser (QIAGEN GmbH, Hilden, Germany). The total RNA from the membrane samples was extracted using the NucleoSpin RNA/protein isolation kit (Macherey-Nagel, Germany). The protein concentration (native and retrieved membrane) was determined using a BCA protein assay kit (Pierce, Thermo scientific, USA). Fifty µg from the protein extract of the native and retrieved membrane samples was prepared in Laemmli sample buffer (Bio-Rad Laboratories, Inc). The samples were heated at 95 °C for 5 min, cooled instantly and loaded on a 10% TGX protean precast gel (Bio-Rad Laboratories, Inc) for gel electrophoresis. The separated protein bands were transferred from the gel to a nitrocellulose membrane (Bio-Rad Laboratories, Inc). The nonspecific binding sites were blocked by rinsing the nitrocellulose membrane with Tris-Buffered Salin-Tween (TBST) containing 2% non-fat skimmed milk powder for 1.5h at room temperature. Blots were then probed, overnight at 4 °C, with the following primary antibodies: rabbit polyclonal anti-BMP-2 (1:1000 dilutions, Abcam, UK) and rabbit polyclonal anti-FGF-2 (1:500 dilutions, antibodies-online, Germany), followed by rinsing with

TBST 3x5 min. As the native membrane for guided bone regeneration was obtained from the small intestinal submucosa of pig, the primary antibodies were selected with respect to species reactivity for both rats and pigs. Protein from rat liver was used as a positive control for BMP-2 antibody. The blots were then incubated with appropriate horseradish-peroxidase secondary goat anti-rabbit antibody (Santa Cruz, Inc) at a dilution of 1:10,000 for 1h at room temperature. All antibodies were diluted in 2% non-fat skimmed milk powder in TBST. Finally, the blots were washed 5-10 min in TBST. The detection of bands was performed using the Chemiluminiscence with Clarity TM Western ECL Substrate detection kit (Bio-Rad Laboratories, Inc). The ChemiDoc XRS+ system with Image Lab Software (Bio-Rad Laboratories, Inc) was used for digital visualization.

Statistics

For histomorphometry and gene expression analyses, statistical comparisons were made between the material groups and between the time periods for each group. A non-parametric Kruskal Wallis test was used to identify statistical differences between groups (Paper I and IV) or time periods (Papers I-IV). Whenever a statistical difference was found, Mann Whitney test was used to determine the statistical difference between two groups. Wilcoxon's signed ranks test was also used to determine the statistical difference between the dependent groups (HA vs SrHA; Paper II, Sham vs membrane; Paper III). Spearman's correlation analysis was carried out to test the dependency between the genes. In Paper I, the analysis was performed between the expression levels of the analyzed genes in each defect type and at the different time points. In Paper III, the correlation analysis was performed between analyzed genes in the defect, with and without membrane after 3, 6 and 28d. The correlation analysis was also performed between the different analyzed genes in the membrane as well as between the genes in the membrane versus the genes in the equivalent defect samples after pooling the expression levels for all healing time periods. All analyses were performed using SPSS Version 10 software (SPSS, Inc., New York, USA), and the significance was set at $p < 0.05$.

4 SUMMARY OF RESULTS

4.1 Paper I

In the first study, the molecular and structural pattern of bone regeneration in trabecular bone defect was investigated after augmentation with synthetic tetrapod-shaped calcium phosphate (TetraB) or natural deproteinized bovine bone (DBB), and compared to Sham defect.

Materials characterization showed that DBB is HA-based granules, their ultrastructure surface composed of aggregates of particles ranging in size from 400 to 500 nm. On other hand, α-TCP and OCP were the main phases in the TetraB granules that revealed porous surface with flake like structures.

After 6d of healing, newly formed osteoid was observed at the peripheral region of all defects. At 14d, substantial amount of mature bone had formed in all defects. TetraB showed a greater degree of bone-granule contact and bone mineralization at 6 and 14d. Also at 14d, coupling activity of osteoblast and osteoclast was observed in conjunction with both granule types. However, resorption-like areas were detected within the bone interfacing TetraB but not DBB. At 28d, the bone area percentage was significantly higher in defects with TetraB compared to the Sham and DBB. No major differences were detected in the granule area percentage between the two materials at any time point.

A major observation, at 3d, was the higher expression levels of bone formation (ALP and OC) and remodeling (CatK) genes in TetraB compared with Sham and DBB. At 14d, DBB revealed significantly lower expression of inflammatory cytokine (IL-1β) and the remodeling gene (TRAP) compared to the Sham and to the TetraB. In the Sham defect, negative and positive correlations were observed, at 3d, for TNF- α with bone formation gene (OC) and bone remodeling gene (TRAP), respectively. On the other hand, DBB and TetraB, revealed a positive correlation between TRAP and CatK. At 6d, TetraB revealed positive relationships between the OC and the remodeling genes, and between the bone formation gene (ALP) and inflammatory cytokine (TNF-α). At 14d, DBB and TetraB defects also displayed several relationships between the remodeling genes and inflammatory genes. At 28d, whereas positive correlations were found between the genes of inflammation and remodeling in all defects, only the Sham and TetraB showed a positive correlation between genes of bone formation and inflammation.

4.2 Paper II

In the second study, we evaluated the healing process in bone defects implanted with hydroxyapatite (HA) or strontium-doped calcium phosphate (SrCaP) granules, in normal (non-OVX) and ovariectomized (OVX) rats.

The XRD revealed that the synthesized HA powder was pure hydroxyapatite. After calcination, the crystallinity of the obtained HA granules increased and a few peaks for tricalcium phosphate appeared. For SrCaP, the particles and granules showed similar crystalline phase (β-tricalcium phosphate). The SrCaP and HA granules had 15 wt% and 11 wt% loss, respectively after 28d in SBF. The release of Sr^{2+} from SrCaP granules increased slightly from 0.03 mM (at 3d) to 0.05 mM (at 7 and 14d).

At 6d of implantation, inflammatory infiltrates were observed in the intergranular spaces of the defect, irrespective of the materials or OVX. Signs of intramembranous osteogenesis were observed, and a low proportion of bone was recorded. Multinucleated giant cells (MNGCs) were frequently encountered in both the HA and SrCaP. At 28d, the percentage of granule area had decreased significantly, with a higher level of reduction in SrCaP defects compared with HA. Considerable amount of mature bone had formed at this late time period. The overall bone formation was comparable between HA and SrCaP, but with topological differences. The bone area was higher in the defect center of the HA, mainly in the OVX, and in the defect periphery of the SrCaP, in both animal groups. MNGCs were still evident in both defects. Other MNGCs appeared on the surfaces of granules, bone and/or the interface zone between granule and bone. These cells had a more osteoclast-like phenotype with close proximity to osteoblast seams on surfaces of newly secreted osteoid. The osteoclast-like cells were mostly restricted to the HA defects and seldom found in the SrCaP.

The ovariectomy induced significant downregulation of bone formation (ALP), and bone remodeling (CatK) genes. In OVX rats, a significantly higher expression of IL-6 was revealed in HA- and SrCaP-filled defects, at 6d, compared with baseline (BL). The expression levels of osteogenic genes, at 6d, were lower in OVX compared to Non-OVX with both materials. HA displayed, at 6d, higher expression of TNF-α in Non-OVX and OVX rats, whereas SrCaP revealed higher level of IL-6 at 6 and 28d, but only in the Non-OVX. SrCaP also displayed significantly lower expression of the osteoclastic genes, compared to HA, both at 6 and 28d in Non-OVX for CR, and at 28d in OVX for CatK.

4.3 Paper III

In the third study, the role of the membrane material for GBR in the underlying defect was investigated. Defects without collagen-derived membrane (Sham) were used as control.

Newly formed osteoid was observed, at 6d, in both defects. Islands of osteoid were more abundant in the top region of the membrane treated defect. A significantly higher amount of bone was detected in the membrane group after 28d in comparison with Sham. At this time point, the top and the central regions of the membrane treated defect exhibited a higher proportion of bone area, compared to the same regions in the Sham defect. This was in parallel with a higher degree of defect restitution and union of the defect margins compared with Sham.

The presence of membrane had induced, at 3d, upregulation of TNF-α and RANKL gene expression. This was switched at 6d, where both genes were detected with lower level in the membrane group compared to Sham. Treatment of the defect with the membrane caused upregulation of cell recruitment (CXCR4), bone formation (OC) and remodeling (CR, CatK) genes at 3 and 28d.

Histology revealed, at 6d, a large population of cells between the collagen strands of the membrane as well as in the periphery of the membrane. The majority of the recruited cells were PMNs and monocytes/macrophages but also included mesenchymal-like cells. At 28d, whereas PMNs were seldom detected, monocytes/macrophages were observed in conjunction with stromal cells. Many osteoclast-like cells were observed on the bone side at the boundary between the membrane and the newly formed bone.

A steady increase in gene expression over time was demonstrated in the membrane compartment for TGF-β, FGF-2 and BMP-2. Opposite trend was observed for VEGF expression, showing the highest expression at 3d. FGF-2 and BMP-2 was also detected at the protein level in the retrieved membranes. This stands in contrast to the finding of FGF-2, but not BMP-2 in the native membrane.

Several correlations were demonstrated between the different genes in the defect and also between genes in the defect and genes in the membrane. At 3d, a strong positive correlation was detected between the pro-inflammatory cytokine gene, TNF-α_{defect}, and the osteoclastic receptor gene, $CR_{defect.}$ At both time periods (3 and 28d), osteoblastic (OC_{defect}) and osteoclastic ($CatK_{defect}$)

genes were found in positive relationship. Moreover, both genes displayed a positive correlation with $CXCR4_{defect}$ (cell recruitment marker).

Importantly, the bone formation and remodeling genes (OC_{defect} and $CatK_{defect}$) in the defect were in positive correlation with analyzed genes of growth factors ($BMP\text{-}2_{membrane}$, $TGF\text{-}\beta_{membrane}$ and $FGF\text{-}2_{membrane}$) in the membrane.

4.4 Paper IV

In the final study, we investigated the early molecular and structural events of bone healing in bone defects treated with the collagen-based membrane (Sham) with and without different bone substitutes (DBB, HA and SrHA050).

Material characterization showed that DBB and SrHA had a homogenous, granular, surface appearance, whereas HA showed a combination of large and small grains. All materials were HA-based; however DBB appeared poorly crystalline with smoother granule surface than HA and SrHA, both of which showed higher crystallinity, comparable surface areas and *in vitro* degradation rates. No major differences in Ca and PO_4 release were observed between the materials and Sr was only released from SrHA (1.5 mM at 7d).

After 12h, all bone substitutes were evenly distributed in the defects, and surrounded by hematoma. PMNs and few CD68-positive, mononuclear cells i.e., monocytes/macrophages and periostin-positive osteoprogenitor cells were observed in all defects. These cells were also revealed inside the membrane throughout the healing period. At 3d, the relative proportion of the monocytes/macrophages in the defect had increased in all groups, but to a lesser extent in the Sham. Diffuse staining of periostin, an early sign of intramembranous bone formation, was detected interstitially in the intergranular tissues in all groups, but less evident in the DBB. At 6d, whereas monocytes/macrophages had increased in all defects, notably in the Sham, CD68-positive multinuclear giant cells were observed either in association with bone (osteoclasts) in all groups or with the surface of materials (FBGCs) in the filled defects. Whereas the material-related multinuclear giant cells were detected more frequently in the SrHA defect, the osteoclasts were less numerous in the SrHA compared to the DBB and Sham defects.

Histomorphometric analysis at 6d showed that woven bone was observed in all defects. A significantly higher bone area was demonstrated in the SrHA defects compared with Sham.

SrHA significantly reduced the expression of IL-6, at 12h, compared to Sham and HA. At 3d, the HA showed higher level of TNF-α compared to the SrHA and DBB. At the same time point, the expression level of CXCR4 was significantly lower in the SrHA compared to Sham. Also, SrHA displayed downregulation of remodeling genes, CR and RANKL compared to the other groups. Comparable level of CatK was observed in the Sham and SrHA, being significantly lower compared to HA. The downregulation of CR in the SrHA was also detected at 6d, compared to HA and DBB. No major differences in the expression of anabolic, osteogenic genes were observed between the groups.

5 DISCUSSION

The present thesis consists of a series of studies investigating the mechanisms of bone regeneration in trabecular bone defect treated with different calcium phosphate materials and/or resorbable collagen membrane. The studies employed a set of cellular and molecular techniques as well as histomorphometric and ultrastructural analyses. These together allowed the determination of the relationship between the material-induced molecular activities and the degree and pattern of bone regeneration. The molecular analyses targeted factors crucial for inflammation, bone formation and remodeling in the defect, as well as in the retrieved membrane. The histological, histomorphometric and ultrastructural analyses were performed on the entire defect site. The studies were performed over different healing periods ranging from 12h up to 28d.

The first studies of this thesis (Paper I and II) investigated the healing events in trabecular bone defects after augmentation with different types of CaP bone substitutes (DBB, TetraB, HA and Sr-doped CaP). In these studies, histological, histomorphometric and molecular investigations were performed in the defect compartment, with and without bone substitutes. The studies provided essential information on the relationship between the molecular activities and the development of bone in the defect. Moreover, the studies demonstrated that a variation in the properties of CaP bone substitutes results in significant changes in the expression of genes involved in bone formation and remodeling. In turn, these molecular changes result in a significant alteration in the amount and/or distribution of the newly formed bone.

The latter studies (Paper III and IV) investigated the healing of bone defects under the concept of GBR, both in the defect compartment and in the overlying membrane. Firstly, in Paper III, it was of importance to explore the mechanism of GBR, without bone substitute in the defect, in order to define the role of the membrane, the main component of GBR. Therefore, in Paper III, a sampling and analytical approach was applied whereby the cellular and molecular healing events were investigated separately in the retrieved GBR membrane and in the underneath defect. The data revealed a bioactive role for the GBR membrane rather than being merely acting as a passive barrier. Secondly, in Paper IV, the mechanism of GBR was further investigated using a combination of membrane and different CaP bone substitutes (DBB, HA and SrHA). Thereby, it was possible to reveal an inward migration of cells into the membrane in parallel with the evaluation of the early cascades of molecular activities, denoting inflammation, bone formation and remodeling,

which together determined the amount and distribution of early formed woven bone inside the defect.

5.1 Methodological considerations

In this thesis, the Sprague Dawley rat was used as an experimental animal model. This model has been extensively used in bone tissue-engineering research, due to several advantages, including the low cost, small size and the well-known age and genetic background[223]. Moreover, there is considerable information about the molecular mechanisms of bone healing that have been obtained from fracture models in rodents[224-226]. On the other hand, there are some drawbacks, which include the relatively high bone turnover. In addition, there are some anatomical disparity with larger animals and humans, such as the lack of Haversian system in the cortical bone[227]. Taken together, it is worth to state that whereas the present studies provided knowledge on fundamental biological processes of healing during bone augmentation and GBR, a direct extrapolation of the data to human conditions should be made with caution.

In all studies of this thesis, standardized cylindrical bone defects in the epiphysis of distal femur were prepared using a trephine. Bone in this site contains a relatively higher proportion of trabecular bone than any other bone site. Regarding the size of the defect, a critical size defect (CSD) has been suggested to produce a challenging environment and increase the clinical relevance of the animal models[228]. The CSD is defined as "a defect that will not heal without intervention". However, the actual defect size that is necessary to preclude healing without intervention varies between the species and the anatomical sites. Moreover, the standardization of the critical size has been mainly made for the segmental defects[229,230]. In this thesis, although the defect was slightly smaller than the suggested critical size in long bone (3mm × 3mm)[231], the morphological observations indicated the lack of complete restitution of the sham defect, up to 28d of healing.

In paper II, ovariectomized (OVX) Sprague Dawley rats were used with the aim to evaluate the bone regeneration in response to HA and Sr-doped CaP substitutes in compromized bone condition. The OVX rat model is widely used to simulate osteoporosis resulting in reduced bone mineral density and deranged bone microarchitecture[232-234]. Experimental studies have shown that estrogen deficiency due to OVX negatively affects fracture healing and the osseointegration of titanium implants[235,236]. In study II, although no major difference was observed between the normal and the OVX rats in response to

the two types of bone substitutes, a major drawback was that a sham defect was not included.

In Paper III and IV, a collagen-derived membrane was used as a model for GBR membrane. This membrane belongs to the second generation of GBR membranes, consisting mainly of collagen and being resorbable. The use of the resorbable membranes, in particular those composed of collagen, has increased over the last years, and they currently represent the main basis for development of newer generation of the barrier membranes[237,238].

In our molecular studies, the selection of target genes for qPCR was based on their relevance to bone healing and regeneration. Furthermore, a major emphasis in this project was put on the selection of reference genes for normalization. The stability of some reference genes has been questioned recently as they might be affected by the experimental conditions[239]. Therefore, a panel of reference genes was validated for each study, from which the most stable reference genes were selected for normalization.

In Paper IV, the HA group, at 6d, was excluded from histological and immunohistochemical analyses due to poor quality sections for the cellular evaluations or counting.

5.2 Bone healing in defects treated with different calcium phosphate-based substitutes and/or membrane

5.2.1 Bone formation and remodeling

A major finding was that in comparison to untreated sham defect, the presence of substitute and/or membrane modulates the molecular activities in the defect, resulting in higher level of bone regeneration and defect restitution. In Paper I, the molecular and structural events of bone regeneration were also affected by the differences in crystallinity, structure and/or surface morphology of the two substitute materials, DBB and TetraB. For instance, a significantly higher level of bone formation was mainly detected in defect treated with TetraB but not with DBB. An important observation was that the early increased level of bone contact with the TetraB compared to DBB was in parallel with a significant upregulation of osteogenic (OC) and resorption (CatK) genes. The early, coupled upregulation and the strong correlation of bone formation and bone remodeling gene expressions were associated with the early, ultrastructural

detection of mineralized bone in response to TetraB substitute. Taken together, the molecular and structural data indicates that a pro-osteogenic and early remodeling microenvironment was rapidly established between the TetraB surface and the adherent cells, and extended throughout the period of implantation. Furthermore, the mere placement of a collagen membrane (Paper III) also promoted the bone response, particularly at the top region of the defect, where the degree of bone formation was significantly increased at the early and the late periods. Interestingly, the early coupled activity induced in the presence of TetraB (Paper I) was also observed in association with the collagen membrane, which induced upregulation of both osteoblastic (OC) and osteoclastic (CatK and CR) markers (Paper III). The higher level of OC in the membrane treated defect was also detected after 28d of healing indicating a continuous process of bone formation.

The increased coupled bone remodeling, induced by the TetraB bone substitute and the collagen membrane, respectively, is in line with observations on other biomaterials. For instance, recent studies have shown that oxidized titanium implants promote higher osteoblastic, osteoclastic and coupling activities in parallel with higher degree of osseointegration, in comparison with machined implants[240]. Moreover, the present morphological and molecular findings of high remodeling activity in association with the OCP-containing TetraB is supported by other *in vivo* histological findings, showing that the increase of bone formation in response to the variation of OCP granule size is linked with more appearance of TRAP- and CatK-positive osteoclastic cells[241]. The observation of active bone formation and remodeling associated with TetraB is also in agreement with previous histological observations *in vivo* showing that different OCP-based substitutes promote more bone formation with frequent appearance of osteoclasts in comparison with HA-based substitutes[242,243]. Remodeling is an essential process for the maturation of bone that forms early during healing. The present results suggest that an accelerated remodeling phase takes place in conjunction with early bone formation and ultimately lead to an increased amount of mature bone in the defects treated with TetraB substitute and collagen membrane, respectively.

Hitherto, it is not known whether osteoblasts in the treated defects are induced firstly, which subsequently stimulate osteoclastic differentiation, or vice versa. The first assumption is supported by the observation of the membrane-induced upregulation of RANKL, the coupling factor and the main modulator of osteoclast formation, which is mainly expressed by osteoblasts (paper III). Regrettably, RANKL expression was not determined in association with TetraB (Paper I). Nevertheless, *in vitro* data have shown

that the presence of OCP, one component of the TetraB, in a co-culture system induces osteoblasts to produce RANKL, which in turn stimulated the differentiation of osteoclasts[244].

On the other hand, the precise role of osteoclasts and their activities on the osteoblastic bone formation at the different biomaterials is unknown. Based on the results of the present thesis tentative mechanisms can be suggested. The present and other studies[241,242,245], indicated that the multinucleated osteoclast-like cells exhibit resorption signs on both the bone and the material sides, particularly in conjunction with the OCP containing materials. Tentatively, such interaction might be associated with the bioresorption of the substitute material, modulation of the Ca and PO4 level in the surrounding environment and consequently the activity of both osteoblasts and osteoclasts. In line with this assumption, a review paper suggested that OCP material in a biological environment exhibit high surface reactivity during the dissolution process[145]. Another possibility is that osteoclasts are able to condition the surface of granules or expose the adsorbed biological factors on the surface after implantation in the recipient sites. This may influence the osteoprogenitors and osteoblasts, upregulating osteogenic gene expression and increasing the level of bone formation. Other roles for osteoclasts in bone regeneration in association with biomaterials can be extrapolated from the events of normal bone remodeling, where the osteoclast is considered as major stimulus for bone formation either by producing osteogenic factors or releasing them from the bone during the active resorption process[89,246,247].

With respect to the membrane-induced osteoclastic activities (Paper III), it was evident that the membrane compartment conveyed signals that promoted high osteoclastic activity, as well as induced high recruitment activity for both osteoblast and osteoclast progenitors in the defect. The membrane induced coupling activity and bone regeneration will be discussed more in detail in section 5.3. Regardless of the type of biomaterial, the multiple positive correlations between the osteoblastic and osteoclastic genes demonstrated in defects treated with TetraB (Paper I) and GBR collagen membrane (Paper III) indicate a common effect of the two different materials by inducing a coupled bone formation and resorption.

The gene expressions of the osteoclastic genes (CatK and TRAP) were downregulated in response to the natural, HA-based, DBB. This indicates that the resorptive activity of the osteoclast in conjunction with this material is reduced at the molecular level. Previous reports indicated that both DBB and HA promote bone formation and defect restitution[248,249]. However, in this thesis, the presence of the DBB did not significantly promote bone formation

in the defect (Paper I). Also during the early phase of guided bone regeneration, DBB and synthetic HA failed to produce any added benefit when combined with the collagen membrane (Paper IV). Taken together, this may indicate that the natural-derived and synthetic HA are not osteoinductive or, at least, not able to rapidly trigger osteogenesis. Another explanation for the finding in Paper IV is that the collagen membrane itself was sufficient to promote bone formation, in the presence or absence of the natural or synthetic HA used in this study. This assumption can be, at least partially, supported by results from a previous human study showing that a collagen membrane for treatment of extraction socket induced larger amount of bone compared to DBB without membrane[250].

5.2.2 Inflammation and role of inflammatory cytokines in the defect

Studies of bone healing around biomaterials have demonstrated that the early inflammation precedes and overlaps with the differentiation of the bone-forming osteoblasts and bone-resorbing osteoclasts[251,252]. In the present thesis, a common observation was that the trigger of osteogenic differentiation and bone formation corresponds to the diminution of the acute inflammation induced by the surgical trauma. For instance, two findings in this thesis indicated that the presence of different types of substitutes and/or collagen membrane does not disrupt the biological switch from inflammation to bone regeneration or, at least, does not prolong the inflammatory phase after implantation. Firstly, in Paper I, the early downregulation of TNF-α from 3 to 6d, was observed in the presence or absence of the substitute materials. Secondly, in Paper IV, during GBR with and without bone substitute, whereas the osteogenic activity increased considerably after 6d of healing, the level of TNF-α was reduced to the baseline level.

The impact of pro-inflammatory cytokines during bone regeneration *in vivo* is controversial. For instance, there are many indications that TNF-α may play different roles during different periods of bone healing and regeneration[253,254]. The significance of the pro-inflammatory cytokines during bone remodeling has been described in previous studies using similar animal model[75,255]. It has been shown that after the downregulation of the initial pro-inflammatory phase, the expressions of pro-inflammatory cytokines increase again during the remodeling phase, about two weeks after injury. This was confirmed in the sham defects (Paper I), where the levels of both TNF-α and IL-1β showed a temporal increase after 14d. In the treated defects, whereas this temporal upregulation was maintained in the TetraB-augmented defects, there was a major downregulation, especially for IL-1β, in the DBB-

augmented defects. This was in parallel with a downregulation of osteoclastic gene expression, and less osteoclast-like cells in the defect treated with DBB. TNF-α and IL-1β bind to respective receptors on osteoclasts and augment their differentiation and resorptive potential[256,257]. The positive role of these cytokines for the osteoclastic activity is also indicated by the strongly positive correlations between the gene expression of these cytokines and the expression of osteoclastic genes at 3d, in the sham defects, and at 14d, both in the sham and in the augmented defects. The latter findings may provide a plausible explanation for the low resorptive capacity of the DBB, which has been discussed in some pre-clinical and clinical studies[258-260].

An association between increased osteoclastic activity and increased expression of TNF-α was also observed, at 3d, in the defects treated with the collagen membrane (Paper III). This finding implies that the regulatory role of inflammatory cytokines in bone remodeling is initiated rather early during GBR. This was not only indicated by the overlapped upregulation of the CR and TNF-α, but also by the significant correlation between these two genes. Firstly, since the osteoblastic activity was also promoted in the membrane treated defect, the upregulation of TNF-α might have been involved in the early recruitment and osteogenic differentiation of mesenchymal cells. Secondly, the expression of TNF-α could also have been implicated in the induction of RANKL expression by the osteoblast and its subsequent effect on osteoclastic differentiation. The first possibility is supported by previous *in vitro* studies showing that TNF-α enhances proliferation of MSCs and promotes higher mineralization[261-263]. The second possibility is supported by observations in Paper III that the RANKL upregulation, at 3d, corresponded with the increased level of CR, in the membrane-treated defect, only when the TNF-α revealed the highest expression level. Further, it has been shown that the role of pro-inflammatory cytokines is not only to augment the RANKL pro-osteoclastic effect[264], but also to directly induce the osteoblastic expression of RANKL[265,266]. Interestingly, it was the membrane-induced expression of TNF-α, but not IL-6, that corresponded with the upregulated expression of RANKL. Similar finding was observed in Paper IV when SrHA simultaneously reduced the expression of TNF-α and RANKL at 3d of healing. These observations are in agreement with previous *in vitro* data showing that IL-1β and TNF-α, but not IL-6, stimulate RANKL expression in human osteoblastic cells[266].

Taken together, the present data suggests an important role for the inflammatory cytokines in bone remodeling during bone healing in conjunction with different bone substitute materials and/or GBR membrane.

This seems to be largely affected by the physicochemical properties of CaP substitutes and presence of the collagen membrane.

5.2.3 The role of strontium in the CaP substitute for bone formation and remodeling

Another major finding was that the modification of the chemical composition of bone substitute, by doping the CaP with strontium ions, strongly modulates the bone remodeling activities. Several *in vitro* studies have shown evidence for both pro-osteogenic (anabolic) and anti-resorptive (anti-catabolic) effects of strontium (Sr) on osteoblasts[267-269] and osteoclasts[270], respectively. This has formed the prevailing hypothesis for using Sr as a dual acting agent in combination with bone substitutes[271,272] and implants[273]. In this thesis, in order to explore the *in vivo* local effect of Sr incorporation in bone substitute material, the bone healing events were investigated in defects treated with SrCaP substitute alone (Paper II) or with a combination of SrHA and GBR membrane (Paper IV). The two studies showed that the Sr-containing materials promoted new bone formation. Firstly, after 6d of healing (Paper IV), among different substitute materials, combining SrHA with the collagen membrane resulted in higher level of woven bone formation compared to the membrane-alone treated defect. Secondly, at late time period, 28d, the newly formed bone at the peripheral region of the defect was significantly higher in conjunction with the SrCaP compared to HA (both in normal and osteoporotic condition) (Paper II). These morphological observations are in agreement with recent *in vivo* data showing that incorporation of Sr with bone substitutes[274-276], collagen membranes[277], and titanium implants[273,278,279] enhance bone regeneration and osseointegration.

The present thesis (Paper II and Paper IV) provided *in vivo* evidence that the presence of Sr in the CaP/HA materials inhibits the osteoclastic activity and reduces the number of osteoclasts. From a mechanistic point of view, the present molecular and cellular findings are in partial agreement with the suggested dual effect of Sr, as no major effect was observed for Sr on the osteoblast anabolic gene expression.

In Paper II, the presence of SrCaP in the defect significantly reduced the expression of osteoclastic genes (CR and CatK) at 6 and 28d compared to HA, whereas no major changes were observed in the osteoblastic gene expression. However, in this study (Paper II), the presence of Sr was not the only variable that may have influenced the osteoclastic activity in the defect. The differences in the crystallinity and biodegradability between the two materials could have affected the osteoclast formation and differentiation,

and may even have masked the effect of Sr on the osteoblastic activity. In study IV, SrHA and HA were synthesized with comparable properties to reduce the confounding effects of other material variables. Interestingly, after 3 and 6d of implantation, the gene expression data, in study IV, confirmed and extended the previous findings and revealed a significant reduction of CR, and CatK in association to SrHA compared with HA. Furthermore, SrHA did not influence the gene expression of bone formation (ALP and OC) markers in the defect differently than the other substitutes. Taken together, it is shown that the presence of Sr, *per se*, exerts profound anti-osteoclastic effect in the augmented defect, irrespective of the crystallinity of the CaP substitute.

Although Sr did not alter the expression of osteoblastic anabolic genes (ALP and OC), it did influence the osteoblastic expression of RANKL, the bone remodeling coupling gene. By virtue of the significant reduction of RANKL expression at 3d in response to SrHA (Paper IV), it is possible that the major effect of Sr on osteoblasts was executed through a de-coupling of the osteoblast-osteoclast cross-talk. In Paper II, the RANKL expression was not affected by Sr at 6d and 28d, which indicates that the Sr effect on the RANKL expression takes place during the very early time stage. The appearance of less osteoclasts in both SrHA (Paper IV) and SrCaP (Paper II) defects is a further supporting evidence of an interaction between Sr and osteoclasts. The anti-osteoclastic effect of Sr demonstrated in the present thesis is in agreement with recent *in vivo* data showing that implantation of Sr-incorporated bioactive glass in rat mandible defect is associated with less number of TRAP-positive osteoclasts and higher degree of bone formation compared to similar bioactive glass without Sr[276]. In the present thesis, the *in vitro* accumulative Sr release from SrHA at 3 and 7d (0.2 and 1.5 mM, respectively) (Paper IV), and Sr release from SrCaP at 7 and 28d (≈ 0.05 mM) (Paper II) was comparable with previously suggested optimal concentrations based on results from *in vitro* studies[280,281]. In line with the present findings, a recent *in vitro* study has demonstrated that 0.01-1mM Sr ranelate or $SrCl_2$ have an anti-osteoclastic effect, but do not stimulate proliferation and activity of osteoblasts[282].

In addition to the down-regulation of osteoclastic and coupling genes, Sr incorporation in CaP and HA also modulates the expression of pro-inflammatory cytokines. The present results of a reduced expression of TNF-α in response to Sr, irrespective of CaP or HA, is in agreement with a recent *in vivo* study, where the administration of Sr ranelate after implantation of titanium particles, in the calvarial bone, reduced the expression of RANKL and pro-inflammatory cytokines, TNF-α and IL-1β[283]. In Paper IV, the lower

level of TNF-α, at 3d, in SrHA compared to HA was in parallel with a significant downregulation of RANKL, CR and CatK. On the other hand, in Paper II, whereas the RANKL expression was not affected after 6d in the SrCaP defects, the expression of TNF-α was significantly downregulated in SrCaP compared to the HA. This down-regulation was associated with lower expression of the osteoclast surface marker, CR. These findings suggest that the Sr inhibitory effects on osteoclasts can occur either directly via the reduction of coupling factor RANKL, or indirectly via the down-regulatory effect on pro-inflammatory mediator, TNF-α.

A large proportion of multinucleated giant cells (MNGCs) was detected in association with both HA and SrCaP granules at 6 and 28d of healing, assuming foreign body type of giant cells (FBGCs) (Paper II). These cells also appeared in association with all bone substitutes (DBB, HA and SrHA) used in Paper IV. This indicates that formation of these cells is an integral process in bone regeneration with CaP substitutes. There is evidence that the FBGCs produce VEGF, which has a fundamental role for angiogenesis during bone healing[284,285]. Yet, the importance of these cells in relation to material degradation, repair and bone regeneration is not clear and needs further investigation. In parallel with a lower number of osteoclasts, SrHA was associated with an increase in the number of FBGCs compared to DBB, which indicates that the presence of Sr inhibits the development of osteoclasts, but does not interfere with the formation of MNGCs.

In Paper II, one major finding was the differential distribution of the newly formed bone in association with the two materials (SrCaP and HA). The SrCaP substitute promoted higher level of mineralized bone in the defect periphery, both in normal and OVX condition. In contrast, the bone area in the central part of the defect was higher in conjunction with HA treatment, mainly in the OVX rats. In Paper IV, a similar trend for bone distribution was observed in the SrHA treated defect. There, the significantly higher amount of new bone demonstrated in the SrHA defect was predominantly distributed in the peripheral region of the defect. A possible explanation for this finding is that the release and bioavailability of Sr during dissolution from SrHA and SrCaP had created a concentration gradient, with low optimal level at the periphery and high level at the center. This assumption is supported by recent *in vivo* and *in vitro* findings showing a multiphasic and dose-dependent effect of systemically administered Sr on bone formation, mineralization and osteoclast formation[280-282,286].

Irrespective of the type of bone substitute, OVX rats revealed an early downregulation of osteogenic bone formation genes during the first 6d of

defect augmentation (Paper II). However, after 28d, the expression of osteogenic bone formation genes was comparable between OVX and non-OVX. At this time point (28d) the OVX rats revealed higher bone fraction in the central and peripheral regions of the defect when treated with the HA and SrCaP granules, respectively. These results are comparable with previous findings on osseointegration in non-OVX and OVX rats[236]. In the latter study, OVX reduced the bone-related gene expression and histomorphometry in T-shaped titanium chambers only at the early time point, however, after 28d, these parameters revealed comparable levels between non-OVX versus OVX rats[236]. Taken together, these studies indicate that OVX mainly impairs the early stage of bone regeneration, whereas at later stage a structural restitution is achieved.

5.3 Cellular and molecular events in the membrane compartment and the mechanism of GBR

In order to explore the role of the membrane for promotion of bone regeneration in the defect, the cellular and the molecular events were further investigated in the membrane compartment (Paper III and Paper IV). Histologically, the membrane was shown to accumulate different cells throughout the early and late stages of healing. In addition to the histological observations of different cell phenotypes, the presence of osteoprogenitors and monocytes/macrophages inside the membrane was verified using periostin and CD68 immunostaining, respectively (Paper IV). The inward migration of the inflammatory cells inside the collagenous membrane may be implicated in the membrane degradation. The analysis of selected proteins revealed that whereas the native membrane contains FGF-2, the retrieved membrane contains both FGF-2 and BMP-2 after implantation *in vivo* (Paper III). The present results related to FGF-2 are in agreement with earlier studies of the membrane, as derived from porcine small intestine submucosa[287]. The findings in this thesis demonstrate that the membrane not only maintains FGF-2 after implantation and during the different phases of GBR, but also accumulates the pro-osteogenic factor, BMP-2 (Paper III). The *de novo* expression of BMP-2, together with FGF-2, TGF-β and VEGF, by the recruited cells in the membrane was further confirmed throughout the healing period. These growth factors possess distinct and synergistic effects on the different processes of bone healing, including inflammation and cell recruitment, bone formation and remodeling[288,289].

The collagen-based biomaterials are generally believed to contain cellular binding and activation motifs, which enhance the binding, migration and activation of cells with different phenotypes[290,291]. The findings of the present thesis show that the collagenous membrane also conveys multiple growth factors, which are crucial for migration, proliferation and differentiation of different cell phenotypes. These factors also play a critical role in angiogenesis and mesenchymal cell mitogenesis[65,292] and are involved in the recruitment and formation of osteoclasts[293,294]. It is therefore strongly suggested that these expressed and secreted factors in the membrane, together with the collagenous support and structure of the membrane, have significantly contributed to the overall healing process in the defect underneath.

A first line of evidence for the above statement was the promoted bone formation and remodeling in the defects treated with membrane, as described earlier in this thesis. Furthermore, the molecular analysis of the defect revealed an enhanced cell recruitment activity in the defect compartment, as indicated by the profound upregulation of fundamental components in cell recruitment axes, MCP-1 and CXCR4 (Paper III). MCP-1 is a chemokine that is majorly responsible for monocyte trafficking[295,296] and recruitment of osteoclast precursors at the sites of bone remodeling[297], whereas CXCR4 is considered as a major element in regulating migration of several progenitor cells including MSCs[298,299] and osteoclast precursors[300]. The early and late upregulation of these factors indicates that the membrane-induced environment enhances the expression of chemotactic signals for different cell phenotypes at different stages of healing. These signals may play a role in the tissue response within and immediately adjacent to the membrane material, e.g. providing cells for bone regeneration in the defect region directly beneath the membrane (i.e. the top region of the defect). An upregulation of MCP-1 has been previously described in association with similar membrane materials, in parallel with increased levels of macrophage inflammatory protein-1 (MIP-1), recruitment of macrophages, and higher rate of graft remodeling[301].

Involvement of MCP-1 and CXCR4 in the recruitment of different cells inside the defect was confirmed by the correlation analysis of different genes in the defect. For instance, MCP-1 revealed strong correlation with TNF-α and CR suggesting an implication of MCP-1 in the recruitment of inflammatory cells and osteoclast precursors. On the other hand, the expression of CXCR4 displayed a strong association with the osteoblastic and osteoclastic activities at the early and late periods. Interestingly, at 28d, osteoclast-like cells were observed at the zone between the membrane and the

newly formed bone. Whereas it was not possible to determine if these cells were involved in the degradation process of the membrane, they appeared to be actively resorbing the underlying bone indicating an ongoing active bone remodeling.

When the membrane was combined with different bone substitutes, the enhanced recruitment activity (MCP-1 and CXCR4) was yet demonstrated after 12h in the treated defects, at least in comparison to the baseline expression (Paper IV). Nevertheless, the type of the substitute may also play a role in addition to the membrane. This was demonstrated by the downregulation of CXCR4, at 3d, in the membrane-SrHA filled defect, which suggests that Sr does not only inhibit the differentiation of osteoclast precursors, but also reduce their recruitment activity.

Given the important biological processes demonstrated inside the membrane compartment, it was judged to be of importance to assess if these activities were related to the expression of factors in the defect, which are involved in the cell recruitment, inflammation, bone formation and bone remodeling. Indeed, the revealed correlations between factors in the membrane and factors in the defect provide strong evidence for the role of the membrane in promoting the defect healing (Paper III). Firstly, the three pro-osteogenic signals (FGF-2, TGF-β1 and BMP-2) in the membrane demonstrated a positive correlation with the bone formation and bone resorption genes in the defect. This observation extends previous finding that implantation of a collagen sponge loaded with recombinant BMP-2 in a rat calvarial defect enhances both osteoclastogenesis and osteoblastogenesis[302]. Further, *in vitro* and *in vivo* studies have revealed regulatory and inducing effects for FGF-2, TGF-β and BMP-2 on the differentiation of both osteoblasts and osteoclasts[303-307] Secondly, the inverse relationship between TGF-β, in the membrane, and MCP-1, in the defect, suggests a role for TGF-β in regulating the inflammatory phase in the defect. TGF-β is a multifunctional growth factor, which has dual pro-inflammatory and anti-inflammatory effects[308].

5.4 Significance and implications of the findings

The present thesis reveals novel mechanisms whereby the GBR collagen membrane and specific types of CaP substitutes modulate the biological processes of bone healing and hence promote the bone regeneration.

Regarding guided bone regeneration (GBR), the application of a membrane to restitute lost bone is a well-established clinical procedure. Recent reviews have estimated that up to 40% of osseointegrated implants required GBR as a part of the treatment[309]. Since the introduction of GBR, the main hypothesis has been that the membrane acts as a passive barrier. However, the lack of proper understanding of the mechanisms and the precise role of the membrane has hindered the development and optimization of the membrane properties, especially for applications other than dentistry. The results of the present thesis provide structural, cellular and molecular evidence suggesting a novel role for the membrane during GBR, by acting as a bioactive compartment rather than a passive barrier. This knowledge is fundamental in order to engineer membranes with specific architectural and chemical cues, which attract, bind to and induce endogenous cells, which acquire phenotypes that contribute both to the membrane degradation as well as the promotion of bone regeneration and defect restitution.

Regarding the bone substitutes, autografts have been regarded, clinically, as a golden standard for bone augmentation. However, problems related to availability, morbidity, rejection and infection remain major drawbacks. Efforts have been made to develop synthetic grafting materials as an alternative option for bone augmentation. However, the knowledge about the interactions between the bone substitute and the bone environment during the sequential phases of inflammation and bone regeneration has been incompletely understood. The results of the present thesis contribute to an increased understanding of the mechanisms of bone healing and regeneration in response to different CaP-based substitutes. The understanding of the activities and signals involved in the processes of inflammation, bone formation and remodeling is fundamental for the design of novel substitute materials that may reduce healing time, failure and complications. Optimized substitute materials would allow improved patient rehabilitation, both in normal and in compromized conditions. For instance, the findings of this thesis provide molecular and structural evidence on the promotion of bone regeneration in response to the novel synthetic substitutes (TetraB and Sr-doped HA). This experimental data forms the basis for clinical trials using these materials as interesting alternatives for augmentation in skeletal defects. Moreover, the present thesis provides molecular, cellular and structural evidence on the promotion of early bone regeneration in response to the synthetic Sr-doped HA substitute in combination with a resorbable collagenous membrane. These findings demonstrate that local effect of Sr *in vivo* is predominantly via the inhibition of osteoclast number and activity and the reduction of osteoblast-osteoclast coupling. This experimental data also forms the basis for clinical studies, using this material as an interesting bone

substitute for GBR. In addition, the reduced osteoclastic activities during bone augmentation using DBB may shed light on the controversy related to the biodegradation and/or resorbability of the natural bovine substitute.

6 SUMMARY AND CONCLUSIONS

The main findings of this thesis are the following:

- The natural (DBB) and synthetic (TetraB) calcium phosphate substitutes promote bone regeneration and restitution of the surgically created defect. The gene expression, morphological and ultrastructural data demonstrates that the bovine substitute (DBB) is osteoconductive and elicits low resorption activity. Further, TetraB induces early osteogenic and osteoclastic activities, resulting in greater bone formation in comparison to DBB.
- Implantation of HA and SrCaP granules result in comparable overall bone formation regardless if the recipient bone was normal (non-OVX) or osteoporotic (OVX). The two materials induced distinctly different inflammatory and bone remodeling responses, as well as differences in the spatial distribution of the newly formed bone.
- The sequence of biological events during GBR encompasses an inward migration of cells, which acquire phenotypes that contribute to a pro-osteogenic and remodeling micro-environment, primarily underneath the membrane.
- The molecular mechanisms whereby the membrane-induced pro-osteogenic and remodeling micro-environment contributes to bone regeneration and healing of the defect, involve the expression and accumulation of pro-osteogenic factors within the membrane, which trigger the molecular cascade for rapid, and greater bone formation and remodeling in the underlying defect.
- Strontium incorporation in the calcium-phosphate bone substitutes promotes bone regeneration in defects, with and without combination with GBR collagenous membrane. The mechanism for the Sr-induced bone formation involves a reduced number of osteoclasts and down-regulation of the osteoclastic CatK and CR, and the osteoblastic RANKL.

It is concluded that different formulations of calcium phosphate bone substitutes induce different molecular cascades involved in the different processes of bone healing, including early inflammation, bone formation and remodeling. This significantly promotes bone regeneration and defect restitution in comparison with the sham defect. Strontium incorporation in a synthetic calcium phosphate substitute reduces the osteoclastic resorptive

activities, and promotes bone formation. Furthermore, the present results provide cellular and molecular evidence suggesting a novel role for the membrane during GBR, by acting as a bioactive compartment rather than as a passive barrier.

Based on the results of the present thesis, a tentative scenario for the guided bone regeneration (GBR), with membrane alone or a combination of membrane and bone substitute, is depicted in the following graph:

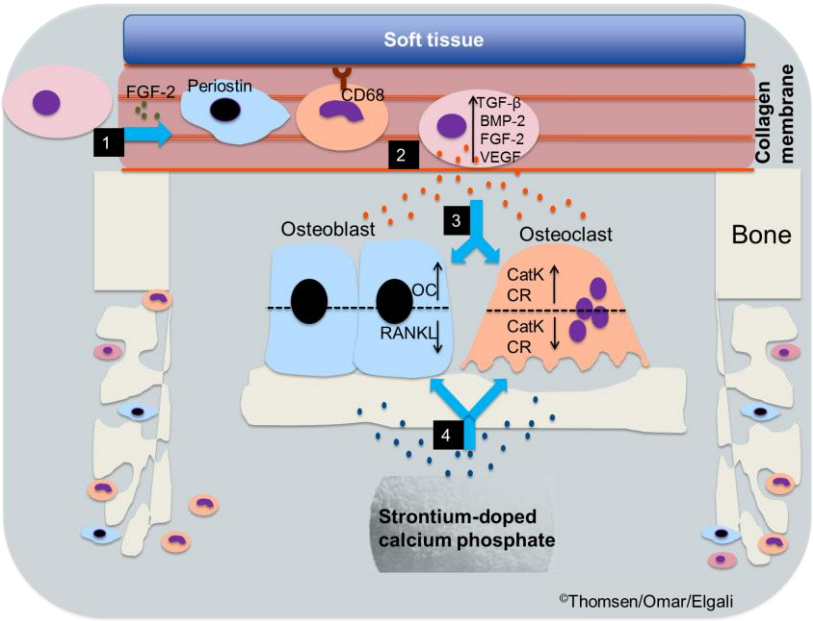


Figure 5. *A schematic diagram showing the cascade of cellular and molecular events taking place during guided bone regeneration. The bone defect is treated with a combination of porcine collagen membrane (with indigenous proteins) and Sr-doped calcium phosphate granules. The events include (1) migration of different cells (e.g. monocytes/macrophages and osteoprogenitors) from the surrounding tissue into the membrane through the peripheral borders, between the separated collagen layers (Papers III & IV). (2) The cells in the membrane express and release factors crucial for bone formation and bone remodeling (Paper III). (3) This promotes the bone regeneration process in the underlying defect by stimulating the activity of osteoblasts and osteoclasts, the main cells of bone remodeling (Paper III). The cellular and molecular activities inside the membrane strongly correlate with pro-osteogenic and bone remodeling environment in the defect underneath, and hence promote higher degree of bone regeneration and restitution of the defect (Paper III). (4) The presence of Sr-doped CaP, presumably via the release of Sr, interferes with the process of bone resorption by reducing the number and activity of bone-resorbing osteoclasts (Papers II & IV). Further, Sr modulates the osteoblastic expression of RANKL, thereby inhibiting osteoblast-osteoclast coupling (Paper IV). Both the membrane-induced and the Sr-induced events lead to increased net formation of bone in the defect (Papers III & IV).*

7 FUTURE PERSPECTIVES

The present thesis demonstrates that the collagen GBR membrane promotes bone regeneration, particularly at the top region of the defect. However, the molecular activities were analyzed in the entire defect site, which may mask possible differences in specific regions of the defect. This raises the question if there is any differential effect on the cellular and molecular activities at different distances from the membrane. To answer this question, site-specific analyses can be employed, for example, using *in-situ* hybridization or laser micro-dissection to obtain samples from different regions of the defect for qPCR analysis. Furthermore, since the current data on GBR is related only to the collagenous type of membranes, further studies are needed to explore the mechanisms whereby other types of membrane (e.g. non resorbable membrane) promote bone regeneration. Moreover, future studies may focus on the effect of specific membrane properties (e.g. porosity, composition, thickness) on the regulation of cellular and molecular activities inside the membrane as well as in the underneath defect. This requires proper control of the physical/chemical/mechanical properties of the membrane.

Combining and correlating the molecular and structural data in the present kinetic studies provides a promising approach for unveiling the mechanisms of CaP osteoinductivity, and identifying the properties of importance for promoting bone regeneration. Therefore, by employing a similar set of correlative analytical techniques (histology, histomorphometry, quantitative-polymerase chain reaction and Western blot), it would be of great interest and significance to further explore and address the following:

- The role of specific material cues (e.g. pore size and shape) of the CaP based substitutes and/or scaffolds on the molecular events of bone healing and the degree of bone regeneration.
- Whether priming of the CaP substitutes and/or scaffolds with biological cues (e.g. mesenchymal stem cells) might be advantageous, where the MSCs might trigger the recipient bone microenvironment and hence promote a favorable bone regeneration.

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REFERENCES

1. Liu J, Kerns DG. Mechanisms of guided bone regeneration: a review. *The open dentistry journal.* 2014;8:56-65.
2. Chai YC, Carlier A, Bolander J, et al. Current views on calcium phosphate osteogenicity and the translation into effective bone regeneration strategies. *Acta biomaterialia.* 2012;8(11):3876-3887.
3. Buckwalter J, Glimcher M, Cooper R, Recker R. Bone biology. I: Structure, blood supply, cells, matrix, and mineralization. *Instructional course lectures.* 1996;45:371.
4. Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. *Nature.* 2014;505(7483):327-334.
5. Buck DW, Dumanian GA. Bone biology and physiology: Part I. The fundamentals. *Plastic and reconstructive surgery.* 2012;129(6):1314-1320.
6. Kini U, Nandeesh BN. Physiology of Bone Formation, Remodeling, and Metabolism. In: Fogelman I, Gnanasegaran G, van der Wall H, eds. *Radionuclide and hybrid bone imaging*: Springer Berlin Heidelberg; 2012:29-57.
7. Clarke B. Normal bone anatomy and physiology. *Clinical journal of the american society of nephrology.* 2008;3(Supplement 3):S131-S139.
8. Sharma RR, Pollock K, Hubel A, McKenna D. Mesenchymal stem or stromal cells: a review of clinical applications and manufacturing practices. *Transfusion.* 2014;54(5):1418-1437.
9. Li Y, Yu X, Lin S, Li X, Zhang S, Song YH. Insulin-like growth factor 1 enhances the migratory capacity of mesenchymal stem cells. *Biochemical and biophysical research communications.* 2007;356(3):780-784.
10. Kovach TK, Dighe AS, Lobo PI, Cui Q. Interactions between MSCs and Immune Cells: Implications for Bone Healing. *Journal of immunology research.* 2015.
11. Kol A, Walker N, Galuppo L, et al. Autologous point-of-care cellular therapies variably induce equine mesenchymal stem cell migration, proliferation and cytokine expression. *Equine veterinary journal.* 2013;45(2):193-198.
12. Choi JJ, Yoo SA, Park SJ, et al. Mesenchymal stem cells overexpressing interleukin-10 attenuate collagen-induced arthritis in mice. *Clinical & experimental immunology.* 2008;153(2):269-276.
13. James AW. Review of signaling pathways governing MSC osteogenic and adipogenic differentiation. *Scientifica.* 2013;2013.
14. Aubin JE. Regulation of osteoblast formation and function. *Reviews in endocrine & metabolic disorders.* 2001;2(1):81-94.

15. Sodek J, McKee MD. Molecular and cellular biology of alveolar bone. *Periodontology 2000.* 2000;24:99-126.
16. Doty SB. Morphological evidence of gap junctions between bone cells. *Calcified tissue international.* 1981;33(1):509-512.
17. Manolagas SC. Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *Endocrine reviews.* 2000;21(2):115-137.
18. Orimo H. The mechanism of mineralization and the role of alkaline phosphatase in health and disease. *Journal of nippon medical school.* 2010;77(1):4-12.
19. Boyce BF. Advances in the regulation of osteoclasts and osteoclast functions. *Journal of dental research.* 2013;92(10):860-867.
20. Franz-Odendaal TA, Hall BK, Witten PE. Buried alive: how osteoblasts become osteocytes. *Developmental dynamics : an official publication of the american association of anatomists.* 2006;235(1):176-190.
21. Bonewald LF. The amazing osteocyte. *Journal of bone and mineral research.* 2011;26(2):229-238.
22. Verborgt O, Gibson GJ, Schaffler MB. Loss of osteocyte integrity in association with microdamage and bone remodeling after fatigue in vivo. *Journal of bone and mineral research.* 2000;15(1):60-67.
23. Teitelbaum SL. Bone resorption by osteoclasts. *Science (New York, N.Y.).* 2000;289(5484):1504-1508.
24. Holtrop ME, King GJ. The ultrastructure of the osteoclast and its functional implications. *Clinical orthopaedics and related research.* 1977(123):177-196.
25. Vaananen HK, Zhao H, Mulari M, Halleen JM. The cell biology of osteoclast function. *Journal of cell science.* 2000;113 (Pt 3):377-381.
26. Saftig P, Hunziker E, Wehmeyer O, et al. Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin-K-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America.* 1998;95(23):13453-13458.
27. Li X, Qin L, Bergenstock M, Bevelock LM, Novack DV, Partridge NC. Parathyroid hormone stimulates osteoblastic expression of MCP-1 to recruit and increase the fusion of pre/osteoclasts. *Journal of biological chemistry.* 2007;282(45):33098-33106.
28. Yu X, Huang Y, Collin-Osdoby P, Osdoby P. Stromal Cell-Derived Factor-1 (SDF-1) Recruits Osteoclast Precursors by Inducing Chemotaxis, Matrix Metalloproteinase-9 (MMP-9) Activity, and Collagen Transmigration. *Journal of bone and mineral research.* 2003;18(8):1404-1418.
29. Kikuta J, Ishii M. Osteoclast migration, differentiation and function: novel therapeutic targets for rheumatic diseases. *Rheumatology (Oxford, England).* 2013;52(2):226-234.

30. Boyce BF, Xing L. Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Archives of biochemistry and biophysics.* 2008;473(2):139-146.
31. Fuller K, Murphy C, Kirstein B, Fox SW, Chambers TJ. TNFα potently activates osteoclasts, through a direct action independent of and strongly synergistic with RANKL. *Endocrinology.* 2002;143(3):1108-1118.
32. Yoshitake F, Itoh S, Narita H, Ishihara K, Ebisu S. Interleukin-6 directly inhibits osteoclast differentiation by suppressing receptor activator of NF-κB signaling pathways. *Journal of biological chemistry.* 2008;283(17):11535-11540.
33. Axmann R, Böhm C, Krönke G, Zwerina J, Smolen J, Schett G. Inhibition of interleukin-6 receptor directly blocks osteoclast formation in vitro and in vivo. *Arthritis & rheumatism.* 2009;60(9):2747-2756.
34. Zhou L, Somasundaram R, Nederhof RF, et al. Impact of human granulocyte and monocyte isolation procedures on functional studies. *Clinical and vaccine immunology.* 2012;19(7):1065-1074.
35. Kuzyk PR, Schemitsch EH. The basic science of peri-implant bone healing. *Indian journal of orthopaedics.* 2011;45(2):108.
36. Hammond ME, Lapointe GR, Feucht PH, et al. IL-8 induces neutrophil chemotaxis predominantly via type I IL-8 receptors. *Journal of immunology (Baltimore, Md. : 1950).* 1995;155(3):1428-1433.
37. Bohnsack JF, Widjaja K, Ghazizadeh S, et al. A role for C5 and C5a-ase in the acute neutrophil response to group B streptococcal infections. *The journal of infectious diseases.* 1997;175(4):847-855.
38. Smolen JE, Boxer LA. Functions of neutrophils. *Williams hematology, 5th ed. New York: McGraw-Hill.* 1995:779-798.
39. Kasama T, Strieter RM, Standiford T, Burdick M, Kunkel S. Expression and regulation of human neutrophil-derived macrophage inflammatory protein 1 alpha. *The journal of experimental medicine.* 1993;178(1):63-72.
40. Kasama T, Miwa Y, Isozaki T, Odai T, Adachi M, Kunkel SL. Neutrophil-derived cytokines: potential therapeutic targets in inflammation. *Current drug targets. Inflammation and allergy.* 2005;4(3):273-279.
41. Bennouna S, Bliss SK, Curiel TJ, Denkers EY. Cross-talk in the innate immune system: neutrophils instruct recruitment and activation of dendritic cells during microbial infection. *Journal of immunology (Baltimore, Md. : 1950).* 2003;171(11):6052-6058.
42. Anderson JM. Biological responses to materials. *Annual review of materials research.* 2001;31(1):81-110.

43. Italiani P, Boraschi D. From monocytes to M1/M2 macrophages: phenotypical vs. functional differentiation. *Frontiers in immunology*. 2014;17;5:514.
44. Shi C, Pamer EG. Monocyte recruitment during infection and inflammation. *Nature reviews immunology*. 2011;11(11):762-774.
45. Wu AC, Raggatt LJ, Alexander KA, Pettit AR. Unraveling macrophage contributions to bone repair. *BoneKEy reports*. 2013;2:373.
46. Alexander KA, Chang MK, Maylin ER, et al. Osteal macrophages promote in vivo intramembranous bone healing in a mouse tibial injury model. *Journal of bone and mineral research* 2011;26(7):1517-1532.
47. Guihard P, Danger Y, Brounais B, et al. Induction of osteogenesis in mesenchymal stem cells by activated monocytes/macrophages depends on oncostatin M signaling. *Stem cells (Dayton, Ohio)*. 2012;30(4):762-772.
48. Nicolaidou V, Wong MM, Redpath AN, et al. Monocytes induce STAT3 activation in human mesenchymal stem cells to promote osteoblast formation. *PloS one*. 2012;7(7):e39871.
49. Ekström K, Omar O, Granéli C, Wang X, Vazirisani F, Thomsen P. Monocyte exosomes stimulate the osteogenic gene expression of mesenchymal stem cells. *PloS one*. 2013;8(9):e75227.
50. Brodbeck WG, Anderson JM. Giant cell formation and function. *Current opinion in hematology*. 2009;16(1):53-57.
51. Thomsen P, Gretzer C. Macrophage interactions with modified material surfaces. *Current opinion in solid state and materials science*. 2001;5(2):163-176.
52. Xia Z, Triffitt JT. A review on macrophage responses to biomaterials. *Biomedical materials (Bristol, England)*. 2006;1(1):R1-9.
53. Singh I. *Textbook of human histology:(with colour atlas & practical guide)*. 6th ed. New Delhi, India: Jaypee Brothers Publishers; 2011.
54. Barbul A. Role of T-cell-dependent immune system in wound healing. *Progress in clinical and biological research*. 1988;266:161-175.
55. Davis PA, Corless DJ, Aspinall R, Wastell C. Effect of CD4(+) and CD8(+) cell depletion on wound healing. *The British journal of surgery*. 2001;88(2):298-304.
56. Park JE, Barbul A. Understanding the role of immune regulation in wound healing. *American journal of surgery*. 2004;187(5a):11s-16s.
57. Singh A, Ali S, Srivastava R, Verma N. Immunological Response to Post-trauma Bone Remodeling. *Journal of postgraduate medicine education and research*. 2012;46(3):148-151.

58. Nam D, Mau E, Wang Y, et al. T-lymphocytes enable osteoblast maturation via IL-17F during the early phase of fracture repair. *PloS one.* 2012;7(6):e40044.
59. Mangashetti LS, Khapli SM, Wani MR. IL-4 inhibits bone-resorbing activity of mature osteoclasts by affecting NF-kappa B and Ca2+ signaling. *Journal of immunology (Baltimore, Md. : 1950).* 2005;175(2):917-925.
60. McNally AK, Anderson JM. Foreign body-type multinucleated giant cells induced by interleukin-4 express select lymphocyte co-stimulatory molecules and are phenotypically distinct from osteoclasts and dendritic cells. *Experimental and molecular pathology.* 2011;91(3):673-681.
61. Ferguson C, Alpern E, Miclau T, Helms JA. Does adult fracture repair recapitulate embryonic skeletal formation? *Mechanisms of development.* 1999;87(1):57-66.
62. Grundnes O, Reikeras O. The importance of the hematoma for fracture healing in rats. *Acta orthopaedica Scandinavica.* 1993;64(3):340-342.
63. Giannoudis PV, Einhorn TA, Marsh D. Fracture healing: the diamond concept. *Injury.* 2007;38:S3-S6.
64. Yellowley C. CXCL12/CXCR4 signaling and other recruitment and homing pathways in fracture repair. *BoneKEy reports.* 2013;2:300.
65. Lieberman JR, Daluiski A, Einhorn TA. The role of growth factors in the repair of bone. Biology and clinical applications. *The Journal of bone and joint surgery. American volume.* 2002;84-a(6):1032-1044.
66. Tsuji K, Bandyopadhyay A, Harfe BD, et al. BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. *Nature genetics.* 2006;38(12):1424-1429.
67. Sakou T. Bone morphogenetic proteins: from basic studies to clinical approaches. *Bone.* 1998;22(6):591-603.
68. Shapiro F. Bone development and its relation to fracture repair. The role of mesenchymal osteoblasts and surface osteoblasts. *European cells & materials.* 2008;15(53):e76.
69. Einhorn TA. The cell and molecular biology of fracture healing. *Clinical orthopaedics and related research.* 1998(355 Suppl):S7-21.
70. Kini U, Nandeesh B. Physiology of bone formation, remodeling, and metabolism. *Radionuclide and hybrid bone imaging.* 2012:29-57.
71. Mackie E, Ahmed Y, Tatarczuch L, Chen K-S, Mirams M. Endochondral ossification: how cartilage is converted into bone in the developing skeleton. *The international journal of biochemistry & cell biology.* 2008;40(1):46-62.
72. Goldring MB, Tsuchimochi K, Ijiri K. The control of chondrogenesis. *Journal of cellular biochemistry.* 2006;97(1):33-44.
73. Oryan A, Alidadi S, Moshiri A. Current concerns regarding healing of bone defects. *Hard tissue.* 2013;2:13.

74. Uchida S, Sakai A, Kudo H, et al. Vascular endothelial growth factor is expressed along with its receptors during the healing process of bone and bone marrow after drill-hole injury in rats. *Bone.* 2003;32(5):491-501.
75. Dimitriou R, Tsiridis E, Giannoudis PV. Current concepts of molecular aspects of bone healing. *Injury.* 2005;36(12):1392-1404.
76. Massague J, Attisano L, Wrana JL. The TGF-beta family and its composite receptors. *Trends in cell biology.* 1994;4(5):172-178.
77. Reddi AH. Bone morphogenetic proteins: from basic science to clinical applications. *The Journal of bone and joint surgery. American volume.* 2001;83-A Suppl 1(Pt 1):S1-6.
78. Cheng H, Jiang W, Phillips FM, et al. Osteogenic activity of the fourteen types of human bone morphogenetic proteins (BMPs). *The journal of bone & joint surgery.* 2003;85(8):1544-1552.
79. Chen G, Deng C, Li Y-P. TGF-β and BMP signaling in osteoblast differentiation and bone formation. *International journal of biological sciences.* 2012;8(2):272.
80. Gaur T, Lengner CJ, Hovhannisyan H, et al. Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. *Journal of biological chemistry.* 2005;280(39):33132-33140.
81. Zernik J, Twarog K, Upholt WB. Regulation of alkaline phosphatase and alpha2 (I) procollagen synthesis during early intramembranous bone formation in the rat mandible. *Differentiation.* 1990;44(3):207-215.
82. Rucci N. Molecular biology of bone remodelling. *Clinical cases in mineral and bone metabolism.* 2008;5(1):49.
83. Raggatt LJ, Partridge NC. Cellular and molecular mechanisms of bone remodeling. *Journal of biological chemistry.* 2010;285(33):25103-25108.
84. Cowin SC, Moss-Salentijn L, Moss ML. Candidates for the mechanosensory system in bone. *Journal of biomechanical engineering.* 1991;113(2):191-197.
85. Sims NA, Martin TJ. Coupling the activities of bone formation and resorption: a multitude of signals within the basic multicellular unit. *BoneKEy reports.* 2014;3.
86. Hauge EM, Qvesel D, Eriksen EF, Mosekilde L, Melsen F. Cancellous bone remodeling occurs in specialized compartments lined by cells expressing osteoblastic markers. *Journal of bone and mineral research.* 2001;16(9):1575-1582.
87. Boyce B. Advances in the regulation of osteoclasts and osteoclast functions. *Journal of dental research.* 2013;92(10):860-7.
88. Tamma R, Zallone A. Osteoblast and osteoclast crosstalks: from OAF to Ephrin. *Inflammation & allergy-drug targets.* 2012;11(3):196-200.

89. Pederson L, Ruan M, Westendorf JJ, Khosla S, Oursler MJ. Regulation of bone formation by osteoclasts involves Wnt/BMP signaling and the chemokine sphingosine-1-phosphate. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105(52):20764-20769.
90. Tang Y, Wu X, Lei W, et al. TGF-beta1-induced migration of bone mesenchymal stem cells couples bone resorption with formation. *Nature medicine*. 2009;15(7):757-765.
91. Xian L, Wu X, Pang L, et al. Matrix IGF-1 maintains bone mass by activation of mTOR in mesenchymal stem cells. *Nature medicine*. 2012;18(7):1095-1101.
92. Howard GA, Bottemiller BL, Turner RT, Rader JI, Baylink DJ. Parathyroid hormone stimulates bone formation and resorption in organ culture: evidence for a coupling mechanism. *Proceedings of the National Academy of Sciences of the United States of America*. 1981;78(5):3204-3208.
93. Arron JR, Choi Y. Osteoimmunology: bone versus immune system. *Nature*. 2000;408(6812):535-536.
94. Warren JT, Zou W, Decker CE, et al. Correlating RANK Ligand/RANK Binding Kinetics with Osteoclast Formation and Function. *Journal of cellular biochemistry*. 2015. doi: 10.1002/jcb.25191. [Epub ahead of print]
95. Feng X. RANKing intracellular signaling in osteoclasts. *IUBMB life*. 2005;57(6):389-395.
96. Luchin A, Purdom G, Murphy K, et al. The Microphthalmia transcription factor regulates expression of the tartrate-resistant acid phosphatase gene during terminal differentiation of osteoclasts. *Journal of bone and mineral research*. 2000;15(3):451-460.
97. Matsumoto M, Kogawa M, Wada S, et al. Essential role of p38 mitogen-activated protein kinase in cathepsin K gene expression during osteoclastogenesis through association of NFATc1 and PU. 1. *Journal of biological chemistry*. 2004;279(44):45969-45979.
98. Zhang Y-H, Heulsmann A, Tondravi MM, Mukherjee A, Abu-Amer Y. Tumor necrosis factor-α (TNF) stimulates RANKL-induced osteoclastogenesis via coupling of TNF type 1 receptor and RANK signaling pathways. *Journal of biological chemistry*. 2001;276(1):563-568.
99. Goldring SR. Bone and joint destruction in rheumatoid arthritis: what is really happening? *The journal of rheumatology*. 2002;65:44-48.
100. Yang C-M, Chien C-S, Yao C-C, Hsiao L-D, Huang Y-C, Wu CB. Mechanical strain induces collagenase-3 (MMP-13) expression in MC3T3-E1 osteoblastic cells. *Journal of biological chemistry*. 2004;279(21):22158-22165.

101. Partridge N, Jeffrey J, Ehlich L, et al. Hormonal regulation of the production of collagenase and a collagenase inhibitor activity by rat osteogenic sarcoma cells*. *Endocrinology.* 1987;120(5):1956-1962.
102. Walker EC, McGregor NE, Poulton IJ, et al. Cardiotrophin-1 is an osteoclast-derived stimulus of bone formation required for normal bone remodeling. *Journal of bone and mineral research.* 2008;23(12):2025-2032.
103. Negishi-Koga T, Shinohara M, Komatsu N, et al. Suppression of bone formation by osteoclastic expression of semaphorin 4D. *Nature medicine.* 2011;17(11):1473-1480.
104. Zhao C, Irie N, Takada Y, et al. Bidirectional ephrinB2-EphB4 signaling controls bone homeostasis. *Cell metabolism.* 2006;4(2):111-121.
105. Lorenzo J. Ephs and ephrins: a new way for bone cells to communicate. *Journal of bone and mineral research.* 2008;23(8):1168-1169.
106. Oryan A, Alidadi S, Moshiri A, Maffulli N. Bone regenerative medicine: classic options, novel strategies, and future directions. *Journal of orthopaedic surgery and research.* 2014;9(1):18.
107. Myeroff C, Archdeacon M. Autogenous bone graft: donor sites and techniques. *The journal of bone & joint surgery.* 2011;93(23):2227-2236.
108. Salyer K, Taylor D. Bone grafts in craniofacial surgery. *Clinics in plastic surgery.* 1987;14(1):27-35.
109. Gazdag AR, Lane JM, Glaser D, Forster RA. Alternatives to autogenous bone graft: efficacy and indications. *Journal of the american academy of orthopaedic surgeons.* 1995;3(1):1-8.
110. Nandi S, Roy S, Mukherjee P, Kundu B, De D, Basu D. Orthopaedic applications of bone graft & graft substitutes: a review. *The indian journal of medical research*. 2010;132:15-30.
111. Misch CE, Dietsh F. Bone-grafting materials in implant dentistry. *Implant dentistry.* 1993;2(3):158-166.
112. Goldberg V, Akhavan S. Biology of bone grafts. In: Lieberman J, Friedlaender G, eds. *Bone regeneration and repair*: Humana press; 2005:57-65.
113. Khan SN, Cammisa FP, Sandhu HS, Diwan AD, Girardi FP, Lane JM. The biology of bone grafting. *Journal of the american academy of orthopaedic surgeons.* 2005;13(1):77-86.
114. Kaveh K, Ibrahim R, Bakar MZA, Ibrahim TA. Bone grafting and bone graft substitutes. *Journal of animal and veterinary advances : JAVA.* 2010;9:1055-1067.
115. Burchardt H. The biology of bone graft repair. *Clinical orthopaedics and related research.* 1983;174:28-34.

116. Greenwald AS, Boden SD, Goldberg VM, Khan Y, Laurencin CT, Rosier RN. Bone-graft substitutes: facts, fictions, and applications. *The journal of bone & joint surgery.* 2001;83(2 suppl 2):S98-103.
117. Glowacki J. A review of osteoinductive testing methods and sterilization processes for demineralized bone. *Cell tissue bank.* 2005;6(1):3-12.
118. Ward WG, Gautreaux MD, Lippert II DC, Boles C. HLA sensitization and allograft bone graft incorporation. *Clinical orthopaedics and related research.* 2008;466(8):1837-1848.
119. Stevenson S, Horowitz M. The response to bone allografts. *The journal of bone & joint surgery.* 1992;74(6):939-950.
120. Siemionow M, Zor F. Allotransplantation. In: Farhadieh R, Bulstrode N, Cugno S, eds. *Plastic and reconstructive surgery: Approaches and Techniques.* 2015:130-143.
121. Chakkalakal DA, Strates BS, Garvin KL, et al. Demineralized bone matrix as a biological scaffold for bone repair. *Tissue engineering.* 2001;7(2):161-177.
122. Bauer TW, Muschler GF. Bone graft materials: An overview of the basic science. *Clinical orthopaedics and related research.* 2000;371:10-27.
123. Turonis JW, McPherson III JC, Cuenin MF, Hokett SD, Peacock ME, Sharawy M. The effect of residual calcium in decalcified freeze-dried bone allograft in a critical-sized defect in the Rattus norvegicus calvarium. *Journal of oral implantology.* 2006;32(2):55-62.
124. Solheim E. Osteoinduction by demineralised bone. *International orthopaedics.* 1998;22(5):335-342.
125. Peterson B, Whang PG, Iglesias R, Wang JC, Lieberman JR. Osteoinductivity of commercially available demineralized bone matrix. *The journal of bone & joint Surgery.* 2004;86(10):2243-2250.
126. Schwartz Z, Mellonig J, Carnes Jr D, et al. Ability of commercial demineralized freeze-dried bone allograft to induce new bone formation. *Journal of periodontology.* 1996;67(9):918-926.
127. Stavropoulos A. Deproteinized bovine bone xenograft. In: Pietrzak W, ed. *Musculoskeletal tissue regeneration*: Humana press; 2008:119-151.
128. Benke D, Olah A, Möhler H. Protein-chemical analysis of Bio-Oss bone substitute and evidence on its carbonate content. *Biomaterials.* 2001;22(9):1005-1012.
129. Yu B, Zhao G, Lim J, Lee Y. Compressive mechanical properties of bovine cortical bone under varied loading rates. *Proceedings of the institution of mechanical engineers, Part H: Journal of engineering in medicine.* 2011;225(10):941-7.
130. Valentini P, Abensur D, Densari D, Graziani J, Hämmerle C. Histological evaluation of Bio-Oss in a 2-stage sinus floor elevation

and implantation procedure. A human case report. *Clinical oral implants research.* 1998;9(1):59-64.
131. De Boever AL, De Boever JA. Guided bone regeneration around non-submerged implants in narrow alveolar ridges: a prospective long-term clinical study. *Clinical oral implants research.* 2005;16(5):549-556.
132. Ewers R, Goriwoda W, Schopper C, Moser D, Spassova E. Histologic findings at augmented bone areas supplied with two different bone substitute materials combined with sinus floor lifting. *Clinical oral implants research.* 2004;15(1):96-100.
133. Stavropoulos A, Kostopoulos L, Mardas N, Randel Nyengaard J, Karring T. Deproteinized bovine bone used as an adjunct to guided bone augmentation: an experimental study in the rat. *Clinical implant dentistry and related research.* 2001;3(3):156-165.
134. Stavropoulos A, Karring ES, Kostopoulos L, Karring T. Deproteinized bovine bone and gentamicin as an adjunct to GTR in the treatment of intrabony defects: a randomized controlled clinical study. *Journal of clinical periodontology.* 2003;30(6):486-495.
135. Stavropoulos A, Kostopoulos L, Nyengaard JR, Karring T. Deproteinized bovine bone (Bio-Oss®) and bioactive glass (Biogran®) arrest bone formation when used as an adjunct to guided tissue regeneration (GTR). *Journal of clinical periodontology.* 2003;30(7):636-643.
136. LeGeros RZ. Calcium phosphate-based osteoinductive materials. *Chemical reviews.* 2008;108(11):4742-4753.
137. LeGeros RZ, Daculsi G, LeGeros JP. Bioactive bioceramics. *Musculoskeletal tissue regeneration*. 2008:153-181.
138. Dorozhkin SV, Epple M. Biological and medical significance of calcium phosphates. *Angewandte chemie international edition.* 2002;41(17):3130-3146.
139. Albee FH. Studies in bone growth: triple calcium phosphate as a stimulus to osteogenesis. *Annals of surgery.* 1920;71(1):32.
140. Monroe E, Votava W, Bass D, Mc Mullen J. New calcium phosphate ceramic material for bone and tooth implants. *Journal of dental research.* 1971;50(4):860-861.
141. Nery E, Lynch K, Hirthe W, Mueller K. Bioceramic implants in surgically produced infrabony defects. *Journal of periodontology.* 1975;46(6):328-347.
142. Al-Sanabani JS, Madfa AA, Al-Sanabani FA. Application of calcium phosphate materials in dentistry. *International journal of biomaterials.* 2013;2013:876132.
143. Zhou AJ-J, Peel SA, Clokie CM. An evaluation of hydroxyapatite and biphasic calcium phosphate in combination with Pluronic F127 and BMP on bone repair. *Journal of craniofacial surgery.* 2007;18(6):1264-1275.

144. Manjubala I, Sivakumar M, Sureshkumar R, Sastry T. Bioactivity and osseointegration study of calcium phosphate ceramic of different chemical composition. *Journal of biomedical materials research.* 2002;63(2):200-208.
145. Suzuki O. Octacalcium phosphate: osteoconductivity and crystal chemistry. *Acta biomaterialia.* 2010;6(9):3379-3387.
146. Komlev VS, Barinov SM, Bozo II, et al. Bioceramics composed of octacalcium phosphate demonstrate enhanced biological behavior. *ACS applied materials & interfaces.* 2014;6(19):16610-16620.
147. Roveri N, Iafisco M. Evolving application of biomimetic nanostructured hydroxyapatite. *Nanotechnology, science and applications.* 2010;3:107.
148. Vahabi S, Amirizadeh N, Shokrgozar M, et al. A comparison between the efficacy of Bio-Oss, hydroxyapatite tricalcium phosphate and combination of mesenchymal stem cells in inducing bone regeneration. *Chang Gung medical journal.* 2011;35(1):28-37.
149. Nandi SK, Kundu B, Mukherjee J, Mahato A, Datta S, Balla VK. Converted marine coral hydroxyapatite implants with growth factors: In vivo bone regeneration. *Materials science and engineering: C.* 2015;49:816-823.
150. Barrère F, van Blitterswijk CA, de Groot K. Bone regeneration: molecular and cellular interactions with calcium phosphate ceramics. *International journal of nanomedicine.* 2006;1(3):317.
151. Salinas AJ, Vallet-Regí M. Bioactive ceramics: from bone grafts to tissue engineering. *RSC Advances.* 2013;3(28):11116-11131.
152. Blokhuis TJ, Termaat MF, den Boer FC, Patka P, Bakker FC, Henk JTM. Properties of calcium phosphate ceramics in relation to their in vivo behavior. *Journal of trauma and acute care surgery.* 2000;48(1):179.
153. LeGeros RZ. Properties of osteoconductive biomaterials: calcium phosphates. *Clinical orthopaedics and related research.* 2002;395:81-98.
154. Combes C, Rey C. Adsorption of proteins and calcium phosphate materials bioactivity. *Biomaterials.* 2002;23(13):2817-2823.
155. El-Ghannam A, Ducheyne P, Shapiro I. Effect of serum proteins on osteoblast adhesion to surface-modified bioactive glass and hydroxyapatite. *Journal of orthopaedic research.* 1999;17(3):340-345.
156. Daculsi G, Hartmann D, Heughebaert M, Hamel L, Le Nihouannen J. In vivo cell interactions with calcium phosphate bioceramics. *Journal of submicroscopic cytology and pathology.* 1988;20(2):379-384.
157. Chai YC, Carlier A, Bolander J, et al. Current views on calcium phosphate osteogenicity and the translation into effective bone regeneration strategies. *Acta biomaterialia.* 2012;8(11):3876-3887.

158. Dvorak MM, Siddiqua A, Ward DT, et al. Physiological changes in extracellular calcium concentration directly control osteoblast function in the absence of calciotropic hormones. *Proceedings of the National Academy of Sciences of the United States of America.* 2004;101(14):5140-5145.
159. Lee YK, Song J, Lee SB, et al. Proliferation, differentiation, and calcification of preosteoblast-like MC3T3-E1 cells cultured onto noncrystalline calcium phosphate glass. *Journal of biomedical materials research. Part A.* 2004;69(1):188-195.
160. Liu Y, Cooper PR, Barralet JE, Shelton RM. Influence of calcium phosphate crystal assemblies on the proliferation and osteogenic gene expression of rat bone marrow stromal cells. *Biomaterials.* 2007;28(7):1393-1403.
161. Honda Y, Anada T, Kamakura S, Nakamura M, Sugawara S, Suzuki O. Elevated extracellular calcium stimulates secretion of bone morphogenetic protein 2 by a macrophage cell line. *Biochemical and biophysical research communications.* 2006;345(3):1155-1160.
162. Liu YK, Lu QZ, Pei R, et al. The effect of extracellular calcium and inorganic phosphate on the growth and osteogenic differentiation of mesenchymal stem cells in vitro: implication for bone tissue engineering. *Biomedical materials.* 2009;4(2):025004.
163. Heymann D, Pradal G, Benahmed M. Cellular mechanisms of calcium phosphate ceramic degradation. *Histology and histopathology.* 1999;14(3):871-877.
164. Lorenz J, Kubesch A, Korzinskas T, et al. TRAP-positive multinucleated giant cells are foreign body giant cells rather than osteoclasts: Results from a split-mouth study in humans. *Journal of oral implantology.* 2014. [Epub ahead of print]
165. Baslé MF, Chappard D, Grizon F, et al. Osteoclastic resorption of Ca-P biomaterials implanted in rabbit bone. *Calcified tissue international.* 1993;53(5):348-356.
166. Ghanaati S, Barbeck M, Detsch R, et al. The chemical composition of synthetic bone substitutes influences tissue reactions in vivo: histological and histomorphometrical analysis of the cellular inflammatory response to hydroxyapatite, beta-tricalcium phosphate and biphasic calcium phosphate ceramics. *Biomedical materials.* 2012;7(1):015005.
167. Barradas A, Yuan H, Blitterswijk CA, Habibovic P. Osteoinductive biomaterials: current knowledge of properties, experimental models and biological mechanisms. *European cells & materials.* 2011;21:407-429.
168. Daculsi G, Laboux O, Malard O, Weiss P. Current state of the art of biphasic calcium phosphate bioceramics. *Journal of materials science: materials in medicine.* 2003;14(3):195-200.

169. Shepherd JH, Shepherd DV, Best SM. Substituted hydroxyapatites for bone repair. *Journal of materials science: materials in medicine.* 2012;23(10):2335-2347.
170. Yang L, Perez-Amodio S, Barrère-de Groot FY, Everts V, van Blitterswijk CA, Habibovic P. The effects of inorganic additives to calcium phosphate on in vitro behavior of osteoblasts and osteoclasts. *Biomaterials.* 2010;31(11):2976-2989.
171. Julien M, Khairoun I, LeGeros RZ, et al. Physico-chemical–mechanical and in vitro biological properties of calcium phosphate cements with doped amorphous calcium phosphates. *Biomaterials.* 2007;28(6):956-965.
172. Aina V, Lusvardi G, Annaz B, et al. Magnesium-and strontium-co-substituted hydroxyapatite: the effects of doped-ions on the structure and chemico-physical properties. *Journal of materials science: materials in medicine.* 2012;23(12):2867-2879.
173. Landi E, Logroscino G, Proietti L, Tampieri A, Sandri M, Sprio S. Biomimetic Mg-substituted hydroxyapatite: from synthesis to in vivo behaviour. *Journal of materials science: materials in medicine.* 2008;19(1):239-247.
174. Patel N, Brooks R, Clarke M, et al. In vivo assessment of hydroxyapatite and silicate-substituted hydroxyapatite granules using an ovine defect model. *Journal of materials science: materials in medicine.* 2005;16(5):429-440.
175. Hulsart-Billström G, Xia W, Pankotai E, et al. Osteogenic potential of Sr-doped calcium phosphate hollow spheres in vitro and in vivo. *Journal of biomedical materials research. Part A.* 2013;101(8):2322-2331.
176. Xia W, Grandfield K, Schwenke A, Engqvist H. Synthesis and release of trace elements from hollow and porous hydroxyapatite spheres. *Nanotechnology.* 2011;22(30):305610.
177. Zahra N, Fayyaz M, Iqbal W, Irfan M, Alam S. A process for the development of strontium hydroxyapatite. *IOP Conf. Series: Materials Science and Engineering.* 2014;60:012056
178. Gallacher S, Dixon T. Impact of treatments for postmenopausal osteoporosis (bisphosphonates, parathyroid hormone, strontium ranelate, and denosumab) on bone quality: a systematic review. *Calcified tissue international.* 2010;87(6):469-484.
179. Peng S, Liu XS, Huang S, et al. The cross-talk between osteoclasts and osteoblasts in response to strontium treatment: involvement of osteoprotegerin. *Bone.* 2011;49(6):1290-1298.
180. Yamaguchi M, Weitzmann MN. The intact strontium ranelate complex stimulates osteoblastogenesis and suppresses osteoclastogenesis by antagonizing NF-κB activation. *Molecular and cellular biochemistry.* 2012;359(1-2):399-407.

181. Retzepi M, Donos N. Guided bone regeneration: biological principle and therapeutic applications. *Clinical oral implants research.* 2010;21(6):567-576.
182. Laurell L, Gottlow J. Guided tissue regeneration update. *International dental journal.* 1998;48(4):386-398.
183. Gottlow J, Nyman S, Karring T, Lindhe J. New attachment formation as the result of controlled tissue regeneration. *Journal of clinical periodontology.* 1984;11(8):494-503.
184. Nyman S, Lindhe J, Karring T, Rylander H. New attachment following surgical treatment of human periodontal disease. *Journal of clinical periodontology.* 1982;9(4):290-296.
185. Line S, Polson A, Zander H. Relationship between periodontal injury, selective cell repopulation and ankylosis. *Journal of periodontology.* 1974;45(10):725.
186. Tatakis DN, Trombelli L. Gingival recession treatment: guided tissue regeneration with bioabsorbable membrane versus connective tissue graft. *Journal of periodontology.* 2000;71(2):299-307.
187. Murphy KG, Gunsolley JC. Guided tissue regeneration for the treatment of periodontal intrabony and furcation defects. A systematic review. *Annals of periodontology.* 2003;8(1):266-302.
188. Wang H-L, Bunyaratavej P, Labadie M, Shyr Y, MacNeil RL. Comparison of 2 clinical techniques for treatment of gingival recession. *Journal of periodontology.* 2001;72(10):1301-1311.
189. Farzad M, Mohammadi M. Guided bone regeneration: A literature review. *Journal of oral health and oral epidemiology.* 2012;1(1):3-18.
190. Becker W, Dahlin C, Becker BE, et al. The use of e-PTFE barrier membranes for bone promotion around titanium implants placed into extraction sockets: a prospective multicenter study. *The international journal of oral & maxillofacial implants.* 1993;9(1):31-40.
191. Bartee BK. Evaluation of new polytetrafluoroethylene-guided tissue regeneration membrane in healing extraction sites. *Compendium of continuing education in dentistry.* 1998;19(12):1256-8, 1260, 1262-4.
192. Rakhmatia YD, Ayukawa Y, Furuhashi A, Koyano K. Current barrier membranes: titanium mesh and other membranes for guided bone regeneration in dental applications. *Journal of prosthodontic research.* 2013;57(1):3-14.
193. Nowzari H, Slots J. Microbiologic and clinical study of polytetrafluoroethylene membranes for guided bone regeneration around implants. *The international journal of oral & maxillofacial implants.* 1994;10(1):67-73.
194. Simion M, Baldoni M, Rossi P, Zaffe D. A comparative study of the effectiveness of e-PTFE membranes with and without early exposure during the healing period. *The international journal of periodontics & restorative dentistry.* 1994;14(2):166-180.

195. McAllister BS, Haghighat K. Bone augmentation techniques. *Journal of periodontology.* 2007;78(3):377-396.
196. Piattelli A, Scarano A, Coraggio F, Matarasso S. Early tissue reactions to polylactic acid resorbable membranes: a histological and histochemical study in rabbit. *Biomaterials.* 1998;19(10):889-896.
197. Hürzeler MB, Quiñones CR, Hutmacher D, Schüpbach P. Guided bone regeneration around dental implants in the atrophic alveolar ridge using a bioresorbable barrier. An experimental study in the monkey. *Clinical oral implants research.* 1997;8(4):323-331.
198. Lekovic V, Camargo PM, Klokkevold PR, et al. Preservation of alveolar bone in extraction sockets using bioabsorbable membranes. *Journal of periodontology.* 1998;69(9):1044-1049.
199. Gielkens P, Schortinghuis J, De Jong J, et al. The influence of barrier membranes on autologous bone grafts. *Journal of dental research.* 2008;87(11):1048-1052.
200. Meinig RP. Clinical use of resorbable polymeric membranes in the treatment of bone defects. *Orthopedic clinics of North America.* 2010;41(1):39-47.
201. Bunyaratavej P, Wang H-L. Collagen membranes: a review. *Journal of periodontology.* 2001;72(2):215-229.
202. Rothamel D, Schwarz F, Sager M, Herten M, Sculean A, Becker J. Biodegradation of differently cross-linked collagen membranes: an experimental study in the rat. *Clinical oral implants research.* 2005;16(3):369-378.
203. Jorge-Herrero E, Fernandez P, Turnay J, et al. Influence of different chemical cross-linking treatments on the properties of bovine pericardium and collagen. *Biomaterials.* 1999;20(6):539-545.
204. Sam G, Pillai BRM. Evolution of barrier membranes in periodontal regeneration-"Are the third generation membranes really here? *Journal of clinical and diagnostic research: JCDR.* 2014;8(12):ZE14-7.
205. Ribeiro N, Sousa SR, van Blitterswijk CA, Moroni L, Monteiro FJ. A biocomposite of collagen nanofibers and nanohydroxyapatite for bone regeneration. *Biofabrication.* 2014;6(3):035015.
206. Liao S, Wang W, Uo M, et al. A three-layered nano-carbonated hydroxyapatite/collagen/PLGA composite membrane for guided tissue regeneration. *Biomaterials.* 2005;26(36):7564-7571.
207. Veríssimo DM, Leitão RF, Figueiró SD, et al. Guided bone regeneration produced by new mineralized and reticulated collagen membranes in critical-sized rat calvarial defects. *Experimental biology and medicine.* 2015;240(2):175-184.
208. Jo J-Y, Jeong S-I, Shin Y-M, et al. Sequential delivery of BMP-2 and BMP-7 for bone regeneration using a heparinized collagen membrane. *International journal of oral and maxillofacial surgery.* 2015;44(7):921-8.

209. Nakahara T, Nakamura T, Kobayashi E, et al. Novel approach to regeneration of periodontal tissues based on in situ tissue engineering: effects of controlled release of basic fibroblast growth factor from a sandwich membrane. *Tissue engineering.* 2003;9(1):153-162.
210. Shim J-H, Yoon M-C, Jeong C-M, et al. Efficacy of rhBMP-2 loaded PCL/PLGA/β-TCP guided bone regeneration membrane fabricated by 3D printing technology for reconstruction of calvaria defects in rabbit. *Biomedical materials.* 2014;9(6):065006.
211. Park YJ, Ku Y, Chung CP, Lee SJ. Controlled release of platelet-derived growth factor from porous poly (L-lactide) membranes for guided tissue regeneration. *Journal of controlled release.* 1998;51(2):201-211.
212. Badylak SF, Record R, Lindberg K, Hodde J, Park K. Small intestinal submucosa: a substrate for in vitro cell growth. *Journal of biomaterials science. Polymer edition.* 1998;9(8):863-878.
213. Hodde JP, Badylak SF, Brightman AO, Voytik-Harbin SL. Glycosaminoglycan content of small intestinal submucosa: a bioscaffold for tissue replacement. *Tissue engineering.* 1996;2(3):209-217.
214. Hodde J, Janis A, Ernst D, Zopf D, Sherman D, Johnson C. Effects of sterilization on an extracellular matrix scaffold: part I. Composition and matrix architecture. *Journal of materials science: materials in medicine.* 2007;18(4):537-543.
215. Chun L, Yoon J, Song Y, Huie P, Regula D, Goodman S. The characterization of macrophages and osteoclasts in tissues harvested from revised total hip prostheses. *Journal of biomedical materials research.* 1999;48(6):899-903.
216. McNally AK, Anderson JM. Foreign body-type multinucleated giant cells induced by interleukin-4 express select lymphocyte co-stimulatory molecules and are phenotypically distinct from osteoclasts and dendritic cells. *Experimental and molecular pathology.* 2011;91(3):673-681.
217. Heinemann D, Lohmann C, Siggelkow H, Alves F, Engel I, Köster G. Human osteoblast-like cells phagocytose metal particles and express the macrophage marker CD68 in vitro. *The Journal of bone and joint surgery. British volume.* 2000;82(2):283-289.
218. Kashima TG, Nishiyama T, Shimazu K, et al. Periostin, a novel marker of intramembranous ossification, is expressed in fibrous dysplasia and in c-Fos–overexpressing bone lesions. *Human pathology.* 2009;40(2):226-237.
219. Jarmar T, Palmquist A, Brånemark R, Hermansson L, Engqvist H, Thomsen P. Technique for preparation and characterization in cross-section of oral titanium implant surfaces using focused ion beam and

transmission electron microscopy. *Journal of biomedical materials research. Part A.* 2008;87(4):1003-1009.
220. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. *Bioinformatics methods and protocols*: *Methods in Molecular Biology*. 1999;132: 365-386.
221. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology.* 2002;3(7):research0034.
222. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer research.* 2004;64(15):5245-5250.
223. O'Loughlin PF, Morr S, Bogunovic L, Kim AD, Park B, Lane JM. Selection and development of preclinical models in fracture-healing research. *The Journal of bone and joint surgery. American volume.* 2008;90(Supplement 1):79-84.
224. Aurégan J, Coyle R, Danoff J, Burky R, Akelina Y, Rosenwasser M. The rat model of femur fracture for bone and mineral research An improved description of expected comminution, quantity of soft callus and incidence of complications. *Bone and joint research.* 2013;2(8):149-154.
225. Histing T, Garcia P, Holstein J, et al. Small animal bone healing models: standards, tips, and pitfalls results of a consensus meeting. *Bone.* 2011;49(4):591-599.
226. Bentolila V, Boyce T, Fyhrie D, Drumb R, Skerry T, Schaffler M. Intracortical remodeling in adult rat long bones after fatigue loading. *Bone.* 1998;23(3):275-281.
227. Gomes P, Fernandes M. Rodent models in bone-related research: the relevance of calvarial defects in the assessment of bone regeneration strategies. *Laboratory animals.* 2011;45(1):14-24.
228. Hollinger JO, Kleinschmidt JC. The critical size defect as an experimental model to test bone repair materials. *Journal of craniofacial surgery.* 1990;1(1):60-68.
229. Poser L, Matthys R, Schawalder P, Pearce S, Alini M, Zeiter S. A standardized critical size defect model in normal and osteoporotic rats to evaluate bone tissue engineered constructs. *BioMed research international.* 2014;2014:348635.
230. Muschler GF, Raut VP, Patterson TE, Wenke JC, Hollinger JO. The design and use of animal models for translational research in bone tissue engineering and regenerative medicine. *Tissue engineering. Part B, Reviews.* 2010;16(1):123-145.

231. Kim J-H, Kim H-W. Rat defect models for bone grafts and tissue engineered bone constructs. *Tissue engineering and regenerative medicine.* 2013;10(6):310-316.
232. Johnston BD, Ward WE. The Ovariectomized rat as a model for studying alveolar bone loss in postmenopausal women. *BioMed research international.* 2015:635023.
233. Kalu DN. The ovariectomized rat model of postmenopausal bone loss. *Bone and mineral.* 1991;15(3):175-191.
234. Turner RT, Maran A, Lotinun S, et al. Animal models for osteoporosis. *Reviews in endocrine & metabolic disorders.* 2001;2(1):117.
235. Yingjie H, Ge Z, Yisheng W, et al. Changes of microstructure and mineralized tissue in the middle and late phase of osteoporotic fracture healing in rats. *Bone.* 2007;41(4):631-638.
236. Ozawa S, Ogawa T, Iida K, et al. Ovariectomy hinders the early stage of bone-implant integration: histomorphometric, biomechanical, and molecular analyses. *Bone.* 2002;30(1):137-143.
237. Dimitriou R, Mataliotakis GI, Calori GM, Giannoudis PV. The role of barrier membranes for guided bone regeneration and restoration of large bone defects: current experimental and clinical evidence. *BMC medicine.* 2012;10(1):81.
238. Bottino MC, Thomas V, Schmidt G, et al. Recent advances in the development of GTR/GBR membranes for periodontal regeneration—a materials perspective. *Dental materials.* 2012;28(7):703-721.
239. Luchsinger C, Arias ME, Vargas T, Paredes M, Sánchez R, Felmer R. Stability of reference genes for normalization of reverse transcription quantitative real-time PCR (RT-qPCR) data in bovine blastocysts produced by IVF, ICSI and SCNT. *Zygote.* 2014;22(04):505-512.
240. Lennerås M, Palmquist A, Norlindh B, Emanuelsson L, Thomsen P, Omar O. Oxidized titanium implants enhance osseointegration via mechanisms involving RANK/RANKL/OPG regulation. *Clinical implant dentistry and related research.* 2015. doi: 10.1111/cid.12276. [Epub ahead of print]
241. Murakami Y, Honda Y, Anada T, Shimauchi H, Suzuki O. Comparative study on bone regeneration by synthetic octacalcium phosphate with various granule sizes. *Acta biomaterialia.* 2010;6(4):1542-1548.
242. Kikawa T, Kashimoto O, Imaizumi H, Kokubun S, Suzuki O. Intramembranous bone tissue response to biodegradable octacalcium phosphate implant. *Acta biomaterialia.* 2009;5(5):1756-1766.
243. Imaizumi H, Sakurai M, Kashimoto O, Kikawa T, Suzuki O. Comparative study on osteoconductivity by synthetic octacalcium

phosphate and sintered hydroxyapatite in rabbit bone marrow. *Calcified tissue international.* 2006;78(1):45-54.
244. Takami M, Mochizuki A, Yamada A, et al. Osteoclast differentiation induced by synthetic octacalcium phosphate through receptor activator of NF-κB ligand expression in osteoblasts. *Tissue engineering. Part A.* 2009;15(12):3991-4000.
245. Kamakura S, Sasano Y, Homma-Ohki H, et al. Multinucleated giant cells recruited by implantation of octacalcium phosphate (OCP) in rat bone marrow share ultrastructural characteristics with osteoclasts. *Journal of electron microscopy.* 1997;46(5):397-403.
246. Teti A. Mechanisms of osteoclast-dependent bone formation. *BoneKEy reports.* 2013;2:449.
247. Kreja L, Brenner R, Tautzenberger A, et al. Non-resorbing osteoclasts induce migration and osteogenic differentiation of mesenchymal stem cells. *Journal of cellular biochemistry.* 2010;109(2):347-355.
248. Paknejad M, Rokn A, Rouzmeh N, et al. Histologic evaluation of bone healing capacity following application of inorganic bovine bone and a new allograft material in rabbit calvaria. *Journal of dentistry (Tehran, Iran).* 2015;12(1):31.
249. Liljensten E, Adolfsson E, Strid KG, Thomsen P. Resorbable and nonresorbable hydroxyapatite granules as bone graft substitutes in rabbit cortical defects. *Clinical implant dentistry and related research.* 2003;5(2):95-102.
250. Carmagnola D, Adriaens P, Berglundh T. Healing of human extraction sockets filled with Bio-Oss®. *Clinical oral implants research.* 2003;14(2):137-143.
251. Omar O, Svensson S, Zoric N, et al. In vivo gene expression in response to anodically oxidized versus machined titanium implants. *Journal of biomedical materials research. Part A.* 2010;92(4):1552-1566.
252. Omar O, Lennerås M, Svensson S, et al. Integrin and chemokine receptor gene expression in implant-adherent cells during early osseointegration. *Journal of materials science: materials in medicine.* 2010;21(3):969-980.
253. Gerstenfeld L, Cho T-J, Kon T, et al. Impaired intramembranous bone formation during bone repair in the absence of tumor necrosis factor-alpha signaling. *Cells tissues organs.* 2001;169(3):285-294.
254. Mountziaris PM, Mikos AG. Modulation of the inflammatory response for enhanced bone tissue regeneration. *Tissue engineering. Part B, Reviews.* 2008;14(2):179-186.
255. Ai-Aql Z, Alagl AS, Graves DT, Gerstenfeld LC, Einhorn TA. Molecular mechanisms controlling bone formation during fracture healing and distraction osteogenesis. *Journal of dental research.* 2008;87(2):107-118.

256. Azuma Y, Kaji K, Katogi R, Takeshita S, Kudo A. Tumor necrosis factor-α induces differentiation of and bone resorption by osteoclasts. *Journal of biological chemistry.* 2000;275(7):4858-4864.
257. Jimi E, Nakamura I, Duong LT, et al. Interleukin 1 induces multinucleation and bone-resorbing activity of osteoclasts in the absence of osteoblasts/stromal cells. *Experimental cell research.* 1999;247(1):84-93.
258. Oliveira R, El Hage M, Carrel J-P, Lombardi T, Bernard J-P. Rehabilitation of the edentulous posterior maxilla after sinus floor elevation using deproteinized bovine bone: a 9-year clinical study. *Implant dentistry.* 2012;21(5):422-426.
259. Yildirim M, Spiekermann H, Biesterfeld S, Edelhoff D. Maxillary sinus augmentation using xenogenic bone substitute material Bio-Oss® in combination with venous blood. *Clinical oral implants research.* 2000;11(3):217-229.
260. Shin S-Y, Hwang Y-J, Kim J-H, Seol Y-J. Long-term results of new deproteinized bovine bone material in a maxillary sinus graft procedure. *Journal of periodontal & implant science.* 2014;44(5):259-264.
261. Hess K, Ushmorov A, Fiedler J, Brenner RE, Wirth T. TNFalpha promotes osteogenic differentiation of human mesenchymal stem cells by triggering the NF-kappaB signaling pathway. *Bone.* 2009;45(2):367-376.
262. Bocker W, Docheva D, Prall WC, et al. IKK-2 is required for TNF-alpha-induced invasion and proliferation of human mesenchymal stem cells. *Journal of molecular medicine (Berlin, Germany).* 2008;86(10):1183-1192.
263. Fu X, Han B, Cai S, Lei Y, Sun T, Sheng Z. Migration of bone marrow-derived mesenchymal stem cells induced by tumor necrosis factor-α and its possible role in wound healing. *Wound repair and regeneration.* 2009;17(2):185-191.
264. Katagiri T, Takahashi N. Regulatory mechanisms of osteoblast and osteoclast differentiation. *Oral diseases.* 2002;8(3):147-159.
265. Hofbauer LC, Heufelder AE. Osteoprotegerin and its cognate ligand: a new paradigm of osteoclastogenesis. *European journal of endocrinology / European Federation of Endocrine Societies.* 1998;139(2):152-154.
266. Hofbauer LC, Lacey DL, Dunstan CR, Spelsberg TC, Riggs BL, Khosla S. Interleukin-1beta and tumor necrosis factor-alpha, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. *Bone.* 1999;25(3):255-259.
267. Su W-T, Chou W-L, Chou C-M. Osteoblastic differentiation of stem cells from human exfoliated deciduous teeth induced by thermosensitive hydrogels with strontium phosphate. *Materials*

science & engineering. C, Materials for biological applications. 2015;52:46-53.
268. Yang F, Yang D, Tu J, Zheng Q, Cai L, Wang L. Strontium enhances osteogenic differentiation of mesenchymal stem cells and in vivo bone formation by activating Wnt/catenin signaling. *Stem cells (Dayton, Ohio).* 2011;29(6):981-991.
269. Peng S, Zhou G, Luk KD, et al. Strontium promotes osteogenic differentiation of mesenchymal stem cells through the Ras/MAPK signaling pathway. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology.* 2009;23(1-3):165-174.
270. Baron R, Tsouderos Y. In vitro effects of S12911-2 on osteoclast function and bone marrow macrophage differentiation. *European journal of pharmacology.* 2002;450(1):11-17.
271. Kumar S, Chatterjee K. Strontium eluting graphene hybrid nanoparticles augment osteogenesis in a 3D tissue scaffold. *Nanoscale.* 2015;7(5):2023-2033.
272. Santocildes-Romero ME, Crawford A, Hatton PV, Goodchild RL, Reaney IM, Miller CA. The osteogenic response of mesenchymal stromal cells to strontium-substituted bioactive glasses. *Journal of tissue engineering and regenerative medicine.* 2015;9(5):619-631.
273. Liang Y, Li H, Xu J, et al. Strontium coating by electrochemical deposition improves implant osseointegration in osteopenic models. *Experimental and therapeutic medicine.* 2015;9(1):172-176.
274. Li Y, Shui X, Zhang L, Hu J. Cancellous bone healing around strontium-doped hydroxyapatite in osteoporotic rats previously treated with zoledronic acid. *Journal of biomedical materials research. Part B.* 2015. doi: 10.1002/jbm.b.33417. [Epub ahead of print]
275. Zhang Y, Cui X, Zhao S, et al. Evaluation of Injectable Strontium-Containing Borate Bioactive Glass Cement with Enhanced Osteogenic Capacity in a Critical-Sized Rabbit Femoral Condyle Defect Model. *ACS applied materials & interfaces.* 2015;7(4):2393-2403.
276. Zhang Y, Wei L, Wu C, Miron RJ. Periodontal regeneration using strontium-loaded mesoporous bioactive glass scaffolds in osteoporotic rats. *PLoS One*. 2014;12;9(8):e104527.
277. Kitayama S, Wong LO, Ma L, et al. Regeneration of rabbit calvarial defects using biphasic calcium phosphate and a strontium hydroxyapatite-containing collagen membrane. *Clinical oral implants research.* 2015. doi: 10.1111/clr.12605. [Epub ahead of print]
278. Zhang J, Liu L, Zhao S, Wang H, Yang G. Characterization and In Vivo Evaluation of Trace Element-Loaded Implant Surfaces in Ovariectomized Rats. *The International journal of oral &*

maxillofacial implants. 2015. doi: 10.11607/jomi.3906. [Epub ahead of print]
279. Newman SD, Lotfibakhshaiesh N, O'Donnell M, et al. Enhanced osseous implant fixation with strontium-substituted bioactive glass coating. *Tissue engineering. Part A.* 2014;20(13-14):1850-1857.
280. Braux J, Velard F, Guillaume C, et al. A new insight into the dissociating effect of strontium on bone resorption and formation. *Acta biomaterialia.* 2011;7(6):2593-2603.
281. Schumacher M, Lode A, Helth A, Gelinsky M. A novel strontium(II)-modified calcium phosphate bone cement stimulates human-bone-marrow-derived mesenchymal stem cell proliferation and osteogenic differentiation in vitro. *Acta biomaterialia.* 2013;9(12):9547-9557.
282. Wornham D, Hajjawi M, Orriss I, Arnett T. Strontium potently inhibits mineralisation in bone-forming primary rat osteoblast cultures and reduces numbers of osteoclasts in mouse marrow cultures. *Osteoporosis international.* 2014;25(10):2477-2484.
283. Liu X, Zhu S, Cui J, et al. Strontium ranelate inhibits titanium-particle-induced osteolysis by restraining inflammatory osteoclastogenesis in vivo. *Acta biomaterialia.* 2014;10(11):4912-4918.
284. Ghanaati S, Barbeck M, Lorenz J, et al. Synthetic bone substitute material comparable with xenogeneic material for bone tissue regeneration in oral cancer patients: First and preliminary histological, histomorphometrical and clinical results. *Annals of maxillofacial surgery.* 2013;3(2):126-138.
285. Ghanaati S, Barbeck M, Orth C, et al. Influence of beta-tricalcium phosphate granule size and morphology on tissue reaction in vivo. *Acta biomaterialia.* 2010;6(12):4476-4487.
286. Querido W, Campos AP, Ferreira EHM, San Gil RA, Rossi AM, Farina M. Strontium ranelate changes the composition and crystal structure of the biological bone-like apatite produced in osteoblast cell cultures. *Cell and tissue research.* 2014;357(3):793-801.
287. Voytik-Harbin SL, Brightman AO, Kraine MR, Waisner B, Badylak SF. Identification of extractable growth factors from small intestinal submucosa. *Journal of cellular biochemistry.* 1997;67(4):478-491.
288. Lieberman JR, Daluiski A, Einhorn TA. The role of growth factors in the repair of bone. Biology and clinical applications. *The Journal of bone and joint surgery. American volume.* 2002;84(6):1032-1044.
289. Poniatowski ŁA, Wojdasiewicz P, Gasik R, Szukiewicz D. Transforming Growth Factor Beta Family: Insight into the Role of Growth Factors in Regulation of Fracture Healing Biology and Potential Clinical Applications. *Mediators of inflammation.* 2015;2015:137823.

290. Badylak S, Liang A, Record R, Tullius R, Hodde J. Endothelial cell adherence to small intestinal submucosa: an acellular bioscaffold. *Biomaterials.* 1999;20(23-24):2257-2263.
291. McPherson TB, Badylak SF. Characterization of fibronectin derived from porcine small intestinal submucosa. *Tissue engineering.* 1998;4(1):75-83.
292. Saadeh PB, Mehrara BJ, Steinbrech DS, et al. Mechanisms of fibroblast growth factor-2 modulation of vascular endothelial growth factor expression by osteoblastic cells. *Endocrinology.* 2000;141(6):2075-2083.
293. Collin-Osdoby P, Rothe L, Bekker S, Anderson F, Huang Y, Osdoby P. Basic fibroblast growth factor stimulates osteoclast recruitment, development, and bone pit resorption in association with angiogenesis in vivo on the chick chorioallantoic membrane and activates isolated avian osteoclast resorption in vitro. *Journal of bone and mineral research.* 2002;17(10):1859-1871.
294. Irie K, Alpaslan C, Takahashi K, et al. Osteoclast differentiation in ectopic bone formation induced by recombinant human bone morphogenetic protein 2 (rhBMP-2). *Journal of bone and mineral metabolism.* 2003;21(6):363-369.
295. Rahimi P, Wang CY, Stashenko P, Lee SK, Lorenzo JA, Graves DT. Monocyte chemoattractant protein-1 expression and monocyte recruitment in osseous inflammation in the mouse. *Endocrinology.* 1995;136(6):2752-2759.
296. Yoshimura T, Robinson EA, Tanaka S, Appella E, Leonard EJ. Purification and amino acid analysis of two human monocyte chemoattractants produced by phytohemagglutinin-stimulated human blood mononuclear leukocytes. *The Journal of immunology : official journal of the American Association of Immunologists.* 1989;142(6):1956-1962.
297. Li X, Qin L, Bergenstock M, Bevelock LM, Novack DV, Partridge NC. Parathyroid hormone stimulates osteoblastic expression of MCP-1 to recruit and increase the fusion of pre/osteoclasts. *The journal of biological chemistry.* 2007;282(45):33098-33106.
298. Kucia M, Ratajczak J, Reca R, Janowska-Wieczorek A, Ratajczak MZ. Tissue-specific muscle, neural and liver stem/progenitor cells reside in the bone marrow, respond to an SDF-1 gradient and are mobilized into peripheral blood during stress and tissue injury. *Blood cells, molecules & diseases.* 2004;32(1):52-57.
299. Zhu W, Liang G, Huang Z, Doty SB, Boskey AL. Conditional inactivation of the CXCR4 receptor in osteoprecursors reduces postnatal bone formation due to impaired osteoblast development. *The journal of biological chemistry.* 2011;286(30):26794-26805.
300. Wright LM, Maloney W, Yu X, Kindle L, Collin-Osdoby P, Osdoby P. Stromal cell-derived factor-1 binding to its chemokine receptor

CXCR4 on precursor cells promotes the chemotactic recruitment, development and survival of human osteoclasts. *Bone.* 2005;36(5):840-853.
301. Aachoui Y, Ghosh SK. Extracellular matrix from porcine small intestinal submucosa (SIS) as immune adjuvants. *PloS one.* 2011;6(11):e27083.
302. Kim RY, Oh JH, Lee BS, Seo YK, Hwang SJ, Kim IS. The effect of dose on rhBMP-2 signaling, delivered via collagen sponge, on osteoclast activation and in vivo bone resorption. *Biomaterials.* 2014;35(6):1869-1881.
303. Chaudhary LR, Hofmeister AM, Hruska KA. Differential growth factor control of bone formation through osteoprogenitor differentiation. *Bone.* 2004;34(3):402-411.
304. Jensen ED, Pham L, Billington CJ, Jr., et al. Bone morphogenic protein 2 directly enhances differentiation of murine osteoclast precursors. *Journal of cellular biochemistry.* 2010;109(4):672-682.
305. Okada Y, Montero A, Zhang X, et al. Impaired osteoclast formation in bone marrow cultures of Fgf2 null mice in response to parathyroid hormone. *The journal of biological chemistry.* 2003;278(23):21258-21266.
306. Sriarj W, Aoki K, Ohya K, Takahashi M, Takagi Y, Shimokawa H. TGF-beta in dentin matrix extract induces osteoclastogenesis in vitro. *Odontology / the Society of the Nippon Dental University.* 2015;103(1):9-18.
307. Szpalski M, Gunzburg R. Recombinant human bone morphogenetic protein-2: a novel osteoinductive alternative to autogenous bone graft? *Acta orthopaedica Belgica.* 2005;71(2):133-148.
308. Sanjabi S, Zenewicz LA, Kamanaka M, Flavell RA. Anti-inflammatory and pro-inflammatory roles of TGF-beta, IL-10, and IL-22 in immunity and autoimmunity. *Current opinion in pharmacology.* 2009;9(4):447-453.
309. Bornstein MM, Halbritter S, Harnisch H, Weber H-P, Buser D. A retrospective analysis of patients referred for implant placement to a specialty clinic: indications, surgical procedures, and early failures. *The international journal of oral & maxillofacial implants.* 2008;23(6):1109.