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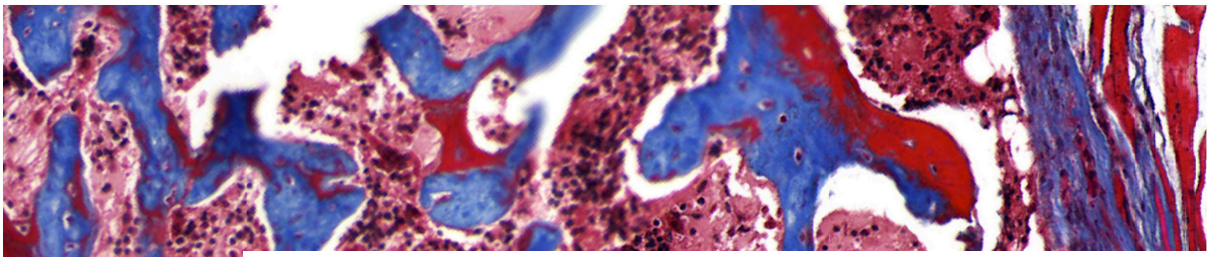
Raina, Deepak

2018



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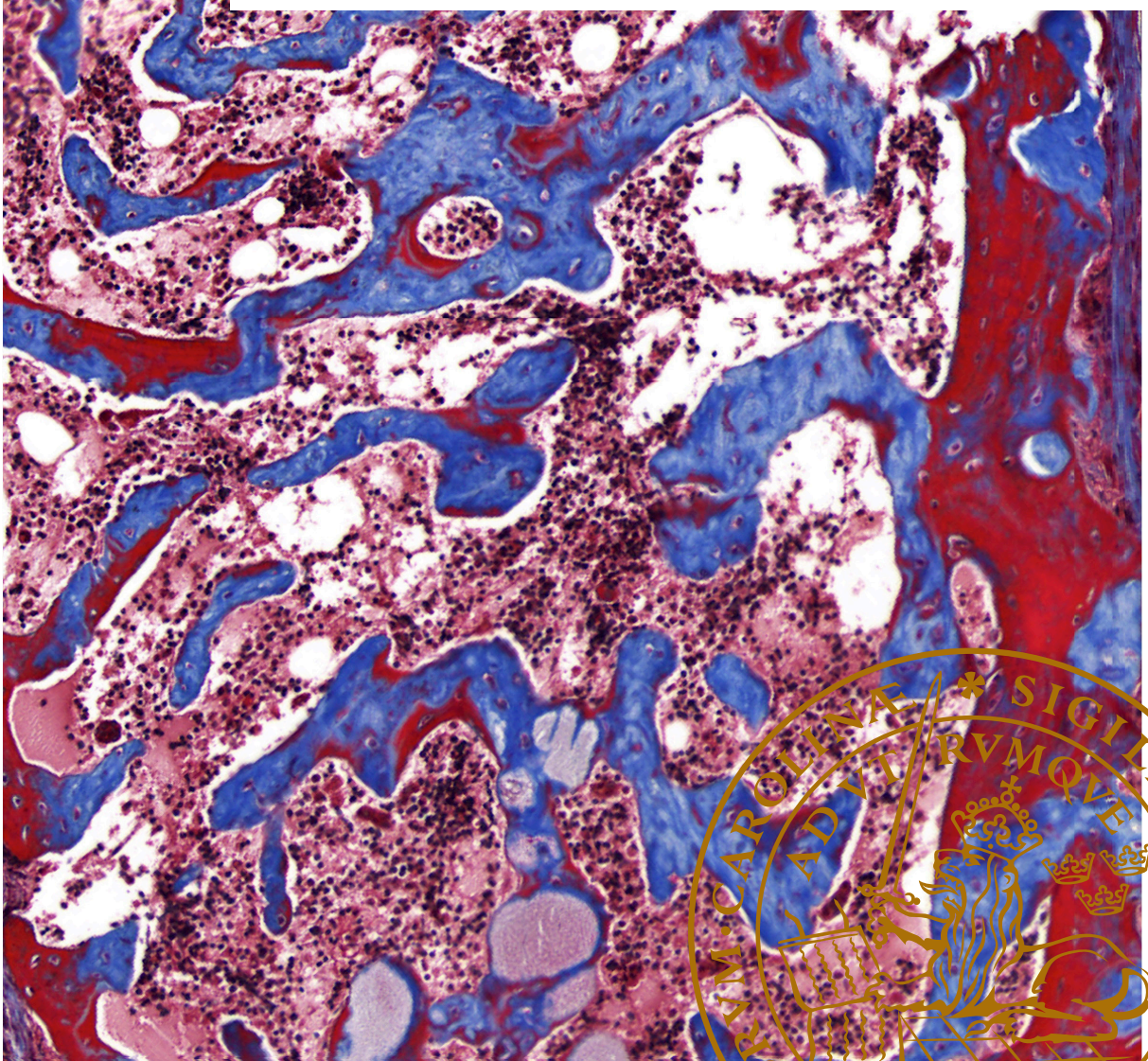
DEEPAK BUSHAN RAINA
DEPARTMENT OF CLINICAL SCIENCES LUND, ORTHOPEDICS | LUND UNIVERSITY



Biomaterials as carriers for bone active molecules

An approach to create off-the-shelf bone substitutes

Deepak Bushan Raina

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Abstract

Bone tissue is commonly transplanted during orthopedic surgeries, primarily for the management of bone defects caused by trauma or various orthopedic conditions including, but not limited to, infections and tumours. Bone grafts are a surgeon's choice, but their associated drawbacks are paving the way for biomaterial based bone graft substitutes. Biomaterials inherently lack the ability to induce significant amount of bone growth due to which they may be combined with cells, biomaterials, growth factors and drugs to regenerate functional bone tissue. This thesis focused on characterizing biomaterial carriers that can locally deliver bone active molecules for bone regeneration and potentially act as an alternative to conventional bone grafting. We focused on the delivery of recombinant bone morphogenic protein-2 (rhBMP-2) as a bone inducing anabolic growth factor. Simultaneously, we have used an osteoclast inhibiting bisphosphonate, zoledronic acid (ZA) to prevent BMP-2 induced premature bone resorption. Three different biomaterials scaffolds; a microporous calcium sulphate (CaS)/hydroxyapatite (HA), a macroporous gelatin-CaS/HA and a collagen membrane were used in distinct animal models of bone regeneration.

The carrier properties of the three biomaterials in the ectopic muscle pouch model (studies 1, 4 and 5) showed that the tested materials were efficient carriers of rhBMP-2 and ZA and that co-delivery of rhBMP-2 and ZA regenerated higher volume of bone compared to rhBMP-2 alone. Studies 2& 3 show that the CaS/HA material locally delivering ZA or ZA+rhBMP-2 could be efficiently used for bone regeneration in clinically relevant bone defect models. These studies also indicated that local delivery of ZA not only has an anti-osteoclast effect but it also has an anabolic role. Study 4 compared the developed porous biomaterial with the current FDA approved collagen sponge and results indicated that the developed biomaterial outperforms the current marketed product for the delivery of rhBMP-2. During this study, it was also established that co-delivery of rhBMP-2 with ZA could reduce the effective rhBMP-2 doses by up to four times, which is crucial to reinstate BMPs into the clinics. Study 5 was a follow-up of study 2 separating the metaphyseal defect healing in two stages; 1) Healing the cancellous bone using a porous material and 2) Guiding cortical regeneration using a thin collagen membrane. Significantly better cortical healing was noted using this approach in comparison to study 2.

In summary, this work describes promising strategies for bone regeneration. It established how the release of bone active molecules can be controlled by the choice of carrier material and how we can decrease the minimally effective dose of rhBMP-2 by up to four times. These findings can potentially be translated from the bench to the bedside. The materials and methods developed within the scope of this work can be used in a variety of orthopedic conditions and can provide the surgeon with an effective off-the-shelf substitute for bone replacement, in turn leading to improved care of the patient.

Key words: Bone, Biomaterials, BMP-2, Zoledronic Acid, Bone tissue engineering

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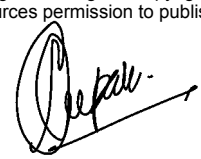
Signature  Date 2018-09-06

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LUND
UNIVERSITY

Contact address

Deepak Bushan Raina
BMC C12, Klinikgatan 28
221 84 Lund, Sweden
Phone: +46-46-2220740
email: deepak.raina@med.lu.se
Lund University,
Faculty of Medicine,
Department of Clinical Sciences Lund, Orthopedics

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Histological image of bone tissue formed by the local delivery of bone active molecules via a biomaterial in the abdominal muscle.

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To My Family and My Teachers….

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Acknowledgements

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List of papers

1. **Raina, D.B.,** Isaksson, H., Hettwer, W., Kumar, A., Lidgren, L., and Tägil, M. A Biphasic Calcium Sulphate/Hydroxyapatite Carrier Containing Bone Morphogenic Protein-2 and Zoledronic Acid Generates Bone. *Scientific Reports* **6** 2016.
2. Horstmann, P.F., **Raina, D.B.**, Isaksson, H., Hettwer, W., Lidgren, L., Petersen, M.M., and Tagil, M. Composite Biomaterial as a Carrier for Bone-Active Substances for Metaphyseal Tibial Bone Defect Reconstruction in Rats. *Tissue Engineering Part A* **23,** 1403-1412. 2017
3. Širka, A*., **Raina, D.B***., Isaksson, H., Tanner, K.E., Smailys, A., Kumar, A., Tarasevičius, S., Tägil, M., and Lidgren, L. Calcium Sulphate/Hydroxyapatite Carrier for Bone Formation in the Femoral Neck of Osteoporotic Rats. *Tissue Engineering Part A*. 2018. https://doi.org/10.1089/ten.TEA.2018.0075

   * Equal first authorship.
4. **Raina, D. B.,** Larsson, D., Mrkonjic, F., Isaksson, H., Kumar, A., Lidgren, L. and Tägil, M. Gelatin- hydroxyapatite- calcium sulphate based biomaterial for long term sustained delivery of bone morphogenic protein-2 and zoledronic acid for increased bone formation: In-vitro and in-vivo carrier properties. *Journal of Controlled Release* **272**, 83-96. 2018
5. **Raina, D. B.,** Qayoom, I., Larsson, D., Zheng, M.H., Kumar, A., Isaksson, H., Lidgren, L. and Tägil, M. Guided Tissue Engineering for Healing of Cancellous and Cortical Bone Using a Combination of Biomaterial Based Scaffolding and Local Bone Active Molecule Delivery. *Under Review.* 2018

Study 1

Aim: Carrier properties of CaS/HA biomaterial for rhBMP-2 and ZA

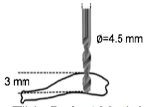
Results:
In-vitro, 90% rhBMP-2 and 10% ZA released after 1-week

Combination of rhBMP-2 and ZA regenerates significantly higher bone than rhBMP-2 delivered alone

CaS/HA alone did not induce any bone formation in the ectopic muscle pouch model

Study 5

Aim: To use the gelatin-CaS-HA (GCH) material to guide cancellous bone regeneration and a collagen membrane (CM) to guide cortical bone regeneration



Tibia Defect Model

Results:
Cancellous bone regeneration was higher in all GCH treated groups

The defect was not critical, all cortices healed in the empty group

Cortical healing was impaired in GCH groups without CM

70% cortices bridged in the group where GCH was covered by a CM releasing low dose rhBMP-2

Study 4

Aim: Carrier properties of gelatin-CaS/HA biomaterial for rhBMP-2 and ZA

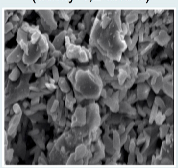
Results: In-vivo, ~65% rhBMP-2 and ~40 % ZA was released after 4-weeks

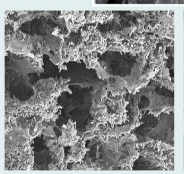
The developed material regenerated more bone than the Commercially available carrier for delivery of rhBMP-2

Co-delivery of rhBMP-2 and ZA could aid in reducing the rhBMP-2 doses by 4-times

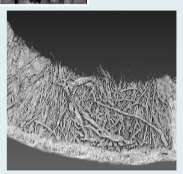
Biomaterials

Microporous
Calcium Sulphate (CaS)/ Hydroxyapatite (HA)
(Study 1,2 and 3)





Macroporous
Gelatin-CaS/HA
(Study 4 and 5)



Thin Collagen
Membrane (Study 5)

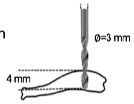
Bone Active Molecules

Bone Morphogenic Protein-2 (rhBMP-2)
Osteoinductive protein to induce bone formation

Zoledronic Acid (ZA)
Anti-osteoclast drug to stop BMP-2 induced osteoclastogenesis

Study 2

Aim: To use the CaS/HA material with or without local ZA or ZA+rhBMP-2 in a metaphyseal bone void



Tibia Defect Model

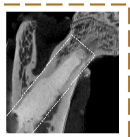
Results:
Local delivery of ZA or ZA+rhBMP-2 via the CaS/HA biomaterial better than the empty control or the defect filled with allograft bone

The defect was non-critical. Empty and allograft treated groups exhibited almost complete cortical bridging but no cancellous bone formation

All CaS/HA treated groups had impaired cortical healing

Study 3

Aim: To use the combination of CaS/HA, ZA and ZA+rhBMP-2 to enhance bone formation and mechanical strength of the femoral neck canal of OVX rats



Femoral Neck Defect in OVX Rats

Results:
Local delivery of only ZA or ZA+rhBMP-2 via the CaS/HA was better than the empty group or the CaS/HA treated group

No difference in peak force to fracture the femoral neck was seen

Abstract

Bone tissue is commonly transplanted during orthopedic surgeries, primarily for the management of bone defects caused by trauma or various orthopedic conditions including, but not limited to, infections and tumours. Bone grafts are a surgeon's choice, but their associated drawbacks are paving the way for biomaterial based bone graft substitutes. Biomaterials inherently lack the ability to induce significant amount of bone growth due to which they may be combined with cells, biomaterials, growth factors and drugs to regenerate functional bone tissue. This thesis focused on characterizing biomaterial carriers that can locally deliver bone active molecules for bone regeneration and potentially act as an alternative to conventional bone grafting. We focused on the delivery of recombinant human bone morphogenic protein-2 (rhBMP-2) as a bone inducing anabolic growth factor. Simultaneously, we have used an osteoclast inhibiting bisphosphonate, zoledronic acid (ZA) to prevent BMP-2 induced premature bone resorption. Three different biomaterials scaffolds; a microporous calcium sulphate (CaS)/hydroxyapatite (HA), a macroporous gelatin-CaS/HA and a collagen membrane were used in distinct animal models of bone regeneration.

The carrier properties of the three biomaterials in the ectopic muscle pouch model (studies 1, 4 and 5) showed that the tested materials were efficient carriers of rhBMP-2 and ZA and that co-delivery of rhBMP-2 and ZA regenerated higher volume of bone compared to rhBMP-2 alone. Studies 2& 3 show that the CaS/HA material locally delivering ZA or ZA+rhBMP-2 could be efficiently used for bone regeneration in clinically relevant bone defect models. These studies also indicated that local delivery of ZA not only has an anti-osteoclast effect but it also has an anabolic role. Study 4 compared the developed porous biomaterial with the current FDA approved collagen sponge and results indicated that the developed biomaterial outperforms the current marketed product for the delivery of rhBMP-2. During this study, it was also established that co-delivery of rhBMP-2 with ZA could reduce the effective rhBMP-2 doses by up to four times, which is crucial to reinstate BMPs into the clinics. Study 5 was a follow-up of study 2 separating the metaphyseal defect healing in two stages; 1) Healing the cancellous bone using a porous material and 2) Guiding cortical regeneration using a thin collagen membrane. Significantly better cortical healing was noted using this approach in comparison to study 2.

In summary, this work describes promising strategies for bone regeneration. It established how the release of bone active molecules can be controlled by the choice of carrier material and how we can decrease the minimally effective dose of rhBMP-2 by up to four times. These findings can potentially be translated from the bench to the bedside. The materials and methods developed within the scope of this work can be used in a variety of orthopedic conditions and can provide the surgeon with an effective off-the-shelf substitute for bone replacement, in turn leading to improved care of the patient.

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Abbreviations

ACS	Absorbable collagen sponge
ALP	Alkaline phosphatase
ANG-1	Angiopoietin-1
ATP	Adenosine triphosphate
CaS	Calcium sulphate
CM	Collagen membrane
CMC	Carboxy methyl cellulose
DKK	Dickkopf related protein
DMSO	Dimethyl sulfoxide
DPD	Deoxypyridinoline
ELISA	Enzyme linked immunosorbent assay
FDA	Food and drug administration
FGF	Fibroblast growth factor
FPPS	Farnesyl pyrophosphate synthase
FTIR	Fourier transform infrared
GCH	Gelatin-calcium sulphate-hydroxyapatite
GTP	Guanosine triphosphate
HA	Hydroxyapatite
HMV	Highly mineralized volume
H&E	Hematoxylin and Eosin
IGF	Insulin growth factor
LRP	Low density lipoprotein receptor related protein
M-CSF	Macrophage colony stimulating factor
Micro-CT	Micro computed tomography
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MV	Mineralized volume
NTX	N-terminal telopeptide
OCN	Osteocalcin
OP-1	Osteogenic protein-1
OPG	Osteoprotegrin

OSX	Osterix
OVX	Ovariectomized
PDGF	Platelet derived growth factor
PLA	Polylactic acid
PMMA	Poly (methyl methacrylate)
PP_i	Inorganic pyrophosphatase
PTH	Parathyroid hormone
qPCR	Quantitative polymerase chain reaction
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
rhBMP	Recombinant human bone morphogenic protein
ROI	Region of interest
RUNX-2	Runt related transcription factor 2
SEM	Scanning electron microscopy
SOST	Sclerostin
SPECT	Single photon emission computed tomography
TCP	Tissue culture plastic
TGF	Transforming growth factor
TRAP	Tartrate resistant acid phosphatase
TV	Tissue volume
VEGF	Vascular endothelial growth factor
XRD	X-ray diffraction
ZA	Zoledronic acid

1. Introduction

Bone is an excellent example of a naturally occurring composite material enabling load bearing while providing us with mobility. The biomechanical properties of bone with respect to its structure and overall weight are eximious. Even with the advances in material sciences, we have still not been able to replicate a bone substitute material (metal alloy, composite carbon fiber, ceramics) that possesses equal or better biomechanical properties than bone. This composite of bone contains an organic phase (approximately 30%) consisting of proteins, and an inorganic phase consisting of hydroxyapatite (approximately 70%) both constituting the extracellular matrix of bone [1, 2]. Approximately 90% of the bone extracellular matrix consists of type I collagen while the rest is composed of non-collagenous glycosaminoglycans such as aggrecan, versican, decorin, biglycan, fibromodulin. Glycoproteins like osteonectin, alkaline phosphatase, bone sialoprotein, osteopontin, fibronectin, gamma carboxyglutamic acid (GLA) containing proteins such as osteocalcin and finally other proteins including bone morphogenic proteins, growth factors and enzymes form the rest of the bone matrix [3, 4]. It must be noted that several of these proteins are also expressed in other tissues as well but their role in bone is speculated to be critical for hydroxyapatite crystal nucleation and maturation [3]. As far as the mineral phase of bone is concerned, hydroxyapatite crystals, a mixture of calcium and phosphate ions forms the mineral mass of bone. Most of the hydroxyapatite crystals in bone are nano-sized, either needle shaped or platelet like and range about 20-50 nm in length, approximately 0-20 nm in width and 2-4 nm in thickness [1, 5, 6]. Evidence of hydroxyapatite nucleation has been provided in the literature and it is widely accepted that nano-hydroxyapatite particle nucleation occurs on and within collagen fibers thereby forming an intertwined composite network of collagen fibers and hydroxyapatite crystals [7, 8]. Long bones in humans exhibit a cylindrical structure with the outer core consisting of the cortical bone while the inside is filled with spongous bone, also called as trabecular or cancellous bone, as per the classical explanation. However, a detailed hierarchical structure has been provided recently in which a 12-level breakup of the hierarchical bone structure begins with bone at the macroscopic level and works its way through to the microscopic level. The stages include: resolving the bone structure starting from the bone itself, cortical and trabecular bone, osteons, lamellar level, collagen bundle, organization of the collagen bundle (random or ordered fibril orientation), mineralized collagen bundle, separating the mineralized collagen fibril bundles into

collagen microfibrils and aggregate of mineral, separating collagen microfibrils into collagen triple helix and the mineral aggregate into stacks of platelets, collagen triple helix into a single chain and stack of platelets into a single platelet, resolving collagen at the amino acid level and a mineral platelet into a acicular crystal followed by the atomic level at the end [9].

1.1 Lack of bone: A genuine orthopedic problem

Bone defects occur due to several reasons including but not limited to, congenital disorders, trauma, infections, tumors and fragility fractures. As a result, bone is the second most commonly transplanted tissue after blood [10, 11] . Some examples of clinical cases requiring bone transplantation are shown in Fig. 1.

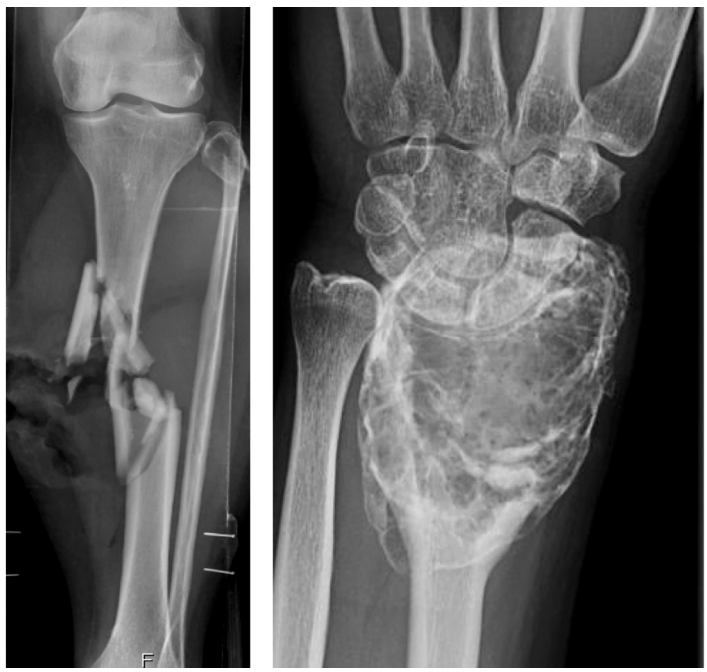


Fig. 1 Clinical cases requiring bone transplantation.
Left, a comminuted tibial fracture. Right, a tumor in the distal radius. Picture: Magnus Tägil, Lund University Hospital, Sweden.

Therefore, there is an obvious need of additional bone during orthopedic surgery to fill the defect. Traditionally, this has been achieved using autograft as the 'gold standard' for bone substitution. Autografts provide a scaffold, the necessary cells and finally necessary signals for the induction of bone growth [11]. However,

autograft harvesting requires additional surgery thereby increasing risk of secondary infection as well as donor site morbidity [12]. Furthermore, harvesting large volumes of autograft can be challenging in the elderly and the young, especially when large volumes of bone are missing. Allograft bone, taken from dead donors has also been widely used, but its lack of osteogenicity, risk of disease transmission, high cost and variable efficacy puts their use into question [11, 13, 14]. This opens up an area of research on efficient bone substitutes that can potentially replace the need for bone grafting in orthopedic surgery.

1.2 Bone at the cellular level

Bone is a highly dynamic tissue in terms of remodeling. It is believed that cortical bone undergoes remodeling at a rate of 3-10% every year while trabecular bone, being metabolically more active remodels at an annual rate of 20-30% [4]. This of course would not be possible without a carefully orchestrated process similar to an assembly line of a big production unit. Three important cell types, *osteoblasts, osteocytes and osteoclasts,* carefully modulate this process of constant bone building, remodeling and the overall maintenance of bone function.

Osteoblasts form the bone matrix (both organic and inorganic) and are recruited from the mesenchymal precursors. The mineralization stage of osteoblasts is divided in two phases. The first phase is characterized by the formation of an *osteoid*, which is a protein rich matrix carefully laid out to form lamellae [15]. The second phase consists of mineralization of the lamellar structures and occurs when congenial conditions for hydroxyapatite crystal formation and propagation are present. A high percentage of this mineralization occurs in the very early phases, but it is shown to take several months to achieve complete mineralization of an osteoid [15].

Osteocytes originate from mineralizing osteoblasts and can be thought of as bone preservers. When osteoblasts approach the end of the mineralization phase, they can get buried in a mineral matrix causing them to become osteocytes. What is remarkable about these cells is that they maintain close contact with each other using their dendrites, often termed as *canaliculi*. Osteocytes can be thought of as biomechanical sensors in bone, which is achieved by an interconnected network of these canaliculi, which sense mechanical strain and convert the signals into biochemical responses [15]. Due to the spread-out network, osteocytes are capable of initiating targeted bone remodeling, often by coordinating with nearby osteoblasts and other hematopoietic cells, involving the Wnt/β-Catenin pathway [16].

Osteoclasts are responsible for resorption of bone. Osteoclasts are multi-nucleated cells which differentiate from mono-nuclear hematopoietic precursors. Their genesis is controlled by two important cytokines; receptor activator of nuclear

factor kappa B ligand (RANKL) and macrophage colony stimulating factor (M-CSF), produced by osteoblasts and their precursors as a response to resorption stimulus [15, 17, 18]. Macrophages are considered as osteoclast precursor and in-vitro studies have indicated that stimulation of macrophages with RANKL and M-CSF leads to osteoclastogenesis [19]. Initiation of bone resorption by osteoclasts is an interesting mechanism. Osteoclasts have ruffled borders and the process begins with affixing or creating a tight seal with the underlying bone controlled via integrins [20, 21]. Once a secure perimeter is established, the osteoclasts discharge a series of proteolytic enzymes including cathepsin K, tartrate resistant acid phosphatase (TRAP), matrix metalloproteases (MMP) including MMP 9 and 13, which digest the organic bone matrix [15, 22, 23]. Osteoclasts also release hydrochloric acid to digest the bone mineral eventually leading to a resorption pit also called as a Howship's lacuna [15, 23].

1.3 Bone remodeling cycle

In healthy individuals, the cycle of bone remodeling is an infinite loop of meticulously executed events which begins with *resorption* of old bone by the osteoclasts followed by a *reversal* phase wherein mononuclear mesenchymal cells appear at a resorbed bone surface and finally the *formation* phase, which involves replacing the resorbed bone by osteoblasts that eventually form osteocytes (Fig. 2) [24]. There is also a resting period after which the next remodeling process begins. This process continues throughout the life of an individual unless, due to the onset of disease, this cycle is discontinued causing an imbalance in bone turnover. It must be noted that the process of bone resorption and bone formation is a tightly coupled process. Osteocytes, the biomechanical-sensors, signal osteoblasts to undergo morphological and biochemical changes. This enables them to secrete RANKL, which then binds to RANK on the osteoclast precursor to form the bone resorbing osteoclast [25]. There are systemic and local regulators of bone remodeling including: parathyroid hormone (PTH), glucocorticoids, transforming growth factor-β (TGF-β), vitamin D_3, estrogen, prostaglandins, which all have a direct effect on RANKL or an indirect effect on RANKL via its decoy receptor, osteoprotegrin (OPG) [24].

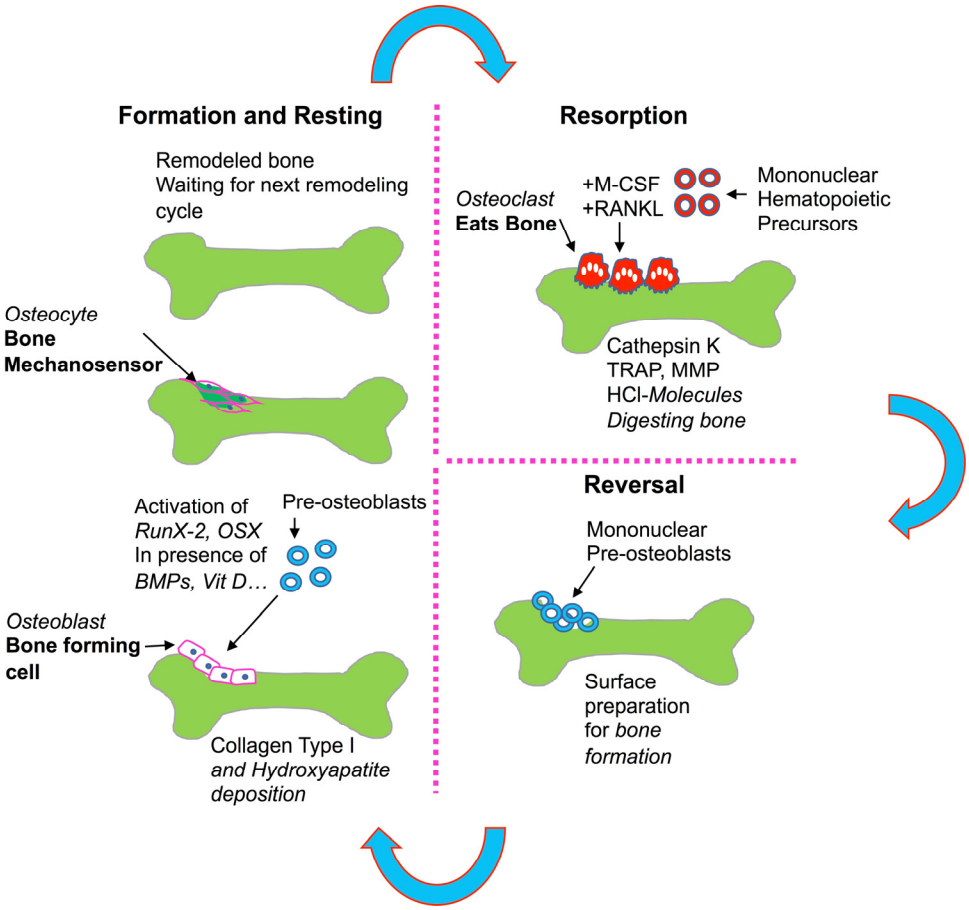


Fig. 2 Bone cells and the cycle of bone remodeling.

1.4 Bone defect and repair

Fracture healing has been classified as *indirect and direct* [26]. Indirect healing is considered to be a natural route of fracture healing as it does not demand absolute mechanical stability. The process of indirect fracture healing begins with *acute inflammation and hematoma* formation. Once the acute inflammation withers away, mesenchymal cells from surrounding tissues like muscle, periosteum, endosteum, marrow cavity and circulating stem cells are recruited to the site [27]. These cells are then re-programmed to lay a *cartilaginous matrix*, which is mineralized at later stages. It must be noted that sub-periosteal cells play a pivotal role in initiating direct bone healing leading to the formation of a hard callus on the outside, which provides initial mechanical stability to the fracture [26, 28]. After endochondral ossification,

a *new vasculature* is provided to the damaged bone. Some of the chondrocytes within the callus undergo apoptosis and the cartilage matrix is degraded in order to make way for new blood vessels via the angiopoietin-1,2 pathway and the vascular endothelial growth factor (VEGF) pathway. The remaining chondrocytes enter hypertrophy leading to *mineralization of the callus matrix*. Finally, the *remodeling of fracture callus* takes place in order to restore original biomechanical properties of bone. This occurs as per the bone remodeling cycle described in section 1.3.

On the contrary, direct bone healing occurs when there is a structural continuity between the bone ends (contact healing) and a rigid mechanical fixation is present. Furthermore, if two ends of the bone are less than 1 mm apart but a rigid mechanical fixation is available, direct bone healing can still be achieved (gap healing). In both cases, osteons are laid out directly, which then remodel into lamellar bone without forming an external fracture callus.

1.5 Engineering bone for bone substitution

To overcome the drawbacks and shortage associated with bone grafts, tissue engineering and regenerative medicine methods have been rigorously researched over the last decade. Tissue engineering involves an interplay of engineering and biomedicine techniques aimed at replacing or repairing a damaged tissue using tissue mimicking structures. These structures can comprise of a natural, synthetic or composite precursor, which can be engineered to restore the functionality of a target tissue. Peter Giannoudis and co-workers have suggested the Diamond Concept, a 4-step intertwined interaction in fracture healing, which to some extent is based on a general 3-step tissue engineering approach [29]. The first three steps include interactions between biomaterial scaffolds (tissue mimicking templates), cells (tissue specific) and growth factors/drugs that promote tissue regeneration to regenerate a functional tissue (Fig. 3). In the case of bone, a fourth step of mechanical stability is also included. The coming sections will focus on the approaches used in this thesis work including an introduction to *biomaterials, growth factors and drugs* used in the area of experimental orthopedics.

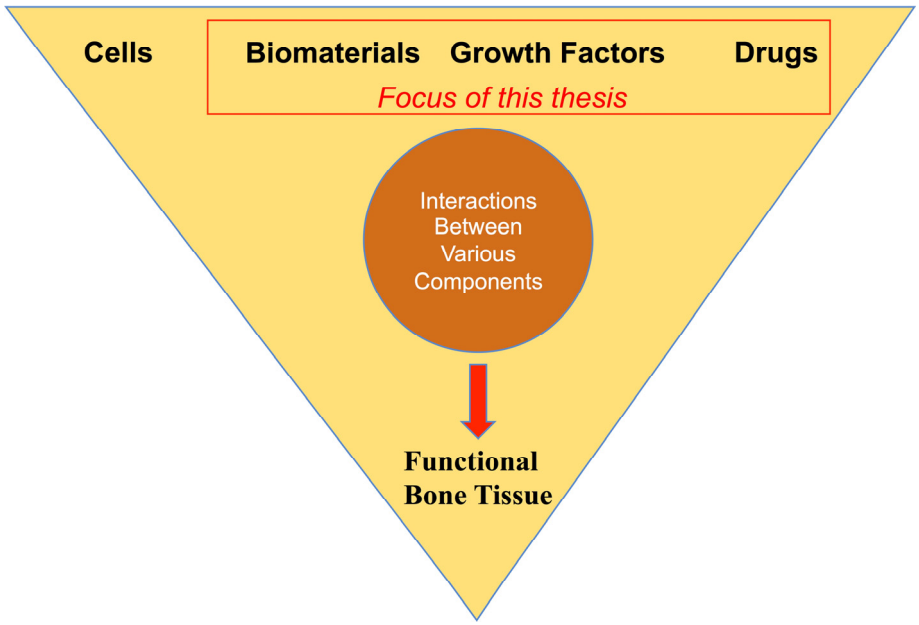


Fig. 3 Tissue engineering based strategy for bone regeneration.

1.5.1 Biomaterials in experimental orthopedics

Biomaterials play a central role in bone tissue engineering and can be used to provide a template or a scaffold to progenitor and committed cells to initiate bone repair and in the recent years, biomaterials have also been used to deliver therapeutic bone active agents locally [30]. Biomaterials for bone tissue engineering consist of either inorganic precursors such as: calcium phosphates, hydroxyapatite, bioactive glass and their several combinations or can be derived from polymeric precursors of natural or synthetic origin like collagen, gelatin, chitosan, hyaluronic acid, poly-fumarates, poly-lactic acid (PLA) etc. A third type of composite bone-based biomaterial includes a combination of inorganic and organic components in a bid to further resemble the native structure of bone, for instance, a composite of collagen and hydroxyapatite [30-32]. Increasing research in the area of implants has also led to the development of metallic biomaterials in bone tissue engineering [33]. Fig. 4 shows ideal properties of biomaterials required for bone regeneration.

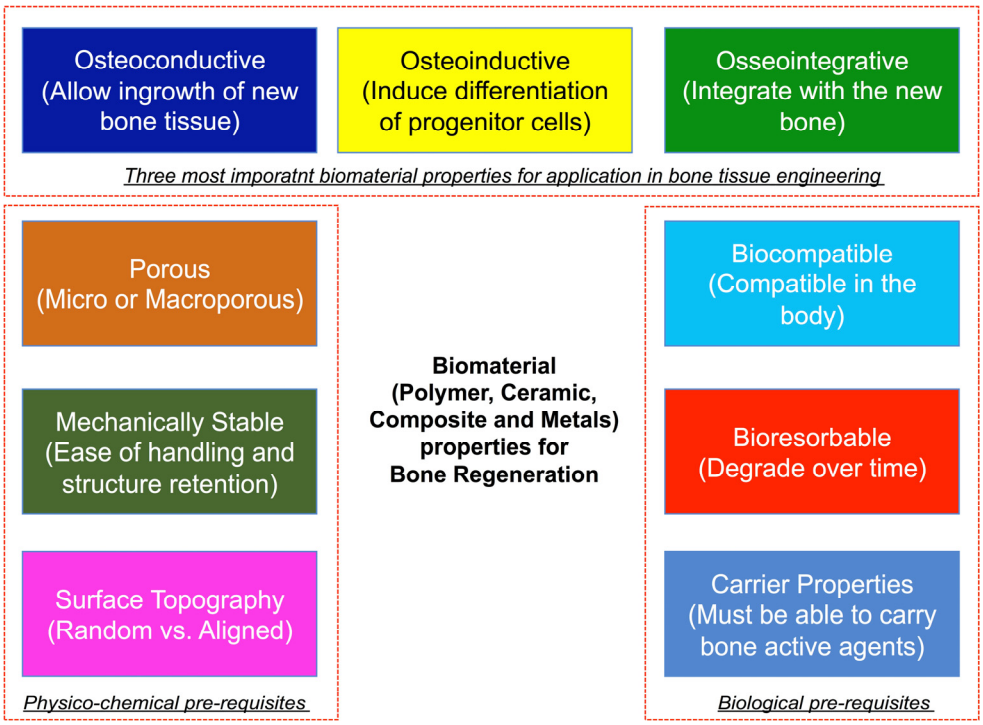


Fig. 4 Ideal properties of biomaterials for bone tissue regeneration.

1. An ideal bone tissue engineering biomaterial possesses three important properties: 1. *Osteoinduction*- The property of a biomaterial or a molecule to induce non-committed progenitor cells into committed bone forming osteoblasts [34]. Several ceramic and composite biomaterials and a limited number of polymeric biomaterials with micro to macroporous structure have been shown to induce bone formation in ectopic bone formation models including sub-cutaneous and muscle models as described by Barradas et al. in their review of osteoinductive biomaterials [35]. It is vital to be attentive to the pore-structure and the species involved in most of the studies describing osteoinductivity. It appears that porosity is an important property for osteoinduction. Furthermore, most of the osteoinductive studies have been carried out in non-human primates and canines emphasizing the need of higher animal species to study osteoinduction. Only a few studies claimed to show biomaterial based osteoinduction in rats but we have not been able to reproduce these results in any of our ectopic models so far.
2. *Osteoconduction*- The ability of a biomaterial to allow ingrowth or surface growth of bone tissue into/onto its structure [34].

3. *Osseointegration*- The ability of a biomaterial to integrate with the surrounding bone. While osseointegration is often defined in context of prostheses and implants, it is important for bone substitutes/biomaterials to integrate with old bone to form a single functional unit after completion of bone remodeling. It must be noted that these three properties of a biomaterial are tightly coupled in a way that osteoinduction is necessary for osteoconduction, which is necessary for osseointegration. Apart from these, other important biomaterial properties for bone tissue engineering include porosity, surface topography, biomechanical properties and biodegradation among others (Fig. 4). Porosity is a beneficial scaffold property and it makes sense to use a porous scaffold that can let bone grow into the structure but there seems to be no consensus on the most optimal pore size. Some studies have indicated that a pore size similar to that of an osteon (approximately 220 μm) is an optimal pore size, while others have shown that even smaller pores (50 μm) are also sufficient for osteonal bone formation in rabbits [36, 37]. It is important to remember that pore size and mechanical strength of a biomaterial are indirectly related, thus to find a balance between pore size and mechanical properties is essential. In terms of topography, it has already been shown that random surface topographical features induces better mesenchymal stem cell (MSC) differentiation and mineral production compared to highly organized topographical features of a material [38]. Biomechanically, we are still far from achieving optimal biomechanical properties from a biomaterial, which could perform complete load bearing without the added stability from a fixation device both in pre-clinical and clinical studies. Lastly, the rate of degradation of a bone tissue engineering scaffold must match the rate of new bone formation in order to not only provide osteoconduction but also restore the native biomechanical properties of the damaged tissue.

1.5.2 Anabolic factors in experimental orthopedics

Different hormones, growth factors, cytokines and proteins have been used in experimental orthopedics to achieve anabolism. Parathyroid hormone (PTH), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), insulin growth factor (IGF), transforming growth factor (TGF-β), anti-sclerostin, and bone morphogenic proteins (BMPs) 2 and 7 are commonly used anabolic factors in experimental orthopedics [39].

PTH works by increasing the proliferation and differentiation of sub-periosteal osteoprogenitors seen via an increase in expression of bone matrix proteins thereby enhancing early fracture healing response and simultaneously stimulating osteoclasts leading to remodeling of fracture callus [40]. A literature review of pre-

clinical and clinical data on PTH and its role in fracture healing by Ellegaard et al. suggests that systemic injections of various PTH doses increases bone strength and bone volume in several animal models including fracture healing models, models of osteoporosis and ageing [41]. In one of the few early clinical trials including a PTH analog teripartide, Aspenberg et al. indicated that low dose PTH administration reduces the time required for fracture repair without measuring any other effect on bone properties involved in fracture healing [42]. A follow up study by the same groups indicated an improvement in early callus formation in patients with distal radius fractures in a small set of patients but recently failed to show any effect on knee implant migration [43, 44].

VEGF promotes angiogenesis during various phases of fracture repair and since bone is a vascular tissue, VEGF has experimentally been shown to enhance fracture repair via an upregulation of blood vessel formation. Using an osteoblast specific Vegfa knockout mice, VEGF appears to have a role in macrophage chemotaxis and angiogenesis in endochondral fracture repair process during the acute inflammatory phase [45, 46]. During the intermediate stages of bone remodeling, VEGF promotes cartilage matrix resorption to enhance angiogenesis in the fracture callus. Finally, during the resorption stages, VEGF stimulates osteoclast formation, which leads to remodeling of the fracture callus. Exogenous VEGF is shown to enhance bone formation in several models of bone formation primarily due to its pro-angiogenic effects, which causes an increase in bone formation [47-49].

PDGF, especially its BB isoform via the PDGF-BB/PDGFR-β pathway together with other signaling molecules like TGF-β and Angiopoietin-1 (Ang1) are responsible for pericyte (mesenchymal cells also capable of undergoing osteochondral differentiation) recruitment, maturation, attachment and detachment [50, 51]. PDGF is produced by endothelial cells, platelets and macrophages among others in a fracture environment early on and acts as a stem cell attractant and mitogen [50, 52]. Experimental usage of PDGF has been documented several years ago and its role in bone regeneration has been almost three decades old [53]. Exogenously applied PDGF has also shown an overall positive effect on bone healing with accelerated fracture repair [54]. Similar results have also been reported in geriatric and osteoporotic pre-clinical model of fracture repair in rats [55].

FGF is a large family of proteins with 22 known members among which FGF-2 has been widely used in bone tissue engineering [56]. FGF-2 knock out mice have reduced trabecular bone and reduction in bone volume and mineral apposition rates [57]. Early studies investigating the role of FGF-2 demonstrated a positive role on endosteal and trabecular bone formation but not on periosteal bone [58]. Fei et al., reported that FGF-2 causes an increase in the osteoblastic differentiation of bone marrow stromal cells by modulating the genes in the canonical Wnt/β-Catenin pathway [59]. Role of exogenous FGF-2 delivered locally via different carrier systems have shown an overall positive influence on bone formation at various

skeletal sites like ulnar defects, periodontal defects, calvarial defects, femoral and tibial defects in non-human primates, rodents and in humans [60-64].

IGF-1 forms the most expressed growth factor in the bone matrix and its precise role in bone matrix has been recently explained in detail by Xian et al [65]. Like several other growth factors, IGF-1 positively affects MSC recruitment and stimulation of osteoblastic differentiation controlled by the activation of mammalian target of rapamycin (mTOR). IGF-1 knock out mice display a failure in osteoblastic development of pre-osteoblast precursors recruited at the skeletal sites [65].

TGF-β plays a role in both intramembranous and endochondral ossification. Out of its three common isoforms, TGF-β1 has been a commonly studied isoform and knockout mice lacking TGF-β1 gene do not report any abnormal development at birth but suffer from a mixed immunological response, tissue necrosis and eventually multiple organ failure a few weeks post birth [66]. TGF-β plays a role in osteogenic and chondrogenic differentiation of precursor cells [67].

Sclerostin is a protein expressed by osteocytes and binds to its receptor on osteoblasts thereby invoking a downstream cascade of molecular events that lead to inhibition of bone formation [68]. Sclerostin initially was thought to be a BMP antagonist but other reports indicate that sclerostin together with Dickkopf related protein-1 (DKK-1) inhibits the Wnt signaling by binding to LRP5/6 [68, 69]. Deletion of the gene (SOST) coding for sclerostin leads to an overwhelmingly high bone turnover both in the metaphysis and in the cortex, resembling to *van Buchems* disease to some extent [70, 71]. This is obvious, especially when osteoblasts are not capable of stopping bone formation when indicated to do so by the osteocytes. Attempts have been made to use sclerostin monoclonal antibodies to prevent sclerostin from binding to its receptor on osteoblasts with an aim to promote osteoblastic bone formation and sclerostin antibody has proven beneficial in different pre-clinical models of orthopedic conditions like osteoporosis, steroid induced bone loss, inflammation, osteotomy models as well as implant fixation. Although the studies are quite recent, almost all studies indicate an increase in bone mass and biomechanical strength [71]. Clinical trials involving systemic injections of anti-sclerostin antibody have shown promising results leading to increased bone mineral density (BMD) in the lumbar spine and the hip with reduced risk of fracture [72-74].

BMPs are the only real osteoinductive molecules capable of inducing bone at both ectopic and entopic locations [75]. They belong to the TGF-β superfamily of proteins and more than a dozen BMP isoforms have been identified with BMP-2, 4, 5, 6 and 7 having strong osteoinductive properties and only recombinant BMP-2 and BMP-7 tried extensively in the area of experimental and clinical bone healing [67, 76]. BMPs in bone serve a dual role with high degree of anabolism and catabolism. 1. They induce differentiation of precursor osteoblasts into mature bone forming osteoblasts, thus anabolic [77, 78] and 2. They also induce differentiation of osteoclasts via the receptor activator of nuclear factor kappa B ligand (RANKL)-

receptor activator of nuclear factor kappa B (RANK) system, thus being catabolic [79, 80]. It must be noted that BMPs also play a role in the development of other tissues. In regards to bone formation, after BMPs bind to their cell surface receptor, a downstream signaling cascade is activated involving their Smads, particularly Smads 1, 5 and 8, which form a complex with Smad 4 and thus translocating into the nucleus where transcriptional factors runt related transcription factor-2 (RunX-2) and osterix (OSX) are activated and differentiation of MSCs and pre osteoblast, respectively, into osteoblasts takes place [67]. SOST and DKK-1 dependent increase in BMP related bone formation has also been proposed [81]. BMP-2 deficient mice are nonviable at birth and have severe cardiac defects during development [82]. Tsuji and co-workers created a mouse line lacking the gene coding for BMP-2 in the limbs using a Prx1cre enhancer model, which avoided the lethal effects of a total BMP-2 knockout [83]. Their results suggest that the mice that lack BMP-2 gene are subjected to spontaneous limb fractures and are incapable of healing since the first few stages of fracture repair in these subjects are missing, thus indicating the necessary role of BMP-2 in fracture repair [83]. The same research group created conditional knock out mice lacking the BMP-7 gene and reported that the deletion of BMP-7 does not impair fracture healing indicating that other BMPs present in the bone are sufficient to overcome BMP-7 deficiency [84]. BMP-2 and BMP-7 are clinically approved BMPs. rhBMP-2 is sold by Medtronic in their InductOS®/Infuse® bone graft kit while rhBMP-7 often termed as OP-1 was sold by Stryker Biotech and later by Olympus Corporation, which in 2014 discontinued OP-1 from the markets in the USA [85]. Both BMP-2 and 7 are intended for local delivery due to their short biological half-life [86]. rhBMP-2 has been a subject of numerous pre-clinical and clinical studies with promising results during pre-clinical trials and debatable results in the clinical trials [76].

1.5.3 Anti-catabolic factors in experimental orthopedics

Since bone homeostasis involves both formation and resorption, another approach to enhance *overall* bone formation/retention during fracture healing is to control bone resorption, which eventually leads to an overall increase in net bone formation. While the majority of research has been carried out on bone anabolics, several anti-catabolic agents like bisphosphonates, osteoprotegrin (OPG) and anti-RANKL (Denosumab) have targeted modulation of bone resorption.

Bisphosphonates are analogues of naturally occurring pyrophosphates and have a strong affinity to hydroxyapatite and thus bone [87-89]. Bisphosphonates have a P-C-P backbone with phosphate groups flanking the structure and the central carbon atom is further linked to R1 and R2 structures where R1 is often replaced by a hydroxyl group, which together with the flanking phosphates create a strong interaction with hydroxyapatite thus giving bisphosphonates strong affinity to bone

[88, 90]. The R2 structure provides the bisphosphonates with the potency to inhibit bone resorption and this is why bisphosphonates are characterized as non-nitrogen containing and nitrogen containing molecules. When a bisphosphonate does not contain nitrogen on the R2 chain (etidronate, clodronate, tiludronate), their relative potency is several orders of magnitude lower than their nitrogen containing counterparts such as alendronate, risedronate, ibadronate, and zoledronate. In terms of the magnitude of osteoclast inhibition, zoledronate or zoledronic acid has the highest potential followed by risedronate, ibadronate, alendronate, tiludronate, clodronate and lastly etidronate. Bisphosphonates selectively induce apoptosis in osteoclasts while it has been indicated that they may have a no effect on osteoblasts or osteocytes [91, 92]. Due to the strong affinity of bisphosphonates to exposed mineral surface, osteoclasts trying to remodel bone end up with huge local concentrations of bisphosphonates in their vicinity causing some of the bisphosphonate to be taken up. Depending on whether the bisphosphonate contains nitrogen or not, two different mechanisms of bisphosphonate induced apoptosis have been suggested. After its uptake by the osteoclast, non-nitrogen based bisphosphonates, which resemble inorganic pyrophosphate (PPi) get integrated with newly synthesized adenosine triphosphate (ATP) molecules. These non-hydrolysable forms of ATP cause cytotoxicity because they block all molecular processes that depend on ATP causing osteoclast apoptosis [88]. Nitrogen containing bisphosphonates affect the mevalonic acid pathway by blocking the activity of farnesyl pyrophosphate synthase (FPPS), which in turn affects guanosine triphosphate (GTP) binding proteins like Rho and Rab [88, 93]. These proteins are important for the osteoclasts in the maintenance of ruffled border with the bone as well as their survival in general and a disturbance in their expression leads to apoptosis in osteoclasts. Clinically, bisphosphonates have been widely used in the treatment of Paget's disease, osteoporosis and in treatment of several malignancies.

OPG is a decoy receptor for RANKL, as a result of which it controls osteoclastogenesis and preserves bone mineral density by binding to the RANK receptor on osteoclast precursor [94]. In principle, OPG works by binding to RANK, causing a blockage in the RANKL-RANK signaling necessary for osteoclastogenesis. OPG knock out mice have been reported to have severe trabecular bone loss and increased cortical bone porosity. Surprisingly, these mice also demonstrate calcification of blood vessels like the aorta and renal arteries [95]. One of the earliest randomized clinical trials using OPG demonstrated a reduction in bone resorption markers like N telopeptides of type I collagen (NTx) and deoxypyridinoline (DPD) in post-menopausal women who were administered a single sub cutaneous injection of OPG, confirming the hypothesis that OPG halts osteoclastic resorption of bone [96].

Denosumab is a monoclonal antibody against RANKL, which acts in a similar fashion as OPG, blocking the RANKL-RANK signaling and reducing osteoclastogenesis, but was preferred since it targets the ligand rather than the

receptor [97]. An animal study comparing the efficacy of bisphosphonate, alendronate with denosumab concluded that both agents have almost similar effect on fracture healing and both increased fracture strength during the course of the treatment but they did not report the fracture callus properties after the treatment was stopped [98]. Denosumab has been commonly used for osteoporosis treatment and prevention of metastatic skeletal lesions. However, recent literature shows that there is a rapid reduction in the bone mineral density and concomitant increase in bone resorption markers when denosumab treatment is discontinued in osteoporosis [99].

1.6 Delivery routes for BMPs and bisphosphonates

Due to a short biological half-life of BMPs, delivery of both rhBMP-2 and rhBMP-7 has been performed locally, via a carrier, at the site of fracture repair. Commercially available rhBMP-7 or OP-1 product has been supplied as a mixture of 3.5 mg rhBMP-7 contained in 1 g of type I collagen granules further supplemented with 230 mg of carboxymethylcellulose (CMC), which forms putty like consistency when mixed with saline [85]. Water soluble rhBMP-2 is delivered via a highly porous absorbable collagen sponge (ACS) sold as Medtronic® Infuse Bone Graft. The protein is reconstituted in saline solution and the ACS is soaked with the solution containing the protein before implantation. The product is available in 5 different doses ranging from 1.05 mg to 12 mg of the recombinant protein (as per manufacturers information) and has been clinically used for lumbar fusion surgeries, bone defects and maxillofacial applications [100]. On the contrary, zoledronic acid (ZA), one of the most potent bisphosphonate, is administered systemically once a year as an intra-venous infusion at a dose of 4-5 mg for prevention of post-menopausal fragility fractures in osteoporosis [101]. In experimental studies, it has been shown that the maximum ZA concentration is found in the blood plasma until a few hours after infusion following which the plasma levels of ZA drop tremendously within the first 96 h since roughly 40% of the drug is processed by the kidneys and excreted through urine [102]. The rest of the drug preferentially binds the skeleton with more affinity to cancellous bone.

1.7 The problem at hand

Although bone grafts are a preferred choice for bone regeneration during orthopedic surgery, concomitant drawbacks including, but not limited to, donor site morbidity, increased risk of infection, shortage and variable efficacy need to be overcome [11-14]. Bone tissue engineering based strategies have shown promising results in

creating bone substitutes that possibly in the near future could replace traditional bone grafts. Biomaterials, cells, growth factors and drugs are the central components of the tissue engineering based approach [29]. Biomaterials alone lack inherent osteoinductivity to form large volumes of bone and thus need to be combined with growth factors like rhBMP-2 to endow a biological action (osteoinduction). Usage of rhBMP-2 has had its fair share of criticism including its inability to outperform the current 'gold standard', the autograft. Due to a short half-life, rhBMP-2 is delivered via an absorbable collagen sponge locally but problems associated with a burst release profile of the protein and the usage of supraphysiological doses have not been paid attention to by the manufacturer [103, 104]. A more recent observation regarding the usage of rhBMP-2 is its ability to promote significant osteoclastogenesis, which in turn leads to pre-mature resorption of bone, thus a low net bone formation [79, 105]. Based on these facts all together, it was evident to us that the problem at hand is the *lack of an optimal carrier biomaterial* for local delivery of rhBMP-2 and further, there is a scope for *reducing* the rhBMP-2 doses. To overcome the excessive osteoclast formation due to the rhBMP-2 usage, a few research groups including ours have tried to silence the osteoclast mediated bone resorption using bisphosphonates such a ZA [106, 107]. ZA binds to the bone mineral and induces apoptosis in osteoclasts when they try to resorb the bone matrix [88]. As a consequence of this, a balance between anabolic effects of rhBMP-2 and anti-catabolic effects of ZA is achieved, which provides an increased net bone turnover. A bisphosphonate like ZA is given systemically and binds to bone mineral throughout the skeleton. Bone remodeling as described earlier is an essential part of bone homeostasis and thus binding of bisphosphonates to all skeletal sites can possibly lead to reduced bone remodeling. Besides this, other reports of ZA side effects including myalgia after infusion, osteonecrosis of the jaw with prolonged usage, and atypical cortical fractures of the long bones have also come forth [108]. In light of these observations, we believe that local, site specific delivery of ZA using biomaterial carriers might have beneficial effects.

2. Research Questions

Within the scope of the thesis, we asked ourselves:

1. *If we can develop/characterize biomaterials that can act as efficient **carriers** for **local** delivery of both **rhBMP-2 and ZA**? Can these materials outperform what already exists?*
2. *Does **local delivery of ZA** combined with rhBMP-2 via the developed carrier materials work?*
3. *What does **locally delivered ZA do** when delivered alone? Besides its anti-catabolic effects, is it also anabolic?*
4. *By improving the carrier and using the combination of rhBMP-2 and ZA, can we **reduce** the high doses of **rhBMP-2**?*
5. *Can the **combination of biomaterials and bone active molecules** be used in clinically relevant models of bone regeneration using small animal models?*

3. Hypotheses

We hypothesized the following:

1. A clinically approved microporous biphasic material consisting of fast resorbing calcium sulphate (CaS) and bone mimicking hydroxyapatite (HA) can chemically interact with both rhBMP-2 and ZA and thus provide controlled release of both bioactive factors in-vivo.
2. A pre-set macroporous biomaterial consisting of gelatin and Cas/HA mimics the micro-architecture of trabecular bone. Both gelatin and HA can interact with rhBMP-2 while ZA can bind to HA. This can provide spatio-temporal delivery of both bioactive molecules. Besides, pre-set material gives an added benefit of shape retention, which is important at critical anatomical locations like the spine.
3. Local co-delivery of rhBMP-2 and ZA will have a dual effect. Firstly, rhBMP-2 will induce osteogenesis and ZA will restrict bone resorption induced by rhBMP-2. Secondly, with local co-delivery of ZA, it will be possible to reduce effective doses of rhBMP-2 and circumvent side effects of systemic ZA delivery.
4. Some cell-culture studies have indicated a possible anabolic role of ZA on mesenchymal stem cells. Thus, we hypothesized that local delivery of ZA can be anabolic and lead to increased bone formation.
5. Bone mimicking materials, which provide controlled release of bone active molecules, can be used as alternatives to bone grafting materials in animal models of bone defects.

4. Experimental design

4.1 Biomaterials

During this thesis work, three different biomaterials have been evaluated for their potential in bone regeneration (Fig. 5).

1. The first biomaterial consists of a biphasic powder of micron sized calcium sulphate (CaS) and micron sized hydroxyapatite (HA) mixed at a ratio of 60/40 by weight %, which sets into a microporous hard mass when it is mixed with a non-ionic radiographic contrast medium (Fig. 5, Top). The material was developed at our laboratory as an injectable bone substitute nearly two decades ago and is approved for clinical use and commercially produced by Bone Support AB, Sweden. This biomaterial has been used in studies 1- 3. *This biomaterial is referred to as CaS/HA hereafter.*
2. To create a macroporous biomaterial mimicking the structure of trabecular bone, we developed a porous biomaterial via cryogelation technology. The material consisted of gelatin based polymeric backbone embedding microparticles of CaS and HA forming a macroporous composite material having a pore size range of a few micrometers to approximately 100 μm (Fig. 5, Middle). Gelatin-CaS/HA was chosen over collagen-CaS/HA at the optimization phase, due to it's greater mechanical strength and improved handleability. This biomaterial has been used in studies 4 and 5 and *will be referred to as GCH*.
3. Lastly, a collagen membrane developed by our collaborators at the University of Western Australia by Prof. Ming Hao Zheng's laboratory was used. The membrane consists of a smooth side which is non-permeable to cells and prevents formation of scar tissue and a rough side consisting of randomly organized collagen fibers leading to a semi-porous structure, which is good for cell penetration and attachment (Fig. 5, Bottom). Although the group has described a similar membrane for healing of tympanic membrane in pre-clinical models, the efficiency of the membrane in bone regeneration has not been tested before. It was thus used in study 5 to test its potential in guiding cortical bone regeneration. The third biomaterial *will be referred to as CM.*

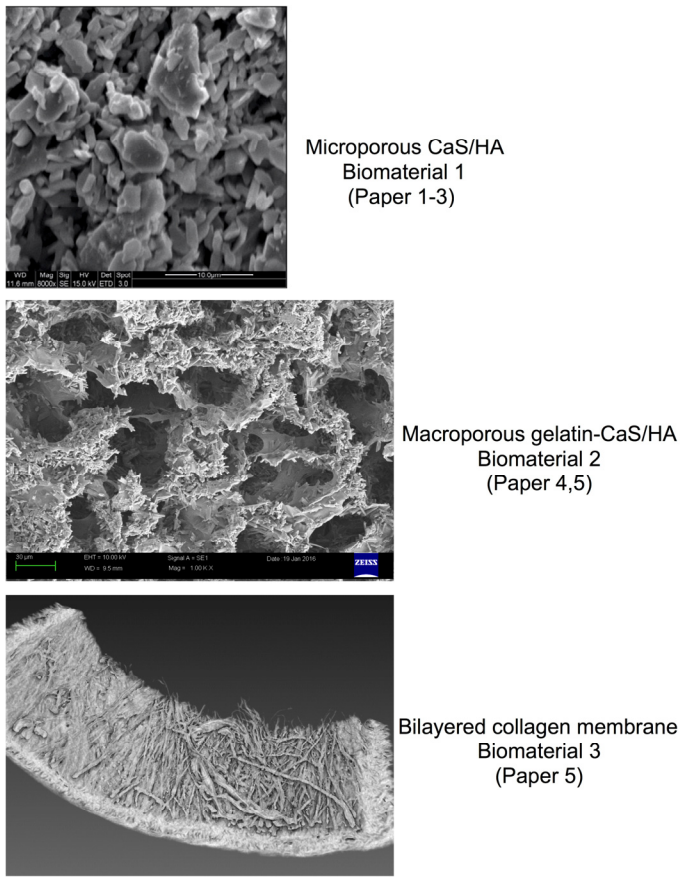


Fig. 5 Biomaterials used in the thesis work.
The CaS/HA biomaterial (Top) was used in studies 1-3, the GCH biomaterial was used in studies 4 and 5 and the CM was used in study 5.

4.2 Animal models

In the five experimental studies, three different animal models for bone regeneration were used. All experiments have been carried out on Sprague-Dawley rats. Male rats have been used in studies 1, 2, 4, and 5 while female rats have been used in study 3.

In studies 1, 4 and partly 5, an ectopic abdominal muscle pouch model was used to test the potential of developed biomaterials in delivering rhBMP-2 and ZA (Fig.6). This model was preferred because any bone formation in this model is *new bone* formation due to the anatomical location of the experimental specimens. Moreover, this model is rather simple to create and involves a small skin incision

near the abdominal midline followed by cleaning the fascia and creating a small pocket in the *rectus abdominis* muscle. The biomaterials with or without bone active molecules which are to be tested are placed in the pocket, the pocket is sutured with a non-resorbable sutures to facilitate identification of the specimens followed by suturing the skin. After 4-weeks, the animals are sacrificed and the biomaterial/bone composite is harvested and subjected to different tests to quantify the amount of bone formation.

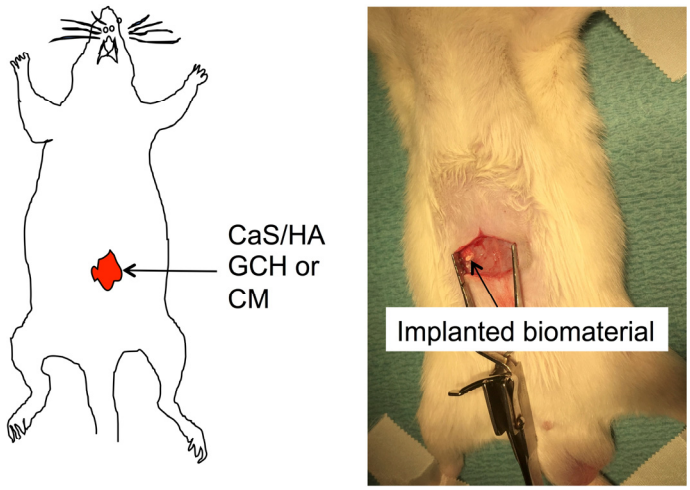


Fig. 6 Abdominal muscle pouch model used in studies 1, 4 and 5.

In studies, 2 and 5, a tibia defect model was used (Fig. 7). After anesthesia, the right knee of the animal was shaved, disinfected and incised near the knee joint. The proximal tibia was exposed, cleaned of the soft tissue and the periosteum scraped in both proximal and distal directions. The model then involved creating a circular hole in the proximal tibial metaphysis, resulting in a circular cortical defect and a cylindrical cancellous defect in the tibia. In study 2, the cortical defect diameter was *3 mm,* which was then increased to a diameter of *4.5 mm* in study 5 since results from study 2 indicated that the chosen diameter did not give a critical defect. Drilling was done with a manual hand-held drill with a drill stop to control depth. In study 2, the cancellous defect was treated with a CaS/HA biomaterial and its different combinations leaking ZA and rhBMP-2 locally and the cortical bone was left open to heal. In study 5, the cancellous defect was filled with a porous GCH scaffold and its combinations while the cortical defect was covered with a CM with or without rhBMP-2 to guide cortical bone healing.

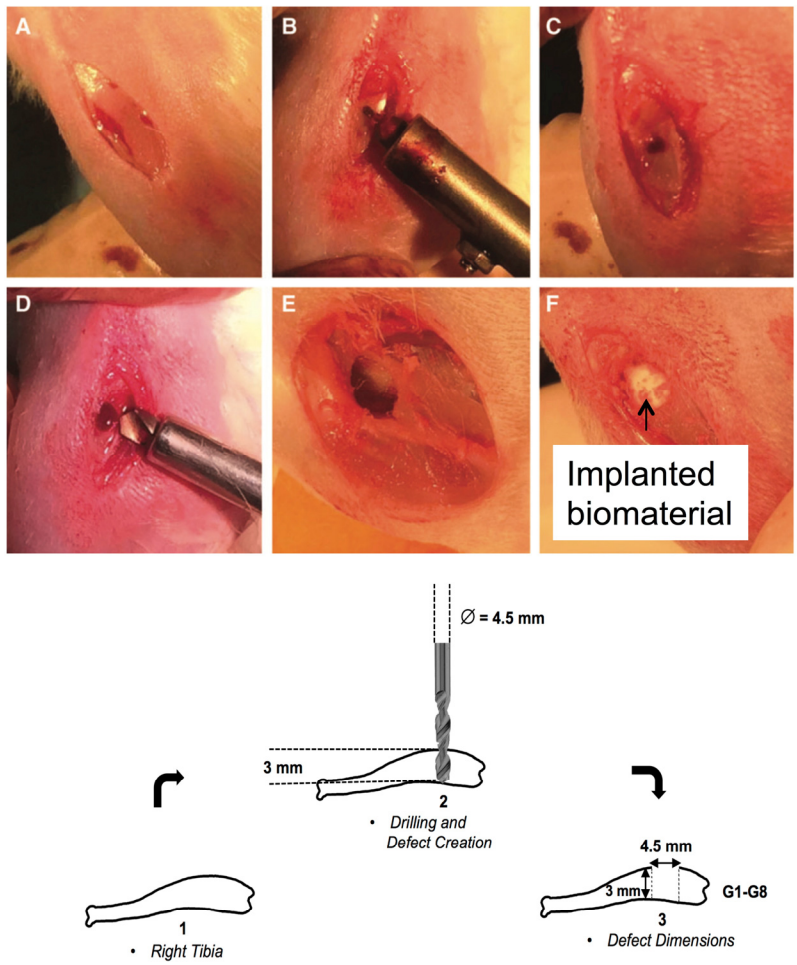


Fig. 7 Tibia defect model used in studies 2 and 5.
Top shows surgical procedure and bottom shows a schematic of the model and defect dimensions used in study 5.

In study 3, female Sprague-Dawley rats, ovariectomized at 12- weeks of age were used to develop osteoporosis-like characteristics in the bone and were operated on at the age of 28-weeks. A defect in the femoral neck canal of these animals was created using a drilling burr. The defect dimensions were 1 mm in diameter and 8 mm in depth, starting at the posterior cortex opposite to the lesser trochanter and ended at the sub-capital zone (Fig. 8). The defect was then filled with CaS/HA biomaterial and its different compositions by impaction. Assessment of bone formation was done in-vivo at 4-weeks post-operation followed by sacrifice at 8-weeks.

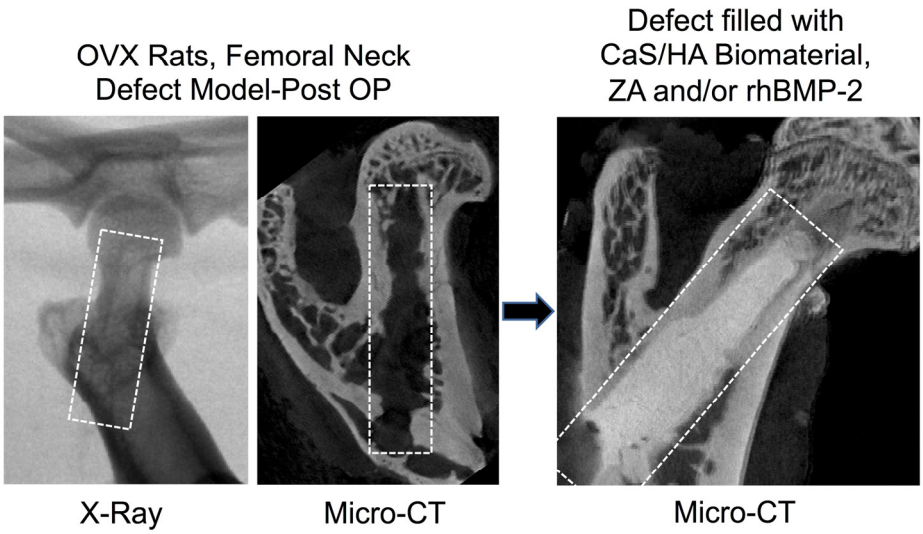


Fig. 8 Femoral neck defect model used in study 3.
Image in the left is an X-ray of the right proximal femur showing the drilled region immediately after defect creation. Image in the middle shows a micro-CT slice indicating the drilled region and image on the right shows a micro-CT slice showing impaction of the CaS/HA material in the femoral neck canal immediately after implantation. The dotted white box is for representation only and indicates the approximate location of interest (defect creation and material impaction).

4.3 Experimental techniques

This thesis work comprises of several different techniques including: engineering methods, cell and molecular biology methods and animal models. The most important techniques used within the framework of this thesis are detailed below.

4.3.1 Micro-computed tomography (Micro-CT)

X-rays interact with matter in different ways, which is predominantly determined by the density of the object under investigation. Micro-CT consists of a rotating object or rotating X-ray source and detector, which take X-ray tomographs of samples at small pre-defined intervals providing a 360° view of the samples. Finally, the X-ray images taken at each step/angle are stacked together to provide a 3-D image of the sample unlike the conventional X-rays, which gives only a 2-D image. Once the image acquisition is complete, the images can then be analyzed to get a volumetric measure of different parameters. Since during this thesis work, we were interested in bone regeneration, once a particular region of interest (ROI) was defined, all pixels in that ROI that had an intensity (in simpler terms, density) greater

than a pre-defined threshold/density were considered as bone or highly mineralized tissue. This gave a volumetric measure of bone regeneration in the defect. This method is more accurate than conventional histomorphometric evaluation where the outcome is dependent on the location from where the specimen is taken and also consumes more time. Furthermore, micro-CT is very detailed and can resolve various bone structures like cortical bone, trabecular bone, trabecular number etc. owing to a high imaging resolution (<100 μm). In-vivo imaging modality on a micro-CT device helps longitudinal follow up of animals without being invasive resulting in more accurate measurements, reducing animal to animal variation and total number of experimental animals.

4.3.2 Single photon emission computed tomography (SPECT-CT)

SPECT-CT is a nuclear medicine imaging modality based on two different imaging approaches. The SPECT system consists of a gamma camera, which rotates around a subject and acquires images from locations with radioactivity or gamma emissions, for instance from ^{125}I, an isotope of iodine with high gamma emission. To calibrate the images with an anatomical location, a CT is also done either before or after the scan. The images from two different acquisitions are then superimposed to look for radioactive emissions from a particular location of interest. Image analysis software then measures the pixel intensity within the ROI to evaluate the activity of a radioactive tracer. In our experiment, SPECT-CT was used to detect the release of radioactively marked rhBMP-2 from the porous gelatin biomaterial. Baseline scans made at Day 1 gave us the maximum radioactive signal at the site of implantation and the same animals were followed longitudinally at different time points. The signal measured at each time point was compared to the signal at baseline to plot a release kinetics curve.

4.3.3 Histology and histomorphometry

Histology was used to analyze the cellular composition of regenerated bone tissue and biomaterial remnants at microscopic levels. Tissue of interest was processed using a series of histological steps involving decalcification and dehydration before the tissues were embedded as a block in paraffin wax. The tissue block was then cut to a thickness of approximately 5 μm, collected on a glass slide, deparaffinized and stained using various stains including hematoxylin and eosin (H&E), tartrate resistant acid phosphatase (TRAP) and picrosirius red. In some experiments, where co-relation of micro-CT results and histological analysis was necessary to ensure what was measured as bone in micro-CT was also histologically positive bone tissue and not just remnants of biomaterials, histomorphometry was done to quantify the area of bone tissue on each slide. This was done using a histomorphometry software,

which differentiated between different tissues and biomaterial remnants on the basis of staining intensity.

4.3.4 Cell culture

Cell culture techniques were used mainly in paper 1 and paper 4. In paper 1, lung cancer cell line A549 was used to indirectly measure the amount of ZA released from the CaS/HA biomaterial. Apoptotic effect of free ZA on A549 cells is well established. Cells were cultured on tissue culture plates (TCP) and supplemented with medium collected from CaS/HA biomaterial containing ZA, with a hypothesis that the released ZA should incur toxicity to A549 cells. The viability of the cells was then assessed using the MTT assay, which interacts with the mitochondrial enzymes of live cells, creating formazan crystals which can be solubilized in dimethyl sulfoxide (DMSO) and spectrophotometrically read at 570 nm. Using a standard curve with increasing ZA concentrations and viability of A549 cells, we could compare the cytotoxicity induce by the released fraction to quantify the total amount of released ZA from CaS/HA material. As a control experiment to confirm the results, A549 cells were cultured directly on discs of CaS/HA containing ZA and their viability was evaluated using MTT assay.

In paper 4, MC3T3-e1 pre-osteoblast cells were used to model the progenitor cell population present during fracture repair. These cells respond to BMP-2 treatment and differentiate into more mature osteoblast like cells. To confirm that rhBMP-2 is functionalized on the developed GCH scaffolds and that it interacts with the scaffolds without losing its functionality, MC3T3 cells were seeded on the scaffold functionalized with rhBMP-2. Alkaline phosphatase (ALP) activity was used as an early biochemical marker of osteoblastic differentiation of MC3T3 cells. Furthermore, real time polymerase chain reaction (qPCR) was performed to study the expression of genes related to osteoblastic differentiation of pre-osteoblasts such as osterix (OSX-marker of pre-osteoblast to osteoblast commitment) and osteocalcin (OCN-a marker of mature osteoblast cells). Same experiments were carried out on cells seeded on scaffolds without rhBMP-2 and plastic plates for comparisons.

4.3.5 Material characterization

Biomaterial characterization was carried out in paper 1 and paper 4. Some of the common techniques and the rationale of using those are provided below:

Scanning electron microscopy (SEM) was performed in both study 1 and 4. SEM works by focusing an electron beam on an object and based on the atomic composition of the material and its topography, a high resolution and high magnification image of the object can be created. In study 1, SEM was used to look

at the structure of the CaS/HA material after incorporating rhBMP-2 and ZA to the material as well as to evaluate the changes in the micro porosity of the material after the CaS phase resorbs over time. SEM was performed on the bone/material composite which was harvested from the ectopic muscle pouch model 4-weeks after implantation to visualize structure of new bone. Similarly, in study 5, SEM was used to evaluate the structure of the GCH material as well as to study cell attachment on the biomaterial surface in-vitro.

Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) were used in study 4. FTIR works by shining lights of different wavelengths at a material and based on the chemical properties of the material, certain wavelengths are absorbed with specific intensity, which is then converted into a spectrum of transmission /absorption using a mathematical process (Fourier transformation). From the spectra, it is possible to infer the nature of the chemical bonds which exist within the sample. FTIR is commonly used in polymer chemistry to confirm crosslinking and formation of monomer to polymer structures. In study 4, GCH scaffold was pulverized in a mortar pestle before performing FTIR analysis. FTIR was performed to primarily confirm the crosslinking of the gelatin structure with glutaraldehyde.

XRD is used to study the atomic structure of a crystal by bombarding X-rays at a sample, which as a result of the crystal's composition are diffracted at different angles and detected by the detector. The device then maps a 3-D picture of electron density in the specimen which can be matched with the atomic structure of a crystal. XRD is widely used in ceramic chemistry, especially to detect the purity of a hydroxyapatite crystal after different chemical and physical treatments. XRD was used in study 4 to confirm the incorporation of hydroxyapatite in the GCH scaffold.

4.4 Design of animal studies

Animal studies have been performed in all 5 papers entailed in this thesis. Groups for comparison were selected as summarised in table 1 below.

Table 1. An overview of the biomaterials and experimental animal models used.

Study	*Biomaterial Used*	*Main Aim*	*Model*	*Groups for Comparisons*	*Sample Size/Group (n)*
1	CaS/HA	Carrier properties of CaS/HA in delivering rhBMP-2 and ZA	Abdominal muscle pouch	1. CaS/HA 2. CaS/HA+rhBMP-2 (10 µg) 3. CaS/HA+rhBMP-2 (10 µg) +ZA (10 µg)	6
2	CaS/HA	CaS/HA and/or rhBMP-2+ZA for healing of metaphyseal bone defect	Tibia defect	1. Empty defect 2. Allograft 3. CaS/HA 4. CaS/HA+ZA (10 µg) 5. CaS/HA+rhBMP-2 (5 µg) +ZA (10 µg)	10
3	CaS/HA	CaS/HA and/or rhBMP-2+ZA for regenerating bone in femoral neck canal of osteoporotic rats	Femoral neck defect model in OVX rats	1. Empty defect 2. CaS/HA 3. CaS/HA+ZA (10 µg) 4. CaS/HA+rhBMP-2 (4 µg) +ZA (10 µg)	12
4	GCH	Carrier properties of GCH in delivering rhBMP-2 and ZA	Abdominal muscle pouch	1. GCH 2. GCH+rhBMP-2 (10 µg) 3. GCH+rhBMP-2 (10 µg) +ZA (10 µg)	8
		In-vivo release of rhBMP-2 and ZA		1. GCH+^{14}C-ZA+ rhBMP-2 2. GCH+^{125}I-rhBMP-2+ZA	6
5	GCH+CM	GCH and/or rhBMP-2+ZA for cancellous bone healing CM and/or rhBMP-2 for cortical bone healing	Tibia defect	1. Empty defect 2. GCH 3. GCH+ZA (10 µg) 4. GCH+ rhBMP-2 (5 µg) +ZA (10 µg) 5. GCH+ ZA (10 µg) +(CM) 6. GCH+ rhBMP-2 (5 µg) +ZA (10 µg) +(CM) 7. GCH+ ZA (10 µg) +(CM+rhBMP-2 (2µg)) 8. GCH+ rhBMP-2 (3 µg) +ZA (10 µg) +(CM+rhBMP-2 (2µg))	10

4.5 Methodological Considerations: Animal models and ethics

It is important to note that this thesis contains results from animal experiments emanating from rat models of bone formation and bone healing and thus similar findings in other animal models are only speculative. Careful interpretation of these results and stepwise implementation of the methods developed in this thesis should be performed before clinical trials. Although differences exist between human bones and rat bones, rat models of bone regeneration and bone healing are widely accepted in experimental orthopedics owing to readily available and economical inbred strains with less inter-animal variation. Most of the animal experiments have been carried out on *male rats* mostly to avoid hormonal fluctuation seen in female rats, which can affect the outcome of the studies and add experimental variations. Although using animal models incur *animal suffering*, the process of fracture healing is complicated and involves a cascade of cells, cytokines and signaling molecules from local surrounding tissues, blood, immune system etc., making it impossible to create a perfect in-vitro fracture healing system. All five studies included in this thesis work have complied with the *3R's principle* with an aim to *reduce and refine* the number of experimental animals without affecting the main message of the thesis. Exclusion of experimental groups wherever deemed necessary has been done to achieve this. *Replacement* of animal models with in-vitro models of bone formation was not possible as a perfect in-vitro model of bone regeneration/defect healing does not exist due to the multi-dimensional nature of the bone healing process. Power calculations and group sizes were based on the previous studies conducted and published by the group. Besides this, all animal studies have been approved by the Swedish board of agriculture (Jordbruksverket, permit numbers M124-14-study 1, 4 and 5, M79-15-study 2 and 5 and M128-16-study 3). All necessary steps to reduce animal suffering were followed and all animals had free access to regular food pellets and water throughout the experimental period. Animals were housed two per cage with hygienic conditions and 12 h long day/night light cycles.

4.6 Statistical methods

In study 1, student's t-test was used to analyze difference between experimental groups in the published study but post-publication, the analysis was also run using a non-parametric test due to a smaller sample size, which resulted in similar statistical differences arising. Again, due to a smaller sample size, in study 4, we used non-parametric Mann-Whitney U test for data emanating from two

independent experimental groups. Paired data were tested using Wilcoxon-matched pair signed rank test. Data emanating from multiple groups (>2) were analyzed using Kruskal Wallis multiple comparison test with corrections for multiple comparisons (Dunn's method). Studies 2, 3 and 5 had comparatively larger sample size and more experimental groups than to that of studies 1 and 4. As a result, data were always tested for normality using Shapiro-Wilk normality test and by looking at the spread of the data (residuals) on Q-Q plots and histograms. Normally distributed data were tested with ANOVA to confirm statistical differences. Post-hoc tests to point out group based differences were chosen based on the homogeneity of variances tested using Levene's method. Group comparisons for homogenous variance data sets were performed using ANOVA with Tukey HSD post-hoc method and non-homogenous variance data sets were tested using ANOVA with Games-Howell post-hoc test. In case of non-normally distributed data, Kruskal Wallis multiple comparison method was used when groups to be tested were greater than 2 and p-values after correction for multiple comparison using Dunn's method were reported. A p-value of less than 0.05 ($p<0.05$) was considered statistically significant. Data were tested either using Prism 7 for MacOS (GraphPad Software Inc. CA, USA) or SPSS statistics (IBM Corporation, NY, USA).

5. Results

5.1 Study 1-Carrier properties of CaS/HA biomaterial

- The CaS/HA biomaterial released 90% rhBMP-2 and approximately 10% ZA in-vitro after one week and was detected using ELISA and MTT assay, respectively.
- In the muscle pouch model, pellets of CaS/HA combined with rhBMP-2 and ZA produced significantly higher volume of highly mineralized tissue when compared to CaS/HA+rhBMP-2 and CaS/HA alone, as seen from micro-CT (21.4±5.5 mm^3 vs. 10.9±2.1 mm^3 vs. 4.9±0.9 mm^3) (Fig. 9). Histomorphometry corroborated well with micro-CT results.
- Histological findings indicated new bone formation throughout the sample in the CaS/HA+rhBMP-2+ZA group compared to the only rhBMP-2 group, which showed bone formation mostly on the edges while the core of the specimen was filled with marrow like tissue.
- CaS/HA material alone failed to show any histological signs of bone formation.
- Biomechanically, both CaS/HA+rhBMP-2 group and CaS/HA+rhBMP-2+ZA group showed significantly higher absorbed energy when compared to only CaS/HA.

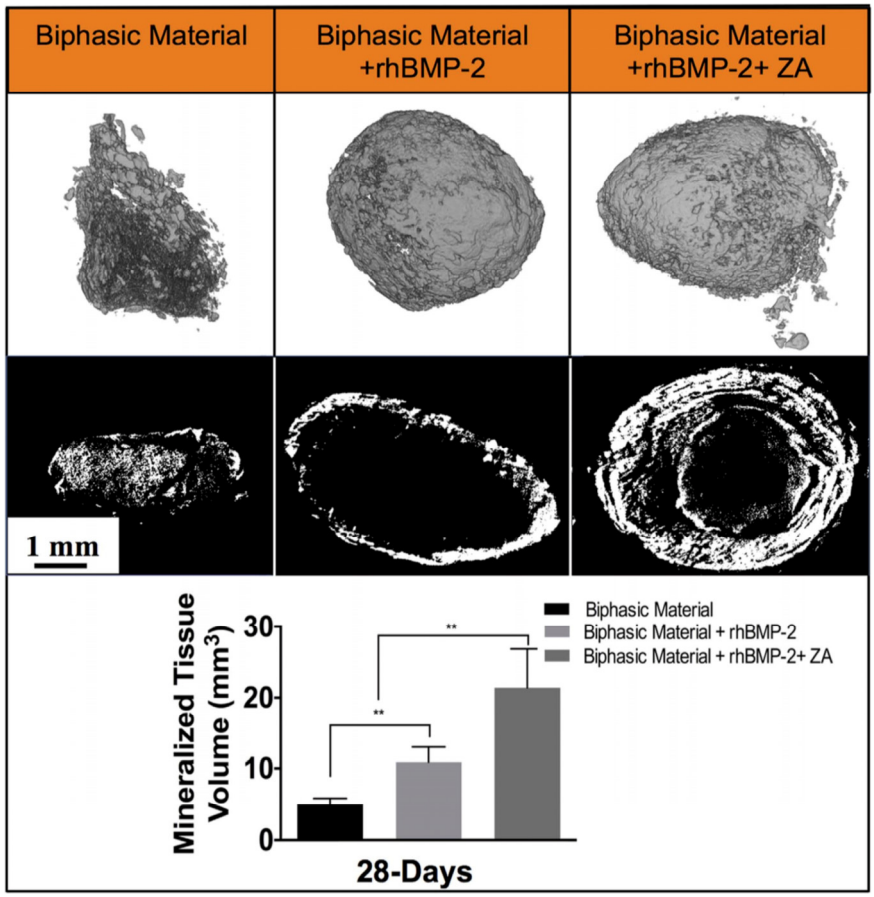
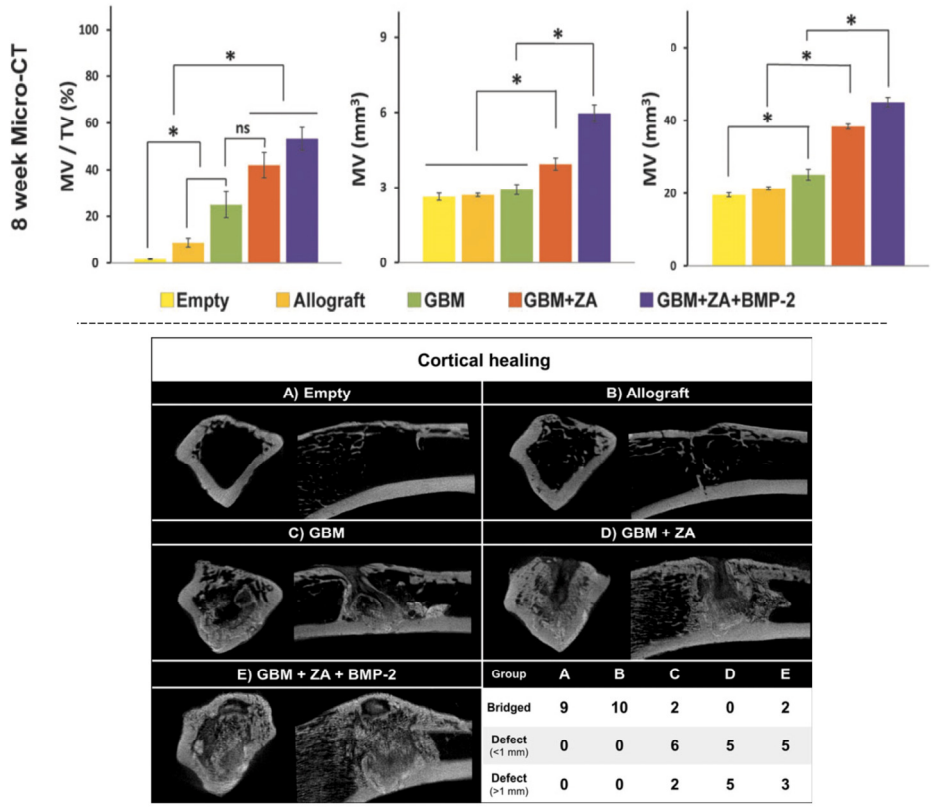


Fig. 9 Micro-CT results from study 1.
Top and middle show representative 3-D reconstructions and 2-D micro-CT slices from different treatment groups, respectively. Bottom panel shows the micro-CT quantifications. ** Indicates $p<0.01$. Figure has been reproduced from [109].

5.2 Study 2- CaS/HA + bioactive molecules in a rat tibia defect

- Radiographically, all specimens treated with CaS/HA biomaterial had higher radiolucency in the defect region compared to the empty and the allograft groups. Addition of ZA or ZA+rhBMP-2 increased the radiolucency of the specimens further.
- Micro-CT results at 8-weeks showed that the MV/TV (%) was higher in CaS/HA+ZA and CaS/HA+ZA+rhBMP-2 groups when compared to the empty and allograft group in the intramedullary ROI. Similar results were seen in cortical ROI and the 3 mm full bone ROI. CaS/HA alone performed better than the empty group in intramedullary ROI and 3 mm full bone ROI (Fig. 10).
- Representative histological findings corroborated well with micro-CT findings. Moreover, what was measured as MV using micro-CT was mostly new bone formation and not only CaS/HA remnants as confirmed by CT and histology slice matching.
- The defect created was non-critical since almost all cortices in the empty group and the allograft group bridged completely (Fig. 10). CaS/HA and CaS/HA+rhBMP-2+ZA group had only two cases with complete cortical bridging. CaS/HA+ZA group had no cases of complete cortical bridging.



Group	A	B	C	D	E
Bridged	9	10	2	0	2
Defect (<1 mm)	0	0	6	5	5
Defect (>1 mm)	0	0	2	5	3

Fig. 10 Micro-CT results and cortical bridging from study 2.
The graph in the top panel shows micro-CT quantifications in different treatment groups after 8-weeks and the panel in the bottom including the table shows the extent of cortical bridging. * Indicates $p<0.05$. Figure has been reproduced from [110].

5.3 Study 3- CaS/HA + bioactive molecules in a femoral neck defect of osteoporotic rats

- In the defect leg, the application of CaS/HA biomaterial increased the MV/TV in ROI 1 i.e. almost the entire drilled region as well as in ROI 2 i.e. the femoral neck compared to the empty defect as seen from micro-CT imaging at 4-weeks.
- At 8-weeks, the MV/TV in the defect leg was significantly higher in the CaS/HA biomaterial group in ROI 1 when compared to the empty group but no differences in ROI 2 were seen. Addition of ZA and rhBMP-2+ZA to the CaS/HA biomaterial led to increased MV/TV compared to both empty and CaS/HA groups in both ROIs.
- No differences between the groups were seen on the contralateral legs. Comparison of defect and contralateral legs in the empty group indicated a reduction in the MV/TV fraction in the defect leg when compared to the contralateral leg. No such changes were seen for only CaS/HA group. Both CaS/HA+ZA and CaS/HA+ZA+rhBMP-2 groups had statistically higher MV/TV in the defect leg compared to the contralateral leg in both ROI 1 and 2.
- Biomechanically, no differences in the peak force to fracture were noticed in the treatment groups on the defect side. The CaS/HA only group had significantly lower peak force to fracture in the defect leg than its contralateral control. No differences between defect and contralateral legs were seen in other groups (Fig. 11).
- In the defect leg, addition of ZA and ZA+rhBMP-2 seemed to create more fractures on the lateral side than the sub-capital side. Empty and CaS/HA groups had a mixed distribution of fracture locations with half on lateral and half on sub-capital side.

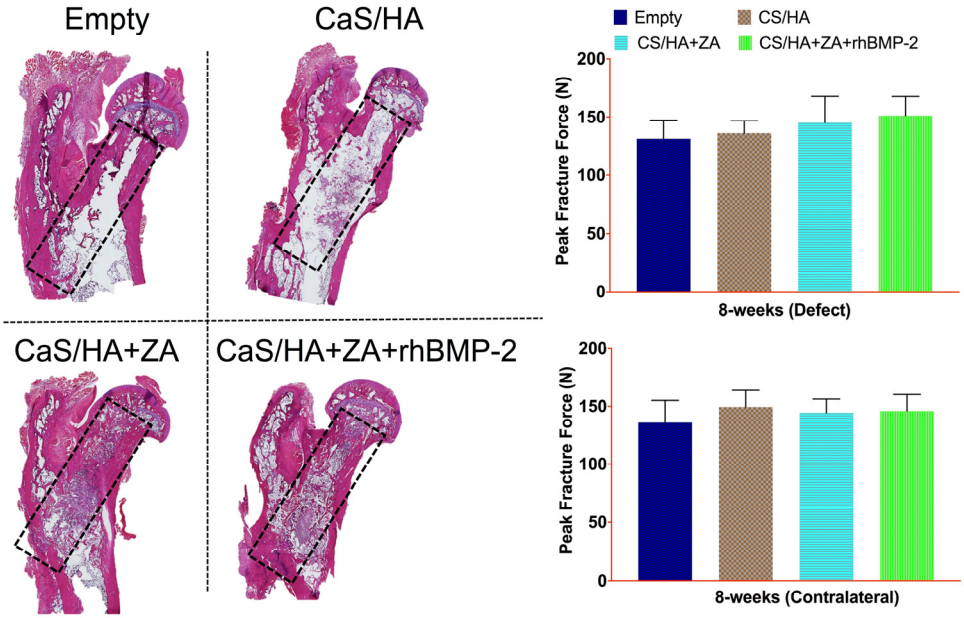


Fig. 11 Histology and mechanical testing results from study 3.
Histological images (Left) and biomechanical testing results (Right) from the femoral neck defect in osteoporotic rats. The rectangular box in the histology images highlights the defect region and is for representation only. Figure has been reproduced from [111].

5.4 Study 4-Characterizing the macroporous GCH carrier material

- The material had a spongy appearance and porous structure with pores ranging up to 100 μm as seen from SEM. Crosslinking of gelatin was confirmed via FTIR, which showed an aldimine peak in the spectra. Incorporation of hydroxyapatite was confirmed via XRD.
- The viability of MC3T3 cells seeded on the scaffolds was good and increased with an increase in time indicated by the MTT assay. Cells seeded on rhBMP-2 functionalized scaffolds differentiated into more mature osteoblasts as shown by the increase in alkaline phosphatase activity as well as the increase in the expression of osteogenic genes OSX and OCN.
- Functionalization of the scaffold with rhBMP-2 and ZA resulted in formation of statistically higher mineralized volume in the muscle pouch when compared to rhBMP-2 alone as seen via micro-CT. Excessive osteoclast activity as seen from an increased TRAP staining in only rhBMP-2 treated samples explained the results.
- The developed GCH scaffold also outperformed the current FDA approved absorbable collagen sponge (ACS) in terms of rhBMP-2 delivery and total volume of bone formed (Fig. 12).
- Co-delivery of rhBMP-2 and ZA with the GCH scaffold led to a reduction of rhBMP-2 doses by a factor of four and yet produced same result as a full dose rhBMP-2 delivered alone via the scaffold.
- In-vitro, the scaffold released approximately 3% rhBMP-2 and 13% ZA over a period of 4-weeks.
- In-vivo, the scaffold released approximately 65% ^{125}I-rhBMP-2 and 40% ^{14}C-ZA in 4-weeks as detected using SPECT-CT and scintillation counting, respectively.

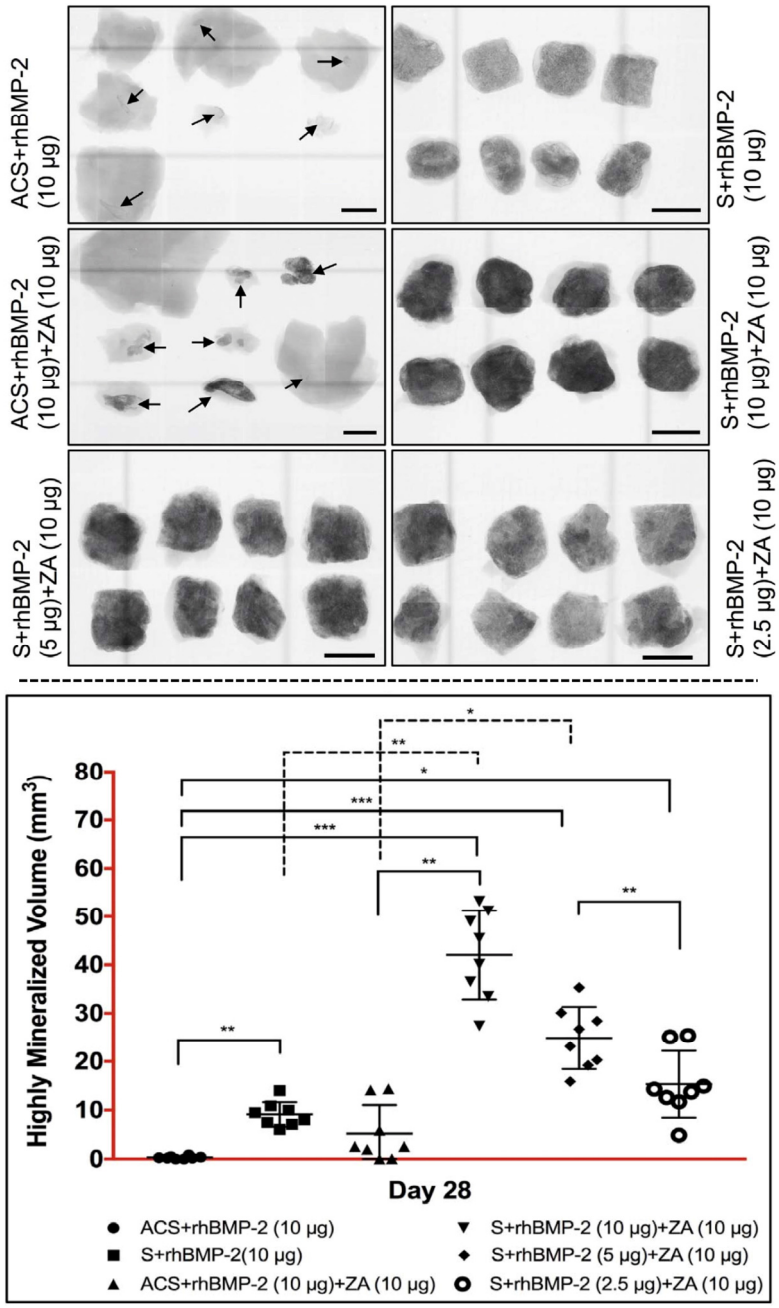


Fig. 12 X-ray and micro-CT results from study 4.
Top panel shows X-ray images while the bottom panel shows micro-CT based quantifications of the bone volume. Figure is reproduced from [105].

5.5 Study 5- GCH and CM + bioactive molecules in a tibia defect

- During the feasibility study, the CM could efficiently deliver rhBMP-2 and ZA within the muscle pouch and the combination of rhBMP-2 and ZA generated significantly higher volume of bone in comparison to rhBMP-2 alone as demonstrated by the micro-CT data.
- In the tibia defect model, all GCH treated groups irrespective of the bioactive molecules or the endosteal CM could regenerate significantly higher MV/TV (%) in the intramedullary defect compared to the empty group as seen using micro-CT.
- All GCH groups treated with ZA or rhBMP-2 and ZA had significantly higher MV compared to the empty group in the 6.5 mm full defect ROI, which took into account the amount of bone proximal and distal to the defect as well.
- In terms of cortical bridging, all cortices in the empty group bridged but almost no cancellous bone regeneration was seen. Cortical defects in the GCH groups with or without bioactive molecules but without the membrane were impaired. Maximum bridging was seen in the GCH+rhBMP-2+ZA group with 2/10 cortices ranked as completely bridged (Fig. 13).
- In GCH groups containing ZA and ZA+rhBMP-2 on the GCH and having CM +rhBMP-2 as an endosteal cover, 5/10 and 7/10 cortices completely bridged, respectively (Fig. 13).
- Histological results corroborated well with the micro-CT results. Complete bridging was seen in the groups that were ranked bridged on micro-CT as well.

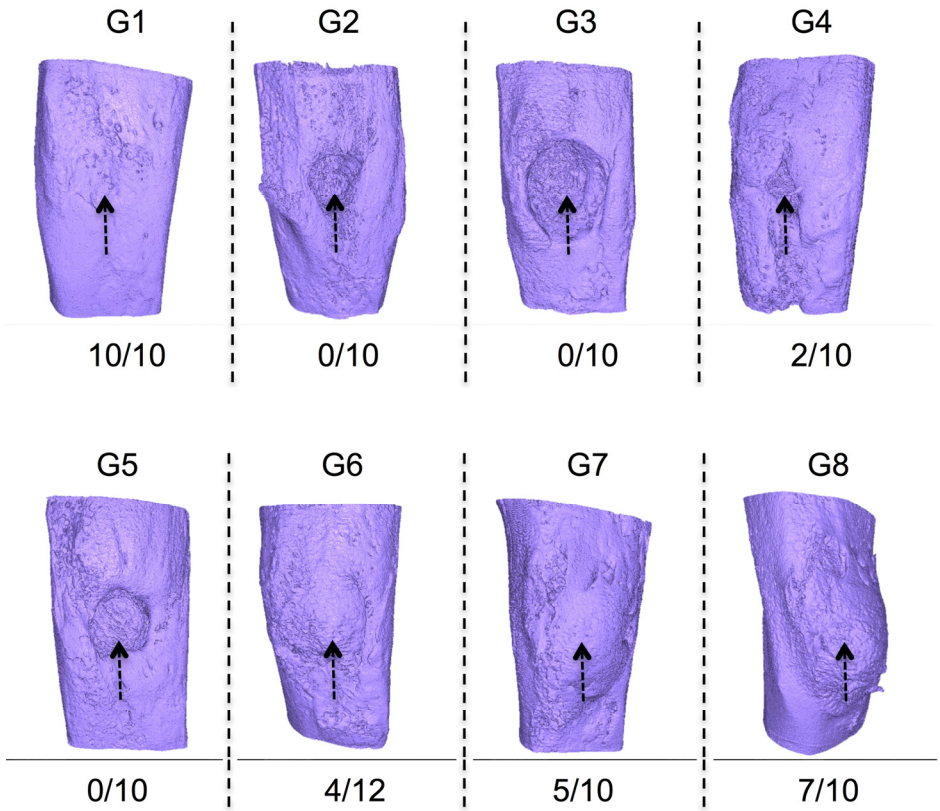


Fig. 13 3-D micro-CT reconstructions of the tibia samples from study 5.
Top panel shows samples from G1-G4 (i.e without the membrane). The bottom panel shows specimens from G5-G8 (i.e with the membrane). The numbers below each image indicate the total cortices healed in each group with respect to the sample size.

6. Connection between the studies

All studies described in this thesis form a continuous pattern. Lessons learnt from preceding studies were implemented in the following studies.

- In study 1, we established that a clinically approved CaS/HA biomaterial can also be used as a tool for delivery of rhBMP-2 and ZA. This rose the question of whether this delivery system could be used to heal clinically relevant bone defects in small animal models of bone regeneration?
- In study 2, it was observed that the CaS/HA biomaterial and its several combinations can heal cancellous bone defects but cortical bone healing was somewhat delayed. This led to two important questions; 1. Can the biomaterial augment biomechanical properties of bone in a challenging defect model? and 2. Could an alternate approach be taken to improvise cortical healing?
- In study 3, the ability of CaS/HA biomaterial with or without bioactive molecules was tested to augment biomechanical properties of osteoporotic femoral neck, a challenging anatomical location.
- In study 4, a novel gelatin-CaS/HA biomaterial was developed with the aim to have a trabecular bone mimicking structure, which could also provide controlled release of bone active substances. Evaluating its performance in a clinically relevant bone defect was the next step.
- Study 5 was built on study 2. Cancellous bone regeneration was achieved using the material developed in study 4 by delivering bone active molecules locally in the defect. The problem of delayed cortical healing from study 2 was overcome by using a thin collagen membrane as an endosteal cover releasing small doses of rhBMP-2.

7. Discussion

7.1 rhBMP-2 and ZA: The present

Owing to a very strong osteoinductive effect caused by rhBMP-2 and a similar strong anti-resorptive effect of bisphosphonates, and our prior experience with these bioactive molecules, this thesis work emphasized the use of rhBMP-2 and ZA and the combination of these compounds.

rhBMP-2 has arguably been the most researched bioactive molecule for bone regeneration in the last decade. Consequently, its usage has been profoundly debated in the orthopedic research community. In 2011, Carragee and co-workers performed a critical review of the existing literature with respect to 13 original clinical trials carried out involving the use of rhBMP-2 in spinal surgery. The authors concluded that the studies exaggerated the morbidities involved with iliac crest bone graft and undermined the side effects such as, local inflammation, osteolysis, urogenital troubles, retrograde ejaculation, ectopic ossification and poorer global outcomes [104, 112]. Later, in 2013, the same group expressed concerns of a higher risk of cancer with the use of rhBMP-2 [113]. Their findings were however considered to be inconclusive by Simmonds et al., who performed a meta-analysis of 11 out of 17 trials sponsored by the manufacturer, Medtronic [114]. Their analysis also concluded that usage of rhBMP-2 increased spinal fusion rates and reduced pain. Another recently published study in 2018 performed an analysis of a larger set of patients (from commercially available insurance database) treated with rhBMP-2, and concluded that rhBMP-2 treatment does not increase the risk of subsequent cancers [115]. Despite the backlash regarding the usage of rhBMP-2, it is important to carefully analyze the problem and maybe even propose a solution. rhBMP-2 is delivered in supraphysiological doses, sometimes at doses several times higher than physiological BMP-2 present during bone remodeling, using mg doses exogenously compared to the ng doses present in bone [85, 116]. The second reason of potential importance is the inability of the collagen carrier to provide a controlled release of the protein at the implantation site. Early experimental studies have indicated that nearly 90% of rhBMP-2 is released from the ACS scaffolds within the first two weeks of implantation [103]. Whether this is a good result or not could still be debated. While we and a few others speculate that controlled and spatio-temporal release of rhBMP-2 characterized by a small burst release early on followed by a controlled release during the entire duration of fracture healing is

important [105, 117], others have recently shown that a burst release of rhBMP-2 regenerates a significantly higher amount of bone compared to slow releasing materials [118]. This forms the first part of this thesis; 1) *To develop and characterize biomaterials for delivery of rhBMP-2 and 2) To reduce rhBMP-2 doses* so they can be attractive for the clinical use again. Hopefully, better results can be achieved when an efficient carrier material is provided.

Regarding bisphosphonate delivery, a systemic approach has widely been used, especially in improving bone quality in osteoporosis. Only a few studies, from us and our Australian collaborators have tried to combine BMPs and bisphosphonates, in a bid to create a net positive balance of anabolic and anti-catabolic events, to successfully modulate fracture healing. As mentioned earlier, BMP usage induces excessive and premature bone resorption and a single systemic infusion of ZA can stop the catabolic effects of BMP and give rise to stronger fracture callus [106]. The timing of a systemic infusion is critical. In experimental rat studies, a single injection 1-2 weeks post fracture has shown most optimal results compared to an injection at the time of fracture [119]. While co-administration of BMP and ZA is indeed beneficial for accelerating fracture repair and maintenance of fracture callus properties, systemic administration of ZA has also been questioned lately. Drawbacks like myalgia, gastro intestinal irritation, osteonecrosis of the jaw and atypical fractures have been reported with prolonged use [108]. Furthermore, since ZA seeks bone mineral at all active remodeling sites, it is safe to presume that these molecules also reduce the overall osteoclast assisted bone remodeling. This made us to evaluate if, similar to rhBMP-2, *local co-delivery of rhBMP-2 together with ZA* could provide equally beneficial results with an aim to *circumvent the side effects associated with systemic ZA delivery*. This forms the second part of this thesis.

7.2 Are CaS/HA and GCH biomaterials good carriers for rhBMP-2?

An injectable CaS/HA biomaterial delivering rhBMP-2 locally provides a minimally invasive platform for delivery of the osteoinductive rhBMP-2 molecule. Injectability is a highly desirable property of a biomaterial for closed fractures not requiring mechanical stability. It must be noted that we were unable to use the CaS/HA biomaterial as an injectable carrier within the scope of this work mainly due to the technical difficulty associated with injecting the CaS/HA biomaterial in relatively small defects in rats. *In-vitro* the CaS/HA biomaterial released 90% of rhBMP-2 within the first week. A lower release of 55% rhBMP-2 was found *in-vivo* after four weeks from the CaS/HA biomaterial (unpublished findings). This could possibly be due to a quicker in-vitro dissolution of calcium sulphate dihydrate in the buffer as compared to the in-vivo situation, wherein the pellet does not come in

contact with similar volumes of body fluids due to protection from hematoma at least during the first few days.

The GCH material was designed with an aim to act as a carrier and a bone graft substitute in invasive surgical procedures. Injectable ceramic materials like CaS/HA do not have inherent macro porosity and the porosity only increases with time due to dissolution of the CaS phase [109]. Moreover, these materials in our experience also tend to lose their shape over time, become soft and acquire irregular shapes. A pre-set material, which can preserve its shape would be much more efficient, for instance, in spinal fusion, thereby reducing the risk of heterotrophic ossification. Gelatin at a specific isoelectric point range is known to electrostatically interact with rhBMP-2 [120, 121]. Apart from electrostatic interactions, chemical interactions based on the 3-D conformation of the protein have also been suggested [120]. HA can also to some extent show affinity for rhBMP-2 [122]. The amino group, the carboxyl group and the hydroxyl group in BMP-2 enable the BMP-2 interaction with HA, which depends on the orientation of the protein. The addition of CaS can provide a burst release of unreacted rhBMP-2, which can be beneficial for early cell chemotaxis and osteogenic differentiation. CaS also provides a calcium ion rich microenvironment, which has been associated with increased hydroxyapatite precipitation and bone formation [123]. With this hypothesis, the GCH scaffold was tested as a carrier for rhBMP-2. It was rather surprising that a very low release of rhBMP-2 from the GCH scaffold in-vitro (~3%) over a period of 4-weeks was observed [105]. Superior release kinetics of rhBMP-2 from the scaffold was seen in-vivo with a burst release early on, followed by a sustained release of the protein over a period of 4-weeks. Nearly 35 % of rhBMP-2 was still left in the scaffold at the end of the experiment. This has two implications. Firstly, the developed GCH carrier provided a better spatio-temporal delivery of rhBMP-2 compared to the FDA approved Medtronic® ACS biomaterial, which releases almost all rhBMP-2 in 2-weeks [103]. Secondly, it might be possible to reduce the rhBMP-2 doses further, since a significant fraction of rhBMP-2 is left behind in the scaffold at 4-weeks.

Both materials managed to induce bone formation when rhBMP-2 was delivered locally. However in both cases, delivery of rhBMP- 2 alone, did not lead to maximum bone formation and in study 4 we verified that rhBMP-2 usage alone causes excessive osteoclastogenesis. Study 4 also described another important finding wherein a head-to-head comparison of the existing ACS material with the developed GCH was performed. Micro-CT results indicated significantly higher volume of bone regenerated by the GCH+rhBMP-2 (10 µg) compared to the ACS+rhBMP-2 (10 µg). Whether these differences exist due to the difference in release of rhBMP-2 from the two scaffolds or due to the differences in structural and physicochemical properties remain unknow and can be pursued later on.

7.3 Are CaS/HA and GCH biomaterials a good carrier for ZA?

ZA has a strong affinity for hydroxyapatite, due to the hydroxyl groups attached at R1 location and the flanking phosphate groups [87-89]. In a recent unpublished study, we were able to demonstrate the uptake of ^{14}C-ZA in a pellet of CaS/HA biomaterial implanted in the abdominal muscle pouch confirming that *ZA seeks HA* irrespective of whether HA is part of a living bone matrix or not. Thus, addition of ZA to the CaS/HA biomaterial and the GCH material both containing HA was no surprise. CaS/HA material contains 40% by weight HA making it an effective injectable carrier for ZA. In-vitro release experiments conducted to demonstrate the release of ZA from the CaS/HA biomaterial have shown very low release of ZA (~10%) whereas, in-vivo data (unpublished) in the abdominal muscle pouch showed 22% ^{14}C-ZA being released from the CaS/HA material over a period of 4-weeks. These results are promising and hold the hypothesis that ZA strongly interacts with the CaS/HA material. What percentage binds to the individual components (CaS and HA) still remains unknown. The porous GCH material also contains HA. In-vitro, the material released approximately 13% ^{14}C-ZA in 4-weeks with a burst release on day 1 followed by a sustained release over the course of the experiment. Compared to the CaS/HA material, the GCH material had a higher burst release of ZA in-vivo (approximately 40%) following which no more ZA release was seen. This may be due to the GCH material containing less HA by weight % when compared to the CaS/HA material. Furthermore, macro porosity of the material may also have played a role. In summary, the two materials are efficient carriers of ZA since it is desirable to have local availability of ZA during early fracture healing so that the BMP mediated bone resorption can be controlled. It must however be noted that long term data on ZA availability in the materials are unavailable. If ZA stays in the materials for a very long time, it might affect the long-term remodeling of the fracture callus, which might be a cause for concern. Further experiments will be necessary to elucidate long term effects of local bisphosphonate usage and such experiments will probably also increase our understanding of what kind of carriers are required for efficient local delivery of ZA in order to prevent premature bone resorption but not long-term bone remodeling.

7.4 Role of local ZA delivery?

This thesis originally aimed at delivering ZA locally to exploit the cytotoxic effects of ZA on osteoclasts to hinder premature bone resorption caused by the use of only rhBMP-2, but also to circumvent side effects associated with systemic ZA usage [108]. Both studies 1 and 4 demonstrate that when the materials are combined with both ZA and rhBMP-2, the volume of new bone formation in the muscle pouch model is significantly higher than when rhBMP-2 is delivered alone. These studies thus indicate that local ZA delivery is feasible in preventing bone resorption due to rhBMP-2 usage. Moving from studies 1 through 3, it was also established that it is important to evaluate if co-delivery of rhBMP-2 with ZA may also allow reducing the rhBMP-2 doses. Study 4 reveals that combined delivery of rhBMP-2 and ZA from the GCH not only produces significantly higher volume of bone but also leads to reduction of the rhBMP-2 doses by 4-times whilst still achieving similar results to a full dose rhBMP-2 delivered via the GCH scaffold or ACS scaffold. This can have important clinical implications. As mentioned earlier, supraphysiological BMP-2 doses are one of the reasons for the skepticism in the clinical use of BMP-2. Our experiments show that there is a hope in reinstating BMPs into clinical practice.

ZA alone did not induce bone in the muscle pouch model (unpublished data, experiments carried out with both biomaterials) thus it is quite safe to say that *ZA is not osteoinductive*. A completely unexpected finding, was the significant amounts of regenerated cancellous bone when only ZA was delivered using the CaS/HA biomaterial in studies 2 and 3 and GCH biomaterial in study 5. It is possible to argue that the pseudo-anabolic and osteoinductive role of ZA alone reduces the rate of resorption of a radio dense CaS/HA biomaterial. This in-turn could lead to an increase in MV/TV or MV as measured by micro-CT, since quantifications are based on density and differentiation of material from living bone is impossible making the interpretation of results more complex. This could then falsely be interpreted as an anabolic effect. Even though representative histology supports the micro-CT findings, no histological quantifications were made. However, in study 5, we used a gelatin-CaS-HA biomaterial, which is a pre-set material with 8% of HA by weight as opposed to the CaS/HA biomaterial, which contained 40% HA by weight. This means that the GCH biomaterial would interfere less with the micro-CT quantifications compared to the pure ceramic CaS/HA material. Even in study 5, a significantly higher amount of bone regeneration was seen with local delivery of ZA only via the GCH material, especially around the defect periphery (ROI 3). *This confirms that delivery of ZA alone leads to significant cancellous bone regeneration indicating a possible anabolic effect of local ZA delivery*. Some studies have indicated a possible true anabolic role of ZA even inducing differentiation of mesenchymal stem cells (MSCs) into osteoblast like cells, but clear molecular

mechanisms underlying this phenomenon are missing [124]. MSCs are often isolated from the medullary canal in both femur and tibia in rodents, which means that the defect created in studies 2 and 5 is surrounded by a rich MSC source [125]. The results from study 2 and 5 to some extent corroborate well with published literature showing an osteogenic impact of ZA on MSCs to induce metaphyseal bone regeneration [124]. We have however not been able to show if the same applies to cortical bone. Cortical healing is often guided by periosteal stem cells and muscle derived stem cells and no studies showing an osteogenic role of ZA on these cells exist [126, 127]. Thus, it is currently not possible to contemplate on an *anabolic role of ZA on cortical bone healing, for instance, in an open fracture model or a critical diaphyseal defect model*. Moreover, this anabolism of ZA on trabecular bone seen in these studies might be dosage related and all studies have been carried using a fixed dosage of ZA (10 µg). It is therefore difficult to speculate regarding the results with lower doses of ZA. High ZA doses given systemically have a so-called ceiling effect and after a specific concentration does not add any significant pro-bone effect, but rather renders the bone brittle [128]. Based on these findings, we speculate that there exists a narrow window when it comes to local ZA doses and too high local doses might have detrimental effects on bone properties both from a qualitative perspective and overall patient safety perspective. Belfrage and co-workers have confirmed this to some extent in an earlier study [129]. Before clinical translation of these results, it is therefore important to identify right doses for local ZA treatment.

7.5 Lessons learnt from using CaS/HA and GCH biomaterials within animal models

CaS/HA biomaterial is a microporous biomaterial, which undergoes fast remodeling both in-vitro and in-vivo. Most of this remodeling is a consequence of calcium sulphate dihydrate crystals solubilizing in buffers and body fluids [130]. This increases the porosity of the material over time (dynamic remodeling) thereby increasing the osteoconductivity of the material [109]. It is likely that due to this phenomenon, histological bone formation was observed even in the middle of the material in study 1. Furthermore, the CaS phase has been shown to resorb in less than two months in-vivo, indicating that the scaffold can degrade up to 60% in two months [130]. HA, which is a natural component of bone, integrates with the new bone and unintegrated HA particles take years to break down.

GCH biomaterial on the other hand is a pre-set and highly crosslinked biomaterial. It does not remodel significantly (although the CaS phase does resorb) over time and in-vitro results have indicated that the GCH biomaterial only degrades slowly and 50% of the scaffold remains after 6-weeks. GCH scaffold also released

more rhBMP-2 and ZA in-vivo compared to the CaS/HA material. In terms of bone formation, the majority of the bone formation we observed using the GCH scaffold was found around the periphery of the scaffold, with some bone tissue also been seen within the pores of the material.

In terms of the animal models, both CaS/HA and GCH biomaterials failed to show osteoinductivity in the rat ectopic muscle pouch model. Due to unknown reasons, they fail to induce ectopic bone in the materials, and require biological signaling from BMPs to induce ectopic bone formation. However, it was learnt that the muscle pouch model is an extremely efficient model to study carrier properties of materials because whatever bone is present after 4-weeks is new bone formation. Furthermore, it is technically less challenging, is reproducible, economical and incurs limited animal suffering.

A significant increase in metaphyseal defects due to tumors, infections and fractures make the tibia defect model an increasingly interesting and clinically relevant model to study bone regeneration. The tibia defect model in rats is a promising model to study bone regeneration in the metaphyseal region. Moreover, it separates cancellous bone healing from cortical bone healing and thus it is possible to study both aspects of defect healing. However, being mechanically somewhat stable, the results cannot be extrapolated to completely stable or unstable fractures. In both studies 2 and 5, it was expected that the defects would be critical and would not heal without treatment. Study 2 demonstrated complete healing of cortices in the empty and allograft group, which made us increase the defect dimensions in study 5 (ϕ 3 mm vs. 4.5 mm). This however did not make a difference and cortices in the empty group healed again. On the contrary, cortical healing in groups treated with CaS/HA or GCH biomaterials, with or without bone active molecules was impaired. It was speculated that the biomaterials protrude out into the cortical defect and block cortical healing. This hypothesis was to some extent confirmed from study 5, wherein a thin collagen membrane with rhBMP-2 covering the GCH scaffold endosteally (preventing it from protruding into the cortical bone) improved the cortical healing rate by 70%. Cancellous bone healing in the empty and the allograft groups in study 2 and in the empty group in study 5 was negligible, indicating a role of scaffolding for cancellous bone regeneration. When CaS/HA and GCH scaffolds with or without bioactive molecules were used for cancellous bone regeneration, significant improvement in cancellous bone formation was seen.

Finally, the last animal model consisting of a femoral neck defect in osteoporotic rats is a novel model to study the process of bone regeneration with the aim of preventing osteoporotic fractures of the hip. Decreasing compliance to osteoporosis prevention treatment programs among patients and a steep increase in osteoporosis related socio-economic burden (morbidity and money) make this strategy innovative [131, 132]. The study was performed as a feasibility analysis of using the combination of CaS/HA biomaterial functionalized with ZA or rhBMP-2+ZA in the clinical studies wherein patients with high risk of osteoporotic fractures could

be injected with such materials to prevent hip fractures. The results from the model were promising and a good agreement between studies 2, 3 and 5 was seen. Bone formation observed via micro-CT and histology did not however translate into increased biomechanical strength. This could be due to the fact that the model focuses on regenerating cancellous bone in the femoral neck canal (since we drilled only the cancellous bone), which has limited contribution to the mechanical strength of the femoral neck under the tested loading condition [133].

7.6 Future work

Recreation of large diaphyseal defects is truly challenging in orthopedic surgery. A relatively recent technique to treat diaphyseal bone defects is to use the Masquelet technique, which involves a two-stage procedure to regenerate missing bone [134, 135]. The first stage involves removing the damaged bone and replacing it with a cement spacer (PMMA), followed by mechanical stabilization using an intramedullary nail and locking pins. A few weeks later, a second stage is performed during which the thin vascularized membrane formed over the spacer is cut open, the cement spacer is replaced with autograft bone and the membrane is stitched together again, and the bone is left to heal. The method is very promising but two stages are required. In the immediate future, an idea would be to utilize the tools developed in this thesis in an animal model of the Masquelet method followed by clinical trials for treatment of large bone defects using a one stage Masquelet model. If successful, this method has advantages both in terms of patient morbidity and hospital related expenses. In the future, pursuing in-situ autograft formation using the combination of the developed biomaterials and bioactive molecules would also be an interesting continuation of this work. The bone graft would be made from the patient's own cells and hence pose no risk of disease transmission or rejection, which will then be transplanted at the desired site. Concerning local bisphosphonate delivery, there is currently no evidence of data existing in relation to the duration of bisphosphonate availability in the said biomaterials. It is believed that an optimal carrier should contain bisphosphonates only during the bone formation phase and most of it should clear out by the time bone remodeling begins in order to achieve physiological bone remodeling. Experiments on bisphosphonate availability, long-term release and re-uptake from the biomaterials and surrounding bone must be carried out in the future. Experiments comparing local delivery of bisphosphonates with latest anti-catabolic molecules like Denosumab to evaluate the efficacy of these molecules head-to-head will also be pursued. The approach of regenerating bone in the femoral neck of osteoporotic rats (study 3) was promising and based on this study, a feasibility study, which involved injecting the CaS/HA material into the femoral neck canal of patients receiving a total hip was performed. Initial

injectability results looked promising and a clinical trial related to the use of the CaS/HA biomaterial with bioactive molecules to prevent fractures of the femoral neck is due to commence within the coming year.

8. Conclusions

- The three biomaterials (microporous CaS-HA, macroporous GCH and CM) described in this thesis are efficient carriers of rhBMP-2 and ZA. Ectopic muscle pouch experiments indicate that the materials are not osteoinductive on their own. Co-delivery of rhBMP-2 and ZA regenerates significantly higher volume of bone than delivering rhBMP-2 alone at an ectopic, non-osseous location.
- In orthotopic osseous defects (tibial metaphysis and femoral neck canal), local delivery of ZA alone induces significant bone formation compared to the controls indicating not only an anti-osteoclastic effect but also an *anabolic* effect on bone formation. This needs to be further confirmed by verifying the underlying molecular mechanisms.
- From the bone defect model and the drug doses used, it was evident that the addition of rhBMP-2 together with ZA does not show any additive effect of rhBMP-2, indicating that it might be sufficient to use local ZA alone.
- From a dose reduction perspective, co-delivery of rhBMP-2 and ZA together can reduce the effective rhBMP-2 doses by at least four times.
- By developing efficient carrier biomaterials, it is possible to achieve a prolonged release of rhBMP-2 and ZA in-vivo, which is an improvement over the existing approved collagen sponge. The developed GCH material also proved to be better than the existing collagen sponge in terms of rhBMP-2 delivery and bone formation in a muscle pouch model.
- An efficient way of treating metaphyseal bone defects is to use two distinct functionalized biomaterials, one aiding in cancellous bone regeneration and the other guiding cortical bone regeneration.

9. Sammanfattning på svenska

Behovet att kunna återskapa benvävnad ökar. Stora bendefekter kan uppstå efter kirurgisk behandling av ben/ledinfektion/tumör eller vid benskörhetsfraktur. Oftast används olika typer av bentransplantat och antingen används patientens egen benvävnad (autograft) eller donators (allograft). Autograft har varit 'the gold standard' och inducerar benbildning även i vävnad som inte är i direkt anslutning till levande benvävnad. Autograft har dock begränsningar och oftast finns det inte tillräckligt att skörda för att fylla större defekter samt en risk för infektion och/eller smärta från tagstället. Allograft har en betydligt sämre benbildningsförmåga och det finns således ett behov av att utveckla alternativa metoder som kan ersätta allo och autograft.

Biomaterial i sig saknar en inneboende förmåga att själv bilda större mängder benvävnad. Man kombinerar därför ofta biomaterial med celler och tillväxtfaktorer. I denna avhandling har vi använt rekombinant humant *Bone Morphogenic Protein-2* (rhBMP-2) som beninducerande (osteoinduktiv) faktor i ett biomaterial. Biomaterialet används som en bärare av rhBMP-2 med en *lokal frisättning* av proteinet parallellt med materialets nedbrytning in situ.

I dag är en absorberbar kollagen-svamp (ACS) Medtronic® den enda produkten godkänd som bärare av rhBMP-2 av Food and Drug Administration. Svampen bryts ner snabbt vilket orsakar en tidig och hög frisättning av rhBMP-2 i suprafysiologiska doser. En hög dos stimulerar också de bennedbrytande cellerna (osteoklasterna) vilket minskar styrkan i den nybildade benvävnaden. Bisfosfonater är ett läkemedel som används i kliniken för behandling av benskörhet. Om vi kombinerar benmaterialet med en bisfosfonat kan vi även blockera den BMP-inducerade osteoklastaktiviteten.

Det övergripande målet med denna avhandling är att studera och optimera benersättningsmaterial. Vi vill kunna sänka dosen av rhBMP-2 men bibehålla samma eller bättre effekt vad gäller bennybildning, såväl i spongiös som kortikal benbildning. Vår hypotes är att en effektivare bärare som bryts ned och frisätter proteinet under längre tid än nuvarande material kan bidra till en längre och mer kontrollerad frisättning av rhBMP-2. Genom att tillsätta en bisfosfonat kan vi även kontrollera den BMP-inducerade resorptionen.

I studie 1, använde vi ett kliniskt godkänt mikroporöst calciumsulfat (CaS)/hydroxyapatit (HA) som bärare av både rhBMP-2 och ZA i en bukmuskelmodell i råtta. Som förväntat, bildades inget ben när materialet användes

ensamt i bukmuskel men vi kunde visa att materialet var en bra bärare av rhBMP-2 och ZA, och att lokal frisättning av dessa båda tillsammans regenererade signifikant större mängder ben jämfört med enbart rhBMP-2. Vi kunde visa att lokalt administrerad ZA förhindrade rhBMP-2-inducerad prematur resorption av nybildad callus.

I studie 2, utvecklade vi en modell med metafysär bendefekt hos råtta. En kombination av CaS/HA biomaterial plus ZA och/eller rhBMP-2 användes för att fylla defekten. Efter 8 veckor hade gruppen som behandlats med CaS/HA plus ZA, samt kombinationen av ZA och rhBMP-2 bildat betydligt mer benmassa jämfört med tom eller allo-transplanterad kontroll. Detta är första gången man kunnat visa att enbart lokalt administrerat ZA förbättrade benbildningen i spongiös bendefekt utan tillsats av beninduktivt protein. Vi fann det även överraskande att kortikal bennybildning var fördröjd trots betydande regeneration av spongiöst ben.

I studie 3, utvecklade vi en djurmodell för att studera bennybildning i lårbenshalsen hos osteoporotisk råtta. CaS/HA användes som bärare av ben-aktiva molekyler. CaS/HA tillsammans med ZA och ZA+rhBMP-2 visade återigen större volym regenererat ben jämfört med gruppen tom lårbenshals eller CaS/HA. Vi kunde visa att behandlingssidan i ZA och ZA+rhBMP-2 grupperna hade ökad mängd ben i det skadade benet jämfört med den obehandlade kontralaterala sidan. Lokal behandling med ZA hade däremot ingen effekt på den kontralaterala sidan vilket bekräftar att lokal administrering av ZA är just specifikt lokal.

I studie 4, skapade vi ett helt nytt makroporöst biomaterial bestående av gelatin-CaS-HA till vilket adderades rhBMP-2 och ZA. Vi mätte frisättningen in-vivo av rhBMP-2 över en 4-veckorsperiod. Vi kunde notera en tidig topp för ZA varefter frisättningen snabbt avtog. En jämförande studie gjordes mellan det nya biomaterialet och den FDA-godkända kollagen-svamp som finns för kommersiellt bruk. Med samma mängd rhBMP-2 i de respektive materialen bildades en signifikant större volym nytt ben med det nya materialet i bukmuskelmodellen. Kombinationen av en bättre bärare av rhBMP-2 och en resorptionshämmande ZA gjorde att dosen rhBMP-2 kunde minskas till en fjärdedel med bibehållen effekt.

Studie 5 var en uppföljning av studie 2 som visade att kortikal benläkning fördröjdes i metafysärt ben när det spongiösa tomrummet fylldes med biomaterial. I studie 5 ville vi därför undersöka regeneration av kortikalt respektive spongiöst ben och använde två olika biomaterial. Det porösa biomaterialet av gelatin-CaS-HA (GCH) användes för utfyllnad av håligheten i det spongiösa benet, och ett tunt kollagenmembran (CM) adderades på utsidan för kortikalläkning. Genom att koppla GCH biomaterialet med ZA eller ZA+rhBMP-2 kunde vi se om det skedde ökad benbildning i den spongiösa håligheten. Som förväntat kunde vi se att kortikal benläkning uteblev med enbart biomaterialet men att de grupper som hade CM med rhBMP-2 visade bättre kortikal benläkning. Vi kunde således visa att det är möjligt att förfina styrningen av benläkningen och adaptera biomaterial, operationstekniker och läkemedel så att spongiös och kortikal benläkning kan optimeras separat.

Med denna avhandling vill vi skapa en teoretisk bas för biomaterial som bärare av benaktiva molekyler vid benläkning. Vi tror att dessa tekniker i framtiden kan ersätta dagens traditionella transplantationstekniker.

10. Summary in English

Bone defects are on the rise with increasing incidence of high-energy trauma, infections, tumors, congenital disorders and impaired bone quality with the aging population. During surgery, some defects require replacement of the lost or eradicated bone, and transplantation with autografts (taken from the same patient) and allografts (donor bone) have been the surgeon's preferred options. Autografts till date remain as the 'gold standard', but limitations including graft site complications, shortage of available volume among the old and very young people and risk of infection at the harvest site makes it impractical and difficult to use autografts in every scenario. Although being osteoinductive, allografts do not provide satisfactory results and there is a risk of disease transmission. To overcome the shortcomings of bone grafts, significant research has been done over the past decades on bone substitutes, and new biomaterials with the potential to replace conventional bone grafting methods.

Biomaterials *alone* lack the ability to induce significant amounts of bone in large defects and thus have often been combined with cells, growth factors and drugs forming the basis for bone tissue engineering approach. This thesis focused on using recombinant human bone morphogenic protein-2 (rhBMP-2) as an osteoinductive factor. The BMP-2 was locally delivered via the developed biomaterials to enhance their bone forming potential. Local delivery of rhBMP-2 is necessary due to its short half-life in-vivo. The only Food and Drug Administration (FDA) approved device for local delivery of rhBMP-2 today is the absorbable collagen sponge (ACS) produced by Medtronic®. BMP-2 delivered via the ACS has not shown consistent convincing outcome and the reasons are believed to be; 1) A rapid degradation and a burst release of the protein, 2) The use of supraphysiological doses and 3) BMPs are strong inducers of secondary osteoclastogenesis which causes premature bone resorption.

We hypothesized that the fabrication of efficient carriers, which can provide a spatio-temporal release of rhBMP-2, could overcome the existing problems with burst release. Furthermore, to overcome the pro-osteoclast effect of rhBMP-2 and problems associated with high BMP doses clinically, we hypothesized that local delivery of zoledronic acid (ZA), a bisphosphonate, would assist in silencing the osteoclast activity, reducing premature bone resorption. ZA in bone is primarily used as a treatment towards osteoporosis, since it blocks excessive osteoclast formation and preserves bone mass. The combination of testing BMPs and ZA

together is not new and has been studied for over a decade. The common approach has been to use BMPs locally and ZA systemically. In the recent years, reports have indicated that systemic delivery of ZA can lead to osteonecrosis of the jaw, reduced bone remodeling and an increased risk of atypical cortical fractures with prolonged use.

Thus, this thesis aimed at creating biomaterials to; 1) Efficiently deliver rhBMP-2, 2) Efficiently deliver ZA locally and evaluate its role on bone formation and simultaneously circumvent the side effects associated with systemic ZA delivery, 3) Reduce the rhBMP-2 doses by using efficient carriers and suppressing enhanced osteoclast activity by ZA, 4) Compare the developed materials with existing approved materials, and 5) Translate the results from the more basic, proof of concept studies into clinically relevant orthopedic problems.

In study 1, a clinically approved calcium sulphate (CaS)/hydroxyapatite (HA) microporous biomaterial was used for co-delivery of rhBMP-2 and ZA in an ectopic muscle pouch model in rats. We were able to show that the material was a good carrier for these molecules and that local co-delivery of rhBMP-2 and ZA regenerated significantly higher amount of bone compared to when rhBMP-2 is delivered alone. The results from this study also indicated that *local* delivery of *ZA* combined with rhBMP-2 works as expected and prevents BMP-2 induced premature resorption. No bone formation was seen when the material was used alone in a rat model of ectopic bone formation. This indicates that the biomaterial is not osteoinductive, at least not in this model.

In study 2, a model of a metaphyseal bone defect in rats was used and the combination of CaS/HA biomaterial together with ZA and/or rhBMP-2 was used for bone void management. At the end of 8-weeks, the groups treated with CaS/HA biomaterial with ZA and the combination of ZA and rhBMP-2 regenerated significantly higher bone when compared to the control groups left empty or filled with allograft bone. We were for the first time able to show that only ZA delivered locally in a cancellous bone environment can lead to improved bone formation. Although significant cancellous bone regeneration was seen, surprisingly problems were encountered with cortical bone regeneration in all CaS/HA treated groups.

In study 3, a novel animal model was developed to study bone regeneration in the femoral neck canal of osteoporotic rats. The aim of the study was to treat a surgically created defect in the femoral neck canal with CaS/HA biomaterial, with or without bone active molecules to reinforce bone formation and mechanical strength. The addition of CaS/HA with ZA and ZA+rhBMP-2 again regenerated significantly higher volume of bone when compared to the empty defect or the CaS/HA group. It was also shown that in the treatment groups, an increased volume of bone in the defect leg was found compared to the untreated contralateral sides. Local treatment of ZA did not have any effect on the contralateral legs emphasizing the effect of local ZA delivery. Surprisingly, the new bone formed in the bone active molecule treated groups did not translate into an increase in the mechanical strength.

In study 4, a novel macroporous gelatin-CaS-HA biomaterial was fabricated and characterized for local delivery of rhBMP-2 and ZA. In-vivo release kinetics of both rhBMP-2 and ZA over a four-week period indicated that the developed biomaterial provided a spatio-temporal release of rhBMP-2. A burst release of ZA was seen early on, followed by a period during which no more ZA was released. The developed biomaterial was compared head to head with the FDA approved collagen sponge. Despite delivering equal amounts of rhBMP-2 via both tested materials, the developed porous material generated significantly higher bone volume than the collagen sponge in an ectopic muscle pouch model. Another important finding of the study was that the co-delivery of rhBMP-2 and ZA could aid in reducing the effective rhBMP-2 doses by four times.

Study 5 was a follow up of study 2 during which cortical healing was impaired when the cancellous cavity in a metaphyseal bone defect was filled with the CaS/HA biomaterial. In study 5, we aimed to test the efficacy of the porous material developed in study 4 in cancellous bone regeneration and use a collagen membrane (CM) to guide cortical regeneration. The membrane restricted the cancellous filler within the cavity and also acted as a template for periosteal cells and muscle stem cells to augment cortical healing. Functionalization of the GCH biomaterial with ZA and ZA+rhBMP-2 led to significant bone formation in the cancellous cavity. Nonetheless, as expected, cortical healing was impaired even when rhBMP-2 was used in the cancellous defect. Treatment groups with a CM functionalized with rhBMP-2 significantly increased cortical healing. Thus, this approach to guide cancellous and cortical bone separately using two biomaterials was an improvement over the approach employed in study 2.

Overall, this thesis provides an approach to regenerate bone by using biomaterials as carriers for bone active molecules, which in future can replace traditional bone grafting methods.

References

[1] A.L. Boskey, Bone composition: relationship to bone fragility and antiosteoporotic drug effects, Bonekey reports 2 (2013).

[2] A.L. Boskey, Mineralization of bones and teeth, Elements 3(6) (2007) 385-391.

[3] M.F. Young, Bone matrix proteins: their function, regulation, and relationship to osteoporosis, Osteoporosis international 14(3) (2003) 35-42.

[4] L.J. Deftos, Clinical essentials of calcium and skeletal disorders, Professional communications1998.

[5] M.J. Glimcher, Bone: Nature of the calcium phosphate crystals and cellular, structural, and physical chemical mechanisms in their formation, Reviews in mineralogy and geochemistry 64(1) (2006) 223-282.

[6] M.J. Turunen, J.D. Kaspersen, U. Olsson, M. Guizar-Sicairos, M. Bech, F. Schaff, M. Tägil, J.S. Jurvelin, H. Isaksson, Bone mineral crystal size and organization vary across mature rat bone cortex, Journal of structural biology 195(3) (2016) 337-344.

[7] Y. Wang, T. Azaïs, M. Robin, A. Vallée, C. Catania, P. Legriel, G. Pehau-Arnaudet, F. Babonneau, M.-M. Giraud-Guille, N. Nassif, The predominant role of collagen in the nucleation, growth, structure and orientation of bone apatite, Nature materials 11 (2012) 724-733.

[8] W.J. Landis, K.J. Hodgens, M.J. Song, J. Arena, S. Kiyonaga, M. Marko, C. Owen, B.F. McEwen, Mineralization of collagen may occur on fibril surfaces: evidence from conventional and high-voltage electron microscopy and three-dimensional imaging, Journal of structural biology 117(1) (1996) 24-35.

[9] N. Reznikov, M. Bilton, L. Lari, M.M. Stevens, R. Kröger, Fractal-like hierarchical organization of bone begins at the nanoscale, Science 360(p.eeao2189) (2018).

[10] O. Faour, R. Dimitriou, C.A. Cousins, P.V. Giannoudis, The use of bone graft substitutes in large cancellous voids: Any specific needs?, Injury 42 (2011) S87-S90.

[11] A.S. Greenwald, S.D. Boden, V.M. Goldberg, Y. Khan, C.T. Laurencin, R.N. Rosier, Bone-graft substitutes: facts, fictions, and applications, The Journal of bone and joint surgery. American volume 83-A Suppl 2 Pt 2 (2001) 98-103.

[12] E.D. Arrington, W.J. Smith, H.G. Chambers, A.L. Bucknell, N.A. Davino, Complications of iliac crest bone graft harvesting, Clinical orthopaedics and related research (329) (1996) 300-309.

[13] L. Wei, M.J. Richard, B. Shi, Y. Zhang, Osteoinductive and osteopromotive variability among different demineralized bone allografts, Clinical implant dentistry and related research 17(3) (2015) 533-542.

[14] Z. Schwartz, A. Somers, J.T. Mellonig, D.L. Carnes, D.D. Dean, D.L. Cochran, B.D. Boyan, Ability of commercial demineralized freeze-dried bone allograft to induce new bone formation is dependent on donor age but not gender, Journal of periodontology 69(4) (1998) 470-478.

[15] H. Zhou, S.S. Lu, D.W. Dempster, Bone remodeling: cellular activities in bone, Osteoporosis in men (Second Edition), Elsevier2010, pp. 15-24.

[16] L.F. Bonewald, M.L. Johnson, Osteocytes, mechanosensing and Wnt signaling, Bone 42(4) (2008) 606-615.

[17] F. Arai, T. Miyamoto, O. Ohneda, T. Inada, T. Sudo, K. Brasel, T. Miyata, D.M. Anderson, T. Suda, Commitment and differentiation of osteoclast precursor cells by the sequential expression of C-Fms and receptor activator of nuclear factor κb (Rank) receptors, The journal of experimental medicine 190(12) (1999) 1741-1754.

[18] N. Udagawa, N. Takahashi, T. Akatsu, H. Tanaka, T. Sasaki, T. Nishihara, T. Koga, T.J. Martin, T. Suda, Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells, Proceedings of the national academy of sciences 87(18) (1990) 7260-7264.

[19] P. Collin-Osdoby, X. Yu, H. Zheng, P. Osdoby, RANKL-mediated osteoclast formation from murine RAW 264.7 cells, in: M.H. Helfrich, S.H. Ralston (Eds.), Bone research protocols, Humana Press, Totowa, NJ, 2003, pp. 153-166.

[20] K.P. McHugh, K. Hodivala-Dilke, M.-H. Zheng, N. Namba, J. Lam, D. Novack, X. Feng, F.P. Ross, R.O. Hynes, S.L. Teitelbaum, Mice lacking β3 integrins are osteosclerotic because of dysfunctional osteoclasts, The journal of clinical investigation 105(4) (2000) 433-440.

[21] F.P. Ross, S.L. Teitelbaum, αvβ3 and macrophage colony-stimulating factor: partners in osteoclast biology, Immunological reviews 208(1) (2005) 88-105.

[22] M. Gowen, F. Lazner, R. Dodds, R. Kapadia, J. Feild, M. Tavaria, I. Bertoncello, F. Drake, S. Zavarselk, I. Tellis, Cathepsin K knockout mice develop osteopetrosis due to a deficit in matrix degradation but not demineralization, Journal of bone and mineral research 14(10) (1999) 1654-1663.

[23] S.L. Teitelbaum, Bone resorption by osteoclasts, Science 289(5484) (2000) 1504-1508.

[24] D.J. Hadjidakis, I.I. Androulakis, Bone Remodeling, Annals of the New York academy of sciences 1092(1) (2006) 385-396.

[25] H. Hsu, D.L. Lacey, C.R. Dunstan, I. Solovyev, A. Colombero, E. Timms, H.-L. Tan, G. Elliott, M.J. Kelley, I. Sarosi, Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand, Proceedings of the national academy of sciences 96(7) (1999) 3540-3545.

[26] R. Marsell, T.A. Einhorn, The biology of fracture healing, Injury 42(6) (2011) 551-555.

[27] T. Kitaori, H. Ito, E.M. Schwarz, R. Tsutsumi, H. Yoshitomi, S. Oishi, M. Nakano, N. Fujii, T. Nagasawa, T. Nakamura, Stromal cell–derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model, Arthritis & rheumatology 60(3) (2009) 813-823.

[28] T.A. Einhorn, L.C. Gerstenfeld, Fracture healing: mechanisms and interventions, Nature reviews rheumatology 11 (2014) 45-54.

[29] P.V. Giannoudis, T.A. Einhorn, D. Marsh, Fracture healing: The diamond concept, Injury 38 (2007) S3-S6.

[30] K.J.L. Burg, S. Porter, J.F. Kellam, Biomaterial developments for bone tissue engineering, Biomaterials 21(23) (2000) 2347-2359.

[31] M.M. Stevens, Biomaterials for bone tissue engineering, Materials today 11(5) (2008) 18-25.

[32] F.J. O'Brien, Biomaterials & scaffolds for tissue engineering, Materials today 14(3) (2011) 88-95.

[33] K.-H. Frosch, K.M. Stürmer, Metallic biomaterials in skeletal repair, European journal of trauma 32(2) (2006) 149-159.

[34] T. Albrektsson, C. Johansson, Osteoinduction, osteoconduction and osseointegration, European spine journal 10 Suppl 2 (2001) S96-101.

[35] A.M. Barradas, H. Yuan, C. van Blitterswijk, P. Habibovic, Osteoinductive biomaterials: current knowledge of properties, experimental models and biological mechanisms, European cells & materials 21 (2010) 407-429.

[36] R.E. Holmes, Bone regeneration within a coralline hydroxyapatite implant, Plastic and reconstructive surgery 63(5) (1979) 626-633.

[37] A.I. Itälä, H.O. Ylänen, C. Ekholm, K.H. Karlsson, H.T. Aro, Pore diameter of more than 100 μm is not requisite for bone ingrowth in rabbits, Journal of biomedical materials research 58(6) (2001) 679-683.

[38] M.J. Dalby, N. Gadegaard, R. Tare, A. Andar, M.O. Riehle, P. Herzyk, C.D.W. Wilkinson, R.O.C. Oreffo, The control of human mesenchymal cell differentiation using nanoscale symmetry and disorder, Nature materials 6 (2007) 997-1003.

[39] E. Nyberg, C. Holmes, T. Witham, W.L. Grayson, Growth factor-eluting technologies for bone tissue engineering, Drug delivery and translational research 6(2) (2016) 184-94.

[40] A. Nakajima, N. Shimoji, K. Shiomi, S. Shimizu, H. Moriya, T.A. Einhorn, M. Yamazaki, Mechanisms for the enhancement of fracture healing in rats treated with intermittent low-dose human parathyroid hormone (1-34), Journal of bone and mineral research 17(11) (2002) 2038-47.

[41] M. Ellegaard, N.R. Jørgensen, P. Schwarz, Parathyroid hormone and bone healing, Calcified tissue international 87(1) (2010) 1-13.

[42] P. Aspenberg, H.K. Genant, T. Johansson, A.J. Nino, K. See, K. Krohn, P.A. Garcia-Hernandez, C.P. Recknor, T.A. Einhorn, G.P. Dalsky, B.H. Mitlak, A. Fierlinger, M.C. Lakshmanan, Teriparatide for acceleration of fracture repair in humans: a prospective, randomized, double-blind study of 102 postmenopausal women with distal radial fractures, Journal of bone and mineral research 25(2) (2010) 404-14.

[43] P. Aspenberg, T. Johansson, Teriparatide improves early callus formation in distal radial fractures, Acta orthopaedica 81(2) (2010) 234-6.

[44] H. Ledin, L. Good, T. Johansson, P. Aspenberg, No effect of teriparatide on migration in total knee replacement, Acta orthopaedica 88(3) (2017) 259-262.

[45] K. Hu, B.R. Olsen, Osteoblast-derived VEGF regulates osteoblast differentiation and bone formation during bone repair, The journal of clinical investigation 126(2) (2016) 509-526.

[46] K. Hu, B.R. Olsen, The roles of vascular endothelial growth factor in bone repair and regeneration, Bone 91 (2016) 30-38.

[47] E. Quinlan, A. Lopez-Noriega, E.M. Thompson, A. Hibbitts, S.A. Cryan, F.J. O'Brien, Controlled release of vascular endothelial growth factor from spray-dried alginate microparticles in collagen-hydroxyapatite scaffolds for promoting vascularization and bone repair, Journal of tissue engineering and regenerative medicine 11(4) (2017) 1097-1109.

[48] D. Kaigler, Z. Wang, K. Horger, D.J. Mooney, P.H. Krebsbach, VEGF scaffolds enhance angiogenesis and bone regeneration in irradiated osseous defects, Journal of bone and mineral research 21(5) (2006) 735-44.

[49] J. Street, M. Bao, L. deGuzman, S. Bunting, F.V. Peale, N. Ferrara, H. Steinmetz, J. Hoeffel, J.L. Cleland, A. Daugherty, N. van Bruggen, H.P. Redmond, R.A.D. Carano, E.H. Filvaroff, Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover, Proceedings of the national academy of sciences 99(15) (2002) 9656-9661.

[50] A.I. Caplan, D. Correa, PDGF in bone formation and regeneration: new insights into a novel mechanism involving MSCs, Journal of orthopaedic research 29(12) (2011) 1795-803.

[51] H. Gerhardt, C. Betsholtz, Endothelial-pericyte interactions in angiogenesis, Cell and tissue research 314(1) (2003) 15-23.

[52] J.O. Hollinger, C.E. Hart, S.N. Hirsch, S. Lynch, G.E. Friedlaender, Recombinant human platelet-derived growth factor: biology and clinical applications, The Journal of bone and joint surgery. American volume 90 Suppl 1 (2008) 48-54.

[53] S.E. Lynch, R.C. Williams, A.M. Polson, T.H. Howell, M.S. Reddy, U.E. Zappa, H.N. Antoniades, A combination of platelet-derived and insulin-like growth factors enhances periodontal regeneration, Journal of clinical periodontology 16(8) (1989) 545-8.

[54] P. Bordei, Locally applied platelet-derived growth factor accelerates fracture healing, The Journal of bone and joint surgery. British volume 93(12) (2011) 1653-9.

[55] J.O. Hollinger, A.O. Onikepe, J. MacKrell, T. Einhorn, G. Bradica, S. Lynch, C.E. Hart, Accelerated fracture healing in the geriatric, osteoporotic rat with recombinant human platelet-derived growth factor-bb and an injectable beta-tricalcium phosphate/collagen matrix, Journal of orthopaedic research 26(1) (2008) 83-90.

[56] P. Charoenlarp, A.K. Rajendran, S. Iseki, Role of fibroblast growth factors in bone regeneration, Inflammation and regeneration 37 (2017) 10.

[57] A. Montero, Y. Okada, M. Tomita, M. Ito, H. Tsurukami, T. Nakamura, T. Doetschman, J.D. Coffin, M.M. Hurley, Disruption of the fibroblast growth factor-2 gene results in decreased bone mass and bone formation, The journal of clinical investigation 105(8) (2000) 1085-1093.

[58] H. Mayahara, T. Ito, H. Nagai, H. Miyajima, R. Tsukuda, S. Taketomi, J. Mizoguchi, K. Kato, In vivo stimulation of endosteal bone formation by basic fibroblast growth factor in rats, Growth factors (Chur, Switzerland) 9(1) (1993) 73-80.

[59] Y. Fei, L. Xiao, T. Doetschman, D.J. Coffin, M.M. Hurley, Fibroblast growth factor 2 stimulation of osteoblast differentiation and bone formation is mediated by modulation of the Wnt signaling pathway, The Journal of biological chemistry 286(47) (2011) 40575-83.

[60] H. Kawaguchi, K. Nakamura, Y. Tabata, Y. Ikada, I. Aoyama, J. Anzai, T. Nakamura, Y. Hiyama, M. Tamura, Acceleration of fracture healing in nonhuman primates by fibroblast growth factor-2, The Journal of clinical endocrinology and metabolism 86(2) (2001) 875-80.

[61] D.A. Oortgiesen, X.F. Walboomers, A.L. Bronckers, G.J. Meijer, J.A. Jansen, Periodontal regeneration using an injectable bone cement combined with BMP-2 or FGF-2, Journal of tissue engineering and regenerative medicine 8(3) (2014) 202-9.

[62] K.S. Hong, E.C. Kim, S.H. Bang, C.H. Chung, Y.I. Lee, J.K. Hyun, H.H. Lee, J.H. Jang, T.I. Kim, H.W. Kim, Bone regeneration by bioactive hybrid membrane containing FGF2 within rat calvarium, Journal of biomedical materials research. Part A 94(4) (2010) 1187-94.

[63] H. Kawaguchi, S. Jingushi, T. Izumi, M. Fukunaga, T. Matsushita, T. Nakamura, K. Mizuno, T. Nakamura, K. Nakamura, Local application of recombinant human fibroblast growth factor-2 on bone repair: a dose-escalation prospective trial on patients with osteotomy, Journal of orthopaedic research 25(4) (2007) 480-7.

[64] P. Aspenberg, L.S. Lohmander, Fibroblast growth factor stimulates bone formation. Bone induction studied in rats, Acta orthopaedica Scandinavica 60(4) (1989) 473-6.

[65] L. Xian, X. Wu, L. Pang, M. Lou, C.J. Rosen, T. Qiu, J. Crane, F. Frassica, L. Zhang, J.P. Rodriguez, J. Xiaofeng, Y. Shoshana, X. Shouhong, E. Argiris, W. Mei, C. Xu, Matrix IGF-1 maintains bone mass by activation of mTOR in mesenchymal stem cells, Nature medicine 18(7) (2012) 1095-101.

[66] M.M. Shull, I. Ormsby, A.B. Kier, S. Pawlowski, R.J. Diebold, M. Yin, R. Allen, C. Sidman, G. Proetzel, D. Calvin, N. Annunziata, T. Doetschman, Targeted disruption of the mouse transforming growth factor-β1 gene results in multifocal inflammatory disease, Nature 359 (1992) 693.

[67] G. Chen, C. Deng, Y.-P. Li, TGF-β and BMP signaling in osteoblast differentiation and bone formation, International journal of biological sciences 8(2) (2012) 272-288.

[68] D.G. Winkler, M.K. Sutherland, J.C. Geoghegan, C. Yu, T. Hayes, J.E. Skonier, D. Shpektor, M. Jonas, B.R. Kovacevich, K. Staehling-Hampton, M. Appleby, M.E. Brunkow, J.A. Latham, Osteocyte control of bone formation via sclerostin, a novel BMP antagonist, The EMBO journal 22(23) (2003) 6267-76.

[69] X. Li, Y. Zhang, H. Kang, W. Liu, P. Liu, J. Zhang, S.E. Harris, D. Wu, Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling, The Journal of biological chemistry 280(20) (2005) 19883-7.

[70] X. Li, M.S. Ominsky, Q.T. Niu, N. Sun, B. Daugherty, D. D'Agostin, C. Kurahara, Y. Gao, J. Cao, J. Gong, F. Asuncion, M. Barrero, K. Warmington, D. Dwyer, M. Stolina, S. Morony, I. Sarosi, P.J. Kostenuik, D.L. Lacey, W.S. Simonet, H.Z. Ke, C.

Paszty, Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength, Journal of bone and mineral research 23(6) (2008) 860-9.

[71] H.Z. Ke, W.G. Richards, X. Li, M.S. Ominsky, Sclerostin and Dickkopf-1 as therapeutic targets in bone diseases, Endocrine reviews 33(5) (2012) 747-783.

[72] P. Desmond, J. Graham, S. Brian, F. Liang, P. Edward, Single-dose, placebo-controlled, randomized study of AMG 785, a sclerostin monoclonal antibody, Journal of bone and mineral research 26(1) (2011) 19-26.

[73] M.R. McClung, A. Grauer, S. Boonen, M.A. Bolognese, J.P. Brown, A. Diez-Perez, B.L. Langdahl, J.Y. Reginster, J.R. Zanchetta, S.M. Wasserman, L. Katz, J. Maddox, Y.C. Yang, C. Libanati, H.G. Bone, Romosozumab in postmenopausal women with low bone mineral density, New England journal of medicine 370(5) (2014) 412-20.

[74] F. Cosman, D.B. Crittenden, J.D. Adachi, N. Binkley, E. Czerwinski, S. Ferrari, L.C. Hofbauer, E. Lau, E.M. Lewiecki, A. Miyauchi, C.A. Zerbini, C.E. Milmont, L. Chen, J. Maddox, P.D. Meisner, C. Libanati, A. Grauer, Romosozumab Treatment in Postmenopausal Women with Osteoporosis, New England journal of medicine 375(16) (2016) 1532-1543.

[75] M.R. Urist, Bone: formation by autoinduction, Science 150(3698) (1965) 893-9.

[76] S.N. Lissenberg-Thunnissen, D.J.J. de Gorter, C.F.M. Sier, I.B. Schipper, Use and efficacy of bone morphogenetic proteins in fracture healing, International orthopaedics 35(9) (2011) 1271-1280.

[77] A. Yamaguchi, T. Ishizuya, N. Kintou, Y. Wada, T. Katagiri, J.M. Wozney, V. Rosen, S. Yoshiki, Effects of BMP-2, BMP-4, and BMP-6 on osteoblastic differentiation of bone marrow-derived stromal cell lines, ST2 and MC3T3-G2/PA6, Biochemical and biophysical research communications 220(2) (1996) 366-71.

[78] E.-C. Kim, T.-H. Kim, J.-H. Jung, S.O. Hong, D.-W. Lee, Enhanced osteogenic differentiation of MC3T3-E1 on rhBMP-2-immobilized titanium via click reaction, Carbohydrate polymers 103 (2014) 170-178.

[79] H. Kaneko, T. Arakawa, H. Mano, T. Kaneda, A. Ogasawara, M. Nakagawa, Y. Toyama, Y. Yabe, M. Kumegawa, Y. Hakeda, Direct stimulation of osteoclastic bone resorption by bone morphogenetic protein (BMP)-2 and expression of BMP receptors in mature osteoclasts, Bone 27(4) (2000) 479-486.

[80] E. Canalis, A.N. Economides, E. Gazzerro, Bone morphogenetic proteins, their antagonists, and the skeleton, Endocrine reviews 24(2) (2003) 218-235.

[81] N. Kamiya, T. Kobayashi, Y. Mochida, P.B. Yu, M. Yamauchi, H.M. Kronenberg, Y. Mishina, Wnt inhibitors Dkk1 and Sost are downstream targets of BMP signaling through the type IA receptor (BMPRIA) in osteoblasts, Journal of bone and mineral research 25(2) (2010) 200-10.

[82] H. Zhang, A. Bradley, Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development, Development (Cambridge, England) 122(10) (1996) 2977-86.

[83] K. Tsuji, A. Bandyopadhyay, B.D. Harfe, K. Cox, S. Kakar, L. Gerstenfeld, T. Einhorn, C.J. Tabin, V. Rosen, BMP2 activity, although dispensable for bone

formation, is required for the initiation of fracture healing, Nature genetics 38 (2006) 1424.

[84] K. Tsuji, K. Cox, L. Gamer, D. Graf, A. Economides, V. Rosen, Conditional deletion of BMP7 from the limb skeleton does not affect bone formation or fracture repair, Journal of orthopaedic research 28(3) (2010) 384-389.

[85] I. El Bialy, W. Jiskoot, M. Reza Nejadnik, Formulation, delivery and stability of bone morphogenetic proteins for effective bone regeneration, Pharmaceutical research 34(6) (2017) 1152-1170.

[86] F. Wegman, A. Bijenhof, L. Schuijff, F.C. Oner, W.J. Dhert, J. Alblas, Osteogenic differentiation as a result of BMP-2 plasmid DNA based gene therapy in vitro and in vivo, European cells and materials 21 (2011) 230-42.

[87] Z.J. Henneman, G.H. Nancollas, F.H. Ebetino, R.G.G. Russell, R.J. Phipps, Bisphosphonate binding affinity as assessed by inhibition of carbonated apatite dissolution in vitro, Journal of biomedical materials research. Part A 85(4) (2008) 993-1000.

[88] M.T. Drake, B.L. Clarke, S. Khosla, Bisphosphonates: mechanism of action and role in clinical practice, Mayo Clinic proceedings. Mayo Clinic 83(9) (2008) 1032-1045.

[89] A. Jung, S. Bisaz, H. Fleisch, The binding of pyrophosphate and two diphosphonates by hydroxyapatite crystals, Calcified tissue research 11(4) (1973) 269-280.

[90] R.G. Russell, Bisphosphonates: from bench to bedside, Annals of New York academy of science 1068 (2006) 367-401.

[91] A. Schindeler, D.G. Little, Osteoclasts but not osteoblasts are affected by a calcified surface treated with zoledronic acid in vitro, Biochemical and biophysical research communications 338(2) (2005) 710-6.

[92] L.I. Plotkin, R.S. Weinstein, A.M. Parfitt, P.K. Roberson, S.C. Manolagas, T. Bellido, Prevention of osteocyte and osteoblast apoptosis by bisphosphonates and calcitonin, The Journal of clinical investigation 104(10) (1999) 1363-1374.

[93] K.L. Kavanagh, K. Guo, J.E. Dunford, X. Wu, S. Knapp, F.H. Ebetino, M.J. Rogers, R.G. Russell, U. Oppermann, The molecular mechanism of nitrogen-containing bisphosphonates as antiosteoporosis drugs, Proceedings of the national academy of sciences 103(20) (2006) 7829-34.

[94] W.S. Simonet, D.L. Lacey, C.R. Dunstan, M. Kelley, M.S. Chang, R. Lüthy, H.Q. Nguyen, S. Wooden, L. Bennett, T. Boone, G. Shimamoto, M. DeRose, R. Elliott, A. Colombero, H.L. Tan, G. Trail, J. Sullivan, E. Davy, N. Bucay, L. Renshaw-Gegg, T.M. Hughes, D. Hill, W. Pattison, P. Campbell, S. Sander, G. Van, J. Tarpley, P. Derby, R. Lee, W.J. Boyle, Osteoprotegerin: A novel secreted protein involved in the regulation of bone density, Cell 89(2) (1997) 309-319.

[95] N. Bucay, I. Sarosi, C.R. Dunstan, S. Morony, J. Tarpley, C. Capparelli, S. Scully, H.L. Tan, W. Xu, D.L. Lacey, W.J. Boyle, W.S. Simonet, Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification, Genes & development 12(9) (1998) 1260-1268.

[96] P.J. Bekker, D. Holloway, A. Nakanishi, M. Arrighi, P.T. Leese, C.R. Dunstan, The effect of a single dose of osteoprotegerin in postmenopausal women, Journal of bone and mineral research 16(2) (2001) 348-60.

[97] D.L. Lacey, W.J. Boyle, W.S. Simonet, P.J. Kostenuik, W.C. Dougall, J.K. Sullivan, J.S. Martin, R. Dansey, Bench to bedside: elucidation of the OPG–RANK–RANKL pathway and the development of denosumab, Nature reviews drug discovery 11 (2012) 401.

[98] L.C. Gerstenfeld, D.J. Sacks, M. Pelis, Z.D. Mason, D.T. Graves, M. Barrero, M.S. Ominsky, P.J. Kostenuik, E.F. Morgan, T.A. Einhorn, Comparison of effects of the bisphosphonate alendronate versus the RANKL inhibitor denosumab on murine fracture healing, Journal of bone and mineral research 24(2) (2009) 196-208.

[99] E. Tsourdi, B. Langdahl, M. Cohen-Solal, B. Aubry-Rozier, E.F. Eriksen, N. Guanabens, B. Obermayer-Pietsch, S.H. Ralston, R. Eastell, M.C. Zillikens, Discontinuation of denosumab therapy for osteoporosis: A systematic review and position statement by ECTS, Bone 105 (2017) 11-17.

[100] W.F. McKay, S.M. Peckham, J.M. Badura, A comprehensive clinical review of recombinant human bone morphogenetic protein-2 (INFUSE(®) Bone Graft), International orthopaedics 31(6) (2007) 729-734.

[101] D.M. Black, P.D. Delmas, R. Eastell, I.R. Reid, S. Boonen, J.A. Cauley, F. Cosman, P. Lakatos, P.C. Leung, Z. Man, C. Mautalen, P. Mesenbrink, H. Hu, J. Caminis, K. Tong, T. Rosario-Jansen, J. Krasnow, T.F. Hue, D. Sellmeyer, E.F. Eriksen, S.R. Cummings, Once-yearly zoledronic acid for treatment of postmenopausal osteoporosis, New England journal of medicine 356(18) (2007) 1809-1822.

[102] H.M. Weiss, U. Pfaar, A. Schweitzer, H. Wiegand, A. Skerjanec, H. Schran, Biodistribution and plasma protein binding of zoledronic acid, Drug metabolism and disposition 36(10) (2008) 2043-2049.

[103] H. Uludag, D. D'Augusta, R. Palmer, G. Timony, J. Wozney, Characterization of rhBMP-2 pharmacokinetics implanted with biomaterial carriers in the rat ectopic model, Journal of biomedical materials research 46(2) (1999) 193-202.

[104] A.W. James, G. LaChaud, J. Shen, G. Asatrian, V. Nguyen, X. Zhang, K. Ting, C. Soo, A review of the clinical side effects of bone morphogenetic protein-2, Tissue engineering. Part B, Reviews 22(4) (2016) 284-297.

[105] D.B. Raina, D. Larsson, F. Mrkonjic, H. Isaksson, A. Kumar, L. Lidgren, M. Tägil, Gelatin- hydroxyapatite- calcium sulphate based biomaterial for long term sustained delivery of bone morphogenic protein-2 and zoledronic acid for increased bone formation: In-vitro and in-vivo carrier properties, Journal of controlled release 272 (2018) 83-96.

[106] D.G. Little, M. McDonald, R. Bransford, C.B. Godfrey, N. Amanat, Manipulation of the anabolic and catabolic responses with OP-1 and zoledronic acid in a rat critical defect model, Journal of bone and mineral research 20(11) (2005) 2044-52.

[107] N. Mathavan, P. Bosemark, H. Isaksson, M. Tägil, Investigating the synergistic efficacy of BMP-7 and zoledronate on bone allografts using an open rat osteotomy model, Bone 56(2) (2013) 440-8.

[108] L. Dalle Carbonare, M. Zanatta, A. Gasparetto, M.T. Valenti, Safety and tolerability of zoledronic acid and other bisphosphonates in osteoporosis management, Drug, healthcare and patient safety 2 (2010) 121-37.

[109] D.B. Raina, H. Isaksson, W. Hettwer, A. Kumar, L. Lidgren, M. Tägil, A biphasic calcium sulphate/hydroxyapatite carrier containing bone morphogenic protein-2 and zoledronic acid generates bone, Scientific reports 6 (2016) 26033.

[110] P.F. Horstmann, D.B. Raina, H. Isaksson, W. Hettwer, L. Lidgren, M.M. Petersen, M. Tägil, Composite biomaterial as a carrier for bone-active substances for metaphyseal tibial bone defect reconstruction in rats, Tissue Engineering Part A 23(23-24) (2017) 1403-1412.

[111] A. Širka, D.B. Raina, H. Isaksson, K.E. Tanner, A. Smailys, A. Kumar, Š. Tarasevičius, M. Tägil, L. Lidgren, Calcium sulphate/hydroxyapatite carrier for bone formation in the femoral neck of osteoporotic rats, Tissue engineering Part A (2018) doi:10.1089/ten.tea.2018.0075.

[112] E.J. Carragee, E.L. Hurwitz, B.K. Weiner, A critical review of recombinant human bone morphogenetic protein-2 trials in spinal surgery: emerging safety concerns and lessons learned, Spine journal 11(6) (2011) 471-91.

[113] E.J. Carragee, G. Chu, R. Rohatgi, E.L. Hurwitz, B.K. Weiner, S.T. Yoon, G. Comer, B. Kopjar, Cancer risk after use of recombinant bone morphogenetic protein-2 for spinal arthrodesis, The Journal of bone and joint surgery. American volume 95(17) (2013) 1537-45.

[114] M.C. Simmonds, J.V. Brown, M.K. Heirs, J.P. Higgins, R.J. Mannion, M.A. Rodgers, L.A. Stewart, Safety and effectiveness of recombinant human bone morphogenetic protein-2 for spinal fusion: a meta-analysis of individual-participant data, Annals of internal medicine 158(12) (2013) 877-89.

[115] G.S. Cooper, T.D. Kou, Risk of cancer following lumbar fusion surgery with recombinant human bone morphogenic protein-2 (rhBMP-2): An analysis using a commercially insured patient population, International Journal of spine surgery (2018) doi:10.14444/50323.

[116] S.C. Gamradt, J.R. Lieberman, Genetic modification of stem cells to enhance bone repair, Annals of biomedical engineering 32(1) (2004) 136-147.

[117] M. Rodríguez-Évora, A. Delgado, R. Reyes, A. Hernández-Daranas, I. Soriano, J. San Román, C. Évora, Osteogenic effect of local, long versus short term BMP-2 delivery from a novel SPU–PLGA–βTCP concentric system in a critical size defect in rats, European Journal of pharmaceutical sciences 49(5) (2013) 873-884.

[118] M.G.L. Olthof, D.H.R. Kempen, X. Liu, M. Dadsetan, M.A. Tryfonidou, M.J. Yaszemski, W.J.A. Dhert, L. Lu, Bone morphogenetic protein-2 release profile modulates bone formation in phosphorylated hydrogel, Journal of tissue engineering and regenerative medicine 12(6) (2018) 1339-1351.

[119] N. Amanat, M. McDonald, C. Godfrey, L. Bilston, D. Little, Optimal timing of a single dose of zoledronic acid to increase strength in rat fracture repair, Journal of bone and mineral research 22(6) (2007) 867-76.

[120] S. Young, M. Wong, Y. Tabata, A.G. Mikos, Gelatin as a delivery vehicle for the controlled release of bioactive molecules, Journal of controlled release 109(1) (2005) 256-274.

[121] M. Yamamoto, Y. Ikada, Y. Tabata, Controlled release of growth factors based on biodegradation of gelatin hydrogel, Journal of biomaterials science, polymer edition 12(1) (2001) 77-88.

[122] X. Dong, Q. Wang, T. Wu, H. Pan, Understanding adsorption-desorption dynamics of BMP-2 on hydroxyapatite (001) surface, Biophysical journal 93(3) (2007) 750-759.

[123] J.-W. Park, K.-B. Park, J.-Y. Suh, Effects of calcium ion incorporation on bone healing of Ti6Al4V alloy implants in rabbit tibiae, Biomaterials 28(22) (2007) 3306-3313.

[124] F. von Knoch, C. Jaquiery, M. Kowalsky, S. Schaeren, C. Alabre, I. Martin, H.E. Rubash, A.S. Shanbhag, Effects of bisphosphonates on proliferation and osteoblast differentiation of human bone marrow stromal cells, Biomaterials 26(34) (2005) 6941-9.

[125] M. Soleimani, S. Nadri, A protocol for isolation and culture of mesenchymal stem cells from mouse bone marrow, Nature protocols 4 (2009) 102.

[126] Y.Y. Yu, S. Lieu, C. Lu, C. Colnot, Bone morphogenetic protein 2 stimulates endochondral ossification by regulating periosteal cell fate during bone repair, Bone 47(1) (2010) 65-73.

[127] R. Liu, O. Birke, A. Morse, L. Peacock, K. Mikulec, D.G. Little, A. Schindeler, Myogenic progenitors contribute to open but not closed fracture repair, BMC musculoskeletal disorders 12 (2011) 288.

[128] S. Pozzi, S. Vallet, S. Mukherjee, D. Cirstea, N. Vaghela, L. Santo, E. Rosen, H. Ikeda, Y. Okawa, T. Kiziltepe, J. Schoonmaker, W. Xie, T. Hideshima, E. Weller, M.L. Bouxsein, N.C. Munshi, K.C. Anderson, N. Raje, High-dose zoledronic acid impacts bone remodeling with effects on osteoblastic lineage and bone mechanical properties, Clinical cancer research 15(18) (2009) 5829-39.

[129] O. Belfrage, H. Isaksson, M. Tägil, Local treatment of a bone graft by soaking in zoledronic acid inhibits bone resorption and bone formation. A bone chamber study in rats, BMC musculoskeletal disorders 13 (2012) 240-240.

[130] J.-S. Wang, M. Tägil, H. Isaksson, M. Boström, L. Lidgren, Tissue reaction and material biodegradation of a calcium sulfate/apatite biphasic bone substitute in rat muscle, Journal of orthopaedic translation 6 (2016) 10-17.

[131] J.A. Kanis, C. Cooper, R. Rizzoli, B. Abrahamsen, N.M. Al-Daghri, M.L. Brandi, J. Cannata-Andia, B. Cortet, H.P. Dimai, S. Ferrari, P. Hadji, N.C. Harvey, M. Kraenzlin, A. Kurth, E. McCloskey, S. Minisola, T. Thomas, J.Y. Reginster, Identification and management of patients at increased risk of osteoporotic fracture: outcomes of an ESCEO expert consensus meeting, Osteoporosis international 28(7) (2017) 2023-2034.

[132] E. Hernlund, A. Svedbom, M. Ivergard, J. Compston, C. Cooper, J. Stenmark, E.V. McCloskey, B. Jonsson, J.A. Kanis, Osteoporosis in the European Union: medical management, epidemiology and economic burden. A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA), Archives of osteoporosis 8 (2013) 136.

[133] G. Holzer, G. von Skrbensky, L.A. Holzer, W. Pichl, Hip fractures and the contribution of cortical versus trabecular bone to femoral neck strength, Journal of bone and mineral research 24(3) (2009) 468-74.

[134] P.V. Giannoudis, O. Faour, T. Goff, N. Kanakaris, R. Dimitriou, Masquelet technique for the treatment of bone defects: Tips-tricks and future directions, Injury 42(6) (2011) 591-598.

[135] P. Bosemark, C. Perdikouri, M. Pelkonen, H. Isaksson, M. Tagil, The masquelet induced membrane technique with BMP and a synthetic scaffold can heal a rat femoral critical size defect, Journal of orthopaedic research 33(4) (2015) 488-95.

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Biomaterials as carriers for bone active molecules

Deepak Raina is a doctoral student at the Department of Orthopedics, Lund University. An engineer by training with strong interests in medicine, who works with bone mimicking biomaterials that can locally release bone active molecules to accelerate and regenerate large volumes of bone.

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