UPPSALA UNIVERSITET

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 1287

Bone Regeneration with Cell-free Injectable Scaffolds

GRY HULSART BILLSTRÖM

ACTA UNIVERSITATIS UPSALIENSIS UPPSALA 2017

ISSN 1651-6206
ISBN 978-91-554-9786-6
urn:nbn:se:uu:diva-310312

Dissertation presented at Uppsala University to be publicly examined in Enghoffsalen, Akademiska sjukhuset, ingång 50, Uppsala, Friday, 24 February 2017 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish. Faculty examiner: Professor Olle Svensson (Umeå Universitet).

Abstract
Hulsart Billström, G. 2017. Bone Regeneration with Cell-free Injectable Scaffolds. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1287. 67 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-554-9786-6.

Bone is a remarkable multifunctional tissue with the ability to regenerate and remodel without generating any scar tissue. However, bone loss due to injury or diseases can be a great challenge and affect the patient significantly. Autologous bone grafting is commonly used throughout the world. Autograft both fills the void and is bone inductive, housing the particular cells that are needed for bone regeneration. However, a regenerative complement to autograft is of great interest as the use of biomaterials loaded with bioactive molecules can avoid donor site morbidity and the problem of a limited volume of material. Two such regenerative products that utilise bone morphogenetic protein (BMP)-7 and -2 have been used for more than a decade clinically. Unfortunately, several side effects have been reported, such as severe swelling due to inflammation and ectopic bone formation. Additionally, the products require open surgery and use of supra physiological doses of the BMPs due to poor localisation and retention of the growth factor. The purpose of this thesis was to harness the strong inductive capacity of the BMP-2 by optimising the carrier of this bioactive protein, thereby minimising the side effects that are associated with the clinical products and facilitating safe and localised bone regeneration. We focused on an injectable hyaluronan-based carrier developed through polymer chemistry at the University of Uppsala. The strategy was to use the body's own regenerative pathway to stimulate and enhance bone healing in a manner similar to the natural bone-healing process. The hyaluronan-based carrier has a similar composition to the natural extracellular matrix and is degraded by resident enzymes. Earlier studies have shown improved properties when adding hydroxyapatite, a calcium phosphate that constitutes the inorganic part of the bone matrix. In Paper I, the aim was to improve the carrier by adding other forms of calcium phosphate. The results indicated that bone formation was enhanced when using nano-sized hydroxyapatite. In Paper II, we discovered the importance of crushing the material, thus enhancing permeability and enlarging the surface area. We wished to further develop the carrier system, but were lacking an animal model with relatively high throughput, facilitated access, paired data, and we were also committed to the 3Rs of refinement, reduction, and replacement. To meet these challenges, we developed and refined an animal model, and this is described in Paper III. In Paper IV, we sought to further optimise the biomaterial properties of the hydrogel through covalent bonding of bisphosphonates to the hyaluronan hydrogel. This resulted in exceptional retention of the growth factor BMP-2. In Paper V, SPECT/PET/µCT was combined as a tri-modal imaging method to allow visualisation of the biomaterial's *in situ* action, in terms of drug retention, osteoblast activity and mineralisation. Finally, in Paper VI the correlation between existing *in vitro* results with *in vivo* outcomes was observed for an array of biomaterials. The study identified a surprisingly poor correlation between *in vitro* and *in vivo* assessment of biomaterials for osteogenesis.

Keywords: bone tissue engineering, hydrogel, computed tomography, positron emission tomography, large femoral bone defect, rat model, hydrogel, in vivo, osteogenesis, bone regeneration, 3R, single-photon emission computed tomography, bone morphogenetic protein 2, calcium phosphates, injectable, bisphosphonate

Gry Hulsart Billström, Department of Surgical Sciences, Orthopaedics, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden.

ISSN 1651-6206
ISBN 978-91-554-9786-6
urn:nbn:se:uu:diva-310312 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-310312)

Till Wictor och Viggo

Illustrations by Amanda Barrow

List of Papers

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

I Hulsart-Billström, G., Hu, Q., Bergman, K., Jonsson, K. B., Åberg, J., Tang, R., et al. (2011) Calcium phosphates compounds in conjunction with hydrogel as carrier for BMP-2: a study on ectopic bone formation in rats. *Acta Biomaterialia*, 7(8):3042–9

II Hulsart-Billström, G., Piskounova, S., Gedda, L., Andersson, B.-M., Bergman, K., Hilborn, J., et al. (2013). Morphological differences in BMP-2-induced ectopic bone between solid and crushed hyaluronan hydrogel templates. *Journal of Materials Science: Materials in Medicine,* 24(5):1201–9

III Hulsart-Billström, G., Bergman, K., Andersson, B., Hilborn, J., Larsson, S., & Jonsson, K. B. (2015) A uni-cortical femoral defect model in the rat: evaluation using injectable hyaluronan hydrogel as a carrier for bone morphogenetic protein-2. *Journal of Tissue Engineering and Regenerative Medicine*, 9(7):799-807

IV Hulsart-Billström, G., Yuen, P. K., Marsell, R., Hilborn, J., Larsson, S., & Ossipov, D. (2013). Bisphosphonate-Linked Hyaluronic Acid Hydrogel Sequesters and Enzymatically Releases Active Bone Morphogenetic Protein-2 for Induction of Osteogenic Differentiation. *Biomacromolecules,* 14(9):3055–63

V Hulsart-Billström, G*., Kumar Selvaraju, R*., Estrada, S., Lubberink, M., Asplund V., Bergman, K., Marsell, R., Larsson, S., Antoni, G. (2014) Non-invasive tri-modal visualisation of recombinant human bone morphogenetic protein-2 retention and associated bone regeneration: A proof of concept. *Submitted manuscript*

VI Hulsart-Billström, G*., Dawson, J. I*., Hofmann, S., Muller, R., Stoddart, M. J., Alini, M. Redl, H., El Haj, A., Brown, R., Salih, V., Hilborn, J., Larsson, S., Oreffo, R. O. (2016) A surprisingly poor correlation between *in vitro* and *in vivo* testing of biomaterials for bone regeneration: results of a multicentre analysis. *European Cells & Materials Journal*. 24;31:312-22.

*Authors contributed equally to this work and should be regarded as joint first authors

Contents

Abbreviations

ALP	Alkaline phosphatase
ANOVA	Analysis of variance
BMC	Bone mineral content
BMD	Bone mineral density
BMP	Bone morphogenetic protein
BMP-2	Bone morphogenetic protein 2
BMV	Bone mineral volume
BP	Bisphosphonate
CaP	Calcium phosphate
CPC	Calcium phosphate cement
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FOV	Field of view
HAA	Hyaluronan aldehyde
HAP	Hydroxyapatite
HA	Hyaluronan
IL-6	Interleukin 6
IL-8	Interleukin 8
K_i	Influx rate constant (plasma to tissue)
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cells
OCN	Osteocalcin
OPG	Osteoprotegerin
PBS	Phosphate buffered saline
PET	Positron emission tomography
pQCT	Peripheral quantitative computed tomography
PVAH	Poly(vinyl alcohol) hydrazide
ROI	Region of interest
RUNX2	Runt-related transcription factor 2
SPECT	Single photon emission computed tomography
SD	Standard deviation
VOI	Volume of interest
µCT	Microcomputed tomography

Introduction

Scope of the thesis

Bone is a remarkable multifunctional tissue with the ability to regenerate and remodel without generating scar tissue. Bone not only functions as a framework for muscles, and protection for vital organs, it is also responsible for the calcium homeostasis and erythropoiesis and has several endocrine functions. The exceptional ability to self-regenerate without scar tissue is mainly due to the presence of multi lineage cells. The osteoclast is a multi-nucleated cell that has the ability to degrade and resorb bone. It communicates with osteoblasts that follow the osteoclast and deposit new osteoid that subsequently becomes calcified. Osteocytes have been described as one of the major regulators of bone-cell activity, functioning as the sensory system of the bone with their interconnected canaliculi, transducing mechanical stimuli, and transporting substances to remote osteocytes embedded within the bone (fig. 1). Immediately following trauma, a complex healing process starts that is orchestrated by a multitude of cytokines from a large spectrum of cell origins (fig. 2).

In most cases, fracture stabilisation is sufficient to result in union. However, bone loss due to injury or disease can be challenging and result in significant patient morbidity. Transplantation of bone graft from one site in the patient to the site of fracture or bone void, i.e. autologous bone grafting is commonly used throughout the world. The transplanted bone not only fills voids, but is also bone inductive, housing the particular cells that are required for bone regeneration. These cells, which are resident within the graft, are able to migrate and commence bone formation. However, a regenerative alternative to autograft is of great interest and importance because the benefits from an off-the-shelf product with regenerative capacity equal to that of autograft will circumvent limitations associated with autograft use. These limitations include patient morbidity associated with harvest, such as infection and nerve damage, increased operative time, a great variety in the quality and healing capacity of the autograft, and the finite volume of graft available. Allograft is an alternative graft material, in which bone from tissue banks, is implanted. This method is widely used and has the benefits of osteoconduction, but the procedure is associated with a risk for transmission of disease, immune response and rejection, poor osteoinduction and poor mechanical strength (Giannoudis et al., 2005). With a tissue-engineering and regenera-

tive-medicine approach, the use of biomaterials loaded with bioactive molecules can avoid donor site morbidity and the problem of limited volume of material. Two such regenerative products that utilise bone morphogenetic protein (BMP)-7 and -2 have been used for more than a decade in the clinic. However, several side effects have been reported, such as severe swelling due to inflammation and ectopic bone formation. Additionally, the products require open surgery, use of supra physiological doses of the BMPs due to poor localisation and retention of the growth factor (McKay et al., 2007, Carragee et al., 2011).

The purpose of this thesis was to harness the strong inductive capacity of the BMP-2 by optimizing the carrier of this bioactive protein, thereby minimizing the side effects that are associated with the clinical products, and facilitating safe and localised bone regeneration at the desired site (Schmidt-Bleek et al., 2016). We focused on an injectable hyaluronan-based carrier developed through polymer chemistry at the University of Uppsala (Bergman et al., 2009). The strategy was to use the body's own regenerative pathway to stimulate, and enhance bone healing in a manner similar to the natural bone-healing process. The hyaluronan-based carrier has a similar composition to the natural extracellular matrix, and is degraded by resident hyaluronidase enzymes. Earlier studies have shown a more controlled release, and improved mechanical properties when adding a weight of 25 percentage of hydroxyapatite, a calcium phosphate that constitutes the inorganic part of the bone matrix. In **Paper I**, the aim was to improve the carrier by adding other forms of calcium phosphate. The results indicated that bone formation was enhanced when using nano-sized hydroxyapatite. In **Paper II**, we discovered the importance of crushing the material, thus enhancing permeability and enlarging the surface area. We wished to further develop the carrier system, but were lacking an animal model with relative high throughput and facilitated access. We also wanted to provide paired data, and were committed to the 3Rs of refinement, reduction and replacement. To meet these challenges, we developed and refined an animal model, and this is described in **Paper III**. **In Paper IV**, we sought to further optimise biomaterial properties of the hydrogel through covalently bonding of bisphosphonates to the hyaluronan hydrogel. The results demonstrated exceptional retention of the growth factor BMP-2. In **Paper V**, the *in vivo* response related to the release of the growth factor was examined by combining a SPECT/PET/μCT imaging method to visualise both the retention of the drug, and the *in vivo* response in terms of mineralisation. Finally, in **Paper VI** the correlation between published *in vitro* results with *in vivo* outcomes was observed for an array of biomaterials. The study identified a surprisingly poor correlation between *in vitro* and *in vivo* assessment of biomaterials for osteogenesis.

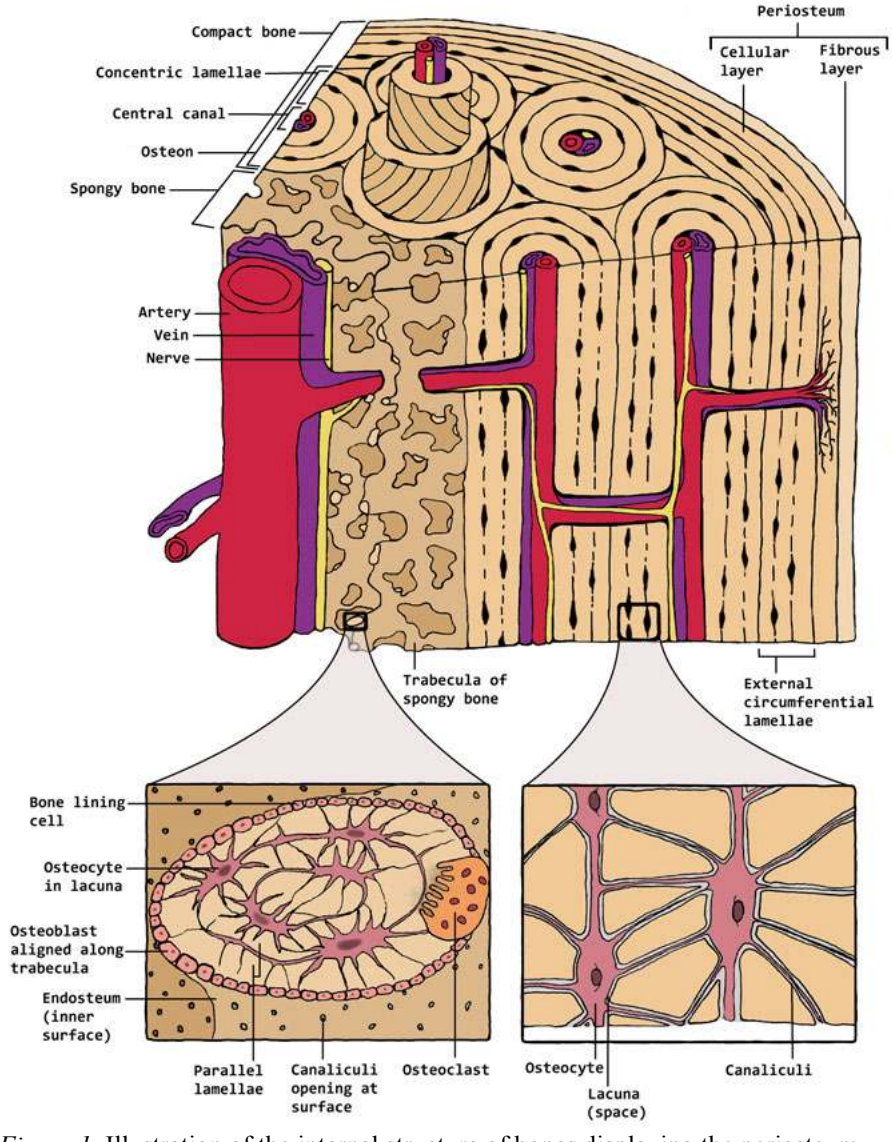

Figure 1. Illustration of the internal structure of bones displaying the periosteum, cortical, and trabecular bone. The cortical bone contains Haversian systems with osteocytes that are embedded within lacunae, and are connected to other osteocytes via canaliculi.

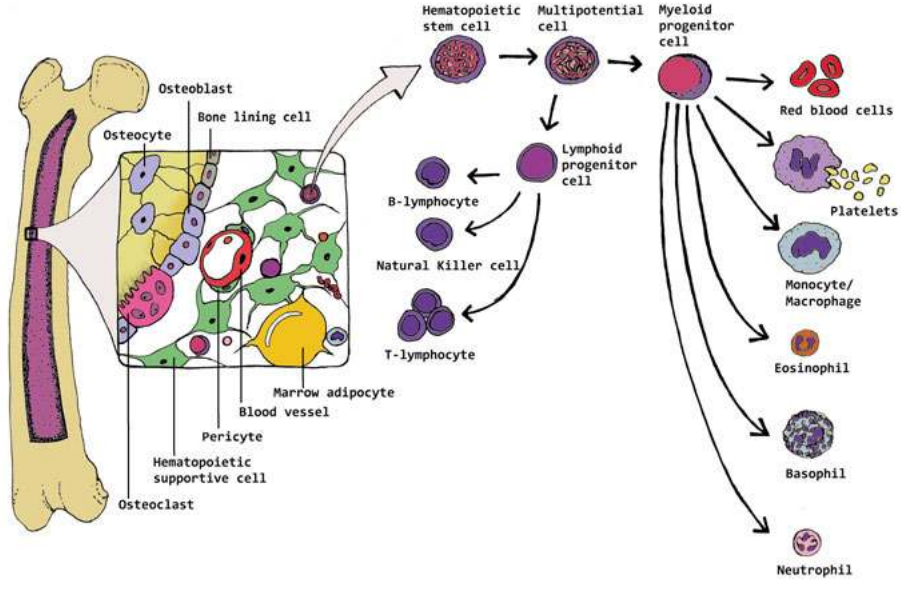

Figure 2. Illustration of the great diversity of cells that originate from bone.

Fracture healing

Bone tissue has the ability to regenerate itself, but only in the presence of at least five factors, these are: osteogenic cells, growth factors, osteoconductive scaffolds, vascularisation, and mechanical stimuli (Giannoudis et al., 2007). Recently, evidence of a sixth factor has emerged, the importance of immune cells as regulators of fracture healing (Loi et al., 2016). Osteogenic cells are cells that have the ability to differentiate into chondrocytes and osteoblasts. These cells can migrate from surrounding tissues such as muscle, bone marrow, and periosteum (Karp and Leng Teo, 2009). Chappuis et al. demonstrated that signalling of bone morphogenetic protein 2 (BMP-2) activates quiescent periosteal cells. When knocking out BMP-2 signalling, embryonic fracture healing was unaffected, while in contrast, the BMP-2 signalling was required for initiation of fracture healing post-natum (Chappuis et al., 2012). These findings stress the importance of the second factor: growth factors. The third factor necessary for bone regeneration is the presence of osteoconductive scaffolds. The hematoma forms a scaffold in normal fracture healing facilitating cell migration, and binding. The fourth factor is vascularisation, which is crucial for the supply of nutrients, such as oxygen, and disposal of waste products. Finally, the mechanical environment is pivotal for bone regeneration as micro motions are critical for fracture healing, an effect that is predominantly mediated through osteocyte signalling (Bonewald, 2006, Klein-Nulend et al., 2013). If the factors are present in appropriate quanti-

ties, bone formation can be observed at the fracture site within weeks of injury, followed by complete bone bridging and healing after only a few months. Clearly, in addition to these five factors healing is dependent on factors such as location and energy of the injury, and patient factors such as age and smoking status. So-called normal fracture healing usually occurs through indirect fracture healing, which is a mixture of intramembranous and endochondral bone formation. An acute inflammation is of major importance in order to produce and release cytokines that recruit mesenchymal stem cells (MSC). In the first stage, a primary callus will develop and become vascularised. The callus matrix will later calcify and undergo remodelling into a normal bone structure (Marsell and Einhorn, 2011, Einhorn and Gerstenfeld, 2015)(fig. 3).

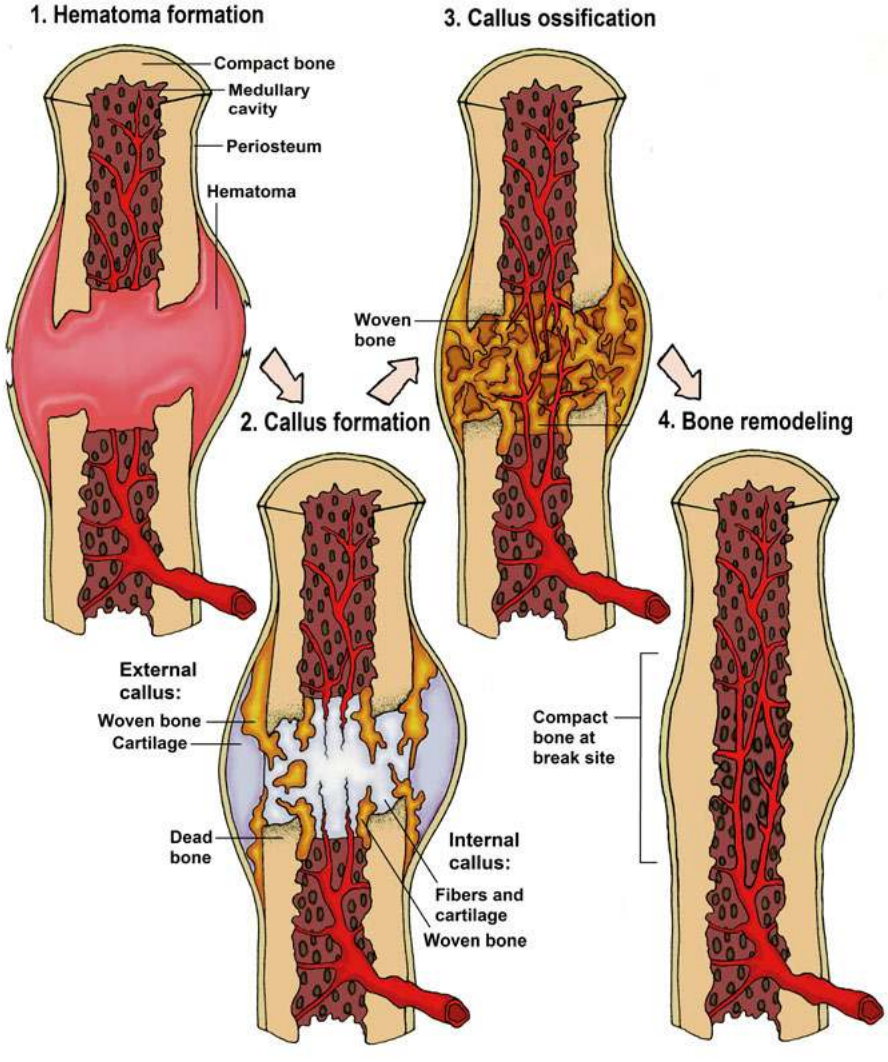

Figure 3. Natural fracture healing. After the initial trauma, a hematoma is formed. The hematoma contains pro-inflammatory cytokines that rapidly recruit immune cells to the site of the fracture. Cartilage is formed that later gets replaced by woven bone. The callus then becomes remodelled into its former structure of compact and spongy bone.

Bone formation

Growth factors

Fracture healing is orchestrated by numerous cytokines; IL-1, TNF-α and IL-6 are some of the first cytokines secreted, and initiate the repair cascade. These factors recruit inflammatory cells, and promote angiogenesis. IL-1 and TNF- α signalling overlaps, and both of them induce production of IL-6 in osteoblasts, and angiogenesis, as well as promote callus formation. In turn, IL-6 induces production of vascular endothelial growth factor (VEGF) that is important for re-vascularisation. TGF-ß is mainly produced by platelets, and induces proliferation of osteoprogenitor cells. TGF-ß is, together with platelet-derived growth factor (PDGF) and BMP-2, responsible for the initiation of callus formation. It has been suggested that GDF-8, SDF-1 and BMP-7 are cytokines responsible for recruitment of MSC to the fracture site. BMP-2 and 4 are highly expressed directly following trauma, and are crucial for bone healing (Chappuis et al., 2012). As indicated earlier, BMP-2 appears to be expressed throughout the entire healing process, but to varying degrees. Another interesting feature of bone healing was discovered by Marsell et al., who demonstrated that reaming a tibia resulted in an early peak of BMP-2 mRNA, expressed within the first 24 hours, and a later peak at day 7. Surprisingly, mRNA of BMP-2 were not only expressed in the endosteal-lining cells, periosteal cells, and the osteocytes of the reamed bone, but also expressed in the healthy contralateral bone at day 7. In addition, an increase of CD73 positive cells was detected in the peripheral blood two days after reaming, suggesting a systemic action of bone regeneration (Marsell and Einhorn, 2011).

WNT signalling

WNT signalling is essential in development and tissue homeostasis. Historically, the pathways have been divided into canonical and non-canonical WNT pathway, and WNT-calcium pathway. All of these pathways are important mediators in development; however, this introduction will focus on a simplified description of the complex canonical WNT pathway, which is the best characterised of the three pathways (fig. 4). The amount of ß-catenin remains low in the absence of WNT ligands. ß-catenin is captured in a destruction complex composed of axin and adenomatous polyposis (APC). Together with GSK-3, this complex phosphorylates serine/threonine residues on the ß-catenin, which renders it a target for the E3 ligase B-TrCP which polyubiquitinatise it for proteosomal destruction. WNT signalling is initialised by WNT ligand binding to the Frizzled receptor that binds to its co-receptor LRP5/6. Dishevelled binds the frizzled receptor and together with FRAT1 recruits axin to the LRP5/6 tail. GSK-3 also binds to this complex,

and the phosphorylation of ß-catenin is thereby prevented, permitting accumulation of ß-catenin in the cytoplasm, that then translocates into the nucleus where it replaces the repressor Groucho, and binds to the TCF/LEF transcription factor, thereby enhancing proliferation and differentiation of osteoblasts. There is growing evidence of the cross talk between WNT, Parathyroid hormone (PTH) and BMP signalling, where PTH appears to have an inhibitory effect on WNT antagonists. The cross talk between BMP-2 signalling seems more complex with opposing effects depending on the cellular context. Crosstalk of BMP-2 and PTH can result in synergistic effects, for example in osteoblast and bone induction. In other contexts, such as bone homeostasis, BMP-2 can have an inhibiting effect on WNT signalling (Baron and Kneissel, 2013).

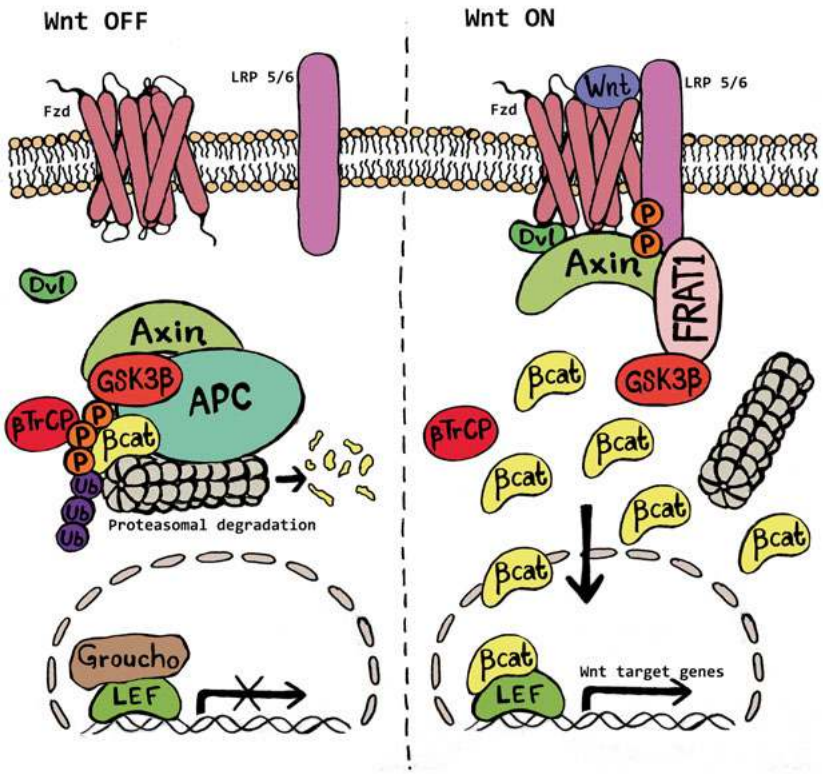

Figure 4. A simplified overview of the canonical WNT signalling pathway. In the absence of WNT signalling, ß-catenin becomes captured and phosphorylated by a destruction complex of APC, axin and GSK-3. The phosphorylated ß-catenin gets ubiquitinised by B-TrCP for proteasomal targeting. The frizzled receptor binds to and phosphorylates its co-receptor LRP5/6 upon WNT ligand binding. Dishevelled and FRAT1 bind to the receptor complex with axin and GSK-3, preventing the targeting and degradation of ß-Catenin. ß-catenin accumulates in the cytoplasm and translocates into the nucleus where it replaces the suppressor Groucho and initiate transcription of WNT target genes.

Biomineralisation

The osteoblast secretes osteoid consisting of a collagenous matrix mainly composed of type 1 collagen. The osteoid also contains proteins such as osteocalcin that facilitate spontaneous mineralisation, and alkaline phosphatase, which is crucial in bone formation and a useful clinical marker for bone turnover. Alkaline phosphatase is a dimeric enzyme that hydrolyses pyrophosphate, an inhibitor of mineralisation, to inorganic phosphate, which triggers biomineralisation. In the absence of pyrophosphate, the saturation of calcium and phosphate in the serum would induce spontaneous mineralisation. Approximately 50-70% of bone is inorganic, comprising of several trace elements referred to as carbonate hydroxyapatite (CHA) (Ca, Na, $Mg)_{10}(PO_4, HPO_4, CO_3)_6(OH, Cl, F)_2$ (LeGeros, 2008). The carbonate hydroxyapatite contributes to the collagen matrix of bone by providing compressive strength, while the collagenous matrix contributes to tensile strength. It is suggested that bone mineralisation occurs initially by amorphous calcium phosphate nanospheres being transported as intracellular mineral-containing vesicles in the osteoblast, followed by their deposition into the extracellular matrix where they later crystallise into carbonated hydroxyapatite, predominantly located inside the collagen fibrils. The average calcium/phosphate ratios are 1.58 ± 0.16 in mineralised extracellular matrix but only 0.75 ± 0.22 in the osteoblastic intracellular mineral-containing vesicles (Mahamid et al., 2011). Osteocalcin, is the second most abundant protein in bone after collagen, and also mediates bone formation. It is expressed in osteoblasts in the latter stages of differentiation, and has been used as a biological marker for bone formation, although its precise function is not fully understood. Osteocalcin binds to the bone matrix, a process governed by the affinity of this negatively charged protein for the positive charge of calcium ions in the hydroxyapatite crystal lattice (Hoang et al., 2003).

Induced bone formation

In cases of extensive bone damage or bone loss, enhancement of bone healing and formation is often needed. If bone loss is too extensive, the healing capacity will not be enough to bridge the defect, usually referred to as a "critical size defect." Osteoinduction can be promoted by growth factors or bone autograft, inducing bone formation by endogenous or transplanted osteoprogenitor cells. Osteoconduction can occur by creating a bone-like structural environment, conducting bone cells from adjacent bone. Fortunately, the majority of fractures heal without complication, however, 5%–15% of fractures do not heal (Kanakaris and Giannoudis, 2007). In cases where bone healing is compromised, or a severe defect is present, the gold standard treatment is autologous bone grafting. The most common technique is

through harvesting of bone from the iliac crest. Even though the use of autologous bone is well established and efficacious, the technique is associated with several limitations including finite volume available, variable bone-inducing capacity, and donor site morbidity such as infection and pain (Dimitriou et al., 2011). An alternative approach is local application of growth factors such as bone morphogenetic proteins (BMP) (Urist, 1965) that are available for clinical use. BMPs are members of the transforming growth factor ß superfamily (Sanchez-Duffhues et al., 2015), and there are presently more than 15 known forms of human BMPs. BMPs 2, 4, 6, 7, 9 and 14 have osteoinductive properties (Wang et al., 2014), and BMP-2 and BMP-7 have been developed into clinical products. BMP-2 and BMP-7 are expressed at different stages of the fracture healing process. BMP-2 seems to be expressed throughout the entire healing process but to different extents. BMP-2 is highly involved both in the initial inflammatory stage, in which it has its largest expression, and in triggering the expression of the other BMPs. It has been demonstrated that knock out mice lacking BMP-2 are unable to heal fractures. Even though it has a crucial role in early fracture healing, BMP-2 is also expressed throughout the later stages: (1) soft callus formation where it is expressed by chondroid progenitors; (2) it is expressed by periosteal bone-lining cells in the ends of the fractured bone where intramembranous ossification occurs; and (3) in low amounts in primary ossification by osteoblast-lining cells where the soft callus is replaced by woven bone. (4) Lastly, it is expressed in secondary ossification by osteoblasts, where the bone is remodelled into ordered bone. BMP-7 is mainly expressed in two peaks at day 14 and 21 and is reported to be of less importance in bone healing (Marsell and Einhorn, 2011). We focused on the BMP-2 for two reasons: the high involvement of BMP-2 throughout the bone-healing process, and the existing expertise in our group of BMP-2 use for bone induction. It is important to stress that success has been shown clinically with BMP-7, and to our knowledge remains uncertain which growth factor is most efficient for clinical use. The advantages with BMP, compared to autograft, are the lack of donor-site morbidity, a homogeneous inducing capacity and unlimited amount of material. Due to BMPs short half-life in the body, it cannot be delivered directly at the fracture site unless mixed with a carrier that ensures a controlled release and containment (Poynton and Lane, 2002). The present carriers have several drawbacks including a bovine origin that creates a risk for immunological reactions in human, poor retention of the BMP necessitating high doses of the protein, and difficulties in containing BMP at the target site during the time it is required to exert its biological activity. In addition, the high dose of BMP-2 itself can increase the risk of immunological response (Keefe et al., 1992, Carragee et al., 2011, Neovius et al., 2013). An additional limitation is that current products are not injectable, and require an open surgical technique for application (McKay et al., 2007). Finally, due to the requirement for such large doses of BMP-2 as a result of the inade-

quate containment and release, the cost per treatment is a concern (Garrison et al., 2007). One overall aim during the last decade has been to develop "Off-the-shelf bone substitute products" that will reduce the requirement for autologous bone graft. For BMPs, there is a need to develop new products with better retention, and sustained release (Schmidt-Bleek et al., 2016). In addition, the rate of degradation, cell-binding capacity and cellular uptake must be considered. The ineffective delivery of BMPs with current strategies is partly because the interplay between each specific carrier, the growth factor, and the surrounding tissue remains to a large extent unknown. The mechanical and chemical properties of the carrier will influence the release profile and stability of the growth factor, directing its site-specific pharmacological action. For biodegradable materials, these properties are dynamic, changing with time due to degradation, and new tissue formation.

Bone morphogenetic protein-2

BMP-2 is a multifunctional cytokine that belongs to the transforming growth factor β-superfamily (Sanchez-Duffhues et al., 2015). BMP-2 has strong osteoinductive properties and is expressed early in the bone-healing cascade where it constitutively activates serine/threonine domains in type II receptors. Phosphorylated BMP-activated type I receptor subsequently activates intracellular-signalling SMAD-proteins by phosphorylating Smad 1, 5 and 8, which form a complex with common mediator Smad-4, and translocates into the nucleus, where the complex regulates transcription factors of BMP-responsive genes, such as Runx2 and Osterix (fig. 5). There are 3 forms of each type 1 and type 2 receptor (Lavery et al., 2008); however, BMP-2 signalling in MSC is predominantly mediated by a homodimer of the type 2 receptor called BMPR2, combined with either a homodimer of the type 1 receptor BMPR1A, or heterodimers of the type 1 receptor BMPR1A/ACVR1A. Other BMP-signalling pathways elucidated include Smad-independent p38 mitogen-activated protein kinase pathway, and the phosphatidylinositol 3-kinase/AKT pathway (Lavery et al., 2008). BMP-2 has a half-life of only a few minutes, and needs to be retained at the site of injury for an extended period to reach clinical effect. A biphasic release is preferred where a burst release of BMP-2 can initiate fracture healing by activating quiescent cells, followed by a slower release to continuously promote differentiation into bone producing cells (Li and Wozney, 2001, Seeherman and Wozney, 2005). Piskounova et al. demonstrated that an intramuscular injection of hydrogel combined with radiolabeled BMP-2 in rats resulted in a burst release of 55% of BMP-2 at day 2, followed by a slow release for 28 days, and resulted in ectopic bone formation. The bone formation was detected at day 8 by pQCT with increasing bone density until the endpoint at day 28 (Piskounova et al., 2014). As with most drugs, BMP-2 has a pharmaceutical window to reach optimal effect. Zara et al. showed, in

a segmental bone defect model in rats, that BMP-2 concentrations within a range of 30-600 mg/mL had no significant difference in the amount of bone formed. However, a difference in morphology occurred, with a large volume of poorly structured bone was observed in the specimens receiving greater concentrations of BMP-2 (Zara et al., 2011, Schmidt-Bleek et al., 2016). Wang et al. reported the same effect, where higher levels of BMP-2 gave the same scoring in bone formation (Wang et al., 1990). In this thesis, a concentration of 150 µg/mL of BMP-2 (approximately a dose of 15 µg/kg) is referred to as high dose, and a concentration of 5 µg/mL (approximately 0.5 µg/kg) as low dose. We based the higher concentration on previous studies from our group using similar hydrogels where robust bone formation was formed. In clinical practice, 12 mg of BMP-2 is administered when using InductOS (InductOS, Medtronic BioPharma, Netherlands), which is equivalent to approximately 200 µg/kg of BMP-2 when applied in a 70 kg human patient. In this thesis, the amount of BMP-2 that was administered was dependent on the size of the defect of the model used, which corresponds to literature stating that the dosage of the BMP-2 should be related to the volume of the defect (Gothard et al., 2014).

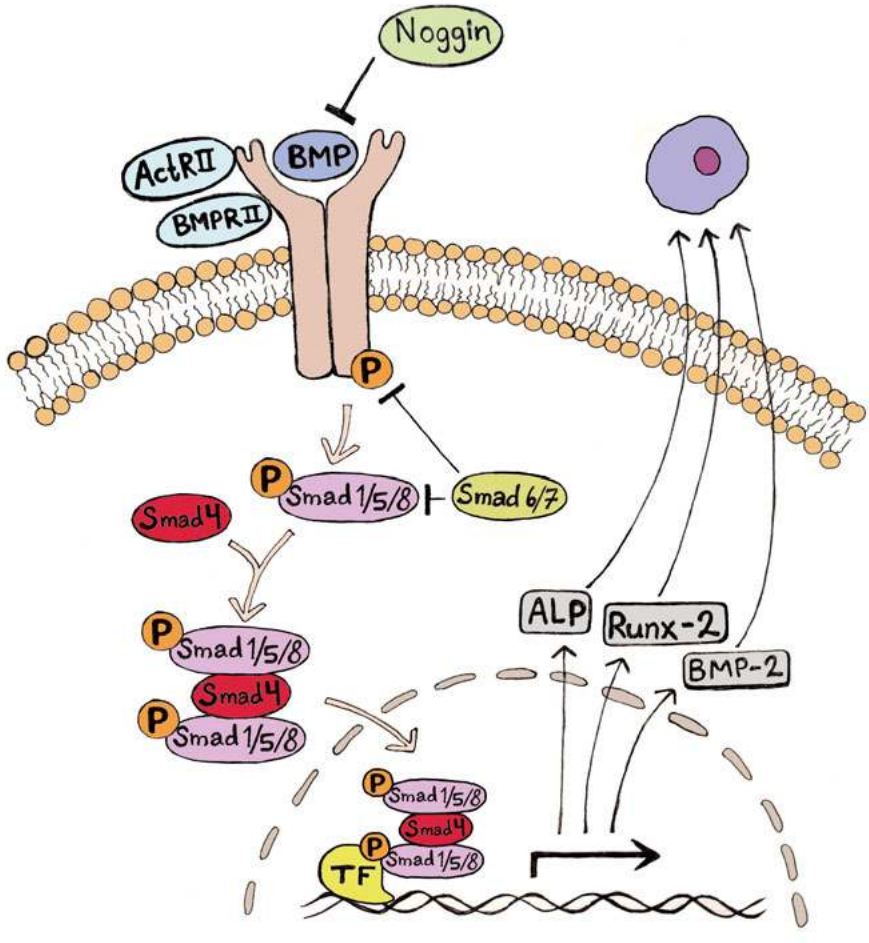

Figure 5. Simplified overview of BMP-2 signalling upon BMP-2 binding. Serine/threonine kinase receptors form a complex of tetramers, leading to phosphorylation of glycine/serine domains of type I receptors by constitutively active serine/threonine domains in type II receptors. BMP-activated type I receptor then activates intracellular-signalling SMAD-proteins by phosphorylating Smad 1, 5 and 8, the phosphorylated proteins form a complex with common mediator Smad-4 and translocates into the nucleus, where the complex regulates transcription factors of BMP-responsive genes, such as Runx2 and Osterix.

Hyaluronan

Hyaluronan (HA) is a naturally occurring, negatively charged and highly conserved glycosaminoglycan (GAG); which is abundant in the extracellular matrix of vertebrates. It was first isolated from the vitreous humor in the 1930s by Karl Meyer (Meyer and Palmer, 1934). HA is comprised of a long, unbranched polysaccharide with repetitive sequences of D-glucuronic acid and N-acetyl-D-glucosamine that in contrast to other GAGs are produced in the plasma membrane. HA has a high turnover in the body. It is endocytosed and degraded in lysosomes by hyaluronidase. This linear polysaccharide has a stiff helical configuration, which creates a large coil structure that entangles and forms a meshwork, functioning as a barrier against rapid water flux, controlling transport of plasma proteins, and facilitating immobilisation and exclusion of larger molecules. It also binds in excess of a thousand-fold of water. It contains several cell-binding motifs for receptors, such as RHAMM, CD44, I-CAM and HARE, and promotes cell migration by interacting with cell surface receptors (Turley, 1991, McCourt et al., 1994, Knudson et al., 2002). High levels of endogenous HA are detected in remodelling and healing tissues with high rates of cell proliferation and migration. HA is involved in a number of physiological processes, e.g. structural support, angiogenesis, extracellular matrix homeostasis and wound healing. (Fraser et al., 1997). HA has also been shown to affect the interaction between osteoclasts and osteoblasts, cells which are pivotal in bone remodelling and fracture healing (Chang et al., 2006). In contrast to other glycosaminoglycans, HA only differs in the degree of polymerisation between the different species, thus making it non-immunogenic, and almost identical in all species. Cells engulf and degrade extracellular HA in stages, generating decreasing sizes of HA. Hyal-2 is a size-dependent hyaluronidase that cleaves high molecular HA, while Hyal-1 has the ability to cleave all sizes of HA (Stern, 2004). Due to this characteristic, along with enzymatically degradability, viscoelastic and physiochemical properties, HA is an attractive tool for development of biomaterials in tissue engineering. The use of endogenous HA to enhance fracture healing initially gave a negative result, probably due to its high turnover. Modified HA with a lower turnover was later successfully used to produce a range of scaffolds for tissue-engineering applications, as well as drug delivery and has resulted in the development of hydrazide- and aldehyde-modified HA hydrogels that form hydrazones (Jia et al., 2004, Zheng Shu et al., 2004). Our collaboration with the Department of Polymer Chemistry at Uppsala University has developed an *in vivo* injectable system consisting of lightly derivatised water-soluble aldehyde-modified HA and hydrazide-modified polyvinyl alcohol (PVAH) that upon mixing results in gelation within 1-2 minutes after injection. They have modified the carboxyl group on the glucuronic acid to either contain an aldehyde (R-CHO) or hydrazide ($R\text{-}CON_2H_2$). The cross-linking produces stable hy-

drazones in aqueous solutions at physiological pH. The cross-linking is highly selective, and the only by-product is water, making it ideal for injection (Bergman et al., 2009). Similar HA-based injectable systems have been developed by others, including Prestwich and Langer (Jia et al., 2004, Zheng Shu et al., 2004). Initially, Bergman et al. injected 0.2 ml of the hydrogel in an ectopic rat model, which gave no immune response and formed local bone when administered with 30 µg of BMP-2 (Bergman et al., 2009). Similar results were reported in a critical size defect in mini pigs with no immune response, and complete resorption of the hydrogel was reported when administered alone. Meanwhile, hydrogel containing 1.25 mg of BMP-2 induced bone formation with complete healing of the defect (Docherty-Skogh et al., 2010). The hydrogel is able to retain, and deliver additives, such as growth factors, and osteoconductive ceramic particles due to its mechanical and electrostatic properties (fig. 6). In earlier experiments, hydroxyapatite (HAP) has been added as 25 weight-% to enhance mechanical properties of the hydrogel. There is also evidence to suggest that HAP has a positive influence on bone formation (Bergman et al., 2009, Docherty-Skogh et al., 2010).

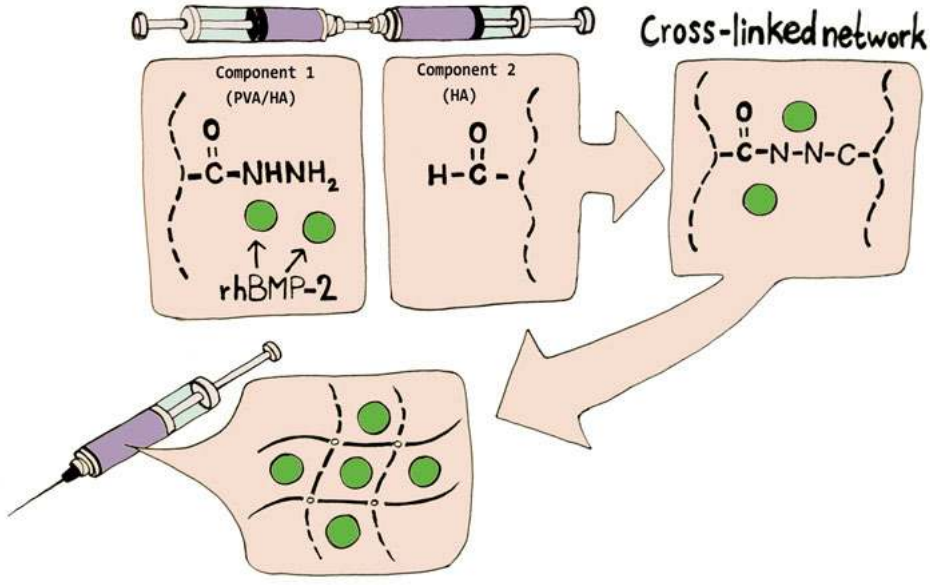

Figure 6. Illustration of an *in vivo* injectable system consisting of aldehyde-modified HA and hydrazide-modified polyvinyl alcohol used as a carrier of BMP-2.

Factors that are proposed to induce bone formation

Bisphosphonate

Bisphosphonates (BP) were first used in industry during the 18th century as chelating agents, corrosion inhibitors, and water softeners. In the mid-1900s, scientists started to investigate the effect on bone of a salt called pyrophosphate ($P_2O_7^{4-}$), which was shown to protect HAP from resorption *in vitro*. The same effect could not be shown *in vivo* due to the rapid hydrolysis of pyrophosphate. The oxygen bridge between phosphates in pyrophosphate is exchanged in BP with a carbon atom, thereby preventing hydrolysis by alkaline phosphatase (Wang et al., 2005). This also makes the BP highly hydrophilic, with a high affinity for calcium ions and HAP. BPs localise to bone following oral or intravenous administration. They demonstrate minimal distribution to soft tissues, but remain in bone tissue over an extended period (Pamidronate 1-2 years) (Cremers et al., 2005). BPs have a net anabolic effect on bone, through inhibition of osteoclasts. This bone effect forms the basis for BPs use in prevention and treatment of osteoporosis.

Impaired bone healing is often caused by systemic factors in the body such as age, and concomitant health conditions that negatively affect bone formation. BMPs are potent anabolic stimulators of bone, serving to enhance the differentiation of osteoblasts, however, they stimulate osteoclasts (Jensen et al., 2010). Harding et al investigated if modulation of bone catabolism would result in an increase in bone formation. Harding applied a mixture of BMP-7 or saline with bone graft to a chamber implanted into a rate tibia, 2 weeks following surgery a subcutaneous injection of either zoledronate 0.1 mg/kg or saline was given. After 6 weeks, the group with BMP-7 had a significant increase in new bone ingrowth, and the single injection of zoledronate significantly increased the trabecular volume from 14% seen in the non-BP control to 40% (Harding et al., 2008). BPs are classified according to the molecules bound to their side chains R1 and R2. Both side chains alter the affinity for bone, and the target of inhibition. We have used the aminobisphosphonate Pamidronate ((3-amino-1-hydroxypropane-1, 1-diyl)bis(phosphonic acid)), which inhibits farnesyl pyrophosphate synthase in osteoclasts, thus impairing GTPase signalling, resulting in osteoclast apoptosis. Previous studies have shown that BPs enhance bone net formation when administered both systemically, and locally (Wilkinson and Little, 2011), with a synergistic effect seen when BPs are combined with BMPs (Little et al., 2005).

Nano Hydroxyapatite

Nano crystals of CaP are the building blocks of biomineralisation. Their nanoscale features not only allow the crystals to act as blocks for biomineralisation, but also their critical length of 20–50 nm prevents the Nano crystals from dissolving (Tang et al., 2004). The nanoscale characteristics also include greater toughness when used as a biomaterial, and increased bioactivity due to their higher surface area. There have been suggestions that nano-sized HAP promotes the synthesis of alkaline phosphates by osteoblasts, which is an important factor in biomineralisation (Cai et al., 2007). Mahamid et al. suggested that bone mineralisation occurs by transportation of amorphous calcium phosphate nanospheres in intracellular vesicles in the osteoblast, followed by deposition into the extracellular matrix where they later crystallise into carbonated HAP (Mahamid et al., 2011). CaPs have the ability to bind various proteins upon exposure to the *in vivo* environment. The nano properties and increase in surface area of nano HAP facilitates binding of BMPs, and other essential endogenous proteins, which in turn can trigger stem cell differentiation into osteoblasts, and thereby promote bone formation. Furthermore, inorganic ion release may also be a direct trigger of osteoinduction (Barradas et al., 2011).

Methods

Biomaterials and substances

Most of the biomaterials or substances that we have investigated as carriers for BMP-2 have already been approved for other clinical applications. Cross-linked HA hydrogels are widely used in the cosmetic industry as fillers, and CaP has been used for decades as bone-void filler. However, *in vitro* testing was required when additives such as BP were added to the carriers, inorder to test for biocompatibility, osteoinductive effects or *in vitro* release of BMP-2.

Alkaline phosphatase assay

Osteoinduction by BMP-2 activity can be readily quantified by alkaline phosphatase assay, in which a substrate for alkaline phosphatase (ALP) p-nitrophenyl phosphate becomes dephosphorylated into a yellow product with absorbance that can be measured at 405 nm wavelength. ALP is crucial for mineralisation, and is highly expressed by osteoblasts. ALP counteracts the effects of inorganic pyrophosphate (PPi), a molecule that normally inhibits spontaneous calcification (Addison et al., 2007, Millan, 2013, Orriss et al., 2016). PPi is a by-product of many intracellular metabolic reactions, e.g., hydrolysis of ATP into AMP in cells. Although the mechanism by which it inhibits crystal growth is not fully understood, PPi is thought to prevent soft tissue mineralisation by binding specifically to mineralization nucleation centres, thus preventing the further nucleation of mineral ions. Addison et al. demonstrated that PPi prevents mineralisation in MC3T3-E1 osteoblast cultures by three mechanisms: direct binding to growing crystals, induction of osteopontin expression, and inhibition of ALP activity (Addison et al., 2007). We have predominantly used rat bone marrow stromal cells (W-20 clone 17, ATCC, Teddington, UK) as their expression of ALP by BMP-2 is close to linear. In the ALP assay, the cells have been cultured with digests of the material for 48 hours and then washed with PBS. Cells were analysed, and ALP activity measured by incubating cell lysate with Alkaline Phosphatase Yellow pNPP Liquid Substrate (Sigma-Aldrich Inc., St. Louis, MO, US) after which the absorbance was measured at 405 nm wavelength. W-20 clone 17 is an ideal cell line for the investigation of BMP-2; however, when

examining other osteoinductive factors, alternative progenitor cell lines should be considered.

Cell biocompatibility assay (MTS assay)

MTS assay is a biocompatibility test, which can be used for cell proliferation, cell viability, and cell attachment. The MTS reagent is reduced to formazan by cell metabolic activity. We used the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay from Promega, Madison, WI, to determine the viability of cells. Osteoblasts (UMR ROS-106) and MSCs (RMSC R492-05) (both from Health Protection Agency Culture Collections, Salisbury, UK) were seeded on 96 well plates, and when 50% confluence was reached, the material that had been previously digested by hyaluronidase were added and incubated for 36 h after which the MTS reagent was added. The MTS reagent is composed of a tetrazolium compound that is mixed with phenazine methosulfate and is bioreduced by cellular metabolic activity of dehydrogenase to a soluble formazan product that is purple and can be quantified by the UV absorbance 492 nm wavelength.

Release assays

Enzyme-linked immuno-sorbent assay (ELISA) was used to measure the release of BMP-2 from hydrogels. In accordance with International Standard for Biological Evaluation of Medical Devices (Parts 5 and 12, ISO10993-5 and 10993-12), a ratio of 1.25 cm^2/ml of extracts from materials was used in ELISA, using a human BMP-2 ELISA Development Kit (PeproTech Inc., Rocky Hill, NJ). The same BMP-2 that was used in the carrier was also used to obtain a standard calibration curve to quantify the amount of BMP-2 released. BMP-2 (InductOs, Medtronic BioPharma, Netherlands) was calibrated in a diluent (0.05% Tween-20, 0.1 % BSA in 1 × PBS) to give standard solutions with concentrations ranging between 0.125 and 8 ng/ml. The standard solutions were analysed by ELISA according to the manufacturer's protocol. Absorbance was measured at 405 nm with correction at 650 nm.

Radio labelling of BMP-2 with ^{125}I was performed according to the chloramine-T method (Hunter and Greenwood, 1962), where ^{125}I-labeled BMP-2 was added to the non-labelled protein at weight ratio 1:6,000. Samples were placed in 2 mL Protein LoBind tubes and covered with 1 mL of sterile PBS. The initial amount of radioactivity at t = 0 was measured using a 1480 Wallac Wizard gamma counter (Perkin Elmer, Wellesley, MA). The hydrogels were then placed at 37°C and the ^{125}I-labeled BMP-2 release was measured over 28 days. For each time point, the old release medium was collected and replaced with fresh PBS. Both the retained and the released amounts of ^{125}I-

labeled BMP-2 were measured to account for any possible protein loss due to pipetting. The acquired counts were corrected for radioactive decay and normalised to radioactivity of the hydrogels at t = 0.

Animal models

As previously described, the interest in biomaterials tailored for bone regeneration has increased, and novel biomaterials are being continuously developed. Such work is critically dependent on suitable animal models to study the regenerative potential of the various materials. In choosing an animal model for a particular biomaterial, several different factors need to be taken into consideration, e.g., cost, ease of surgery, relevance to the clinical question, size of animal, bone structure of the animal, and ethics.

A number of animal models have been developed for evaluating the ability of novel biomaterials in inducing bone formation. Rabbits, sheep, pigs, dogs, rats, and mice have frequently been used in orthopaedic research. For the larger animals, a major advantage is the ease to mimic clinical problems. The drawbacks are the greater cost for animal housing, and increased difficulty in handling. Our group has focused on rats for the following reasons: (1) their size is adequate for small defects and several subcutaneous implants; (2) the available strains are not aggressive; (3) their curiosity makes them easy to handle; (4) there is a low cost for both the animal and housing; and finally, (5) there is a great amount of orthopaedic literature using rat models. Drawbacks with rat models, compared to larger animal models, include reduced relevancy to clinical practice, and the relative difficulty to translate data from rat models to humans. For example, modest changes in enzyme function between species can result in major pharmacologically effects. An attempt to prevent difficulties in translation is to run an *in vitro* study on human tissues and cells, and combine the *in vivo* data with the *in vitro* data. The trend, at least in Europe, is to reduce the use of animals, and consequently there is a drive to develop complex *in vitro* cultures mimicking human tissue (Langley et al., 2007). Scientists have become more aware of animals' capabilities, and continue to publish results showing the awareness and neurophysiology of animals. Pankseep et al. found adolescent vocalisations that seemed to have evolutionary relations to the joyfulness of human children's laughter (Panksepp and Burgdorf, 2003, Bering, 2012). Ben-Ami Bartal showed empathy and prosocial behaviour in rats that learned to free their cage mates (Ben-Ami Bartal et al., 2011). Recently, Ishiyama and Brecht confirmed that tickled rats both evoked vocalisations and spontaneous jumps, and by monitoring the stimuli in the brain; they could correlate this behaviour to what is defined as ticklishness in humans (Ishiyama and Brecht, 2016). However, animal models remain a critical part of preclinical studies because the complexity of the living organism is still superior to even

complex *in vitro* setups. *In vitro* experiments therefore, in the foreseeable future, will not be sufficient to fully replace the knowledge that can be gained through animal models.

Our project has adhered to the 3Rs declared by Russell et al. in the book "The Principles of Humane Experimental Technique" (Russell and Burch, 1959). We have used animal models where controls and samples have been placed in the same animal to allow paired analysis, providing greater statistical power to the study while lowering the number of animals used. All animal procedures were approved by the Uppsala (Sweden) ethical committee (C249/8, C317/10, C305/8, C76/13) according to the Federation of European Laboratory Animal Science Association's guidelines. We focused on ectopic intramuscular injections, subcutaneous injections or incisions, and orthotopic defects in a uni-cortical femoral defect model. Ectopic and subcutaneous models can be adequate for testing osteoinductivity of biomaterials, as the inductivity is needed for any kind of bone formation in these ectopic areas. Intramuscular injections have the advantage of the presence of cell populations and vascularisation, which is more representative of the bony environment than subcutaneous pouches. However, several subcutaneous pouches can be used in one rat, allowing paired analysis between different groups, which is an especially useful technique for screening of various materials. We have during this project developed a uni-cortical rat femoral defect that does not require stabilisation. The model is based on earlier studies from Melcher, Bay and Wong. Melcher et al. drilled a 2-mm-bilateral femoral diaphyseal defect in rats with a dental drill (Melcher and Irving, 1962). Bay et al. developed a cortical window in the radii of dogs, (Bay et al., 1993) and Wang et al. used a rabbit radius defect model that did not require stabilisation (Wang et al., 2011). We found that a simple drill defect in a rat femur was too small to mimic the large defect that we required. In addition, the size of the drill hole was inadequate for many injectable biomaterials. Therefore we developed a defect that was as large as possible without compromising the mechanical integrity, i.e. there was no need for stabilisation. Such a model is in accordance with the principle of the 3Rs (Russell and Burch, 1959). This model was also easier to perform than models that require stabilisation. Use of bilateral femoral anterolateral defects allowed paired comparison, making this defect ideal for screening osteoinductive biomaterials (fig. 7).

We have also used a more complex critical size defect model for evaluation of osteoinductive properties. In that model, a 5 mm-long segmental femoral shaft defect was created in which the femur was stabilised with an external fixator. The defect could be filled with various compounds to study the ability to enhance bone formation. The advantage of this defect is the critical size that prevents self-healing. Critical size defects are of great clinical relevance as they simulate challenging defects seen in clinical practice. The major drawback with the large segmental bone defect model is the in-

fluence on the animals' well being, as an external fixator, which is needed for stabilisation of the bone is used. Use of external fixators is associated with risks such as inflammation, and infection at the pin sites, or even loss of fixator pins. While critical-size models such as rat-femoral defects greater than 6 mm (Einhorn et al., 1984) and rat-calvarial defects greater than 8 mm are often used (Ray and Holloway, 1957, Takagi and Urist, 1982); generally, only one defect can be performed per animal. This negates the possibility of bilateral defects, and paired analysis that permits a reduction in the number of animals used to achieve reliable data. The reason why we chose to also use a critical-sized segmental defect was to have a sufficient volume of material to be able to detect the radiolabelled BMP-2 with the SPECT camera (fig. 8). The defect size needed to be at least as large as the pixel size of the camera to prevent some of the artefacts. With *in vivo* non-invasive imaging comes the issue of radiation dose. Excessive radiation leads to decrease in vascularisation, and cellular apoptosis (Takahashi et al., 1994). This makes it very important to carefully design the study with respect to number and timing of scans, to be able to minimise the radiation dose, and still obtain reliable data. A maximum total exposure of 1 gray in rats is considered to be good practice (Klinck et al., 2008, Laperre et al., 2011).

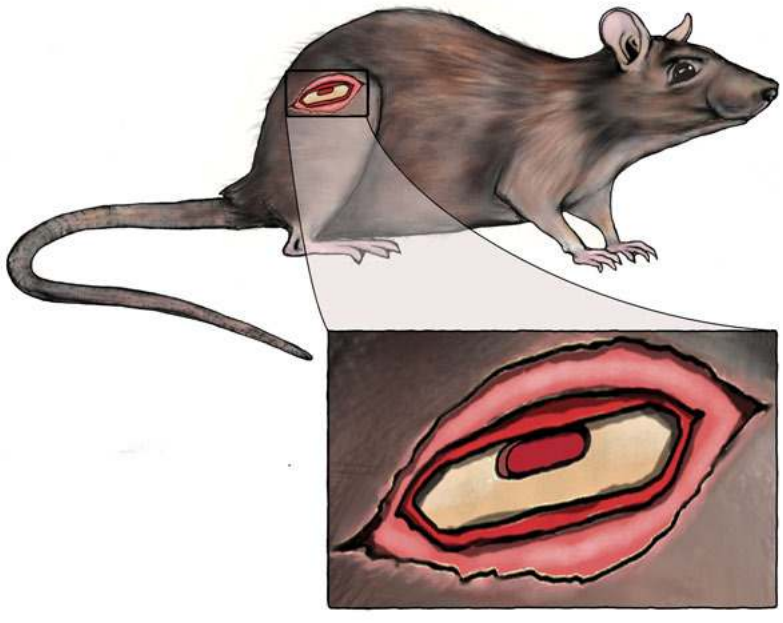

Figure 7. Illustration of a uni-cortical defect, a drilled defect with the dimensions of 6 × 2 mm placed just distal to the third trochanter on the anterolateral side of the femoral shaft

.

Figure 8. Critical-size defect. The 5 mm long femoral segmental defect was stabilised by an external fixator.

Imaging

Computed tomography

CT revolutionised the world of imaging and its developers, Cormak and Hounsfield, received the Nobel Prize in 1979. The ability to build cross-sections by filter-back projection from a projection allowed detailed imaging of internal structures and the technique quickly advanced to 3D imaging and resolution in the micro scale. Both peripheral quantitative computed tomography (pQCT) and micro computed tomography (μCT) operate in the same way. An X-ray beam is emitted from an X-ray source and a detector on the opposite side detects the attenuation of the X-ray beam. pQCT and μCT are powerful tools, but both techniques come with limitations. When measuring biomaterials with some kind of radiopacity such as Sr, HAP or CaPs, there is a risk of misinterpreting the material as bone. We circumvent these problems by measuring the biomaterial alone in order to assess to what extent it might influence the data.

Peripheral computed tomography

pQCT scans were obtained using an XCT Research SA+bone scanner (Stratec Medizintechnik, Pforzheim, Germany) with software v5.50D (XCT Stratec Console software, Stratec Medizintechnik). Scans with a minimum pixel size of 70 µm was separated by 0.5 mm slices and acquired to cover the sample and two slices were also located outside the sample to ensure that the whole sample was scanned; these two slices were later excluded from the calculations. The analysis was performed with the CALCBD algorithm (CortMode 1, PeelMode 2, threshold 1 0.280 g/cm^3). A region of interest on each scan was set to cover the whole cross-section of the sample. The data was tabulated and converted to BMD, which was calculated by taking the mean of each slice that included a part of the explant. Bone mineral content (BMC) and bone mineral area were calculated by summing the values of all slices for each specimen.

Micro computed tomography

In this thesis, a SkyScan 1176 (Bruker MicroCT, Kontich, Belgium) was used that had a minimum spatial resolution of 14 µm with three optional pixel sizes, 35 µm, 18 µm and 9 µm. The sample was placed inside the scanner between a detector and an X-ray source that rotated around the sample. Radiography is based on the same principles as photography, but it uses X-rays instead of visible light. A source produces an X-ray beam and a camera detects and counts the hits of photons per camera pixel. When an object is placed between the camera and the X-ray source, some of the photons will be attenuated by the object, leading to fewer detected photons in the camera.

Partial absorption of the X-rays is required and can be obtained by adjusting the voltage or the energy of the photons. Low voltage results in less energetic photons, which will be attenuated more easily, while high voltage results in more energetic photons that will be attenuated less. The attenuation of the X-rays is proportional to the atomic number Z, in this way, the CT can capture a 2D density map of the object. By means of back-projection a new data set of cross-sectional images are obtained on which 3D analysis can be performed. To allow comparison of results from different scans, calibration phantoms with a known density are required.

The optimisation of the scanner parameters makes it possible to scan a wide range of objects and tissues—from lungs to cortical bone. This non-invasive imaging method allows repetitive scanning of the same object. In the case of scanning live animals, the radiation dose needs to be addressed in every study design to avoid biological effects. After scanning and reconstruction, the cross-sectional images were binarised to separate the bone from the background. In some cases, a volume of interest (VOI) was selected to analyse only the defect while excluding the surrounding bone.

Radionuclide-based imaging

Molecular imaging is a non-invasive process, which visualises and quantifies physiological and biochemical processes *in vivo* at the molecular level. This is based on tracers that specifically bind to the target protein of interest, or are incorporated as a substrate in a biochemical process. The tracers will accumulate at the specific site where the process occurs or the protein is expressed. The tracers are usually composed of small molecules that are labelled with a specific radionuclide. It is important for the study design to consider the half-life of the radionuclide, and radioactivity that can be administered to the animal, and the timeframe of interest. In this thesis, single photon emission tomography (SPECT) and positron emission tomography (PET) was used in combination with µCT. This combination made it possible to monitor the material containing BMP-2 that was labelled with radionuclide iodine-125 (^{125}I), which is a gamma-emitting tracer, in the same animal using SPECT. The *in vivo* response was at the same time studied by PET with sodium [^{18}F]fluoride. This data could then be combined with µCT to quantify the amount of bone formed, and to follow the *in vivo* kinetics of BMP-2. PET and SPECT have several advantages and complement each other. SPECT can detect gamma rays in the range of 45-300 keV that are directly emitted from the radioactive atom, and only one photon is detected per event (Vallabhajosula, 2009)(table 1). PET tracers emit a positron that annihilates when it hits a nearby electron. Two gamma rays with an angle of 180° opposite of each other are emitted, and 511 keV is detected in the ring detectors of the PET camera. SPECT is a semi quantitative modality, whereas the PET camera provides quantitative measurements of tissue radioactivity, which can be used, for example, to determine the rate of a specific biological process. The PET data was analysed to give an influx rate constant as a measure of incorporation of [^{18}F]fluoride in bone tissue. This rate is called K_i and in our case, it measures how much, biomineral is available, for tracer binding. This means that if a large amount of biomineral is available [^{18}F]fluoride will bind faster to that tissue and the rate K_i will become higher. Ventura et al. (Ventura et al., 2014b) compared PET and SPECT as methods for quantifying bone formation by using PET tracer [^{18}F]fluoride and SPECT tracer 99mTc-HDP. The PET quantification was superior to SPECT with a higher sensitivity and less variation between samples. Iodine-125 labelled BMP-2 has also been investigated in SPECT by Kempen et al. that could follow the retention of the BMP-2 until day 28 in a rat femoral implant (Kempen et al., 2009).

Table 1. Small Animal PET/SPECT imaging modalities

	SPECT	**PET**
Form of energy used	γ-photons	Annihilation photons
Energy detected	45-300 keV	2 x 511 keV
Spatial resolution	1–4 mm	1–3 mm
Acquisition time	60–2000 s	1–300 s

Histology

The specimens were decalcified in modified formic acid in a Decalcifier System (Sakura Finetek Europe, Netherlands), and subsequently dehydrated in an ethanol series, after which they were embedded in paraffin. Histological sections (5–7 μm) were prepared, paraffin removed, and hydrated. Staining was performed with either hematoxylin-eosin or Masson's trichrome, which stain connective tissue: collagen is stained blue, nuclei are stained dark red or purple and cytoplasm is red pink. With our Masson's trichrome staining, the slightly mineralised bone stained blue, and dense crystalline bone stained red. In the literature, the opposite has been described, i.e. mineralised bone is blue and osteoid is red (Asonova and Migalkin, 1996). However, our findings were confirmed both by pQCT and μCT. Safranin O stains nuclei black, subchondral mineralised bone green, and cartilaginous tissue from orange to red. Another useful, and beautiful staining is Picro Sirius red, this provides different information when using polarised or non-polarised light. The Picro Sirius red stains collagen, and in polarised light, the thickness of the collagen layer can be seen in a colour range from green to red, where they represent thinner and thicker layers respectively. (Junqueira et al., 1979).

Statistics

Computational modelling and replacement models are emerging with the potential to fully replace *in vivo* models in the future. Still, to this date, the *in vivo* milieu with the combination of complex cell interactions between the regenerative system and the immune system is critical, in order to evaluate the *in situ* activity of drugs and biomaterials. This thesis is based on *in vivo* results stemming from studies committing to the 3 R's with a focus on reduction and refinement (Russell and Burch, 1959). The last decade there has been a paradigm shift where the over-use of animals has been strongly considered as inappropriate and unethical. The experimental study should however be explicitly designed to minimize the number of animals used without sacrificing experimental efficacy, which in turn can be detrimental for any conclusion related to the undertaken study. Power calculation is necessary prior to all studies, both *in vitro* and *in vivo*, and both in animal and human studies, in order to estimate the necessary sample size "n" needed to detect predefined levels of statistical significance and clinical relevance. As the materials had not been tested before the differences between groups and the variability within a group could only be estimated by literature research of related biomaterials. Based on these results, sample sizes and study designs were chosen. The choice of using parametric tests to analyse the data was also largely based on previous study results.

A selection of statistical tests was used to analyse the results based on the type of data and number of groups in the study. All data were analysed using Prism 5.0 (GraphPad Software, La Jolla, CA)

Paper I used one-way ANOVA, along with Tukey's adjustment for multiple comparisons to test the following hypothesis: bone induction can be altered by using calcium phosphate combinations with HA-hydrogels as a carrier for BMP-2 in an ectopic animal model. Five different ceramic additives were tested. Each sample (0.2 mL hydrogel containing 50 µg CaP and 30 µg BMP-2) was injected intra-muscularly and cross-linked *in situ* in both hind limbs. Three rats were used per group resulting in six samples per group with two samples in each rat. In this study the rats were assumed to be identical biological systems and each sample was treated as a single observation (n=6). Since observations that are not independent carry the risk of affecting the variance within that group and without the appropriate methodological considerations to adjust the analysis, the precision of the estimate may be falsely improved which could increases the potential risk of a biased estimate. In fact, Bryant et al. (Bryant et al., 2006) conducted a systemic review of high-impact-factor orthopaedic journals and found that 42% of the clinical studies used multiple observations from single individuals. They concluded that this could potentially bias the result as most statistical tests is based on the assumption that each observation is independent of all other observa-

tions. Taking this into consideration, an independent statistician was contacted to test and validate the statistical evidence behind the conclusions drawn from the papers in this thesis. In **Paper I**, to evaluate the treatment of observations as independent also within the same rat, the mean of the two treatments within each rat was presented as the sample outcome resulting in a number of three observations per treatment. Interestingly, even though the degree of freedom had radically been reduced the results were still significant with a p-value changing from 0.008 to 0.010 Therefore we could still accept the research hypothesis; that nano-sized HAP formed bone with higher density compared to other forms of HAP compounds.

Paper II's hypothesis stated: crushed hydrogels will induce more bone formation than solid hydrogels, due to easier cell infiltration and the shorter diffusion distance required for the release of BMP-2, as well as through the increase of surface area. Crushed and solid forms of the hydrogels were analysed in vivo in a subcutaneous ectopic bone model in rats. Similarly to **Paper I**, the rats were, in the Paper II study, also assumed to be an identical biological system and each sample was treated as a single observation where 12 samples/group were distributed over three rats (giving four dependent samples per rat of each test group). In order to allow paired observations each animal received four subcutaneous implantations of solid hydrogels (0.2 mL) on the right side and four crushed hydrogel implants on the left side. The difference was tested with Student's paired t-test, which gave a p=value of 0.0107. By the independent statistician, each rat's response - in terms of a joint mean value from the single rat's samples rather than each sample was tested with Student's paired t-test, as in **Paper I,** with a maintained significant difference (p=0.038), but with a slightly higher p-value. This p-value was still below 0.05 and we kept the conclusion of the significant changes in morphologies where solid hydrogels induced the formation of a dense bone shell around non-degraded hydrogel, while crushed hydrogels demonstrated a uniform bone formation throughout the entire sample.

In **Paper III** the hypothesis was the following: A femoral non-critical-sized cortical defect rat model could be used to measure the differences in bone formation between defects filled with BMP-2 hydrogel and empty defects. Uni-cortical bilateral defects were drilled to allow paired comparisons between hydrogel treated (left leg) or untreated (right leg). Six rats were sacrificed at each time point: day 10, 20, 30 and 40. The results were evaluated by two-way ANOVA with Bonferroni's post hoc test to explore if time and treatment had any effect on the healing. It was not possible to account for the paired data when running two-way ANOVA in GraphPad Software, which made the paired data more a hamper in this statistical test. Therefore, Student's paired t-test was also used to test the difference between treated and untreated defects at each time point and here the paired data of each rat pro-

vided an advantage, as the paired data was accounted for, the rat as a co-factor was circumvented. The relative difference in bone healing was most prominent at day 10 and day 20 and decreased as the defect healed in the control legs, which is in line with normal healing. We could conclude that this animal model enabled the assessment of biomaterials tailored for acceleration of bone healing.

Paper IV and **Paper V** did not include any statistics, as both were observational studies. The model developed in **Paper V** is planned to be employed for monitoring growth factor retention of tunable smart materials implementing comparative studies with sample numbers estimated by power calculus.

Finally, **Paper VI** was a multicentre review to investigate the correlation between existing *in vitro* results with *in vivo* outcomes observed for a range of biomaterials. Members from the European consortium BioDesign collated the data, which included 36 *in vivo* experiments, 47 *in vitro* experiments and 93 tested biomaterials that was used as the variables. The outcome of each variable for each *in vitro* and *in vivo* assay was scored out of 5 (1 = poor, 5 = very good). The overall correlation between *in vitro* and *in vivo* outcomes across the entire dataset was characterized by sorting and then categorizing the data according to *in vitro* score. In addition the *in vitro* outcomes were sorted into subgroups representing individual classes of *in vitro* assays. The *in vitro* outcome scores of the material were subsequently correlated with the *in vivo* outcome scores. Further, the *in vitro* assay groups were combined in pairs to investigate if a combination of *in vitro* assays provided a better prediction of *in vivo* outcome over single assays for correlation. The Hypothesis was: Specific *in vitro* parameters or a combination of *in vitro* parameters can be used to predict material *in vivo* outcomes. To answer this, a Pearson's correlation was used to test for significant linear relationships between the *in vitro* and *in vivo* outcomes (n=5-24). Coefficients of determination (R^2) were used to express the proportion of shared variance and a p-value of < 0.05 was considered significant.
The data for each *in vitro* assay groups was split into quartiles in an attempt to distinguish false positives, false negatives, true positives and true negatives. Here a positive result was defined as one that scored higher than 2.5 and a negative result was defined as one that scored lower than 2.5. Fisher's exact test was performed to measure the sensitivity and specificity of the various groups. A confidence interval of 95% was used with no observed significance, thus, the hypothesis was rejected.

Results and Discussion

In **Paper I**, the major finding was that the 20 nm HAP yielded a higher bone density than other additives (P = 0.0008, ANOVA with Tukey's multiple comparison test). We hypothesise that the higher bone density induced by nanoHAP might be due to Nano crystals of CaP acting as direct building blocks for biomineralisation. Five different ceramic additives were tested in aldehyde modified HA and hydrazide modified polyvinyl alcohol, beta-tricalcium phosphate, and four types of HAP, (nanoHAP, HAP, clods of HAP larger than 100µm and a commercial biomimetic HAP product (Ostim35). The compounds were injected into the rat quadriceps muscle, in which it gelated. Bone formation was evaluated after four weeks by pQCT and histology. All materials in this study were CaP compounds, including nanoHAP. However, nanoHAP had defining feature: the ability to enter the mineralisation pathway as a direct building block, as it is similar in size amorphous CaP secreted from osteoblasts (Mahamid et al., 2011). This was a major advantage of nanoHAP compared to the other groups of CaP; that depended on cellular activity to degrade them, prior to mineralisation. There are a few things that would have been beneficial in this study. Firstly, a larger sample size would have been beneficial to offset the large sample variations observed in this animal model. The intramuscular injections resulted in samples of different shapes, and consequently surface area, a major determinant in BMP-2 release, and thus cellular response. Retrospectively, investigation of clods greater than 100 µm resulted in no significant findings, and this group could have been excluded from the study. The results from the nanoHAP, however, are very interesting, and our hypothesis that they underwent indirect mineralisation could have been confirmed by staining TRAP and OPG, to measure osteoclast activity. Furthermore, all the samples should have been run in pQCT prior to injection to analyse the radiopacity of the CaP compounds, which can be misinterpreted as bone. Finally, nanoHAP showed excellent properties, and it would have been advantageous to develop its use in further studies. Unfortunately, this was not possible as the nanoHAP was developed by our collaborator only in small quantities.

Stenfelt et al. evaluated the effects of the pre-incubation of HA hydrogels for various lengths of time following the initiation of chemical crosslinking prior to injection (Stenfelt et al., 2014). This was achieved by investigating the release kinetics of *in vitro* BMP-2 from the hydrogel, and by evaluating

ectopic bone formation in rats. From the curing profile, obtained from rheological analysis, pre-incubation times (1 min, 5 h and 3 days) were selected to prepare slightly, moderately and fully cured hydrogels. Comparable release profiles were observed for all three test-groups *in vitro*. Furthermore, longer pre-incubation times resulted in greater bone volume and graft organisation, but a decrease in bone density. One drawback of this study was the inability to distinguish between the effect of cured hydrogel, and that of BMP-2 inactivation during storage. Both factors affected the study, but we can only hypothesise as to the relative magnitudes of these effects. The results from this study highlight the importance of standardised handling, and the effect of variation in mechanical resistance on determining bone density. An effect, likely to result from enhanced cell infiltration. These differences inspired us in **Paper II** to compare crushed and solid forms of the same hydrogels *in vitro* via the same release study described by Stenfelt et al. using ^{125}I radioactive labelling of BMP-2, and *in vivo* in a rat subcutaneous ectopic bone model. In this study, we discovered dramatically different morphologies in *in vivo* ectopic bone formation for the two gel types, despite virtually identical BMP-2 release profiles observed *in vitro*. Solid hydrogels induced the formation of a dense bone shell surrounding non-degraded hydrogel, while crushed hydrogels demonstrated a uniform bone formation throughout the entire sample. We hypothesised that crushing the hydrogel disrupted the construct's three-dimensional network. This may have exposed unreacted functional groups, making the fragments' surfaces reactive and allowing limited chemical fusion between the crushed hydrogel fragments, leading to similar *in vitro* release profiles. However, enzymes present *in vivo* degrade these bonds, creating a macroporous structure that enhanced cell infiltration, and uniform bone formation throughout the gel. Dadsetan et. al., showed that porous photo-crosslinked hydrogels with 75% porogen content triggered significantly greater ALP expression and Ca^2 deposition by MSC compared with MSCs cultured on tissue culture polystyrene (Dadsetan et al., 2008). Similar results were published by Betz et al. who used macroporous polyethylene glycol hydrogels to up-regulate the expression of BMP-2 and ALP in MSCs, compared to MSCs cultured in non-porous controls (Betz et al., 2010). A technical limitation in this study was the detection of the hydrogel containing HAP, which gave a strong signal in the pQCT with a density of 335 mg/cm^3. Histology revealed a large undegraded core of hydrogel in the solid hydrogel group, and this could be seen in the pQCT data from the solid hydrogel group that had a greater amount of trabecular bone compared to crushed hydrogel. In fact, the hydrogel was measured and interpreted as bone. If it would have been possible to exclude the signal from the hydrogel, there could be a chance of greater differences between the groups in total bone density, total bone content and total bone volume. This problem may be solved when using μCT; however, as the density of the hydrogel is simi-

lar to newly formed rat bone (280 mg/cm^3) distinguishing the two remains challenging.

In Paper III a new, femoral non-critical-sized cortical defect model was developed and evaluated. We drilled, standardised, and elongated unilateral cortical defects in the rat femur, which did not require stabilisation, and could be created bilaterally, permitting paired comparisons of biomaterials. In a time-course experiment, the bone defects were treated with 40 µl of the aforementioned hydrogel loaded with 10 µg HAP (25%), and a dose of 6 µg BMP-2 (150 µg/mL). The defect in the right leg was left untreated as a control, while the left defect was filled with the BMP hydrogel. As determined by pQCT analysis, the treated defects had a greater bone mineral content, bone area, and bone density than the control defects. The relative difference was greatest between the groups at 10 and 20 days, and diminished as the defect healed in the controls, demonstrating that this defect is suitable for detecting of acceleration of bone formation. Bilateral models facilitate greater sensitivity of the test. Melcher was one of the first to describe a uni-cortical defect (Melcher and Irving, 1962); however, the defect was small and empty defects healed at day nine, and began remodelling at day 21. Bay et al. described a uni-cortical defect in dogs similar to our defect, but the defect required metal fixation even though it was not a segmental defect. In conclusion, our model represents an important improvement over previously described cortical defect models.

In **Paper IV,** we used the bone defect model that we developed in **Paper III** to evaluate a slightly different HA hydrogel. In this hydrogel, the hydrazide polyvinyl alcohol was changed to a modified hydrazide HA component where the carboxyl group on the glucoronic acid had been amidated and linked to a hydrazide to allow crosslinking to the aldehyde modified HA component. In addition, 15% of the carboxyl groups had been covalently linked with BP groups. The hydrogel was prepared with the *in situ* entrapped BMP-2. The *in vitro* performance of the BP-grafted hydrogel (HA-BP hydrogel) was investigated in comparison with its analogue lacking BP groups (HA hydrogel). First, we found that, while conventional HA hydrogel releases 100% of BMP-2 over two weeks, less than 10% of the BMP-2 was released from the HA-BP hydrogel according to the enzyme-linked immunosorbent assay (ELISA). Following the release studies, hydrogels were degraded with hyaluronidase (Hase), and the degradation products analysed using BMP-2 specific ELISA. In accordance with the release studies, 10 fold greater amounts of the growth factor were detected among the degradation products of HA-BP hydrogel compared with the digest obtained from enzymatic degradation of the l HA hydrogel control. These results demonstrate the strong binding of the growth factor to HA-BP hydrogel. Moreover, BMP-2 is likely to be bound to BP groups of the hydrogel-derived HA-BP

degradation product, and is thus protected against deactivation. This has been confirmed by ELISA of the growth factor formulated with soluble cross-linkable HA-BP-hydrazide derivative. When equal quantities of BMP-2 were incubated for one hour in pure PBS, and in the buffer containing HA-BP-hydrazide, a greater amount of the growth factor was detected by ELISA in the later. BMP-2 recovered from the hydrogels by Hase-mediated degradation was demonstrated to induce alkaline phosphatase (ALP) expression in MSCs. Bioactivity of BMP-2 recovered from HA-BP hydrogel was greater compared with the bioactivity of the same quantity of BMP-2 liberated from the HA hydrogel control. The hydrogels' degradation products were not toxic to MSCs and osteoblasts at concentrations up to 0.5%. Finally, we have also demonstrated the increased cell adhesion of rat MSCs and osteoblasts on the surface of HA-BP hydrogel compared to tissue culture plastic and on the surface of control HA hydrogel. It seems like a burst release is critical for initiation of fracture healing and if the *in vivo* retention of BMP-2 is similar to *in vitro*, there is a risk that HA-BP hydrogel gives a release that is not sufficient for enhanced bone formation. Bhakta et al. used HA hydrogels functionalised with heparin for controlled release of BMP-2. Their major finding was that a slower release of BMP-2 over longer periods gave 40% less bone compared to the control that lacked heparin and that did not support a sustained release (Bhakta et al., 2012). This shows that the initial burst release of BMP-2 in the first few days is critical for improved bone formation, which corroborates earlier findings (Li and Wozney, 2001, Seeherman and Wozney, 2005). On-going *in vivo* studies will tell if there is a need of tuning the retention of BMP-2 by modifying the HA-BP hydrogel to contain less functional groups of BP.

We wanted to be able to visualise the different responses to the biomaterials and therefore the aim of **Paper V** was to develop a non-invasive method for the visualisation and the quantification of the bone healing process induced by biomaterials. Sodium [^{18}F]fluoride and positron emission tomography (PET) is a non-invasive method for the study of bone remodelling. The high uptake of [^{18}F]fluoride in bone can be explained by the substitution of fluoride for hydroxide and phosphate where mineralisation is occurring (Aoba et al., 2003, Toegel et al., 2006). A small uptake can also be seen in the normal skeleton with an increase in the growth plates of the long bones. Ventura et al. explored PET as a method to monitor *in vivo* response to the release of a BMP-2 from calcium phosphate cements in a calvarial defect. The [^{18}F]fluoride uptake correlated with the bone volume of the de novo bone (Ventura et al., 2014a).

In this study, we combined these two techniques, SPECT and PET to apply a double-isotope approach. We used SPECT to monitor the action of the HAA/PVAH system used as a BMP-2 carrier in **Paper I-III**. SPECT was used to measure the *in vivo* release of BMP-2 that was radiolabelled with iodine-125 targeting tyrosine residues on the BMP protein, according to

Hunter and Greenwood (Hunter and Greenwood, 1962). The osteoblast activity was measured by the [^{18}F]fluoride uptake in PET as the *in vivo* response to the material. The outcome was measured as bone formation in the μCT. A critically sized rat segmental femoral defect was used as a longitudinal *in vivo* model with the advantage of reducing the animal number and eliminating the individual variation between the time points by using each animal as its own control. Combination of SPECT/PET/μCT permitted a detailed non-invasive longitudinal monitoring of bone healing of the critical defect. The presence of the radio labelled BMP-2 was visualised by SPECT and a decrease of the iodine-125 could be detected until week six. During the same period, an increased uptake of [^{18}F]fluoride was observed at the defect site using PET. This was first visible at the second PET scan, two weeks after surgery, with a continuous elevation during the first six weeks, and a continuous uptake seen at the site of the pinholes. This could be due to the mechanical stimulation from the external fixator. Our results corroborate earlier findings where the uptake of [^{18}F]fluoride in calvarial defects was investigated following treatment with calcium phosphate cement discs soaked in 10 μg of BMP-2 (Ventura et al., 2014a). One drawback with this method was the issue of radiation dose. The animals were exposed to radiation from all these methods. Radiolabelled BMP-2 protein, [^{18}F]fluoride, and X-rays from both the CT and μCT scanners would affect the animals, thus making it crucial to minimise the number of scans used in the study. The total exposure for each animal was kept under 1 gray. This has been shown to be a critical radiation dose (Laperre et al., 2011).

Paper VI we hypothesised that "specific *in vitro* parameters or a combination of *in vitro* parameters can be used to predict material *in vivo* outcomes". To answer these questions we conducted a multicentre review with members from the European consortium BioDesign. The data included 36 *in vivo* experiments, 47 *in vitro* experiments and 93 biomaterial variables. The outcome of each variable for each *in vitro* and *in vivo* assay was between 1 = poor, 5 = very good. The *in vitro* and *in vivo* outcomes across the entire dataset were sorted and then categorised according to *in vitro* score. Furthermore, were the *in vitro* outcomes additionally sorted into subgroups representing individual classes of *in vitro* assays. The *in vitro* outcome scores of the material were subsequently correlated with the *in vivo* outcome scores. Further, the *in vitro* assay groups were combined in pairs to investigate if a combination of *in vitro* assays provided a better prediction of *in vivo* outcome over single assays for correlation. Pearson's correlation was used to test for significant linear relationships between the *in vitro* and *in vivo* outcomes with a range of n numbers (n=5-24). Coefficients of determination (R^2) was used to express the percentage of shared variance and a p-value of < 0.05 was considered significant. Surprisingly, the correlation between *in vitro* and *in vivo* outcome was poor, stressing the need for improved *in vitro* assays with higher predictive value.

Summary

Paper I

Hypothesis: Bone induction can be altered by using HAP in combination with a HA and polyvinyl alcohol hydrogel as a carrier for BMP-2 in an ectopic animal model.

Method: We used intra-muscular injections through a double compartment syringe with 0.2 mL hydrogel containing 50 µg CaP and 30 µg BMP-2 cross-linked *in situ*. Five different ceramic additives were tested, including ß-tricalcium phosphate and four types of HAP: nano-sized HAP, HAP, clods of HAP >100 µm and the biomimetic HAP Ostim35. Rats were sacrificed after four weeks. The samples were analysed with pQCT, radiography and histology.

Results and Conclusion: Nano-sized HAP formed bone with greater density compared with other HAP compounds (fig. 9). This could be due to the nano-sized HAP acting as building blocks for the mineralisation.

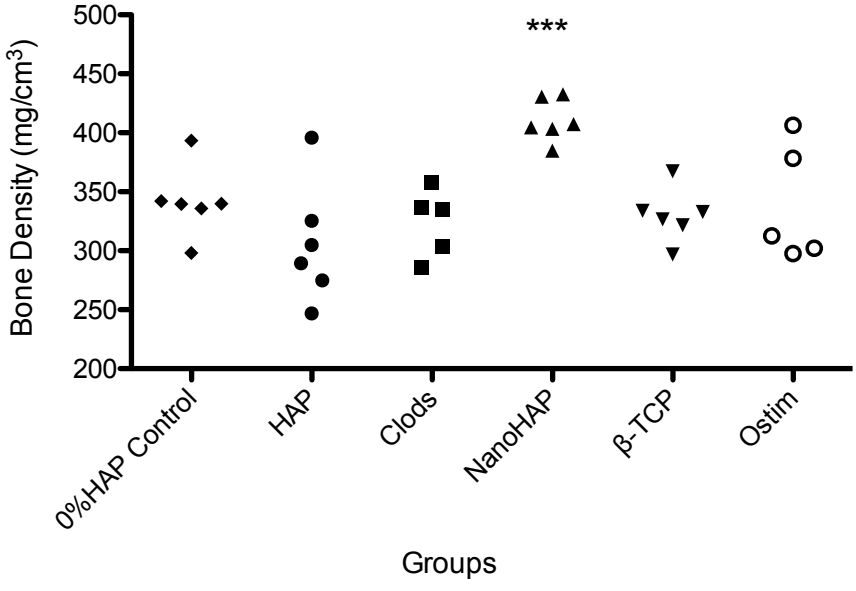

Figure 9. Mean density of ectopic bone four weeks after bilateral injection into rat quadriceps muscle. The specimens were assessed using pQCT. Each value represents one leg. The nanoHAP group had a significantly higher bone density than the other groups (***P = 0.006, ANOVA with Tukey's multiple comparison). Note the sample (384 mg/cm^3) included in NanoHAP, this sample was excluded in the article because it was only observable in 3 pQCT cross sections. The addition of this value resulted in the p-value going from 0.008 to 0.006.

Paper II

Hypothesis: Crushed hydrogels will induce greater bone formation than solid hydrogels, due to easier cell infiltration and the shorter diffusion distance required for the release of BMP-2, as well as resulting from the increase in surface area.

Method: Hydrogel of 0.2 mL containing 50 µg CaP and 30 µg BMP-2 was used. Crushed and solid forms of the hydrogels were analysed *in vitro* via a release study using ^{125}I radioactive labelling of BMP-2, and *in vivo* in a subcutaneous ectopic bone model in rats.

Results and Conclusion: Dramatically different morphologies were observed for the ectopic bone formed *in vivo* in the two types of gels, even though virtually identical release profiles were observed *in vitro*. Solid hydrogels induced the formation of a dense bone shell surrounding non-degraded hydrogel, while crushed hydrogels demonstrated uniform bone

formation throughout the entire sample (fig. 10). Crushing of the hydrogel facilitated the cell migration and bone formation.

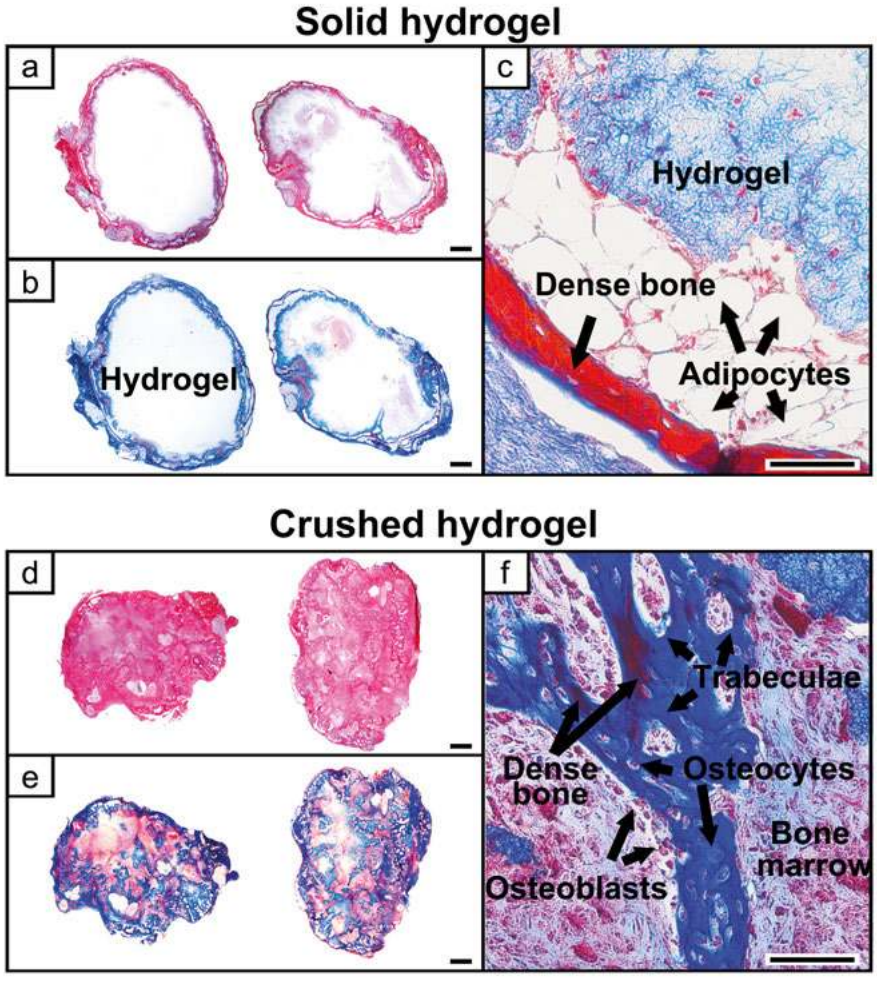

Figure 10. Histological sections of explants after 28 days, stained with hematoxylin/eosin (a, d) and Masson's Trichrome (b, c, e, f). Solid hydrogels formed a dense shell of bone with large amount of undegraded hydrogel inside (a, b). Bone shell with mineralised bone, adipocytes and collagen network emerged in the hydrogel (c). Crushed hydrogel formed dense bone in the whole sample (d, e). Newly formed trabeculae of bone with bone marrow are visible in (f).

Paper III

Hypothesis: A femoral non-critical-sized cortical defect rat-model could be used to measure the differences in bone formation between defects filled with BMP-2 hydrogel and empty defects.

Method: Elongated uni-cortical defects with a size of 6 x 2 mm were created in the shaft of rat femurs. No stabilisation was required and bilateral defects could be used to allow paired comparisons. Defects were filled with either 40 µl hydrogel with 10 µg HAP and 6 µg BMP-2 (left leg) or left untreated as a control (right leg). At 10, 20, 30 and 40 days, the rats were sacrificed and their femurs were evaluated by radiography, pQCT and histology.

Results and Conclusion: The treated defects had a greater bone mineral content, bone area and bone density than the control defects. Relative difference was greatest between the groups at 10 and 20 days and diminished as the defect healed in the controls. Histology revealed a distinction in bone healing between the hydrogel treated defects and the controls (fig. 11). We conclude that this animal model allows the assessment of biomaterials for bone regeneration in cortical femoral defects without requiring fixation.

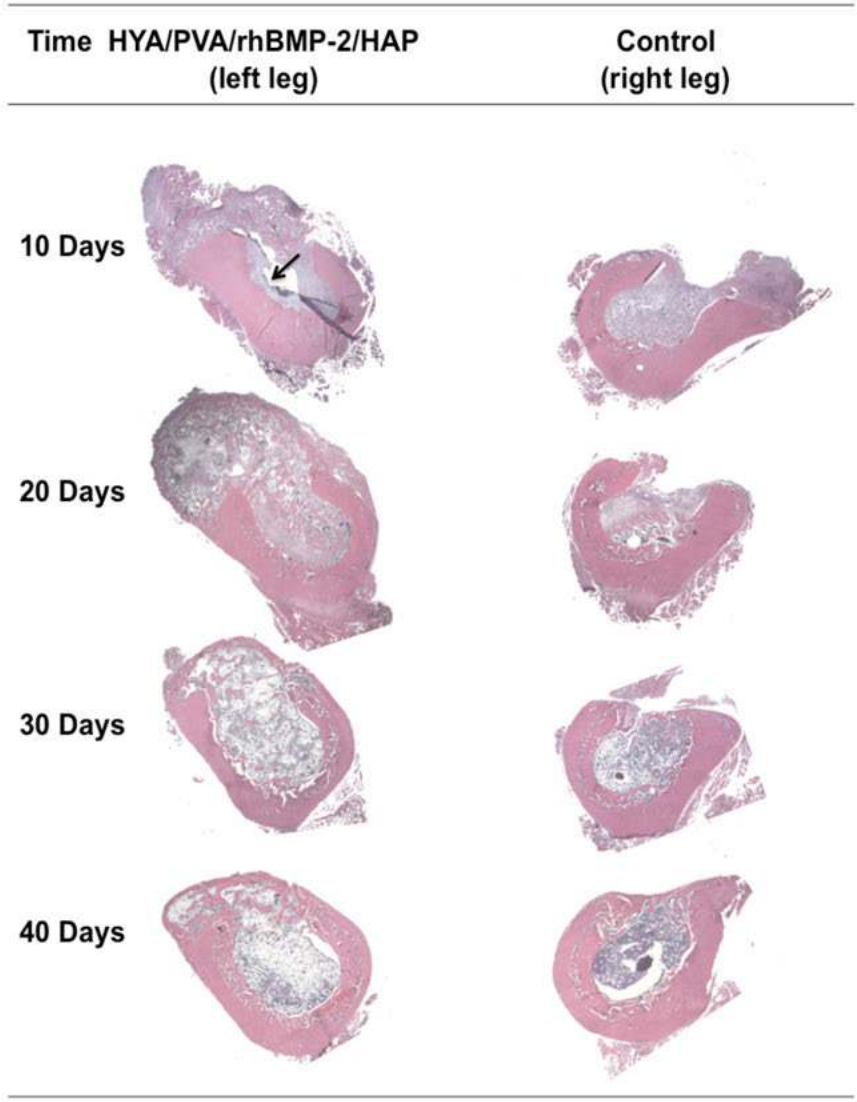

Figure 11. Histological analysis of bone formation at the defect site. Cross-sections of defects at 10, 20, 30 and 40 days were stained with H&E. The large external callus formation seen in HYA–PVA–rhBMP-2–HAP hydrogel-treated specimens at early time points was remodelled during the study period. Remnants of HYA–rhBMP-2–HAP could only be seen at day 10 (light-blue spaces at the arrows). Extensive and early extra-medullary callus formation was seen in HYA–PVA–rhBMP-2–HAP hydrogel-treated specimens, whereas a slow endosteal and intramedullary bone formation occurred in the controls.

Paper IV

Hypothesis: Bisphosphonate covalently linked to hyaluronan hydrogel may retain greater quantities of BMP-2 compared to its BP lacking analogue.

Method: The *in vitro* performance of the hydrogel containing BP was compared with its BP lacking analogue using ELISA, a biocompatibility test (MTS assay), and assessment of osteoinduction with the ALP assay.

Results and Conclusion: Covalently linked BP resulted in a dramatically slower release of BMP-2, while there was no change in the biocompatibility when compared with hydrogel lacking BP (fig. 12).

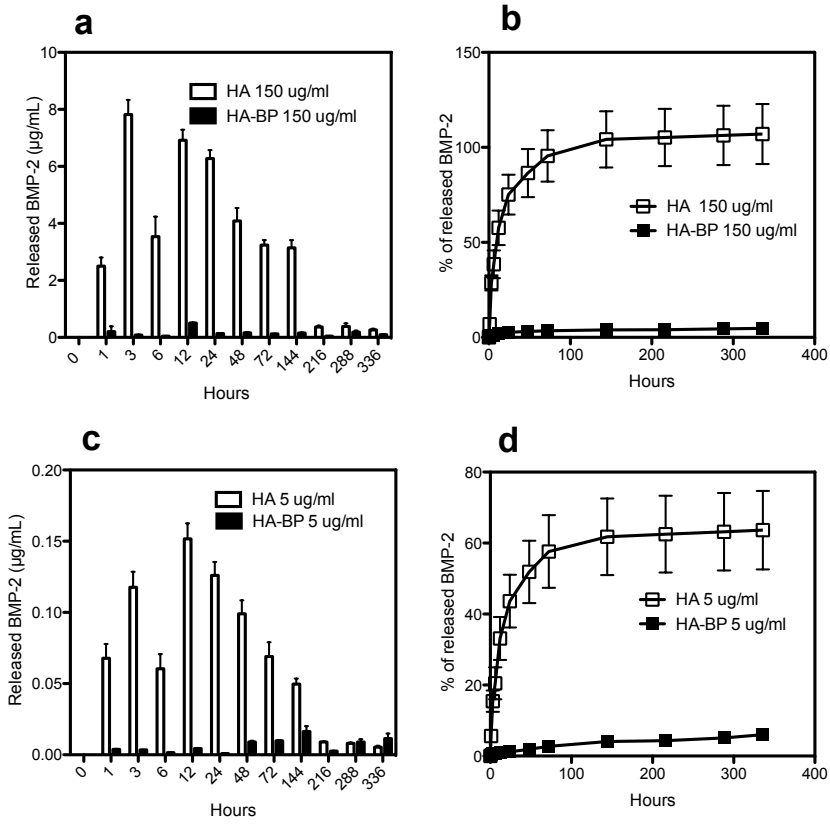

Figure 12. The release of BMP-2 from high- and low-dose of BMP-2 from HA hydrogels (white) and HA-BP hydrogels (black). Mean concentration ± SD of BMP-2 in the samples of release medium obtained at different time points of incubation of the high-dose BMP-2 hydrogels (a) and low-dose BMP-2 hydrogels (c). The cumulative release profile of the entire amount of BMP-2 from the high-dose BMP-2 hydrogels (b) and the low-dose BMP-2 hydrogels (d).

Paper V

Hypothesis: To develop a double-isotope approach using SPECT to monitor the *in vivo* release of iodine radiolabelled BMP-2 from a hyaluronan hydrogel and PET to visualise the osteoblast activity.

Methods: SPECT measured the *in vivo* release of radiolabelled BMP-2. Osteoblast activity was measured by the [^{18}F]fluoride uptake in PET as the *in vivo* response to the release of [^{125}I]BMP-2 from the hydrogel. The hydrogel with and without [^{125}I]BMP-2 was tested in a critically sized rat-femoral with the endpoint bone formation at the defect assessed with µCT over a 12 week study duration.

Results and Conclusion: Our functional, non-invasive imaging method permitted visualisation of the retention of BMP-2 and the resulting *in vivo* response, and should hereby be considered a novel tool for investigation of bone regeneration using smart biomaterials (fig. 13).

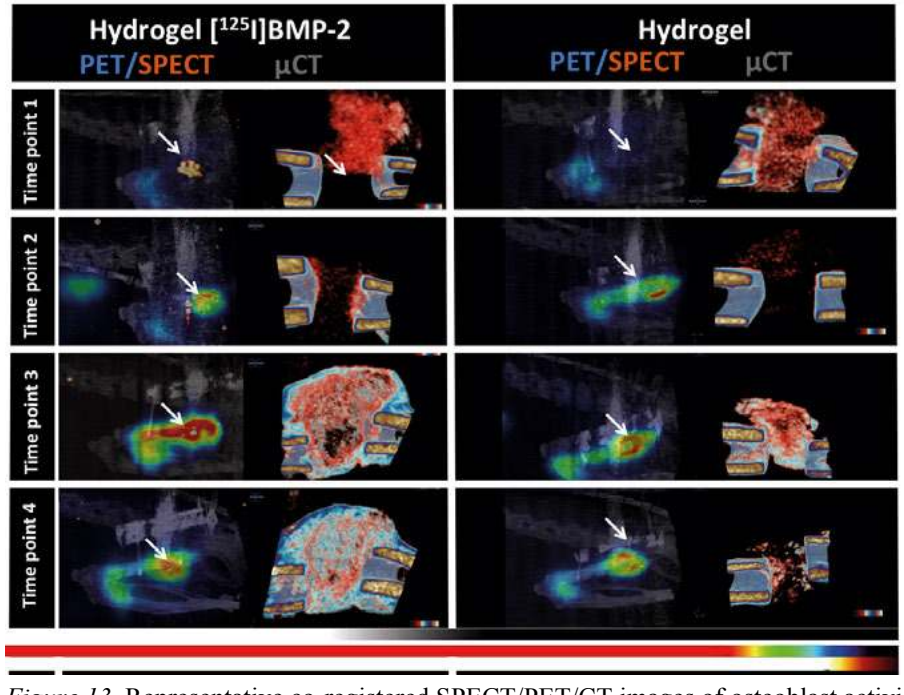

Figure 13. Representative co-registered SPECT/PET/CT images of osteoblast activity in femoral defects filled with hydrogel+BMP-2 at day 3, week 2, 4 and 6 in a segmental femoral defect. To the left are µCT images that display the formed bone in close time frame to the PET/SPECT imaging. µCT scans were undertaken at day 0 and week 1, followed by forth-nightly scans, the threshold of the image was set to include low densities in order to display the material post-surgery.

Paper VI

Hypothesis: Specific *in vitro* parameters or a combination of *in vitro* parameters can be used to predict material *in vivo* outcomes.

Methods: The data included 36 *in vivo* experiments, 47 *in vitro* experiments and 93 biomaterial variables. The outcome of each variable for each *in vitro* and *in vivo* assay was between 1 = poor, 5 = very good. The *in vitro* and *in vivo* outcomes across the entire dataset were sorted and then categorised according to *in vitro* score. Furthermore, were the *in vitro* outcomes additionally sorted into subgroups representing individual classes of *in vitro* assays. The *in vitro* outcome scores of the material were subsequently correlated with the *in vivo* outcome scores. Further, the *in vitro* assay groups were combined in pairs to investigate if a combination of *in vitro* assays provided a better prediction of *in vivo* outcome over single assays for correlation. Pearson's correlation was used to test for significant linear relationships between the *in vitro* and *in vivo* outcomes with a range of n numbers (n=5-24). Coefficients of determination (R^2) was used to express the percentage of shared variance and a p-value of < 0.05 was considered significant.

Results and Conclusion: The hypothesis "specific *in vitro* parameters or a combination of *in vitro* parameters can be used to predict material *in vivo* outcomes" was rejected. Surprisingly, the correlation between *in vitro* and *in vivo* outcome was poor, stressing the need for improved *in vitro* assays with higher predictive value.

Acknowledgement

Först av allt vill jag tacka min käre handledare, Sune Larsson. Sune, här kommer en varning för att texten nedan kan kännas obehaglig för en gammal norrlänning; Tack Sune för att du har varit världens bästa handledare. Du är min stora förebild och det är ofta jag tänker ”hur skulle Sune göra?” när jag befinner mig i kniviga situationer. Du har försökt lära mig att ha tålamod och ödmjukhet och du har visat att man kan vara både ödmjuk, generös och anspråkslös utan att tappa integritet och respekt. Du kommer säkert ihåg att jag har råkat jämföra dig med Örnen nebulosan och till din stora förfäran, så gör jag det fortfarande. Orsaken är att du har gett mig en fantastisk frihet och samtidigt en trygg plats där du och Brittmarie har funnits precis lagom tillhands för att låta mig finna min egen gravitation och hitta mitt eget spinn. Nu är det upp till mig att visa att jag kan bli mer än ett virvlande dammoln.

Jag vill också tacka mina underbara bihandledare som alla har sina fantastiska egenskaper. Jöns, du har tagit mig under dina vingar och du har lotsat mig in i ditt enorma nätverk av fantastiska labb och coola forskare. Utan dig och din entusiasm hade jag aldrig ens kommit på tanken att vilja doktorera. Tack för att du har varit min forskarpappa genom åren. Kenneth, tack för att din dörr alltid har stått öppen så man har kunnat sticka in huvudet och säga precis vad man känner eller tänker, om det så var ångest eller glädje. Håkan, tack för att du har lärt mig om strategi, jag har alltid beundrat hur du har kunnat jobba strategiskt och samtidigt varit öppen och rakt på sak. Richard, tack för att du har hoppat in och ställt upp när jag har behövt dig. Jag hoppas vi kan hitta på lite roliga studier i framtiden.

Nu till min underbara livscoach och kompanjon genom dessa 4 år, Brittmarie. Jag vill tacka dig för att du har varit min sidekick. Du har snällt tagit på dig Robin-rollen och jag har gladeligen gått runt och låtsats som Batman. Du har genom åren agerat som en förstaklassig labbtekniker som har lärt mig allt det praktiska på labbet samtidigt som du också har varit min extramamma, dryckespartner och mycket goda och nära vän. Du har dessutom räddat mig från att tillbringa alla jular på labbet då du varje november snällt påmint mig om att vi inte ska starta en ny *in vivo* studie i vecka 52; Viggo tackar och hälsar. Du ska veta att jag inte kommer släppa taget om dig!

Mina fina studenter Elin, Pik Kwan, Nick, Björn och Kia utan er hade jag aldrig lyckats få ihop dessa artiklar ni har alla varit enastående och ett mycket bättre alternativ än mitt första förslag till Sune som involverade kloning.

Ni har haft så många goda idéer, kommit med lösningar och ifrågasatt mina idéer, vilket har gjort våra projekt så mycket bättre. Särskilt Tack till Elin som har gått från student till min kära labbsyster. Du är underbar och jag saknar våra ”djupa” samtal över för mycket vin.

Min fina labbrorsa Anders, tack för att du kommer in med all din energi, glädje och fantastiska idéer och anekdoter. Vilket futtigt labb vi hade haft utan alla dina uppfinningar. Det spretiga Benlabbsgänget; Brittmarie, Elin, Katarina, Nils, Nikos, Navya, Annica, Thomas, Östen, Håkan, Andreas, Monica och Margareta; Tack för att vi har hållit ihop och gett varandra känslan av gemenskap som en grupp fast vi har jobbat med så olika saker i olika grupper. Jag har verkligen uppskattat alla benmöten vi haft, den glada och positiva stämningen och sättet vi har hjälpt varandra med lösningar, nya spännande artiklar eller en klapp på axeln när det har känts tufft. Länge leve Bengänget! Ett särskilt tack till Nils Hailer, tack för att du alltid har haft tid för att svara på frågor och lagt tid på mig!

Alla galningar FoA3. Ni har förgyllt mina raster med en massa hetsiga diskussioner (ja, Maija ta åt dig) och retsamma kommentarer (Hans, Lotta, Lina, Elin, Hanna, Brittmarie, Olle, Carin, Eva, Ann och Bettan, ta åt er allihop), goda kakor och råd om protokoll (Anna Lena och Inger). Det har varit ett rent nöje att hänga med er. Extra Tack till Olle för våra legendariska fajv vajners. Jag vill tacka Susanne, Reza och Siv för att ni på något magiskt vis (kanske för att det alltid varit så tidigt på morgonen, att jag aldrig har sett er) har städat och skämt bort oss forskare med rena och sterila instrument. Med er support har det varit en ren lyx att labba!

Hela Polymer-teamet på Ångström, tack för att ni har lärt mig så mycket och att vi har haft så många fina samarbeten. Jag vill särskilt tacka Cecilia för att du lärt mig allt om cellkultur. Sonya, för att du lärt mig allt annat man behöver veta om allt inom forskning. Jonas för shotsen i Budapest. Kristoffer, Tim, Ida och Sujit för roligt skitsnack om både forskning och allt och alla. Dmitri och Oommen för alla härliga ivriga diskussioner vi har haft om forskning och nya studier. Alla ni på Materials in Medicin på Ångström, tack för fina samarbeten men också för goda burgare på Bullit.

Jag vill särskilt rikta ett tack från Mr Hulsart-Billstrom till Miss Ohman för alla trevliga… var det hotell eller? Vi har bott billigt, i alla fall på våra jobbresor och snälla lär dig tyska för guds skull! Även tack till Mr Öhman-Mägi för att se åt andra hållet när frugan “varit på jobbresa”.

Min kära Mentor Cecilia, ja fatta du är min mentor om du så vill det eller inte. Jag har lärt mig jättemycket av dig och dina tips har varit till stor nytta, OCH du blir inte av med mig! Mentor for LIFE!

Serre, Vonkan, kRam, MagicMark och Räven på Preklinisk PET och PET centrum, Tack för att jag har fått känna mig så hemma hos er! Jag har verkligen stortrivts i ert kök och skulle lätt kunna komma förbi en dag bara för att få sitta där och skoja med er. Särskilt tack till kRam som har varit en Superb Co-författare och räddare i nöden.

Jag vill tacka Iors förskola för att ni har tagit så otroligt bra hand om min son. Ni har gett mig en gnutta mindre dåligt samvete för Viggos ibland långa dagar, då jag har vetat att han har varit i goda händer med fantastiska människor som har utvecklat och utmanat honom samtidigt som ni låtit honom få känna sig omtyckt och respekterad.

Tack till mina underbara vänner! Jag älskar hur vi har kunnat hjälpa och stötta varandra samtidigt som vi kunnat glädjas åt våra framgångar både privat och professionellt. Ni är en viktig del av mitt liv, där oförskönad ärlighet och öppenhet har varit grunden. Jag är så glad att jag har er och att jag kan dela hela mig och mina funderingar med er. Anna Sara, tid biter inte på vår vänskap även om vi har levt väldigt olika liv så har vi alltid haft en massa gemensamt. Liza och Elvira, er vänskap har varit som ett elixir, ni har gett mig så otroligt mycket energi och glädje. Liza du är helt jävla otrolig! Jag är såååååå stolt över dig! Tur att du finns i mitt liv och lämna mig aldrig! Lina, tack för att du har delat din familj och släkt med mig och fått mig att känna mig som en riktig Uppsaliensare. Jocke och Fredrik, tack för att jag kan vara mitt värsta jag med er, alltså ettrig, grov och allmänt barnslig.

A big special thank you to the Bone and Joint group! Richard for teaching me so much and being my biggest role model! Ines Moreno for just giving me so much energy and so much tough love. May, Gibbs and Nick for being a role model in work and life and for showing that you can be caring, intelligent and dedicated without compromising your own personality. Edo, Ewa and Stef for being so loving, caring and… sarcastic. I admire you all tremendously and I have learned so much from all of you!

Leila and Patrick for adopting us and showing us the best of Hampshire and British culture. Banistor School, with Mr. Cook and Mr. Balcombe for giving my son so much knowledge, while I was at work.

Jag vill tacka Wictors familj för trevliga middagar och mysiga stunder. Särskilt vill jag tacka Pia som har ställt upp i ur och skur som jourhavande farmor/skräddare/stylist/juvelerare.

Min galna familj med Mathias, Line, Anja, Toffe , Kira, Louie, Tack för att ni är så förbannat jobbiga och alltid har åsikter, ifrågasätter, lägger er i och bryr er. Ni ger mig perspektiv på livet och jag älskar er för det. Tack Anja för att du har varit min extramamma genom åren och Tack Kira för att du är lika mycket min nära vän som syster. Tack mamma för att du alltid har hoppat upp och tagit tag i närmsta uppslagsverk direkt jag hade en fråga som liten. Du har visat mig att all kunskap finns bara en bok bort och att om man frågar får man svar. Pappa, tack för att du alltid finns där, ett samtal bort och att du förstår hur jag tänker, även när jag är arg. Du har genom åren förvandlats till en god vän och självutnämnd kapten på… var det min båt?

Tack för att ni två har ställt upp till tusen för vår familj, med barnpassning, matlagning, korrekturläsning och en och annan uppgift på labbet. FAMILJEN HULSART ÄGER!!!!

Sist men allra mest vill jag tacka min alldeles egna familj, Wictor, Viggo och Snoddas. Wictor, tack för att du mestadels genom åren har orkat med mig. Jag vet att jag inte är lätt att leva med. Tack för att du har funnits där när jag har varit en liiiiiten blöt fläck men även när jag har haft storhetsvansinne och trott att jag var Napoleon själv. Jag älskar dig för att du inte har backat för en karriärstokig kvinna utan sett mig som en parhäst där du och jag har axlat livets ekipage och rusat mot vår framtid och framgång. Du är min största kritiker och du är den som har fått mig att växa allra mest som person. Jag älskar dig precis hur mycket som helst! Viggo min älskade son, tack för alla trevliga pratstunder vi har haft vid alla nattningar där vi ibland råkat prata lite för länge om allt från ljus och genomskinlighet till döden. Förhoppningsvis får jag natta dig några år till! Jag vill också tacka dig för ditt enorma tålamod alla de gånger du har följt med på jobbet, varit med på möten och föreläsningar, skannat diverse prov och alla gånger jag har suttit med näsan nere i datorn och inte hört vad du har sagt. Du har fått betala ett stort pris för att jag ville bli doktor. Jag ser framemot att få se dig växa vidare och utvecklas till den coola starka och intelligenta person du redan har visat att du är. Jag älskar dig av hela mitt hjärta. Sist men inte minst Snoddas, tack för att du har värmt mina fötter, mage och hjärta i alla år.

Sammanfattning på svenska

Fördröjd eller utebliven läkning av benbrott och utfyllnad efter stora bendefekter är tillstånd som vållar både lidande och stora kostnader. Det är därför två mycket angelägna problem att hitta lösningar till. På senare år har konstgjort ben och ämnen som stimulerar ben-nybildning utvecklats och ett flertal produkter finns numera i kliniskt bruk. Den prekliniska ortopediska forskningen har på senare år fått se en utveckling med fokus på nya behandlingsmöjligheter med injicerbara kroppsegna material för applikation av läkningsstimulerande ämnen. Det övergripande syftet med denna avhandling är att vidareutveckla detta område och därmed bidra till lösningen av ett betydande problem; att skapa ben där det saknas och åstadkomma läkning där denna uteblivit. Sedan lång tid är den etablerade metoden att transplantera ben från en annan del av skelettet till området där benläkningen har uteblivit. Man kan då packa in benflisor tagna från till exempel höftbenskammen hos den aktuella patienten för att stimulera till läkning eller fylla ut defekten. Med denna metod får man både utfyllnadsmaterial och benläkningsstimulans via de transplanterade bencellerna. Metoden har dock begränsningar vad gäller både benmängd och möjligheterna att på ett förutsägbart sätt åstadkomma tillräcklig benbildning. Dessutom skapas onödigt lidande i form av smärta från platsen där benet tas ifrån.

Regenerativa produkter innehållande naturliga tillväxtfaktorer för ben praktiseras kliniskt idag. Dessa produkter administreras lokalt vid benet med hjälp av en kollagenbärare som ska underlätta appliceringen på rätt plats och bibehålla rätt mängd samtidigt som nedbrytning ska undvikas. De bärare som finns idag fungerar inte tillfredsställande av flera orsaker: de kan inte injiceras utan de måste tillföras vid en öppen operation, bärarna är också svåra att hantera på grund av att deras konsistens inte är optimal. Önskvärt vore därför en injicerbar bärare som bibehåller sin position där man önskar få effekten av det benläkningsstimulerande ämnet och dessutom frisätter det i en kontrollerad takt. Hyaluronsyrabaserade polymerer, så kallade hydrogeler, är ett lovande alternativ då de kan uppfylla dessa krav. De liknar den naturliga extracellulära matrisen vilket är byggnadsmaterialet mellan cellerna som återfinns i bindväv hos alla människor och djur. De kan dessutom tjäna som mall för benbildande celler att bygga vidare på samtidigt som de kan brytas ner av naturliga mekanismer i kroppen. I detta avhandlingsarbete har den benbildande förmågan hos olika former av injicerbara hyaluronsyrabaserade biomaterial innehållande läkningsstimulerande ämnen studerats.

Då benläkningen är beroende av såväl bäraren som den aktiva substansen blev arbetet en kartläggning av de båda. Fokus låg dock på att optimera bärarens egenskaper för att kunna reducera mängden av läkningsstimulerande ämnen. Biomaterialet består av en hyaluronsyrapolymer som är uppbyggt av ett tvåkomponentssystem. Detta system kan antingen appliceras via en injektion där man blandat vätskorna innan eller en dubbelspruta där vätskorna blandas vid injektionsögonblicket i sprutpipen. Komponenterna var modifierade med kemiska kopplingar, så kallade hydrazider och aldehyder, som reagerar med varandra vid kontakt, det vill säga polymeriserar, och bildar vatten som biprodukt. Den aktiva molekylen bone morphogenetic protein (BMP-2), som är en naturlig tillväxtfaktor för ben, tillsätts i en av komponenterna och blandas ut i hydrogelen under blandningen av de två komponenterna. Efter injektion polymeriserar vätskan till fast gel som frisätter BMP-2 under en längre period samtidigt som det kroppsegna systemet ersätter gelen med nybildat ben. Man kan även tillsätta ämnen till hydrogelerna och i detta fall innehåller hydrogelen även 25 % hydroxyapatitpulver, vilket har påvisat en positiv effekt på benläkning. Detta studerades i delarbete I, där hydroxyapatitpulvret byttes ut mot hydroxyapatit i nanostorlek vilket gav en positiv effekt på benbildning genom att ge benet en tätare karaktär. För att då kunna utröna effekten av de olika materialegenskaperna var själva hanteringen av hyaluronsyrapolymeren viktig att utvärdera och standardisera. I delarbete II studerades de positiva effekterna av att låta materialet förpolymeriseras innan injektion vilket gav en markant skillnad i benbildning. Detta doktorsarbete har genomsyrats av Russell och Burchs principer om de 3 R:en. Refinement innebär förfining av djurmodeller för att minska stress och lidande och höja den vetenskapliga precisionen. Reduction står för att minska antalet djur som används och Replacement syftar till att i mån av möjlighet kunna byta ut djurmodeller mot till exempel provrörsförsök. Vår strävan har varit att förfina djurmodellerna för att minimera eller helt utesluta stress hos djuren och samtidigt höja den statistiska styrkan samtidigt som man reducerar antalet djur. Detta lyckades vi med i delarbete III där vi utvecklade en lårbensdefekt i form av ett expanderat borrhål, ett så kallat unikortikalt fönster, som inte behövde stabiliseras med fixatorer och kunde appliceras i båda lårbenen. Detta möjliggjorde en jämförelse mellan de olika materialen i samma individ, vilket i sin tur gav en högre statistisk styrka och ett kraftigt minskat antal försöksdjur per studie. Genom att individen var sin egen referens blev modellen känsligare för små förändringar i materialets egenskaper såsom tillsatsen av andra benläkningstimulerande ämnen. Efter delarbete III fanns en klar bild av hur hanteringen kunde optimera materialets effekt och ett naturligt nästa steg var då att i delarbete IV minska mängden av det aktiva ämnet BMP-2 och kontrollera frisättningen av ämnet från hydrogelen. Detta gjordes genom att utrusta gelen med bisfosfonat som var kovalent bundet till hyaluronsyrapolymeren. Bisfosfonaten gav en tydlig skillnad genom en minskad och kontrollerad

frisättning av det benläkningsstimulerande ämnet. Vi hade nu ett verktyg för att kunna kontrollera frisättningen och detta gav upphov till en önskan att både kunna följa frisättningen och registrera den kroppsegna responsen på det benläkningsstimulerande ämnet. I delarbete V utvecklades en ny metod för att samtidigt kunna följa frisättningen av det benläkningsstimulerande ämnet och nybildningen av ben över tid. Detta möjliggjordes genom att använda två radioaktiva markörer som kunde detekteras med hjälp av single photon emission computed tomography och positron emission tomography. Sammanfattningsvis, frågade vi oss om resultat från cellstudier på biomaterial för ben, kunde förutspå materialets effekt i djurstudier. I delarbete VI samlade vi data på olika biomaterial från ett europeiskt projekt, och korrelerade, resultat från cellstudier och djurstudier på samma material. Studien visade däremot väldigt liten korrelation mellan cell och djurstudier.

Denna avhandling har bidragit med mer kunskap om hur man kan förbättra och justera injicerbara biomaterial för att optimera benläkningsförmågan. Resultaten i denna avhandling kan ligga till grund för ytterligare förbättringar av biomaterial för benbildning som i en nära framtid kan appliceras kliniskt.

References

Addison, WN, Azari, F, Sorensen, ES, Kaartinen, MT & McKee, MD. 2007; Pyrophosphate inhibits mineralization of osteoblast cultures by binding to mineral, up-regulating osteopontin, and inhibiting alkaline phosphatase activity. *J Biol Chem,* 282**:** 15872-83.

Aoba, T, Shimazu, Y, Taya, Y, Soeno, Y, Sato, K & Miake, Y. 2003; Fluoride and apatite formation in vivo and in vitro. *J Electron Microsc (Tokyo),* 52**:** 615-25.

Asonova, SN & Migalkin, NS. 1996; [Use of Masson's trichrome method for staining decalcified bone tissue]. *Arkh Patol,* 58**:** 66-7.

Baron, R & Kneissel, M. 2013; WNT signaling in bone homeostasis and disease: from human mutations to treatments. *Nat Med,* 19**:** 179-92.

Barradas, AM, Yuan, H, van Blitterswijk, CA & Habibovic, P. 2011; Osteoinductive biomaterials: current knowledge of properties, experimental models and biological mechanisms. *Eur Cell Mater,* 21**:** 407-29; discussion 429.

Bay, BK, Martin, RB, Sharkey, NA & Chapman, MW. 1993; Repair of large cortical defects with block coralline hydroxyapatite. *Bone,* 14**:** 225-30.

Ben-Ami Bartal, I, Decety, J & Mason, P. 2011; Empathy and pro-social behavior in rats. *Science,* 334**:** 1427-30.

Bergman, K, Engstrand, T, Hilborn, J, Ossipov, D, Piskounova, S & Bowden, T. 2009; Injectable cell-free template for bone-tissue formation. *J Biomed Mater Res A,* 91**:** 1111-8.

Bering, J 2012. The Rat that Laughed. *Scientific American.* Nature Publishing Group.

Betz, MW, Yeatts, AB, Richbourg, WJ, Caccamese, JF, Coletti, DP, Falco, EE & Fisher, JP. 2010; Macroporous hydrogels upregulate osteogenic signal expression and promote bone regeneration. *Biomacromolecules,* 11**:** 1160-8.

Bhakta, G, Rai, B, Lim, ZX, Hui, JH, Stein, GS, van Wijnen, AJ, Nurcombe, V, Prestwich, GD & Cool, SM. 2012; Hyaluronic acid-based hydrogels functionalized with heparin that support controlled release of bioactive BMP-2. *Biomaterials,* 33**:** 6113-22.

Bonewald, LF. 2006; Mechanosensation and Transduction in Osteocytes. *Bonekey Osteovision,* 3**:** 7-15.

Bryant, D, Havey, TC, Roberts, R & Guyatt, G. 2006; How many patients? How many limbs? Analysis of patients or limbs in the orthopaedic literature: a systematic review. *J Bone Joint Surg Am,* 88**:** 41-5.

Cai, Y, Liu, Y, Yan, W, Hu, Q, Tao, J, Zhang, M, Shi, Z & Tang, R. 2007; Role of hydroxyapatite nanoparticle size in bone cell proliferation. *Journal of Materials Chemistry,* 17**:** 3780–3787

Carragee, EJ, Hurwitz, EL & Weiner, BK. 2011; A critical review of recombinant human bone morphogenetic protein-2 trials in spinal surgery: emerging safety concerns and lessons learned. *Spine J,* 11**:** 471-91.

Chang, E-J, Kim, HJ, Ha, J, Kim, HJ, Ryu, J, Park, K-H, Kim, U-H, Lee, ZH, Kim, H-M, Fisher, DE & Kim, H-H. 2006; Hyaluronan inhibits osteoclast differentiation via Toll-like receptor 4. *Journal of Cell Science,* 120**:** 166-176.

Chappuis, V, Gamer, L, Cox, K, Lowery, JW, Bosshardt, DD & Rosen, V. 2012; Periosteal BMP2 activity drives bone graft healing. *Bone,* 51**:** 800-9.

Cremers, SC, Papapoulos, SE, Gelderblom, H, Seynaeve, C, den Hartigh, J, Vermeij, P, van der Rijt, CC & van Zuylen, L. 2005; Skeletal retention of bisphosphonate (pamidronate) and its relation to the rate of bone resorption in patients with breast cancer and bone metastases. *J Bone Miner Res,* 20**:** 1543-7.

Dadsetan, M, Hefferan, TE, Szatkowski, JP, Mishra, PK, Macura, SI, Lu, L & Yaszemski, MJ. 2008; Effect of hydrogel porosity on marrow stromal cell phenotypic expression. *Biomaterials,* 29**:** 2193-202.

Dimitriou, R, Mataliotakis, GI, Angoules, AG, Kanakaris, NK & Giannoudis, PV. 2011; Complications following autologous bone graft harvesting from the iliac crest and using the RIA: A systematic review. *Injury,* 42 Suppl 2**:** S3-S15.

Docherty-Skogh, AC, Bergman, K, Waern, MJ, Ekman, S, Hultenby, K, Ossipov, D, Hilborn, J, Bowden, T & Engstrand, T. 2010; Bone morphogenetic protein-2 delivered by hyaluronan-based hydrogel induces massive bone formation and healing of cranial defects in minipigs. *Plast Reconstr Surg,* 125**:** 1383-92.

Einhorn, TA & Gerstenfeld, LC. 2015; Fracture healing: mechanisms and interventions. *Nat Rev Rheumatol,* 11**:** 45-54.

Einhorn, TA, Lane, JM, Burstein, AH, Kopman, CR & Vigorita, VJ. 1984; The healing of segmental bone defects induced by demineralized bone matrix. A radiographic and biomechanical study. *J Bone Joint Surg Am,* 66**:** 274-9.

Fraser, JR, Laurent, TC & Laurent, UB. 1997; Hyaluronan: its nature, distribution, functions and turnover. *J Intern Med,* 242**:** 27-33.

Garrison, KR, Donell, S, Ryder, J, Shemilt, I, Mugford, M, Harvey, I & Song, F. 2007; Clinical effectiveness and cost-effectiveness of bone morphogenetic proteins in the non-healing of fractures and spinal fusion: a systematic review. *Health Technol Assess,* 11**:** 1-150, iii-iv.

Giannoudis, PV, Dinopoulos, H & Tsiridis, E. 2005; Bone substitutes: an update. *Injury,* 36 Suppl 3**:** S20-7.

Giannoudis, PV, Einhorn, TA & Marsh, D. 2007; Fracture healing: the diamond concept. *Injury,* 38 Suppl 4**:** S3-6.

Gothard, D, Smith, EL, Kanczler, JM, Rashidi, H, Qutachi, O, Henstock, J, Rotherham, M, El Haj, A, Shakesheff, KM & Oreffo, RO. 2014; Tissue engineered bone using select growth factors: A comprehensive review of animal studies and clinical translation studies in man. *Eur Cell Mater,* 28**:** 166-207; discussion 207-8.

Harding, AK, Aspenberg, P, Kataoka, M, Bylski, D & Tagil, M. 2008; Manipulating the anabolic and catabolic response in bone graft remodeling: synergism by a combination of local BMP-7 and a single systemic dosis of zoledronate. *J Orthop Res,* 26**:** 1245-9.

Hoang, QQ, Sicheri, F, Howard, AJ & Yang, DS. 2003; Bone recognition mechanism of porcine osteocalcin from crystal structure. *Nature,* 425**:** 977-80.

Hunter, WM & Greenwood, FC. 1962; Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature,* 194**:** 495-6.

Ishiyama, S & Brecht, M. 2016; Neural correlates of ticklishness in the rat somatosensory cortex. *Science,* 354**:** 757-760.

Jensen, ED, Pham, L, Billington, CJ, Jr., Espe, K, Carlson, AE, Westendorf, JJ, Petryk, A, Gopalakrishnan, R & Mansky, K. 2010; Bone morphogenic protein 2 directly enhances differentiation of murine osteoclast precursors. *J Cell Biochem,* 109**:** 672-82.

Jia, X, Colombo, G, Padera, R, Langer, R & Kohane, DS. 2004; Prolongation of sciatic nerve blockade by in situ cross-linked hyaluronic acid. *Biomaterials,* 25**:** 4797-804.

Junqueira, LC, Bignolas, G & Brentani, RR. 1979; Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochem J,* 11**:** 447-55.

Kanakaris, NK & Giannoudis, PV. 2007; The health economics of the treatment of long-bone non-unions. *Injury,* 38 Suppl 2**:** S77-84.

Karp, JM & Leng Teo, GS. 2009; Mesenchymal stem cell homing: the devil is in the details. *Cell Stem Cell,* 4**:** 206-16.

Keefe, J, Wauk, L, Chu, S & DeLustro, F. 1992; Clinical use of injectable bovine collagen: a decade of experience. *Clin Mater,* 9**:** 155-62.

Kempen, DH, Yaszemski, MJ, Heijink, A, Hefferan, TE, Creemers, LB, Britson, J, Maran, A, Classic, KL, Dhert, WJ & Lu, L. 2009; Non-invasive monitoring of BMP-2 retention and bone formation in composites for bone tissue engineering using SPECT/CT and scintillation probes. *J Control Release,* 134**:** 169-76.

Klein-Nulend, J, Bakker, AD, Bacabac, RG, Vatsa, A & Weinbaum, S. 2013; Mechanosensation and transduction in osteocytes. *Bone,* 54**:** 182-90.

Klinck, RJ, Campbell, GM & Boyd, SK. 2008; Radiation effects on bone architecture in mice and rats resulting from in vivo micro-computed tomography scanning. *Med Eng Phys,* 30**:** 888-95.

Knudson, W, Chow, G & Knudson, CB. 2002; CD44-mediated uptake and degradation of hyaluronan. *Matrix Biol,* 21**:** 15-23.

Langley, G, Evans, T, Holgate, ST & Jones, A. 2007; Replacing animal experiments: choices, chances and challenges. *Bioessays,* 29**:** 918-26.

Laperre, K, Depypere, M, van Gastel, N, Torrekens, S, Moermans, K, Bogaerts, R, Maes, F & Carmeliet, G. 2011; Development of micro-CT protocols for in vivo follow-up of mouse bone architecture without major radiation side effects. *Bone,* 49**:** 613-22.

Lavery, K, Swain, P, Falb, D & Alaoui-Ismaili, MH. 2008; BMP-2/4 and BMP-6/7 differentially utilize cell surface receptors to induce osteoblastic differentiation of human bone marrow-derived mesenchymal stem cells. *J Biol Chem,* 283**:** 20948-58.

LeGeros, RZ. 2008; Calcium phosphate-based osteoinductive materials. *Chem Rev,* 108**:** 4742-53.

Li, RH & Wozney, JM. 2001; Delivering on the promise of bone morphogenetic proteins. *Trends Biotechnol,* 19**:** 255-65.

Little, DG, McDonald, M, Bransford, R, Godfrey, CB & Amanat, N. 2005; Manipulation of the anabolic and catabolic responses with OP-1 and zoledronic acid in a rat critical defect model. *J Bone Miner Res,* 20**:** 2044-52.

Loi, F, Cordova, LA, Pajarinen, J, Lin, TH, Yao, Z & Goodman, SB. 2016; Inflammation, fracture and bone repair. *Bone,* 86**:** 119-30.

Mahamid, J, Sharir, A, Gur, D, Zelzer, E, Addadi, L & Weiner, S. 2011; Bone mineralization proceeds through intracellular calcium phosphate loaded vesicles: a cryo-electron microscopy study. *J Struct Biol,* 174**:** 527-35.

Marsell, R & Einhorn, TA. 2011; The biology of fracture healing. *Injury,* 42**:** 551-5.

McCourt, P, Ek, B, Forsberg, N & Gustafson, S. 1994; Intercellular adhesion molecule-1 is a cell surface receptor for hyaluronan. *J Biol Chem,* 269**:** 30081-4.

McKay, WF, Peckham, SM & Badura, JM. 2007; A comprehensive clinical review of recombinant human bone morphogenetic protein-2 (INFUSE Bone Graft). *Int Orthop,* 31**:** 729-34.
Melcher, AH & Irving, JT. 1962; The healing mechanism in artificially created circumscribed defects in the femora of albino rats. *Journal of Bone and Joint Surgery,* 44 B**:** 928-936.
Meyer, K & Palmer, JW. 1934; The polysaccharide of the vitreous humor. *Journal of Biological Chemistry*.
Millan, JL. 2013; The role of phosphatases in the initiation of skeletal mineralization. *Calcif Tissue Int,* 93**:** 299-306.
Neovius, E, Lemberger, M, Docherty Skogh, AC, Hilborn, J & Engstrand, T. 2013; Alveolar bone healing accompanied by severe swelling in cleft children treated with bone morphogenetic protein-2 delivered by hydrogel. *J Plast Reconstr Aesthet Surg,* 66**:** 37-42.
Orriss, IR, Arnett, TR & Russell, RG. 2016; Pyrophosphate: a key inhibitor of mineralisation. *Curr Opin Pharmacol,* 28**:** 57-68.
Panksepp, J & Burgdorf, J. 2003; "Laughing" rats and the evolutionary antecedents of human joy? *Physiol Behav,* 79**:** 533-47.
Piskounova, S, Gedda, L, Hulsart-Billstrom, G, Hilborn, J & Bowden, T. 2014; Characterization of recombinant human bone morphogenetic protein-2 delivery from injectable hyaluronan-based hydrogels by means of 125I-radiolabelling. *J Tissue Eng Regen Med,* 8**:** 821-30.
Poynton, AR & Lane, JM. 2002; Safety profile for the clinical use of bone morphogenetic proteins in the spine. *Spine (Phila Pa 1976),* 27**:** S40-8.
Ray, RD & Holloway, JA. 1957; Bone implants; preliminary report of an experimental study. *J Bone Joint Surg Am,* 39-A**:** 1119-28.
Russell, WMS & Burch, RL 1959. *The principles of humane experimental technique,* London,, Methuen.
Sanchez-Duffhues, G, Hiepen, C, Knaus, P & Ten Dijke, P. 2015; Bone morphogenetic protein signaling in bone homeostasis. *Bone,* 80**:** 43-59.
Schmidt-Bleek, K, Willie, BM, Schwabe, P, Seemann, P & Duda, GN. 2016; BMPs in bone regeneration: Less is more effective, a paradigm-shift. *Cytokine Growth Factor Rev,* 27**:** 141-8.
Seeherman, H & Wozney, JM. 2005; Delivery of bone morphogenetic proteins for orthopedic tissue regeneration. *Cytokine Growth Factor Rev,* 16**:** 329-45.
Stenfelt, S, Hulsart-Billstrom, G, Gedda, L, Bergman, K, Hilborn, J, Larsson, S & Bowden, T. 2014; Pre-incubation of chemically crosslinked hyaluronan-based hydrogels, loaded with BMP-2 and hydroxyapatite, and its effect on ectopic bone formation. *J Mater Sci Mater Med,* 25**:** 1013-23.
Stern, R. 2004; Hyaluronan catabolism: a new metabolic pathway. *Eur J Cell Biol,* 83**:** 317-25.
Takagi, K & Urist, MR. 1982; The reaction of the dura to bone morphogenetic protein (BMP) in repair of skull defects. *Ann Surg,* 196**:** 100-9.
Takahashi, S, Sugimoto, M, Kotoura, Y, Sasai, K, Oka, M & Yamamuro, T. 1994; Long-term changes in the haversian systems following high-dose irradiation. An ultrastructural and quantitative histomorphological study. *J Bone Joint Surg Am,* 76**:** 722-38.
Tang, R, Wang, L, Orme, CA, Bonstein, T, Bush, PJ & Nancollas, GH. 2004; Dissolution at the nanoscale: self-preservation of biominerals. *Angew Chem Int Ed Engl,* 43**:** 2697-701.

Toegel, S, Hoffmann, O, Wadsak, W, Ettlinger, D, Mien, LK, Wiesner, K, Nguemo, J, Viernstein, H, Kletter, K, Dudczak, R & Mitterhauser, M. 2006; Uptake of bone-seekers is solely associated with mineralisation! A study with 99mTc-MDP, 153Sm-EDTMP and 18F-fluoride on osteoblasts. *Eur J Nucl Med Mol Imaging,* 33**:** 491-4.

Turley, E. 1991; Hyaluronan-binding proteins and receptors. *Advanced drug delivery reviews*.

Urist, MR. 1965; Bone: formation by autoinduction. *Science,* 150**:** 893-9.

Vallabhajosula, S 2009. *Molecular imaging : radiopharmaceuticals for PET and SPECT,* Berlin ; New York, Springer-Verlag.

Ventura, M, Boerman, OC, Franssen, GM, Bronkhorst, E, Jansen, JA & Walboomers, XF. 2014a; Monitoring the biological effect of BMP-2 release on bone healing by PET/CT. *J Control Release,* 183**:** 138-44.

Ventura, M, Franssen, GM, Oosterwijk, E, Boerman, OC, Jansen, JA & Walboomers, XF. 2014b; SPECT vs. PET monitoring of bone defect healing and biomaterial performance in vivo. *J Tissue Eng Regen Med*.

Wang, D, Miller, SC, Kopeckova, P & Kopecek, J. 2005; Bone-targeting macromolecular therapeutics. *Adv Drug Deliv Rev,* 57**:** 1049-76.

Wang, EA, Rosen, V, D'Alessandro, JS, Bauduy, M, Cordes, P, Harada, T, Israel, DI, Hewick, RM, Kerns, KM, LaPan, P & et al. 1990; Recombinant human bone morphogenetic protein induces bone formation. *Proc Natl Acad Sci U S A,* 87**:** 2220-4.

Wang, RN, Green, J, Wang, Z, Deng, Y, Qiao, M, Peabody, M, Zhang, Q, Ye, J, Yan, Z, Denduluri, S, Idowu, O, Li, M, Shen, C, Hu, A, Haydon, RC, Kang, R, Mok, J, Lee, MJ, Luu, HL & Shi, LL. 2014; Bone Morphogenetic Protein (BMP) signaling in development and human diseases. *Genes Dis,* 1**:** 87-105.

Wang, Y, Wang, X, Li, H, Xue, D, Shi, Z, Qi, Y, Ma, Q & Pan, Z. 2011; Assessing the character of the rhBMP-2- and vancomycin-loaded calcium sulphate composites in vitro and in vivo. *Arch Orthop Trauma Surg,* 131**:** 991-1001.

Wilkinson, JM & Little, DG. 2011; Bisphosphonates in orthopedic applications. *Bone,* 49**:** 95-102.

Zara, JN, Siu, RK, Zhang, X, Shen, J, Ngo, R, Lee, M, Li, W, Chiang, M, Chung, J, Kwak, J, Wu, BM, Ting, K & Soo, C. 2011; High doses of bone morphogenetic protein 2 induce structurally abnormal bone and inflammation in vivo. *Tissue Eng Part A,* 17**:** 1389-99.

Zheng Shu, X, Liu, Y, Palumbo, FS, Luo, Y & Prestwich, GD. 2004; In situ crosslinkable hyaluronan hydrogels for tissue engineering. *Biomaterials,* 25**:** 1339-48.

Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 1287

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title "Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine".)

Distribution: publications.uu.se
urn:nbn:se:uu:diva-310312

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2017