From Karolinska Institutet, Department of Molecular Medicine and Surgery,
Karolinska University Hospital,
Department of Reconstructive Plastic Surgery, Stockholm, Sweden

Craniofacial bone reconstruction with Bone Morphogenetic Protein-2

Ann-Charlott Docherty Skogh

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“This is not the end. It is not even the beginning of the end. But, perhaps, the end of the beginning.”

Sir Winston Churchill

To Andrew, Alexander, Filippa and Isabelle
with love

ABSTRACT

Bone defects in the craniofacial area are a clinical challenge and can be the result of trauma, tumour resection or congenital malformations. The golden standard for reconstruction is autologous bone grafts, but bone may not always be readily available and donor-site morbidity might follow. Alternatives to autologous tissue are sought in the field of tissue engineering, where a range of biomaterials, bone forming cells and growth factors are combined, searching to engineer the missing tissue. The use of bone morphogenetic proteins (BMPs) together with different carriers has been explored ever since Marshall Urist discovered the BMPs in 1965. In this thesis we use BMP-2, together with different carriers in an attempt to reconstruct cranial defects in different species.

In paper Ia we look into ectopic bone induction with BMP-2 and heparin/chitosan in a rat model. The resulting bone induction is compared to BMP-2 and type I collagen, and found to be superior in the BMP-2 and heparin/chitosan group. In the following clinical study, paper Ib, BMP-2, heparin/chitosan and titanium mesh are used for the reconstruction of large cranial defects in humans. The patients demonstrate a postoperative inflammatory reaction and week bone formation, and the results are disappointing and discourage the use of heparin/chitosan in a clinical setting. The healing of cranial defects in minipigs with BMP-2 and hyaluronan-based hydrogel is studied in paper II. The defects treated with BMP-2 and hyaluronan-based hydrogel demonstrate 119 percent ossification, indicating complete healing and bone overgrowth to some extent. Animals treated with hydrogel alone show 58 percent ossification and 53 percent ossification in the control group, showing a significant difference in induced bone volumes between the BMP-treated animals and animals treated with hydrogel alone. Bone healing of cranial defects in rats comparing hyaluronic acid hydrogel and type I collagen is studied in paper III. Immunohistochemistry and histomorphometric analysis show more active bone formation in the BMP-2 and hydrogel group with significant increase in bone formation two to four weeks after surgery compared to BMP-2 and collagen or hydrogel alone. In the last study (paper IV) cranial reconstruction after neurosurgery with BMP-2 and hydrogel is studied. Boreholes are randomized into treatment with BMP-2 and hydrogel, hydrogel alone, autologous bone and Tisseel™ or Spongostan™ (negative control). Bone healing in holes treated with BMP-2 and hydrogel or autologous bone and Tisseel™ is significantly increased compared to negative control.

In conclusion tissue engineering of bone with heparin/chitosan and hyaluronan-based hydrogel with BMP-2 show good bone inductive capacity, superior to type I collagen and BMP-2. Hyaluronan-based hydrogel has more attractive qualities regarding the inflammatory response and BMP-2 and hydrogel produce bone comparable to bone autografts.

Keywords: Bone morphogenetic protein, bone induction, heparin, chitosan, hyaluronan, hydrogel

LIST OF ORIGINAL PAPERS

This thesis is based on the following original studies which will be referred to by their Roman numerals.

Ia. Thomas Engstrand, Riikka Veltheim, Claes Arnander, **Ann-Charlott Docherty Skogh,** Anders Westermark, Claes Ohlsson, Lars Adolfsson, Olle Larm
A novel biodegradable delivery system for Bone Morphogenetic Protein-2
Plastic and Reconstructive Surgery 2008;121:1920-1928

Ib. **Ann-Charlott Docherty Skogh,** Thomas Engstrand
Bone morphogenetic proteins in cranial reconstructions: Clinical evaluation of heparin-chitosan as a carrier for BMP-2
Plastic and Reconstructive Surgery 2009;123:192e-193e

II. **Ann-Charlott Docherty Skogh,** Kristoffer Bergman, Marianne Jensen Waern, Stina Ekman, Kjell Hultenby, Dimitri Ossipov, Jöns Hilborn, Tim Bowden, Thomas Engstrand
Bone Morphogenetic Protein-2 delivered by hyaluronan-based hydrogel induces massive bone formation and healing of cranial defects in minipigs
Plastic and Reconstructive Surgery 2010;125:1383-1392

III. **Ann-Charlott Docherty Skogh,** Kristoffer Bergman, Mats O Beckman, Jöns Hilborn, Thomas Engstrand
Time-point study of cranial regeneration with hyaluronic acid hydrogel and BMP-2 in rats
Manuscript

IV. **Ann-Charlott Docherty Skogh,** Lars Kihlström, Erik Neovius, Cecilia Persson, Mats O Beckman, Thomas Engstrand
Prospective randomized study of cranial bone regeneration after neurosurgery with BMP-2 and new carrier
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ALP	Alkaline Phosphatase
ASC	Adult stem cell
BMP	Bone morphogenetic protein
DBM	Demineralized bone matrix
ECM	Extra cellular matrix
ESC	Embryonic stem cell
FGF	Fibroblast growth factor
HA	Hyaluronic acid
HAP	Hydroxyapatite
HU	Hounsfield units
IGF	Insulin-like growth factor
iPSC	Induced pluripotent stem cell
MB	Mineralized bone matrix
MSC	Mesenchymal stem cell
NGF	Nerve growth factor
OB	Osteoblast
OC	Osteocyte
OI	Osteoid
PAA	Polyallylamine
PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
PEO	Polyethylene oxide
PGA	Polyglycolic acid
PLA	Polylactic acid
PLGA	Polylactic-co-glycolic acid
PRP	Platelet-rich-plasma
PVA	Polyvinyl alcohol
TCP	Tricalcium phosphate
TGF-beta	Transforming growth factor beta
TNF-alpha	Tumor necrosis factor-alpha
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

1.1 TISSUE ENGINEERING

The term “tissue engineering” dates back to the 1980s when scientists with backgrounds in engineering, medicine and biology started to look into the concept of tissue creation and repair. The most cited definition was proposed by Robert Langer and Joseph P. Vacanti in a review paper, published in Science in 1993: “Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function”[1]. This thesis is based on five papers looking into tissue engineering of bone, ectopic and in the craniofacial area, based on the cooperation between scientists in the different fields of medicine, chemistry and veterinary medicine. It ranges from preclinical studies in rats and minipigs to clinical studies in humans.

1.2 BONE TISSUE

Bone tissue contains three cell types which synthesize, remodel and maintain the intercellular mineralized matrix. *Osteoblasts* produce the organic part of the matrix called osteoid. It is composed of type I collagen, proteoglycans and glycoproteins, and contributes to the deposition of the inorganic components like hydroxyapatite. Endogenous glycoproteins present in bone are alkaline phosphatase (ALP), osteonectin, osteopontin, bone sialoprotein, fibronectin and osteocalcin[2,3] . The collagen fibers are initially haphazardly arranged and when the osteoid is mineralized it is referred to as woven bone. When the bone is remodeled the collagen fibers are deposited in the long axis of the bone and mineralization of this mature osteoid produces lamellar bone. *Osteoclasts* are multinucleated giant cells, responsible for resorption and remodeling of the matrix, a constantly ongoing process in living bone. *Osteocytes* are matured osteoblasts, trapped within cavities of the bony matrix, and are actively involved in the maintenance of the matrix. The osteocytes are connected by a network of canaliculi (small canals) enabling cellular communication through thin cytoplasmic extensions. Blood vessels transverse the matrix and provide nutrients and oxygen to the cells. The bone forming cells take part in matrix turnover as well as in the production and secretion of regulating growth factors such as insulin-like growth factors, transforming growth factors and bone morphogenetic proteins.

The skeleton is formed by two distinct processes, intramembranous and endochondral ossification. Intramembranous ossification involves primarily the development of flat bones such as the skull, and mesenchymal cells differentiate directly into osteoblasts. Endochondral ossification refers to the process of epiphyseal cartilaginous growth of the long tubular bones. The cartilage is progressively replaced by osteoid, which is then mineralized[4].

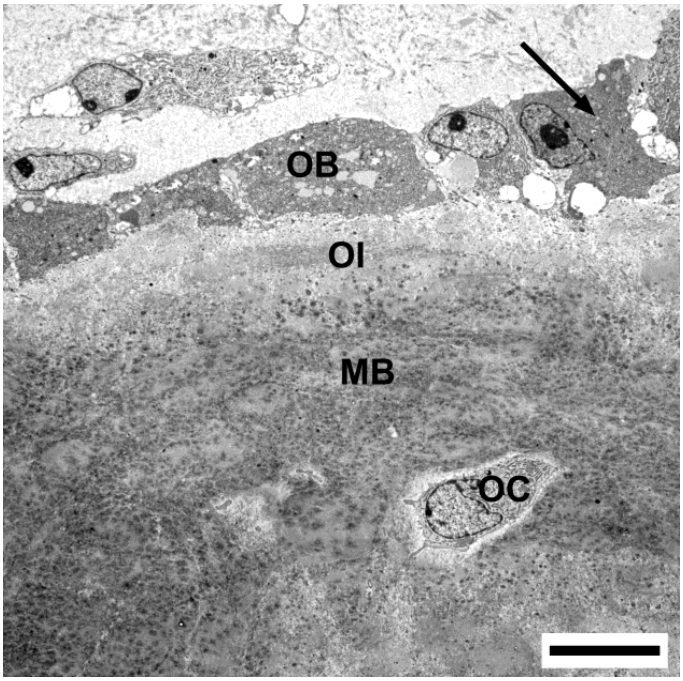

Figure 1. Transmission electron microscopy showing direct bone formation. Osteoblasts (*OB*) are polarized toward the bone matrix displaying an active morphology with extensive endoplasmic reticulum (*arrow*), producing type I collagen, osteoid (*OI*).An osteocyte (*OC*) is trapped in the mineralized bone matrix (*MB*). Scale bar 10 μm.

Bone is composed of compact (cortical) bone and trabecular (cancellous) bone. Compact bone has a porosity of 5–30% and accounts for 80% of the total bone mass of an adult skeleton. Trabecular bone accounts for the remaining 20% of total bone mass and has nearly ten times the surface area of compact bone and a porosity of 30–90% [5]. Bone is composed of 60% mineral. The mineral content of bone is mostly hydroxyapatite, with small amounts of carbonate, magnesium, and acid phosphate[6]. All bones are covered with a periosteum on the outer surface and lined with an endosteum along the bone marrow cavity. These well vascularized and sensate connective tissues contain bone progenitor cells, capable of differentiation towards osteoblastic lineage and secretion of growth factors.

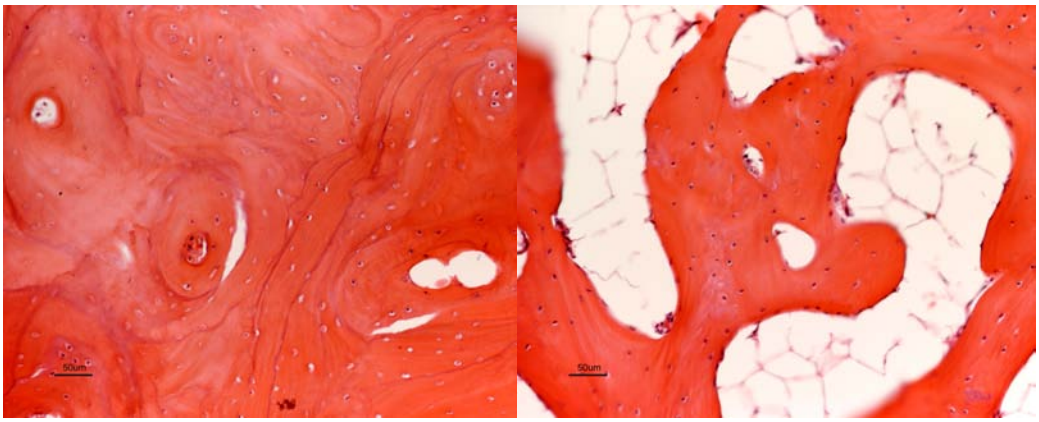

Figure 2. Compact lamellar bone (left), trabecular wowen bone (right). Scale bar 50 µm.

1.3 STEM CELLS

Stem cells are defined by their capacity of self-renewal, and the capacity to differentiate into different cell lineages under appropriate conditions. Stem cells divide asymmetrically, into a more differentiated daughter cell and into a clone to maintain the stem cell population. Human stem cells can be categorized into three main types: embryonic stem cells (ESCs), adult stem cells (ASCs) and induced pluripotent stem cells (iPSCs). A stem cell is totipotent if it is capable of giving rise to a whole animal, including germ cells, all types of the three germ layers (i.e. ectoderm, mesoderm and endoderm) and extraembryonic tissues. Zygotes are totipotent. A pluripotent cell can produce all cell types of the germ layers but not extraembryonic tissues, while a multipotent cell can produce cells of the same germ layer only. Adult stem cells are described as multipotent [7]. Bone marrow derived stem cells were the first adult stem cells shown to have multi-lineage potential. Bone marrow contains at least two distinct stem cell populations; hematopoietic stem cells giving rise to all blood cell types, and mesenchymal stem cells (MSCs). MSCs can differentiate into tissues of the embryos mesoderm including bone, adipose, cartilage and muscle[8-10] . Markers for skeletal stem cells have been identified and isolated based on a minimal surface phenotype noted for expression of CD146, CD105 and alkaline phosphatase [11]. Skeletal stem cells represent a central model system for investigating skeletal diseases, as tools for in vitro and in vivo models, for cell therapy- based strategies, or as targets for drugs.

1.4 BONE MORPHOGENETIC PROTEINS

Bone morphogenetic proteins (BMPs) are biologically active signaling molecules which were first described by Dr Marshall Urist in 1965. He discovered that extracts of

bovine bone induced ectopic bone formation subcutaneously in rats[12]. BMPs are naturally present in the extracellular matrix (ECM) and can bind to heparin sulfate, heparin and type IV collagen[13,14]. Molecular cloning of BMP-1 through BMP-4 in 1988 was an important step in BMP research and opened up new possibilities in research and clinical trials with BMPs[15] . BMPs are members of the transforming growth factor-beta (TGF-beta) superfamily and have great osteoinductive potential[16]. At least 40 different subtypes in the TGF-beta family have been described to date, and these have been divided into groups according to their primary amino acid sequence[17]. The human BMP family has 15 members (BMP-1 through BMP-15) and among these BMP-2, BMP-4 to BMP-7 and BMP-9 have been shown to induce intramembranous and endochondral bone formation[18]. Of these, BMP-2, BMP-6 and BMP-9 appear to have important roles in the induction of mesenchymal cell differentiation into osteoblasts[19]. In terms of bone formation, BMPs regulate intramembranous as well as endochondral ossification through chemotaxis and mitosis of mesenchymal cells, induction of mesenchymal commitment to osteoblasts or chondrocytes, promotion of further osteoblast or chondrocyte differentiation, and programmed cell death[20]. Other members of the TGF-beta family, such as chordin and noggin, have inhibitory effect on bone formation by inactivating BMPs[21]. BMPs are synthesized by osteoblasts as 400-500 amino acid peptides, each consisting of a leader sequence, a propeptide, and a mature osteoinductive domain at the carboxy-terminal. Prior to secretion from the osteoblasts, BMP molecules are cleaved between the propeptide and mature regions to release the active BMP dimer[22]. BMP then binds to serine/threonine kinase receptors (BMP type I and II) that are displayed on the stem cells' surface. Following ligand binding, the type II receptor homodimer cross-phosphorylates the type I receptor in the GS region, activating its kinase domain. The type I receptor kinase then initiates downstream signaling by phosphorylating and activating intracellular messenger proteins called Smads. Distinct type I and type II receptors have been identified. The specific Smad protein to be activated depends on the type of BMP ligand and the type I receptor it binds to. BMP-2, for example binds to BMP-Ia and BMP-Ib receptors, whereas BMP-7 binds to ALK-2 or BMP-Ib receptors. Osteoinduction appears to be mediated by R-Smads (Smads 1, 5 and 8). Smads 1 and 5 are activated by BMP-Ia and BMP-Ib receptors, whereas Smads 1, 5 and 8 can be activated by ALK-2 receptors. Once activated, R-Smads combine with Smad 4 to form a nuclear signaling complex that is capable of altering specific patterns of gene expression to promote cell proliferation and stimulate the concentration dependent transformation of daughter cells into

chondroblasts or osteoblasts. Smads 6 and 7 compete for phosphorylation of Smad 4, and appear to be involved in the inhibition of osteoinduction[23].

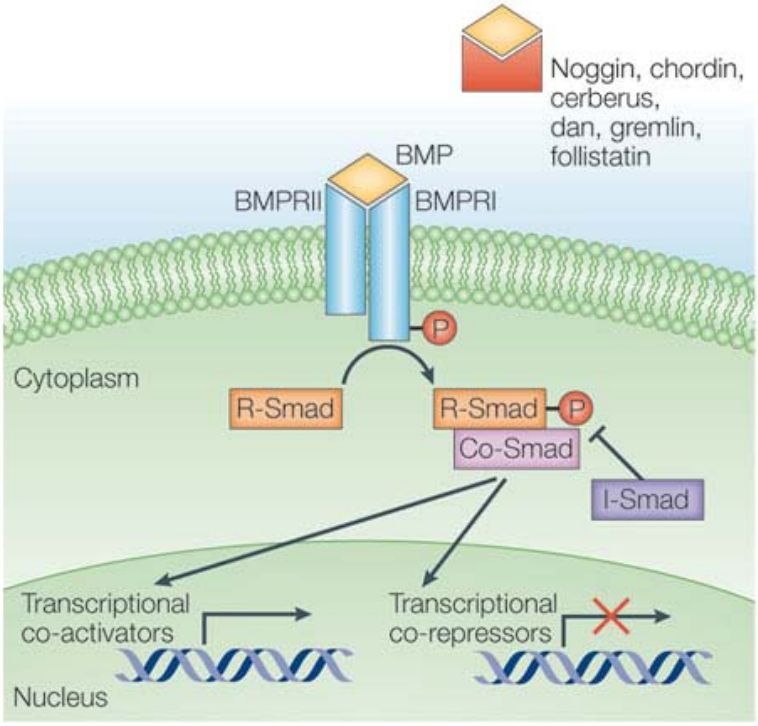

Figure 3. Bone morphogenetic protein (BMP) ligands bind to the BMP receptors BMPRI and BMPRII, and BMPRII then phosphorylates and activates BMPRI. Phosphorylated BMPRI subsequently phosphorylates receptor-activated Smad proteins (R-Smads), which associate with common mediator-Smad (co-Smad) and enter the nucleus, where they regulate gene expression. The Smad proteins regulate promoter activity by interacting with transcriptional co-activators or co-repressors to positively or negatively control gene expression. The BMP signal can be blocked by extracellular antagonists, such as noggin, which bind BMP ligands and prevent their association with the BMP receptors, as well as by intracellular proteins, such as inhibitory Smads (I-Smads), which prevent the association between R-Smads and co-Smads. *From Liu A., Niswander L.A. Bone morphogenetic protein signaling and vertebrate nervous system development, Nature Reviews Neuroscience 6, 945-954 (December 2005)*

BMPs also promote angiogenesis during ossification via a mechanism that involves osteoblast-derived VEGF-A[24]. Studies in cell and animal models have shown that BMP-induced neovascularisation is critical for bone induction, probably playing an essential role in enabling the recruitment of BMP receptor-positive cells[22].

1.5 BIOMATERIALS

A biomaterial is any matter, surface, or construct that interacts with biological systems. The development of biomaterials, as a science, is about fifty years old.

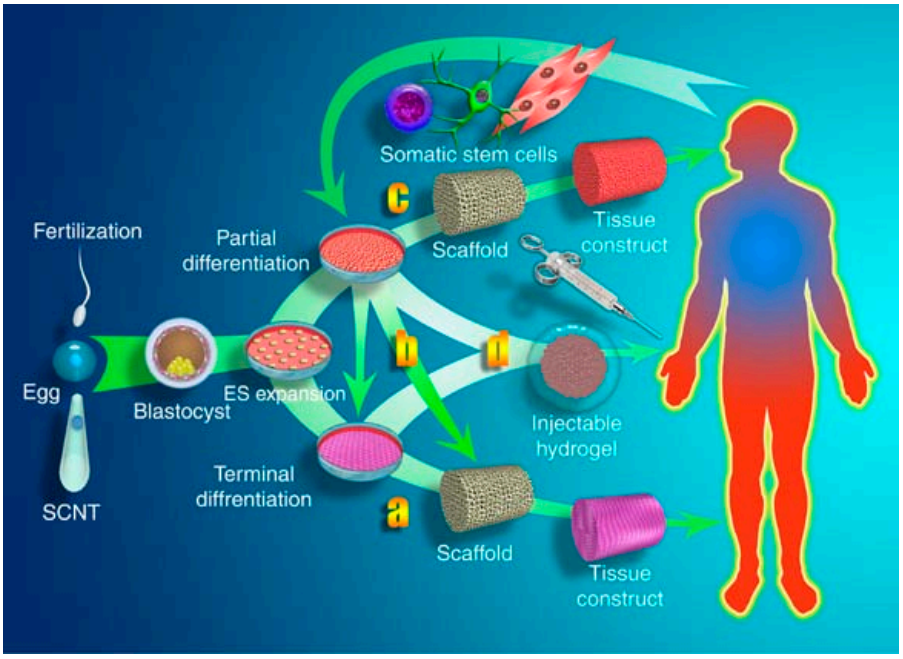

Figure 4. Multiple roles for biomaterials in stem cell tissue engineering, source: http://www.nature.com/

Biomaterials can be used as vehicles or carriers for growth factors, scaffolds for tissue regeneration with three dimensional properties providing a volume in which vascularization, new tissue formation and remodeling can occur. The different types of biomaterials can be divided into four different groups comprising inorganic materials, naturally-derived polymers, synthetic polymers and composite materials[25].

1.5.1 Inorganic materials

Inorganic materials include ceramics, such as compositions of calcium phosphate like hydroxyapatite (HAP) and tricalcium phosphate (TCP). It also includes non-ceramics like calcium phosphate based cements. Other inorganic materials include calcium sulfates, metals and bioglasses. HAP has been used clinically in prosthetics since the 1980s[26]. HAP is brittle and difficult to mould, and HAP alone with BMP-2 shows lack of bone induction because of the lack of resorption of HAP and the tight binding affinity to BMPs. It is therefore often combined with TCP for a more resorbable and porous BMP carrier with greater deal of bone formation[27]. The porosity of the scaffold is important for the ingrowth of cells, and it has been demonstrated that the minimum

channel (or pore) diameter required for cell penetration into HAP scaffolds is approximately 80 µm[28].

1.5.2 Naturally-derived polymers

Naturally-derived polymers include collagen, hyaluronic acid, fibrin, chitosan, heparin, alginate and other animal or plant-derived polysaccharides. The most widely used natural polymer is type I collagen, which is derived from bovine or porcine bone, skin or tendon. It can be used as demineralized bone matrix (DBM), as gels and cross-linked sponges, but considering its origin there is a risk of pathogen transmission. Collagen scaffolds loaded with BMPs have been successfully used to achieve bone formation. This has led to the development of collagen-based BMP products for the treatment of long bone fracture non-unions and lumbar interbody fusion[29]. For BMP-2 a collagen sponge reconstituted from bovine tendon is used in the commercially available form (InductOs, Wyeth). Hyaluronic acid (HA), or hyaluronan, is a non-sulfated, linear polysaccharide composed of repeating disaccharide units of glucuronic acid-N-acetyl-D-glucosamine. It is a major component of the extra cellular matrix (ECM) and present in nearly every mammalian tissue and fluid. It plays a role in wound healing[30] and it has been found in high concentrations in the early fracture callus, in lacunae surrounding hypertrophic chondrocytes in the growth plate and in the cytoplasm of osteoprogenitor cells[31,32].It is typically derived from rooster comb for commercial purposes. Hyaluronic acid is negatively charged and can form ionic bonds with positively charged BMPs to increase affinity[33,34]. Disadvantages of hyaluronic acid include rapid resorption unless crosslinked or chemically modified to decrease its intrinsic hydrophilicity[25,33,35-37]. Hyaluronic acid scaffolds have been used for the delivery of various growth factors including TGF-beta and BMP-2[38-40]. Hyaluronan has previously been shown to induce the expression of its own receptor, CD44, specifically in mesenchymal stem cells[41]. The hyaluronan/CD44 interaction induces adhesion and migration of mesenchymal stem cells to hyaluronan, which suggests a dual capacity of hyaluronan-based biomaterials by functioning as both a matrix for attraction of mesenchymal stem cells and as a carrier and protective container for differentiation factors. Chitosan is a linear polysaccharide of (1-4)-linked D-glucosamine and N-acetyl-D-glucosamine residues that is commercially derived by partial deacetylation of chitin obtained from crustacean shells[42]. It´s a non toxic, resorbable material that has been shown to promote wound healing. Due to its cationic nature, it forms water insoluble ionic complexes with a wide range of polyanionic compounds. Chitosan

increases the mechanical strength when incorporated into a collagen scaffold due to ionic complex formation between the positively charged chitosan and the negatively charged collagen ions[43]. Heparin is a natural polymer that occurs in the human body at very high concentrations in the tissues surrounding the capillaries of the lungs and the liver. Heparin is a mucopolysaccharide with a high negative charge due to the presence of sulfate groups on the back bone. It is used clinically to delay blood clotting, and it also takes part in various biological activities such as cell adhesion, migration and recognition[44]. Many growth factors show a very high affinity for heparin owing to specific binding sites for growth factors on heparin. This binding stabilizes growth factors and enables their sustained release at the site of tissue regeneration. In addition to this, the bioactivity of some growth factors is enhanced upon binding with heparin[45]. Heparin has been shown to stabilize growth factors (including BMP-2), protect them from enzymatic degradation and inactivation, and enhance their biological activities by enhancing BMP-induced osteoblast differentiation in vitro and in vivo and by protecting BMPs from degradation and inhibition by BMP antagonists[46]. Fibrin is derived from blood, and fibrin formation takes place during the activation of coagulation, stabilizing the haemostatic plug and providing the temporary matrix for subsequent cellular responses of wound and vessel repair [47]. Fibrin glue and fibrin-base scaffolds allow the immobilization of a wide range of growth factors for controlled delivery, including bFGF, nerve growth factor (NGF), VEGF and BMP-2[48] .

1.5.3 Synthetic polymers

Synthetic polymers have the advantage of being well-characterized materials that can be modified for specific uses in tissue engineering and are free of viral and infectious agents. The most predominant polymer is the class of poly(alfa-hydroxy acids) that are approved by the Food and drug administration (FDA)[49]. Of these the most used polymer for growth factor delivery are polylactic acid (PLA), polyglycolic acid (PGA) and their copolymers poly(lactic-co-glycolic) acid (PLGA). These scaffolds can be processed into highly porous scaffolds or microspheres[50]. They can be used for delivery of definite amounts of growth factors. These polymers break down by hydrolysis producing acidic breakdown products that might affect the wound healing detrimentally due to a decrease in the local pH and inflammatory response. Polyethylene glycol (PEG) is one of the most popular carrier materials for drug delivery due to its biocompatibility, hydrophilicity and rapid biodegradability[51].

1.5.4 Composite materials

Composite materials include varying combinations of ceramics, natural polymers and synthetic polymers to build optimized systems that take advantage of the benefits of each material. In this way the controlled release properties of synthetic polymers can be combined with the biocompatibility of natural polymers. Examples of this strategy include hyaluronic acid-impregnated PLA-sponges, collagen-PLG-alginate composites, PLGA-gelatin composites and alginate-PLA composites[51,52]. Mineral components can also be combined with natural polymers such as collagen or hyaluronic acid.
Ideally, a carrier material would enhance retention of BMP at the local site, stimulate bone healing by osteoconduction, provide mechanical support, be readily available, would not cause an inflammatory reaction, would not be very expensive and would resorb over time. In this way the effectiveness and handling properties of BMPs would be enhanced and the applied dosage could be lowered, leading to lower costs[53].

1.6 DELIVERY SYSTEMS

To deliver drugs in a controlled manner over extended periods of time different delivery systems are used. In a delivery system, growth factors are encapsulated in a polymeric covering which modulates the rate of release[54]. The growth factors are also protected from the proteolytic environment which can cause a cleavage in the protein structure. Some well-studied delivery systems are microspheres, hydrogels, membranes, granules, foams and implant coatings using a variety of materials such as gelatin[55], collagen[56], fibrin, chitosan[57] and PLGA[58].

1.6.1 Microspheres

Microspheres are spherical particles ranging from 1-100 µm that can be implanted at the site of injury or injected into the wound. Both degradable and non degradable polymeric materials can be used in the microspheres[58]. They are usually used together with a different scaffold material. In recent publications 3D scaffolds made of poly(propylene fumarate)/diethyl fumarate photopolymer incorporating BMP-2 loaded PGLA microspheres were used to reconstruct bony defects[59]. In another study an injectable calcium phosphate-chitosan fibrous scaffold was used to deliver BMP-2 and umbilical cord mesenchymal stem cells in alginate microbeads[60].

1.6.2 Scaffolding systems

Scaffolding systems serve as a structure for cell attachment/growth and also as a growth factor delivery system. The scaffold retains and locally releases the protein during formation of new tissue[25,61-63]. The delivery of growth factors is controlled either by passive diffusion from the scaffold, or by the degradation rate of the scaffold. The composition of the scaffold and the subsequent release of growth factors can be altered to match the healing process, and the magnitude of release can be controlled by the amount of growth factor added in the scaffold. Examples in the recent literature include apatite-coated collagen scaffolds shown to increase the bone formation with BMP-2 compared to non-modified collagen scaffolds[64]. In another study of bone healing in calvarial defects in rats a calcium-deficient hydroxyapatite porous scaffold with sulfated chitosan coating was used for improved release of BMP-2[65]. The mechanical behavior of polymer scaffolds is important for their use in tissue engineering, and new ways of testing are being presented. Image-guided failure assessment (IGFA), which combine synchrotron radiation computed tomography (SR CT) and in situ micro-compression testing is a useful tool for assessing structural and mechanical scaffold properties[66]. Multiple examples of new scaffolding systems are continuously presented in the literature trying to create the optimal conditions for tissue engineering.

1.6.3 Hydrogels

Hydrogels are cross-linked polymeric structures that are water-expandable, biocompatible, tissue-like in elasticity and permeability, which make them favorable candidates for tissue engineering applications[38]. The high water content allows diffusion of low-molecular weight compounds and reduces the interfacial tension with other fluids, but also leads to low mechanical strength. To compensate for this cross-linking and copolymerization of hydrogels with bulky hydrophobic monomers has been performed. Hydrogels have been mixed with aqueous solutions of poly(vinyl alcohol-vinyl acetate) and poly(acrylic acid) in different ratios, and then studying the effects of cross-linking agents such as glyoxal and glutaraldehyde on the mechanical properties of the resulting hydrogels. The results demonstrated that cross-linked hydrogels showed higher storage modulus values when compared with non cross-linked hydrogels[67]. Synthetic as well as natural materials have been used to form hydrogels that can be used as scaffolds for tissue engineering. The former includes materials such as polyethylene oxide (PEO), polyvinyl alcohol (PVA) and poly allylamine (PAA)[68] and the latter includes alginate, chitosan, collagen, agarose, hyaluronic acid, fibrin and

many more[38,69-71]. Biocompatibility, biodegradability, sustained release, protection from proteolytic cleavage and choice of polymerization with large numbers of synthetic and natural materials make hydrogels good candidates for growth factor-delivery systems. In the recent literature there are examples of hydrogels used for surface modification of free form-based scaffolds followed by BMP-2 loading to enhance bone regeneration[72]. Another study presents a nanofiber mesh tube for guiding bone regeneration of segmental bone defects in a rat model combined with peptide-modified alginate hydrogel injected inside the tube for sustained release of BMP-2[73].

1.7 CLINICAL APPLICATIONS OF BONE TISSUE ENGINEERING

In cranial reconstructions after neurosurgery healing problems can occur, because the blood supply to the bone flap is disturbed, which ultimately may lead to necrosis in approximately 5% of the bone flaps[74]. Different techniques are used to reconstruct calvarial defects including bone transplants and a variety of inert or bioactive biomaterials[75]. The use of autologous bone grafts may be restricted due to limited amounts of donor bone, the enhanced morbidity and significant bone resorption[76,77]. Complications are mainly related to the non-vascularised nature of an implant or free bone graft and therefore, vascularised bone grafts are state of the art for the treatment of large bone defects[78]. In 2004 Lendeckel et al. healed a large calvarial defect in a child with the use of resorbable macroporous sheets, fibrin glue and autologous fat derived stem cells[79]. The healing was uneventful despite long lasting infection and CT-scans showed near complete calvarial continuity three months after the reconstruction. Reconstruction of facial bones is another challenge due to their three dimensional properties. In 2004, Warnke et al. reported a case study, where an extended mandibular discontinuity was replaced by a custom made bone transplant composed of mineral blocks, bone marrow and BMP 7[80]. The contents were placed within a titanium mesh cage and implanted in the latissimus dorsi muscle of the patient to allow bone formation and blood vessel infiltration. Seven weeks later the vascularized composite graft was transplanted to the jaw using microvascular techniques. Mesimäkis research group used a microvascular flap using autologous fat derived stem cells, beta-tricalcium phosphate and bone morphogenetic protein-2 to reconstruct a defect following hemimaxillectomy[81]. Other investigators believe that adequate delivery of growth factors will recruit enough local or migrating precursor cells. In 2001 Moghadam et al. showed that critical size bone defects in the mandible can be healed with only growth factors and scaffolding materials[82]. This approach was further investigated by Arnander

et al. in 2006, creating a composite graft suitable for microvascular transfer to the forehead using an acellular degradable scaffold containing BMP-2[83].

In craniofacial cleft repair there are some studies describing the use of BMP-2 and different scaffolding materials[84-87]. The dose of BMP-2 is high (1.5 mg/mL), and associated with local swelling in some of the patients. The challenge still remaining is to get closer to physiological doses of growth factor, and still achieve healing of the defect without adverse side effects. Another issue when it comes to larger defects is the need for vascular supply. Accelerating angiogenesis as well as osteogenesis would be a significant advance in the field of tissue engineering.

2 HYPOTHESIS AND AIMS

The main hypothesis is that bone induction with BMP-2 differs in temporal aspects, bone forming capacity and mechanism depending on the carrier material used.

The main objective of this thesis is evaluation of different carriers for BMP-2 in cranial bone reconstruction in different species.

The specific aims are:

Ia and b. To evaluate heparin/chitosan as a carrier for BMP-2 in bone induction in a preclinical and clinical setting.

II. To evaluate hyaluronan-based hydrogel as a carrier for BMP-2 in bone induction in a large animal study.

III. To compare hyaluronan-based hydrogel to type I collagen as a carrier for BMP-2 in bone induction in a small animal study.

IV. To compare hyaluronan-based hydrogel and BMP-2 to autografts in a clinical study.

3 MATERIALS AND METHODS

3.1 SCAFFOLDS AND SCAFFOLD PREPARATION

3.1.1 Heparin/Chitosan gel

The heparin/chitosan gel was used in **study Ia**. A suspension of chitosan (4.5 g) (Primex, Haugesund, Norway) with a degree of *N*-acetylation of 16% was dissolved in water (100 g) by drop-wise addition of hydrochloric acid (4 M). The reaction was performed at room temperature and under stirring until a clear solution with a pH value of 4.7 was obtained. The solution was kept overnight in a closed vessel. Heparin (1.8 g) (Pharmacia, Uppsala, Sweden) was dissolved in water (25 g). The heparin solution was added to the chitosan solution under stirring and a viscous gel was obtained. One milliliter of the resulting gel contained 12 mg of heparin and 30 mg of chitosan. The product was macroscopically homogenous and stable. Within 30 minutes, BMP-2 (Wyeth Lederle, Madison, N.J.) was added by stirring to final concentrations of 50 µg of BMP-2 per mL of gel or 250 µg of BMP-2 per mL of gel. The gels were transferred to 1-mL syringes and kept at room temperature for approximately 10 to 15 minutes.

3.1.2 Heparin/Chitosan sponge

The heparin/chitosan sponge was used in **study Ia and Ib**. The heparin/chitosan complex (40 g), see above, was poured into Petri dishes with a diameter of 9 cm. The Petri dishes were freeze-dried, which resulted in a white, sponge-like material.

3.1.3 Collagen gel

The collagen gel was used in **study Ia**. Bovine type I collagen (Vitrogen 100, Cohesion, Palo Alto, Calif.),was used as a BMP-2 carrier and prepared as described by the manufacturer. Briefly, 8 ml of chilled Vitrogen collagen was mixed with 1 mL of 10x phosphate buffered saline solution and 1 mL of 0.1 M sodium hydroxide. The pH of the mixture was monitored and adjusted to 7.4 by adding a few drops of either 0.1 M hydrochloric acid or 0.1 M sodium hydroxide. The neutralized collagen solution was stored at 4ºC. Within 30 minutes, recombinant human BMP-2 (Wyeth Lederle, Madison, N.J.) was added by stirring to final concentrations of 50 µg of BMP-2 per mL of gel or 250 µg of BMP-2 per mL of gel. In one group, the BMP-2 was treated with 1000 IE of heparin (Pharmacia, Uppsala, Sweden), before mixing with the collagen gel

as described above. The solutions were transferred to 1 mL syringes and kept at room temperature for 10 to 15 minutes.

3.1.4 Collagen sponge

In study III type I collagen sponge (InductOs, Wyeth Pharmaceuticals, UK) was used. The sponge was cut into circles with a diameter of 8 mm and treated with 25 µg BMP-2 (InductOs, Wyeth Pharmaceuticals, UK).

3.1.5 Aldehyde-modified hyaluronic acid hydrogel

Aldehyde-modified hyaluronic acid was used **in study II, III and IV** and was prepared as described by Bergman et al[88]. Aldehyde-modified hyaluronic acid was dissolved in phosphate buffered saline to a concentration of 26 mg/mL and filter sterilized. A BMP-2 solution of 0.5 mg/mL was prepared by adding 24 mL of deionized water to a lyophilized powder containing 12 mg of recombinant human (rh) BMP-2 in a formulation buffer of pH 4.5 (InductOs; Wyeth, Madison, N.J.). Hydrazide-modified polyvinyl alcohol was prepared as described previously by Ossipov et al[89]. Hydrazide modified polyvinyl alcohol was dissolved to a concentration of 4 mg/mL in the BMP-2 solution and filter sterilized. For gels without BMP-2, hydrazide modified polyvinyl alcohol was dissolved in the formulation buffer alone, which contained 0.833% glycine, 0.167% sucrose, 0.033% polysorbate 80, 0.01% sodium chloride, and 0.025% L-glutamic acid. Heat-sterilized hydroxyapatite in powder form was added to each polymer solution to an amount of 0.25 g/mL and the resulting suspensions were mixed vigorously. Equal volumes of aldehyde-modified hyaluronic acid/hydroxyapatite and hydrazide modified polyvinyl alcohol/hydroxyapatite suspensions were added to 3-mL dual-cartridge syringes that were sealed and stored at 4°C until use. Gels of 2.5 mL were formed in situ by injecting the suspensions using the dual cartridges equipped with mixing tips **(study II)**. **In study III** 0.1 mL of premixed hydrogel with or without 25 µg BMP-2 was used. **In study IV** 1 mL of premixed hydrogel with or without 250 µg BMP-2 was used.

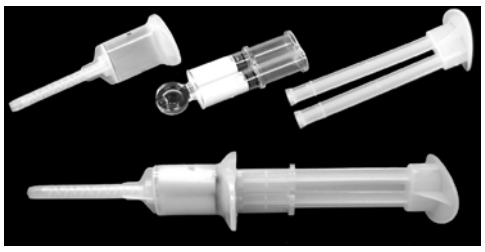

Figure 5. The dual-compartment syringe was used for the preparation of aldehyde-modified hyaluronic acid hydrogels by mixing equal volumes of the gel precursors. The syringe is equipped with a static mixing tip.

3.2 ANIMAL STUDIES

3.2.1 Ethical permission animal studies

In study Ia the ethical application was approved by the local ethical committee at Karolinska Institute (registration no. N70/05). **In study II** all procedures were approved by the Ethical Committee for Animal Experimentation, Uppsala, Sweden (Dnr C35/8). **Study III** was approved by the local ethical committee in Stockholm, Sweden (Dnr N 30/08).

3.2.2 Animals and surgical procedures (Paper Ia, II and III)

In study Ia adolescent male Sprague-Dawley rats weighing 250 to 300 g were used in the in vivo experiments. The rats were anesthetized with Temgesic (0.16 mL/kg body weight; Reckitt Benckiser, Slough, United Kingdom). Three carriers in gel formulation were tested: type I collagen, heparin/type I collagen, and heparin/chitosan. Each carrier was mixed with 0 µg of BMP-2 (buffer alone), 10 µg of BMP-2, or 50 µg of BMP-2 before the animal procedures. Forty-five rats were divided into nine groups, with ten implantations per group. Both hind legs of each animal were used for the injections. The results were derived from two independently performed experiments. The gels were injected into the quadriceps muscle using a 22-gauge needle. The animals were allowed to move freely after the procedure. They were killed by means of carbon dioxide four weeks after injection. Freeze-dried formulations of the same carriers (type I collagen, heparin/type I collagen, and heparin/chitosan) were tested with 0 µg of BMP-2, 10 µg of BMP-2, or 50 µg of BMP-2 by implantation into both legs in 15 rats with five implantations per group. Heparin/chitosan and type I collagen freeze-dried sponges were cut into 4 x 6 mm pieces. The 4 x 6 mm heparin/chitosan sponges contained 6.1 mg of chitosan and 2.42 mg of heparin. Buffer with 0 µg of BMP-2, 10 µg of BMP-2, or 50 µg of BMP-2 were added to the sponges. For the heparin/type I collagen carrier, BMP-2 was treated with 1000 IE of heparin before being added to the collagen sponge. The sponges were kept at room temperature for at least 15 minutes before implantation into the quadriceps muscles in the rats through a 15 mm skin incision.

In study II 14 male health-certified minipigs (Ellegaard Göttingen Minipigs, Dalmose, Denmark) 14 months of age and with a mean weight of 23.2±3.4 kg were used. They were allowed to acclimatize for 19 days before surgery at the Department of Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden. The pigs

were fed a diet without antimicrobials (sodium dodecyl sulfate–sodium nitro prusside) and had free access to water. The pigs were randomized into three groups: group 1, craniotomy and application of 5 mL of hydrogel with 1.25 mg of BMP-2 (n=6); group 2, craniotomy and application of 5 mL of hydrogel without BMP-2 (n=6); and group 3, craniotomy and no further treatment (n=2). The protocol ran for a total of 15 weeks. Anesthesia was induced with a combination of medetomidine (Domitor; Pfizer, New York, N.Y.), tiletamine, and zolazepam (Zoletil; Virbac Animal Health, Carros, France). Intramuscular buprenorphine (Temgesic; Reckitt Benckiser, Parsippany, N.J.) was provided for additional analgesia at a dosage of 0.1 mg/kg body weight. One dose of antibiotics (Ceftiofur 0.5 g; Pfizer) was administered intramuscularly. The animals were intubated and general anesthesia was maintained with isoflurane. A skin flap measuring 4 x 5 cm was raised including the underlying periosteum under local anesthesia (Marcaine; AstraZeneca Pharmaceuticals, Wilmington, Del.) A cranial bone defect measuring 2 x 4 cm was marked on the parietal and frontal bone including the sagittal and coronal sutures. A craniotomy was performed using Midas Legend equipment (Medtronic, Minneapolis, Minn.) on the inner layer of bone overlying the dura mater. The dura was injured and mended with Surgicel (Ethicon, Inc., Somerville, N.J.) in three pigs. To achieve a closed defect, we put in bone transplants using the bone that was removed from the same individual to cover the lateral sinuses, and we made a periosteal flap to cover the anterior sinuses. Five milliliters of hydrogel was applied to the defect per pig, except for animals in the control group, where the defect was left without treatment. The skin overlying the defect was sutured subcutaneously and intracutaneously with resorbable 2-0 Vicryl (Ethicon). Buprenorphine was administered intramuscularly twice daily for three days. After surgery, the pigs were weighed two times per week and blood samples were taken four weeks after surgery and before the animals were euthanized. At the end of the study (i.e., three months after surgery), the pigs were euthanized under general anesthesia with an overdose of pentobarbital sodium. All animals underwent a complete necropsy within 30 minutes after death, and the heads were separated from the bodies for further analyses.

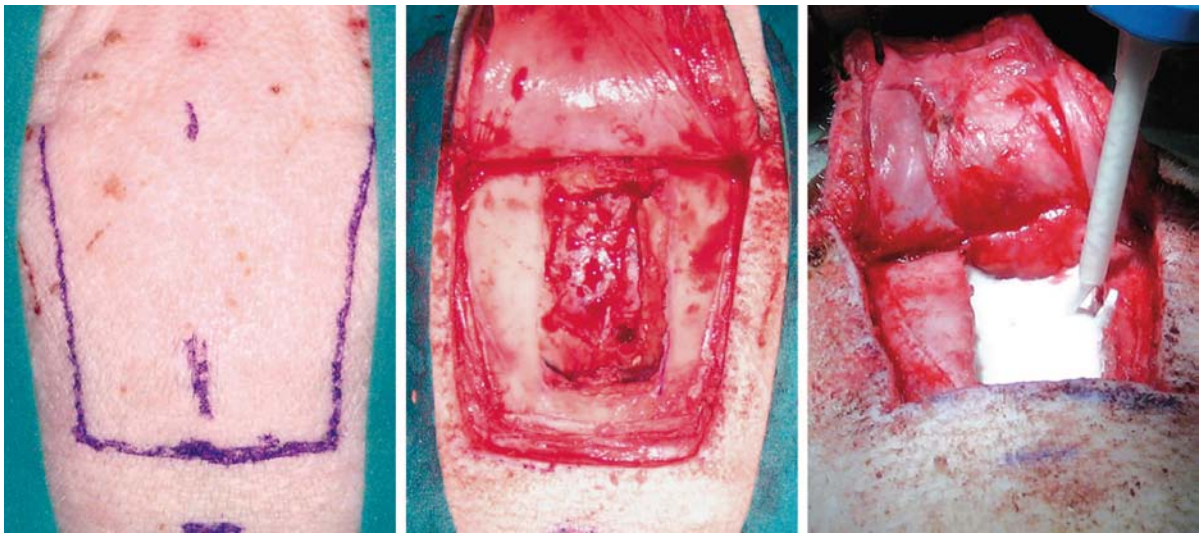

Figure 6. The craniotomy and reconstructive procedure. (Left) A skin flap is designed over the parietal and frontal area. (Center) The flap is raised including the underlying periosteum. (Right) Craniotomy measuring 2 x 4 cm is performed and the underlying dura mater is exposed. The defect is filled with 5mL of hydrogel. The gelation time is less than 2 minutes, and the defect is closed.

In study III adolescent male Sprague Dawley rats (n=45) weighing 250-300 g were used. They were kept at the animal department at Karolinska University Hospital in cages with free access to food and water. A 12:12 h light/dark schedule (lights on at 7.00 a.m.) was used and the room temperature was 20 ± 2°C. There were three to four animals per cage, and they received veterinary supervision. The rats were divided into four groups. Group 1 (N= 15): Defect filled with 0.1 mL gel with 25 µg BMP-2; Group 2 (N=15): Defect filled with 0.1 mL gel without BMP-2; Group 3 (N=12): Defect filled with type I collagen sponge and 25 µg BMP-2, and Group 4 (N=3): Defect was left empty.

A critical size cranial defect was created in anesthetized (Isofuran, Abbott Scandinavia, Sweden) adolescent male Sprague Dawley rats. A skin flap was raised and a craniotomy with 8 mm diameter was created in the parietal and frontal bones using Midas Legend equipment (Medtronic). Constant irrigation with saline was applied and care taken to avoid injury to the underlying dura. The defect was treated according to the protocol. The skin flap was sutured in place using resorbable 3-0 Vicryl (Ethicon). The animals were allowed to move freely after the procedure. Subcutaneous buprenorphine (Temgesic, Schering-Plough) was provided for additional analgesia at a dosage of 0.05 mg/kg b.w. every eight hours for three days postoperatively. Three animals per group were sacrificed at one-, two-, three-, and four-week time-point by CO_2 and the skull was fixed in formaldehyde. In the hydrogel groups, with and without BMP-2, three animals per group were kept for 10 weeks.

3.3 HUMAN STUDIES

3.3.1 Ethical permission human studies

The study Ib was approved by the local ethical committee at Karolinska Institute (Dnr 03-244). **The study IV** was approved by the local ethical committee in Stockholm, Sweden (Dnr 2010/118-31/3). Informed consent was obtained from the patients.

3.3.2 Patients and surgical procedures (Paper Ib and IV)

In study Ib three patients were treated. The first patient had a frontotemporal defect (56 cm2) after a postoperative infection and necrosis of the bone following surgery for brain tumor. She had received radiation to the operating field. The patient underwent reconstruction with titanium mesh and heparin-chitosan with 12 mg of recombinant human BMP-2 (InductOs; Wyeth-Lederle, Ltd.). The second patient had a frontotemporal defect (54 cm2) after unsuccessful reconstruction following surgery for a cerebral aneurysm. She underwent reconstruction with titanium mesh and heparin-chitosan sponge with 12 mg of recombinant human BMP-2. The third patient had a parietal defect (117 cm2) after a postoperative infection following partial hemicraniectomy resulting from cerebral hemorrhage. The reconstruction was performed with titanium mesh and heparin-chitosan sponge with 10 mg of recombinant human BMP-2.

In study IV 12 patients treated for meningeoma, cerebral aneurysm or cerebral cyst were randomized into either treatment group or control group. In the treatment group the holes made during craniotomy were randomly treated with hydrogel with BMP-2 (250 µg/mL) or hydrogel alone. The remaining hole/s, used as paired controls in the same patient, were treated with Spongostan™ (Ethicon) or Tisseel™ (Baxter) mixed with autologous bone dust obtained from the drilled holes. In the control group the holes were treated according to local standard procedure; either with Spongostan™ (Ethicon) as haemostatic agent or with Tisseel™ (Baxter) mixed with autologous cranial bone collected from the drilled holes.

Craniotomy was made using the Midas Legend equipment (Medtronic). Three to five separate boreholes of 14 mm diameter in the tabula externa and 12 mm in tabula interna were interconnected with a rotating drill pin. Tacoseal™ (Nycomed), Surgicel™ (Ethicon) and bipolar coagulation were used for topical haemostasis on the dura, avoiding the site of the boreholes. The dura was stitched to the edges of the craniotomy

defect with 4-0 Vicryl™ (Ethicon) and Tisseel™ (Baxter) was applied under the bone edges for haemostasis. The dura was incised and the tumour or the aneurysm was removed. Floseal™ (Baxter) or Surgicel™/Tisseel™ was applied in the cavity; the cavity was rinsed and filled with water. The dura was subsequently mended producing a sealed closure using a running 4-0 Vicryl™. In some cases after tumor removal, bovine dura substitute replaced the normal dura (Lyodura™, B Braun). The bone flap was repositioned and the flap was attached to the surrounding bone by using Craniofix™ (Aesculap) or micro plates (Synthes). The holes were treated according to the protocol. The periosteum or muscle was stitched back in place over the craniotomy, and the skin flap was repositioned and sutured with 3-0 Vicryl™ (Ethicon) subcutaneously followed by skin staples (Covidien).

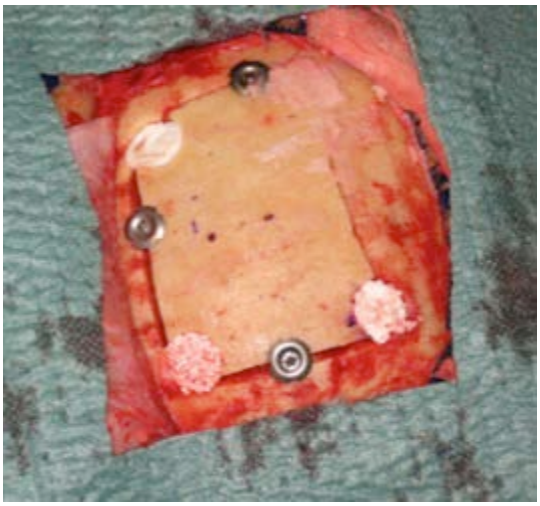

Figure 7. The holes made during craniotomy were treated according to the protocol. In this patient the top left hole was randomized to treatment with Spongostan™, the top right hole to Tisseel™ and autologous bone, the lower left hole to treatment with hydrogel and BMP-2 and the lower right hole to hydrogel alone.

3.4 RADIOLOGICAL ANALYSES

3.4.1 Radiography

In paper Ia radiography of the hind legs was performed after four weeks.

3.4.2 Computed tomography

In paper Ib computed tomography was performed one to 13 months postoperatively.

In paper II the cranium was examined with high-resolution computed tomography. The technique was used to compare the bone volume of bony regenerates at the site of the former calvarial defect. Computed tomography was performed with a 64-slice spiral

computed tomography scanner (Somatom Definition; Siemens, Forchheim, Germany) with the following scan parameters: collimation, 0.6 mm; pitch, 0.6; 200 effective mA; 120 kV; reconstructed slice thickness, 1.0 mm; reconstruction increment, 0.6 mm; kernel, H30. To estimate the volumes of the created defects and to determine volumes of new bone formation, measurements were performed from serial computed tomographic scans over an area of 20 x 40 mm centered over the sagittal suture and 10 mm from the dorsal aspect of the cranium. The volume was calculated using the software volume program in the workstation multimodality workplace (Siemens). The density of the regenerated bone was calculated using the same program and equipment.

In paper III the skulls were examined with computed tomography using a General Electric Lightspeed 64 channel VCT. Exposure settings were 120 Kv, Ma 80, and Pitch 0.531. Slices were reconstructed with soft algorithm 0.625 thickness and increment. The acquired dataset was then analysed in Advantage Windows workstation Volume viewer version 7.4.71.

In paper IV all the patients went for postoperative CT scans. After three and six months the bone healing was assessed by new CT scans. The skulls were examined with computed tomography using a General Electric Lightspeed 64 channel VCT. Spiral exposure settings were 100 Kv, Ma 70, and Pitch 0.96. Some examinations were acquired using axial mode and equivalent exposure settings resulting in DLP (Dose Length Product) in the range of 74-90. Slices were reconstructed with bone plus algorithm 0.625 thickness and increment. The acquired dataset was then analysed in Advantage Windows workstation Volume viewer version 4,5_02. The boreholes were analysed after thresholding for > 80 Hounsfield units (HU). The holes were rotated to be seen “en face” and their area measured. The substance filling the holes > 80 Hounsfield units (HU), which was interpreted as bone, was also analysed in orthogonal planes.

3.4.3 Volumetric Bone Mineral Density and Bone Volume

In paper Ia computed tomographic scanning was performed on dissected specimens with gel implants to calculate induced ectopic bone volume and bone mineral density four weeks after implantation. The muscles were scanned using multiple computed tomographic scans, with an interscan distance of 1 mm. The volumetric bone mineral density is given as the average of all scans detecting calcified tissue. The bone volume (in cubic millimeters) was calculated as the average area (in square millimeters) of all

computed tomographic scans detecting calcified tissue multiplied by the number of scans detecting calcified tissue (one scan per millimeter).

3.5 TISSUE PROCESSING AND IMAGING

3.5.1 Light microscopy

In paper Ia the quadriceps muscles were dissected out and the tissues were demineralized in decalcifying solution (Stephens Scientific, Riverdale, N.J.) for 48 hours. Demineralized specimens were fixed in 4% paraformaldehyde and embedded in paraffin. 5-µm thick sections were cut and stained in hematoxylin and eosin.
In paper II the heads of the minipigs were cleaved sagittally in the middle of the reconstructed area in the parietal and frontal cranial bone. The macroscopic appearances were documented by digital images and the craniotomy area was further cut into smaller pieces and placed in 4% aqueous solution of phosphate-buffered formaldehyde. The specimens were decalcified in 3.4% sodium formate (weight/volume) and 15.1% formic acid (weight/volume), trimmed, dehydrated, embedded in paraffin, cut into approximately 6-µm-thick sections, coded, and stained with hematoxylin and eosin and Mason Trichrome for light microscopy.
In paper III the skulls were decalcified in formic acid and sodium citrate for one week followed by dehydration and embedding in paraffin. 4 µm-thick sections were stained with Masson Trichrome for histological analysis.

3.5.2 Transmission Electron Microscopy

In paper II samples from the craniotomy area were preserved in 2% glutaraldehyde plus 1% paraformaldehyde in 0.1 M sodium cacodylate buffer. The specimens were then decalcified in 4% ethylenediaminetetraacetic acid containing 0.5% glutaraldehyde, postfixed in 2% osmium tetroxide, dehydrated, and embedded in LX-112 resin. Approximately 5 µm-thick sections were cut, stained with toluidine blue, and examined with light microscopy. Areas of interest, identified with light microscopy, were further subjected to thin-section and transmission electron microscopic analyses. Sections were analyzed using a Tecnai 10 microscope (Fei Company, Eindhoven, The Netherlands) and documented by a Megaview III digital camera (SiS Company, Münster, Germany).

3.5.3 Immunohistochemistry

In paper III immunohistochemistry was performed on paraffin-embedded sections using rabbit anti-CD146 monoclonal antibodies (Abcam) and peroxidase-DAB staining. Photographs were taken with Nikon Eclipse TE 2000-U using NIS Elements F 3.0 program.

3.5.4 Histomorphometric analysis

In paper III the histomorphometric analysis was based on photomicrographs taken with a Zeiss dissection microscope (Zeiss, Germany) and analyzed by use of Adobe Photoshop CS4 (Adobe Systems Inc.) The areas of newly formed osteoid and mineralized bone were measured on pictures from representative sagittal sections in the central part of the reconstructed cranial defect and measurements were divided by area of corresponding created original bone defect.

3.6 BLOOD ANALYSES

In paper II ethylenediaminetetraacetic acid–preserved blood was analyzed for hemoglobin, hematocrit, and total and differential white blood cell counts with an electronic cell counter validated for porcine blood (Cell-Dyn 3500; Abbott, Wiesbaden, Germany). Serum amyloid A (in micrograms per milliliter) was measured in serum samples using a commercially available enzyme-linked immunosorbent assay (Tridelta Phase range serum amyloid A kit; Tridelta Development Ltd., Greystones, Co. Wicklow, Ireland). Serum samples were analyzed for activities of aspartate aminotransferase, serum alanine aminotransferase, glutamyl transpeptidase (in microkatals per liter), and glutamate dehydrogenase (in nanokatals per liter) by use of automated equipment (Roche Cobas Mira; Roche Diagnostics, Indianapolis, Ind.).
In paper IV blood samples were taken daily for three days postoperatively analysing C reactive protein and white blood cell counts. After three and six months blood samples were repeated.

3.7 STATISTICAL ANALYSES

In paper Ia the non-parametric, one-sample Wilcoxon test was used. Statistical significance was calculated using the R statistical program (Advanced Research Computing at Virginia Tech, Blacksburg, Va.).
In paper II the statistical analysis was performed on the volume and density variables between the three experimental groups using analysis of variance. Because the number

of observations was small, the nonparametric Kruskal-Wallis test was used as a supportive statistical method. In addition, a t test and Mann-Whitney U test were used to determine where the differences were between the groups. All statistics were calculated using the R statistical program version 2.7.2 (http://www.R-project.org).

In paper III a General Linear Model was used to analyze the data in IBM SPSS Statistics (Version 19). Interactive effects were analyzed and Scheffe's post-hoc test was used for multiple comparisons to determine statistical significance between specific groups. The results are presented as mean +/- standard deviation. A p-value < 0.05 was considered as statistical significant.

In paper IV SPSS19 (IBM Statistics) was used. Not enough data was available for a repeated measures analysis. Subsequently, using all available data, a Univariate General Linear Model Analysis with 2 fixed factors was performed with 1 output; bone area, and 1 covariate; patient, as it may be a confounding factor. The analysis was made with 2 levels: time (3 and 6 months), and with 4 levels: material (1: hydrogel and BMP-2, 2: hydrogel alone, 3: Tisseel™ and autologous bone, 4: Spongostan™) and 1 covariate; patient, as it may be a confounding factor.

4 RESULTS AND COMMENTS

4.1 BONE INDUCTION WITH BMP-2 AND HEPARIN/CHITOSAN (IA AND IB)

Different doses of BMP-2 were evaluated together with type I collagen alone, type I collagen and heparin, and heparin/chitosan both in gel formulation and in sponge. The carriers and growth factor were implanted in the quadriceps muscle of rats and evaluated after four weeks. Previous to the animal studies the heparin/chitosan interaction was evaluated with enzymatic assays showing that heparin in complex with chitosan is not hydrolyzed by heparinase I. On treatment with heparinase III, at least 50 percent of heparin remained after 10 days indicating a slower enzymatic degradation of heparin in complex with chitosan, thus potentially stabilizing the growth factor and enhancing the activity of BMP-2. The animal studies showed abundant ectopic bone formation in the heparin/chitosan group together with 50 µg of BMP-2. No ectopic bone was detected for any BMP-2 doses when using type I collagen alone. When heparin was added to the type I collagen system, minor amounts of bone were induced using 50 µg of BMP-2 but not at lower doses. The heparin/chitosan complex, without exogenous BMP-2 added, induced a small amount of ectopic bone, which implies the stabilization in situ of endogenous bone-inductive factors by the complex. When using the heparin/chitosan complex as a carrier for BMP-2, ectopic bone was formed with a good yield in a dose-dependent fashion.

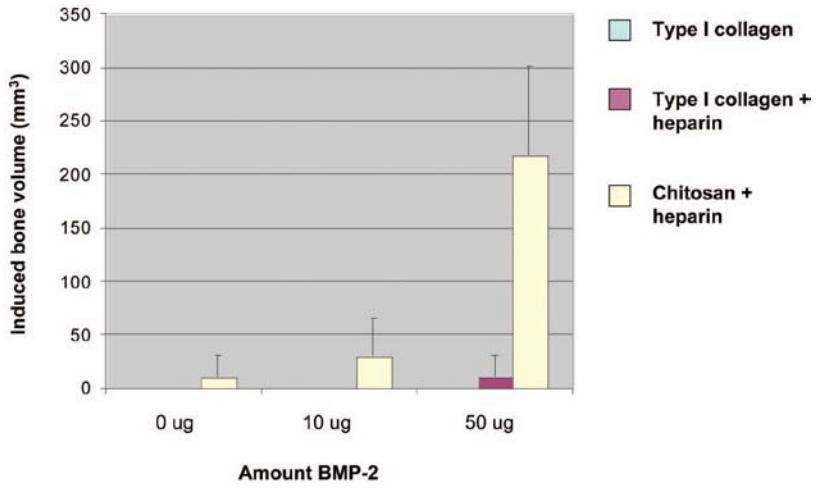

Figure 8. The volumes of induced bone formation as calculated from small-animal computed tomographic scans are presented. Gel formulations of the carriers were used with10 implantations per group. The differences in induced bone volumes between heparin/type I collagen plus 50 µg of BMP-2 and heparin/chitosan plus 50 µg of BMP-2 was analyzed statistically (p= 0.0019).

Similar bone formation and a more coherent bone structure was observed when using the sponge formulation of the heparin/chitosan complex compared to the gel. There was no difference in bone mineral density (330 to 350 mg/cm3) between the heparin/type I collagen and heparin/chitosan groups. Histological examination demonstrated osteoid with osteoblasts indicating active bone formation at this time point. No remains of implanted heparin/chitosan sponges were detected. A low to moderate inflammatory reaction, with lymphocyte infiltration but without the presence of giant cells, was seen in the surrounding muscular tissue.

In the clinical study titanium mesh combined with heparin-chitosan as a BMP-2 delivery vehicle was used to reconstruct cranial defects. The three included patients all demonstrated a postoperative inflammatory reaction, and two out of three patients had exposure of the titanium mesh. Week bone formation was seen in one patient.

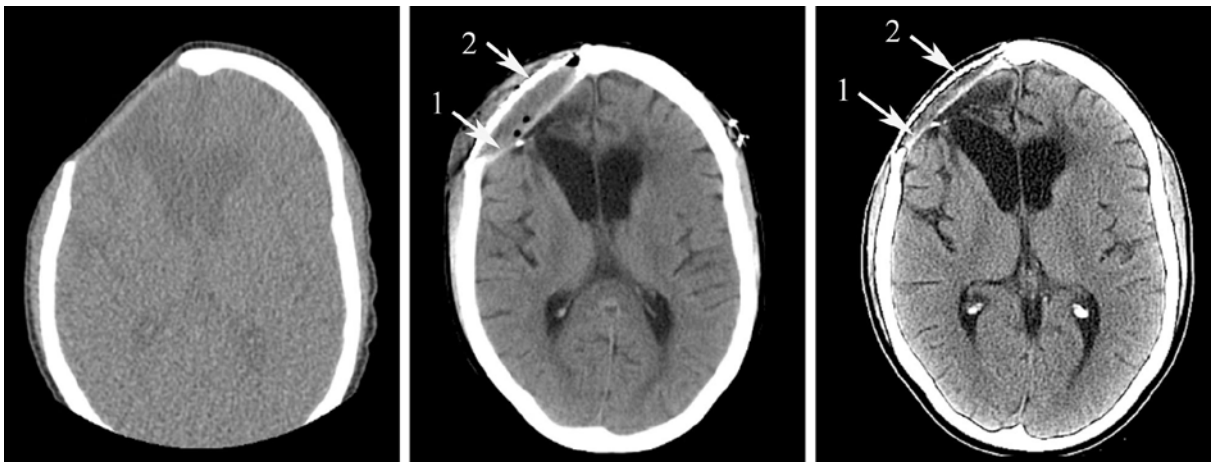

Figure 9. Computed tomographic scans of the frontotemporal defect in the first patient before surgery (*left*), four weeks after surgery (*center*), and 12 months after surgery (*right*) reveal weak bone formation. Although a calcified dura mater was seen after four weeks (*arrow 1*), no evidence of new bone formation was present at that time point or after 12 months. The dense computed tomographic signal at the reconstructed area corresponds to the titanium implant (*arrow 2*).

Although the preclinical study showed good bone formation in an ectopic site with the tested carrier, the results in the clinical study are disappointing. The lack of uniform bone formation at the implantation site and the induced inflammatory reaction make this composition unfavorable in a clinical setting.

4.2 HEALING OF CRANIAL DEFECTS IN MINIPIGS WITH BMP-2 AND HYALURONAN-BASED HYDROGEL (II)

After three months, computed tomographic and histological examinations of the minipig skulls were performed. The volumes of the created defects measured 19.6 ±7.2 cm3 and varied due to different bone heights. The volumes of newly formed bone in animals treated with hydrogel and BMP-2 were 23.4 ±6.3 cm3 (119 percent ossification of the defects) as determined from serial computed tomographic scans indicating the complete healing of the defects and bone overgrowth to some extent. The volumes of new bone in animals treated with hydrogel alone and in animals with untreated defect were 11.4 ±3.4 cm3 (58 percent ossification) and 10.3 ±2.3 cm3 (53 percent ossification), respectively.

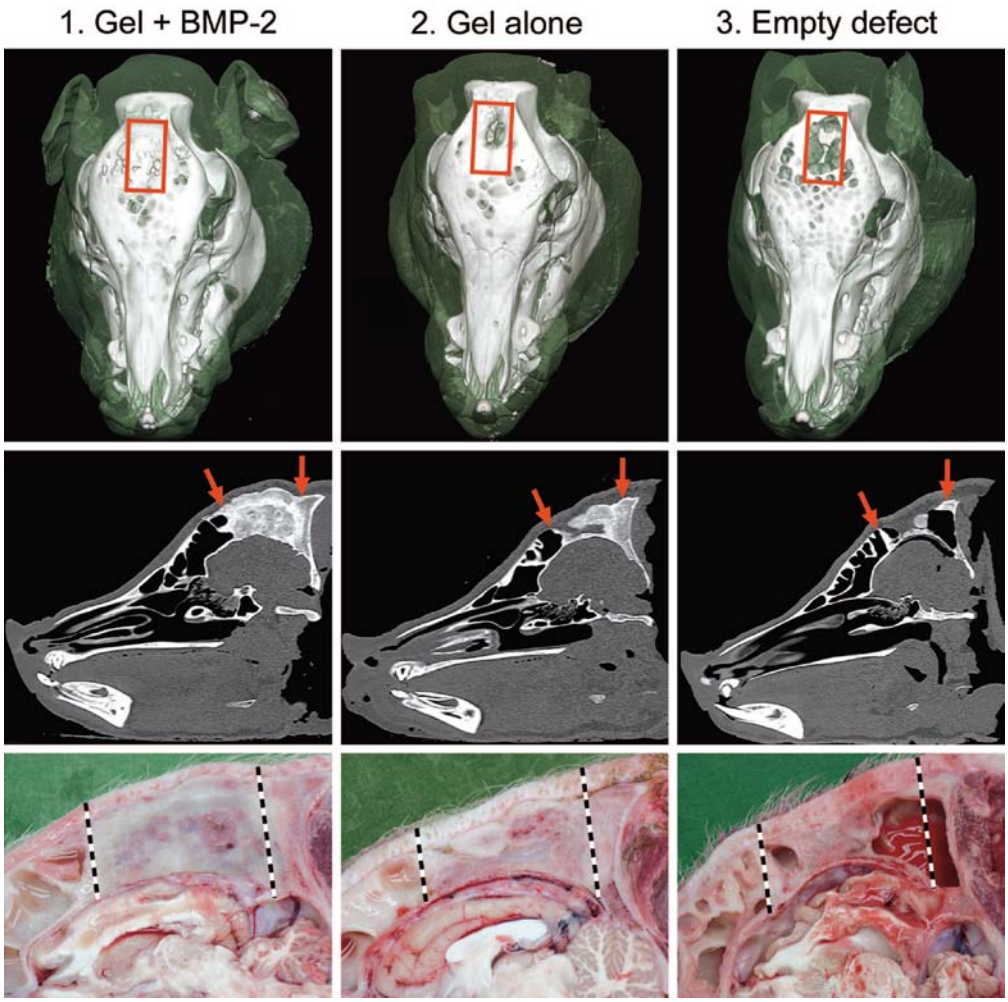

Figure 10. Three-dimensional computed tomography (*above*) and sagittal computed tomography (*center*) with *red rectangle* and *arrows* indicate the location of the defect in representative animals of groups 1 through 3. Sagittal cross-sections (*below*) show the macroscopic appearance of the reconstructed area.

There was a statistically significant difference in induced bone volumes between the BMP-treated animals and the animals treated with hydrogel alone. The densities in Hounsfield units were measured in areas comprising compact bone in the defects, with no significant differences seen between the groups.

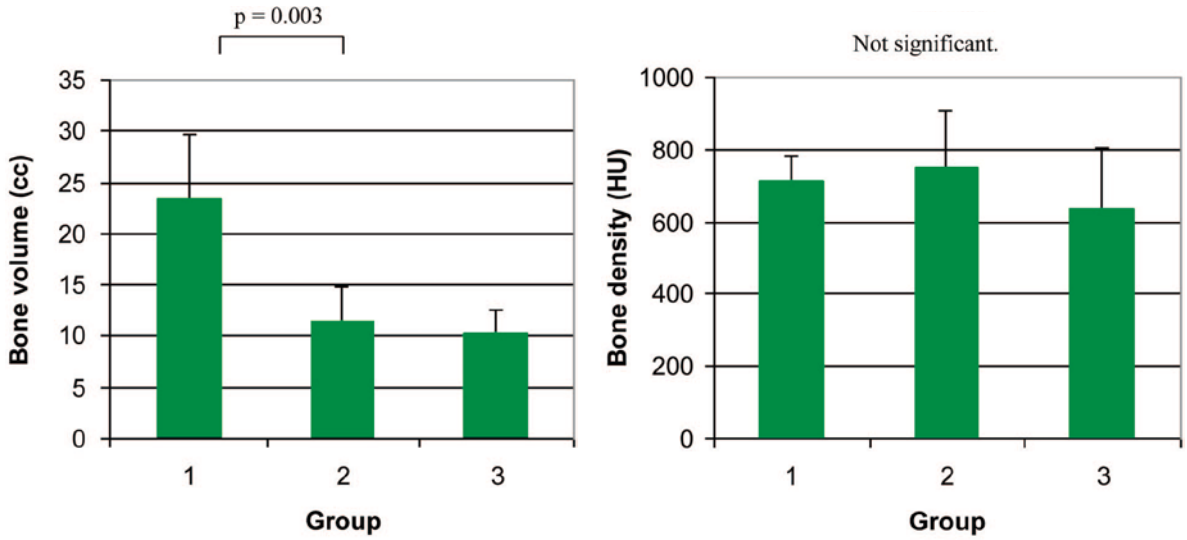

Figure 11. (*Left*) Bars showing differences in induced bone volume between the treatment groups. Group 1, hydrogel with BMP-2; group 2, hydrogel alone; group 3, empty defect. There is a significant difference in bone volume between the groups. Analysis of variance, $p = 0.003$; Kruskal-Wallis, $p = 0.013$. The independent *t* test gives the *p* values between groups as follows: groups 1 and 2, $p = 0.003$; groups 1 and 3, $p = 0.040$; and groups 2 and 3, $p = 0.702$. Mann-Whitney *U* test gives the *p* values between groups as follows: groups 1 and 2, $p = 0.004$; groups 1 and 3, $p = 0.095$; and groups 2 and 3, $p = 0.643$. Both the *t* test and the Mann-Whitney *U* test give a significant difference between groups 1 and 2. Only the *t* test gives a significant difference between groups 1 and 3. (*Right*) Bars showing bone densities. There is no significant difference in density between the groups. Analysis of variance, $p = 0.583$; Kruskal-Wallis, $p = 0.614$.

Histological examination revealed compact lamellar bone in the BMP group without intertrabecular fibrous tissue, as was seen in the other groups. The hydrogel was resorbed completely within three months and, importantly, caused no inflammatory reaction.

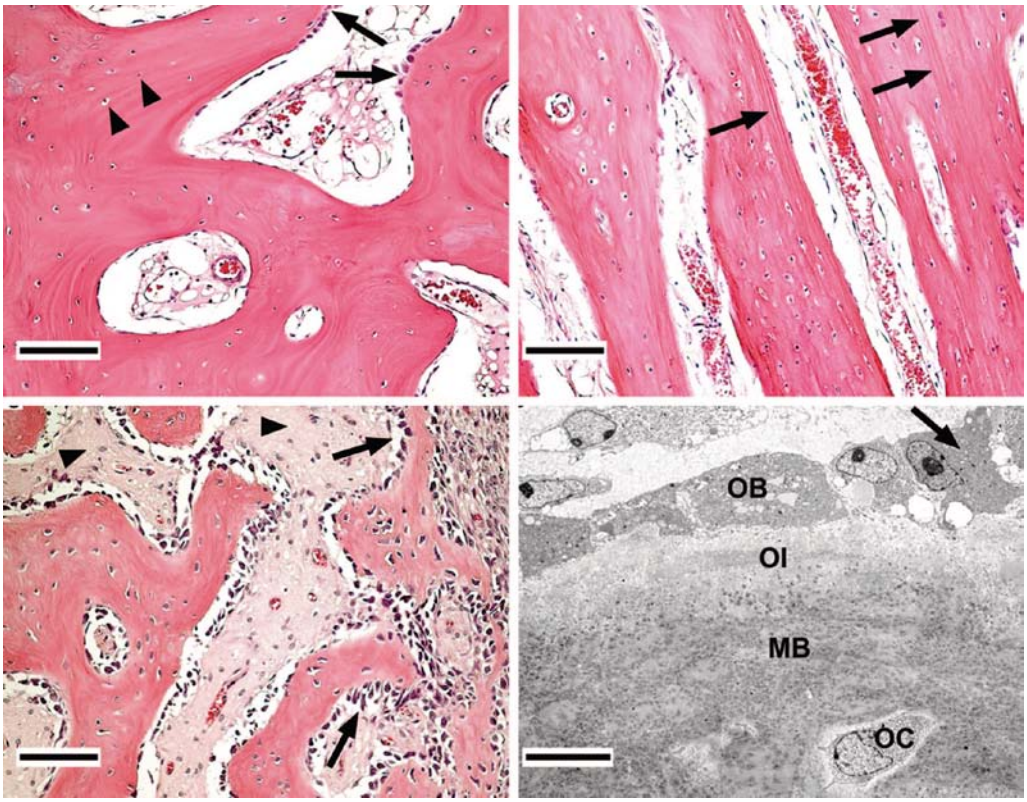

Figure 12. Sections of bone illustrating the histological picture. In the group treated with hydrogel and BMP-2 (*above*, *left*), trabecular and compact bone with a dominating structure of lamellar tissue is seen. The bone contains osteocytes (*arrowheads*) and is lined by osteoblasts (*arrows*). Intertrabecular adipose tissue with blood vessels is also seen. In the group treated with hydrogel alone (*above*, *right*), trabecular woven bone with areas of lamellar structure (*arrows*) is present. In empty defects (*below*, *left*), trabecular woven bone with high osteoblastic activity (*arrows*) and intertrabecular fibrous tissue (*arrowheads*) is observed (hematoxylin and eosin stain; scale bars=100 µm). Transmission electron microscopy from the BMP group showing direct bone formation (*below*, *right*). Osteoblasts (*OB*) are polarized toward the bone matrix displaying an active morphology with extensive endoplasmic reticulum (*arrow*), producing type I collagen, osteoid (*OI*).An osteocyte (*OC*) is trapped in the mineralized bone matrix (*MB*). Scale bar =10 µm.

The minipigs initially weighed 18 to 29 kg and gained an average of 9 kg during the experimental period. The laboratory tests showed raised liver counts, mainly aspartate aminotransferase, in four animals before the start of the study. These tests were normalized during the experimental period, and all other tests including hematocrit and white blood cell counts were normal. The examination of the pigs under anesthesia four weeks after surgery showed good healing of the flap and no swelling or redness in any of the cases except for one treated with hydrogel and BMP-2. Necropsy of the inner organs including brain, heart, liver, kidney, and bowels showed no pathologic changes.

4.3 BONE HEALING OF CRANIAL DEFECTS IN RATS COMPARING HYALURONIC ACID HYDROGEL AND COLLAGEN CARRIER (III)

Temporal cellular and tissue morphological changes at the reconstruction sites were studied on histological sections from each group. In the hydrogel and BMP-2 treated animals bone was formed along the dura after one week, with larger quantities at the edges of the 8 mm defect. The hydrogel was seen in the defect and also in the subcutaneous layer, where large cavities were observed. At one and two week time-points, undefined mononuclear cells infiltrated the hydrogel from the periphery. After three weeks the hydrogel was partly resorbed and replaced by increasing amounts of osteoid and smaller cavities. The bone volume increased further, and after four weeks more mature mineralized bone with bone marrow cavities were observed within the treated area, but also an overgrowth at a subcutaneous location occurred. No extended growth of bone tissue towards the dura mater and the brain was observed.

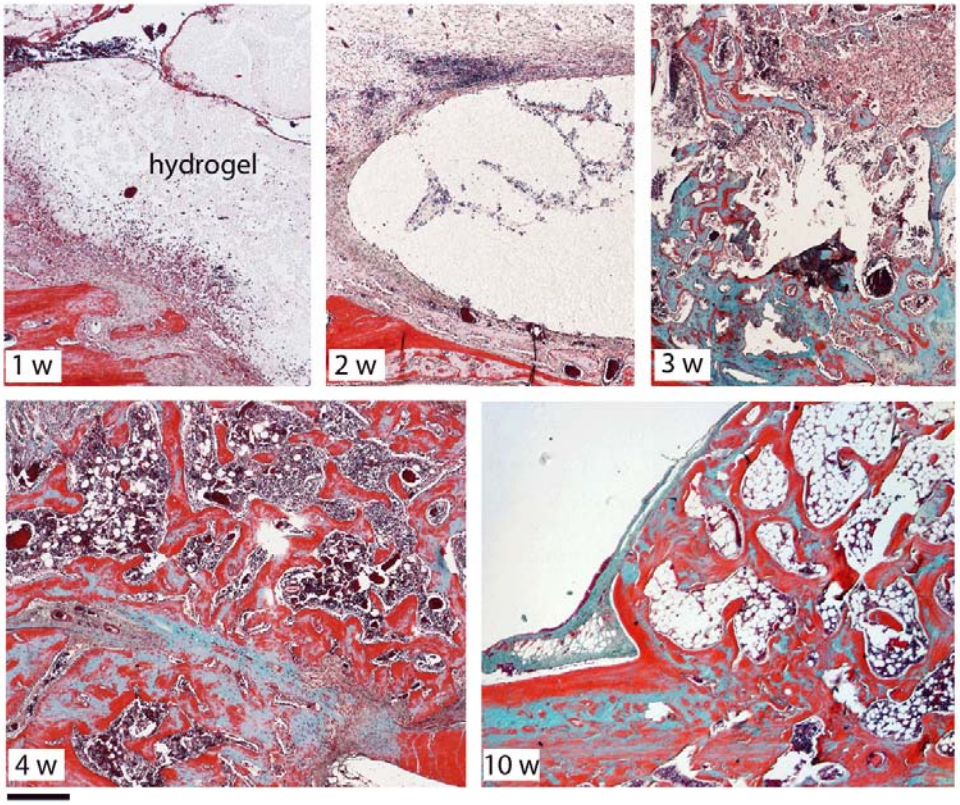

Figure 13. Masson Trichrome-stained histological sections from cranial defects treated with hydrogel and BMP-2 demonstrate the temporal cascade during bone formation. The hydrogel is initially infiltrated by undefined mesenchymal cells, resorbs, and ultimately replaced by osteoid and mineralized bone after three to four weeks. A 10-week time point was included showing mature, fully integrated bone with fat-rich bone marrow cavities. Bar = 500 µm.

In the collagen and BMP-2 group bone was initially formed along the dura. A transient presence of hypertrophic chondrocytes was seen after one week but not at later time points. This was in contrast to bone induced by hydrogel-delivered BMP where no sign of endochondral ossification was observed histologically. A minor overgrowth of bone at subcutaneous location was present after three and four weeks.

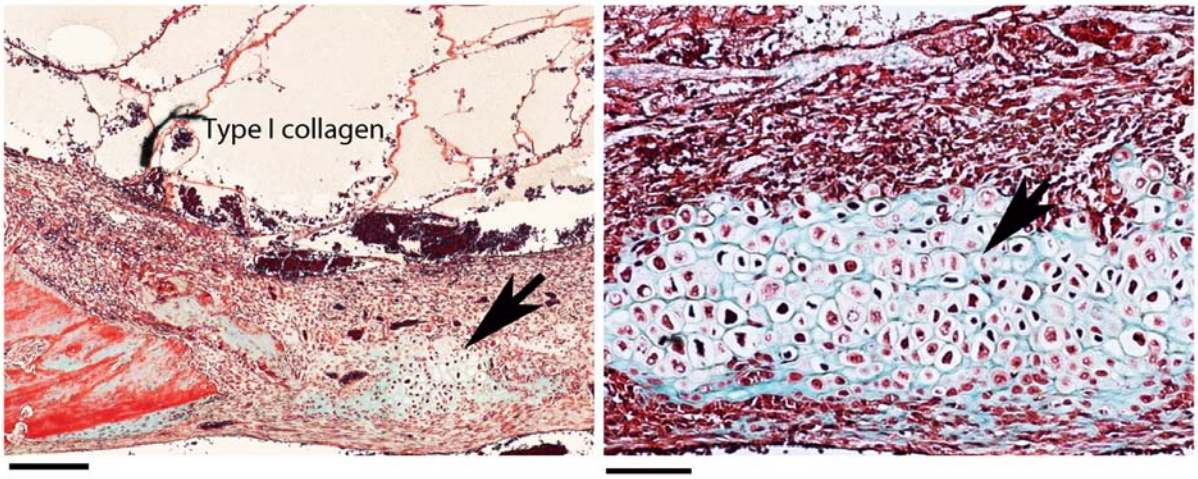

Figure 14. Histological examination from cranial defects treated with type I collagen and BMP-2 demonstrates areas with hypertrophic chondrocytes (arrows) after one week at lower (left, bar =500 µm) and higher magnifications (right, bar =100 µm). This indicates endochondral ossification in this group in contrast to defects treated with hydrogel and BMP-2 where no cartilage was present at any time point.

In the hydrogel group, without BMP-2, the picture was similar as in the collagen-BMP-2 group after one week, although showing a more limited increase in bone volume over the following three weeks. In the empty sham operated animals some bone formed along the dura, but incomplete bony healing of the defect was seen (data not shown). The final four week results from implants are further demonstrated by representative 3D-CT scans of bone development.

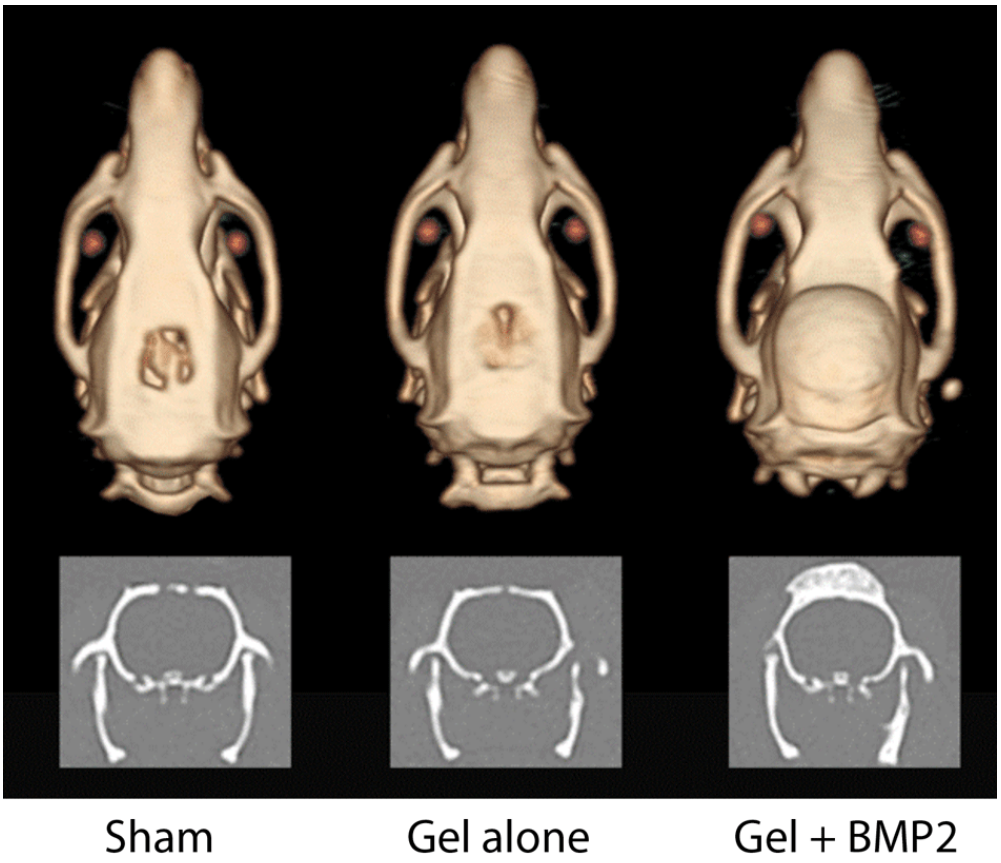

Figure 15. 3D CT-scans illustrate the gross appearance of cranial defects in rats four weeks after treatment with hydrogel, with and without 25 µg BMP-2, and compared to untreated sham operated controls. The lower pictures are coronar cross-sections from CT-scans, which further show the pronounced overgrowth of bone in animals treated with hydrogel and BMP-2.

Immunohistochemistry showed staining of the mesenchymal osteoprogenitor cell marker CD146 in the newly formed bone tissue in sections from BMP-2 treated animals, both in the hydrogel and the collagen groups. Weaker signals were seen from non-BMP treated animals. After one week CD146 positive cells co-localized with infiltrating blood vessels in the newly formed mesenchymal tissue. After three weeks the staining was less restricted to vessels and more intense in the newly formed bone marrow cavities in the hydrogel and BMP-2 groups whereas the presence of CD146 diminished in the collagen and BMP-2 group at this later time point. A slightly different tissue distribution of CD146 was seen in animals treated with type I collagen and BMP-2 showing a line of positive cells along the edge of the bone. In the hydrogel group some staining was seen in the mesenchymal tissue next to the newly formed bone with little difference over time. A similar pattern was also seen in the sham operated control group (data not shown).

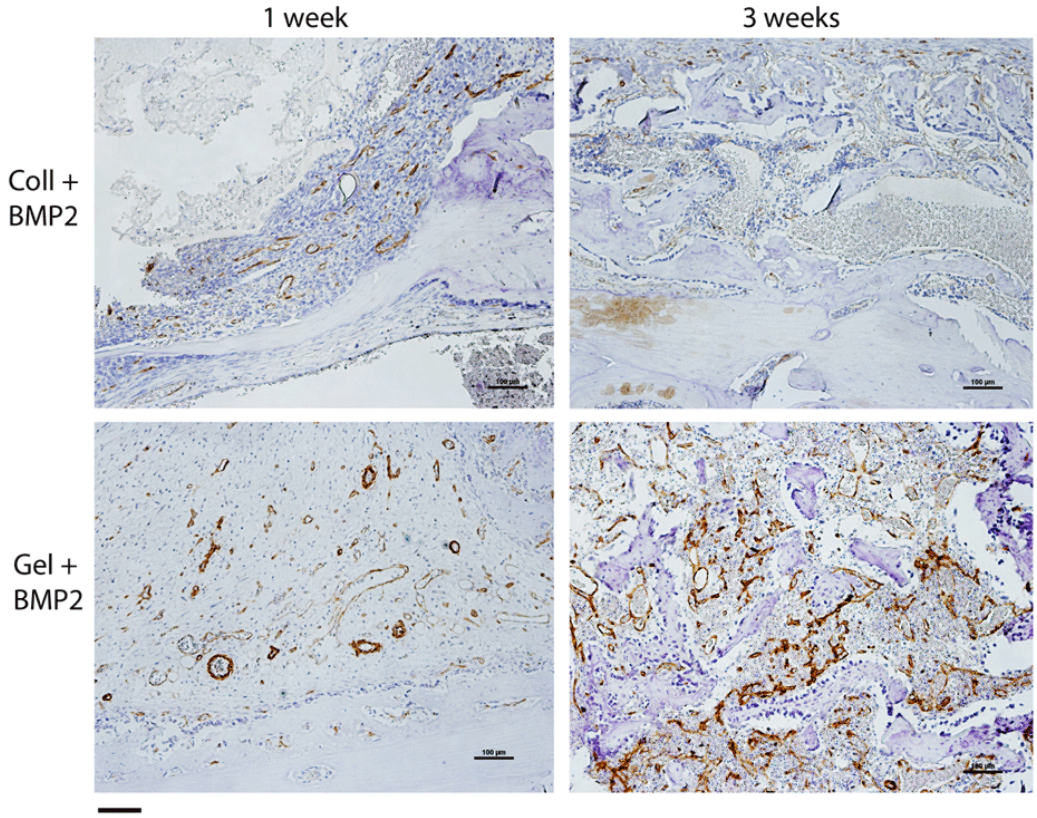

Figure 16. Spatial and temporal distribution of the osteoprogenitor marker CD146 was investigated by immunohistochemistry and comparison was made between cranial defects treated with hydrogel and BMP-2 and treatment with type I collagen and BMP-2. The results reveal a vascular staining pattern in both groups after one week, which indicates that osteoprogenitor cells are recruited through blood vessel formation during early bone formation. After three weeks the signals are even higher in the hydrogel and BMP-2 group and the distribution of CD146 positive cells are different, now localized within bone marrow-like tissue in between newly formed bone. CD146 staining intensity declined in the collagen and BMP-2 group after three weeks, which may indicate a lower BMP activity at this time point. Bar = 100 µm.

Histomorphometric analysis revealed an increasing amount of bone over time in the different treatment groups. Interactive effects were found between time and material ($p=0.002$), as well as main effects of time ($p<0.001$) and material ($p<0.001$). The multiple comparisons test (Scheffe's) showed significantly higher bone area for hydrogel and BMP-2 compared to the other materials and also higher bone area for weeks two to four in comparison to week one. The linear model predicted an increase in bone area of 4.02 for hydrogel and BMP-2 in comparison to the sham control group ($p<0.001$). Type I collagen and BMP-2 also gave an increase in bone area in comparison to control only of 0.74, but this increase was not statistically significant ($p=0.27$). For hydrogel only, a decrease in bone area of -0.15 was predicted, but this was not significant either ($p=0.83$).

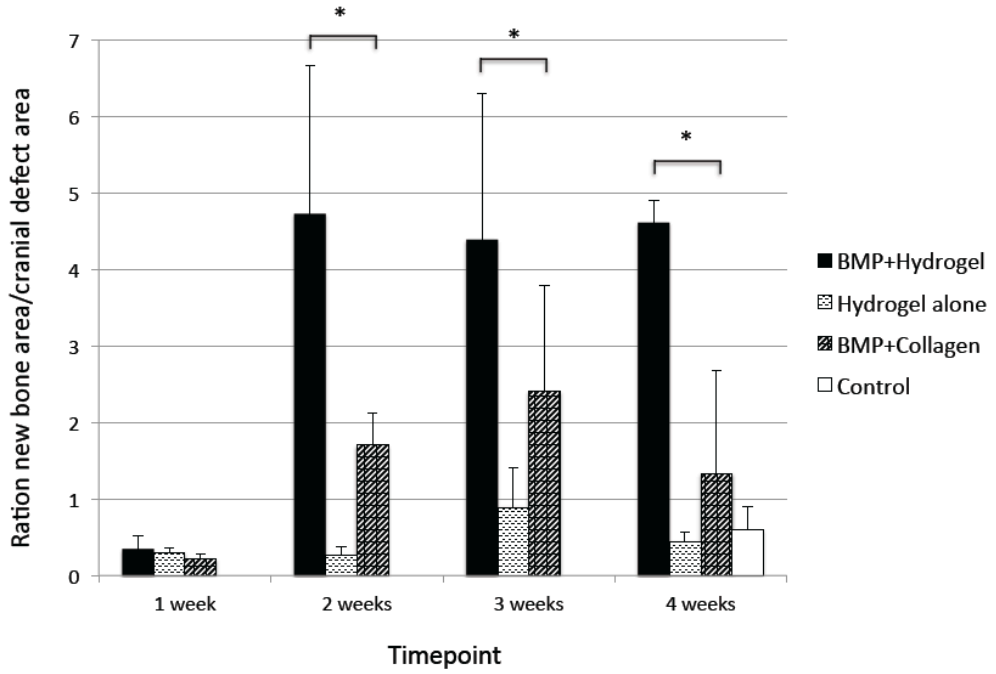

Figure 17. Areas of new bone formation were measured from histological pictures from each experimental group and different time-points and related to the calculated area of corresponding created cranial defect. A ratio > 1 thus means that excessive bone formation was induced. Histomorphometric analysis show the significant increase in bone formation in the hydrogel and BMP-2 groups as compared to other treatment groups two to four weeks after surgery. * $p < 0.001$.

4.4 PROSPECTIVE RANDOMIZED STUDY OF CRANIAL RECONSTRUCTION AFTER NEUROSURGERY WITH BMP-2 AND A HYDROGEL CARRIER (IV)

Bone healing was evaluated with CT scans after three and six months. Bone healing in holes treated with Tisseel™ with autograft or hydrogel with BMP-2 was significantly increased compared to negative controls ($p < 0.001$ and $p = 0.008$, respectively) whereas holes treated with hydrogel only did not heal significantly better ($p = 0.066$). Material was found to have a significant effect ($p<0.001$) whereas time and patient were found not to have a significant effect ($p=0.14$ and 0.28, respectively). In holes treated with hydrogel and BMP-2 an increase in bone area of approximately 49 mm2 were obtained. Holes treated with Tisseel™ with autograft showed an increase in bone area of approximately 66 mm2. Holes treated with hydrogel without BMP-2 showed an increase in bone area of approximately 33 mm2. In one of the patients we noticed complete healing of all the holes after six months.

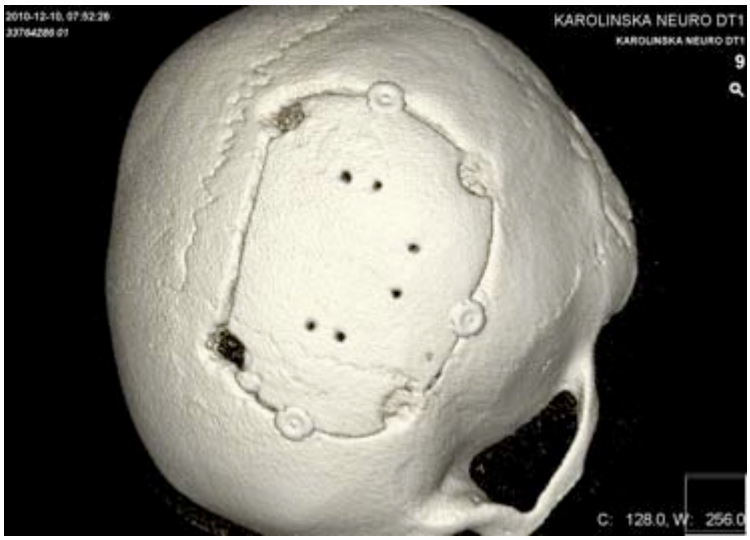

Figure 18. In one of the patients we noticed complete healing of all the holes after six months independent of treatment although the thickness of bone varied between the groups. A thin layer of bone is observed in the top left hole treated with hydrogel alone, as in the bottom left hole treated with Spongostan™. In the top right hole, treated with Tisseel™ and autologous bone, a thicker layer of bone is observed, as in the lower right hole treated with BMP-2 and hydrogel.

The increase in bone area from three to six months was not significant in any group (p=0.72). Six patients had treatment with both hydrogel and BMP-2 and hydrogel without BMP-2 in different cranial holes. A paired analysis comparing bone formation showed a significant difference between the groups (p=0.043).

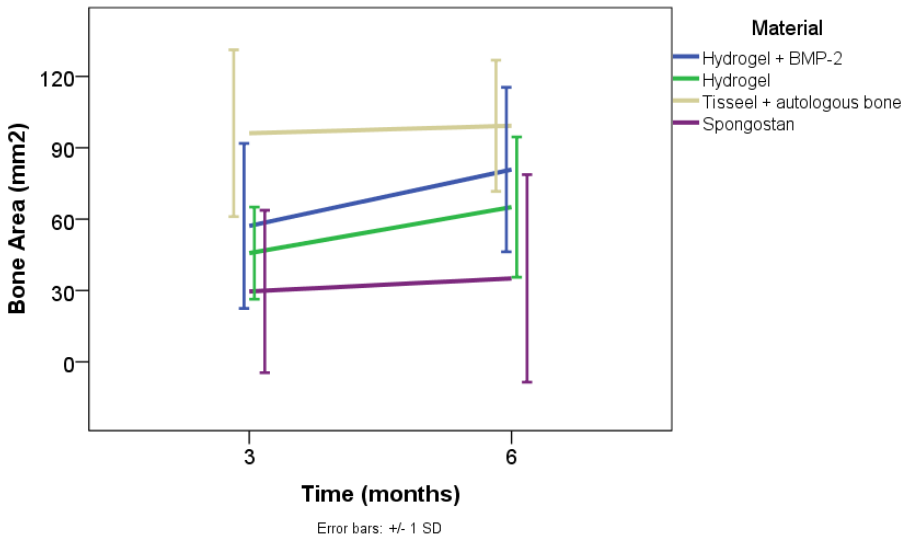

Figure 19. A significant increase in healing of bone were seen in holes treated with Tisseel™ and bone transplants ($p < 0.001$) and in holes treated with hydrogel and BMP-2 (p=0.008) as measured from CT-scans and compared to Spongostan™ , negative control group, and independent of time-points.

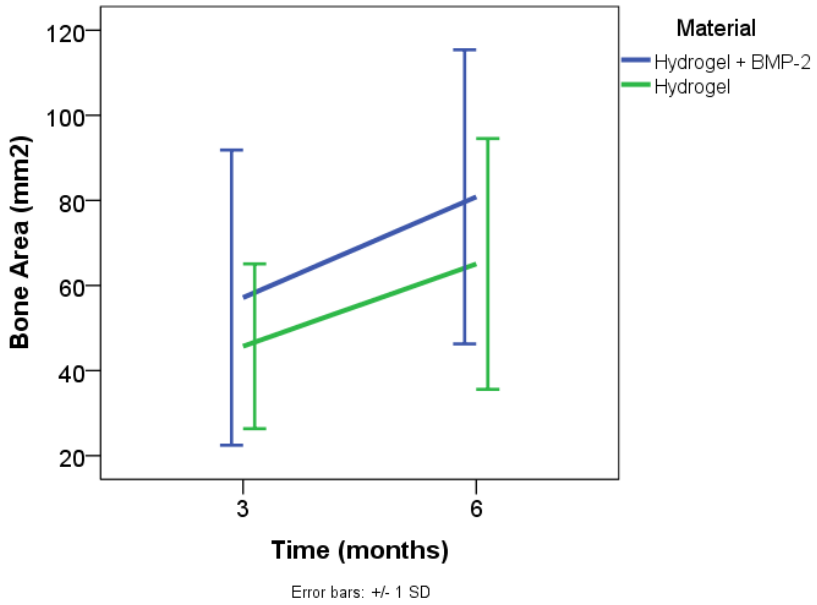

Figure 20. A second study was performed comparing hydrogel and BMP-2 to hydrogel only. Six patients had both materials and were included in a repeated measures general linear model. Time and material was evaluated as within-subject factors. A statistically significant difference between the results at three and six months was found (p=0.016) and between hydrogel and BMP-2 and hydrogel only (p=0.043). No interactive effect was found between time and material (p=0.786). The observed statistical power was 81% for time and 59% for material.

Blood analyses showed slightly raised levels of C reactive protein and white blood cells in the early postoperative period. No difference was detected between the patients in hydrogel and BMP-2 group compared to controls. After three and six months the blood tests were normal in all the patients. No local or systemic side effects, including excessive bone over-growth or inflammatory reaction, were seen in treated patients. Three patients, one in the treatment group and two in the control group, had postoperative infections that required antibiotic treatment. One patient in the control group had the bone flap removed due to infection.

5 GENERAL DISCUSSION

5.1 HEPARIN/CHITOSAN IN TISSUE ENGINEERING

Heparin is known to have a positive effect in tissue engineering by stabilizing and activating growth factors, such as BMPs. In wound healing studies heparin is used to sustain the release of growth factors with affinity for heparin, for example in Platelet-rich-plasma (PRP). PRP contains fibroblast growth factor 2 (FGF-2), platelet-derived growth factor-BB, and vascular endothelial growth factor (VEGF) that enhances the healing of skin wounds in mice[90]. Studies, in vitro, in a human wound-healing assay show that heparin does not stimulate reepithelialization, whereas the heparin/chitosan complex stimulates wound healing in human skin[91].

In a recent review article by Hudalla et al, it is stipulated that biomaterials covalently or non-covalently modified with heparin glycosaminoglycans can augment growth factor releasing strategies. In addition, recent studies demonstrate that biomaterials modified with heparin-binding peptides can sequester cell-secreted heparin proteoglycans and, in turn, sequester growth factors and regulate stem cell behavior[92]. Johnson et al. conclude that the addition of heparin alone to collagen does not promote bone ingrowth and the addition of heparin to collagen does not improve BMP-mediated bone regeneration. Delivery of precomplexed BMP-2 and heparin in a collagen matrix, on the other hand, results in new bone formation with mechanical properties similar to those of intact bone[93]. This is in accordance with our study where heparin is used to stabilize BMP-2 and chitosan in turn stabilizes heparin thus prolonging the half-life of heparin and the BMP-2 induced bone formation.

The interest in heparin is not only limited to enhancement of wound healing and bone formation, but also its effects on angiogenesis. In a recent study by Mammadov et al. a synthetic peptide functionalized with biologically active groups to mimic heparin is presented. Like heparin, the molecule has the ability to interact with growth factors and effectively enhance their bioactivity. The nanofibers formed by these molecules were shown to form a 3D network mimicking the structural proteins in the extracellular matrix. Because of heparin mimicking capabilities of the peptide nanofibers, angiogenesis was induced without the addition of exogenous growth factors in vitro[94].

Chitosan has played a major role in bone tissue engineering over the last two decades, being a natural polymer obtained from chitin, which forms a major component of

crustacean exoskeleton. In recent years, considerable attention has been given to chitosan composite materials and their applications in the field of bone tissue engineering due to its minimal foreign body reactions, an intrinsic antibacterial nature, biocompatibility, biodegradability, and the ability to be molded into various geometries and forms such as porous structures, suitable for cell ingrowth and osteoconduction. The composite of chitosan including hydroxyapatite is very popular because of the biodegradability and biocompatibility in nature[95]. The majority of studies regarding chitosan are performed in cell culture or in animal studies, with very few examples of human application. In our clinical study we experienced pronounced inflammatory reaction in all the patients and poor bone forming capacity leaving questions about chitosan as a carrier material in a clinical setting.

5.2 HYALURONAN-BASED HYDROGEL IN CRANIAL BONE FORMATION

Hyaluronic acid hydrogel has all the benefits of a hydrogel; it is biocompatible and injectable, allowing for minimally invasive procedures. It can be cross-linked to improve its mechanical qualities, and it produces no evident inflammatory reaction at the site of reconstruction or at the systemic level. Different growth factors can be delivered by the hydrogel and released in a sustained manner. In **study II, III and IV** aldehyde-modified hyaluronic acid was used in a small animal study, a large animal study and a human study to deliver BMP-2. The limitations of the hydrogel are the problems of defining the bone volume in size and shape; it depends on a surrounding wall or scaffold to limit the bone formation.

The amount of BMP-2 needed for bone formation in different species is another issue. Comparison of healing rates between different species is difficult because higher order animals are known to be less responsive to a given concentration of BMP-2 than lower order animals[96]. In our studies we used 0.1 mL with 25 µg of BMP-2 (250 µg/mL) in the rat study, 5 mL with 1.25 mg of BMP-2 (250 µg/mL) in the minipig study, and 1 mL with 250 µg (250 µg/mL) in the human study. This generated excess bone in both animal studies, but not in the human study where the bone healing was slower and probably could have benefitted from a slightly higher dose.

The inflammatory response in **study II and IV** were measured. In the minipig study the level of serum amyloid A (in micrograms per milliliter), the equivalent of the human acute phase protein, C reactive protein, was measured before surgery, at four weeks after surgery, and at termination of the study. Normal levels of serum amyloid A and clinical examinations of the operation field without signs of redness and swelling,

except for one animal with infection, suggest that BMP-2 in conjunction with the hydrogel does not induce inflammation in minipigs. In the human study the levels of C reactive protein and white blood cells were slightly raised in the early postoperative period with no difference seen between the patients in hydrogel and BMP-2 group compared to controls. After three and six months the blood tests were normal in all the patients. No local or systemic side effects, including excessive bone over-growth or inflammatory reaction, were seen in treated patients.

5.3 TYPE I COLLAGEN AS A CARRIER FOR BMP-2

In **study Ia and III** type I collagen was used in comparison to heparin/chitosan and aldehyde-modified hyaluronic acid. As previously mentioned, type I collagen sponge is the only commercially available carrier for BMP-2 (InductOs, Wyeth). In the first study a poor result with very little bone formation in an ectopic location was seen, inferior to heparin/chitosan with BMP-2. In **study III** a significantly lower amount of bone was seen after two to four weeks comparing collagen to hydrogel and BMP-2, and immunohistochemistry showed a faster decrease in bone forming intensity in the collagen group. All together type I collagen seems to induce weaker bone formation than both heparin/chitosan and aldehyde-modified hyaluronic acid. One advantage of collagen compared to hydrogel is that bone formation is more restricted to the sponge and does not spill over into the surrounding tissue, an important issue when reconstruction is done close to vital structures.

5.4 STUDY DESIGN – CRITICAL SIZE DEFECTS

In **study II, III and IV** different types of critical size cranial defects were used. In **study II** a minipig model with a critical size defect of 2 x 4 cm was used. Because of the minipigs head anatomy with large frontal sinuses, this defect often interferes with the sinuses. Defects communicating with sinuses are prone to infection[75], and in this study one out of fourteen pigs were excluded because of this. Another point is the different thickness of the minipigs´ cranium, something that affects the volume that needs to be reconstructed. This did not seem to affect the outcome of the study, since all the defects healed with over 100% ossification of the defect. In **study III** a circular defect with 8 mm diameter was made in the rat cranium in the frontal and parietal bones. This standardized defect works well as the resulting bone formation is easily compared to other studies with the same experimental design. In **study IV** we randomized the patients into two groups to ensure that there were no systemic side

effects of the treatment given in the experimental group. In the experimental group in turn, the patients were their own controls with the cranial holes randomized to treatment with BMP-2 and hydrogel, hydrogel alone or control. This model has not been presented earlier, and we found that it worked well in comparing different compounds in a clinical setting.

5.5 BMP-2 TOGETHER WITH OTHER GROWTH FACTORS

The bone forming process is a cascade of events which include the involvement of different types of cells and growth factors. Peptide growth factors stimulate the activity of osteoprogenitor cells and osteoblasts and may enhance osteogenesis[97]. The generation of a functional microvascular network within the generated tissue is important to provide oxygen and nutrients to facilitate growth, differentiation and tissue functionality[98,99]. Fibroblast growth factor (FGF) and VEGF are strongly expressed during fracture repair[100] and the importance of angiogenesis in fracture healing and bone formation has previously been described[101-103]. During endochondral bone formation VEGF modulates angiogenesis, chondrocytes apoptosis, cartilage remodeling, osteoblast migration and endochondral growth plate ossification[101,104-106]. It has been stated that BMPs produce bone by a complex series of events involving BMPs 2, 3, 4 and 6. Concurrently other cytokines may facilitate bone formation in other ways, e.g. FGF, which has an angiogenic effect that promotes neovascularisation, and PDGF and IGF-1 acting as local modulators[96].

In order to improve the effect of BMPs in tissue engineering and to lower the doses for bone regeneration, a combination with other factors is possible. There seems to be a synergistic effect of dual delivery of VEGF and BMP-2 in early bone regeneration described by Patel and others[107-110]. Platelet rich plasma (PRP) naturally includes FGF, TGF-beta1, PDGF, VEGF and IGF-1. The combination of PRP and BMP has been shown to result in improved vascular perfusion around bone defects, enhanced bone healing and density as well as a possibility of lowering the doses of BMP-2[111]. NELL1 is another molecule that has been discussed by Aghaloo et al[112]. It plays a key role as a regulator of craniofacial skeletal morphogenesis, especially in committed chondrogenic and osteogenic differentiation, and might be an ideal molecule for combination with BMP-2 in calvarial defect regeneration.

5.6 INFLAMMATORY RESPONSE EVOKED BY BMP-2

An inflammatory response, with elevated expression of pro-inflammatory cytokines such as interleukin-1, interleukin-6 and tumor necrosis factor-alpha (TNF-alpha), is proposed to initiate the bone healing cascade and induce bone healing[113]. The use of systemic factors such as non-steroidal anti-inflammatory drugs (NSAIDs, which inhibit cyclooxygenase and therefore prostaglandins required in the inflammatory phase) influence bone healing together with age (with decreased expression of mediators, hormonal changes, impaired osteoblast function)[113], smoking[114] and local factors. The role of TNF-alpha is debated. According to Lehmann et al. TNF-alpha signaling contributes to the regulation of chondrocyte apoptosis and a lack of TNF-alpha signaling leads to a persistence of cartilaginous callus and delayed resorption of mineralized cartilage, thus delaying bone healing[115]. Glass et al. state that TNF-alpha promotes muscle derived stromal cells (MDSC) migration, then osteogenic differentiation at low concentrations. However, TNF-alpha inhibits bone healing at high concentrations[116]. Thus, modulation of the inflammatory response is important, and different drugs that suppress the TNF-alpha induced NF-κB activation, leading to reduced inflammatory response are studied[117]. The inflammatory response evoked by BMPs in certain applications has been found to be disadvantageous, especially in areas close to vital structures such as the brain or the spine. The difficulties of restraining the osteoinductive effects avoiding potential deleterious excessive bone growth balances with the need to obtain sufficient bone formation[118,119]. The safety of BMP-2 and BMP-7 in spine surgery and cranial reconstruction is debated, especially when high doses of growth factor are being used[120,121]. In children with craniosynostosis facial oedema has been reported after reconstruction with BMP-2[122]. High doses of BMP-2 has been shown to induce inflammatory reaction, tissue swelling and increased osteoclast activity in a rat model[123], further emphasising the need for carriers that allow doses closer to physiological levels. In cervical spine surgery high doses of BMP-2 are associated with adverse effects. Lowering the dose most likely reduces the risks of local inflammatory reaction, which is known to be BMP-2 dose-dependent[124].

6 CONCLUSIONS

Ia and b. Heparin in complex with chitosan has the ability to stabilize and activate BMP-2 in vivo in a preclinical setting and generate bone in good amounts. In a clinical setting the bone inducing capacity is poor and an inflammatory reaction is seen.

II. Hyaluronan-based hydrogel and BMP-2 induce good amounts of cranial bone with no evident inflammatory reaction in a large animal study.

III. Hyaluronan-based hydrogel presents a good alternative to type I collagen as a carrier for BMP-2 with more pronounced bone formation and a possibility of lowering the BMP-2 dose in a small animal study.

IV. Hyaluronan-based hydrogel and microgram doses of BMP-2 can heal smaller critical size cranial defects in humans with effects that are comparable to bone transplants.

The presented studies investigate different carrier materials for BMP-2 in different species. The examined carrier materials heparin/chitosan, type I collagen and hyaluronan-based hydrogel show different bone forming capacity and growth factor carrying potential, in favour of the hyaluronan-based hydrogel also in terms of evoked inflammatory response. In study IV we present a novel study design for clinical evaluation of cranial reconstruction in humans.

7 FUTURE PRESPECTIVES

The use of growth factors in future tissue engineering applications is promising. Many different aspects will need to be considered including the material used as a carrier device. The development of more effective, sustained and controlled delivery systems in the near future is crucial. The material and the form in which it is used determines the properties of the growth factor delivery system such as bioactivity, release kinetics of the growth factor, biocompatibility, biodegradability, nonimmunogenicity, efficiency and cost-effectiveness of the overall delivery system. An ideal growth factor-delivery system should mimic the natural healing process which involves the complex participation of multiple growth factors that perform their functions in a specific sequence and at specific concentrations. Therefore, moving from single growth factor-delivery systems to multiple growth factor-carrier devices is probably the next step in the development of growth factor-delivery systems[51].

In this thesis different carrier materials are used to deliver rhBMP-2 to a bony defect. Bone healing depends on the recruitment to and presence of mesenchymal stem cells in the area of reconstruction. When the number of MSCs is low precursor cells can be added to the site of reconstruction as described by Lendeckel and Mesimäki, where autologous fat derived stem cells were added[79,81]. This complicates the procedure, especially when cell culture is needed, and limits the use of the method.

Bone allograft contains osteoinductive growth factors and other non-collagenous proteins present in the matrix, that support new bone formation[125]. However, the osteoinductive capacity of massive allografts is very frail. The high rate of fractures observed in clinical practice in structural bone allograft is the result of micro-cracks that cannot be repaired by the necrotic bone because there is no vascular supply[126,127]. The bone formation has been shown to be significantly greater using BMPs added to the allograft. Numerous animal studies have demonstrated increased bone-allograft integration when rhBMPs, mixed with a collagen type I carrier, were added to the site of interest[128,129]. When BMPs are used to induce bone formation around an implant, long-term observation studies have found evidence of bone lysis. Some authors report that BMPs are able to up-regulate osteoclast-like activity, leading to greater allograft porosity, stimulating graft remodelling and enhanced resorption of bone[130,131]. This has lead to studies of anti-catabolic bisphosphonates that interfere with osteoclast activity. Studies have shown that treatment of cancellous bone grafts with BMP-7 and

zoledronate, a bisphosphonate, increases both the bone formation rate and bone density [132]. This might prove important in further clinical orthopaedic studies.

Instead of supplying exogenous BMPs to the site of reconstruction the growth factor can be locally produced by cells treated with gene therapy. The advantage of gene delivery include the ability to establish a local, endogenous synthesis of therapeutic substances produced by local cells[133]. Gene transfer requires vectors, that can be viral (adenovirus, parvovirus, retrovirus, lentivirus or herpes simplex virus for instance) or non-viral (naked DNA, DNA-protein complexes, DNA-polymer complexes, plasmid-DNA)[134]. Problems included are immunogenicity, cytotoxicity, mutagenicity and general safety issues. Gene therapy for the regeneration of bone has so forth been studied in small animal models using a variety of different transgenes, including those encoding morphogens, growth factors, angiogenic factors, and transcription factors. A small number of studies demonstrate efficacy in large animal models. Developing these promising findings into clinical trials will be a long process, constrained by economic, regulatory and practical considerations[135].

Many different methods of tissue engineering can be used in combination or as a separate option. I personally believe in a combination of carriers and scaffold materials that work together with the growth factors needed, enabling a sequential release of the growth factors to imitate the natural healing process, and also reducing the dose of growth factor to more physiological levels. This will adjust the inflammatory response to a level where it works in conjunction with the growth factors initiating bone healing. The possibility to enhance the result by autologous fat derived stem cells is interesting, especially if the cells can be used in an autologous situation without pre-expansion or culture.

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