Non-viral gene delivery of BMP-2 for bone regeneration

Cell-free and cell-based strategies

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Cel-vrije en cel-gebaseerde strategieën

(met een samenvatting in het Nederlands)

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Introduction

CLINICAL NEED FOR BONE GRAFTS

Severe trauma as well as tumor removal can result in major musculoskeletal defects. If the void is large, the mechanical stability unsatisfactory or the bone biology impaired, the body may fail to heal the bone defects. Treatment often consists of surgery, in which stability is provided and the defect is filled with material that allows for bone regeneration locally: a bone graft. Bone graft has been successfully applied in many locations, including spinal fusion surgery. The graft facilitates ingrowth of bone, which leads to vertebrae fusion. Another frequent application of bone graft is found in maxillofacial surgery, in which local stimulation of bone formation is mainly needed to provide grip for dental implants. Every year, more than 200 000 bone graft procedures are carried out worldwide. As the world's population grows and ages its prevalence is increasing [1].

CURRENT BONE REGENERATION STRATEGIES AND THEIR LIMITATIONS

Autograft

To date, autologous bone is considered the golden standard for bone graft procedures. It contains the ideal matrix, pro-osteogenic factors and living cells. The extracellular matrix is cell-generated and functions as a framework to which osteoblasts can attach and generate new bone. When existing matrix is colonized by osteoblasts and mineralized, we speak of osteoconduction. A second process observed in autograft is osteoinduction: it involves pro-osteogenic factors, including bone morphogenetic proteins (BMPs) that stimulate precursor cells to further differentiate towards the osteogenic lineage. Furthermore, living cells, present in the graft may deposit new bone tissue, which is the third process: osteogenesis. An undeniable disadvantage of applying autograft, however, is the need for a second operation in order to harvest bone. Although the bone tissue is harvested at locations where the harm is relatively limited such as the iliac crest, fibula or parts of the mandibular bone, harvesting is associated with local injury resulting in pain, deformity, scarring and surgical risks such as excessive bleeding, inflammation and infection. Moreover, in many cases the amount of available harvestable bone tissue is limited and insufficient for the defect site [2,3].

Allograft

Allografts consist of bone either harvested during regular surgery (e g. the femoral head removed after total hip replacement) or derived from cadaveric material. The explanted bone is usually frozen and stored and in some cases even devitalized by irradiation or freeze-drying. Allografts have less regenerative potential than autografts, probably due to

both the freeze-thaw cycle and its storage. In addition, there is a risk of severe immune reactions and transmission of diseases. However, as long as no good alternative is available, allograft is being used. To date, it makes up 20-30% of all bone transplantation procedures [1,4,5].

Demineralized bone matrix (DBM)

Demineralized bone matrix is produced by means of decalcification of cortical bone. The structure of the original tissue is thus retained and serves as an osteoconductive scaffold. The growth factors that are present in the bone, on the other hand, stay (hypothetically) unaffected. The osteoinductive effect of DBM appears to be greater than that of standard mineralized allograft. Clinical results vary widely, partly as a result of non-uniform processing methods, and they are not satisfactory in all respects probably due to loss of structural strength previously contributed by the minerals in the bone. Yet, DBM is widely used as an additive to current bone grafts [6].

Distraction

Other strategies to treat bone defects include so called bone transport techniques in which the bone defect is closed by approximation while at the same time, distraction is applied in a healthy area. This technique, first described by Ilizarov, uses the regenerative capacity of healthy bone [7]. The affected bone is cut in an area away from the defect site, where it is distracted at a speed equal to the speed of bone apposition, aiming to finally reach the original length. Unfortunately, only certain cases are eligible for this strategy. Downsides are the major surgical intervention and the burdensome postoperative trajectory as a result of the external fixator and the regular need for adjustment of the distraction [8].

Synthetic (ceramic) scaffolds

A scaffold generates a structural component, providing shape, porosity and possibly mechanical stability. It provides a surface for cell attachment, migration, matrix deposition and, moreover, serves as depot for biological stimuli.

Scaffolds used for bone regeneration are composed of a variety of synthetic and natural materials. Firstly, metals and alloys, which have excellent mechanical properties and could be coated with bioactives, but which are non-resorbable. Secondly, synthetic ceramic scaffolds, which closely mimic the mineral phase of natural bone, but lack elasticity and load bearing properties. Thirdly, synthetic polymers, which are versatile and adaptable for excellent biomechanical characteristics. Some of these polymers are bioresorbable, but they are not very cell-friendly in most cases. And lastly, natural hydrogels, which resemble

tissue matrix components and are compatible with cells and other biologics, but lack mechanical strength [9].

Due to their superior osteoconduction characteristics ceramic materials are most frequently applied clinically. The once pompous ceramic blocks have now been developed towards products that mimic the mineral structure of natural bone tissue such as tricalciumphospate (TCP), hydroxyapatite (HA) and combinations of both, biphasic calcium phosphate (BCP); these products highly differ in bio-resorbability. By adjusting the ratio and the sintering temperature of these synthetic ceramics, the surface structure as well as the porosity can be tailored. Porosity is essential for tissue ingrowth; it enables colonization of the graft by blood vessels and distribution of biologics including bone-forming cells. These scaffolds have an excellent capacity to bind proteinaceous tissue factors, which have provoked osteoinduction in several animal models [10,11] Ceramics are very brittle compared to bone, as collagen and elastin are lacking, resulting in low torsional and bending properties. These characteristics make ceramics not suited to function as a replacement for bone tissue in a mechanical sense, but more in a biological sense, as a bone filler, to be replaced by bone tissue over time. Since the value of ceramics is mainly based on their osteoconductive capacities, they are used primarily to supplement autologous bone grafts and not as a stand-alone therapy [12,13].

Optimal biological properties can be achieved by combining the porous ceramics with natural hydrogels to fill the pores; the ceramic provides some mechanical protection and functions as surface for bone conduction whereas the hydrogel functions as optimal extracellular matrix (ECM) and delivery vehicle for osteogenic cells and/or growth factors [14]. Hydrogels used for this purpose comprise synthetic polymers (such as Polyethylene Glycol, PEG) and natural polymers such as alginate, chitosan and hyaluronic acid derivatives and proteins such as collagen and fibrin. Natural polymers are biocompatible and have shown to enhance cell adhesion and differentiation. Therefore they are frequently applied in pre-clinical and clinical studies [15].

Growth factors (BMPs)

Growth factors are bioactive proteins that control migration, differentiation and proliferation of cells. They act in a concentration- and time dependent manner [16]. As early as 1966, Marshall Urist discovered that the induction of bone formation in devitalized bone grafts could be attributed to proteins, the bone morphogenetic proteins (BMPs). Since then, bone regeneration by means of growth factors became a large field of interest [17,18]. Proteins with varying roles in bone formation properties have been isolated such as different BMPs, transforming growth factor-beta (TGF-β), fibroblast growth factor (FGF), insulin-like growth factor I (IGF-I), vascular endothelial growth factor (VEGF), platelet-

derived growth factor (PDGF), epidermal growth factor, parathyroid hormone (PTH) and interleukins (IL). Table 1 shows an overview of the occurrence of these factors during different phases of fracture healing [19].

	Phases in fracture healing			
Signaling molecules	*1) Inflammation*	*2) Soft callus*	*3) Primary bone*	*4) Remodeling*
Cytokines				
IL-1	↑↑↑			↑↑
IL-6	↑↑↑			
TNF-α	↑↑↑		↑↑	↑↑
TGF-β superfamily				
BMP-2	↑↑↑	↑↑↑	↑↑↑	↑↑↑
BMP-6	↑	↑↑↑	↑↑↑	↑
BMP-7			↑↑↑	
Angiogenic Factors				
VEGF-(A,B,C,D)		↑	↑↑	
Angiopoietin (1&2)	↑↑	↑↑	↑	↑

Table. 1: Based on published results: molecular regulators and their prevalence in the consecutive bone generation phases [19-22]

Bone morphogenetic proteins (BMPs)

To date, at least 20 isotypes of BMPs have been identified in mammals. BMP-2, -6, -7 and -9 are the most promising, as these are associated with orthotopic and ectopic ossification [21,23,24]. BMP-2 and -7 have been approved by the US Food and Drug Administration (FDA) for certain clinical applications: BMP-2 is approved for treatment of acute open tibial shaft fractures and for single-level fusion of lumbar spine in patients with degenerative disk disease and BMP-7 is approved as an alternative to autografting in recalcitrant long bone non-unions and for certain cases of spinal fusion [25,26]. BMP therapies are efficacies comparable to autologous bone graft, the golden standard [27,28]. The downside of BMP use is the incidence of severe side effects such as ectopic bone formation at unwanted locations and soft tissue swelling [25,29-31]. The latter has led to life-threatening situations when occurring in the cervical region. A review of its application in spinal surgery is found here [32]. To our opinion, the use of BMP is only justified in specific difficult cases and is therefore only sporadically applied in the Netherlands. Nevertheless, the strong

osteogenic potential of BMPs is unsurpassed and serves as a benchmark for future therapies.

BMP-heterodimers

BMPs exist as homodimers of two subunits connected via a disulfide bond. There is also evidence for the existence of BMP heterodimers in nature consisting of two different BMP subunits [33,34]. BMP-2/6 and BMP-2/7 are best known and they turned out to have a stronger osteogenic potential, compared to their homodimeric counterparts [35-39].

BMPs make use of a common signal transduction pathway involving type-I and II cell surface receptors (BMPRI and BMPRII), which in turn induce the SMAD signaling pathways leading to transcriptional activation [40,41]. BMP-2 has a high affinity with BMPR-I, BMP-6 and BMP-7 show a high affinity for BMPRII. BMP-2/6 and BMP-2/7 combine these properties; they show a high bioactivity, which could be attributed not only to the high affinity for both BMPRI and BMPRII but also to the ability to upregulate the BMP receptor genes and to decrease the synthesis of BMP inhibitors [35,37,42]. Thanks to their very strong potency to induce bone formation, the use of BMP-2 and BMP-7 in bone healing has been subject of a large number of studies, both cellular and clinical. Other BMPs and BMP-heterodimers have not been applied yet.

MSCs

Cell-based therapies for bone regeneration have been extensively studied and most attention has been paid to multipotent mesenchymal stromal cells (MSCs). MSCs and their potential to differentiate towards osteoblasts were discovered in the 1960s [43]. At a later date their multipotency, e g. their ability to differentiate towards adipogenic and chondrogenic lineages was discovered [44]. MSCs are present in virtually all tissues, mostly as pericytes, and are easy to harvest and expand, wherein their ability to differentiate towards different lineages, even up to multiple population doublings, maintains. Interestingly, MSCs also fulfill a role in immune regulation, and can even be used successfully in allogeneic applications, as they do not evoke a strong graft-versus-host response [45].

In multiple preclinical models MSCs have shown to be beneficial for bone regeneration, also in relevant size long bone defects in sheep and goats [46]. Clinical studies, however, are scarce and the results not overwhelming [47-49]. Some examples: Marcacci et al. describe convenient results in three patients whose large segmental defects were treated with marrow derived MSCs combined with a hydroxyapatite scaffold. In a 7 year follow-up, to determine the HA absorption rate, the fractures were found to be healed. Nevertheless,

the additional osteogenic effect of the MSCs on top of the osteoconductive effect of the scaffold can be debated. Hernigou and Beaujean treated femoral head osteonecrosis by means of marrow derived MSCs; they administered cells before the femoral head was completely collapsed. Only 9 of 145 cases required total hip joint replacement, however, no control group was present [50]. Kim et al. injected pre-differentiated MSCs into fracture sites, which resulted in an increase of callus formation in the first weeks after injection. As baseline characteristics were highly variable, the implications of this work are debatable [51]. Lendeckel et al. used autologous MSCs derived from adipose tissue in a dental application in a 7-year old patient, aimed to repair calvarial defects, with success. However, the role of the MSCs remains unclear [52]. Meijer et al. placed pre-cultured MSCs in jaw defects in humans. Biopsies after 4 months showed bone formation in 3 out of 6 patients of which only in 1 patient bone formation was induced by the implanted cells [53].

These above mentioned examples are the result of decades of research on MSCs: sporadically occurring minor osteo-inductive effects. Large clinical trials confirming the effectivity of MSCs do not exist. Some researchers consider the heterogeneity of MSCs due to a lack of isolation and culture protocols as cause of the disappointing outcomes regarding osteo-inductivity of MSCs [49]. In their opinion, the capacity of MSCs to regenerate bone is influenced by many factors including harvesting location, animal species, donor age, culture methods and passage number. They state that therefore donor cells should be carefully selected and cultured [54,55]. For example, when comparing rat MSCs with human MSCs, large differences in bone formation are observed; the latter result in fibrous tissue formation only [56]. Furthermore, screening of the cells is difficult, as there is no correlation between *in vitro* osteogenicity and *in vivo* bone formation when using human MSCs [57]. Another explanation of the disappointing outcomes with respect to cell-based bone tissue engineering could be that larger implants are troubled by the lack of vascularization and the presence of a non-cell-friendly hematoma upon implantation, both leading to cell senescence or even cell death [58,59].

In conclusion, it can be stated that more than five decades of cell-based research did not result in a widespread clinically applicable therapy. As long-term cell engraftment after implantation is only found in a limited number of preclinical studies, opportunities may be found in harnessing short-term effects of cells. An attractive strategy might be to modify cells to temporarily overexpress certain cytokines, which in turn drive the bone-regeneration process.

ISSUES THAT OUGHT TO BE ADDRESSED IN FUTURE STRATEGIES

Vascularization:

The presence of blood vessels is a prerequisite for bone formation to occur. In mature bone the distance between tissue cells and vascular networks varies between 100 to 200 μm as exchange of oxygen, nutrient and waste is limited to these distances [60]. The bone grafts researched in this thesis consist of cell-seeded porous constructs with proportions of at least 3x3x3 mm. Although this is more than 10 fold larger than the diffusional limit for nutrients and oxygen, the interconnected pores of 100-200 μm width ensure a certain degree of diffusion of oxygen and nutrients throughout the construct. Clinically relevant sized bone defects are generally larger, in the order of magnitude of centimeters. This means that embedded cells in the center of cm-sized implants will have less chance to survive the avascular phase and are unlikely to participate in the bone formation process.

Various options are introduced to increase survival of grafts. In the first place, transplantation of vascularized grafts combined with anastomosis of graft vessels to host vasculature. Fibula vascularized grafts and, to a lesser extent, iliac crest and ribs have been successfully applied [61]. These transplantations, however, have a high risk of failure and are technically difficult to apply, especially in small bone constructs, where the need for microsurgery is evident. Moreover, this strategy is not applicable everywhere, as host vasculature in the vicinity is needed.

A second possibility is to facilitate the penetration of new host vasculature. This blood vessel ingrowth, which is a response to signals secreted by ingrowing or transplanted cells provoked by hypoxia, is limited to several tenths of micrometers per day. The ingrowth process can take days or weeks depending on the size of the implant. Local release of some of the signals, of which VEGF is best known, can be harnessed to stimulate angiogenesis, and has shown to reduce the time of vascularization [62].

A third option is to engineer a network, to which the host vasculature could connect. Constructs with tubular networks formed by endothelial progenitor cells proved capable of doing so [63].

A last option is adding porosity to the graft on a macro scale; this decreases diffusion distances, resulting in better oxygenation of residing cells [64]. For ceramic materials this means providing adequate pore size and pore interconnectivity [61]. Hydrogels, being an excellent matrix candidate for both osteogenesis and angiogenesis, can be produced in a porous fashion by means of 3D printing.

In conclusion, the strategies to ensure survival of grafts rely predominately on stimulating host vasculature ingrowth. Even when angiogenesis starts immediately, vascularization is

limited to tenths of micrometers per day, which hampers cell survival in the middle of the tissue.

Wound bed and hematoma:

When implanting a graft the local wound bed should be taken into account. The damage to the vasculature caused by either the initial trauma or the implantation procedure results in an influx of blood, containing several types of cells, mainly erythrocytes and some white blood cells. The latter initiate the regeneration process of bone by the excretion of numerous factors. The influx of erythrocytes on the contrary results in a high local concentration of potassium, sodium and a decrease of the pH, which is likely to jeopardize cell survival [65].

Adequate mechanical stability:

Regeneration strategies have to consider mechanical conditions, such as axial and rotational stability, at least during the first few weeks. Fractures of the long bones in the lower leg for example, frequently show healing problems, when the minimal soft tissue cuff is inadequate in providing enough stability. After several weeks during the healing process, when the callus mineralizes, stability develops [66,67]. Absolute rigidity on the other hand is contra indicated, as some micro-motion of the fragments is beneficial for bone formation. An example is bone loss due to stress shielding around prosthesis. The biomechanical and biophysical environment needed for bone formation is reviewed elsewhere [68].

CURRENT STRATEGIES: IN SUMMARY

Bone regeneration in the clinical environment has to deal with rather challenging circumstances: large defects, unsatisfactory mechanical stability and the presence of a fracture hematoma. Moreover, patients have often comorbidities such as diabetes and metabolic syndrome that negatively influence healing capacity that are even more present in the aging population. The goal of bone tissue-engineering strategies is to replace the injured bone by implanting tissue consisting of the main components of bone tissue, cells, growth factors and ceramics. Despite more than three decades of research in the field and over 15,000 papers published in the last 10 years, in clinical applications, bone tissue engineering failed to show strong effect. Most strategies relied on the osteogenic properties of cells; they ignored the above-mentioned problems concerning vascularization and wound bed. The ever-repeated failures of the cell-based strategies can be attributed to this. Attempts to optimize the scaffold on which the cells are seeded, as far as possible mimicking the natural bone extracellular niche, or addition of signals that stimulate cells to differentiate did not lead to better results [69,70]. Cell-free strategies such as use of BMPs,

have proven to be more effective, repeatedly showing strong osteogenicity. Their application although is limited due to variable efficiency in humans and association with serious adverse events [71,72]. This is the reason that in most cases autologous bone remains the golden standard [73].

Summarizing one could state that in the clinical environment only some strategies have been successful to regenerate bone, being: transplantation of autologous or allogeneic bone, BMP delivery, and distraction. These strategies could be complemented by synthesized ceramic materials, including HA, TCP and BCP and demineralized bone matrix.

Strategies that have stranded in pre-clinical trials, or did not prove beneficial to bone regeneration in clinical applications include among others all cell-based strategies, such as those combined with ceramic scaffolds and/or growth factors, the use of growth factors other than BMPs and growth factors combined with ceramics

Interestingly, one could state that successful strategies rely on the regenerative capacity of the body, which is in contrast with the original bone tissue engineering strategies in which a piece of tissue is implanted. The focus of future bone regeneration should be on smart ways to stimulate and facilitate the body to regenerate bone tissue. The term Bone Tissue Engineering should be replaced for Bone Regenerative Medicine.

GENE DELIVERY OF BMP FOR BONE REGENERATION

Rationale for gene delivery approach

Clinical use of BMP-2 and BMP-7 has appeared to be efficient in inducing bone formation. But to accomplish the intended effect extremely high dosages are needed. Doses between 1.95 and 40 mg of BMP-2 per application are used in the clinic. Despite these high doses, BMP disappears from the implant site within a few days due to the quick wash out of protein upon implantation and the short BMP half live owing to proteinases [74,75]. Furthermore, the nature of the material in which the BMP is incorporated, a collagen sponge, causes the protein to release in a large initial burst [76]. The high dosage as well as the inadequate release profile is assumed to be the cause of severe adverse effects, such as edema, ectopic bone formation and bone resorption around the graft [25,77].

Much research is directed towards the development of scaffolds that deliver growth factors in a controlled way to mimic as much as possible the release pattern observed in fracture healing, in which BMPs are present up to several weeks [78,79]. By using microspheres, a sustained BMP release over time could be effectuated [80,81], resulting in bone regeneration equal to conventional therapies with lower but sustained BMP dosages [82]. The use of scaffolds and microspheres is not ideal in all respects; to mention are the

difficult BMP loading, the decrease in bioactivity of BMP due to multiple manipulations and, despite all efforts, still a burst release of BMP [80,81].

A more elegant strategy is gene delivery to cells in order to produce the desired protein. Physiological dosages can be produced and the disadvantages of short half-life and washout of protein can be evaded [83]. In using cells for bone regeneration the transient character of transplanted cells must be taken into account. It could be harnessed and improved by genetically engineering the cells to overexpress BMPs.

Local gene delivery strategies

Gene delivery or gene therapy is the process in which genetic material is introduced into cells to enforce transgene expression and subsequent protein production. The produced transgenic proteins either affect the producer cells directly (autocrine signaling) or they stimulate other cells to do so (paracrine effect). The advantage of gene therapy over protein therapy is a local, prolonged production of protein in physiologic dosages. Furthermore, the bioactivity of newly synthesized proteins might be increased due to optimal posttranslational modification, which is not achieved with recombinant protein. Another advantage is the possibility to co-express multiple genes simultaneously, which might result in the creation of heterodimers in case of BMPs [84].

Currently several local gene delivery methods are being investigated. Below the different approaches are compared: systemic vs. local approaches; viral versus non-viral methods and *in vivo* versus *ex vivo* approaches.

Viral vs. non-viral gene delivery strategies

Viral gene delivery methods make use of a lentivirus, a retrovirus, an adenovirus or an adeno-associated virus. The use of viral methods results, generally speaking, in high transduction efficiencies and prolonged protein production. But viral delivery strategies are associated with severe complications, giving them a negative connotation; oncogenic mutations [85] and strong immune responses, which has even led to the unexpected death of two patients [86]. Since then, new generations of viral vectors were developed, increasing their safety profile. As of now, more than 500 clinical studies did not show any side effects [83,87]. Currently, five phase I trails for osteoarthritis are ongoing or have recently been finished, which elucidates the confidence in viral gene delivery strategies even for non-lethal disorders [88]. The majority of studies using viral gene delivery strategies, however, address lethal diseases in which the risk of an immune response is outweighed by the chance of healing. In view of the desirable bone regeneration to be addressed in this thesis, viral methods are not the preferred choice.

Non-viral gene delivery methods generally use plasmid DNA (pDNA), circular double stranded DNA structures. An asset of pDNA is that it is easily produced in bacteria and stable when stored [89]. Non-viral delivery strategies to mammalian cells are considered safe; no immune responses or other potential side effects have been reported, not even at high dosages [90]. Since the DNA inserts into the host genome at very low frequency only, the likelihood of mutagenesis is low. Nevertheless, these strategies come with drawbacks such as low transfection efficiencies and short periods of gene expression. Enhancing the efficiency of transfection, which is highly dependent on the transfection method, is subject of much research. The pDNA is often complexed to liposomes or polymers (e g. alginate). To achieve adequate protein production more hurdles have to be addressed such as preventing endosomal degradation and increasing nuclear entry, optimizing the promoter region etc. Nowadays, these issues are subject of much research [91]. Two main delivery methods of non-viral gene delivery of pDNA can be distinguished in a clinical application: pDNA can be introduced either directly into the desired location in the body (*in vivo* delivery) or extracorporeal into cells that are subsequently implanted into the site of injury (*ex vivo* delivery) [89,92].

In vivo vs. *ex vivo* gene delivery strategies

In vivo gene delivery

In vivo gene delivery strategies consist of administration of pDNA either directly or released via a scaffold. The goal is to make endogenous cells, which are present at the location of implantation, take up the pDNA and express the protein of interest. The transfection efficiency of endogenous cells is the main hurdle; potential target cells are scarce as the damaged tissue often suffers from considerable cell death and most of these cells are in a post-mitotic phase, which is associated with low transfection efficiency. Other hurdles are a fast washout of pDNA, active degradation by DNA-ases and a immune reaction to the CpG sequences of the pDNA [93], reviewed here [94,95].

Direct local injection of pDNA

Direct local injection of pDNA in either muscle or bone defect results in gene expression [96,97]. The expression levels are low, probably due to quick washout and degradation of pDNA. Nevertheless, local bone formation and systemic measurable levels of protein have been demonstrated as result of multiple intramuscular injections of pDNA [96,97]. However, it should be noted that a muscle of a healthy mouse, is not comparable with a clinical relevant bone defect environment in humans. Other strategies to accomplish higher gene expression, such as administering electrical shocks (electroporation) or ultrasonic shocks (sonoporation) to the tissue immediately after injection of DNA, are associated with

abundant tissue damage and have a limited penetration depth, which make them unsuitable for clinical application [98,99]. Also strategies in which delivery of pDNA is mediated via liposomes [100] or microspheres [101] have been tried and were reviewed extensively [102]. Despite all these efforts, local transfection efficiencies and ensuing protein levels remain disappointing; they resulted in minimal increase of bone, even if applied in rodents.

Gene activated matrix (GAM)

The most promising *in vivo* gene delivery strategy uses a matrix in which pDNA is encapsulated, a gene activated matrix (GAM). These GAMs can provoke a gradual release of DNA aimed at transfecting cells that invade the matrix, and shield the pDNA from washout and degradation [103-107]. GAMs that have been successfully applied in segmental defects in rats and dogs (resulting in some osteogenesis), consists of a collagen sponge loaded with pDNA encoding parathyroid hormone and BMP-4 [104,108]. Attempts to further improve the GAM, to ensure better gene delivery, resulted in the use of other polymers including chitosan hydrogel, PEI and alginate hydrogel [104,106,107,109,110]. Based on our results, the alginate hydrogel scaffolds loaded with pDNA-BMP-2 have proven to be a promising application for bone regeneration.

Alginate is a natural polysaccharide derived from seaweed. Alginate polysaccharides form a hydrogel when combined with water, which is widely used as extracellular matrix in bone tissue engineering. The polysaccharides are composed of glucuronic acid and mannuronic acid that gelates with bivalent cations such as Ca^{2+} [111]. Alginate is considered to be compatible with cell seeding; it is non-toxic and non-immunogenic. Degradation takes place in weeks, and depending on the concentration used it disappears from the implant location within weeks due to surface erosion. Alginate hydrogel is considered to be a suitable carrier for cells and growth factors. Moreover, inside these gels adequate bone formation could be seen, which makes them suited for bone regeneration [112,113]. Besides this function as a temporary matrix for seeded cells [100,114], alginate has the capacity to deliver plasmid DNA to cells. The exact mechanism behind this transfection has not yet been elucidated, but our view is as follows: the alginate forms condensed complexes with pDNA based on surface charge, thus allowing for a smaller molecules to be ingested in the cells via endocytosis. The complexes protect the DNA-structures against nucleases and other blood components. Finally, The release of pDNA from the complexes with alginate hydrogel into the surrounding tissue occurs in a timely fashion which results in a continuous controlled presence [115]. All these features make alginate a promising GAM. Previously, efficient transfection of seeded cells has been shown by researchers from our group [116,117]. Others have used similar alginate GAMs for gene delivery [118,119]. When combining the hydrogel with BMP-2 plasmid DNA and MSCs, bone was formed in

subcutaneous pockets in mice [116,117]. To date, the role the embedded cells in the alginate GAM remains unveiled. In this thesis the MSC containing constructs are compared to cell-free strategies, in order to elucidate the contribution of the MSCs in these constructs to bone formation and to apply this strategy in more demanding models.

Ex vivo gene delivery

Ex vivo gene delivery has been applied to overcome the low transfection efficiency of current *in vivo* methods. Hereby cells, harvested during surgery or received from a donor bank, are transfected to overexpress BMP coding regions (which are smaller than the complete BMP gene) and are locally implanted in the area of interest where the cells can release protein in a timely fashion [120,121].

Apart from viral strategies, which are not discussed here, many *ex vivo* transfection methods have been introduced, including the use of naked pDNA [106,110], liposome based transfections [122,123], and the use of synthetic polymers such as PEI [100]. Furthermore physical methods have been developed, such as sonoporation, magnetic transfection and electroporation. Nucleofection, a method of electroporation, has proven to be efficient in delivering pDNA into primary cells and established cell lines. Thanks to the high transfection efficiency as well as the prolonged expression and protein production, there is a strong interest in this strategy. Introduction of pro-osteogenic genes via nucleofection in MSCs and fat derived stem cells has resulted in increased levels of bone formation when compared to controls [124-128]. In this work we will further investigate the applicability of this strategy in the clinic. Compared to *in vivo* transfection methods, the *ex vivo* gene delivery shows improved protein production, no local tissue damage and no off-target effects.

An obvious drawback of *ex vivo* methods is the use of transplanted cells. As elucidated before, the role of the transplanted cells as bone generator is questionable, as long-term engraftment of donor cells is scarce [129-131]. Only a temporary function of the cells in the *ex vivo* gene delivery strategies might be of interest. Future methods should focus on investigating this paracrine role, thus aiming at strategies without need for long-term presence and engraftment of the seeded cells [132].

The need for cell isolation as well as culture expansion and the need for transgene expression testing before implantation make *ex vivo* transfection strategies elaborate and highly expensive, which forms a real drawback [133]. To overcome this drawback, one-stage procedures have been tried, in which cells are harvested, genetically modified and used in a single procedure. To date, one-stage viral applications have shown promising results, e g. transduction of BMP-2 cDNA to freshly isolated bone marrow [134].

Concluding, as stated above, the role of the cells in these *ex vivo* gene delivery strategies remains unclear. Whether the MSCs, that overexpress BMP, actually participate

in bone formation has yet to be investigated. The applicability of cells other than osteo-precursors is unclear.

AIMS AND OUTLINE OF THIS THESIS

Despite decades of research, bone tissue engineering has resulted in only a handful of applications that are used clinically, such as the local application of BMP. BMPs are the most potent pro-osteogenic factors known to date and the common factor in all parts of this work. Clinically they are applied as alternatives to autografting. However, the use of BMPs has its drawbacks: bone formation at unwanted locations and swelling of tissues around the implantation site, which has led to life-threatening situations. Due to the quick washout of protein upon implantation and the short BMP half-life owing to proteinases, extremely high dosages of protein are needed clinically. To overcome the disadvantages associated with direct BMP use and to effectuate the pro-osteogenic effects, we aim for a non-viral way of gene delivery of BMPs. A multitude of these non-viral gene delivery strategies exist, applied as well *in vivo* as *ex vivo*. Most of them, however, have only been tested in so called 'proof of concept' studies, which does not guarantee feasibility in clinically relevant situations.

In line with this, the subject and aim of this thesis are defined as follows:
To investigate the suitability of non-viral gene delivery of BMPs as a means to (re)generate bone tissue in clinical applications.

A stepwise approach was used to systematically investigate the suitability of non-viral gene delivery of BMPs. Several sub-aims were identified, as discussed in more detail below.

A pre-selection of promising non-viral gene delivery strategies was made based on literature and previous work. First, the most challenging, but most obvious strategy was investigated: a cell-free approach in which a BMP gene loaded matrix was locally applied *in vivo* (aim 1).

When this strategy appeared to result in gene-expression levels inadequate to induce bone formation, consecutive research was directed toward a strategy supplemented with cells, both in an ectopic and in an orthotopic environment. Both an ectopic and an orthotopic environment were investigated (aim 2 and 3). Subsequently, we investigated methods to improve the bioactivity of (the) seeded cells by tailoring the construct design (aim 4). An *ex vivo* transfection method was tested to further enhance the osteogenicity, because seeding of cells has proven necessary, and resulted in much higher gene expression levels (aim 5). Different combinations of BMP genes were applied in the hope that osteogenicity would increase (aim 6). Different cell sources that did not possess the

ability to differentiate toward osteoblasts were used as BMP producers (aim 7). This made the step toward a single stage approach more feasible, which was investigated successively (aim 8). To get a better understanding of the stages in our research and the development of the research topics, the topics of this thesis will be elaborated and elucidated hereafter.

Aim 1: Assessment of local in vivo BMP-gene delivery without seeded cells

Is cell seeding a prerequisite for transgene expression and subsequent bone formation?

In *chapter 2* we investigated *in vivo* transfection using an alginate GAM; we assessed the capacity of cell-free pDNA loaded alginate hydrogel to provoke transfection in the cells that are present at the implant location. A cell-free approach has obvious advantages, as elucidated previously. To test and optimize this *in vivo* transfection, we varied the pDNA dosage, as literature showed little consensus on pDNA concentrations. Subsequently we aimed to compare cell-free to cell seeded constructs (MSCs and Fibroblasts, see Aim 2). The matrix that we used was alginate hydrogel. This has proven to be best suited because of its compatibility to cells, its slow degradation with associated release pattern, its high transfection efficiency and its ability to protect pDNA against degradation. In view of clinical applicability we investigated gene expression in ectopic as well as orthotopic locations, in this case a small bone defect in the spine of the rat.

Aim 2: Assessment of a BMP-gene loaded matrix combined with MSCs

Is it possible to use any cell with the capacity to produce BMPs to induce bone formation, or are osteogenic precursor cells (such as MSCs) indispensable?

The next step was to enhance the osteogenicity of the above-described alginate GAM. Therefore our focus directed toward a pDNA-BMP-2 loaded alginate matrix supplemented with recipient cells. In previous work we had observed that osteoprogenitor cells (MSCs) could function as recipient cell expressing the transgene. Applied in a nude mouse model, this pDNA-BMP-2 loaded GAM with MSCs resulted in abundant bone formation [135]. The concept is simple: mix the pDNA and the cells with the hydrogel and implant or inject the mixture at the location of interest. Therefore we aimed to compare cell free to cell seeded constructs, and subsequently investigate the role of the transfected cells in the bone formation process. We compared transfected MSCs with transfected fibroblasts in a rat model *(chapter 2).* Surprisingly, bone formation could not be demonstrated in both conditions. Therefore we addressed this issue again in *chapter 6,* with a different transfection strategy. It should be noted that ectopic bone formation by BMP producing MSCs has so far been demonstrated in nude mice only. Factors that might have played a role causing the absence of bone, even in the condition with MSCs, will be discussed in *chapter 2*: a possible dysfunction of the alginate gel, variations in the osteogenic capacity of

MSCs, or the normothymic nature of the rat model, causing an immune response to the implanted pDNA.

Aim 3: Assessment of BMP-gene loaded matrix combined in an orthotopic location

Is cell seeding a prerequisite for effective gene delivery in the orthotopic location? In *chapter 3* we investigated the pDNA-BMP-2 loaded alginate GAM in orthotopic defects in the iliac bone of goats with and without the addition of MSCs. We hypothesized that the efficiency of *in vivo* gene delivery might improve compared to the ectopic location, as many osteogenic progenitors are present in a bone defect site. This cell population mainly consists of MSCs, which have shown to be adequate target cells for gene delivery.

Aim 4: Assessment of upscaling the size of the construct by means of adding porosity

Can we 3D bioprint a BMP-gene loaded matrix with pores?

Enlargement of the size of the construct jeopardizes the survival of the cells within the scaffold due to limited diffusion of nutrients and oxygen to the construct center. Previous work has shown that by adding porosity to the implanted tissue the seeded cells have a higher survival rate and better biological function [64]. We hypothesized that by adding certain porosity to the scaffold, gene delivery, and subsequent BMP production would not be hindered by lack of nutrients and oxygen. In *chapter 4* we investigated the possibility to add porosity to the construct by means of 3D printing through extrusion deposition. We aimed to design a porous alginate hydrogel loaded with pDNA-BMP-2 and containing seeded MSCs. We tried to obtain the optimal gene expression and mechanical stability of the 3D-printed constructs.

Aim 5: Assessment of the ex vivo BMP-gene delivery to regenerate bone

What is the most efficient ex vivo gene delivery method with regard to inducing bone formation?

We compared the most promising *ex vivo* gene delivery strategies both *in vitro* as *in vivo*. The alginate transfection was compared with Lipofectamin and Nucleofection. The latter appeared superior to other appealing strategies *in vitro* as well as *in vivo*; resulting in more bone, when using pDNA-BMP transfected MSCs in a rat model. These data were not (yet) published.

Aim 6: Assessment of several BMP genes and combinations of these

Are heterodimers formed upon co-delivery of BMP genes? Are heterodimers superior with regard to inducing bone formation?

In *chapter 5* we optimized the different settings and conditions used during the nucleofection process; we varied in transfection program, transfection buffer, cell

concentration and pDNA concentration. Subsequently, we compared different BMP isoforms with respect to their potency to induce bone formation. By co-expressing isoforms that bind to different BMP receptors, BMP heterodimers were formed, with the aim to induce osteogenic differentiation more efficiently.

Aim 7: Assessment of non-osteogenic progenitors as target cells for BMP expression.

Is it possible to use any cell with the capacity to produce BMPs to induce bone formation, or are osteogenic precursor cells (such as MSCs) indispensable?

In *chapter 6* we elucidated the paracrine and autocrine roles of the MSCs in an *ex vivo* application. The clinical applicability of non-osteogenic progenitor is largely determined by the degree of participation in osteogenesis. If cell survival and long-term engraftment are prerequisites for bone formation to occur, then the current *ex vivo* strategy is very similar to the bone tissue engineering strategies that have repeatedly failed. Both rely on cell survival and long-term engraftment of cells, which is unlikely in clinical applications.

Aim 8: Assessment of a one-step ex vivo gene delivery strategy

If bone formation could be induced by transient expression of a sufficiently high dose of BMPs, in theory, any transfectable cell can be harnessed to produce protein.

In chapter 6 we investigated the application of a one-stage procedure in which cells are isolated, transfected and implanted in a single session. The cell sources chosen were bone marrow and fat. This strategy offers great clinical advantage as with this approach the elaborate cell culture can be omitted.

Chapter 2

BMP-2 gene delivery in cell-loaded and cell-free constructs for bone regeneration

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ABSTRACT

To induce osteogenicity in bone graft substitutes, plasmid-based expression of BMP-2 (pBMP-2) has been successfully applied in alginate polymer-based cell-loaded constructs. Here, we investigated whether cell seeding is necessary for non-viral BMP-2 gene expression *in vivo*. Furthermore, to gain insight in the role of BMP-producing cells, we compared bone progenitor cells with non-osteogenic producer cells in gene delivery constructs.

Plasmid DNA encoding GFP (pGFP) was used to trace transfection of host tissue cells and seeded cells in a rat model. Transgene expression was followed in both cell-free constructs as well as constructs seeded with syngeneic fibroblasts or multipotent mesenchymal stromal cells (MSCs). Titration of pGFP revealed that the highest pGFP dose resulted in frequent presence of positive host cells in the constructs. Both cell-loaded groups were associated with transgene expression, most effectively in the MSC-loaded constructs.

Subsequently, we investigated effectiveness of cell-free and cell-loaded constructs with pBMP-2 to induce bone formation. Local BMP-2 production was found in all groups containing BMP-2 plasmid DNA, and was most pronounced in the groups with MSCs transfected with high concentration pBMP-2. Bone formation was only apparent in the recombinant protein BMP-2 group.

In conclusion, we show that non-viral gene delivery of BMP-2 is a potentially effective way to induce transgene expression *in vivo*, both in cell-seeded as well as cell-free conditions. However, alginate-based gene delivery of BMP-2 to host cells or seeded cells did not result in protein levels adequate for bone formation in this setting, calling for more reliable scaffold compatible transfection methods.

INTRODUCTION

Cell-based strategies for bone regeneration have been extensively investigated [43,138] and the applicability of multipotent mesenchymal stromal cells (MSCs) has been demonstrated in several preclinical models [46]. Despite this achievement, translation to clinical practice appears to be difficult and only few clinical studies have been reported, which consisted mainly of case series. Main limiting factors when upscaling the technique include metabolic stress due to hypoxia and lack of early vascularization and activation of the host immune system [58,59]. Until now the precise fate of implanted cells is not clear. Tracing studies have shown contradictory results for long-term engraftment of MSCs [139]. The use of MSCs for bone regeneration has not been adopted in clinical practice but is regarded as a feasible option with several clinical trials ongoing [48,49].

To induce bone in clinical situations, growth factor-based strategies have been more frequently applied than cell-based applications. Members of the TGF-β superfamily of growth factors, such as bone morphogenetic proteins (BMPs) have been successfully used in spinal fusion surgery and tibial fracture healing [140-142]. In order to achieve clinically meaningful effects, BMP-2 is applied on a collagen sponge at a dosage that is much higher than the minimum effective dose to compensate for wash-out and rapid degradation by proteinases [26]. Despite this supraphysiological dose, BMP-2 remains present for only a few days [74]. BMP-2 use is associated with serious complications probably due to these supraphysiological doses [142,143]. In natural circumstances, during uncomplicated fracture healing, the BMP-2 levels are elevated for up to three to four weeks [21,22]. This supports the strive for therapies associated with a sustained and more physiological BMP-2 release to reduce the risk of adverse effects [144,145]. Gene delivery is one of the approaches that enables BMP-2 production over a period of several weeks [83].

Gene delivery strategies consist of viral and non-viral methods. For bone regeneration non-viral transfection strategies are preferred over virus-based methods as they are considered to be safer. Immune responses or complications as a result of mutagenesis have not been described in clinical trials, even at high plasmid DNA dosages [146]. Furthermore, the transient nature of plasmid-based transgene expression is desirable in bone regeneration, as only a temporary BMP production is required. The main limitation of non-viral strategies is the transfection efficiency seen for most methods. The resulting low amounts of transgene expression in the target area have hampered clinical translation.

The combination of local gene delivery with the implantation of cells, as a target for the gene delivery, has been proven successful in preclinical models [147]. For this purpose, MSCs have been used most frequently as they are efficiently expanded, transfected and implanted [83]. Other attractive cell sources that can be harvested and re-implanted during surgery are

bone marrow mononuclear cells or fat tissue-derived cells [128,134]. The fate of these implanted transfected cells is however still unclear. Only a few studies showed long-term engraftment of donor cells via genetic tracing [129,130]. In general, donor cells are rarely found in the regenerated tissue [131]. It remains unclear whether the levels and duration of BMP-2 protein production, which are reached by non-viral gene therapy, are sufficient for osteogenesis. Cell-free plasmid-based gene delivery, which omits the need for the steps of cell isolation, transfection and implantation targets the host cells for transgene expression and might be an elegant alternative [84].

In previous work, we developed a non-viral gene therapy technique using alginate hydrogel loaded with plasmid DNA containing the human BMP-2 coding region (pBMP-2). The alginate hydrogel functions both as a delivery vehicle for plasmid DNA to cells [118,148], and as temporary matrix for seeded cells [100,114], making alginate a promising gene activated matrix (GAM) [148,149]. Using this approach, MSC-seeded constructs have resulted in measurable protein levels *in vitro* and abundant bone formation in mice [147]. However, to date, the role of transfected MSCs in relation to bone formation remains unclear. Other cell types have not been investigated to date and a single cell-free approach failed to show an increased bone formation [150]. This could be due to suboptimal dosage of pDNA, which has shown to be of great importance to transfection efficiency in other work [149].

In this study, we investigated the conditions for optimal transgene expression and secondly addressed whether cell seeding is indispensable for non-viral BMP-2 gene expression *in vivo*. Furthermore, to gain insight in the role of BMP-producing cells, we compared bone progenitor cells with non-osteogenic producer cells (i.e. fibroblasts) in plasmid-based gene delivery constructs.

MATERIALS AND METHODS

Scaffold components

The ceramic scaffold was composed of porous biphasic calcium phosphate (BCP, Xpand biotechnology, Bilthoven the Netherlands) that was used in previous gene delivery studies [149,150]. The 3x3x6 mm scaffolds consisted of 80±5% (w/v) hydroxyapatite and 20±5% (w/v) β-tricalcium phosphate, which were 70±5% porous. The pore size was 200-800 μm. BCP scaffolds were cleaned in ultrasonic baths and autoclaved and subsequently used without further treatment.

Autoclaved high-viscosity alginate powder (International Specialty Products, ISP, Memmingen, Germany) was dissolved in alpha minimum essential medium (α-MEM, Gibco, Breda, The Netherlands) and used at a final concentration of 10 mg/ml, as previously described [149].

Plasmid DNA containing the GFP and BMP-2 genes

Vectors consisted of pEGFP-N1 (BD Biosciences, Franklin Lakes, NJ, USA), pVAX1/rhBMP-2 (Fig. 1A) and control vector was pVAX1 (Invitrogen) without insert. The pBMP-2 construct contained the full-length human recombinant BMP-2 cDNA. Plasmid DNA was isolated, purified and cleared from endotoxins (EndoFree Plasmid Maxi kit, Qiagen K.K., Tokyo, Japan).

Isolation of fibroblasts and MSCs

Fibroblasts were isolated from skin samples, collected and pooled from male Fischer 344 inbred rats (Charles River Laboratories, France). Fur was shaved and skin was disinfected with 70% ethanol. A 1 cm^2 skin sample was obtained aseptically and cut in smaller fragments of approximately 1 mm^2. These fragments were then digested by treatment with a collagenase mix over 30 minutes under constant stirring. Subsequently cells were pelleted and washed in DMEM/F12 (Gibco) with 10% (v/v) fetal calf serum (Cambrex, Charles City, IA, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, 50 mg/mL gentamicin and 0.5 mg/mL fungizone (Sigma). Thereafter cells were cultured in fibroblast expansion medium (DMEM/F12 with 10% (v/v) fetal calf serum, 100 U/ml Penicillin and 100 μg/ml Streptomycin) at 37°C and 5% CO_2 in a humidified incubator.

MSCs were harvested and pooled from the marrow of tibias and femurs of the same animals. The bones were aseptically removed and placed in PBS. The epiphyses were cut, while the diaphyses were pierced with a sterile 16-gauge needle. Subsequently the marrow was flushed out with 5 mL PBS. The collected marrow was filtered, pelleted and

resuspended in MSC expansion medium (DMEM, fetal calf serum, 100 U/ml Penicillin and 100 μg/ml Streptomycin) and cultured at 37°C and 5% CO_2 in a humidified incubator.

Group	Ceramic	Alginate	Cells	pVAX1-BMP-2	pVAX1	rhBMP-2	Location
pBMP-2	+	+	-	500 μg	-	-	i.m.
pBMP-2	+	+	-	100 μg	-	-	i.m.
pBMP-2 + fibroblasts	+	+	10^7	100 μg	-	-	i.m.
pBMP-2 + MSCs	+	+	10^7	100 μg	-	-	i.m.
rhBMP-2	+	+	-	-	-	25 μg	i.m.
Control	+	+	-	-	100 μg	-	i.m.
Fibroblast control	+	+	10^7	-	100 μg	-	i.m.
MSC control	+	+	10^7	-	100 μg	-	i.m.

Table 2: Study groups used to assess effectiveness of cell seeding using pBMP-2 (eight weeks in rats).

Construct composition

A two weeks gene tracing study was performed in rats using pGFP at different concentrations, with or without cells as detailed in Table 1. Expression and effectivity of pBMP-2 was studied in an eight-week experiment in rats as outlined in Table 2. An additional group was added with a 5-fold higher pDNA concentration compared to the gene tracing study because a plateau concentration was not reached yet and high dosages of pDNA were not disadvantageous for bone formation previously [104]. BMP-2 protein at a final concentration of 25 μg/ml was used as the positive control.

Cryopreserved fibroblasts and MSCs were thawed, washed and cultured for three days in expansion medium. Cells were detached using 2 mL of 0. 25% (v/v) trypsin solution (Sigma), washed, and resuspended at 10^7 cells/ml in alginate (10 mg/ml). Subsequently pGFP, pBMP-2 or empty control plasmid was added to alginate, with and without cells, according to the groups in Tables 1 and 2. The alginate mixtures were then repeatedly pipetted on the porous BCP scaffolds, ensuring a complete distribution of cells and alginate. A total volume of 40 μl was added to each BCP scaffold. To polymerize the alginate, the constructs were submerged in 100 mM aqueous $CaCl_2$ supplemented with 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.4). After 10 minutes the Ca-solution was replaced by MSC expansion medium.

Group	Ceramic	Alginate	Cells	pEGFP	pVAX1	Location
pGFP	+	+	-	100 μg	-	i.m., s.c. o'topic
pGFP	+	+	-	10 μg	-	i.m. & s.c.
pGFP	+	+	-	1 μg	-	i.m. & s.c.
pGFP + fibroblasts	+	+	10^7	100 μg	-	i.m. & s.c.
pGFP + MSCs	+	+	10^7	100 μg	-	i.m. & s.c.
Control	+	+	-	-	100 μg	i.m. & s.c.

Table 1: Study groups for gene tracing study using pGFP (two weeks in rats)

In vitro experiments

To assess the *in vitro* plasmid DNA release profiles, constructs were made consisting of BCP scaffold, alginate hydrogel and pBMP (100 μg/ml). These constructs were kept in PBS (500 μl per sample) at 37°C in a humidified incubator for 14 days. Samples were taken at day 1, 2, 3, 7 and 14. The pDNA was separated from other DNA fragments via agarose gel (1% w/v) electrophoresis containing 0.5 μg/ml EtBr (Sigma). EtBr intensity of the plasmid DNA bands was quantified via pixel comparison with a calibration curve made with known concentrations of the same pDNA.

To demonstrate alginate-mediated transfection, fibroblasts or MSCs (10^7 cells/ml) and pGFP or empty control plasmid (100 μg/ml) were suspended in 100 μl alginate (10 mg/ml) plugs without BCP scaffold. Plugs were polymerized and kept at 37°C in a humidified incubator, and the medium was changed every three days. At day 7, GFP expression was assessed using an inverted fluorescence microscope (AMG EVOS, Fisher).

To quantify the efficiency of BMP-2 synthesis, fibroblasts or MSCs (10^7 cells/ml) and pBMP-2 or empty control plasmid (100 μg/ml) were suspended in 100 μl alginate (10 mg/ml) plugs without BCP scaffold. Constructs were kept at 37°C in 2 ml expansion medium in a humidified incubator. At day 7 the gel was depolymerized with citrate buffer (150 mM NaCl, 55 mM sodium citrate and 20 mM EDTA in H_2O) for 15 minutes at room temperature. The BMP-2 concentration in the buffer and the medium was measured with ELISA (Quantikine #DBP200 from R&D Systems) following standard protocol.

Animals and implantation

After approval of the local animal care committee, 14 male Fischer 344 inbred rats (126–150 g; Charles River Laboratories, L'Arbresle, France) were used. Food and water were

given ad libitum. The laboratory animal welfare officer monitored the animals' general health and care. All procedures were performed under general anesthesia by inhalation of isoflurane and the incisions were closed using a vicryl 5-0 suture. Pain relief was given by subcutaneous injections of buprenorphine (Temgesic; Schering-Plough, Utrecht, The Netherlands), 0.05 mg/kg bodyweight, every 8h until 48h postoperatively. After shaving and disinfecting the back of the rats, a posterior midline incision was made to expose the paraspinal muscles. For the gene tracing study (groups outlined in Table 1), six subcutaneous and six paraspinal intramuscular pockets were made. In addition, one orthotopic spinous process defect location was created at the lumbosacral junction. For the pBMP-2 expression study (groups in Table 2) ten paraspinal intramuscular pockets were made per animal, of which eight were used for this study. In both studies, all groups were implanted according to a randomized block design. After two and eight weeks, respectively, the animals were euthanized with CO_2 asphyxiation and constructs were retrieved.

Post-mortem sample acquisition and processing

After retrieval, samples were fixed in 4% (w/v) formalin and processed for 5 μm thick decalcified paraffin sections through 0.5 M EDTA and alcohol dehydration series for GFP immunohistochemistry and hematoxylin and eosin (HE) staining. For the pBMP-2 expression study: after retrieval, samples were fixed and cut in half. One half was processed for paraffin embedding as described above for BMP-2 immunohistochemistry, tartrate-resistant acid phosphatase staining and HE staining. The other half was embedded in poly-methyl-methacrylate (PMMA), for undecalcified histology. Sections were stained with methylene blue and basic fuchsin.

GFP immunohistochemistry

Immunohistochemistry was used to demonstrate GFP presence, as its intrinsic fluorescence was lost during the tissue processing. Paraffin sections were rehydrated, permeabilized with 0.1% Triton X-100 in PBS and incubated in 0.3% (v/v) H_2O_2 for 10 min and in 5% (w/v) bovine serum albumin (BSA) in PBS for 30 min. Sections were incubated for two hours at room temperature with chicken anti-GFP (ab13970, Abcam) 20 μg/ml in PBS/5% BSA and subsequently washed and incubated with goat anti-chicken Alexa Fluor® 594 (ab150176, Abcam) at 8 μg/ml in PBS/5% BSA for 30 min at room temperature. Nuclei were stained with DAPI and sections were mounted in aquamount. GFP presence was assessed with a fluorescence microscope (E600, Nikon). Per sample the first section was stained; and three pre-designated fields of view (ROI) were photographed. Two observers scored the blinded specimens on a scale from 0-3 corresponding to the number of positive

cells per ROI (0 = negative; 1 = 1-5 GFP positive cells ; 2 = 6-20 GFP positive cells; 3 = >20 GFP positive cells). Mean scores of both observers were compared. When the scores differed, agreement was reached after discussion.

BMP-2 immunohistochemistry

BMP-2 protein detection was performed as described before [150]. Per sample, the first section was stained; three pre-designated ROIs were photographed, a representative image was chosen. In short, paraffin sections were rehydrated and subsequently permeabilized with 0.1% Triton X-100 in PBS and then incubated in 0.3% (v/v) H_2O_2 for 10 min. Antigen retrieval steps were performed in 10 mM sodium citrate buffer (pH 6.0) for 30 min at 95^0C. The sections were then incubated in 5% (w/v) bovine serum albumin (BSA) in PBS for 30 min and subsequently for two hours at room temperature with rabbit-anti-BMP-2 (C43125, LifeSpan BioSciences, Province, RI, USA) at 2.5 μg/ml in PBS/5% BSA. Control stainings were performed with non-immunized rabbit immunoglobulin (IgG) 2.5 μg/ml in PBS (Dako, X0903, Glostrup, Denmark). Subsequently samples were incubated with goat-anti-rabbit horseradish peroxidase (Dako, P0448, Glostrup, Denmark) at 1 μg/ml in PBS/5% BSA for 60 min at room temperature. The staining was developed with 3,3'-diaminobenzidine. Counterstaining was performed with Mayer's hematoxylin.

Tartrate-resistant acid phosphatase

Osteoclasts, macrophages, and dendritic cells expressing tartrate-resistant acid phosphatase (TRAP) were visualized using the Kit 386A-1KT (Sigma–Aldrich) according to the manufacturer's instructions on decalcified paraffin sections [151]. TRAP-positive cells appear in red.

Statistics

The statistical significance of differences between experimental groups in Fig. 1D was assessed using the two-factor ANOVA and Tukey post-hoc test. Data are represented as mean ± standard deviation. P values <0.05 are considered statistically significant.

RESULTS

In vitro assessment of construct performance

The release of pDNA to the surrounding environment was assessed to estimate release kinetics of pDNA from the constructs, which would allow invading and surrounding cells to be transfected after the implantation phase.

Up to 50% of the initially loaded plasmid DNA was released in the first three days, which increased to more than 60% after two weeks (Fig. 1B). This implies a prolonged availability of plasmid DNA for seeded cells but also for tissue-resident cells.

To demonstrate transfection of cells by the alginate GAM, fibroblasts or MSCs were included in alginate constructs with pGFP and assessed for *in vitro* GFP expression. GFP-positive cells were observed in both the fibroblast and MSC containing groups (Fig. 1C) but were absent from the control samples (containing empty plasmid, results not shown).

To quantify BMP-2 production as a result of gene transfer, both cell types were transfected with pBMP-2 and cumulative production was measured at day 7. Unpublished work has shown that at this time point gene expression is optimal. Transfection of MSCs resulted in higher BMP-2 production than the other conditions, although fibroblasts also produced a substantial amount of BMP-2. In the MSC control group (transfected with empty plasmid) low levels of endogenously produced BMP-2 were measured. Note that the ELISA detects human BMP-2 as well as endogenous rat BMP-2. In previous studies we have shown that the produced BMP-2 is biologically active [149].

Histological and immunological aspects of the implants

No surgical complications occurred in the rats, and all samples were uneventfully retrieved. To evaluate the host response against the implants as well as infiltration of host cells, immunohistochemistry on decalcified tissue was done. Microscopic analysis of the pGFP tracing study after two weeks revealed tissue ingrowth with connective tissue throughout the entire scaffold, seen for every group and in all three implant locations. Distinct differences in cell morphology or cell density were not found. Multinucleated giant cells (MGCs) were frequently observed in all groups (Fig. 2). Microscopic analysis after eight weeks of the pBMP-2 expression study showed extensive tissue ingrowth throughout the entire scaffold in all groups. Sporadically MGCs were present in the tissue, more evident in the groups with pDNA than the corresponding controls (Fig. 2). No clear signs of an active immune response could be found at the time of explantation, as lymphoid clusters or fibrous capsules were not observed.

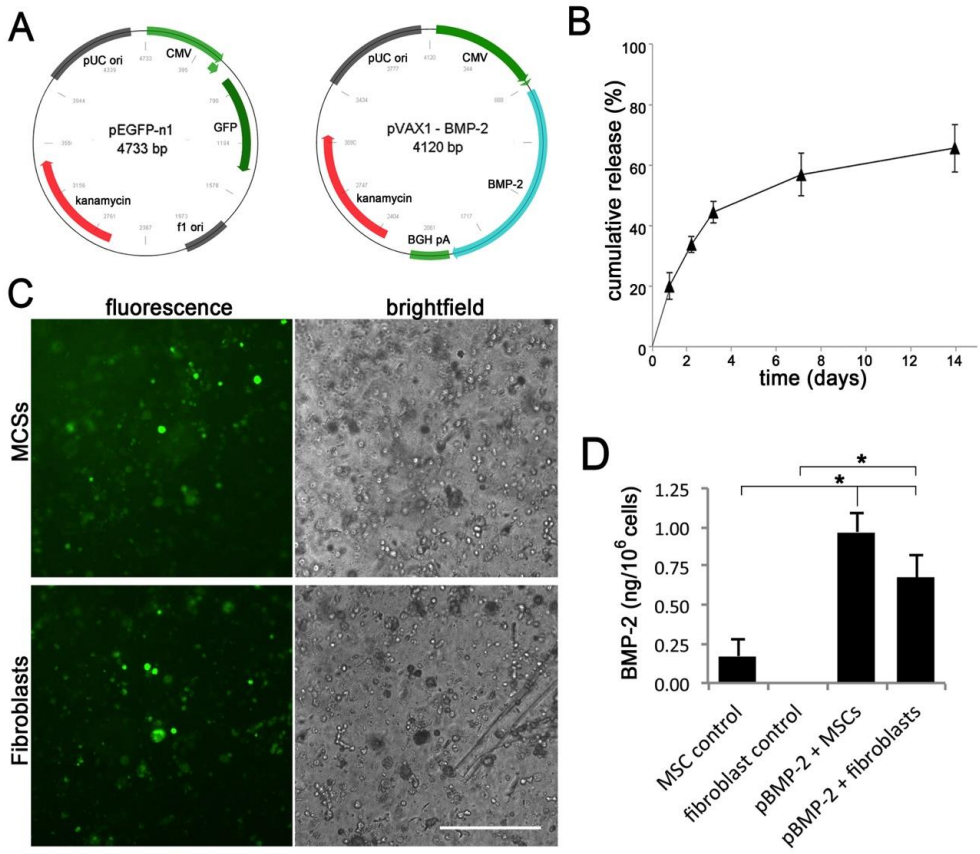

Figure 1: Plasmid retention and transgene expression *in vitro*. A) Vector maps of the pGFP and pBMP-2. B) *In vitro* plasmid DNA release over 14 days from constructs consisting of BCP scaffold, alginate hydrogel and pBMP-2. Initial loading concentration was 100 μg/ml. Data are shown from a single experiment (technical triplicate), which was repeated with similar results. C) Microscopic images of pGFP transfected MSCs as well as fibroblasts after 7 days incubation in alginate. Transfection efficiency was 21 ± 4% for the MSC group and 16 ± 3% fibroblasts group (controls not shown). Representative images are shown for two individual experiments. Scale bar = 200 μm. D) BMP-2 protein release from constructs containing MSCs and fibroblasts transfected with pBMP-2 (100 μg/ml) and empty control DNA at day 7. Results represent mean ± SD of technical triplicates, the experiment was repeated with similar results. * = $p < 0.05$

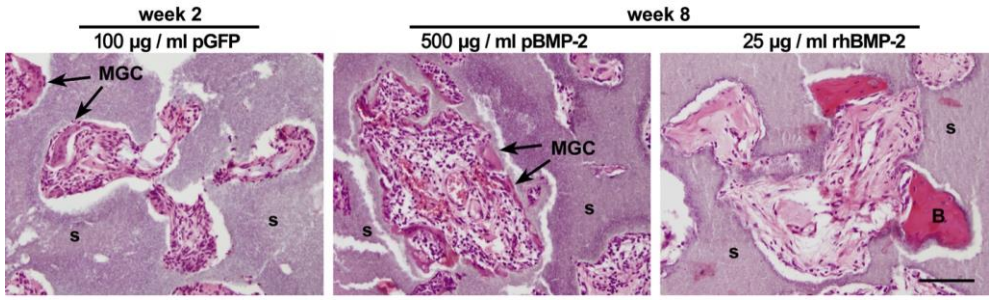

Figure 2: Tissue ingrowth and bone formation after two and eight weeks *in vivo*. The images show H&E-stained sections of i.m. samples, containing 100 µg/ml pGFP (week 2), 500 µg/ml pBMP-2 or 25 µg/ml rhBMP-2 (week 8) without seeded cells. A representative image of each group is shown. After two weeks, complete ingrowth of tissue could be observed as well as frequent presence of multinucleated giant cells (MGC). After eight weeks, alginate was no longer detectable in any of the constructs. In all groups containing pDNA a high cell density could be seen together with the frequent presence of MGCs. s = scaffold; B = bone; MGC = multinucleated giant cell. Scale bar = 100 µm.

Transgene expression in cell-free and cell-seeded implants

Presence of GFP expression as a result of transfection was assessed with a semi-quantitative scoring on 4 animals. The results varied with respect to the type of cell being transfected, and was influenced by pDNA dosage and the presence of seeded cells. The two week implantation period was chosen based on *in vitro* work, and the notion that *in vivo* degradation and thereby plasmid release would be faster than *in vitro*. Figure 3 and Table 3 show representative images and quantified data respectively. It appears that for the cell-free groups the pGFP concentration of 100 µg/ml clearly resulted in abundant GFP transgene expression. The groups containing seeded cells, both fibroblasts and MSCs, also showed frequent expression of GFP. Overall observations of all samples indicated that GFP was mainly present within the construct, but in two intramuscular samples (100 µg/ml pGFP without seeded cells) tissue outside the construct boundaries stained positive as well. Intramuscular implants showed overall frequent transgene-positive cells. The orthotopic location was also associated with frequent GFP-positive cells. The control samples did not show any GFP, neither within the construct boundaries nor in the surrounding tissue.

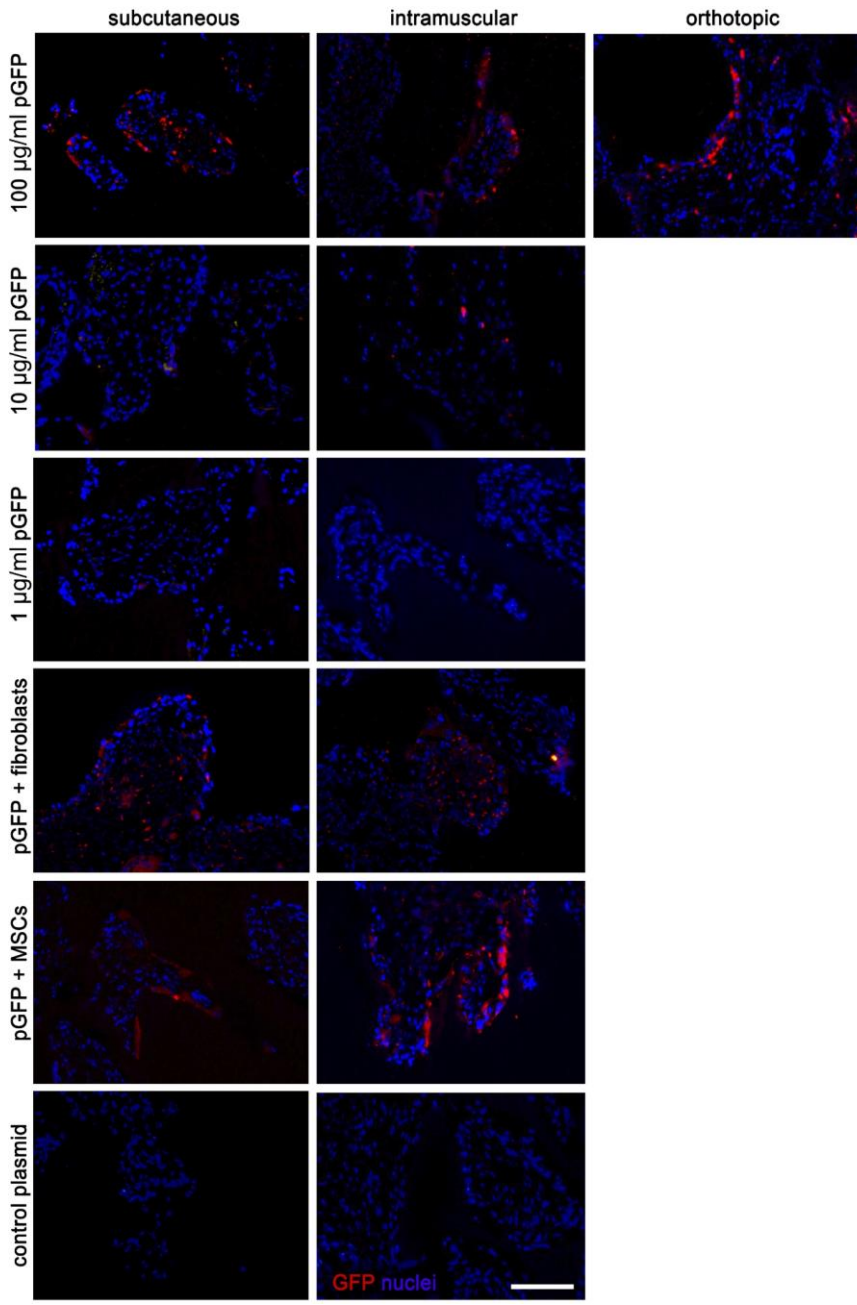

Figure 3: Qualitative imaging of *in vivo* gene expression. A representative image of the GFP immunohistochemistry (in red) for each group implanted in the subcutaneous (left column panels), intramuscular (middle panels) or orthotopic (right panel) locations. Each row represents an experimental group, as outlined in Table 2. Nuclei are DAPI stained (in blue). Scale bar = 100 μm for all panels.

	Rat 1			Rat 2			Rat 3			Rat 4			Sum of scores		
	i.m.	s.c.	ort.	i.m.	s.c.	ort.	i.m.	s.c.	ort.	i.m.	s.c.	ort.	i.m.	s.c.	ort.
pGFP 100 μg/ml	2	0	1	2	0	3	2	1	3	0	1	1	**6**	**2**	**8**
pGFP 10 μg/ml	0	0		1	0		0	0		0	0		**1**	**0**	
pGFP 1 μg/ml	0	0		0	0		0	0		0	0		**0**	**0**	
pGFP + fibroblasts	2	2		1	1		0	1		2	0		**5**	**4**	
pGFP + MSCs	1	1		3	0		3	1		2	3		**9**	**5**	
Control	0	0		0	0		0	0		0	0		**0**	**0**	

Table 3: Scoring of transgene expression at the different implant locations.
Each implant was given a score (0, 1, 2 or 3) for the amount of GFP expression (as detailed in the M&M section). The sum of the total scores per group is depicted. i.m.: intramuscular; s.c.: subcutaneous; ort.: orthotopic.

Bone formation and assessment of BMP-2 presence

As transgene expression was pDNA dependent but a plateau was not reached, a 5 fold higher dosage was included in the pBMP-2 expression study. Furthermore the i.m. location was selected because transgene was more frequently observed in that location. BMP-2 immunohistochemistry was performed to detect the presence of BMP-2 and localize the producing cells, both in and around the implants. The antibody used is human-specific but, as a result of highly conserved sequence, cross-reacts with rat BMP-2. BMP-2 was found in all groups and appeared to be mainly present in and directly around cells that align the BCP scaffold material (Fig. 4). The expression of BMP-2 varied among the individual groups. BMP-2 presence was most obvious in the groups seeded with MSCs and 100 μg/ml pBMP-2 and in unseeded samples containing 500 μg/ml pBMP-2. The groups seeded with fibroblasts (100 μg/ml pBMP-2) and unseeded samples with 100 μg/ml pBMP-2 resulted in frequent BMP-2 positive cells. The positive control with rhBMP-2 showed positively stained osteoblasts bordering the newly formed bone. The negative control groups, both with and without seeded cells, only rarely showed BMP-2 positive cells.

The MMA sections showed no bone formation in any of the groups with pBMP-2, whereas in seven out of eight rhBMP-2 containing constructs, bone was present (Fig. 5). As we found plasmid-derived BMP-2 expression to be associated with TRAP-positive macrophophages in goat studies [150], we performed TRAP stainings on the rat intramuscular constructs. The positively stained multinucleated giant cells (MGC) were visible in regions directly contacting the scaffolds (Fig. 6A), coinciding with the localization of BMP-2 positive cells (Fig. 6B).

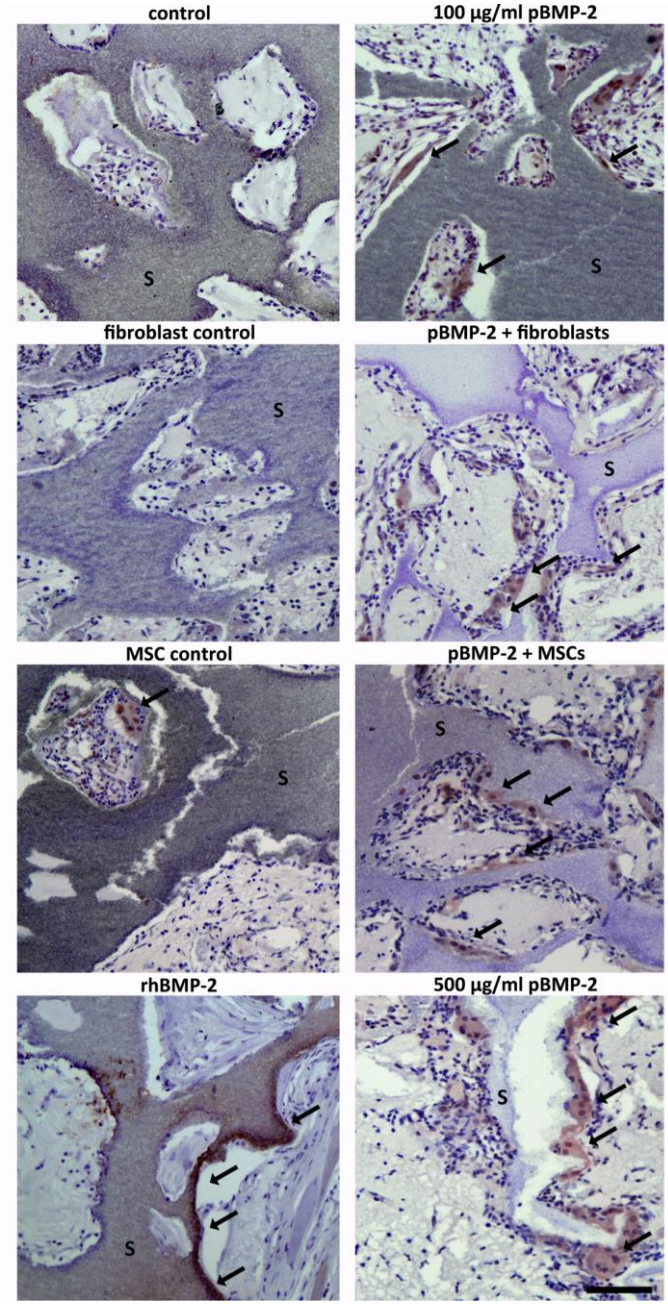

Figure 4: Identification of BMP-2 protein within the sample boundaries. A representative image of the BMP-2 immunohistochemistry is shown for each group. BMP-2 (brown staining, indicated with arrows) is present in all the groups containing pBMP-2, as well as the rhBMP-2 positive control group. The empty vector control groups showed BMP-2 presence sporadically, also when MSCs or fibroblasts were seeded. The BMP-2 antibody recognizes rat endogenous and transgene derived human BMP-2. s = scaffold. Scale bar = 100 µm for all panels.

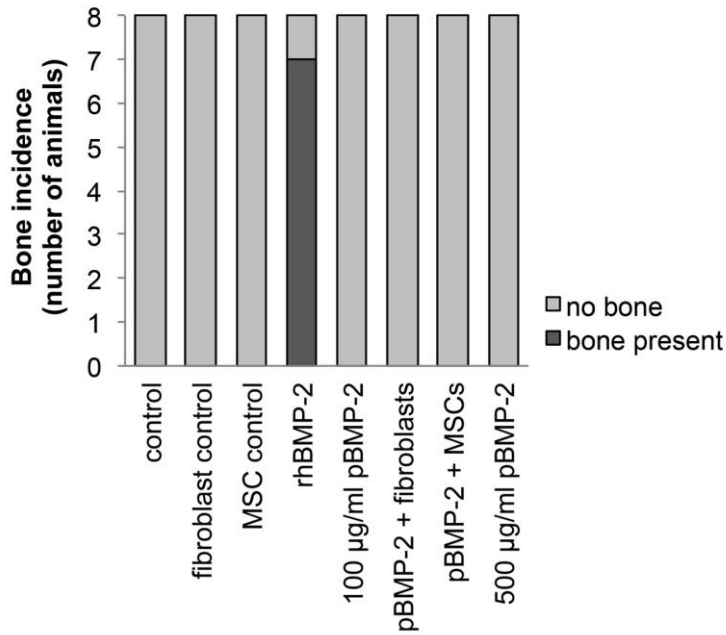

Figure 5: Frequency plot showing the incidence of the bone within the construct boundaries.

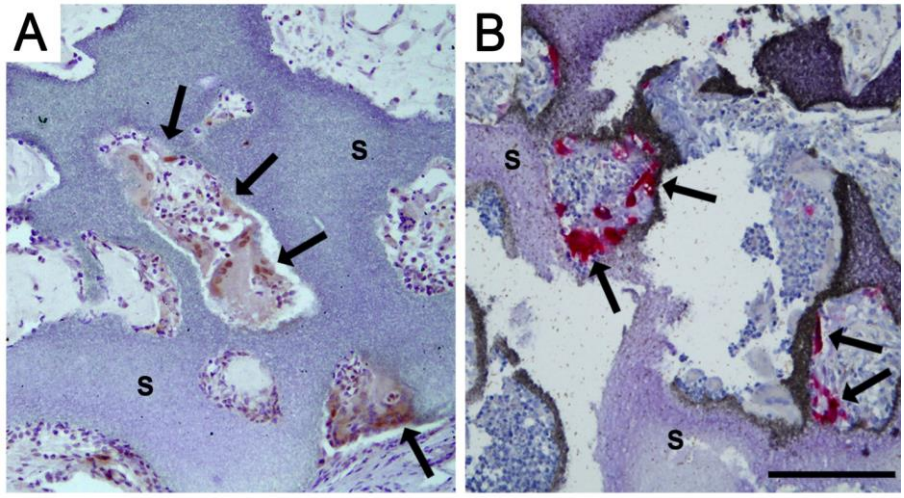

Figure 6: Characterization of the cells expressing BMP-2. BMP-2 immunohistochemistry (A) and TRAP staining fo r cells of the macrophage lineage (B) were performed on sections of the same group (pBMP-2 + MSCs). Cells aligning the scaffold, indicated with the arrows, are stained BMP-2 positive (brown in A) and TRAP positive (red in B). s = scaffold. Scale bar = 200 µm.

DISCUSSION

In current studies, the potentials and limitations of the alginate-based gene delivery method were investigated. The boundary conditions for efficiency of transgene expression using pGFP and pBMP-2 were determined *in vitro*. Then *in vivo* performance of cell free and cell containing constructs elucidated that cell-free constructs loaded with pDNA in an alginate matrix can locally transfect the host cells. Furthermore, co-seeding of MSCs with this technique resulted in enhanced BMP-2 presence in the tissue. Despite evidence that local BMP-2 production was increased in the conditions with pBMP-2, this did not lead to ectopic bone formation. Detectible ectopic bone formation only occurred with the use of rhBMP-2.

To ensure adequate gene transfer and BMP-2 production in the *in vivo* bone-induction experiment, *in vitro* BMP-2 release was first determined. Although fibroblasts and MSCs both produced BMP-2 after transfection, the average BMP-2 secretion was higher in MSCs. This could possibly be explained by an autocrine feedback loop in MSCs, in which BMP-2 induces differentiation and endogenous BMP-2 production, as transfection efficiencies for with MSCs and fibroblasts were similar (data not shown) [152].

The tracer gene GFP was used to assess transgene expression and to establish optimal plasmid DNA concentration *in vivo*. This marker is widely known for its sensitivity and great signal to noise ratio in tissue samples by immunohistochemistry. Using pGFP, the transfection of host cells was demonstrated after two weeks. Furthermore, the gene tracing experiment was set up to determine optimal plasmid DNA dosage. As a plateau effectivity of pDNA dosage could not be reached in the cell free groups and because high plasmid concentrations are not associated with severe side effects [104], we included one group with an even higher dose of pBMP-2 (500 µg/ml) in the bone-induction experiment.

To gain insight in the role of BMP-producing cells, MSCs and fibroblasts as producer cells were compared in plasmid-based gene delivery constructs. We hypothesized that fibroblasts could be suitable target cells for gene therapy, thereby functioning as a temporary BMP-2 delivery system. This role of fibroblasts stands in contrast to the role of MSCs, which function both as BMP-2 producer and subsequently become bone-forming cells.

Although we found differential expression of BMP-2 in several groups, a clear correlation with bone formation was absent. This could be explained in several ways: First, the BMP production levels as a result of the transfection may have been insufficient. Studies with viral transductions show that the amount of produced BMP is key in bone formation in gene therapy [153]. To optimize host cell transfection, the timing of pDNA release together with the presence of host cells is essential [154]. From the present study it is

unknown whether the release of plasmid DNA did or did not match with the presence and/or invasion of target cells. The first pre-clinical experiments with pDNA that applied a single extremely high dosage only resulted in a minimal increase of gene expression [104,108]. Interestingly, repeated injections, ensuring prolonged presence of plasmid DNA, did show a strong positive effect on bone formation [96,97]. Since we observed that pDNA is readily released from alginate *in vitro* and then becomes subject to washout, we suspect that a limited cellular invasion into the constructs was the cause of low BMP-2 expression and absence of bone formation. Second, timing of BMP protein release is of great importance. As physiological BMP-2 expression in fracture repair starts already within hours, an early onset of BMP-2 production may be essential [21,22,155]. The quick release of BMP-2 when using commercial products and *ex vivo* transfection strategies mimic the early expression pattern and have successfully resulted in bone formation [128,156]. In theory, alginate-mediated gene delivery starts when the components are combined, and cumulative production is ongoing for at least several weeks, at least *in vitro* [117,149]. A conclusion about availability of BMP-2 in time and amounts *in vivo* is notoriously hard to draw.

Despite the fact that the BMP-2 levels appeared too low to induce bone formation, our results indicate that cell seeding is not obligatory for transgene expression. Most of the BMP-2 expression was associated with multinucleated giant cells, even in the conditions with seeded target cells. Active degradation of the alginate/pDNA complex by the observed MGCs might have diminished the transfection and subsequent BMP-2 production of other resident cells (Fig. 6), thus affecting the sustained pDNA release profile as observed *in vitro* (Fig. 1B). We and others have shown that the application of genetically engineered MSCs could in some cases lead to long-term engraftment or result in temporary local BMP production as the majority of the cells disappear from the implant area [130,131]. Furthermore, it has been shown that bone formation as a result of viral BMP gene therapy seems to be independent of cell type [157]. These observations underline that the amount of BMP-2 production, together with its timing, are more essential to the process of bone formation than the presence of seeded bone-forming cells during gene delivery.

In conclusion, the conditions for optimal transgene expression using the alginate delivery system in rats were determined and secondly we found that cell seeding is dispensable for non-viral BMP-2 gene expression *in vivo*. When investigating the role of BMP-producing cells, we found that bone progenitor cells outperformed non-osteogenic producer cells (i.e. fibroblasts) in plasmid-based gene delivery constructs. Other strategies, including more efficient *ex vivo* transfection methods and/or the use of hydrogel systems that allow cellular infiltration and show sustained release kinetics of plasmid DNA, might be interesting options for increasing efficacy in bone regeneration strategies.

ACKNOWLEDGEMENTS

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Chapter 3

Bone morphogenetic protein-2 non-viral gene therapy in a goat iliac crest model for bone formation.

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ABSTRACT

Treatment and reconstruction of large bone defects, delayed unions and non-unions is challenging and has resulted in an ongoing search for novel tissue-engineered therapies. Bone morphogenetic protein-2 (BMP-2) gene therapy is a promising strategy to provide a sustained production of BMP-2 locally. Alginate polymer based non-viral gene therapy with BMP-2 plasmid DNA (pBMP-2) in constructs with multipotent mesenchymal stromal cells (MSCs) has resulted in prolonged gene expression and bone formation *in vivo*. To further translate this technology towards larger animal models, important issues remain to be investigated, such as the necessity of seeded cells as a target for gene therapy. For that purpose, a large animal-screening model in an orthotopic location, with fully separated chambers, was investigated.

Four cylinder shaped implants were placed in the iliac crests of ten goats. Polycaprolactone tubes around each implant allowed bone ingrowth from the underlying bone and bone marrow and ensured separation of the experimental conditions. An empty tube showed low levels of spontaneous bone ingrowth and implantation of autologous bone indicated proper bone function with respect to remodeling and resorption.

Control ceramic scaffolds were compared to scaffolds containing pBMP-2 either or not combined with seeded MSCs. Fluorochrome incorporation, evaluated at three, six and nine weeks and histomorphometry at twelve weeks after implantation revealed clear differences between the groups, with pBMP-2 combined with MSCs being most effective. BMP-2 protein was demonstrated in a variety of bone-residing cells through immunohistochemistry. Further analysis indicated that multinucleated giant cells might have an important role in transgene expression.

Taken together, this work introduces a large animal model for studying bone formation at multiple sites simultaneously in an orthotopic location. The model appeared robust, showed no neighboring effects and demonstrated effectivity of combined cell-and gene therapy.

INTRODUCTION

Current bone graft treatments for large bone defects, delayed unions and non-unions are often insufficient. Therefore a search for novel tissue-engineered therapies as a substitute for current bone grafts is ongoing. Bone morphogenetic protein-2 (BMP-2) has shown to be an excellent stimulus for bone growth, resulting in improved bone formation in several orthopedic locations [158-160]. When using BMP-2, fusion rates and functional outcomes are comparable to autologous bone grafts [161], omitting the harvesting of autologous bone, which can result in complications. However, BMP-2 protein is expensive [162], and additionally, high amounts of protein are used clinically, presumably needed due to fast degradation and clearance in clinical applications [163]. High dosage is possibly associated with the occurrence of several severe side effects such as ectopic bone formation and cervical soft tissue swelling [164,165].

Gene therapy is a well-known strategy that can result in a sustained production of the desired protein [166]. Gene therapy can be performed by viral or non-viral DNA delivery. Non-viral gene therapy is not hampered by disadvantages associated with viral gene therapy, such as mutagenesis and severe immune responses, since the plasmid DNA is not integrated in the host genome and the viral proteins invoking immune responses are lacking [167,168].

In previous work we developed a non-viral alginate hydrogel-based gene therapy applying the BMP-2 coding region (pBMP-2). *In vitro* expression of this gene therapy resulted in an increased bioactive protein production over a period of five weeks [149]. When implanted subcutaneously in mice, BMP-2 gene therapy combined with MSCs resulted in effective bone formation [169].

To further translate this technology, important issues remain to be investigated in larger preclinical models, for example the need for cell seeding in an orthotopic location and the optimal construct composition. From previous experiments, it appears that the immediate availability of cells is favorable for expression of the transgene. This was however only studied in small (mm-size) implants, not in more relevant (cm-size) constructs [170]. Also, it is not clear whether direct contact to the complexity of bone tissue influences in any way local gene therapy. Furthermore, it would be interesting to determine endogenous target cells for gene therapy as this could eventually lead to a cell-free strategy. Finally, a screening model would allow easy optimization of dosage and construct composition, and provide a platform to study safety and potential off-target effects.

Previously we developed a screening model in goats that allowed comparison of multiple groups at the location of posterolateral fusion [171]. Although successful, this model

is technically difficult and highly invasive. Moreover, in this transverse process cassette model insufficient separation of the samples might lead to neighboring effects when soluble factors such as BMP-2 are investigated.

Here we describe the development of an animal model located at the iliac crest of goats, which is easily accessible and allows for comparison of different groups in fully separated chambers. The model was used to investigate the feasibility of alginate-based BMP-2 gene therapy in a setting with bone contact and the necessity of cell seeding.

MATERIALS AND METHODS

Study design (Fig. 1A)

Per goat (n=10), four iliac crest implant locations were made (two per side). One location received either autologous bone graft or was left empty (n=5). In the other 3 locations, the experimental groups (n=10) where tested: (1) biphasic calcium phosphate (BCP) control, (2) BCP with alginate, pBMP-2 and allogeneic MSCs and, (3) BCP with alginate and pBMP-2. Fluorochrome markers were administered at 3, 6 and 9 weeks and the animals were terminated after 12 weeks. Bone formation was evaluated by (immuno) histology, fluorescence microscopy and histomorphometry.

Scaffold

Porous biphasic calcium phosphate (BCP) cylinders were used as it is a highly biocompatible material that facilitates osteoconduction, and provides a mineralization surface in alginate based constructs, also in combination with BMP-2 gene therapy [147,170]. BCP scaffolds consisted of 80±5% (w/v) hydroxyapatite and 20±5% (w/v) β-tricalcium phosphate, with overall porosity of 70±5%, macroporosity of 55±5% and microporosity of 20±5% (Xpand biotechnology, Bilthoven the Netherlands) and a pore size of 200-800 μm. BCP cylinders (diameter 6 mm and height 10 mm) were cleaned in ultrasonic baths and autoclaved.

To prevent neighboring effects of the constructs, their longitudinal borders were shielded by a poly(ε-caprolactone) (PCL) tube, thereby allowing nutrient supply and tissue ingrowth only from the top and the bottom of the cylinders. PCL was chosen, because it is stable for the time duration of the experiment, is biocompatible and can be 3D printed in the desired shape (inner diameter 6 mm, wall thickness 0.2 mm and height 10 mm) (see Fig. 1 and Fig. 2A) [172].

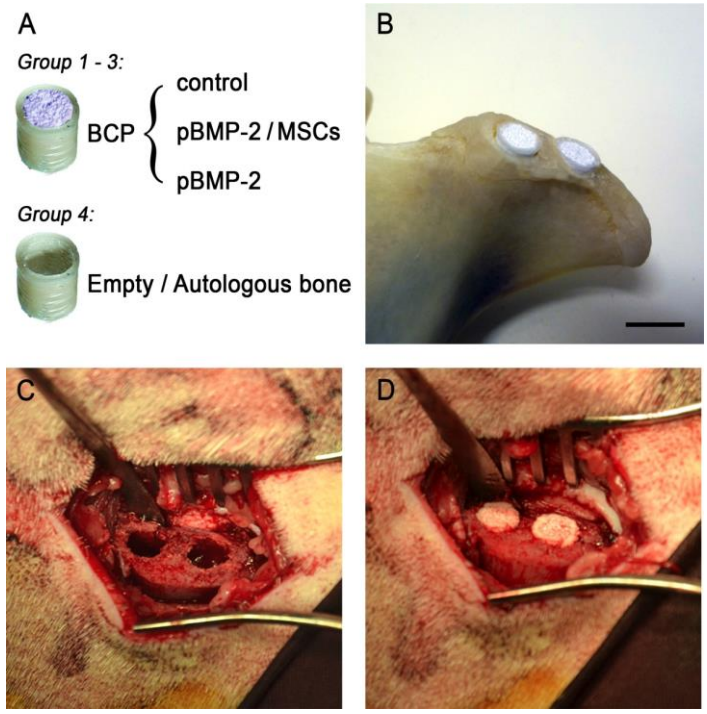

Figure 1: Overview of the experimental design and the surgical procedure. **(A)** At four locations per goat, implants were placed in a plastic cylinder. Group 1, 2 and 3 consisted of a BCP scaffolds; either left unseeded, or with pBMP-2/MSCs, or pBMP-2. The 4th location was left empty or was filled with autologous bone. **(B)** Two implants (6.4 mm diameter and 10 mm high) per iliac wing. Scale bar = 10 mm. **(C)** Intraoperative image of the holes, with **(D)** implants press fit in situ.

Alginate gel

Autoclaved high-viscosity alginate powder (International Specialty Products, ISP, Memmingen, Germany) was dissolved in alpha minimum essential medium (α-MEM, Gibco, Breda, The Netherlands) and used at a final concentration of 10 mg/ml. Alginate hydrogel is an FDA approved, non-toxic, biocompatible delivery gel and transfection agent frequently used for preclinical orthopedic applications [148].

BMP-2 coding plasmid DNA

For expression of BMP-2 the plasmid pcDNA3.1/rhBMP-2 (pBMP-2) was used [149]. Plasmid DNA was isolated and purified using an endotoxin free kit (EndoFree Plasmid Maxi kit, Qiagen K.K., Tokyo, Japan). pBMP-2 was mixed with alginate hydrogel 1% (w/v) to a final concentration of 10 μg/ml plasmid DNA. The optimal concentrations of alginate hydrogel and pBMP-2 were determined previously [149].

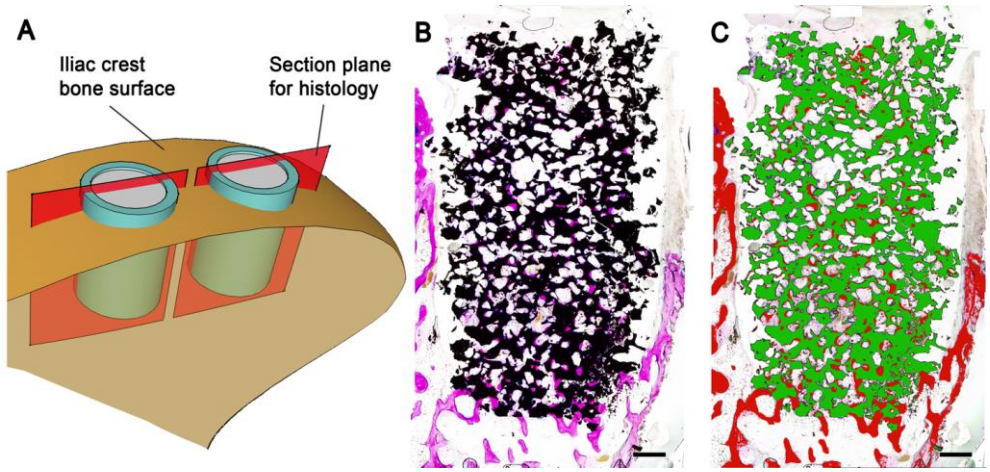

Figure 2: Overview of the section plane and undecalcified histology and pseudocoloring. **(A)** Computer generated image of the goat iliac crest and the implants indicating the sectioning plane. **(B)** Low magnification histology image of a stained (methylene blue/basic fuchsine) construct with pBMP-2/MSCs (bone in pink and the ceramic in black). **(C)** Pseudo-colored version of (B), to highlight the bone (red) and the scaffold (green). Scale bar = 1 mm.

Animals, cell harvest and culture

After approval of the local animal care committee, ten adult female Dutch milk goats aged 24–27 months, 50-60 kg, were used. Food and water were given ad libitum. The animals' general health and care groups were recorded in a diary of well-being for each goat separately and monitored by the laboratory animal welfare officer. Bone marrow was aspirated from both iliac wings (10 ml each) of 10 goats using a bone biopsy needle seven weeks before the actual implantation surgery. MSCs were isolated from the bone marrow by adherence to tissue culture plastic and expanded in αMEM (Gibco), supplemented with 15% (v/v) fetal calf serum (FCS, Hyclone, Perbio, Belgium), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mm L-glutamine (Glutamax, Gibco) [173,174]. The MSCs were maintained at 37°C and 5% CO_2 in a humidified incubator. After five passages, MSCs were cryopreserved in 1 ml aliquots of $2x10^6$ MSCs. Every goat received allogeneic MSCs isolated previously.

Construct composition

Before implantation MSCs were thawed, washed and suspended in alginate (10^7 cells/ml) with pBMP-2. Subsequently, alginate with pBMP-2 either with or without MSCs was

dripped on the porous BCP scaffolds, ensuring that the complete scaffold contained the hydrogel. After seeding of the constructs, alginate was crosslinked by submerging them in 100 mM $CaCl_2$ for 10 minutes.

Implantation

Additionally to the current study, the goats received implants for unrelated studies that will not be discussed: bilateral transverse process cassettes at L2, L3 and L4 and bilateral intramuscular implants. The surgical procedures were performed under standard conditions. After shaving and disinfection of the dorsal pelvic area, bilateral 5 cm skin incisions were made over the well visible and palpable iliac crest to expose the fascia layer. The fascia was split and the periosteum was carefully removed, exposing the bone of the posterior superior iliac spina. Under constant saline cooling, two 6.2 mm diameter and 10 mm deep holes were drilled in each of the posterior superior iliac spinae at a distance of 4 mm using a dental drill (Fig. 1C). The trabecular bone obtained with reaming was collected for the autologous implant group. The PCL tubes alone, required for the empty and autologous bone group, appeared too flexible to press fit in the drilled holes. Therefore BCP containing tubes were placed instead, where after the BCP was drilled out. The implants were placed press fit in the four locations (Fig. 1D). Finally the soft tissues were closed in layers. Postoperatively, pain relief was given by ibuprofen and buprenorphine (Schering–Plough, The Netherlands). The goats received sequential fluorochrome labels at 3 weeks (calcein green, 10 mg/kg intravenously, Sigma), 6 weeks (oxyteracycline, 32 mg/kg intramuscular, Engemycine, Mycofarm, The Netherlands) and 9 weeks (xylenol orange, 80 mg/kg intravenously, Sigma) after the implantations [175]. At 12 weeks the animals were killed by an overdose of pentobarbital (Organon, The Netherlands) and the implants were retrieved.

Post-mortem sample acquisition and processing

After retrieval, samples were fixed in 4% (w/v) formalin and cut in half (red plane in Fig. 2A). One half of the samples was processed for 5 μm thick decalcified paraffin sections through 0.5 M EDTA and alcohol dehydration series for immunohistochemistry using haematoxylin/eosin staining. The other half was embedded in poly-methyl-methacrylate (PMMA), from which 20 μm thick sections were prepared using a sawing microtome (SP1600, Leica, Nussloch, Germany) starting from the center of each implant (Fig. 2). Sections remained unstained for epifluorescence microscopy or were stained with methylene blue and basic fuchsin for histomorphometry.

Histomorphometry

High-resolution (300 dpi) digital photographs of the PMMA embedded sections were made. Adobe Photoshop CS3 was used to pseudocolor the bone red and the scaffold green (Fig. 2), subsequently bone area% (bone area% = bone area/[total area-scaffold area]) x 100%) was determined. Axiovision software (version 3, Zeiss, Nussloch, Germany) was used to measure bone contact%, the percentage of available scaffold perimeter in contact to bone (contact% = bone scaffold contact length/scaffold perimeter length x 100%).

Fluorescence microscopy

Fluorescence microscopy, with a light microscope (Olympus BX51, Japan) equipped with a quadruple filter block (XF57, dichroic mirror 400, 485, 558 and 640nm, Omega filters, The Netherlands), was used to study the dynamics of bone formation [175]. The presence of fluorochromes was scored to determine the time of mineralization. To do this, a translucent overlay displaying an upper, middle, and lower third was created (see Fig. 5) which was superimposed on the section. Data were given in a frequency plot for presence of the fluorochromes.

Tartrate-Resistant Acid Phosphatase (TRAP),
BMP-2 and Cathepsin-K (immuno)histochemistry

Osteoclasts, macrophages and dendritic cells express TRAP [151], which was analyzed using the Kit 386A (Sigma–Aldrich, France) according to the manufacturer's instructions. TRAP positive cells appear in red.

BMP-2 and Cathepsin-K, a marker for osteoclasts [176], detection was performed paraffin sections that were rehydrated and subsequently permeabilised with 0.1% Triton X-100 in PBS and then incubated in 0.3% (v/v) H_2O_2 for 10 min. Antigen retrieval steps were performed in 10 mM sodium citrate buffer, pH 6.0, for 30 min at 95°C. The sections were then blocked in 5% (w/v) bovine serum albumin (BSA) in PBS for 30 min and subsequently incubated for two hours at room temperature with rabbit-anti-human BMP-2 (C43125, LifeSpan BioSciences, Province, RI, USA) at 2.5 μg/ml or rabbit-anti-Cat-K (ab19027-100 Abcam, Cambridge, UK) at 2 μg/ml in PBS/5% BSA. Negative control stainings were performed with non-immunized rabbit immunoglobulin (IgG) (2.5 and 2 μg/ml in PBS, Dako, X0903, Glostrup, Denmark). Subsequently samples were incubated with goat-anti-rabbit horseradish peroxidase (Dako, P0448, Glostrup, Denmark) at 1 μg/ml in PBS/5% BSA for 60 min at room temperature. The staining was developed with 3,3'-diaminobenzidine (DAB). Counter-staining was performed with Mayer's hematoxylin. Digital photographs were made of the BMP-2 immunohistochemistry to quantify BMP-2

per implant via a computerized pixel count (Aperio ImageScope), according to the Positive Pixel Count algorithm. The percentage of positive pixels/total area of the implant was determined. The isotype-matched control staining section was used as a reference.

Statistical analysis of the data

Based on previous data [177], a sample size of n=10 was calculated to identify a >20% difference in bone quantity (bone contact%), with a power of 90%. This power analysis is conservative since we did paired comparisons, excluding inter animal variance. Statistical analyses were performed using statistical software, IBM SPSS 20 (IBM SPSS Inc., Chicago, IL, USA). Data were tested for homogeneity of variances using Levene's test. Subsequently an ANOVA was done to identify differences in bone contact% and bone area%. After Bonferroni correction, significance was assumed when $p<0.05$.

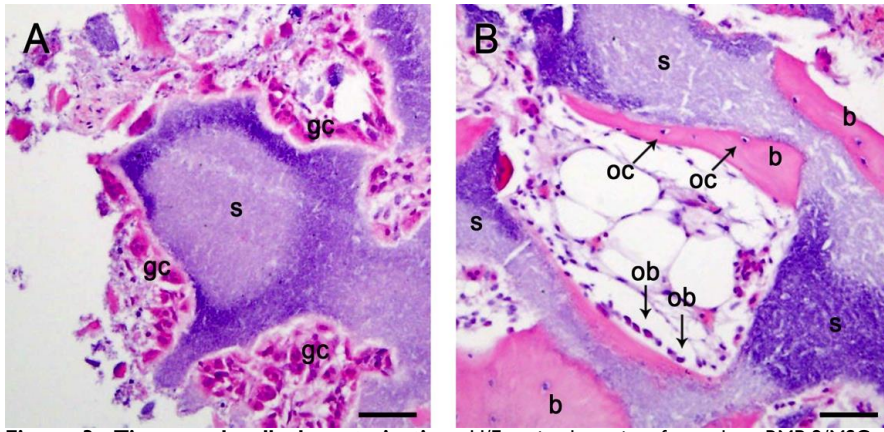

Figure 3: Tissue and cell characterization. H/E stained section from the pBMP-2/MSCs group. The structures and cells depicted are visible in all groups containing BCP scaffolds. **(A)** Multinucleated giant cells (gc) lying against the scaffold surface (s), and **(B)** bone (b) lining the scaffold material with osteoblasts (ob) lining the bone and osteocytes (oc) in lacunae. Scale bar = 50 μm.

RESULTS

Surgery and recovery

During surgery implants were placed well separated. During the postoperative period two goats developed superficial wounds at the location of the iliac crest incisions due to scratching and biting the sutures and skin. After treatment with zinc-containing lotion, the wounds healed uneventfully.

Macroscopic and microscopic assessment of the implants

During the entire implantation period, all constructs remained firmly in place and well separated. Microscopic analysis of the basic fuchsin / methylene blue stained MMA-embedded sections revealed full tissue ingrowth with connective tissue, bone and bone marrow in all implanted groups.

Giant cells were often observed aligning the BCP scaffold where no bone was formed (Fig. 3A). At sites of bone formation osteoblasts were aligning the newly formed bone and osteocytes were embedded in the bone (Fig. 3b). These observations occurred in all the BCP containing implants.

Transgene expression and identification of target cells

In order to assess the efficiency of the local gene therapy, BMP-2 immunohistochemistry was performed on paraffin embedded implant sections. The antibody used is human-specific, but cross-reacts with goat as a result of the highly conserved sequence. Changes in BMP-2 levels within the construct boundaries were thus assumed to be the result, either direct or indirect, of transgene expression. All bone containing implants showed BMP-2 in two distinct cell types: multinucleated giant cells aligning the scaffold surface and osteoblasts aligning the newly formed bone (Fig. 4B and 4C). Quantification of the BMP-2 staining using image analysis software resulted in relative expressions of (mean ± SD): control 4±4 area%, pBMP-2/MSCs 9±6%, and pBMP-2 alone 7±4% area%, no significant differences (Fig. 4A). TRAP histochemistry and Cathepsin-K immunohistochemistry identified multinucleated cells expressing BMP-2 in consecutive sections, confirming that these cells are osteoclasts (Fig 4c-e). In the bone tissue around the implants these BMP-2 positive osteoclasts were only sporadically observed.

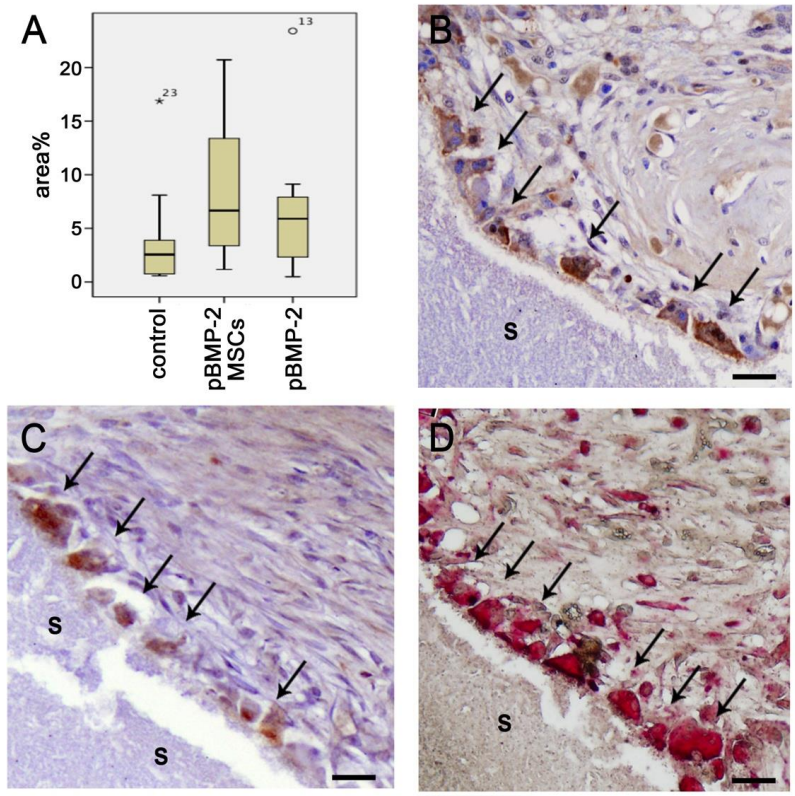

Figure 4: Quantification of BMP-2 protein within the sample boundaries and identification of BMP-2 producing cells. (A) Quantification of BMP-2 immunostained positive tissue within the implant, expressed in area% per group, outliers are indicated. No significant differences. **(B)** BMP-2 immunohistochemistry shows BMP-2 positive cells, many of which align the scaffold (s)**. (C)** and **(D)** are consecutive sections showing Cathepsin-K immunohistochemistry **(C)**, and TRAP staining **(D).** Arrows indicate positive cells for the staining confirming that these cells are osteoclasts. Scale bar = 25 μm.

Bone formation dynamics

Analysis of the fluorochrome labels showed that bone formation occurred from the bottom upwards, except for 2 out of 40 cases, both in the pBMP-2/MSCs group, where the early markers were also present in the upper part (Fig. 5). In 8 (out of 10) samples bone had formed at twelve weeks in the upper third of the constructs containing pBMP-2/MSCs, compared to only two and three samples in the control and pBMP-2 alone groups, respectively. The speed of bone ingrowth was also higher in the pBMP-2/MSCs group as bone formation in the middle third of the construct had occurred in four samples at week six and eight samples at week nine, where the control and pBMP-2 group had both in only one sample bone at week six and three and five sample at week nine

respectively. The autologous and empty groups showed a similar pattern of ingrowth from the bottom. In all cases bone formation was seen in the lower part of the construct after three weeks. The model appeared challenging even for the autologous bone graft as ingrowth height was 65±22% (mean±SD) compared to 60±29% in the empty group.

Bone formation analysis

The contact% was used for primary analysis because it is more sensitive for bone apposition than area%, since it has relatively little volume and always occurs on the material surface [178]. Quantification of bone contact% (mean±SD) resulted in the experimental groups in: 5.1±3.7% (control), 8.5±3.7% (pBMP-2/MSCs), and 5.0±3.5% (pBMP-2). The bone area% in these groups was 3.6±2.6%, 6.1±2.6%, and 3.5±2.5% respectively. The group containing pBMP-2/MSCs showed a higher contact% as well as area% than the control and the pBMP-2 alone (Fig. 6). In the autologous bone group 8.3±2.5% bone area% was observed compared to 5.3±2.6% in the empty group.

DISCUSSION

In this study we introduce a new iliac crest model for bone regeneration in multiple groups in an environment where the distance to underlying bone and nutrition is comparable to the environment for bone regeneration in a clinical situation. As intended, without grafting the defect did not heal. With autologous bone graft only 8% bone was observed compared to 15-20%, normal for iliac crest bone. This low amount indicates that bone formation in this model is demanding, comparable to established bone chamber models [179], whereas differences in bone volumes could still be measured, indicating that this model is robust for studying bone formation. Shielding from mechanical influences is limited and possibly results in less bone remodeling, whereas the observed bone ingrowth seemed not to be hampered by the presence of soft tissue. Therefore this model should not be considered a functional model, but a model for screening different experimental conditions that promote bone formation.

As expected, the BCP cylinder showed osteoconduction from the underlying bone, with a significant increase of bone formation in the combined pBMP-2/MSCs group (1.7-fold increase in area% compared to control). Whether this effect is a result of BMP-2 plasmid DNA expression in the seeded cells, cannot be verified. Because MSCs alone have shown a limited contribution to increased bone volume in the orthotopic cassette model, we ascribe the increase in bone formation mainly to the pBMP-2 [177,179].

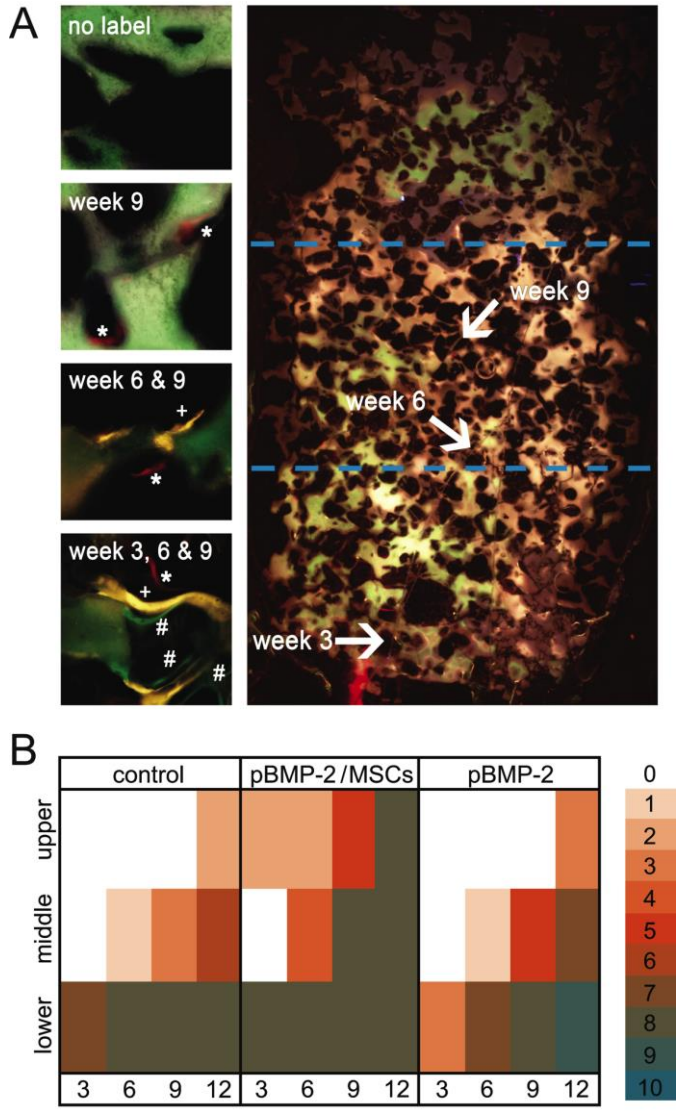

Figure 5: Dynamics of bone formation. (A) Fluorochrome incorporation at week 3 (calcein green #), week 6 (tetracycline yellow, +), and week 9 (xylenol orange, *). A representative image from pBMP-2/MSCs group is shown. **(B)** Frequency plot showing fluorochrome incorporation scored for weeks 3, 6, and 9 and bone (histology) for week 12 of implantation. Vertical axis: lower, middle, and upper third of the cylinders. The color-coding indicates the frequency of a given fluorochrome or bone (n = 10).

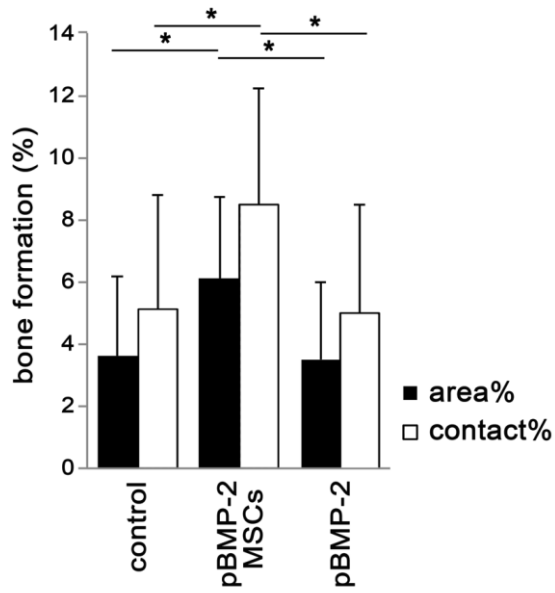

Figure 6: Bone formation expressed as bone contact% and bone area%. The results are represented as mean ± SD. Significance between the groups is indicated by * ($p < 0.05$).

In the pBMP-2/MSCs group bone formation started from the top in two out of ten cases, which was observed previously when using MSCs alone [177,179]. BMP-2 immunohistochemistry showed no significant increase in BMP-2 protein in the pBMP-2 group. Interestingly the observed BMP-2 was mainly present in osteoblasts and multinucleated giant cells, identified as osteoclasts. Osteoblasts and osteoclasts endogenously express BMP-2 [167,180], which is reflected in the staining observed in the controls and the underlying bone. We have no reliable information about the duration of gene expression in an alginate-based setting as is used here. It is expected that transgene expression takes mainly place during the early phase of implantation, which could be an explanation for the absence of significant difference of levels of BMP-2 protein found after 12 weeks. Contrary to this, BMP-2 is known to activate a positive feedback loop, which would result in enhanced BMP-2 levels long after the plasmid-induced expression has resided. At the moment it is unknown, which of these processes underlie the observed results, and this can only be answered after further in-depth investigations.

In the group where pBMP-2 was included without seeded cells, it could be that the amount of plasmid DNA has been too low over a prolonged period to allow for sufficient

transfection and transgene expression of the host cells to induce more bone formation. Plasmid DNA is known to be quickly degraded once in the circulation and by locally infiltrating cells, which is recognized by several groups using naked plasmid DNA [96,97]. Furthermore, the presence of specific target cells for gene therapy seems mandatory to allow for high transfection efficiency, transgene protein production and finally increased bone formation. MSCs are excellent target cells for gene therapy [149], but also other cell types, such as muscle cells have shown high transgene expression and ectopic bone formation locally [23,97]. When bone progenitor cells, such as MSCs, are not mandatory target cells for gene therapy, this opens doors for studying other cell types that can function as local BMP-2 producer. The current model is very suited to further optimize the conditions for endogenous cell transfection and subsequent bone induction.

CONCLUSION

The current study demonstrates a relatively easy model to investigate multiple conditions for bone regeneration simultaneously in a clinically relevant environment. The model appeared robust, has minimized the risk of neighboring effects, and it allows for easy assessment of bone formation and its dynamics because this process is restrained to a one-directional activity. Applying the model for studying combined plasmid DNA/cell-based constructs resulted in effective increase in bone formation.

Chapter 4

Porous bioprinted constructs in BMP-2 non-viral gene therapy for bone tissue engineering

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ABSTRACT

A well-known osteogenic agent in the field of regenerative medicine is bone morphogenetic protein-2 (BMP-2). Non-viral delivery of a plasmid containing the gene encoding BMP-2 has shown to induce bone formation *in vivo*. In order to develop gene activated matrices into larger constructs, we created porosity in a hydrogel using bioprinting technology, thereby allowing better diffusion and blood vessel ingrowth. We were able to produce 3D constructs that were accurate and reproducible in size, shape and pore geometry.

Constructs consisting of alginate supplemented with multipotent stromal cells (MSCs) and calcium phosphate particles were printed either in a porous or a non-porous/solid fashion. The plasmid DNA encoding BMP-2 was included in the constructs. Porous constructs were reproducibly bioprinted and remained intact for at least 14 days in culture. Cells were efficiently transfected by the plasmid DNA, and differentiated towards the osteogenic lineage as shown by elevated BMP-2 and ALP production. Porous constructs performed in the first week better in producing BMP-2 than solid constructs. However, after implantation for six weeks subcutaneously in nude mice, no bone formation was seen, which calls for optimization of the biomaterials used.

In conclusion, we show for the first time a model in which 3D printing and non-viral gene therapy can be combined.

INTRODUCTION

Autologous and allogeneic bone grafts are the most used treatment options for challenging fractures, large osseous defects and fusion of vertebrae. However, these techniques are associated with a number of drawbacks, including the limited graft material available for autografts and the high failure rate of allografts [181]. These limitations have initiated the search for tissue engineering/regenerative medicine techniques for bone repair.

Small tissue engineered bone grafts have led to good bone formation when implanted in rodents, but upscaling to clinically relevant-sized, i.e.cm-sized grafts remains challenging [182]. Methods to enhance vascularization of the tissue-engineered bone grafts in order to improve cell survival in larger cell seeded constructs are essential for the development of larger grafts [139].

Bioprinting is a 3D fiber deposition technology allowing production of cm-sized constructs. Layers of cell-laden hydrogel strands are deposited according to a computer design. The

resulting 3D scaffolds are highly accurate and reproducible in size, shape, porosity, pore-geometry, and orientation. Pores enhance oxygen/nutrient supply and waste product removal in the first days after implantation and allow blood vessel ingrowth after prolonged implantation times [113].

The printable hydrogel alginate, a seaweed-derived ion-sensitive hydrogel, is a well-suited matrix for cell encapsulation and supports viability and differentiation of embedded cells [113]. In addition, alginate supports the encapsulation of growth factors or naked DNA, such that it forms a vehicle for non-viral gene therapy [117,147].

Gene delivery using non-viral vectors is relatively new in the field of bone tissue engineering and when compared to viral gene therapy. The latter one results in high transfection efficiencies but is associated with problems such as immunogenicity, virus-dependent recombination risks and prolonged protein expression exceeding the timeframe required for bone defect healing [167,183]. Non-viral gene therapy is considered much safer and usually provides a transient expression of the desired gene. However many approaches result in a low transfection efficiencies.

In the field of bone regeneration three studies show effective gene delivery using a non-viral gene delivery method. Acetylated polyethylenimine and cationic polysaccharide complexes are used to transfect cells with plasmid DNA containing genes that give an osteogenic stimulus [105,117,184].

One of the most efficient stimuli for bone formation is bone morphogenetic protein-2 (BMP-2), which has already been used extensively in clinical applications. Concerns about the safety of the high doses of BMP-2 used in the clinic and their possible side effects have called for different ways of applying and delivering BMPs [29,185]. A slow constant release profile would match natural levels seen in for instance fracture healing. To achieve that, we have developed a non-viral plasmid based gene delivery system for BMP-2, obviating the need for expensive protein [117]. *In vitro* a sustained level of BMP-2 in the order of nanograms was produced by transfected cells in a period of up to 5 weeks. *In vivo* gene therapy efficiently stimulated bone formation to a level similar to a clinical doses of BMP-2 protein [147].

The aim of current study was to introduce porosity in DNA-based alginate constructs by applying the bioprinting technology. We investigated whether printing has added value to the gene delivery system in terms of cell survival rates and osteogenic differentiation of seeded cells, and ultimately to bone formation when implanted *in vivo*.

MATERIALS AND METHODS

Alginate gel

Autoclaved high-viscosity non-medical-grade alginate powder (International Specialty Products, ISP, Memmingen, Germany) was dissolved at different concentrations in alpha minimum essential medium (α-MEM, Gibco, Breda, The Netherlands). Gelation was achieved by immersion in 100 mM $CaCl_2$ buffer for 10 minutes at room temperature.

Cell culture

MG-63 cells (osteosarcoma cell-line, ATCC #CRL-1427; ATCC, Wesel, Germany) were cultured in expansion medium consisting of Dulbecco's modified Eagle's Medium (Gibco) supplemented with 15% (v/v) new-born calf serum (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco).

Bone marrow was aspirated from the iliac wing of adult Dutch milk goats (aged between 20-28 months). Mesenchymal stem cells (MSCs) were isolated from the bone marrow by adherence to tissue culture plastic and cultured in αMEM (Gibco), supplemented with 15% (v/v) fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Glutamax, Gibco). The cells were maintained at 37 °C and 5% CO_2 in a humidified incubator. After four to eight passages cells were cryopreserved in 1 ml aliquots of $1x10^7$ cells according to standardized protocols [186,187]. For the *in vivo* experiments MSC of one goat donor were used.

BMP-2 gene

For expression of BMP-2 the plasmid pcDNA3.1/rhBMP-2 (pBMP-2) was used. For studies on transfection efficiency pEGFP-N1 (BD Biosciences, Franklin Lakes, NJ, USA) was used. Plasmid DNA was isolated and purified using an endotoxin free kit (EndoFree Plasmid Maxi kit, Qiagen K.K., Tokyo, Japan).

Construct composition

For plasmid DNA concentration optimization, goat MSCs (10^7 / ml) were suspended in alginate hydrogel of 1% (w/v), which yielded high transfection efficiency in previous work [117], and 10 μg/ml, 1 μg/ml 100 ng/ml, 10 ng/ml or 0 ng/ml pBMP-2 was added. Hydrogel plugs (100 μl/plug) were formed and polymerized in 100 mM autoclaved $CaCl_2$ supplemented with 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4 (Gibco) for 10 minutes. Ca^{2+}-solution was then replaced by 0.5 ml culture medium. After 7 and 14 days of culture, medium and dissolved gel were analyzed by ELISA for BMP-

2 production.

To study the effect of higher plasmid DNA concentrations goat MSCs (10^7/ml) were suspended in alginate hydrogel plugs of 1% (w/v) and pEGFP-N1 at 0-50 μg/ml was added. After 7 days of culture, the cells were analyzed by FACS for GFP production and transfection efficiency as well as GFP expression levels were determined.

For alginate concentration range, 10 concentrations ranging from 10% (w/v) to 1% (w/v) of alginate in medium were prepared. Hydrogel plugs (100 μl/plug) were formed containing both 10 μg/ml pEGFP-N1 (pEGFP) and cells: goat MSCs or MG-63 both at 10^7 cells/ml. After mixing alginate with cells and pEGFP, gels were polymerized in Ca^{2+}-solution for ten minutes and were then cultured in 0.5 ml expansion medium. After five days of incubation, GFP positive cells were counted using a fluorescence microscope (E600, Nikon) and given as percentage of total cells.

Alginate hydrogel plugs (1% w/v) containing 10 μg/ml pEGFP and 10^7 goat MSCs/ml were formed and incubated for 10 days. Plugs were dissolved using citrate buffer (150 mM NaCl, 55 mM sodium citrate and 20 mM EDTA in H_2O) for 15 min at 37°C. Cells were pelleted, resuspended in PBS, and analyzed for green fluorescence by FACS Calibur.

Pre-cross-linked alginate gel for bioprinting: By precrosslinking alginate with solution a bioprintable hydrogel was formed. The constructs contained 3% alginate (w/v), 25 mM Ca^{2+},10^7 gMSC/ml, 10 μg/ml pBMP-2 and 5 % (w/v) BCP particles (size 106-212 μm). BCP particles consisted of 80% ± 5% (w/v) hydroxyapatite and 20 ± 5% (w/v) β-tricalcium phosphate, and total porosity was 70 ± 5%, macroporosity 55 ± 5% and microporosity 20 ± 5% (Xpand biotechnology, Bilthoven). The BCP particles were cleaned in an ultrasonic bath and sterilized by autoclave and finally suspended at 5% (w/v).

Bioprinting presets

Constructs were produced using the 3D fiber deposition technique using the BioScaffolder system (SysEng, Hünxe, Germany) under sterile conditions. The constructs were printed with an 18 gauge (0.84 mm inner diameter) conical printer head as described before [182]. Construct contents are described below. Printer settings summarized in short: For the *in vitro* part: porous and solid constructs consisted of 10 successive layers (dimensions 20 x 20 x 5 mm) either with 16 vertical pores with a strand distance of 3.0 mm and a strand thickness of 1.0 mm or printed without pores with a strand distance of 1 mm. For the *in vivo* part: porous and solid constructs consisted of 10 successive layers (dimensions 10 x 10 x 5 mm) either with 4 vertical pores with a strand distance of 3.0 mm and a strand thickness of 1.0 mm or printed without pores with a strand distance of 1 mm.

Construct properties

Mechanical characterization

After polymerization, bioprinted constructs were mechanically analyzed using a DMA 2980 dynamic analyzer (TA instruments, New Castle, DE, USA) in controlled force mode. Scaffolds were placed between the parallel plates and a static force was applied. For alginate, this force increased from 0 to 1 N at a rate of 0.2 N min^{-1}. Young's modulus (E) was determined by measuring the variation of the apparent stress/strain ratio, as described previously [188].

Swelling and degradation

Printed gels were weighed and either directly freezedried or placed in 5 ml 50 mM phosphate buffer (pH 7.4), and incubated at 37 °C. The gels remained in the buffer solution 1, 3, 7 or 14 days. At each time point samples were weighed and freezedried to measure dry weight. Five samples of each group per time point were printed and analyzed.

Bioprinted groups

For the *in vitro* and *in vivo* experiments all constructs consisted of alginate hydrogel (10 mg/ml), 10^7 goat MSCs/ml and BCP particles. Groups were as follows: 1. porous control constructs without pBMP-2, 2. porous with pBMP-2 and 3. solid constructs with pBMP-2. After printing the gel was fully polymerized by immersion in Ca^{2+}-solution for 10 min and then implanted in separate subcutaneous dorsal pockets in mice or cultured in expansion medium for 7 and 14 days.

Analysis of cultured printed constructs

BMP-2 protein production was analyzed by ELISA in medium and gel. The collected medium was stored at -20°C. Gel constructs were divided in specific parts and separately analyzed.

A quarter of the gel was used for viability analysis using a LIVE/DEAD assay according to the manufacturer's recommendations (Invitrogen, L3224, USA). Living and total cell numbers were scored using a fluorescence microscope. The cell viability was calculated as the average ratio of vital over total cells in a sample, determined from four randomly chosen fields per sample. The rest of the gel was depolymerized with citrate buffer for 15 minutes at 37°C. Cells were centrifuged and the supernatant containing the dissolved protein was stored at -20°C. The ELISA (Quantikine #DBP200 from R&D Systems) was performed on thawed samples following the standard protocol.

A part of the cell pellets was resuspended in lysisbuffer (PBS containing 0.1% triton-X 100) for 30 minutes. As one of the early differentiation markers, alkaline phosphatase

(ALP) activity was determined via colorimetric assay kit (Abcam plc, Cambridge, UK) by using p-nitrophenyl phosphate (pNPP) as a substrate. The assays were performed in triplicate and the enzyme activity was expressed as U/ml, normalized for DNA content. DNA analysis was performed using Picogreen kit (Quant-iT™ PicoGreen dsDNA Reagent Kit, Invitrogen, USA).

A fraction of the cells was used to make cytospins, which were (immuno) stained for osteocalcin and ALP. The activity of alkaline phosphatase was determined by 60-minute staining with the Fuchsin Substrate-Chromogen system (K0624, Dako, Carpinteria, USA). The sections were counterstained with Weigert's hematoxylin, and mounted with Aquatex. The presence of alkaline phosphatase-positive cells was analyzed with a light microscope equipped with an Olympus DP70 camera. For osteocalcin immunohistochemistry, samples were permeabilised with Triton X-100 and blocked in 5% (w/v) BSA for 5 min, then incubated with 5 μg/ml anti-osteocalcin antibody (TAKARA, Piscataway, NJ, USA, A00613) in Tris-buffered saline supplemented with bovine serum albumin (TBS/BSA 1%) for 1 hour and as secondary antibody 3.3 μg/ml Streptavidin/ horse radish peroxidase (HRP) was used. The stainings were developed with diaminobenzidine (DAB) and Mayer's hematoxylin was used for counterstaining. After a dehydration protocol, sections were mounted in Depex.

Animals and implantation

Female nude mice (Hsd-cpb:NMRI-nu, Harlan) were anaesthetized with 1.5% isoflurane, after which the three implants were placed in separate subcutaneous dorsal pockets per mouse (n=6). A porous construct with pBMP-2, a porous control construct without pBMP-2, and a solid control construct with pBMP-2 were printed and implanted the same day.

The animals were postoperatively treated with the analgesic buprenorphine (0.05 mg/kg, sc; Temgesic, Schering-Plough) and housed together at the Central Laboratory Animal Institute, Utrecht University. At 3 weeks after implantation calcein green was administered s.c. (10 mg/kg, Sigma, Zwijndrecht, Netherlands). At 4 weeks after implantation xylenol orange was administered s.c. (30 mg/kg, Merck, Amsterdam, Netherlands) [189].

The mice were terminated after 6 weeks and the constructs were retrieved. Experiments were conducted with the permission of the local Ethical Committee for Animal Experimentation and in compliance with the Institutional Guidelines on the use of laboratory animals.

Explantation and embedding

Six weeks after implantation the constructs were retrieved and cut in half to analyze general morphology, transfection and bone histomorphometry. One half was fixed overnight in 4% (v/v) formalin containing 100 mM $CaCl_2$ and processed for 5 μM thick decalcified paraffin sections through alcohol dehydration series. The other half was fixed in 4% formalin, dehydrated by ethanol series and embedded in polymethylmethacrylate (MMA). From this half 30 μm thick sections were cut from the center and side of each implant using a sawing microtome (Leica, Nussloch, Germany). Half of the MMA sections were stained with basic fuchsine/methylene blue and half remained unstained for fluorochrome analysis.

Histology analysis of osteogenic differentiation

General histological staining was performed with hematoxylin and eosin (HE) and Goldner's trichrome. Presence of collagen type I was assessed by immunocytochemistry. For this, the paraffin sections were rehydrated and incubated in 0.3% (v/v) H_2O_2 in TBS for ten minutes followed by boiling in citrate buffer for 20 minutes as antigen retrieval. The sections were then blocked in 5% (w/v) BSA in TBS for 30 min and incubated overnight at 4°C with mouse anti-collagen type I antibody (20 μg/ml in 5% (w/v) BSA in TBS, clone I-8H5, Calbiochem, Darmstadt, Germany). A biotinylated secondary antibody was applied (5 μg/ml in 5% (w/v) BSA in TBS, biotinylated sheep anti-mouse, RPN1001V1, GE Healthcare, Diegem, Belgium) for one hour and the staining was enhanced by incubation with streptavidin-peroxidase for an additional hours (2 μg/ml, PN IM0309, Immunotec, Montreal, Canada). The staining was developed with DAB and counterstained with Mayer's hematoxylin.

Statistics

The statistical significance of differences between experimental groups in Figures 1, 3, 4 and 5 was assessed using two-factor ANOVA and Tukey post hoc test. Data are represented as mean ± standard deviation. P values < 0.05 are considered statistically significant.

RESULTS AND DISCUSSION

DNA dose determination

In order to determine the optimal amount of pBMP-2 needed for the highest BMP-2 protein production, four concentrations of pBMP-2 were tested, ranging from 0-10 μg/ml. After 14 days, the BMP-2 production, as determined by ELISA, was measured (Fig. 1). BMP-2 production was the highest in the plugs with 10 μg/ml pBMP-2 resulting in a BMP-2 protein production of 8.4 ng/ml. Lower amounts of pBMP-2 resulted in lower BMP protein production. In an additional experiment transfection efficiency with higher concentrations of pDNA was determined. Increasing the plasmid concentration to 50 μg/ml did not result in a higher transfection efficiency nor in higher expression levels per cell of the tracer gene GFP (results not shown). Therefore in following experiments 10 μg/ml pBMP-2 was used (Fig. 1).

For every cell large amounts of pBMP-2 are available, which increases the chance on internalization of pBMP-2. Other authors report optimal doses of pDNA within the same range as the higher dosages tested [100,148,184].

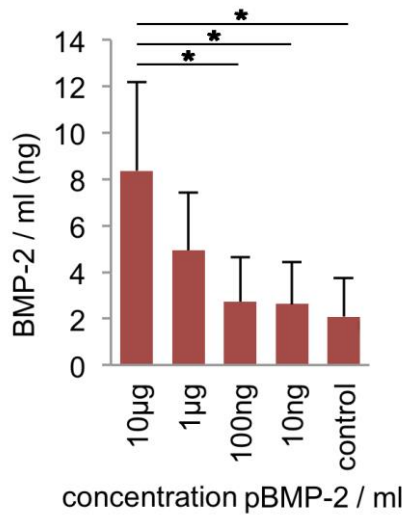

Figure 1: rhBMP-2 release after 14 days from transfected gMSCs in alginate. BMP-2 release from alginate constructs containing 10^7 cells/ml and pBMP-2 at the indicated concentrations. BMP-2 release was analyzed in two independent experiments in duplicate in the medium and in the gel separately by ELISA. The results represent mean ± SD, * indicates a significant difference ($p < 0.05$).

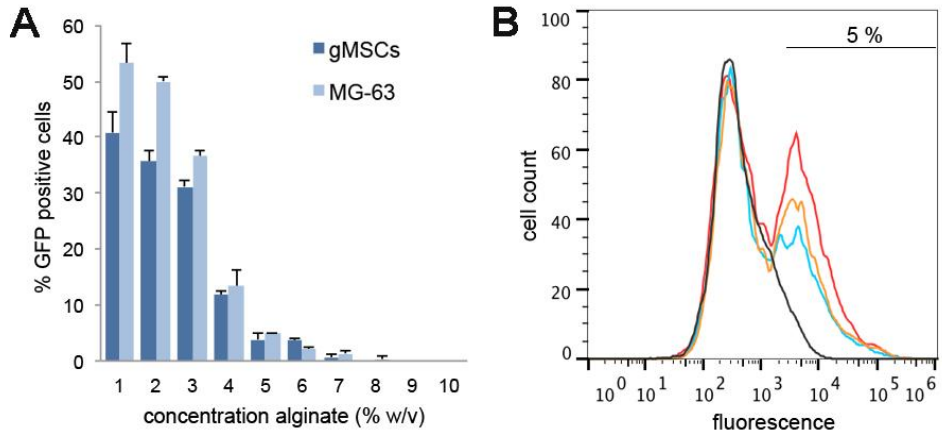

Figure 2: Transfection efficiency with pEGFP as a function of alginate concentration and determined by flow cytometry. A) Green fluorescence was analyzed after 10 days in two independent experiments containing either MSCs or MG-63 cells and 10 μg/ml pEGFP. Fluorescent cells were counted under a fluorescence microscope and compared to the number of non-transfected cells using bright field microscopy. In three different areas/well the transfection efficiency was determined. The results represent mean ± SD. B) Green fluorescence was analyzed 10 days after transfection of MSCs with 10 μg/ml pEGFP. Gels were dissolved in citrate buffer and cells were analyzed by FACS. Control constructs were left untransfected. The horizontal black region excludes 95% of the control cells. The three curves represent 3 donors (blue 32% transfected cells, yellow 38%, and red 46%).

Alginate concentration

The concentration of alginate present in the final construct is of great influence on the printing, handling and degradation properties of the constructs. At high concentrations of alginate hydrogel printability increases, whereas cell viability and function decreases [64].

By measuring transfection efficiency in different concentrations of alginate we aimed to find optimal conditions for both printing and gene therapy. Transfection efficiency was determined using pEGFP-N1 in ten different concentrations of alginate. A low alginate concentration resulted in the highest transfection efficiency. For 1% (w/v) alginate hydrogel the transfection was 40.8% for 2% (w/v) gel 35.7% and for 3% (w/v) gel 31.2% with higher concentrations of alginate transfection efficiency decreased to 11.8% for 4% (w/v) gel and <4% for higher concentrations of alginate (Fig. 2A).

In a second experiment results were confirmed by flow cytometry, resulting in transfections of 32%, 38%, and 46% in 1% alginate hydrogel (Fig. 2B). When comparing to other non-viral gene therapies, this is an efficient transfection mechanism. Hoelters et al. transfected human MSC (hMSC) with pEGFP using Lipofectamine™2000 and showed a 50% fraction of fluorescent cells [190]. Other naturally derived hydrogels suitable for gene

therapy, chitosan-DNA complexes have been applied on human MSCs, always leading to slightly lower transfection efficiencies than the Lipofectamine™2000 control [191,192]. From these results we conclude that optimal dose is 10 μg/ml. A higher dose has no negative influence.

Printability was assessed for all concentrations of alginate hydrogel. With low alginate concentrations (<3%), the hydrogels were too liquid and pores diminished after printing. To overcome the need for high concentrations, alginate hydrogel was pre-cross-linked before printing and subsequently directly fully cross-linked after printing. With this technique alginate was printable at a concentration of 3% (w/v) (see also Fig. 4).

We conclude from these experiments that transfection efficiency is relatively high when low concentrations of alginate (1, 2 and 3%) are used, rapidly decreasing with higher concentrations. Since alginate <3% was suboptimal for printing, 3% alginate was chosen.

Mechanical characteristics

Degradation & swelling

Porous and solid constructs swelled up to respectively 182% and 170% of the initial weight after 14 days (Fig. 3A). Dry mass measurements indicating degradation of the constructs resulted in dry masses of 89% and 91% at day 14 for respectively porous and solid constructs (Fig. 3B). Compressive modulus of the porous and solid constructs were 5.6±0.36 and 7.3±0.49 respectively (Fig. 3C).

3D printing

Defined 10-layer alginate constructs (20 x 20 x 5 mm; Fig. 4B) were bioprinted by fiber deposition technology. Vertical pores were regular throughout the samples. The weight of the porous constructs measured 60±7% (n=6) of the solid grafts, resulting in average porosity of 40%. Cell viability of the printed constructs assessed by live/dead assay after 7 and 14 day of culture showed respectively for day 7 and 14: 90±6% and 79±5% for the porous control: 91±4% and 80±12% for the porous with pBMP-2; and 85±4% and 69±9% for the solid constructs (Fig. 4).

Protein production in bioprinted constructs

BMP-2 protein was produced by cells in the printed scaffolds respectively at day 7 and 14 in ng/ml: 1.3±0.7 and 1.6±1.3 for the porous control, 6.5±1.2 and 7.2±1.1 for the porous with pBMP-2, and 1.7±1.6 and 4.3±2.4 for the solid constructs (Fig. 5). A significant difference in protein production is observed between the porous constructs with pBMP-2 and the solid constructs ($p<0.05$) on day 7, which is not significantly different on day 14. The porous control groups produce significantly less protein than the porous constructs

containing pBMP-2 on both time points ($p<0.05$).

From these results we conclude that introducing porosity promotes a higher BMP-2 protein production in the first week in culture. This could be caused by a better cell functionality due to better nutrient supply and waste removal due to shorter diffusion distances. After 14 days also the solid constructs start producing physiological levels of BMP-2, which is in accordance with our previous findings [193]

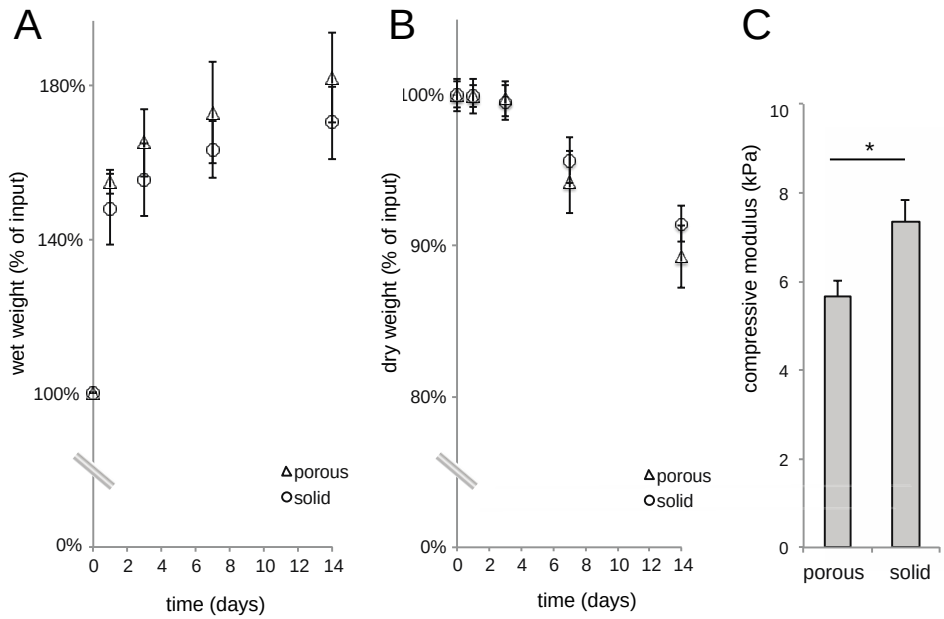

Figure 3: Swelling and degradation characteristics of bioprinted constructs. A) Hydrogel swelling over 14 days. Shown as the increased percentage of wet weight compared to input. B) Hydrogel degradation over 14 days. Represented as a percent dry mass compared to initial dry mass. C) Mechanical properties of printed constructs. Compressive modulus is shown for the porous and solid constructs. Data are presented as mean ± standard deviation (n=5); * indicates $p < 0.05$.

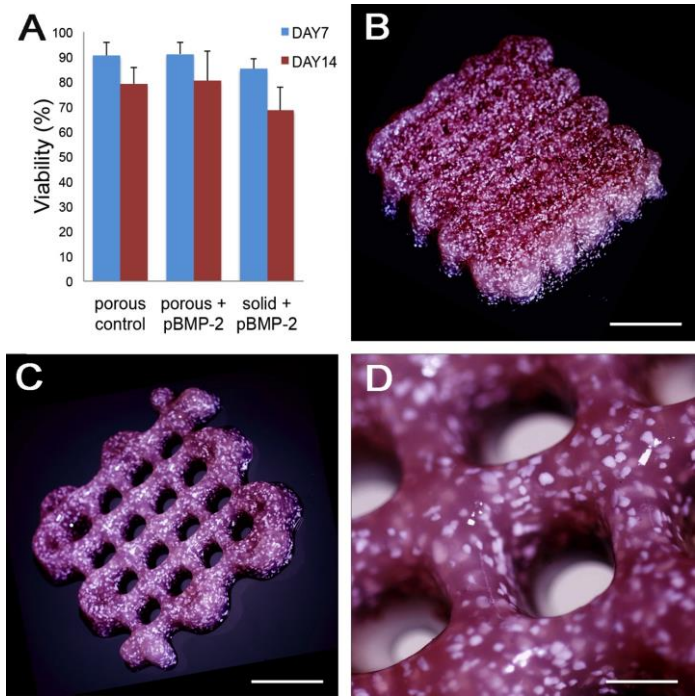

Figure 4: Bioprinted constructs containing MSCs, ceramic particles with/without plasmid DNA encoding BMP-2. A) Viability of MSC in bioprinted constructs after 7 and 14 days, determined by Live/dead staining. The results represent mean ± SD. No significant differences. B) a solid construct and C) a porous construct directly after printing and polymerization. Porous constructs with and without DNA have the same macroscopic appearance (C). Scale bars B and C: 500 μm. D) A close up of a porous construct. Scale bar: 100 μm.

Bioactivity of produced BMP-2 in the bioprinted constructs

In order to determine the bioactivity of the BMP-2 produced in printed gene-activated constructs, alkaline phosphatase (ALP) activity and osteocalcin (OCN) expression were measured as markers for osteogenic differentiation. After 14 days of culture ALP activity was significantly higher in both groups containing pBMP-2, although there was no difference between the porous and the solid groups containing pBMP-2 (Fig. 6A). As we measured a single time-point, it is not known whether for each group the peak activity of ALP was reached. It was shown before that during osteogenic differentiation with dexamethasone on human MSCs ALP production peaks around day 10 to 14, where after levels decrease slightly [194].

Osteocalcin expression was seen on fractions of the bioprinted MSCs alginate scaffolds. gMSCs encapsulated in porous constructs exhibited a higher degree of early osteogenic marker expression than cells in solid scaffolds, measuring around 70% and 50% OCN-

positive cells, respectively. In the porous control samples only a small amount of cells <2% was positive (Fig. 6 B-D). Considering the later osteogenic marker osteocalcin, which is only produced by osteoblasts or osteocytes, we observed much more pronounced differences between the groups.

From these results we conclude that porosity led to a better cell functionality, stressing the importance of scaffold porosity.

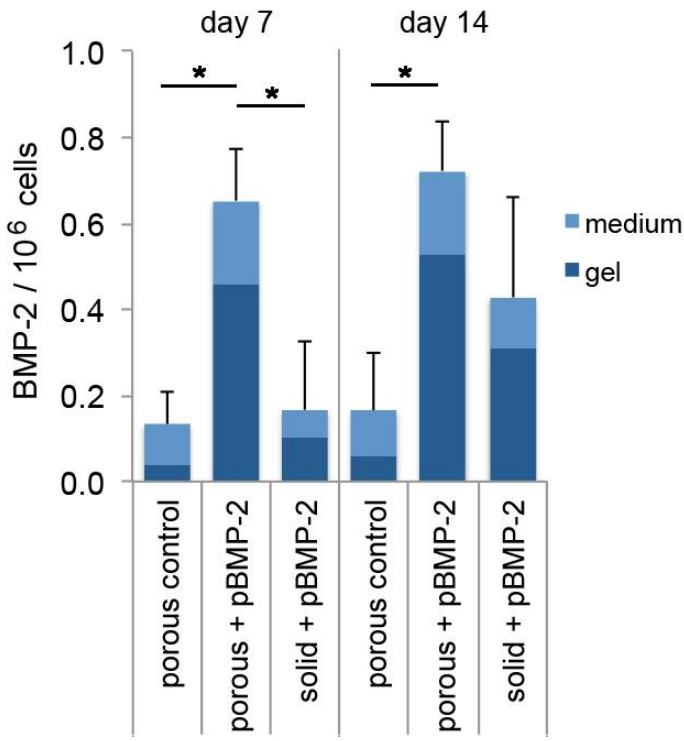

Figure 5: BMP-2 production in medium and gel after 7 and 14 days. BMP-2 release from bioprinted alginate constructs. Media were stored at each medium change, and the constructs were dissolved in citrate buffer at the end of the experiment. BMP-2 concentrations were measured in medium and gel separately by ELISA (n=3 for gel and medium). The results represent mean ± SD, * indicates a significant difference ($p < 0.05$).

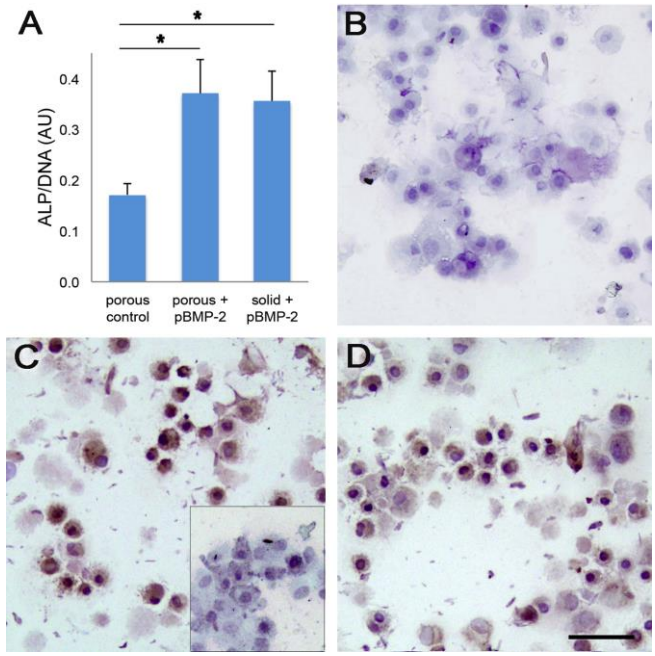

Figure 6. Osteogenic differentiation in bioprinted constructs. A) Alkaline phosphatase (ALP) activity after 14 days *in vitro* in printed constructs containing MSCs, normalized for DNA-content of the sample (n=3). The results represent mean ± SD, * indicates a significant difference ($p < 0.05$). B-D) Osteocalcin immunocytochemistry on cytospins of cells after dissolution of the construct, B) Porous control, C) Porous with pBMP-2 (70% positive cells), Inset: isotype-matched control antibody staining on section from C, D) Solid with pBMP-2 (50% positive cells). Scale bar: 100 µm.

	Integrity of construct	Bone formed	Collagen I presence	Alginate remnants	Blood vessel density	Fibrous capsule formation	Cellularity	Flurochrome incorporation
Porous control	+	-	+	+/-	+	-	+	-
Porous with pBMP-2	+	-	+	-	++	-	+	-
Solid with pBMP-2	++	-	-	++	++	+	++	-

Table 1: Performance of bioprinted constructs after 6 weeks implantation *in vivo*. Several construct-related parameters were scored, as described in M&M. Scorings were performed by two independent observers on blinded sections, n=6 per group.

Tissue development *in vivo*

To investigate whether transfection in printed constructs *in vivo* would take place and is able to induce osteogenic differentiation, constructs were implanted in subcutaneous pockets in immunodeficient mice. Analysis of the histological sections after an implantation period of 6 weeks showed matrix deposition around the ceramic particles in the porous constructs with or without pBMP-2, which was not seen in the solid constructs (Fig. 7 A-F). The solid constructs contained large areas of alginate remnants (Fig. 7) and some of the solid constructs were partially encapsulated (see also Table 1).

Ingrown tissue exhibited a certain level of maturation in time showing development of fibrous tissue and blood vessels, as evidenced by the presence of erythrocyte-filled vessels (Fig. 7 E and F).

Immunohistochemistry on collagen type I was performed to determine whether the expressed levels of BMP-2 are sufficient to induce osteogenic differentiation (Fig. 7 G-I). Abundant collagen formation was seen in all constructs. Collagen I deposition in the porous scaffolds was mostly seen around the ceramic particles, whereas in the solid scaffolds it was mainly seen between the alginate remnants (Fig. 7 G-I). The implants also contained connective tissue as shown in green by a Goldner's trichrome staining but no highly cellularized tissue like osteoid, which would be indicated in red (Fig. 7 D-F).

To monitor alginate morphology changes, blood vessel formation and tissue response, HE stained sections were scored on several features (see Table 1). It appears that solid constructs had partially degraded, leaving remnants alginate material, surrounded by highly vascularized tissue. The porous constructs had almost completely degraded, leaving only minor traces of alginate after 6 weeks. The degradation rate likely correlates to access of surrounding tissue and subsequent cellular ingrowth. A faster degradation would imply a faster and more complete release of DNA and also an increase in cell-produced BMP-2. These factors would lead to a higher availability of BMP-2 to possible target cells and therefore a faster onset of osteogenic differentiation in the porous constructs, which is substantiated by the increase in collagen deposition around the BCP particles.

Whereas we found efficient bone formation of gene activated alginate at 6 weeks in previous experiments [147], in this particular setting the bone formation was not (yet) apparent. This might be explained by the timing of the BMP-2 release, which is expected to be slower due to the higher alginate concentration used for the bioprinting (3% versus 1% in previous work [117,147]. Also, other studies report an higher availability of BMP-2 protein after gene-expression at early time points than our results show [195]. The *in vitro* production at day 7 is still relatively low, but we expect the *in vivo* production to be faster due to hydrogel dissolution. Apart from the effect of alginate concentration on transfection

efficiency, the high concentration as such (3% w/v) might have a negative effect on bone formation [112,195].

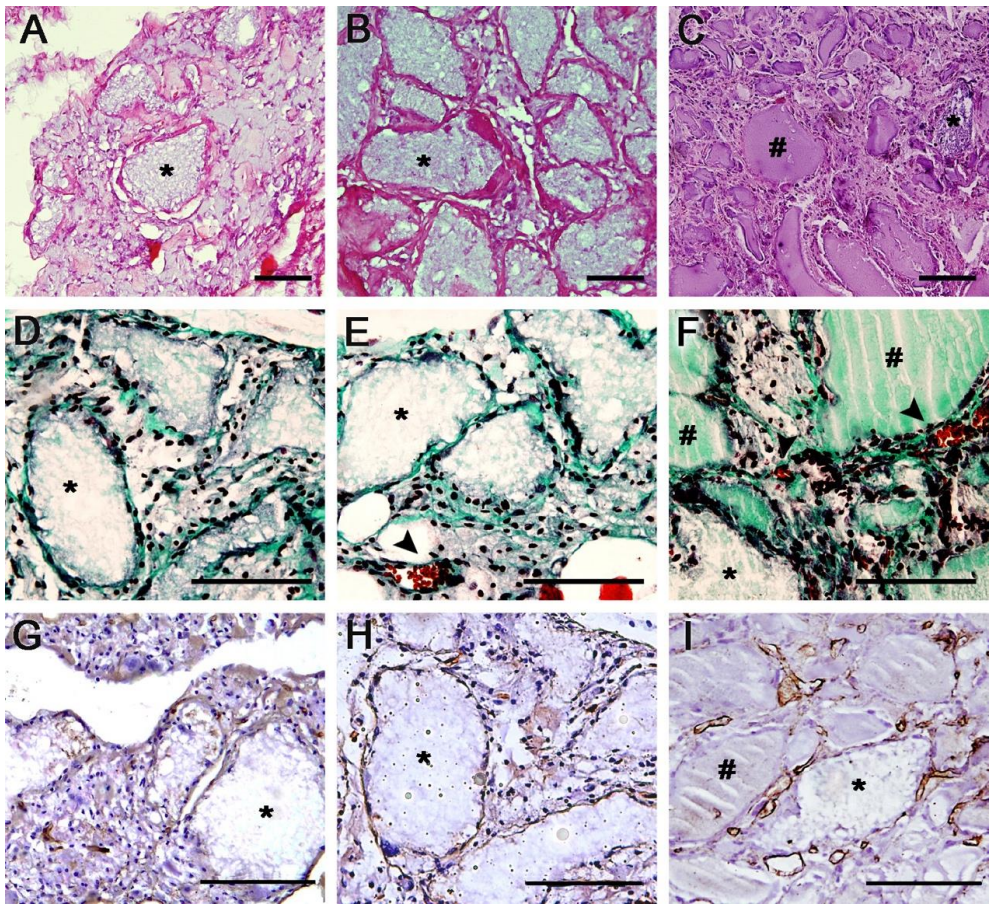

Figure 7: *In vivo* performance of bioprinted constructs containing pBMP-2. After 6 weeks of implantation subcutaneously in mice, histological sections were prepared as described in M&M. A,D,G) porous control samples; B,E,H) porous with pBMP-2 and C,F, I) solid with BMP-2. A,B,C) H/E staining, D,E,F) Goldner's trichrome staining, G,H,I) Collagen I immunohistochemistry, collagen I in brown, counterstained with hematoxylin (blue). # indicates undissolved alginate hydrogel, * are BCP particles, arrows indicate blood vessels. Representative pictures for each group (n=6) are shown. Scale bars: A-C 100 µm, D-I, a larger magnification, scale bars: 100 µm.

CONCLUSION

The present study used printed pores in TE constructs as an effective strategy to increase gene therapy efficiency and osteogenic differentiation of embedded cells. We showed that 3D bioprinted alginate hydrogel constructs support and enhance the efficiency of non-viral gene therapy for BMP-2.

The combined application of gene delivery and bioprinting is feasible; the gene therapy delivering human BMP-2 induces osteogenic differentiation in bioprinted constructs, as seen by induced ALP activity and osteocalcin expression. Introduction of porosity further promotes BMP-2 production and subsequent osteogenic lineage commitment.

In vivo matrix deposition is seen around ceramic particles although no bone formation could be observed after *in vivo* implantation of the bioprinted constructs in mice.

Chapter 5

Bone formation by hetero-dimers through non-viral gene delivery of BMP-2/6 and BMP-2/7

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ABSTRACT

Non-viral gene delivery is a safe technique that can result in a sustained release of physiologic dosages of BMP. Co-delivery of multiple BMPs can result in the formation BMP heterodimers which are more potent. In this study, we assessed non-viral co-delivery of BMP-2/6 and BMP-2/7 as a means to produce heterodimers.

Goat MSCs were non-virally transfected with plasmid DNA encoding BMP isoforms (pBMP), known to be relevant for osteogenesis: BMP-2, -6 or -7. Combined delivery of pBMP-2 with pBMP-6 or pBMP-7 was used as a means to create BMP2/6 and BMP2/7 heterodimers. As a result of transfection, BMP-2, -6, and -7 were produced and were detectible up to 14 days. Formation and secretion of heterodimer proteins in the pBMP2/6 and pBMP2/7 conditions was shown by sandwich ELISAs. Produced BMPs and heterodimers were biologically active as concluded from induced differentiation of reporter cells and MSCs. To assess bone formation, transfected MSCs were seeded on ceramic scaffolds and implanted subcutaneously in nude mice. Bone formation was significantly enhanced in the pBMP-2/6 condition and a trend for more bone was observed in the pBMP-2/7 and pBMP-6 homodimer condition. No bone was found in the conditions with pBMP-2, pBMP-7 or in the control condition.

In conclusion, simultaneous delivery of pBMP-2 with pBMP-6 or -7 resulted in the production of heterodimers, which were beneficial for bone formation as compared to BMP homodimers. Combination of BMP sequences can reduce the need for high BMP protein dosages and may enhance prolonged availability of the growth factors.

INTRODUCTION

Autologous bone grafting is frequently performed in cases of non-union and in cases of spinal fusion surgery. Although autologous bone is the best grafting material, only limited amounts are available and harvesting is associated with local morbidity [161]. Therefore, other strategies to induce bone formation and regeneration are being explored. These strategies focus on delivering the different components involved in bone regeneration such as (pro)osteogenic cells, calcium phosphate-based scaffold materials and growth factors.

Multipotent stromal cell (MSC) based approaches to repair bone tissue have shown to be effective in small and large animal models [46]. However, the exact role of MSCs is not fully elucidated as the few cell-tracing studies available show contradicting results with respect to long-term engraftment, albeit a cell-dependent performance of the implants is often apparent [59,178]. This has led to the theory that at least part of the pro-osteogenic effect of MSCs lies in the temporary excretion of factors beneficial to bone regeneration [49,58]. This property may be utilized even further by genetically modifying seeded cells to overexpress pro-osteogenic proteins [83].

Interesting candidates are bone morphogenetic proteins (BMPs) isotypes, as they have shown to play an important role in bone formation [23]. To date, at least 20 isotypes of BMPs have been identified in mammals, among which BMP-2 and BMP-7 are best known and clinically approved for certain clinical applications [196,197]. Other BMPs have also shown potential for bone regeneration [23], such as BMP-6, which is related to both chondrogenesis and osteogenesis [198,199]. Besides these well known homodimeric BMPs, there is evidence for the existence of BMP heterodimers [33,34]. Heterodimers such as BMP-2/6 [35] and BMP-2/7 [39] have been produced *in vitro*. Compared to their homodimeric counterparts heterodimers showed increased activity [36-39].

Despite their high potency to induce bone, BMP based therapies have been hampered by short protein half-life and quick protein degradation *in vivo* [74,75]. Non-viral delivery of BMP-encoding sequences to cells/tissue could overcome this drawback, as this strategy ensures a transient, sustained protein release. Furthermore, non-viral methods are less immunogenic and safer than viral approaches [200]. The major drawback of non-viral methods however, is the low transfection efficiencies seen *in vivo* [96]. This drawback could be omitted by combining gene delivery with cell based regeneration: donor cells, harvested during surgery [134] or received from a donor bank [201] can be efficiently transfected *ex vivo* to overexpress BMP before implantation [202]. Co-delivery of multiple BMPs can enhance the potency of cell based gene delivery strategies even more [38,203,204]. Nucleofection, a method of electro-permeabilization, is an efficient physical method of delivering plasmid DNA into both primary cells and established cell lines. The high transfection efficiencies observed

with nucleofection are related with prolonged expression and protein production of osteogenic factors, and result in bone formation *in vivo* [126,128].

In this study we assessed *ex vivo* non-viral gene delivery of BMP-2/6 and BMP-2/7 to MSCs as a means to produce heterodimers. Subsequently, the capacity to induce osteogeneic differentiation and bone formation was assessed and compared to gene delivery of single BMP isotypes.

MATERIALS AND METHODS

Isolation and culture of goat MSCs

After approval of the local animal care committee, bone marrow was aspirated from adult female Dutch milk goats aged 24–27 months, 50– 60 kg. MSCs were isolated from the bone marrow by adherence to tissue culture plastic and cultured in normal expansion medium, consisting of α-MEM (Gibco), 10% (v/v) fetal bovine serum (FCS; Hyclone), 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine (Glutamax; Gibco). The MSCs were maintained at 37°C and 5% CO_2 in a humidified incubator. The osteogenic, chondrogenic and adipogenic differentiation potential of the MSCs was determined using appropriate media, as detailed in previous work [177]. MSCs were cryopreserved and used between passage nr 4-6. MSCs from different goat donors were used for the *in vitro* experiments. For the *in vivo* experiment cells from a single donor were used for transplantation in nude mice. Goat MSCs were chosen because they are well characterized and can be applied in future translational goat studies. Previously these cells were characterized for their osteogenic differentiation capacity *in vitro* and cryopreserved according to established protocol [205].

Vector composition

Vectors used were pEGFP-N1 (BD Biosciences, Franklin Lakes, NJ, USA), and vector pVAX1 (Invitrogen) as such or subcloned to contain full-length rhBMP-2 (using HindIII and BamHI restriction sites) or rhBMP-7 (using HindIII and NotI restriction sites); the resulting products were designated pBMP-2 and pBMP-7 in the experimental set-up. pcDNA3.1 (Invitrogen) containing the mouse BMP-6 cDNA (NM_007556) insert using the HindIII and XhoI restriction sites was kindly provided by Prof. Peter ten Dijke, Leiden University Medical Center, Leiden The Netherlands). This construct is referred to as pBMP-6. Expression of each of the BMP isoforms was driven by the CMV promoter. The murine BMP-6 sequence is highly similar to the human isoform with only two amino acids difference in the seven-cysteine region and has five differences in the basic amino-terminal domain; amino acid identity overall is 91%. All plasmid DNA constructs were confirmed by

sequencing. Plasmid DNA was isolated, purified and cleared from endotoxins (EndoFree Plasmid Maxi kit, Qiagen K.K., Tokyo, Japan) prior to cell and animal studies.

Gene delivery by nucleofection

With the aid of Amaxa Nucleofector™ technology (Lonza ltd, Basel, Switzerland), MSCs were transfected with the different vectors, according to the manufacturer's instructions. Immediately after electroporation, the cells were transferred to tissue culture plates, containing expansion medium supplemented with 20% FBS. Nucleofected MSCs were harvested by adding trypsin–EDTA at least 24 h after gene transfer. All the living and dead cells in the culture medium, washing steps, and trypsin steps were collected and analysed during the optimization period. Viability and transfection efficiency were assessed by staining with 10 μg/ml of 7-AAD (Sigma-Aldrich, Germany) in PBS on ice for 30 minutes and directly analysed for the expression of EGFP by using Gallios Flow Cytometer (Beckman Coulter, Inc., Germany). Using FlowJo software (FlowJo Inc., Oregon, USA), the percentage GFP positive cells within the gated living cells and their mean fluorescence intensity were determined.

The different parameters of the nucleofection protocol were optimized for the goat MSCs using the pEGFP-N1 vector. Six different buffers were compared, as follows: Buffer 1 [5 mM KCl, 15 mM $MgCl_2$, 120 mM Na_2HPO_4/NaH_2PO_4 (pH 7.2), 50 mM NaCl]; Buffer 2 [5 mM KCl, 15 mM $MgCl_2$, 15 mM HEPES, 150 mM Na_2HPO_4/NaH_2PO_4 (pH 7.2), NaCl 50 mM]; Buffer 3 [5 mM KCl, 15 mM $MgCl_2$, 15 mM HEPES, 90 mM NaCl, 10 mM Glucose, 0.4 mM $Ca(NO_3)_2$, 40 mM Na_2HPO_4/NaH_2PO_4 (pH 7.2)]; Buffer 4 [OptiMEM]; Buffer 5 [The Nucleofector™ solution / OptiMEM (1:1 v/v)]; Buffer 6 [The Nucleofector solution]. Subsequently six different nucleofection programs were compared (G22, U28, U23, T30, X01, X05), which were recommended in the preliminary protocol provided by Amaxa and were previously used in literature for MSCs. Protocols differ basically in intensity of the electric pulse (field strength) and pulse length. The concentration of plasmid DNA was optimized using 2, 5, 10, 15, 20 and 40 μg of pDNA per reaction (in a total volume of 100 μl). Cell density was varied from 0.5, 1, 2, 4, and 8 x 10^6 cells per reaction.

In vitro BMP protein production

MSCs ($2x10^6$) were transfected with pBMP-2, pBMP-6, pBMP-7, or combinations thereof (pBMP 2/6 and 2/7 each at 1:1 ratio of the respective vectors) and empty vector (total amount of 20 μg of pDNA per reaction) according to the nucleofection protocol, using buffer 1 and program G22. Thereafter, cells were kept for 24 hours in expansion medium with 20% FBS, then medium was replaced with normal expansion medium and maintained up to two weeks. Medium samples from the nucleofected MSCs were collected at day 1, 2, 4, 7 and 14. Production of BMP-2, 6, 7 homodimers and BMP-2/6 and -2/7 heterodimers was assessed via commercially available ELISA kits (BMP-2: PeproTech Ltd. London, UK; BMP-6 and 7: Duo-Set ELISA Development kit, R&D Systems, Inc., Minneapolis, USA). All ELISAs detect both human and mouse BMP, and show high cross reactivity between species, such as goat BMP, due to highly conserved BMP amino acid sequence. Endogenous BMP production by the goat MSCs is therefore detected, but may be an underestimation due to possible lesser activity.

In vitro heterodimer experiment

To assess the presence of BMP-2/6 and -2/7 heterodimers, a sandwich ELISA was performed using the BMP-6 or -7 capture antibody and BMP-2 detection antibody. Medium samples of MSCs which were transfected with pBMP-2, -6, -7, -2/6, -2/7 or empty vector were collected two days after transfection and added to the ELISA plates coated with capture antibody coated in duplicate wells. Calibration curves were made with recombinant BMP-2/6 and -2/7 (R&D Systems Inc., Minneapolis, USA). Duplicate control samples with 4 ng/ml recombinant BMP-2, -6, -7 (R&D Systems Inc., Minneapolis, USA) were used to establish possible cross-reactivity of the antibodies. Subsequently BMP-2 detection antibodies were used for development of the ELISAs.

Bioactivity assay of secreted transgene products

The bioactivity of the secreted BMPs by transfected MSCs with BMP-2, -6, -7, -2/6, -2/7 and empty vector were determined using mouse chondrogenic ATDC-5 reporter cells, which express ALP upon exposure to a variety of BMPs[206]. Medium from different conditions of MSCs transfected with BMPs or control vector was harvested 48 hours after nucleofection and was added to subconfluent ATDC-5 cells in duplicate. Parallel positive controls were included consisting of a range of increasing concentrations (0 to 250 ng/ml) of rhBMP-2, 6 and 7 protein. Three days after the addition of the conditioned medium or fixed amounts of rhBMP protein, cells were lysed in PBS containing 0.5% Triton-X100. ALP activity was determined via a colorimetric assay kit (Abcam plc, Cambridge, UK) by using

p-nitrophenyl phosphate (pNPP) as a substrate. The assays were performed in duplicate and the enzyme activity was expressed as U/ml, relative to the empty vector control.

Osteogenic differentiation of MSCs overexpressing BMPs

MSCs overexpressing different BMPs or combinations of BMPs were assayed for induction of osteogenicity. After transfection and medium refreshment at day 1, cells were placed in expansion medium supplemented with 5 mM L-ascorbic acid-2-phosphate (AsAP), and 10 mM β-glycerophosphate (βGP) and cultured for 10 or 20 days to assess early (ALP) and late (mineralization) osteogenic differentiation, respectively. The medium was refreshed every fourth day. DNA analysis was performed using Picogreen kit (Quant-iT PicoGreen dsDNA Reagent Kit, Invitrogen, USA). ALP activity was expressed as U/ml, normalized for DNA content, relative to the empty vector control. Alizarin red staining was performed to evaluate mineralization in the differentiated conditions. The cells were washed with PBS, fixed in cold 10% formaldehyde, rinsed with distilled water. After being washed, staining was performed with Alizarin Red Solution (2% w/v, pH 4.2) for 10 min, followed by extensive washing with distilled water to remove the remaining stain.

Construct composition of *in vivo* implants

Six different constructs were prepared that comprised of a 3x3x6 mm porous biphasic calcium phosphate ceramic scaffolds (BCP, Xpand biotechnology, Bilthoven the Netherlands). This material is composed of 80±5% (w/v) hydroxyapatite and 20±5% (w/v) β-tricalcium phosphate and has a porosity of 70±5% with a pore size of 200-800 μm [149,150]. The scaffolds were seeded with $0.5x10^6$ goat MSCs transfected with 20 μg BMP-encoding plasmid DNAs or control vector as follows: empty vector, pBMP-2, -6, -7, -2/6 or -2/7. After nucleofection, the MSCs were kept in expansion medium for 1 hour, then counted, pelleted and seeded on the ceramic scaffolds one day prior to implantation.

Ectopic transplantation of nucleofected MSCs into NOD/SCID mice

Experiments were conducted with the permission of the local Ethical Committee for Animal Experimentation and in compliance with the Institutional Guidelines on the use of laboratory animals. Ten female nude mice (Hsd-cpb:NMRI-nu, Harlan) were anaesthetized with 1.5% isoflurane.

Five separate subcutaneous dorsal pockets were created per animal in which implants were placed in a randomized manner (n=10 for pBMP-2/6, -2/7 and empty control; n = 7 for pBMP-2 and BMP-7 and n = 6 for BMP-6). The incisions were closed using a Vicryl 5-0 suture. The animals were postoperatively treated with the analgesic buprenorphine (0.05 mg/kg, sc; Temgesic, Schering-Plough) and housed together at the Utrecht University

Animal Laboratory. Three weeks after implantation calcein green was administered s.c. (10 mg/kg, Sigma, Zwijndrecht, Netherlands). At 4 weeks after implantation Alizarin red was administered s.c. (30 mg/kg, Merck, Amsterdam, Netherlands) [175]. The mice were terminated after 5 weeks and the constructs were retrieved.

Explantation and processing

After retrieval, samples were cut in halves to analyse general morphology, transgene expression and bone histology. One half was fixed overnight in 4% (v/v) formalin and decalcified in 0.36 M EDTA (pH 7.4) and processed for 5 μm thick paraffin sections through alcohol dehydration series. The other half was fixed in 4% formalin, dehydrated by ethanol series and embedded in polymethylmethacrylate (Sigma). From this, 30 μm thick sections were cut from the centre and side of each implant using a sawing microtome (Leica, Nussloch, Germany). Half of the sections were stained with basic fuchsine/methylene blue. High-resolution (300 dpi) digital photographs of the 3rd and 7th section were made. Adobe Photoshop CS5 was used to pseudocolor the bone scaffold areas, hereafter bone area% (bone area% = bone area/[total area-scaffold area]) · 100%) was determined. Unstained sections were kept for fluorochrome analysis. A fluorescence microscope (Olympus BX51) equipped with a quadruple filter block (XF57, dichroic mirror 400, 485, 558, and 640 nm, Omega filters), was used to determine fluorochrome incorporation at several timepoints during the study.

BMP-2 and BMP-7 immunohistochemistry

BMP-2 and BMP-7 detection in the constructs was performed on paraffin sections, which were rehydrated and subsequently permeabilized with 0.1% Triton X-100 in PBS and then incubated in 0. 3% (v/v) H_2O_2 for 10 min. Antigen retrieval steps were performed in 10 mM sodium citrate buffer, pH 6.0, for 30 min at 95°C. The sections were then blocked in 5% (w/v) bovine serum albumin (BSA) in PBS for 30 min and subsequently incubated for 2 h at room temperature with rabbit-anti-human BMP-2 (C43125; LifeSpan BioSciences) at 2.5 μg/ml or rabbit-anti-human BMP-7 (ab56023; Abcam) at 2 μg/ml in PBS/5% BSA. Negative control stainings were performed with non-immunized rabbit immunoglobulin (IgG; 2.5 and 2 μg/ml in PBS, Dako, X0903). Subsequently, samples were incubated with goat anti-rabbit horseradish peroxidase (Dako; P0448) at 1 μg/ml in PBS/5% BSA for 60 min at room temperature. The staining was developed with 3,3'-diaminobenzidine (DAB). Counterstaining was performed with Mayer's hematoxylin.

Statistics

SPSS version 20 (IBM SPSS, Inc.) software was used for statistical analyses, significance was assumed when $p < 0.05$. *In vitro* data (Fig. 1, 2, 3, 4 and 5): Levene's test was used to assess the equality of variances. A square root data transformation was performed on the data in Fig. 2 and Fig. 4. ANOVA with Tukey correction for post hoc analysis was performed to assess differences between conditions. Kruskal–Wallis test by ranks was performed on the data in Fig. 3A as the data were not normally distributed (based on the observation of skewed data and a significant Shapiro-Wilk test). *In vivo* scoring of bone incidence (Fig. 6A): The Fisher exact test was used to examine whether the incidence of bone was random within the control, pBMP-2/6 and pBMP-2/7 condition. Post hoc pairwise comparison of these conditions was applied using a Bonferroni correction. *In vivo* bone area% (Fig. 6B): Data were not normally distributed. A Friedman test was performed, with a randomised block design only for the pBMP-2/6, pBMP-2/7 and control conditions. Post hoc pairwise comparison of pBMP-2/6 and pBMP-2/7 conditions to the control was performed with a Wilcoxon signed rank test with Bonferroni correction.

RESULTS

Optimization of transgene expression by MSCs

To examine the efficiency of non-viral gene delivery into MSCs, we used Amaxa Nucleofector technology for EGFP gene delivery to MSCs. Settings and ingredients for the nucleofection were optimized via a stepwise approach. Primarily, six buffers were compared, consisting of one provided by the manufacturer, and five others which were suggested in literature. Buffer 1 resulted in a transfection efficiency and viability of respectively 65±9% and 34±12%, whereas the recommended buffer 6 resulted in 72±8% and 26±9%. Buffer 1 was chosen for further experiments, because the ingredients are known (compared to the Amaxa buffer 6) and viability as well as transfection efficiency were high (Fig. 1A). Subsequently nucleofection programs were assessed. Programs were either suggested by the manufacturer or used for MSCs by others. Programs differ basically in intensity of the electric pulse and pulse length (details are proprietary information of Lonza). Program G22 (transfection efficiency 53±3% and viability 48±19%) was considered optimal, although program X01 and X05 had similar results (Fig. 1B). Thereafter pDNA concentrations were assessed. In the condition with 20 μg pEGFP-N1, incidence of gene expressing cells was the high (77±19%) and viability (45±17%) still acceptable (Fig. 1C). Finally, the amount of cells per reaction was varied.

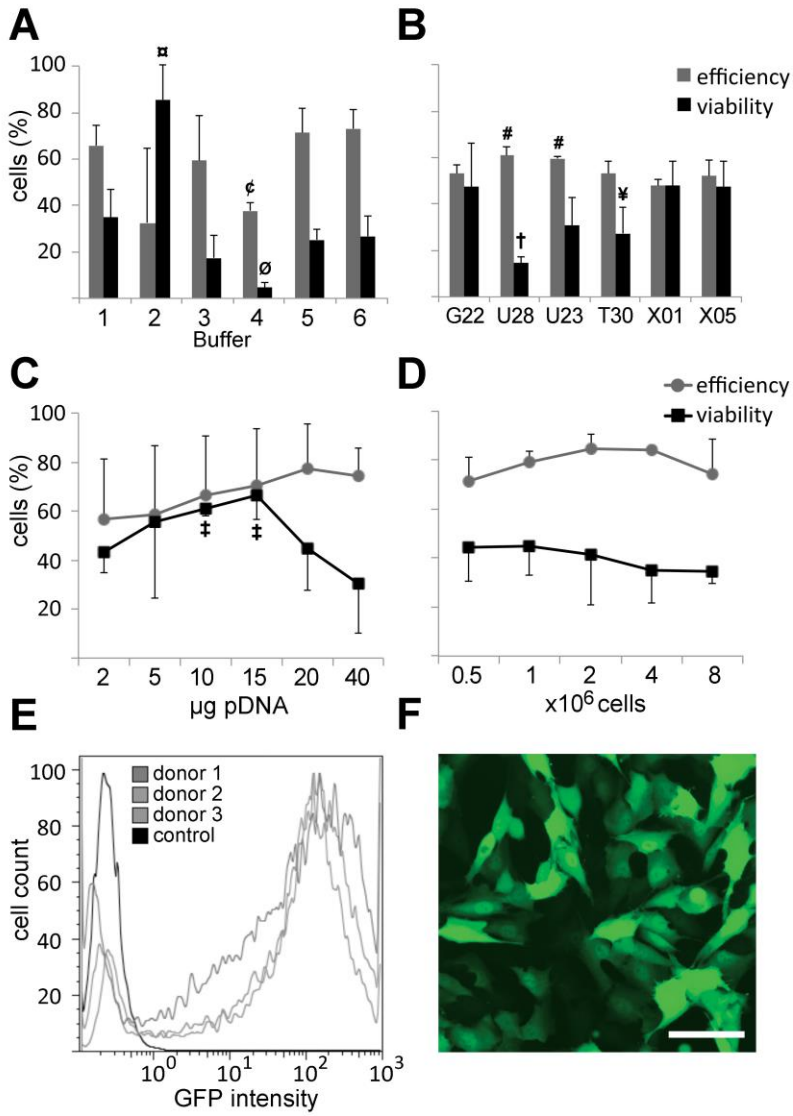

Fig. 1: Optimization nucleofection regarding cell viability and transfection efficiency. A: nucleofection buffers are varied, with nucleofection program U23, $5*10^5$ cells and 5 µg pEGFP-N1. B: nucleofection programs are varied, with nucleofection buffer 1, $5*10^5$ cells and 5 µg pEGFP-N1. C: pDNA concentration is varied, with nucleofection buffer 1, nucleofection program G22 and $5*10^5$ cells. D: cell density is varied, with nucleofection buffer 1, nucleofection program G22 and 20 µg pEGFP-N1. E: Cells expressing GFP were measured with via flow cytometry and compared to MSCs-mock (cells that underwent nucleofection without DNA) (Buffer 1, program G22, 20 µg of pDNA and $1*10^6$ cells). F: Fluorescence microscopy of monolayer at day 1. Scale bar = 50 µm. Duplicate measurements are assessed from three different donors (mean ± SD). *a* significantly higher than all other conditions. *b* significantly lower than buffer 1, 5 and 6. *c* significantly lower than all other conditions. *d* significantly higher than X01 and X05. *e* significantly lower than G22, X01 and X05. *f* significantly lower than X01 and X05. *g* significantly higher than the condition with 40 µg.

No evident changes in viability or transfection efficiency were observed by increasing the number of cells per reaction (Fig. 1D).

In vitro BMP production

ELISAs for BMP-2, -6 and -7 were performed on media collected from cultures of nucleofected cells to assess the extent of protein secretion as a result of BMP overexpression. In general, BMP protein production was higher in the conditions that contained cells overexpressing the BMP coding sequence than control. Endogenous expression levels of BMP-6 and BMP-7 in the control condition were below the detection limit of the ELISA. Some endogenous BMP-2 was detected (< 2 ng/ml, up to day 3) which could be underestimated due to reduced reactivity with goat BMP-2 compared to human BMP-2. In the pBMP conditions, highest levels of BMP were determined at day 2 or 3 after transfection and measured respectively for pBMP-2, -6, and -7: 14±11, 16±5 and 16±6 ng /10^6 cells. At day 4, medium was refreshed, thereafter BMP concentrations were lower. As expected, co-delivery of pBMP-2/6 and pBMP-2/7 resulted in decreased production of each BMP isotype, which was most evident in the BMP-7 ELISA. Please note that the ELISAs used, detect homodimers and heterodimers (see Fig. 2).

Co-delivery of BMPs resulted in heterodimer formation

To ascertain the presence of heterodimers upon co-delivery of combinations of BMPs, sandwich ELISAs were performed using BMP-6 (Fig. 3A) or BMP-7 (Fig. 3B) capture antibodies and BMP-2 detection antibodies. Controls comprised of recombinant BMP-2, -6 and -7 homodimers. In both sandwich ELISAs, the BMP-6 and -7 capture antibodies showed some cross reactivity with recombinant BMP-2, which was estimated to be no more than 10-20 % of the total signal. The BMP-2 antibody was specific. Co-delivery of pBMP-2 and -6 as well as pBMP-2 and -7 resulted higher values than the homodimer conditions. The pBMP-2/7 condition was significantly higher than the pBMP-2 and pBMP-7 condition (Fig. 3B). For the pBMP-2/6 condition, in one donor almost no heterodimers were detected. A non-parametric test had to be performed which showed no statistical significant difference from the other pBMP conditions (Fig. 3A). The other two donors showed high ELISA values that could not be found in the pBMP-2 and pBMP-6 conditions. Therefore we are confident BMP-2/6 heterodimers were formed. The exact ratio of heterodimers and homodimers formed could not be determined, due to some cross reactivity.

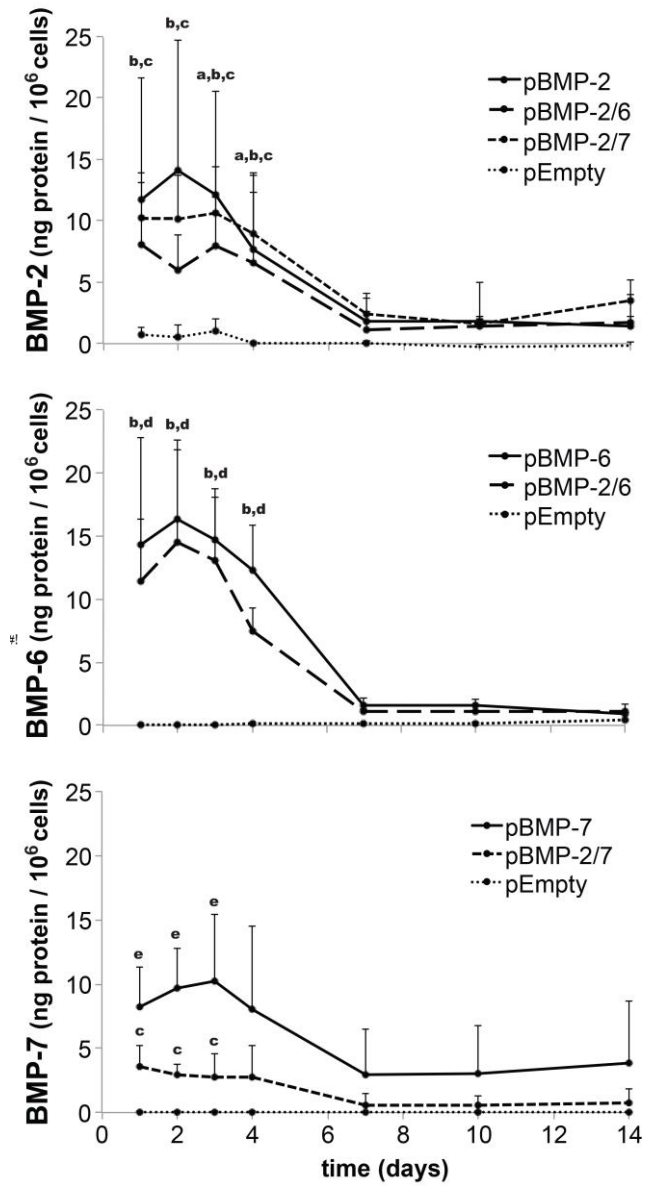

Fig. 2: Secretion of rhBMP-2 and rhBMP-7 from nucleofected MSCs. Cumulative BMP-2, -6 and -7 release of MSCs overexpressing BMPs is shown. After day 4 medium was refreshed. When assessing overall BMP production a peak can be observed between day two and three, after which protein production decreases gradually. Control conditions showed negligible BMP-2, -6 and -7 levels. The co-delivery of pBMP-2/6 and pBMP-2/7 resulted in BMP production, likely of both homo-and heterodimers. Duplicate measurements are assessed from three different donors (mean ± SD). Significant differences as follows: *a* pBMP-2 condition is higher than pEmpty. *b* pBMP-2/6 condition is higher than pEmpty. *c* pBMP-2/7 condition is higher than pEmpty. *d* pBMP-6 condition is higher than pEmpty. *e* pBMP-7 condition is higher than pEmpty.

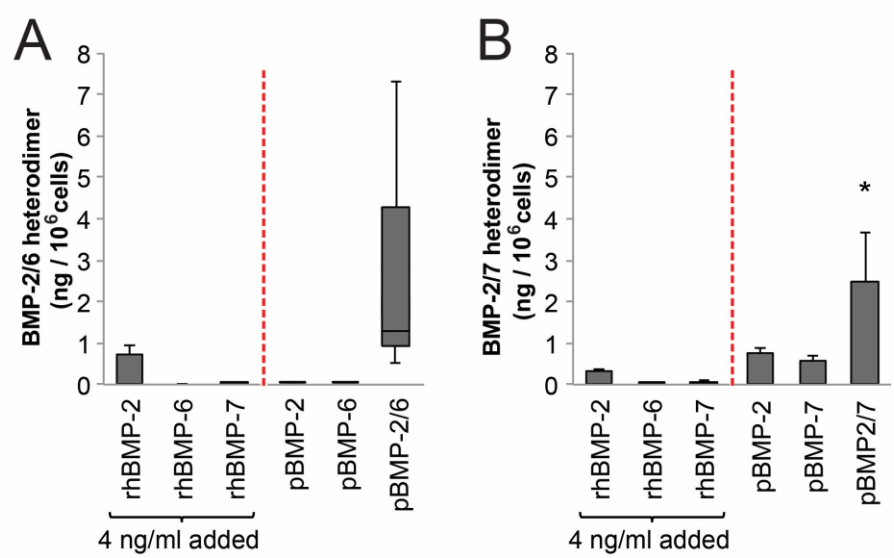

Fig. 3: Heterodimer formation via co-delivery of pBMP-2 with pBMP-6 or pBMP-7. Sandwich ELISAs were performed on the medium of MSCs overexpressing a single or two BMP isotypes (day 2 after transfection) using BMP-6 (A) or BMP-7 (B) capture antibodies and BMP-2 detection antibodies. Calibration curves were made with recombinant BMP-2/6 and BMP-2/7 heterodimers respectively. Controls comprised rhBMP-2, -6 and -7 homodimers shown on the left. BMP-2 is recognized by anti-BMP-6 or anti-BMP-7 capture antibodies, although at low affinity whereas BMP-6 and BMP-7 are not recognized by the anti-BMP-2 antibody. This means that the sandwich ELISA in A and B detect the produced heterodimers and to a much lesser extent homodimers of BMP-2 but not BMP-6 or -7. Data given are the median and interquartile range (IQR) (Fig 3A) and mean ± SD (Fig 3B), from three independent donors, measurements are performed in duplicate. * significantly different from the other pBMP conditions.

Bioactivity of BMPs on producer cells and reporter cells

Protein bioactivity was assessed with ATDC-5 reporter cells that differentiate upon stimulation with BMPs by producing ALP as early osteogenic differentiation marker. Positive controls with rhBMP-2, -6 and -7 were included. In both co-delivery conditions a significant ALP increase was found. Similar trends could be observed for the transfection with single conditions, although not significant (Fig. 4). In consecutive experiments we found that MSCs are still capable of differentiation after transfection. Ten days after nucleofection and incubation in their own produced BMP, ALP produced by MSCs is significantly higher in conditions with co-delivery of pBMP-2/6 and pBMP-2/7 than in the single transfections (Fig. 5A). These results are in concordance with the results from the Alizarin red stainings performed on day 20, that showed increased mineralization in the co-delivery conditions as compared to the pBMP-2 and pBMP-6 single transfections (Fig. 5B).

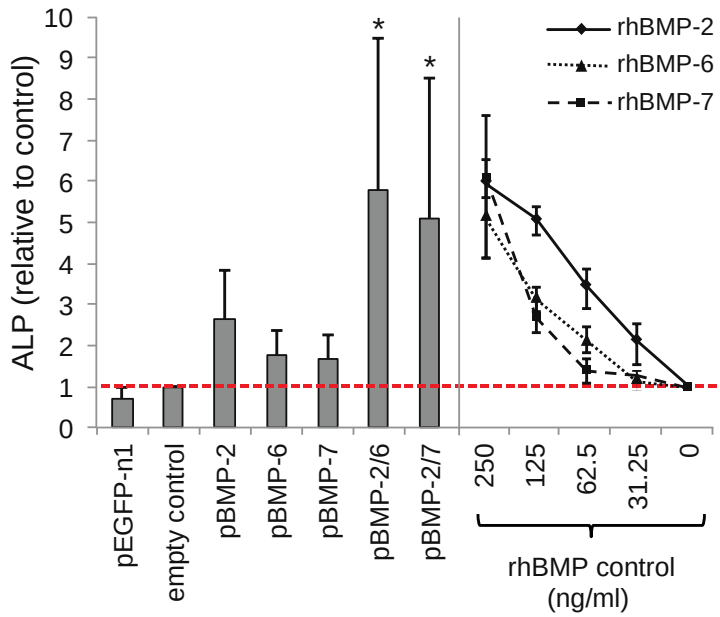

Fig. 4: Bioactivity of produced BMPs. ALP assays of ATDC reporter cells was done after stimulation with conditioned medium from MSCs transfected as indicated. ALP activation is depicted in fold change relative to empty plasmid control. Results are mean ± SD for n = 4 donors. * = significantly different from all other conditions except pBMP-2 and other co-delivery condition.

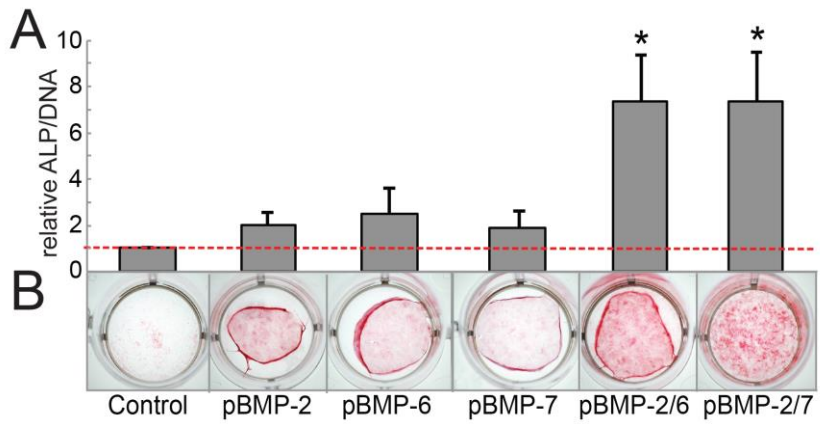

Fig. 5: Osteogenic differentiation of MSCs overexpressing pBMP. A: ALP levels of MSCs overexpressing BMP genes relative to control. * significantly different from all other conditions except the other co-delivery condition. B: A representative image is shown of qualitative alizarin red staining for calcium deposition at day 20. Results are mean ± SD for *n* = 4 donors.

Bone formation and immunological aspects of the implants

To assess the effectivity of BMP heterodimers with respect to bone formation, transfected MSCs were seeded on ceramic scaffolds and implanted subcutaneously in mice for 5 weeks. Postoperatively one animal died, possibly due to perioperative hypothermia. The other animals fulfilled the implantation period without complications. Microscopic analysis revealed tissue ingrowth with connective tissue throughout all scaffolds. Distinct differences in cell morphology or cell density were not found (Fig. 7). Bone was present in the pBMP-2/6, the pBMP-2/7, and the pBMP-6 condition. No bone was found in the pBMP-2, pBMB-7 or control condition (Fig. 6A). The incidence of bone was not randomly distributed over the conditions (Fisher exact test: $p<0.001$). When assessing individual groups, no significant difference in incidence was found. Histomorphometry showed bone area% of 1.8±1.8%, 1.7±2.8% and 0.15±0.4% (mean ± SD) in pBMP-2/6, the pBMP-2/7 and pBMP-6 conditions, respectively. Statistical analysis was only performed on the pBMP-2/6, pBMP-2/7 and control condition (n=9) due to the loss of one animal resulting in n=5 for the other conditions. Bone area% was significantly higher in the pBMP-2/6 condition compared to control (Fig. 6B). Fluorochrome markers showed that bone formation had started before week 3 in most of the cases, which was not associated with a specific condition (results not shown).

BMP production in implants as a result of gene therapy

BMP-2 and BMP-7 immunohistochemistry was performed to detect the presence of the BMPs and localize the producing cells *in vivo*. The antibodies used are human-specific but, as a result of highly conserved sequences, cross-react with endogenous mouse BMPs. BMP-2 was detected in all groups and appeared to be mainly present in and directly around cells that align the BCP scaffold material (Fig. 8). Furthermore, positive cells were present in the tibia control samples and in and around cells aligning the newly formed bone (results not shown), indicating cross reactivity with endogenous BMP-2. The expression of BMP-2 varied among the individual conditions, and was most obvious in the pBMP-2 and pBMP-2/7 conditions. BMP-7 was not detected in the control and pBMP-2 condition. Positive cells were only observed in the pBMP-7 and pBMP-2/7 conditions (Fig. 8).

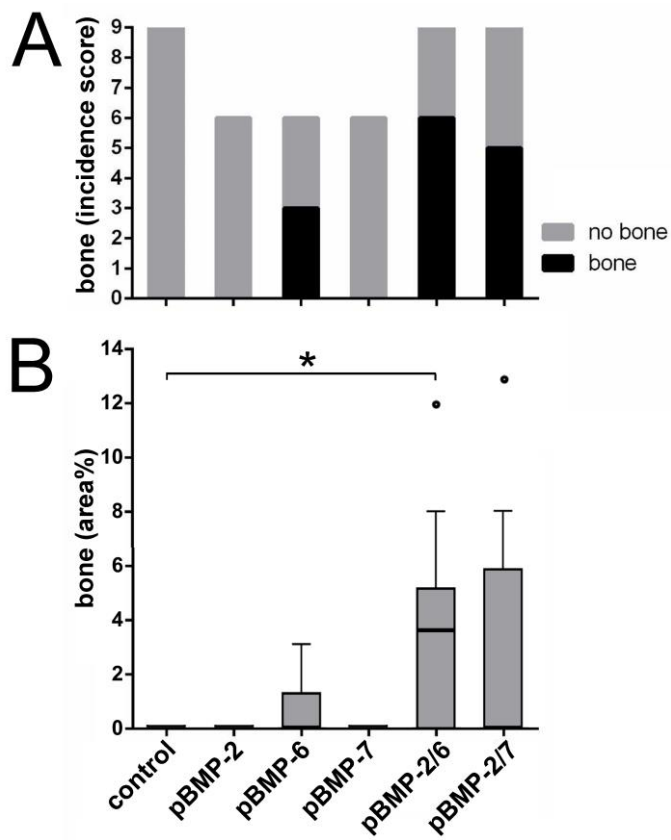

Fig. 6: Frequency plot and quantification of bone formation in mice. A: Frequency plot showing the incidence of the bone within the construct boundaries. B: Bone formation expressed as bone contact%. The results are represented as median and interquartile range (IQR). * significantly different from the control condition.

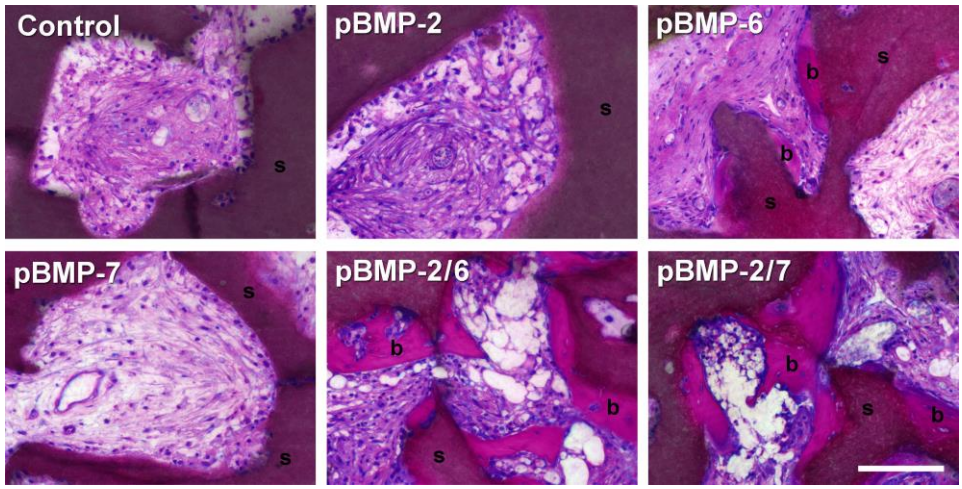

Fig. 7: Tissue ingrowth and bone formation after two and five weeks *in vivo*. Images show basic fuchsin and methylene blue stained sections containing ceramic scaffolds seeded with MSCs that overexpress BMPs. A representative image of each group is shown. Complete tissue ingrowth can be observed with frequent presence of blood vessels. No signs of fibrous capsule formation or immune responses were seen. In the pBMP-2/6, pBMP-2/7 and pBMP-6 conditions bone formation is observed, which lamellar bone positioned at the ceramic scaffold surface. Sporadic bone marrow is observed in the pBMP-2/6 and pBMP-2/7 conditions. s = scaffold; b = bone. Scale bar = 100 μm.

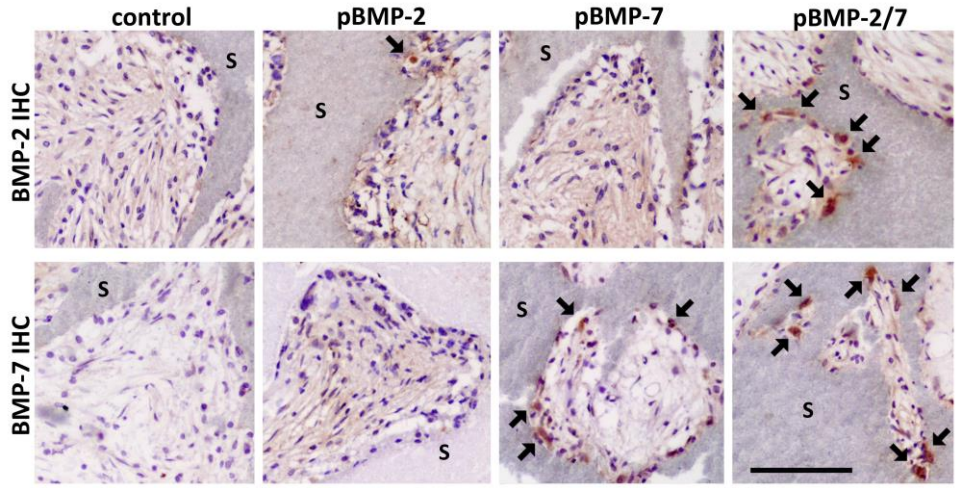

Fig. 8: identification of BMP expressing cells within construct boundaries. A representative image of the BMP-2 (upper row) and BMP-7 (lower row) immunohistochemistry is shown for each group. In the conditions where bone was present, images were taken of areas away from the bone (showing abundant BMP presence). BMP is detected in brown as indicated with arrows. BMP-2 was found in all conditions mainly present in and directly around cells that align the ceramic scaffold. The number of positive regions and intensity of BMP-2 staining varied among the individual conditions, but was most obvious in the pBMP-2 and pBMP-2/7 conditions. BMP-7 was not seen in every sample. s = scaffold. Scale bar = 100 μm.

DISCUSSION

This study demonstrates that non-viral delivery of plasmid DNA encoding BMP-2 together with BMP-6 or 7 results in the formation of heterodimers in recipient MSCs. We optimized an *ex vivo* gene delivery method to MSCs with pDNA and showed that co-delivery of pBMP-2/6 and pBMP-2/7 prevails over single pBMP delivery, regarding *in vitro* differentiation and *in vivo* bone formation.

Upon non-viral co-expression of pBMP2/6 and pBMP2/7 heterodimers were formed, as shown by sandwich ELISAs. Both BMP-2/6 and BMP-2/7 heterodimers were biologically active as shown by the increased differentiation and presence of bone formation in the co-transfection conditions. In these conditions, two heterodimers of BMP-2/6 and BMP-2/7 were formed along with their respective homodimers. It remains uncertain to which extend homodimers and heterodimers were present in one cell. BMP-7 detection was evidently lower in the co-delivery condition: less than a third of those in the homodimer condition. In the other conditions this effect was less apparent. Despite the lower protein content, the osteogenic differentiation *in vitro* and bone formation *in vivo* were increased in all cases of co-delivery of different BMP isoforms, leading to the conclusion that the heterodimers present have a stronger biological activity than the corresponding homodimers, which is in line with earlier findings [35,36,207,208]. Compared to previous non-viral gene delivery work, which mainly focuses on combining pBMP-2 and pBMP-7, we show that non-viral co delivery of BMP-2/6 has comparable beneficial effects on bone formation.

To increase heterodimer production further, BMP coding sequences have been combined in one vector, either separated by an intra ribosomal entry site or using separate promoters. This resulted however in a lowering of downstream expression and competition in the CMV promoter region, resulting in lower heterodimer production [203,204]. Therefore co-delivery of separate plasmids remains an effective strategy [42,209]. Alternative promoters that improve expression yields in adult stem cells might enhance heterodimer production further [210].

The mechanism underlying the increased biological activity of heterodimeric BMPs has not yet been fully elucidated [211]. BMPs make use of a common signal transduction pathway involving type-I and II cell surface receptors. The binding of BMP to the receptor results in activation of the SMAD signalling pathway [40]. BMP-2 has a high affinity for BMPRIA/IB (ALK3 and ALK6) whereas, BMP-6 and BMP-7 show high affinity for ACVR1 (ALK2) [212]. The increased bioactivity of cells to which the BMP-2/6 and -2/7 heterodimers bind, can be explained in three ways or a combination of these: First, the affinity for both receptors mentioned above combined, causes a greater SMAD activation [35,37]. Second, the signals

generated by heterodimers lead to up-regulation of the BMP receptor gene(s), such as the BMPR2 gene [35]. Third, heterodimeric and homodimeric BMPs differ in their ability to regulate the synthesis of BMP inhibitors and/or are differentially affected by these inhibitors. The BMP-2/7 heterodimer was reported to be resistant to Noggin, and to be a weaker inducer of Noggin compared with homodimeric BMPs [42].

The absence of bone in the BMP-2 and BMP-7 single conditions was unexpected (Fig. 6), as our group has repeatedly shown that implantation of goat MSCs results in bone formation in similar models [129]. Apart from inter-experiment variations, mainly attributed to the differences between supplied animal groups, it might be that the nucleofection procedure is the main cause. Although it was noted that *in vitro* the transfected cells proliferated and differentiated normally, nucleofection possibly influenced cell engraftment and performance upon implantation. In vivo, the total amount of BMP protein produced might have been below the threshold for bone formation, or timing of BMP release was inadequate to induce bone formation, despite evidence that produced BMP was present after five weeks in the BMP-2 and BMP-7 conditions.

This nucleofection strategy with MSCs overexpressing BMPs has some other considerations: First, the ratio of homodimers and heterodimers produced by the same cell cannot be controlled. Although we have some indications that the heterodimer formation in case of co-delivery is very efficient, we presume that also homodimers are formed in the same cells. Under conditions of saturated protein production, this lowers the maximal attainable BMP activity. By controlling the number of transfected cells seeded in a construct, overdosing of BMP is prevented, which is seen as an advantage. Due to the single hit nature of this transfection method, gene expression is transient, lasting at most 2 weeks *in vitro* and possibly somewhat longer *in vivo* due to decreased proliferation rate. A system that allows renewed uptake of plasmid DNA, such as seen for some gene-activated matrices, could prolong the gene expression period if necessary [150]. Lastly, an important issue is the use of easily transfectable MSCs, making either autologous or allogeneic strategies within reach.

The electroporation procedure is relatively harsh, and the implanted cells may not survive for a very long time under conditions of limited vascularization. Using gene-marked MSCs, long-term engraftment in the defect area and actual bone production by the transduced cells, along with the host cells has been observed in small animal models [129]. Others have shown that BMP-2 transduced MSCs can function as a temporary delivery vehicle stimulating host cells to form new bone [157] and that cells finally disappear from the implant area [213]. With respect to clinical translation, it would be interesting to further elucidate the contribution and fate of BMP-producing cells in larger animal models.

In conclusion, simultaneous delivery of pBMP-2 with pBMP-6 or -7 results in actual heterodimer formation, and these are beneficial for induction of osteogenic differentiation and subsequent bone formation when compared to applying BMP homodimers.

ACKNOWLEDGEMENTS

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Chapter 6

Osteoinduction by *ex vivo* non-viral BMP gene delivery is independent of cell type

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ABSTRACT

Ex vivo non-viral gene delivery of bone-inductive factors has the potential to heal bone defects. Due to their inherent role in new bone formation, multipotent stromal cells (MSCs) have been studied as the primary target cell for gene delivery in a preclinical setting. The relative contribution of autocrine and paracrine mechanisms, and the need of osteogenic cells, remains unclear. This study investigates the contribution of MSCs as producer of transgenic BMPs and to what extent the seeded MSCs participate in actual osteogenesis. Rat-derived MSCs or fibroblasts (FBs) were co-transfected with pBMP-2/pBMP-6 or pBMP-2/7 via nucleofection. The bioactivity of BMP products was shown through *in vitro* osteogenic differentiation assays. To investigate their role in new bone formation, transfected cells were seeded on ceramic scaffolds and implanted subcutaneously in rats. Bone formation was assessed by histomorphometry after 8 weeks. As a proof of principle, we also investigated the suitability of bone marrow derived mononuclear cells (BM-MNCs) and the stromal vascular fraction isolated from adipose tissue (SVF) for a one-stage gene delivery strategy. Bone formation was induced in all conditions containing cells overexpressing BMP heterodimers. Constructs seeded with FBs transfected with BMP-2/6 and MSCs transfected with BMP-2/6 showed comparable bone volumes, both significantly higher than controls. Single stage gene delivery proved possible and resulted in some bone formation. We conclude that bone formation as a result of *ex vivo* BMP gene delivery can be achieved even without direct osteogenic potential of the transfected cell type, suggesting that the transfected cells mainly function as a production facility for the osteoinductive proteins. In addition, single stage transfection and re-implantation of cells appeared feasible, thus facilitating future clinical translation of the method.

INTRODUCTION

Clinically, there is a strong need for strategies that can heal bone defects in a compromised biological environment. Autologous bone is considered the golden standard bone grafting material, as it possesses osteogenic and osteoconductive properties. However, its limited availability, donor site morbidity and the desire for controllable osteogenic effectivity resulted in a search for alternatives [214,215].

The most important factors that induce bone formation are found in the group of bone morphogenetic proteins (BMPs). Recombinant human BMPs have shown the most potent osteoinductive agents available today, and their efficacy has been shown in several clinical trials [216,217]. However, the variable results, reports of serious adverse events and also the high costs have dampened the initial enthusiasm [218,219]. The avoidance of supraphysiological BMP dosage, together with a prolonged release profile, could likely overcome these shortcomings.

In this respect, *ex vivo* gene delivery of BMP isotypes may be more efficient and/or effective compared to direct protein use. This procedure entails that cells, harvested during surgery or obtained from a donor bank, are transfected to overexpress one or more BMP isotypes. Subsequently, the cells are combined with a carrier and delivered at the defect site to release protein in a timely fashion [83]. Both viral and non-viral methods have been applied to produce bone-inductive constructs via *ex vivo* gene delivery. However, clinical use of viral therapy raises many concerns regarding potential insertional mutagenesis and the immune response to viral particles. Non-viral strategies in turn may be superior in terms of safety, but are hampered by low transfection efficiencies and accordingly limited local protein production [220-222]. These drawbacks might be circumvented by applying nucleofection. Nucleofection, a transfection method based on electro-permeabilization, has proven to be an effective non-viral way of delivering BMP plasmid DNA to cells. For example, it has been reported that the delivery of BMP-2 coding region in multipotent stromal cells (MSC) leads to upregulation of gene expression up to three weeks [125], and protein production up to two weeks [126,128].

The role of the transfected cells in the gene delivery applications could be twofold. The produced transgenic BMP could act in an autoinductive way when bone progenitors are used, or be purely paracrine when other cell types such as the non-osteogenic fibroblasts are used. MSCs have been well-studied for use in cell based bone tissue engineering, mainly in preclinical work. However, the ability to heal clinically relevant bone defects has not been demonstrated convincingly to date [48,49]. The exact reason why is unclear, although it is presumed that cells are not maintained long enough as a result of insufficient vascularization [59,223]. Although a few small animal studies were able to show long-term

engraftment of donor cells via genetic tracing, host cells nevertheless appeared predominant in the regenerated tissue [129-131]. This suggests that the role of the transplanted cells as bone generator could be limited, especially in clinically-relevant defects.

An alternative role of transplanted cells could be to induce the host cells to generate bone. By relying on such a paracrine effect long-term cell engraftment may be less crucial, while cell types other than MSCs could be harnessed depending on the ease with which they can be harvested and transfected [157,224,225]. Ideally, these cells are harvested and re-implanted during a single procedure. For this purpose, cells isolated from bone marrow and adipose tissue are interesting candidates, as protocols to isolate, manipulate and re-implant within a short time have already been established [134,226,227]. Similarly, efficient methods to isolate stromal cells from fat, the so-called adipose tissue derived stromal vascular fraction (SVF) are available [228]. This SVF consists of a heterogeneous mixture of cells (part of which are endothelial cells, fibroblasts and MSCs), which can form a suitable recipient for gene delivery [228].

The main aim of this study was to establish the feasibility of *ex vivo* BMP gene delivery for new bone formation. For this purpose, we determined the relative importance of autoinduction and paracrine signaling following recombinant BMP production by transfected cells. The feasibility of a one-stage procedure was assessed by transfecting and re-implanting different freshly-isolated cell sources.

MATERIALS AND METHODS

Study design:

Ceramic blocks were seeded with different transfected cells and implanted subcutaneously in Fisher 344 rats for eight weeks. BMP-2 plasmid was co-delivered with BMP-6- or BMP-7 plasmid based on its increased potential to induce new bone formation, which we observed previously [229] and compared to the delivery of a single BMP-encoding plasmid. Recipient cells were either cultured cells (MSCs and FBs) or freshly isolated cells (SVF and bone marrow mononuclear cells, BM-MNC), which were all isolated from syngeneic animals. Furthermore a negative (empty scaffold) and positive control condition (rhBMP-2) were applied, as well as a condition with pre-differentiated MSCs, to assess the contribution to bone formation of non-transfected differentiated MSCs (Table 1).

Scaffold components

Porous biphasic calcium phosphate (BCP, Xpand biotechnology, Bilthoven, the Netherlands) was used as used in previous gene delivery studies [149,150]. The 3x3x6 mm scaffolds consisted of 80±5% (w/v) hydroxyapatite and 20±5% (w/v) β-tricalcium phosphate and had a porosity of 70±5% The pore size was 200-800 μm [230]. The scaffolds were cleaned in an ultrasonic bath, autoclaved at 121 °C for 30 min, and dried at 60 °C.

Cell isolation

For the isolation of multipotent stromal cells (MSCs), bone marrow was obtained from the pooled marrow of tibias and femurs of syngeneic animals. The bones were aseptically removed and the marrow was flushed with 5 mL PBS. The collected marrow was filtered, pelleted and resuspended in expansion medium, which consisted of: DMEM/F12 (Gibco, WA, USA) with 10% (v/v) fetal calf serum (Cambrex, Charles City, IA, USA), 100 U/ml penicillin (Sigma-Aldrich, Germany), 100 μg/ml streptomycin (Sigma-Aldrich, Germany). Cells were cultured at 37°C in a humidified incubator with 5% CO_2. Cells were passaged 3-5 times and cryopreserved until use.

For the isolation of skin fibroblasts (FBs), a 1 cm^2 skin sample was obtained aseptically from the back of a rat and cut in fragments of approximately 1 mm^2. These fragments were digested with a liberase DL mixture (Roche, Indianapolis, IN, USA) at 37 °C under agitation for 40 min. Subsequently, cells were pelleted and washed in expansion medium supplemented with 50 mg/mL gentamicin (Sigma-Aldrich, Germany) and 0.5 mg/mL fungizone (Sigma-Aldrich, Germany). Thereafter, cells were cultured in expansion medium in a humidified incubator with 5% CO_2 at 37°C. Cells were passaged 2-4 times and cryopreserved until the experiment.

Bone marrow-derived mononuclear cells (BM-MNC) and adipose tissue derived stromal vascular fraction (SVF) were harvested from syngeneic animals and used the same day for either the *in vitro* or *in vivo* experiments. For isolation of BM-MNCs, bone marrow was harvested as described above, and after Ficoll-Paque (Sigma-Aldrich, Germany) separation, the leukocyte band (containing all cells except the erythrocytes) was taken, washed with PBS and immediately used for experiments. The SVF was obtained from the adipose tissue of the hump in shoulder region. The tissue was washed in PBS, minced manually, and enzymatically digested with liberase TM mixture (Roche) at 37 °C under agitation for 40 min. After digestion, the tissue collagenase sample was centrifuged, buoyant adipocytes were removed and the cell fraction of the pellet was retrieved to obtain the SVF. Cells were washed with PBS and immediately used for the experiments.

Condition	Plasmid	N
Empty scaffold	-	5
1.5 μg rhBMP-2	-	15
Pre-differentiated MSCs	-	15
MSC transfection	control vector	15
	pBMP-2 + pBMP-6	15
	pBMP-2 + pBMP-7	15
FB transfection	control vector	15
	pBMP-2 + pBMP-6	15
	pBMP-2 + pBMP-7	15
SVF transfection	control vector	5
	pBMP-2 + pBMP-6	10
BM-MNC transfection	control vector	5
	pBMP-2 + pBMP-6	5

Table 1: overview of conditions studied *in vivo*.
MSC, multipotent stromal cells; BMP, bone morphogenetic protein; p, plasmid; FB, fibroblast; SVF, stromal vascular fraction; BM-MNC, bone marrow mononuclear cells

Vector composition

The following vectors were used: pEGFP-N1 (BD Biosciences, Franklin Lakes, NJ, USA; referred to as pGFP), pVAX1/hBMP-2, pcDNA3.1/mBMP-6 and pVAX1/hBMP-7 (referred to as pBMP-2, -6 and -7) and control vector, pVAX1 (Invitrogen) without insert. The pBMP-2 and -7 constructs contained the full-length human recombinant BMP cDNAs, which were cloned into the pVAX1 vector, described in more detail elsewhere [229]. The pBMP-6 construct contained mouse BMP-6 cDNA in the pcDNA3.1 vector. The murine BMP-6 sequence is highly similar to the human isoform with only two amino acids difference in the seven-cysteine region and has five differences in the basic amino-terminal domain [229]. Plasmid DNA was isolated, purified and cleared from endotoxins prior to any experiments (EndoFree Plasmid Maxi kit, Qiagen K.K., Tokyo, Japan).

Gene delivery by nucleofection

Cells were transfected with the different vectors with the aid of Amaxa Nucleofector™ technology (Lonza ltd, Basel, Switzerland). According to a previously optimized protocol 15 μg pDNA and 2-4 x 10^6 cells were diluted in the 100 μl nucleofection buffer composed of 5 mM KCl, 15 mM $MgCl_2$, 120 mM Na_2HPO_4/NaH_2PO_4 (pH 7.2), 50 mM NaCl [229]. Nucleofection programs were optimized for each cell type as follows: Program U-23, A-24, G-22, T-30, V-13 and T-16 were tested on both MSCs as well as FBs. Considering the low yield after isolation and the fact that BM-MNCs are relatively similar to MSCs, only G-22 and U-23 were compared. For SVF U-23, A-24, G-22 and T-16 were compared.

Immediately after electroporation, the cells were transferred to expansion medium supplemented with 20% FBS, and cultured at 37°C in a humidified incubator with 5% CO_2. The transfection efficiency and cell viability were determined 24 h after pGFP transfection via fluorescence microscopy and FACS analysis using Gallios Flow Cytometer (Beckman Coulter, Inc., Germany). Exclusion of 7-AAD (Sigma-Aldrich, Germany) was used to quantify live/dead cells. Data were analyzed by FlowJo software (FlowJo Inc., Oregon, USA).

Bioactivity assay of secreted transgene products

The bioactivity of the secreted BMPs by transfected MSCs and FBs with BMP-2/6, -2/7 and control vector was determined using mouse chondrogenic ATDC5 reporter cells, which demonstrate a high sensitivity to a variety of BMPs in terms of elevated alkaline phosphatase (ALP) activity [206]. The supernatant of MSCs and FBs transfected with BMPs or control vector was harvested 48 hours after nucleofection and was added to subconfluent ATDC5 cells. Positive controls consisted of a rhBMP-2, -6 and -7 protein at a dose range of 0-500 ng/ml. After three days, cells were lysed in PBS containing 0.5% Triton-X100. ALP activity was determined by means of a colorimetric assay kit (Abcam plc, Cambridge, UK) using p-nitrophenyl phosphate (pNPP) as substrate. Values were normalized to a standard ALP measurement using serial dilutions of calf intestinal ALP (Sigma). The assays were performed in duplicate.

Osteogenic differentiation assay

MSCs and FBs overexpressing different BMP combinations or control vector were assayed for autoinduction of osteogenicity. The following non-transfected control conditions were included: negative controls that remained untransfected and positive controls that were stimulated with medium spiked with 200 ng/ml rhBMP-2. After transfection and medium refreshment at day 1, cells were placed in expansion medium supplemented with 5 mM L-ascorbic acid-2-phosphate (AsAP) and 10 mM β-glycerophosphate (β-GP). Cells were

cultured for 10 or 20 days to assess early (ALP) and late (mineralization) osteogenic differentiation, respectively. The ALP activity was determined as mentioned above, and normalized for DNA content using a Picogreen kit (Quant-iT PicoGreen dsDNA Reagent Kit, Invitrogen, USA). The assays were performed in duplicate, To evaluate the matrix mineralization at day 20, the cells were washed with PBS, fixed in cold 10% formaldehyde, and rinsed with distilled water. Thereafter, staining was performed with Alizarin red Solution (2% w/v, pH 4.2) for 10 min, followed by extensive washing with distilled water to remove the unbound stain.

Construct preparation for animal experiments

Cryopreserved MSCs or FBs were thawed, washed and cultured in expansion medium for 5 days. To obtain pre-differentiated MSCs, the cells were cultured in osteogenic differentiation medium (ODM) for 10 days, which consisted of expansion medium supplemented with 5 mM AsAP, 10 mM β-GP and 10 nM dexamethasone. BM-MNCs and SVF were isolated as described above.

MSCs, FBs, BM-MNCs and SVF were transfected with pDNA as described in Table 1 using nucleofection program U-23 for MSCs, FBs and BM-MNCs, and nucleofection program G-22 for SVF. Immediately after electroporation, the cells were transferred into tubes containing expansion medium supplemented with 20% FBS, cytospun and washed to remove the transfection buffer. For the preparation of cell loaded BCP constructs, solutions of 10^7 cells/mL in PBS were prepared. Subsequently, 50 µl of this solution or PBS alone (empty control) was pipetted onto the scaffolds. After 30 min in a humidified incubator, the constructs were submerged in α-MEM medium and kept until implantation at 37 °C and 5 % CO_2. For the preparation of rhBMP-2 loaded constructs, 50 µl of a 30 µg/mL BMP-2 in PBS stock solution was pipetted on the scaffolds (1.5 µg in total/scaffold).

Animals and implantation

After approval of the local animal care committee, 15 male Fischer 344 inbred rats (126–150 g; Charles River Laboratories, L'Arbresle, France) were used. Food and water were given *ad libitum*. The laboratory animal welfare officer monitored the animals' general health. All procedures were performed under general anesthesia by inhalation of isoflurane. After shaving and disinfecting the back of the rats, subcutaneous pockets were created on each side of the spine through 5 mm skin incisions, at least 25 mm apart. Following implantation of the samples according to a randomized design, the incisions were closed with sutures. Pain relief was given by subcutaneous injections of buprenorphine (0.05 mg/kg Temgesic; Schering-Plough, Utrecht, The Netherlands) every 8 h until 48 h postoperatively. Fluorochrome labels were injected subcutaneously to

determine the onset and location of new bone formation [175]: calcein green at week 4 (10 mg/kg in 0.2 M $NaHCO_3$, Sigma), oxytetracycline at week 6 (25 mg/kg in 50/50 PBS/demineralized water, Merck Millipore, Billerica, USA).

During the implantation period one animal was euthanized due to unrelated reasons and was excluded from the study. All other animals fulfilled the implantation period without complications. After eight weeks, the animals were euthanized with CO_2 asphyxiation and the constructs were retrieved.

Post-mortem sample acquisition and processing

After retrieval, the samples were fixed in 4% (w/v) formalin and cut in two. One half was processed for paraffin embedding. The other half was dehydrated by an ethanol series and embedded in methyl methacrylate (MMA, Merck Millipore). Subsequently, 30 μm-thick sections were cut using a sawing microtome (Leica, Nussloch, Germany) and stained with basic fuchsin and methylene blue. The samples were completely sectioned. Images of the mid-section were pseudo-colored using Adobe Photoshop CS6 (Adobe Systems, San Jose, USA) to quantify the percentage of bone in the available pore space (bone area %). One MMA section per BCP sample was left unstained for fluorochrome detection by fluorescence microscopy (Olympus BX51 with DP70 camera, Olympus, Shinjuku, Tokyo, Japan).

Statistics

Based on previous data, a sample size of n=15 was calculated to identify a >20% difference in bone quantity (bone area%), with a power of 90%. This power analysis is conservative since we did paired comparisons, excluding inter animal variance. Statistical analyses were performed using SPSS 20 (IBM SPSS Inc., Chicago, IL, USA). Data were tested for homogeneity of variances using Levene's test. Subsequently, differences in bone area % were analyzed using a linear mixed-model approach, with randomized block design (Fig. 4). Post hoc comparisons with a Bonferroni correction were performed to assess differences between the conditions of interest. For the *in vitro* results in Fig. 1 a student's T-test was applied to compare the FB en MSC conditions. For Fig. 2 and Fig. 3 a one-way ANOVA with Bonferroni correction for post hoc analysis was performed to assess differences between conditions of interest.

RESULTS

Optimization of transgene expression

To examine the efficiency of non-viral gene delivery to MSCs, FBs, BM-MNCs and SVF, we used plasmid DNA encoding GFP. Several transfection programs were compared (Fig. 1). Program U-23 was most optimal when considering both transfection efficiency and viability for MSCs and FBs. The transfection efficiency of MSCs and FBs were 75.6% and 72.5% and the viability 80.3% and 91.1% respectively (Fig. 1). No statistical differences were found. For the BM-MNCs we also used program U-23 and for the SVF program G-22 was best suitable. Transfection efficiencies were lower than the MSCs and FBs: BM-MNCs 53.6% and for SVF 51.1% which was not surprising considering the presence of a multitude of cell types, but 76.1% and 88.9% of the cells remained viable.

Assessment of the paracrine mechanism

ATDC5 reporter cells were cultured in conditioned medium derived from transfected cells to study the paracrine effects of BMP transfection and the bioactivity of the produced BMP isoforms. The ATDC5 cells were shown to be highly responsive to exogenous rhBMP-2, -6 and -7 in terms of dose-dependent increases in ALP activity (results not shown). The conditioned media derived from transfected MSCs or FBs both induced a more than 5-fold increase ($p < 0.05$) in ALP activity in the ATDC5 cells when pBMP-2 was co-delivered with pBMP-6 or pBMP-7 (Fig. 2).

Assessment of the autoinductive mechanism

The potential of BMP-transfected cells to differentiate through autoinduction was assessed in osteogenic differentiation assays for intermediate (ALP at day 10) as well as late osteogenic markers (matrix mineralization at day 20). After 10 days of culture, ALP activity was significantly higher in the MSCs transfected with pBMP-2/6 or pBMP-2/7, whereas ALP activity in the FB condition was comparable to the controls (Fig. 3A). As a control, untransfected MSCs and FBs were exposed to exogenous rhBMP-2. MSCs showed a strong increase in ALP activity ($p < 0.01$). In contrast, no significant increase was found for the FBs, confirming that FBs remain devoid of osteogenic differentiation despite presence of an adequate osteoinductive stimulus. In agreement to the early changes in ALP activity, MSC transfected with pBMP-2/6 or pBMP-2/7 showed abundant calcified matrix deposition after 20 days of culture, whereas the fibroblasts did not (Fig. 3B). Taken together, these data show that the nucleofection procedure did not hamper MSCs' osteogenic capacity, while FBs did not show any signs of osteogenic differentiation.

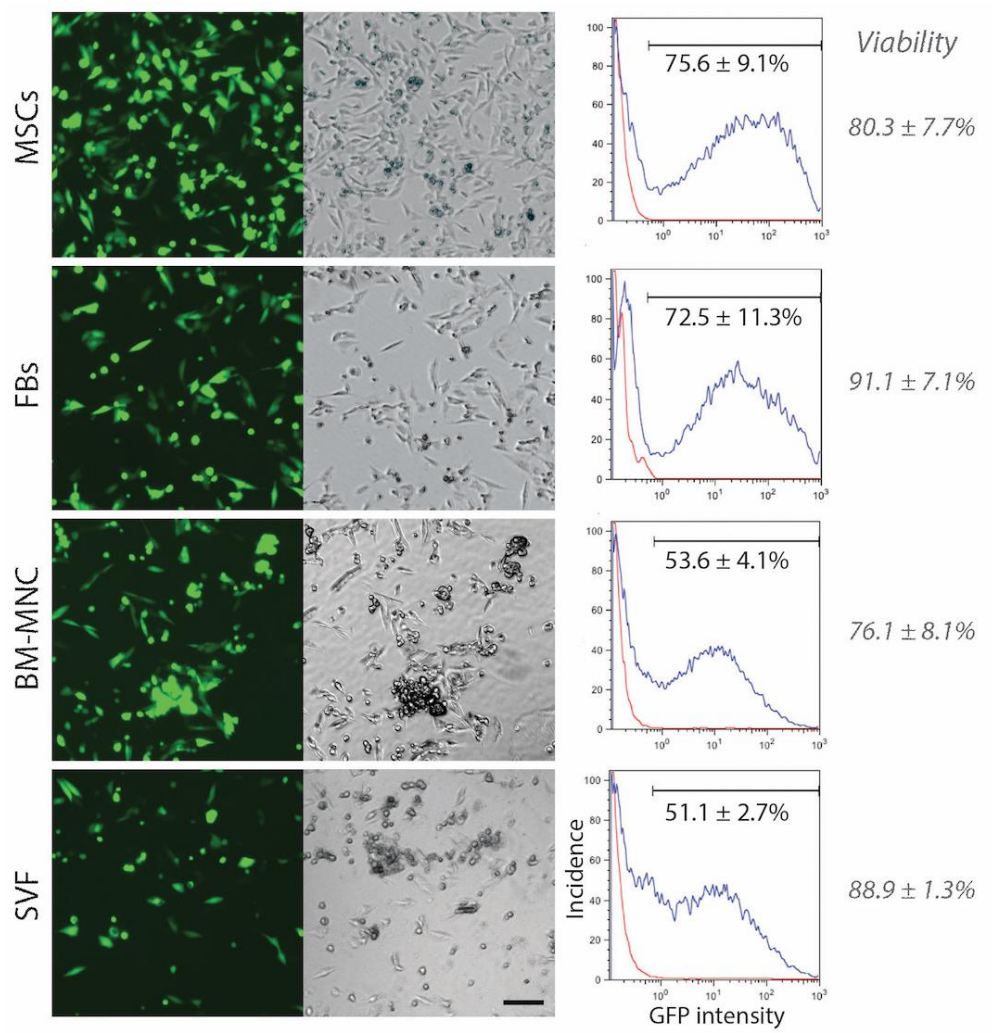

Figure 1: Nucleofection of MSCs, FBs, BM-MNCs and SVF with pEGFP. Fluorescence and corresponding brightfield images (column 1 and 2) of the different cell types after 24 hours. Scale bar = 100 µm. Transfection efficiency of pEGFP is indicated in each histogram (pEGFP transfected cells in blue and empty vector control in red). Transfection efficiency and viability are represented as mean ± SD (n=3).

In vivo tissue response and bone formation

The osteogenic potential of the different BMP transfected cell sources was investigated by implantation of cell-seeded BCP constructs in subcutaneous pockets in rats. We provided evidence that bone formation by transfected non-osteogenic cells – in this study skin fibroblasts – is as effective as bone formation by transfected MSCs.

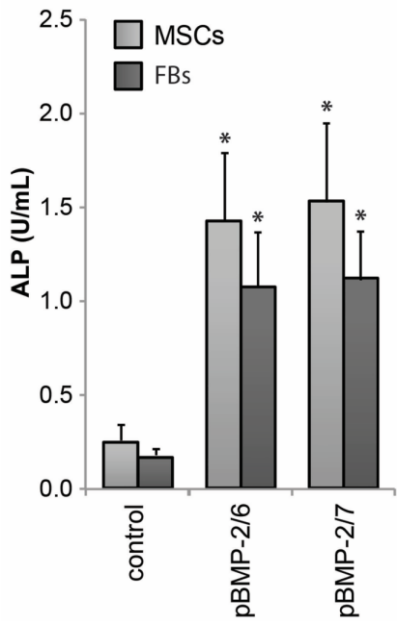

Figure 2: Paracrine osteogenic effect of BMP transfected MSCs and FBs. ATDC5 reporter cells were cultured in medium derived from multipotent stromal cells (MSCs) or fibroblasts (FBs) transfected with control vector, BMP-2/6 or BMP-2/7. Alkaline phosphatase (ALP) activity was measured after 3 days. The results are represented as mean ± SD (n=3). *p<0.05 compared to control vector.

After 8 weeks connective tissue ingrowth was observed in all scaffolds. There were no apparent differences in cell morphology or cell density between the conditions. Qualitative assessment of bone formation showed small/focal areas of bone tissue formation in the majority of the samples. As an exception, the empty scaffolds and the group with untransfected fibroblasts (FB control) did not show any bone formation (Fig. 4).

Quantification of the bone volume by histomorphometry showed different amounts of bone formed in the three control conditions (.), depicted as bone area% (mean ± SD): the positive control (rhBMP-2) showed only 6.0±5.3% bone; the pre-differentiated MSCs resulted in little bone formation (1.7±1.8%), and the empty scaffold remained devoid of any bone. Cell transfection was shown to enhance new bone formation. Constructs containing BMP-transfected MSCs induced more bone (MSCs+pBMP-2/6: 3.1±2.6% and MSCs+pBMP-2/7: 2.2±2.4%) than the MSC control constructs (0.5±1.3%), indicating that local levels of BMP produced were effective in enhancing osteogenesis. Importantly, the pro-osteogenic effects of BMP-transfected cells may be largely the result paracrine mechanisms, since transfected fibroblasts induced new bone formation, which was significant in the pBMP-2/6 condition (2.7±2.0%; $p < 0.01$).

To investigate transgene delivery in a one-stage procedure, the BM-MNCs and SVF conditions were included. Bone formation in both BMP expressing BM-MNCs and SVF showed a trend towards more bone than the corresponding control conditions, but were not statistically significant (Fig. 5).

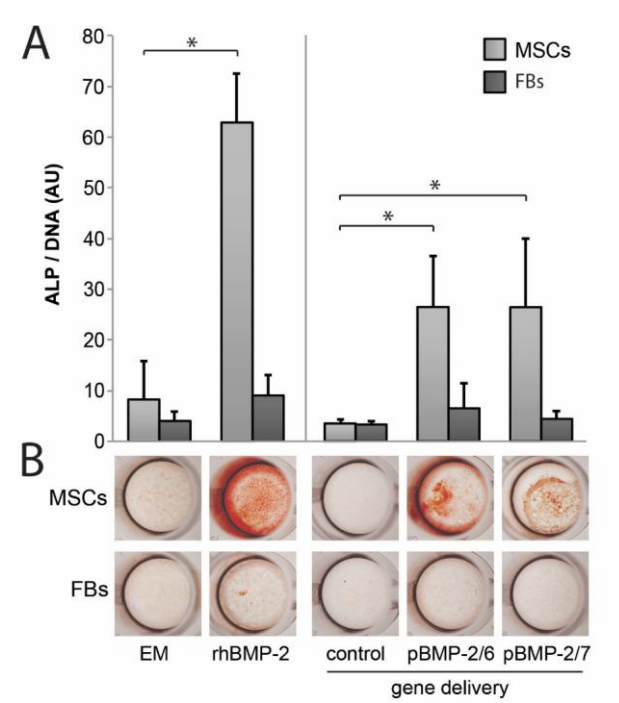

Figure 3: Autocrine osteogenic effects of BMP transfected MSCs and FBs. Day 10 ALP activity in MSCs and skin fibroblasts overexpressing control vectors or BMP heterodimers (right column). As a control, untransfected cells were cultured in expansion medium (EM) or medium with rhBMP-2 were added (left column). The results are represented as mean ± SD (n=3). * $p<0.01$ compared to the different from the MCS control condition (A). Alizarin red staining for calcium deposition at day 20. Representative images are shown (n=3 for MSCs, n=2 for FBs) (B).

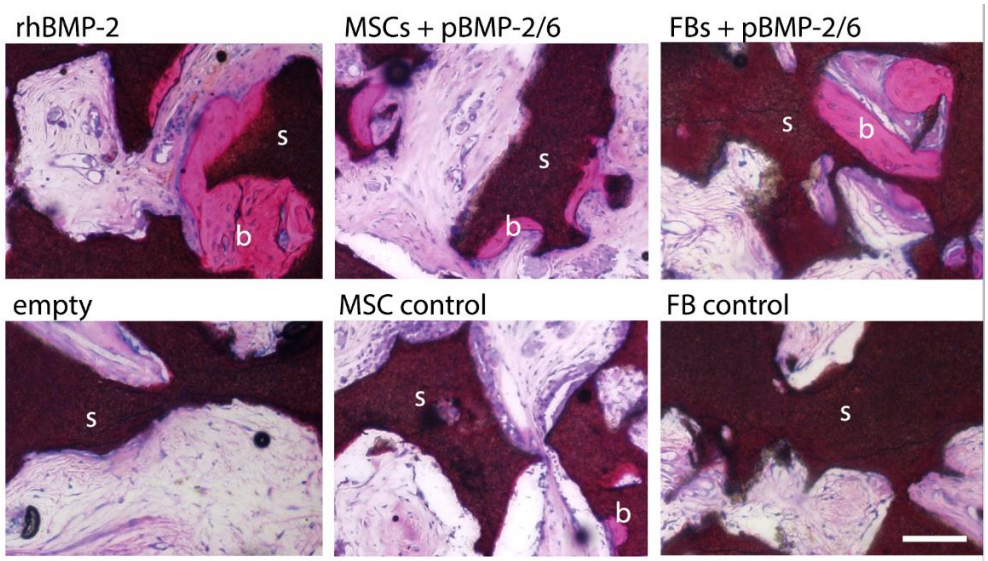

Figure 4: Tissue ingrowth and bone formation *in vivo* after eight weeks.
Representative images of basic fuchsin and methylene blue stained BCP sections. s = scaffold; b = bone. Scale bar = 50 µm.

DISCUSSION

In this study we showed that bone formation is induced by both autoinductive as well as paracrine mechanisms of transfected cells. Due to their osteogenic activity, MSCs may act as producer and effector cells for BMPs, thereby acting in an autoinductive way. In comparison, transfected cell sources that support effective paracrine stimulation would raise possibilities to also apply various cell types that can be obtained in a one-stage procedure, independent of their osteogenicity. To represent a situation confined to only paracrine stimulation, we transfected non-osteogenic cells in parallel and studied their paracrine effects on *in vitro* and *in vivo* osteogenesis. All together, the results show that local application of an osteogenic signal, through a combination of overexpressed BMP isoforms, can be achieved by non-osteogenic cells.

We provide evidence that bone formation induced by transfected non-osteogenic cells in the form of skin fibroblasts is comparable to bone formation mediated by transfected MSCs. We confirmed the non-osteogenic nature of the fibroblasts to strengthen hypothesis that BMP transfection would only mediate osteogenic processes via a paracrine mechanism. *In vitro*, fibroblasts did not show the potential to differentiate towards the osteogenic lineage and similarly *in vivo,* the fibroblast control condition did not show any bone. Bone tissue formed by application of transfected fibroblasts can therefore only be

attributed to the induction of host cells. The comparable osteogenic response found for MSCs and FBs in terms of established bone volume hints towards the possibility that paracrine stimulation is the major contributor of bone induction. To further strengthen these data, quantification of the amounts of BMPs produced *in vivo* would be helpful, but is virtually impossible. Together, these data indicate that non-osteogenic cells can serve as an instrument for BMP secretion after transfection.

Our work is in line with previous work that investigated the autoinductive and paracrine roles of genetically modified cells using viral transduction methods. Other reports show that human MSCs and human skin fibroblasts overexpressing BMP-2 induce comparable amounts of bone in an orthotopic mouse model [157]. Disputable however is whether fibroblasts are unresponsive to BMP-2 and completely non-osteogenic, as some studies showed that fibroblasts isolated from other tissues than skin might have a role in bone formation. Krebsbach et al. showed that human gingival fibroblasts were present within newly formed bone, although sporadic, when transduced with adenovirus based BMP-7 expression vector [231]. However, skin fibroblasts have repeatedly shown devoid of any osteogenic potential [224,232]. Therefore, in line with previous work with viral vectors, we conclude that also non-viral delivery methods rely on the paracrine effect of cells, when regarding bone formation.

In an attempt to design a one-stage *ex vivo* gene delivery procedure, cells derived from fat tissue and bone marrow were applied. We found that single-step isolates of cells, although heterogeneous in nature, can be transfected and implanted the same day, and subsequently can induce bone. This one stage procedure should be further optimized before clinical application comes within reach [233]. It replaces the costly and time-consuming culture expansion of stem cells needed in a two-step *ex vivo* approach. Furthermore, concern associated with *in vitro* expansion of bone marrow stem cells including the potential risk introducing an infection is omitted [234,235].

As a proof of principle, we aimed to investigate the feasibility of a one-stage approach. Accordingly, it was beyond the scope of the current study to identify significant differences between the pBMP-2/6 transfected BM-MNCs and SVF and their corresponding control conditions. Interestingly, BM-MNCs and SVF have a subtle osteoinductive potential. This is not surprising, as the hematopoietic fraction of bone marrow contains platelets, monocytes and macrophages which has shown to be a source of osteoinductive and angiogenic factors during fracture repair [236], and SVF contains monocytes, endothelial cells, and mesenchymal stromal cells [237]. The latter two are known to have a beneficial effect on bone formation, which has to be taken into account when interpreting these results.

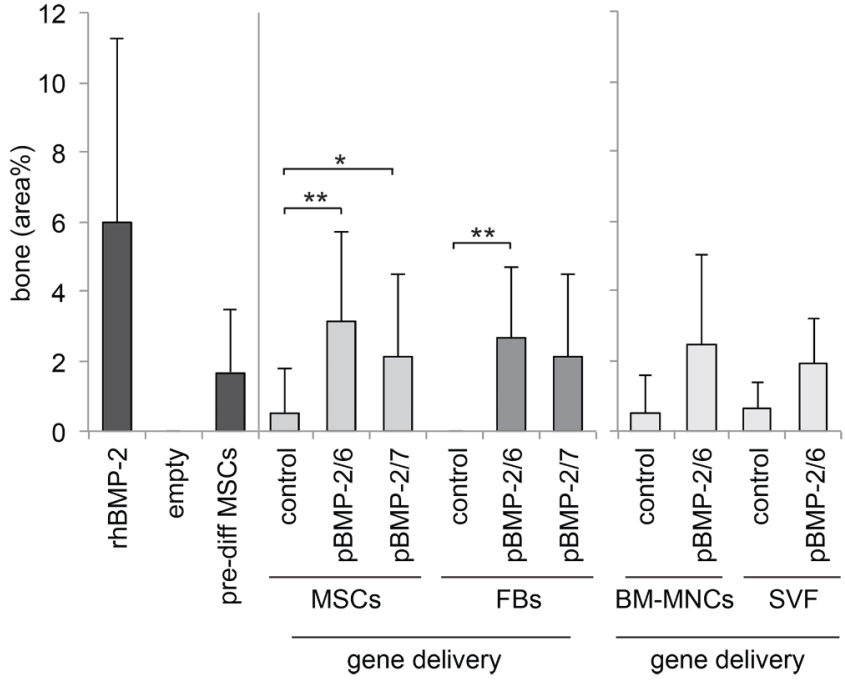

Figure 5: Bone formation in rat ectopic constructs after 8 weeks, expressed as bone area%.
The results are represented as mean ± SD (A: n=14 for all conditions, except: empty scaffold n=5; B: n=4). *p<0.05, **p<0.01.

With respect to translating the results of this work into the clinical context, caution should be exercised, since the ectopic animal model does not recapitulate the more challenging bone healing environment in clinical situation in terms of defect size. In our model a small dose of only 1.5 µg of rhBMP-2 is sufficient to induce bone formation, whereas milligrams of BMP are applied clinically. Furthermore, due to the small size of the implants, cell survival throughout the whole scaffold is more likely than in clinically relevant defects. On the other hand, in the orthotopic location endogenous growth and cell sources are present that can lead to a higher osteogenic response than in the ectopic location. We therefore think that the findings have positive implications for the clinical situation. As BMP activation of responsive cells leads to a positive feedback loop, a single stimulation lasting no longer than several weeks is expected to be sufficient. This can be accomplished by transient plasmid based gene expression by any cell. Therefore, long term presence and finally engraftment of the seeded cells is not a necessity and the absence of early vascularization, observed clinically, might be less important. This may be of interest

as the latter is thought to be one of the main obstacles to long-term survival and engraftment of implanted cells [238].

In conclusion, we show that non-osteogenic cells can be rendered osteoinductive by *ex vivo* BMP-2/6 or BMP-2/7 gene delivery. The use of non-virally transfected cells may be particularly feasible as a delivery vehicle to induce paracrine BMP signaling in host cells. Finally, we extend the clinical applicability of the current findings by demonstrating the applicability of a single stage transfection procedure.

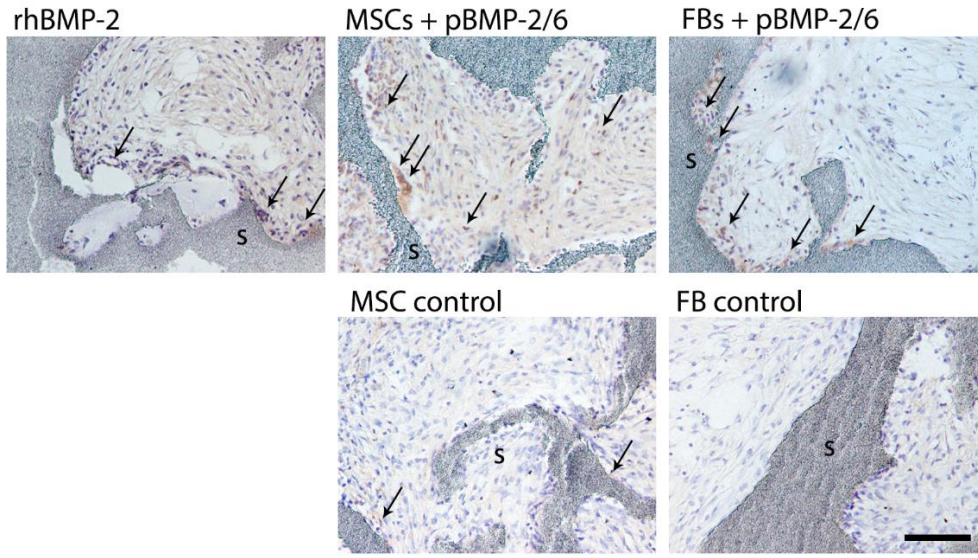

Figure 6: Identification of BMP expressing cells within construct boundaries.
BMP-2 immunohistochemistry for each group. In the conditions where bone was present, images were taken of areas away from the bone (containing abundant BMP presence). BMP is detected in brown as indicated with arrows. BMP-2 was found in all conditions mainly present in and directly around cells that align the ceramic scaffold. The number of and intensity of BMP-2 staining varied among the individual conditions, but was most obvious in the pBMP-2/6 and pBMP-2/7 conditions. s = scaffold; b = bone. Scale bar = 50 μm.

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Chapter 7

Discussion, conclusions, future perspectives

SHORT SUMMARY

In this thesis, we investigated the suitability of non-viral gene delivery of BMPs as a means to (re)generate bone tissue in a clinical application. A multitude of different non-viral gene delivery strategies (*ex vivo* and *in vivo* applied) and a multitude of transfection methods have been developed over the past decades. These strategies, however, have mainly been tested in a 'proof of concept' setting and steps toward a potential application in a clinical setting have rarely been fully explored. We addressed several aspects of the non-viral gene delivery of BMPs to explore and achieve an optimal design of a gene-activated matrix (GAM).

C*hapter 2* details an *in vivo* gene delivery approach using an alginate hydrogel GAM. Local gene delivery appeared to result in transfection of endogenous cells that migrated into the construct boundaries. The amount of transfected cells could be increased by co-transplanting cells that function as a recipient for the transgene. When using pDNA-BMP-2, in this study, no bone formation could be demonstrated, neither in the cell-free nor in the cell-seeded conditions. This was surprising as in previous work with a similar animal model, comparable cells (MSCs), without treatment have shown to result in bone formation.

From previous work we observed that in a goat animal model, the application of seeded MSCs at an orthotopic location have only little effect on bone formation. In *chapter 3* we showed bone formation in an orthotopic location that was shielded from the underlying bone to be dependent on additional seeding of cells: gene delivery with addition of MSCs resulted in higher bone volumes than control and was accompanied with faster ingrowth from the perimeters of the implant throughout the scaffold.

In *chapter 4* bioprinted constructs were investigated: we showed that the 3D geometry of the printed constructs consisting of a grid like shape with horizontal strands and vertical pores proved highly reproducible, but highly dependent on the polymer concentration of the print. The concentration of alginate polymer was found to affect not only the transfection efficiency, but also the biological activity of cells. In comparison with solid constructs, introducing porosity has a positive influence on the biological function of the cells including the expression of the BMP-2 transgene. *In vivo,* however, this difference could not be objectified as bone formation was not observed.

In *chapter 5*, we aimed to further enhance the expression level and effectivity of osteogenic BMP isoforms via an *ex vivo* approach, in which cells were nucleofected before implantation. Simultaneous delivery of pBMP-2 with either pBMP-6 or pBMP-7 resulted in the production of heterodimers, that have a favorable effect on bone formation compared

to BMP homodimers. The combination of BMP sequences appeared to reduce the need for high BMP protein dosages and may extend the availability of the growth factors.
In *chapter 6* we provided evidence that bone formation by BMP transfected non-osteogenic cells –*fibroblasts*– was nearly as effective as by transfected MSCs. As we also showed that fibroblasts are not susceptible for BMP stimulation, this study indicates that transplanted cells may serve as an instrument for localized BMP secretion by means of gene therapy.

In addition, we argued that a single stage procedure applying gene therapy, if further optimized, seems to be within reach. We showed that both cells derived from fat tissue and bone marrow can be isolated, transfected and implanted within a single day, and, subsequently, be used to induce bone *in vivo*.

GENERAL DISCUSSION

Local *in vivo* BMP-gene delivery

A cell-free approach with an alginate gene-activated matrix

The goal of this study as described in *chapter 2* was to induce bone formation via *in vivo* gene delivery. Alginate hydrogel was used and loaded with plasmid DNA with the BMP-2 gene. Previous work by our group has shown that alginate hydrogel functions as a gene-activated matrix, inducing very efficient transfection of seeded cells [117]. Others have shown that alginate hydrogel also functions as a carrier for pDNA that can gradually release pDNA *in vivo* [111,112,239]. Based on this previous work and the extensive knowledge about the use of alginate in combination with pDNA-BMP-2 and promising results in our group we chose to continue investigations on alginate-based bone regeneration.

Different dosages of pDNA were applied to optimize the transfection efficacy of the target cells present at the implant location (subcutaneous, intramuscular and on spinal defect locations in a rat model). We showed that the highest pDNA-GFP concentration was associated with the highest prevalence of GFP positive cells. Therefore, in a subsequent bone-induction experiment an even higher dose of pDNA-BMP-2 (500 µg/ml, resulting in an absolute local dosage of 20 µg) was used, as the optimal pDNA dosage was not yet reached. As expected, local gene expression resulted in BMP-2 production, which was detectable in the samples up to eight weeks, but bone formation was absent. The main reason for the lack of bone formation was sought in the insufficiently produced amounts of BMP-2. This assumption remains unvalidated, as *in vivo* quantification of BMP production is nearly impossible with current techniques. Nevertheless, we have indications some BMP-2

was produced locally: in comparison with background staining in the control condition, BMP-2 immunohistochemistry showed clear staining in the pDNA-BMP-2 conditions.

Limitations of immunohistochemistry: GFP and BMP-2 immunohistochemistry showed signs of gene delivery and gene expression. The method used to quantify the presence of GFP must be regarded as semi-quantitative: due to several enhancement steps in the immunohistochemistry procedure, even very small amounts of transgenic protein are detected in the tissue sections. Therefore it is hard to interpret a positive signal: it may well be that only small amounts of BMP-2 have been present locally. Furthermore, the immunohistochemistry does not differentiate between endogenous and transgenic BMP-2. Therefore the cumulative effect is detected, resulting from both the direct production from the transgene and the indirect BMP-2 production caused by its stimulation of BMP-2 responsive cells. Nevertheless, the assumption that local gene delivery has been inadequate to induce sufficient local BMP levels is in line with observations by others, who observed only small amounts of gene expression regardless of the local *in vivo* gene delivery strategy used [94,240].

pDNA release pattern: previous work has shown that alginate acts as a transfection agent, through an unknown mechanism: are DNA-alginate complexes or is naked DNA ingested by the cells? If naked plasmid DNA can enter the cells, it must first be released from the alginate. The release pattern is important because it has to coincide with the presence of the target cells. Previous work showed that simply increasing the pDNA-BMP-2 dosage is not sufficient; pre-clinical experiments that applied a single extremely high pDNA dosage only resulted in a minimal increase of gene expression. Optimizing the timing of pDNA release, such that the release coincides with the presence and/or invasion of host cells, is considered to be of great importance, although little is known about the optimal timing [241]. The release pattern of pDNA from alginate can be easily adjusted by forming more or stronger crosslinks, but the effect on gene transfer remains uninvestigated [16,111]. Methods such as repeated injections of plasmid DNA, ensuring prolonged presence, did show a strong positive effect on bone formation [96,97], but have to be seen as a proof of concept rather than a clinically applicable strategy, due to the extent of the treatment.

Presence of recipient cells: in accordance with the previous argument, it is important to realize that the cells present in the fracture hematoma as well as the cells invading the hematoma to initiate the regeneration process are unfavorable as target cell as they mainly consist of immune system cells, namely T-cells and monocytes/macrophages [242]. An additional factor is that in the surrounding healthy tissue, mainly mature postmitotic tissue cells are present such as osteocytes, myocytes, fibroblasts etc., which are generally hard to transfect [243].

Clearing of pDNA by the immune system: on the implanted scaffolds frequently multi-nucleated giant cells (MNGC) were found. The presence of these cells is part of a foreign body response. Coinciding with the presence these MNGCs is activation of the immune system, which plays an important role in clearing the implant site from pDNA via excretion of DNases [241]. Delivery of pDNA by means of PEI or CaP nanoparticles, as performed by other researchers, provides better shielding of pDNA from degradation, resulting in only a slight increase of gene expression [118,119].

Early presence of BMP-2: an alternative explanation for the absence of an adequate osteogenic stimulus could lie in the timing of the BMP-2 protein release. Given the fact that physiological BMP-2 expression in fracture repair already starts within hours, an early onset of BMP-2 production may be essential [21,22,155]. By using a gene activated matrix the expression of BMP-2 occurs late, after a few days is starts and increases over weeks *in vitro*, which is a disadvantage of this methodology.

Conclusions regarding local *in vivo* BMP-gene delivery

Current *in vivo* gene delivery strategy by means of a BMP gene activated matrix is not suitable for clinical bone regeneration, as the osteoinductive signal is too weak. The theory is simple: the pDNA vector has to enter the nucleus of locally present cells, which express the encoded gene. In reality many additional extracellular and intracellular barriers are present [91,244-249].

Can we improve current strategy? Some of the hurdles discussed above can be overcome by adjusting the transfection strategy. In theory, the release pattern of the pDNA can be optimized to better coincide with the presence of target cells. By altering the crosslinking density of the hydrogel or via incorporation of the pDNA in nanoparticles the release pattern can be tailored [16,111,145]. Nevertheless, knowledge is lacking about optimal release patterns to better coincide with the presence of endogenous target cells. Furthermore, DNA vectors can be optimized to increase transfection efficiency, thereby altering the amount of BMP production and possibly the onset of osteogenesis. By stripping the classical plasmid DNA backbone to the bare necessities 'mini cirle' DNA has been created, which contains only the therapeutic gene of interest and regulatory sequences. Minicircle DNA has already displayed greatly improved transgene expression in several *in vivo* studies [250,251]. Other hurdles include the lack of transfectable cells in the defect area. Local release of soluble factors to attract MSCs to the defect area, such as CXCL12, might result in an improvement of the osteogenic signal [252]. Nevertheless, other issues are inherent to the *in vivo* strategy and have to date no solution such as active degradation of the pDNA by the immune system and the lack of adequate target cells.

In summary, we must conclude that with these results with respect to osteogenicity, current cell-free *in vivo* strategies are too weak for translation to the clinic. When the above-mentioned improvements are applied, it remains questionable if the osteogenicity of *in vivo* non-viral strategies will be strong enough to be of any clinical relevance.

BMP-gene loaded alginate hydrogel with MSCs

Anticipating on the *in vivo* gene delivery strategy to be little effective with respect to bone formation other aspects of the gene therapy were investigated simultaneously. Constructs were equipped with cells to function as a recipient for transfection, which provided the following benefits: the release pattern of pDNA did not have to be timed with cell invasion. Furthermore, recipient cells could be chosen based on their ease of transfection, compared to the cells present in the implant location, which are generally post mitotic and therefore hard to transfect. Simultaneous seeding of cells and pDNA enables the gene of interest to reach the target cells before degradation by the host immune system.

In previous work MSCs have proven to be good candidates. *In vivo* pDNA-GFP loaded alginate hydrogels seeded with MSCs resulted in transfection percentages of up to 80% [117]. A pDNA-BMP-2 GAM combined with goat MSCs resulted in bone formation in subcutaneous pockets in nude mice [169]. Important to emphasize is: when the implants are small, as is generally the case in small animal models, cell-based gene therapy leads to positive results that are hard to reproduce in larger constructs. With MSCs, the observed bone formation might not only be the result of autoinduction by the transgenic BMP, but also from secondary, untransfected MSCs targeted by the produced BMP. In clinically relevant sized implants, applied in human patients or in large animal models, the survival and role in bone formation of the added cells is still unclear, but is clearly less extensive compared to small rodent models. Therefore we aimed to investigate to what extent seeded MSCs contribute as producer of transgenic BMP and to what extent they actually participate in osteogenesis.

Role of seeded cells

In *chapter 2* we investigated the contribution of MSCs in current gene delivery strategy. *Do MSCs induce bone formation by producing transgenic BMP or do they also actively participate in osteogenesis by engraftment and differentiation toward osteoblasts?* To answer this question, we compared the effect of MSCs with that of skin fibroblasts in chapter 6, as the latter did not show osteogenic differentiation potential *in vitro*. Transfection with pDNA-GFP showed both cell types to be transfectable in a comparable manner. *In vitro*, transfection with pDNA-BMP-2 resulted in a higher BMP-2 production in the MSC condition compared to the fibroblast condition. This could be explained by both the autocrine effect of

transgenic BMP-2 on MSCs, which stimulates their differentiation, and the endogenous BMP-2 production (as a result of the induced differentiation), which supplements the transgenic BMP-2. However, differences in BMP-2 production by the two cell types cannot be confirmed by our data, as the used BMP-2 ELISA does not distinguish between transgenic and endogenous BMP-2. The *in vitro* BMP-2 production could not be associated with bone formation: bone was only observed in the positive control condition with added BMP-2.

The absence of bone was surprising since MSCs previously had been shown to be able to produce bone tissue, namely in a similar rat model without the intervention of gene therapy [129]. A possible explication of this aberrant outcome in cell performance may be sought in the process of cell isolation and culturing before implantation, which is known to strongly influence the osteogenic signal [54,55]. On top of this, the osteogenic stimulus of the combination of MSCs and pDNA-BMP-2 did not appear strong enough to induce bone. The contrast with previous work is striking. Then abundant bone formation was observed, using a similar study design, using the same alginate batch, the same pDNA-BMP-2 and the same BCP, but goat MSCs in a nude mouse model [117,169]. The frequent presence of multinucleated giant cells in our immunocompetent rat samples is an indication of a strong foreign body response, which has effects on pDNA clearance [241]. The reason for the absence of bone in the current rat model might be attributed to an active clearing of the pDNA by the host immune system.

Even though this study has its limits, we can conclude with reasonable firmness: in an ectopic environment, current alginate GAM based BMP-2 gene delivery strategy with addition of MSCs shows too little osteogenicity to be of interest for clinical translation.

Bone formation in an orthotopic environment

In *chapter 3* we investigated the contribution of cell-seeding to gene-activated matrix induced osteogenicity in an orthotopic environment. The effectivity of pDNA-BMP-2 gene loaded alginate hydrogel with or without the addition of MSCs was investigated in the iliac bone of goats. The cylindrical constructs were shielded from direct bone contact from the sides, preventing cell recruitment and tissue ingrowth, whereas from the underlying bone marrow and from the tissue covering the constructs ingrowth was still possible.

In this study bone formation appeared to be dependent on cell seeding: gene delivery with addition of MSCs resulted in higher bone volumes than the cell-free gene delivery condition, and was accompanied with faster ingrowth from the perimeters of the implant throughout the scaffold. We assume that the BMP-2 gene delivery to MSCs was efficient and resulted in local presence of BMP-2 which provided an osteogenic stimulus, resulting

in bone formation. But as a condition with seeded MSCs that were not treated with gene delivery was lacking, the added osteogenic effect of seeded MSCs could not be assessed. Moreover, in previous work in comparable orthotopic models did show an added effect of seeded MSCs that were not treated with gene delivery compared to control conditions that not contained cells. The ingrowth speed in which bone was formed from the perimeter of the implant was faster in cell-based conditions compared to control [177,179,253]. After 9 weeks *in vivo* more bone was found in cell based samples whereas this effect diminished after 12 weeks [254].

Therefore, the amount of bone formed is might be the result of temporary BMP production by the cells, but the attribution of MSCs to bone formation cannot be ruled out.

Interestingly, in current application the size of the defects exceeded the critical diffusion distance of oxygen and nutrients - estimated to be several tenths of a millimeters - making the survival of the majority of the MSCs unlikely [255]. Therefore the role of seeded cells is arguably mainly a temporary one, namely as producer of pro-osteogenic factors, which are presumably partially the result of BMP gene delivery.

Construct porosity

When scaffold size exceeds several millimeters, cell survival within the scaffold becomes problematic. By adding porosity to the scaffold, diffusion distances are reduced and oxygen and nutrient supply ameliorated [64].

In *chapter 4* we showed the possibility to fabricate constructs using 3D fiber deposition, resulting in alginate hydrogel constructs loaded with both ceramic particles and MSCs and pDNA-BMP-2. The 3D geometry of the printed constructs proved highly reproducible.

When printing cell-seeded structures, hydrogel properties have to be adjusted to the printing process and the presence of living cells: the gel should be quickly polymerized to retain its shape directly after printing, its stiffness should be suitable for the envisioned tissue type, and should have minimal cytotoxicity. The concentration of alginate polymer correlates with its stiffness and thus to the shape retention of the bioprinted construct. A disadvantage of alginate is that by increasing the polymer concentration the transfection efficiency decreases. The optimal concentration had to be found: by pre-crosslinking the gel before printing alginate polymer concentrations as low as 3% resulted in a bioprintabele construct. The construct remained intact during several weeks *in vitro* culture and could be handled and implanted without losing its integrity. This alginate polymer percentage is much lower than previously used in 3D alginate printing [64].

In vitro, alginate concentrations of 3% (w/v) appeared to be the most suitable, resulting in transfection efficiencies greater than 30%. Furthermore, adding porosity resulted in higher BMP-2 production and more osteogenic differentiated cells compared to solid constructs. However, when implanted in a subcutaneous nude mouse model, no bone was formed. The absence of bone could be explained by the increased stiffness due to a denser network as result of a higher alginate concentration. Previous work with the same cell source and exactly the same nude mouse model, source of alginate, BCP scaffold and pDNA, but using 1% (w/v) of alginate resulted in >20% bone formation [169]. The increase of alginate polymer concentration and consequently the denser network affects the release pattern of protein: the majority of the produced BMP stayed entrapped within the hydrogel during the (two weeks) *in vitro* culture. The effect of increased crosslinking was clearly visible, where patches of undissolved alginate were frequently observed after 5 weeks implantation.

To conclude: we were able to bioprint porous constructs and maintain the printed shape up to several weeks. The alginate concentration was reduced to only 3% without losing printability. Nevertheless, regarding bone formation, the alginate polymer concentration was arguably still too high. This makes the bioprinting 'window of opportunity' very small: at low viscosity the integrity of the 3D printed constructs is easily lost, whereas using high alginate concentrations interferes with transgene expression. It remains questionable whether bioprinted gels a stand-alone scaffold will ever become clinically applicable, since they provide little biomechanical support, the production process is elaborate, and cell performance within printable gels is compromised [256].

Conclusions regarding BMP-gene loaded GAM with and without MSCs

The results with respect to BMP-2 gene loaded GAM induced bone formation can be summarized as follows. Cell-free approaches for gene therapy have not resulted in bone formation, to date. With added MSCs the induction of bone formation varies:

1) In the subcutaneous location in a nude mouse model bone formation was observed repeatedly, varying between 4% and 30% [117,169,252].

2) In the intramuscular location in normothymic rats no bone was found (chapter 2).

3) In iliac crest defects in goats the MSCs with the BMP-gene loaded GAM exhibited an added effect on bone formation compared to the cell-free approach (chapter 3).

4) Increasing the concentration of alginate hydrogel in the GAM to allow bioprinting resulted in osteogenic differentiation but not in bone formation (chapter 4).

Conclusions that can be drawn from these observations are:
First, these studies indicate that reproducibility of animal experiments is sensitive to numerous probably small variations, which may lie in the batch of animals (feeding regimes, exposure to pathogens or stress) or stability of the components of the implant. The latter has become apparent when reproducing some of the *in vitro* work with different batches of alginate. The efficacy of the gene delivery appeared to be easily disrupted, as shown by the quick decrease in transfection efficiency and resultant osteogenicity with higher polymer concentrations.

Second, if the use of cells is needed for gene delivery strategies to induce bone formation, then any *ex vivo* transfection method can be applied. This makes it attractive to consider other strategies that generally result in even higher efficiencies.

As said, we have observed a decrease in transfection efficiencies of MSCs in both the *in vivo* studies as well as our *in vitro* work. It is very likely that the quality of the alginate has decreased over the years, thereby reducing its capacity to induce transfection. Replication of experiments with the same alginate has resulted in a variety of transfection efficiencies. An even larger variety in transfection efficiency is found when different alginate batches are compared. These findings are not surprising as alginate is a natural product, with variable composition and impurities, which might influence the transfection efficiency. Stability of the dry product with respect to transfection efficiency should therefore be reevaluated.

All in all, the alginate GAM is not a gene delivery strategy with perfect reproducibility, the osteogenic signal *in vivo* is rather weak, and its use has several drawbacks. Therefore, the remainder of the discussion will be focused on *ex vivo* gene delivery to cells, using different transfection strategies. Furthermore the exact role of the MSC should be investigated, as in this work, their role in the bone formation process remained unveiled.

Ex vivo BMP-gene delivery

The continuation of our research consisted of comparing the most promising gene delivery strategies chosen on the basis of their gene transfer efficiency, cytotoxicity to the transfected cells, duration of transgene expression, applicability to both primary and differentiated cells and safety profile. The used data have been derived from literature as well as our own work.
In unpublished work we compared the previously described alginate based transfection with both nucleofection (a type of electroporation) and liposome transfection. Nucleofection of MSCs with pDNA-GFP resulted in high transfection efficiencies and corresponding large amounts of transgenic protein. In a rat model, bone formation was greater with nucleofected MSCs that were transfected with pDNA-BMP than MSCs treated with liposome gene delivery or MSCs in combination with an alginate GAM.

Optimizing the *ex vivo* gene delivery

In *chapter 5* we describe the optimization of the nucleofection process, which is essentially a form of electroporation creating small pores in cell membranes by applying electrical pulses. The electroporation procedure has several advantages: it is quick, the electroporation itself takes only milliseconds and the target cells can conveniently remain suspended in a test tube. Transfection buffer as well as transfection program, cell concentration and pDNA concentration influence the transfection efficiency. These factors have to be optimized for each cell type. Nucleofection might be suitable for clinical practice, as large amounts of cells can be effectively treated in a small timeframe.

We made use of the ability to create BMP heterodimers by co-delivery of plasmids coding for BMP-2 and BMP-6 or BMP-7 to MSCs. BMP heterodimers are known to possess stronger biological activity than the corresponding homodimers [36-39]. *In vivo*, co-delivery of BMP isoforms to MSCs resulted in significantly higher bone volumes compared to control. Other researchers have also demonstrated this effect, using the combination of BMP-2/7 with viral vectors [36-39].

In MSC control conditions as well as in pBMP-2 and pBMP-7 single gene conditions, bone formation was absent. This could mean that the nucleofection procedure has some negative effect on MSCs, as their intrinsic osteogenicity appeared to be decreased. Apart from the diminished osteogenic effect of the MSCs, the levels of produced BMP-2 and BMP-7 homodimers were probably inadequate. A possible explanation is a rapid decline in protein production upon implantation due to lack of nutrients and oxygen for the MSCs, compared to the well-nourished and oxygenated *in vitro* situation.

Taking into account all the above-mentioned results, it is questionable whether non-viral gene delivery of a single BMP isoform is robust enough to ensure adequate BMP production in clinical relevant models. Sporadically BMP production of up to 20 nanograms per 10^6 cells per day has been shown [125], whereas, by using viral transfection strategies BMP production of 200 nanograms per day per 10^6 cells can be reached [157]. The latter was shown to be adequate for spinal fusions in rats [257].

In conclusion, nucleofection of BMPs to MSCs is an interesting candidate for clinical bone regeneration, as its application is easy, efficient, and it shows bone formation in a small animal model. Furthermore, some limitations of gene therapy can be alleviated by co-delivery of certain BMP isotypes, which result in formation of the more potent BMP heterodimers. Before translation into clinically relevant models becomes realistic, possible limitations have to be further investigated, namely the role of the recipient cell (as discussed in the next part), and the adequacy of BMP release: the amount and pattern of produced protein.

The role of the 'transgenic' cell

In *chapter 6* both roles of MSCs were investigated: as producer of transgenic BMP (for other cells) and as osteoblast precursor. We showed evidence that bone formation by BMP transfected non-osteogenic cells –*fibroblasts*– was as effective as bone formation by transfected MSCs. As we showed that fibroblasts are not susceptible for BMP stimulation, this study indicates that the transplanted cells may serve as an instrument for BMP secretion only. Therefore osteogenic differentiation and engraftment of the seeded cells is not a prerequisite for bone formation. This finding has only been shown in one other study in which viral transfection methods were used [157].

As the osteogenic effect of this *ex vivo* gene delivery strategy does not rely on engraftment of cells we consider this strategy to be possibly feasible in the clinical context. Nevertheless, to ensure adequate BMP production locally, temporary survival may be a prerequisite. The local survival time after implantation of the seeded cells might be longer in small animals and therefore remains to be investigated in larger models. Furthermore, caution should be exercised when translating these results to a clinical setting. Only 1.5 µg of rhBMP-2 was sufficient to induce bone formation in the rat and mouse animal models. The BMP dosages as a result of gene expression resulting in bone formation have been even lower. In the clinic much higher dosages are applied. Although no direct comparison with the clinical setting can be made, the dosages used in current clinical applications are illustrative: products vary between 1.9 to 4 milligram of rhBMP-2 [26-28]. It remains unclear if cells will be able to produce adequate amounts of transgenic protein via *ex vivo* gene delivery.

Is a single stage procedure using *ex vivo* gene delivery feasible?

If bone formation is the result of temporary excretion of BMPs, in theory any cell can be harnessed to produce protein. In the second part of *chapter 6* we showed that cells derived from fat tissue and bone marrow can be isolated, transfected and implanted within a single day, and that these cells are able to induce bone *in vivo*. The explantation and re-implantation was done in no more than 4 hours and the cells were harvested from syngeneic animals. Nevertheless, to perform the procedure within the duration of a regular spinal surgery, such as the posterior fusion of two lumbar vertebrae, is technically possible. The harvesting of the cells and subsequent implantation can be performed within two hours as shown by Helder et al [227]. Adding to this, the electroporation procedure of the cells gives only a few minutes delay. Provided that further optimization steps are done, this single stage procedure seems to be within reach. It can replace the costly and time-consuming culture expansion of stem cells and reduces the risks associated with a second

operation seen with the classical two-step approach [258,259]. It remains to be seen if fibroblast produced BMP heterodimers lead to functional bone repair in an orthotopic setting.

GENERAL CONCLUSIONS

Despite the apparent benefits of cell-free BMP gene delivery method, a cell-free strategy proved inadequate for clinical bone regeneration. Most difficulties encountered with our approach can be extrapolated to all *in vivo* strategies, such as the activation and active pDNA clearance by the immune system and the reluctance of the locally present cells to get transfected. With current knowledge and experiences we can state that *in vivo* gene delivery strategies have very little chance of becoming feasible in the clinic.

Secondly, despite promising *in vitro* results, the results of alginate-based delivery with added MSCs in rats were disappointing, as no bone was formed. On the contrary, the orthotopic goat study provided some proof of increased bone formation by gene delivery with MSCs. These two contradictory outcomes could be coincidental or could indicate a differential role of the MSCs. Our tentative conclusion is that the stimulus provoked by alginate based BMP-2 gene delivery with MSCs, though showing osteogenicity, was too weak to provide potential for clinical application.

Finally, to improve the osteogenicity of current strategies, MSCs were genetically manipulated *ex vivo* to overexpress combinations of BMPs. The bone in these studies proved to be the result of BMP production (partially consisting of heterodimers), and did not depend on the osteogenic capacity of the producer cells: BMPs acted in a paracrine manner, inducing the host cells to form bone. These findings indicate that gene therapy has potential to be applied in a single-stage procedure, which we have shown in the last study presented in this thesis.

The aim of this thesis was *to investigate the suitability of non-viral gene delivery of BMPs as a means to regenerate bone tissue in clinical applications.* For now we can state that an *ex vivo* non-viral gene delivery through a combination of BMPs applied to autologous cells or allogeneic MSCs has the potential to become clinically applicable.

FUTURE PERSPECTIVES

The *ex vivo* gene delivery to autologous or allogeneic cells should not be considered as a cell-based strategy depending on the osteogenic effect of the cells. Cell-based strategies have repeatedly failed over the past decades. Instead current gene delivery strategies should be considered as an alternative way to induce bone formation via soluble factors. The intended effect lies in the temporal overexpression of pro-osteogenic growth factors, by the implanted cells, which can have a catalyzing effect on the regeneration process of bone tissue.

To improve current *ex vivo* gene delivery strategies, we have identified important issues:

Increasing the effectivity of the osteogenic signals: the combinations of, and the ratios of the different BMP genes can be optimized to provide an optimal osteogenic signal. Combinations with other growth factors can be made, comparable to the orchestra of growth factors that are observed in natural bone healing (see Introduction, table 1). Besides optimizing the types of growth factors released, the timing of growth factor release should be considered, as in natural fracture healing some factors are present within minutes, others within hours and some factors are expressed after weeks. To mimic the natural bone healing environment, a combination of timed release strategies can be made: gene expression combined with controlled delivery of certain proteins [252].

Increasing the amount and duration of protein synthesis: the main hurdle of all non-viral gene delivery strategies preventing progression towards the clinic is the lack of sufficient gene expression. Further research should focus on epigenetics: which factors influence gene expression over a prolonged period? And how can we ensure a longer presence of the pDNA in the cell, preventing it from degradation? Other issues that could be addressed are strategies that can extend cell survival upon implantation, with as main goal prolongation of protein synthesis. Recently, oxygen-releasing materials have shown to improve *in vivo* cell survival after implantation [260]. Furthermore the added value of adding different kinds of macroporosity to the implant could be further investigated [64].

Test the clinical feasibility in a meaningful way: to properly test improved bone formation of the newly developed gene delivery strategies, an early switch to more demanding models is needed: investigate gene therapy in a large animal model, in critical sized bone defects, in which long-term cell survival and engraftment is not plausible [261]. When small animal models are used it should be kept in mind that the results can be misleading as cells might survive longer than they would in clinical applications. For bone regeneration, an experimental setup that resembles the clinical situation is in my view only possible in large animal models.

Appendices

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LIST OF ABBREVIATIONS

α-MEM	alpha minimal essential medium
AAV	adeno associated virus
Ab	antibody
ALP	alkaline phosphatase
Asap	L-ascorbicacid-2-phosphate
BCP	biphasic calciumphosphate
bFGF	basic fibroblast growth factor
BM	bone marrow
BMP	bone morphogenetic protein
BSA	bovine serum albumin
BTE	Bone Tissue Engineering
cDNA	complementary deoxyribonucleic acid
DAB	Diaminobenzidin
DBM	Demineralised bone matrix
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ELISA	enzyme-linked immuno sorbent assay
EM	expansion medium
EPC	Endothelial progenitor cell
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FCS	fetal calf serum
FDA	food and drug administration
GAM	gene activated matrix
GFP	green fluorescent protein
HA	Hydroxyapatite
H/E	hematoxylin/eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
IGF-1	insulin like growth factor-1
i.m.	intra muscular
MFI	mean fluorescence intensity
miRNA	micro ribonucleic acid
(p)MMA	(poly) methylmetacrylate
MSC	multipotent stromal cell

OCN	osteocalcin
OM / ODM	osteogenic (differentiation) medium
PBS	phosphate buffered saline
pDNA	Plasmid DNA
PEI	polyethylenimine
PNPP	p-nitrophenyl phosphate
PTH	parathyroid hormone
rhBMP-2	Recombinant human BMP-2
RNAi	ribonucleic acid interference
RT	room temperature
s.c.	subcutaneous
SDF-1α	stromal cell derived factor-1α
siRNA	short interfering ribonucleic acid
TBS	Tris buffered saline
TCP	tricalcium phosphate
TE	tissue engineering
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-α
VEGF	vascular endothelial growth factor

NON-VIRALE GENTHERAPIE MET BMP-2 VOOR BOTREGENERATIE

Introductie

Als strategie om wervels te fuseren en grote botdefecten op te vullen wordt momenteel vooral gebruik gemaakt van het transplanteren van lichaamseigen bot. Het "oogsten" van bot voor de transplantatie verloopt echter niet probleemloos; de patiënt houdt vaak chronische pijn op de plaats van wegname en de hoeveelheid bot is veelal ontoereikend voor het beoogde doel [2,3]. Er is dan ook grote behoefte aan nieuwe strategieën voor botaanmaak.

Ondanks decennialang onderzoek naar alternatieven is het resultaat teleurstellend; slechts enkele methoden hebben hun weg gevonden naar de kliniek [4,5]. Veelbelovend is het gebruik van biologisch actieve eiwitten, de bone morphogenetic proteins, zoals BMP-2 en BMP-7 [6,7]. Deze eiwitten kunnen stamcellen aanzetten tot differentiatie naar bot producerende cellen. Ze worden lokaal geïmplanteerd door een sponsje doordrenkt met deze BMPs op de gewenste locatie aan te brengen. Het resultaat, de vorming van bot, is veelal gelijk aan dat van bot transplantaties. Om deze reden is toepassing van BMP een goed alternatief [8-10]. Het grote nadeel is echter het risico op levensbedreigende complicaties [11-13]. Het vermoeden bestaat dat deze complicaties veroorzaakt worden door de extreem hoge dosering eiwitten die nodig is omdat de eiwitten uit de spons weglopen en zeer snel worden afgebroken in het lichaam.

Gentherapie is een veelbelovende methode om de positieve eigenschappen van BMPs te behouden en de negatieve te voorkomen. Hierbij wordt het BMP-gen (pDNA-BMP-2) in cellen, zowel in het lichaam als *in-vitro* ingebracht, waar het wordt afgelezen en waar vervolgens het betreffende eiwit wordt geproduceerd (genexpressie). Vergeleken met het direct toedienen van BMPs is het voordeel van gentherapie dat er een kleine hoeveelheid BMP over een langere periode geproduceerd wordt. Daarnaast kan pDNA in tegenstelling tot BMP zeer lang bewaard worden; het is zowel in als buiten het lichaam zeer stabiel. Bovendien is pDNA makkelijk en goedkoop te produceren [14,15].

Er zijn virale en non-virale strategieën om het BMP-gen (pDNA-BMP-2) op de gewenste locatie tot expressie te brengen. Virale strategieën leiden weliswaar tot een hogere eiwit productie, maar brengen het risico op hevige immuunreacties en de kans op schadelijke mutaties met zich mee[16,17]. Non-virale gentherapie is veilig, maar heeft als grote nadeel de geringe eiwitproductie. Deze is veelal het gevolg van een moeizaam transfectieproces (het proces van intrede van pDNA in de cel). Het lichaam heeft immers natuurlijke barrières om lichaamsvreemd materiaal te weren [18,19].

In het algemeen gesteld richt dit proefschrift zich op: Het onderzoek van de mogelijkheid om in de kliniek botvorming te induceren middels non-virale gentherapie met BMP-2.

In de loop van de jaren zijn diverse non-virale methoden ontwikkeld om cellen te transfecteren. Zo is er een methode waarbij DNA door fysieke perforatie van de cel wordt ingebracht. Een andere methode maakt gebruik van polymeren om DNA in de cel te brengen. Voor beide geldt dat de klinische toepasbaarheid voor botregeneratie nog onduidelijk is. In voorgaande jaren ontwikkelde onze onderzoeksgroep een methode voor gentherapie met gebruikmaking van alginaat hydrogel. Deze gel bezit de potentie cellen te transfecteren en is bovendien zeer geschikt voor biologische toepassingen.

In dit proefschrift testen wij deze transfectiemethode, al dan niet gecombineerd met het inbrengen van extra (stam)cellen op de klinische haalbaarheid. De celvrije toepassing heeft onze voorkeur. Vervolgens onderzoeken wij gentherapie in combinatie met celtransplantatie. In deze strategie fungeert de cel niet alleen als producent van BMP maar transformeert hij in theorie tevens tot botcel.

Celvrije gentherapie

H*oofdstuk 2* beschrijft de toepassing van lokale gentherapie zonder tegelijkertijd cellen te implanteren. Het doel is de lichaamseigen cellen ter plekke te transfecteren en BMP te laten aanmaken om zo het botvormingsproces in gang te zetten. In een diermodel wordt door middel van een alginaat hydrogel een grote hoeveelheid pDNA aangebracht. Wij tonen aan dat lichaamseigen cellen het pDNA opnemen en tot expressie brengen.

Om de grootst mogelijke genexpressie te bereiken zoeken we de optimale dosis pDNA. Toch lukt het niet om botvorming te induceren. Weefselonderzoek in het gebied van de implantatie toont de aanwezigheid van een grote hoeveelheid cellen van het immuunsysteem. Mogelijk werd het pDNA grotendeels opgeruimd door het immuunsysteem. Bovendien is uit eerder onderzoek gebleken dat het volwassen karakter van lichaamseigen cellen het transfectieproces in de weg staat. Aanpassingen van de methode zullen bovenstaande problemen niet of nauwelijks kunnen verhelpen, waardoor er weinig zicht op significante verbetering van genexpressie. Gezien de huidige gentherapie mogelijkheden is deze methode dan ook niet geschikt voor de kliniek.

Gentherapie met toegevoegde cellen

Aangezien bovengenoemde celvrije aanpak niet effectief bleek, kozen we ervoor een alginaat hydrogel te gebruiken en hieraan stamcellen toe te voegen. Eventueel ontstane botvorming kan het resultaat zijn van BMP-2-productie als gevolg van gentoediening, maar ook van differentiatie van stamcellen tot osteoblasten (botvormende cellen) die het implantatieproces hebben overleefd en actief bot vormen. In eerder onderzoek bleek deze

overleving van getransplanteerde cellen in diermodellen mogelijk, maar in de kliniek onwaarschijnlijk. Dit kan worden toegeschreven aan de grootte van het botdefect en de tijdsduur nodig voor vascularisatie, waardoor mee-geïmplanteerde cellen uiteindelijk niet overleven.

In *hoofdstuk 2* bespreken we de rol van de stamcellen in de alginaat-gentherapie. We maken een vergelijking met bindweefselcellen die qua transfectie vermogen en BMP-productie gelijkwaardig zijn maar die niet kunnen differentiëren tot osteoblast. *In-vitro* blijken beide celtypes na adequate transfectie in staat BMP te produceren. In de dierproef echter blijken beide groepen niet in staat bot vorming te realiseren . De onderzoeksvraag blijft hierdoor vooralsnog onbeantwoord.

In *hoofdstuk 3* bespreken we de alginaat- gentherapie met en zonder toevoeging van stamcellen, maar nu in een klinisch relevant diermodel: een centimeter diep botdefect in de bekkenkam van de geit. Zonder toegevoegde cellen is de botvorming gelijk aan die van de negatieve controle groep, dus verwaarloosbaar. Mét toevoeging van stamcellen daarentegen is de hoeveelheid gevormd bot en ook de ingroeisnelheid groter dan in de controlegroep. Mogelijk is dit het resultaat van een tijdelijke BMP-2 productie door getransfecteerde cellen, welke een lokaal effect bewerkstelligen op de cellen in de omgeving. Echter een actieve rol van de geïmplanteerde cellen zelf, als osteoblast, is niet uit te sluiten [24,25].

In voorgaand onderzoek is aangetoond dat getransplanteerde cellen beter functioneren door het aanbrengen van porositeit in de implantaten [26]: In een blokje van enkele kubieke centimeters worden enkele verticale kanalen opengelaten waardoor de diffusie-afstand van zuurstof en nutriënten gereduceerd wordt.

In *hoofdstuk 4* bespreken we de invloed van porositeit op gentherapie en dus op botvorming. Poreuze implantaten blijken een grotere productie van BMP-2 op te leveren dan niet-poreuze. Overigens blijkt het grootste deel van het geproduceerde eiwit in het alginaat netwerk gevangen te zitten. Dit wordt waarschijnlijk veroorzaakt door de hoge alginaat concentratie die nodig is om de gel voldoende stevigheid te geven. Een dierproef laat geen botvorming zien. Waarschijnlijk wordt botvorming belemmerd door de hoge concentratie alginaat. Weefselonderzoek laat inderdaad grote delen onopgelost alginaat zien.

Samenvattend: de potentie van pDNA-BMP-2 geladen alginaat hydrogel gentherapie om bot te vormen valt tegen en de rol van stamcellen in het botvormingsproces blijft onduidelijk. Ook blijkt de botvorming bij deze vorm van gentherapie gemakkelijk verstoord te worden, zoals in deze experimenten: door het immuunsysteem en door een hoge concentratie alginaat.

Optimalisatie van gentherapie met cellen

Vanwege de bevinding dat voor een effectieve BMP-productie cellen nodig zijn, zochten we de meest effectieve methode om buiten het lichaam cellen te transfecteren. We vergeleken enkele methoden van gentherapie, waaronder alginaat-toepassing en nucleofectie, zowel *in-vitro* als in een dierproef. Nucleofectie is een transfectie-technologie die tijdelijk doorgang verleent aan pDNA door kleine gaatjes in de celmembraan. Wij tonen aan dat deze methode superieur is.

In *hoofdstuk 5* bespreken wij de optimalisatie van het nucleofectie proces. Daarnaast tonen we aan dat pDNA-BMP-2 gecombineerd kan worden met zowel pDNA-BMP-6 als pDNA-BMP-7. Deze combinatie leidt tot de vorming van combinatie BMPs met een sterkere osteogene stimulus en uiteindelijk meer botvorming dan de controle groep.

In *hoofdstuk 6* bespreken wij, evenals in *hoofdstuk 2,* het onderzoek naar de rol van de stamcellen door deze te vergelijken met bindweefselcellen, maar nu met een effectievere transfectie methode. Stamcellen en bindweefselcellen blijken *in-vitro* gelijke hoeveelheden BMP te produceren. Bij stamcellen leidt deze BMP-productie tot differentiatie naar botvormende cellen; bij bindweefselcellen niet. In een dierproef wordt in de groep stamcellen evenveel botvorming aangetroffen als in de groep bindweefselcellen. Hieruit kan geconcludeerd worden dat botvorming in deze studie een gevolg is van lokale BMP-productie en dat de botvormende capaciteit van cellen geen voorwaarde is.

Wetende dat de geïmplanteerde cel fungeert als een tijdelijke BMP-producent, onderzochten we de mogelijkheden om andere, gemakkelijker te oogsten celtypen te gebruiken, cellen die idealiter in één enkele procedure geïsoleerd, getransfecteerd en geïmplanteerd kunnen worden. We laten zien dat het mogelijk is, om in een paar uur tijd: de cellen uit zowel vetweefsel als beenmerg te isoleren, middels nucleofectie te behandelen en tenslotte te implanteren. Met als resultaat: botvorming.

Conclusie

In dit proefschrift tonen wij aan dat het gebruik van cellen voorwaarde is om een zodanige hoeveelheid eiwit te produceren dat botvorming tot stand kan komen. Een combinatie van genen resulterend in een combinatie van BMPs blijkt een sterkere stimulus dan een enkel gen. Het onderzoek laat zien dat meerdere celtypen als producent van eiwit kunnen fungeren en botvorming kunnen induceren. Daarnaast blijkt het proces van cel-isolatie, transfectie en implantatie te reduceren tot enkele uren en mogelijk toe te passen in een enkele operatie.

Wat op korte termijn de aandacht verdient is het toetsen van bovengenoemde ‘single procedure’ strategie in een groter diermodel. De mogelijkheid van cellen om voldoende

BMP te produceren zal waarschijnlijk de beperkende factor zijn voor verdere toepassing in de kliniek.

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CURRICULUM VITAE

Loek David Loozen werd geboren op 21 maart 1986 in Amsterdam. Na de middelbare school (Stedelijk Gymnasium, Haarlem) startte hij de opleiding geneeskunde aan de Universiteit van Utrecht. Tijdens zijn studie nam hij deel aan onderzoek op de afdeling Sportgeneeskunde van het UMC Utrecht onder begeleiding van dr. M. Moen en prof. F. Backx. Na zijn afstuderen aan de faculteit geneeskunde in 2011 werkte hij als promovendus aan het onderzoeksproject 'Gene-delivery strategies for bone regeneration' onder supervisie van prof. W.H.A. Dhert, prof. F.C. Öner, dr. J. Alblas en dr. M.C. Kruyt. Het onderzoek resulteerde in verschillende wetenschappelijke publicaties waarop dit werk is gebaseerd. In 2016 begon hij een zesjarige opleiding tot orthopedisch chirurg in regio Noord-West.