NON-VIRAL GENE THERAPY FOR BONE TISSUE ENGINEERING

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Non-viral gene therapy for bone tissue engineering
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GENERAL INTRODUCTION AND AIMS OF THIS THESIS

INTRODUCTION TO BONE TISSUE ENGINEERING

Bone is one of the few tissues capable of renewing and healing itself. However, to heal large defects, such as complicated fractures or spinal fusions, autologous or allogeneic bone grafts may be necessary to support the new bone formation. With 2.2 million bone grafting procedures performed annually worldwide, bone is the second most transplanted tissue after blood [1].

Autograft has several advantages in stimulating new bone formation. It contains all the elements necessary for bone repair such as osteoconductivity, by providing extracellular matrix with suitable compliance for bone to grow into and growth factors to stimulate osteoinduction and vascularization of the newly formed bone. In addition, it provides living cells fundamental to osteogenicity. This combination of scaffold, growth factors and cells is very successful at healing bone defects. However, disadvantages such as limited availability, donor site pain, longer operating procedures, and possible non-unions demonstrate the necessity of alternatives [2 3]. Allograft can overcome some of these issues but presents an increased risk in transmitting diseases, and has limited active cells and growth factors due to the production process which includes freezing and sterilization [4].

Tissue-engineered bone constructs represent a possible alternative for auto- or allograft bone repair. Classical bone tissue engineering combines osteogenic cells, such as multipotent stromal cells responsible for bone matrix deposition, with osteoinductive growth factors and an osteoconductive or -inductive scaffold providing a framework in which bone formation can occur [5 6].

In the following paragraphs these three crucial components will be discussed more in depth.

MULTIPOTENT STROMAL CELLS

One of the most studied cell types for bone tissue engineering is the multipotent stromal cell (MSC), also known as mesenchymal stem cell. These cells were discovered for the first time almost 40 years ago in murine bone marrow. It was found that bone marrow contains a population of adherent colony-forming cells which differed from hematopoietic stem cells, and were able to reconstitute a hematopoietic environment [7 8]. Later it was shown that these cells were able to differentiate along three main cell lineages: the adipogenic, chondrogenic and osteogenic lineages [9]. It also became clear that MSCs could not exclusively be isolated from bone marrow [10 11], but are also present in adipose tissue [11], dental pulp [12], umbilical cord [13] and cord blood [14], synovial membranes [15] and placenta [16]. To be able to compare different studies using MSCs, more systematic research has been performed leading to minimal criteria describing MSCs. In short MSCs are plastic adherent, express CD73, CD90 and CD105, and lack expression of hematopoietic, leukocyte and monocyte markers such as CD14, CD34 and CD45. However their main feature is the ability to differentiate towards at least all three previously described cell types [9].

As the characterization of MSCs advanced it was observed that isolated MSCs as such are a very heterogeneous cell population of which a small fraction contains genuine stem cell characteristics, including multilineage potential, unlimited and asymmetric cell division and repopulating capacity. This led to concerns regarding the name mesenchymal stem cell [9 17 18].

Therefore MSCs are nowadays mainly referred to as multipotent stromal cells [17]. In 2008, Da Silva et al. proposed the now widely accepted theory stating that all MSCs are pericytes, but not all pericytes are MSCs [18]. Although different names are still being used and isolated MSCs are mainly defined by a combination of morphological, phenotypical and functional characteristics, they are a popular cell source within the field of regenerative medicine and tissue engineering. This is due to a combination of factors such as easy isolation procedures from adult tissues (BM aspirate or liposuction), fast proliferation and multipotency. Furthermore these cells are immune privileged making it possible to use allogeneic cells [19], and have the ability to home to injured tissues throughout the body [20 21]. *In vitro* expanded allogeneic MSCs are also capable of locally forming new bone without being rejected [22 23 24].

Although the use of MSCs for bone tissue engineering has provided good results in pre-clinical settings, mainly in ectopic implants in rodents, only a limited number of studies have been published which confirm the relevance of cell-based constructs for human clinical use. The necessity for cell seeding in bone tissue engineering has been debated. [24 25]. It is hypothesized that in orthotopic locations, cells will be recruited from the underlying bone whereas this is not possible in ectopic locations [25]. Therefore implant location is considered one of the main determining factors in the success of cell-based tissue engineering [24]. Furthermore, cellular tracing of implanted MSCs revealed that the long-term cell engraftment is variable, depending on the animal model, implant location and activity of the immune system [26].

MSCs are very suitable to explore the osteogenic potential of materials or growth factors both *in vitro* and *in vivo*. By adding the target cells, in this case MSCs, the biological response to certain growth factors can be investigated, providing clues for optimization of bone inducing constructs, and also for the development of cell-free strategies [27].

GROWTH FACTORS

In bone regeneration several bioactive molecules, including growth factors, are involved. Growth factors are able to initiate a number of processes important to bone formation, vascularization and cell recruitment. By addition of single growth factors to tissue engineered constructs it is possible to stimulate one or more of the above mentioned tissue responses. Well-known growth factors, which are frequently applied in bone tissue engineering are members of the BMP family, VEGF, IGF-I, bFGF and PDGF [28 29 30]. The BMPs were discovered in 1965 by Marshall Urist. He launched the growth factor induced tissue engineering field with the discovery that factors in implanted bone matrix could induce new bone formation ectopically [31]. Approximately 20 years later advances in molecular biology allowed the purification and subsequent cloning of the growth factors involved in bone regeneration. Once advances in recombinant DNA technology made their efficient production possible, research started to focus on the use of growth factors for bone tissue engineering [32 33 34].

Many growth factors are administered locally because they have relatively short half lives *in vivo* and their desired action is at the defect site [35]. Controlled delivery systems are therefore of great interest and have to-date led to a vast number of publications [36 37].

There are two main strategies to deliver growth factors at the desired regeneration site. One is based upon (slow) release of the growth factor protein from a biomaterial such as

polymer gel, or nano-/microparticle. By tailoring the pharmacokinetics it is possible to mimic the growth factor release profile which for example occurs after a bone fracture. During this process of fracture healing, several bone morphogenetic proteins (BMP-2, -4, -6, -7) known to be associated with ectopic osteoinduction are upregulated over a prolonged period of time, with expression levels peaking at 21 days. During this period, MSCs are recruited, and directed into the osteogenic lineage to form new bone [38 39 40 41]. Locally applied controlled release systems have several advantages over systemic delivery, including better retention of the growth factor, which leads to reduction of the total dose applied and thereby reduction of the costs. Challenges lie in the optimization of controlled delivery materials with respect to cytotoxicity, degradation and tissue responses.

Growth factors are often delivered as recombinant purified proteins, but can also be produced on the spot by local application of a gene encoding a growth factor. Incorporation of the DNA encoding the gene into cells occurs either before the cells are implanted or *in situ*. A number of viral and non-viral gene delivery systems have been developed and are all aimed at local expression of the target genes, often growth- or transcription factors. This strategy has several advantages over traditional protein release. First of all DNA is easily manipulated, stable *in vitro* and the isolation is simple, fast and low-cost. It can be used in combination with a number of dedicated delivery systems such as liposomes or viral vectors, which may be tailored to target specific cell types [42] . Furthermore it provides the possibility of expressing a combination of genes from a single cell, such that potent combinations of growth factors can be produced (e.g. BMP-2/7 heterodimers). Secretion of the dimers will then target BMP-receptor positive cells, leading to bone formation [43].

DNA can be transferred via viral or non-viral delivery methods. Viral gene delivery is considered the most efficient way of gene transfer for many cell types. DNA is transported to the nucleus via viral envelope elements and can either integrate into the host genome, leading to a stable expression of the introduced gene, or remain present as an episomal vector, which is gradually lost after cell division. However, the viral envelope proteins can trigger immune responses, and DNA incorporation into the genome can lead to severe side-effects such as oncogenic transformation.

Non-viral gene delivery induces transient expression of the desired protein. For bone tissue engineering this may be very suitable because expression over a short period of time is sufficient to reach the desired effect of osteogenesis. Furthermore, localized delivery of DNA is very feasible, because it shows a high turnover rate in the blood stream which minimizes unwanted diffusion to off-target tissues, there is no limit to the amount of DNA which can be delivered, and gene transfer is efficient in a large number of cell types [44 45 46].

BMP-2

BMP-2 is one of the main growth factors used for bone regeneration. It is part of the transforming growth factor β (TGF-β) superfamily and involved in committing MSCs towards the osteogenic lineage [47]. A large number of studies have addressed the application of several BMPs for bone healing, which has also resulted in widespread clinical application. To ensure enough active protein at the regeneration site, high doses are applied clinically and despite the effectivity of these treatments, concerns regarding the applied

supraphysiological doses have emerged [48 49]. Side effects such as ectopic bone formation around or in the spinal canal, hematoma, soft tissue swelling, inflammation or neural problems due to protein diffusion have been reported for interbody fusions, and ectopic bone formation for fracture healing [50 51]. This has led to extensive discussions about the safety of BMPs, calling for investigation of the complex biology of BMP-2 release, and for further experiments analyzing dose-response [35 52].

In line with these findings our approach is to administer BMP-2 in the form of plasmid DNA either combined with a delivery vehicle such as collagen or alginate, or without such a vehicle. Immediate diffusion of the DNA from the implant site can be prevented and relatively sustained delivery of protein can be achieved [53]. This has been successful at inducing bone formation *in vivo* in animal studies [48 54 55].

SDF-1α

Growth factors are not only used to differentiate MSCs towards the osteogenic lineage, but also to recruit cells towards the site of injury where new bone formation should occur, and to induce vascularization [56 57]. Vascularization is important for the delivery of nutrients, oxygen and cells, and the removal of waste products from the newly formed bone. The chemokine stromal cell derived factor 1α (SDF-1α or CXCL12) promotes vascularization *in vitro* [58 59] as well as *in vivo* [60 61] and facilitates the recruitment of MSCs towards fracture sites. This has been shown to be regulated by the CXCR4-SDF-1 axis [62]. SDF-1α is known to induce homing of hematopoietic cells towards the bone marrow during embryogenesis, and to attract immune cells such as lymphocytes during adult stages. The role of SDF-1α in recruitment of host cells has yet to be investigated properly. Only a few studies show recruitment of endogenous cells to SDF-1α loaded scaffolds [63 64 65].

When SDF-1α is combined with BMP-2, a more positive effect on bone formation and regeneration has been reported when compared with either growth factor alone, leading to improved bone regeneration *in vivo*[66 67]. The mechanism behind this increased bone formation has been described as a combination of enhanced mobilization and homing of bone marrow-derived osteoprogenitor cells to the implant leading to increased numbers of cells available for bone regeneration at ectopic bone implants [68 66 67].

SCAFFOLDS

The third major element of tissue engineering is the scaffold. One of the main functions of scaffolds for bone tissue engineering is to mimic the natural extracellular matrix and provide mechanic stability. This includes providing a stable structure for ingrowth of cells and vessels and offering a source of biological stimuli such as surface characteristics, and growth factor adhesion, necessary for bone tissue formation [69 70 71]. It is important, as with every implant, that a scaffold is biocompatible, meaning that it performs with an appropriate host response. To allow new bone to replace the scaffold, and form a stable bone mass, it is desirable to use a scaffold composition that allows for degradation over time. For bone tissue engineering this process needs to be slow enough to retain sufficient stability while replacing parts of the scaffold with bone. Several features such as pore size and interconnectivity of

the pores can influence cell adhesion, ingrowth and differentiation as well as degradation of the scaffold present in the construct. Porosity also allows vascularization and thereby cell survival within the construct [72]. Tailored calcium phosphates can combine the above mentioned qualities and are therefore popular in bone tissue engineering.

Natural or synthetic polymer hydrogels are also often used as scaffolds in bone tissue engineering. Although these hydrogels lack good mechanical properties they have several other features making them suitable for bone regeneration. Firstly they are able to retain growth factors, DNA and cells [73]. As explained in the previous paragraphs, both seeded cells and growth factors can contribute to osteogenic differentiation or vascularization. Furthermore they have the ability to be injected instead of implanted and the desired shape can be produced by a rapidly increasing technology called '3D bioprinting' [74]. The field of bioprinting focuses on optimizing scaffold design by using a 3D bioprinter. Using this method, cell-, DNA- or growth factor-laden hydrogels are integrated with the so-called rapid prototyping technology, which is based on computer-assisted design and manufacturing of layered structures [75]. A model of the implant is created on a computer, the dispensing material is loaded into the 3-axis robot arm and material is extruded layer-by-layer enabling formation of 3D structures of defined external shape and internal morphology. With this method, porous structures have been printed and tailored to optimize transfection efficiency, growth factor release, possible vascularization and eventually bone formation [76] [77].

A combined use of ceramics and hydrogels creates hybrid scaffolds providing stability and a surface for bone formation to start on, as well as the controlled release and 3D tailoring capacity provided by hydrogels.

Various combinations of scaffolds have been applied in the last decades in experimental and clinical settings for bone tissue engineering. The choice for a specific (combination of) material(s) is often determined by multiple factors such as desired location, addition of seeded cells, release profile of added growth factor, possibility to deliver DNA, or 3D structure.

Calcium phosphates

One of the most common calcium phosphates used for bone tissue engineering is biphasic calcium phosphate (BCP). BCP is a combination of hydroxyapatite (HA) with low biodegradability and tricalcium phosphate (TCP) with high biodegradability. Both HA and TCP are (very similar to) the inorganic components of bone and therefore highly biocompatible. HA crystals, when applied in bone TE constructs, are strong and able to resist mechanical loading, but are poorly resorbed *in vivo*. TCP, on the other hand is rapidly resorbed, but too fragile to sustain mechanical loading. By combining these two materials into BCP at different ratios the mechanical strength and resorbing ability can be tailored [78]. Furthermore, to be suitable for bone tissue engineering, BCP has to contain sufficient mechanical strength to match the compressive strength of bone. This can be altered by changing the porosity for example. Pores are necessary to aid cells to migrate into the scaffolds, support vascularization and allow for diffusion of oxygen, nutrients and waste products. BCP is generally highly osteoconductive, allowing cell migration and thereby new bone formation into the construct and ensuring an intimate bond between scaffold and bone [79] [80] [81]. Some calcium phosphates are osteoinductive in the context of a certain tissue or animal species, where they induce

differentiation of immature cells towards the osteogenic linage. Implantation at an ectopic location, without access to osteoblasts, is often performed to establish the protein binding capacity and osteoinductive nature of a certain material. The question is whether these results can be extrapolated to other locations or animal models [82 83].

BCP is not suitable as weight bearing bone replacement because in contrast with natural bone the collagen component is missing. This makes the material resistant to compression but not to bending and shear stress. Therefore this material is often used in non-weight bearing areas or combined with other materials such as metals. In these locations BCP has successfully been used as bone filler in oral and maxillofacial, reconstructive and orthopedic surgeries [84].

Alginate

Alginate contains a unique combination of features making it very suitable for DNA based bone tissue engineering. It is a hydrogel which is FDA approved, non-toxic, biocompatible and extensively used in the food and drug industry. It consists of anionic linear copolymers of β-D-mannuronic acid and α-L-glucuronic acid residues that gelates with bivalent cations like Ca^{2+} [85]. Alginate is never used for structural purposes since it quickly loses its mechanical properties *in vitro* (within days), most likely due to an outward flux of ions into the surrounding medium [86]. This dissolution behavior however makes it suitable for release of growth factors. Alginate hydrogel is also compatible with cell seeding, and bone ECM formation has been shown to occur inside alginates *in vitro* [87 88 89] , subcutaneously between the bone and periosteum [90], and in cranial bone defects [91]. In addition to cells and growth factors, alginate can act as a carrier for gene delivery. It has been used as a vehicle for plasmid DNA in the form of nanoparticles, combined with other hydrogels, or as described in this thesis combined with BCP particles [92 93]. It is suitable for bioprinting, allowing it to be printed into 3D designed porous structures. It is even possible to print cell-, DNA- or BCP particle-laden alginate. This means that a printed alginate construct can combine all key elements for bone tissue engineering [76].

Different aspects of bone tissue engineering have been researched extensively over the past decades, including growth factor delivery by gene therapy. In order to develop a safe and accessible system, we explored the possibility of generating a non-viral gene delivery system, focusing on the delivery of human BMP-2. As to our knowledge no human DNA based therapies are available for bone tissue engineering currently, this implies that many questions, technical limitations and safety issues have to be addressed before actual translation to the clinic. In this thesis we have addressed a number of the steps necessary for the development of this therapy.

RESEARCH AIMS AND OUTLINE OF THIS THESIS

This thesis will focus on the development of a non-viral gene delivery method of BMP-2 plasmid DNA to promote the induction of bone formation. The general aim of this thesis was to investigate whether delivery of BMP-2 by non-viral gene therapy is feasible and effective for bone tissue engineering.

Firstly, a non-viral gene delivery vehicle appropriate for bone tissue engineering was developed and optimized. Secondly, the performance of this gene delivery vehicle with respect to bone formation *in vivo* was established. Finally, the bone-forming construct was optimized in order to function in a preclinical setting.

The introduction and aims as described here in Chapter 1 are followed by Chapter 2 which is a literature overview of the use of non-viral gene delivery for bone tissue engineering. The advantages of delivering growth factors via non-viral gene therapy, the commonly used types of non-viral gene delivery, the main delivery routes, different gene delivery vehicles, and most important target genes will be discussed.

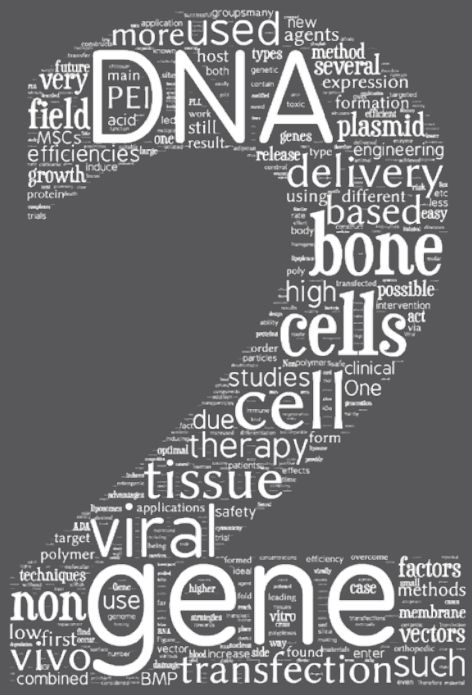
In Chapter 3 the development of a new alginate based plasmid DNA delivery system is described. The optimal conditions to introduce BMP-2 cDNA in MSCs are determined by analyzing transfection efficiencies, protein release from the construct, duration of the transfection period and *in vitro* and *in vivo* osteogenic response of MSCs and other cells with osteogenic potential. Chapter 4 describes the optimization of alginate-based plasmid DNA constructs for bone tissue engineering by adding ceramic material for bone to grow on. Furthermore, bone formation in ectopic implantation locations was compared to the clinically highly relevant spinal cassette model in goats.

In Chapter 5 the optimized BMP-2 plasmid DNA-laden alginate constructs are compared to the more translational setting in which BMP-2 protein instead of cDNA is delivered for bone regeneration. Osteogenic differentiation and bone formation in mice was analyzed and compared to investigate the efficacy of the strategy.

In an attempt to generate cell-free constructs Chapter 6 focuses on recruitment of host cells, mainly MSCs, towards the BMP-2 gene-based construct, by controlled delivery of SDF-1α. In this study, the contribution of cell seeding was studied. In addition, synergy between SDF-1α and BMP-2 was investigated.

Chapter 7 is focused on the bioprinting of alginate based scaffolds and how the bioprinting process affects transfection efficiency, cell survival, protein production as a result of the transfection and eventually osteogenic differentiation.

Finally Chapter 8 discusses and summarizes the results and conclusion described in this thesis, and addresses their implications for future research.



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ABSTRACT

The possibilities of using gene therapy for bone regeneration have been extensively investigated. Improvements in the design of new transfection agents, combining vectors and delivery/release systems in order to diminish cytotoxicity and increase transfection efficiencies have led to several successful *in vitro, ex vivo* and *in vivo* strategies. These include growth factor or siRNA delivery, or even enzyme replacement therapies and have led to increased osteogenic differentiation and bone formation *in vivo*. These results provide optimism to consider use in man with some of these gene delivery strategies in the near future.

INTRODUCTION

Bone (re)generation is one of the major focus points within the field of regenerative medicine. To create bone constructs, many strategies have been developed, including different cell based techniques, bioactive materials and various growth factors [94 95]. One of the possibilities is the use of genetic intervention techniques to stimulate bone formation. This review will discuss the main gene therapy strategies, their possible application and some future directions in the field of bone tissue engineering.

For engineering of bone tissue several components play a key role, such as scaffold materials, bone-forming cells or their progenitors and other biological stimuli, often growth factors. Depending on its composition, a hybrid bone replacement construct will elicit a number of molecular signals at the implantation site, leading ideally to osteogenic differentiation followed by bone formation. Scaffolds mainly function to provide a three-dimensional structure, a biomechanical component and function as vehicle for biological components. Recent discussions on bone replacement materials and scaffolds can be found elsewhere [96 97 95]. Many studies have investigated the feasibility of addition of growth factors [98], either as protein or delivered as plasmid DNA via gene therapy. The best known growth factors used in tissue engineered constructs are BMP-2 and -7, IGF-I, SDF-1α and TNF- α. The most commonly used cell type in bone tissue engineering constructs is the multipotent mesenchymal stromal cell (MSC). MSCs can be isolated from various adult tissues such as bone marrow, adipose tissue, dental pulp, muscle and umbilical cord blood and are known for their ability to differentiate into different lineages such as cartilage, adipose tissue and bone. Furthermore, they elicit immune repressing capacities, making them also suitable for allogeneic transplantation [99 19]. These features make MSCs also the main targeted cell type for gene intervention techniques in the field of bone tissue engineering [100].

GENE THERAPY: HISTORY AND BASIC CONCEPTS

Gene therapy comprises the introduction of functional genes into cells in order to enhance or enforce their expression. This most common form of additive gene therapy is now used in patients for some rare conditions. An inhibitory approach using siRNA or miRNA is still under development and may have potential in the field of bone tissue engineering as well. Different strategies to deliver DNA can be divided in viral transduction and non-viral transfection, both of which can be performed *in vivo* and *ex vivo*. The main difference between the application of a viral packaging unit, which may be cell-specific, versus the use of naked oligomeric DNA or plasmid DNA, which is often complexed to a transfection agent, is the ability to obtain prolonged transgene expression by viruses that incorporate their genetic material in the host genome, whereas the chance this happens with non-viral vectors is nil [101 102] and therefore a transient effect is achieved. The importance of this aspect only became clear in 1999, when the first gene therapy based clinical trial started with ADA-SCID patients. These patients have a severely impaired adaptive immune system due to a single gene mutation coding for the enzyme adenosine deaminase (ADA). Delivery of this gene in virally transduced white blood cells can cure these patients. However 4/10

patients in this study eventually developed leukemia, because the new ADA gene got incidentally inserted into the genome close to the site of a proto-oncogene. This caused the FDA to ban all clinical trials using gene therapy for nearly a decade, which damaged the image of gene intervention techniques. With safer viral vectors and the development of non-viral delivery methods these techniques are again being investigated at the moment, in more than 1840 clinical trials for various disorders (clinical trial database). An improved version of the ADA trial was published in 2009 and described feasibility in 50% of the included patients but is still experimental due to several vector related side effects [103]. No application of gene therapy in the field of bone (re)generation has been reported so far.

GENE THERAPY IN BONE TISSUE ENGINEERING

Use of gene intervention techniques for generation of bone tissue does not involve the replacement of a non-functional gene as is the case in genetic disorders but involves the delivery of transcription - or growth factors. Enforced expression of growth factors has several advantages compared to the addition of protein growth factors. Many proteins have a short half-life at the site of application, resulting in the delivery of up to milligram dosages in order to provide a sufficient stimulus that lasts longer than a few days in order to induce osteogenic differentiation and eventual bone formation. As a result of these supraphysiological local doses, protein diffusion is more likely to happen and systemic effects may occur. Recently, the negative side effects of bone-morphogenetic protein-2 (BMP-2) in spinal fusion procedures were reported [104 105 106]. Gene therapy as a means to administer growth factors might overcome the use of high protein doses, because a sustained delivery of protein can be achieved [54]. Because of their ability to induce bone formation *in vivo*, BMPs (in particulary BMP-2, -4 and -7) are candidates for this kind of gene intervention techniques. One of the earliest studies however, used PTH1-34 in gene activated collagen matrices to transfect cells both *in vivo* and *ex vivo* [107 108]. Later, growth factors such as IGF-1, VEGF or TNF-α have been shown to contribute to osteogenic differentiation or bone formation (Table 1) [109].

One of the possibilities of gene therapy is the expression of heterodimeric proteins. During this process, multiple genes are expressed in the same cell, ensuring that translation and protein dimerization occur simultaneously for both genes. For instance in the case of combining different BMPs, it is known that the BMP-2/BMP-7 heterodimer is more potent in inducing bone formation than any of the homodimers [43]. Secretion of the dimer will then target BMP-receptor positive cells.

Apart from growth factors, bone regeneration via gene therapy can also work on constitutive expression of proteins that are rapidly degraded, such as the transcription factors Runx2/Cbfal, Sox9 and Osterix, or on underlying genetic diseases such as hypophosphatasis (HPP), with enzyme replacement therapies [110 111 112 113 109].

VIRAL VERSUS NON-VIRAL GENE DELIVERY

As we have already stated, genes can be delivered both virally and non-virally. Both delivery routes have their own advantages and disadvantages and the choice of suitability depends

Table 1. Overview of viral and non-viral gene delivery methods in the bone field.

gene delivery method	(therapeutic) gene	targeted cell/tissue	biological effect	reference
liposomes	Osterix	MSC	earlier mineralization, more bone formation	(Lai, 2011)
	Plekho1 casein kinase-2 interacting protein-1	osteogenic cells (rats)	RNAi is targeted towards bone formation surface, bone resorption (osteoporosis) prevented	(Zhang, 2012)
	GFP	MSC	40% transfection efficiency, 90% cell viability	(Madeira, 2010)
	BMP-2	MSC (rat)	induced bone formation *	(Blum, 2003)
synthetic polymers	Human parathyroid hormone	osteoblasts (dog)	bridging of bone defect #	(Bonadio, 1999)
	Cbfa1	MSC	enhanced osteogenicity #	(Park, 2012)
	BMP-2	C2C12 (mouse) myoblasts	osteogenic differentiation	(Reckhenrich, 2012)
	GFP	MSC	increased transfection efficiency due to synergistic effect PLLA + liposome	(Clements, 2007)
	Different pDNA	osteoblasts (rat)	increased DNA or RNA delivery via targeted endosomes	(Wolff, 2008)
	GFP	ADSC	increased transfection efficiency	(Ahn, 2008)
natural polymers	BMP-2	MSC (rat)	high differentiation and bone growth, dental area #	(He, 2012)
	BMP-2	MSC (rat)	increased bone formation in goat spinal muscle # high differentiation and bone growth, dental area #	(Wegman, 2012)
	BMP-2	MSC cranial defect (rat)	longer transfection period because of slow release system DNA #	(Chew, 2011)
inorganic nanoparticles	GFP	MSC	longer transfection period and increased transfection efficiencies	(Park, 2010)
	GFP	MSC	increased transfection efficiencies	(Uchimura, 2007)
physical transfection methods	GFP LacZ	MSC	increased transfection efficiency, without affecting biologic MSC characteristics, expression of reportergene for over 10 days	(Ferreira, 2008)
	BMP-2/7	MSC (rat)	bone formation in skeletal muscle (rat) #	(Kawai, 2009)
	GFP	MSC	custom tailored DNA delivery via nanosyringe	(Park, 2009)

*** Ex vivo transfection # In vivo transfection**

highly on the target cell type and desired expression period. Viral gene delivery is considered the most efficient way of gene transfer for many cell types. It relies on viral envelope proteins to transfer the gene of interest into the cytoplasm where it is transported to the nucleus and subsequently expressed. The viral vector can either integrate into the host genome, leading to a stable expression of the introduced gene, or remain present as an episomal vector, which is gradually lost after cell division. The commonly used viral vectors are retroviruses, adenoviruses, adeno-associated viruses (AAVs) and lenti- and herpes viruses [114 115 116]. The main concern of using viral transduction is triggering an immune response to the viral

proteins. To avoid this, the vectors are modified to contain only the minimally necessary viral genes. In the case of retroviruses and AAVs, there still remains a risk that normal gene function can be disturbed based on insertional mutagenesis [117] (*Figure 1*).

Non-viral gene delivery is mostly used in the form of plasmid DNA, which can easily be isolated from bacteria. One of the main advantages of using bacterial DNA is that this does not stimulate any pre-existing adapted immune reaction. Cytotoxicity is only seen when unmethylated cytosine guanine dinucleotide motifs (so-called CpG motifs) are combined with liposomes [118]. Furthermore, localized expression of plasmid DNA is very feasible, because it shows a high turnover rate in the blood stream, which minimizes unwanted diffusion to off-target tissues. There is no limit to the amount of DNA, which can be delivered, and gene transfer is efficient in a large number of cell types [44 45 119]. Plasmid DNA vectors do contain viral promoter sequences but do not integrate in the host genome, leading to transient expression patterns (*Figure 1*). Since bone regeneration usually does not require a permanent expression of the desired gene, non-viral tissue engineering is very suitable for this application. The disadvantage however is that transfection efficiencies are often very low, particularly *in vivo*. Therefore, a considerable effort in the field is dedicated to optimizing the different transfection methods, which are described in detail below. To extend the transgene expression period and prevent loss of transgene mRNA, minicircle DNA can be used instead of regular plasmid DNA. This new method of producing vectors increases transgene expression up to a 1000-fold and can be considered as a candidate to replace regular plasmid DNA in the future [120].

NON-VIRAL GENE DELIVERY METHODS

Non-viral gene transfer is usually performed using plasmid DNA. These small circular double stranded DNAs show a stable chemistry, are easily produced in bacteria and may contain a variety of promoters and therapeutic cDNAs [121]. The plasmid DNA has to reach the nucleus of the cell to be transcribed and therefore several barriers have to be overcome. First, degradation and body clearance (*in vivo*) must be prevented. Then it has to cross the cell- and nuclear membrane to enter the nucleus and the DNA has to be released from any possible transfection complexes [122 123]. The efficiency of non-viral gene delivery is dependent on the preparation, purification and composition of the DNA and also on the chosen transfection method, the cell type and its cell cycle phase (*Table 1*).

In the following sections, the main non-viral methods for gene delivery are described, together with their possible applications in bone tissue engineering models.

Liposome-based transfection

Cellular uptake of plasmid DNA via endocytosis is enabled by formation of lipoplexes, cationic lipids that self-assemble into liposomes due to their polar heads and non-polar tails, combined with plasmid DNA. The DNA is either entrapped in the internal aqueous core or bound to the surface [124 122]. The interaction of liposomes with the cell membrane induces endocytosis. After endocytosis, it is thought that the endosomal membrane destabilizes, causing the release of DNA into the cytoplasm [125] (*Figure 1*).

Even though liposome-based DNA delivery was one of the first methods to introduce exogenous DNA into eukaryotic cells, this method is hardly used in the field of bone tissue engineering. This is possibly due to the fact that lipoplexes are cytotoxic at higher concentrations, because they are able to interact with and destabilize the cell membrane [126 127]. High concentrations however are needed to induce high transfection efficiencies. This limits their suitability for DNA-based gene therapy [128 129 130]. Lipoplexes however are also used for RNA interference (RNAi) delivery, which appears to be more successful at inducing bone formation. In case of gene silencing using RNA interference, a temporary block of the negative regulatory genes is achieved. In this way often multiple genes are targeted at relatively low concentrations. Zhang *et al.* [131] where able to target siRNA towards bone forming areas based on liposome surface properties. The targeted RNA stimulated osteoblast activity without interfering with bone resorption, so less bone remodeling and breakdown took place, leading to higher quantities of bone. These results indicate that the use of targeted delivery of lipoplexes is a promising future strategy for bone tissue engineering.

Synthetic polymer-based transfections

Polymers can be designed to act as gene carriers. They are very versatile molecules that can vary in chemical composition, weight, size, 3D architecture, side chain length and branching, density, etc. [132]. Most polymers investigated for gene therapy are cationic and contain a high density of positively charged groups, mainly amines [100]. These positive groups interact with the negatively charged phosphate groups of DNA to form condensed structures called polyplexes. The structures are similar to lipoplexes and can be endocytosed by cells (*Figure 1*).

The first cationic polymer-based vector used was poly-L-lysine (PLL). At first it appeared to be a poor transfection agent, due to a low transfection efficiency and a tendency to aggregate and precipitate, causing cell death [133]. Therefore in later studies PLL is combined with other chemicals such as palmic acid (PA) or other transfection agents such as liposomes. After conjugation of PLL to PA, the polyplexes showed improved interactions with the cell membrane thereby increasing internalization of the polyplexes. The DNA uptake and thus transfection efficiencies were enhanced by 10-fold, up to 22 %, making it comparable to liposome-based transfection efficiencies, which were used as a control [134]. The combination of both increased transfection efficiencies even more [135].

Other examples of synthetic polymer-based delivery agents are the biodegradable polylactic acid (PLA), poly(lactide-co-glycolide) (PLG) and poly(lactic-co-glycolic acid) (PLGA) [122 136]. These are interesting systems because they are degraded into non-toxic waste products after delivery of the plasmid DNA. PLG was first described as gene activated matrix from which DNA could be released slowly *in vivo* leading to higher transfection efficiencies, longer exposure of cells to the DNA and eventually more bone ingrowth [137]. To tailor degradation rate and release profile, PLGA is often used in the form of microspheres. The advantages of the microspheres are low cytotoxicity and long-term transgene expression [138 139] probably as a result of the gradual release of DNA. Other advantages are high cell viability of different types of stem cells such as embryonic, adult adipose and cord blood derived MSCs (80-90 %). Transfection efficiencies up to 60 % (embryonic stem cells) can be achieved with an ideal poly-end modified derivate [140].

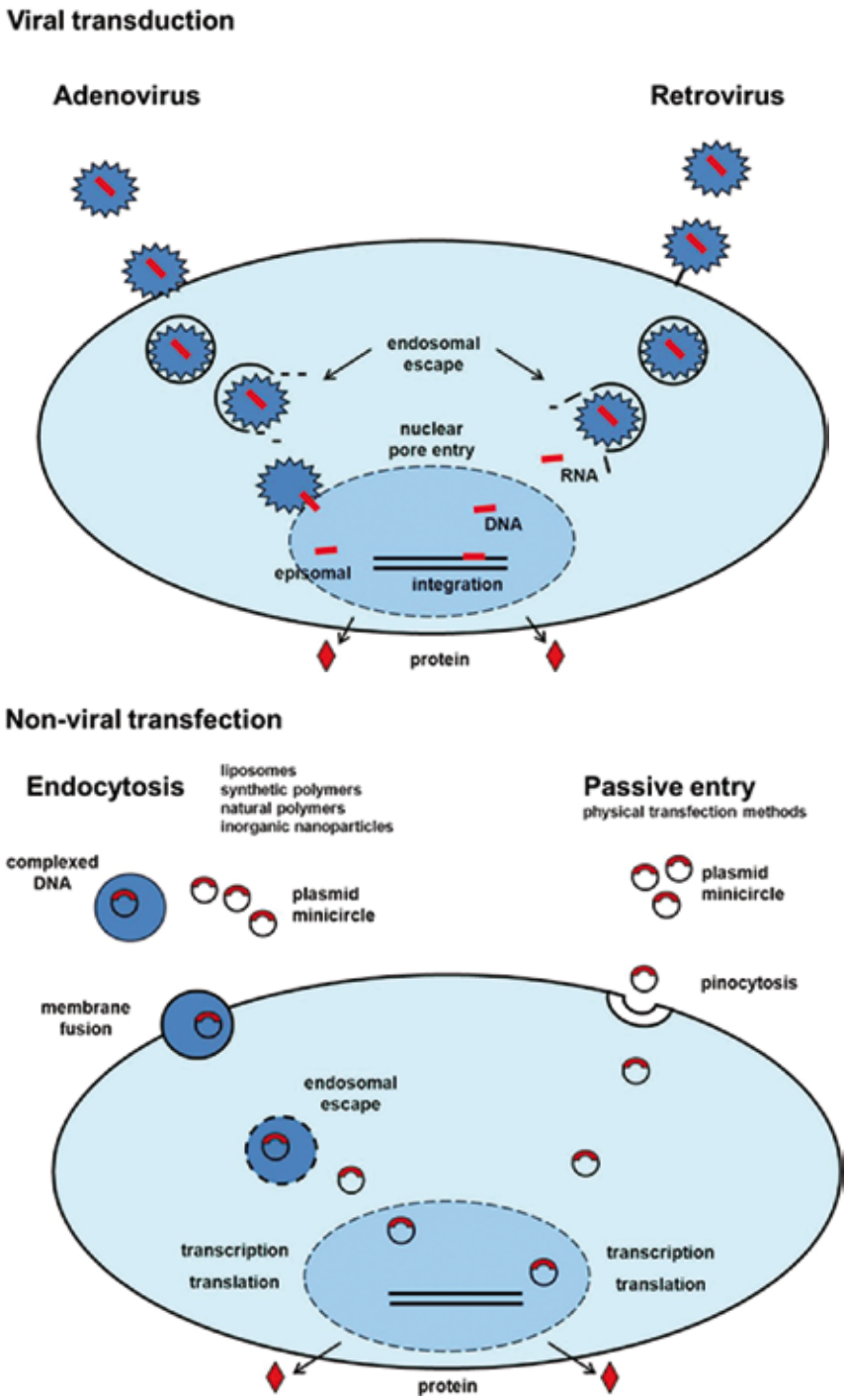

Figure 1. The different viral and non-viral gene delivery methods.

One of the most popular polymer based vectors is based on polyethylenimines (PEI). The widely used commercial form often serves as a control in gene delivery studies, and is pursued as one of the most successful transfection agents [141]. After endocytosis, PEI increases the pumping of protons and influx of anions into the internal cell organelles, leading to osmotic rupture. PEI/DNA complexes then enter the nucleus and it is thought that PEI-complexation prevents premature breakdown of the DNA. It has been studied as a gene carrier over a wide range of molecular weights (0.4-800 kDa), but 12-70 kDa appears to be most effective [142] [143]. In MSCs the maximum transfection efficiency for tissue engineering applications has been 50 % [144]. Besides the molecular weight range, N/P ratio (N = molar number of primary amines in the polymer; P = molar number of phosphate groups in the pDNA backbone) also influences transfection efficiencies. A lower N/P ratio induces higher transfection rates, but the cytotoxicity is also increased [145]. In order to increase the transfection efficiency and decrease the cytotoxicity, several studies focus on combining PEI with other biologicals with varying success. When PEI is covalently bound to hyaluronic acid (HA), polyplexes are formed which indeed increase transfection efficiencies comparable to lipofectamine (up to 34 %). This can be explained by the fact that HA improves cell targeting by binding to CD44 expressed by many cell types including human MSCs at their surface. It also reduces the toxicity of PEI because the cationic amine groups from PEI are combined with the carboxylic groups present in HA resulting in higher cell viability levels [146].

The perfect synthetic polymer-based vector has yet to be found. It depends on the delivery method, target cells, host, etc. which polymer or combination of polymers induces the best biological effect. In the future polymers might become very efficient gene carriers. They are relatively efficient, easy to handle, can be combined with other carriers, their degradation can be tailored for optimal release profiles and the delivery route can be manipulated. For example, it is possible to design delivery agents that release the DNA as a result of external stimuli through chemical bond cleavage. This makes DNA targeting into specific cell types *in vivo* more efficient [147].

Despite all these promising features, hardly any of these strategies has been translated to the clinical practice. Similar to what is seen with liposomes, dosage determines the optimal transfection efficiency, but coincides with cell damage due to cell membrane manipulation.

Natural polymer-based transfection

Natural non-viral polymer-based vectors are often used in gene therapy applications due to their biodegradability, biocompatibility, low/non-toxicity and because they can be modified to increase the functionality of these vectors [122].

One of the most studied natural polymers is chitosan, a polysaccharide that is formed by deacetylating chitin. It is studied as a gel and as micro/nanoparticles [148] [149]. Chitosan forms complexes with the DNA but the exact mechanism behind the transfection is still unknown. When compared to liposomes, it is found that the transfection efficiency of chitosan is always a little bit lower, comparable to naked DNA, but the compound is significantly less toxic than liposomes and easy to work with [150]. To improve the transfection efficiencies, chitosan is often combined with other materials. For orthopedic applications it can be incorporated into titanium films with BMP-2 plasmid DNA, or incorporated in alginate hydrogel as nanoparticles [93].

Alginate as such is also used for gene delivery. This anionic linear copolymer of β-D-mannuronic acid and α-L-glucuronic acid residues gelates with bivalent cations like Ca^{2+} [85]. It is considered non-toxic and biocompatible, and can be used in the form of nanoparticles or combined with other hydrogels [92 93]. High transfection efficiencies, a slow release of biologically active BMP-2 and osteogenic differentiation *in vitro* and bone formation *in vivo* were recently found as a result of alginate mediated transfections with plasmid DNA [53 151].

Another well-known natural polymer is gelatin. Gelatin is formed after hydrolysis of collagen and easy to work with. It is very biocompatible and is mostly used as microparticles formed by emulsion techniques. After gelatin cross-linking by glutaraldehyde, the stronger cross-links prevent fast degradation *in vitro* and *in vivo*. It is a widely used release system for growth factors, but can also be used to release DNA [152 153 154].

Natural polymers in general have in common that they are often easy to work with, hardly trigger immune responses and are easily available. However, the notion that these natural polymers can be used as gene delivery systems is only slowly being appreciated. In most cases the hydrogels that they form are used as delivery vehicles for other DNA-complexes. Besides lipo- or polyplexes, these gels are also often combined with other materials such as synthetic polymers or ceramics for biomechanical and osteopromotive reasons. Although batch variation in some hydrogels might greatly influence transfection efficiencies, the addition of ceramic particles does not [151].

Inorganic nanoparticles

A novel, non-viral gene delivery methods is based on the use of inorganic nanoparticles [155 156]. Plasmid DNA is coupled to small material particles such as gold, silica, iron oxide or calcium phosphate. These particles are endocytosed, thereby delivering the plasmid DNA intracellularly. Transfection efficiencies for these methods are very moderate, but due to several advantages such as low toxicity, good shape control and easy storage ability, more research is being conducted on these types of delivery agents [157]. When combined with a different kind of carrier, it was found that gold particles covered with DNA/JetPEI complexes can increase transfection efficiencies 2.5-fold [158]. A similar result was found for silica nanoparticles combined with PEI used to transfect human MSCs. This combination of plasmid DNA delivery agents is more successful at inducing transfection than silica or PEI alone [159].

One of the disadvantages of these kinds of transfection enhancements is that it is still unclear whether all particles are cleared from the body, and if eventually tissue damage will occur if these particles remain in place. Therefore, further research should focus more on safety issues.

Physical transfection methods

With physical transfection methods, cell membranes are temporarily permeabilized to allow plasmid DNA to enter the cells. To carefully permeabilize the cell membrane, different methods are used, such as electroporation, which uses a high intensity electric pulse. As a result, DNA can enter the cell and induce transfection before the pores of the cell close again [160]. Unfortunately this is not a very cell friendly method, therefore several groups are trying to optimize the ideal voltage and duration of the procedure to increase transfection rate and reduce cell death [160 161 162]. Similar to electroporation is sonoporation,

which uses ultrasound to disrupt the cell membrane and induce transfection. It is still a highly experimental procedure and not very successful yet, since the majority of the cells do not survive the procedure and a very limited number of cells can be reached in tissues because only the cells at the surface receive enough ultrasound to be transfected.

In an effort to find a noninvasive transfection method without irreparable damage of the cells, electric field-induced molecular vibration is being tested. Hereby, cells are brought into suspension together with plasmid DNA in an *in vitro* setting. The vibration induces the cells and DNA to colloid allowing the cells to become transfected. Different cell types including MSCs were efficiently transfected without disturbance of differentiation capability [163]. In case of the ex-vivo microinjection method using nano-needles which are manipulated using an atomic force microscope, transfection efficiencies were even higher (up to 70 %), while no irreparable membrane damage did occur [164]. To be able to microinject multiple cells at a time, hollow carbon nanotubes are lined up vertically and inject multiple cells simultaneously. The carbon tubes act as a nano-syringe for plasmid DNA to enter the cells [165].

All physical methods however, destabilize the plasma membrane temporarily, which in many cases result in low cell survival. The difficulty of these techniques is to find the optimal conditions. Another difficulty is to reach deep into the tissue. Most of these techniques are only capable of penetrating the skin and maybe reach the muscle and adipose tissue just below the skin, but bone cannot be reached non-invasively, making it less optimal for orthopedic applications.

Ex vivo transfections

For non-viral gene therapy to be applied in bone (re)generation, many hurdles need to be overcome. Most techniques are based on controlled cell membrane damage or particle uptake. The main disadvantages for *in vivo* application are high levels of cell death/tissue damage, low penetration depth, the risk of particle migration and the risk of off-target effects. One way to overcome this problem is by doing *ex vivo* transfections. Hereby the DNA is not transferred into the body to transfect targeted host cells, but the desired host cells are isolated from the body, transfected *in vitro*, often selected afterwards, and reinfused to act as bone-forming cells or as protein factories. The two major advantages over classic *in vivo* transfections are preselection of the target cells, and post-selection of the transfected cells. Prior quality control of the transfected cells becomes possible, which might increase safety. As safety is the primary concern, especially in the orthopedic field, *ex vivo* transfections models appear to be more popular at the moment [166 162 110 167 168]. A disadvantage arises with the harvesting of autologous cells, which might be time-consuming and warrants additional surgery [169]. Knowledge on the use of allogeneic MSCs is increasing and several institutes are initiating safety studies for MSCs, which might open up new possibilities in orthopedic gene interventions.

SAFETY AND FUTURE OUTLOOK

Despite all the promising small and large animal studies performed, it is still a huge challenge to translate these studies to human clinical practice. Safety will be the first and foremost aspect of this road but no safety study has been reported so far for orthopedic applications. This is due to several possible reasons including the fact that gene therapy is

a relatively new field of medicine, which suffered from an enormous image problem after severe side effects occurred in one of the first clinical gene therapy based trials. Secondly, in a field where non-lethal diseases or injuries are treated, the possible health risks are considered too high to justify human experimentation. As soon as several gene therapy studies in other areas have proven to be effective and safe, this will add to the attempts in the field of bone tissue engineering. Since vectors are more innovative and safe, it is possible that over the next few years, non-viral gene therapy trials will be set up for orthopedic applications. However, in order to design safety studies, the following aspects have to be taken into account: a. Insertion of the DNA in the host genome must be minimized to avoid possible mutagenesis, b. Cytotoxicity of the vector must be prevented, c. Delivery method/route needs to be optimized to prevent degradation of the DNA in the bloodstream or tissue, d. GMP protocols need to be designed to warrant the quality of the construct. These safety studies are laborious and time-consuming.

The area that most likely will be the first to proceed towards clinical gene intervention technique will possibly be the craniofacial field. Not only can the jaw be considered a relatively isolated tissue, but also it is also feasible to take biopsies of the newly formed bone from a patient, since this is part of the standard procedure to place a dental implant. Histological and genetic analysis of those biopsies would generate sufficient amount of safety and efficacy knowledge, necessary for future developments in other applications such as spinal fusions or large bone defects.

CONCLUDING REMARKS

Although the use of gene therapy for bone tissue engineering is an upcoming field, no ideal delivery agent has yet been found. There is a thin optimum between inducing high transfection efficiencies and cytotoxicity. For the orthopedic field it is hard to find an ideal technique for efficient and safe gene transfer, which is also biocompatible and mechanically suitable for bone tissue engineering. In an optimal construct for induction of bone formation, several components will need to be combined. This leads to constructs, which contain transfection agents in different forms, coated on scaffolds or mixed to hydrogels with or without the addition of ceramic or other carriers. This approach has led to some promising results *in vitro* in small and large animal studies. To proceed towards clinical application the next step will be designing and performing safety studies to finally establish gene therapy as a possible new therapy to induce bone (re)generation.

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OSTEOGENIC DIFFERENTIATION AS A RESULT OF BMP-2 PLASMID DNA BASED GENE THERAPY *IN VITRO* AND *IN VIVO*

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An error in this manuscript regarding the His-tag has been discovered.
See appendix: Correction chapter 3 and 4 regarding the His-tag

ABSTRACT

Bone regeneration is one of the major focus points in the field of regenerative medicine. A well-known stimulus of bone formation is bone morphogenetic protein-2 (BMP-2), which has already been extensively used in clinical applications. We investigated the possibility of achieving osteogenic differentiation both *in vitro* and *in vivo* as a result of prolonged presence of BMP-2 using plasmid DNA-based gene therapy. By delivering BMP-2 cDNA in an alginate hydrogel, a versatile formulation is developed.

High transfection efficiencies of up to 95% were obtained in both human multipotent stromal cells (MSCs) and MG-63 cells using naked DNA *in vitro*. Over a period of 5 weeks, an increasing amount of biologically active BMP-2 was released from the cells and remained present in the gel. In vivo, transfected cells were found after both two and six weeks implantation in naked mice, even in groups without seeded cells, thus indicating *in vivo* transfection of endogenous cells. The protein levels were effective in inducing osteogenic differentiation *in vitro*, as seen by elevated alkaline phoshpatase (ALP) production and *in vivo*, as demonstrated by the production of collagen I in a mineralized alginate matrix.

We conclude that BMP-2 cDNA incorporated in alginate hydrogel appears to be a promising new strategy for minimal-invasive delivery of growth factors in bone regeneration.

INTRODUCTION

Bone regeneration is one of the major focus points within the field of regenerative medicine. To create bone tissue and augment defects, many strategies have been used, such as different cell based strategies, material development and growth factor delivery [94 170].

An important growth factor in bone tissue engineering is bone morphogenetic protein 2 (BMP-2), member of the transforming growth factor β (TGF-β) superfamily. BMP-2 is involved in committing multipotent stromal cells (MSCs) towards the osteogenic lineage and known to induce new bone formation [171]. Both *in vitro* and *in vivo* BMP-2 has shown strong osteoinductive activity. However, large amounts of rhBMP-2 protein are administered clinically for an osteogenic effect mainly due to fast degradation by proteinases. Animal studies have shown a half-life of 7-16 minutes systemically, and up to 8 days locally when implanted on a collagen sponge [48]. The possible negative side effects for patients due these high doses of BMP-2 may result in vertebral osteolysis, ectopic bone formation, radiculitis and cervical soft tissue swelling [104].

BMP-2 gene therapy is a low-cost alternative that might be able to overcome the use of high doses of BMP-2, because applied to cells a relatively sustained delivery of protein can be achieved [54]. Gene therapy can be performed by viral or non-viral DNA delivery. Non-viral has several advantages over viral gene therapy, the latter one resulting in high transfection efficiencies but associated with problems such as immunogenicity, virus-dependent recombination risks and the protein expression can exceed the timeframe required for bone defect healing [172 173 174]. Non-viral gene delivery is considered much safer and usually provides a transient expression of the desired gene. However many approaches result in a low transfection efficiencies. Therefore much effort is invested into the development of an efficient non-viral gene delivery method, with the use of various transfection agents. Particularly liposomes, phosphilipid bilayer vesicles which can easily merge with the cell membrane to deliver encapsulated plasmid DNA, are well studied [175 176 177].

Another carrier for gene delivery, which is compatible with simultaneous cell seeding, is alginate hydrogel. Alginate is an anionic linear copolymer of β-D-mannuronic acid and α-L-glucuronic acid residues that gelates with bivalent cations like Ca^{2+} [85]. It is FDA approved, non-toxic, biocompatible and extensively used in the food and drug industry. It has been used as a vehicle for plasmid DNA in the form of nanoparticles or combined with other hydrogels [92 93] and was applied in bone tissue engineering applications both in vitro and in vivo [178 90] This makes alginate very suitable both as gene activated matrix (GAM), and for cell seeded constructs. Cell seeded constructs might improve bone formation at an earlier time point because bone forming cells do not have to home first. GAMs without seeded cells however can be used as an "of the shelf" product.

Our aim is to develop an injectable hydrogel for bone regeneration, in which progenitor cell delivery and plasmid-based expression of BMP-2 are combined. The present study investigates the transfection efficiency of epitope-tagged BMP-2 plasmid DNA in primary human MSCs and the MG-63 cell line in alginate, and the amount of protein produced and released from the gel *in vitro* as well as its bioactivity. Furthermore we studied the possibility of creating a cell-free construct, and determined *in situ* transfection efficiency of

non-seeded constructs in comparison with cell-seeded constructs as well as osteogenicity of the resultant BMP-2 in mice.

MATERIALS AND METHODS

Alginate gel

Autoclaved high-viscosity non-medical-grade alginate powder (International Specialty Products, ISP, Memmingen, Germany) was dissolved at a concentration of 20 mg/ml in alpha minimum essential medium (α-MEM, Gibco, Breda, The Netherlands). The gel was polymerized by adding an equal volume of 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-solution was then replaced by 1 ml culture medium.

Cell culture

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

Human MSCs were isolated from bone marrow, aspirated from the iliac crest or acetabulum of patients after their informed consent and collected in heparin coated tubes. The mononuclear fraction was isolated using Ficoll density gradient centrifugation. The MSCs are isolated by adherence to tissue culture plastic, cultured in αMEM, supplemented with 10% (v/v) fetal calf serum (Cambrex, Charles City, IA, USA), 100 U/ml penicillin, 0.2 mM L-ascorbic acid-2-phosphate (AsAP) (Sigma) and 1 ng/ml bFGF (R&D Systems).

Goat MSCs were isolated similarly from bone marrow (BM), aspirated from the iliac wings of Dutch milk goats and cultured in αMEM (Gibco), supplemented with 15% (v/v) fetal calf serum (Cambrex, Charles City, IA, USA), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (glutamax, Gibco). All cells were maintained at 37 ˚C and 5% CO_2 in a humidified incubator.

Transfection procedures

For expression of BMP-2 the plasmid pcDNA3.1/His/hBMP-2 was used, in which full-length human recombinant BMP-2 is in frame preceded by a 18 nucleotide polyhistidine region (His tag). This tag is used to detect transfected cells and discriminate between BMP-2 produced by certain celltypes and BMP-2 produced as a result of transfection. During secretion of BMP-2 from cells, the tag may be removed together with the signal sequence.

Plasmid DNA (0-50 µg/ml of pcDNA3.1/His/hBMP-2 or pEGFP-N1(BDBiosciences, Franklin Lakes, NJ, USA))/ Lipofectamine2000 (Invitrogen, Breda, The Netherlands) complexes were prepared according to the manufacturer's instructions. Per sample, the DNA, either or not complexed with liposomes and 10^6 trypsinized cells was mixed with 50-100 µl of alginate hydrogel (20 mg/ml). This gel mixture was polymerized by adding 100-200 µl $CaCl_2$/HEPES solution. After 10 min, supernatant was removed and replaced with culture medium. The gels were cultured at 37 ˚C and 5% CO_2 in a humidified incubator for up to 5 weeks before analysis of gene expression. For preparation of the *in vivo* implants, see below.

FACS analysis of BMP-2, ALP and GFP

Constructs of 100 µl gel containing plasmid DNA (pcDNA3.1/His/hBMP-2) and 10^6 cells were depolymerized using citrate buffer (150 mM NaCl, 55 mM sodium citrate and 20 mM EDTA in H_2O) for 15 minutes at 37 ˚C. Cells were pelleted and fixed in 4% (v/v) formalin (Clinipath, Duiven, The Netherlands) in PBS for 10 minutes. All subsequent washing steps were done with PBS/1% (w/v) BSA. The cells were washed with PBS/BSA three times and permeabilized with 0.1% (v/v) Triton-X100 (Fluka, Zwijndrecht, The Netherlands) in PBS for 10 minutes. The cells were washed again and incubated with 2 µg/ml anti-His (recognizing His-tagged BMP-2) antibody (Genscript, Piscataway, NJ, USA) in PBS/BSA for 1 hr at RT. Control samples were incubated with a mouse isotype-matched control IgG (2 µg/ml) (Dako, Glostrup, Denmark). After antibody incubation, cells were washed with PBS/BSA and incubated in the dark with the secondary antibody goat-anti-mouse-Alexa488 (Invitrogen, Breda, The Netherlands), diluted to 20 µg/ml in PBS/BSA for 30 min at RT. After secondary antibody incubation cells were washed and taken up in PBS/BSA and analyzed for green fluorescence by FACS calibur. For ALP analysis the same procedure was used, with the primary antibody B4-78-C (Developmental studies Hybridoma Bank of Iowa, USA) diluted 1:100 in PBS/BSA and incubated for 1 hr at RT, followed by incubation in the dark for 30 min at RT with the secondary antibody goat-anti-mouse-PE (Southern Biotech, Birmingham, Alabama, USA), diluted to 0.25 µg/ml in PBS/BSA. For GFP transfected cells, a similar cell isolation from gel was performed and cells were analysed for GFP intensity at day 7 (ideal timepoint for GFP transfected cells in alginate) using the FACS calibur.

BMP-2 ELISA

Constructs of 100 µl gel were cultured for up to 5 weeks and medium (2 ml) was changed weekly. The collected medium was stored at -20 °C. Gel samples were prepared by depolymerizing alginate 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 Inc., Minneapolis, USA) was performed on thawed samples following the standard protocol.

Preparation *in vivo* implants

6 different constructs of 300 µl alginate (10 mg/ml) gel each were constructed and polymerized with 1 ml of 100 mM autoclaved $CaCl_2$, supplemented with 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4 (Gibco), for 10 minutes. One group consisted of only alginate, one group contained 3 µg of His/BMP-2 plasmid DNA in alginate, four groups consisted of alginate with 3 µg His/BMP-2 plasmid DNA combined with 3 million MSCs from 4 different donors. The latter four contained cells from two human MSC donors, or two goat MSC donors. The gels were implanted immediately after polymerization.

Animals and implantation

18 female nude mice (Hsd-cpb:NMRI-nu, Harlan) were anaesthetized with 1.5% isoflurane, after which the implants were placed in 4 separate subcutaneous dorsal pockets per mouse (n=6 per group / time point). 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. 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

Two and six weeks after implantation the constructs were retrieved to analyze transfection, osteogenic differentiation and alginate properties *in vivo*. Samples were fixed overnight in 4% (v/v) formalin containing 100 μM $CaCl_2$ and processed for 5 μM thick paraffin sections through alcohol dehydration series.

Histology

To analyze the alginate properties *in vivo* and investigate blood vessel formation, hematoxylin/eosin (HE) stainings were performed on all samples. Samples were scored on morphology, calcification and blood vessel ingrowth by two observers. A scale of 0-2 was applied; 0 indicating absence of the readout parameter, 1 for some morphology changes, calcification or blood vessel presence, and 2 for significant/abundant presence. To detect His/BMP-2-transfected cells in the implants, anti-His immunohistochemistry was performed. Sections were permeabilized with Triton X-100 and blocked in 3% H_2O_2 for ten minutes and 5% BSA for 30 min. Antigen retrieval steps were performed using 1 mg/ml pronase and 10 mg/ml hyaluronidase for 30 min each. Sections were subsequently incubated with 5 μg/ml biotinylated anti-His antibody (Genscript, Piscataway, NJ, USA, A00613) for 1 hour and as second antibody 3.3 μg/ml streptavidin/ horse raddish peroxide (HRP) was used. For collagen I, a similar protocol was performed using rabbit-anti-collagen I primary antibody (Abcam, Ab34710) at 3.3 μg/ml and a goat-anti-rabbit-streptavidin/HRP (Invitrogen, 624320)) secondary antibody at 6.6 μg/ml. Both stainings were developed with diaminobenzidine (DAB) and Mayer's hematoxilin was used for counterstaining. A double staining was also performed using the same primary antibodies and concentrations but with fluorescent secondary antibodies. First the collagen I antibody was added to samples for 1 hour and after several wash steps the matching goat-anti-rabbit-Alexa488 (Invitrogen, A52700) at 13.3 μg/ml was incubated. Then 5 μg/ml biotinylated anti-His antibody was added for 1 hour, followed by anti-biotin streptavidin/Alexa594 (Molecular Probes, S11227) at 6.6 μg/ml for 30 min. All antibodies were diluted in PBS/BSA.

Von Kossa staining

Sections were deparaffinized and incubated with a 1% (w/v) silver nitrate solution (Fisher Scientific) under a 60-100 watt light bulb for 1 hour. After washing with destilled water, unreacted silver is removed by 5% (w/v) sodium thiosulphate solution for 5 minutes. Slides were counterstained with nuclear Fast Red.

Alizarin Red staining

Sections were covered for 1-2 min with 2% (w/v) Alizarin Red (Merck) and 0.5 % (v/v) ammonium hydroxide in distilled water, final pH 4.0. After incubation sections were washed with distilled water and mounted in Depex.

Goldner's Trichrome staining

Sections were deparaffinized and incubated with heamatoxilin (weigherts) for 5 min. Sections were washed with tap water for 5 minutes and incubated with Goldner's 1 solution, containing 0.2% (v/v) glacial acetic acid, 0.033% (w/v) acid fuchine and 0.13% (w/v) Ponceaux de xilidine (Fluka), for 30-45 min. After rinsing with 1% (v/v) acetic acid, sections were stained with Orange G (Fluka, 75380) for 7 minutes and washed again with 1% acetic acid. Sections were counterstained with Light green (Sigma L5382) for 7 minutes and after rinsing with 1% acetic acid were dehydrated and mounted in Depex.

Safranin O staining

Sections were deparaffinized and incubated with hematoxilin (Weigert's) for 5 min. After washing in running tap water, sections were dipped in distilled water and counterstained in 0,4% (w/v) Fast Green solution. Sections were rinsed in 1% (v/v) acetic acid until color no longer runs and couterstained again in freshly prepared 0.125% (w/v) Safranin O (Merck) staining. After short dehydration protocol, sections were mounted in Depex.

Statistics

The statistical significance of differences between experimental groups was assessed using a Fisher exact test. Multiple comparisons were generated using a Boneferroni correction to calculate the p-values. A p-value of < 0.05 was considered significant.

RESULTS

We developed an alginate gene delivery system for use with osteoblasts and their precursors, including MSCs. To show that cell transfection in alginate is an effective method, the tracer gene enhanced green fluorescent protein (EGFP) was transfected in hMSCs or MG-63 cells, either or not after complexation with liposomes. After seven days, the transfected cells and untransfected controls were isolated from the alginate and analyzed for GFP expression. On DAPI counterstained cytospins, the positive cells were scored under a fluorescence microscope, resulting in transfection efficiencies ranging from 35-50% (Fig 1A,B). For further quantification, FACS analysis was performed on transfected and untransfected cells. Cells were analysed for their fluorescence intensity level; the cells were considered transfected at a fluorescence intensity level that excluded 95 % of the untransfected cells. To compare the average amount of protein produced/ cell between different groups, the mean fluorescence intensity (MFI) was analysed. For GFP this resulted in a MFI of 156-157 compared to 25 for the control (Fig.2A). The transfection efficiency was confirmed for both the liposome-complexed group and the naked plasmid DNA group.

Transfections using the His/BMP-2 cDNA were similarly performed on MG-63 cells. Immunocytochemistry on cytospun cells transfected with or without lipofectamine-complexed plasmid DNA was performed with an antibody against the His-tag preceding the BMP-2 sequence (Fig. 1D) to discriminate between cells producing BMP-2 and transfected cells. The same cells stained negative using an isotype-matched control antibody (Fig. 1E), whereas untranfected cells also remained unstained (Fig. 1F). FACS analysis of His/BMP

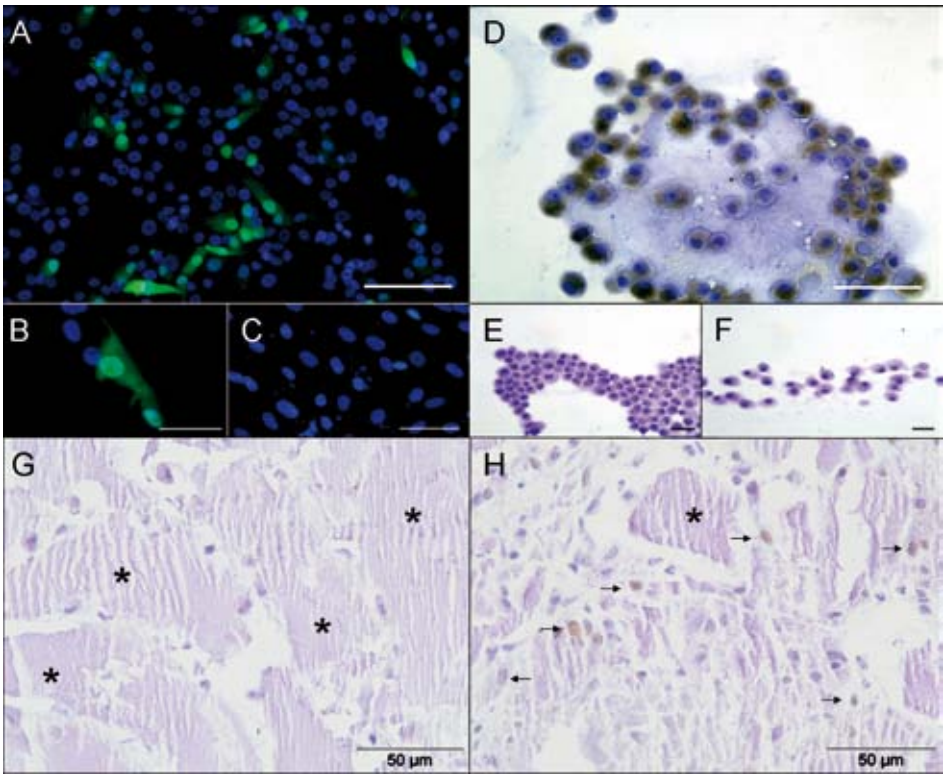

Figure 1. The alginate delivery system: GFP and BMP-2 transfected cells *in vitro* and *in vivo*/ A, B) GFP transfected, C) Untransfected D) His-tagged BMP-2 transfected, α-His staining, E) His-tagged BMP-2 transfected, isotype-matched control Ab staining, F) Untransfected control, α-His staining. Pictures A-F are representative of 3 independent experiments on MG-63 cells, performed in duplicate. G) Anti-His staining of no cells/ no DNA 6 weeks *in vivo* sample. H). Anti-His staining of hMSCs + BMP-2 plasmid DNA 6 weeks *in vivo* sample. Pictures G and H are representative for 6 implants. Arrows indicate transfected cells, * marks alginate, scalebars represent 50 µm.

stained MG-63 cells, human and goat MSCs showed high transfection efficiencies ranging from 64% up to 95% with a MFI of 643-713 compared to 38 in the control. This was consistent in both the liposome-complexed and the naked DNA group (fig 2).

To determine the optimal DNA concentration for BMP-2 with and without liposome complexation, hMSCs and MG-63 cells were transfected with different plasmid DNA concentrations ranging from 0.008 to 5 µg DNA/gel (100 µl). We also performed a FACS analysis of the His-BMP stained cells, which showed little differences in transfection efficiencies and expression levels between all different concentrations. (results not shown) Even the lowest concentration tested resulted in significant expression of BMP-2. We also could not find a consistent positive effect of liposome complexation in all concentrations.

To determine BMP-2 levels released by the cells and the duration of production, alginate constructs with His-BMP-2 plasmid DNA and MG-63 cells (Fig.3) or hMSCs (three

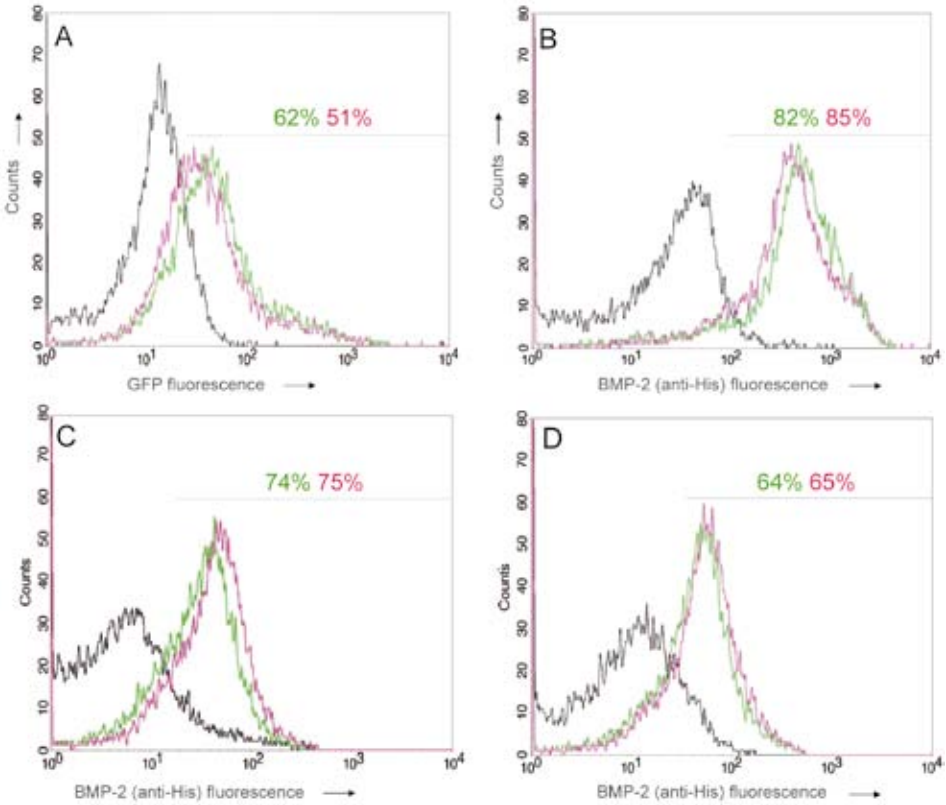

Figure 2. FACS analysis of GFP- or His/BMP-2 transfection efficiency of MG-63 cells in alginate. A) GFP fluorescence of untransfected cells (black line), GFP transfection with liposomes (green line), GFP transfection without liposomes (pink line). B, C, D) anti-His staining of His/BMP-2 transfected MG-63 cells (A), goat MSCs (C) or human MSCs (D) with liposomes (green line) or without liposomes (pink line); staining of transfected cells with isotype-matched control antibody (black line). The horizontal black region excludes 95% of the control cells. Graphs are representative of 3 independent experiments performed in duplicate.

timepoints, results not shown) were cultured for a period up to 5 weeks. The culture medium was collected on a weekly basis and the cultured gels were depolymerised to determine the amount of BMP-2 associated with the gel by ELISA. This revealed a weekly increase of BMP-2 levels in the medium and a much higher weekly increase of BMP-2 in the gel (Fig.3), indicating that the bulk of the produced BMP-2 remained in the gel. The complexation of DNA with liposomes did not have a further additional effect on the amount of released BMP-2.

To evaluate the biological activity of the produced BMP-2, enzymatic stainings on cell monolayers and a FACS analyses of gel samples were performed for the early osteogenic marker alkaline phosphatase. Monolayers of MG-63 cells and goat- and human MSCs were either transfected with the BMP-2 cDNA and cultured in expansion medium, or cultured in osteogenic differentiation medium as a positive control. As a negative control,

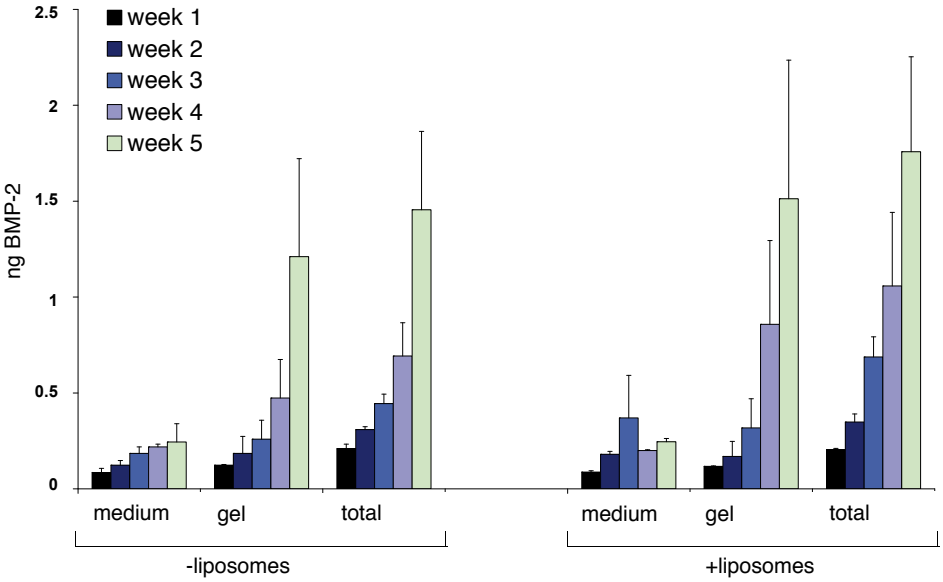

Figure 3. rhBMP-2 release/week from transfected MG-63 cells in alginate. BMP-2 release/week from 0.1 ml alginate constructs containing 1 million cells and either naked (-lipo) or liposome complexed (+lipo) His/BMP-2 plasmid DNA. BMP-2 release was analysed in two independent experiments in duplicate in the medium and in the gel separately by ELISA. The results represent mean ± SD.

untransfected cells were cultured in expansion medium. In both positive controls and in the BMP-2 transfected group red staining revealed the production of ALP after a week, while the unstimulated cells remained negative (Fig. 4B). Similar results were found for MG-63 cells in gel samples which were analyzed by FACS analysis using an anti-ALP antibody. The BMP-2 transfected cells even showed a higher increase in MFI (212) than the positive control, cultured in osteogenic differentiation medium (OM) (MFI=178) compared to untransfected cells cultured in expansion medium (EM) (MFI=72), and the transfected cells stained with an isotype matched control antibody (MFI=100) (Fig 4A).

To investigate whether transfection *in vivo* would take place and is able to induce osteogenic differentiation, we implanted gel samples subcutaneously in immunodeficient mice. Analysis of the histological sections after an implantation period of either 2 or 6 weeks showed that *in vivo,* transfection in alginate was successful as shown by immunohistochemistry on the BMP-2 coupled His-tag. In both the human- and goat MSC seeded groups, numerous positive BMP-2 transfected cells could be found at both the two and six weeks timepoints. (Fig 1G, H) The samples that received BMP-2 plasmid DNA but without seeded cells also expressed the transgene (Fig 1H). At two weeks, 4/6 samples and at six weeks 5/6 samples stained positive for His-tagged BMP-2. This means that resident mouse cells are able to take up the plasmid DNA from the constructs and express it. This proves that transfection in vivo occurs. To determine whether the expressed levels of BMP-2 are sufficient to induce osteogenic differentiation, immunohistochemistry

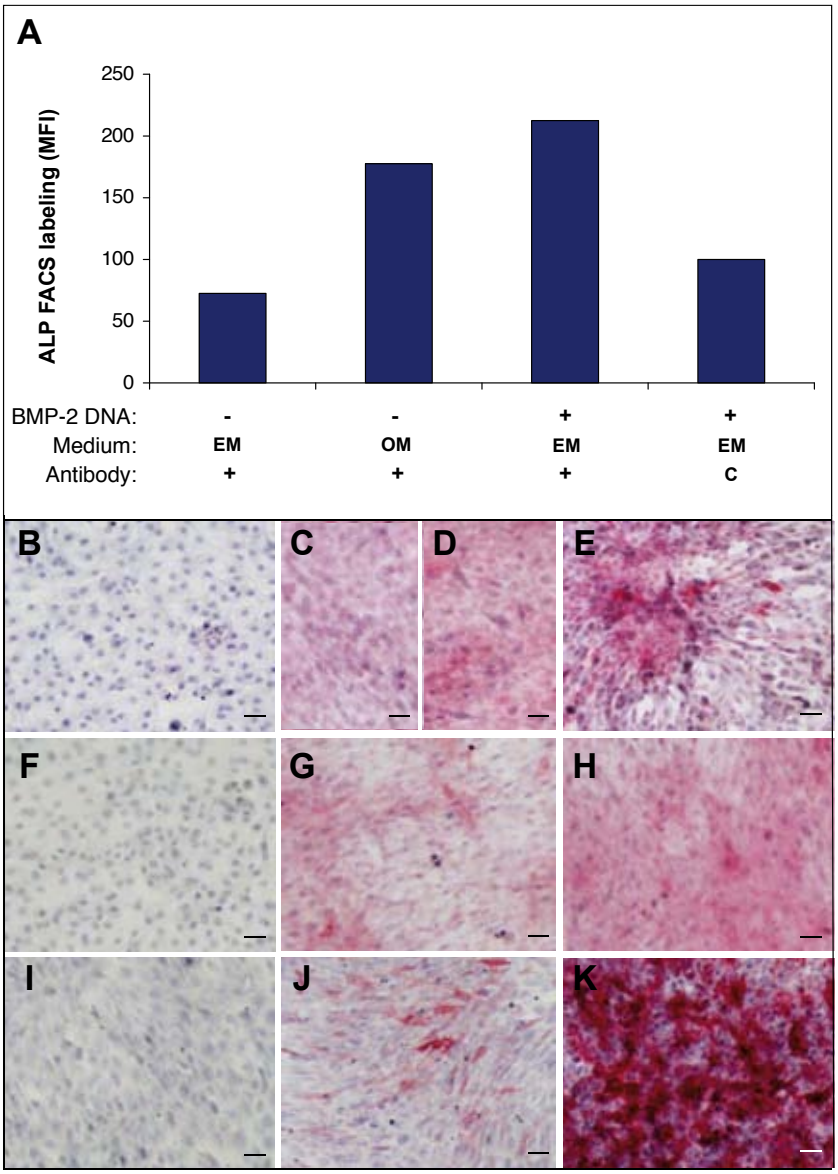

Figure 4. Alkaline phosphatase expression in transfected hMSCs after 7 days. A) FACS analysis using anti-ALP Ab on cells, cultured in alginate for 7 days either in expansion medium (EM) or osteogenic medium (OM). Mean fluorescence intensity (MFI) represents the average fluorescent signal of 10,000 cells. Cells were either untransfected (-) or transfected with His/BMP-2 plasmid DNA (+) and stained with an anti-His antibody or an isotype matched control antibody (C). B) ALP activity staining (red) on monolayers of MG-63 cells and human or goat MSCs after 7 days of culturing. Cells were either untransfected and cultured in expansion medium as a negative control, untransfected and cultured in osteogenic medium as a positive control or transfected and cultured in expansion medium. Pictures are representative of 2 independent experiments performed in duplicate. The scalebars represent 50 μm.

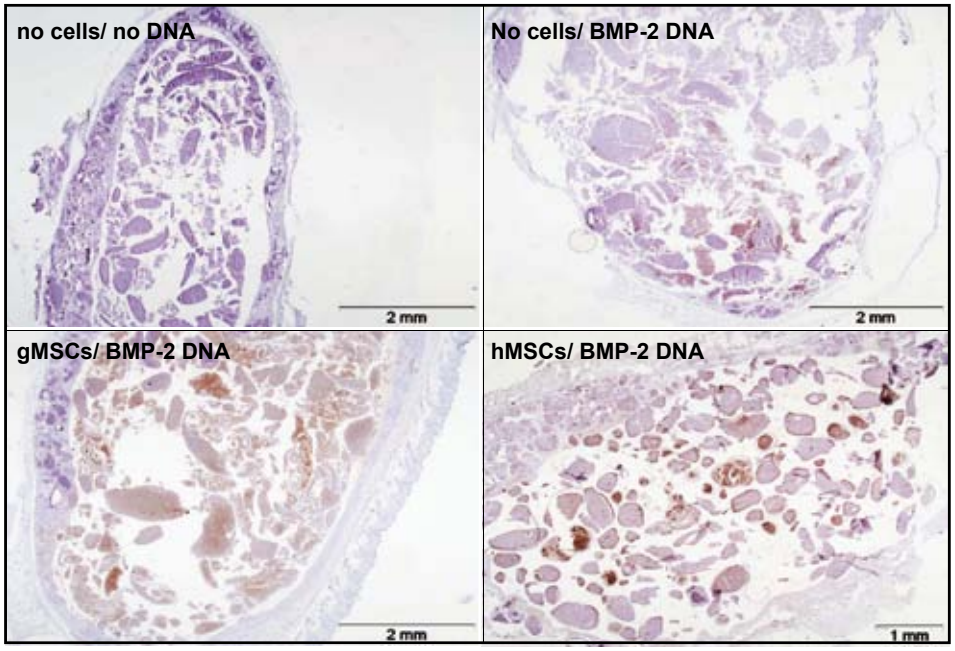

Figure 5. Osteogenic differentiation in alginate constructs *in vivo.* Collagen I staining on mouse implants after 6 weeks. Collagen I staining in brown, counterstained with hematoxylin (blue). Representative pictures for each group (n=6) are presented.

on collagen I was performed (Fig. 5). At two weeks, none of the groups had produced large amounts of collagen I but some staining was visible, mainly in one of the hMSC donors. At 6 weeks, differences between the groups with and without BMP-2 plasmid DNA were far more evident. The groups receiving plasmid DNA showed significantly more collagen I production than the control group without BMP-2 DNA ($P< 0.05$). With the exception of hMSC donor 2, in all the groups in which plasmid DNA was combined with seeded cells, we found the significant highest amount of collagen I staining ($P<0.05$) (Fig. 5). To investigate whether the cells expressing the plasmid DNA are also the cells that produce collagen I, or that neighbouring cells are also osteogenically induced, we performed a double staining for both His-tagged BMP-2 and collagen I (Fig. 6). The results show that the His-BMP-2 and collagen I staining are present within the same regions in the implants, but only in a few cells co-localize. This suggests that the majority of cells differentiating towards the osteogenic linage do so through a paracrine mechanism (Fig 6).

To look at alginate morphology changes, HE stained sections were scored for degradation or calcification of the gel, and blood vessel formation. It appears that the gels were slowly degraded, leaving remnants of calcified material, interleaved with ingrown highly vascularized tissue. All of these aspects were found in the implants but occurred independent of the cell type or presence of DNA. (Table 1). Alizarin red and von Kossa staining of the samples also confirmed that almost all of the alginate implants calcify between 2 and 6 weeks of

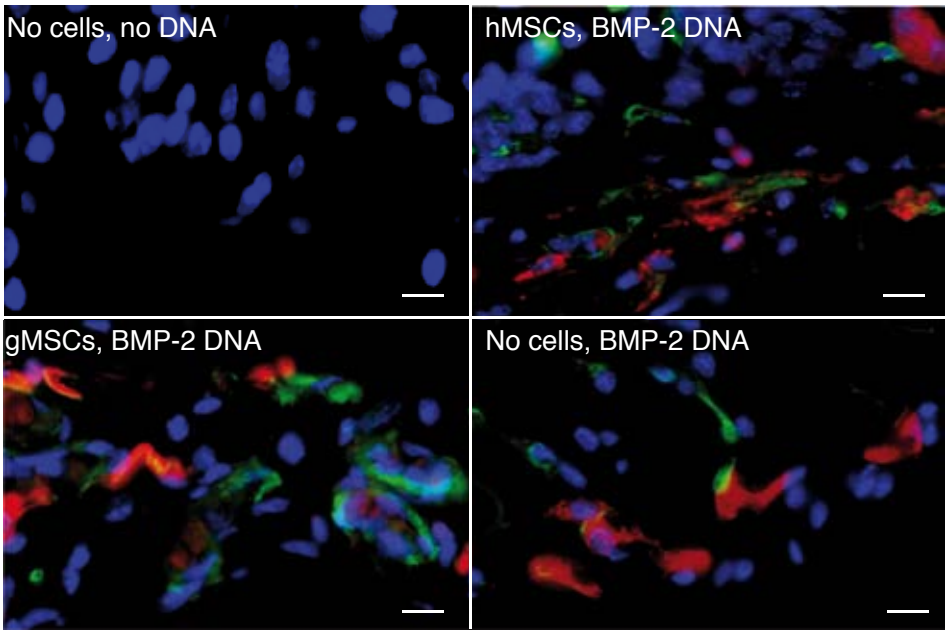

Figure 6. Paracrine action of His/BMP-2-transfected cells *in vivo.* Pictures represent triple staining of collagen I in green, anti-His/BMP-2 staining in red, and nuclei in blue. Co-localization of collagen I and His/BMP-2 appears yellow, pictures are representative for 6 implants/group. The scalebars represent 10 µm.

implantation, which could contribute to the osteogenic potential of the implants.(Fig 7). The alginate implants also contains connective tissue as shown in green by a goldners trichrome staining but no higly cellularized tissue like osteoid, which would be indicated in red (Fig 7).

At 6 weeks however cartilaginous tissue is present in the human MSC seeded group as shown by the cell cluster morphology, collagen matrix surrounding the cells and the heavy Glycosaminoglycan (GAG) staining, indicated in red by a Safranin O staining.(Fig 7).

DISCUSSION AND CONCLUSIONS

This study investigates the possibility of creating injectable bone tissue engineering constructs by transfecting cells with BMP-2 plasmid DNA in alginate hydrogel. Both goat and human MSCs and MG-63 cells were found to be capable of efficiently expressing BMP-2 from plasmid DNA and show a sustained release of relevant levels bioactive BMP-2. *In vitro*, results from MG-63 cells were shown because these are fully differentiated osteoblasts which is a relevant cell type for bone tissue engineering. Besides MG-63 cells, human MSCs were used because they are one of the most interesting candidates for translation towards a clinical application. Goat MSCs were used because goats are a well known large animal model in bone tissue engineering. Data on gMSCs can be relevant if further in vivo studies on orthotopic locations will be performed.

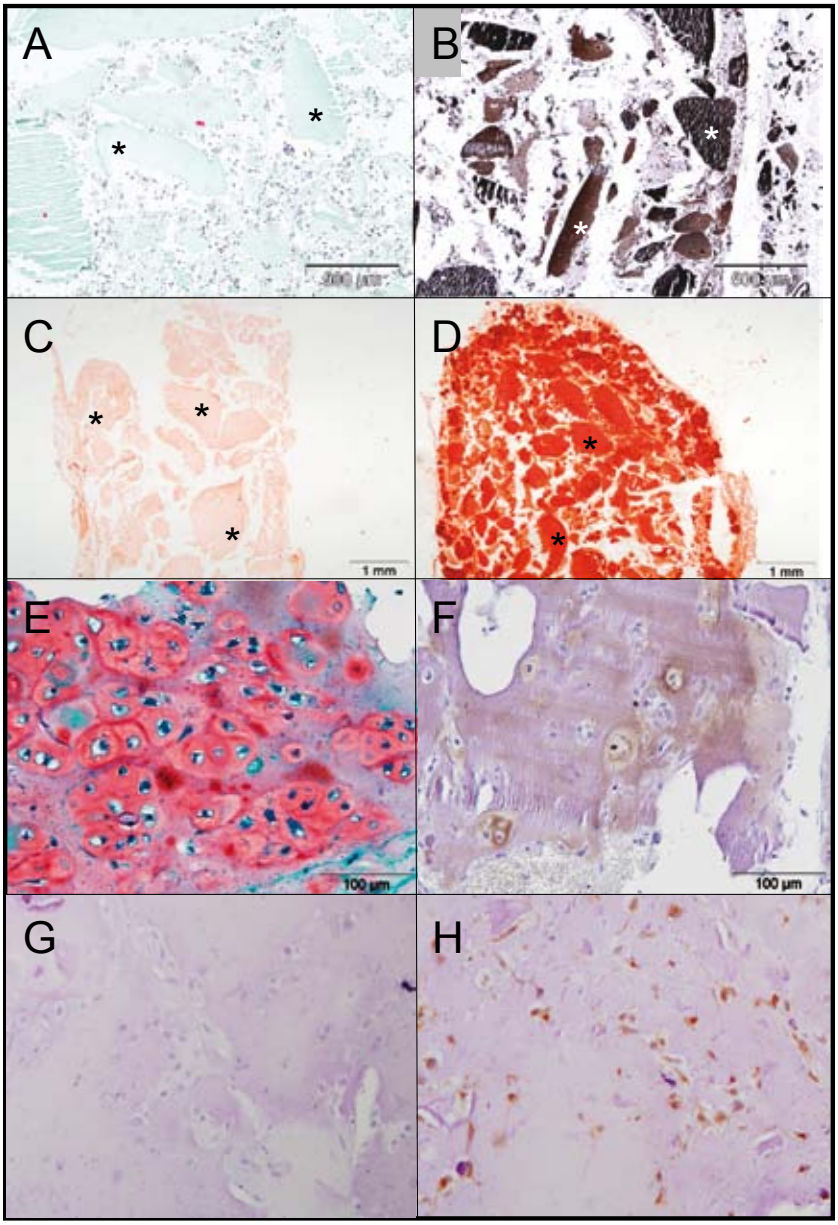

Figure 7. Alginate mineralization after *in vivo* implantation. A) Goldner's trichrome staining for connective tissue after 6 weeks, collagen appears in green. B) Von Kossa staining after 6 weeks, mineralization in black or brown. C) Alizarin red staining for Ca^{2+}-salts after 2 weeks, calcified tissue in red (hardly present), D) Alizarin red staining after 6 weeks, calcified tissue in red, A-D) Pictures are representative for all samples, * indicates alginate patches.E) Safranin O staining after 6 weeks on hMSC seeded group, glucosaminoglycans are stained in red, Fast Green counterstaining. F) Collagen I staining after 6 weeks on hMSCs seeded group. Collagen I in brown, methylene blue counterstaining.

Table 1. Presence of transfected cells and histological parameters *in vivo*.

	transfected cells		alginate morphology		alginate calcification		blood vessels		collagen I	
weeks	2	6	2	6	2	6	2	6	2	6
no cells/no DNA	0	0	7	7	6	8	5	6	4	3
No cells/BMP-2	6	8	9	7	8	9	3	4	4	6
hMSCs donor 1	7	8	6	6	9	9	0	3	8	8
hMSCs donor 2	7	10	7	10	5	10	3	6	5	3
gMSCs donor 1*	4	9	5	6	4	6	3	5	1	10
gMSCs donor 2	5	4	7	3	7	4	4	6	5	8

Scoring of transfected cells, alginate properties, blood vessels and collagen I (n=6 per group) of the *in vivo* samples after 2 and 6 weeks, see M&M for details. Sections were scored by two independent observers.

Gene therapy based on non-viral plasmid DNA is pursued by many groups because of reportedly low immunogenicity, transient expression without genetic recombination, and the production of large protein quantities at low cost. However, non-viral gene therapy is also known to have variable and low transfection efficiencies [179]. Most groups transfecting in alginate also report low transfection efficiencies and do not comment on the amount of protein produced by their constructs [180 181].

Our results demonstrate that the combination of plasmid DNA with alginate hydrogel however, induces high transfection efficiencies with different constructs, at different DNA concentrations in different cell types. The alginate incorporation might be an important factor in the mechanism behind these successful naked DNA transfections. Two possible explanations for high transfection efficiencies can be considered. The first theory describes DNA uptake via calcium phosphate precipitation. The alginate gels are polymerized by a 100 mM calciumchloride solution. This is a 50-100 times higher amount of calcium than in the bloodstream or culture medium. Combined with phosphates, calciumphosphate/DNA co-precipitates might be formed, inducing DNA uptake into the cells [182 183 184]. Macropinocytosis, a different transfection method, transfers naked DNA into the cell, mediated by proteoglycans and other proteins present in culture medium [185]. In the context of alginate, we did not observe a clear additive effect from the use of liposomal transfection agent, even though literature describes this as an even more successful transfection method than the use of calcium phosphate precipitation [186]. Normally the positively charged liposome complexes make it easier for DNA liposome complexes to access the negatively charged cell membrane [135]. The sugarchains of alginate however, might neutralize the effect of liposome complexation on the cell membrane. Even though an optimum for both naked and complexed DNA was expected to be within the range of 5 to 0.008 µg/gel, titration of plasmid DNA in the context of alginate revealed that the transfection remains successful at both high- or very low DNA concentrations.

An increased release of BMP-2 protein production over a period of 5 weeks was found by ELISA analysis. This was a surprising result, considering the transient nature of non-viral

transfections [187]. Since not only culture medium samples were collected but BMP-2 levels were also measured in the depolymerized alginate, we found that a large percentage of the produced protein is contained in the gel. This probably creates a release system in which protein is produced by the transfected cells and retained in the gel. Degradation of the gel the protein then mediates its release into the medium over time which could be favourable *in vivo* since BMP-2 protein in the bloodstream has a half-life of several minutes. Cells might also be able to retain the plasmid DNA for several weeks, especially if little cell division takes place [188 189].

When investigating whether osteogenic differentiation could be induced by BMP-2 transfection, the expression of early osteogenic marker ALP both on monolayers and in cells from depolymerized gel was determined. The level of ALP expression in BMP-2 transfected cells cultured in EM was similar to that in the positive control group cultured in ODM containing the osteogenic dexamethasone [190]. Based on this assay, we conclude that BMP-2 produced from plasmid DNA transfected cells is at least as effective as ODM in directing stem cells into early osteogenic differentiation.

We found that transfection is not only possible *in vitro* but also *in vivo*. Transfected cells were found in all groups containing BMP-2 plasmid DNA, either combined with or without human and goat MSCs, at both the 2 and 6 weeks time points. This means that the delivered BMP-2 plasmid DNA is also available for resident cells to take up and produce BMP-2, creating an opportunity to design an off-the-shelf construct without seeded cells. This could overcome several problems such as the risks involved in bone marrow aspirations, or the costs and time involved with culturing stem cells. Similar to the *in vitro* experiments, osteogenic differentiation also occurs in the *in vivo* implanted samples, demonstrated by collagen I staining in the groups containing BMP-2 plasmid DNA, whereas little collagen I staining is visible in the control group without seeded cells and plasmid DNA. This is in accordance with several studies in which implanted plasmid DNA induces differentiation [107]. This makes it plausible that the induction of collagen I expression is the result of the BMP-2 transfection. During the implantation period in mice, no bone was formed. This might be explained by the timing of the BMP-2 release, which might be slower compared to other studies [191 92]. A large amount of produced BMP-2 is retained in the alginate and will not be accessible for the cells until the alginate degrades sufficiently. After 6 weeks, we still found patches of alginate present in the implants. It is possible that at later timepoints bone could be found, since the alginate calcifies between week 2 and week 6, providing a much more favourable surrounding to deposit bone on for differentiated osteoblasts. In a goat model, in which constructs similar to the ones described here were implanted in the muscle, abundant bone was formed after a longer implantation period [151].

Remarkably, cartilaginous tissue was formed in the human MSC seeded group. MSCs are known to be able to produce bone via the endochondral bone route, so it would be interesting to see whether the cartilage is eventually mineralized and replaced by bone [192].

Another explanation for the lack of bone tissue could be that the concentration of produced BMP-2 is insufficient to induce bone formation. The amount of produced BMP-2, as determined from the in vitro study is in the range of nanograms, whereas the dosage used in the clinic is in the range of milligrams [193]. And even though large doses are used in the clinic, those are

considered supraphysiological and possibly harmful [194]. Alginate is more and more used as a delivery vehicle for BMPs and turned out to be a good matrix for osteogenic differentiation and reportedly for bone formation [90]. As seen in the histological stainings, blood vessel ingrowth necessary for oxygen and nutrient delivery to the cells is efficient, and it calcifies in vivo, creating suitable mineralized matrix for cartilage and eventually bone formation.

Another interesting result is the fact that we found hardly any transfected cells which also produced collagen I. It is possible that other cells surrounding the implants e.g. macrophages take up the plasmid DNA in their attempt to engulf the alginate samples. These cells might then become BMP-2 producing cells, attracting and differentiating nearby osteogenic precursors, but are probably not BMP-2 responsive. This could explain the presence of limited numbers of transfected cells, which are themselves differentiating towards the osteogenic linage.

In summary, the use of gene therapy in bone tissue engineering to deliver osteogenic factors like BMP-2 has several advantages over the use of rhBMP-2 protein. Not only does it overcome the problems of short half-life and instability of BMP-2 resulting in the administration of supraphysiological protein dosages, but in some cases it can also result in more and better bone formation than the administration of rhBMP-2 [195 196].

We developed an economical, efficient non-viral BMP-2 gene delivery method by combining alginate with osteogenic cells and BMP-2 plasmid DNA. The alginate hydrogel serves multiple roles as an injectable scaffold, but also functions as a transfection agent and release system, releasing protein for at least 6 weeks. The produced BMP-2 protein has been proven to be biologically active and present at an effective dose to induce early osteogenic differentiation, but not yet bone formation by both human and goat MSCs and MG-63 cells *in vitro* and *in vivo*. Based on these results we conclude that after optimization, this alginate-based gene delivery system might be a promising new development in the field of bone regeneration.

ACKNOWLEDGEMENTS

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GENE DELIVERY OF BONE MORPHOGENETIC PROTEIN-2 PLASMID DNA PROMOTES BONE FORMATION IN A LARGE ANIMAL MODEL

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Jacqueline Alblas

An error in this manuscript regarding the His-tag has been discovered.
See appendix: Correction chapter 3 and 4 regarding the His-tag

ABSTRACT

In the field of bone regeneration, BMP-2 is considered one of the most important growth factors, because of its strong osteogenic activity, and is therefore extensively used in clinical practice. The short half-life of BMP-2 protein however necessitates the use of supra-physiologic doses, leading to severe side effects. This study investigated the efficiency of bone formation at ectopic and orthotopic sites as a result of a low-cost, prolonged presence of BMP-2 in a large animal model. Constructs consisting of alginate hydrogel and BMP-2 cDNA, together acting as a non-viral gene activated matrix, were combined with goat multipotent stromal cells (gMSCs) and implanted in spinal cassettes, or together with ceramic granules intramuscularly in goats, both for 16 weeks. Bone formation occurred in all cell-seeded ectopic constructs, but the constructs containing both gMSCs and BMP-2 plasmid DNA showed higher collagen I and bone levels, indicating an osteogenic effect of the BMP-2 plasmid DNA. This was not seen in unseeded constructs even though transfected, BMP-2 producing cells were detected in all constructs containing plasmid DNA. Orthotopic constructs showed mainly bone formation in the unseeded groups. Besides bone, calcified alginate was present in these groups, acting as a surface for new bone formation.

In conclusion, transfection of seeded or resident cells from this DNA delivery system lead to stable expression of BMP-2 during 16 weeks, and promoted osteogenic differentiation and subsequent bone formation in cell-seeded constructs at an ectopic location and in cell-free constructs at an orthotopic location in a large animal model.

INTRODUCTION

Bone regeneration is one of the major focus points within the field of regenerative medicine. To create bone tissue and repair bone defects, many strategies such as different cell based strategies, material development and growth factor delivery have been used [94 170]. One of the main growth factors used in bone tissue engineering is bone morphogenetic protein 2 (BMP-2), a member of the transforming growth factor β (TGF-β) superfamily. BMP-2 is known for inducing new bone formation and differentiating multipotent stromal cells (MSCs) towards the osteogenic lineage [171]. Both *in vitro* and *in vivo,* BMP-2 has shown strong osteoinductive activity and has been used in the clinical practice during the past decade [197]. Clinically however, large amounts of recombinant BMP-2 protein are administered for an osteogenic effect because of fast degradation by proteinases. BMP-2 has a half-life of 7-16 minutes systemically, and up to 8 days locally when implanted on a collagen sponge in animal studies [48]. Clinically relevant complications such as ectopic bone formation, vertebral osteolysis, radiculitis, retrograde ejaculation and soft tissue swelling are possibly related to these extremely high doses of BMP-2 [105 104].

A possible alternative is BMP-2 gene delivery, as is it low-cost and could provide sustained delivery of the protein at a more physiological concentration [107 54]. Non-viral gene therapy is highly preferred, as viral gene therapy is associated with several disadvantages such as host immunogenicity, virus-dependent recombination risks, and the prolonged protein expression exceeding the timeframe required for bone healing [54 173 174]. Non-viral gene therapy is considered much safer and usually provides a transient expression of the desired gene, but in many cases is associated with low transfection efficiencies. We recently developed an efficient non-viral gene delivery method using alginate, which acts as vehicle and transfection agent and is compatible with simultaneous cell seeding [53]. Alginate is an anionic linear copolymer of β-D-mannuronic acid and α-L-glucuronic acid residues that gelates with bivalent cations like Ca^{2+} [198 85]. It is FDA approved, non-toxic, biocompatible and extensively used in the food and drug industry. It has been used as a vehicle for plasmid DNA in the form of nanoparticles or combined with other hydrogels [85 92 93] and was applied in bone tissue engineering applications both *in vitro* and *in vivo* [178 90]. High transfection efficiencies up to 85%, slow release of biologically active BMP-2 and osteogenic differentiation both *in vitro* and *in vivo* were recently found as a result of alginate mediated transfections with plasmid DNA [53].

To increase stability and osteoinductivity of the hydrogel-based ectopic constructs, biphasic calcium phosphate (BCP) particles may be added. These particles are known for their osteoconductive and sometimes even osteinductive properties, and when seeded with MSCs, they show efficient bone formation [199 82]. MSCs are also effective in allogeneic transplantations because they do not elicit a strong immune response *in vivo,* and result in bone formation comparable to autologous MSCs [22 23 24].

To study the possibility of using plasmid DNA for posterolateral spinal fusion, cassettes that are placed on the transverse processes of goats are very attractive. This relevant bone formation model is partially comparable to posterolateral spinal fusions or critical size bone defects, since the newly formed bone is not only originating from the decorticated underlying transverse

processes but also from the more ectopic surrounding of the paraspinal muscles. In the past, the cassettes have mainly been used to study ceramic or titanium implants but have been adapted to contain hydrogel constructs as well, to investigate their performance in a bone environment [200].

In the present study we investigated the feasibility of our recently developed BMP-2 gene delivery system in a large animal model (goat), both at ectopic and spinal/orthotopic implant locations. Alginate-based constructs supplemented with BMP-2 cDNA, ceramic particles and/or MSCs were implanted in the muscle of goats to investigate the osteoinductive effect of transfection with BMP-2 plasmid DNA on bone formation in absence of bone and bone forming cells. In spinal cassettes on the transverse processes, the use of BMP-2 plasmid DNA combined with other components such as MSCs, BCP and alginate was investigated, to design an ideal bone inducing construct for future spinal fusion studies. These constructs were implanted in 4 animals as a pilot study. The expression of the transgene, onset of bone formation, tissue reaction, osteogenic differentiation and bone formation were analyzed.

MATERIALS AND METHODS

MSC isolation and culturing

Bone marrow was aspirated from the iliac wing of a Dutch milk goat, plated and cultured to isolate and expand the MSCs. These allogeneic cells were characterized and cryopreserved after a second passage in 1 ml aliquots of $1x10^7$ cells according to standardized protocols [24]. Cells were cultured in αMEM (Gibco), supplemented with 15% (v/v) fetal calf serum (Cambrex, Charles City, IA, USA), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (Glutamax, Gibco). All cells were maintained at 37 ˚C and 5% CO_2 in a humidified incubator.

Preparation of ectopic and orthotopic constructs

The ectopic constructs contained 20% (w/v) 100-200 µm BCP particles, consisting of 80%±5% (w/v) hydroxyapatite and 20±5% (w/v) β-tricalcium phosphate with total porosity of 70±5%, macroporosity of 55±5%, and microporosity of 20±5% (Xpand biotechnology). The scaffolds were cleaned in an ultrasonic bath, and sterilized by autoclave. Then placed in 8 x 8 x 8 mm chambers of Permanox chamberslides (lab-tec, Nunc) that acted as a mold. 300 µl autoclaved alginate (10 mg/ml) gel was combined with either 3 µg His/BMP-2 plasmid DNA (1 µg/ µl in water) and/or 3 million pelleted allogeneic gMSCs. The gel with cells and/or plasmid DNA was pipetted with a gelpipet on the BCP scaffolds. Subsequently the constructs were polymerized with 1 ml of sterile $CaCl_2$ solution [100 mM autoclaved $CaCl_2$, supplemented with 10 mM of sterile 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4 (Gibco)], for 10 minutes, and then were kept in culture medium until implantation. All samples were assembled in a sterile fashion. The four different ectopic groups consisted of 300 µl alginate (10 mg/ml) gel combined with 20% (w/v) BCP particles (100-200 µm) and : 1) nothing; 2) 3 µg His/BMP-2 plasmid DNA; 3) 3 x 10^6 gMSCs; 4) 3 µg His/BMP-2 plasmid DNA and 3 x 10^6 gMSCs. After mixing in cells and/or DNA, the constructs were polymerized with 2 ml of $CaCl_2$ solution, for 10 minutes, and then kept in medium until implantation.

The four orthotopic implant groups consisted of 200 µl alginate (10 mg/ml) gel combined with: 1) nothing; 2) 2 µg His/BMP-2 plasmid DNA; 3) 2 x 10^6 gMSCs; 4) 2 µg His/BMP-2 plasmid

DNA and 2 x 10^6 gMSCs. After mixing in cells and/or DNA, the constructs were polymerized with 2 ml of $CaCl_2$ solution, for 10 minutes, and then kept in medium until implantation. A gelpipet was used to inject these samples into the chambers of the spinal cassette.

Animals

After approval of the local animal committee, 4 adult female Dutch milk goats were used. The animals weighed between 51-66 kg and were between 20 and 28 months of age. Food and water were given *ad libitum* and general health was recorded per goat and monitored by the laboratory animal welfare officer. Each goat received two lumbar spinal cassettes (Figure 1) and 5 implants in the paraspinal muscles for this study. In the same animals, an instrumented lumbar spinal fusion and two iliac implants for different unrelated studies were also performed in sites unrelated to this study (not described here).

Surgery and implantation

The procedure was performed under general anesthesia using an isoflurane in air-gas mixture (Abbott Laboratories, AST Pharma, Oudewater, The Netherlands) preceded by dexmedetomidine sedation (Pfizer, Capelle aan de IJssel, The Netherlands). After shaving and disinfecting the dorsal thoracolumbar region, a midline incision was made to expose

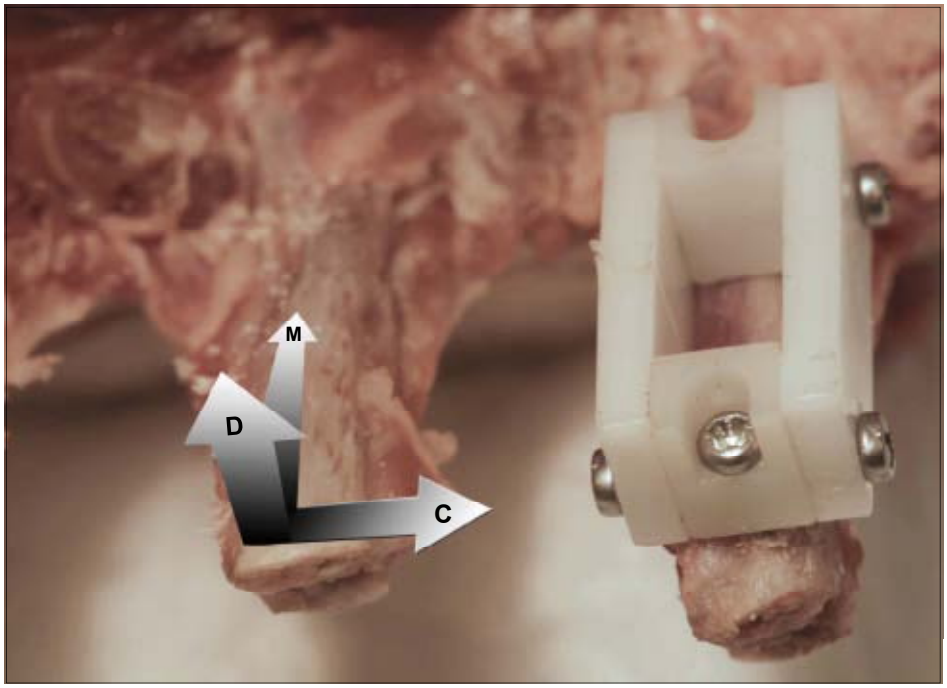


Figure 1. Spinal Cassette. Cassette placed on the dorsal side of the transverse process. M = medial, D = dorsal and C = cranial.

the paraspinal muscles. Separate bilateral incisions were made through the paraspinal muscles to expose the transverse processes of the L1 vertebra on both sides. The L2-5 vertebrae were used in an unrelated instrumented posterolateral spinal fusion study where all animals received the same treatment regiment without involvement of any growth factors. The processes of L1 were decorticated with an angled bone rasp until flat, bleeding surfaces were obtained. Two polyacetal spinal cassettes designed for fixation on the dorsal side of the transverse process of the goat lumbar spine [200] were aseptically assembled and screwed on each L1. To avoid contact between different implants/treatments, the sample compartements were separated by filling the middle compartment with acrylic bone cement (Osteonics, Stryker). The cement was molded in place between two tightly fitting Teflon sheets. After a 20 min cool down period of the cement, the prepared alginate gel samples (described under 2.2) were placed in the remaining chambers using a gelpipet (Microman, Gilson). The inserted alginate samples were in direct contact with the underlying bone and the overlying muscle (see Figure 1).

For ectopic implantation, intramuscular pockets were created through separate fascia incisions, and the four constructs (described under 2.2) were then placed in these pockets, two on each side of the spine according to a randomized block schedule. After implantation the pockets were separately closed by nonresorbable sutures. The muscle fascia, subcutaneous tissues, and skin were subsequently closed in layers. For postoperative pain relief Durogesic 25 patches (fentanyl transdermal patches; Janssen-Celag, Beers, Belgium) were used.

To investigate the dynamics of bone growth, the goats received fluorochrome labels at week 3 (calcein green, 10mg/kg, i.v.; Sigma), week 5 (oxytetracyclin, 32mg/kg, i.m.; Mycofarm, de Bilt, The Netherlands), and week 9 (xylenol orange, 80mg/kg, i.v.; Sigma). At week 16, the animals were killed by an overdose of pentobarbital (Organon, Oss, The Netherlands), and all implants were retrieved.

Histological processing and histomorphometry

After explantation, ectopic samples and cassettes with the underlying transverse process were fixed in 4% formalin, dehydrated by ethanol series and embedded in polymethylmethacrylate (PMMA). Sections were sawn from the center and side of each implant using a sawing microtome (Leica, Nussloch, Germany). The first sections remained unstained for fluorochrome analysis whereas the following sections were either stained with basic fuchsin/ methylene blue or Goldner's trichrome. The stained sections were analyzed and photographed using a light microscope (E600, Nikon, Amstelveen). The fluorochrome markers were analyzed from the unstained sections using a fluorescence microscope (E600, Nikon) equipped with a quadruple filter block (XF57, dichroic mirror 400, 485, 558, and 640nm, Omega optics, Brattleboro, VT). Fluorochromes incorporated in the newly formed bone were scored. To discriminate between the bone and the underlying scaffold, the fluorescence signal was compared to the brightfield signal.

Immunohistochemistry

Samples were fixed overnight in 4% (v/v) formalin containing 100 mM $CaCl_2$ and processed for 5 μM thick paraffin sections through alcohol dehydration series. To detect His/BMP-2-

transfected cells in the implants, anti-His immunohistochemistry was performed. Sections were permeabilised with Triton X-100 and blocked in 3% H_2O_2 for 10 minutes and 5% BSA for 30 min. Antigen retrieval steps were performed using 1 mg/ml pronase and 10 mg/ml hyaluronidase for 30 min each. Sections were subsequently incubated with 5 µg/ml biotinylated anti-His antibody (Genscript, Piscataway, NJ, USA, A00613) for 1 hour and after washing incubated with 3.3 µg/ml Streptavidin/ horse radish peroxide (HRP) for 1 hour. For collagen I, a similar protocol was performed using rabbit-anti-collagen I primary antibody (Abcam, Ab34710) at 3.3 µg/ml and a goat-anti-rabbit-Streptavidin/HRP (Invitrogen, 624320)) secondary antibody at 6.6 µg/ml. Both stainings were developed with diaminobenzidine (DAB) and Mayer's hematoxylin was used for counterstaining.

Scoring of collagen I, calcified alginate and bone

To determine differences per group in the amount of collagen formed, scorings were assigned as follows: 0= no collagen; 1= moderate collagen; 2= abundant amounts of collagen. Two independent observers scored all blinded sections. Results were compared and in cases of inconsistencies a third independent observer was added. Due to a small sample size no reliable statistical analysis could be performed.

Calcified alginate and bone were scored in a similar fashion on basic fucsin/methylene blue stained MMA sections: 0 =absence of bone or calcified alginate; 1= some bone or calcified alginate; 2= abundant amounts of bone or calcified alginate.

Bone and calcified alginate histomorphometry

High-resolution digital photographs were made from HE stained sections for histomorphometric analysis. Three pictures at predefined positions within the explant were taken from each sample. Bone, calcified alginate and scaffold were pseudocolored using Adobe Photoshop CS3, whereafter histomorphometry was performed using Axiovision software (version 3; Zeiss, Nussloch, Germany). A custom macro was used to measure the region of interest, the area of scaffold, the area of bone/alginate, the scaffold outline available for bone apposition, and the contact length of bone and scaffold. This allowed the calculation of the percentage bone/alginate in available space, called bone area% (bone area%=(bone area/[total area - scaffold area])100%). And the percentage bone apposition was indicated as bone contact% (bone contact% = (bone-to-scaffold contact length/scaffold outline)100%).

Statistics

The statistical significance of differences between experimental groups in Figure 4 and 6 was assessed using a Fisher exact test. Multiple comparisons were generated using a Bonferroni correction to calculate the p-values. A p-value of < 0.05 was considered significant.

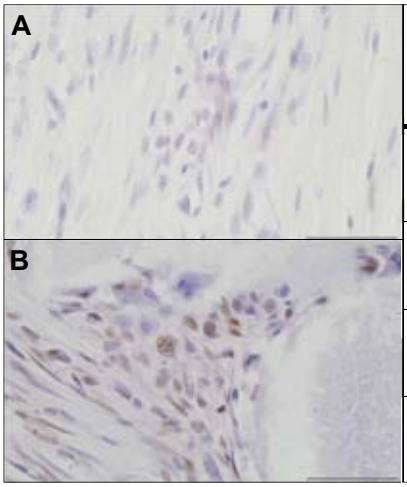
The statistical significance of differences between experimental groups in Figure 7 was assessed using a randomized block design. Multiple comparisons were generated using a tukey posthoc correction to calculate the p-values. A p-value of < 0.05 was considered significant.

RESULTS AND DISCUSSION

We investigated the bone forming capacity of BMP-2 plasmid DNA based alginate constructs at ectopic and orthotopic implant locations. Alginate/BCP constructs containing BMP-2 plasmid DNA and/or gMSCs were implanted ectopically in the paraspinal muscles of goats for 16 weeks. After 16 weeks half of the samples were embedded in MMA for fluorochrome analysis and the other half in paraffin for immunohistochemistry. Analysis of the histological sections demonstrated that the transfection in alginate was successful as shown by immunohistochemistry of the BMP-2 coupled His-tag. All samples containing BMP-2 plasmid DNA, both cell seeded and non-seeded, contained numerous His-tag positive cells (Figure 2). This is a strong indication that resident goat cells are able to take up the DNA and express BMP-2 protein up to 16 weeks after implantation. Since plasmid DNA provides transient expression, these are remarkable results after an implantation period of 16 weeks, even though comparable results were found after 6 weeks in previous mouse studies or *in vitro* [53]. Alginate however, besides being used for bone tissue engineering *in vitro* and *in vivo* is also used for slow release strategies, and might therefore retain the plasmid DNA until invading and surrounding cells endocytose it [90 201].

To determine further morphology and tissue response of the implanted samples, hematoxylin/eosin (HE) staining was performed on all paraffin embedded samples (Figure 3A,B). Despite the use of allogeneic cells, hardly any immune response was detected. In a few samples some lymphoid clusters were present, but no encapsulation of the implants were seen and hardly any multinucleated giant cells were lining the scaffolds. The BCP particles were not degraded and were surrounded by either bone or fibrous tissue.

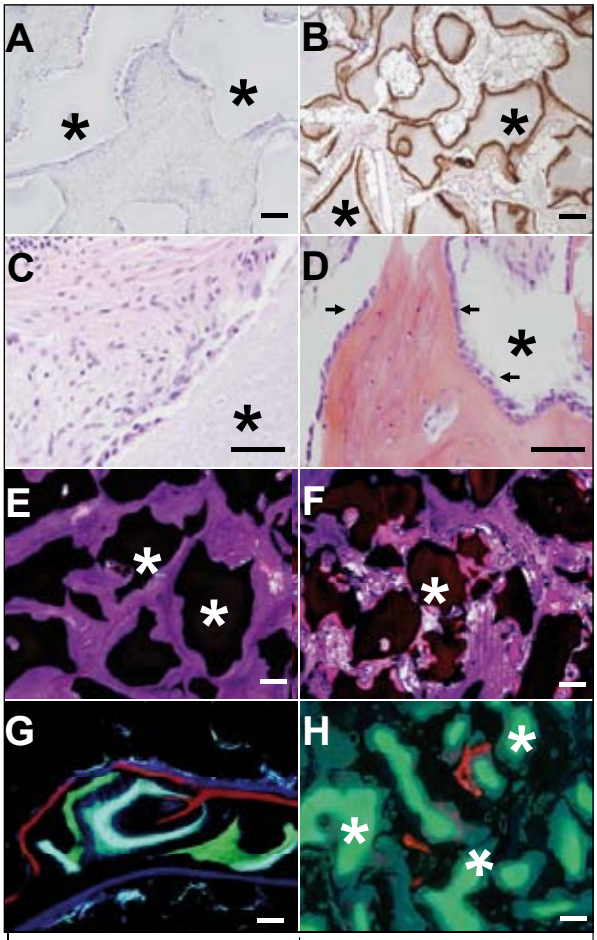
To determine the amount of collagen I, the main organic component of bone, immunohistochemistry was performed on all ectopic, paraffin embedded samples



C

Group	α-His
Control	0/4
gMSCs	0/3*
His/BMP-2	4/4
gMSCs + His/BMP-2	3/3*

Figure 2. BMP-2 expression as a result of in vivo transfection. A) Untransfected control sample and B) His/BMP-2 cDNA loaded alginate sample immunostained for the His-tag (in brown). C) Scoring for presence/absence of His-positive cells in all implants. *= missing sample. Scalebar = 50 µm.



Group	Xylenol Orange (9 w)
Control	2/4
gMSCs	1/4
His/BMP-2	1/4
gMSCs + His/BMP-2	0/4

Figure 3. Bone and collagen presence in ectopic implants. A) Interface between BCP scaffold and fibrous tissue; HE staining of control sample. B) gMSCs + His/BMP-2 seeded sample, HE staining; bone in pink, arrows indicate osteoblasts. C) Control sample stained for collagen I. D) gMSCs + His/BMP-2 seeded sample with Collagen I staining in brown. E-F) Bone formation seen after basic fuchsin/methylene blue staining; control sample in E), gMSCs + His/BMP-2 seeded sample in F); bone in pink, fibrous tissue in purple. G-H) Detection of fluorochromes in the spine in G) and in gMSCs + His/BMP-2 seeded sample in H), week 9 label in orange, background staining of scaffold in green. A-H) * = BCP scaffold, scalebar = 100 µm. I) Scoring of number of samples with positive for xylenol orange / total number of samples

(Figure 3C,D). The unseeded groups either with or without BMP-2 plasmid DNA contained hardly any collagen I. Even though we found transfected cells in the plasmid DNA containing samples, no differences in collagen I production were detected between the control group and the BMP-2 plasmid loaded group.

In the cell seeded groups collagen I was present in all samples. The amount of collagen I was most abundant in the group containing gMSCs and BMP-2 plasmid DNA. It appears that in the presence of seeded cells, the production of BMP-2 as a result of the transfection induces more osteogenic differentiation than in the group without BMP-2 plasmid DNA, as seen by collagen I production. To assess bone formation in these samples, PMMA embedded sections were stained with basic fuchsin/methylene blue staining (Figure 3E, F). The bright pink stained bone is easily detected and scored and coincides with the results of the collagen I staining. Both cell-free groups contained hardly any bone or collagen staining, whereas the cell seeded groups showed high amounts of both collagen I and bone formed between the BCP particles of the implants (Figures 3B,E,F, 4). This corresponds with previous studies in which BCP combined with seeded MSCs also elicits a higher bone forming capacity compared to cell-free constructs [24]. Allogeneic MSCs can be used, inducing comparable effect as autologous MSCs with respect to bone formation. When BMP-2 plasmid DNA is added to the MSC seeded implants, more collagen I production and bone formation was scored than with MSCs alone. This is a remarkable result considering the fact that the construct containing MSCs is already osteoinductive. It seems that the addition of BMP-2

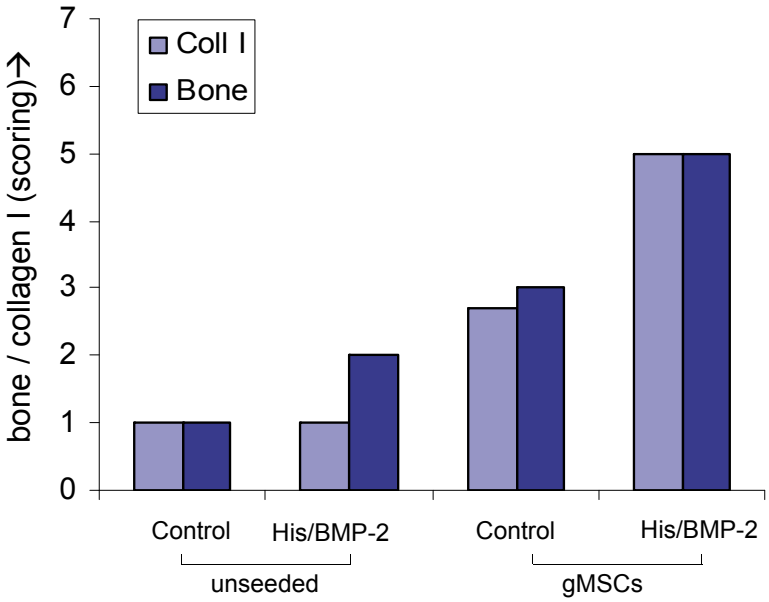


Figure 4. Osteogenicity of the ectopic implants. Scoring of the presence of collagen I and bone (n=4 per group) after 16 weeks implantation. See M&M for details. Sections were scored by two independent observers.

can further increase bone formation. In the cell-free groups, even though resident cells did take up the plasmid DNA and produced His-tagged BMP-2, no additive effect on either collagen I production or bone formation was detected. Fluorochrome analysis revealed that the onset of bone formation in all groups occurred later than previously shown at our lab [24]. None of the groups contained either the green or yellow label and only a few samples contained the orange label. The presence of the orange label indicates bone formation started between week 5 and week 9. In the absence of the orange label bone formation only took place after week 9 (Figure 3G,H, I). This might have a multifactorial cause. The fact that BCP particles instead of a solid BCP scaffold were used, might have delayed bone formation due to possible movement and friction between the different particles [202]. Another possibility could be that alginate retains a lot of produced proteins and growth factors, making them only available after the alginate degrades [203].

To determine bone formation in the orthotopic surrounding, MMA sections of the spinal cassettes were stained with basic fuchsin/methylene blue (figure 5A,C). In all samples some bone ingrowth from the decorticated transverse processes was detected but in contrast to the ectopic implants, no additive effect of the BMP-2 plasmid DNA was found in either the cell seeded or non-seeded groups. Bony structures in the center of the construct were almost exclusively found in samples without seeded cells. This tissue appears similar to bone but with a less organized morphology and less incorporated cells. This is most likely calcified alginate, which is known from the literature to form a bone-like calcified matrix, both with respect to biomechanical and biological properties [204 205 206]. The calcified alginate appears to act as a scaffold for new bone to grow on since layers of bone are deposited on the alginate. In our study both bone and calcified alginate were found mainly in the non-cell seeded samples. This could indicate that alginate is being degraded differently in the presence of MSCs, and that a mineralized substrate might promote bone formation. This bone formation occurred only after 9 weeks since none of the three administered fluorochromes were present in any of the orthotopic samples.

To determine whether this calcified tissue also contains collagen, a Goldner's trichrome staining was performed (Figure 5B, D). We found that besides the newly formed bone, the calcified alginate also stains for collagen, but in this staining a difference in morphology between calcified alginate and bone is also visible. It is remarkable however that the highest bone quantities can be found in groups containing a lot of calcified alginate. The groups with seeded MSCs show low amounts of calcified alginate and bone (Figure 7). Besides acting as a scaffold for bone to be deposited on, the calcified alginate also triggers a foreign body response, seen by the multinucleated giant cells lining the alginate especially at sites where calcified alginate is not covered with bone. The giant cells are not seen in ectopic constructs, where the alginate is degraded much faster, probably due to better tissue infiltration from all sides. The presence of multinucleated giant cells however is very common in tissue engineering [207]. It is possible that they are attracted after calcification.

Besides the fact that BCP was only used in the ectopic implants, the differences seen between ectopic and orthotopic constructs might also be caused by the use of alginate in our constructs. Ectopically, the seeding of cells resulted in the highest scored bone formation.

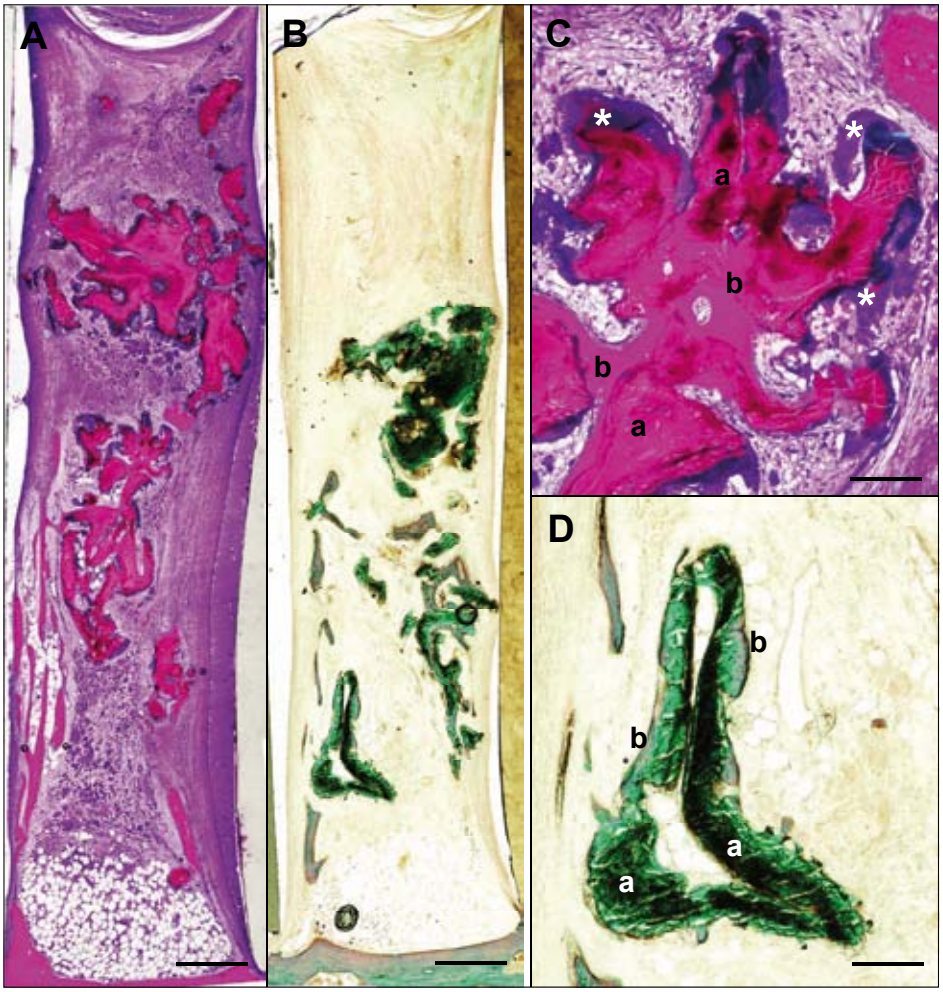


Figure 5. Bone and alginate in spinal cassettes. A) Overview control sample with bone and calcified alginate in pink, stained by basic fuchsin/ methylene blue staining; B) Overview unseeded His/BMP-2 sample with bone and calcified alginate in green (collagen), stained by Goldner's trichrome; C) detail of A; D) detail of B; a: alginate, b: bone, *: multinucleated giant cells. A, B) scalebar = 1 mm, C,D) scalebar = 100 µm.

Orthotopically however, these constructs only showed minor bone formation, whereas the cell-free samples performed better. We previously described that in constructs directly in contact with bone, the presence of seeded cells does not contribute to bone formation [24]. Both in the cell-seeded en cell-free constructs however, no additive effect of the BMP-2 plasmid DNA was found. Since the cassette model creates a confined space in which cells from the underlying bone migrate into the chamber to colonize the scaffold and induce bone formation, it might be possible that the alginate present in the cassette hampers this cell migration process. Therefore degradation of the alginate might be slower than in the

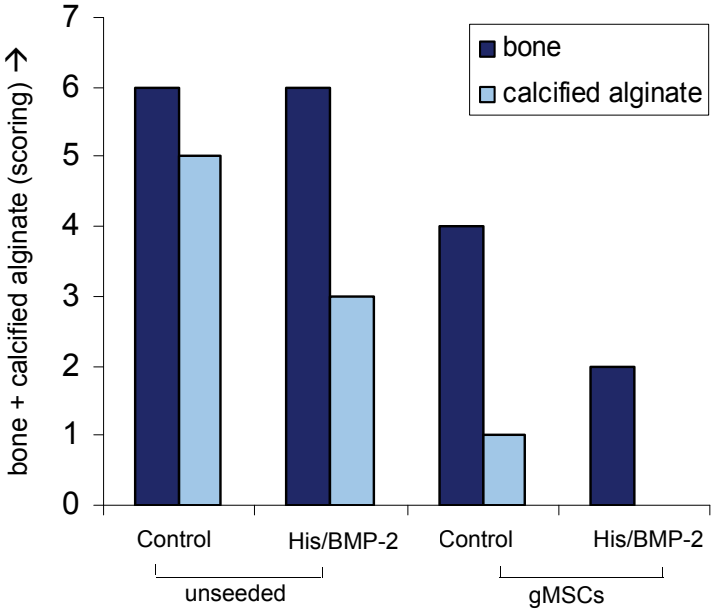


Figure 6. Calcified alginate and bone in orthotopic implants. Scoring of the presence of calcified alginate and bone (scale 0-2, n=4 per group) after 16 weeks implantation, see M&M for details. Sections were scored by two independent observers.

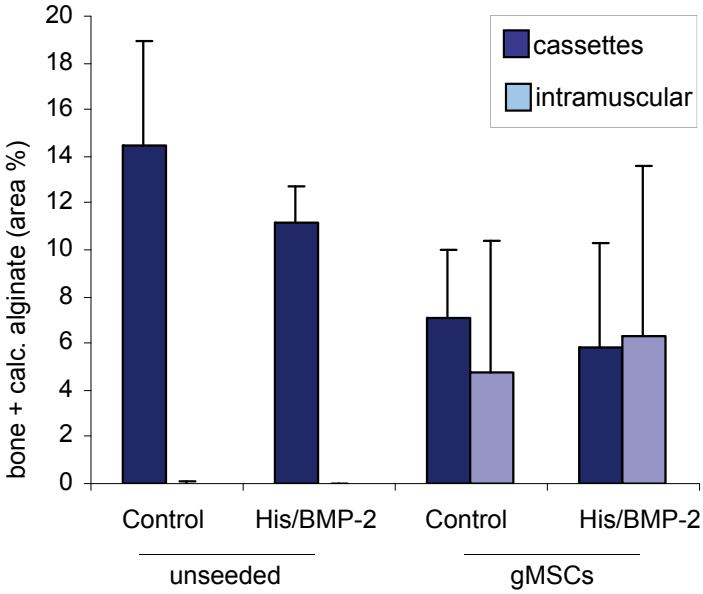


Figure 7. Quantification of bone and calcified alginate. Total bone and calcified alginate area % indicated for each group (mean + standard deviation of 4 samples in the ectopic (intramuscular) and orthotopic (cassettes) constructs).

ectopic constructs, and access to the plasmid DNA might be less efficient and blood vessel ingrowth and influx of bone forming cells from the underlying bone could be delayed. This would decrease the amount of produced BMP-2 and delay the onset of bone formation.

In summary, the use of BMP-2 plasmid DNA delivery in alginate gel could be a promising new strategy for bone tissue engineering. We have previously shown its potential in immunodeficient mice and extend these studies now to a widely used large animal model. Ectopically, BMP-2 plasmid DNA increases the osteoinductivity of constructs containing BCP particles combined with MSCs. In this setting, cell-free constructs have no benefit from the BMP-2 DNA, since hardly any bone was formed. In the cassette model, the calcified alginate triggered bone formation mainly in the unseeded groups. The calcified alginate provided a scaffold with bone-like properties for new bone to grow on. Based on these results we conclude that after optimization, the delivery of BMP-2 plasmid DNA via alginate might be a promising new approach for bone tissue engineering.

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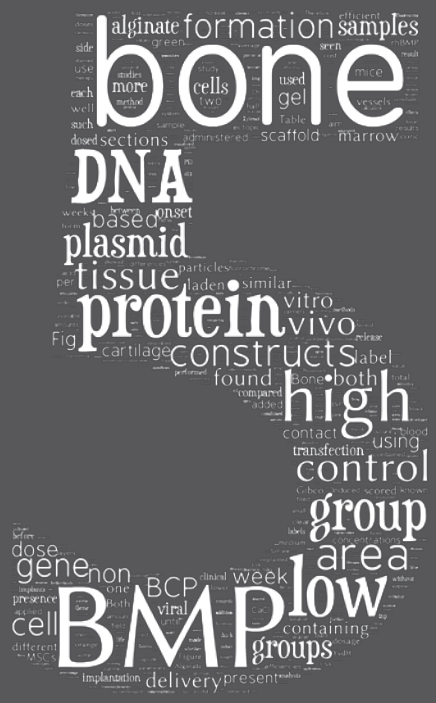


BMP-2 PLASMID DNA AS A SUBSTITUTE FOR BMP-2 PROTEIN IN BONE TISSUE ENGINEERING

Fiona Wegman
Yvonne van der Helm
Cumhur Öner
Wouter Dhert
Jacqueline Alblas

ABSTRACT

Bone regeneration is one of the focus points in the field of regenerative medicine. A well-known stimulus of bone formation is bone morphogenetic protein-2 (BMP-2), which has already been extensively used in clinical applications. However due to a short half-life, supraphysiological doses are applied resulting in severe side effects such as ectopic bone formation or even loss of bone. We compared the effectivity of transient BMP-2 gene delivery with BMP-2 protein at clinical (high) and physiological (low) doses by subcutaneously implanting alginate based constructs in mice. After 6 weeks of implantation, both the protein-laden constructs and BMP-2 plasmid DNA based constructs showed similar early bone onset and elevated bone formation compared to controls without any BMP-2 added. We found no differences in efficiency by using BMP-2 plasmid DNA or any of the BMP-2 protein dosages. Therefore we conclude that BMP-2 plasmid DNA based gene therapy in alginate is a promising new strategy for BMP-2 administration for bone (re)generation.

INTRODUCTION

Bone morphogenetic protein (BMP-2) is one of the major growth factors in the field of bone tissue engineering. This member of the transforming growth factor β (TGF-β) superfamily is involved in committing multipotent stromal cells (MSCs) towards the osteogenic lineage and is known to induce new bone formation [99 171]. Both *in vitro* and *in vivo,* BMP-2 has shown strong osteogenic activity. Recombinant BMP-2 in combination with a collagen sponge has been approved for the treatment of open long bone fractures and combined with a metal cage for spinal fusions [208 48 105]. Due to the very short half-life of only 7-16 minutes in vivo, large amounts of the protein have to be administered in combination with carriers to prevent immediate diffusion from the desired site [48]. The possible negative side effects for patients as a result of high dosage of BMP-2 are vertebral osteolysis, ectopic bone formation, radiculitis and cervical soft tissue swelling [105 104 106]. As an alternative for recombinant BMP-2, gene therapy might overcome the use of high doses because applied to cells a relatively sustained delivery of protein can be achieved [54].

Gene therapy is most often applied by viral gene delivery, which results in high transfection efficiencies but is associated with problems such as host immunogenicity and virus-dependent recombination risks [174 173 172]. Non-viral gene delivery techniques aim at transient expression of the desired gene and are therefore not hampered by the risks of integration-dependent mutations. A disadvantage is that non-viral transfection methods are notoriously inefficient. Therefore much effort has been invested in the development of an efficient non-viral gene delivery method, with the use of various transfection agents [209] and application matrices [107]. Our group developed a method for efficient gene delivery based on alginate hydrogel, which is compatible with simultaneous cell seeding [53]. Alginate was chosen, because this anionic polysaccharide that gelates with bivalent cations such as Ca^{2+} [85] is FDA approved, non-toxic and biocompatible. Combined with other hydrogels or in the form of nanoparticles it has been used as a vehicle for plasmid DNA before [93 210 211].

In comparison to established non-viral gene delivery methods such as PEI, PLGA and Chitosan, Alginate is able to induce similar transfection efficiencies (PEI 50% [144], PLGA 80% [140], Chitosan 20% [148] Alginate 60% [53]). To reach these transfection efficiencies, the proteins or polymers are often combined with other materials. The main problem is namely that an increase of material in nescecary to increases the percentage of transfected cells, which often leads to cytotoxic concentrations. For alginate this phenomenon has not been described, as low concentrations appear to be successful at inducing cell transfections [53]. In terms of practicality, the alginate gel will act as a combination of cell carrier, transfection agent and protein release system. This combination of factors is not found in the other transfection agents. It has been succesfully applied in bone tissue engineering applications both in vitro and in vivo [90 178], which makes it very suitable as gene activated matrix (GAM) for cell seeded constructs.

Our aim is to apply BMP-2 plasmid DNA based constructs for bone tissue engineering. The present study investigates whether BMP-2 plasmid DNA based constructs can be as efficient in inducing bone formation as recombinant BMP-2 protein.

MATERIALS AND METHODS

Alginate gel

Autoclaved high-viscosity non-medical-grade alginate powder (International Specialty Products, ISP, Memmingen, Germany) was dissolved at a concentration of 10 mg/ml in alpha minimum essential medium (α-MEM, Gibco, Breda, The Netherlands). The gel was polymerised by adding an equal volume of 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 1 ml expansion medium.

Cell culture

Goat MSCs were isolated from bone marrow (BM), aspirated from the iliac wings of Dutch milk goats. The MSCs are isolated by adherence to tissue culture plastic and cultured in expansion medium, containing αMEM (Gibco), supplemented with 15% (v/v) fetal calf serum (Cambrex, Charles City, IA, USA), 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. All cells were between p3-p6 in the experiments described here.

Preparation *in vivo* implants

Four different constructs were made, each consisting of 200 μl alginate (10 mg/ml), 10^7 goat MSCs/ml gel and 20 % (w/v) BCP particles (size 106-212 μm). The 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% (kindly provided by Xpand biotechnology, Bilthoven NL). The BCP particles were cleaned in an ultrasonic bath and sterilized by autoclaving. The BMP-2 plasmid DNA construct contained the full-length human recombinant BMP-2 cDNA, whose expression was driven by the cytomegalovirus promoter in pcDNA3.1 (Invitrogen). Recombinant human BMP-2 protein (rhBMP-2) was obtained from a clinical kit (Infuse, Medtronic, Minneapolis USA).

The four different constructs were further supplemented as follows: group 1) buffer (control), group 2) 25 ng/ml rhBMP-2 protein (BMP-2 low), group 3) 25 μg/ml rhBMP-2 protein (BMP-2 high), group 4) 10 μg/ml of pcDNA3.1/hBMP-2 plasmid DNA. BMP-2 protein or plasmid DNA was added before alginate polymerization with 1 ml of 100 mM autoclaved $CaCl_2$, supplemented with 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES pH 7.4, Gibco), for 10 minutes. The supernatant was removed and all implants were kept in MSC culture medium until implantation (within 2 hours)

Animals and implantation

Female nude mice (Hsd-cpb:NMRI-nu, Harlan) were anaesthetized with 1.5% isoflurane, after which the implants were placed in 4 separate subcutaneous dorsal pockets per mouse (n=6 per group). To prevent mutual influence of neighboring samples, they are implanted in a randomised fashion. 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), and at 4 weeks after implantation Xylenol orange was administered s.c. (30 mg/kg, Merck, Amsterdam, Netherlands) [212]. 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 to analyse general morphology, transfection and bone histomorphometry. Samples were cut in two, one part 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% (v/v) phosphate buffered formalin, dehydrated by ethanol series and embedded in polymethylmethacrylate (MMA), after which 10 µm thick sections were sawn from the center and side of each implant using a sawing microtome (Leica, Nussloch, Germany). These sections remained unstained for fluorochrome analysis. The presence of each fluorochrome label was scored for all implants.

Immunocytochemistry

Paraffin sections were stained with hematoxylin/eosin (HE). Blinded samples were scored for morphology and presence of blood vessels by two observers (FW, YvdH).

Safranin O staining

Sections were deparaffinized and incubated with hematoxylin (Weigert's) for 5 min. After washing in running tap water, sections were dipped in distilled water and counterstained in 0.4% (w/v) Fast green solution. Sections were rinsed in 0.17 M acetic acid until colour no longer runs and counterstained again in freshly prepared 0.125% (w/v) Safranin O (Merck) staining dissolved in distilled water. After a short dehydration protocol, sections were mounted in Depex. Two independent observers (FW, YvdH) scored for presence of cartilage.

Histological scoring

HE stained sections were scored for several general histological parameters (Table 1). Bone, bone marrow and cartilage were scored for presence or absence of these features. For blood vessels more than 10 vessels/microscopic field was scored as positive. For capsule formation the criterium was a thickness of 5 cell layers; more than 5 cell layers received a positive score and less than 5 cell layers a negative score. All histological scorings were performed by two independent observers (FW, YvdH) in a blinded fashion. The incidences of parameters are calculated as % of the total number of animals.

Bone histomorphometry

High-resolution digital photographs were made from HE stained sections for histomorphometric analysis. Three pictures at predefined positions within the explant were taken from each sample. Bone and scaffold were pseudocolored using Adobe Photoshop CS3, where after histomorphometry was performed using Axiovision software (version 3; Zeiss, Nussloch,

Germany). A custom macro was used to measure the region of interest, the area of scaffold, the area of bone, the scaffold outline available for bone apposition, and the contact length of bone and scaffold. This allowed the calculation of both the percentage bone per available space, called bone area% [= (bone area/(total area-scaffold area))*100%], and the percentage bone apposition, called contact% [= (bone-to-scaffold contact length/scaffold outline)*100%](2).

Fluorochrome analysis

The fluorochrome markers were analyzed from the unstained sections using a fluorescence microscope (Olympus BX51 microscope, Olympus DP70 camera, Hamburg, Germany) equipped with a quadruple filter block (XF57, dichroic mirror 400, 485, 558, and 640nm, Omega optics, Brattleboro, VT). Fluorochromes incorporated in the newly formed bone were scored for presence/absence. To discriminate between the bone and the underlying scaffold, the fluorescence signal was compared to the brightfield signal.

Statistics

The statistical significance of mutual differences between the control group, the low dosed BMP-2 protein group, the high dosed BMP-2 protein group and the plasmid BMP-2 group in Figure 1 were assessed using a randomized block design. Multiple comparisons were generated using a Tukey posthoc correction to calculate the p-values. A p-value of < 0.05 was considered significant.

For Table 1 a Chi-square test was performed to evaluate statistical differences between the control group, the high and low dosed BMP-2 protein groups, the BMP-2 plasmid DNA group, and the precultured plasmid DNA group for presence or absence of several histological features. Multiple comparisons were generated using a Bonferroni correction to calculate the p-values. A p-value of < 0.05 was considered significant.

RESULTS

From previous studies in which alginate gel was combined with BMP-2 plasmid DNA and MSCs, it has become clear that this is a successful method to transfect cells. When BCP particles are added, osteogenic differentiation is induced *in vivo* as well [53].

To investigate whether bone formation induced by plasmid DNA can reach an efficiency similar to bone formation induced by BMP-2 protein, alginate based samples were implanted subcutaneously in mice. Histology of the implanted constructs revealed abundant quantities of bone in all BMP-2 containing constructs. In the control group without BMP-2 in any form present, some bone formation was seen on the BCP particles. Large quantities of bone bridging the BCP particles was mainly found in the BMP-2 containing groups (Fig. 1a-d). Both the BMP-2 protein groups and the plasmid DNA group appear to induce similar amounts of bone. Besides bone, bone marrow consisting of adipose tissue and heterogenous small nucleated cells, was present in all groups containing extensive bone formation. This was seen more often in the BMP-2 protein groups (Table 1, Fig 1f). All constructs were well vascularized, as seen by abundant blood vessels and no signs of an inflammatory response were present. Alginate remnants were not detected in any of the samples indicating that it had been degraded completely. Hardly any

Table 1. Incidence of bone and histological parameters in vivo (scorings given as % of total animals)

	Bone	Bone marrow	Cartilage	Blood vessels	Capsule
Control	60 [2]	20	20	100	0
BMP-2 low	100	67 [3]	17	100	0
BMP-2 high	100	67 [3]	17	100	0
Plasmid DNA	83	33	67 [4]	100	0

[1] Significantly lower bone incidence compared to all other groups
[2] Significantly lower bone incidence compared to BMP-2 low and BMP-2 high
[3] Significantly higher bone marrow incidence compared to the control, plasmid DNA and precultured plasmid DNA groups
[4] Significantly higher cartilage incidence compared to all other groups

capsule formation was detected and if there was some fibrous tissue surrounding the implant this was less than 5 cell layers thick. In all groups containing bone, some cartilage formation was detected as well (Fig 1g-h). Both the control group and the high and low dosed BMP-2 protein groups presented cartilage formation. In the plasmid DNA group all implants containing bone also exhibited areas with cartilage formation (Table 1, Fig 1g-h).

The onset of bone formation was investigated by fluorochrome analysis. Calcein green (green fluorescent label) was administered at week 3 and Xylenol orange (orange/red fluorescent label) was administered at week 4. The fluorochromes showed a general onset of bone formation in all BMP-2 constructs before week 3. No clear differences between the two different protein concentrations and the plasmid DNA based group were found, although the BMP-2 protein high concentration group was the only group in which we found both labels in all samples within the group. The control group contained one sample showing early bone formation and all other samples did not incorporate any of the labels (Fig 2).

To quantify the amount of bone in the implants, bone histomorphometry was performed on the HE stained sections for determination of both bone area% and bone contact% (Fig 3). In the control group an average of 15% bone area and 20% bone contact was found. Compared to the control, the BMP-2 protein low concentration group showed a trend, though not statistically significant, towards a higher amount of bone formed: up to 30% bone area and 40% bone contact. Very similar results were found for the high dosed BMP-2 protein group with an average of 31% bone area and 41% bone contact ($p<0.05$ compared to control). The plasmid DNA laden group presented the highest amount of bone formation with an average bone area of 38% and bone contact of 44% ($p<0.05$ compared to control).

DISCUSSION

This study investigated whether BMP-2 plasmid DNA based gene delivery can be as efficient at inducing bone formation as rhBMP-2 protein application. To this end, we used a gene delivery method that was recently developed by us to express BMP-2 in hybrid constructs implanted subcutaneously in mice and compared this to two BMP-2 protein concentrations and unloaded controls. After six weeks *in vivo,* BMP-2 added in the form of plasmid DNA

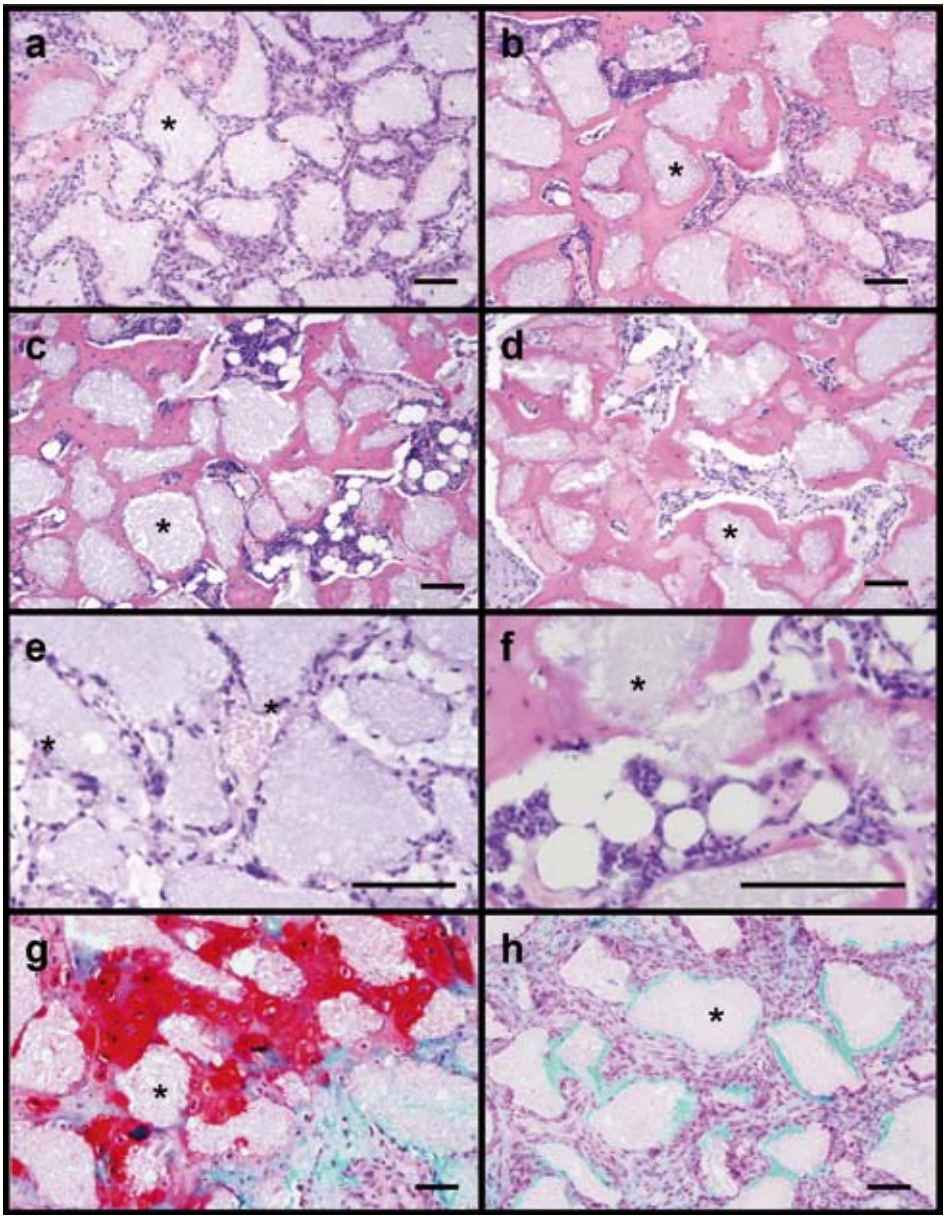
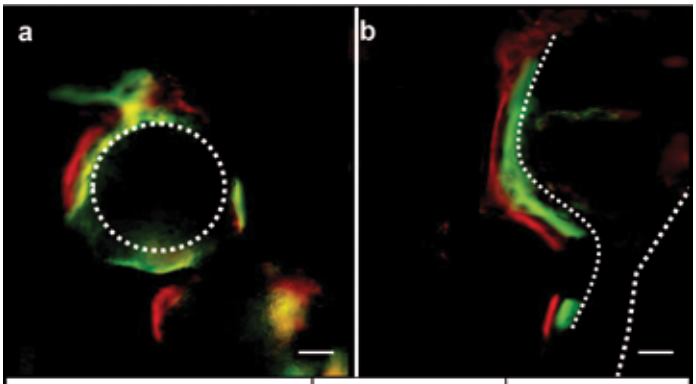


Figure 1. Bone and cartilage formation *in vivo*. a-f) HE staining, bone in pink. a) control b) BMP-2 protein low conc., c) BMP-2 protein high conc., d) BMP-2 plasmid DNA, e) detail of blood vessel f) detail of bone marrow from b,c,d. g-h) Safranin O staining, cartilage in red, bone in blue/green, g) BMP-2 plasmid DNA laden sample containing cartilage tissue, h) control sample without cartilage tissue. a-h) Representative samples were chosen for each group. BCP particles indicated with *, scalebar = 100 µm.



c	week 3	week 4
control	20	20
BMP-2 low	83	50
BMP-2 high	100	83
BMP-2 plasmid DNA	67	67

Figure 2. Bone onset analysed by fluorochrome incorporation. Detection of Calcein green, administered at week 3 after implantation and Xylenol orange, administered at week 4 after implantation. a) BMP-2 protein high conc. sample displaying both the 3 and 4 week label. b) BMP-2 plasmid DNA sample displaying both the 3 and 4 week label. a-b) The outline of the BCP scaffolds is indicated by a white dotted line, scalebar = 50 µm. c) Frequency (%) of samples with fluorochrome incorporation.

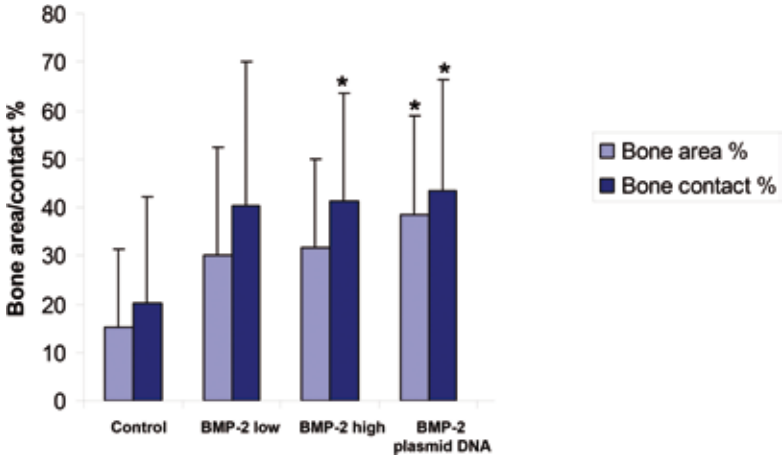


Figure 3. Quantification of bone formation. Analysis of bone area% and bone contact% (for details see Materials and Methods). * = significantly different from control group, $p<0.05$.

was very efficient in stimulating bone formation and performed at similar levels as BMP-2 protein at both concentrations tested.

Gene therapy based on non-viral plasmid DNA is pursued by many groups because of its clear advantages, like transient expression, efficient protein production at low cost, but is also known for low transfection efficiencies [179]. Gene delivery using alginate has proven to result in high (up to 62% based on GFP expression) transfection efficiencies in different cell types *in vitro*, and osteogenic differentiation *in vivo* [53]. In the present study the design of the constructs was changed in order to stimulate bone formation in this ectopic setting. The addition of ceramic particles optimized osteogenicity while still allowing sensitivity to BMP-2. The plasmid DNA group has shown to be successful and is clinically relevant, reason why we focused on comparing different BMP-2 delivery methods to a BMP-2 free control.

When monitoring bone onset in the various constructs using fluorochromes, we found that bone formation had started before week 3 in BMP-2 laden samples. Almost all of these samples showed the presence of the green fluorescent label, which is in line with literature [213]. Early onset was not always followed by the incorporation of the orange label, a week later. This might be explained by the notion that bone formation was already so robust that available space for expanding bone formation was filled, halting further label incorporation after week 3. Another explanation could be that pores in the constructs were often filled with cartilaginous and/or bone marrow-like tissue, which do not bind the fluorescent labels (Table 1).

To optimize the constructs and provide a mineralized surface for bone formation, BCP particles were added to the alginate in the present study. The particles, known to promote osteogenesis, were combined with gMSCs, which contribute to bone formation in tissue engineered constructs [26]. Therefore some bone was also formed in the control samples, which is in accordance with previous results [214] [24]. The addition of BMP-2 in any form at least doubled the amount of bone present. Both the high and low dose of BMP-2 protein induced similar amounts of bone. The use of a supraphysiological dose of BMP-2 protein is highly debated. Several severe side effects including bone loss have been reported, and insufficient research has been performed to establish an optimal dose [105]. Therefore inclusion of a low concentration protein group alongside the plasmid DNA group is relevant when considering efficacy and safety of BMP-2. In literature little is found on the comparison of different concentrations of BMP-2 to establish a minimal effective dose, and furthermore a few published results suggest that a higher dose does not automatically increase bone formation [215] [216]. The majority of bone tissue engineering based on BMP-2 focuses on prolonged release of BMP-2 from different hydrogel or ceramic carriers, in order to lower the initially applied dose of BMP-2 [53] [217]. *In vitro* the responsiveness of several cell lines such as the MC3T3-E1 cells was established up to 50 ng/ml, which is more than 4 decades lower than the clinically administered dose which is 1.5 mg/ml scaffold. When translated to mice, the local dosages applied are the used 25 ng and 25 µg of rhBMP-2/ml construct, representing physiological (low) and clinical (high) doses. The *in vitro* dosage of released BMP-2 protein after transfection with 1 µg BMP-2 plasmid is in the order of 10-20 ng/ml and thus more comparable to the lower administered protein dose with respect to the BMP-2 quantities, but not the release or delivery kinetics [53]. In mice, no significant

differences in bone onset or amount of bone were found when comparing high and a low dosage of BMP-2 protein and the BMP-2 plasmid laden group. We conclude that applying a low dose is sufficient to significantly enhance bone formation in this setting. In addition, it is known from previous experiments and literature that alginate is a slow release system for BMP-2, and that most of the protein is retained in the gel [53 217]. *In vitro* studies indicated a BMP-2 release in and from the gel up until 5 weeks. While taking into account that *in vitro* and *in vivo* protein expression are not always comparable, the presence of the gel likely functions as a controlled release system preventing high BMP-2 dosage.

Significant differences in bone formation were found between the high dosed BMP-2 group and the plasmid DNA group compared to control samples. This illustrates the additive effect of BMP-2 on cell laden BCP constructs, which presented a basal average bone area of 20%. The fact that BMP-2 plasmid DNA induced bone formation ectopically is consistent with the majority of studies described in literature. Both intramuscular and ectopic, either transfected MSCs or delivered BMP-2 plasmid DNA significantly increase bone formation compared to negative controls. These studies all reported considerably lower bone volume percentages than we found and did not mention the presence of cartilage or bone marrow [107 211 213 218]. Semiquantitative scoring revealed that bone marrow was seen more frequently in the BMP-2 protein group as compared with the the BMP-2 plasmid DNA and simultaneously more cartilaginous tissue was visible. This might indicate that in samples containing BMP-2 plasmid DNA endochondral bone formation plays a more important role than in the protein groups. This may be the result of different dosage at the start of the implantation, because it takes a few days for the cells to become transfected and start to produce BMP-2, whereas the protein-laden groups have BMP-2 available immediately after implantation. Since there is some bone marrow present in the plasmid DNA group and some cartilage in the protein groups, the most likely explanation is that both forms of bone formation occured in all groups but one is favoured over the other, which is in accordance with other studies [219 220]. As only small amounts of plasmid DNA are necessary, this method creates a cheap, non-viral substitute for the clinically used BMP-2 protein treatment for bone regeneration.

CONCLUSION AND FUTURE DIRECTION

Using a recently developed gene delivery system, BMP-2 protein was substituted with BMP-2 plasmid DNA which resulted in efficient bone formation in an ectopic mouse model. We conclude that BMP-2 plasmid DNA is a low-cost, non-viral alternative for BMP-2 protein used in bone tissue engineering and in the clinic. Studies establishing its minimal effective dose and safety, as well as effectivity in orthotopic locations will be necessary before clinical translation can be made possible.

ACKNOWLEDGEMENTS

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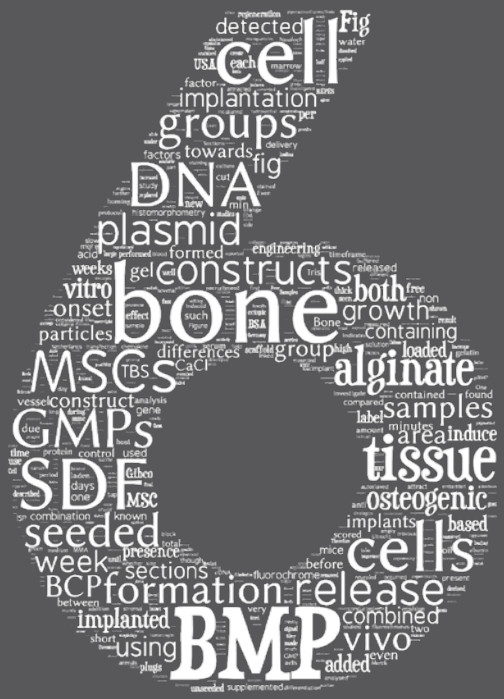

COMBINATION OF BMP-2 PLASMID DNA WITH SDF-1α CREATES AN ADDITIVE EFFECT ON BONE ONSET AND VOLUME *IN VIVO*

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Jacqueline Alblas

ABSTRACT

BMP-2 gene delivery has shown to induce bone formation *in vivo* in cell-based tissue engineering. In addition, the chemo attractant stromal-derived factor 1α (SDF-1α) is known to recruit MSCs towards its release site where it enhances vascularization and contributes to osteogenic differentiation. To investigate the feasibility of cell-free constructs, we investigated combined release of BMP-2 and SDF-1α on ectopic bone formation in mice. MSC-seeded and cell-free constructs with BMP-2 and /or SDF-1α were implanted subcutaneously in mice for a period of 6 weeks. Histological analysis and histomorphometry revealed that the onset of bone formation and the bone volume formed were induced by the combination of BMP-2 and SDF-1α compared to controls in cell-seeded constructs. Samples without seeded MSCs failed to induce any bone formation.

We conclude that the addition of SDF-1α to a cell-seeded alginate based BMP-2 plasmid DNA construct has an additive effect on bone formation and can be considered a promising combination for bone regeneration.

INTRODUCTION

Bone tissue engineering is one of the major focus points in the field of bone regeneration and tissue engineering. Novel strategies are being developed combining new materials with cell based strategies and growth factor delivery [94 170]. Bone morphogenetic protein 2 (BMP-2) is involved in committing multipotent stromal cells (MSCs) towards the osteogenic lineage and known to induce new bone formation and is therefore considered one of the main growth factors in bone tissue engineering [171]. In clinical practice large amounts of rhBMP-2 are administered to overcome the fast washout of protein due to degradation by proteinases. These high dosages have recently been under debate due to several serious adverse effects such as vertebral osteolysis, ectopic bone formation, radiculitis and cervical soft tissue swelling [105 104]. As an alternative for the high dosages of BMP-2 protein, BMP-2 based non-viral gene therapy can be considered. Our group developed a method for efficient gene delivery based on alginate hydrogel, which is compatible with simultaneous cell seeding [53]. The basis of the delivery is alginate, an anionic polysaccharide that gelates with bivalent cations such as Ca^{2+} [85], is FDA approved, non-toxic and biocompatible. Combined with other hydrogels or in the form of nanoparticles it has been used in combination with plasmid DNA before [93 210 211]. Alginate has been successfully applied in bone tissue engineering applications both in vitro and in vivo [90 178], which makes it very suitable as gene activated matrix (GAM) for MSC seeded constructs.

Besides osteogenic growth factors such as BMP-2, other growth factors such as stromal-cell derived factor 1α (SDF-1α) become increasingly important in the field of bone tissue engineering. During embryogenesis SDF-1 directs the migration of hematopoietic cells towards the bone marrow and induces the formation of large blood vessels [221]. In adults SDF-1α is crucial for MSC recruitment to fracture sites and for induction of bone regeneration [222 212]. In addition to homing of cells, SDF-1α has also been shown to induce vessel formation *in vitro* as well as *in vivo* which is important in bone tissue regeneration. Various studies have applied SDF-1α and showed that endogenous cells can be recruited to these constructs in a tissue engineering setting [223]. Furthermore, the chemotactic properties of SDF-1α also account for activation of endochondral bone formation during the acute phase of bone formation after injury [222 57].

When SDF-1α is combined with BMP-2, a positive effect on bone formation and regeneration has been reported in literature [68 66 67]. The recruitment of MSCs towards BMP-2 protein or genetically engineered BMP-2 producing MSCs, via SDF-1α or -β induces osteogenic differentiation [224]. When both growth factors are released from a combined construct, a synergistic effect on bone formation has even been reported [67]. The mechanism behind this increased bone formation has been described as a combination of enhanced mobilization and homing of bone marrow-derived osteoprogenitor cells to the implant leading to increased numbers of cells available for bone regeneration at ectopic bone implants [66].

This study investigates the ability of SDF-1α protein to attract MSCs towards the BMP-2 plasmid DNA laden alginate construct, leading towards transfection and osteogenic differentiation of the cells, and eventually increased bone formation in the implanted constructs.

MATERIALS AND METHODS

Alginate gel

Autoclaved high-viscosity non-medical-grade alginate powder (International Specialty Products, ISP, Memmingen, Germany) was dissolved at a concentration of 10 mg/ml in alpha minimum essential medium (α-MEM, Gibco, Breda, The Netherlands). The gel was polymerised by adding an equal volume of 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 1 ml expansion medium.

Cell culture

Goat MSCs were isolated from bone marrow (BM), aspirated from the iliac wings of Dutch milk goats. The MSCs are isolated by adherence to tissue culture plastic and cultured in expansion medium, containing αMEM (Gibco), supplemented with 15% (v/v) fetal calf serum (Cambrex, Charles City, IA, USA), 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.

Production of gelatin microparticles (GMPs)

Gelatin microparticles (GMPs) were produced using an adapted protocol first described by Y. Tabata et al [225 226]. In short: hydrated gelatin type B (Sigma, St Louis, MO, USA) was added to refined olive oil (Arcos Organics, NJ, USA) to create a water-in-oil emulsion. The emulsion was stirred, rapidly cooled down, washed with acetone and filtered under pressure. Particles were sieved to obtain a size range of 50-75 μm ø. Finally the particles were freeze-dried. SDF-1α was dissolved (at 200 μg/ml?) in Tris buffered saline (TBS) supplemented with 1% bovine serum albumin (BSA) and then10 μl was loaded onto the 10 mg microspheres overnight at 4°C by diffusional loading.

Release of SDF-1α from GMPs

SDF-1α release from GMPs was measured after loading 5 μl of SDF1α-solution (R&D, MN, USA) per sample (n=3) onto 5 mg GMPs to a total amount of SDF1α (final conc. 100 ng/scaffold). The microspheres were then incorporated into a 100 μl alginate gel (2% w/v) (IMCD, Amersfoort, The Netherlands) that was cross-linked for 5 minutes with 100 mM $CaCl_2$ solution [100 mM aqueous $CaCl_2$ supplemented with 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4 (Gibco)] to create a plug. $CaCl_2$ was removed and replaced by 1ml TBS/BSA 1% and incubated up to 19 days. At each time point GMPs were centrifuged at 100 g for 5 minutes, supernatant was removed and replaced with 1ml TBS/ BSA 1%. The supernatant was measured using the standard enzyme linked immuno sorbent assay (ELISA, R&D) protocol.

Preparation *in vivo* implants

Four different constructs were made, each consisting of 200 μl alginate (10 mg/ml), 10^7 goat MSCs/ml gel, 20 % (w/v) BCP particles (size 106-212 μm) and 10 mg gelatin microparticles (GMPs). The 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. The BMP-2 plasmid DNA construct contained the full-length human recombinant BMP-2 cDNA, whose expression was driven by the cytomegalovirus promoter in pcDNA3.1 (Invitrogen) [53].

The four different constructs further received: 1) empty GMPs; 2) 10 μg/ml of pBMP-2 and empty GMPs (in short BMP-2); 3) 10 μg/ml of pBMP-2 plus 10 mg GMPs loaded with 2 μg SDF-1α (in short: BMP-2/G-SDF-1α); or 4) 10 mg G-SDF-1α (in short: G-SDF-1α). The alginate constructs were polymerized and implanted the same day. In groups 1 and 2 GMPs were soaked in TBS overnight and added before polymerization, and in group 3 and 4 GMPs were loaded with 10 μl TBS containing 2 μg of SDF-1α overnight and also added before polymerization.

All implants were polymerized with 1 ml of 100 mM autoclaved $CaCl_2$, supplemented with 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES pH 7.4, Gibco), for 10 minutes before implantation. The supernatant was removed and all implants were kept in MSC culture medium until implantation.

Animals and implantation

Female nude mice (Hsd-cpb:NMRI-nu, Harlan) were anaesthetized with 1.5% isoflurane, after which the implants were placed in 5 separate subcutaneous dorsal pockets per mouse (n=6 per group). 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), and at 4 weeks after implantation xylenol orange was administered s.c. (30 mg/kg, Merck, Amsterdam, Netherlands). Experiments were conducted with the permission of the Ethical Committee for Animal Experimentation at Utrecht University and in compliance with the Institutional Guidelines on the use of laboratory animals.

Explantation and embedding

Six weeks after implantation the constructs were retrieved to analyse general morphology, transfection and bone histomorphometry. Samples were cut in two. One part 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), after which 10 μm thick sections were cut from the centre and side of each implant using a sawing microtome (Leica, Nussloch, Germany). These sections remained unstained for fluorochrome analysis. The presence of each fluorochrome label was scored for all implants.

Histology

Paraffin sections were stained with hematoxylin/eosin (HE). Samples were scored on morphology and the presence of blood vessel ingrowth by two observers. To detect His/BMP-2-transfected cells in the implants, anti-His immunohistochemistry was performed. In short, sections were permeabilised with Triton X-100 and blocked in 3% H_2O_2 for 10

minutes and 5% BSA for 30 min. Antigen retrieval steps were performed using 1 mg/ml pronase and 10 mg/ml hyaluronidase for 30 min each. Sections were subsequently incubated with 5 µg/ml biotinylated anti-His antibody (Genscript, Piscataway, NJ, USA, A00613) in Tris-buffered saline supplemented with bovine serum albumin (TBS/BSA 1%) for 1 hour and as second 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.

Safranin O staining

Sections were deparaffinised and incubated with hematoxylin (Weigert's) for 5 min. After washing in running tap water, sections were dipped in distilled water and counterstained in 0.4% (w/v) Fast green solution. Sections were rinsed in 0.17 M acetic acid until colour no longer runs and counterstained again in freshly prepared 0.125% (w/v) Safranin O (Merck) staining dissolved in distilled water. After a short dehydration protocol, sections were mounted in Depex. The presence of cartilage was scored by two independent observers.

Bone histomorphometry

High-resolution digital photographs were made from HE stained sections for histomorphometric analysis. Three pictures at predefined positions within the explant were taken from each sample. Bone and scaffold were pseudocolored using Adobe Photoshop CS3, where after histomorphometry was performed using Axiovision software (version 4.8; Zeiss, Nussloch, Germany). A custom macro was used to measure the region of interest, the area of scaffold, and the area of bone. This allowed the calculation of both the percentage bone in available space, called bone area% [= (bone area/(total area-scaffold area))*100%].

Fluorochrome analysis

The fluorochrome markers were analysed from the unstained sections using a fluorescence microscope (E600, Nikon) equipped with a quadruple filter block (XF57, dichroic mirror 400, 485, 558, and 640nm, Omega optics, Brattleboro, VT). Fluorochromes incorporated in the newly formed bone were scored for presence/absence. To discriminate between the bone and the underlying scaffold, the fluorescence signal was compared to the brightfield signal.

Statistics

The statistical significance of differences between experimental groups in Figure 3 was assessed using a randomized block design. Multiple comparisons were generated using a LSD posthoc correction to calculate the p-values. A p-value of < 0.05 was considered significant.

RESULTS

In order to simultaneously deliver the chemokine SDF-1α and the osteogenic stimulus BMP-2, the previously described gene delivery technique based on alginate was used [53]. This provides a long-lasting production and release of BMP-2. In the current study GMPs containing SDF-1α were added to these constructs.

To investigate the SDF-1α release profile from the alginate plugs, *in vitro* release studies were performed. The sustained release of SDF-1α was measured during a time period of 19 days (Figure 1), resulting in the gradual release of 50 % of the loaded protein after 19 days. The alginate plugs remained intact during 19 days.

GMP containing constructs were implanted subcutaneously in mice for 6 weeks to investigate whether bone formation can be induced in cell-free constructs by chemokine attraction of host MSCs or if bone formation can be enhanced in seeded constructs due to a synergistic BMP-2/SDF-1α combination [67].

Transfection of seeded MSCs with BMP-2 plasmid DNA is possible in alginate constructs. By releasing SDF-1α from the same constructs, this process might be optimized and accelerated since vascularization, important for bone formation, is stimulated and more osteogenic cells are attracted to the construct. Because of this homing of osteogenic cells towards the BMP-2 plasmid DNA laden constructs, groups without seeded cells have been implanted as well. With this experimental setting, it was investigated whether osteogenic cells could be attracted to induce transfection with BMP-2 plasmid DNA, and as a result of the transfection induce osteogenic differentiation and eventually bone formation.

In the unseeded constructs bone formation was absent, and no differences could be detected in scoring of vessel like structures, number of recruited cells or fibrous tissue formed around the BCP particles (fig. 3A, B). The cell-seeded experimental setup however resulted in bone formation, and was further analysed for tissue responses, onset of bone formation and bone histomorphometry. Therefore we will mainly discuss the MSC-seeded samples.

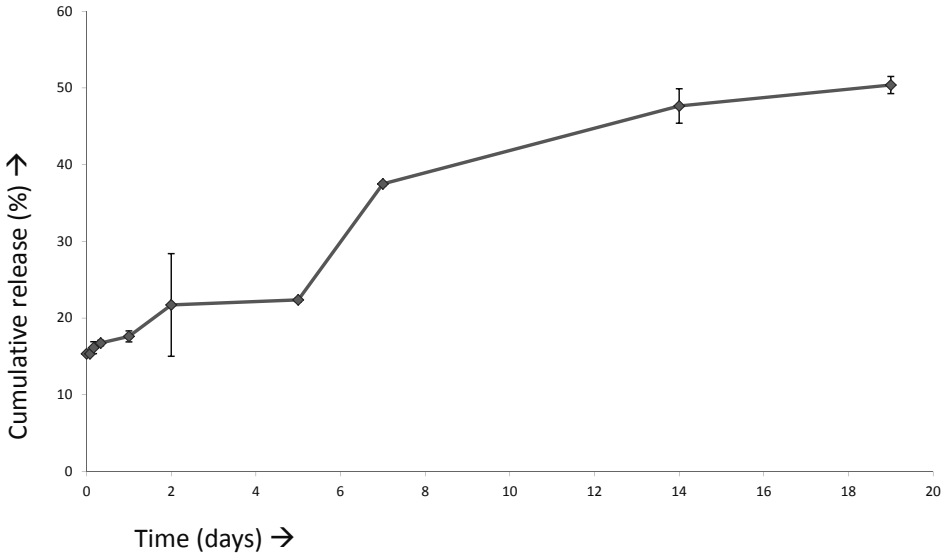
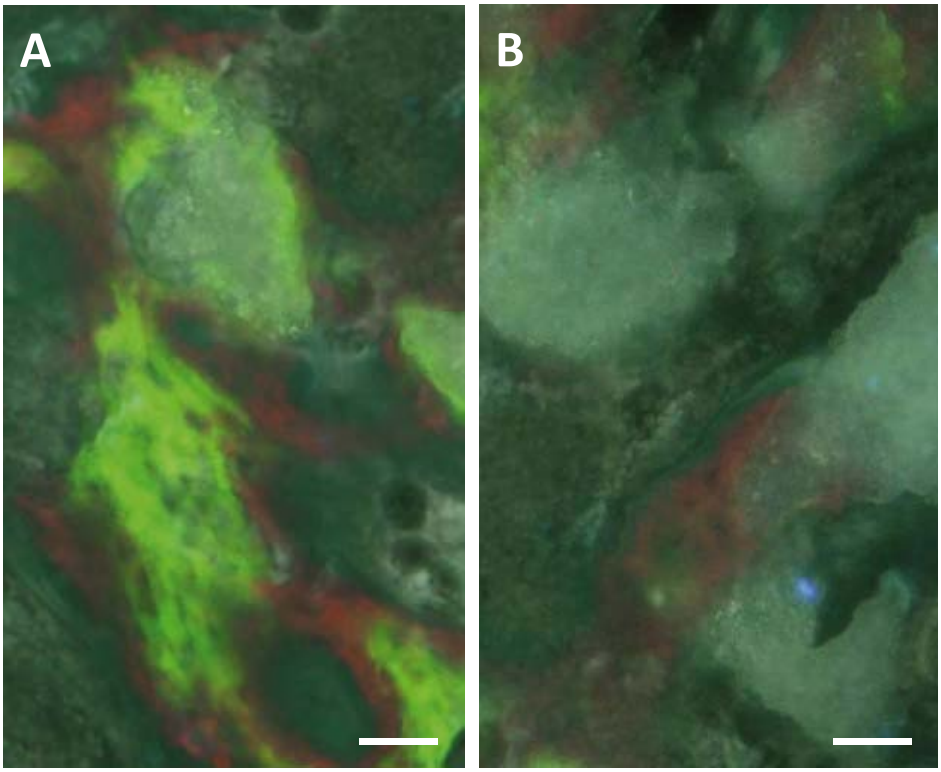


Figure 1. Cumulative release of SDF-1α from GMPs. Cumulative release of SDF-1α from GMPs in a 200µl alginate plug into TBS/BSAover a period of 19 days.

All groups, both seeded and unseeded contained some samples with remnants of the alginate present (fig 3C, D). However, no presence of the GMPs was detected. Furthermore no signs of an inflammatory response were present. Hardly any capsule formation was detected and if there was some fibrous tissue surrounding the implant this was less than 5 cell layers thick.

To determine whether differences in bone onset occurred, fluorochrome incorporation was analysed. Except for the BMP-2/G-SDF-1α group and one sample in the BMP-2 group no fluorochromes have been detected (fig 2). Since hardly any of the week 3 and week 4



	Week 3	Week 4
Empty GMPs	0	0
BMP-2	0	17
G-SDF-1α	0	0
BMP-2/G-SDF-1α	33	67

Figure 2. Bone onset analysed by fluorochrome analysis. Detection of Calcein green, administered at week 3 after implantation and Xylenol orange, administered at week 4 after implantation. A) Combined BMP-2/SDF-1α sample displaying both the 3 and 4 week label. B) Control sample without BMP-2 or SDF-1α, no fluorochromes present. Scale bar represents 25 µm

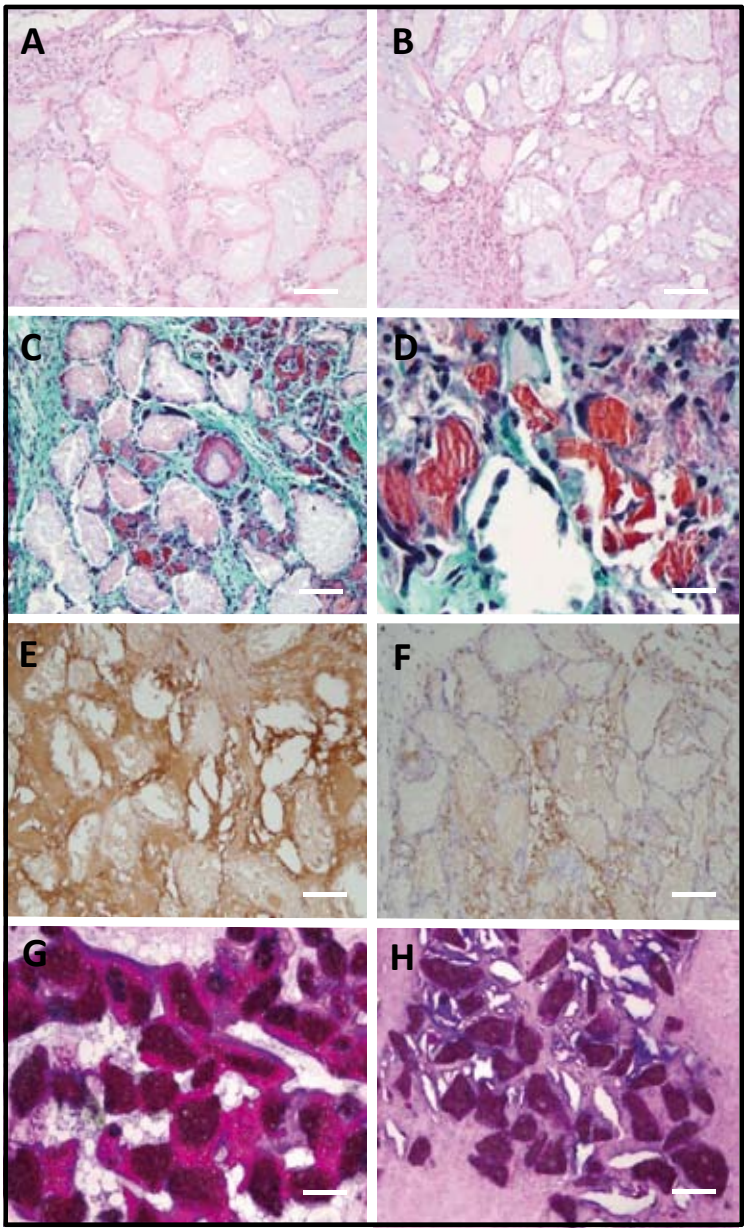


Figure 3. General Histology and bone formation of MSC seeded samples. A,B) HE staining, bone in pink, A)Combined BMP-2/SDF-1α sample, B) control sample. C, D) Safranin O staining, fibrous tissue in green, BCP in pink and alginate remains in red. C) representative sample containing alginate remains, overview. D) representative sample containing alginate remains Close-up. E,F) Collagen I staining, collagen I in brown. E) Combined BMP-2/SDF-1α sample, F) control sample. G, H) Basic fuchsin/methylene blue staining, Bone in pink. G) Combined BMP-2/SDF-1α sample, H) control sample. A-C and E-H) Representative samples were chosen for each group, scale bar represents 100 μm, D) scale bar represents 20 μm.

labels were detected, the onset of bone formation must have been between week 4 and 6 after implantation in most animals. In the BMP-2/G-SDF-1α group however, 33% of the samples contained the orange label but not the green 3 week label. Therefore we conclude that bone formation occurred between week 3 and week 4 in these samples, which indicates an earlier bone onset as a result of combined growth factor release of BMP-2 and SDF-1α (Fig. 2).

To investigate not only onset but also amount of newly formed bone as a result of combined SDF-1α and BMP-2 release, matrix and bone formation have been analysed. In the MSC seeded groups, differences between the implanted constructs were apparent. In the BMP-2/G-SDF-1α constructs abundant collagen I staining was seen (Fig.3E,F). To quantify the amount of bone in the seeded implants, bone histomorphometry was performed on basic fuchsin/methylene blue stained MMA sections for determination of bone area% (fig 3G,H). This revealed that all groups contained several samples showing new bone. In the control samples however very small volumes were detected. In the groups with either BMP-2 or G-SDF-1α bone formation was clearly present. The BMP-2 /G-SDF-1α constructs however induced significantly more bone formation compared to the control or single growth factor groups. An average of 6.4% bone volume was detected (Fig. 4).

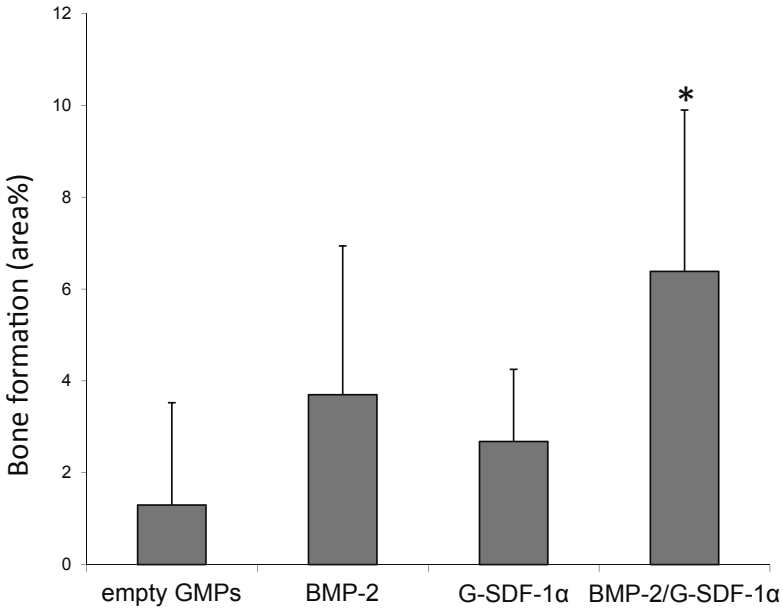


Figure 4. Quantification of bone formation in MSC seeded constructs. Analysis of bone area% (for details see Materials and Methods). * = significantly different from control and single growth factor groups, $p<0.05$.

DISCUSSION

This study investigated the use of SDF-1α for bone tissue engineering using alginate/BMP-2 plasmid DNA laden constructs. We studied the ability of SDF-1α to sufficiently induce the homing of host MSCs towards unseeded BMP-2 plasmid DNA containing constructs. From previous experiments it is known that MSCs are able to take up and express the BMP-2 cDNA, thereby inducing osteogenic differentiation *in vitro* and subsequent bone formation *in vivo*.

The implanted constructs consisted of alginate plugs combined with BCP particles and GMPs loaded with SDF-1α. The alginate provides the basis of the construct and acts as a transfection agent for BMP-2 plasmid DNA. The BCP particles were added to provide a mineralized surface for bone formation to start on. These particles, when combined with MSCs, are known to promote osteogenesis in previous studies [214 24]. The GMPs were added as a slow release system for SDF-1α. Several groups have shown that SDF-1α can be successfully released over a longer period of time from GMPs [226 58]. To ensure successful release of SDF-1α, loaded GMPs when embedded in alginate-based constructs that were implanted in mice, combined with release experiments conducted on these alginate/GMP constructs (Fig. 1).

After 19 days a sustained release was still apparent. About 50% of all incorporated SDF-1α was released by then (Figure 1). This seems rather slow for a chemokine which is supposed to attract MSCs to the construct directly after implantation. However, as known from literature *in vivo* release is faster than *in vitro* release [98], possibly due to degradation of the alginate construct. In vitro, the constructs were completely intact after 19 days of incubation in buffer whereas in vivo some fragmented alginate and no GMPs have been found after explantation (Fig 3C,D).

In the explanted samples no differences have been found in histological scorings of general morphology and immunological processes such as capsule formation or presence of multinucleated giant cells (fig 3A,B). And even though most SDF-1α appears to be released, no increase in vessel formation was observed in both seeded and unseeded samples. This finding differs from findings in literature where the presence of SDF-1α increases vessel formation [58 227]. It is possible that the alginate surrounding the GMPs loaded with SDF-1α prevented or delayed vessel ingrowth. Another possibility is that the timeframe of implantation (6 weeks) exceeds the timeframe in which vascular changes can be detected since these are most often seen during early stages of the implantation period.

To investigate whether SDF-1α and/or BMP-2 had an effect on the onset of bone formation in cell-seeded constructs, fluorochrome analysis was performed (Fig. 2). But even though we found some bone in the control and SDF-1α laden group, no fluorochromes were detected with the exception of one sample in the BMP-2 plasmid DNA group, expressing the week 4 label. This indicates that bone formation mainly occurred after the administration of last label at week 4 but before explantation at week 6. The samples containing both growth factors and expressing the highest amount of bone, showed both the week 3 and week 4 label in several samples. This indicates that the combination of BMP-2 and SDF-1α advances bone onset compared to the other three groups. Bone is first formed sometime around 3 to 4 weeks after implantation. To our knowledge this effect of the combined growth factors has not yet been reported before.

Consistent with the fluorochrome analysis, no bone was present in any of the unseeded constructs. In the in vitro release study we found that SDF-1α was released, however in

vivo our constructs contained plasmid DNA and BCP. Even though it is very unlikely that this affected growth factor release, since the BCP particles had not been soaked in growth factor, we cannot exclude the possibility that insufficient MSCs have been attracted towards the constructs because the release of SDF-1α exceeded the time frame of implantation [228].

In the MSC-seeded groups, differences between the implanted groups, containing either SDF-1α, BMP-2 plasmid DNA, both, or neither (control) were observed. Because BCP particles, known to promote osteogenesis when combined with MSCs, were added to the implanted constructs, some bone had formed in the control samples, which is in accordance with previous results [214 24]. Some bone also had formed in the groups containing either BMP-2 plasmid DNA or SDF-1α alone, but even though a higher average of bone volume was detected, no significant differences in bone formation occurred compared to the control samples. However, when BMP-2 plasmid DNA and SDF-1α are combined, an in literature described additive effect occurres due to the combination of osteogenic cells recruitment from the circulation with SDF-1α and induction of *in vivo* bone formation in the presence of BMP-2 (fig 4). [68 66 67]. This increase in bone formation was consistent with an increase in collagen I (fig 3E,F) .

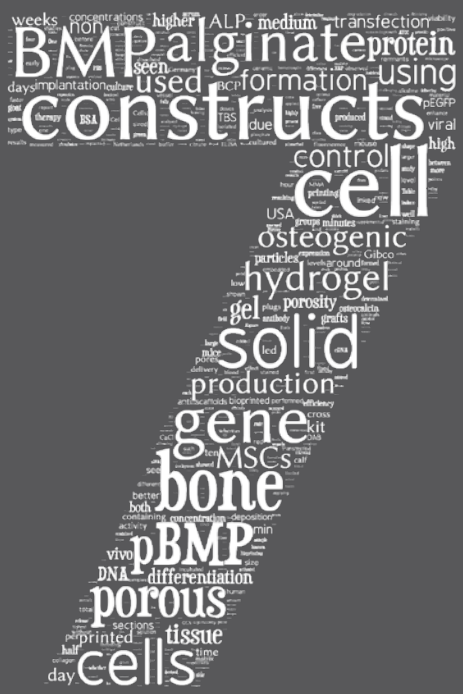
CONCLUSION

The addition of the chemokine SDF-1α to a cell-free alginate based, BMP-2 plasmid DNA laden construct does not recruit enough osteogenic cells from the circulation to induce uptake and expression of BMP-2, osteogenic differentiation and eventually bone formation in a six weeks implantation period.

When cultured MSCs however are seeded in constructs, the combination of SDF-1α and pBMP-2 induced an additive effect on bone formation, leading to an earlier onset of bone formation and a significantly higher amount of bone volume. Therefore we conclude that adding SDF-1α further optimizes bone regeneration *in vivo* of previously established BMP-2 plasmid DNA laden alginate constructs.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the support of the TeRM Smart Mix Program of the Netherlands Ministry of Economic Affairs and the Netherlands Ministry of Education, Culture and Science ,and Kelly van Deventer for her contribution to the performed histology.



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BMP-2 NON-VIRAL GENE THERAPY IN 3D BIOPRINTED CONSTRUCTS FOR BONE TISSUE ENGINEERING

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ABSTRACT

A well-known osteogenic stimulus in the field of regenerative medicine is bone morphogenetic protein-2 (BMP-2). Non-viral delivery of the gene encoding BMP-2 has shown to induce bone formation *in vivo*. To further develop the gene activated matrices towards larger scale constructs, introduction of porosity was introduced in the hydrogel, thereby allowing better diffusion and blood vessel ingrowth. This was achieved by applying the bioprinting technology, resulting in 3D constructs that are accurate and reproducible in size, shape and pore geometry.

Constructs (10x10x7 mm) 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 cDNA 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 cDNA, and differentiated towards the osteogenic lineage as shown by elevated BMP-2 and ALP production. Porous constructs performed significantly better in producing BMP-2 than solid constructs. After implantation for six weeks subcutaneously in nude mice, bone formation could not be confirmed, which calls for an optimization of the biomaterials used.

In conclusion, we show for the first time a model in which 3D printing and non-viral gene therapy are 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 [229]. 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 up scaling to clinically relevant-sized, i.e.cm-sized grafts remains challenging [230]. 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 [231 232].

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 allowing blood vessel ingrowth after prolonged implantation times [74].

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 [74]. In addition, alginate supports the encapsulation of growth factors or naked DNA, such that it forms a vehicle for non-viral gene therapy [53 151].

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 a different way of applying and delivering BMPs [208 105]. A slow constant release profile would match natural levels seen in for instance fracture healing. To achieve that, we have developed a non-viral gene delivery system for BMP-2 [53]. *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 [233].

Gene delivery using non-viral vectors is a relatively new in the field of bone tissue engineering and only a few studies have been published on this subject [234 235 236 237].

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).

Goat MSCs were isolated from bone marrow (BM), aspirated from the iliac wings of Dutch milk goats. The MSCs are isolated 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.

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) and 10 µg/ml, 1 µg/ml 100 ng/ml or 10 ng/ml of pBMP-2 was added. Hydrogel plugs (100 µl/plug) were formed and polymerised 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 14 days of culture, medium and dissolved gel were analysed by ELISA for BMP-2 production.

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 polymerised 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 fibre 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 [230]. Printer settings are summarised in short: 10 successive layers (dimensions 10x10x7 mm) either with 4 vertical pores with a strand distance of 3 mm and a strand thickness of 0.75 mm or without pores; strand distance 1 mm. 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 polymerised by immersion in Ca^{2+}-solution for 10 min and then cultured in expansion medium for 7 or 14 days.

Analysis of cultured printed constructs

BMP-2 protein production was analysed 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) [212]. 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 analyse 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 3 and 4 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 0.84 ng/10^6 cells. Lower amounts of pBMP-2 resulted in lower BMP protein production: 1 µg /ml 0.49 ng/10^6 cells, 100 ng/ml 0.27 ng/10^6 cells, 10 ng/ml 0.26 ng/10^6 cells, and control 0.21 ng/10^6 cells. Therefore in following experiments 10µg/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 higher optimal doses of pDNA at 40µg/10^6 cells [236] or pDNA polymer complex of 50 µg/10^6 cells [93] or inject 25 µg in the hind limb of mice [85].

Alginate concentration

The concentration of alginate present in the final hydrogel 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 [232]. 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) (Fig. 3).

By measuring transfection efficiency in different concentrations of cross-linked 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%

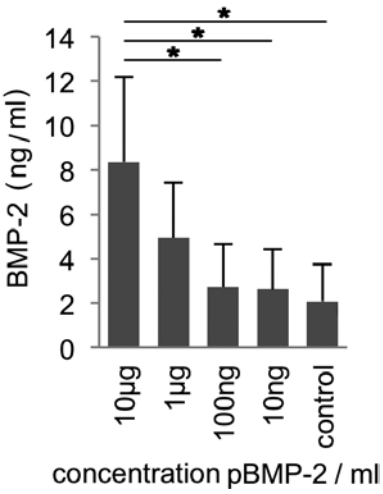

Figure 1. rhBMP-2 release after 14 days from transfected gMSC cells in alginate. BMP-2 release from 0.1 ml alginate constructs containing 10^6 cells and pBMP-2 at the indicated concentrations/ml. BMP-2 release was analyzed in two independent experiments in duplicate in the medium and in the gel separately by ELISA. The results represent mean ± SD.

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.[238] transfected human MSC (hMSC) with pEGFP using Lipofectamine™2000 and showed a 50% fraction of fluorescent cells. 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 [150 239].

3D printing

Defined 15-layer alginate constructs (10 x 10 x 4 mm; Fig. 3B) were bioprinted by fiber deposition technology. Vertical pores were regular throughout the samples, whereas transversal pores fused due to the relative softness of the material. 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% (CI 85-96) and 79% (CI 73-86) for the porous control, 91% (CI 87-96) and 80% (CI 68-92) for the porous with pBMP-2, and 85% (CI 81-89) and 69% (CI 59-78) for the solid constructs (Fig. 4). No statistical differences were seen.

Protein production in bioprinted constructs

BMP-2 protein was produced by cells in the printed scaffolds respectively at day 7 and 14 in nanogram per million cells: 0.13±0.07 and 0.16±0.13 for the porous control, 0.65±0.12

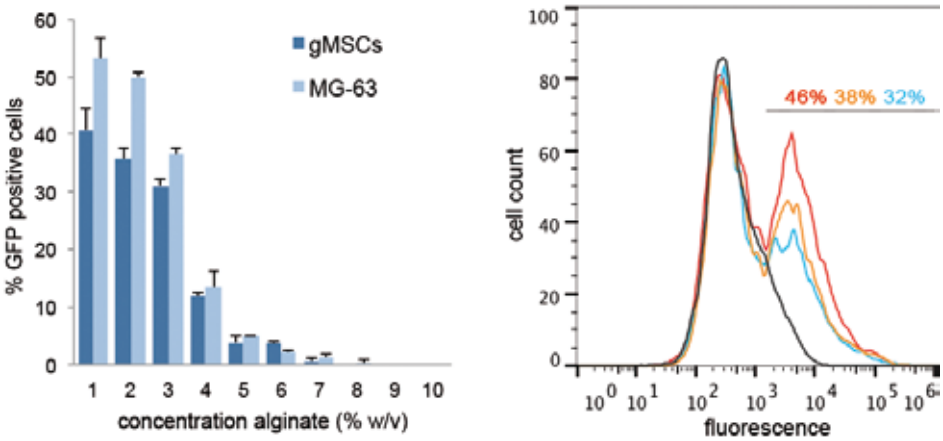

Figure 2. Transfection efficiency with pEGFP as a function of alginate concentration and determined by flow cytometry. A) Green fluorescence was analyzed after 10 days in two independent experiments containing either MSCs or MG-63 cells and 10 µg/ml pEGFP. Fluorescent cells were counted under a fluorescence 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%).

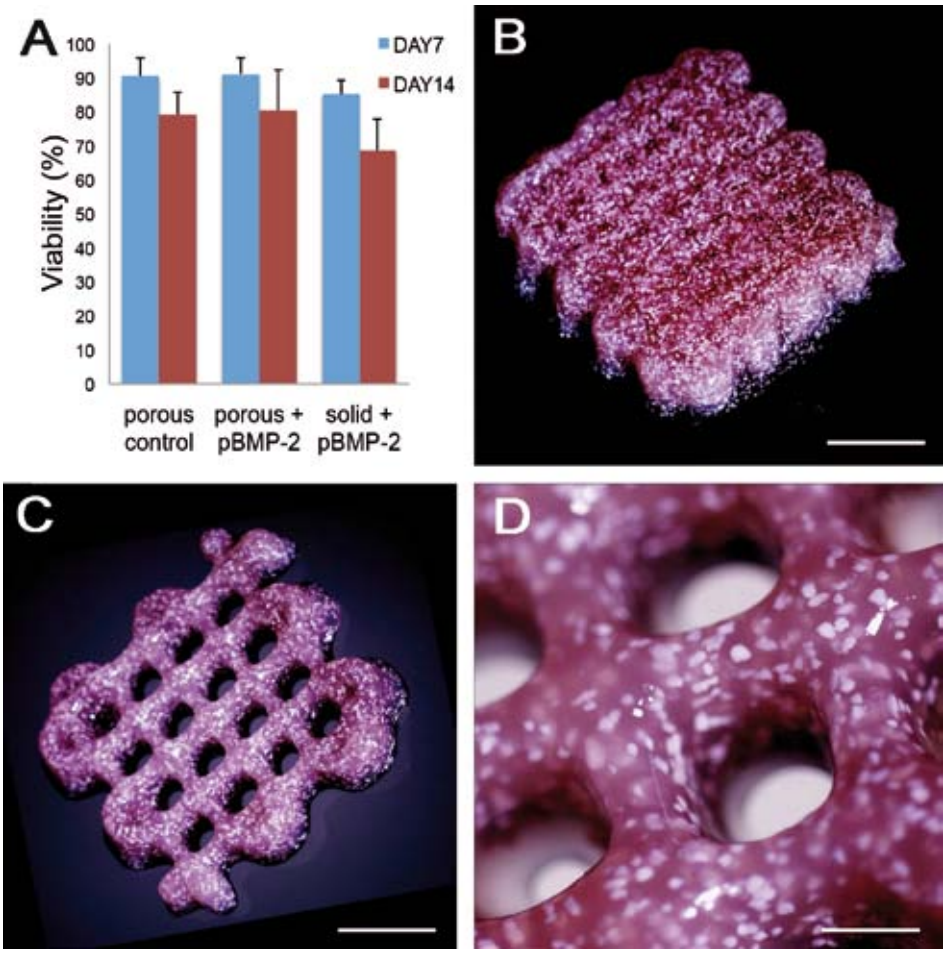

Figure 3. Bioprinted constructs containing MSCs, ceramic particles and/or plasmid DNA encoding BMP2. A) Viability of MSC in bioprinted constructs after 7 and 14 days, determined by Live/dead staining. The results represent mean ± SD. B and C) Constructs directly after printing and polymerization. Porous constructs with and without DNA have the same macroscopic appearance (C). Scale bar: 250 µm. D) A close up of a porous construct. Scale bar: 75 µm.

and 0.72±0.11 for the porous with pBMP-2, and 0.17±0.16 and 0.43±0.24 for the solid constructs (Fig. 4). 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. This could be caused by a better cell functionality due to better nutrient supply and waste removal due to shorter diffusion distances. The low background levels of BMP-2 protein produced in the control group is normal and reported before [240].

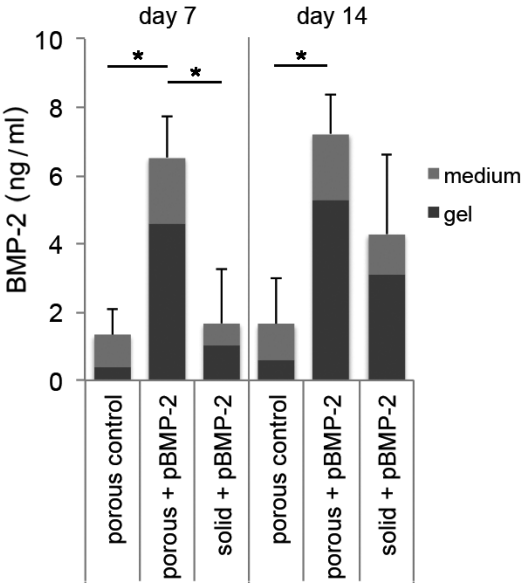

Figure 4. BMP-2 production in medium and gel after 7 and 14 days.BMP-2 release from bioprinted alginate constructs. Media were stored at each medium change, and the constructs were dissolved in citrate buffer at the end of the experiment. BMP-2 concentrations were measured in medium and gel separately by ELISA (n=3 for gel and medium). The results represent mean ± SD, * indicates a significant difference ($p < 0.05$).

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. 5A). 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 [241].

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 (see figure B-D).

Considering the later osteogenic marker osteocalcin, which is only produced by osteoblasts or cells with osteoblastic nature, we observed much more pronounced differences between the groups.

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

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 immuno deficient 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. 6 A-F). The solid constructs contained large areas of alginate remnants (Fig. 6) 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. 6 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. 6 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. 6 G-I). The implants also contained connective tissue as shown in green by a Goldner's trichrome staining but no highly cellularised tissue like osteoid, which would be indicated in red (Fig. 6 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 increased 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 [242], in this particular setting the bone formation was not (yet)

Table 1. Performance of bioprinted constructs after 6 weeks implantation *in vivo*.

	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	++	-	-	++	++	+	++	-

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.

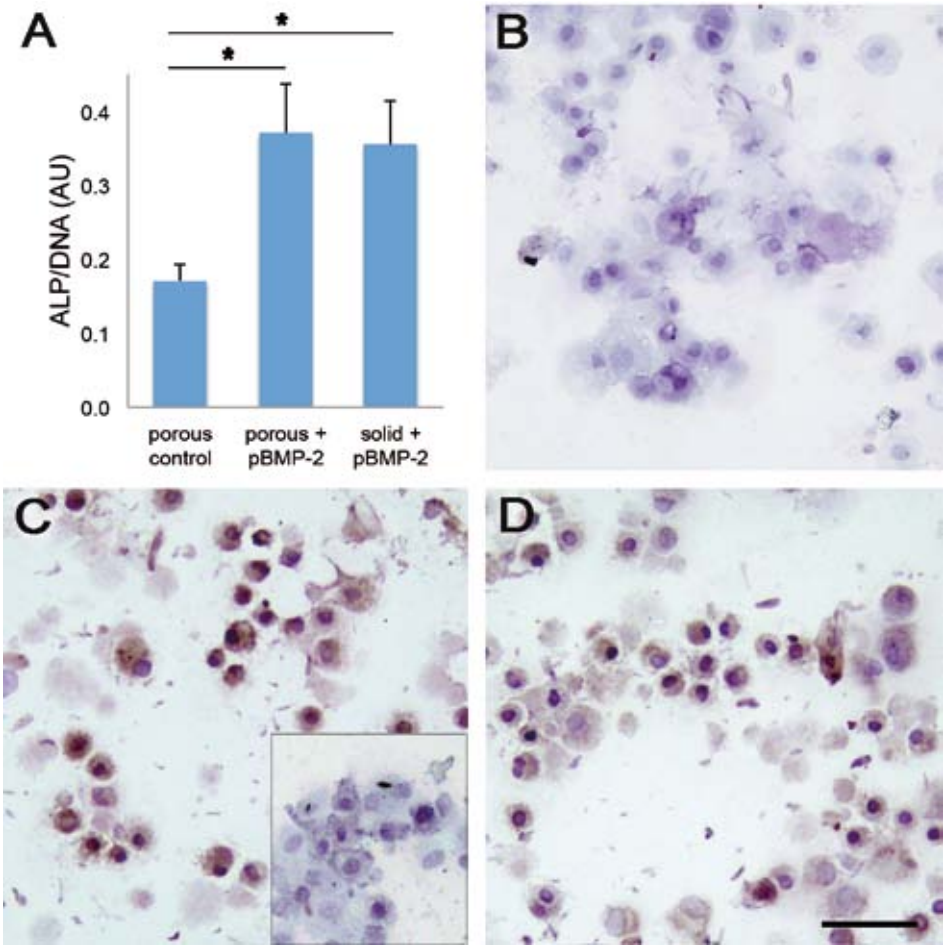

Figure 5. Osteogenic differentiation in bioprinted constructs. A) Alkaline phosphatase (ALP) activity after 14 days *in vitro* in printed constructs containing MSCs, normalized for DNA-content of the sample (n=3) and * indicates a significant difference ($p < 0.05$). B-D) Osteocalcin immunocytochemistry on cytospins of cells after dissolution of the construct, B) Porous control, C) Porous with 10 µg/ml pBMP-2, Inset: isotype-matched control Ab staining on section from C. D) Solid with pBMP-2, Scale bar: 100 µm.

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 [53 151]. Also, other studies report an higher availability of BMP-2 protein after gene-expression at early time points than our results show [92]. 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 [90 92].

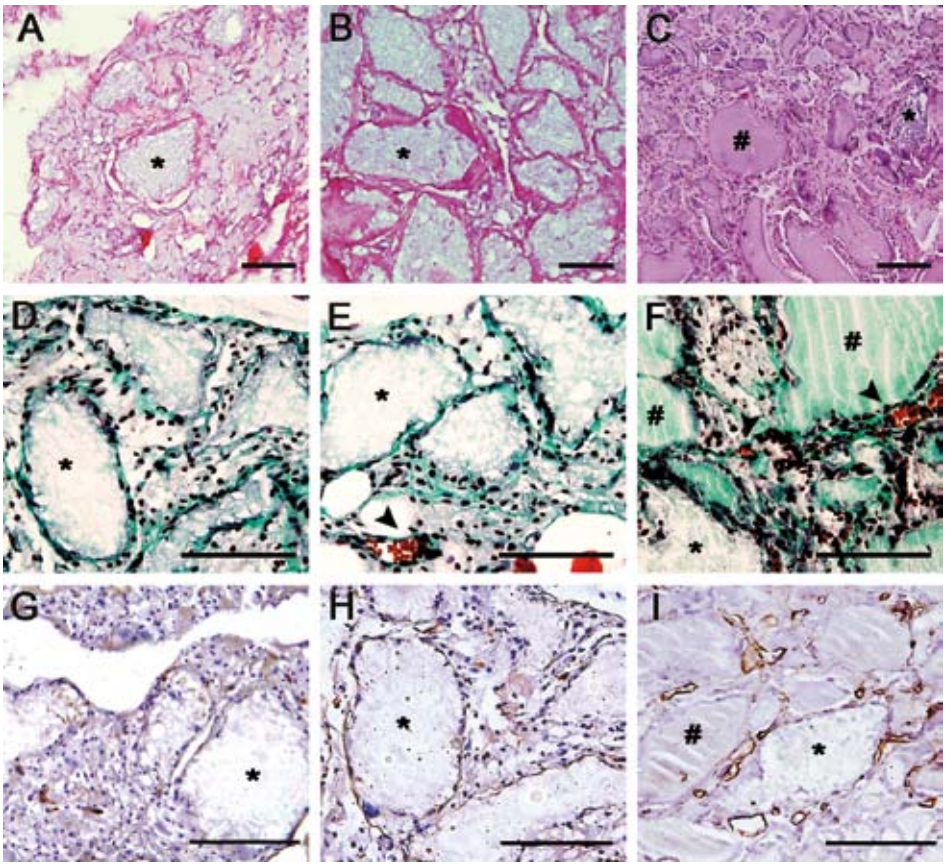

Figure 6. *In vivo* performance of bioprinted constructs containing pBMP-2. After 6 weeks of implantation subcutaneously in mice, histological sections were prepared as described in M&M. A,D,H) porous control samples; B,E,I) porous with pBMP-2 and C,F, I) solid with BMP-2 samples. A-C) H/E staining, D-F) Goldner's trichrome staining, H-J) 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.

CONCLUSIONS

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 construct

ACKNOWLEDGEMENTS

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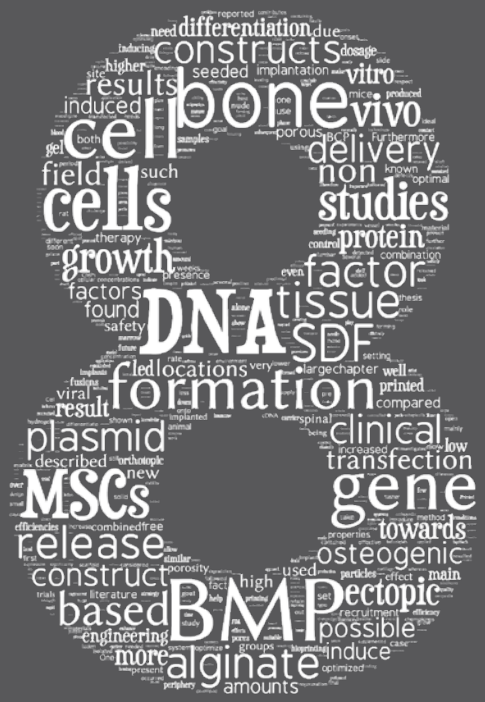


GENERAL DISCUSSION AND SUMMARY

Bone tissue engineering has been investigated extensively for decades focusing on the use of cells, scaffold materials and growth factors. Its proof of concept has been clearly shown in animal studies at different ectopic and orthotopic locations [170 94]. For cell based and material based bone tissue engineering some clinical case reports have been published but it is not yet being used on a regular basis [243 244 245 246 247]. In the field of growth factor based bone regeneration, more progress has been booked with the application of BMP-2 and -7. BMP-2 is FDA approved for interbody fusions, lumbar spinal fusions, lumbar spine pseudo arthrosis, open tibial shaft fractures and maxillofacial augmentations, whereas BMP-7 is approved for the revision of posterolateral intertransverse lumbar spinal fusions and long bone nonunions.

BMPs enhance the formation of new bone, as shown in numerous preclinical [248 249] and clinical studies [250 251]. Physiological doses of BMP-7 induce cellular responses *in vitro*, however, when administered *in vivo*, rapid degradation and consequently insufficient and improper tissue regeneration occurred [252].

BMP-2 is considered the most effective growth factor for bone regeneration. It has proven to stimulate osteogenic differentiation *in vitro* and bone formation *in vivo* in a large number of publications (reviewed in [253 254]). It is effective at inducing new bone formation when applied either as recombinant purified proteins, or produced on the spot by local application of the gene encoding BMP-2. One of the factors that has greatly influenced its effectivity, is the release rate, which can be tailored by combining BMP-2 with different scaffold materials such as natural or synthetic polymers. *In vitro* we found a sustained release over a period of at least 5 weeks. *In vivo* however, the release is most likely faster due to faster degradation of the alginate gel. Compared to literature fast release might have a positive effect on bone formation. An initial burst release followed by some sustained release of BMP-2 is found to be the most productive release profile for inducing bone formation [255 256 257 258]. Probably due to the fact that MSCs are initially attracted towards the fracture/bone inducing construct and then differentiated towards osteogenic cells [40]. BMP release timing is closely associated with finding the BMP dosage necessary and sufficient to induce bone healing. Despite this widespread use, reported complications might be associated with the dosage [35 49]. Therefore the field of BMP-2 based bone tissue engineering is more and more focusing on different delivery methods such as protein release systems or gene therapy based treatments.

THE DEVELOPMENT AND OPTIMIZATION OF A NON-VIRAL GENE DELIVERY SYSTEM

To deliver and retain BMP-2 plasmid DNA locally and to induce bone tissue engineering a gene carrier is needed. We found that alginate is very suitable as a transfection agent and protein release system, and furthermore is known to be biocompatible, and a suitable matrix to induce bone formation [93 92 90 178]. When combined with MSCs and BMP-2 plasmid DNA, it becomes a low cost, non-viral gene delivery method which displays high transfection efficiencies of over 60%. Especially the transfection efficiencies are unusually high for non-viral gene therapy compared to other gene activated matrices (GAMs) [181 180 179]. Upon further *in vitro* characterization, we observed that BMP-2 protein was increasingly produced

and released up to a period of 5 weeks as a result of transfection with plasmid DNA. The majority of the produced protein however was retained in the gel creating an alginate based release system. Even when the DNA concentrations were titrated down to 0.08 µg/ml gel, similar protein levels were expressed independent of the DNA concentration. The produced protein proved bioactive by inducing osteogenic differentiation *in vitro*.

The main question remained whether *in vivo* the gene delivery would perform as well. In mouse and goat experiments it was found that besides osteogenic differentiation, bone formation including bone marrow and cartilaginous tissue has been induced as a result of the transfection with BMP-2 plasmid DNA. This is consistent with literature describing ectopically induced bone formation after transfections with BMP-2 plasmid DNA [259].

Transfected MSCs or delivered BMP-2 plasmid DNA significantly increase bone formation both intramuscularly and at orthotopic locations compared to negative controls. These published studies all reported considerably lower bone volume percentages than the ones we found, and did not mention the presence of cartilage or bone marrow [107 211 212 215]. The amounts of bone induced by the alginate/BMP-2 construct were similar to those induced by a BMP-2 protein dosage similar to what is used in the clinic. In the same experiment, it appeared that a 1000-fold lower dose of BMP-2 protein in alginate was potent enough to induce comparable amounts of bone. This implicates that using lower doses of BMP-2 should be possible when all other construct conditions are optimized. In our studies constructs were optimized by adding BCP particles. These were added to increase the stability and osteoinductivity of the hydrogel-based constructs. The particles are known for their osteoconductive and sometimes even osteoinductive properties and, when seeded with MSCs they show efficient bone formation [199 82]. This was confirmed by the fact that bone formation was only induced ectopically in the presence of these particles.

CONTRIBUTION OF SEEDED MSCS TO BONE FORMATION

MSCs play an important role in gene based bone tissue engineering [260 261 262 100]. MSCs have the ability to differentiate into several lineages including cartilage, adipose tissue and bone, can be isolated from different easily accessible tissues and are efficiently transfected. MSCs also possess immune repressing as well as immune evading capacities which make them suitable for allogeneic transplantations [19 99]. These features combined make MSCs the main targeted cell type for gene intervention techniques in the field of bone tissue engineering [100]. However, the results from *in vivo* studies are varying. Several factors contribute to this inconsistency, such as differences in gene carrier and heterogeneity of the isolated MSCs. The main factor however appears to be implant location [263 24]. At orthotopic locations, bone forming cells are present in the underlying bone and can be recruited towards the construct, making cell seeding often redundant. In ectopic locations, cells need to be recruited from the periphery and to differentiate *in situ*, and therefore cell seeding at heterotopic or ectopic sites contributes to bone formation [264 265 26]. In clinical situations bone formation is mainly needed in orthotopic locations however, there are some partly ectopic locations, such as posterolateral spinal fusions. Also, in some pathologic situations, contact to the underlying bone may not be optimal, in which case cell seeding might also be beneficial. During preclinical studies,

the ectopic implantation site remains very important to help understand which factors and conditions best regulate recruitment and osteogenic differentiation of seeded MSCs in an environment that is less influenced by the presence of bone-derived factors and cells.

In our studies we consistently found ectopic bone formation in cell seeded constructs. The combination of BCP particles with MSCs has shown some bone formation in the control groups as well, however in all studies the amount of bone was significantly higher in the BMP-2 laden groups (either plasmid DNA or protein) than the control. This confirms that MSCs, which are the main target cells in orthotopic locations, respond to transfection with BMP-2 plasmid DNA by differentiating into the osteogenic lineage and depositing bone matrix. Unseeded ectopic samples failed to show bone formation in mice. Nevertheless, those samples contained osteogenic cells indicating that host cells migrated into the construct and became transfected. Since alginate is known to be a poor substrate for cell migration, it is possible that not enough cells were present to start bone formation. A more optimal cell recruitment from the periphery and/or improving migration characteristics of the hydrogel carrier might solve this.

Orthotopically, cells do not have to be recruited from the periphery. This might explain why, in contrast to the ectopic implants, bone was mainly found in the cell-free samples even though this was not significantly higher than the control without BMP-2 plasmid DNA. In the samples that contained bone, calcified alginate was present. It is known from literature that alginate has the ability to calcify and become a bone like matrix with respect to both biomechanical and biological properties [204 205 206]. It appeared to act as a scaffold for new bone to grow on, since layers of bone are deposited on the alginate. This could indicate that alginate is being degraded differently in the presence of MSCs and that a mineralized substrate promotes bone formation. Furthermore it might be possible that the cassette model prevented proper cell migration into the alginate due to the confined space. Degradation of the alginate was slower than in the ectopic constructs, and access to the plasmid DNA as well as blood vessel ingrowth and influx of bone forming cells from the underlying bone might be less efficient. This would decrease the amount of produced BMP-2 and delay the onset of bone formation.

In our studies high amounts of bone have only been induced *in vivo* in the presence of seeded MSCs. These MSCs most likely take up the plasmid DNA present in the gel, become transfected, produce BMP-2 and differentiate towards osteoblasts. Subsequently bone is deposited on the surface of BCP particles in the construct. The studies however have been performed in nude mice, from which it is known that cells are needed to stimulate bone formation. Other animal models such as rat, goat and rabbit are also inducible with an osteogenic material or growth factor [266].

For clinical translation, a cell-free construct would be ideal because harvesting, isolation and culturing of MSCs are time-consuming and costly. Cell-free constructs or constructs containing allogeneic cells can be used as off-the-shelf products, which is optimal for one-stage procedures and quality control. As mentioned before, cell recruitment is also an attractive possibility.

CELL RECRUITMENT TOWARDS BONE TE CONSTRUCTS

To recruit MSCs from the periphery towards a fracture, a commonly used strategy is to increase local concentrations of cytokines and/or chemokines at the target site, because cytokines and chemokines are important factors in regulating mobilization, trafficking and homing of stem/progenitor cells [267 268].

When SDF-1α is incorporated into scaffolds, endogenous cells can be recruited to these constructs in a tissue engineered setting (in preparation). The release of SDF-1α for longer periods of time has been achieved by loading it onto controlled release systems such as gelatin microparticles (GMPs) [226 58]. Based on this knowledge SDF-1α was loaded onto gelatin microparticles (GMPs) and combined with BMP-2 plasmid DNA into an alginate constructs to attract MSCs towards the plasmid DNA. The constructs were left cell-free or combined with MSCs and implanted ectopically in nude mice. No differences in vascularization were detected, possibility due to the timeframe of implantation (6 weeks) that exceeds the timeframe in which vascular changes can be detected (mostly 10-12 days) [58 227]. This implantation period however is ideal to detect changes in bone formation. The main result found in the MSC seeded samples is that the combined addition of BMP-2 plasmid DNA and SDF-1α led to significantly higher bone formation compared to either of the single growth factors. This confirms what is described in literature, namely that SDF-1α recruits multipotent cells from the circulation and that BMP-2 induces their osteogenic differentiation and subsequent bone formation *in vivo* [68 66 67]. Several groups have shown that the combination of SDF-1α with BMP-2 promotes higher bone formation compared to SDF-1α or BMP-2 alone [68 66 67]. The mechanism behind this increased bone formation has been described as a combination of enhanced mobilization and homing of bone marrow-derived osteoprogenitor cells to the implant, leading to increased numbers of cells available for bone regeneration at ectopic bone implants [66 224]. The finding that a combination of BMP-2 and SDF-1α also advances bone onset compared to BMP-2 or SDF-1α alone might be another possible explanation. To our knowledge this effect of the combined growth factors has not yet been reported.

PRINTING DNA

To optimize delivery of BMP-2 encoding cDNA at the site of implantation, construct design with respect to porosity was investigated using a 3D bioprinter. Hereby cell-, DNA- or growth factor-laden hydrogels are integrated with so-called rapid prototyping technology [75]. With this method porous structures have been printed and tailored to optimize transfection efficiency, growth factor release and eventually bone formation. The pores enhance oxygen/nutrient supply and waste product removal in the first days after implantation and allow blood vessel ingrowth after prolonged implantation times [74]. When using this technology, a major challenge is the combination of material printability and biological performance. The printed hydrogels must have a certain stiffness to maintain integrity of the printed structure, but need to be of sufficiently low viscosity to be dispersed through a small needle. Furthermore, the hydrogel's polymerization rate must allow fusion with the previously printed layer. This leads to a narrowing of design choices, which in the case of alginate led to

optimizing concentrations for printing and for cell transfection [230]. By pre-crosslinking the alginate, providing extra mechanical stability, printing was possible at a low 3% concentration of alginate, which also provided acceptable transfection efficiencies and growth factor release. Once the protocol for printing DNA was established, the role of porosity in construct design was further investigated. Printed pores in the alginate construct containing BMP-2 plasmid DNA and MSCs increase the production and release of BMP-2 as a result of the transfection compared to solid printed constructs. Furthermore, a higher amount of osteocalcin, an osteogenic marker, was produced by cells in porous constructs. This indicates that porosity promotes gene expression and subsequent osteogenic differentiation.

Printed constructs were implanted subcutaneously in nude mice for 6 weeks. The constructs were supplemented with MSCs and BCP particles, as described for the earlier experiments. In the group of solid constructs, large pieces of alginate remained intact whereas all alginate degenerated/dissolved in the porous groups. In spite of the quick degradation, BMP-2 plasmid DNA did not induce bone formation in the porous and non-porous groups. It is possible that the higher alginate concentration used for the bioprinting (3 % versus 1 % in previous work) decreased the transfection efficiency and thereby resulting in suboptimal BMP-2 concentrations.

The main conclusion that can be drawn from this study is that tailoring material properties for printing affects plasmid DNA transfection, growth factor release, osteogenic differentiation and eventually bone formation. The presence of pores in the printed construct led to increased growth factor production, release and osteogenic differentiation *in vitro*, but the results cannot be translated towards the *in vivo* environment yet. However, when optimized in the future, the porous 3D printed constructs might be up scaled towards clinically relevant sizes to fill large bone defects and induce bone formation locally.

SAFETY AND FUTURE OUTLOOK

Despite promising results in small and large animal studies, described both in literature and in this thesis, it is unlikely that gene therapy based growth factor delivery will be used in a clinical setting soon. It is still a huge challenge to translate the animal studies' results to human clinical practice. One of the problems is that gene therapy is a relatively new field of medicine which suffers from a damaged image after severe side effects occurred in one of the first clinical gene therapy based trials [103]. Years after the trial more and more studies are now back in a clinical trial phase.

The field of orthopedics however mainly deals with non-lethal diseases or injuries. Therefore possible health risks which might arise during clinical trials do not justify human experimentation. The field is now in the phase where safety studies need to be performed in order to proceed with gene therapy in a more clinical setting but no safety study has been reported so far for orthopedic applications. As soon as safety and efficacy of gene therapy studies in several other areas have been proven, this will add to the attempts in the field of bone tissue engineering. In the context of safety it is most likely that over the next few years, non-viral gene therapy trials will be set up for orthopedic applications, since the vectors and tools are generally considered more innovative and safe. To set up proper safety studies

several aspects need to be taken into account. Firstly the GMP protocols need to be designed to warrant the quality of the construct, furthermore the insertion of the DNA in the host genome must be minimized to avoid possible mutagenesis, cytotoxicity of the vector must be prevented, distribution of the DNA to other organs needs to be ruled out, and finally optimal dosage needs to be established together with in depth monitoring of possible side effects.

The craniofacial field will probably be the first area to meet all these criteria and proceed towards clinical gene intervention technique. This is due to the fact that the jaw can be considered a relatively isolated tissue, but also because it is possible to biopt the newly formed bone and investigate it with respect to most orthopedic procedures. These types of studies might contribute to safety and efficiency knowledge, since biopting is part of the standard procedure to place a dental implant. The rest of the bone tissue engineering field can then take advantage of this knowledge and apply the future developments in other applications such as spinal fusions or bone defects.

SUMMARY AND FINAL REMARKS

The goal of this thesis was to develop and optimize a BMP-2 gene based bone inducing construct as a means to tightly control timing and dosage of BMP-2 availability in tissue engineered bone implants.

In chapter 2 we reviewed the existing literature regarding non-viral growth factor delivery for bone tissue engineering. A large variety of materials, cells, transfection agents and delivery strategies are being investigated at the moment. Some developed protocols provide very promising results in terms of transfection efficiencies, growth factor delivery, osteogenic differentiation and bone formation. Furthermore, efficacy studies at ectopic and orthotopic locations are emerging for some of the new strategies, and these will soon be followed by safety studies to investigate the possibility to apply these techniques in a clinical setting.

As discussed in chapter 3, our strategy is based on a very simple formulation consisting of naked plasmid DNA encoding BMP-2 infused in alginate hydrogel. The optimal conditions to introduce BMP-2 cDNA into MSCs were established leading to a construct which induces high transfection efficiencies *in vitro*. Furthermore growth factor production and release from the alginate was accomplished and subsequent osteogenic differentiation was induced.

To optimize bone forming properties of the construct at orthotopic or ectopic implantation locations, BMP-2 plasmid DNA based constructs were implanted in goats intramuscularly and in spinal cassettes. The results as described in chapter 4 indicate that contact to a highly cellular environment largely determines alginate degradation and concomitant transgene expression. The implanted cells were able to differentiate as a result of the transfection with BMP-2 and a positive effect on bone formation was induced as a result of the transfection with BMP-2.

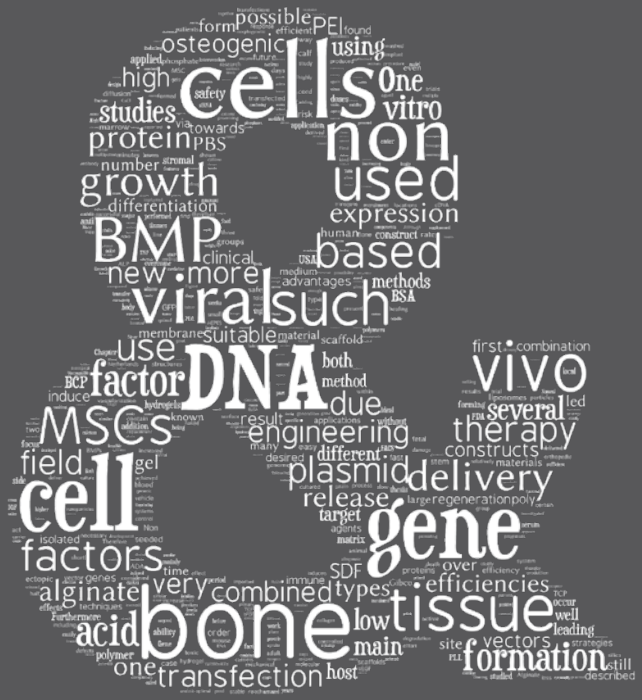
To relate these results to clinically applied protein dosage, BMP-2 effectivity as a result of gene delivery was compared to both high and low doses of BMP-2 protein, as described in chapter 5. This study showed similar amounts of bone formed irrespective of the delivery mode of BMP-2, thus demonstrating the effectivity of the method. We acknowledge that the results were only apparent in small ectopic constructs under optimized conditions.

The constructs described in chapter 5 were supplemented with seeded MSCs. To investigate possible recruitment of MSCs from the circulation, SDF-1α was added to the BMP-2 plasmid DNA based constructs in chapter 6. The controlled release of SDF-1α combined with BMP-2 resulted in not only higher amounts of bone formation, but also in an earlier onset of bone formation than what was seen with either factor alone. We conclude that SDF-1α is a valuable factor in bone tissue engineered constructs but that in this mouse ectopic implantation model it cannot replace cell seeding.

Scaffold form and porosity has great impact on transfection efficiency, growth factor release, vascularization and bone formation. In chapter 7 the effect of 3D morphology from printed constructs is described. The creation of a porous construct has led to the increased release of BMP-2 protein as a result of the transfection compared to a solid construct. The bioprinting of DNA encoding BMP-2 resulted in increased osteogenic differentiation, as evidenced by osteocalcin expression. From these results we conclude that bioprinting of the gene delivery system is feasible and that the resulting porosity contributes to transgene expression and subsequent osteogenicity of the construct..

Overall this thesis took a step in developing a non-viral gene delivery strategy for BMP-2 application in bone tissue engineering. The goal of creating and optimizing a gene delivery vehicle and incorporating it in a bone inducing construct has been achieved. We developed and optimized a BMP-2 plasmid DNA based constructs in alginate which has been able to efficiently transfect MSCs and induce osteogenic differentiation *in vitro* and *in vivo*. These constructs have been further optimized by combining them with SDF-1α, and introduce porosity with 3D bioprinting techniques. The latter results will help in future up scaling of the constructs towards larger implants. Furthermore bone formation has been successfully induced *in vivo* in amounts comparable to protein delivery.

In the future safety and efficacy studies at orthotopic locations will have to determine whether this gene delivery strategy is ready to be translated to clinical settings.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ADDENDUM

APPENDIX: CORRECTION CHAPTER 3 AND 4 REGARDING THE HIS-TAG

An error has been discovered in the manuscript "osteogenic differentiation as a result of BMP-2 plasmid DNA based gene therapy *in vitro* and *in vivo*", and in the manuscript "Gene delivery of bone morphogenetic protein-2 plasmid DNA promotes bone formation in a large animal model".

After subcloning of the coding region and sequencing of the, in the manuscript, discussed His/BMP-2 plasmid DNA, it appears that the His-tag is not present in this construct. The BMP-2 coding region is still intact. This implies that some of the published data concerning His detection need reconsideration.

In chapter 3, figure 1, panels 1D-F represents anti-His staining on *in vitro* His/BMP-2 transfected MG-63 cells to establish transfection efficiency. His positive cells were detected in the transfected group whereas the transfected, isotype stained, and untransfected cells remained negative. Similar findings are shown in figure 1, panels 1G-H. His positive cells were detected in implanted alginate constructs *in vivo* in mice, but only in samples containing His/BMP-2 plasmid DNA. In both experiments the controls remained negative and the transfected samples stained positive.

In chapter 4, figure 2, His positive cells were detected in implanted alginate constructs *in vivo* in goats and scored for presence or absence. In the experiment the control samples without plasmid DNA remained negative whereas the His/BMP-2 transfected samples stained positive as demonstrated after incubation with an anti His antibody(genscript).

If the fact that BMP-2 protein contains two His enriched regions is combined with information from the product sheet stating that 4x Histidines can be detected by the His antibody, the hypothesis was formed that an unexpected cross reaction between the His antibody and the produced BMP-2 protein occurred.

This hypothesis was confirmed by incubating purified human BMP-2 protein (2μg/μl, Medtronic) spotted on nitrocellulose with the anti-His antibody as described in the above manuscripts (anti-His antibody, Genscript) for 1 hr at RT. As a positive control, His-tagged polyUbiquitin chains (10μg/μl, His-Ub3-7 K48 linked, Boston Biochem) were used and as a negative control bovine serum albumin (BSA, 50μg/μl, Roche). The results are presented in figure 1 hereunder, and clearly show cross reactivity towards BMP-2.

Therefore the conclusions in chapter 3 that a high percentage of cells in alginate can be transfected (Fig 1A-C and 2A), and that the the majority of the cells produce BMP-2 protein as a result of the transfection with BMP-2 plasmid DNA, (Fig 1D-H) remains plausible. Furthermore in Fig. 6 a paracrine effect has been demonstrated. It is not possible to discriminate between endogenous BMP-2 and BMP-2 produced as a direct result of the transfection, but since this effect was only shown in samples containing BMP-2 plasmid DNA, and not in the untransfected samples, it is still highly likely that the described His-staining actually reflects BMP-2 produced as a result of the transfection.

The conclusion in chapter 4 that the detected His-staining represents BMP-2 protein produced as a result of the transfection (Fig 2) remains plausible. Also here it is not possible to discriminate between endogenous and BMP-2 produced as a direct result of the transfection anymore, but since this effect was only shown in samples containing BMP-2

plasmid DNA, and not in the control samples, it is still highly likely that the described His-staining actually represents BMP-2.

Since the main conclusions in chapter 3 and 4 is not dependent on His-stainings, but on independent measurements of BMP-2 concentrations by ELISA and induction of osteogenic narkers , and transfection efficiencies have been determined with GFP-encoding plasmid DNA as well, and quantifications of newly formed bone, the conclusion remains unchanged.

To sum up: all our main conclusions remain intact. We have developed an efficient non-viral gene delivery method in alginate that produces and releases bioactive BMP-2 for at least 6 weeks. As a result of that, osteogenic differentiation is induced *in vitro* and *in vivo*. We are still convinced that the use of BMP-2 plasmid DNA for bone tissue engineering in orthotopic and ectopic locations might be a promising new approach for bone tissue engineering. Transfection efficiency percentages are based on GFP expression and remain in the same order of magnitude. The only conclusion that is not substantiated by independent measurements concerns the paracrine effect of BMP-2 produced as a result of the transfection.

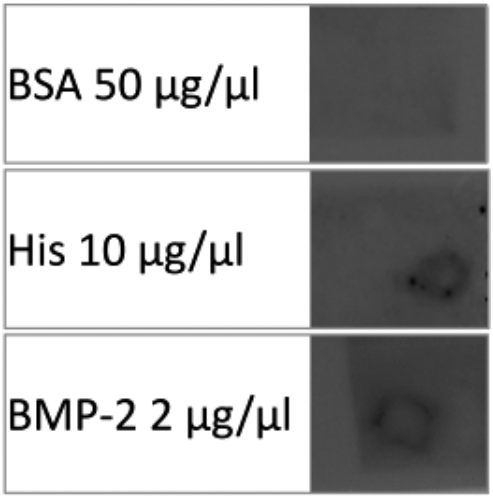

Figure 1. Immunohistological staining of purified recombinant BMP-2 protein on a spotblot, using His-tagged polyUbiquitin as a positive control and BSA as a negative control.

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NEDERLANDSE SAMENVATTING

Het onderzoek beschreven in dit proefschrift richt zich op het ontwikkelen en optimaliseren van niet-virale gen therapy voor bot tissue engineering. Hierbij ligt de focus op het toedienen van de groeifactor BMP-2 in de vorm van plasmide DNA. BMP-2 is een van de meest gebruikte groeifactoren op het gebied van bot tissue engineering. Het induceert de differentiatie van mesenchymale stromale cellen (MSCs) naar botvormende cellen. Op dit moment wordt BMP-2 met name toegdiend in de vorm van eiwit. Door o.a. een snelle afbraak in het lichaam word een hoge dosis gebruikt waarvan men inmiddels vermoedt dat dit leidt tot diverse complicaties. Een andere toedienings methode is via plasmide DNA. Dit is een vorm van niet-virale gentherapie, waarbij voor BMP-2 coderend DNA door de cel wordt opgenomen, afgeschreven en tenslotte wordt een eventueel geproduceerd eiwit uitgescheiden. Voordelen van deze methode van BMP-2 toediening is dat het goedkoop, relatief simpel en in een lage dosis toe te dienen is.

Uit literatuuronderzoek blijken er zeer veel verschillende methodes te worden onderzocht om dit DNA in de cel te krijgen waarin met name het dragermateriaal en/of transfectie agentia wisselen, uiteenlopend van fysieke perforatiemethoden tot allerhande polymeren.

Het in dit proefschrift beschreven onderzoek richt zich op de combinatie van BMP-2 plasmide DNA met een alginaat hydrogel. Alginaat is een natuurlijke polymeer waarin cellen overleven en het is al eerder gebruikt voor tissue engineering doeleinden. Wanneer MSCs worden gecombineerd met BMP-2 plasmide DNA in de alginaat hydrogel krijgt het naast de functie van scaffold ook de functie van transfectie reagent en wordt ruim 62% van de cellen getransfecteerd. Na transfectie wordt BMP-2 uitgescheiden in het medium, maar voornamelijk in de gel. Het alginaat werkt als een gecontroleerd afgifte systeem en tot 5 weken na de transfectie wordt er BMP-2 geproduceerd en af gegeven. Er komt voldoende biologisch actief BMP-2 vrij om in vitro en in vivo osteogene differentiatie te induceren.

Om botvorming te stimuleren door middel van de transfectie zijn keramische BCP partikels toegevoegd aan de constructen als ondergrond voor botcellen om zich op te hechten en botmatrix op af te zetten. Deze constructen zijn vervolgens zowel ectopisch, waar de cellen nog niet aanwezig zijn maar gerekruteerd moeten worden uit de circulatie, als orthotopisch geimplanteerd in geiten. Het resultaat is een toename van botvorming ectopisch. Orthotopisch wordt dit niet gezien wat verklaard zou kunnen worden doordat het alginaat niet calcificeerd wanneer er geen cellen zijn gezaaid in de constructen. Wanneer er wel MSCs worden gezaaid hebben de ze zelfs een negatief effect op de botvorming.

Om de botvorming, geïnduceerd door BMP-2 plasmide DNA, te kunnen vergelijken met het toedienen van BMP-2 eiwit zijn beiden ectopisch in naakte muizen geïmplanteerd. Het eiwit is in twee doses toegediend; een hoge dosis, vergelijkbaar met wat klinisch wordt toegediend en een lage dosis vergelijkbaar met wat er vrijkomt na transfectie. De constructen met BMP-2 plasmide DNA bleken het meest succesvol in het induceren van nieuw bot. In deze constructen is de grootste hoeveelheid bot gevonden en daarnaast start deze botvorming eerder. Dit impliceert dat BMP-2 plasmide DNA een goedkoop, makkelijk toepasbaar alternatief zou kunnen zijn voor het hoog gedoseerde BMP-2 eiwit wat nu in de kliniek wordt gebruikt. Om de stap naar de kliniek te kunnen maken is het van belang dat het gebruik van plasmide DNA ook toepasbaar wordt in grotere defecten waarbij vascularisatie een rol gaat spelen.

Mede hiervoor is SDF-1α toegevoegd aan de alginaat/BMP-2 constructen. Deze chemokine is in staat cellen vanuit de circulatie naar implantaten te rekruteren. Op deze manier zou het theoretisch overbodig worden MSCs toe te voegen aan het construct. Naast de chemotactische functie van SDF-1α wordt ook de vorming van bloedvaten gestimuleerd wat zoals eerder genoemd cruciaal is voor grotere tissue engineering constructen die niet meer voldoende worden voorzien van zuurstof en voedingstoffen door middel van diffusie. Tevens zijn er synergistische effecten op de botvorming beschreven in de literatuur wanneer SDF-1α wordt gecombineerd met BMP-2.

Voor implantatie is SDF-1α eerst gekoppeld aan gelatine micropartikels, op die manier kan een gecontroleerde afgifte van een aantal dagen tot weken worden bereikt. De gelatine partikels zijn samengevoegd met alginaat en BMP-2 plasmide DNA en geïmplanteerd in naakte muizen. Na 6 weken blijkt echter dat de toevoeging van SDF-1α onvoldoende effect heeft op de aantrekking van cellen om vascularisatie/botvorming te stimuleren. Wanneer er MSCs aan het construct worden toegevoegd blijkt echter dat de aanwezigheid van SDF-1α, wanneer het gecombineerd wordt met BMP-2 wel een toegevoegd positief effect op de botvorming te hebben. Niet alleen wordt er meer bot gevormd dan met alleen SDF-1α of BMP-2 maar de botvorming start ook eerder.

Een andere strategie om grotere constructen te kunnen implanteren is het toevoegen van poriën met behulp van een 3d bioprinter. De poriën ondersteunen de uitwisseling van gassen en voedingstoffen en faciliteren ingroei van bloedvaten. Tijdens het print proces worden lagen van MSCs bevattend alginaat op elkaar geprint volgens een computer ontwerp. De geprinte constructen zijn reproduceerbaar in bijvoorbeeld vorm, grootte en porositeit.

Om te kunnen printen, maar ook transfecteren in alginaat, is optimalisatie nodig. Om een poreus printbaar construct te kunnen maken is een hogere mate van viscositeit en stevigheid vereist. Voor een optimale transfectie daarentegen is een lage viscositeit en dus een lage alginaat concentratie vereist. Door te pre-polimeriseren met een iets verhoogde alginaat concentratie (3% tov 1%) zijn deze twee eigenschappen in een construct mogelijk. Na incubatie van een week in vitro is de eiwit productie en release significant groter in een poreus versus solide geprint construct. Ook osteogene differentiatie treed meer op in poreuze constructen zowel in vitro als in vivo. Botvorming is nog niet waargenomen in zowel de poreuze als niet poreuze constructen. Hiervoor is verdere optimalisatie van de vorm, alginaatconcentratie en BCP partikel eigenschappen vereist. Toch zijn de eerste resultaten waarin eiwitproductie en zelfs osteogene differentiatie plaatsvindt hoopgevend.

Ondanks de veelbelovende resultaten in diverse diermodellen, zoals beschreven in de literatuur en in dit proefschrift, is het onwaarschijnlijk dat groeifactoren op korte termijn via gen therapie zullen worden toegediend. Gentherapie kampt met een slechte reputatie nadat ernstige bijwerkingen zijn opgetreden in een van de eerste klinische trials. Daarnaast zijn de meeste orthopedisch aandoeningen niet letaal, waardoor de risico's van een experimentele behandeling niet opwegen tegen de risico's van de aandoening zelf.

Wanneer veiligheids- en rendementsstudies positief zijn afgerond in andere vakgebieden, zal het veld van bot tissue engineering ongetwijfeld volgen. Zeer waarschijnlijk zullen de eerste studies op het gebied van de kaakchirurgie zijn, wat redelijk geïsoleerd is van de rest van het

lichaam en waar biopteren een mogelijkheid is. De resultaten van die studies kunnen worden gebruikt in de wervelchirurgie of voor het helen van botdefecten elders in het lichaam.

Concluderend hebben we met dit proefschrift een stap gezet in de ontwikkeling en optimalisatie van niet-virale gentherapie op basis van BMP-2 plasmide DNA en alginaat. Deze DNA constructen zijn in staat om vergelijkbare botvorming te induceren als de hoge doseringen BMP-2 eiwit die op dit moment veelvuldig in de kliniek worden gebruikt. Verdere optimalisatie door de combinatie met SDF-1α, of introductie van porositeit met 3D print technieken, kan bijdragen aan toekomstige opschaling van de constructen om grote defecten te kunnen helen. Maar toekomstige veiligheids- en rendementsstudies op orthotopische locaties zullen bepalen of de in dit proefschrift beschreven niet-virale gentherapie methode vertaald zal worden naar klinische toepassingen.

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Prof. Dr. W.J.A Dhert, beste Wouter

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Onmisbare analisten, en andere ondersteuning

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CURRICULUM VITAE

Fiona Wegman was born on august 28th, 1984 in Zevenaar, The Netherlands.
In 2002, she graduated from high school (VWO, Thomas a Kempiscollege, Arnhem) and started the study Biomedical Sciences at Utrecht University, The Netherlands. In 2006 she joined a research project studying the influence of endothelial progenitor cells and platelet gel on tissue engineered bone in goats at the department of Orthopaedics at the University Medical Center in Utrecht, The Netherlands (supervisors: Dr. R. E. Geuze, Dr. J. Alblas). After graduating from Biomedical sciences (mastertrack biology of disease) in august 2007 she continued working at the department of Orthopaedics at the University Medical Center in Utrecht, The Netherlands on a PhD project called "Novel instructive scaffolds for the regeneration of bone tissue" (Supervisors: prof. W.J.A. Dhert, prof. F.C. Öner and dr. J. Alblas). The research resulted in several publications, presentations at international conferences and this thesis. Currently, she is working as a teacher and supporting educational staff at the department of biomedical technology at Eindhoven University of technology.
Fiona Wegman lives in Utrecht with Jordi Hoogendoorn and their son Jip Hoogendoorn.

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LIST OF ABBREVIATIONS

α-MEM	alpha minimal essential medium
AAV	adeno associated virus
Ab	antibody
ADA – SCID	adenosine deaminase –severe combined immune deficiency
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
cDNA	complementary deoxyribonucleic acid
CXCL 12	chemokine (C-X-C Motif) ligand 12
CXCR 4	chemokine (C-X-C Motif) receptor 4
DAB	diaminobenzidin
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ELISA	enzyme-linked immuno sorbent assay
EM	expansion medium
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FCS	fetal calf serum
FDA	food and drug administration
GA	glutaraldehyde
GAG	glycosaminoglycan
GAM	gene activated matrix
GFP	green fluorescent protein
GMP	good medical practice
GMPs	gelatin microparticles
HA	hydroxy apatite
HA	hyaluronic acid
HE	hematoxylin/ eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
IGF-1	insulin like growth factor-1
MFI	mean fluorescence intensity
miRNA	micro ribonucleic acid
M&M	materials and methods
(p)MMA	(poly) methylmetacrylate
MSC	multipotent stromal cell
OCN	osteocalcin

OM/ODM	osteogenic (differentiation) medium
PA	palmic acid
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PEI	polyethylenimine
PLA	polylactic acid
PLG	poly(lactide-co-glycolide)
PLGA	poly(lactic-co-glycolic acid)
PLL	poly-L-lysine
PNPP	p-nitrophenyl phosphate
PTH	parathyroid hormone
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