TRANSLATIONAL CHALLENGES IN BONE TISSUE ENGINEERING

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LIST OF ABBREVIATIONS

α-MEM	Alpha modified minimum essential medium
ALIF	Anterior lumbar interbody fusion
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
ASAP	Ascorbic acid-2-phosphate
AU	Arbitrary units
Av	Average
BCP	Biphasic calcium phosphate, mixture of β-TCP/ HA
BFGF	Basic fibroblast growth factor
BGP	β-glycerophosphate
BLI	Bioluminescence imaging
BM	Bone marrow
BMP	Bone morphogenetic protein
BMSCs	Bone marrow stromal cells
BSA	Bovine serum albumin
CaP	Calcium phosphate
CFU-F	Colony forming unit fibroblast
CFU-E	CFU-Efficiency
CMV	Cytomegalovirus
cPPT	Central polypurine tract
CT	Computed tomography
DAB	3,3'-diaminobenzidine
DBM	Demineralized bone matrix
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
env	Envelope protein (viral)
EPC	Endothelial progenitor cell
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FTIR	Fourier transform infrared spectroscopy
GFP	Green fluorescent protein
HA	Hydroxyapatite
HEK293	Human embryonic kidney cells
HLA	Human leukocyte antigen
IGF	Insulin-like growth factor
IRES	Internal ribosome entry site

KXA	Ketamine, xylazine, atropine
Luc2	Luciferase 2
LV	Lentivector
MMA	Methyl methacrylate
MNGC	Multinucleated giant cell
MSC	Multipotent stromal cell/ Mesenchymal stem cell
n	Sample size of statistical group
NK cells	Natural killer cells
p	Probability of statistical finding
P	Power (1-β)
PBS	Phospate buffered saline
PDGF	Platelet derived growth factor
PG	Platelet gel
PGA	Polyglycolic acid
PLF	Posterolateral fusion
PLG	Platelet leukocyte gel
PLIF	Posterolumbar interbody fusion
PMMA	Polymethylmethacrylate
PRE	Posttranscriptional regulatory element
PRP	Platelet rich plasma
RNA	Ribonucleic acid
ROI	Region of interest
RRE	Rev-responsive element
RT	Room temperature
SD	Standard deviation
SEM	Standard error of the mean
SIN	Self-inactivating
TCP	Tricalcium phospate
TE	Tissue engineering
TGFβ	Transforming growth factor β
TU	Transducing units
VEGF	Vascular endothelial growth factor
XRD	X-ray diffraction

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GENERAL INTRODUCTION AND AIMS OF THIS THESIS

CLINICAL PROBLEM

Bone tissue has the unique ability to heal itself perfectly. However when complicated fractures, pathological defects or large bone defects have to be bridged, healing processes may fail. This might be a result of several causes like insufficient blood supply, infections or even a systemic disease and could potentially lead to nonunions. This causes a need for bone grafts or bone graft substitutes, and the therapeutic strategy of choice is the implantation of autologous bone grafts. Bone is the second most transplanted tissue after blood, with more than 500.000 annual bone grafting procedures taking place in the United States and over 2.2 million worldwide in order to repair bone defects in orthopedic surgery, maxillofacial surgery, trauma surgery and neurosurgery (1).

Autograft bone contains all key elements required for bone repair. Firstly, cells with proliferative and/ or osteogenic potential; secondly growth factors for osteoinduction and vascularization of the new bone; and lastly an osteoconductive scaffold which is the extracellular matrix. When implanting graft material in a bony defect not all these elements are always necessary, for example cells can migrate from the surrounding bone and cells can create their own matrix, which can then be modified to form the bone tissue. The percentage of success of autograft is high, but disadvantages like lengthening the overall surgical procedure, limited availability and complications like donor-site pain, infections and nonunions are common, and when used in spinal fusion surgery, pseudoarthrosis rates have been reported to range from 5 - 44% (2-4). An entire industry has risen, providing substitutes for autologous bone grafts, which (partially) mimic natural bone repair. Allograft bone has been used to avoid the complications of donor-site morbidity. Furthermore substitutes such as demineralized bone matrix (DBM), collagen and synthetic options like ceramics are being used. However they all have their own disadvantages. For example, allograft bone contains little or no cells depending on the harshness of the production process of freezing and sterilization, and another popular substitute based on collagen is also cell-free and has poor mechanical properties (5, 6). Tissue engineering is a relatively new technology aiming at replacement of tissues, which are created by combining cells, growth factors and scaffolds. Tissue engineering has been described as an interdisciplinary field that applies the principles of engineering and the life sciences to the development of biological substitutes that restore, maintain, or improve tissue function (7). In the following paragraph the key elements and their relationship with bone tissue engineering will be discussed in further detail.

KEY ELEMENTS FOR CELL-BASED BONE TISSUE ENGINEERING

Cells important in bone tissue engineering

The multipotent stromal cell: the introduction

More than 35 years ago, Friedenstein and colleagues described that murine bone marrow contained a population of adherent fibroblast-like cells, which were able to form colonies,

were nonphagocytic, and differed from the hematopoietic stem cells. Furthermore, when transplanted subcutaneously, they were able to make bone and reconstitute a hematopoietic microenvironment (8, 9). These early studies on mesenchymal stem cells (MSC, later more aptly renamed as multipotent stromal cells) were progressed by others (10). In 1991 the MSC was proposed for the first time as part of a therapeutic concept, especially for orthopedic indications (11). Over the years it became progressively clear that these MSCs, which can differentiate *in vitro* into a variety of mesenchymal lineages such as osteoblasts, chondrocytes and adipocytes, are not an exclusive feature of the bone marrow. They can also be isolated from other organs and tissues like placenta (12), cord blood and umbilical cord (13, 14), synovial membrane (13), adipose tissue (15), or dental pulp (16). In 2008 Caplan stated that all MSCs are pericytes, however that not all pericytes are MSCs (17), an idea that becomes more and more accepted.

Characterization of MSCs

It took until 1999 before the first systematic investigation of human bone marrow derived MSCs that confirmed their multipotent differentiation capacity was published (18). Later, more detailed investigations of MSCs raised concerns regarding the term "stem cell", as MSC did not match the criteria defined for "stemness" without restriction (19, 20). Nowadays the term mesenchymal stromal cell (MSC) is also often used (21), but multipotent stromal cells is strictly taken a more accurate description. In 2006, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, proposed minimal non-exclusive criteria to define human MSCs, mainly to create the opportunity for scientists to compare their work with each other: 1) MSCs are plastic-adherent when maintained in standard culture conditions; 2) MSCs must express CD105, CD73, and CD90, and lack expression of CD45 (a pan-leukocyte marker), CD34 (expressed by primitive hematopoietic progenitors and endothelial cells), CD14 or CD11b (expressed on monocytes and macrophages), CD79a or CD19 (markers of B-cells) and HLA-DR surface molecules; 3) MSCs have multilineage potential: they differentiate to osteoblasts, adipocytes and chondroblasts *in vitro* (22). Much of the preclinical research is being performed with MSCs derived from various species like mice, rats, dogs and goats. Characterization of these cells is much more challenging making it very difficult to compare the different studies.

MSCs used in an allogeneic setting

Autologous MSC transplantation in a clinical setting has several downsides, like aspiration of bone marrow from the patient several weeks before the actual implantation surgery, needed to expand the cells and perform quality control tests. In the last few years, i*n vitro* studies have shown that allogeneic human MSCs are capable of suppressing T-cell immune responses, by directly inhibiting $CD3^+CD4^+$ T cell proliferation, and secretion of T_H1 lymphokines, such as IL-2 and IFN-γ (23-25). Besides the direct suppression, MSCs are also considered immune privileged, as they are not removed by the immune system because they lack the co-stimulatory molecules CD80, DC86 and CD40 (23). It has been suggested, that *ex vivo* expanded allogeneic MSCs are capable of bone formation and engrafting in other tissues without being rejected when applied locally (26, 27). Systemically administered allogeneic MSCs have been used to treat several diseases like severe acute graft-versus-

host disease (28), osteogenesis imperfecta (29) and amyotrophic lateral sclerosis (30-33). These results suggest the possibility to isolate MSCs from donors, culture expand them and cryopreserve them in a cell bank, creating the opportunity to use the MSCs as an off-the-shelf therapy.

Endothelial progenitor cells and vascularization

Bone formation is an angiogenesis-dependent process (34, 35). As a result of that, vascularization has been recognized to be a crucial factor in the field of bone tissue engineering during the last years (36, 37). The seeded MSCs in TE implants are expected to suffer from severe hypoxia in the first week after implantation, because oxygen transport is limited to approximately 200 μm from a blood vessel, and therefore may not survive (38, 39). Several strategies have been described to accelerate the establishment of a functional vascular network in bone-engineered tissues, like optimization of scaffold architecture and the addition of angiogenic growth factors. As extensive paracrine and direct communication between osteoprogenitor cells and endothelial cells has beneficial effects for both cell types with respect to proliferation and factor secretion, it is an interesting idea to combine both cell types within one bone tissue engineered construct (34, 40, 41). So far, most vascularization strategies relied either on the ingrowth of vasculature from the surroundings, which will take some time until appropriate blood supply and waste removal is reached, or the use of endothelial cells, which is so far not clinically applicable (37, 42-44). Highly proliferative endothelial progenitor cells (EPC) (45, 46) however, can be isolated from bone marrow (or from other sources, such as peripheral blood or adipose tissue), implicating that bone forming MSCs and blood vessel-forming EPCs can be acquired from the same source and will only take a single bone marrow biopsy (47, 48). Coculture of MSCs and EPCs to enhance bone formation in a tissue engineered construct is expected to be a next step in bone regenerative therapies.

Bioactive factors

In bone regeneration, many growth- and differentiation factors play a role. Urist (49) already described the process of osteoinduction in 1965. He found that devitalized, demineralized bone was able to induce ectopic bone formation and subsequently, that a mixture of proteins named *bone morphogenetic proteins (BMPs)* was responsible for this bone induction. This resulted in the strategy of bone tissue engineering using bioactive molecules (50). Since then, various proteins were isolated and investigated for their therapeutic potential in bone regeneration, including specific BMPs (51-53), vascular endothelial growth factor (VEGF) (54), transforming growth factor-β (TGFβ), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF), which are most well-known and best studied. In the following paragraphs several bioactive factors applied in this thesis will be highlighted. Furthermore some other growth factors involved in bone formation are discussed.

Bone morphogenetic proteins (BMPs)

BMPs are bioactive molecules that bind to cell-surface receptors and regulate the differentiation, maturation, and proliferation of MSCs into osteogenic and/ or chondrogenic cells (55-57). They are widely studied in developmental and skeletal biology. BMPs belong

to the TGFβ superfamily, which also includes other growth factors such as activins and inhibins. Members of the BMP family, comprising over 30 members, half of whom in mammals are divided into different subgroups based on their amino acid sequences (58, 59). Not all of the members of the BMP family possess osteogenic properties: BMP 2, 4, 6, 7, and 9 are osteogenic (60-64). Research showed, that BMPs in bone are produced by; osteoprogenitor cells, osteoblasts, endothelial cells, chondrocytes and platelets, and are involved in several developmental and pathophysiological processes. Because of their osteoinductive capacities and early availability as recombinant proteins, this research mainly focuses on BMP-2 and BMP-7 (51, 65, 66). *In vitro*, BMPs are able to initiate both bone and cartilage progenitor cell differentiation, and *in vivo* they control the formation of new bone through both endochondral and intramembranous ossification (67, 68). Recombinant DNA technologies have allowed the optimization of BMP-2 and BMP-7 protein preparation (51, 69). Extensive pre-clinical studies have supported the safety of these BMPs (70-72), which has already resulted in clinical successes (73, 74) and many clinical studies are ongoing to expand the indications for the use of BMPs (75, 76). However over time and with longer follow-up, several authors report multiple serious adverse effects, including local inflammation, neuroinflammation, osteolysis, implant migration and soft-tissue swelling (77-79). Recently retrograde ejaculation has been described in more detail, especially in anterior lumbar interbody fusion (ALIF) (80). This paper resulted in extensive discussions between researchers, editors and spinal surgeons (81-86), mainly about the importance of mentioning adverse events in the published papers. This emphasizes that the complex biological actions (like release) of BMPs are still not completely clear, and needs more research.

Vascular endothelial growth factor (VEGF)

Vessel formation is mainly induced by VEGF, and is important for transport of nutrients, oxygen, and cells towards, and removal of waste products from the newly formed bone. As a result of its importance in angiogenesis, VEGF was shown to be necessary during early fracture repair, and endochondral and intramembranous ossification (54, 87, 88). In line with this, experimental models have shown that normal fracture healing is disturbed if VEGF is inhibited (89, 90). Communication between osteoprogenitor and endothelial cells is mediated by several mechanisms: the direct process of gap-junctional communication and the indirect process involving soluble factors like VEGF and BMP-2 (34, 35). VEGF has been described to interact synergistically with BMP-2 and BMP-4 in both bone formation and bone healing by increasing cell survival, angiogenesis and enhancing cell recruitment (91). Furthermore, VEGF can stimulate chemotaxis and differentiation of osteoblasts (92), osteoclasts (93) and MSCs (94) and finally it may also directly contribute to bone formation by enhancing BMP-2 mRNA and protein expression (95). Moreover, osteoblasts are known to synthesize VEGF when oxygen levels are low and in this way actively enhance the process of angiogenesis (40). Given the importance of the angiogenesis-osteogenesis coupling, combining the most important bioactive molecules involved in these processes might be beneficial for bone regeneration (96).

Other growth factors active in bone formation

Transforming growth factor beta (TGFβ) is known to control cell proliferation, cell differentiation, and extracellular matrix synthesis (97, 98) and furthermore to stimulate migration of osteoprogenitor cells. Osteoinductive capacities have mainly been described in the nonhuman primate *Papio ursinus* (99).

Fibroblast growth factors (FGFs) are initially important in the condensation of MSCs in areas of embryos destined to become bone and subsequently in growth of the endochondral skeleton (100). Furthermore, they have a mitogenic effect on many cell types and they are involved in angiogenesis and cell differentiation (101-103). FGF-2 alone, in bone regeneration the best studied member of the FGF family, does not seem to be capable of inducing bone formation, however it plays an important role in the regulation of normal bone healing at all ages (104).

Platelet-derived growth factor (PDGF) is a potent mitogenic and chemotactic factor for various cells like MSCs, fibroblasts and vascular smooth muscle cells. It can be produced by various cell types and is mainly stored in the α-granules of platelets (105). PDGF is one of the crucial biological factors in general tissue repair, however is also responsible for bone healing (106).

Platelet gel (PG)

During wound and fracture healing, platelets accumulate at the site of an extravasation, and when activated by thrombin, they secrete alpha granules. These granules release growth factors involved in the initiation and promotion of wound and fracture healing. Among these are platelet-derived growth factor (PDGF), transforming growth factor beta (TGFβ), fibroblast growth factors (FGFs), and insulin-like growth factor (IGFs). Platelet-leukocyte rich plasma, which may be isolated from a patient's peripheral blood, and becomes platelet gel after activation with thrombin and calcium chloride, contains all of these growth factors, making it an obvious next step to use it in bone regeneration. *In vitro,* PG has been shown to have a dose-dependent mitogenic and chemotactic effect on MSCs and osteoblast-like cells (107, 108) and an inhibiting effect on differentiation (109). *In vivo* results so far in animal and clinical studies are ambiguous (110-112). In an animal study of distraction osteogenesis a positive effect was seen of the use of PG in combination with MSCs. Furthermore more intense bone formation was found in a study using mandible defects in Beagle dogs (113). In a human study it was shown that adding PG to autologous bone when performing spinal fusion, did not result in higher fusion rates (110) and that supplementation of PRP in long bone nonunions treated by external fixation failed to show effectiveness (114). However, little results are known concerning results of PG in tissue engineered construct, making PG an interesting product to use.

Scaffolds

The major function of scaffolds is similar to that of the natural extracellular matrix (ECM) that assists proliferation, differentiation and biosynthesis of cells. There are various scaffold materials that are used in clinical and experimental studies for bone tissue engineering. These include metals, ceramics, and biological and synthetic polymers. In this thesis we

focus on the use of calcium phosphate ceramic materials. They were the first ceramics that have been specifically developed for bone repair. In 1920 the first paper was published describing the repair of a bony defect in rabbits (115) using ceramic scaffolds. After this period, many different calcium phosphate biomaterials have been developed and used in clinical practice (116-118). Calcium phosphates are salts of phosphoric acid (H_3PO_4), and thus can form compounds that contain $H_2PO_4^-$, HPO_3^{2-} or PO_4^{3-} (119). Examples of calcium phosphates are hydroxyapatite (HA, structural formula: $Ca_{10}(PO_4)_6(OH)_2$, calcium to phosphorous ratio 1.67), a material with a low solubility; tricalciumphosphate (TCP, structural formula: $Ca_3(PO_4)_2$, Ca/P ratio of 1.5) a material with a high solubility, and a combination of these two materials is biphasic calcium phosphate (BCP), whose solubility depends on the percentage of both materials. To achieve the goal of bone tissue engineering *in vivo*, the ideal scaffold has several requirements, among which are sufficient mechanical strength to match the intended site of implantation and handling, porosity to allow for cell migration, vascularization and diffusion of nutrients and waste products (120). Scaffolds should have a controlled biodegradability so that bone will eventually replace the scaffold. The materials should also be biocompatible to avoid inflammatory or toxic responses upon implantation. Furthermore, they need to be osteoconductive to facilitate cellular invasion (121, 122) and ideally they are osteoinductive, meaning they have the intrinsic ability to induce osteogenic differentiation both *in vitro* as well as *in vivo*. These characteristics will be discussed in more detail in the next paragraphs.

Biocompatibility

The definition of biocompatibility has changed over the years with the emergence of tissue engineering and regenerative medicine. It changed from a passive process to an active process. Previously, the definition was a list of characteristics by default. A biocompatible material should be non-toxic, non-immunogenic, non-thrombogenic, non-carcinogenic, non-irritant and so on. This was changed in the nineties to the definition that biocompatibility means: the ability of an implanted material to perform while inducing a host response, which is appropriate in a specific application (123-125). According to the old definition, no host response is raised towards a biocompatible material, making the material bioinert. However, hybrid constructs usually include cell therapy and/ or the addition of bioactive molecules to a material, which are aimed at regenerating the tissue in which it is implanted, rather than replacing it with a synthetic material, therefore making the host response part of the process. This creates a distinct difference between biocompatibility in relation to implantable devices, and to hybrid constructs. A medical device, such as drug delivery systems and intravascular devices, should achieve a physical or mechanical function without eliciting an unusual response from the relevant tissue, while a hybrid construct may benefit from such a response, making the material either biotolerant (e.g fibrous tissue between implant and bone) or bioactive (e.g. osseointegration between implant and bone) (126). No foreign material implanted within a living body is completely biocompatible. This only accounts for substances produced by the body itself. Because ceramic materials, and in particular calcium phosphate ceramics have great similarities to the mineral component of bones and teeth, they are expected to induce mild tissue reactions. We have used

these materials, knowing that they induce a reaction involving multinucleated giant cells (MNGCs) recruited to the implanted materials or formation of a fibrous layer around the implant, and monitored the possible presence of a host response (127-130).

Biodegradability

Both *in vitro* and *in vivo* degradation of calcium phosphate ceramics depend on the composition, particle size, crystallinity, porosity, and preparation conditions of the construct. Because of the low degradation rate, HA is mainly used in maxillofacial surgery for pulp capping and filling of periodontal defects. This material can stay *in situ* for years before it is completely degraded. In orthopedics it is mainly used for filling defects remaining after tumor resection, tibial plateau fractures or as coating (131, 132). TCP degrades 10-20 times more quickly (depending on porosity and preparation conditions) *in vivo* and is therefore only used to augment defects where a resorbable material is needed (117, 133, 134). The degradation rate of BCP, consisting of combinations of HA and TCP, is between that of pure HA and pure TCP. By choosing the appropriate composition, degradation rate of the implant, which preferably coincides with the rate of bone formation (135), can be tuned.

Osteoconduction and integration capacities

Osteoconduction in calcium phosphate materials is the bioactive behavior and capacity to allow migration of cells into the construct, resulting in integration of an implant into living tissue. Osteoconduction is important to stimulate new bone ingrowth in several ways, like cell migration and adhesion, which can subsequently result in proliferation, maturation and matrix mineralization (136). An active process in remodeling of healthy bone can lead to an intimate bond between the implants and bone, termed osseointegration (137). Osteoconduction is dependent on porosity, pore size and pore interconnectivity and of surface characteristics of the scaffold material.

Osteoinduction

Osteoinduction is described as the process of the differentiation of immature cells towards osteoblasts (138). There is an ongoing discussion about when it is proper to speak of osteoinductivity of a material and how to test this. A material can be implanted at an ectopic site ensuring that no osteoblast contact is available, and bone formation must be the result of progenitor recruitment from the circulation and subsequent differentiation. Osteoinduction is considered to be intrinsic to the material, i.e. the property of the material to induce osteogenic differentiation and as a result bone formation *de novo* ectopically (i.e., in non-bone forming sites) independent of the animal model. Question remains whether osteoinductivity of a material shown at one ectopic location could be extrapolated to other locations or other animal models. There are various studies attributing osteoinductive properties to calcium phosphate materials (139-142). Although these materials have the same chemical composition, some are claimed to be osteoinductive and others are not. The mechanism behind this claimed osteoinduction is not yet revealed. Suggestions have been made about the microenvironment around the implant being crucial (143), microstructures of calcium phosphate biomaterials could influence their osteogenicity (140), and micropores that increase the surface area of the materials and thus enhance the protein adsorption capacity and cell adhesion (144).

Porosity and pore interconnectivity

Many fabrication techniques are available to produce calcium phosphate scaffolds with varying architectural and physical forms: powders, particles, granules, dense blocks, porous scaffolds, and much more. Pores are defined as a void space within a scaffold, and porosity is a collection of these pores (119). Macro-pores (>10 μm) especially influence tissue function and pores bigger than 300 μm are suggested for bone ingrowth and efficient *in vivo* blood vessel formation. Micro-pores (< 10 μm) are suggested to influence cell function, as cells are around 10-20 μm and furthermore to provide a sufficiently large area for protein adsorption. In 1979 it was already suggested that the optimal pore size was 200-400 μm in consistency with the size of the human osteon of approximately 223 μm (145). For increasing total protein adsorption, microporosity is important (<10 μm).

Mechanical properties

The major limitation of the use of calcium phosphates is that these materials cannot be used as a load-bearing biomaterial, as they have poor mechanical properties. They have a ceramic origin (the word ceramic comes from the Greek word keramikos meaning pottery), making the mechanical properties far less than that of bone, which consists of organic minerals combined with collagen triple helices, bone forming cells and other extracellular matrix proteins (145). Ceramics are, just like the mineral phase of bone, resistant to compression, but not to bending and shear stresses. The collagen is responsible for this quality. This implicates that ceramic implants can only be used in non-weight bearing areas. In cases when material degradability is designed to coincide with bone regeneration and remodeling, other implantation sites may be considered.

Limitations of (cell based) bone tissue engineering

After thirty years of extensive research, the number of autologous bone transplantations is still increasing. Millions of dollars have been spent to search for an alternative bone graft. The last decades the research area of tissue engineering and regenerative medicine is achieving results. MSCs have been used in clinical trials and/ or in clinical practice in various fields of regenerative medicine like treatment of graft versus host disease (146), diabetes mellitus, liver cirrhosis or osteonecrosis of the femoral head (147). However, to our knowledge, no human MSC-based technology is currently available for bone tissue engineering purposes, meaning still many limitations and questions remain that prevent the actual clinical use of cell based bone tissue engineering.

Research aims and questions to be addressed in this thesis

The overall aim of this thesis was to create strategies to improve bone graft substitutes for future clinical applicability. First, the value of cell based tissue engineered constructs was investigated. Second, other aspects of construct optimization involving material improvement and additives were investigated.

Aim 1: To investigate the value of cell based bone tissue engineering constructs *in vivo*. The following research questions are addressed:

- What is the contribution to bone formation of implanted MSCs at both ectopic and spinal fusion locations in rats?
- What is the feasibility of off-the-shelf components like allogeneic MSCs and freshly isolated bone marrow in tissue engineered constructs, in terms of bone quantity and immune response by the host?
- How does implantation location influence effectiveness of TE constructs containing autologous or allogeneic MSC?
- Are endothelial progenitor cells able to enhance bone formation in coculture at an ectopic location?
- What is the additional value of MSCs seeding on bone formation in a clinically relevant instrumented posterolateral spinal fusion model in goats?

Aim 2: To investigate the creation of cell-free bone substitutes by either using bioactive molecules, or by the application of improved scaffold materials. The following research questions are addressed:

- Is platelet gel effective in enhancement of bone formation in tissue engineered constructs?
- Is timing of sequentially released BMP-2 and VEGF of importance in bone formation in a large animal model?
- Are we able to design an effective implant based on β-TCP for spinal fusion in a clinically relevant instrumented posterolateral spinal fusion model in goats, in terms of fusion results compared to autologous bone, degradation and adverse reactions of the scaffold material?

OUTLINE OF THIS THESIS

For decades now, autologous bone has been the golden standard for several surgical indications in which bone grafts are needed, like spinal fusion. Several disadvantages like donor-site pain have been described, which press the need for alternatives without these disadvantages, but which perform equally well as autologous grafts. Great potential has been attributed to cell-based bone tissue engineering, because these hybrid constructs combine bioactive factors with bone forming cells, seeded on porous osteoconductive and -inductive scaffolds.

Chapter 1 provides an overview of the background of the bone tissue engineering field. It highlights the different components of a bone tissue engineered construct, like cells, soluble factors and scaffold material. An ideal graft should have at least the following characteristics: it should be biocompatible, biodegradable, have good mechanical properties, be osteoconductive, osteoinductive and preferably be off-the-shelf. Furthermore aims and research questions are highlighted.

It remains unclear what the contribution of cells is in hybrid constructs placed at ectopic (non bony surroundings) as well as orthotopic locations. In **Chapter 2** bioluminescence

imaging (BLI), a non-invasive *in vivo* imaging technique for detection of a marker gene, linked to specific promoters, is used to try and answer these fundamental questions. This technique allows non-invasive tracking of viable luciferase-transduced cells implanted in living small animals. In this study, the contribution of implanted MSCs on bone formation was assessed, by using this BLI technique at both an ectopic as well as a spinal fusion location in rats.

In **Chapter 3** the possibility to create an off-the-shelf product for bone tissue engineering *in vivo* is analyzed. This was done, by adding two different off-the-shelf components, allogeneic MSCs, and freshly isolated bone marrow, to scaffolds, and implanting those seeded scaffolds ectopically in goats. To notice possible adverse reactions, the animals were monitored closely and furthermore histological analysis focused on signs of immunological/ inflammatory tissue response. In **Chapter 4** the possibility to use the off-the-shelf allogeneic MSCs was analyzed in a more clinically relevant model in bone growth chambers in goats and this was compared to the ectopic location. This study compared allogeneic MSCs to autologous MSCs to see whether bone growth showed obvious differences especially considering immunological tissue response.

It is known that for the development of functional large bone tissue constructs optimal oxygen and nutrients supply of seeded MSCs is needed and this process is likely dependent on vascularization. Therefore in **Chapter 5,** we tested the hypothesis that addition of the highly proliferative endothelial progenitor cells (EPCs), through their reported stimulation of MSCs and their possible effect on vascularization, might increase total bone formation in ectopic constructs implanted in goats.

In three of the experimental chapters (**Chapters 3, 4 and 5**) platelet leukocyte gel was added to the various tissue engineered constructs. This platelet leukocyte gel has been described to contain several growth factors like VEGF, PDGF, TGFβ and FGF2, which are known to have a positive effect on both bone formation and vascularization.

Subsequent *in vivo* investigations are aimed at translation to clinical practice. Firstly, in **Chapter 6**, the importance of timing of sequentially released BMP-2 and VEGF for bone formation was analyzed by making use of fast and sustained release systems for these proteins. This study was performed in a clinically relevant, segmental (ulnar) defect in dogs and compared to implants at the ectopic location. Secondly, in **Chapter 7,** a new osteoconductive and -inductive β-TCP is used in a clinically relevant pilot study, an instrumented posterolateral spinal fusion model in goats. The aim was to assess whether the osteoconductive capacities of this material alone are sufficient to promote a bony fusion (determined by histology and CT scans) and whether the addition of allogeneic MSCs enhances the onset of bone formation and the total amount of newly formed bone.

Finally in **Chapter 8**, the work as described in this thesis is discussed and summarized.

2

LUCIFERASE LABELING FOR MSC TRACKING IN SPINAL FUSION VERSUS ECTOPIC BONE TISSUE ENGINEERING IN MICE AND RATS

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ABSTRACT

Tissue engineering of bone, by combining multipotent stromal cells (MSCs) with osteoconductive scaffolds, has not yet yielded any clinically useful applications so far. The fate and contribution of the seeded cells are not sufficiently clarified, especially at clinically relevant locations. Therefore, we investigated cell proliferation around the spine and at ectopic sites using noninvasive *in vivo* bioluminescence imaging (BLI) in relation to new bone formation. Goat MSCs were lentivirally transduced to express luciferase. After showing both correlation between MSC viability and BLI signal as well as survival and osteogenic capacity of these cells ectopically in mice, they were seeded on ceramic scaffolds and implanted in immunodeficient rats at two levels in the spine for spinal fusion as well as subcutaneously. Nontransduced MSCs were used as a control group. All rats were monitored at day 1 and after that weekly until termination at week 7. In mice a BLI signal was observed during the whole observation period indicating survival of the seeded MSCs, which was accompanied by osteogenic differentiation *in vivo*. However, these same MSCs showed a different response in the rat model, where the BLI signal was present until day 14, both in the spine and ectopically, indicating that MSCs were able to survive at least 2 weeks of implantation. Only when the signal was still present after the total implantation period ectopically, which only occurred in one rat, new bone was formed extensively and the implanted MSCs were responsible for this bone formation. Ectopically, neither a reduced proliferative group (irradiated), nor a group in which the cells were devitalized by liquid nitrogen and the produced extracellular matrix remained (matrix group) resulted in bone formation. This suggests that the release of soluble factors or the presence of an extracellular matrix is not enough to induce bone formation. For the spinal location, the question remains whether the implanted MSCs contribute to the bone regeneration or that the principal mechanism of MSC activity is through the release of soluble mediators.

INTRODUCTION

Autologous bone grafting procedures, with its obvious disadvantages like donor-site pain and nonunions, are performed regularly, for example, in posterior lateral spinal fusions to enhance and stimulate the local bone formation process. Traditional materials like ceramics, titanium, and polymers are used for new bone formation to circumvent the use of autologous bone grafts but generally lack biological signals necessary for the bone formation process. Tissue engineered constructs, for example, a hybrid construct consisting of a scaffold, stem, or progenitor cells or bioactive molecules, have been a major research topic in the field of regenerative medicine for decades now (148-150). A key success has been the identification of bioactive bone-stimulating growth factors like bone morphogenetic proteins-2 and -7. They have been used extensively over the last years and have proven their efficacy in clinical practice (73, 74). The addition of multipotent stromal cells (MSCs), which can be derived from various sources (9, 15), to porous scaffolds to create hybrid constructs have been described to contribute to bone formation, especially when seeded on biomaterials such as hydroxyapatite and biphasic calcium phosphate (BCP) at ectopic locations. This clear effect of cell-based bone tissue engineering ectopically (128, 148, 151) however, is not representative for similar constructs orthotopically (127, 128) when using similar materials.

Therefore, the function of MSCs used in cell-based bone tissue engineering at these two different locations (ectopic and orthotopic) needs to be revealed. Specific questions that should be clarified such as the following: (1) do these implanted MSCs proliferate and are they actually the cells that produce the newly formed bone? (2) To what extent are growth factors and other soluble factors, produced by the implanted MSCs, responsible for stimulating bone formation by host cells (a paracrine process)? (3) Can the presence of the extracellular matrix (ECM) itself produced by seeded MSCs explain bone formation by endogenous MSCs?

Bioluminescence imaging (BLI), a non-invasive *in vivo* imaging technique for detection of a marker gene, linked to specific promoters, is an appropriate technique to answer these fundamental questions. This technique allows tracking of viable luciferase-transduced cells implanted in living small animals at different time points. It is mainly used in analysis of tumor growth (152) and therapy efficacy (153) in *vivo,* but also for bone tissue engineering purposes (154-156). Several aspects of bone formation in small animals like proliferation, and differentiation of MSCs, can be monitored by using specific promoters, for example, a cytomegalovirus (CMV) promoter or human osteocalcin promoter to drive the expression of luciferase. Next to that, the luciferase present makes it possible to use immunohistochemistry to identify implanted cells after explantation in histological slides.

In this study we report the first use of BLI to clarify the contribution of implanted MSCs at both ectopic and spinal fusion locations in rats. Osteogenicity and survival of the implanted MSCs were proven in mice and the possibility to translate a successful mouse model to a larger rat model was evaluated. To investigate the possibility of paracrine effects or ECM effects by the seeded cells, irradiation, and liquid nitrogen treatment were used to treat MSC-seeded scaffolds.

MATERIALS AND METHODS

MSC isolation and culture

Bone marrow (BM) was aspirated from both iliac wings of one goat, as previously described (129). These MSCs have been used extensively in bone tissue engineering studies. The relative numbers of MSCs in this BM was assessed, by performing a colony-forming efficiency assay (CFU-F) (9). For this, two fractions of $1x10^5$ mononuclear cells (MNCs)/cm^2 were cultured in standard medium containing minimal essential medium (αMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mM L-glutamine (all Invitrogen), and 15% heat inactivated fetal bovine serum (FBS; Cambrex, Verviers, Belgium), which was refreshed every 3 or 4 days. After 9 days the colonies were washed, fixed in 4% formalin, stained using methylene blue, and counted under an inverted microscope. The remaining aspirate was plated, and the isolated MSCs were expanded and cryopreserved after passage one, in 1 ml aliquots of $1x10^7$ cells according to standardized protocols until viral transduction (157).

Lentiviral vector

The lentivector (LV) used in this study was a modified version of the LV pRRL-cPPT-CMV-IRES-GFP-PRE-SIN (kindly provided by Prof. Hoeben, Leiden University Medical Center, Leiden, The Netherlands (158)) in which a 1.7kb Luc2 fragment was inserted. The 1.7kb Luc2 fragment was isolated from pGL4.10(*luc2*) vector (Promega, Madison, WI, USA) using *XhoI* and *XbaI* restriction sites. Subsequently, this Luc2 fragment was cloned in the LV pRRL-cPPT-CMV-IRES-GFP-PRE-SIN using the same restriction sites, downstream of the CMV promoter, to generate pRRL-cPPT-CMV-Luc2-IRES-GFP-PRE-SIN (Figure 1). Expression of the luciferase gene driven by the CMV promoter was confirmed with *in vitro* assays (159, 160).

Transfection of virus-producing cells

For virus production, $6x10^6$ human embryonic kidney (HEK) 293T cells were plated on 15-cm-diameter culture dishes and cotransfected the following day with equal molar amounts of a packaging vector (psPAX2, Invitrogen), an envelope vector (pLP-VSVG), and the LV vector containing the luciferase gene (pRRL-cPPT-CMV-Luc2-IRES-GFP-PRE-SIN). A total amount of 35 µg of DNA per dish was dissolved in a total volume of 575 µL of distilled H_2O. Subsequently, the same amount of 0.5 M $CaCl_2$ was added. This DNA/$CaCl_2$ mixture was added slowly in a drop-wise method to 1150 µL of 2x HBS (5 mM Na_2HPO_4, 10 KCl, 280 mM $NaCl_2$, 12 mM D-Glucose, and 50 mM HEPES; pH 7.1) while making air bubbles in the 2x HBS buffer. This solution was incubated for 60 min at room temperature, and subsequently 20 ml medium (Dulbecco's modified Eagle's medium [DMEM], 10% FBS, P/S) was added and transferred to the cells. The transfection medium was replaced by MSC expansion medium 18 h posttransfection. The medium containing LV supernatant was collected at 48 and 72 h after transfection and filtered through a 0.45 µm pore filter. These supernatants were pooled afterwards. Transfection efficiency was determined by fluorescence-activated cell sorting (FACS), analyzing green fluorescent protein (GFP^+) HEK

Figure 1: Schematic outline of the lentiviral vector. Schematic outline of the lentiviral vector pRRL-cPPT-CMV-Luc2-IRES-GFP-PRE-SIN. The schematic overview shows the multiple elements in the self-inactivating (SIN) lentivirus. The enhancer region contained in the 3'LTR is deleted. Also indicated are the positions of the Rev-responsive element (RRE), the central polypurine tract (cPPT), and the posttranscriptional regulatory element (PRE). The promotor is derived from the human cytomegalovirus (CMV) and drives the expression of Luciferase2 (Luc2) and green fluorescent protein (GFP). Between these genes of interest, an internal ribosome entry site (IRES) is located that allows for translation initiation in the middle of the messenger RNA seqence. Scheme adapted with permission of Carlotti *et al.* (158).

293T cells. The titer of the virus supernatant was estimated by transducing 293T cells with different dilutions and the transduction efficiency was determined using FACS analysis of GFP. Titer was calculated using the following formula: (cell concentration at the day of transduction) x (virus supernatant dilution) x (%GFP positive cells)/total volume.

Transduction with LV vector

MSCs were thawed and plated in 175 cm^2 Falcon filter cap culture flasks (Becton Dickinson, Franklin Lakes, NJ, USA) at a density of 5000 MSCs/cm^2 and transduced at ~50% confluence. Transductions were carried out in the presence of 8 μg/ml hexadimethrine bromide (Polybrene; Sigma, Zwijndrecht, The Netherlands) and 1:3 diluted LV vectors. After 24 h, the transduction medium was replaced with fresh medium. At 90% confluence cells were harvested and GFP^+ cells were analyzed using FACS to determine transduction efficiency. After this, MSCs were cryopreserved according to standardized protocol until implantation.

Scaffolds and seeding conditions

One day before surgery, luciferase-transduced MSCs and untransduced MSCs were thawed on ice, thoroughly washed in culture medium containing 30% FBS. The described quantities of cells (Table 1) were seeded on BCP porous particles of diameter 2-3 mm sintered at 1150°C (BCP; Progentix, Bilthoven, The Netherlands (161)) and allowed to attach for 4 h, each implant consisting of three (ectopic) or six particles (spine). The ceramics consisted of 80±5% (w/v) hydroxyapatite and 20±5% (w/v) b-tricalciumphosphate, and total porosity was 70±5%, macroporosity was 55±5%, and microporosity was 20±5%. Scaffolds were cleaned in an ultrasonic bath and sterilized by autoclave.

After 4 h attaching to the scaffolds, the medium was added until surgery. Just before this surgery, 24 h after seeding, two conditions were prepared separately. In the first condition (reduced proliferative), constructs were gamma-irradiated with a dose of 30 Gy. In the second condition (matrix), constructs were dipped in liquid N_2 three times for 30 s, and then all scaffolds were implanted.

Animals

Immunodeficient RAG-2$^{-/-}$ γc$^{-/-}$ Balb/c mice were originally obtained from the Academic Medical Center (Amsterdam, The Netherlands). The mice were bred and housed in the specific pathogen-free breeding unit of the Central Animal Facility of the University of Utrecht. Four RAG-2$^{-/-}$ γc$^{-/-}$ Balb/c male mice, and 14 male nude Harlan Sprague Dawley rats (HsdHan: RNU-Foxn1-rnu, Harlan, Horst, The Netherlands), all ten weeks old, were used. The animals were maintained in filtertop cages and supplied with autoclaved sterilized food pellets and distilled water *ad libitum*. Experiments were conducted after permission of the local Ethics Committee for Animal Experimentation and in compliance with the institutional guidelines on the use of laboratory animals and according to current Dutch Law on Animal Experimentation.

Surgery and implantation

All procedures (mouse and rat) were performed under general anesthesia using an intramuscular injection of ketamine (35 mg/kg), xylazine (6 mg/kg), and atropine (0.025 mg/kg) (KXA) mixture. Pain relief was given by subcutaneous injections of buprenorphine (Temgesic; Schering-Plough, Utrecht, The Netherlands), 0.05 mg/kg body weight, and every 8 h until 72 h postoperatively.

After shaving and disinfecting the back of the mice, scaffolds were implanted in separate subcutaneous pockets of the mice to minimize interference of the bioluminescence signals. Each animal (n=4) received five different implants; the results of only one of them are described in this paper. Influence of the other implants, containing the same cells as the ones described in this paper, however, with different virus batches (produced from the same viral vectors) and at various transduction efficiencies, was not expected. Upon assessment that the BLI signal was positive and the MSCs implanted were shown to have osteogenic capacity, analyzed after explantation at 6 weeks in this mice study, cells from the same batch of transduced MSCs were used for the rat study.

After shaving and disinfecting the back of the rats (n=14), a posterior midline incision was made to expose the paraspinal muscles. Two separate paravertebral fascial incisions were made parallel to the spinous processes of L4-5 and L1-2, and the facet joints were exposed using sharp and blunt dissection. These facet joints and spinous processes were decorticated using a diamant file and a curette. Per level one construct (Table 1) was implanted bilaterally. Per two rat all four conditions, (1) no cells, (2) MSCs, (3) MSCs reduced proliferative, and (4) MSCs matrix, for the spinal fusion were implanted. The muscle fascia, subcutaneous tissue, and skin were subsequently closed in layers. For ectopic implantation in rats, seven subcutaneous pockets were created, four on the right side of the spine and three on the left. All groups (Table 1) were implanted according to a randomized block schedule, after which the pockets were closed.

Bioluminescence

Before starting the BLI, all animals were anesthetized in the same way as described above and shaved, and an intraperitoneal injection of D-luciferin (Synchem Chemie, Kassel,

TABLE 1: Seeding conditions and treatments in mice ectopically and rats ectopically and in the spine.

Condition	Animal	Amount of MSCs/ implant	Implant	Luciferase transduced	n
Spinal implants					
1. no cells	rat	no cells	BCP 6 particles	no	7
2. MSCs	rat	3.0 million	BCP 6 particles	yes	7
3. MSCs rp	rat	3.0 million	BCP 6 particles	yes	6
4. MSCs matrix	rat	3.0 million	BCP 6 particles	yes	6
Ectopic implants					
1. MSCs	mouse	1.0 million	BCP 3 particles	yes	4
2. no cells	rat	no cells	BCP 3 particles	no	13
3. MSCs control	rat	1.0 million	BCP 3 particles	no	13
4. MSCs high conc	rat	1.0 million	BCP 3 particles	yes	13
5. MSCs middle conc	rat	0.5 million	BCP 3 particles	yes	13
6. MSCs low conc	rat	0.1 million	BCP 3 particles	yes	13
7. MSCs rp	rat	1.0 million	BCP 3 particles	yes	13
8. MSCs matrix	rat	1.0 million	BCP 3 particles	yes	13

Rp= reduced proliferative, MSC= multipotent stromal cell, BCP=biphasic calcium phosphate, conc= concentration

Germany) in phosphate-buffered saline (PBS; 125 mg/kg body weight) was given at day 1, and then weekly. BLI images were acquired over a period of 10 minutes. Exposure conditions (time, aperture, stage position, binning, and time after injection) were kept identical in all measurements. For quantification, standard regions of interes were defined for the spine and the ectopic implants separately. Measurements were expressed in arbitrary units. The mice were euthanized after 6 weeks by cervical dislocation, and the rats after 7 weeks by an overdose of pentobarbital (Organon, Oss, The Netherlands) and all implants were retrieved.

Histological processing and identification of implanted MSCs

After retrieval the scaffolds were fixed in 4% glutaraldehyde for 8 h, decalcified in 12.5% w/v EDTA in demineralized water, dehydrated by ethanol series, and embedded in paraffin. Ten-micrometer sections were stained with hematoxylin and eosin (H&E) to analyze bone formation and vascularization.

Luciferase-transduced cells were identified by immunohistochemistry using a rabbit antiluciferase antibody (CR 2029 RAP; Cortex Biochem, San Leandro, CA, USA), 1:100 (0.5 μg/ml IgG) in PBS/5% bovine serum albumin (Roche, Woerden, The Netherlands). As an isotype control, rabbit IgG (0.5 μg/ml, DAKO, X0903, Glostrup, Denmark) was used. Sections were pretreated with 1.5% H_2O_2 and incubated with the antibodies overnight at 4°C. Finally, samples were incubated with goat-anti-rabbit horseradish peroxidase

(DAKO, P0448, Glostrup, Denmark) at 2.0 µg/ml in PBS/5%bovine serum albumin for 60 min at room temperature and detected using 3,3'-diaminobenzidine (DAB) staining. Counterstaining was performed with Mayer's hematoxylin.

RESULTS

Preparation of hybrid constructs

From the iliac crests of a female Dutch milk goat, 4.5 ml of bone marrow was aspirated, which contained $12x10^6$ MNCs/ml. The CFU-F of the aspirate was determined. We found an average of 2.8 colonies per 100,000 MNCs, meaning ~1 out of 35,000 cells had attached to the tissue culture plastic and formed a colony and was considered to be a MSCs. MSCs were expanded and cryopreserved for further experiments.

During preparation of the lentivirus, we found a 100% transfection efficiency of the HEK 293T cells (analyzed by FACS), and the subsequent viral titer of the culture supernatant was $1.5x10^6$ transducing units/ml. The multiplicity of infection that was used in this experiment was 1.5, resulting in a transduction efficiency of the MSCs of ~60% on the basis of GFP expression, analyzed by FACS (Figure 2). Correlation was found between cell viability of these cells and BLI signal during 6 weeks *in vitro* culture (data not shown). A batch of transduced cells was frozen in aliquots and used for all further experiments in mice and in rats. The day before surgery (of both mice and rats) MSCs were seeded on the BCP scaffolds and allowed to attach for 4 h after which supplementary medium was added. To dertermine the relation between cell number, BLI signal and bone formation, three different concentration groups were used. In addition, to analyze whether a paracrine effect of MSCs could be responsible for bone formation, seeded scaffolds were sublethaly irradiated with a dose of 30 Gy before implantation (reduced proliferative group), such that the proliferative capacity and osteogenic potential of MSCs was substantially decreased (162), while the capacity to produce growth factors was preserved (163, 164). To devitalize the constructs while keeping the ECM intact, seeded scaffolds were dipped in liquid N_2 three times for 30 s (matrix group), and all groups were implanted in the animals.

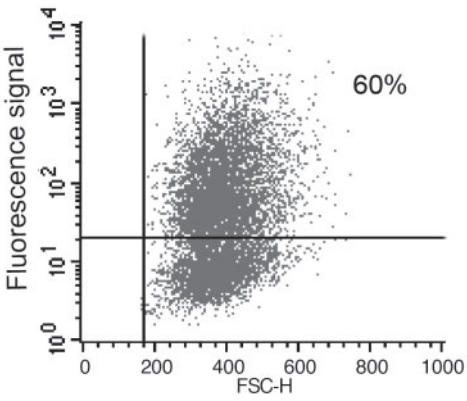


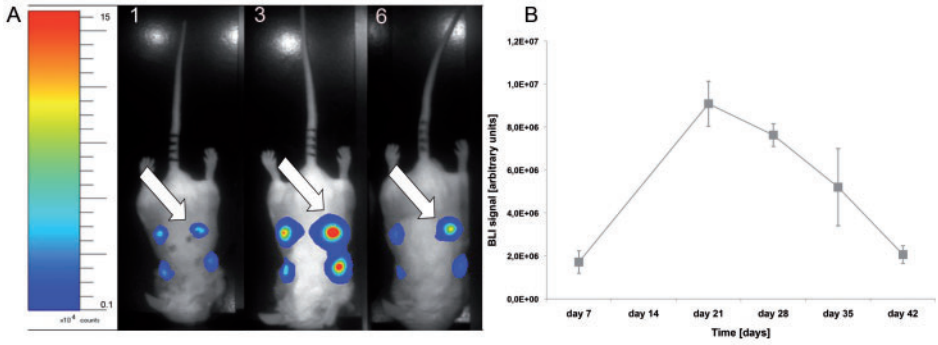
Figure 2: Analysis of GFP-luciferase transduced multipotent stromal cells. Expression of GFP, upper right quadrant: ±60% of the MSC population, analyzed by fluorescence-activated flow cytometry (FACS).

In vivo monitoring and ex vivo detection of luciferase-transduced MSCs in mice

All mice recovered well from the surgery, without any complications. From day 7 to 21, the BLI signal increased 5.3-fold. After day 21 the signal gradually decreased to a level 1.2-fold higher at day 42 than at day 7 (Figure 3A, B). After sectioning, H&E staining showed a border of fibrous tissue surrounding the scaffolds. Most pores of the scaffolds were filled with bone, connective tissue, fat cells, and blood vessels. All bone was lining the scaffold material (Figure 3C). No signs of degradation of the scaffold material and no immune response were observed by histology. Furthermore, the antiluciferase immunohistochemistry showed that gene-marked seeded cells were lining the borders of the newly formed bone and were also present in the bone matrix, appearing as osteoblasts and osteocytes, respectively (Figure 3D).

In vivo monitoring and ex vivo detection of luciferase-transduced MSCs in rats ectopically

During surgery, one rat died, and a second rat did not recover from the anesthesia during the BLI procedure at day 42, but the results are included in this study. In both cases, pathology did not reveal the cause of death. The remaining surgeries and BLI procedures were without complications and all animals recovered well. During the complete implantation period, no BLI signal was found in the MSC matrix and control group. The average BLI signal of the



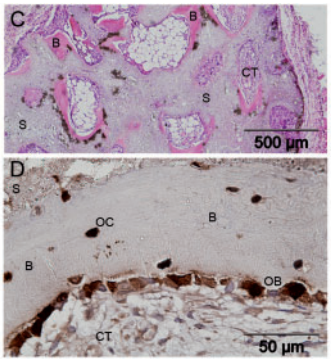


Figure 3: BLI signals, bone formation, and identification of seeded MSCs ectopically in mice. A: monitoring *in vivo* BLI signals at week 1, 3 and 6. Arrows indicate the implanted hybrid construct described here. **B:** BLI signals (Av±SEM) were calculated at five time points during the 42 days. **C:** histological section after 7 weeks (H&E stained). Newly formed bone (B) lining the scaffold (S) material. Connective tissue (CT) inside the scaffold pores, and fat cells (FC) are present within this connective tissue. **D:** Antiluciferase immunohistochemical staining: osteocytes (OC) are situated in the extracellular matrix of the newly formed bone (B); osteoblasts (OB) are lining this newly formed bone.

three MSC concentration groups and MSC-reduced proliferative group ectopically followed the same trend during their complete implantation period (Figure 4A, B). The signal first increased from day 1 to day 7. Only the $0.1x10^6$ group showed almost no decrease from day 7 to day 14; the other three showed a rapid decrease. When looking at the different concentrations, it was noticed that the signal of the $1.0x10^6$ group is clearly higher than the $0.5x10^6$ group, the lowest being the $0.1x10^6$ group. Analyzing the BLI signals of separate $1.0x10^6$ implants showed that rat 10 has a completely different signal pattern compared to the implants in the other rats. The moderate signal at day 7 increased to a peak at day 21, after which the signal reduced gradually (Figure 4C), a pattern very comparable to the BLI signals in mice (Figure 3B). H&E staining after explantation revealed that this rat 10 was the only one in which bone had formed extensively. This bone was lining the scaffold material, bone-lining cells were present, and osteocytes were found in this bone. Furthermore, the pores were filled with blood vessels and connective tissue (Figure 4D). The antiluciferase immunohistochemistry suggests that the newly formed bone was mainly formed by the implanted (luciferase-positive) MSCs, for osteocytes and osteoblasts stained positive

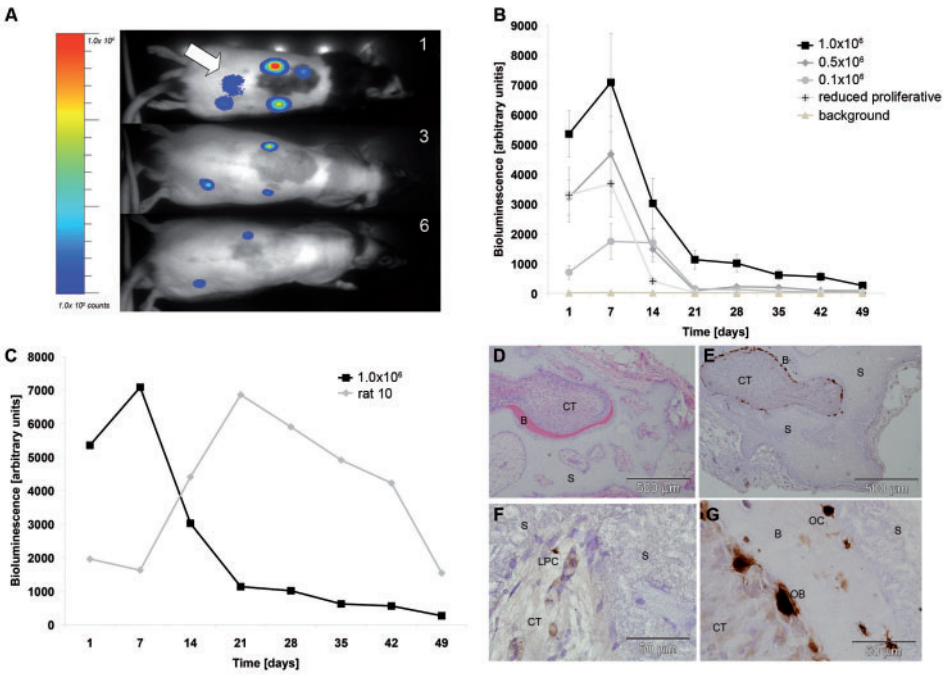


Figure 4: BLI signals, bone formation, and identification of seeded MSCs ectopically in rats. **A:** monitoring *in vivo* BLI signals at week 1, 3, and 6. The white arrow indicates the orthotopic implant. **B:** BLI signal (Av±SEM) of the luciferase-transduced groups implanted during 49 days. **C:** BLI signal of the average of the MSC $1.0x10^6$ groups vs. BLI signal of the MSC $1.0x10^6$ implant in rat 10. **D:** Upper left: H&E staining: bone (B) lining the scaffold (S), connective tissue (CT) inside the scaffold pores and a fibrous border around the scaffold. **E, G**: antiluciferase immunohistochemistry (rat 10): bone lining the scaffold, positive bone lining cells (osteoblasts, OB), and positive cells inside the bone matrix (osteocytes, OC), **F**: antiluciferase immunohistochemistry: luciferase positive cell (LPC) in connective tissue (group MSC $0.5x10^6$).

(Figure 4E, 4G). H&E staining of the remaining samples of the $1.0x10^6$ group showed that in 4/13 constructs bone was formed, though in a small amount. These small spots of bone did not contain luciferase-positive cells. However incidentally, some of the seeded cells remained present in the connective tissue formed within the pores of the groups: $0.5x10^6$ (4/13)(Figure 3F), $0.1x10^6$ (3/13), and MSC reduced proliferative (2/13). No bone formation was found in the other ectopic groups; these scaffolds were completely filled with connective tissue. In 9/13 constructs of the control MSC group (non-transduced) bone was formed. As expected, all of these control constructs were negative for luciferase, as assessed by immunohistochemistry.

In vivo monitoring and ex vivo detection of luciferase-transduced MSCs in the rat spine

All BLI signals in the spine are lower than the ectopic signals because the implants are placed ~1cm deep below the paraspinal muscles of the rat on the facet joint. Comparable to the ectopic implants, also in the spine no BLI signal was found during the complete implantation period in the MSC matrix and control groups. The BLI signals of the MSC group and the MSC-reduced proliferative group first showed an increase in signal from day 1 to day 7, when the signal of the MSC group was 2.6-fold higher than that of the reduced proliferative group (Figure 5A, 4B). From day 7 to 14 the signal of the MSC group

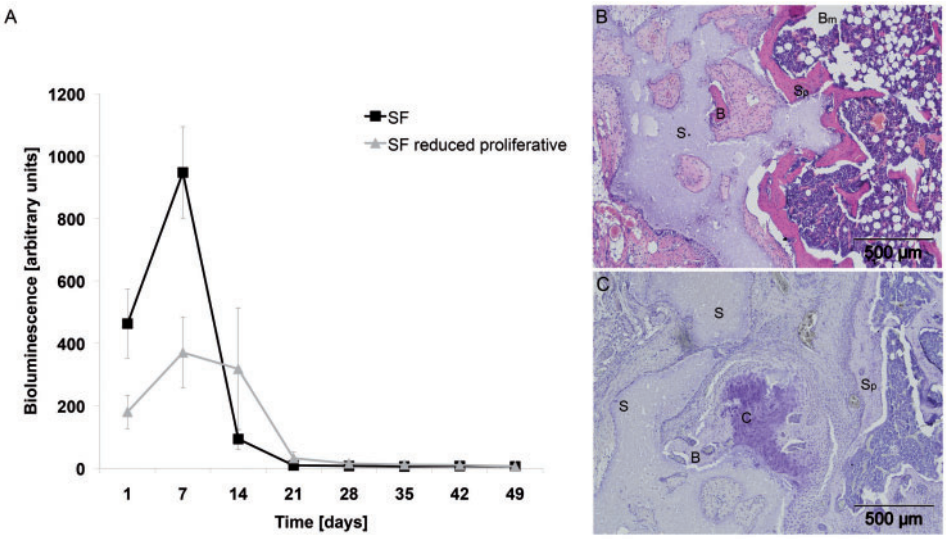


Figure 5: BLI signals, bone formation, and identification of seeded MSCs in spinal implants in rats. A: BLI signal (Av±SEM) of spinal fusion (SF) MSC- and SF-reduced proliferative groups implanted during 49 days. **B:** histological section at week 7 (H&E stained): scaffold (S) placed against the spinous process (Sp) with bone marrow (Bm) inside. A minimal amount of bone (B) is growing into this scaffold. **C:** antiluciferase immunohistochemistry: no positive cells were found in these stainings (counterstaining hematoxylin). Cartilage (C) is formed between the spinous process (Sp) on the right, and the scaffold (S).

showed a sharp decline, whereas the signal of the reduced proliferative group remained approximately constant during this period (being 3.4-fold higher than the MSC group) and decreased from day 14 onward. Histological sections (H&E stained) showed that most scaffolds aligned the spinous processes. Frequently, cartilage formation was seen between the spine and the scaffolds, without any signs of a bony fusion. Comparable to the ectopic implants almost no bone formation was observed, apart from some ingrowth from the decorticated spinous processes and facet joints (Figure 5B). When analyzing this bone formation in all implanted groups, no luciferase-positive MSCs were found (Figure 5C). This coincided with the BLI signal that was decreased to baseline level at day 21.

DISCUSSION

In this study we have used BLI to clarify the contribution of implanted MSCs to bone formation at both ectopic and posterior spinal fusion locations in rats. The implanted MSCs in mice showed a positive BLI signal during the complete implantation period and osteogenic differentiation *in vivo,* in accordance to several other mouse studies (156, 165). However, these same MSCs did not show comparable results in the rat model used for this study, with one exception. In the rats the BLI signal was present until day 14 both in the spine and ectopically, indicating that MSCs were able to survive several weeks of implantation, but apparently this was not sufficient to produce bone. The persistence of a BLI signal in one of the rats until the end of the experiment (day 49), with a concurrent substantial bone formation, suggests that long-term survival of the MSCs on the scaffold might be essential. Overall, we conclude that translation from a small to a larger animal model, in this case the rat model, has been proven to be very difficult, and it should be realized that a rat still is a relatively small animal model.

Therapeutic infusion and implantation of MSCs is being used in trials for the treatment of several human diseases (e.g., heart disease (166), graft-versus-host disease (28, 167), and osteogenesis imperfecta (168)) and in large animal models (169), which makes them ideal candidates for bone tissue engineering studies. In previous studies, we showed a significant contribution of MSC seeding on the amount of bone formed ectopically in a goat implantation model (127, 128). Orthotopically, MSCs advanced the onset of bone formation, shown by fluorochrome incorporation, but their implantation did not result in more bone compared to non-cell-seeded constructs after 16 weeks. As BLI is impossible in goats due to their size and depth of the orthotopic location (170), we decided to use a rat model in which the ectopic and the spinal implantation are both possible. For this study several hybrid constructs, consisting of luciferase positive (and negative as a control) MSCs seeded on BCP scaffolds, were created. In control experiments, we showed a positive relationship between MSC growth and the BLI level *in vitro* and thus activity of the luciferase gene product (data not shown), but we cannot exclude either direct or indirect regulation of the CMV promoter *in vivo*. Seeding increasing concentrations of MSCs resulted during the first week in an equivalent higher BLI signal, implicating that the BLI signal is proportional to the number of luciferase-positive cells present. In contrast to the increasing signal in the mice up to week 3, signals of all groups in rats decreased after 1 week. This could be due to

the phenomenon that MSCs start differentiating and thus stop proliferating earlier in this model and next to this cell death plays a role. It has been described before that a certain minimal cell load is necessary to result in bone formation (171). This early differentiation would result in minimal amounts of bone-forming cells. An alternative explanation is that luciferase-positive MSC survival in these rats is shorter compared to survival in mice, which is substantiated by the finding that after explantation only small areas of bone were observed, both ectopically and in the spine. This was highly unexpected, because the same MSCs survived for 6 weeks in mice and were proven to be osteogenic. Strikingly, in one rat, the BLI signal course was comparable to the signal observed in mice (Figure 4C) and only in this rat extensive bone formation was found. Moreover, in this rat the immunohistology of the explanted scaffolds revealed the presence of luciferase-positive cells (osteoblasts and osteocytes), suggesting that after initial proliferation *in vivo*, these MSCs substantially contributed to the osteogenic process. It remains unclear why cell survival was so poor in our rat study. In the non-transduced control group bone formation was also less compared to what is common in mice. This means the viral transduction may have played a role; however, it is not the sole cause of the suboptimal bone formation; moreover in mice there is no obvious difference in the amount of bone formation in constructs containing transduced or non-transduced MSCs (data not shown). This was the reason to omit a group containing empty-vector-transduced MSCs in the rat study, which would have made it possible to distinguish whether the luciferase construct or the viral transduction itself was responsible for the suboptimal bone formation. Further, the immune status of the rats might be of importance in this model. We used immune-deficient rats (which do have natural killer [NK] cell activity) in this study to prevent loss of the MSCs by an immune response; however, this does not imply that the conditions for goat MSCs to grow are optimal; longer pre-culturing might have been a solution (172). The RAG2γc KO mice that we used lack all T, B, and NK cell activity. Although we did not find any lymphoid cells surrounding the implants in the rats, NK cell activity may have played a role in the survival of the goat MSCs. For future experiments, it might be a possibility to use immunocompetent rats and autologous MSCs to investigate whether the immune system is the confounding factor.

By irradiating MSC-seeded scaffolds, we wanted to create hybrid implants of which the MSCs were still able to excrete paracrine factors, but with a reduced capacity to proliferate. This makes it possible to see whether MSC proliferation and differentiation itself is the main factor determining bone formation, or that the production of soluble factors like chemo-attractants or other growth factors (e.g. bone morphogenetic protein-2 or vascular endothelial growth facor) also is sufficient to develop a favorable environment for bone regeneration. From the results obtained in this study it is not possible to completely answer this question. From day 1 to day 7 the signal of the irradiated samples (reduced proliferative group) increased both ectopically and in the spine, as a result of proliferation of MSCs, approximately comparable to the $0.5x10^6$ groups, while twice as many cells were seeded. The observation that the signal did increase during this time span is probably due to the fact that after irradiation not all MSCs were in their G0 phase of the cell cycle yet, which we found earlier when performing CFU-F assays on irradiated MSCs, where ~0.002-0.004% of MSCs was still possible to form a new colony, while normally this is around 0.1-0.2%.

This phenomenon, that a fraction of the stromal cells with clonogenic potential survived and showed the capacity to regenerate was described before, and depended on irradiation dose (164). At day 21 these signals reduced to background and antiluciferase staining after explantation showed no newly formed bone or luciferase-positive cells. However, in the only rat where extensive bone formation was found in the $1.0x10^6$ group ectopically, no bone formation was found in the reduced proliferative group, which is in line with earlier work, stating that the initial proliferation and survival of seeded cells are prerequisites for bone formation (173). In the matrix group no BLI signal was seen during the 49 days of follow-up; this was not enough for endogenous cells to initiate bone formation. This was confirmed by the absence of luciferase-positive cells after explantation. From these results we can conclude that the presence of the MSC matrix alone is not enough to initiate bone formation by endogeneous MSCs.

CONCLUSION

We show that BLI of luciferase-marked MSCs can be applied to monitor survival and proliferation of MSCs in ectopic constructs in mice, and in ectopic and spinal fusion constructs in rats. Seeding cells at higher densities leads to increased BLI signals and persistence of the signal throughout the implantation period correlates with new bone formation. Immunohistology confirmed the contribution of the implanted MSCs to bone formation in this process. For the spinal location, the question whether the implanted MSCs contribute to the bone formation is unresolved and it cannot be excluded that the principal mechanism of MSC activity is through the release of soluble mediators.

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3

COMPARING VARIOUS OFF-THE-SHELF METHODS FOR BONE TISSUE ENGINEERING IN A LARGE-ANIMAL ECTOPIC IMPLANTATION MODEL: BONE MARROW, ALLOGENEIC BONE MARROW STROMAL CELLS, AND PLATELET GEL

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ABSTRACT

Construction of bone grafts for regenerative medicine would highly benefit from off-the-shelf components, such as allogeneic bone marrow stromal cells (BMSCs) and blood-derived growth factors from platelet concentrate. Although allogeneic BMSCs are considered immunosuppressive, their use in transplantation studies is still cautioned. In this study, we used off-the-shelf goat allogeneic BMSCs, per-operatively aspirated bone marrow (BM) and platelet gel (PLG). Ten goats received six different hybrid constructs consisting of biphasic calcium phosphate scaffolds seeded with PLG or plasma that were mixed with BM, allogeneic BMSCs or left without cells. All constructs were implanted in the paraspinal muscles for 9 weeks. Fluorochromes were administered at 2, 3, and 5 weeks to assess onset of bone formation. Analysis revealed that the scaffolds without cells yielded small amounts of bone. Allogeneic BMSCs had a positive effect on the amount and early onset of bone formation. Fresh BM did not enhance ectopic bone formation. The PLG, which contained increased levels of transforming growth factor beta than plasma, did not result in more bone either. Fluorochrome incorporation results indicate that the presence of seeded cells in the constructs accelerates bone formation. This study shows a potential role of allogeneic BMSCs in bone tissue engineering research.

INTRODUCTION

Bone grafting is critical for healing of large defects and spinal fusion (174). Autologous sources for bone grafts are mostly used for several reasons, including their osteoconductive (116, 175-177), osteogenic (175), and osteoinductive (49, 116, 175) properties and the lack of immunogenicity (178) or disease transmission when used. However, many disadvantages have also been reported on harvesting autologous bone grafts, including chronic donor-site pain (3), hematoma, limited availability, infection, and longer operation time (179). Moreover, when used in spinal fusion surgery, pseudoarthrosis rates with autograft have been reported to range as high as 5-44% (180).

Tissue engineering (TE) of bone by combining bone marrow stromal cells (BMSCs) with porous osteoconductive scaffolds (150, 181, 182) is considered a promising alternative to these autologous bone grafts, although this technique has some important drawbacks. When using autologous BMSCs, there is generally a delay of at least 2 weeks due to necessary culture expansion of the cells before re-implantation, and thus it always requires a two-step procedure. Furthermore, expansion in culture makes it logistically difficult to prepare the scaffold- cell construct ready at the same time as the surgery.

In recent literature, the use of allogeneic BMSCs has been described as alternative for autologous BMSCs. *In vitro* studies have shown that allogeneic human BMSCs are capable of directly suppressing T-cell immune responses (24, 25). Furthermore, it has been suggested previously that *ex vivo*-expanded allogeneic BMSCs are capable of bone formation and engrafting in other tissues without being rejected (26, 27). Even when injected intravenously they were well tolerated (183). Furthermore, allogeneic BMSCs have been used to treat severe, acute graft-versus-host disease (28). It has been suggested that prostaglandin E_2, transforming growth factor beta (TGF-β), and indoleamine 2,3-dioxygenase (IDO) secreted by the allogeneic BMSCs were responsible for the observed modulation of inflammation (25, 184, 185).

Another way to create a bone graft substitute is to use an osteoconductive ceramic material combined with osteoinductive factors like bone morphogenetic protein-2 (BMP-2), BMP-7, and other biologically active molecules. Several TGF-β/BMP family members were proven beneficial for bone formation and healing at ectopic (186) and orthotopic locations (73, 74, 187, 188). Apart from the beneficial effects of human recombinant BMP-2 and -7, they also have disadvantages, like short shelf life, the need for supraphysiologic concentrations, and high cost. Other growth factors known to be involved in the osteoinductive response, fracture- or wound-healing processes are platelet-derived growth factor (PDGF) (189) and basic fibroblast growth factor (bFGF) (190).

Platelets accumulate at the site of an extravasation and, when activated by thrombin, secrete alpha granules. These granules release growth factors involved in the initiation and promotion of wound healing and fracture healing. Among these are PDGF, TGF-β, bFGF, and epidermal growth factor (EGF). The addition of platelet concentrate to an autologous cancellous bone graft resulted in a faster bone maturation rate and ultimately in more bone (191). This effect was attributed to the release of a wide range of growth factors, including TGF-β and PDGF, which are known to stimulate vascular in growth and cell migration.

We hypothesize that the use of platelet gel (PLG), a mixture of platelet- and leukocyte-rich plasma and thrombin, could also lead to a higher bone formation in tissue engineered constructs at an ectopic location than the use of plasma.

To investigate the bone-forming capacity of off-the-shelf products, allogeneic BMSCs and freshly isolated bone marrow (BM) were used in an ectopic implantation model in goats. Second, we assessed the potential additional effect of PLG to these constructs in the same model. Therefore we created six hybrid constructs, four of which were cell-based (Table 1).

TABLE 1: Seeding conditions.

Condition	Seeded Cells	Resuspended in	Scaffold size & material
1	No cells	Plasma 150 µl	7x7x7mm BCP
2	No cells	PLG 150 µl	7x7x7mm BCP
3	Concentrated BM	Plasma 150 µl	7x7x7mm BCP
4	Concentrated BM	PLG 150 µl	7x7x7mm BCP
5	Allogeneic BMSCs 8x 10^6/cm^3 scaffold	Plasma 150 µl	7x7x7mm BCP
6	Allogeneic BMSCs 8x 10^6/cm^3 scaffold	PLG 150 µl	7x7x7mm BCP

PLG= platelet-leukocyte gel, BM=bone marrow, BMSCs=bone marrow stromal cells

MATERIALS AND METHODS

Animals

After approval of the local animal care committee, 10 adult female Dutch milk goats aged 24 to 27 months were used. Food and water were given *ad libitum*. The animals' general health and care conditions were recorded in a diary of well-being for each goat separately and monitored by the laboratory animal welfare officer.

BMSC culture and seeding conditions

On the day of surgery, cryopreserved, passage 2, allogeneic BMSCs were thawed, washed, and resuspended in standard culture medium containing 30% fetal calf serum (FCS) and taken to the operating room. Before cryopreserving, these allogeneic BMSCs had been cultured in standard culture medium containing 15% FCS (Cambrex, Landen, Belgium). The allogeneic BMSCs used were from an earlier experiment with female Dutch milk goats without a known direct family relationship to the goats in the present study.

During surgery, BM was aspirated (±10 ml per goat) from the right iliac wing. Two aliquots of 4 ml each were centrifuged for 10 min at 300 *g* to concentrate the BM cells for direct seeding. Subsequently, the suspensions of allogeneic BMSCs were centrifuged at the same speed, the medium was discarded, and the two pellets and the samples of concentrated BM and allogeneic BMSCs were re-suspended in 150 µL autologous plasma or platelet-leukocyte rich plasma (PRP, see below) and seeded on different scaffolds. Another two scaffolds were seeded with plasma and PRP only. After seeding, the PRP-containing

constructs were activated, by adding 90 mM of calcium chloride ($CaCl_2$) and recombinant bovine thrombin (50 U/ml; Jones Pharma Inc., St Louis, Mo), and the constructs were allowed to polymerize before implantation. This way six hybrid constructs, listed in Table 1, were prepared for implantation in the paraspinal muscles of the goat.

After the operation, fractions of $5x10^6$ mononuclear cells of the remaining BM were plated in two T25 cm^2 flasks in standard culture medium containing 15% FCS (Cambrex) (9) to assess the proportion of BMSCs in the aspirates in a colony-forming unit efficiency (CFU-E) assay. The medium was changed every 3 or 4 days. After 9 days, the medium was discarded, and the cells were fixed with 4% formalin. The colonies were stained with methylene blue and counted macroscopically. The remainder of the aspirate was plated and cultured according to a standardized protocol to retrieve, expand, and cryopreserve the BMSCs (192).

PLG and plasma preparation method

PRP preparation was performed with the Angel Whole Blood Processing SystemT (AWBPS; Sorin Group, Milan, Italy), a semi-automated tabletop centrifuge system using a flat disc, with a variable blood volume ranging from 60 to 180 ml, to sequester the blood in three components. The predonated whole blood volume of 100 ml was injected in the blood collection reservoir of the AWBPS. After a 19-min spin at 1200 *g*, platelet-poor plasma (PPP) was removed in a transfer bag, PRP was collected in a collection syringe, and the remaining erythrocyte concentrate was collected in a retransfusion bag. PLG was prepared by mixing the PRP, with 90 mM $CaCl_2$ and bovine thrombin (50 U/ml; Jones Pharma Inc). The PPP and erythrocyte concentrate were discarded. Autologous plasma was prepared by centrifuging 10 ml of peripheral blood at 1200 *g* in a Falcon tube (Greiner) for 10min. Aliquots were withheld to analyze the concentration of TGFβ.

TGF- β1 determination

TGFβ-1 concentrations in the circulating whole blood (i.e., plasma) and PLG supernatant were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis MN) that had been validated for measuring TGFβ-1 (Quantikine TFG-β), according to the manufacturer's instructions. The ELISA procedures were programmed in an automated analyzer (Coda Automated EIAT analyzer; Bio-Rad Laboratories, Hercules CA). Samples were measured in duplicate and were prepared as required in appropriate dilutions for fitting the respective calibration curves. Repeat analysis was performed when differences between duplicates were larger than 10%. Furthermore several other growth factors were tested using human-specific ELISA's, but none of the tested methods showed cross-reactivity with goat factors like PDGF and IGF.

Scaffolds

Cubes of 7x7x7 mm of a biphasic calcium phosphate (BCP)(161) were used. The ceramic consists of 80±5 % (w/v) hydroxyapatite (HA) and 20±5 % (w/v) β-tricalciumphosphate (βTCP), total porosity was 70 ± 5%, macroporosity 55 ± 5%, and microporosity 15 ± 5% (Progentix, Bilthoven, The Netherlands).

Surgery and implantation

The surgical procedures were performed under general inhalation anesthesia of an isoflurane gas mixture (Ebbott, Oudewater, The Netherlands), preceded by intravenous detomidine (Pfizer, Capelle an den Yssel, The Netherlands) sedation. After anesthetics were induced, 100 ml of whole blood was aspirated from the external jugular vein for PLG and plasma preparation, and 10 ml was used to centrifuge for 10 min at 4°C to prepare plasma for later use. After shaving and disinfection of the dorsal thoracolumbar area, a midline skin incision from T10-L5, which extended along the right iliac wing, was made to expose the paraspinal muscles and the iliac wing. This incision also allowed the implantation of transverse process implants that are not discussed in the present manuscript. The fascia was opened to create three intramuscular pockets on each side using blunt dissection, with approximately 2 cm between the implants. All constructs were placed in the pockets according to a randomized block schedule. The fascia, subcutis, and skin were closed separately. Post-operative pain relief was given using Buprenorphine (Shering-Plough, Maarssenbroeksedijk, The Netherlands).

At 2 (calcein green; 10 mg/kg intravenously, Sigma, Zwijndrecht, The Netherlands), 3 (oxytetracyclin; 32 mg/kg intramuscularly, Engemycin, Mycofarm, The Netherlands), and 5 weeks (xylenol orange; 80 mg/kg intravenously, Sigma, The Netherlands), fluorochrome markers were administered to allow assessment of the onset and dynamics of bone growth. At 9 weeks, the animals were killed using an overdose of pentobarbital (Organon, Oss, The Netherlands), and the implants retrieved.

Post-mortem sample acquisition, histology and histomorphometry

After explantation, the samples were fixed in 4% glutaraldehyde for 8 h and dehydrated using ethanol series. Next, all samples were embedded in polymethylmethacrylate MMA). Two centrally located 10-µm-thick sections were made using a sawing microtome (Leica, Nusslochh, Germany). The first was left unstained for epifluorescence microscopy, and the second was stained with methylene blue and basic fuchsin for routine histology and histomorphometric analyses. Tissue response and bone formation were observed using a light microscope (Olympus-BX50). Fluorochrome markers were analyzed using a light/fluorescence microscope (E600, Nikon, Amstelveen, The Netherlands) equipped with a quadruple filter block (XF57, dichroic mirror 400, 485, 558 and 640 nm, Omega Optics, Brattleboro, VT). Only the fluorochromes incorporated in the newly formed bone were scored, by comparing the fluorescent signal with the visible light illumination. High-resolution digital scans made of the stained sections for histomorphometry using a photographic film scanner. Bone and scaffold were pseudocoloured yellow and green using Adobe Photoshop 7.0 (Adobe Systems Incorporated, San José, CA).

A custom macro was used to measure the area of interest, the area of the 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 the contact% ((bone-to-scaffold contact length/scaffold outline) x100%). The contact% was previously shown to be more sensitive than the bone area% (193), because new bone forms exclusively by apposition on the scaffold

surface. Quantification of clusters of lymphocytes was performed quantitatively by counting all the clusters present, either in the pores or in the tissue surrounding the scaffold, in one midsection per scaffold. We classified them into three groups: 1= no lymphoid clusters, 2= ≤ two lymphoid clusters/scaffold, 3= >two lymphoid clusters/scaffold**.** An immunologist and a pathologist evaluated the lymphoid clusters histologically.

Statistics

For histomorphometry contact% (percentage of bone apposition on the available scaffold surface) was analyzed using a univariate analysis of variance according to a randomized block design. Interactions between the groups were analyzed, and then we tested whether PLG had an effect on bone formation. Next we tested whether there was difference between the construct after adding the two cell suspensions (allogeneic BMSCs or BM) and the constructs without cells. Bonferroni corrected *post hoc* tests were performed to analyze differences between these groups. For the TGF-β data, a two-sided Students *t*-tes*t* was performed. The intraclass correlation coefficient (ICC) was used to assess a possible correlation between bone contact% and the CFU-E. A Pearson Correlation test was performed to assess correlation between bone contact% and lymphoid clusters.

RESULTS

We operated 10 mature 2-year-old Dutch milk goats. During surgery, six different conditions (see Table 1) for ectopic implantation were created. First BM was aspirated from the iliac wing and yielded $11.5\pm2.84\times10^6$ nucleated cells/ml. This BM was concentrated 12 and used directly. From the remaining BM, the fraction of plastic adherent BMSCs was determined using CFU-E. We found 1.6±0.9 colonies per 100.000 mononuclear cells, meaning approximately one of 50.000 mononuclear cells in the aspirate had attached and formed a colony and thus was considered a BMSC. Aliquots of cryopreserved allogeneic BMSCs, derived from an earlier study performed in goats, were thawed and prepared for direct seeding. Peripheral blood was aspirated from the external jugular vein to produce PLG and plasma. To verify the success of the PLG preparation, ELISA analysis of TGFβ-1 was performed. The TGFβ-1 concentration of the PLG produced was significantly ($p<0.001$) greater than in circulating blood levels (108.8 ±31.1 and 16.2 ± 4.1 pg/ml, respectively). This confirmed an increase of this growth factor of approximately 7 times the baseline level.

BCP scaffolds of 7x7x7 mm were used for seeding of the six different conditions. Before use, the biphasic nature of the material was confirmed by x-ray diffraction and Fourier transform infrared spectroscopy; the material did not contain any impurities (161). All constructs created were randomly implanted in the paraspinal muscles of the goat. At 2, 3 and 5 weeks, fluorochrome markers were administered to allow assessment of the onset and dynamics of bone growth.

Results of implants

No surgical complications occurred, and all implanted samples were retrieved after nine weeks.

Bone formation. Analysis of the explanted samples by basic fuchsin and methylene blue staining of (MMA)-embedded sections revealed that bone was present in various amounts in all groups (Figure 1). Statistical analysis of the histomorphometry data showed no interactions between the various groups. Irrespective of the application of PLG, bone was found in 100% (20/20) of the allogeneic BMSCs-seeded scaffolds, in 85% (17/20) of the BM-seeded scaffolds, and in 30% (3/10) of the plasma non-cell-seeded scaffolds. Bone was seen in 40% (4/10) of the constructs containing PLG without the addition of cells. The seeding of allogeneic BMSCs resulted in a significantly greater bone formation independent of further PLG addition than in the BM-seeded constructs and the non-cell-seeded constructs (group 5 plasma/allogeneic BMSCs (8.1%) and 6 PLG/allogeneic BMSCs (4.8%) together, compared with group 3 plasma/BM (3.6%) and group 4 PLG/BM (1.5%) together; p=0.03 and with group 1 plasma/no cells (0.2%) and group 2 PLG/no cells (0.6%) together; p=0.001). Freshly isolated BM, however, did not result in greater ectopic bone formation than in the non-cell-seeded constructs using PLG or plasma (groups 3 and 4 compared with groups 1 and 2; p=0.553). Addition of PLG to the scaffold did not result in bone formation significantly different from plasma. This was the case in all treatment groups (groups 1,3, and 5 together vs. groups 2,4, and 6 together; p=0.21). No correlation (ICC=0.15) was found between bone contact% in the BM groups and the amount of seeded CFU-Fs present.

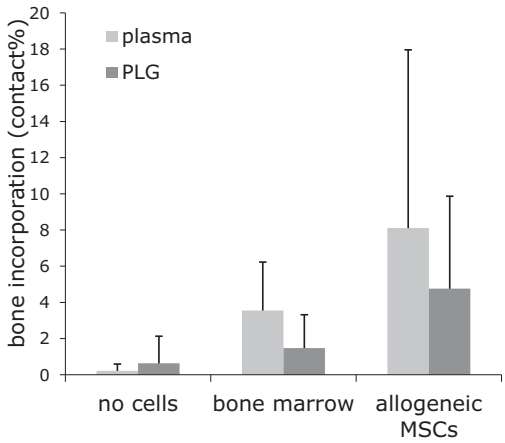

FIGURE 1: Bone formation in ectopic scaffolds: bone contact% after 9 weeks ectopic implantation in the goat. The results are represented as mean ± standard deviation (n=10). Allogeneic BMSCs have a significantly greater positive influence on bone formation than the bone marrow and non-cell-seeded constructs (p=0.03 and 0.001). PLG (groups 2,4,6) does not show a significant effect on bone formation compared to plasma seeding in groups 1,3 and 5 (p=0.205). PLG= platelet-leukocyte gel, BM=bone marrow, BMSCs=bone marrow stromal cells.

Onset of bone formation. Fluorescence microscopy showed the fluorochrome incorporation in various amounts in all six conditions. All fluorescence microscopy results are shown in Table 2. None of the constructs showed the two-week (calcein green) label, which suggests that no bone mineralization had started before 2 weeks. The 3- (tetracycline) and 5- week (xylenol orange) labels were found in the allogeneic BMSC seeded constructs and the BM-seeded constructs irrespective of the addition of plasma or PLG. The 5-week label was found in only one no-cell-seeded scaffold, indicating that, in most cases bone, formation had started in

TABLE 2: Onset of bone formation: the frequency of fluorescent labels built in the ectopic scaffolds.

Condition	Calcein green 2 weeks label	Tetracyclin 3 weeks label	Xylenol orange 5 weeks label
1) no cells/ plasma	0/10	0/10	1/10
2) no cells/ PLG	0/10	0/10	0/10
3) BMSCs/ plasma	0/10	8/10	8/10
4) BMSCs/ PLG	0/10	5/10	7/10
5) BM/ plasma	0/10	5/10	8/10
6) BM/ PLG	0/10	2/10	5/10

Calcein Green (2-week label) was not visible; Tetracyclin (3-week label); Xylenol Orange (5-week label). PLG= platelet-leukocyte gel, BM=bone marrow, aBMSCs=allogeneic bone marrow stromal cells.

these groups after 5 weeks. The fluorochrome label order indicated that bone formation had started from the scaffold surface and was directed towards the pore centre.

Immunological aspects. Histology of the implants, as indicated in Figure 2, showed a border of fibrous tissue surrounding the scaffolds. Most pores of the scaffolds were filled with blood vessels and loose connective tissue. None of the scaffolds showed signs of degradation of the ceramic material. All hybrid constructs showed foreign body multinucleated giant cells lining the border of the scaffold material, where bone was not present (Figure 3 E, F). At sites of new bone formation, osteoblasts were lining the surface of the bone, and osteocytes were lying in lacunas in the newly formed bone (Figure 3 A, B). In 50% of hybrid constructs containing the allogeneic BMSCs, clusters of lymphoid cells (Figure 3 C, D) were present, which was only seen in 2.5% of the constructs not containing allogeneic BMSCs. They did not contain neutrophils or promyelocytes, indicating no acute inflammation was present at the time of explantation. Unfortunately, we were not able to further define these clusters of lymphoid cells because basic fuchsin and methylene blue staining is not able to discriminate between various cell types, and MMA embedding does not allow immunohistochemistry for difference in T- and B-cells. After counting the lymphoid clusters, we found a correlation between the number of lymphoid clusters per scaffold and the bone contact% (CC=0.568) in the allogeneic BMSC containing constructs (Figure 3).

DISCUSSION

In the current study performed in goats, we investigated off-the-shelf constructs for bone tissue engineering. The first aim was to investigate the bone-forming capacities of allogeneic BMSCs and freshly isolated BM. Second; we assessed the potential additional effect of PLG on these constructs. Therefore, we created six hybrid constructs, four of which were cell-based.

Previous cell-based bone tissue engineering studies performed in our group showed no major differences in the amount of bone formation between expanded autologous and expanded allogeneic BMSCs (128). Based on these promising results, we investigated

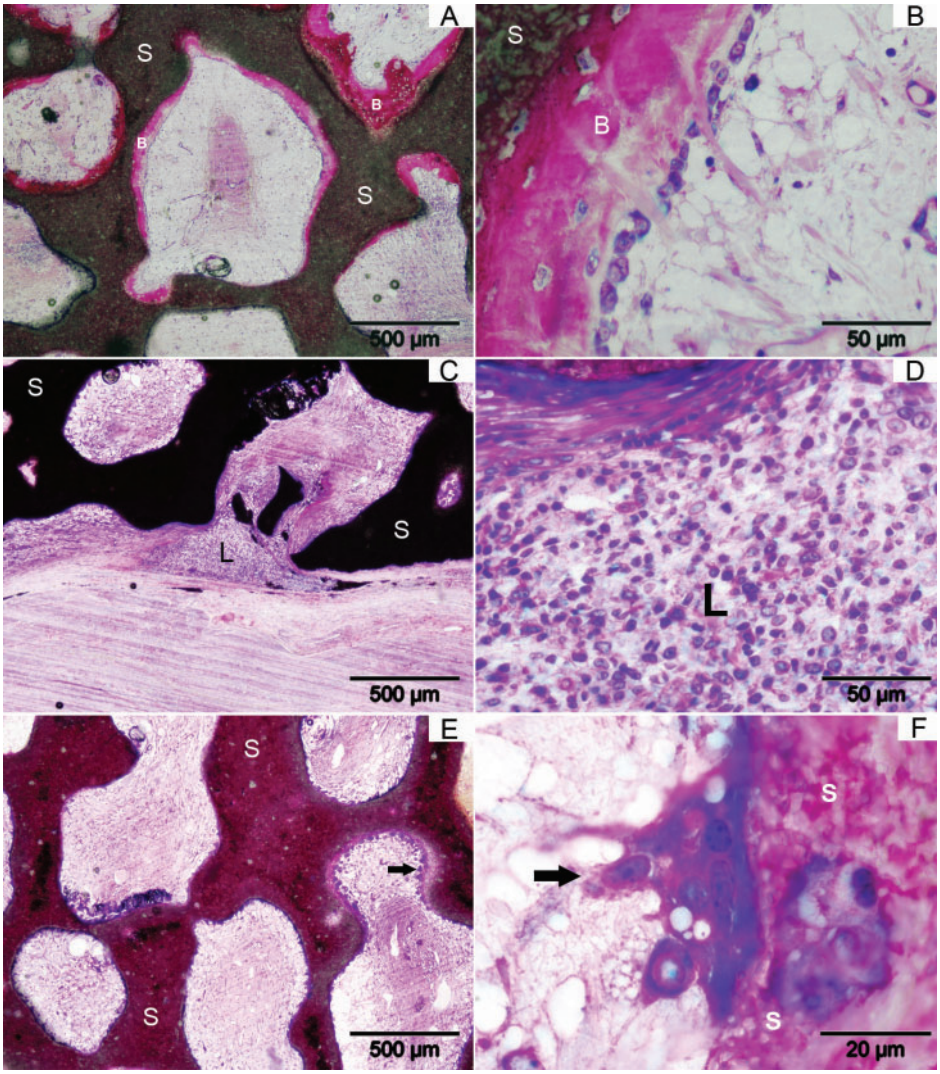

FIGURE 2: Histology. A) bone lining the scaffold material, B) enlargement of A; osteoblasts lining the bone and osteocytes in lacunae, C) lymphoid cluster, D) enlargement of lymphoid cells, E) multinucleated giant cells lying against the border of the scaffold, F) enlargement of a multinucleated giant cell. The structures and cells depicted in A, B, E and F are visible in all groups. Lymphoid clusters as shown in C and D are visible only in scaffolds seeded with allogeneic BMSCs. B= bone; S= scaffold; L= lymphoid cluster.

the feasibility of other off-the-shelf constructs. In our study, we found that the off-the-shelf allogeneic BMSC constructs had a clear positive effect on bone formation compared with the non-cell-seeded constructs and the BM-seeded constructs. CFU-E results can partially explain the results in the BM groups. There were only 58±30 BMSCs on constructs seeded with BM, compared with $2.75x10^6$ on the scaffolds seeded with allogeneic BMSCs.

The difference between 58 and 2.75x10^6 cells is not reflected in the amount of bone that was formed. It is likely that not all expanded allogeneic BMSCs have the potential to differentiate towards the osteoblastic lineage, and most of the allogeneic BMSCs will not survive the implantation process because of the harsh conditions of wound healing; furthermore, apart from cells, the fresh BM may contain various unknown osteoinductive factors not present in expanded allogeneic BMSCs.

The PLG, which contained high amounts of growth factor, turned out to be ineffective as an osteogenic stimulus in the current study. The published clinical and experimental data regarding bone-healing potential of PLG are however controversial. A few authors have reported a positive influence of PLG on bone regeneration in humans undergoing orthopedic (194, 195) surgery and only an early effect (after 3 months) in maxillofacial surgery (196). On the other hand, our results are in agreement with a number of clinical and experimental studies demonstrating no additional effect of PLG on bone healing (197-199). Also, *in vitro* experiments are ambiguous with respect to a dose-dependent mitogenic and chemotactic effect of PLG on BMSCs (107) and osteoblast-like cells (108), whereas osteogenic differentiation was inhibited (109). These opposite actions of PLG might explain our *in vivo* results. We seeded expanded allogeneic BMSCs, which need to receive an osteoinductive signal for differentiation, amongs others delivered by the ceramic scaffold used. In our study, we added PLG to these allogeneic BMSCs seeded on BCP, thereby possibly inhibiting their differentiation toward the osteoblast lineage. However, these results were obtained in an ectopic implantation model. In an orthotopic cassette model (200) performed by our group in the same goats, where PLG was added to BCP without the addition of allogeneic BMSCs, PLG did have a positive effect on bone formation (201). Furthermore, in a study by Nash *et al.,* PDGF delivered in a collagen gel had a positive effect on the treatment of tibial osteotomies is rabbits (202). At the orthotopic location, factors present in PLG are able to recruit bone-forming cells from the underlying bone by chemotaxis (107). Another explanation for the absence of a positive effect of PLG ectopically, when compared with the plasma-impregnated groups, might be that the PDGF, which is present in the PLG, induces fibroblast migration, proliferation, and synthesis of new connective tissue (189). When connective tissue has formed in pores and around the scaffold before bone has grown, it can be more difficult for bone to be formed at these sites. We hypothesize that a net effect on bone formation in this ectopic cell-based bone tissue-engineering model is the sum of bone cell recruitment and the negative effect on differentation that probably occurred. When we compare results from this study to the ectopic and heterotopic bone formation when using other growth factors like BMP-2 and -7, it is clear that the additive effect of these BMPs in both human (203) and animal (186, 204) studies has been proven to be beneficial as opposed to the PLG used here. However, the human study was performed in one patient only and thus a case study with little evidence. A comprehensive review by Cancedda *et al.,* discusses in detail strategies for reconstructive surgery of large bone defects (169).

The present study furthermore supports the opinion that tissue engineered BMSCs can be used in an allogeneic setting (27, 184) in goats, because there was not a complete adverse reaction to the seeded allogeneic BMSCs by the host, for we did not see any signs of acute

inflammation, like swelling, redness of the implantation site, or fever. Histologically, we found a positive correlation between the amount of lymphoid clusters and the bonecontact%. This effect of immunoreactions on the amount of bone formation has been described before. In an animal study, using rats, a mid-diaphyseal osteotomy and fracture of the femoral bone was created and then nailed. Induction of an inflammatory response mimicked by the addition of lipopolysaccharides (LPS) from gram-negative bacteria systemically or locally resulted in increased callus production (205), although this callus was hypertrophic and immature. Furthermore, the strength of the callus was far from optimal in the group where LPS were injected systemically. However, when LPS were injected locally, greater callus production appeared to compensate for the immaturity, resulting in stronger bone. These results show that the presence of a contained inflammatory response may be beneficial for the amount of newly formed bone, but the quality of this bone remains doubtful.

CONCLUSION

These results concerning allogeneic BMSCs provide us two major advantages. First, it creates the opportunity to use allogeneic BMSCs for *in vivo* bone tissue engineering research in this animal model, in an off-the-shelf way. This is less time-consuming than the two-step procedure when using autologous BMSCs. Second, it suggests the possibility for future application of allogeneic cells in a clinical setting, although before this step can be taken, several issues have to be addressed. After using allogeneic BMSCs in an ectopic implantation site, implantation in an orthotopic location, followed by a challenging critical-size defect in goats should be carried out. Furthermore, additional studies to assess the alloreactivity of these cells and the immunological consequences in the bone tissue receiving them have to be performed. Finally, the addition of PLG was unable to enhance bone formation at an ectopic implantation site.

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4

ORTHOTOPIC LOCATION HAS LIMITED BENEFIT FROM ALLOGENEIC OR AUTOLOGOUS MULTIPOTENT STROMAL CELLS SEEDED ON CERAMIC SCAFFOLDS

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ABSTRACT

Improvement of tissue engineered grafts is still a challenge in the field of regenerative medicine. By using multipotent stromal cells (MSCs), which have immunosuppressive qualities in an allogeneic situation, off-the-shelf implants can be created. This study compared allogeneic and autologous MSCs at an orthotopic (L1 transverse process model) and ectopic (intramuscular) implantation location in 2-year-old goats. Further, the possible additional effect of platelet-leukocyte gel (PLG) as a source of growth factors on bone formation was investigated. For the orthotopic implantation, cassettes were implanted in nine goats bilaterally on the lumbar transverse processes, either with PLG-seeded or with plasma-seeded constructs. To assess the onset of bone formation, fluorochromes were administered at weeks 3, 5, and 9. Their incorporation in newly formed bone indicated that seeded cells enhanced bone formation in the first weeks. Nevertheless, after 16 weeks no beneficial effects of cells were found in the cassettes in contrast to the ectopic location. No effect of PLG on bone formation was shown at either location. Finally, we show no significant difference in bone formation between autologous and allogeneic MSCs, an important finding when considering the use of allogeneic cells as an off-the-shelf component in tissue engineered bone in goats.

INTRODUCTION

Posterior lateral spinal fusion is a commonly performed and successful procedure for orthopedic and neurological indications. To create a bony bridge between two transverse processes, autologous bone grafts are frequently used; however, still several disadvantages like donor-site pain and nonunion persist (3). To find alternatives for autologous grafts, a substantial amount of research has focused on material development, applied in animal and human studies. A promising strategy may come from tissue engineered (TE) bone. A hybrid construct is created, consisting of bone marrow-derived multipotent stromal cells (MSCs), seeded on porous osteoconductive and osteoinductive scaffolds (150, 181, 182). This procedure usually requires two interventions: bone marrow aspiration and the actual implantation operation; however, it would be preferable to create this hybrid construct in a one step procedure. A possibility would be to use the mononuclear fraction from lipo-aspirate or concentrated bone marrow. Although promising, the extra intervention of liposuction and bone marrow aspiration results in a longer operation time, and not many studies have addressed this strategy (129). Therefore, an opportunity would be to use allogeneic MSCs. In the past years, it has become increasingly clear that allogeneic MSCs do not elicit a strong immune response *in vitro* (24, 25) and *in vivo* (26, 27), and are therefore interesting candidates for bone TE. Bone marrow can be aspirated from patients undergoing, for example, total hip arthroplasty and be the source from which MSCs are isolated, expanded, and stored for later use. In an earlier study, we found significantly more bone formation in a biphasic calcium phosphate (BCP) scaffold when seeding allogeneic MSCs compared to empty or concentrated bone marrow seeded constructs, implanted at an ectopic implantation location (129).

The site of implantation has great consequences for the anticipated bone formation. At orthotopic locations, osteoblasts may be recruited from the underlying bone, while this is not possible at the ectopic implantation location. To further stimulate recruitment of cells from underlying bone, platelet leukocyte gel (PLG) may be added to different hybrid constructs. PLG has been shown to have a dose-dependent mitogenic and chemotactic effect on MSCs and osteoblast-like cells (107, 108) and an inhibiting effect on differentiation (109) *in vitro.* Furthermore, PLG, which contains platelet-derived growth factor, fibroblast growth factor, and transforming growth factor β (TGF-β), has been described to improve bone formation *in vivo* (191), also when added to different materials tested in the cassette model (201). However, it is unknown how PLG effects bone formation in an orthotopic setting in the presence of MSCs.

An adapted form of the earlier described transverse process model will be used to analyze the effect of allogeneic and autologous MSCs, and PLG (200). This model is highly suitable to evaluate bone incorporation in multiple conditions simultaneously, and besides this, it has a strong resemblance with the initial bone formation processes relevant to posterior spinal fusion, because the newly formed bone is originating and progressing from the decorticated transverse processes. Therefore, we used this model in goats to investigate whether allogeneic and autologous MSCs are efficient in bone formation at an orthotopic location, and compared the results to an ectopic location. In both of these models, non-cell-seeded constructs served as controls. Second, we tested the additional effect of PLG on these constructs.

MATERIALS AND METHODS

Animals

After approval of the local animal care committee, 9 adult female Dutch milk goats aged 24-27 months, 60-70 kg, were used. Food and water were given *ad libitum*. The animals' general health and care conditions were recorded in a diary of well-being for each goat separately and monitored by the laboratory animal welfare officer.

MSC culture and seeding conditions

Bone marrow (2x10 ml) was aspirated from both iliac wings using a procedure needle (Cadence Science, New Hyde Park, NY) 4 weeks before the actual implantation surgery. The amount of MSCs in bone marrow was assessed with performing a colony-forming assay (9). For this, fractions of $1x10^5$ mononuclear cells/cm^2 were cultured in standard culture medium containing 15% fetal bovine serum (FBS; Cambrex, Landen, Belgium), which was refreshed every 3 or 4 days. After 9-11 days, colonies were washed, fixed in 4% formalin, stained using methylene blue, and counted under an inverted microscope. The majority of the aspirate was plated and cultured to isolate and expand the MSCs, and next cryopreserve them after the second passage in 1 ml aliquots of $1x10^7$ cells according to standardized protocols (192). Pluripotency of the cell cultures was regularly tested by incubation in osteogenic, adipogenic, or chondrogenic media, using the following protocols (data not shown, see also (18, 156, 206).

Osteogenic differentiation: Osteogenic differentiation was induced using standard expansion medium, mentioned above, supplemented with 0.1 mM ascorbic acid (Sigma, Zwijndrecht, The Netherlands), 10 mM β-glycerophosphate (Sigma), and 10 mM dexamethasone (Sigma). Cells were plated at a density of 5000 cells/cm^2. Medium was changed twice a week. Osteogenicity was analyzed by performing an alkaline phosphatase staining after 7 and 14 days.

Chondrogenic medium for pellet culture: About $0.5x10^6$ MSCs were centrifuged at 300 *g* to form a pelleted micromass. These pellets were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA), 2% FBS, 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen), 0.1 mM ascorbic acid, 2% insulin-transferrin-selenium supplement (Invitrogen), and 10 ng/ml TGF-β2 (R&D Systems, Minneapolis, MN). Medium was changed twice a week. Chondrogenic capacity was analyzed by Safranin-O staining.

Adipogenic differentiation: For adipogenic differentiation, we used medium containing DMEM, 10% FCS, 100 U/ml penicillin and 100 U/ml streptomycin, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 0.2 mM indomethacin (Sigma), and 10 μM insulin (Sigma). Cells were plated at a density of 4000 cells/cm^2, and medium was refreshed twice a week. Adipogenic capacity was analyzed by an Oil-red-O staining.

At the day of surgery, the MSCs (which were either used as autologous or as allogeneic MSCs) were thawed on ice, thoroughly washed in standard culture medium containing 30% FBS, and aliquoted to $8x10^6$ MSCs/cm^3 scaffold for preoperative seeding, before being taken to the operating room. The allogeneic MSCs were obtained from an earlier experiment with female Dutch milk goats (aged 24-27 months) without a known direct family relationship. In

total, six conditions were created for ectopic and transverse process implantation (Table 1). During surgery, the cell suspensions were centrifuged at 300 *g*, medium was decanted, and autologous and allogeneic MSCs were resuspended in either 150 µl PLG (for preparation see PLG and plasma preparation section) or plasma for the ectopic constructs and 100 µl PLG or plasma for the orthotopic cassette samples. The suspension (=$8x10^6$ MSCs/cm^3) was drop-seeded on the scaffolds ($2.75x10^6$ MSCs/ ectopic scaffold and $2x10^6$ MSCs/cassette scaffold), and the PLG-containing constructs were subsequently activated by addition of 90 mM calcium chloride and recombinant bovine thrombin (50 U/ml Jones Pharma Inc, St Louis, MO). After this the constructs were allowed to polymerize at room temperature for approximately 15 min before implantation.

TABLE 1: Seeding conditions

Condition	Seeded cells	Resuspended in	Scaffold size & material	N	Material used
1.	No cells	Plasma	7x7x7 & 12x7x3 mm	9	BCP
2.	No cells	PLG	7x7x7 & 12x7x3 mm	9	BCP
3.	Autologous BMSCs	Plasma	7x7x7 & 12x7x3 mm	9	BCP
4.	Autologous BMSCs	PLG	7x7x7 & 12x7x3 mm	9	BCP
5.	Allogeneic BMSCs	Plasma	7x7x7 & 12x7x3 mm	9	BCP
6.	Allogeneic BMSCs	PLG	7x7x7 & 12x7x3 mm	9	BCP

PLG: platelet-leukocyte gel, MSCs: multipotent stromal cells
All 6 conditions are implanted in the paraspinal muscles (ectopic) and in orthotopic transverse process cassettes.

Cassettes

Polyacetal cassettes, designed for fixation to the dorsal side of the transverse processes of the goat lumbar spine, were described previously (200). Each cassette consisted of two sidewalls, two end pieces, four stainless steel screws for assembly, and two self-tapping screws to attach the cassette to the decorticated transverse process (Figure 1). All components and screws were sterilized by autoclave. Four BCP scaffolds (one will not be described in this paper) separated by a 0.5 mm-thick Teflon® sheets (Impreglon B.V. Beuningen, The Netherlands) fitted tightly within each cassette. The scaffolds, when placed in the mounted cassettes, were in contact to the underlying bone and overlying soft tissues.

Scaffolds

BCP scaffolds (161) (Progentix, Bilthoven, The Netherlands) were used. The ceramic consists of 80±5% (w/v) hydroxyapatite and 20±5% (w/v) β-tricalciumphosphate, and total porosity was 70±5%, macroporosity 55±5%, and microporosity 20±5%. Scaffolds, measuring 12x7x3 mm, for cassette implantation and 7x7x7 mm for ectopic implantation were cut, cleaned in an ultrasonic bath, and sterilized by autoclave.

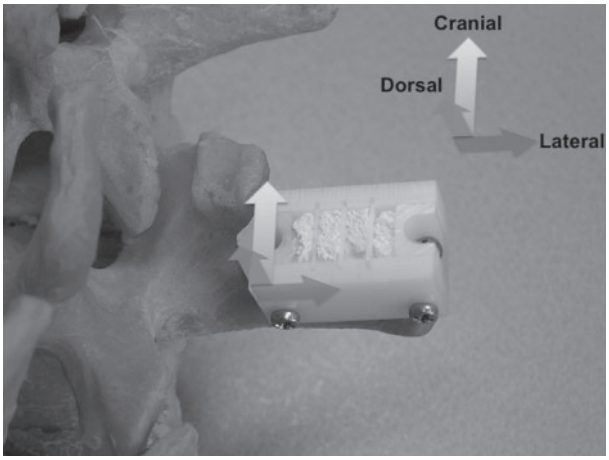

FIGURE 1: Cassette placed on the dorsal side of the transverse process of the goat.

PLG and plasma preparation method

PLG preparation was performed using the Angel Whole Blood Processing SystemT (AWBPS; Sorin Group, Milan, Italy), a semi-automated tabletop centrifuge system using a flat disc, with a variable blood volume ranging from 60 to 180 ml, to sequester the blood in three components. Two 60 ml syringes were prefilled with dextrose A solution. Fifty-three milliliters of whole blood was slowly withdrawn via the infusion line from the external jugular vein of the goats and injected in the blood collection reservoir of the AWBPS. After a 19-min spin at 1200 *g*, platelet-poor plasma was removed in a transfer bag, the platelet-leukocyte plasma fraction was collected in a syringe, and the remaining erythrocyte concentrate was collected in a retransfusion bag. PLG was prepared by mixing the platelet-leukocyte plasma fraction with 90 mM calcium chloride and bovine thrombin (50 U/ml; Jones Pharma Inc.). The platelet-pore plasma and erythrocyte concentrate were discarded.

Autologous plasma was prepared by centrifuging 10 ml of peripheral blood at 1200 *g* in a Falcon tube (Greiner, Frickenhausen, Germany) for 10 min.

Surgery and implantation

The procedures were 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 the paraspinal muscles. Separate bilateral incisions were made through the paraspinal muscles to expose both transverse processes of the L1 vertebrae, and bilateral incisions were made to expose the transverse processes of the L2-5 vertebrae. The L2-5 vertebrae were used for a double-instrumented posterolateral spinal fusion of L2-3 and L4-5, not described here. Morbidity of the L2-5 levels was not observed, and all animals received the same treatments, reason why influence of L2-5 implants on L1 is not expected, but cannot

be fully excluded. The processes of L1 were decorticated with an angled bone rasp until flat, bleeding surfaces were obtained. Two spinal cassettes for each animal were aseptically assembled. Cassettes were screwed on the processes. Slight finger pressure was applied to the top of the blocks of the cassette to ensure direct contact of all constructs with the underlying bone. Shortly before the PLG cassette was filled, 0.5 ml of PLG was put on the bottom; subsequently it was filled with the PLG scaffolds, separated by tightly fitting Teflon sheets. An additional 3 ml of PLG was applied into the wound after skin closure via a 14-gauge Tuohy epidural needle to ensure that the PLG was in a contained environment. At the contralateral side, the same procedure was carried out with plasma-seeded scaffolds. For ectopic implantation, intramuscular pockets were created through separate fascia incisions, and the six constructs were then placed in these pockets, three on each side of the spine according to a randomized block schedule, after which they were closed by nonresorbable sutures. The muscle fascia, subcutaneous tissues, and skin were subsequently closed in layers. Postoperative pain relief was given with Durogesic 25 patches (fentanyl transdermal patches, Janssen-Celag, Beers, Belgium).

To monitor the onset and dynamics of bone growth, the goats received fluorochrome labels at week 3 (Calcein Green, 10 mg/kg, i.v.; Sigma), week 5 (Oxytetracyclin, 32 mg/kg, i.m. Mycofarm, de Bilt, The Netherlands), and week 9 (Xylenol Orange, 80 mg/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 (MMA). Two 10-µm-thick longitudinal sections were cut through the center of each cassette using a sawing microtome (Leica, Nussloch, Germany). The first section remained unstained for epifluorescence microscopy, and the second section was stained with methylene blue and basic fuchsin. An additional slide was made from the constructs seeded with allogeneic MSCs for hematoxylin staining, to be able to better analyze the lymphoid clusters. Tissue response and bone formation were observed using a light microscope (Olympus-BX50, Olympus, Zoeterwoude, The Netherlands). High-resolution digital photos were made from the stained sections for histomorphometric analysis. Bone and scaffold were pseudocoloured yellow and green using Adobe Photoshop CS3. Histomorphometry was performed using KS400 software (version 3, Zeiss, Nussloch, Germany). A custom macro was used to measure the area 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 the percentage bone in available space, called bone area%=(bone area/[total area -scaffold area]) x100% and the percentage bone apposition, contact%=(bone-to-scaffold contact length/scaffold outline) x100%. The contact% was previously shown to be more sensitive, as new bone exclusively forms by apposition to the scaffold surface (193, 207). To investigate bone distribution, the measurements were performed separately for the upper (ectopic), middle, and lower (orthotopic) thirds of the cassette scaffolds, comprising 4 mm each.

Fluorochrome markers were analyzed using a light/fluorescence microscope (E600; Nikon, Amstelveen, The Netherlands) equipped with a quadruple filter block (XF57, dichroic mirror 400, 485, 558 and 640 nm, Omega Optics, Brattleboro, VT, USA). The presence or absence of each of the three fluorochrome labels was evaluated for all implants. For the slides of the cassettes, a translucent overlay displaying an upper, middle and lower third was created. This overlay was superimposed on the slide after which a frequency plot for presence/absence of the labels was made. Only the fluorochromes incorporated in the newly formed bone were scored, by comparing the fluorescent signal with the visible light illumination, for better discrimination between the fluorochromes incorporated in the newly formed bone versus the scaffold material. Quantification of lymphoid clusters was performed by counting all the clusters present, either in the pores or in the tissue surrounding the scaffold, in one midsection per scaffold. We classified them into three groups: 1, no lymphoid clusters; 2, ≤ two lymphoid clusters/scaffold; 3, >two lymphoid clusters/scaffold. An immunologist and a pathologist analyzed the lymphoid clusters histologically.

Statistics

Histomorphometric data were analyzed by SPSS version 13.0 software (Chicago, Illinois). Interactions between the groups were analyzed. The effects of platelet gel, the condition (autologous MSCs, allogeneic MSCs, or non-cell-seeded) and the level (upper, middle, or lower) were analyzed using repeated measurements. *Post hoc* tests were performed using a Bonferroni correction. A Pearson Correlation test was performed to assess correlation between bone contact% and lymphoid clusters. Significance was assumed when $p<0.05$.

RESULTS

Bone marrow (BM) was aspirated from nine mature 2-year-old Dutch milk goats, and yielded 15.0 $x10^6$±7.5 $x10^6$ (mean±SD) nucleated cells/ml. Colony-forming efficiency of the aspirates was determined. We found 1.5±0.6 colonies per 100.000 mononuclear cells, meaning approximately 1 out of 50.000 cells in the aspirate had attached and formed a colony, and thus was considered a MSC. The following treatments were investigated for ectopic (paraspinal muscles) and cassette implantation (transverse process) in the goats: autologous MSCs, allogeneic MSCs, and non-cell-seeded groups, either seeded with PLG or plasma. All goats recovered well within 2 days of surgery. All implanted samples were retrieved successfully after sixteen weeks of implantation.

Results of ectopic implants

Immunological aspects and histology. Analysis of the explanted samples by basic fuchsin/methylene blue staining of MMA-embedded sections revealed that a border of fibrous tissue surrounded the scaffolds. Most pores of the scaffolds were filled with blood vessels (Figure 2A), connective tissue, and bone (Figure 2B). The constructs showed no obvious signs of degradation. Foreign body multinucleated giant cells were often lining the border of the scaffold material, where newly formed bone was not present (Figure 2C). At sites of bone formation, osteoblasts were lining the surface of the bone, and osteocytes were lying in

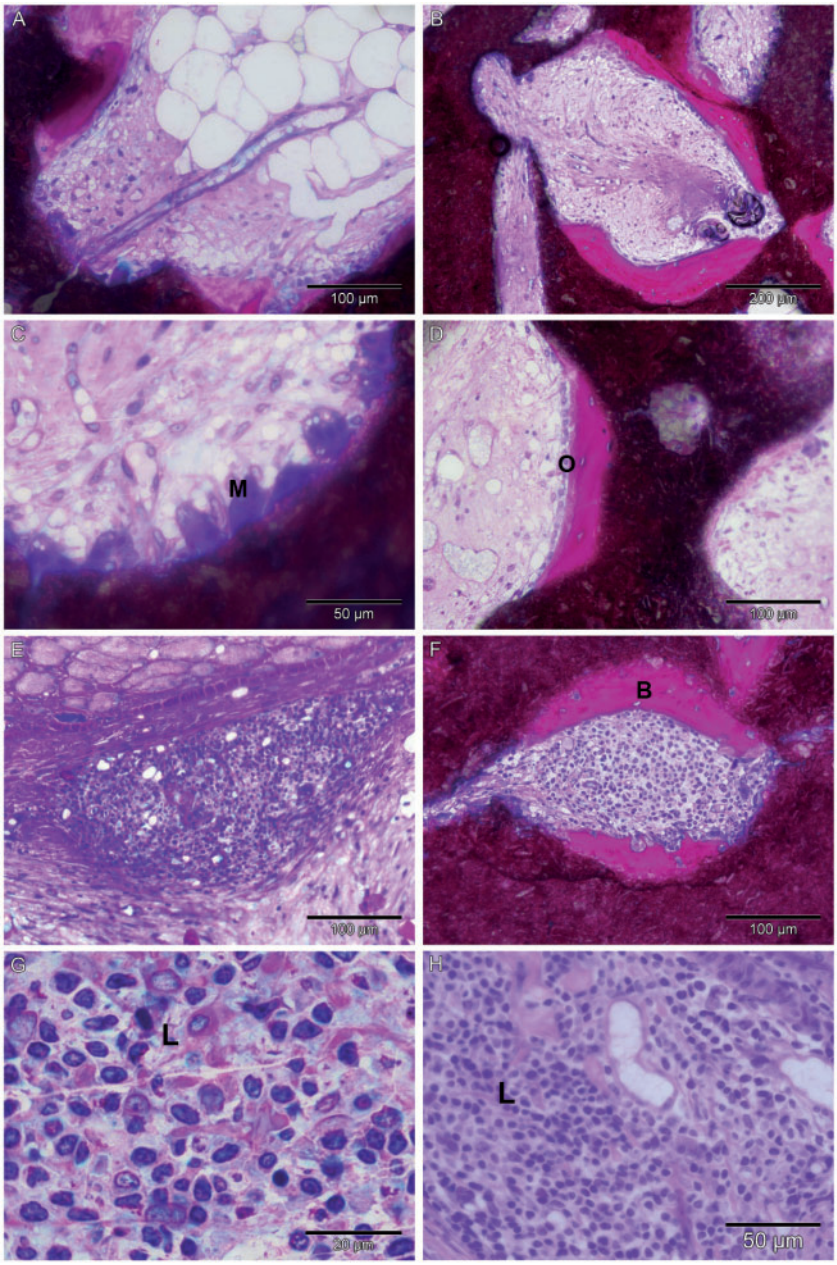

FIGURE 2: Histology shown in all implanted constructs. A) vessel formation, B) bone formation C) multinucleated giant cells against the border of the scaffold, D) osteoblasts lining the newly formed bone, osteocytes in lacunae. E) to G): histology only seen in constructs seeded with allogeneic MSCs: E) lymphoid cluster present outside the scaffold, F) lymphoid cells present within the pores of the scaffold, G) enlargement of lymphoid cells, H) idem. Panel A) to G): Methylene blue-Basic fuchsin staining. Panel H): Hematoxylin staining. B: bone; L: lymphoid cluster; M: multinucleated giant cell; O: osteoblast; S: scaffold.

lacunas in the newly formed bone (Figure 2D). Similar to previous work, clusters of lymphoid cells were present in the allogeneic MSC-seeded constructs both outside (Figure 2E) and in (Figure 2F) the scaffold (129). The cell clusters did not contain neutrophils or promyelocytes (Figure 2G&H), which can be interpreted as absence of an acute inflammation at the time of explantation. The lymphoid clusters were not present in the scaffolds seeded with autologous MSCs and control scaffolds. Unfortunately we were not able to further define these clusters of lymphoid cells for T- and B-cells, due to lack of antibodies for the goat.

Onset of bone formation. Analysis of the fluorochrome labels of the ectopic control samples showed that bone formation never started before week 3, and only in 4 out of 18 controls had started before week 5 (Figure 3A). This in contrast to the MSC-seeded constructs, where bone formation started in 34 out of 36 constructs before week 3 (Figure 3B). The contribution of seeded cells was still obvious when looking at the 9-week label, present in 11 out of 18 control constructs and in all MSC-seeded constructs. Table 2 shows an overview of all fluorescence incorporation results in the ectopic constructs. Further, it was apparent that bone formation had started at the outer rim of the scaffolds, as the green label was only visible at the periphery.

Bone formation analysis. Analysis of the explanted samples by basic fuchsin/ methylene blue staining of MMA-embedded sections revealed that bone was present in various amounts in all groups (Table 3). Control scaffolds without cells showed bone formation in 8/9 plasma controls and in 7/9 PLG control scaffolds. Bone formation in these controls was variable, with a contact% of 5.3±6.6% (mean±SD) for the plasma control and 5.7±5.4% for the PLG control. Histomorphometry showed the effect of MSCs, with a bone incorporation (contact%) of 18.3±10.2% (autologous MSCs) and 19.0±5.9% (allogeneic MSCs) for the plasma seeded constructs. Similar results were found for the PLG seeded constructs. This positive effect on bone formation of autologous and allogeneic MSCs was significantly different compared to the non-cell-seeded controls (both $p<0.01$), while no significant difference was found between the amount of newly formed bone in constructs containing autologous or allogeneic MSCs ($p=0.55$). Addition of PLG on the scaffolds did not significantly influence bone formation when compared to plasma (groups 1, 3, and 5 together vs. groups 2, 4, and 6; $p=0.13$).

Results of the orthotopic implantats

Immunological aspects and histology of cassettes. Upon explantation, we found that all cassettes were still firmly attached to the underlying bone. In all treatments bone had formed in approximately similar amounts, and the appearance of the bone was similar in all constructs. While we described the presence of lymphoid clusters when seeding ectopic constructs with allogeneic MSCs (Figure 2 and (129)), in this orthotopic setting the lymphoid clusters were not visible. This means that based on our histology results, no obvious differences were found between the groups containing allogeneic or autologous MSCs.

Onset of bone formation. Analysis of the bone growth dynamics in the cassettes by fluorochrome labels in the control construct showed the early label calcein green in the lower third of the implant contacting the transverse process and never in the upper third of

TABLE 2: Onset of bone formation in ectopic constructs.

Condition	Calcein Green week 3 label	Tetracyclin week 5 label	Xylenol Orange week 9 label	Histology week 16
1. Control plasma	0	1	5	8
2. Control PLG	0	3	6	7
total % FI/ Histology control	**0%**	**22%**	**61%**	**83%**
3. Autologous MSCs plasma	8	9	9	9
4. Autologous MSCs PLG	9	9	9	9
5. Allogeneic MSCs plasma	9	9	9	9
6. Allogeneic MSCs PLG	8	9	9	9
total % FI/ Histology MSC seeded	**94%**	**100%**	**100%**	**100%**

The frequency (in nr. of positive scorings out of 9) of fluorescent labels built in at week 3, 5 and 9 and bone apposition at week 16. PLG: platelet-leukocyte gel, MSCs: multipotent stromal cells, FI: fluorochrome incorporation.

the scaffold (Figure 4). This suggests that initial bone formation at the site of bone contact was a result of osteoconduction. In the MSC-seeded constructs, however, the calcein green label was also visible in the submuscular part of the scaffold in 10 out of 36 constructs, which indicates early MSC-related osteogenesis. This difference still seems to be present at weeks 5 and 9; however it was not possible to statistically analyze these data. There was no

TABLE 3: Bone formation in ectopic scaffolds and cassettes.

Plasma	control		autologous MSCs		allogeneic MSCs	
	Ectopic	Cassette	Ectopic	Cassette	Ectopic	Cassette
Total scaffold	5.3±6.6	35.8±13.8	18.3±10.2*	40.5±13.9	19.0±5.9*	36.9±11.2
Upper 3rd		5.4±4.2		8.1±6.0		6.2±4.0
Middle 3rd		7.9±3.4		6.4±3.46		7.2±1.5
Lower 3rd		22.5±7.3		26.0±8.3		24.1±8.3
PLG	**control**		**autologous MSCs**		**allogeneic MSCs**	
	Ectopic	Cassette	Ectopic	Cassette	Ectopic	Cassette
Total scaffold	5.7±5.4	36.7±23.3	20.9±9.2*	45.3±17.9	18.5±7.3*	44.3±22.3
Upper 3rd		6.8±5.7		7.7±4.6		7.1±4.8
Middle 3rd		6.2±4.5		8.9±3.0		7.6±5.0
Lower 3rd		23.7±13.8		28.8±12.7		29.6±13.8

Bone apposition in the different constructs at week 16, calculated from the percentage scaffold surface occupied by bone (contact%, mean±SD). Apposition of bone in the total scaffold and in the lower, middle, and upper third part of the cassette. Asterisk indicates a significant effect compared to the control.

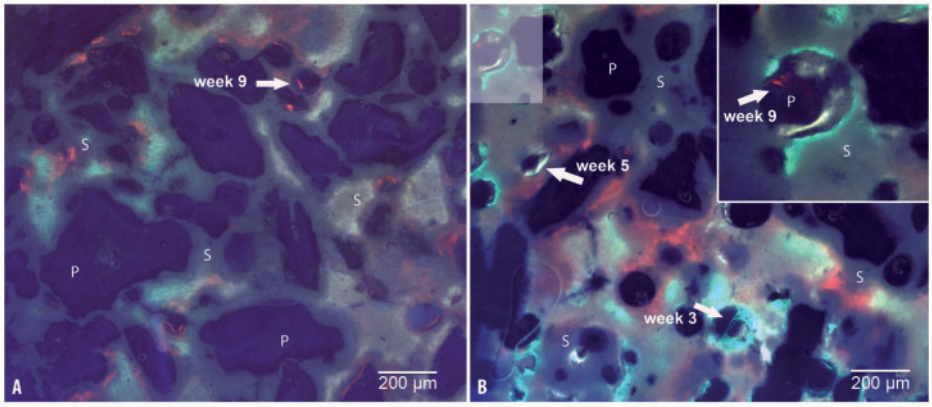

FIGURE 3: Fluorescence microscopy of the ectopic implants. Fluorochromes injected at weeks 3 (Calcein Green, green), 5 (Tetracyclin, yellow) and 9 (Xylenol Orange, orange). A) Non-cell-seeded construct, showing only the Xylenol Orange label in the newly formed bone, indicating bone formation started between week 5 and 9, B) MSC-seeded construct. All labels are present, indicating bone formation started before week 3. P: pore, S: scaffold.

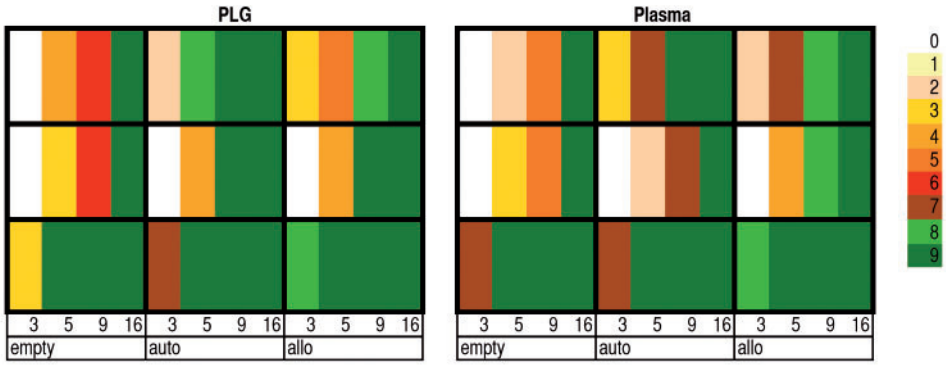

FIGURE 4: Frequency plot of fluorescence and histology. Fluorochrome incorporation at weeks 3, 5 and 9 and bone (histology) after 16 weeks of implantation. Vertical axis: lower, middle and upper third of the cassettes. The color-coding indicates the frequency a given fluorochrome (for fluorescence microscopy) or bone (for histology) was scored positive in each construct (out of 9 goats).

obvious difference in bone growth dynamics when looking at PLG-seeded versus plasma-seeded constructs.

Bone formation in the cassettes. All scaffolds remained intact. In some scaffolds we found minor signs of degradation of the BCP, mostly in the upper part of the material, where muscles lie dorsal to the cassette. After histomorphometric analysis, it appeared that in all implants similar amounts of bone were deposited on the scaffold (Table 3). We analyzed the upper (ectopic), middle, and lower (orthotopic) third separately, as we found a significant difference in the effect of cell-based bone tissue engineering was observed between these

two locations, in previous work (207). Statistical analysis of the histomorphometry data showed no interactions between the various groups. A significantly higher amount of bone was incorporated in the lower third of the cassette compared to the middle and upper third ($p<0.01$) in each of the constructs. In contrast to the ectopic MSC-seeded implants, there was no significant influence of the seeded cells on bone incorporation (contact%, $p=0.325$) after 16 weeks in the entire cassette, or when measuring lower, middle, and upper parts of the cassettes separately. Analogous to the ectopic constructs, addition of PLG did not enhance bone formation.

DISCUSSION

The primary aim of this study was to assess the bone forming capacity and immunological response of constructs carrying allogeneic MSCs in a cassette model in goat. These were compared side-by-side to autologous MSCs as well as to ectopically placed constructs containing the same components. The second aim was to assess the potential additional effect of PLG on bone formation (191, 208) in an orthotopic context in the presence of MSCs.

The early, positive osteogenic effects of MSC (either autologous or allogeneic) seeding, analyzed by calcein green fluorescence, were clearly present at the ectopic location, and in the submuscular part of the cassettes when compared to the non-cell-seeded controls. Ectopically, bone formation started before 3 weeks in 94% of all MSC-seeded constructs. It never started before this time point in the control constructs. In the submuscular part of the cassettes, this phenomenon was also observed: bone was found at this location in 28% of the MSC-seeded constructs but never in the control constructs. This early effect of MSCs was not observed in the lower part of the cassettes. In a 9-week study performed by our group in a similar cassette model, a positive influence of MSCs was observed by histomorphometry of the submuscular part (207). Our present data could not confirm this observation after 16 weeks of implantation although an obvious effect was shown for the intramuscular construct. This positive effect of cell-based bone TE ectopically has been described many times in various animal models such as mouse (209) and rat (148), and has been attributed to the osteogenic effect of the seeded MSCs. The fact that no positive effect of seeded MSCs on bone apposition was seen after 16 weeks in the cassettes is most likely explained by a difference in location. Bone-forming stem cells are recruited from the underlying transverse process to the BCP. However, the lack of an additive effect of MSCs after 16 weeks of implantation is not in agreement with several studies performed in large animal models and even studies in clinical practice recently reviewed (169). Difference between these studies is that we used a scaffold that showed osteoinductivity at the ectopic location, which has not been done in several other studies (150). This might mean that inductive and conductive characteristics of the materials used in published studies were inferior and thus cells do provide an additive effect. We therefore conclude that the material used in this study has such good osteoinductive and -conductive characteristics, that the addition of cells does not influence the total bone content in the transverse process model, although it does accelerate bone formation in the upper part of the cassette.

Use of PLG in this study did not enhance bone formation, neither at the orthotopic nor at the ectopic location, irrespective of cell seeding. However, it cannot be assumed that both groups are equal since no power analysis was performed in advance. These results are contrary to our previous findings where we evaluated the cassettes after 9 weeks (201). We expected a positive effect because PLG is known to be able to recruit bone-forming cells from the underlying bone by both chemotaxis and stimulation of cell proliferation (107). Possibly, the effect of PLG is limited to early bone formation. In the present study, there might have been an effect after 9 weeks; however, the fluorescence incorporation results as shown are a rough estimate when comparing to histomorphometry, and insufficient to determine an effect of PLG. TGFβ-1 levels were analyzed, and this PLG preparation method was validated in an earlier study, resulting in approximately 7 times higher TGFβ-1 levels in PLG compared to whole blood.

Allogeneic MSCs were previously shown efficient in forming significantly more bone as compared to non-cell-seeded and bone marrow seeded scaffolds in an ectopic implantation model (129). In the present study, we extended this study with allogeneic and autologous MSCs in a direct comparison at an orthotopic implantation location without the use of immunosuppressive medication (210). We found that at either location no significant difference in bone formation between autologous and allogeneic MSCs was present; however, equality is not shown because no power analysis for showing equality was done in advance. The lack of obvious difference in bone formation, however, is an important finding when considering the use of allogeneic cells as an off-the-shelf component in bone TE in goats.

In addition to the earlier described ectopic implantation of allogeneic MSCs, these cells can also be used at the orthotopic location in goats, especially as no signs of an adverse reaction by the host to the seeded allogeneic MSCs was observed. Allogeneic MSCs have been described to be immuneprivileged cells because of their lack of certain surface markers responsible for the host T-cell response and even more, they inhibit T-cell activation by the host (24). Unfortunately, it has not been possible in this study to verify whether surface makers like CD40, CD80, and CD86 were present on the MSCs used, for it is difficult to find antibodies which show reactivity with these goat surface markers. We previously described the presence of lymphoid clusters in BCP constructs seeded with allogeneic MSCs implanted in goats, not observed in the autologous bone marrow and control scaffolds. Although these lymphoid clusters were most likely the result of an immune response after allogeneic transplantation, they acted beneficial for bone formation, as a positive correlation between the numbers of lymphoid clusters and the amount of newly formed bone was found (129). In the present study, these lymphoid clusters were seen in the ectopic implants, but not in the cassettes. However, a positive correlation between the amount of clusters and bone incorporation ectopically was not found. This may be explained by the longer implantation time. Shorter follow-up periods will be needed to determine whether lymphoid cells were never present in the cassettes during the 16 weeks of implantation.

To our knowledge, we are the first to show that autologous and allogeneic MSCs do not obviously differ in their bone forming capacity in a direct comparison, both at the ectopic

implantation location and in the cassette model. Results in the current study concerning these allogeneic MSCs provide us a major advantage. It creates the opportunity to use allogeneic MSCs for *in vivo* bone tissue engineering research in goats and maybe additional animal models as well. The time-consuming operation to aspirate bone marrow and isolate MSCs from the same animal might not be necessary, and the resulting cryopreserved cells can potentially be used in an off-the-shelf way. In the future, allogeneic MSCs may be useful for clinical applications; however, before this step can be taken, more in-depth investigations and clinical studies are required.

CONCLUSION

Our data indicate that the site of implantation and the material used greatly defines the contribution of seeded MSCs in hybrid constructs. This implicates that when at an orthotopic location a ceramic material is used, with good osteoconductive and osteoinductive characteristics, MSCs might not be necessary; that no obvious differences were found between autologous and allogeneic MSCs with respect to bone formation, although tissue response was more pronounced at the ectopic location; and that PLG is not effective at orthotopic MSC-seeded sites during a long follow-up period.

ACKNOWLEDGEMENTS

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5

INFLUENCE OF ENDOTHELIAL PROGENITOR CELLS AND PLATELET GEL ON TISSUE ENGINEERED BONE ECTOPICALLY IN GOATS

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ABSTRACT

For the development of functional large bone tissue constructs, optimal oxygen and nutrients supply of seeded multipotent stromal cells (MSCs) is likely dependent on vascularization. The introduction of endothelial progenitor cells (EPCs) to MSC cultures might enhance vascularization and therefore increase bone formation. In this study we cocultured MSCs and EPCs and investigated performance and bone formation both *in vitro* and *in vivo*. The EPCs used were characterized by uptake of acetylated low-density lipoproteins, binding of isolectin B4 and expression of platelet endothelial cell adhesion molecule. EPC/MSC *in vitro* coculture showed that both cell types exerted a positive effect on proliferation of the other. For the *in vivo* studies, we applied platelet-leukocyte gel (PLG), containing several growth factors, as a means to further induce vascularization and thereby enhance bone formation. Cocultures and monocultures were combined with either PLG or plasma, seeded on ceramic scaffolds, and implanted intramuscularly in nine goats. After 16 weeks of implantation, it turned out that seeding MSCs and EPCs both resulted in significantly more bone lining the scaffold than the unseeded controls, and MSCs and cocultures with highest MSC/EPC ratio were most competent. Cocultures did not show a higher bone content than the monoculture of MSCs. Fluorochrome incorporation results showed that the presence of seeded cells, either MSCs or EPCs, in the constructs accelerated bone formation. Finally, the addition of PLG instead of plasma did have a positive influence on the quantity of incorporated bone.

INTRODUCTION

Finding a reliable alternative for autologous bone graft transplantation with its obvious disadvantages (e.g., donor-site morbidity and limited graft quantity) is a major goal within the field of regenerative medicine. Tissue engineering (TE) of bone, by combining multipotent stromal cells (MSCs) with porous osteoconductive scaffolds, has been a promising technique for years, but without clinical realization. One of the possible reasons for this failure might be the inadequate transport of nutrients and oxygen to the construct and insufficient removal of waste products from the construct, due to a delay in vascularization. Especially oxygen transport is limited to approximately 200μm from a blood vessel, and thus MSCs are expected to suffer from severe hypoxia and therefore may not survive the implantation process (38, 39). Nevertheless, it has been shown that viable cells are necessary for bone formation in tissue engineered constructs, especially when implanted at an ectopic location (156, 173). As bone formation is an angiogenesis-dependent process (34, 35), vascularization might be a crucial factor and accordingly received attention within the field of regenerative medicine during the past years (37, 211).

As extensive growth factor and gap junctional communication between osteoprogenitor cells and endothelial cells has been described, it is an interesting idea to combine both cell types within one bone tissue engineered construct (34, 40, 41). So far, most vascularization strategies relied either on the ingrowth of vasculature from the surroundings, which will take some time before appropriate blood supply and waste removal conditions are achieved, or the use of endothelial cells, which is not clinically applicable (37, 42-44). Highly proliferative endothelial progenitor cells (45, 46), however, can be isolated from bone marrow (BM), which means that bone-forming MSCs and blood vessel-forming EPCs can be acquired from the same source and will only take one BM biopsy (47, 48). For this reason, this study assessed the coseeding of MSCs and EPCs to enhance bone formation in a tissue engineered construct.

The second strategy we studied was the use of growth factors to accelerate ingrowth of surrounding blood vessels from the periimplant region. Several experimental studies have shown that inducing early vascularization through vascular endothelial growth factor (VEGF) creates more oxygenation and nutrition of the surrounding tissue, increasing cell survival and eventually enhancing bone formation (212). For this purpose we used autologous platelet-leukocyte gel (PLG) in this study, a known source of (autologous) growth factors. Upon activation, the platelets release growth factors like VEGF, but also platelet-derived growth factor, transforming growth factor-β, endothelial growth factor, and basic fibroblast growth factor. These factors are known to have a positive effect on both bone formation and vascularization (191, 208).

In short, this study tested the hypothesis that addition of EPCs, through their reported stimulation of MSCs and their possible effect on vascularization, might enhance final bone formation in ectopic constructs. Furthermore, we studied the effectiveness of PLG as a source of multiple stimuli for MSC and EPC proliferation and differentiation.

MATERIALS AND METHODS

Animals

After approval of the local animal care committee, nine 60-70 kg adult female Dutch milk goats aged 23-28 months were used for this study. Food and water were given *ad libitum*. The animals' general health and care conditions were recorded in a diary of wellbeing for each goat separately, and monitored by the laboratory animal welfare officer.

BM aspiration, MSC, and EPC isolation

BM was aspirated from both iliac wings of the goats and collected in heparin-coated tubes. The amount of MSCs in BM was assessed with performing a colony-forming efficiency assay (9). Fractions of $1x10^5$ mononuclear cells/cm^2 were cultured in standard culture medium containing α minimum essential medium (αMEM; Invitrogen, Carlsbad, CA) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin, 2 mM L-glutamine (all Invitrogen), and 15% (v/v) fetal bovine serum (FBS; Cambrex, Landen, Belgium), which was refreshed every 3-4 days. After 9-11 days, colonies were washed, fixed in 4% formalin, stained using methylene blue, and counted under an inverted microscope. The remaining of the aspirate was used to isolate MSCs and EPCs. MSCs were isolated from BM by adherence to tissue culture plastic, and cultured in standard culture medium. Culture medium was refreshed twice a week. For the isolation of EPCs the mononuclear cells (MNCs) were obtained by Ficoll density gradient centrifugation. BM aspirates were diluted 1:3 with phosphate-buffered saline PBS, layered on top of a Ficoll (Sigma, Zwijndrecht, The Netherlands) layer and spun down 20 min at 300 *g*. The interphase containing the MNCs was obtained and washed twice with 3 mM ethylenediaminetetraacetic acid/PBS. Finally, cell numbers were determined using a cytocounter (Cell-Dyne 1800, Abbott Diagnostics, Hoofddorp, The Netherlands) and the MNCs were resuspended in endothelial basal medium (EBM-2; Clonetics, San Diego, CA) supplemented with the endothelial growth mediums bullet kit (Clonetics) and 20% FBS, at the desired concentration and seeded on fibronectin (Harbor Bioproducts, Noorwood, MA)-precoated culture flasks and maintained in this medium. After 2 days the nonadherent cells were discarded and EPCs were isolated after 5-7 days. All cell cultures were maintained in a humidified incubator, containing air and 5% CO_2 at 37°C.

EPC characterization: Early markers

After 4 days of culture, cells were characterized for two early endothelial markers, uptake of DiI-acetylated LDL (acLDL; Invitrogen) and binding of isolectin B4 (Vector Laboratories, Peterborough, UK), and as a control MSCs were stained for the same markers. About 15.000 EPCs were seeded on fibronectin-coated glass coverslips, washed with PBS, incubated in DiI-labeled acLDL (diluted 1:400 in serum-free medium), and in fluorescein labeled isolectin B4 (diluted 1:100 in serum-free medium), for 1h at 37°C. Coverslips were washed twice with PBS and mounted with Vectashield mounting medium containing 4',6-diamindine-2-phenylindole (DAPI; Vector Laboratories, Amsterdam, The Netherlands) and analyzed by fluorescence microscopy using a Leica DMIRBE microscope. The percentage of positive

cells was determined by counting the number of cells positive for the marker and the number of total cells visible via DAPI staining in three fields per sample, manually.

EPC characterization: Late marker

After 2 weeks of culturing, cells were characterized for platelet endothelial cell adhesion molecule (PECAM-1) using fluorescence-activated cell-sorting (FACS) analysis. Cells were trypsinized 13 days after start of culture, resuspended in culture medium, and kept in a falcon tube over night at 37 °C. Cells were washed with ice-cold PBS and incubated for 45min on ice with mouse anti-ovine PECAM-1 (Serotec, Oxford, UK) diluted 1:10 in 1% bovine serum albumin/PBS. Cells were washed with PBS twice and incubated with Alexa488-goat-anti-mouse Ab (Invitrogen) diluted 1:100 in 1% bovine serum albumin / PBS for 30min. Cells were washed with ice-cold PBS twice, resuspended in 100 µl PBS, and analyzed using FACSCalibur system (BD Biosciences, Breda, The Netherlands).

Analysis of proliferation in coculture

This experiment was performed with passage 2-3 cells (n=3). Cell cultures were maintained in a humidified incubator, containing air and 5% CO_2 at 37°. At day 0, MSCs and EPCs were trypsinized and washed with PBS twice. The cell pellet was resuspended in 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE, 0.5 mg/ml in dimethyl sulfoxide), diluted 1:100 in PBS. This was mixed gently and incubated for 10 min at 37°C. One hundred percent FBS was added 1:1 and cells were incubated for 2 min at room temperature. Next, cells were washed with culture medium three times. CFSE-labeled MSCs and unlabeled EPCs were cocultured. In parallel experiments CFSE-labeled EPCs are cocultured with unlabeled MSCs. The medium used was a 1:1 mixture of MSC medium (see above) and EGM-2. Three different coculture ratios were tested, MSCs/EPCs=0.25, MSCs/EPCs=1, MSCs/EPCs=4, and MSCs and EPCs alone, all groups at a total concentration of 30.000 cells per well. At day 0, 1, or 3, cells were trypsinized and CFSE signal of either MSCs or EPCs was measured by FACS analysis.

Scaffolds

Cubes of 7x7x7 mm biphasic calcium phosphate (BCP)(161) were used. The ceramic consists of 80±5 % (w/v) hydroxyapatite and 20±5 % (w/v) β-tricalciumphosphate, total porosity was 70 ± 5%, macroporosity 55 ± 5%, and microporosity 20 ± 5% (Progentix, Bilthoven, The Netherlands). Before use, the biphasic nature of the material was confirmed by X-ray diffraction. Fourier transform infrared established that the material did not contain any impurities.

MSC and EPC seeding conditions in vivo

At the day of surgery, passage 2 autologous MSCs and EPCs were trypsinized, washed, and resuspended in standard culture medium containing 30% FBS. They were mixed to obtain the coculture ratios as described for the *in vitro* coculture experiments, at a final concentration of $1x10^7$cells/cm^3 scaffold. During surgery the cell suspensions were centrifuged for 10 min at 300 *g* to concentrate the cells for direct seeding. Subsequently, the medium was discarded

and the cells were resuspended in 150 µl autologous plasma or platelet-leukocyte rich plasma (PRP preparation, see below), and seeded on the scaffolds. Another two control scaffolds were seeded with plasma and PRP alone. After seeding, the PRP-containing constructs were activated by adding 90 mM $CaCl_2$ and recombinant bovine thrombin (50 U/ml; Jones Pharma Inc., St Louis, MO) after which all constructs were allowed to polymerize for 30 min at room temperature before implantation. This way 12 constructs, listed in Table 1, were prepared for implantation in the paraspinal muscles of the goat. As we did not have enough EPCs from one of the goats, from the four groups we have thus missed one hybrid construct.

Platelet gel and plasma preparation method

Platelet-leukocyte rich plasma (PRP) was prepared as described before (129) using the Angel Whole Blood Processing SystemT (AWBPS; Sorin Group, Milan, Italy), a semi-automated tabletop centrifuge system using a flat disc, with a variable blood volume ranging from 60 to 180 ml, to sequester the blood in three components. The whole blood volume of 100 ml was injected in the blood collection reservoir of the AWBPS. Following a 19-min spin at 1200 *g*, platelet-poor plasma (PPP) was removed in a transfer bag, PRP was collected in a collection syringe and the remaining erythrocyte concentrate was collected in a retransfusion bag. PLG was prepared by mixing the PRP with 90 mM $CaCl_2$ and bovine thrombin (50 U/ml; Jones Pharma Inc). Autologous plasma was prepared by centrifuging 10 ml of peripheral blood at 1200 *g* in a Falcon tube (Greine, Alphen a/d Rijn, The Netherlands) for 10 min at 4°C.

TABLE 1: Seeding conditions and onset of bone formation in ectopic constructs.

Conditions	MSCs	EPCs	n	Resuspended in	Calcein green Week 3	Tetracyclin Week 5	Xylenol Orange Week 9	Histology Week 16
1.	no	no	9	Plasma	0/9	1/9	6/9	7/9
2.	no	no	9	PLG	0/0	1/9	8/9	8/9
3.	0%	100%	8	Plasma	7/8	7/8	7/8	8/8
4.	0%	100%	8	PLG	6/8	7/8	8/8	8/8
5.	20%	80%	8	Plasma	6/8	7/8	8/8	8/8
6.	20%	80%	8	PLG	7/8	8/8	8/8	8/8
7.	50%	50%	9	Plasma	8/9	9/9	9/9	9/9
8.	50%	50%	9	PLG	8/9	9/9	9/9	9/9
9.	80%	20%	9	Plasma	9/9	9/9	9/9	9/9
10.	80%	20%	9	PLG	9/9	9/9	9/9	9/9
11.	100%	0%	9	Plasma	8/9	9/9	9/9	9/9
12.	100%	0%	9	PLG	9/9	9/9	9/9	9/9

All 12 conditions are implanted in the paraspinal muscles (ectopic, 7x7x7 mm biphasic calcium phosphate blocks). The frequency of fluorescent labels built in at week 3, 5, and 9 and bone formed at week 16 was given. PLG: platelet-leukocyte gel, MSCs: multipotent stromal cells, EPCs: endothelial progenitor cells.

Implantation of ceramic constructs

The surgical procedures were performed under general inhalation anesthesia of an isoflurane gas mixture (Ebbott, Amersfoort, The Netherlands) preceded by intravenous detomidine (Pfizer, Capella a/d Ijssel, The Netherlands) sedation. After inducing anesthesia 100 ml of whole blood was aspirated from the external jugular vein for PLG and plasma preparation, 10 ml was used to prepare plasma for later use. After shaving and disinfection of the dorsal thoracolumbar area, a midline skin incision from T10-L5 was made to expose the paraspinal muscles. This incision also allowed the implantation of transverse process implants, which are not discussed in the present manuscript. The fascia was opened to create multiple intramuscular pockets on each side using blunt dissection. All constructs were placed in the pockets according to a randomized implantation schedule. The muscle, fascia, subcutis, and skin were closed separately. Postoperative pain relief was provided with Durogesic 25 patches (fentanyl transdermal patches, Janssen-Celag, Beers, Belgium). To monitor the onset and dynamics of bone growth, the goats received fluorochrome labels at week 3 (Calcein Green, 10 mg/kg, i.v.; Sigma, The Netherlands), week 5 (Oxytetracyclin, Engemycine 32 mg/kg, i.m.; Mycofarm, De Bilt, The Netherlands), and week 9 (Xylenol Orange, 80 mg/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.

Post-mortem sample acquisition, histological processing and histomorphometry

After explantation, the samples were fixed in 4% glutaraldehyde for 8 h and dehydrated by ethanol series. Next, all samples were embedded in polymethylmethacrylate (MMA). Two centrally located 10-µm-thick sections were made using a sawing microtome (Leica, Nusslochh, Germany). The first was left unstained for epifluorescence microscopy and the second was stained with methylene blue and basic fuchsin for routine histology and histomorphometric analyses. Tissue response and bone formation were observed using a light microscope (Olympus-BX51, Zoeterwoude, The Netherlands), and high-resolution digital photos were made from the stained section for histomorphometric analysis. Bone and scaffolds were pseudocoloured yellow and green using Adobe Photoshop CS3. A custom macro was used to measure the area of interest, the area of the scaffold, the area of bone, the scaffold outline available for bone apposition, and the contact length of bone and scaffold (193). This allowed the calculation of the bone contact% = (bone-to-scaffold contact length/scaffold outline) x100%, and bone area% = (bone apposition/available pore surface) x100%. To analyze fluorochromes, a fluorescence microscope (X-Cite; EXFO, Mississauga, Canada) was used, equipped with a triple filter block (Chroma 104158, Chroma Technology, Rockingham, VT).

Statistics

For histomorphometry, bone contact% and bone area% were measured and analyzed using a mixed effects two-way ANOVA with fixed effects for PLG/plasma, cells, their interaction and a random effect for goat. First we tested if PLG had an effect on bone formation. Next we tested if there was an influence of adding the two cell types and cocultures compared to

the constructs without cells. LSD corrected *post hoc* tests were done to analyze differences between groups. Earlier results with measurements of less samples showed no significant differences for the PLG vs plasma.

RESULTS

In vitro results

From the nine mature 2-year-old Dutch milk goats BM was aspirated, which yielded $15.0x10^6$ ±7.5 (mean±SD) nucleated cells/ml. The colony-forming efficiency assay (of the MSCs) resulted in 1.5±0.6 colonies per 100.000 MNCs, meaning approximately 1 out of 70.000 cells in the aspirate had attached and formed a colony, and thus was considered a MSC. From the remaining BM, MSCs and EPCs were isolated. Early outgrowth, elongated, spindle shaped colonies of EPCs were visible already at day 3-4. MSCs and EPCs were used for further *in vitro* characterization and cryopreserved at passage 2 for later implantation *in vivo*. From one goat we were not able to culture enough EPCs before the time of implantation.

Early and late characterization of EPCs. For the early characterization of the EPCs in this study, immunocytochemistry was performed after 4 days, for the uptake of acLDL and binding of isolectin B4. MSCs were used as a control. Over 95% of all the cells were positive for isolectin B4 and both markers were found in 4±2% (mean±SD) of the cells (Figure 1A). MSCs were also 85% positive for isolectin B4, but were not able to take up acLDL. After two weeks PECAM-1, a highly expressed adhesion molecule on differentiated endothelial cells, was analyzed by FACS. The curve representing the specific PECAM-1 Ab showed a shift to the right (higher fluorescence signal) compared to the isotype-matched control

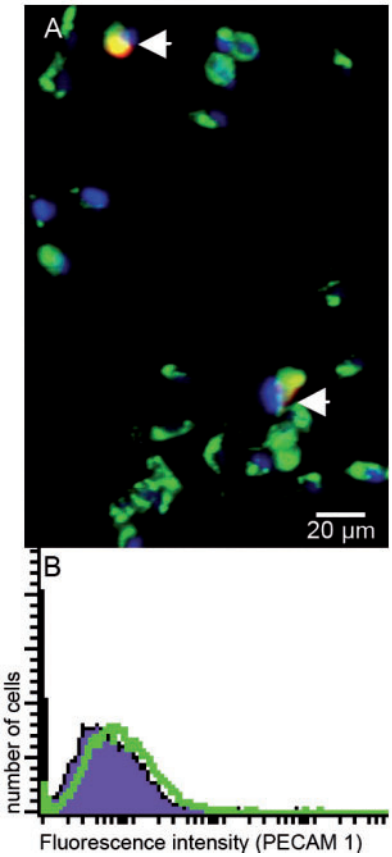

FIGURE 1: In vitro characterization of EPCs. A: early EPC characterization by isolectin B4 binding/ acLDL uptake. Blue: nuclear staining, red: acLDL staining, green: isolectin B4 staining. Over 95% of all EPCs are isolectin B4 positive. The arrows show the double positive cells (4%±2%). **B:** expression of PECAM-1 analyzed by flow cytometry (open peak) and isotype-matched antibody control (filled peak). EPCs, endothelial progenitor cells.

Ab of the total population indicating modest expression of PECAM-1 on this EPC population (Figure 1B).

Proliferation of cocultures. To determine whether coculturing in different ratios influences the proliferation of either MSCs or EPCs, a CFSE assay was performed. The CFSE label equally divides over daughter cells during mitosis, resulting in a halved fluorescence signal after each cell division. CFSE-labeled MSCs and unlabeled EPCs and vice versa were cocultured in parallel experiments (n=3). CFSE signal of either MSCs or EPCs was quantified by FACS analysis at different time points after labeling. Directly after labeling (filled peaks in Figures 2A-D) the highest fluorescence intensity per cell was observed. At day 1 (Figure 2A, B) and day 3 (Figure 2C, D) more cells with a lower signal intensity were detected, indicating cell division. The ratio of MSC/EPC input greatly determined proliferation of the cell type studied. Results showed that seeding more MSCs resulted in a higher proliferation rate of EPCs and seeding more EPCs resulted in a higher proliferation rate of MSCs at day 1 (Figure 2A, B), indicated by the lower fluorescence signal. This effect was still observed at day three (Figure 2C) for the proliferation of EPCs, however not for the MSCs (Figure 2D).

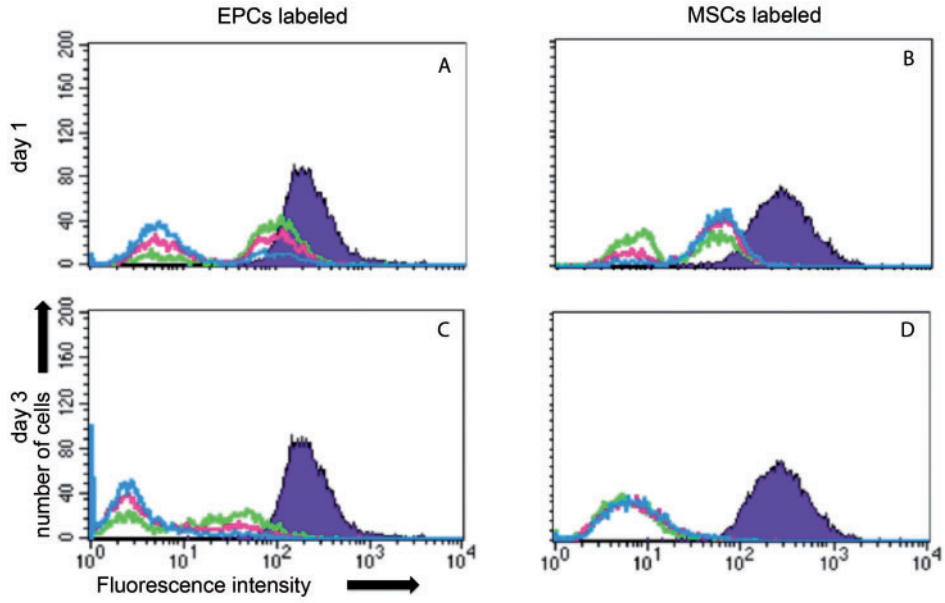

FIGURE 2: MSC and EPC proliferation assay by CFSE analysis. A, C: EPCs labeled; B, D: MSCs labeled; A, B: CFSE analysis at day 1; C, D: CFSE analysis at day 3. Blue line: MSCs/EPCs=4, pink line: MSCs/EPCs=1, green line: MSCs/EPCs=0.25, purple filled peak: CFSE-labeled cells at day 0 (either MSCs or EPCs). MSCs: multipotent stromal cells, EPCs: endothelial progenitor cells, CFSE: carboxy-fluorescein diacetate succinimidyl ester.

Scaffold preparation for ectopic implantation

At the day of surgery, MSCs and EPCs, passage 2, were thawed and prepared for direct seeding. From the external jugular vein peripheral blood was aspirated for the production of PLG and plasma. BCP scaffolds were used for seeding of the 12 different conditions (Table 1). All created constructs were implanted in the paraspinal muscles of the goat, according to a randomized implantation schedule. All goats recovered well within 2 days of surgery and further follow up was without complications. At 3, 5, and 9 weeks, fluorochrome markers were administered to allow assessment of the onset and dynamics of bone growth. Implanted constructs were retrieved after 16 weeks of implantation.

Results of the implantation studies

Histological aspects of the implants. Analysis of samples stained with basic fuchsin and methylene blue of methylmethacrylaate (MMA)-embedded sections showed a border of fibrous tissue was surrounding the scaffolds. The pores of all 12 implanted groups contained connective tissue, bone, and blood vessels. Furthermore, foreign body multinucleated giant cells were frequently seen lining the scaffold border. The scaffold did not show any signs of degradation. Obvious differences with respect to the amount of bone formation were seen between the cell-seeded scaffolds (Figure 3B-F) and the non-cell-seeded scaffolds (Figure 3A). Extensive bone formation was seen in the cell-seeded groups and little bone formation in the non-cell-seeded groups. However, no clear differences were seen between the constructs containing the cocultures and the constructs only containing MSCs or EPCs (Figure 3B-F).

Onset and growth dynamics of newly formed bone. Fluorescence analysis of the implants showed that the 3-week label was incorporated in newly formed bone in the cell-seeded constructs (Figure 4B) but not in the non-cell-seeded scaffolds (Figure 4A). This indicates that bone formation started already before 3 weeks in the cell-seeded implants. After 5 weeks bone formation was present in 2/18 (11%) of the non-cell-seeded constructs, after 9 weeks in 14/18 (77%) of the constructs, and after 16 weeks histology showed bone formation in 15/18 (83%) of the implants. However, in 77/86 (90%) cell-seeded constructs, with either MSCs or EPCs, bone formation already started at 3 weeks of implantation; this increased to 83/86 (97%) in week 5 and 85/86 (99%) at 9 weeks of implantation. After 16 weeks of implantation all cell-seeded samples showed bone formation. Further, the 3-week label was always visible in the outer rim of the scaffolds, while the 9-week label was also visible in the center of the scaffolds. In the samples without cells, however, bone formation did not occur before week 5 and did not show much increase anymore after week 9 (Table 1).

Amount of bone formation in ectopic scaffolds. Analysis of the explanted samples by basic fuchsin/ methylene blue staining of MMA-embedded sections revealed that bone was present in various amounts in all groups (Figure 5). Control scaffolds without cells showed bone formation in 15 out of 18 constructs. The amount of bone in the control hybrid constructs was variable, with a contact% of 5.3±6.8% (mean±SD) for the plasma construct and 5.7±5.4% for the PLG constructs. Significantly more bone was found in all cell-seeded constructs compared to the controls ($p<0.001$). Furthermore, in the MSCs group, significantly more bone had formed compared to the EPCs group and MSCs/EPCs=0.25

constructs (p=0.003 and 0.001). Seeding the cells with PLG has a significant positive effect on bone formation (p=0.038) compared to plasma.

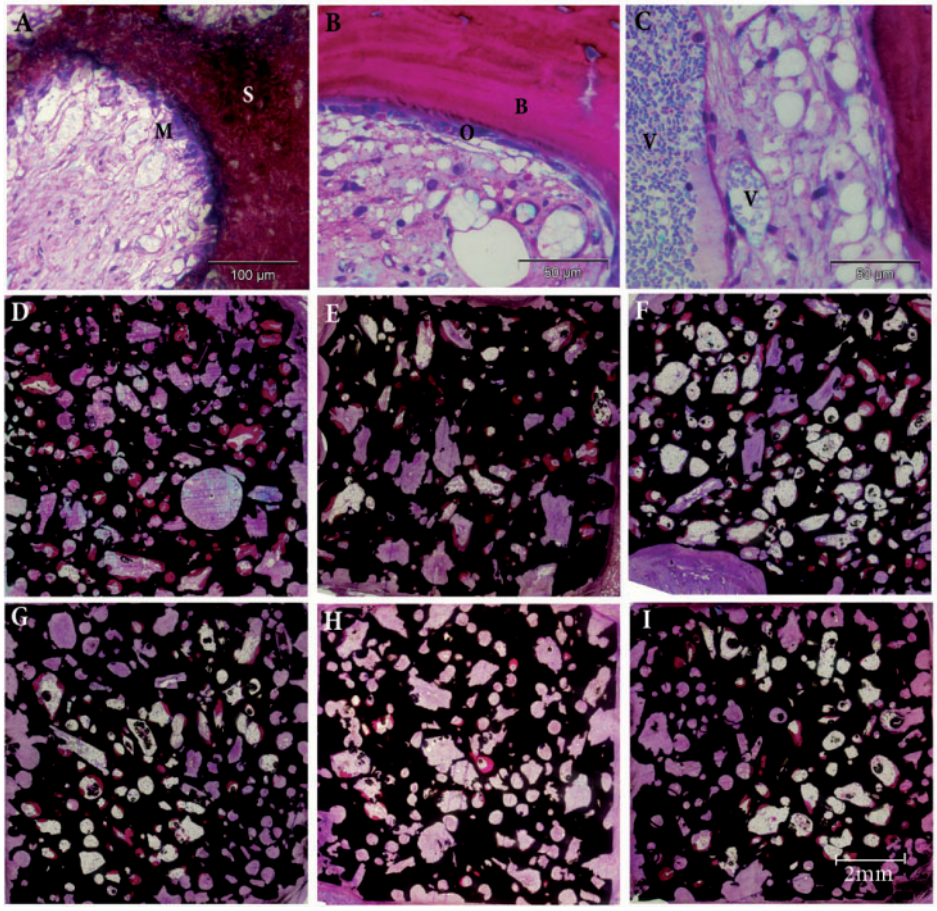

FIGURE 3: Histological images of the different implanted groups and plasma- and PLG-seeded scaffolds. A-C: photographs, representative for all different implanted groups, A: MGCs lining the scaffold border, B: bone with osteocytes in lacunae and osteoblasts lining the newly formed bone, C: vessel formation within the scaffold; D-I different implanted groups, scale bar in I accounts for figure D-I; D: No cells seeded, E: MSCs, F: EPCs, G: MSCs/EPCs=4, H: MSCs/EPCs=1, I: MSCs/EPCs=0.25. S= scaffold, M=MGC= multinucleated giant cells, V= vessel, B= bone, O= osteoblasts.

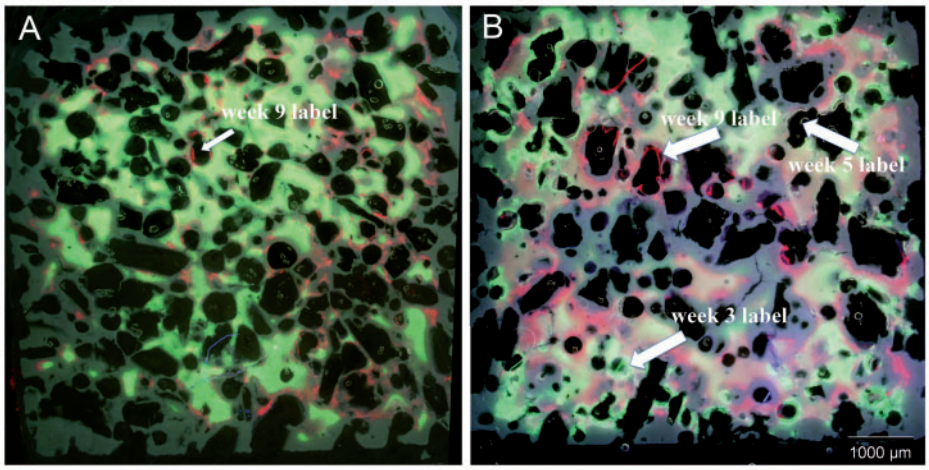

FIGURE 4: Onset of bone formation by fluorochrome incorporation. A: scaffold without cells, only the red (week 9) label is visible in the newly formed bone, B: in a scaffold seeded with cells (MSCs/EPCs=1) the green (week-3 label), yellow (week-5 label) and red labels (week-9 label) are visible. Arrows are pointing towards the different labels.

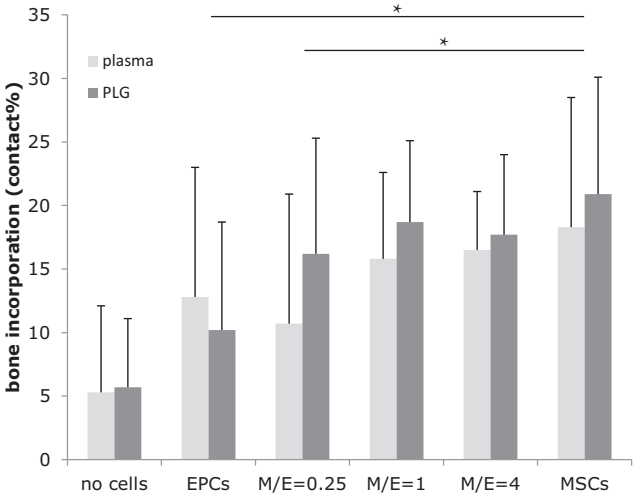

FIGURE 5: Bone formation in ectopic scaffolds. Bone contact% after 16 weeks ectopic implantation in the goat. The results are represented as mean ± standard deviation (n=8 in 4 groups, n=9 in 8 groups). All cell-seeded implants show a significant higher bone contact% compared to the non-cell-seeded implants and MSCs show significantly higher bone contact% compared to the EPCs and MSCs/EPCs=0.25 (*$p<0.05$). M=MSCs, E=EPCs.

DISCUSSION

In this study we demonstrated that MSCs benefit for their *in vitro* proliferation from the presence of EPCs and vice versa. We also found that when EPCs alone were implanted *in vivo,* they were able to stimulate bone formation. However, EPCs added in different ratios to MSC-seeded scaffolds, did not enhance bone formation ectopically in goats.

Both MSCs and EPCs were isolated from BM. The EPCs were positive for the early endothelial markers Isolectin B4, uptake of acLDL, and the late endothelial marker PECAM-1, and they have been shown in another study to form tubular networks on Matrigel whereas MSCs did not (213). However, the uptake of AcLDL was limited compared to what has been described in literature for EPCs and ECs (214, 215). The MSCs, which were not expected to express endothelial markers (214), were negative for acLDL uptake, but positive for Isolectin B4 binding.

An early, positive osteogenic effect, analyzed by calcein green fluorescence, was clearly present in all cell-seeded constructs compared to the non-cell-seeded constructs. No obvious differences were seen in the PLG-seeded groups compared to the plasma-seeded groups at this early timepoint. Bone formation never started before 5 weeks in the control constructs and started before 3 weeks in 77/86 of the cell-seeded implants, without obvious differences between EPCs, MSCs, and coculture groups. Although the early osteogenic effect of MSCs has been extensively reported (169, 207), to our knowledge, this was not yet described for EPCs.

This osteogenic effect of the EPCs was confirmed by histomorphometric analysis. Significantly more bone was present as compared to the non-cell-seeded control scaffolds. This was remarkable because the seeded EPCs were clearly different from the MSCs isolated from the same BM, as seen by acLDL uptake and PECAM-1 expression and tubular network formation on Matrigel (45, 213, 214), and were not expected to contribute to *in vivo* bone formation. These findings may be explained by several mechanisms. First, the relatively homogeneous EPC cell populations used may still contain progenitor cells giving rise to osteoblast differentiation. These cells may: a) be the common ancestors for EPC and MSC also known as mesangioblasts (216), b) have dedifferentiated to the common ancestor just mentioned and then giving rise to osteoblasts, c) themselves have osteogenic capacity, which we find highly unlikely but cannot exclude. Osteogenic differentiation of the so-called peripheral blood (PB) $CD34^+$, $CD133^+$, circulating, BM-derived cells, has been described before *in vitro*, and *in vivo* (216, 217). Unfortunately, we were not able to verify whether the surface markers CD133/ CD34 or progenitor marker CD130 were expressed by our EPCs, due to lack of specific antibodies cross-reacting with goat cells. Another mechanism explaining bone formation after EPC seeding might be indirect bone formation due to the release of paracrine factors. EPCs would then act as a producer of chemoattractants or other cytokines (e.g., BMP-2, VEGF), developing a favorable environment for bone formation by host cells (216). Last, the seeded cells probably consisted of an inhomogeneous population, for we had a low uptake of AcLDL and expression of PECAM-1 and a positive isolectin B4 staining. This might mean MSCs were still present and responsible for bone formation.

In spite of the positive effect of EPCs alone on bone formation, no additive effect of these cells to MSCs was found *in vivo*, when keeping total numbers of seeded cells constant. A possible explanation might be that the transplanted EPCs are the previously described early EPCs, found in peripheral blood and originating from the BM, because colonies were already formed after 4 days of culture. Late EPCs typically form colonies after 2-4 weeks and have cobblestone morphology. Early EPCs have been described to contribute to neovasculogenesis by secreting angiogenic cytokines, whereas late EPCs enhance neovasculogenesis by providing a sufficient number of endothelial cells based on their high proliferative capacity (218). We hypothesize that the excretion of angiogenic cytokines and possible induced neovasculogenesis by the host was too late to make a difference in the perfusion of the constructs. Late EPCs however, might have a positive effect on bone formation through their faster effect. Therefore, we will analyze late outgrowth EPCs in future experiments.

Addition of PLG when seeding the cells enhanced bone formation in this study. A phenomenon we did not find when seeding BM, autologous and allogeneic MSCs (128, 129). However, when using different materials without cells in a transverse process model we did find a significant positive effect on bone formation. From this we conclude that the results are highly variable, not making this strategy very reliable for bone regeneration in this setting, but PLG in this form is not contra-indicated.

CONCLUSION

We found that in spite of the positive effect that both cell types exerted on proliferation of the other *in vitro* and of EPCs alone on bone formation *in vivo,* coseeding of MSCs and EPCs did not give rise to enhanced bone formation, though EPCs alone did reveal enhanced bone formation as compared to control implants without cells.

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6

A DIFFERENTIAL EFFECT OF GROWTH FACTOR RELEASE TIMING ON OSTEOGENESIS AT ECTOPIC AND ORTHOTOPIC SITES

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ABSTRACT

In bone tissue engineering, growth factors are widely used. Bone morphogenetic proteins (BMPs) and vascular endothelial growth factor (VEGF) are the most well known regulators of osteogenesis and angiogenesis. We investigated whether the timing of dual release of VEGF and BMP-2 influences the amount of bone formation in a large animal model. Poly(lactic-co-glycolic acid) (PLGA) microparticles (MPs) were loaded with BMP-2 or VEGF to create sustained release profiles, and rapidly degrading gelatin was loaded with either growth factor for fast release profiles. To study *in vivo* osteogenicity, the two delivery vehicles were combined with biphasic calcium phosphate (BCP) scaffolds and implanted in 10 Beagle dogs for nine weeks, at both ectopic (paraspinal muscles) and orthotopic sites (critical size ulnar defect). The 9 ectopic groups contained combined or single BMP/VEGF dosages, in sustained or fast release profiles. In the ulnae of 8 dogs, fast VEGF and sustained BMP-2 was applied to one leg, the other received the opposite release profiles. The two remaining dogs received bilateral control scaffolds. Bone growth dynamics was analyzed by fluorochrome injection at week 3, 5, and 7. Post-operatively and post-euthanization X-rays of the ulnar implants were taken. After 9 weeks of implantation bone quantity and bone growth dynamics were studied by histology, histomorphometry, and fluorescence microscopy. The release of the growth factors resulted in both an enhanced orthotopic and ectopic bone formation. Bone formation started before three weeks and continued beyond 7 weeks. The ectopic BMP-2 fast groups showed significantly more bone compared to sustained release, independent of the VEGF profile. The ulna implants revealed no significant differences in the amount of bone formed. This study shows that timing of BMP-2 release largely determines speed and amount of ectopic bone formation independent of VEGF release. Furthermore, at the orthotopic site growth factors enhanced bone formation, however no significant effect on bone formation was found from a timed release of these growth factors. This implicates that timed release is location dependent.

INTRODUCTION

Auto-induction of bone is a specialized form of wound healing that occurs during grafting procedures and fracture healing, and is normally a successful process of tissue regeneration. This process is active when autologous bone grafting procedures are used in orthopedic surgery, for example in spinal fusion and when bone repair mechanisms fail. Unfortunately, several disadvantages are associated with graft harvesting, such as limited availability of grafts and donor-site morbidity (219, 220). Therefore, alternative strategies have been developed for the regeneration of bone, e.g. the use of bioactive molecules known to be involved in the process of auto-induction and osteoblast differentiation.

Bone morphogenetic proteins (BMPs), bioactive molecules first found in demineralized bone, are widely studied in developmental and skeletal biology. They have initially proven their inductive capacity by inducing bone formation at ectopic sites (69, 221). Later, they have been described to initiate both cartilage and bone progenitor cell differentiation, and to control the formation of new bone through both endochondral and intramembranous ossification (67, 68). Recombinant DNA technologies have allowed the optimization of BMP-2 and BMP-7 protein preparation, which has already resulted in clinical successes (73, 74). A disadvantage however is that the current clinical available delivery vehicles of these BMPs which are promising, but still need to be optimized. The collagen powder or sponges used show large initial burst release, while during fracture repair BMP expression increases until day 21, suggesting that it is needed for a longer period of time (95, 222, 223).

Apart from induction by BMPs, bone formation is an angiogenesis dependent process. This vessel formation is important for transport of nutrients, oxygen, and cells towards, and removal of waste products from the newly formed bone. Many studies focus on the use of the bioactive molecule vascular endothelial growth factor (VEGF), the key mediator in angiogenesis. As a result of being important in angiogenesis, VEGF has shown to be important during early fracture repair, and endochondral and intramembranous ossification (54, 88, 224). In line with this, experimental models have shown that normal fracture healing is disturbed if VEGF is inhibited (89, 90).

Communication between osteoprogenitor and endothelial cells is mediated by several mechanisms: the direct process of gap-junctional communication and the indirect process involving the soluble growth factors VEGF and BMP-2 (34, 35). VEGF has been described to interact in a synergistic manner with BMP-2 and BMP-4 in both bone formation and bone healing by increasing cell survival, angiogenesis and enhancing cell recruitment (91). Furthermore, VEGF can stimulate chemotaxis and differentiation of osteoblasts (92), osteoclasts (93) and mesenchymal progenitor cells (94) and furthermore VEGF may also directly contribute to bone formation by enhancing BMP-2 mRNA and protein expression (95). Moreover, osteoblasts are known to be able to synthesize VEGF when oxygen levels are low and in this way actively enhance the process of angiogenesis (40). Given the importance of the angiogenesis-osteogenesis coupling, combining the most important bioactive molecules involved in these processes can be beneficial for bone regeneration (96, 225).

One of the major problems considering release of growth factors from delivery vehicles is the loss of biological activity. Since these growth factors have short biological half-lives,

keeping the concentration of growth factor in the therapeutic window for a sufficient period of time is suggested to be important in mimicking natural bone regeneration (226). Additionally, the timing of this release could be important to simulate the natural expression pattern of these growth factors. There are several studies that use a dual release system of VEGF and BMP-2 for the enhancement of bone healing, especially in small animal models, but they do not consider the timing of this release (227, 228). One study does investigate timing of this release in a small animal model, but only uses fast delivery of VEGF and sustained delivery of BMP-2, based on their expression pattern in normal bone repair (225).

Therefore we investigated whether the timing of sequentially released BMP-2 and VEGF is of importance in bone formation in a large animal model. We made use of fast and sustained release systems for proteins that were earlier described and validated for both BMP-2 and VEGF sequentially (225, 229). The effects of VEGF and BMP-2 were studied in ectopic and orthotopic implantation models in dogs and after 9 weeks of implantation bone quantity and bone growth dynamics were studied.

MATERIALS AND METHODS

Experimental design

A total of 10 dogs were used for the experiment after approval of local Ethical Committee for Animal Experimentation and in compliance with the Institutional Guidelines on the use of laboratory animals. A rapidly degrading hydrogel (gelatin) served as a delivery vehicle for fast (*f*) release, whereas poly(lactic-co-glycolic acid) (PLGA) microparticles (MPs) served as a sustained (*s*) release vehicle. Bone forming capacity of constructs containing these growth factors was studied *in vivo* at both ectopic (intramuscular) and orthotopic (critical size ulnar defect) locations. Eight animals received 9 ectopic implants and 2 ulnar constructs: 1) BMP-2 slow / VEGF fast and 2) BMP-2 fast / VEGF slow (Table 1). Two animals received scaffolds without growth factors in the ulnar defect, serving as controls

TABLE 1: Implantation groups

Condition	MPs	Gelatin	Location (n)
BMP*f*/ VEGF*s*	VEGF	BMP	i.m (8)/ ulna(8)
BMP*f*/ VEGF*f*		BMP+VEGF	i.m. (8)
BMP*s*/ VEGF*f*	BMP	VEGF	i.m (8)/ ulna(8)
BMP*s*/ VEGF*s*	BMP+VEGF		i.m. (8)
BMP*s*	BMP		i.m. (8)
VEGF*s*	VEGF		i.m. (8)
BMP*f*		BMP	i.m. (8)
VEGF*f*		VEGF	i.m. (8)
Control			i.m (4)/ ulna(8)

The total amount of BMP-2 was 12 mg/i.m., and 120mg/ulnar implant. The amount of VEGF was 0.4 mg/i.m., and 4 mg/ ulnar implant. f: fast, s: sustained, i.m.: intramuscular.

for scaffold-mediated bone regeneration. Fluorochrome labels were administered after 3, 5 and 7 weeks to analyze bone growth dynamics. Directly after implantation and after euthanization X-rays were taken again to analyze bone formation around the implants. Furthermore histology, histomorphometry, and fluorescence microscopy were performed to analyze bone formation in and around the implants in more detail.

Construct design and preparation

The constructs consisted of three major components. PLGA microparticles (Medisorb®, Lakeshore Biomaterials, AL, USA) for sustained release, loaded with either rhBMP-2 (INFUSE ®, Medtronic, Memphis, TN, USA) or rhVEGF-165 (R&D systems, Minneapolis, MN, USA). A rapidly degrading hydrogel (gelatin from porcine skin, Sigma-Aldrich, St. Louis, MO, USA) for fast release, loaded with either rhBMP-2 or rhVEGF and biphasic calcium phosphate (BCP) (Progentix, Bilthoven, The Netherlands) was used as a scaffold and combined with these delivery vehicles.

The BCP consisted of 80±5%(mean ± SD) (w/v) hydroxyapatite (HA) and 20±5% (w/v) β-tricalciumphosphate (βTCP), total porosity was 70±5%, macroporosity 55±5% and microporosity 20±5%. For ectopic implantation, particles of ∅ 2-3 mm were used and for ulnar implantation cylinders of 22 mm height, 10 mm diameter and a 4 mm diameter central canal. Finally they were cleaned in an ultrasonic bath and sterilized by autoclave.

PLGA microparticles (Medisorb®, Lakeshore Biomaterials, AL, USA) for slow release were loaded with rhBMP-2 (Medtronic) or rhVEGF-165 (R&D systems) and prepared using a previously described water-in-oil-in-water (W1-O-W2) double-emulsion-solvent-extraction technique (230, 231). Four different groups of microparticles were produced: BMP high, BMP low, VEGF high and VEGF low. For this, 100 µl of a BMP-2 stock (8 mg/ml, or 0.8 mg/ml) or 135 µl of a VEGF stock (0.2 mg/ml, or 0.02 mg/ml) or a control solution (BMP-2 buffer, INFUSE®, Medtronic, Memphis, TN, USA) was emulsified in a solution of 500 mg of PLGA (L:G ratio of 50:50, Mw 23 kDa) and in 1.25 ml dichloromethane. These amounts were based on the entrapment efficiency in earlier studies (231). This mixture was re-emulsified for 30 seconds in 2 ml of 1% (w/v) aqueous poly(vinyl alcohol) (PVA, 87-89% mole hydrolyzed, Sigma-Aldrich, St. Louis, MO, USA) solution to create the double emulsion. This double emulsion was then added to 100 ml of a 0.3 % (w/v) aqueous PVA solution and 100 ml of a 2% (w/v) aqueous isopropanol solution and stirred for one hour. The microparticles were centrifuged, collected, washed twice with ddH_2O, vacuum-dried overnight into a free flowing powder system (Savant Speedvac systems, Holbrook, NY, USA) and stored at -20°C.

The hydrogel for fast release was made of a 1 ml aqueous gelatin solution (10% w/v) crosslinked with 40 µL aqueous glutaraldehyde (10% w/v) solution as described before (225). For the orthotopic implants, hydrogel (1 ml) was pipetted in the central canal of the BCP scaffold and served as a plug when it was polymerized. The MPs (for sustained release) were premixed with the gelatin and pipetted in the central canal of the scaffold on top of the plug (1 ml). After 1 hour of cross-linking at 4°C, scaffolds were put in 100 mM aqueous glycine solution for 1 hour to block the residual aldehyde groups of glutaraldehyde (Figure 1A).

For the ectopic implants, chamber slides (Sigma-Aldrich, St. Louis, MO, USA) were used as a mould to make them equally sized (8x8x8 mm, Figure 1B). The chamber slides were filled with BCP particles and the hydrogel mixed with MPs containing VEGF/ BMP-2 was pipetted into the chamber slides (0.5 ml). They underwent the same cross-linking procedure as the orthotopic implants. The fast-released growth factor was then injected into the hydrogel (cylinder and blocks) to allow it to diffuse in the material, and all implants were stored at -20°C prior to implantation. Based on previous studies, the entrapment efficiency was estimated to be 85% or 1.36 µg BMP-2 per mg PLGA that resulted in a total BMP-2 loading of 12 µg per ectopic implant and 120 µg per ulnar implant (both 88 mg PLGA). The same was done for the VEGF implants resulting in 0.4 µg per ectopic implant was and 4 µg for ulnar implants (225). The calculated concentrations of growth factors in these implants are much higher than the *in vitro* effective dosage known for the growth factors (ED_{50} of VEGF is 2-6 ng/ml, ED_{50} of BMP-2 is 20-50 ng/ml, as stated in the product sheets) to make sure enough BMP-2/VEGF was available *in vivo* during the 9 weeks implantation period. The ectopic control groups consisted of BCP mixed with gelatin/ control MPs without growth factor and orthotopic control implants consisted of empty BCP scaffold with gelatin/ control MPs. All scaffolds were stored at -20°C until use.

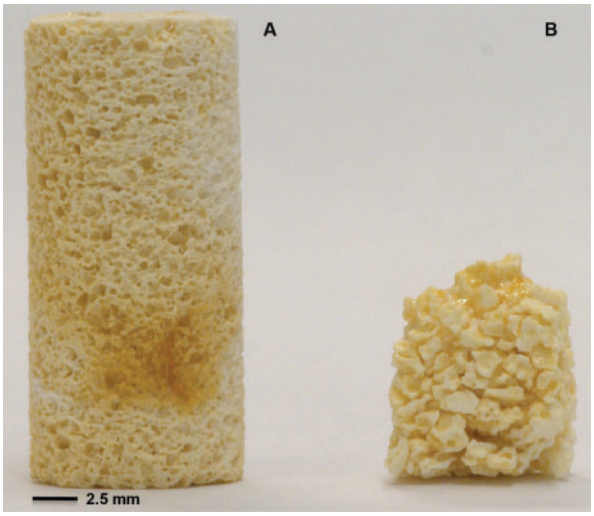

FIGURE 1: Samples before implantation. A) BCP-cylinder *h* 22 mm, ∅ 10 mm, core 4 mm. B) BCP-particles ∅ 2-3 mm, moulded in an 8x8x8 mm chamber slide.

Animals and surgical procedure

Ten beagle dogs were used for the *in vivo* experiment. The age of the dogs was 1.5±0.27 years and weight 13.7±2.1 kg. The procedures were performed under general anesthesia. Anesthesia was initiated using intravenously administered medetomidine (Domitor, Pfizer

Animal Health B.B., Capelle a/d Ijssel, The Netherlands) and propofol (Rapinovet, Schering-Plough Animal Health N.V., Brussels, Belgium). After intubation, it was maintained by inhalation anesthesia with isoflurane, nitrous oxide and oxygen. Amoxicillin with clavulanic acid (Agmentin, SmithKline Beecham Farma B.V., Rijswijk, The Netherlands) were administered intravenously (20 mg/kg body weight) as prophylactic antibiotics before the start of the operation. Both front limbs were shaved, disinfected and draped in a sterile fashion. The ulna was exposed through a craniolateral approach using a longitudinal skin incision ending 4 cm proximal of the ulnar styloid process. The periosteum was preserved, by making a centimeter longitudinal incision, and careful periosteal elevation. A 22.5 mm bone segment was removed using an oscillating saw and ample lavage. In this way a critical-sized bone defect was created (232). The prepared implants were placed in the defects according to a randomized design. The periosteal cylinder was approximated with absorbable suture material. Closure of the subcutaneous tissues and skin was done in a routine fashion. A protective bandage was applied for 3 days. Next, a midline incision was made to expose the paraspinal muscles. For ectopic implantation, intramuscular pockets (in the paraspinal muscle) were created through separate fascia incisions and the constructs were placed in these pockets according to a randomized block schedule (Table 1), after which they were closed by non-resorbable sutures, for localization purposes. The muscle fascia, subcutaneous tissues and skin were closed subsequently in layers. Directly postoperatively X-rays were taken. The dogs received analgesics (Temgesic) for 3 days postoperatively. Full loading of the legs was permitted immediately after surgery. The dogs were nursed until complete recovery from their surgery. After this, they went back to their kennel and fed a standard commercial dog food twice a day with water available *ad libitum*.

Fluorochromes were administered at week 3, (Calcein Green, 10 mg/kg, i.v., Sigma, Zwijndrecht, The Netherlands), week 5 (Oxytetracyclin, Engemycine 32 mg/kg, i.m., Mycofarm, de Bilt, The Netherlands), and week 7 (Xylenol Orange, 80 mg/kg, i.v., Sigma Zwijndrecht, The Netherlands) to analyze onset of bone formation. After nine weeks all animals were euthanized with an overdose of pentobarbital (Organon, Oss, The Netherlands) and radiographs were taken in anterior posterior and medial lateral direction. Samples were retrieved for later histological and histomorphometric analysis.

Histology

The orthotopic and subcutaneous implants were explanted after nine weeks and fixed in 4% formalin, dehydrated by ethanol series and embedded in polymethylmethacrylate (MMA). Two 10-µm-thick longitudinal sections were cut through the middle of each implant using a sawing microtome (Leica, Nussloch, Germany). The first section remained unstained for fluorescence microscopy and the other section was stained with methylene blue and basic fuchsin. High-resolution digital photos were made from the stained sections for histomorphometric analysis. Bone and scaffold were pseudocolored yellow and green respectively, using Adobe Photoshop CS3. KS400 software (version 3, Zeiss, Nussloch, Germany) was used to perform histomorphometry. A custom macro was used to determine the area 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 the percentage bone in available space, called bone area%=(bone area/(total area –scaffold area)) x100% and the percentage bone apposition against the scaffold, contact%=(bone-to-scaffold contact length/scaffold outline) x100%. Fluorochrome labels were analyzed using a light/ fluorescence microscope (X-Cite, EXFO, Mississauga, Canada) equipped with a quadruple filter block (Chroma 104158, Chroma Technology, Rockingham, VT, USA). The presence, or absence, of each of the three fluorochrome labels, was scored for all implants (233).

Statistical analysis

Sample size was determined by performing a power analysis based on histomophometry from earlier studies. Power of 0.9 was used, α 0.05 and minimal difference in bone contact% and area% between the implanted groups was expected to be 40% (with a variance of 30%). For this study at least eight animals were needed. For statistical evaluation of the bone quantification of ectopic implants, a repeated measures analysis was performed to analyze statistical differences between the implanted groups. For the orthotopic implants a Student's t-test was used to analyze statistical differences in bone formation between the two implanted groups. Results are reported as means ± standard deviation and were considered statistical significant at $p < 0.05$.

RESULTS

Animals and surgical procedure

Surgery of the 10 Beagle dogs was performed without any complications (OR time 111.6 ± 16.0 min.) and they recovered well. After surgery the dogs received pain relief and were almost immediately able to stand on both their legs. No dogs were lost during the 9 weeks follow up and no complications in wound healing were observed. Almost all implants were retrieved successfully after 9 weeks of implantation.

Results of the ectopic implants

Histology of the ectopic implants. Explanted ectopic samples were analyzed using basic fuchsin/ methylene blue staining of MMA-embedded sections and showed various amounts of bone in all groups. Strikingly we found extensive bone bridging between different particles especially when BMP-2 fast (BMP*f*) was implanted (Figure 2A). When BMP-2 sustained (BMP*s*) was implanted, newly formed bone was present mostly against the borders of the scaffolds (Figure 2B). No clear signs of degradation of the BCP scaffolds could be observed. No signs of remnants of the MPs or gelatin were found, suggesting they fully degraded. Scaffolds were surrounded by newly formed bone with osteoblasts lining the surface and osteocytes lying in lacunas (Figure 2C). Foreign body multinucleated giants cells were often lining the surface of the scaffold at sites where bone was not present (Figure 2C) and in rare cases they were also found on the bone (Figure 2D). In addition, tissue resembling bone marrow could be seen in the implants (Figure 2D). The newly formed tissue within the implants was well vascularized.

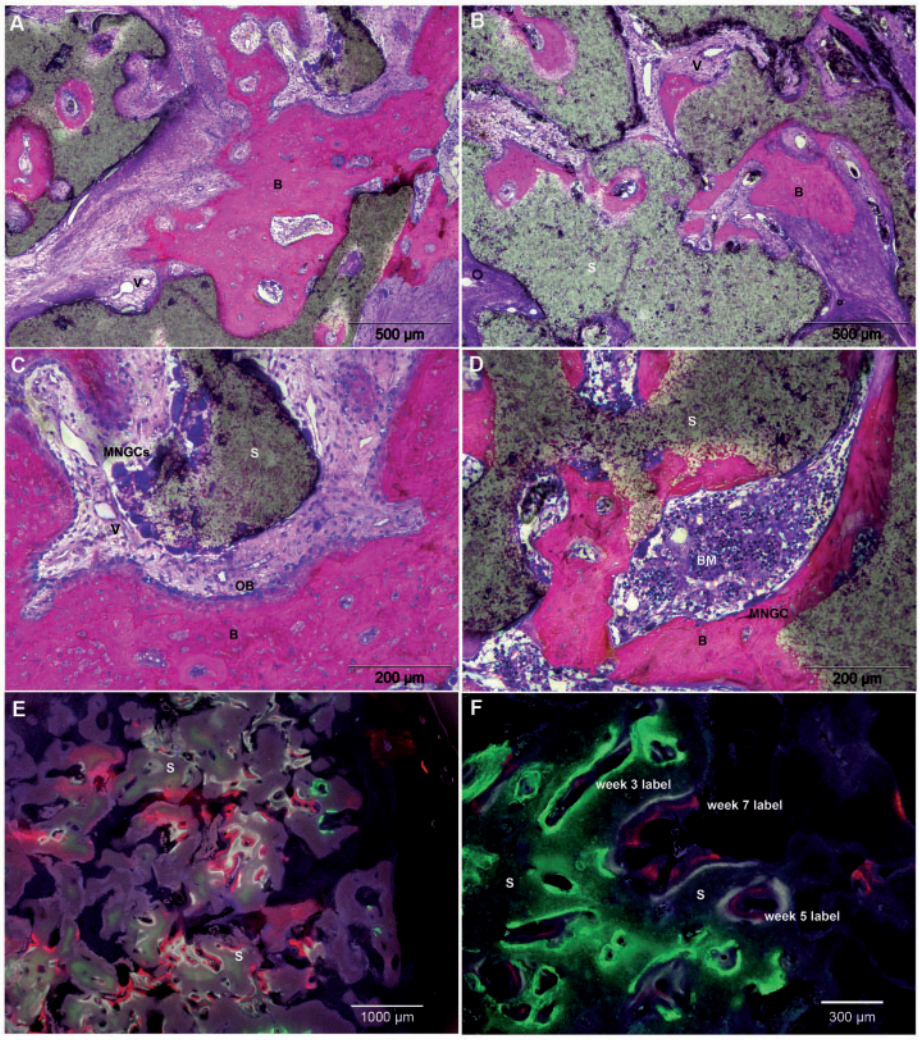

FIGURE 2: Bone formation in the ectopic implants. A) BMP*f*/VEGF*f* group: note the bone bridging between the particles, B) BMP*s*/VEGF*s* group: less to no bridging, C) all groups: bone lining cells/ osteoblast (OB), multinucleated giant cells (MNGCs), D) especially BMP*f* groups: bone marrow, and note the MNGC lying against the bone, suggesting it is an osteoclast. Panel A) to D): Methylene blue/ basic fuchsin stainings. Panel E) and F): fluorochrome detection, green=3-week label, orange=5-week label, red=7 week label. B: bone, MNGC: multinucleated giant cell, OB: osteoblast, OC: osteocyte, V: vessel, BM: bone marrow, S: scaffold.

Onset and growth dynamics of newly formed bone. Analysis of bone growth dynamics by incorporation of fluorochrome labels in the ectopic samples (Table 2) revealed that bone formation almost always started within the first 3 weeks after implantation (in 7/8 or 8/8 implants), except in the VEGF*s* and control groups, where the three-week label (calcein

TABLE 2: Onset of bone formation in ectopic constructs

Condition	Calcein Green week 3 label	Tetracyclin week 5 label	Xylenol Orange week 7 label
BMP*f*/ VEGF*s*	8/8	8/8	8/8
BMP*f*/ VEGF*f*	8/8	8/8	8/8
BMP*s*/ VEGF*f*	7/8	8/8	8/8
BMP*s*/ VEGF*s*	8/8	8/8	8/8
BMP*s*	8/8	8/8	8/8
VEGF*s*	4/7	4/7	4/7
BMP*f*	7/7	7/7	7/7
VEGF*f*	7/8	7/8	7/8
Control	3/8	3/8	3/8

Incorporation of fluorochrome labels at weeks 3, 5 and 7, presence scored per implant.

green) showed less presence. Bone formation had started in the outer rim of the scaffolds, as seen by the green label that was usually present in the periphery of the implants. The yellow (tetracycline) and red label (xylenol orange) was present all over the scaffolds (Figure 2E). A remarkable difference in onset of bone formation was found in the VEGF*s* and control group. Whereas bone formation started within the first 3 weeks after implantation in some scaffolds, it did not start before 7 weeks in the others as indicated by the absence of all 3 fluorochromes.

Bone formation analysis. The amount of bone formed in each group was determined by histomorphometric analysis (Figure 3). Bone contact% and bone area% showed approximately similar results. Largest bone contact% was seen in BMP*f* release groups, ranging from 15.2% ± 8.1 to 25.8% ± 16.1. BMP*f* groups showed significantly higher bone contact% than BMP*s* release groups ($p<0.01$) (area%, 22.2% ± 10.9 to 34.0% ± 8.4, $p<0.01$). The addition of VEGF had no statistically significant effect on bone contact% ($p= 0.239$), or on area% ($p= 0.132$) (Figure 3). Overall, BMP*f* groups induced more bone than BMP*s* groups. BMP*s* groups showed bone contact% of 7.0% ± 4.7 to 9.9% ± 7.5 and bone area% of 4.8% ± 4.5 to 10.6% ± 7.8, which was higher than VEGF*s* and VEGF*f* groups. Furthermore, no significant differences in bone area% and bone contact% were seen between VEGF*s* and VEGF*f* groups. Control scaffolds without growth factors showed little bone formation in only 3/8 samples. Bone formation in these controls was variable, 0.5% ± 0.27 bone area% and 1.17% ± 0.65 bone contact%.

Results of the ulnar implantation

Radiographs of the ulnar implants, postoperative and after euthanization. The postoperative radiographs showed similar placement of all ulnar implants approximately 2 cm above the styloid process of the ulna (Figure 4A). Radiographs after termination showed bone formation in the direction of the implants (Figure 4B, C). Some of the implants had been broken as was seen in the proximal part of the scaffold in Figure 4C. A radiolucent line

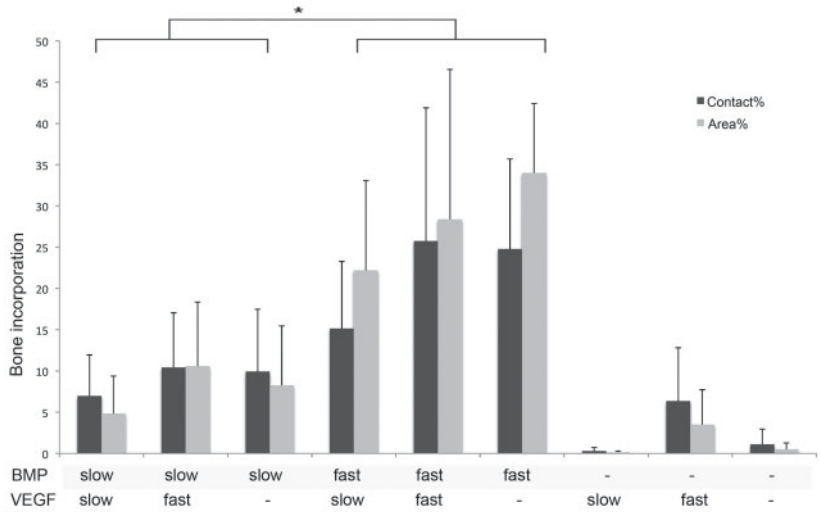

FIGURE 3: Bone quantification in ectopic implants: bone contact% and bone area%, both after 9 weeks of ectopic implantation in the dog. The results are represented as mean ± standard deviation. BMP*f* groups show significant higher bone contact% and area% compared to the BMP*s* groups (*p<0.01), without an effect of VEGF. *f*=fast, *s*=sustained.

(Figure 4C, arrow) was seen in all radiographs mainly at the proximal part of the scaffolds, which might indicate pseudoarthrosis.

Histology of the ulnar implants. Although some of the implants had broken edges, no clear signs of scaffold degradation were observed. Bone formation could be seen in all growth factor-loaded implants. Most of it was found in the scaffold material and less in the central canal of the cylinders (Figure 5A). Overall, aspect of the newly formed bone was comparable to the ectopic implants and contained bone lining cells, multinucleated giant cells (MNGCs) and blood vessels (Figure 5B). In contrast to the ectopic implants, cartilage had formed outside the proximal border between the scaffold and ulna (Figure 5C). This could indicate endochondral ossification taking place. The controls showed only small amounts of newly formed bone in 3 out of 4 implants.

Onset and growth dynamics of newly formed bone. For analysis of bone growth dynamics, the ulnar implants were divided into three regions (Table 3): 1) the scaffold material itself, 2) the central core of the scaffold and, 3) outside the scaffold. In 6 out of 8 dogs, bone formation started in the BMP*s*/VEGF*f* groups before 3 weeks (calcein green label) in the pores of the scaffold (Figure 5D). In the BMP*f*/VEGF*s* it always started before 3 weeks. Outside the scaffolds bone was always present independent of which group it was. In the central canal of the scaffold bone formation started almost always between 3 and 5 weeks. Only in the control groups, bone formation started later than 7 weeks in 50 % of the cases.

Bone formation analysis. Bone was quantified within the pores of the implant. Bone growth in the central canal and outside the scaffold was excluded for the calculation of bone contact% and bone area%. Comparable to the ectopic implants, results of bone contact%

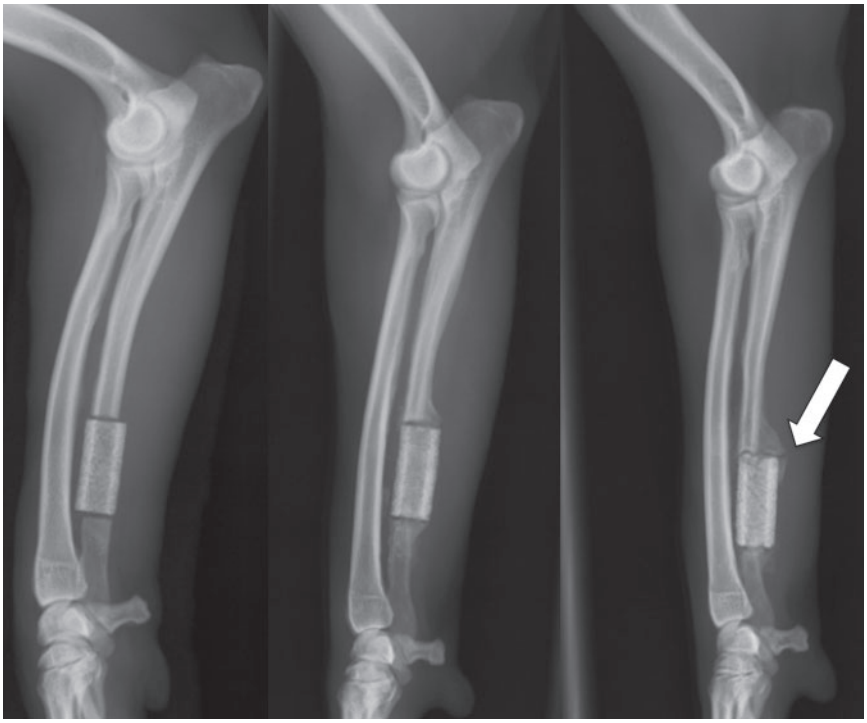

FIGURE 4: Medio-lateral X-rays. A) post operatively, B) post euthanization, intact scaffold with obvious bone formation, C) post euthanization, broken scaffold with obvious bone formation towards the scaffold.

TABLE 3: Onset of bone formation in orthotopic (ulnar) constructs.

Condition Within scaffold	Calcein Green week 3 label	Tetracyclin week 5 label	Xylenol Orange week 7 label
BMP*s*/ VEGF*f*	6/8	8/8	8/8
BMP*f*/ VEGF*s*	8/8	8/8	8/8
Control	2/4	3/4	3/4
Central canal			
BMP*s*/ VEGF*f*	1/8	7/8	7/8
BMP*f*/ VEGF*s*	1/8	8/8	8/8
Control	1/4	2/4	2/4
Outside scaffold			
BMP*s*/ VEGF*f*	8/8	8/8	8/8
BMP*f*/ VEGF*s*	8/8	8/8	8/8
Control	4/4	4/4	4/4

Incorporation of fluorochrome labels at weeks 3, 5 and 7, presence scored per implant.

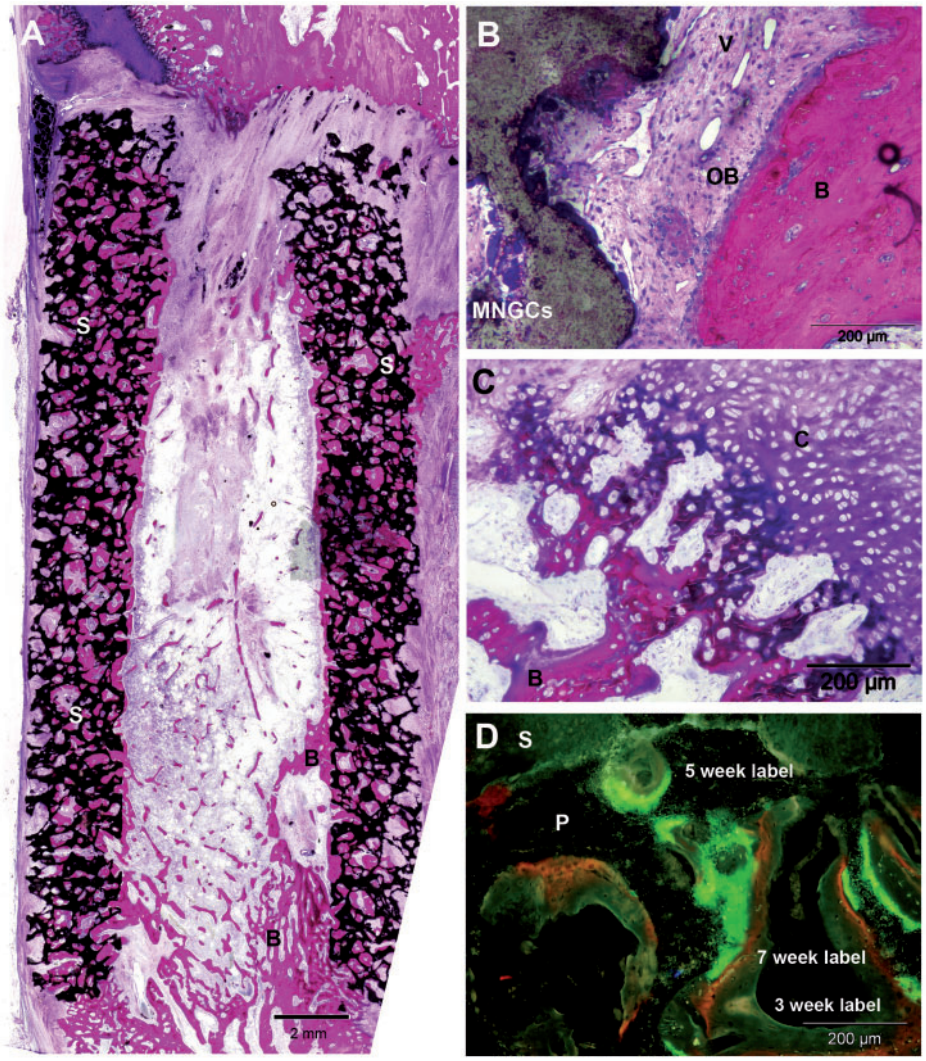

FIGURE 5: Histology shown in orthotopic (ulnar) implants. A) BMP*f*/VEGF*s* group: complete scaffold, B) extensive bone formation with bone lining cells/ osteoblast, C) cartilage formation seen in especially the proximal parts of several scaffolds, D) Fluorochrome detection. Panel A) to C): Methylene blue/basic fuchsin stainings. Panel D): fluorescence microscopy, green=3-week label, orange=5-week label, red=7 week label. B: bone, MNGC: multinucleated giant cell, OB: osteoblast, V: vessel, S: scaffold, C: cartilage.

and area% are quite similar. No significant differences in bone quantity between BMP*f*/VEGF*s* and BMP*s*/VEGF*f* groups could be observed (P=0.506). Bone contact% for BMP*s*/VEGF*f* was 21.5% ± 8.4 and 19.4% ± 12.3 for BMP*f*/VEGF*s*. Both growth factor containing groups did show significant differences compared to the control groups (P<0.01) (Figure 6).

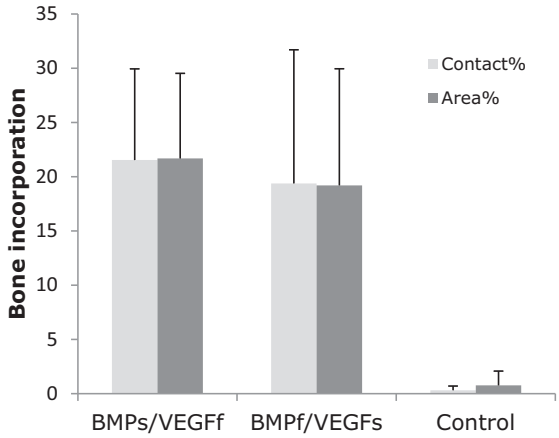

FIGURE 6: Bone quantification in orthotopic (ulnar) implants: bone contact% and bone area%, both after 9 weeks of orthotopic implantation in the dog. The results are represented as mean ± standard deviation. BMP*f*/VEGF*s* and BMP*s*/VEGF*f* groups both show significant higher contact% and area% compared to the control groups ($p<0.01$), but no significant effect compared to each other ($p=0.506$).

DISCUSSION

The aim of this study was to assess whether the timing of a dual release of BMP-2 and VEGF is important in enhancing bone formation in an ectopic and orthotopic (ulnar) implantation model in dogs, since they are the most important regulators in osteogenesis and angiogenesis. To obtain a dual release profile, BCP scaffolds were combined with PLGA microspheres and a gelatin hydrogel as controlled delivery vehicles. MPs were used for the sustained release, and gelatin for a more rapid release. These constructs were implanted at an ectopic as well as an orthotopic (ulnar) location in dogs. The amount of growth factor incorporated in the MPs was based on the entrapment efficiency measured in an earlier study by radiolabeling the BMP-2 and the fact that they showed an almost complete release without retention (229). We have presumed a complete release of growth factors in this larger animal model, because no signs of the MPs or gelatin were found in the histological samples. Previously it has been shown that incorporation of growth factors in gelatin results in a rapid release in a two-week period (225, 234, 235) and PLGA has been widely used for sustained release profiles of VEGF and BMP-2, capable of releasing growth factors for an eight-week period *in vivo* (225, 235, 236).

The early osteogenic effect of both growth factors, analyzed by calcein green fluorescence, was clearly present at both the ectopic and orthotopic location (within the scaffold). Although there was no significant influence of the VEGF on bone area and contact percentage after 12 weeks, fluorochrome analysis suggested an effect of timing of VEGF in the absence of BMP-2. Whereas only 4 out of 7 VEGF*s* scaffolds showed the presence of the 3-week label, calcein green was present in 7 out of 8 scaffolds in the VEGF*f* group. The difference in early bone formation in both ectopic VEGF groups might be attributed to both the vasculogenic and the chemotactic properties of VEGF on osteoblasts, osteoclasts and endothelial cells (92, 237). These properties are important during the first weeks of the implantation period and the gelatin has shown a high initial burst release during the

first few days in our previous study. Therefore the release from the MPs, which had a more sustained release profile over 56 days in this same study, might be too slow to exhibit the chemotactic effect (225). In the orthotopic implants, bone formation within the growth factor containing scaffolds also started earlier compared to the control group. However also in these control groups 50% of implants showed some early bone formation especially at the periphery, probably resulting from an osteogenic effect of the periost covering these implants. The later onset of bone formation observed in the cavity of the orthotopic implants is most likely due to the lack of cells and blood vessels in that location.

The role of the dual delivery of VEGF and BMP-2 on the total amounts of *in vivo* bone formation ectopically, analyzed by histomorphometry, is diverse. A very clear effect of timing of BMP was seen. Significantly more bone was found in the BMP*f* groups compared to the BMP*s* groups without an influence of VEGF in either form. Since there is little difference between the 5 and 7-week incorporation of fluorochromes, initiation of bone formation appears to be the determining factor in these experiments. Therefore, the lower BMP-2 concentrations at early time-points in the sustained delivery vehicles are less effective to initiate bone formation. Since previous studies showed a similar sustained BMP-2 release profile from a microsphere/polymer composite was more efficient than the rapid BMP-2 release from the gelatin hydrogel, this effect cannot be exclusively attributed to the pharmacokinetics (225, 231). The prolonged local BMP-2 retention in the microsphere containing gelatin hydrogel might make it more susceptible to spontaneous and/or enzymatic degradation and might result in lower total dose of BMP-2 available from sustained delivery vehicles. As a result, the microsphere-gelatin composite is less attractive as a sustained delivery vehicle for tissue engineering.

As mentioned previously, no significant additional effect of VEGF was seen on the amount of ectopic bone formation, which is similar to results of earlier studies on bone formation in a rat critical-size cranial defect model (227, 238), where even higher amounts of VEGF were used. Nevertheless, VEGF groups without BMP-2 showed some bone formation in this study, which might be attributed to the earlier described chemotactic properties of VEGF (92, 237). This only account for the VEGF*f* group, and was not significant. Other studies did see an additional effect of VEGF ectopically (225, 228, 239). The discrepancy might be that VEGF was not a restrictive factor in our study and furthermore the use of a different kind of scaffold material. In this study we used the osteoconductive ceramic BCP, as opposed to polymer scaffolds in other studies. This ceramic induced differentiation of multipotent stromal cells (MSCs) towards osteoblasts, which efficiently synthesize VEGF and thereby render the addition of extra VEGF ineffective (239, 240). Furthermore the surgical procedures resulted in local bleeding and haematoma formation in the muscle of the dogs, which could have served as a local source for other chemotactic and angiogenic growth factors contributing to early bone regeneration both at the orthotopic and ectopic location (241, 242).

In the orthotopic location, no statistical significant differences were seen between BMP*f*/VEGF*s* and BMP*s*/VEGF*f* groups, indicating that timing of release of BMP-2 and VEGF has no influence on orthotopic bone regeneration in this setting. The different effects of

growth factor release in the ectopic and orthotopic site might be explained by the location. Overall, the orthotopic defect is less challenging with regards to MSC recruitment. The periost covering the ulnar implants and the exposed marrow cavity contain MSCs, which could have contributed to this bone formation making it not a very challenging model. The osteoinductive properties of the scaffold material allow these cells to differentiate into osteoblasts to form bone within the pores of the scaffold (173). It is possible that the BMP-2 and VEGF were both needed to initiate the bone healing cascade, but that the timing of this release was not important since other (growth) factors and MSCs had a more prolonged effect on this bone formation. Although bone quantification was only performed within the pores of the implant, bone formation was also observed along the borders and the central cavity of the implant. However, radiological and histomorphometric analysis showed no large differences between these groups.

CONCLUSION

This study showed a positive effect on bone formation, however differential results on bone formation induced by timed release of BMP-2 at the orthotopic and ectopic locations. No significant influence of timing of dual VEGF and BMP-2 delivery was seen on orthotopic bone regeneration, whereas at the ectopic site timing of BMP-2 release significantly influences bone formation.

ACKNOWLEDGEMENTS

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7

INSTRUMENTED POSTEROLATERAL SPINAL FUSION IN GOATS USING ALLOGENEIC MSCS IN ADDITION TO A NEW β-TCP

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ABSTRACT

After decades now, bone tissue engineering by combining mesenchymal stem cells and a synthetic scaffold has still not resulted in randomized controlled trials, implying no clinical success so far. In this pilot study the value of multipotent stromal cells (MSCs) to establish bone bridging was investigated in the context of tricalcium phosphate (TCP) scaffold used for instrumented posterolateral spinal fusion (PLF) in goats. Constructs for 4 study groups were implanted during double level instrumented spinal fusion of L2-3 and L4-5 (n=4). To see whether the model is applicable, decortication alone was applied as a negative control and MSC-seeded TCP was compared to cell-free TCP and autologous bone. The onset of bone formation was monitored by fluorochrome administration. After four months the animals were euthanized and CT scans were performed, after which the samples were processed for histology. The negative control group showed hardly any bone formation and fusion occurred in one sample, consisting of a thin layer of bone over the facet joint. In the implantation groups there was extensive bone formation and a mean fusion rate of 67%. The onset of bone formation, indicated by fluorochrome incorporation, was not enhanced by the presence of MSCs. Osseointegration was seen in histological sections of all implant groups, showing woven bone and clear characteristics of active bone formation like osteoblasts, osteocytes, vessel formation and bone marrow. Furthermore, there were signs of biodegradation of the calcium phosphate particles indicated by fluorochrome incorporation and histology. These results indicate that the application of well-performing TCP material might preclude cell seeding for PLF.

INTRODUCTION

In orthopedics instrumented posterolateral spinal fusion is a commonly performed and successful procedure in a wide range of spinal disorders (243). Usually autologous bone grafts are harvested from the iliac crest to create a bony bridge between the posterior elements and in this way stabilize the spine. However, the use of autologous bone has several disadvantages like donor-site pain and when used in spinal fusion surgery, pseudoarthrosis rates have been reported to range from 5 to 44% (3). For many years researchers are trying to create an alternative in the form of tissue engineered bone. An ideal graft meets at least the following characteristics: it is biocompatible, biodegradable, has suitable mechanical properties, and stimulates bone formation by osteoconduction and preferably osteoinduction. Great potential has been attributed to cell-based bone tissue engineering. Tissue engineered bone constructs, in which multipotent stromal cells (MSCs) are seeded on porous osteoconductive and/or -inductive scaffolds, are successful in small and some large animal models (150, 181, 182). When autologous MSCs are used, they need to be harvested, isolated and expanded, which takes considerable time and implicates that it cannot be off-the-shelf. To address this issue, allogeneic MSCs may be used, which can be isolated, processed and stored in advance and applied in clinically relevant models. Strong indications that allogeneic MSCs do not elicit a strong immune response *in vitro* (24, 25) and *in vivo* have become available (26, 27). When comparing autologous and allogeneic MSCs, no significant differences were found in the amounts of bone formed depending on the implantation site. At ectopic implantation locations, both cell sources enhanced bone formation, but in a spinal cassette either cell source did not result in more bone compared to cell-free scaffolds (128). The results of these studies indicate great potential for allogeneic MSCs in preclinical research, in particular at implantation locations with limited contact to underlying bone.

So far, many preclinical studies have been performed trying to mimic natural bone formation by combining autologous MSCs with porous scaffold materials, which showed some promising results especially in segmental defects (150, 182, 244). However, this has not yet reached clinical application on a large scale, and only some case studies are available (245, 246). This may be partly explained by the scaffold properties used in the aforementioned studies. Most ceramic materials are not osteoinductive, and therefore need osteogenic factors or cells to promote and induce bone formation at an ectopic location. A recently developed ceramic material, which consists entirely of β-tricalcium phosphate (TCP), shows excellent bone formation when analyzed *in vivo* (141). This TCP was shown to be osteoinductive in ectopic implantations in dogs and sheep, and the material was used for spinal fusion as well as in a critical size model in the iliac wing in sheep. An important advantage of the TCP is the far better bioresorbability as compared to HA and BCP which allows for complete resorption of the material in time, leaving natural bone only.

In this pilot study we used the new ceramic material β-TCP in a clinically relevant instrumented posterolateral spinal fusion in goats. The aim was to assess whether the osteoinductive and -conductive capacities of this material alone results in a bony fusion (determined histologically and on CT scans) and whether the addition of allogeneic MSCs enhances the onset of bone formation and the total amount of newly formed bone after 16 weeks.

MATERIALS AND METHODS

Animals

Four adult female Dutch milk goats, aged 20-28 months, 51-66 kg, were used, after approval of the local Ethical Committee for Animal Experimentation and in compliance with the Institutional Guidelines on the use of laboratory animals. Food and water were given ad libitum. The animals' general health and care conditions were recorded in a diary of well-being for each goat separately and monitored by the laboratory animal welfare officer.

MSC isolation and culture

Bone marrow (BM) was aspirated from both iliac wings of one goat used in an earlier study (129). The relative numbers of MSCs in this bone marrow were assessed, by performing a colony forming efficiency assay (CFU-E) (9). Two fractions of $1x10^5$ mononuclear cells (MNCs)/cm^2 were cultured in standard medium containing αMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin, 2 mM L-glutamine (all Invitrogen), and 15% fetal bovine serum (FBS, Cambrex, Verviers, Belgium), which was refreshed every 3 days. After 9 days the colonies were washed, fixed in 4% formalin, stained using methylene blue and counted under an inverted microscope. The remaining aspirate was plated and the isolated MSCs were expanded and cryopreserved after second passage in 1 ml aliquots of 10^7 cells. Multipotency of MSCs was established in parallel experiments using routine assays (128).

Scaffolds and seeding conditions

TCP ceramics were prepared from TCP powder (Plasma Biotal, Buxton, Derbyshire, United Kingdom) and sintered at 1100°C. Ceramic particles (1-2 mm) were prepared, cleaned ultrasonically with acetone, 70% ethanol and demineralized water, dried at 80°C, and sterilized by gamma irradiation prior to use (141). For each spinal implant 10 ml of TCP particles was used. At het day of surgery, the passage 2 MSCs were thawed on ice, thoroughly washed in culture medium containing 30% FBS and aliquoted tot $5x10^6$ MSCs/ cm^3 scaffold for peroperative seeding, before being taken to the operating room (247). During surgery blood was drawn from the goats (25 ml) and autologous plasma was prepared by centrifuging at 1200 *g* in a Falcon tube (Greiner, Frickenhausen, Germany) for 10 min. Next, the cell suspensions were centrifuged at 300 *g*, medium was decanted and the MSCs were resuspended in the previously prepared plasma. The suspension was drop-seeded on the scaffolds, $50x10^6$ MSCs/ spinal implant (Table 1). After this the constructs were allowed to polymerize at room temperature for approximately 15 minutes before implantation.

Surgery and implantation

The procedures were performed under general anesthesia using 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 the paraspinal muscles from L1 to L5. Next the fascia was opened and the paraspinal muscles

TABLE 1: Implantation groups.

Group	Function	MSCs	Material	Amount
Empty	negative control			
Autologous bone	positive control		Autologous bone	10cc
TCP	experimental group 1		TCP 1-2 mm particles	10cc
TCP+ MSCs	experimental group 2	$50x10^6$	TCP 1-2 mm particles	10cc

TCP= β-tricalciumphosphate, MSCs= multipotent stromal cells

were subperiosteally stripped from the posterior spinous elements and retracted laterally. The available cortical surfaces were decorticated, including the transverse processes, spinous processes, laminae and the dorsal and lateral aspects of the pars interarticularis. Pedicle screws were inserted and interconnected with rods (BWM-system, Stryker Howmedica Osteonics, Allendale, NJ, USA) at the levels L2-3 and L4-5, leaving L6 uninstrumented. For autologous bone harvesting, both iliac crests were cleared of muscle tissue. Under constant saline cooling, a central Kirchner wire was drilled to guide a ∅19 mm trephine. The holes were filled with TCP putties that will not be discussed in the current paper. The cores were morcellized and provided about 10 ml of autologous bone graft. Based on a prefilled allocation sheet, the implants were placed at one side of fusion segment in the decorticated dorsolateral gutter. In addition to the spinal fusion experiment, several intramuscular implants were investigated and on L1 spinal cages were placed. All implant groups were present in all animals and placed in a randomized fashion and therefore we expected no interference with the spine fusion experiment. To monitor the onset and dynamics of bone growth, the goats received fluorochrome labels at week 3 (Calcein Green, 10 mg/kg, i.v., Sigma, Zwijndrecht, The Netherlands), week 5 (Oxytetracyclin, Engemycine 32 mg/kg, i.m., Mycofarm, de Bilt, The Netherlands), and week 9 (Xylenol Orange, 80 mg/kg, i.v., Sigma). After 16 weeks all animals were euthanized with an overdose of pentobarbital (Organon, Oss, The Netherlands).

Radiological examination

At 16 weeks, the lumbar spine was explanted and fluoroscopy X-rays were made. Most of the soft tissue was stripped of the spines, instrumentation was removed, and computer tomography (CT)-scans were performed on a 64-slice scanner (Philips Brilliance; Philips Medical Systems, Best, The Netherlands). Sagittal and coronal reconstructions were made. These scans were blindly evaluated by two investigators (RG and MK) and were graded as fused or not fused or undetermined in both planes.

Histological processing

After scanning, the segments were divided into a left and a right side by sawing in the middle of, and parallel to, the spinous processes (sagittal plane). After explantation the

spinal implants were fixed in 4% formalin, dehydrated by ethanol series and embedded in polymethylmethacrylate (MMA). Two 10 µm thick sections were made in the transverse plane using a sawing microtome (Leica, Nussloch, Germany) on three different levels, a proximal, middle and distal section (Figure 2). The proximal and distal sections were in the proximity of the pedicle screws. The first section remained unstained for epifluorescence microscopy and the second section was stained with methylene blue and basic fuchsin. The purpose of the histology was to assess the overall morphology of *de novo* bone, the residual implant material, the maturity of the bone and the presence of osteoblasts, osteocytes, osteoclasts/ multinucleated giant cells (MNGCs) as well as the presence of other cell types, like lymphocytes, at the fusion sites. Analysis was by regular light microscopy (Olympus-BX51).

Fluorochrome markers were scored using a light/fluorescence microscope (E600, Nikon, Amstelveen, The Netherlands) equipped with a quadruple filter block (XF57, dichroic mirror 400, 485, 558 and 640 nm, Omega Optics, Brattleboro, VT, USA).

RESULTS

From the iliac crests of a female Dutch milk goat in a previous experiment, 10.5 ml of bone marrow was aspirated, which contained $14x10^6$ MNCs/ml. The CFU-E of the aspirate was determined; resulting in an average of 1.8 colonies per 100.000 MNCs, meaning approximately one out of 55.000 had attached and formed a colony (128), and thus was considered a MSC. The PLF location was used to analyze whether TCP alone can provide a spinal fusion and whether MSCs contribute to bone formation. This was compared to the 'golden standard' autologous bone derived from the iliac crest. The effect of decortication alone was analyzed in an empty group.

A*nalysis of the fusion mass using CT*. In the empty group, in three out of four (25%) animals no fusion and no bone were found in the coronal and sagittal plane. In one animal a fusion was seen with minimal bone formation over the facet joint. In the autologous bone group fusion was found in two out of four animals (50%) and in all animals moderate bone formation was found (Figure 1B, C), only in one animal in the coronal plane extensive bone formation. The cell-free TCP group showed fusion and extensive bone formation in both planes in three animals (75%). In the cell-seeded TCP group fusion was found in both planes in two animals. In the other two animals fusion was found in either the sagittal or the coronal plane, meaning that in the other direction no fusion was observed. Nevertheless, in all animals receiving TCP extensive bone formation was found (Table 2), without any obvious differences between the MSC-seeded (Figure 1A) groups and cell-free groups (1B, C).

Bone formation in the spinal fusion mass. Analysis of the explanted samples by basic fuchsin/methylene blue staining of MMA-embedded sections revealed that various amounts of bone were present in the different groups. In all groups, a small layer of connective tissue had formed around the pedicle screws and the connecting rods. When bone was present, it was predominantly medial from the connecting rod, which was in accordance with the placement of the TCP material or autologous bone (Figure 2A, B, D). The group where decortication took place during surgery, but no material was implanted (empty group),

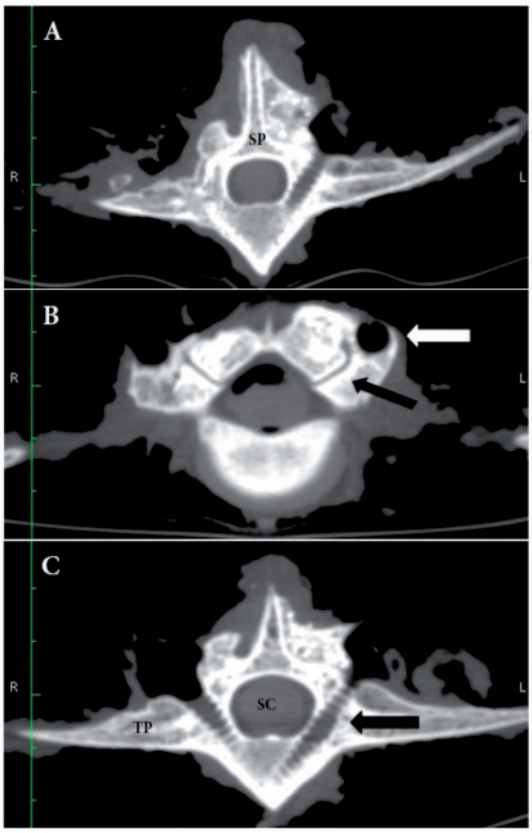

FIGURE 1: CT-scans of the spinal fusion mass. A) MSC-seeded TCP group (L2-3, right side) and empty group (left side), note the extensive fusion mass and the placement of pedicle screws, B) TCP group (L4-5, right side) and autologous bone group (left side) with a fusion mass covering the facet joint (black arrow), opening of connecting rod visible (white arrow), C) TCP group (L4-5, right side) and autologous bone group (left side), note extensive fusion mass and pedicle screw placement (black arrow). SP: spinous process, TP: transverse process, SC: spinal canal.

showed limited bone formation. In this group one animal showed fusion of the facet joint, little bone formation and radiological fusion. The autologous bone implants showed lamellar bone and extensive formation of bone marrow and blood vessels (Figure 2C). Osseointegration between the newly formed bone/scaffold material and the host bone was seen between the new woven bone and the host lamellar bone in both the autologous bone group and the TCP based groups (Figure 2A, B, C). Between the two TCP containing experimental groups no obvious differences were found. Woven bone had formed against the border of the scaffold material and between the scaffolds (all figures containing TCP). At the sites of bone formation often osteoblasts were lining the surface of the bone and osteocytes were lying in lacunas in the newly formed bone (Figure 2E, F). Around the scaffolds, in the scaffold pores and in between the scaffolds also bone marrow and vessel formation was seen (Figure 2D). At places where less material was placed disintegrated scaffolds were seen. At sites where extensive degradation of the scaffold material was apparent, often no bone was present, and at sites of extensive bone formation little degradation was seen (Figure 2A, B). No signs of immunologic response were seen similar to previous work (128), for we did not see any signs of acute inflammation, like swelling, redness of the implantation

TABLE 2: Bony fusion in CT scans.

Group	Animal	Sagittal fusion	Sagittal bone	Coronal fusion	Coronal bone
Empty	1	no	nihil	no	nihil
	2	no	nihil	no	nihil
	3	no	nihil	no	nihil
	4	yes	*	yes	*
Autologous	1	no	*	no	*
	2	yes	*	yes	*
	3	no	*	no	*
	4	yes	*	yes	**
TCP	1	yes	**	yes	**
	2	yes	**	yes	**
	3	no	*	no	*
	4	yes	**	yes	**
TCP+ MSCs	1	no	**	yes	**
	2	yes	**	yes	**
	3	yes	**	no	*
	4	yes	**	yes	**

Scans were performed in two directions, a sagittal and coronal direction. Scorings were performed for the amount of bone (* moderate bone formation, ** extensive bone formation) and fusion/non-fusion.

site and fever. Furthermore no clusters of lymphoid cells were present in the allogeneic MSC-seeded constructs when implanted at this spinal fusion location, compared to the ectopic implantation locations seen previously (129). Furthermore little foreign body multinucleated giant cells (MNGCs) were present compared to earlier studies (Figure 2G), where they were often seen when using other materials like BCP or pure HA (128).

Onset of bone formation during spinal fusion. Analysis of bone growth dynamics by incorporation of fluorochrome labels in the spinal fusion samples (Figure 3 and Table 3) revealed that bone growth started before three weeks in 50 % of the implants, except for the empty group where the green label was absent in newly formed bone. In one animal in this group there was a fusion over the facet joint, in which only the week-9 label (red signal) was present (Figure 3E). No obvious differences were seen in the amount of labeling between the autologous bone and the TCP-based groups (Figure 3A, B and F). Bone formation most often started (as indicated by the week-3 green label) in the transition zone from the host bone to newly formed bone (all groups) and interestingly also around the TCP particles (Figure 3B, white arrow) in proximity to the host bone. This observation was not related to the seeding of the scaffold with cells. The fusion mass most distant from host bone contained primarily the week-9 label (red, Figure 3E, arrow) and occasionally the week-5 label (yellow, Figure 2A). In some areas we found an inversion of the label order

TABLE 3: Onset of bone formation in spinal constructs.

Condition	Calcein Green week 3 label	Tetracyclin week 5 label	Xylenol Orange week 9 label
Empty	0/4	1/4	3/4
Autologous	2/4	4/4	4/4
TCP	2/4	4/4	4/4
TCP+ MSCs	2/4	4/4	4/4

Incorporation of fluorochrome labels at weeks 3,5 and 7, presence scored per implant.

(Figure 3B), the red label being closest to the scaffold material, which is in contrast to earlier studies (128). These were areas where degradation of the scaffold was apparent and may indicate that at those locations the ceramic scaffold is actively resorbed and replaced by newly formed bone.

DISCUSSION

The aim of this study was to assess whether the osteoinductive and -conductive capacities of TCP alone results in a bony fusion (determined histologically and on CT scans) and whether the addition of allogeneic MSCs enhances the onset of bone formation and the total amount of newly formed bone after 16 weeks. We found that autograft did not result in a bony fusion in 100% of the cases, which is comparable to clinical and experimental practice (248). Decortication alone resulted in very little bone formation and a 25% fusion rate. Cell-free TCP implants as well as MSC-seeded TCP implants resulted in comparable fusion amounts without a clear contribution to bone formation by the MSCs.

The increased use of bone graft substitutes to replace autologous bone with its negative traits stimulated the technology of cell-based bone tissue engineering, however its practice is still far from standard. A posterolateral spinal fusion is challenging with respect to its combination of ectopic and orthotopic bone formation, with the ectopic part of this location not directly contacting bone.

We examined whether the posterolateral spinal fusion model is representative for the clinical situation in terms of fusion rate and bone formation without adding graft. Therefore, we set up a negative control group of decortication alone. The fusion rate in this group turned out to be very low, and only a bony ridge covering the facet joint with barely any bone formation present in the sagittal and coronal planes was seen both on CTs as well as histologically. From this, in combination with the autograft rate, we concluded that the model is comparable to clinical practice and suitable for spinal fusion efficiency studies. A great advantage of this model is the possibility to implant four different groups in one animal, by performing a multilevel fusion and contralateral different groups. Contralateral groups can be used because the instrumentation reduces (micro-) movement and therefore spinal fusion rate is not influenced at the contralateral side. This creates the opportunity to

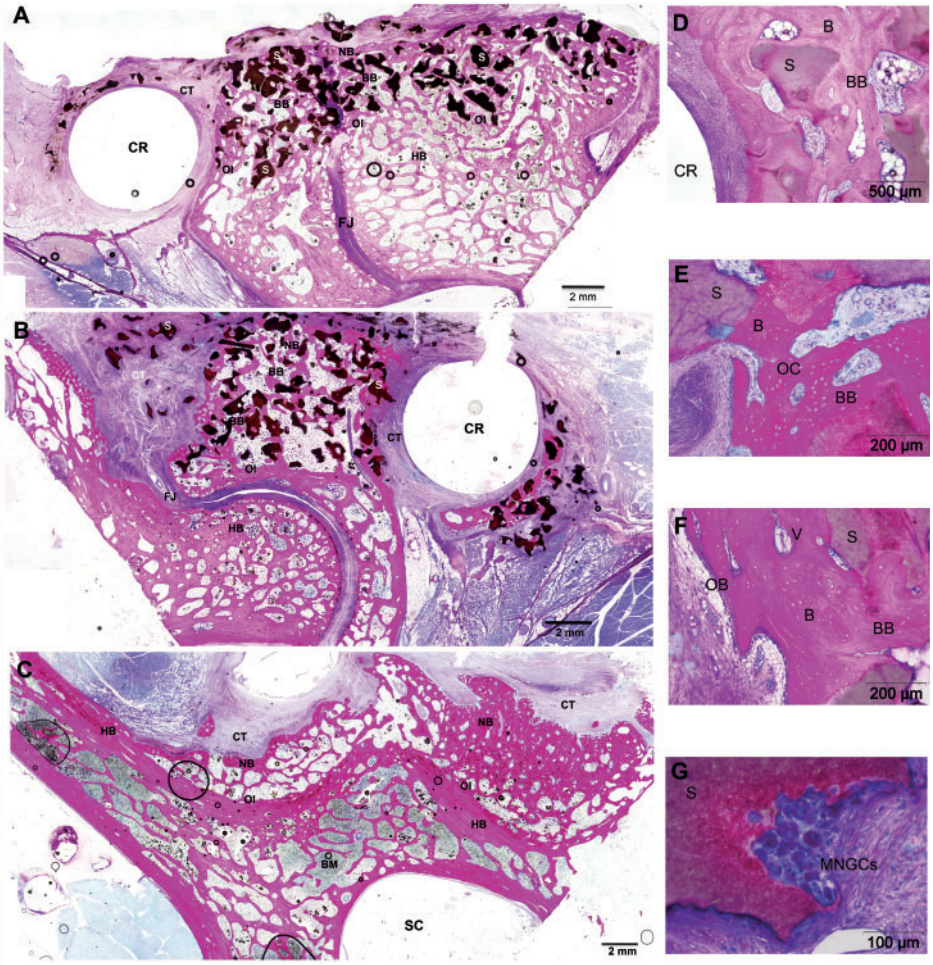

FIGURE 2: Bone formation in spinal implants. A) MSC-seeded TCP group, middle section, fusion over facet joint, osseointegration between scaffold and host bone, B) TCP group, middle section, no fusion over facet joint, osseointegration between scaffold and host bone, C) autologous bone group, proximal section, D) MSC-seeded TCP group: bone bridging, connecting rod with connective tissue on the left, E) MSC-seeded TCP group, detail: bone bridging, osteoclasts lying in lacunas in newly formed bone, F) TCP group, detail: bone bridging, vessel formation, osteoblast lining the surface of the newly formed bone, G) MSC-seeded TCP group, detail: multinucleated giant cells lining the scaffold material. HB: host bone, NB: newly formed bone, FJ: facet joint, CR: connecting rod location, CT: connective tissue, BM: bone marrow, OI: osseointegration, SC: spinal canal, BB: bone bridging, B: bone, S: scaffold, V: vessel, OB: osteoblast, OC: osteocyte, MNGCs: multinucleated giant cells.

perform paired measurements when performing statistical analysis, and reduce the amount of animals used per experiment by 50%.

The potential of the TCP in terms of functional and histological behavior (osseointegration, bone bridging and scaffold degradation) was analyzed. It was not

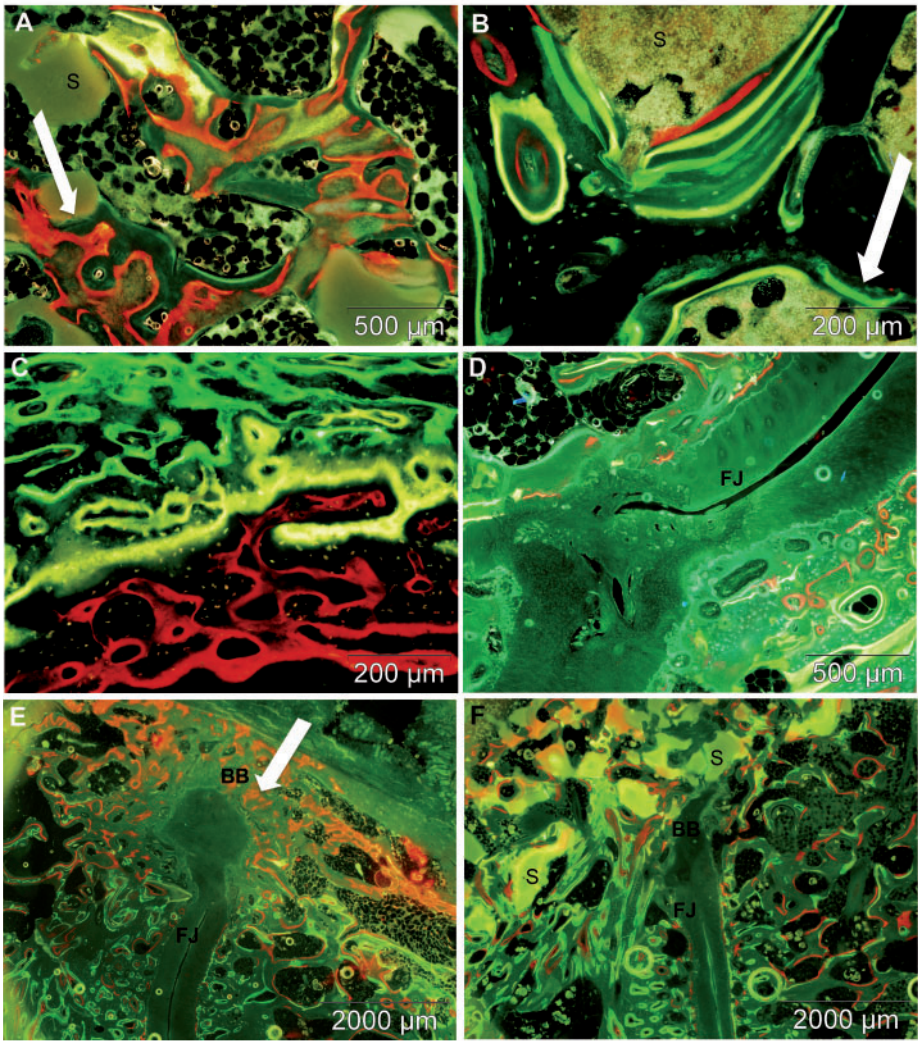

FIGURE 3: Fluorescence microscopy of spinal implants. Fluorochromes injected at week 3 (Calcein green, green), 5 (Oxytetracyclin, yellow) and 9 (Xylenol orange, red). A) MSC-seeded TCP group, bony bridges between scaffolds containing mainly the red label, B) TCP group, nearby the transitional zone of host and newly formed bone, all labels are present, note the label order in the direction of the scaffold, C) autologous bone group, lamellar bone formation in the transverse process, D) empty group, facet joint, no fusion and no label distally, E) empty group with fusion, only the red label is present in bone bridge, F) MSC-seeded TCP group, fusion over the facet joint, all labels present. P: pore, S: scaffold, B: bone, BB: bone bridge, FJ: facet joint.

possible to draw any statistical conclusions from this pilot study. However, when looking at both CT and histological findings we saw a tendency of TCP to perform comparable to the autograft. It must be emphasized that there was a follow-up of 4 months, and that spinal fusion percentages might be higher at later time points. A bony fusion was found in 75% in

both the cell-seeded TCP and cell-free TCP groups. Extensive osseointegration with host bone was observed, which is of importance for spinal fusion; furthermore bone bridging between the TCP particles was found, which is in contrast to earlier studies with ceramic materials (HA and BCP) where bone was found merely against the border of the scaffold material and not crossing over to other particles (128, 233). Ideally the calcium phosphate compound provides a scaffold for bony ingrowth and the development of a fusion, but degrades when the fusion develops. When looking at the fluorochrome order we found for the first time that the red label was closest to the scaffold material followed by the yellow, green and again yellow label (Figure 3B). This might mean that the above-described process had taken place, resorption of scaffold material and subsequently replaced by new bone. A second interesting observation is that bone formation is seen in two directions: away from the scaffold and towards the scaffold, indicated by the yellow label being present on both sides of the green label. Furthermore it seems that certain minimal amounts of TCP are necessary for bone formation, for in the areas where less TCP particles were placed (lateral from the connecting rod), more degradation was seen, which coincided with minimal amounts of bone. This might indicate that high contact area of TCP with soft tissue precludes bone formation and resorption. It is not known whether a causative relationship exists between these parameters. This phenomenon of fast degradation was seen in earlier studies with calcium sulphate and calcium carbonate (182). In this study we found that scaffold degradation seemed slower at locations of new bone formation, which coincided with huge areas of woven bone and a bony fusion between the two transverse processes in almost all groups. The observation that differences in dissolution behavior (changes of chemical behavior or changes in structural properties) of ceramics might be associated with osteoinductive potential *in vivo* has been mentioned before (249), and seems to be applicable in this study. Furthermore it has been hypothesized that calcium release plays a role in this process (141), but the exact mechanism has not been identified yet.

It is known that cell seeding promotes bone formation at ectopic locations (128) and because spinal fusion can be regarded as partially ectopic, we investigated whether seeding allogeneic MSCs on TCP scaffold could advance the onset and total amount of bone formation. When looking at fluorochrome incorporation no obvious differences between the two TCP-based groups were seen. The first label given (green label) is also present in the non-cell-seeded scaffolds and almost always around the particles, indicating that in all groups bone formation started before week 3. This is in contrast to studies performed before showing that MSC seeding lead to earlier onset and in some cases to larger total volume of bone formation (127, 150). The spinal fusion location provided contact to the underlying bone which has probably provided enough cells to colonize the highly osteoconductive TCP scaffolds.

CONCLUSION

We demonstrated in a two level-instrumented posterolateral fusion model that the autologous bone performed as expected concerning fusion rates. This indicates the model is applicable for preclinical studies investigating bone replacement constructs. The fusion

rates and histological quality of newly formed bone in the two experimental groups were comparable and no clear influence of MSCs was seen. Signs of active resorption of the ceramic scaffold and replacement by newly formed bone were seen. These results implicate that addition of osteoprogenitors like MSCs is not necessary in a setting of PLF and highly osteoconductive ceramic material.

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GENERAL DISCUSSION AND SUMMARY

Tissue engineering of bone by using multipotent stromal cells has been investigated for decades and its proof of concept has been clearly shown, especially in animal models at ectopic implantation sites. However, despite numerous clinical case reports in highly regarded peer reviewed journals (245, 246, 250-252), it is not yet being used in clinical practice routinely. In this thesis, the value of cell based tissue engineered constructs was investigated, followed by studies on translational aspects of construct optimization, involving material improvement and additives.

THE CONTRIBUTION OF STEM CELLS

When in 1966 the bone forming capacity of fresh bone marrow was demonstrated by Friedenstein and colleagues (253), and in the early 90s Caplan patented the observation that MSCs could be used to engineer bone, cartilage and other mesenchymal tissues (11), it was hardly imaginable that thousands of researchers would be using this knowledge to progress on their work trying to create constructs with bone forming potential. The use of cells has been extended to cells from various sources, including, but not limited to, allogeneic cells, cells from adipose- and placental tissue and cord blood. In addition, endothelial cells and their progenitors are widely applied to promote vascularization in tissue engineered constructs. The combined efforts are largely focused on creation of a tissue engineered bone graft that can replace autologous bone grafts. Cell-based bone tissue engineering has shown variable results in many preclinical models, and is especially effective in rodents at ectopic locations (128, 148, 254-256). For human clinical practice, only a limited number of reports have been published in which the applicability of cell-based constructs was shown (245, 246, 257-259). The question is what underlies this discrepancy? First of all, the component 'cells' is very complex, and thus are the interpretations of the results associated with them. By applying cells, one adds also extracellular matrix and growth factors produced by these cells. Cell preparations are often very heterogeneous, difficult to characterize and reproduce, and they can evoke a number of reactions in the tissues they reside in through direct and indirect interactions with the environment. This makes it hard to compare results from different studies. Furthermore, although many preclinical studies have shown beneficial effects of cell seeding, cellular tracing revealed that the majority of cells cannot be found back, indicating that long-term engraftment is not the case (260, 261). Therefore definite conclusions about the role of MSCs in bone tissue engineered constructs are still impossible. Based on indications from earlier studies (127), we hypothesized that the implantation location is a determining factor in the contribution of cells to a bone tissue engineered construct. At orthotopic locations, bone-forming cells may be recruited from the underlying bone, while this is not possible at ectopic implant sites, where bone progenitor cells can be recruited from the periphery and differentiate *in situ*. In the clinical situation, bone formation is foremost needed at orthotopic locations, although sometimes a combined ectopic component may play a role, e.g. during spinal fusion procedures. Our results underscore that living cells are a prerequisite for bone formation at the ectopic location in small rodents (148, 254-256). This we infer from experiments, in which deposited extracellular matrix or irradiated - reduced-proliferating - cells appeared unable to initiate bone formation. The implanted

MSCs were luciferase-marked to monitor longitudinal cell survival and proliferation using bioluminescence imaging (BLI). Bone formation occurred only when a prolonged increasing BLI signal could be detected, confirming the importance of proliferating and surviving cells. The implanted cells were found back as bone-lining cells or as osteocytes, implicating they actually produced the new bone. Several attempts were made to apply the bioluminescence technology in rats, but this turned out to be very complicated, probably due to the much larger size of the animal, thicker skin and deeper implantation site. Nevertheless, also in the rat, the persistence of a BLI signal coincided with substantial bone formation. Together these data suggest that either long-term survival of the MSCs or the attainment of a certain cell load is essential for subsequent bone formation (262).

To establish whether cell seeding contributes to bone formation at the spinal fusion location, two different models were used in goats. In the first model, spinal cassettes, adapted from an earlier described spinal transverse process model (200), were used to analyze the effect of MSCs at the orthotopic location and directly compared to the ectopic location (128). Cell seeding was redundant in the cassettes, as opposed to the intramuscular site, which we explained by the direct contact between the inserted samples and the underlying bone. In the upper part of the cassettes contacting the paravertebral muscles, cells did have an effect: they advanced the onset of bone formation. After the full implantation period of 3 months, the observed contribution of cell seeding on advanced onset of bone formation was not reflected in the total amount of bone formed.

The second model, in which we investigated the contribution of seeded MSCs, was an instrumented posterolateral spinal fusion model with a granular implant harnessing more extensive contact with soft tissue. After 4 months, results were comparable to the cassette studies: MSCs did not increase total amount of bone being formed. It appeared that cells are not a limiting factor in orthotopic locations in these types of experiments, most likely because they are readily recruited from the underlying bone. In conclusion, seeded MSCs do advance the initiation of bone formation and increase the amount of bone being formed at ectopic location, but are redundant when the implant is contacting the underlying bone. Cell seeding might therefore still be relevant in challenging environments, like for example defect nonunions, where cell recruitment from nearby bone is hampered. These results are in agreement with earlier studies, which also showed that cell seeding does not contribute to bone formation at orthotopic locations (127, 193). The limited effect of cell seeding at the orthotopic location emphasizes the importance of understanding the cellular en molecular processes involved in bone formation.

ALLOGENEIC MSCS AS OFF-THE-SHELF COMPONENT FOR BONE TISSUE ENGINEERING

At this moment indications for using MSCs are still increasing every year, both for local and systemic cell therapy. The indications vary from cardiovascular, neurological, epithelial, graft-versus-host disease, autoimmune disease to musculoskeletal problems. In bone regeneration, examples of phase I trials using MSCs include the implantation of MSCs for treatment of osteonecrosis of the femoral head and in distal tibial fractures (147). In Europe,

especially in the Netherlands, Belgium, Greece, Isreal, Norway, Slovenia, and Switzerland cellular transplant therapies are frequently reported. An interesting trend, arising since last year in cardiovascular disorders, was a cell source shift from entirely autologous to allogeneic in 36% (from 2008 to 2009) of the cases (257). In this thesis, we also focused on using the allogeneic cells because one of the problems in autologous cell-based bone tissue engineering is the two-step procedure, resulting from culture expansion of cells before implantation, and these allogeneic MSCs can be used as an off-the-shelf component. Rejection can be a major issue in the transplantation of allogeneic cells, however, *in vitro* studies (24, 25) as well as *in vivo* studies (26, 27) have suggested otherwise for MSCs. Soluble factors secreted by the allogeneic MSCs were suggested to be responsible for the observed dampening of inflammation (25, 184, 185) when treating severe acute graft-versus-host disease (28). We applied allogeneic MSCs in various models (ectopic, cassette and spinal fusion) to study their immunologic host response and feasibility with respect to bone formation (128, 129). In side-by-side comparisons with autologous MSCs, results showed that allogeneic MSCs are indistinguishable from autologous MSCs with respect to bone formation in goats. Furthermore severe adverse reactions to the seeded allogeneic MSCs, such as swelling, redness of the implantation site and fever, were not seen. The only observed difference between allogeneic and autologous cells was the presence of lymphoid clusters in the ectopic implants, but not in the cassettes and spinal fusion location (129). The number of lymphoid clusters correlated with the bone contact percentage, indicating these immune cells may have a positive influence on the amount of bone being formed. This is particularly interesting for the field of osteoimmunology, which describes the complex interaction between the immune system and bone. Researchers have focused mainly on bone resorption and osteoclasts, but become more and more interested in osteoblast action (263). While there are many aspects needing further clarification and research, it would be interesting to analyze whether it is possible to use this interaction in a positive way for bone tissue engineering purposes. Also in clinical practice, aspects of the immune system could be used to improve the quality and specificity of therapies available to stimulate bone formation in patients with impaired bone healing (264). The application of allogeneic cells can have great influence on further research and developments, as they can be stored in a cell bank and are ready to use also in other fields where MSCs are being applied, like osteogenesis imperfecta and the treatment of graft versus host disease (265, 266). Before this is possible, there are still various challenges associated with cell therapy using allogeneic MSCs. The exact mechanism by which MSCs exert their immunomodulatory and reparative effects *in vivo* are still poorly understood (31) and in addition, it has been hypothesized that the potential systemic immunosuppression mediated by MSCs after *in vivo* administration suppresses the host antitumor immune response, thus favoring tumor formation (267). This potential risk of allogeneic transplantation needs to be clarified further before using these cells in clinical practice.

PREVACULARIZATION STRATEGIES USING EPCS

In bone tissue engineering, it has been proven to be very difficult to develop large (cm-scale) constructs, because enlarging a construct from a few mm^3 to cm^3 likely results in the inadequate transport of nutrients and oxygen to the construct and insufficient removal of waste products from the construct, due to the time needed for vascularization. As living bone is highly vascularized (34, 35), this is thought to be a crucial factor and accordingly received attention in the field of bone tissue engineering during the past years (37, 211). So far, most vascularization strategies relied either on vessel ingrowth from the surrounding host tissue, which will take some time before appropriate blood supply and waste removal conditions will be achieved, or the use of endothelial cells, which were shown not to be clinically applicable (37, 42-44). Highly proliferative endothelial progenitor cells (now often denoted as endothelial colony forming cell (ECFC) (45, 46, 268, 269) however, can be isolated from various sources like bone marrow (215), peripheral blood (45), and umbilical cord blood (270). When using bone marrow, bone forming MSCs and blood vessel-forming EPCs can be acquired from the same source (47, 48). In a coculture study, using both these cell types, we demonstrated that MSCs benefit for their *in vitro* proliferation from the presence of EPCs and vice versa. *In vivo*, EPC addition to MSCs did not result in enhanced bone formation, which can possibly be explained by the nature of the early outgrowth EPC used in this study (130). These cells, derived from bone marrow, showed limited proliferative ability and little expression of early and late endothelial markers, compared to late outgrowth EPCs (the ECFC), which are characterized by more consistent endothelial marker expression and exponential proliferation. Comparative studies showed that bone marrow-derived early EPCs have limited proliferative capacity and angiogenic potential, as opposed to peripheral blood late outgrowth EPCs (213). Several studies hypothesize that early outgrowth EPCs do not build into newly formed vessels, but only exert a paracrine function thus supporting vasculogenenis by secreting angiogenic cytokines, this unlike late outgrowth EPCs that do directly contribute to vasculogenesis by incorporation into vessels (218, 271). In our experiments, the secretion of angiogenic cytokines and subsequent induction of neovasculogenesis by the host was probably not enough to stimulate vascularization and subsequent bone formation in the constructs. For future research, the combined addition of early and late EPCs might be interesting when they have a positive effect on bone formation through their complementary actions. Taken together, we found that in spite of the positive effect that both cell types exerted on proliferation of the other *in vitro* and the observed bone formation induced by EPCs alone, the co-seeded constructs of MSCs and EPCs did not show an enhancement of bone formation compared to the addition of a single cell type. We propose the use of late outgrowth EPCs or combinations of those with early EPCs in future experiments.

PLATELET GEL AS COMPONENT IN CONSTRUCTS FOR BONE TISSUE ENGINEERING

Many growth- and differentiation factors that play a role in bone regeneration are also involved in the special form of local inflammation seen during callus formation. Platelets accumulating at sites of injury are a rich source of osteogenic growth factors. Therefore we hypothesized that the use of a concentrated platelet preparation, platelet-leukocyte gel (PLG), in which a number of growth factors are present at high concentrations, could result in increased bone formation in tissue engineered constructs. PLG, when added to a number of different hybrid bone replacement constructs, showed variable results. Bone formation was unchanged after PLG addition and only in one out of three studies a significant positive influence of PLG supplementation was found on the total amount of bone compared to plasma seeded constructs (128-130). Contradictory findings are also reported in the literature, where several authors report a positive influence of PLG on bone regeneration in humans undergoing orthopedic surgery (194, 195), maxillofacial surgery (196, 272) and in several animal studies (273-275). The positive effects are mainly seen during the initial osteogenic response (276), using human PLG. In our studies we used goat PLG and we did not study bone formation in the first three weeks of implantation. On the other hand, a number of clinical and experimental studies demonstrate the absence of PLG stimulation of bone healing (197-199, 277). Also, *in vitro* experiments are ambiguous with respect to a dose-dependent mitogenic and chemotactic effect of PLG on MSCs (107) and osteoblast-like cells (108), while osteogenic differentiation was either inhibited (109) or absent (278). These opposite actions of PLG *in vitro*, most of the time explained by the variations in growth factor concentrations (279), might explain our *in vivo* results, for when differentiation is inhibited, bone formation will subsequently be inhibited. In conclusion, PLG quality is unpredictable with respect to composition and has resulted in conflicting data in recent literature.

GROWTH FACTOR DELIVERY

Bone morphogenetic proteins (BMPs) and vascular endothelial growth factor (VEGF) are the most well known regulators of osteogenesis and angiogenesis. BMPs have initially proven their inductive capacity by stimulating bone formation at ectopic sites (69, 221). Recombinant DNA technologies have allowed the optimization of BMP-2 and BMP-7 protein production, which has already resulted in clinical applications (73, 74). Also, many studies focus on the use of the bioactive molecule vascular endothelial growth factor (VEGF), being the key mediator in angiogenesis. As a result of being important in angiogenesis, VEGF has shown to be important during early fracture repair, and endochondral and intramembranous ossification (54, 88, 224). In the past it was shown that not only release of BMP to initiate bone formation was important, but also its prolonged presence (223, 280). In mimicking natural bone formation, the order and timing of growth factors were also mimicked (226) by controlled release of both BMP and VEGF (225, 229). We showed in a large animal model, that timing of BMP-2 release largely determined speed and amount of

bone formation. Fast release of BMP-2 resulted in significantly more bone than slow release of the same dose, without an influence of VEGF in either form. During segmental defect healing, reciprocal release profiles of BMP-2 and VEGF were compared: no differences were found between BMP*fast*/VEGF*slow* and BMP*slow*/VEGF*fast* groups. These data implicate that growth factor order was not important in this setting. This differential effect of growth factor release at ectopic and orthotopic sites, is most probably explained by the differences in cellular make-up, a returning subject in this thesis. The orthotopic location is less challenging with regard to MSC recruitment, due to the proximity of the periost covering the implants and the exposed ulnar marrow cavity, and provides a rich source of MSCs able to migrate towards the scaffold material. We conclude that BMP-2 and VEGF were both needed to initiate the bone-healing cascade in the ulnae, but that the timing of this release was not crucial. Again this study shows a location dependence of treatments, in this case of growth factor timed-release effects.

SCAFFOLD IMPROVEMENT

Bone substitute materials have changed over the last decades. Materials evolved from being biologically passive to active, initiating tissue response. Many preclinical studies have been performed trying to mimic natural bone formation by combining MSCs or osteogenically differentiated cells with porous scaffold materials (150, 182, 244), and case reports have been published applying this technique (245, 246), however without large scale clinical application. A crucial point in these studies might lie in the scaffold materials themselves, which were not proven to be osteoinductive and therefore need osteogenic factors or cells to promote and induce bone formation, depending on the implantation site. New ceramic materials have high potential for translational successes, because of their claimed osteoinductive capacities, resulting in induction of osteogenic differentiation of progenitor cells. One of these new materials is the recently developed new β-TCP, which is resorbable and performs well in dogs and sheep in various defects (141). We showed in an instrumented posterolateral spinal fusion in goats, that the TCP construct did not differ substantially from the golden standard autologous bone. Especially fusion rates were compared to studies performed in rabbits (281-283), sheep (284) and macaques (285). In most studies high spinal fusion rates are reported when using ceramic materials consisting of β-TCP or mineralized collagen used together with local bone from decompression surgery or bone marrow aspirates (286-290). This is comparable to clinical practice where local bone from decompression surgery is used as autograft material. Only one study found very high fusion rates of 95% in a transforaminal lumbar interbody fusion (TLIF)/PLF using interbody cages with a cell-free collagen/ceramic material (291). These last results are in agreement with our study where the TCP used had very good physiological characteristics in terms of osseointegration, bone bridging and scaffold degradation as shown by the histological results. The osteoconductive capacities of the TCP material eliminate all need for cell seeding, making it a candidate for a replacement for autograft in spinal fusion.

SUMMARY AND FINAL REMARKS

The research performed for this thesis focused at strategies to improve bone graft substitutes for future clinical applicability. We started by investigating the value of cell based tissue engineered constructs. First we showed that at the ectopic location, bone formation was only present when BLI signal was present until the end of the implantation period, indicating that living MSCs were necessary for bone formation. By immunohistochemistry we showed, that the implanted MSCs were present in the newly formed bone, either as osteoblast or osteocyte. For the spinal fusion location the question whether the implanted MSCs contribute to the bone regeneration remained unanswered. Next, we created hybrid constructs by using allogeneic MSCs as off-the-shelf components, and showed their effectivity in goat models both at the ectopic as well as the orthotopic location. Before allogeneic MSCs can be used in clinical practice further studies need to be performed to analyze safety in man. In other studies performed in goats we found that location has great influence on the effectiveness of MSCs. They clearly show an additive value in enhancing bone formation at the ectopic location, e.g. in rats, but at the orthotopic location this beneficial effect is only present during the first few weeks (in one study), and not after implantation of 3-4 months (cage model and spinal fusion model). From this we can conclude that MSC seeding is not necessary for bone formation at clinically relevant orthotopic locations. Furthermore, early EPCs were coseeded with MSC to stimulate vasculogenesis, which is a prerequisite for osteogenesis. We found that in spite of the positive effect that both cell types (MSCs and EPCs) exerted on proliferation of the other *in vitro*, co-seeding of MSCs and EPCs did not improve bone formation. Bone-marrow-derived EPCs alone did reveal enhanced bone formation as compared to control implants without cells.

The second aspect of this thesis emphasized on construct optimization, involving material improvement and investigation of bioactives. PLG, containing a wealth of relevant growth factors, was used in three different studies in combination with (allogeneic) MSCs and EPCs to enhance bone formation. Results were highly variable, which was also reported in animal and human studies in the literature, making PLG in our current opinion an unreliable component for bone tissue engineering purposes. Next, the value of timing of sequentially released BMP-2 and VEGF was analyzed at ectopic and orthotopic sites in dogs. A positive effect of growth factors on bone formation was seen at both locations, which is in agreement with reports from literature. With respect to timing of the growth factors, we were the first to show that a differential timing of dual VEGF and BMP-2 delivery did not influence orthotopic bone regeneration, whereas at the ectopic site early release of BMP-2 significantly enhanced bone formation. Again, implant location played an important role. Finally we performed an instrumented posterolateral fusion, using a new TCP, and demonstrated that the cell-free TCP constructs and MSC-seeded TCP implants performed not different from autograft, implying a future role for osteoinductive materials such as the TCP for spinal fusion. From preclinical studies we conclude that cell seeding is almost never needed in bone regeneration.

In this thesis we addressed the value of cell seeding and demonstrated that they only provide beneficial effects at ectopic sites, not at orthotopic locations. When using

appropriate osteoconductive and -inductive scaffolds, it might not be necessary at all to use *ex vivo* expanded MSCs for bone tissue engineering, especially when scaffolds are able to attract progenitor cells from the surrounding tissue to differentiate towards osteoblasts and/or endothelium. This said it remains to be seen whether this also holds true for human patients. Understanding the biology of MSCs *in vivo* remains critical for the development of new bone repair strategies especially in healthy and diseased tissues. The design of bioactive agents can help optimize cell-free treatments (292), in particular those biochemical parameters, which can influence cell behavior. Biomaterial characteristics could also influence the local environment in such a way, that cells are recruited towards the defect and are initiated to differentiate towards bone cells. Chemokines are such soluble factors involved in MSC recruitment, and begin to attract some attention in the field of bone tissue engineering (293-295).

The discussion with respect to the value of growth factor based technologies is far from over. After years of positive results from clinical trials, e.g. in tibial non unions and open tibial fractures (73, 74), it has been shown recently in a large randomized clinical trial, that there was no additional effect of BMPs in open tibial fractures treated with intramedullary reaming (296). In spinal fusion BMPs seemed more effective compared to autograft, which resulted in an increased usage from 0.7% in 2002 to 25% in 2006 (297, 298). Recently these results are under discussion due to underreporting of complications (80, 299), probably as a result of supraphysiologic dosages and drawbacks of the current – collagen based - carriers being used, which release very high dosages in a short time frame. From the literature and our own work, it has become clear that lowering the concentrations of BMP-2 100-1000 fold is still effective for bone formation in several animal models. Lowering the concentration of growth factors used, in combination with optimized timing is a challenge for treatment of human bone defects.

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NEDERLANDSE SAMENVATTING

Samenvatting en slotopmerkingen

Het onderzoek beschreven in dit proefschrift richt zich vooral op strategieën om bottransplantaat substituten voor toekomstige klinische toepasbaarheid te verbeteren. Het eerste deel van het proefschrift beschrijft de onderzoeken die de waarde van geïmplanteerde cellen in tissue engineering (TE) constructen beschrijven. Dat hebben we gedaan door te kijken naar de overleving van cellen op verschillende locaties, een niet bot omgeving (ectopisch) en een bot omgeving (orthotopisch), in diermodellen en vervolgens door het gebruiken van niet lichaamseigen cellen (allogeen) op deze verschillende locaties.

Ten eerste hebben we op een ectopische locatie in ratten en muizen laten zien dat botvorming alleen aanwezig was als het licht signaal tot het einde van de implantatie periode aanwezig was, wat impliceert dat er levende botvormende cellen (MSC's) nodig waren voor de botvorming. Met behulp van een immunohistochemische kleuring toonden we aan dat de geïmplanteerde botvormende cellen aanwezig waren in het nieuw gevormde bot, hetzij als osteoblast of osteocyt. Voor de wervelfusie locatie is de vraag of de geïmplanteerde cellen bijdragen aan de regeneratie van bot onbeantwoord gebleven. Vervolgens hebben we hybride constructen gemaakt met behulp van allogene cellen als off-the-shelf component, en toonden hun effectiviteit in geitenmodellen, zowel op ectopische als de orthotopische locatie. Voordat allogene cellen gebruikt kunnen worden in de klinische praktijk zullen verdere studies moeten worden uitgevoerd met name om de veiligheid van deze cellen te analyseren in de mens. In volgende studies, uitgevoerd in geiten, vonden we dat met name implantatie locatie een grote invloed heeft op de effectiviteit van cellen. Ze tonen duidelijk een additionele waarde ten aanzien van de hoeveelheid botvorming op de ectopische locatie, bijvoorbeeld in ratten. Dit gunstige effect is op de orthotopische locatie echter alleen aanwezig gedurende de eerste paar weken (in een studie), en niet meer na implantatie van 3-4 maanden (cage-model en spinale fusie model). Hieruit kunnen we concluderen dat het zaaien van cellen niet noodzakelijk is voor de botvorming op klinisch relevante orthotopische locaties. Omdat bloedvatvorming een voorwaarde is voor botvorming werden vaatvormende cellen (EPC's) samen gezaaid met botvormende cellen in de hoop botvorming verder te stimuleren. We vonden dat ondanks het positieve effect dat beide celtypes (botvormend en vaatvormende cellen) uitoefenden op elkaars celdeling *in vitro*, het zaaien van botvormende cellen samen met vaatvormende cellen de botvorming niet verbeterde t.o.v. elk van de celtypes apart. De implantaten met de uit beenmerg geïsoleerde vaatvormende cellen toonden een licht verbeterde botvorming in vergelijking met implantaten zonder cellen.

In het tweede gedeelte van dit proefschrift werd de nadruk gelegd op construct optimalisatie, waarbij materiaal verbetering en onderzoek van bioactieve stoffen centraal stond. Bloedplasma verrijkt met groeifactoren afkomstig uit bloedplaatjes (PLG), werd gebruikt in drie verschillende studies in combinatie met (allogene) botvormende cellen en vaatvormende cellen om botvorming te verbeteren. De resultaten waren variabel, wat ook het algemene beeld uit de literatuur was, zowel bij toepassing in patiënten en diermodellen. Hieruit concluderen wij, dat PLG op dit moment een onbetrouwbare component is voor

bot TE. In de volgende studie stonden de groeifactoren BMP-2 (ter stimulatie van de botvorming) en VEGF (ter stimulatie van de bloedvatvorming) centraal en werd de waarde van de timing van sequentieel afgegeven BMP-2 en VEGF geanalyseerd op ectopische en orthotopische locaties in honden. In de literatuur wordt beschreven dat biomimicking (het nadoen van de natuur) van belang is. In dit geval betekent dat VEGF in de natuur snel wordt afgegeven en BMP-2 gedurende een langere periode. Wij wilden kijken of biomimicking echt zo belangrijk is. Een positief effect van groeifactoren op de botvorming werd gezien op beide locaties, wat in overeenstemming is met resultaten bekend uit de literatuur. Met betrekking tot de timing van afgifte van deze groeifactoren, vonden we dat het niet uitmaakt of eerst de ene en dan de ander groeifactor wordt afgegeven en dus geen invloed had op orthotopische botregeneratie. Dit in tegenstelling tot de ectopische locatie waar snelle afgifte van BMP-2 botvorming aanzienlijk verbeterde. Wederom speelde implantaat locatie een belangrijke rol. Tenslotte hebben we een klinisch relevant model gebruikt in geiten uitgevoerd (geïnstrumenteerde posterolaterale fusie) om een nieuw dragermateriaal (tricalcium fosfaat, TCP) te testen voor wervelfusie als vervanging voor lichaamseigen bot uit de bekkenkam. In deze studie hebben we aangetoond dat het gebruik van het celvrije dragermateriaal en dragermateriaal met botvormende cellen gezaaid implantaten dezelfde resultaten gaven als het gebruik van lichaamseigen bot als fusie massa, wat een definitieve rol impliceert in de toekomst voor deze materialen in spinale wervelfusie. Uit deze preklinische studies concluderen we dat de toevoeging van cellen bijna nooit nodig is voor botregeneratie.

Samengevat hebben we de waarde van cellen onderzocht en aangetoond dat ze alleen een gunstig effect hebben op ectopische en niet op orthotopische locaties. Als een geschikt osteoconductief en -inductief materiaal wordt gebruikt, is het niet nodig om *ex vivo* geëxpandeerde cellen te gebruiken voor bot tissue engineering, met name als deze materialen in staat zijn om stamcellen uit het omringende weefsel aan te trekken om te differentiëren naar botvormende en vaatvormende cellen. Het valt echter nog te bezien of een dergelijke strategie ook toepasbaar is in patiënten. Het begrijpen van de biologie van cellen *in vivo* blijft cruciaal voor de ontwikkeling van nieuwe strategieën voor botherstel in zowel gezonde als zieke weefsels. Wellicht kunnen celvrije behandelingen ontworpen worden door het toevoegen van bioactieve stoffen met name die biochemische parameters, welke celgedrag kunnen beïnvloeden. Ook biomateriaal eigenschappen kunnen de lokale omgeving beïnvloeden op een zodanige wijze, dat cellen worden aangetrokken naar het defect en worden gestimuleerd om te differentiëren naar botcellen. Chemokines zijn zulke factoren die betrokken zijn bij MSC aantrekking uit de omgeving en zij beginnen zo langzamerhand wat aandacht te krijgen binnen het gebied van bot tissue engineering. De discussie over de waarde van de groeifactor gebaseerde technologieën is nog lang niet voorbij. Jarenlang werden positieve resultaten beschreven in klinische studies, zoals in tibia non-unions en open tibiafracturen, echter onlangs is er in een grote gerandomiseerde klinische trial aangetoond, dat er geen extra effect van de BMP's is in open tibiafracturen die behandeld werden met intramedullair reamen. In spinale fusies leken BMP's ook effectiever in vergelijking met autologe bot transplantaten, wat resulteerde in een toename

van gebruik van BMP's in de spinale chirurgie van 0,7% in 2002 tot 25% in 2006. Onlangs zijn deze resultaten ter discussie gesteld met name door onderrapportage van complicaties, welke waarschijnlijk het gevolg zijn van het gebruik van suprafysiologische doseringen gecombineerd met een snelle afgifte uit de drager. Uit de literatuur en ons eigen werk is duidelijk geworden dat een verlaging van de BMP-2 concentraties met 100-1000 keer nog steeds effectief is voor botvorming in verschillende diermodellen. Een verlaagde en verlengde afgifte zal in de toekomst hopelijk de nadelige bijwerkingen in patiënten kunnen voorkomen.

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CURRICULUM VITAE

The author of this thesis was born on april 4th, 1978 in 's-Hertogenbosch, The Netherlands. In 1998, she graduated from high school (VWO, St Janslyceum, 's-Hertogenbosch) and started the study HBO-V in the same year at the Hogeschool van Utrecht. In 1999, she started the study of medicine at the University of Utrecht, The Netherlands. In 2004 she joined a research project studying the effect of donor tissue origin, cartilage defect related changes and intra-articular factors on cartilage regeneration at the department of Orthopaedics at the University Medical Center in Utrecht, The Netherlands (supervisors: Dr. K.G. Auw Yang, Prof Dr. D.B.F. Saris). In august 2005 she graduated from medical school. In october 2005 she started a PhD project on bone tissue engineering, creating smarter scaffolds, at the Department of Orthopaedics at the University Medical Center in Utrecht, The Netherlands (Head: Prof. A.J. Verbout, later F.C. Öner, and W.J.A. Dhert; Co-supervisor: J. Alblas). The latter two projects have resulted in several publications, a number of presentations at (inter-)national conferences, the AOspine European Young investigators award, the Dutch Spine Society award, and this thesis. From September 2009 she started her residency at the Department of Surgery at the Diakonessenhuis in Utrecht, The Netherlands (head: Dr. G.J. Clevers), after which she started her orthopaedic surgery training in September 2011 at the Sint Antonius Ziekenhuis, Nieuwengein, The Netherlands (head: Dr. M.R. Veen).

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