TISSUE ENGINEERING OF BONE: THE APPLICABILITY OF CELL-BASED STRATEGIES

Cover illustration: Fluorescence microscopy of tissue engineered bone formation in a goat that received sequential fluorochrome labels.
After injection, fluorochromes are believed to adhere to free, unbound calcium, which is present at physiologic concentrations in the body fluids. When the free calcium is used for the mineralization of new bone, the label is irreversibly bound and incorporated in the newly formed bone. The fluorochrome that is not incorporated is excreted by the kidneys. Therefore, bone will only be labeled on the day of fluorochrome admission. The goat received fluorochromes at 3 (green), 5 (yellow) and 7 (orange) weeks after implantation of the tissue engineered cell/ scaffold hybrids. Detailed observation of the image shows the green line very close to the scaffold surface and the yellow and orange lines more towards the pore center (away from the scaffold surface) with about equal distances between the lines. This allows the interpretation that bone formation started before 3 weeks on the scaffold surface and grew centripetally at a steady pace. At some locations, bone formation started later (no green line) or stopped before 7 weeks (no orange line).

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TISSUE ENGINEERING VAN BOT: DE TOEPASBAARHEID VAN CEL-GERELATEERDE METHODEN

(met een samenvatting in het Nederlands)

THESIS

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Utrecht, op gezag van Rector Magnificus, Prof. Dr. W.H. Gispen, in gevolge het besluit van het College voor Promoties in het openbaar te verdedigen op maandag 15 december 2003 om 12:45 uur.

Door

Moyo Chicondi Kruyt

Geboren 2 september 1973 te Nguludi (Malawi)

Promotoren	Prof. dr. A.J. Verbout Faculteit Geneeskunde, Universiteit Utrecht Prof. dr. C.A. van Blitterswijk Faculteit Chemische Technologie, Universiteit Twente
Co-promotoren	Dr. W.J.A. Dhert Faculteit Geneeskunde, Universiteit Utrecht Dr. J.D. de Bruijn IsoTis SA, Bilthoven

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1 **Kruyt MC**, van Gaalen SM, Oner FC, Verbout AJ, de Bruijn JD, Dhert WJA. Bone tissue engineering and spinal fusion: The potential of hybrid constructs by combining osteoprogenitor cells and scaffolds. In press, Biomaterials 2003.

2 **Kruyt MC**, Dhert WJA, Oner FC, van Blitterswijk CA, Verbout AJ, de Bruijn JD. The osteogenecity of autologous bone transplants in the goat. In press, Transplantation 2003.

3 **Kruyt MC**, de Bruijn JD, Wilson CE, Oner FC, van Blitterswijk CA, Verbout AJ, Dhert WJA. Viable osteogenic cells are obligatory for tissue-engineered ectopic bone formation in goats. Tissue Engineering 2003; 9 (2): 327-36.

4 **Kruyt MC**, de Bruijn JD, Veenhof M, Oner FC, van Blitterswijk CA, Verbout AJ, Dhert WJA. Application and limitations of chloromethyl-benzamidodialkylcarbocyanine for tracing cells used in bone tissue engineering. Tissue Engineering 2003; 9 (1): 105-15.

5 **Kruyt MC**, Stijns MMC, Fedorovich N, de Bruijn JD, van Blitterswijk CA, Verbout AJ, Rozemuller H, Hagenbeek A, Dhert WJA and Martens ACM. Genetic marking with the LNGFR-gene for tracing cells in bone tissue engineering. Submitted to Journal of Orthopaedic Research 2003.

6 **Kruyt MC**, Dhert WJA, Oner FC, van Blitterswijk CA, Verbout AJ, de Bruijn JD. Optimization of bone tissue engineering in goats. In press, Journal of Biomedical Materials Research: Applied Biomaterials 2003.

7 **Kruyt MC**, de Bruijn JD, Wilson CE, Yuan H, van Blitterswijk CA, Verbout AJ, Oner FC, Dhert WJA. Optimization of bone tissue engineering in goats: A peroperative seeding method using cryopreserved cells and localized bone formation in calcium phosphate scaffolds. In press, Transplantation 2003.

8 **Kruyt MC**, Dhert WJA, Yuan H, van Blitterswijk CA, Verbout AJ, de Bruijn JD. Bone tissue engineering in critical size defect compared to ectopic implantations in the goat. Submitted to Journal of Orthopaedic Research 2003.

Abbreviations and definitions

List of Abbreviations

αMEM	Alpha modified minimum essential medium
AG	Autologous bone graft
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
AS	Autologous serum
ASAP	Ascorbic acid-2-phosphate
BCP	Biphasic calcium phosphate
BFGF	Basic fibroblast growth factor
BGP	β-glycerophosphate
BM	Bone marrow
BMP	Bone morphogenic protein
BMSC	Bone marrow stromal cells
C	Calcein green
CaP	Calcium phosphate
CFU-F	Colony forming unit fibroblast
CFU E	CFUefficiency
CM-DiI	Chloromethylbenzamido dialkylcarbocyanine
CP cells	Cryopreserved cells
CSD	Critical size defect
DAB	3'3diaminobenzidinetetra-hydrochloride/hydrogen peroxidase
DBM	Demineralized bone matrix
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulfoxide
DOPC	Determined osteoprogenitor cell
ECM	Extra cellular matrix
eGFP	Enhanced green fluorescent protein
env	Envelope protein (viral)
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FITC	Fluoroscein iso-thiocyanate
FTIR	Fourier transformed infrared
G	Gravity force (9.8 m/s^2)
GALV	Gibbon ape leukemia virus
Gam-lgG_1	Goat anti-mouse immunoglobulin G_1
HA	Hydroxyapatite
IOPC	Inducible osteoprogenitor cell
LacZ	Bacterial β-galactosidase
LNGFR	Low affinity nerve growth factor receptor
MoMuLV	Moloney murine leukemia virus
MRI	Magnetic resonance imaging
MTT	Dimethylthiazol diphenyl tetrazoliumbromide
n	Sample size of statistical group
OS	OsSatura®
OTC	Oxytetracycline
p	Probability of statistical finding
P	Power (1-β)
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PE	Phycoerythrine
PHO	Pathologic heterotopic ossification
PLF	Posterolateral fusion
PLIF	Posterolumbar interbody fusion
PO	Peroperative
SEM	Scanning electron microscopy
SF	Serum free
SV40	Simian virus 40 promoter
TCP	Tri-calcium phosphate
TE	Tissue engineering
TGFβ	Transforming growth factor β
TRITC	Tetramethyl-rhodamine iso-thiocyanate
TPC	Tissue progenitor cell
US	Ultroser®
USS	Constructs kept in Ultroser® for<24h
X	Xylenol orange
XRD	X-ray diffraction

Definitions as used in this thesis

Tissue engineering (TE)

1988, the National Science Foundation (USA): TE is the application of the principles and methods of engineering and the life sciences toward the fundamental understanding of structure/function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve functions.
1993, referred to by Langer and Vacanti:[1,2] "TE is an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain or improve tissue function".
1999, Caplan[3] described this "*interdisciplinary application of basic biological principles"* as biomimetics. "TE is the application of biomimetics toward the development of biological substitutes that restore, maintain or improve tissue function".

Stem cell

Defined as quiescent ancestral cell capable of asymmetric cell division to allow both self renewal (to prevent depletion of the stem cell pool) and the production of a proliferating daughter cell, that becomes the progenitor cell for one or more tissue types.[4-7]
The diversity of tissues that can be derived determines the potency of the stem cell:
Totipotent: capable of complete regeneration of an entire individual (blastocyst);
Multipotent: capable of regeneration of the tissues confined to one of the embryological layers (endo, ecto and mesoderm);
Pluripotent: capable to regenerate cells of several lineages (haemaopoietic stem cells);
Mono/unipotent: capable to generate only one type of progeny (spermatogonia).

Osteoconduction

The ability of a material/graft to allow ingrowth of vessels and osteoprogenitor cells from the recipient bed,[8] or spreading of bone over the surface proceeded by ordered migration of differentiating osteogenic cells.[9] An important aspect is the direct bonding of bone to the material surface without fibrous tissue interposition, so-called contact or bonding osteogenesis.[9]

Osteoinduction

1967 Urist: The mechanism of cellular differentiation towards bone of one tissue due to the physicochemical effect or contact with another tissue,[10] at that time only tissues (like demineralized bone matrix and uroepithelium) were known to have this ability. The last decades this ability is also recognized for some biomaterials.[11]
1968, Friedenstein: The induction of undifferentiated inducible osteoprogenitor cells (IOPC's) that are not yet committed to the osteogenic lineage to form osteoprogenitor cells.[12]

Osteogenesis

1966, Friedenstein: Osteogenesis is bone formation by determined osteoprogenitor cells (DOPC's).[13]
The generation of bone by a certain tissue/cell by itself i.e. in the absence of host cells e.g. in diffusion chambers.[14]

Autograft

Tissue transplanted within one genetic individual.

Iso/syngeneic/homologous graft

Tissue transplanted between genetically similar individuals.

Allograft

Tissue transplanted between genetically different individuals of the same species.

Xenograft

Tissue transplanted between different species.

Biomaterial

Was described as a material that can be used for any period of time as a whole or as part of a system that treats, augments or replaces any tissue, organ or function in the body.[15] The interaction between biomaterials and bone has been classified as[16]

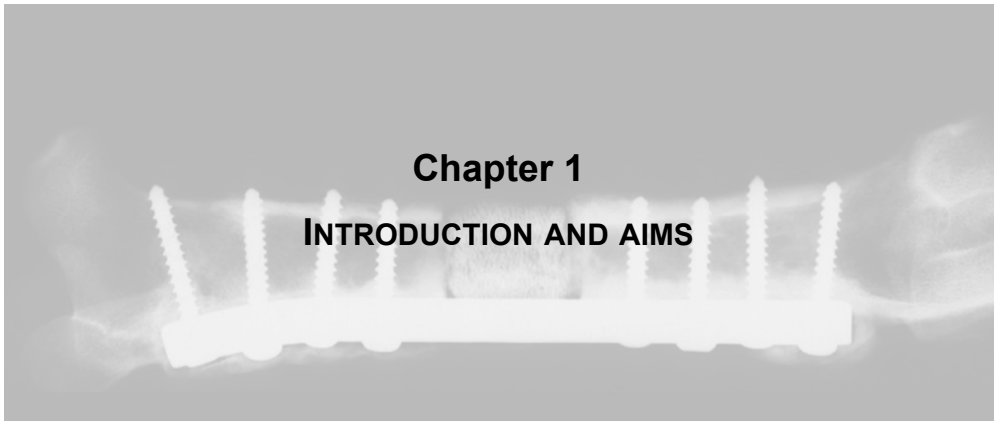
Biotolerant: characterized by a distant osteogenesis with the formation of a fibrous tissue layer between implant and bone;

Bioinert: characterized by surrounding bone growing up to the material surface but without a strong bonding;

Bioactive: characterized by bonding osteogenesis, meaning that bone formation starts at the material surface and has a very tight and direct bond with the material. This typically occurs when an apatite layer can precipitate on the material surface.[17,18]

Hybrid construct

Combination of elements of different offspring. In this thesis: the combination of a synthetic porous scaffold and (biological) cells.



Chapter 1

INTRODUCTION AND AIMS

In orthopaedic surgery, the autologous bone graft is currently considered the golden standard for many procedures that replace or reinforce bone. As is also the case with many other empirically developed therapies, little is known about the mechanism by which the autologous graft functions, especially regarding the unknown role of the cells that are present inside these grafts. Disadvantages of the autologous bone graft, which is often harvested from the pelvic bone, are many and include donor site pain and a limited availability. Therefore, an alternative without these disadvantages, that performs as well as the autologous graft is highly desirable. Tissue engineering of bone has this potential.

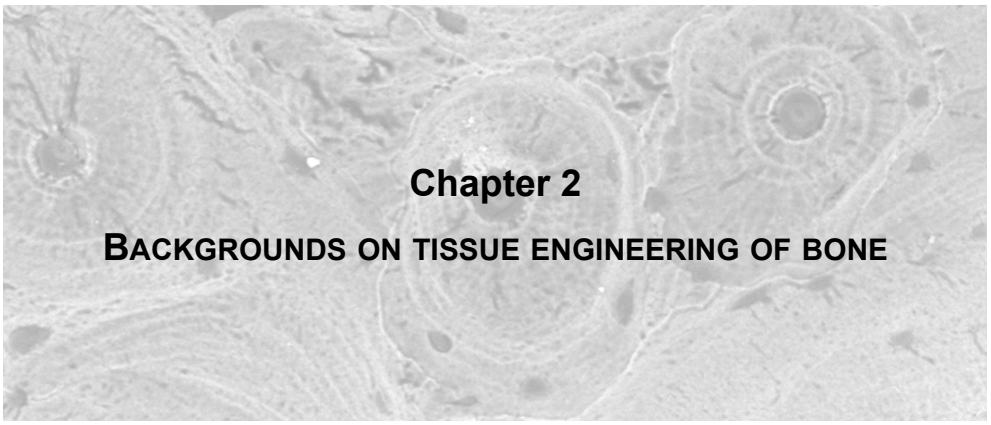
Tissue engineering is defined as a new, interdisciplinary research field that strives to create (artificial) tissues or biologic stimuli to restore or reinforce existing tissue. The cell-based method that combines cells that can generate certain tissues with specifically developed scaffolds to create a so-called hybrid construct, is a promising example. Such hybrid constructs can potentially function in a similar manner as the tissue to be replaced and therefore obviate the need for using the patient's own tissue, such as bone, for grafting procedures.

The proof of the concept of cell-based tissue engineering has recently been shown for tissues of all three embryological lineages (ectoderm, endoderm and mesoderm). Especially the mesodermal i.e. mesenchymal lineage, that includes bone, cartilage and tendons, has extensively been investigated. This may partially be due to the enormous commercial potential, but crucial was the identification of mesenchymal cells with stem-cell characteristics within the adult bone marrow. Currently, bone tissue engineering has advanced so far that some investigators already made the step towards clinical application. Although there is a great desire

for the clinical application of tissue engineered bone, some fundamental questions on cell performance have not yet been answered.
At first: will the cells survive after transplantation? Actually, this is quite unlikely with regard to the fact that vascularization of implants may take weeks, depending on the graft volume that can exceed several cubic centimeters in the clinical situation. Also for autologous bone grafts this is an unanswered question.
At second: what tissue will the surviving cells produce? Until today, this cannot be predicted, because the differentiation of the multipotent cells that are used cannot be fully controlled. Again also in the autologous bone graft this is largely unknown.
It is not possible to entirely investigate these two issues in so-called "small animal" models (mice and rats), where the survival potential is different, because implants cannot be of clinically relevant size. Therefore, our research focused on "large animal" models (goats) that allow clinically relevant sized implants to investigate and optimize bone tissue engineering. We expected the potential role of the cells in tissue engineered implants to be comparable to autologous bone grafts, from the point of view that, although many other factors are involved, bone grafts may resemble a 3D scaffold with osteoprogenitor cells. Therefore, we commenced with an attempt to elucidate the role of cells in clinically relevant sized grafts (both bone grafts and TE grafts). This was done by both an indirect method where control implants were devitalized, and by a direct method that identified the cells after transplantation. Furthermore, the method of tissue engineering was optimized and the feasibility in a critical size bone defect was investigated. The aims of this thesis were formulated as follows.

The aims of this thesis:

1 To investigate the role of cell viability in autologous bone grafts
2 To investigate the role of cell viability in TE constructs
3 To develop a labeling technique to trace cells used for TE
4 To investigate methods to optimize bone tissue engineering
5 To investigate the applicability of TE in clinically relevant models



Chapter 2

BACKGROUNDS ON TISSUE ENGINEERING OF BONE

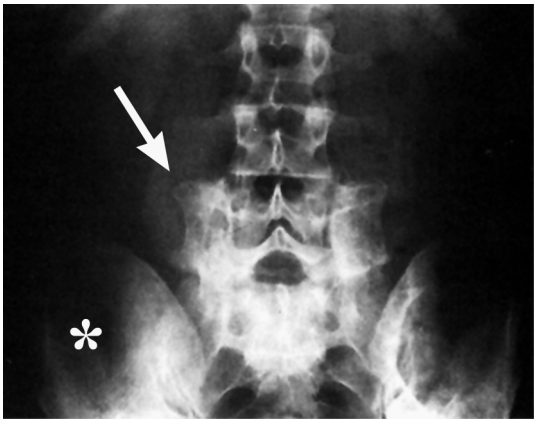
Need for grafting material

Bone transplantation has been the solution for replacement or reinforcement of bone defects for centuries. In a publication in the Netherlands Journal of Medicine, Patka *et al*.[19] refer to a historical description (1668) by the Dutch surgeon Van Meek'ren of such an orthopedic intervention. In this description, Russian priests ban the duke Butterlijn that had received a piece of dog skull-bone to heal his skull defect that was caused by a Tartar sword. The duke recovered well but the combination of a Christian head with dog bone was unacceptable for the church. Therefore, he finally decided to have it removed again. This anecdote is a typical example of xenogenic transplantation (animal to human). With the current knowledge of immunology, rejection by the host can be predicted and therefore these grafts are no longer applied, unless thorough measurements to remove all immunogenic proteins have been employed.[10,20] This also accounts for allografts, that concern human donor bone.[10,21-23] By lyophilization or freezing at very low temperature (–80ºC) and/or defatting the allograft, the immunogenicity decreases and is considered acceptable.[24,25] Another alternative is the use of biomaterials for grafting, these neither elicit an immune reaction, nor carry the risk of transmitting infectious diseases. Many biomaterials have been developed the last decades, ranging from free accessible materials like steel, coral, bamboo, plaster of Paris or methyl methacrylate,[26-31] to sophisticated composites of copolymers and/or ceramics.[32-35] However, in general these materials do not perform as good as the autologous bone graft, because bone conduction over the material surface and the replacement by bone are usually less and biomechanically most materials are inferior to bone.[8,36-40]

If the above had been the only grafting materials, orthopaedic surgery would never have developed to what it is now. The great success of grafting procedures to solve

problems related to congenital, traumatic, or infectious origin must be attributed to the autologous bone graft, which is until today the golden standard for grafting in many orthopaedic interventions.[41-44] Although the theory of evolution predicts a highly efficient use of everything the body consists of, empirical knowledge dictates quite some bone can be missed to use for grafting. Bone from the iliac crests and the fibulae for example is often applied (Fig. 1). However, despite the fact that this bone can be missed from a biomechanical point of view, harvesting is not without complications, of which donor site pain is the most prominent.[45,46] Other disadvantages include the still limited availability and the extended time of surgery. Therefore, creating a substitute for the autologous bone graft with identical or better performance has been a great challenge for decades.

Figure 1 *X-ray radiograph of lumbar spinal fusion with autologous bone graft (arrow) The image also clearly shows the defect in the iliac wing which was the source of the graft (*).*



Autologous bone graft substitute

It is conceivable that a successful substitute of the autologous graft (AG) will need those features that make it superior to the present alternatives (see also Chapter 3).[47] Unfortunately, these features cannot be identified easily, because little is known about the exact mechanisms involved in AG functioning.[42,43] Nevertheless, it is likely that the osteoconductive and osteoinductive properties together with the presence of viable osteoprogenitor cells which are expected to be responsible for osteogenesis, are crucial for the clinical success of AG. Bone tissue engineering techniques therefore, aim at combining two or more of these features.

Two important findings in the previous century have greatly propelled the enthusiasm for bone tissue engineering.

First, the identification of bone morphogenic proteins (BMP's) by Urist.[48,49] These proteins can induce host cells to form bone in and around an appropriate scaffold. This led to a progressive research program, of which the first clinical studies have recently been reported.[50-53] A disadvantage of this currently most successful technique is the very high, unphysiologic BMP doses, that appeared to be necessary for successful application in primates.[54,55] Despite many studies that showed the technique to be safe,[55] the potential risk of uncontrolled bone formation as a result of this unphysiologic stimulus should be considered with great care.[56]

The second revolutionary finding was the identification of osteoprogenitor cells within bone marrow aspirates of adults.[13,57-60] These cells appeared to have stem cell characteristics and could be culture expanded.[61,62,63-65] Especially when combined with an appropriate 3D scaffold, these so-called hybrid constructs behaved like bone.[66-68] A potential advantage of the cell-based technique is the possibility to engineer almost unlimited quantities of synthetic/autologous constructs with theoretically, but not proven, less risk on uncontrolled bone growth, when compared to the bone induction (BMP) strategy. In this thesis, bone tissue engineering strategies based upon the combination of mesenchymal "stem" cells with an appropriate synthetic scaffold to constitute hybrid constructs were investigated.

Mesenchymal stem cells

The existence of such a cell anywhere else than in early embryogenesis remains to be proven formally, and therefore these cells need to be considered as putative stem cells. The extensive work on identification of this putative mesenchymal stem cell that was done by Alexander Friedenstein and colleagues at the Gamalaya Institute in Moscow can hardly be over-estimated.[69-71] In a series of admirable papers, they identified, characterized, and thoroughly investigated the cells they referred to as colony forming unit fibroblast (CFU-F). The following summary of their research delineates the fundamental concepts on which our research was based.

In 1966, they showed the bone forming capacity of fresh bone marrow.[13] The bone that formed in host mice after transplantation of a certain minimum amount of cells per surface area, was unequivocal of donor origin, because bone formed inside so called diffusion chambers. Inside diffusion chambers the content was

shielded from the host by a semi-permeable membrane that allowed fluid diffusion but not cell passage.[60]

In 1968, the osteogenic potential of the bone marrow was attributed to the presence of determined osteoprogenitor cells (DOPC's). Osteogenic bone formation by these DOPC's was considered different from osteoinductive bone formation, that could only be accomplished with inducing substances (like transitional epithelium or demineralized bone), together with so-called inducible osteoprogenitor cells (IOPC's).[12] In subsequent studies on bone induction, they showed bone marrow contained both IOPC's and DOPC's.[61,70,72] Together with observations of induction of lymphoid cells to form bone, Friedenstein concluded that contrary to whole bone marrow, haematologous cells contained only IOPC's . These observations were actually in conflict with Urist's hypothesis of inductor cells and inducible cells that, according to him, were both not blood born.[10,48,73]

In a series of sophisticated studies using guinea pigs, rabbits and different strains of mice with specific (sex) karyotypes, semi-syngeneic F1-hybrids of these strains, and radiochimeras (bone marrow transplantation of one strain into the another after sublethal irradiation), the donor origin of the bone that formed was again shown. The donor cells were also shown to be subject to immunologic rejection when allogeneically transplanted.[74] Interestingly, the haematopoietic marrow (without the osteoprogenitors therein) that populated the newly formed ossicles was always derived from the recipient. This finding clearly demonstrated the presence of two separate cell lineages within the bone marrow organ.

In 1971, they showed in a remarkable experiment, that the newly formed bone marrow inside the ectopic tissue engineered ossicles could be retrieved and was again capable of ectopic bone formation. This could be repeated for four passages, always showing the original (initial) donor origin of the bone forming cells.[74-76]

In the consecutive period, they reported the presence of colony forming unit fibroblasts (CFU-F's) within the bone marrow at 1-10 per 100.000 nucleated cells.[61] For the first time, they showed that these CFU-F's were responsible for bone formation, even after 20 population doublings. CFU-F's adhered to plastic within 90 minutes and transferred from their resting (G0) phase to a proliferating state within 24 hours after adherence. Monoclonal colonies of the CFU-F's emerged, that could be culture expanded. These CFU-F's constitute a large proportion of the cells that most researchers are currently using and refer to as Bone Marrow Stromal Cell (BMSC), Mesenchymal Stem Cell (MSC) Tissue Progenitor Cell (TPC) and many other descriptions.

In 1974, the efficiency of colony formation was shown to depend on several characteristics, i.e. species (guinea pig BM yielded more colonies than mice BM);

location (from the guinea pig iliac wing more colonies than from the femur); marrow disruption, or blood loss 2 hours before aspiration (this resulted in increased CFU efficiency as a result of a haematopoietic stimulus).[70,77] *In-vitro*, the presence of feeder cells or the natural non-adherent bone marrow cells at an optimal density of $2x10^5$ nucleated human BM cells per cm^2 was determined.[78]

In 1982, they reported successful ectopic bone formation in hybrid constructs of CFU-F's and gel foam sponges, if these "scaffolds" did not resorb too fast.[66] They found a linear relationship between seeded cell number and bone formation.

In 1987, rabbit CFU-F's from single colonies were transplanted into diffusion chambers, 20% formed bone, cartilage and reticular (fibrous) tissue, 20% formed bone only and 27% formed only reticular tissue, indicating the pluripotency but also the heterogeneity of these cells.[70,79] By weighing the dry weight of constructs that formed bone in diffusion chambers, they calculated that bone marrow derived from 0.5mg pelvic rabbit bone could generate 30 kg of bone.[79] The single cell origin of the colonies that formed and the potential of these single colonies to generate both cartilage and bone was shown by mixing male and female bone marrow aspirates prior to culture. The colonies that formed from this mix were always only XX or XY, this cannot be explained if the colonies were derived from cell aggregates, that should at least in a proportion, contain both XX and XY cells. [79]

In 1992, they optimized the culture conditions; by trypsinizing instead of mechanically disaggregation, the colony forming efficiency could be increased a tenfold. The presence of haematologic (non-adherent) cells appeared to stimulate colony formation from the adherent single cells dramatically, in their experiments with mice CFU-F's. This suggests the haematologic cells have a "feeder" function and indicates the importance of plating the whole BM aspirate, including the naturally haematologic feeder cells.[80]

In 1995, experiments with human CFU-F's that showed colony formation requires participation of at least four growth factors: PDGF, BFGF, TGF-beta and EGF.[81]

This CFU-F assay as it was developed by Friedenstein, to select and identify the BMSC's, remained standard until today. To facilitate the selection, others developed BM purification methods by either selection on density gradient (by centrifugation over a ficoll gradient),[63] or immunologically with antibodies that select the cells from the haematopoietic cells (SH2, SH3 and SH4[82]), or to select the primitive cell phenotype (e.g. HOP26[83] and STRO1[84,85]). Although the CFU-F populations definitely show stem cell characteristics, i.e. self-renewal, clonogenicity and the potential to form many mesenchymal tissues, they are not homogeneous (of one single cell type). This is reflected by the differences in growth potential (colony size)

and spontaneous differentiation pathways.[5,7,60,86-88] Another deviation from the definition of mesenchymal stem cell is that not all types of mesenchymal tissues can be generated from (all) the CFU-F's. Finally, the cells appear not to be committed to one lineage, because they can shift to other phenotypes during and even after their differentiation (plasticity).[5,7,89]

Therefore, an important issue is to identify markers that can distinguish the different cell types, e.g. bone forming from fibrous tissue forming cells or multipotent stem cells. Several markers have been developed to identify the cells in the various stages of osteogenic differentiation.[4,62] An important marker for early osteogenic differentiation appears to be CBFA1, which is linked to the osteocalcin promotor.[90] However, to my knowledge neither the existence of the ultimate multipotent mesenchymal stem cell, nor a specific marker for such a cell has been shown, and many researchers refer to *progenitor* cells instead.[6,91,92]

Development of hybrid constructs

No less than 20 years after Friedenstein's first publication, many research groups, of which Caplan and co-workers are well known, realized the mesenchymal "stem" cell could be utilized to engineer bone, cartilage and other mesenchymal tissues for grafting and patented several related technologies to obtain and apply these cells.[93] The concept of Caplan's group was to combine the "stem" cells with an appropriate porous scaffold and rely on paracrine and host derived factors after transplantation, to stimulate differentiation and subsequent bone formation. As a scaffold material they preferred porous biphasic ceramics,[68,94] which are well known for their bioactivity i.e. the capacity for bonding osteogenesis.[17,18,95,96] After implantation of calcium phosphate ceramics such as BCP, but also pure hydroxyapatite (HA), some physicochemical dissolution of the surface occurs, which is followed by reprecipitation of a biological carbonated apatite layer. In addition, organic compounds are incorporated in this layer that may be responsible for selective adhesion and stimulation of osteoprogenitor cells.[97-101] Other groups, including our group, applied somewhat different strategies for bone tissue engineering, especially with regard to the scaffold material (we initially used only pure HA) and preparation of the hybrid construct.[102-105] By culturing the construct for one or more weeks, the cells can already differentiate into the osteogenic lineage and generate extracellular matrix.[106,107] The proof of the concept of Caplan's approach was convincingly shown in ectopic rodent models.[63,67,68,94,108-116] Also orthotopic application proved to be feasible in small animals,[108,117] even with human MSC's in a segmental femur defect in athymic rats.[118] In 1998 they

showed an effect of bone tissue engineering in the healing of a segmental femur defect in the dog.[119] This success was repeated with allogeneic cells in the same model.[120] The cells were labeled with the fluorescent membrane marker CM-DiI and could be traced in the newly formed bone. This outstanding result however, can be interpreted in two ways:

1) The concept of tissue engineering is feasible, even with allogeneic cells in a clinically sized defect.

2) The contribution of the cells in terms of participation in tissue engineered bone formation is limited, because the allogeneic cells will be destroyed by the immune system.[74] The presence of labeled cells does not proof the survival of these cells, as it was shown later that the CM-DiI label is subject to transfer to neighboring cells[121] (see Chapter 6). Further progress of bone tissue engineering in terms of clinical trials or at least a comparison with the autologous bone graft have not yet been reported and only a limited number of other groups also reported on the feasibility of this procedure in critically sized defects in large animals.[35,47,122-126] Interestingly, these groups have a relatively short history of research on bone tissue engineering and they do not show the osteogenicity of their constructs (ectopically) in the animal where the orthotopic functioning is investigated. In fact, the group of Cancedda implanted constructs ectopically, but did not observe osteogenicity of these implants.[127] It is surprising that since the early work of Urist and Friedenstein in the seventies of the last century, ectopic implantations in rodents are considered as proof of the concept of osteogenicity and osteoinductivity,[48,49,67,116,128,129] whereas only a few groups showed this proof of osteogenicity for cell-based TE constructs in larger mammals.[130,131] As long as the mechanism of functioning of the technique is unclear and the survival of the cells is questionable, such an ectopic control seems to be desirable. Based upon the literature that is available and our own experience, bone TE appears to be unexpectedly challenging with regard to clinical application where large grafts are required.

Potential pitfalls of bone tissue engineering

Upscaling cell-based bone tissue engineering from rodent models to the clinical situation involves many pitfalls that can be related to the size of the construct, to the location of application and to species related characteristics like the tendency for bone formation and age.

Size

Increasing the size from the cubic mm-range (in mice) to the clinical, cubic cm-range (1000 fold) has dramatic repercussions on both the implant and the wound bed. It is generally accepted that in order to stay alive, organisms that exceed the volume of several mm^3 need vascularization for oxygen and nutritional supply (e.g. osteoblasts die within two hours when deprived of oxygen).[132] This axioma exists in many disciplines of life sciences. Typical examples are the limited size of nonvascularized animals such as insects and the ingenious mechanisms that evolved to compensate for this lack of vascularization. The early development of active perfusion in the human embryo already during the fifth week of gestation, when it is about 3mm long, is another example.[133] Finally, a promising strategy to prevent tumor growth is by inhibiting neo-angiogenesis. Without vascularization, tumors will not grow larger than several mm^3.[134-136]

When regarding the scarce literature on the relationship between the size of tissue engineered constructs and the survival of the cells, it can be concluded that the unvascularized volume that cells can survive in is limited. The acceptable distance from nutrition by diffusion ranges from 100µm to 5mm.[137,138] By improving vascularization of tissue engineered constructs an advantage of *in-vivo* performance has been found.[139,140] Therefore, methods to improve diffusion in scaffolds are promising and may be essential for further progress.[1,141,142]

Another disadvantage of an increased implant size is that the injury to tissues will be extensive, which will result in a profound wound healing reaction. Inside the wound haematoma, cell survival will be compromised, due to the high potassium concentration.[143] This was also the explanation by Takushima *et al* for their observation that in a rabbit segmental defect model, bone formation by injected progenitor cells was only possible when the defect was created by distraction, to prevent the occurrence of a haematoma.[144] The physiologic mechanism of wound repair actually does not rely on the presence of cells inside the wound bed before any vascularization, as can be deduced from the description of normal wound healing by Dvorak[145] and Anderson[146] (Fig. 2). This implicates that the concept of tissue engineering deviates from mother nature's strategy of wound repair and may not be supported, or may even interfere with this mechanism. Whether, despite of this, the TE strategy has a chance in the clinical situation remains to be proven, but it may be a solution when mother nature's strategy alone is inadequate.

Figure 2 *Steps in wound repair*

1) The excudative phase (immediately after injury, minutes to hours): fluid, proteins and cells escape from the damaged vessels and a haematoma forms. A provisional matrix is made of fibrin. Components within or released from this matrix initiate the resolution (of the clot) and repair processes such as cell recruitment and neovascularization;
2) Acute inflammatory phase (minutes to days): more excudation (edema) and organized leukocyte influx;
3) Chronic inflammation phase (granulation, initiates after 3 to 5 days): blood vessel and fibroblast (tissue) proliferation in the fibrin clot.

Location

In the bony environment, the wound healing response has a specific appearance known as the fracture repair mechanism. The cascade of histological events leading to the regeneration of the normal osseous anatomy was described in detail by Bolander and Jingushi (Fig. 3).[147,148]

Figure 3 *Steps in fracture repair*

1) Immediate injury response: haematoma, proliferation of surrounding mesenchymal cells and influx of inflammatory cells. The fibrous clot is organized into a reparative granuloma, called external callus.
2) Intramembranous ossification (first days): differentiation of precursor cells from the periost adjacent to the fracture. Membranous trabecular bone formation called hard callus.
3) Chondrogenesis (days to weeks): Mesenchymal cells inside the granulation tissue are induced to chondrogenesis called soft callus.
4) Endochondral ossification: Capillaries from surrounding bone invade into the cartilage, followed by osteoblasts that synthesize osteoid on the calcified cartilage.

According to the description of fracture healing, it can be concluded that two mechanisms of new bone formation are present: intramembranous bone formation from the periosteal “stem” cells and endochondral bone formation preceded by chondrogenesis from mesenchymal “stem” cells that migrated into the haematoma.[148] This phenomenon of osteogenic cell influx was elegantly demonstrated by Mizuno *et al*,[149] who showed that only 4-days-old fracture haematoma’s were osteogenic ectopically in rats whereas 2-days-old fracture haematoma’s were not (indicating the absence of progenitor cells in the 2-day haematoma’s). Again, progenitor cells are initially not playing a role inside the wound bed, but play their role from the surrounding tissue after the start of revascularization, which may be a limitation for the tissue engineering approach.

Species

The osteogenicity of BMSC's of human origin in diffusion chambers, or seeded on constructs, has often been reported to be inferior to BMSC's of other species origin, e.g. mouse.[128] Regardless of the reason for this, species differences for bone formation/reparation are common, as also reflected by the differences in tendency for pathologic heterotopic ossification (PHA),[150,151] the differences in the ability to generate bone upon inductive stimuli,[152-155] and the various success rates of spinal fusion surgeries between different species (Chapter 3).[156] Usually primates are found to be less prone for bone formation, although Ripamonti showed bone formation inside ectopically implanted ceramic scaffolds to occur more frequently in baboons.[152,157] An inevitable factor in humans is the relatively high age at which the treatment will be performed. There is an age related decrease in the osteogenic potential of marrow aspirates that is normally not considered in the animal experiments.[7,158,159] This may be due to the decreased yield of BMSC's in fresh bone marrow, from 100 at birth down to 1 per million in elderly people,[93,160,161] although theoretically culture expansion (of the adherent BMSC's) would compensate for this matter.[65] Another factor is the bone forming potential of the BMSC's *per se*.[162] This might be diminished as a result of the replicative senescence (the total number of potential cell divisions is restricted as a result of breaking down of the telomeres that cap the chromosomes).[7] As an experimental approach to overcome this problem, two research groups showed that transducing BMSC's with telomerase which partially restores the telomeres, inhibited senescence of the BMSC's and allowed up to 10 times more population doublings (from 20 to 200) Indeed, *in-vivo* bone formation was significantly higher in the constructs with transduced cells.[163,164]

In conclusion, the many pitfalls are challenging and may need a radical change in the strategy for cell-based tissue engineering, for example, to seed cells on prevascularized scaffolds or to implant the cells in a temporary dormant state with minimal metabolic demands. However, as a first step, more insight in the possibilities of the transplanted cells to survive and function will be needed. Eventually, the pitfalls should be considered in clinically relevant animal models.

Comparison of tissue engineered bone to the autologous bone graft

As already mentioned, the approach of bone TE may partially resemble the cellular processes in the autologous bone graft. Besides other important factors such as inductivity and an ultimate template, the cancellous bone graft may be considered as a porous calcium phosphate scaffold loaded with cells that are related to bone formation i.e. (lining) osteoblasts and progenitor cells. Therefore, biological restrictions to autologous graft functioning probably also will account for the tissue engineered graft. Unfortunately, little is known about these restrictions and in fact the biological performance of the autologous bone graft is largely unknown.[42,43] Especially whether in clinically relevant sized AG the cells at first survive the transplantation, and secondly participate in new bone formation (osteogenesis) is controversial.[42,43,165-168] Because this fundamental question could not be answered from the current literature, the following (indirect) sub-questions were formulated and investigated in the available literature.

- Do cells survive inside the autograft after transplantation?
- Does vitality of the graft influence bone formation?
- Are the transplanted cells present in the newly formed bone?

Do cells survive inside the autograft after transplantation?

Direct observations of the cells in bone grafts range from all cells being death, as was shown in studies on autologous grafts of dogs, rabbits and human,[49,57,151,169] to limited cell survival up to a diffusion depth of 300µm.[41,42,170-173] Even substantial survival around 50% of the cells in child rib grafts, that were stored in the back muscles for two weeks during a multi-stage spinal fusion procedure, was reported[174] In posterior intertransverse spinal fusion, where donor bone is laid between the transverse processes, a typical lag in new bone formation was shown in the central part of the bone mass, whereas close to the transverse processes (and blood supply) an early osteogenic response was shown, which is expected to be partially the result of surviving cells.[175-177] It can be concluded that cells can survive inside autografts of yet undetermined size.

Does vitality of the graft influence bone formation?

An indirect method to investigate cell survival is to study the influence of graft viability at transplantation. This has been studied by several authors in small animal models ectopically and orthotopically [49,57,178-180] and occasionally orthotopically in large animals.[23,181]

Although some effect of graft viability was reported by all authors, many authors ascribe this not to osteogenesis, but to other mechanisms such as the release of bone stimulating factors prior to death.[57] Therefore, we conclude that based on indirect evidence even in large bone grafts, cells survive at least the first period after transplantation to release stimulating agents, or maybe longer to participate in new bone formation (osteogenesis).

Are the transplanted cells present in the newly formed bone?

By using ^{3}H labeling, Ray[182] showed in mice that cells inside ectopically transplanted AG survived and were present in the newly formed bone. Boynton[183] showed the survival and subsequent new bone formation in human bone grafts, when implanted ectopically in immune-compromised mice. Observations in rats and rabbits though, made others to conclude that viable graft cells were only responsible for stimulating the host tissue to form bone.[57,172] However, in larger animal models this has never been investigated.

In conclusion, cell functioning in autologous bone grafts will be indicative for the feasibility of cell-based tissue engineering. However, cell survival and participation in bone formation has as yet only been shown in small sized implants (mm^3) in rodents, although some indirect evidence suggest transplanted cells do play a role in large sized grafts.

Tracing the implanted cells by labeling

In order to answer many of the uncertainties discussed above, the ability to trace the transplanted cells in clinically relevant sized models is required. This may sound easy, but the prerequisites for an accurate label are difficult to fulfil. The label should neither effect, nor select the cells in any way, it should not fade and must be compatible with (large) bone histology and naturally, the label should be specific and not transfer to neighboring cells. Currently there are only a few labels available, which are still far from ideal.

Labeling cells of TE constructs is relatively easy, because the BMSC's are in culture and therefore easy to handle before transplantation. Most well known labels are fluorescent dyes that can be incorporated inside the membrane bilayer,[184-186] or inside the cytosol.[187,188] These labels are divided equally at each division over the daughter cells, which allows tracing of the cell progeny, but results in a decrease in label intensity until the label becomes undetectable. Another disadvantage is the

incompatibility with histology procedures like dehydration, which make hard tissue histology impossible.[189] In recent years, genetic marking, by using for instance (retro)viruses has become available and has shown to be compatible with bone histology.[116,190-193] This technique applies the ability of (retro)viridae to introduce (marker) genes into the cells without apparently disturbing these cells[194,195] Contrary to adenoviral labels, which stay outside the nucleus, retroviral markers integrate in the cell genome and are copied at each cell doubling. Therefore the retroviral labels maintain relatively stable in proliferating cells. By using sophisticated methods to generate the infectious particles, replication of the original virus in the infected target cells is prevented and therefore infection of the host is prevented.[196,197] This approach is promising, although tracing the label in large bone grafts remains difficult with regard to the histological procedures. An exciting potential of retroviral cell marking is to integrate functional genes that are traceable *in-vivo*. These can be genes that produce substances that can be measured in the blood e.g. interleukin 3,[116,198] or even more exiting, genes derived from the firefly that are responsible for bioluminescence. Because of the long wavelength of the light (>500nm) the light can penetrate through tissues and bone.[199-201]

It will be more difficult to label cells in autologous bone grafts, because the cells are incorporated inside the tissue, not easily accessible for labeling procedures. In this situation, retroviral transfection also appeared to be successful to label cells in cruciate ligament auto-transplants.[193] Because retroviral transduction is restricted to dividing cells, alternative transductions with lentivirus or adeno-associated virus can be considered in that situation.[195,197, 202]

Because no adequate labeling methods were available at the moment that we started our research, an important aim of this thesis was to develop a label to trace the cells in tissue engineered constructs in clinically relevant models.

Chapters of this thesis

In *Chapter 3*, in addition to the general backgrounds, a review on the investigation of bone tissue engineering in spinal fusion models is presented;
In *Chapter 4*, we investigated the role of viability of autologous bone grafts both ectopically and orthotopically in the goat;
In *Chapter 5*, we addressed the role of cell viability in tissue engineered constructs ectopically in the goat;
In *Chapter 6*, the off-the-shelf fluorescent intramembranous CM-DiI label was investigated as a potential marker for tracing cells in bone tissue engineering;
In *Chapter 7*, the more sophisticated retroviral labeling technique with a cell surface marker gene was addressed;
In *Chapter 8*, a study on optimization of tissue engineered bone formation with respect to scaffold material and culture period of the constructs is reported;
In *Chapter 9*, another study on optimization with a new scaffold material and a peroperative seeding method is addressed;
In *Chapter 10*, we investigated the feasibility of bone tissue engineering orthotopically in critically sized iliac wing defects;
In *Chapter 11*, the results of all studies are discussed;
In *Chapter 12*, we make an effort to draw some general conclusions.

Chapter 3

BONE TISSUE ENGINEERING AND SPINAL FUSION: THE POTENTIAL OF HYBRID CONSTRUCTS BY COMBINING OSTEOPROGENITOR CELLS AND SCAFFOLDS: A REVIEW

Summary

In this review paper we discuss the current knowledge and achievements on bone tissue engineering with regard to spinal fusion and highlight the technique that employs hybrid constructs of porous scaffolds with bone marrow stromal cells (BMSC's). These hybrid constructs potentially function in a way comparable to the present golden standard, the autologous bone graft, which comprises besides many other factors, a construct of an optimal biological scaffold with osteoprogenitor cells. However, little is known about the role of the cells in autologous grafts, and especially survival of these cells is questionable. Therefore, more research will be needed to establish a level of functioning of hybrid constructs to equal the autologous bone graft. Spinal fusion models are relevant because of the increasing demand for graft material related to this procedure. Furthermore, they offer a very challenging environment to further investigate the technique. Anterior and posterolateral animal models of spinal fusion are discussed together with recommendations on design and assessment of outcome parameters.

The need for graft material in spinal fusion

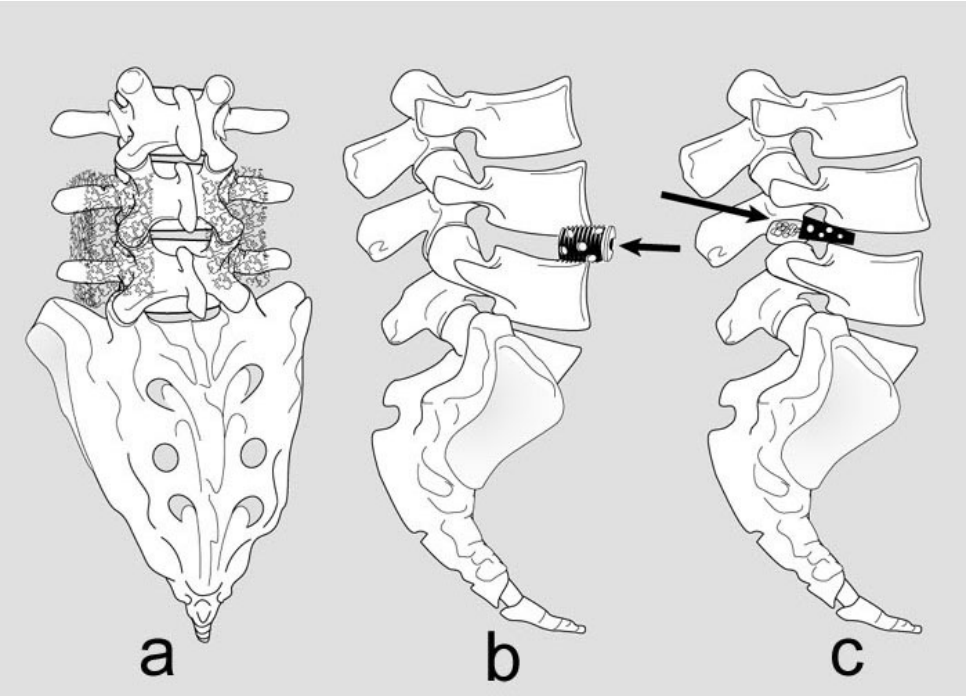
Spinal surgery is performed with increasing frequency for many orthopaedic and neurosurgical indications. It is estimated that more than 200.000 spinal fusions are performed each year in the United States.[44,203] The standard technique of recent years combines pedicle screw instrumentation or intervertebral joint screw-fixation with bone grafting between transverse processes and laminae if available (PosteroLateral Fusion – PLF, Fig. 1).[204] Another technique, with stabilization and (some) distraction of the disc space, performs an anterior arthrodesis between the vertebral bodies (Anterior Interbody Fusion – AIF, Fig. 1). A third technique, applying smaller cages from a posterior approach, is the so-called Posterior-Lumbar Interbody Fusion (PLIF, Fig. 1). Anterior constructs, alone or in combination with posterior instrumentation in the case of PLIF's are thought to be mechanically more stable, as in humans the majority of the compressive loads are conducted through the anterior column.

The increasing frequency of anterior spinal fusion can partially be attributed to the invention and commercial promotion of the so-called 'cages' for inter-body spinal fusion.[203,205] These cages were originally developed to distract and stabilize the cervical spine of horses suffering from the "wobbler syndrome".[206,207] Additional advantages of cages are a reduction of the required graft volume, the potential improvement in fusion rate, and the possibilities to insert cages using less invasive surgery. None of the current procedures is perfect, as reflected by the nonunion rates ranging from between 7-30%.[208] Furthermore, the use of cages has not obviated the need for graft material, although a reduction was achieved from ±15cc for PLF to 4-6cc for AIF.[204]

As a graft material, the golden standard for most orthopaedic applications is the autologous bone graft, which is however inherent to a limited availability and an additional surgical procedure with potential complications.[45,46] Therefore, many substitutes have been developed which all have specific disadvantages, like immunogenecity for allogeneic bone grafts and unfavorable biomechanical properties for most synthetic biomaterials. To be more successful, it is conceivable that these substitutes will need one or more of the features that determine the superior functioning of the autologous bone graft. Although little is known about the exact mechanisms involved in the use of autologous bone graft,[43] the osteoconductive and osteoinductive properties, in addition to viable osteoprogenitor cells are likely to be such features, and crucial for their clinical success. Therefore, substitutes using tissue-engineering techniques aim at combining two or more of these features.

Two important findings have propelled the enthusiasm for bone tissue-engineering research: (1) The identification of bone morphogenic proteins (BMP's) by Urist *et al.*[48,49] which led to a progressive research program of which the first clinical studies have recently been reported,[50-53] and (2) the presence of osteoprogenitor cells in bone marrow aspirates, and the development of techniques to culture and expand these cells.[58,59,61,63] In the present paper, we will further address the bone tissue-engineering strategies based upon the combination of these osteoprogenitor cells with synthetic scaffolds to constitute the so-called "hybrid constructs".

Figure 1 *Spinal fusion techniques*
a): Posterior view of posterolateral fusion (PLF). Graft material is bilaterally applied between the transverse processes and on the facet joints.
b): Lateral view of anterior interbody fusion (AIF). The perforated cage is inserted between the vertebral bodies to distract and stabilize. The cage is filled with graft material to stimulate bony fusion through the cage.
c): Lateral view of posterior lumbar interbody fusion (PLIF). Two cages are inserted bilaterally from a posterior approach. To create space the facet joints need to be removed.



Bone Tissue engineering with Hybrid constructs

History

The osteogenic capacity of viable fresh bone marrow was already demonstrated in 1955 by de Bruyn *et al.*, and confirmed during subsequent years by others.[57,58,209] However, this bone formation appeared to be limited in larger animals.[38,130,131,210] Probably this was related to the more challenging environment combined with a very low density of osteoprogenitor cells in fresh bone marrow (1-30 per 100.000 nucleated cells).[5,123,211] Friedenstein and co-workers identified in bone marrow aspirates the colony forming unit fibroblast (CFU-F), with bone forming capacity.[61,76,79] These cells, having bone forming capacity, were easily culture-expanded to increase the yield of tissue-engineered bone.[105,114,123,211] Since the early nineties, many investigations concerning identification and purification of the CFU-F's or mesenchymal "stem" cells have been performed.[4,63,66,105,212-215] These cells will be further referred to as Bone Marrow derived Stromal Cells (BMSC's). At present, several studies demonstrated the feasibility of hybrid constructs in rodent and large animal models.[119,122-125,130,131] However, studies comparing tissue-engineered bone to autologous bone grafts in a clinically relevant model, or in controlled studies in primates, have not yet been reported. This relatively slow progression towards clinical application may be due to the absence of a clear understanding of how tissue-engineered bone should function, especially when considering the impaired vascularization after transplantation.

Potential mechanism of bone formation in hybrid constructs

In studies where fluorochrome markers were used[105,110,131] (see also Fig. 2), it was demonstrated in porous, ceramic based hybrid constructs that ectopic bone formation starts at the material surface, a mechanism similar to bonding osteogenesis.[9] This observation however, does not explain the exact mechanism by which the bone is formed. To answer this question, first the well-known mechanisms of enchondral (with cartilage intermediate) and intramembranous (without cartilage intermediate) bone formation[216,217] have to be distinguished. The occurrence of these two mechanisms, together with other contributing factors, may indicate the similarity of bone formation in hybrid constructs with aspects of the following empirically known processes of "spontaneous" post-embryonic bone formation:

- **Fracture repair**
- **Osteoinduction**
- **Medullar bone formation**
- **Bone formation in autografts**

In hybrid constructs, the cartilage intermediate has never been described convincingly, although chondrogenesis from BMSC's without replacement by bone has been consistently observed, especially in less perfused areas, such as central regions in diffusion chambers, or inside relatively large implants in animal models.[63,79,125,158,191] Therefore, intramembranous bone formation is expected to be the predominate mechanism.

Fracture repair is unique when compared to other post-embryonic repair mechanisms that result in scar formation, instead of tissue regeneration. The osteogenic process, that commences after the inflammatory phase, under the influence of bone-derived bioactive factors,[147] is initiated by precursor cells from the periosteum adjacent to the fracture, that generate the hard callus by intramembranous bone formation. The majority of bone formation however, is by enchondral ossification of the soft callus that appears after infiltrated mesenchymal cells are induced to chondrogenesis.[147,148,216,218] Therefore, fracture repair is probably not a predominate process in TE bone formation.

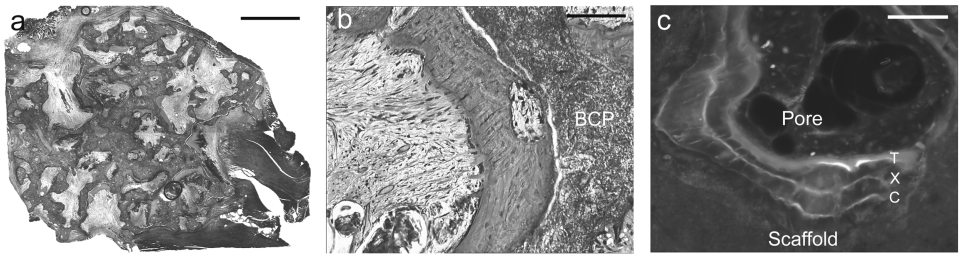
Osteoinduction is most apparent as bone formation in a non-bony environment, described for pathologic heterotopic ossification (PHO)[150] and artherosclerous plaques.[219,220] This phenomenon was consistently observed in ectopically implanted demineralized bone matrix (DBM) and investigated extensively by Urist *et al.*[48,49,129] They identified bone morphogenetic proteins (BMP's) that induce undifferentiated mesenchymal cells to form bone. Depending on the BMP concentration, both enchondral and intramembranous bone formation mechanisms are considered to be involved in this process.[221,222] An interesting and less clearly understood observation is that porous ceramics alone, which are often applied as a scaffold for TE, can induce bone formation, without a cartilage intermediate.[98,152,223] Theoretically this material based osteoinduction, or the presence of additional inductive factors in the extracellular matrix, could be the responsible mechanism of bone formation in hybrid constructs. However, this is unlikely as in many studies bone was absent in the control ceramics,[110,224] even when cell matrix without viable cells was present.[131]

Medullar bone formation is an intriguing phenomenon of intramembranous bone formation that was observed inside rodent femora, after disruption of the bone marrow stromal cavity. Amsel and others described in detail how the femoral cavity was entirely filled with bone that constituted a preliminary scaffold for bone marrow restoration.[225,226] Even when the femora were transplanted ectopically, the origin of the bone was typically from the (stromal) donor cells while the haematopoietic cells appeared to be host derived.[225-227] This resembles bone formation described for hybrid constructs in many ways. Especially the stromal origin of the bone forming cells and the tendency to create a haematopoietic environment.[66,67,75,113,228] However, medullar bone formation depends on precursor cells that are capable of extensive proliferation, as was determined by irradiation studies.[226,229,230] This implicates that a well-vascularized environment is obligatory, which is definitively not the situation during the first period after implantation of clinically relevant sized grafts.[172,173,231]

Bone formation in autologous bone grafts was shown in ectopically transplanted grafts in rodents. The necessity for viable cells in the graft (no bone formation when devitalized grafts were implanted), but also the relative contribution of these cells to the newly formed bone were demonstrated.[57,172,178,179,182,183] We demonstrated in goats a similar necessity of implant viability for ectopic, membranous osteogenesis in clinically sized autologous bone grafts.[232] This observation, that viable cells are required initially, does not necessarily implicate the presence of these cells in the newly formed bone. In fact, a mechanism where donor cells do not survive, but release prior to death a bone inducing substance that activates invading mesenchymal cells, has been proposed by many authors.[42,57,170,172]

In conclusion of this section we assume that (the initiation of) bone formation in hybrid constructs is dependent on, or at least enhanced by viable cells, and progresses with appositional bone formation without a cartilage intermediate.

Figure 2 *Micrographs of hybrid constructs implanted intramuscularly in goats* **(☞ p. 179)**
a): Low magnification micrograph of 7x7x7mm hybrid construct. New bone is clearly present as bright red on the biphasic calcium phosphate (BCP) ceramic scaffold surface. Note the typical distribution of bone that is present only in the interior of the scaffold (bar=1.3mm).
b): High magnification micrograph showing newly formed bone lined by osteblasts (arrows), on the surface of the BCP scaffold (bar=100µm).
c): High magnification with fluorescent microscopy showing the sequential fluorochrome labels in newly formed bone: C=calcein green at 4 weeks; X=xylenol orange at 6 weeks; T=tetracyclin at 8 weeks. Mineralization initiated around four weeks and was directed centripetally (bar=200µm).



Towards spinal fusion

Spinal fusion is considered to be one of the most challenging applications for bone graft substitutes, since even autologous bone, the golden standard, has a relatively high rate of failure. For research purposes, this can be advantageous as it allows for critical examination of both positive and negative effects. Recently, the necessary burden of proof was discussed to decide for clinical use of osteoinductive (BMP based) bone graft substitutes.[44,56] For spinal fusion, it was recommended by Boden *et al.* to follow an extensive establishment of efficacy. This starts with the proof of the concept, normally in small animal models, followed by feasibility studies in larger animals and efficacy studies in non-human primates. In principle, this thorough pathway of pre-clinical evaluation should also be applicable for the use of hybrid constructs. Particularly for spinal fusion, where the use of an osteoconductive scaffold material alone is not regarded as an acceptable alternative for the autologous bone graft[38,40,44] and thus the surplus value of osteoprogenitor cell addition becomes essential. In the following sections we will discuss our view on the sequential steps needed in the development of a cell-based tissue-engineering technique for spinal fusion.

Proof of the concept

The diffusion chamber assay, where cells are shielded from invading host cells by a semi-permeable membrane, can be considered as proof of cell osteogenicity.[79,128,158] The subcutaneous nude-mouse model that allows implantation of hybrid constructs of other species' cells, due to the immune-compromised status of these mice, is a scientifically well-established test for construct osteogenicity.[67,116,128]

Rodents however, do not fully represent the situation in larger mammals. For example, we observed that hybrid constructs of goat BMSC's with a certain type of hydroxyapatite ceramic scaffold yielded always bone in nude mice, but almost never when implanted autologously in the goat muscles. Another scaffold type showed comparable osteogenicity in both species.[131] To investigate osteogenicity in larger mammals, and to optimize the characteristics of a construct, ectopic implantation models are of great value, as these allow paired comparisons of many parameters without the disturbing influence of surrounding bone.[129,233] However, successful ectopic performance does not guarantee adequate orthotopic activity. For instance, we found consistent TE bone formation ectopically in 12 out of 12 goats, whereas identical constructs showed no bone formation when implanted in a femoral segmental defect.[131] On the other hand, other investigators claimed the opposite experience in sheep (orthotopic functioning without ectopic bone formation).[122,127]

Feasibility studies

To investigate the feasibility of tissue-engineered grafts, models in larger animals, where both donor cell survival and host response are more comparable to the human situation are required. Critical-size defect studies with hybrid constructs have been promising, and there are indications of an enhancing effect of cell-based bone TE compared to the scaffolds only for spinal fusion.[35,119,123,124,234] However, as long as the mechanism of TE bone formation is largely unknown, and therefore the functioning cannot be predicted, the comparison with the autologous bone graft seems more than relevant. In the paragraph below, considerations and prerequisites for spinal fusion models are discussed.

Spinal fusion models

Since the investigations of Albee[235] on canine spinal fusion almost a century ago, many animal models for spinal fusion have been developed and used. This has resulted in increased knowledge of the healing process and the role of surrounding tissue. For example, Hurley *et al.* showed, by interposing permeable and impermeable sheets between the host and grafted bone, the essential role of the surrounding tissue in the provision of nutrients through diffusion.[236] It was demonstrated in rabbit posterolateral fusion studies, that the vascular supply of the fusion mass originated from the decorticated transverse processes.[176,237] Furthermore, histology revealed that bone formation in the fusion mass was mainly intramembranous, but with a central “lag effect”, meaning that maturation of the fusion was most advanced near the transverse processes and delayed centrally.[175,238] Largely unknown, but crucial for cell-based TE, is the mechanism of bone formation in autologous bone grafts, especially with regard to osteogenicity as a function of residing osteoprogenitor cells.

Bone tissue engineering is primarily a biologic process, in which various other aspects such as biomechanics play an important role. Therefore, to study the feasibility of this technique in spinal fusion, biologic similarity and non-union rates analogous to the human situation are considered to be of utmost importance.[44,239,240] Furthermore, understanding of the many factors that influence spinal fusion is essential to optimize and identify the effect of TE. In Table 1a and 1b we summarize some potential posterior and anterior spine fusion models.

Table 1 ***A*** *Selected animal models for posterolateral spinal fusions*
** AG is Autograft derived from iliac crest, rib or proc.spinosus*
*** DBM is Demineralized bone matrix*
**** BMP is Bone morphogenetic protein 2,7 or extract*
 # PLA is Polylactic acid

Species (Surg. Model)	Author year [ref]	Segment	Follow up time (weeks)	Conditions
Rabbit (Decort. non instr.)	Boden 1995[175]	L5-L6	2-10	Sham+/- decort, AG*
	1995[238]		1-10	AG
	1995[250]		5	AG, DBM**, Ceramics, +/-BMP***
Mini Pig (Decort. instr.)	Christensen 2000[251]	L3-L4	12	AG, screw materials
Dog-Mongrel (Decort. non instr.)	Frenkel 1993[252]	T1-8	6	AG, DBM
	Lovell 1989[253]	T6-13	3,6,12	AG, PLA# +/-BMP
	Cook 1994[254]	T13-L7	6,12,26	AG, Collagen +/-BMP, No graft
Dog-Beagle (Decort. non instr.)	Sandhu 1997[255]	L4-5	12	AG, PLA+/-BMPs
	David 1999[256]		4,8,12	AG, Collagen, PLA +/-BMP
Goat (Decort. instr.)	Johnston 1990[244]	10 Consecutive T Levels	6,12	AG, instr. stiffness
	Johnston 1995[257]	L3-5	12	AG, instr. stiffness
Sheep (Decort. +/- instr.)	Kotani 1996[245]	L2-3 3-4 4-5 5-6	16	Sham(- instr.), AG (+instr.)
	Kanayama 1999[168]		8,16	AG (+/- instr.)
	Kanayama 1998[258]		16	Sham(+/- instr.), AG (+instr.)
(Decort. + instr.)	Kanayama 1997[259]		4,8,12,16	AG, No graft
	Baramki 2000[260]		20	AG, Ceramics, No graft
	Walsh 2000[210]		24	AG, Collagen/ceramic mix
				+/- Bone marrow
	Steffen 2000[261]		12,20,36,72	AG, Ceramics, No graft
Non-human primate	Boden 1995[250]	L4-5	12	DBM+/- BMP
(Decort. non instr.)	Boden 1999[262]		24	AG, Ceramics+/-BMP

Table 1 ***B*** *Selected animal models for anterior spinal fusions*
** AG is Autograft derived from iliac crest or proc.spinosus*
*** Allo is Allograft material*
**** BMP is Bone morphogenetic protein 2,7 or extract*

Species	Author year [ref]	Segment	Follow up time (weeks)	Conditions
Dog	Cook 1986[263]	C2-3 5-6	1-24	Stand alone Ceramics
	1994[264]		6,12,26	Stand alone AG*, Ceramics
Goat	Zdeblick 1992[265]	C2-3 3-4 4-5	12	Sham, AG, Allo**
	1994[40]			AG, Allo, Ceramics +/- plate
	1998[266]			Cyl. cage with AG or BMP***
	Toth 1995[267]	C2-3 5-6	12,24	Stand alone AG, Ceramic
	Pintar 1994[243]	2x separate cervical 2x separate lumbar	6,12,24	Stand alone AG, Ceramics
	Brantigan 1994[34]	L4-L5	24,48, 96	Stand alone Allo, Flex. box cage
	Van Dijk 2002[268]	L3-4	12,24	Flex. and Titanium box cage
Sheep	Cunningham 1998[269]	T5-6 7-8 9-10	16	AG+/- plate, Cyl. cage with AG
	1999[270]			Cyl. cage with AG or BMP
	Steffen 2000[261]	L2-3 4-5	8,32	Box cage with Ceramics
	2001[271]		8,16,32	Box cage, empty with AG or Ceramics
	Sandhu 1996[272]	L4-5	24	Sham, AG dowel, Cyl. Cage with AG
Non-human primate	Hecht 1999[155]	L7-S1	12,24	Allo dowel with AG or BMP
	Boden 1998[54]	L6-S1	12,24	Cyl. cage with Coll+/-BMP

Factors that influence experimental spinal fusion

There are numerous factors that influence the success rate of experimental spinal fusion. These can be divided into animal related and surgery related factors. Animal related factors are the variations between different species, as a result of evolutionary complexity and the varying pace and degree of skeletal maturity. For example, in rats the fusion rate is much higher than in mature non-rodent mammals, including humans, where spontaneous fusion after decortication of the facet joints and lamina is exceptional.[241] Surgery related factors include the location and number of fused segments,[236,242,243] and the use and rigidity of instrumentation.[168,244,245] For instance, the lumbosacral junction is exceptionally prone to non-union, potentially as a result of excessive motion.[242] Vascularization is critical for the osteogenic process and is greatly influenced by the regime of decortication. In the case of PLF, the pars inter articularis preferentially is not decorticated,[237] contrary to the facet joints and transverse processes, where decortication facilitates vascular ingrowth into the fusion mass.[176] Medication is another important issue, for example non-steroidal anti inflammatory drugs (NSAID's), that are normally used for post-operative pain relief, have shown to negatively affect osteogenesis.[246-248] Finally, postoperative handling also appeared to influence the outcome, as was shown by the negative effect of regularly lifting rabbits that underwent non-instrumented PLF from their cages in comparison to a non disturbed group.[249]

Posterolateral fusion (Table 1a)

Spine surgeons are aware of the difficulties of achieving successful fusion, therefore they meticulously decorticate the facet joints, transverse processes and laminae, and commonly use rigid instrumentation to keep the segments fixed during the critical period during which the fusion mass forms. The animal models simulating this challenging surgical practice, however, have not consistently reproduced these surgical techniques. In the frequently used rabbit model, decortication is restricted to the intertransverse processes and no instrumentation is applied. In most sheep models, extensive decortication, facetectomy and rigid instrumentation (although not always[168]) have been reported. One general advantage of the PLF model for tissue engineering research is that it can be regarded as both orthotopic and ectopic.[241] This allows to some extent the evaluation of a potential osteogenic process per se, with limited confusion by osteoconductive bone formation. Although dogs and sheep approximate the human size and anatomy better, they do not simulate the graft-healing environment of humans any better than rabbits.[238,240] This was one of the reasons why Boden *et al.* chose to validate a non-instrumented

posterolateral intertransverse model in rabbits (see table 1a). In dogs and sheep no such validated models exist.

Anterior interbody fusion (Table 1b)

Most AIF study models are reported for larger mammals, like dogs and goats. The erected goat cervical spine is popular for its analogy with the human cervical spine. Zdeblick and others developed a three level goat cervical AIF model and investigated the effect of plate-instrumentation on stand-alone grafts and the use of interbody cages. When ceramics were used as stand alone grafts, fragmentation was shown for porous and even dense blocks.[40,243,263] Cages offer a unique, well-nourished, relatively small and shielded area, which is especially interesting for the application of hybrid constructs. Considering the much larger graft volumes applied for other spinal fusion techniques, cell survival inside the TE constructs in cages is more likely and little restrictions on scaffold biomechanical properties exist.

Study design

The design can vary from multilevel left-right comparisons to a single condition per animal. In pilot experiments, left/right comparisons can be performed. However, especially when no instrumentation is used, the left/right comparison can be misleading, because fusion on one side will influence the mobility and thus the fusion rate on the contralateral side.[240] Potential biomechanical differences between segmental levels should also be carefully balanced in the design.[242] Most authors leave one segment intact between two operated levels. As with all experiments, a conservative power analysis should be made. Based on histomorphometry results that were published on TE bone, the coefficient of variance (SD/average) was about 0.2 in the experimental groups compared to about 0.5 in the scaffold-only groups.[118,119,123,273] Precise power calculations therefore cannot be made, but unpaired studies exceeding twenty animals per treatment group are not overdone.

Outcome parameters

Radiographs, CT and MRI can provide relevant information on fusion mass and progression. However, determining spinal fusion is complicated and controversial.[274] Particularly when ceramics or hardware are used, current imaging techniques become unreliable and other techniques such as manual palpation or more sophisticated biomechanical testing become necessary.[44,238,260,265,266,274] Brantigan *et al.* demonstrated that post mortem radiographs and CT scans correlate well with histology, when radiolucent cages were used.[34] This supports the opinion that histology is of great importance as an outcome tool to evaluate the quality and quantity of bone formation in spinal fusion.[56,274] Additionally, other tissues like cartilage can be identified and a detailed examination of the host response and scaffold behavior can be made. Finally fluorochrome labels can be applied which indicate the direction and rate of mineralizing bone (Fig. 2).[131,275,276]

Prerequisites for research on TE hybrid constructs

To investigate bone tissue engineering in any spinal fusion model, a reproducible osteogenic construct should be selected. Although the well established rabbit spinal fusion model is highly preferable, our experience with rabbit BMSC's is that the criterion of reproducibility could not be fulfilled. Rabbit BMSC's are extremely variable both *in-vitro* and *in-vivo*, as was also shown by others.[277] In addition, animal losses exceeding 10% are not exceptional. Sheep and goat BMSC's are increasingly popular in the literature.[103,122-125,131] In our own experiments using goat bone marrow aspirates, we found these cells to be easily selected and culture-expanded. When seeded on appropriate scaffolds and implanted ectopically, the hybrid constructs were almost 100% osteogenic.[131,278]

From a clinical perspective, it is debatable whether the TE construct needs to proof osteogenicity ectopically in the animal of investigation. It can be argumented that the local conditions are so different between the ectopic and orthotopic implant location that this influences local osteogenicity. Furthermore it should be realized that not all constructs allow ectopic evaluation, such may be the case with fast degrading scaffolds like hydroxy carbonate.[123]

Discussion

In the present paper we reviewed the current knowledge and achievements on cell-based bone tissue engineering with regard to spinal fusion. Because cell survival in large graft applications is questionable, and therefore a simple cell derived bone formation is unlikely, emphasis was put on the potential mechanisms by which the technique should function. Although the exact mechanism is not elucidated, there are indications that TE bone formation resembles the appositional osteogenesis described to occur in ectopic autologous bone grafts[178,232] and in disrupted bone marrow cavities.[225-227] In both processes, viable cells were shown to be crucial. This might be a serious limitation for the TE technique, because in many studies substantial loss of viability has been reported in the weeks after transplantation of autologous bone grafts that, with respect to cell survival, can be considered as cell-constructs.[42,169,231] Furthermore, in studies where autografts were implanted orthotopically for several months, the added value of viable cells in the grafts was considered insignificant. In these studies, viable autografts were compared to autografts that were devitalized by freezing in liquid nitrogen, before transplantation.[23,181,231,279] On the other hand, the functioning of hybrid constructs might be possible with relatively few surviving cells, as an increasing number of reports of successful critical-size defect studies in large animals has appeared.[119,122-125] These findings are promising and make spinal fusion studies a logical next step in research on feasibility of the technique.

Although anterior interbody fusion with the application of cages has many advantages, researchers should realize that the ability to discriminate between the long-term effects of TE and simple osteoconduction may diminish. Therefore, the more challenging posterolateral model seems initially more appropriate. Besides construct osteogenicity, successful spinal fusion will also rely on functionality of the newly formed bone and integration in the surrounding bone. This is a concern, because hybrid constructs have the tendency to form individual ossicles[66,67,75,113,228] with mainly central bone formation and only occasional bridging of individually implanted granules (Fig. 2).[131] Therefore not only the percentage of bone, but also these aspects of functional behavior should be studied.

As soon as the feasibility of the technique has been established, the question will arise whether safety and efficacy studies in non-human primates are necessary, as was done extensively for BMP's.[54,155] According to our opinion, the need for primate models is defendable for BMP studies, where the risks of (uncontrolled) bone formation may be higher as compared to cell-based TE. Especially when non-stimulated cells are applied, this risk seems reasonably low and from this

perspective, does not necessarily legitimate primate models. On the other hand, the risk of inadequate bone formation in human applications of hybrid constructs cannot be ignored, as the differentiation of the cells cannot be predicted. This is reflected by the finding that only half of the constructs from patients above 50 years showed to be osteogenic in the nude mice model.[159]

In conclusion, since the potentials of cell-based (bone) TE are only emerging recently, a humble research attitude to primarily gain insight in the technique should be applied. Then, the challenge is to establish a level of functioning at least as good as the autologous bone graft in a feasibility model of spinal fusion.

Chapter 4

THE OSTEOGENICITY OF AUTOLOGOUS BONE TRANSPLANTS IN THE GOAT

Summary

Introduction: Little is known about the specific mechanisms that make autologous graft bone (AG) superior to the current alternatives. A potential mechanism is the active bone formation by the osteoprogenitor cells within the AG. However, whether these cells survive the transplantation is questionable, especially in non-vascularized clinically sized grafts. In the present study we investigated the role of viability in AG, implanted ectopically and orthotopically in the goat.

Methods: Eight goats were operated on twice. At the first operation, pieces of vital or devitalized autologous cortical bone were implanted in the paraspinal muscles. Eight weeks later, corticocancellous plugs were taken from the femoral condyles, morselized and re-implanted as either vital or devitalized orthotopic grafts. The goats received fluorochrome labels at 5, 7 and 9 weeks after the first operation. At twelve weeks the goats were killed and the samples were examined histologically.

Results: Ectopically, new bone had formed in both the vital and devitalized grafts. In the vital grafts, all three fluorochrome labels were present, indicating an early osteogenic mechanism. Within the devitalized grafts, only the 9 weeks label was observed. Histomorphometry indicated significantly more new bone in the vital grafts, 10.3 vs. 1.7% in the devitalized grafts. Orthotopically, both vital and devitalized grafts showed new bone. Again, graft viability was advantageous in terms of new bone formation (14.5 vs. 9.3%).

Conclusion: The cells inside the autologous bone transplants most likely survived transplantation and were capable of initiating and sustaining new bone formation.

Introduction

Currently, bone is one of the most frequently transplanted tissues and applied in many orthopedic, neurosurgical and maxillofacial interventions. The non-vascularized autologous bone graft (AG), as a structural graft or morselized, is the gold standard for most applications. However, many disadvantages, such as donor site pain and limited availability, are inherent to this graft.[45] A fundamental difference with alternative graft materials, such as allograft bone, is the presence of a viable osteoprogenitor cell pool in the autograft at implantation. Besides many other important factors, subsequent bone formation by these cells may be responsible for the superiority of the AG. However, contrary to vascularized bone grafts,[280] cell survival and participation in bone formation in non-vascularized grafts is largely unknown and highly controversial.[42,43,49,166] After transplantation, the cells are exposed to the harsh environment of a haematoma[143] and may be deprived of vascular supply for weeks.[173] Some authors report an almost total loss of vital cells in the AG,[49,57,169] others report substantial survival, although limited to a diffusion depth of 300µm.[170,172,173] Elves and Gray[178,179] reported the osteogenicity of ectopically implanted AG in rats. The implants were not osteogenic if devitalized by freezing before transplantation. As direct proof of osteogenicity, Boynton *et al.*[183] used immunohistology to identify human cells within new bone appositions on human bone chips implanted ectopically in immune-compromised mice. These studies indicated that the cells inside small (cubic mm's) grafts have the potential to survive and form new bone ectopically. To our knowledge, this has never been shown in larger mammals with transplants of clinically relevant size (cubic cm's), where cell survival is expected to be more difficult because of the 1000-fold increased volume and subsequently delayed re-vascularization.[173]

Considering orthotopic bone transplants, only a few experiments are reported that compared vital and devitalized autografts in large mammals.[23,181] In these experiments, the contribution of viable cells in the autografts was considered insignificant, however, these grafts were analyzed after incorporation in the host bone. We did not find studies that analyzed grafts early after orthotopic transplantation, before incorporation, when bone formation as a function of transplanted progenitor cells is more likely to be determined.

At present, much research is focussed on cell-based tissue engineered bone as an alternative for autologous bone.[123,131,281] The concept of this technique is to form a hybrid construct from an osteoconductive scaffold and cells that have bone forming capacity. In rodent studies, bone that formed in these hybrid constructs contained the implanted cells.[116] Therefore, the success of tissue engineering is likely to rely on survival and subsequent functioning of these cells. As for the

autologous bone graft, this is questionable when clinically sized constructs are applied. Therefore, as a first step to obtain a better understanding regarding the importance of cell survival for bone grafting, we performed a study on autologous bone grafts in a large animal model. The aim of this study was to investigate whether the viability of autologous bone grafts influenced new bone formation, ectopically and orthotopically in the goat, which would be an indication for cell survival and functioning in these grafts.

Materials and Methods

Experimental design and groups

A total of 8 adult Dutch milk goats were operated on twice during the experiment, for which the local animal care committee gave approval. At the first operation, autologous cortical bone grafts, obtained from a femoral diaphyseal segment, were implanted in the paraspinal muscles after one of the following treatments:

1 Vital cortex – no specific treatment;
2 Devitalized cortex – by freezing in liquid nitrogen;
3 Morselized vital cortex and
4 Morselized devitalized cortex.

This resulted in a sample-size of eight for each treatment group. Fluorochrome labels were administered at 5, 7, and 9 weeks.
Eight weeks after the first operation the goats were operated on again. A corticocancellous plug was taken from both the medial and lateral aspects of the left femoral condyle. Both plugs were morselized and implanted in the trephine shaft opposite the location of origin after one of the following treatments:

1 Vital corticocancellous chips – no specific treatment;
2 Devitalized corticocancellous chips – by freezing in liquid nitrogen.

The animals were killed 4 weeks later (12 weeks after the first operation). Ectopic and orthotopic bone formation was investigated by normal histology, fluorescence microscopy, and histomorphometry of nondecalcified sections.

General procedures

Goats (19-26 months) were obtained from a professional stockbreeder, at least four weeks prior to surgery. Surgery was performed under general inhalation anesthesia, preceded by i.v. detomidine sedation (Domosedan®, Pfizer, The Netherlands). Thiopental 10mg/kg (Rhone-Merieux, The Netherlands) was introduced i.v. and anesthesia was maintained by a halothane gas mixture (Sanofi, The Netherlands). Postoperatively, pain relief was provided by buprenorfine hydrochloride (Shering-Plough, The Netherlands). Three fluorochrome labels (Sigma-Aldrich, The Netherlands) were administered intravenously: Alizarin Red (30mg/kg) at 5 weeks, Calceine green (10mg/kg) at 7 weeks and Xylenol orange (100mg/kg) at 9 weeks.[131] After twelve weeks the animals were killed by an overdose of pentobarbital (Euthesate®, Organon, The Netherlands).

Ectopic grafts

Autologous cortical bone was derived from a femoral segment that was excised as part of a segmental defect model studied in the same goats. The 2.3cm, diaphyseal segment was excised by sawing under constant saline cooling and cleaned of periosteum and medullary contents. It was then sectioned in the sagittal and frontal planes to provide four grafts with a volume of approximately $1cm^3$ each. Two grafts were morselized with a mortar and pestle into bone chips (approximately 2x2x2mm). One intact and one morselized grafts was then devitalized by freezing twice for 5 minutes in liquid nitrogen.[282] The grafts were implanted according to a randomized scheme into separate pockets, created by blunt dissection, in the paraspinal muscles.[131] The intact grafts were cut into two 1.1cm pieces before implantation. The fascia was closed with a non-resorbable suture to facilitate implant localization at explantation and the skin was closed in two layers.

Orthotopic grafts

At the second operation, eight weeks after the first operation, both condyles of the left femur were exposed. Corticocancellous bone plugs of 10mm length were taken[283] with a Ø9.5mm hand trephine and extractor (Mathys, Bettlach, Switzerland) and stored in warm saline. A 12mm Ø1.2mm Kirschner wire was placed centrally in the trephine shaft to serve as a reference during explantation and histology. Per goat, one of the plugs was devitalized in liquid nitrogen. Both plugs were then morselized (approximately 2x2x2mm) and implanted press-fit in the opposing location. The origin of the devitalized plugs and the operative order of the

condyles were randomized. The periosteum was closed carefully before closing the skin in two layers.

Validation of osteogenicity

To determine the osteogenicity of the applied bone grafts in an established model of osteogenicity,[49,121,183] vital and devitalized pieces (1-5mm^3) of both the cortical and corticocancellous grafts of each goat were implanted subcutaneously in nude mice for four weeks.

Validation of devitalization

Cortical (*n*=8) and corticocancellous (*n*=6) grafts, as used for ectopic and orthotopic transplantation, were morselized and cultured under standard conditions for goat bone marrow stromal cells (BMSC's)[131] in 10cm^2 culture dishes (Nalge Nunc, Denmark) for 30 days to detect for surviving adherent cells. Another eight vital and devitalized corticocancellous grafts were cultured for one week to expand the number of any surviving cells. A 48h alamarBlue™ (AB. Biosource, Camarillo, US) assay was then done to detect metabolic activity.[284]

Post-mortem sample acquisition, histology and histomorphometry

At explantation, the ectopic implants were localized and excised together with surrounding muscle. The orthotopic implants were sawed en-bloc from the condyles. Explants were fixated in a 4%paroformaldehyde-5%glutaraldehyde mixture, then dehydrated by alcohol series and embedded in polymethylmethacrylate. Semi-thin sections (10µm) were obtained with a sawing microtome (Leica, Nussloch, Germany) and analyzed with fluorescent microscopy using a fluorescence/light microscope (E600 Nikon, Japan) with double filter block (dichroic mirror 505nm and 590nm). Other sections were stained with methylene blue and basic fuchsin for routine histology and histomorphometry. The bright red, highly cellular, newly formed bone was identified and distinguished from the pale, mainly acellular grafted bone. The mid-section through the implants was used for histomorphometry. For the ectopic implants, this was the section providing the largest sample area. For the orthotopic implants, the explanted condyle blocks were ground in the sagittal plane until the outer (circular) margin of the grafted defect appeared. Then the block was cut 5mm below and parallel to the ground surface through the middle of the 10mm defect. Image analysis was done by a blinded

observer using a VIDAS system (KS400, Zeiss, Munich, Germany) coupled to a light microscope. For the ectopic implants, the area of interest was defined by outlining the graft. Within this area the percentage of newly formed bone was measured. In the orthotopic implants, the area of newly formed bone was measured within four quadrants of the Ø9.5mm outlined defect at 25x magnification. The area percentage newly formed bone was then calculated.

Statistics

Results are given as mean±standard deviation (SD). Two-tailed paired student t-tests were performed after the data were analyzed for normal distribution with SPSS10 software (p<0.05 was considered significant).

Results

Efficiency of devitalization and osteogenicity of the cells

The devitalized grafts did not show remaining viable cells during one month of culture. The vital grafts, however, showed outgrowing fibroblastic cells within one week. When these cells were detached, seeded onto 70% porous hydroxyapatite scaffolds (Cam Implants, The Netherlands) and implanted in nude mice, these constructs were shown to be osteogenic.[121] The 48 hour alamarBlue™ assay also did not indicate any vitality in the devitalized grafts, whereas, in the viable control grafts, metabolic activity could be measured within 1 hour of incubation. The subcutaneous implants of cortical and corticocancellous grafts in mice showed new bone formation only in the vital implants, indicating a cell-dependent osteogenicity of these small grafts (Table 1).

General

Two of the goats developed a painful hoof disease for which they were terminated after 10 weeks instead of 12 weeks. Autopsy and cultures of blood and tissue did not indicate a cause related to the procedure. The ectopic implants of these two goats were used for qualitative measurements and for paired comparisons within the animal. The orthotopic grafts were excluded from quantitative analysis because these had been implanted for only half the planned evaluation time.

Table 1 *Bone formation in the study groups*
The fraction of samples showing new bone and the area percentage new bone (Mean±SD) are shown. The time points at which the observed fluorochrome labels were administered to the goats are shown in the right column.

Group	New bone ectopically in mice	New bone ectopically in goats (area%)	Fluorochrome labels (weeks)
Vital morselized cortex	N.A.	8/8 (18.9±8.7%)	5,7,9
Vital intact cortex	8/8	8/8 (10.3±5.0%)	5,7,9
Devitalized intact cortex	0/8	8/8 (1.7±1.2%)	9
Devitalized morselized cortex	N.A.	Resorbed	Resorbed
Vital corticocancellous chip	8/8	N.A.	N.A.
Devitalized corticocan. chip	0/8	N.A.	N.A.

Ectopic grafts (Table1)

At retrieval, all devitalized morselized cortex chips had been resorbed. All other implants were surrounded by well-vascularized muscle tissue without signs of inflammation. Histology showed new bone formation in all retrieved implants ranging from minute spots on the devitalized implants to abundant trabecular bone formation in the morselized vital implants (Fig. 1).

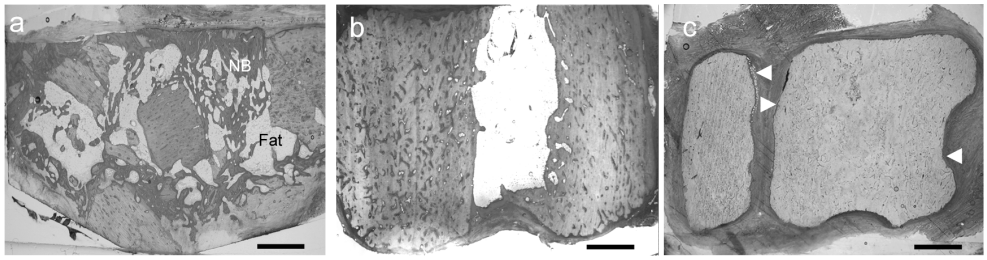
The vital morselized cortex typically formed rigid ossicles (Fig. 1) as a result of extensive new bone bridging between the implanted chips. The interior of these ossicles was filled with fat cells (Fig. 2a).

Within the vital intact cortex, new bone had formed between the two implanted pieces, however, bone apposition on the walls of resorbed haversian channels was responsible for most of the newly formed bone (Fig. 2c).

The devitalized intact cortex had decreased in size substantially when compared to the vital intact implants. Inside these implants, minimal resorption was found. On the periphery, however, extensive resorption was present and small amounts of bone had formed on the bottom of resorbed cavities in all samples (Fig. 2e).

Fluorochrome analysis of the vital morselized cortex indicated that mineralization was present within the newly formed bone at 5, 7 and 9 weeks after transplantation (Fig. 2b). In the vital intact cortex, the 7 and 9 weeks labels were typically present in the remodeled bone within the osteons (Fig. 2d). Within the bone that had formed on the devitalized implants only the 9 weeks label was detectable (Fig. 2f).

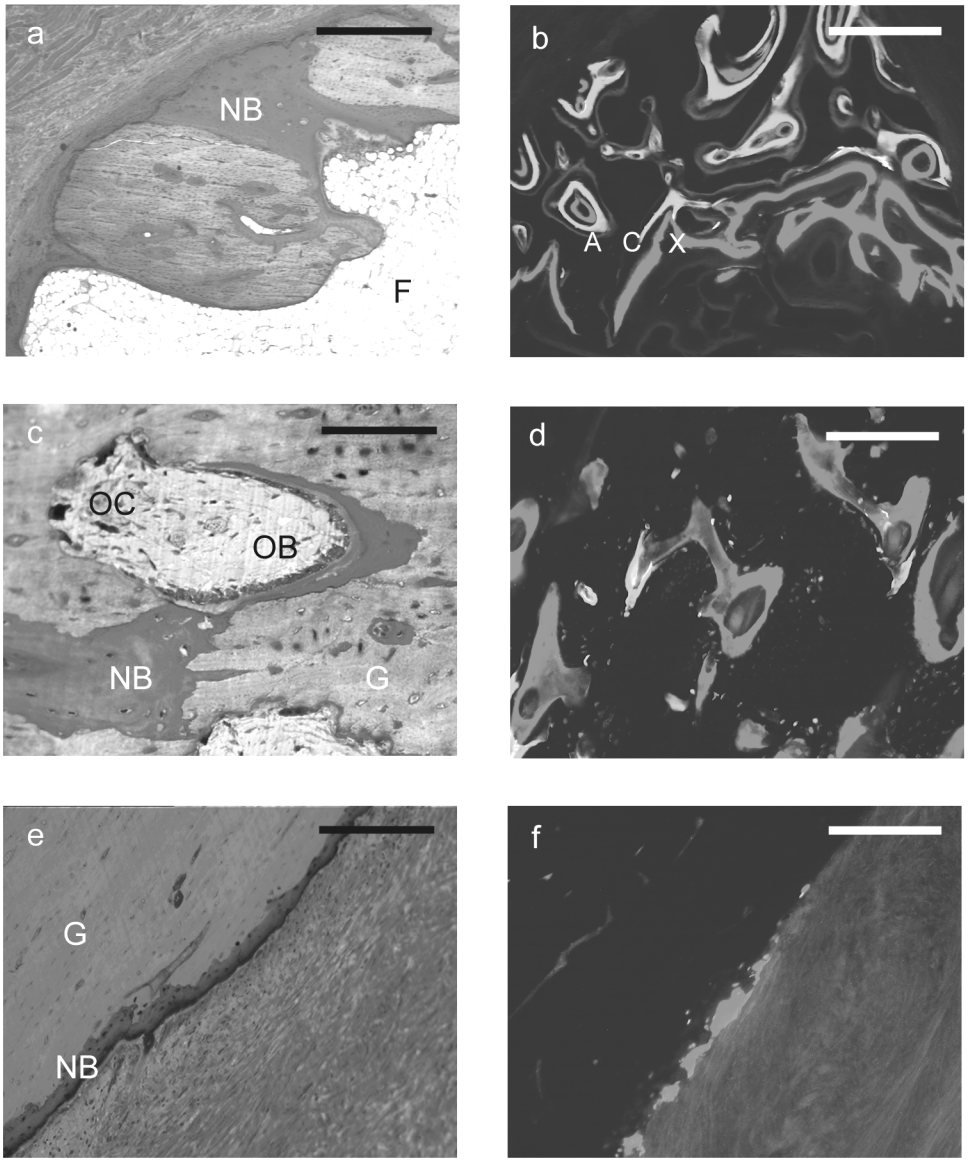
Figure 1 *Micrographs of ectopic autologous implants in goats.* **(☞ p. 180)**
a): Vital morselized cortical implants had formed ossicles as a result of new bone (NB) bridging the grafted chips (bar=250µm).
b): Vital intact cortical implants showed new bone bridging the two implanted pieces and appositioned in the resorbed haversian channels (bar=250µm).
c): Devitalized intact cortical implants showed minute spots of new bone on the periphery (triangles). Note the absence of resorption around the haversian channels (bar=250µm)



Histomorphometry of the samples that were retrieved from the goat, indicated an area% of 18.9±8.7% (Mean±SD) new bone within the vital morselized cortex sample and 10.3±5.0% within the intact vital cortex. The devitalized intact cortex showed only 1.7±1.2% new bone, which was significantly less when compared to the vital intact cortex (p<0.01 Fig.3a). The histomorphometry data of the morselized cortex indicated that morselization resulted in a higher yield of new bone. However, statistical comparison with the intact cortex would be difficult, because in the intact cortical grafts the available area for bone formation was different and restricted to the framework that this structural graft provided. Meaningful histomorphometrical comparisons with respect to the influence of viability of morselized grafts could not be made, because the morselized devitalized cortex was completely resorbed.

Figure 2 *Detailed histology of ectopic autologous implants in goats* **(☞ p. 180)**
a): Vital morselized graft. Detail of new bone (NB) bridging the chips that were implanted vital. The interior of the ossicles was filled with fat cells (F) (bar=400µm).
b): Fluorescent image of morselized vital implant showing all labels A=Alizarine red (5 weeks) C= Calcein green (7 weeks) X= Xylenol orange (9 weeks) (bar=400µm) .
c): Vital intact implant at high magnification. Bone resorption by osteoclasts (OC) and new bone apposition by osteoblasts (OB) is visible. The distinction between pale grafted (G) and red new bone (NB) is obvious (bar=100µm).
d): Fluorescent image of the vital intact implant showing the 7 and 9 weeks labels in the remodelled bone around haversian channels (bar=200µm).
e): Devitalized intact graft (G) with a thin layer of new bone present on the periphery (bar=400µm).
f): Fluorescent image of the devitalized implant showing only the 9 weeks label (bar=400µm).

Figure 2



Orthotopic grafts

Histology of the orthotopic implants showed good integration of both the vital and devitalized grafts in the surrounding bone without an apparent difference between the two groups at first observation (Fig. 4a-b). A distinct layer of new bone covered most surfaces of the grafted bone (Fig. 4c), occasionally reaching the center of the defect. The variance between goats was considerable, however, detailed microscopic and histomorphometric analyses of the paired samples indicated more new bone inside the defects grafted with vital bone, (14.5±3.5 vs. 9.3±4.9% p<0.02). (Fig. 3b). The fluorochrome label given one week after implantation was occasionally present, both in the vital and devitalized grafts, without an obvious difference between the groups.

Figure 3 *Area% new bone in vital and devitalized grafts implanted in goats*
Statistical analysis with paired t-tests, error bars indicate the standard deviation.
a): The area% newly formed bone within the vital and devitalized ectopic cortical implants (n=8, p<0.01).
b): The area% newly formed bone covering the vital and devitalized orthotopically grafted corticocancellous bone chips (n=6, p=0.02).

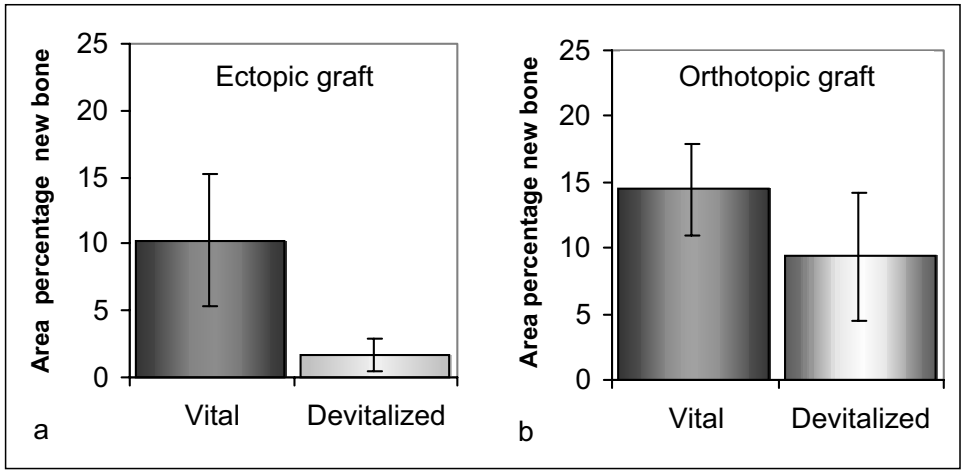
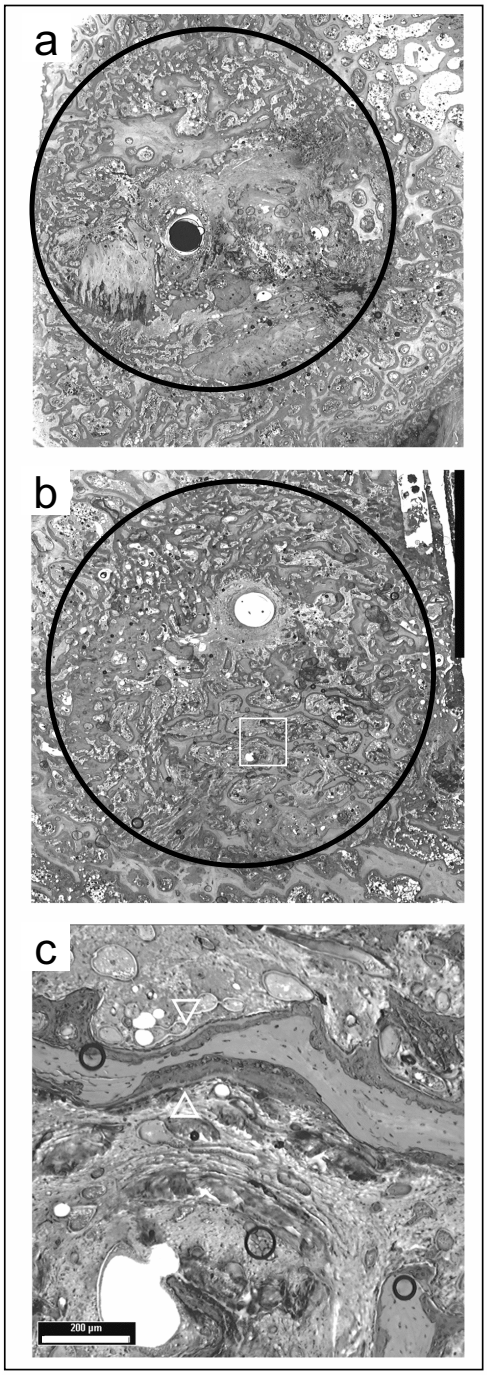


Figure 4 *Histology of orthotopic autologous grafts implanted for 4 weeks.*
a): Overview of the outlined Ø9.5mm defect showing the grafted devitalized chips embedded in soft tissue and surrounded by host bone.
b): Overview of the defect showing the vital graft.
c): High magnification micrograph of the rectangle in b. At this magnification newly formed bone can be distinguished from the grafted bone. Triangles point to osteoblast linings (white bar=200µm)

Figure 4



Discussion and conclusions

In a study on transplants of human bone pieces (0.5mm^3) ectopically in nude mice, Urist *et al.* observed that graft cell survival was exceptional and limited to bone-tumor transplants.[49] Whether cell death was due to insufficient oxygen and nutrition or the result of a residual humoral immunologic response was not clear. In the present study, we investigated new bone formation in ectopically and orthotopically transplanted autologous bone grafts. After implantation of these grafts, with a volume of about 1cm^3, poor oxygen and nutrition conditions can also be expected. To study the role of vital cells present in the graft, we devitalized the control grafts by freezing in liquid nitrogen.[282] We chose this method as other methods, such as lysing, gamma irradiation or heating, might be insufficient or interfere with osteoinduction by devitalized bone matrix.[42,48,285,286] Although freezing has minimal effect on bone morphogenetic protein (BMP) related osteoinductivity,[48] we cannot rule out that freezing interfered with new bone formation in any other way. Oklund *et al.* postulated that freezing could result in calciolysis of bone grafts, thereby promoting ECM exposure and resorption.[181] In our study, new bone formation in the frozen grafts was typically found on the periphery where mineralized bone had been partially resorbed, indicating a positive effect of organic matrix exposure. This is in agreement with the finding of Ripamonti that bone induction occurred on the resorbed surface of partially demineralized allografts in baboons.[11] For these reasons, freezing probably did not negatively influence the osteoinductive capacity of the grafts in our study.

In pilot studies, we found that corticocancellous bone (as used for the orthotopic investigations) could not be used in the ectopic model. Although abundant new bone formed in viable corticocancellous grafts, all devitalized grafts were completely resorbed. We cannot exclude the possibility that the devitalized grafts resorb faster as a result of freezing.[181] It is therefore possible that bone formation in the viable grafts may be the result of bone induction that initiated after the frozen samples had resorbed. Consequently, we chose cortical bone, which was more resistant to resorption, for ectopic investigations in the current study.

Increased bone formation was found in the viable ectopic implants, which showed the early fluorochrome label. This suggests an early, osteogenic mechanism,[287] which indicates that cells do survive after transplantation and are involved in new bone formation. However, we cannot be sure how long cells survive or if they only initiated host derived bone formation by unknown or not fully understood mechanisms.[57,172]

Another interesting finding was the absence of bone resorption around the haversian channels in the devitalized cortical grafts. In the vital cortical grafts,

resorption and subsequent new bone formation were abundant after 5 weeks of implantation, based on the presence of the fluorochrome labels (Figs. 1 and 2). Apparently, bone resorption for remodeling, according the principles of so-called "creeping substitution",[166,287] requires an environment of vital bone, as was also postulated by others,[57] or the residence of vital osteoclasts within the transplant.

The aim of studying the orthotopic model was not for comparison to the ectopic model but to investigate whether the findings on cell survival and function in the ectopic location were of any relevance orthotopically. Irrespective of cell osteogenicity, we expected progressive bone formation in the orthotopic grafts as a result of osteoconduction and induction. Therefore, we chose an evaluation time that would be most distinctive for cell related osteogenesis. In rat studies, consistent osteogenic bone formation was shown two weeks after subcutaneous implantation of an autologous bone graft.[179] In the goats, we expected new bone formation to occur later due to a longer re-vascularization period.[173] We therefore estimated that an implantation period of 4 weeks would allow us to easily detect bone formation. After this period, new bone was indeed present in both conditions, even centrally in the defects. Apparently, host initiated bone formation is the predominant mode of repair in this model. However, histomorphometry indicated a significantly higher percentage of new bone in the defects grafted with vital bone. We therefore conclude that at least a proportion of the grafted cells survived to promote bone formation, leading to an acceleration of this process. However, in agreement with observations by others,[23,181] no advantage of graft viability should be expected for such a model in the longer term.

In conclusion, the cells present in autografts in our ectopic and orthotopic models most likely survived transplantation and were capable of initiating new bone formation. With respect to tissue engineering of bone, these observations suggest that the combination of vital osteogenic cells with an appropriate scaffold may be advantageous to non-vital graft materials. This may be especially important in applications where host bone formation is limited, such as spinal fusion.

Acknowledgements

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Chapter 5

VIABLE OSTEOGENIC CELLS ARE OBLIGATORY FOR TISSUE ENGINEERED ECTOPIC BONE FORMATION IN THE GOAT

Summary

Introduction: Cell survival in tissue engineered (TE) implants is questionable in large animal models, therefore we investigated the significance of vitality, and thus whether living cells instead of only the potentially osteoinductive extracellular matrix are required to achieve bone formation.

Methods: Vital TE constructs of porous hydroxyapatite (HA) with bone marrow stromal cells (BMSC's) within an extracellular matrix (ECM), that resulted from one week *in-vitro* culture, were compared with identical constructs that were devitalized before implantation. Furthermore, we evaluated HA impregnated with fresh bone marrow and HA only. Two types of HA granules were investigated: HA70/800, a microporous HA with 70% interconnected macroporosity and an average pore size of 800µm and HA60/400, a smooth HA. Two granules of both types were combined and then treated as single units. To devitalize, TE constructs were frozen in liquid nitrogen according to a validated protocol. Fresh bone marrow (BM) impregnation was performed peroperatively. All study groups were implanted in the bilateral paraspinal muscles. After 12 weeks, the units were explanted and analyzed by histology.

Results: Bone was present in all vital TE implants. None of the other groups showed any bone. Histomorphometry indicated the microporous HA70/800 yielded more bone than the HA60/400.

Conclusion: Tissue engineered bone formation in the goat can be achieved only with viable constructs of an appropriate scaffold and sufficient BMSC's.

Introduction

Bone tissue engineering (TE) has the potential to provide us with a promising substitute for the autologous bone graft. To be successful, sufficient numbers of potential bone forming cells, an appropriate scaffold to seed the cells, a vasculary supply, and factors to stimulate the cells *in-vivo* are prerequisites.[63,105,288] Bone Marrow Derived Stromal cells (BMSC's) are the cells most frequently applied, and these have been characterized and investigated extensively by Friedenstein *et al.*[75,76] To date, many studies have been performed to optimize the selection, culturing, and seeding of the BMSC's to increase the bone forming capacity.[68,115,130,214] It has now been demonstrated that combining BMSC's with a porous ceramic scaffold is a feasible concept to generate bone ectopically, or in critical sized defects in rodents.[67,103,109,118,128,224] However, upscaling this knowledge to larger mammals is challenging, and only few reports have actually demonstrated bone formation orthotopically[119,122,123] or ectopically in rabbits and dogs,[130,289] with limited control observations. In general, such implants will be larger, and after implantation the cells will be inside the harsh environment of a heamatoma.[143] This, in combination with the expected lack of vascularization until the first week after implantation may compromise cell survival.[173,290] With our method of bone tissue engineering, the hybrid construct consists of (1) living osteoprogenitor cells, (2) non mineralized extracellular matrix (ECM) that already has been produced by these cells during the culturing process,[103,105,291] (3) a biocompatible porous scaffold and (4) media and supplements that have been used during culturing and seeding. We think that a better understanding of the potential separate roles of these parameters is essential in the upscaling of bone tissue engineering to a larger animal, where cell survival is a major concern. To investigate these parameters, it should be considered that osteoinduction is a potential mechanism for bone formation, in addition to the osteogenicity of the BMSC's. Osteoinduction has been described for ECM,[292] that is also produced by the BMSC's after seeding on our scaffolds, and for porous ceramics that were implanted ectopically, either with or without the addition of bone marrow.[98,293,294]
The purpose of the present study was to determine the osteogenicity of tissue engineered constructs in goats, taking into consideration the individual contributions of the parameters mentioned above. Therefore we compared for two different ceramics the bone forming capacity of vital hybrid constructs (scaffold+ECM+BMSC's), devitalized hybrid constructs (scaffold+ECM), scaffolds only and scaffolds loaded with fresh bone marrow in a large animal model (goat). To rule out osteoconduction or periosteal bone formation[119,122,295] as disturbing mechanisms in this fundamental approach, we chose ectopic implantation sites.

Materials and Methods

Experimental design

Eight adult Dutch milk goats were used for the experiment, for which approval was given by the local animal care committee. Two scaffolds types, HA70/800 and HA60/400, were investigated. First, 32 treatment-units were made by combining two granules of each material type. Each of these units was then subjected to one of the following four treatments: (1) TE-vital: seeding of culture expanded BMSC's followed by *in-vitro* culturing of the constructs in the presence of differentiation supplements; (2) TE-devitalized: like treatment 1, but followed by devitalization; (3) BM: loaded with fresh bone marrow; and (4) Control: scaffold treated as in treatment 1 but without cells. One unit of each treatment was randomly allocated to one of four implantation positions in the paraspinal muscles of the goat from which the BMSC's were derived (autologous implantation). This resulted in a sample-size of eight for each treatment group. Fluorochrome labels were administered after 5, 7, and 9 weeks and the animals were killed after 12 weeks. Ectopic bone formation was investigated by histology and histomorphometry of nondecalcified sections.

Harvesting and culture of the cells

BMSC's were derived from the iliac wing and culture expanded as described in detail previously.[103] In brief, 30ml aspirates were plated in culture flasks ($5x10^5$ nucleated cells per cm^2) and cultured in a standard culture medium[103] supplemented with 1ng/ml basic fibroblast growth factor (Instruchemie, The Netherlands). When colonies of adherent cells had formed, these were replated at 5000 cells/cm^2. After another passage, the cells were resuspended at $5x10^5$ cells/ml in seeding medium containing 2% Ultroser (US. Life Technologies, Grand Island, US) as a replacement for the calf serum in the standard medium.

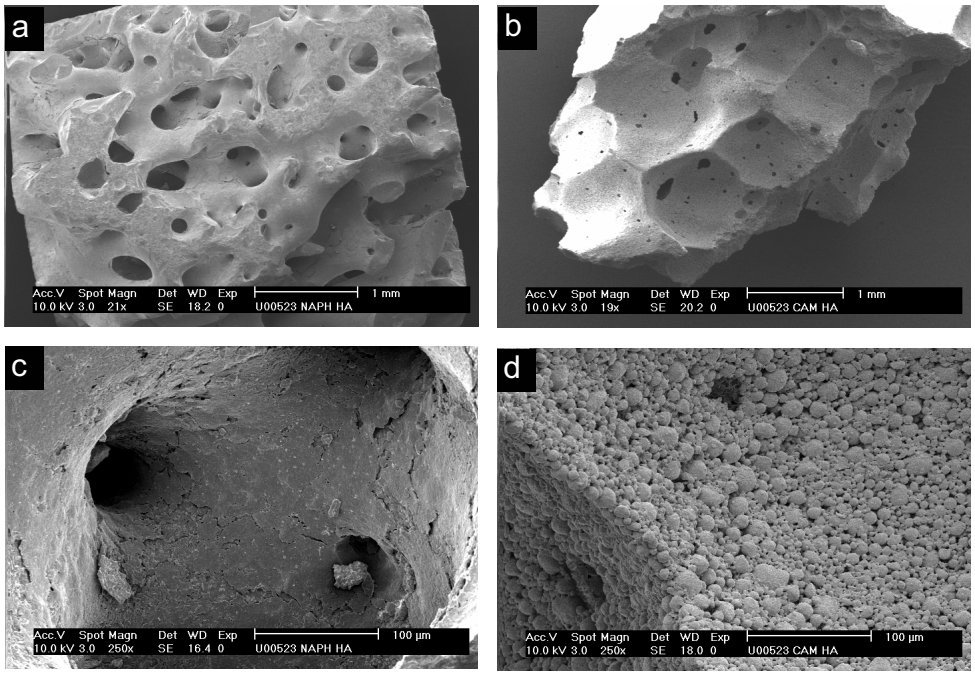
Scaffolds

Two different scaffolds were used in the current experiment (Fig. 1): HA70/800, a 70% porous hydroxyapatite with an average pore size of 800µm was provided by CAM Implants (Leiden, The Netherlands). According to the manufacturer, these scaffolds were produced with spray-dried HA powder. The lamellar interconnected pores were created using a H_2O_2 foaming method and the ceramic was sintered at a temperature above 1100°C. This resulted in a marked microporosity as shown in Fig. 1d. HA60/400, a 60% porous hydroxyapatite with an average poresize of 400µm was provided by IsoTis (Bilthoven, The Netherlands). This scaffold was produced with commercial HA powder (Merck, Darmstadt, Germany) using wax bead incorporation in addition to a MMA foaming method to create a porous structure with interconnections in all three dimensions.[296] The sintering temperature was 1200ºC. Microporosity was much less remarkable in this scaffold (Fig. 1b). The chemical composition of the materials was analyzed by X-ray diffraction (XRD) and Fourier Transformed Infrared (FTIR) spectroscopy. Both scaffold types were sieved until granules of about $40mm^3$ (3x3x4mm) remained. These were cleaned in an ultrasonic bath and then autoclaved. Two granules of each scaffold type (total four granules) were combined and processed as one unit for all further treatments.

Seeding and differatiation

The units were seeded with a load of 10^7 cells per cm^3 scaffold ($1.6x10^6$ cells per unit), overnight (14h) in 10ml tubes on a roller bank. To estimate the seeding efficiency, eight constructs of each scaffold type were digested overnight in 500µl of buffered proteinase K solution (Sigma, St. Louis, MO) at 56ºC. The DNA content was quantified after RNA'se treatment with a CyQUANT® kit (Molecular Probes, Eugene, OR).[297] The same assay was done with six-well plates seeded with known cell numbers to provide the calibration curve. After seeding, the units were cultured for another 6 days in medium supplemented with 10^{-8}M dexamethasone and 0,5mg/ml β-glycerophosphate (both from Sigma) to promote osteogenic differentiation.[106] Control units (scaffold only) were maintained under the same conditions. Cell increase during the culture period was assayed by a second DNA quantification of eight, 6-day cultured constructs. Scanning electron microscopy (SEM) of the constructs was performed to visualize the ECM and related cells. To provide bone marrow impregnated units, fresh aspirate (4ml) without anti-coagulant was loaded on the units for one hour at 37ºC during the operation.

Figure 1 *SEM micrographs of HA 60/400 and HA70/800*
a and c): At low magnification the interconnected porosity of HA60/400 (a) and HA70/800 (c) is visualized. Note the more lamellar pore orientation and interconnections of the HA70/800.
b and d): At higher magnification the smooth microstructure of the HA60/400 (c) is clearly different from the microporous structure of the HA70/800 (d).



Devitalized samples

Tissue engineered constructs with living cells were devitalized by freezing in liquid nitrogen.[59,282,298] The constructs were frozen for 5 minutes and then thawed in saline. This was done twice, to increase the efficiency.[282] To demonstrate this efficiency, we performed the following procedures after devitalization: Eight units were morselized and cultured to detect adhering cells.[299] Another eight units were cultured for one week to expand the potentially surviving cells, and then a 48h alamarBlue™ (AB. Biosource, Camarillo, CA) assay was done to demonstrate possible metabolic activity of cells.[284] Finally SEM of frozen and non-frozen constructs was done to detect for intact cells and to investigate the appearance of the ECM.

Animals and surgical procedure

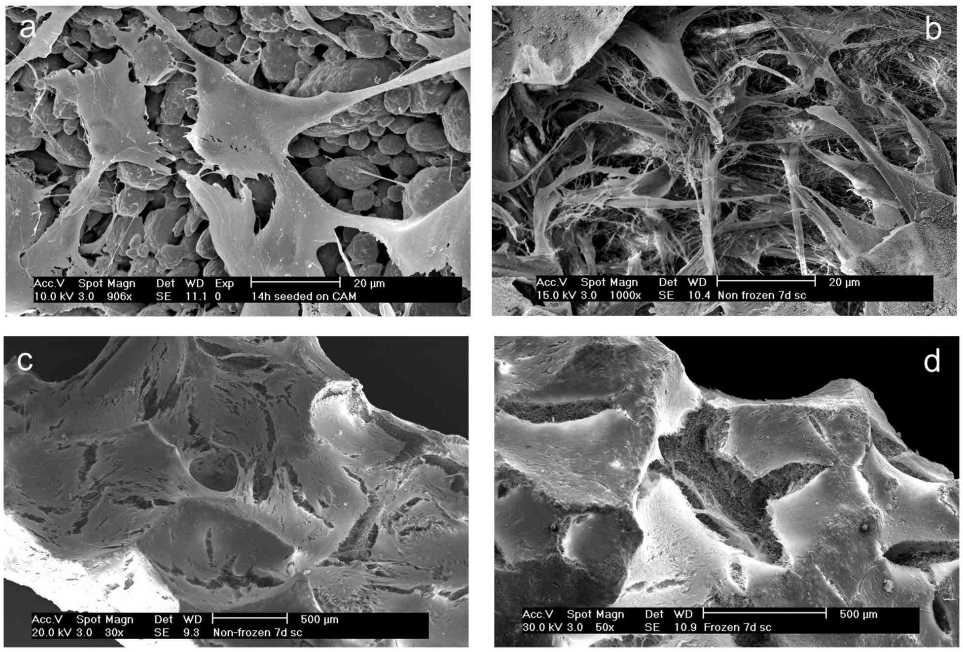
Goats (19-26 months) were obtained from a professional stock-breeder, at least four weeks prior to surgery. The surgical procedures were performed under general inhalation anesthesia, preceded by intravenous detomidine sedation (Domosedan; Pfizer, New York, NY). Thiopental 10mg/kg (Nesdonal; Rhone-Merieux, Athens, GA) was introduced intravenously and anesthesia was maintained by a halothane gas mixture (Sanofi; Paris, France). After shaving the lumbar area and disinfection with iodine, 3cm skin incisions were made at the four implant locations. The muscle fascia was exposed and cut. Using blunt dissection, an intramuscular pocket was created which was filled with one of the four treatment units (autologous implantations). Subsequently, the fascia was closed with a non-resorbable suture to facilitate implant localization at explantation. The skin was closed in two layers. During the postoperative period, pain relief was given by buprenofine (Temgesic; Shering-Plough, Kenilworth, NJ). Fluorochromes (all from Sigma) were administered intravenously: at 5 weeks, Alizarin Red (30mg/kg), at 7 weeks Calceine green (10mg/kg) and at 9 weeks Xylenol orange (100mg/kg).[275,300] After 12 weeks, the animals were killed by an overdose of pentobarbital (Euthesate; Organon, Oss, The Netherlands) and potassium chloride. Besides the intramuscular implantations the goats were subjected to a femur defect operation[301] that is not discussed in this article.

Post-mortem sample acquisition, histology and histomorphometry

At explantation, the units were localized, excised and then fixated in 1.5% glutaraldehyde. The units were dehydrated by alcohol series and embedded in polymethylmethacrylate. Semi-thin sections (10µm, made with a sawing microtome[302]; Leica, Nussloch, Germany) stained with methylene blue and basic fuchsin for routine histology and histomorphometry, or left unstained for epifluorescence microscopy. As the units consisted of four separate granules, several sections were made to provide sections through the center of each granule. The sections were evaluated for general tissue response, bone formation and the fluorochrome labels, using a fluorescence/light microscope (E600 Nikon, Tokyo Japan) with a double filter block (dichroic mirror 505nm and 590nm). The mid-section through the single granules was chosen for histomorphometry. The percentage bone occupying available pore space within the two granules of one scaffold type was measured using a VIDAS image analysis system (KS400, Zeiss, Munich, Germany) coupled to a light microscope. First, the area of interest was defined by outlining the specific granule. Pores that interrupted the exterior scaffold

contour were crossed with straight lines. Next the pore area and the bone area were identified and measured.

Figure 2 *SEM micrographs of HA70/800 granules seeded with 10^7cells/cm^3 material (HA60/400 constructs were comparable in appearance)*
a): After seeding overnight, cells attached to the HA surface were distributed homogeneously over the scaffold.
b): After culturing the constructs for another 6 days, a multilayer of cells within an ECM had formed on the HA surface.
c): After culturing 6 days, SEM at low magnification showed the granules were fully covered with ECM and cells. Only the contour of the peripheral pores remained.
d): After devitalizing the constructs, the ECM covering the HA surface remained visually unaffected.



Statistics

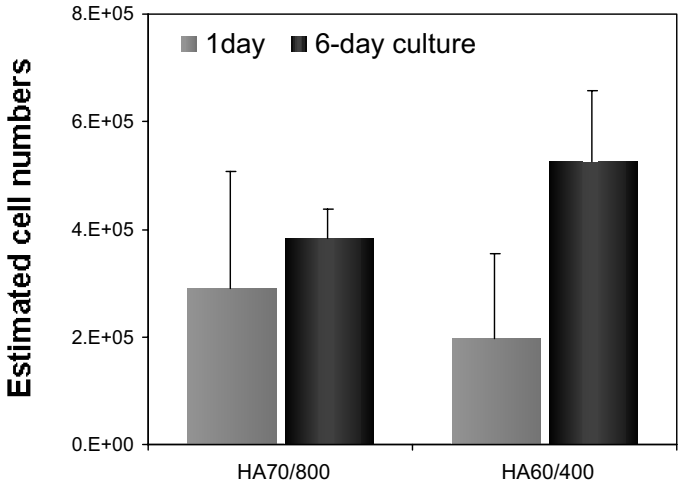
Paired two-tailed student t-test were done to analyze differences in DNA contents between the scaffold materials. The Wilcoxon signed rank test was used to analyze the difference of bone formation between the two scaffold materials (significance level, p=0.05).

Results

In-vitro results (Scaffolds, TE-vital and TE-devitalized samples)

XRD and FTIR analyses were comparable for the two scaffold materials. No additional phases or impurities were detected and both materials were comparable to HA standards (Ca/P ratio of 1.67).[303] The bone marrow aspirates yielded (1.65±0.4)x10^8 (mean±SD) nucleated cells that were plated with 5.8±1.6x10^5 cells per cm^2. After 11±2 days, colonies had formed. After the cells were replated, the population doubling time was 24±2 hours. Cells were seeded on the scaffolds 21 days after the aspirate. Dynamic seeding resulted in an equal distribution of the cells inside the scaffolds as shown by SEM (Fig. 2a). The average seeding efficiency according to the CyQUANT® assay was about 60%, without a significant difference between the two scaffold materials (Fig. 3). After the 6-day postseeding culture period, the total DNA had doubled once on average, with significantly more DNA in the HA60/400 scaffolds as compared with the HA70/800 scaffolds (p=0.02). By then the scaffolds were fully covered with a multilayer of cells within an extracellular matrix, as visible with SEM (Fig. 2b). Examination of the devitalized units with the alamarBlue assay and by culturing the morselized granules did not indicate any vitality. SEM imaging showed the granules were covered with ECM, without the presence of intact cells in these constructs.

Figure 3 *DNA quantify of the HA70/800 and HA60/400 scaffolds seeded overnight with 4x10^5 cells/granule and after culturing the constructs for 6 days (n=8).*
The seeding efficiency was approximately 60% and not significantly material dependent. After 6 days, cell amounts had once doubled on average and the HA60/400 contained significantly more DNA (p=0.02).



In-vivo histology and histomorphometry

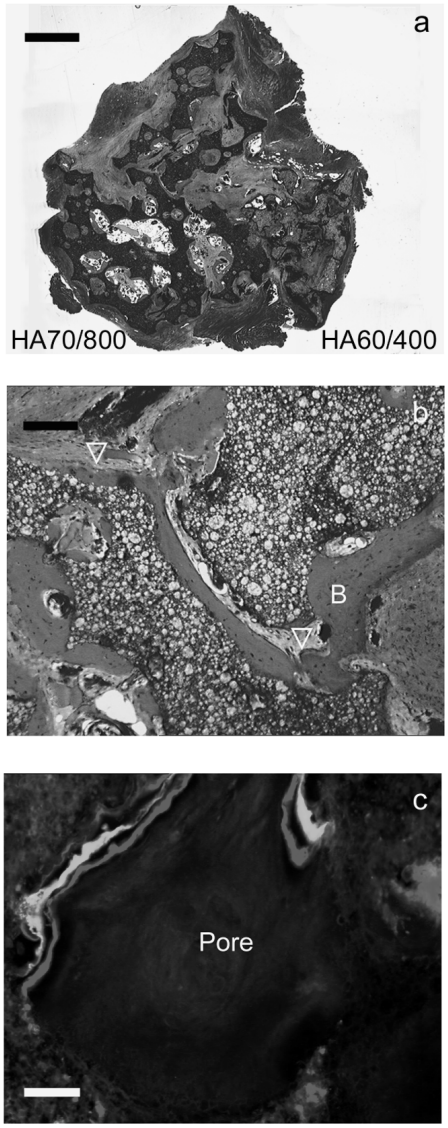
There were no surgical complications, however, two goats developed a painful hoof disease for which they were terminated after 10 weeks instead of 12 weeks. Autopsy and cultures of blood and tissue did not indicate a cause related to the procedure. The data of these to goats was applied for qualitative analysis and for paired comparisons within each animal.

At retrieval, all samples were surrounded by well vascularized muscle tissue. Microscopy showed no signs of an inflammatory tissue response related to the implants. The different appearance of the HA70/800 and HA60/400 was clearly visible under the microscope as a result of the different microporosity (Fig. 4a). In all of the TE-vital implantation units, ectopic bone was present distributed unevenly throughout the granule close to the HA surface with a strong preference for the interior. This was characterized both as woven bone and bone of lamellar appearance, with osteocytes inside the matrix and osteoblast linings. In addition, bone marrow was often present within small voids in the newly formed bone. Occasionally, bone bridging between the separate granules was found (Fig. 4b). Fluorescence microscopy of the newly formed bone showed the five weeks' Alizarine red label closest to the implant surface, while the 7 and 9 weeks labels were present more distant from the surface, indicating centripetal bone formation (Fig. 4c). Histomorphometry of the bone inside the pores revealed an average 13.2±15.9% (±SD) for the HA70/800 scaffolds and 3.4±4.0% (±SD) for the HA60/400 scaffolds (Table 1). Despite the high variation between goats, paired comparisons demonstrated this difference to be significant (p=0.03). In none of the TE-devitalized samples, neither the BM nor the control samples of both scaffold materials, was any sign of bone formation detected. In these samples the pores were occupied with well vascularized, fibrous tissue without signs of adverse tissue response.

Table 1 *Ectopic bone formation in goats*
Incidence of bone formation and percentage of bone area (mean±SD) in the two scaffold materials for each of the four treatment groups after twelve weeks implantation.

Group	Bone formation	HA70/800	HA60/400
Vital TE	8/8	13.2 (15.9)	3.4 (4.0)
Devitalized TE	0/8	-	-
Bone marrow	0/8	-	-
HA only	0/8	-	-

Figure 4 *Histology of tissue engineered constructs implanted for 12 weeks (☞* **p. 181)**
a): Overview showing the distribution of bone on the HA surface of the granules implanted as one unit. Both scaffold types can be distinguished (bar = 1mm).
b): Detail of bone (B) bridging between two granules of HA70/800. The triangles point at osteoblast zones (bar = 100µm).
c): Fluorescent microscopy showing the fluorochromes in the bone that formed: Alizarin red (given at 5 weeks) directly on the HA surface, Calcein green (7 weeks) in the middle and Xylenol orange (9 weeks) on the pore side (bar = 50µm).



Discussion and Conclusions

In the present fundamental study, we clearly demonstrate that the presence of living, culture expanded osteoprogenitor cells inside our porous calcium phosphate scaffolds is a prerequisite for *in-vivo* ectopic bone formation in the goat. The scaffolds alone or when combined with either devitalized ECM or undifferentiated/ non-expanded bone marrow were never successful with respect to bone formation in the current setting. The potential synergistic influence of ECM with cells and the differentiation state of the cells was studied separate and are reported elsewhere.[233] On the basis of our own experience and on the scarcity of comparative literature on the subject, bone tissue engineering appears to be much more challenging in larger mammals. This might be due to impaired cell survival, as the metabolic rate of these animals is lower and/or larger implants that are required. This potential reason is supported by previous studies that report negligible cell survival and delayed revascularization of bone grafts, after autotransplantation.[43,169,173,180,290,304] To study if cell vitality is crucial for bone formation, we compared our TE-vital implants with identical implants that were devitalized by a freezing protocol. Other methods for devitalization such as lysing, gamma irradiation or heating might be insufficient or interfere with the expected osteoinduction.[42,73,220,285,286,305] According to literature, freezing has a minimal effect on osteoinductivity.[48,292] We demonstrated by scanning electron microscopy the presence of intact ECM on the scaffold surfaces after freezing, however, we cannot completely rule out that freezing did not interfere with new bone formation in another way. Oklund *et al.* postulated that freezing resulted in calciolysis of bone grafts, thereby exposing the ECM and promoting matrix resorption.[181] In our study this is not likely to be important, as the ECM is exposed at implantation of the TE samples. Furthermore, the presence of the 5 week label in the newly formed bone suggests an early osteogenic mechanism.[287] Therefore, our findings strongly indicate that vitality is crucial for bone generation, and imply that cells do survive, at least the first period after implantation in which they might secrete osteoinductive factors or longer to finally form bone. It has not been proven yet whether the implanted cells are indeed present in the bone that formed, this will be difficult as a proper label for tracing these cells remains to be found.[306] The bone that formed typically initiated at the HA surface and extended towards the center of the pores as shown with the fluorochrome labels. This finding is in agreement with other reports regarding osteoblast differentiation and bonding osteogenesis,[114,291] and emphasizes the importance of an appropriate scaffold[68] allowing cell attachment, proliferation and differentiation. The difference in bone formation we found between the different scaffolds can not, however, be easily explained by these

characteristics. FTIR and XRD did not indicate differences between the chemical compositions of the bulk materials. The pore sizes were different but as a result of different manufacturing techniques are difficult to compare. However, the pores were interconnected in both materials and >100µm which is regarded as a minimal size.[307] Furthermore, quantitative DNA analysis did not show differences in seeding efficiency and in fact showed a DNA amount in favor of the HA60/400 after the 6-day culture period. The most prominent difference between the scaffolds was the microporosity, as shown in Fig. 1. As postulated by others, the larger surface area might result in increased ion exchange and bone like apatite surface formation by the dissolution and re-precipitation process. Furthermore, more proteins and inducing factors could be adsorbed.[98] From that perspective, it should be noted that the scaffold alone did not show any bone induction. As the scaffold type appears to be of major importance, more specific material research is needed that is beyond the scope of this article.

The addition of bone marrow to scaffolds in order to reach ectopic bone formation has been the topic of many investigations in the past, with various levels of success. A limited number of studies that demonstrated bone formation, applied high amounts of bone marrow,[109,209,273] and it should be realized that the presence of osteoprogenitor cells in fresh bone marrow is limited.[211] We attempted to mimic the clinical situation where the amount of BM is limited by using only 1ml of BM per 40mm^3 granule. That could explain why no bone formation occurred in our implants. In agreement with earlier publications,[105,114,211] this finding emphasizes the value of cell-expansion or at least the necessity for a larger number of precursor cells in the scaffolds.

It is concluded from the current investigation that consistent tissue engineered bone formation in the goat can be achieved with vital constructs of an appropriate scaffold and sufficient BMSC's.

Acknowledgements

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Chapter 6

APPLICATION AND LIMITATIONS OF CM-DIL FOR TRACING CELLS IN BONE TISSUE ENGINEERING

Summary

Introduction: Despite extensive research on bone tissue engineering (TE), little is known about the survival and function of the cells after implantation. To monitor the cells *in-vivo*, labeling is the method of choice. In this study we investigated the use of the fluorescent membrane marker Chloromethyl-benzamidodialkylcarbocyanine (CM-DiI) to label cells used in bone tissue engineering.

Methods: Different label protocols were evaluated and concentrations up to 50μM appeared without negative effects on cell vitality, proliferation or bone forming capacity. Porous hydroxyapatite scaffolds were seeded with labeled cells, and traced up to 6 weeks after implantation in nude mice. To determine transfer of the label *in-vivo*, devitalized, labeled constructs were implanted for different time periods. The presence of vital labeled cells inside these constructs would indicate transfer of the label.

Results: Cells could be traced at all time points, also inside tissue engineered bone. However, contrary to other reports concerning intramembranous labels, transfer of the label from labeled to unlabeled cells was found. Transfer occurred both *in-vitro* and *in-vivo* between vital cells and from dead to living cells. Transfer was found at 7 days when 40μM label was applied while 10μM labeled constructs showed transfer 10 days after implantation.

Conclusion: The CM-DiI label is useful for *in-vivo* tracing of cells for follow-up periods up to 10 days. This makes the label particularly useful for cell-survival studies in tissue engineered implants.

Introduction

Despite its drawbacks, autologous bone is the graft material of choice for many orthopaedic and maxillofacial reconstructions. It is hypothesized that the superiority of autologous bone is partially derived from the osteogenic potential from the cells inside the graft. However, their role - if present - is controversial.[42,43] Some authors report an almost total loss of cells in the autologous graft material after implantation,[169] whereas others report substantial survival and function of these cells.[174] There is no doubt, however, that inside large sized grafts the survival of cells will be compromised, as the cells will be inside the harsh environment of a heamatoma,[143] without vascularization during the first week after implantation.[173,290]

Bone tissue engineering potentially provides us with an autologous bone substitute. The concept studied most extensively is the combination of bone marrow derived mesenchymal cells with a biocompatible porous scaffold. Since the early nineties, many reports have been published on bone formation ectopically or in critical sized defects, using this concept.[113,119,122,123,130,137] However, only a few reports address the question whether the implanted cells survive and are functional.[120,144] As for autologous bone transplants, cell survival is also questionable in tissue engineered constructs of clinically relevant size. To address this question, monitoring the cells *in-vivo* by labeling is the method of choice.

Elegant labeling methods that have been reported include the application of quail cells which have a specific nucleolar marker, or the use of transgenic cells. Both methods showed the donor origin of tissue engineered bone.[67,128] These approaches, however, are limited to xenografts and allografts and cannot be used for autologous cells. Recently, genetic markers have shown to be compatible with the process of bone histology.[116,190] Genetic labeling though, requires special facilities and precautions, and a substantial amount of cells is lost during the procedure.[116] Another drawback is a possible immune reaction of the host towards the viral products,[308,309] thus interfering with the intended feasibility of the graft. As yet, the ideal label for labeling cells effectively, does not exist.

A promising alternative is the use of intramembranous fluorescent labels. This technique has already been applied for tracing cells in soft tissues.[184,310-314] Once incorporated within the membrane bilayer, the label becomes trapped due to its water insolubility. At cell division it is distributed equally between daughter cells. A drawback of this method could be the transfer of label to neighboring cells. For application in soft tissue, several authors reported the absence of this transfer to cocultured cells.[185,312]

As compared to soft tissue, tracking cells with fluorescent membrane label in tissue engineered bone introduces two problems: First, bone and the commonly used calcium phosphate scaffold hydroxyapatite have a relatively high auto fluorescence, which means the label has to be specific and intense to allow adequate discrimination. Second, the label must be maintained during (un)decalcified histology processing. Andrade *et al.* showed the CM-DiI label (chloromethyl-dialkylcarbocyanine) is maintained within the cell membrane after dehydration for paraffin embedding, therefore this label is potentially suitable for bone histology.[315] Recently, Ferrari *et al.* introduced CM-DiI as a label for tracing cells in bone tissue engineering. They did not find label transfer when labeled cells inside a scaffold were cultured next to unlabeled fibroblasts for 10 days.[316] Unfortunately this study did not investigate transfer from dead to living cells or label transfer *in-vivo*.
In our research on bone tissue engineering we chose to study CM-DiI as a label for investigation of cell survival and differentiation. As we expected some of the implanted cells to loose vitality and disintegrate after implantation, the transfer of label both *in-vitro* and *in-vivo* from dead labeled cells to neighboring cells as well as the transfer between living cells was examined thoroughly. The purpose of the present study was to determine an optimal label protocol for bone marrow derived goat cells. We defined optimal as the highest label intensity without noticeable effect on cell vitality, proliferation and bone forming capacity. Furthermore we investigated the limitations of the label with respect to label transfer to host cells *in-vitro* and *in-vivo*.

Materials and Methods

Design

Culture-expanded bone marrow cells were divided into 11 groups (one control, eight groups that were treated with different labeling procedures and two groups that were treated with high concentrations of DMSO). All groups were analyzed for cell vitality, label intensity and label efficiency. Fluorescence activated cell sorting (FACS) analysis of label maintenance was done for all groups at every passage. Groups labeled with 10, 50 and 60μM CM-DiI and the controls were analyzed for cell proliferation. Groups labeled with 10, 30, 40, 50 and 60μM CM-DiI and the controls were seeded on porous hydroxyapatite (HA) scaffolds and implanted in nude mice. The implants were evaluated at ten different time points to determine survival and differentiation of the cells, and the maintenance of the label. To investigate label transfer, cells labeled with 10 and 40μM CM-DiI were cocultured with unlabeled cells. To assess *in-vivo* label transfer from dead to living cells,

constructs of cells labeled with 10 and 40µM CM-DiI were devitalized before implantation. Analysis of these implants was done after various periods to determine when transfer occurred.

Harvesting of cells

Goat bone marrow cells were obtained from an iliac wing bone marrow aspirate. The cells were cultured in a standard culture medium[106] supplemented with 1ng/ml basic fibroblast growth factor (BFGF; Instruchemie, Hilversum, The Netherlands). Cells were cryopreserved at the end of the first passage (p=1). Within 6 months, the cryopreserved cells were thawed and replated. When confluent, the cells were trypsinized and a trypan blue exclusion was performed to identify dead cells. The cells were distributed over eleven 10ml tubes, each containing 10 million cells, and centrifuged. The pellets were treated as indicated in Table 1.

Table 1 *CM-DiI labeling procedure*
Cells were labeled with various concentrations of CM-DiI (column 1) by the following procedure: 50µg CM-DiI was diluted in different volumes of DMSO (column 2), these solutions were diluted further in HBSS (column 3) and then added to cell pellets resuspended in HBSS (column 4) to yield final DMSO (column 5) and CM-DiI (column 1) concentrations. The incubation time was either 2 or 4 minutes at 37ºC plus 15 minutes on ice.

Group	Vol. of DMSO to solute 50µg CM-DiI	Vol. of CM-DiI solution per vol. HBSS	Vol. of HBSS to resuspended cell Pellet	% DMSO	Incubation time
Control	-	-	1.0ml	-	4+15
5µM	50µl	10µl/1.0ml	1.0ml	0.5%	4+15
10µM	50µl	10µl/0.5ml	0.5ml	1.0%	4+15
20µM	25µl	10µl/0.5ml	0.5ml	1.0%	4+15
30µM	25µl	15µl/0.5ml	0.5ml	1.5%	4+15
40µM	25µl	20µl/0.5ml	0.5ml	2.0%	4+15
40µM	25µl	20µl/0.5ml	0.5ml	2.0%	2+15
50µM	25µl	25µl/0.5ml	0.5ml	2.5%	2+15
60µM	25µl	30µl/0.5ml	0.5ml	3.0%	2+15
4% DMSO	DMSO only	40µl/0.5ml	0.5ml	4.0%	2+15
6% DMSO	DMSO only	60µl/0.5ml	0.5ml	6.0%	2+15

Labeling procedure (Table1)

The pellets were resuspended in Hanks' buffered salt solution (HBSS; Gibco, Paisley, Scotland). Chloromethyl-benzamidodialkylcarbocyanine (CM-DiI, mol. wt. 1051.5; Molecular Probes, Eugene, US) was used as labeling agent. This long-chain carbocyanine membrane probe has a peak excitation at 553nm and emits at 570nm. First, 50µg of CM-DiI was reconstituted in 25 or 50µl dimethylsulfoxide (DMSO; Sigma, St. Louis, MO) to make a stock solution. Subsequently, the CM-DiI stock solution was added in varying amounts to 0.5 or 1ml HBSS and immediately combined with the cell suspension. This final suspension was pipetted thoroughly to obtain equal mixing and incubated in a 37ºC water bath. We used incubation times of 2 minutes for label concentrations above 40µM and 4 minutes for lower concentrations, which we determined did not effect cell vitality negatively. However, during this study we found that labeling for 4 minutes negatively influenced cell proliferation, therefore later experiments used a maximum incubation time of 3 minutes. The cells were then incubated for another 15 minutes on ice. According to the manufacturer, incubating at this lower temperature allows the dye to label the membrane, but slows down endocytosis. After incubation, the reaction was stopped by adding 9ml of phosphate buffered saline (PBS, Gibco), and the cells were washed and centrifuged. After centrifugation, the cells were counted and a trypan blue exclusion was done. To study the effect of DMSO only on cell vitality, 2x10 million cells were incubated with high concentrations of DMSO.

Label efficiency and maintenance

All groups were analyzed by FACS (FACScalibur; Becton & Dickinson, San Jose CA). Fluorescent intensity was analyzed on 10.000 gated events of every group in the Fl2 channel (585±21nm). The cells with an intensity higher than 97.5% of control cells were considered to be labeled. The mean fluorescent intensity and the percentage of these labeled cells were registered. All groups were kept in culture and were plated on chamber slides at every new passage (Nalge Nunc, Naperville, IL.) for analysis with an epifluorescence microscope (E600, Nikon, Tokyo, Japan) using the TRITC filter set (excitation 540nm dichroic mirror 565nm). To study the *in-vivo* implants we made use of a double filter block (dichroic mirror 505nm and 590nm) to allow good discrimination between labeled and unlabeled cells. Micrographs were taken with a digital camera system.

Cell proliferation

To monitor cell proliferation of the control, 10, 50 and 60μM labeled groups, each group was plated in two six-well plates. At 3 and 5 days, the cells were counted (*n*=6). The cells obtained after 5 days were again plated in three six-well plates and counted after another 3, 5 and 6 days. Cells were counted twice in duplicate with a Coulter® counter Z2 (Coulter, Miami FL). The proliferation was expressed as the amount of population doublings [^{2}Log(increase)].

In-vitro label transfer

From the cells labeled with 10μM for 3 minutes and 40μM for 2 minutes, 6 aliquots of 100.000 cells were frozen in liquid nitrogen and thawed in PBS at room temperature.[59,282] This was done twice, to effectively devitalize the cells. Another 6 aliquots of each label concentration were frozen and then lysed in demineralized water to obtain the actual label content of these cells in suspension. A final group of six aliquots from each label concentration was left untreated. Three of the untreated (vital) aliquots were cultured single, the remaining three were mixed with an equal amount of vital unlabeled cells and cocultured to determine transfer between vital cells. The frozen and the lysed aliquots were also mixed with unlabeled cells, to determine label transfer from devitalized to vital cells. At 4 days, cultures were replated. All cultures were analyzed at 1, 2, 4, 7, 8 and 11 days by FACS analysis after washing the cells. To assess the percentage of labeled cells, at each time point, we defined regions for labeled and unlabeled cells in the dot plot of the unlabeled cells that were applied to the other dot plots. As labeled cells were mixed with an equal amount of unlabeled cells, the expected percentage of labeled cells in the cocultures was half the percentage found in the labeled cultures at the same time point. Higher than expected percentages indicated transfer of the label to unlabeled cells. When labeled cells were detected in the cocultures of vital unlabeled and devitalized labeled cells, transfer was assumed. These cells were then plated in chambers slides for evaluation by fluorescent microscopy. To ensure the efficiency of devitalization, the remaining frozen and lysed aliquots were cultured for two weeks and checked for vital adhering cells.

In-vivo traceability

We selected the control, 10, 30, 40, 50 and the 60µM groups as representatives for *in-vivo* tracing. Per group, one million cells were dynamically seeded on ten hydroxyapatite granules of 3x3x4mm with a 70% interconnected porosity. (HA; CAM Implants, Leiden, The Netherlands). The constructs were cultured for one week in medium containing 10^{-8}M dexamethasone and 0,5mg/ml β–glycerophosphate (both from Sigma) to promote osteogenic differentiation.[106] The granules were implanted subcutaneously in nude mice (per mouse one granule of each group), and explanted after 2, 3, 4, 5, 7, 10 days and 2, 3, 4 and 6 weeks. Another five granules were implanted for decalcified histology at 10 days. All samples were fixated in 1.5% buffered glutaraldehyde and dehydrated in alcohol. For the undecalcified procedure samples were embedded in methyl-metacrylate (MMA; Merck, Darmstadt, Germany) and sectioned (10µm with a sawing microtome; Leica, Nussloch, Germany). After epifluorescence microscopy, the sections were counterstained with methylene blue and basic fuchsin for routine light microscopy. For decalcified histology the samples were decalcified in a 10% EDTA solution, embedded in glycol methacrylate (GMA; Merck) and sectioned at 10µm on a standard microtome.

In-vivo label transfer

Constructs of cells labeled with 10 and 40µM were devitalized by freezing in liquid nitrogen. To evaluate the devitalization, three frozen constructs of each condition were morselized and cultured for 30 days to determine the appearance of adhering cells (when vital constructs were cultured this way, adhering cells appeared within 7 days). Devitalized samples were implanted in duplo into six nude mice. As a control, vital labeled constructs were implanted in the same mice. At 3, 5, 7 and 10 days, and at 2 and 4 weeks, the samples were explanted and analyzed as described before. When labeled vital cells (as judged by morphology) were detected in the devitalized samples, label transfer was assumed.

Statistics

Statistical calculations were done with the SPSS 9.0 software package. The type 1 error was set at 5%. Cell count data per group at different time points were analyzed by multiple factor analysis of variance (ANOVA). In case of significant interaction between time and group, multiple comparisons per time-point were done using Bonferroni-corrected t-tests.

Results

Labeling procedure (Table 2).

Before labeling, 11% of the cells obtained after trypsinization were indicated non-viable by the trypan blue exclusion method. After labeling this was on average 9%, without large differences between the groups. This indicated that cell vitality was not influenced by the procedure itself, the concentration of CM-DiI, or higher concentrations of DMSO (Table 2). Cell counting after the whole procedure revealed that 20±6% of the cells was lost in all groups; no relation with label intensity was found. FACS analysis after labeling and at each cell passage indicated the difference in intensity and efficiency. This was visualized with epifluorescent microscopy of the plated cells (Fig. 1).

Table 2 *Analysis label maintenance and cell growth results*

[1] *Cell mortality is expressed as the percent difference of dead cells before and after the procedure as measured by trypan blue exclusion.*

[2] *Percentage of cells lost is calculated on the basis of cell count data taken before and after the procedure.*

[3] *Proliferation is expressed as the number of population doublings = ^{2}Log(increase) in 11 days.*

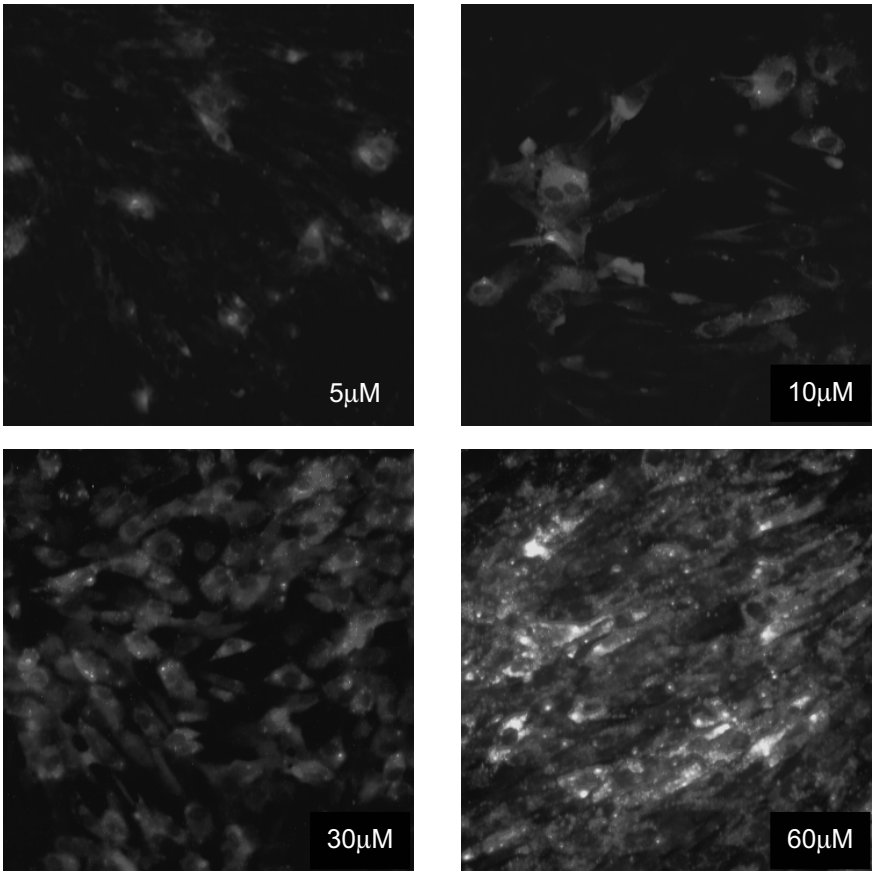
**Cells labeled with 60µM had proliferated significantly less as shown in Fig. 2.*

Group	Cell Mort.[1]	Cells Lost[2]	Results of FACS analysis on CM-DiI labeled cells percentage labeled and mean intensity								Proliferation[3]
			t=0		t=1		t=3		t=4		
			Labeled	Mean	Labeled	Mean	Labeled	Mean	Labeled	Mean	
Control	-1.5%	28%	2.5%	11	2.5%	12	2.5%	19	2.5%	30	8.4
5µM	0.4%	18%	65%	53	61%	58	14%	34	1.1%	43	
10µM	-4.9%	13%	62%	55	57%	54	9%	33	3.2%	41	
20µM	-3.1%	11%	80%	87	84%	133	30%	58	2.6%	52	8.7
30µM	2.4%	14%	75%	60	76%	92	19%	40	1.8%	60	
40µM (2min)	-1.3%	27%	62%	64	70%	92	19%	45	2.3%	43	
40µM (4min)	-4.2%	21%	84%	162	81%	132	27%	66	4.0%	56	
50µm	-1.3%	27%	84%	213	87%	151	28%	65	2.1%	49	8.6
60µM	-2.5%	22%	88%	329	90%	171	37%	83	3.1%	60	7.1*
DMSO 4%	-3.8%										
DMSO 6%	-4.0%										

Label maintenance (Table 2)

FACS analysis after the first trypsinization showed no loss of label intensity. At the second trypsinization, after more than seven population doublings, the label intensity had decreased. At the third trypsinization only few labeled cells were detected. The population had by then doubled about 10 times. In the chamber slides individual cells could be detected by fluorescent microscopy even after 10 population doublings. Cells that we identified as labeled had typically many fluorescent spots. According to the manufacturer, the label spontaneously aggregates at concentrations above 5μM, which could explain this phenomenon. No fading of the label was seen when exposed to the excitation light of the fluorescence microscope.

Figure 1 *Epifluorescent images of cells labeled with varying concentrations of CM-DiI Higher label concentrations resulted in higher intensities and efficiencies of the label. All micrographs were taken after 5 days culture on microslides at the same magnification (200x).*

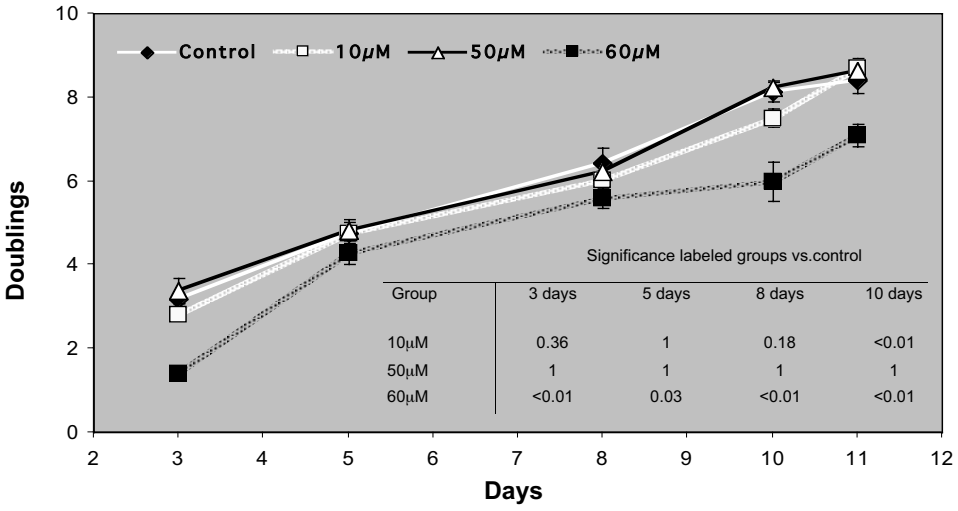


Proliferation (Fig. 2)

Statistical analysis of the six-well counts showed a weak, though significant interaction between group and time (data not shown). Therefore, multiple comparisons were done to examine all relevant group and time combinations. Analysis indicated a significantly lower rate of proliferation at all time points of the cells labeled with 60µM, compared with the control. The group labeled with 50µM was never significantly different from the control group. Cells labeled with 10µM (incubated for 4 minutes) had proliferated significantly less than the control group at 10 days.

Figure 2 *Cell proliferation*

Expressed as the amount of population doublings (^{2}Log increase) ± SD, n=6 for each time point. At all time points the 60µM group had proliferated significantly less than the control



In-vitro label transfer (Figs. 3 and 4)

Devitalization was efficient as no vital, adherent cells were detected in the control cultures. Fig. 3 shows the combined histograms of the unlabeled cells, the labeled cells and the coculture of unlabeled cells with devitalized 10μM labeled cells at 2 and 4 days. The fluorescence intensity in the coculture was progressively higher than the control group. At 2 and 4 days, 20% and 43% of these cells, respectively, had label intensities higher than 97.5% of the control cells. These findings indicate transfer of the label between dead and vital cells. When the devitalized cells were labeled with 40μM CM-Dil, the amount of transfer was higher, 47% and 79% respectively (data not shown). The method of devitalizing by itself (freeze thaw freeze or lysing) did not appear to influence the amount of transfer. Transfer of the label was confirmed by fluorescence microscopy of the cells when cultured on chamber slides.

Fig. 4 shows the result of region-statistics on the FACS dot plots. Vital cells labeled with 10μM CM-Dil did not show any transfer to the unlabeled cells during the 11-day period during which they could be detected. In the coculture with 40μM labeled vital cells, the percentage of labeled cells exceeded the expected percentage, indicating transfer of the label. However, the percentage of false positive cells [(actual-expected)/actual x 100%)] did not increase between 1 and 8 days. This suggests transfer had mainly occurred after plating the cells and before analysis on day 1.

Figure 3 *Combined histograms of FACS results from the in-vitro transfer experiment*
a) After two days of coculturing with devitalized 10mm labeled cells, 20% of the initially unlabelled cells had label intensities higher than 97.5% of the control (within M1).
b) After 4 days this percentage had increased to 43% indicating progressive label transfer.

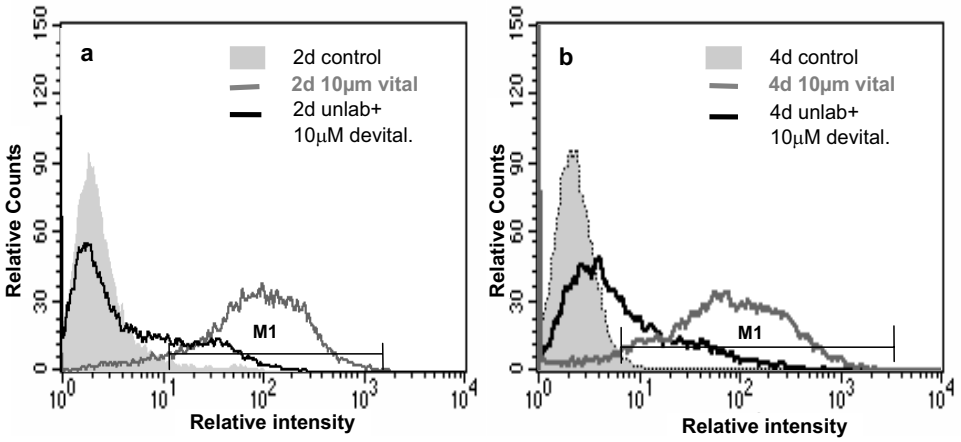
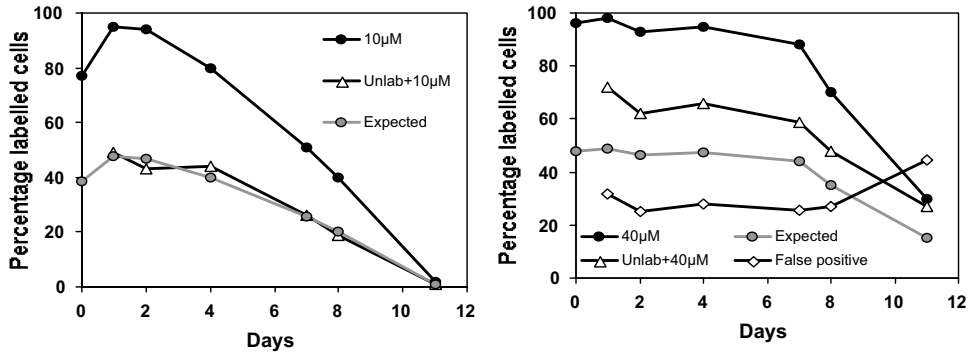


Figure 4 *Percentages of labeled cells at different time points*
Assessed by statistical analysis of regions of FACS dot plots. Data are from labeled cell cultures and 50/50 cocultures of labeled and unlabeled cells.
a) 10μM label concentration. Expected values were calculated from data of labeled cells. Coculture percentages were in agreement with expected values, indicating no transfer.
b) 40μM label concentration. Coculture percentages exceeded expected values, indicating transfer of label. The relationship between actual (coculture) and expected values, indicated by the false positive line, did not change during the first 8 days indicating that no additional label transfer occurred in this time period.



In-vivo label transfer

During the first 5 days after implantation, no label transfer from the dead labeled cells to host cells could be determined. At 7 days, labeled fibroblastic cells could be detected in the 40μM devitalized implants, indicating label transfer. This was not found for the implants that contained 10μM devitalized cells. However, by 10 days, the 10μM devitalized implants also contained occasionally labeled fibroblastic cells. After two weeks label had almost disappeared from the 10μM samples, by then, in vital and devitalized 40μM samples fluorescent cells could be found in equal numbers (Fig. 5e). This was also seen at 4 weeks.

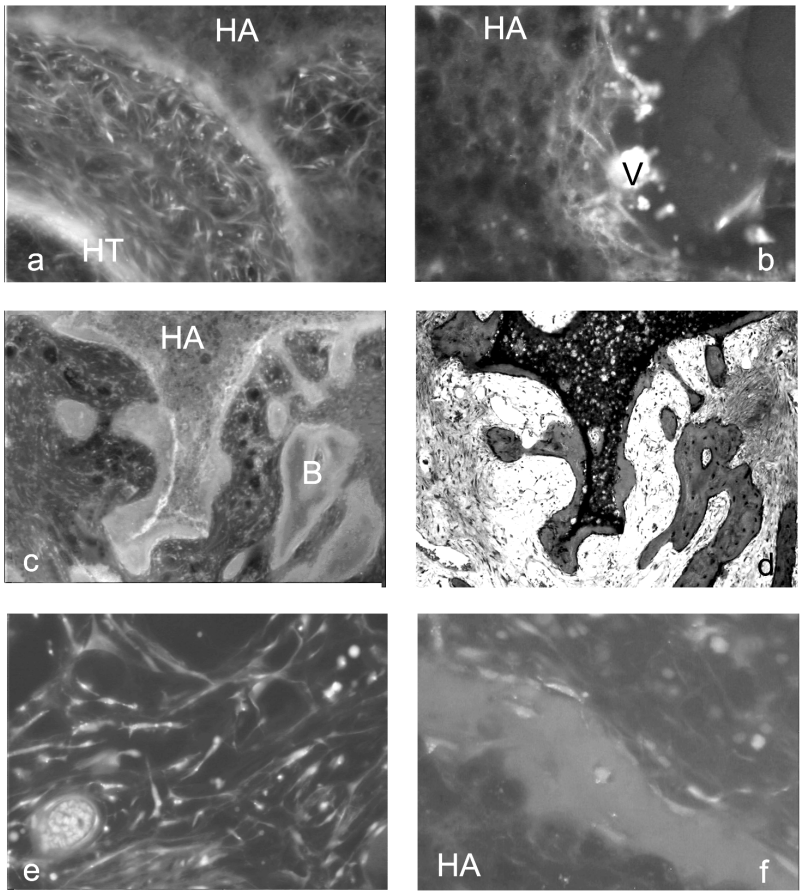
Histology (Figs. 5a-d)

Fluorescence microscopy of the samples implanted for 2 days showed invasive host tissue on the periphery of the scaffold that could be distinguished from the labeled cells (Fig. 5a). At 3 days, the first small blood vessels had invaded in the periphery of the implants. By 4 days, blood vessels were visible throughout the scaffolds adjacent to vital labeled cells (Fig. 5b). After 10 days, the pores were filled with loose connective tissue containing abundant blood vessels. Cells within this reticular tissue were labeled, however, their origin remains uncertain. On the

periphery of all scaffolds (five of five), osteoid formation could be identified with light microscopy. After 2 weeks bone was present at the periphery of all scaffolds. After three weeks bone was present mainly inside the scaffolds (five of five). In some pores, cartilage was found. Counterstaining of the slides, demonstrated the double-filter fluorescent image corresponded well with normal histology (Figs. 5c and d). The four and six weeks' samples demonstrated lamellar bone in all vital samples (24 of 24), occasionally containing bone marrow. Inside the bone, labeled osteocytes were visible (Fig. 5f). Histological evaluation of decalcified samples after 10 days implantation, showed labeled cells, though less bright and in lower frequency.

Figure 5 *Micrographs of samples after various implantation periods* **(☞ p. 182)**

a) 2 days after implantation: Invading host tissue (HT) next to 40µM labeled cells on the HA surface (original magn. x200).
b) 4 days after implantation: 40µM Labeled cells were found next to small bloodvessels (V) within the fibrin network inside the pores (original magn. x100).
c) 3 weeks after implantation: New bone formation was visible (B) and appeared as light green, more mature bone appeared brown when compared with (d) (original magn. x100).
d) sample shown in (c), counterstained with methylene blue and basic fuchsin.
e) 2 weeks after implantation sample with devitalized 40µM labeled cells: labeled fibroblastic cells were abundant indicating label transfer by this time (original magn. x200).
f) 6 weeks after implantation: 50µM labeled cells within the newly formed bone (green) were clearly visible. (original magn. x400).



Discussion and Conclusions

In the present study, we evaluated a method for labeling and subsequently, histological identification of cells used for bone tissue engineering. The labeling procedure took approximately 30 minutes, cell loss was approximately 20%, and no effect on cell vitality was observed. With label concentrations above 20μM, more than 75% of cells maintained the label for at least 4 population doublings. *In-vivo*, the label could be traced with undecalcified histology at all follow-up periods. Labeled cells were detectable in the newly formed bone and surrounding fibrous tissue. No effect on bone forming capacity was observed. According to these findings, labeling with 50μM for 2 minutes, could be considered optimal. However, the *in-vitro* transfer experiments demonstrated label transfer between vital cells when the label concentration was increased from 10μM to 40μM. Furthermore, we found label uptake when cells were cocultured with devitalized cells labeled with both 10 and 40μM CM-DiI. These findings are in contrast to reports where no transfer was found.[184,312,316] Ferrari *et al.* did not detect label transfer from cells labeled with 20μM CM-DiI. They mention, however, that they could not rule out label transfer from dead labeled cells or via direct membrane contact, as we found in our study. Although transfer between vital cells might be controlled, since this occurred only within the first day after coculturing with unlabeled cells (Fig. 4) and only with high label concentrations, the transfer from dead cells cannot. In tissue engineered constructs, where cell death and subsequent release of the label can be expected, the question apparently is not whether transfer occurs, but rather when this occurs. To determine when the label transfer from dead cells became visible, we analyzed samples after different implantation periods. We found label transfer after 7 days in the samples labeled with 40μM, and after 10 days in those labeled with 10μM CM-DiI. These findings imply that the label is applicable for discrimination between implanted and host cells only during the first week after implantation. Therefore, a satisfactory method for tracking cells for longer periods to determine autologous cell differentiation and survival in large immunocompetend animals still must be found.

From the current investigation it can be concluded that fluorescent labeling with CM-DiI has many advantages and is compatible with bone histology. At concentrations that should be defined for specific cells populations, the CM-DiI label is stable in vital cells and useful for tracing these cells. However, transfer does occur from dead labeled cells to host cells at all concentrations. *In-vivo* this was found one week after implantation of dead labeled cells. Therefore, studies for cell survival using the CM-DiI label are only considered feasible during the first days after implantation.

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Chapter 7

GENETIC MARKING WITH THE ΔLNGFR-GENE FOR TRACING GOAT CELLS IN BONE TISSUE ENGINEERING

Summary

Introduction: Little is known about the survival and differentiation of cells used for bone tissue engineering (TE). The aim of this study was to develop a method to trace goat BMSC's *in-vivo* by retroviral genetic marking.

Methods: Goat BMSC's were subjected to an amphotropic envelope containing a MoMuLV-based vector expressing the human low affinity nerve growth factor receptor (ΔLNGFR). Labeling efficiency and effect on the cells were analyzed. Furthermore, transduced cells were seeded onto porous ceramic scaffolds, implanted subcutaneously in nude mice and examined after successive implantation periods.

Results: Flow cytometry indicated a transduction efficiency of 40-60%. Immunohistochemistry showed survival and subsequent bone formation of the gene marked cells *in-vivo*. Besides, marked cells were also found in cartilage and fibrous tissue. These findings indicate the maintenance of the precursor phenotype following gene transfer as well as the ability of the gene to be expressed following differentiation.

Conclusion: Retroviral gene marking with ΔLNGFR is applicable to trace goat BMSC's in bone tissue engineering research.

Introduction

Tissue engineering (TE) by combining specific cells with an appropriate scaffold has gained much attention during the last decades. The proof of the concept has been shown for many tissues types.[1] Especially the mesenchymal lineage has been investigated extensively, since the identification of putative stem cells in the adult bone marrow.[63,75,79] These Bone Marrow Stromal Cells (BMSC's) can be cultured and directed towards several lineages of differentiation[7,63,79,317] e.g. bone,[123,131] cartilage,[79,188] and tendon.[318] Furthermore, these cells have been investigated as a delivery vehicle for gene therapy.[116,198,319] The application of BMSC's in tissue engineering of bone has progressed fast and some investigators already made the step towards clinical application.[320,321]

However, with regard to the clinical application of bone TE, there are two important gaps in the current knowledge of cell performance. First, the differentiation of BMSC's cannot be predicted, because they constitute a heterogeneous population.[7,79,317] At second, little is known about cell survival. Although survival was shown convincingly in implants of the mm^3-volume range in rodents,[67,75,116,128] this will be compromised in the clinical situation, due to the dramatically increased volume (several cm^3), and delayed vascularization.[322]

To investigate these two issues, monitoring of BMSC's in an established "large animal" model for bone TE is the method of choice. Recently, we developed such a model for ectopic bone TE in the goat.[131,278] Together with a more challenging, critical sized defect model in the same animal,[36] this gives the opportunity to extensively evaluate both differentiation and survival of the BMSC's.

To trace cells efficiently, a variety of labeling methods has been developed. The use of fluorescent membrane markers is relatively simple,[121,184,188] but inherent to dilution with cell division and the risk of label transfer to neighboring cells.[121] More reliable methods are the use of transgenic cells,[128] x-y mismatches[75] or xenogenic transplantation.[67] These approaches however, are not compatible with autologous transplantation. Recently, genetic marking has become a standard tool and it was shown to be compatible with bone histology.[116,191-193,323] Although an immune response cannot be excluded, this approach seems to be most optimal.[193,323]

To trace cells after long implantation periods in large bone samples, the label must be compatible with histologic procedures, such as dehydration and decalcification, and not depend on the diffusion of a substrate into the sample for enzymatic conversion. Therefore, a retroviral label (which integrates stable in the cell genome) that can be traced with routine immunohistochemistry is most appropriate. The truncated human nerve growth factor receptor (ΔNGFR) has been applied

successfully for tracing many cell types with both flow cytometry and immunohistochemistry.[323-326] The purpose of the present study was to develop a method to retrovirally label goat BMSC's with the NGFR label without noticeable effect on cell viability and bone forming capacity. Furthermore, the process of tissue engineered bone formation by implanted goat BMSC's in the ectopic nude mice model was investigated.

Materials and Methods

Study design

A key consideration in retroviral transduction is the choice of the viral surface envelope - pseudotype -, which determines the vector tropism. In a first experiment we therefore compared Amphotropic, GALV, RD114 and VSV-G vector pseudotyped virus, expressing the EGFP gene for the transduction efficiency of goat BMSC's, and selected the best for further studies with the NGFR marker gene. The transduction efficiency, i.e. the percentage of NGFR positive goat BMSC's was determined using flow cytometry. Long-term stability of NGFR expression *in-vitro* was determined by weekly analysis for up to 6 weeks. In addition, a population of transduced cells was co-cultured with mock-transduced cells to determine relative differences in cell proliferation.

To analyze *in-vivo* traceability, transduced and mock-transduced cells were seeded on porous ceramic scaffolds and implanted subcutaneously in nude mice. The implants were evaluated at different time points.

Cell cultures

Cryopreserved goat BMSC's that had shown to be osteogenic in a previous study[131] were thawed and replated in culture medium containing 30% Fetal Bovine Serum (FBS, Gibco, Paisly, Scotland, lot# 3030960S).[131] When confluent, the cells were detached and replated at 5000 cells/cm^2 in standard culture medium containing 15% FBS.

Phoenix-ampho retrovirus packaging cells were cultured in DMEM (Gibco), supplemented with 10% FBS, penicillin (100U/ml) streptomycin (100µg/ml) and 2 mM L-glutamine (Gibco). Cultures were passaged twice a week and selected for gag, pol, env expression every 8 weeks using Hygromycin B (300µg/ml) (Roche Diagnostics, Mannheim Germany) and Diphtheria Toxin A (1µg/ml) (Sigma-Aldrich, Zwijndrecht, The Netherlands).

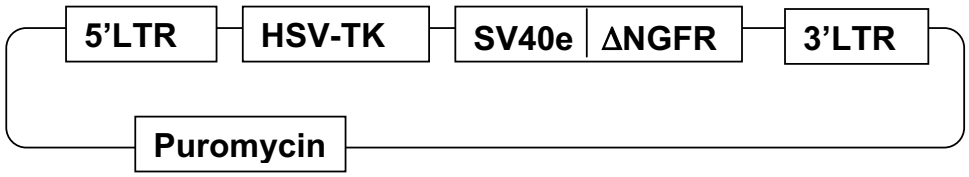
Construction and packaging of retroviral vector containing NGFR

The construction of the retroviral vector, pLZRS-TK, (Fig. 1) has been described previously.[325] The ΔLNGFR marker gene is under the control of Simian Virus 40 early promoter (SV40), flanked by the Moloney Murine Leukaemia Virus (MoMuLV) long-terminal repeats. All experiments for evaluation of the NGFR label were done with amphotropic pseudotyped viral particles, generated in Phoenix-Ampho packaging cell line as follows: 20µg DNA of a retroviral plasmid construct was transfected into Phoenix-Ampho packaging cells that had grown to 70% confluence by using calcium phosphate precipitation.[327] Twenty-four hours after transfection, medium was replaced with fresh culture medium. The following day, retroviral supernatant was collected, filtered through a 0.45µm filter and stored at –80ºC. For an additional harvest of retroviral supernatant, transfected Phoenix-ampho cells were cultured for 3 days in the presence of puromycin (1µg/ml, Sigma) followed by 2 days in culture medium without puromycin.

Selection of vector pseudotype

To select an optimal vector pseudotype, we used the pSFFV-EGFP- vector created and characterized by Baum *et al*,[328] containing the Enhanced Green Fluorescent Protein (EGFP) under the control of a hybrid promoter of Spleen Focus Forming virus and Stem Cell Virus. EGFP was chosen as the transgene because of the ease of detection and quantification of expression by flow cytometry. Two goat BMSC batches were transduced with vectors from four different retrovirus producing cell lines (see also Fig. 2): 1) The Phoenix-Ampho packaging cell line expressed the amphotropic envelope;[327] 2) The PG 13 packaging cell line expressed the Gibbon-Ape Leukemia Virus envelope (GALV);[329] 3) The 293 GPG expressed the vesicular stomatitis virus G envelope (VSVG)[196] and 4) The FLYRD18 packaging cell line expressed the Feline Endogenous Virus envelope (RD114).[330] Viral supernatant containing the different pseudotyped vector particles was generated according to standard procedures. The viral titers were determined on 293T cells, calculated from the linear part of the curve in which the percentage of fluorescent cells (transduction efficiency) was plotted against the dilution of supernatant. The viral titer was adjusted to 1.0×10^5 Transduction Units (TU) per ml.

Fig. 1 *The retroviral construct*
The retroviral vector LZRS-TK as described by Weijtens et al.[325] contains the HSV-tk suicide gene under the transcriptional control of the 5'LTR and the truncated nerve growth factor receptor gene (ΔLNGFR) under the control of the SV40 early promotor that is constitutively switched on. The puromycin resistance gene which is driven by the PGK-1 promoter is present in the nonretroviral portions of the plasmid to allow selection of packaging cells.



Retroviral transduction of goat BMSC's

For comparative goat BMSC transduction studies, the BMSC's were plated in six-well plates (Nalge Nunc, Roskilde, Denmark) at 10^5 cells per well the day before transduction. Transduction was performed by replacing the standard culture medium with a 3-fold dilution of the retroviral supernatant in culture medium supplemented with 6µg/ml polybrene (Sigma). Culture medium supplemented with polybrene was used for mock-transductions. The plates were cultured for another 24 hours, after which the medium was refreshed. Two days later the cells were harvested and analyzed.

Flow cytometry

After transduction, aliquots of cells were withheld for flow cytometry to determine the transduction efficiency. The cells were labeled with primary mouse αNGFR monoclonal antibody (20.4 culture supernatant) for 20 minutes at 4ºC, washed and then incubated with goat anti-mouse Phycoerythrine (PE) conjugated IgG1 (Southern Biotechnologies, Birmingham, US). Cells were washed in PBS-1%FBS and resuspended in standard culture medium, immediately before analysis of 10.000 events on a flow cytometer (FACS Calibur, Becton and Dickinson, San Jose, US).

Immunomagnetic purification of the NGFR positive BMSC's

The presence of NGFR on the cell surface allows in-vitro selection of transduced cells by the use of immunomagnetic microbeads. Transduced goat BMSC's were incubated with diluted αNGFR Mab 20.4 (20µl/1x10^6 cells) and subsequently incubated with goat anti-mouse IgG microbeads (20 µl/10^7 cells) for 10 minutes at 4ºC. Then, cells were washed and separated using a miniMACS separation column according to the manufacturers protocol (Miltenyi Biotec, Bergisch Gladbach, Germany).

Long-term evaluation and co-culture assay

To investigate long-term label expression, two populations of transduced cells were plated in 25cm^2 flasks, cultured for six weeks and analyzed by FACS at each passage. Mock-transduced populations were analyzed parallel. To determine a potential difference in proliferation between transduced and mock-transduced cells, a 50/50% mixture was also investigated.

In-vivo traceability of gene-marked BMSC's

Tissue engineered constructs with transduced (*n*=14) and mock-transduced (*n*=14) cells were prepared as described before.[121] Briefly, 10^5 cells were seeded on 50%-porous, 4x4x3mm ceramic scaffolds (OsSaturaBCP™, IsoTis, Bilthoven, The Netherlands). The hybrid constructs were cultured for one week before implantation in separate subcutaneous pockets on the back of fourteen NMRI nude mice[121] (two mice per evaluation period). Additional constructs with transduced cells (*n*=7) were devitalized by freezing in liquid nitrogen before implantation, (one mouse per evaluation period) to investigate the specificity of the label for viable cells.[121] The implants were retrieved after 2, 4, 7 and 10 days, and 2, 4 and 6 weeks. All animal experiments were according to the regulations of the local committee for animal research.

Immunohistochemistry

Samples were fixated in a 4% formaldehyde solution and decalcified for 1-2 days in 50% formic acid. Dehydration to allow paraffin embedding, was performed by graded alcohol series. The decalcification procedure was based on series of experiments with increasing concentrations of formic acid or EDTA (data not shown). Sections of 5µm were dewaxed, rehydrated and endogenous peroxidase

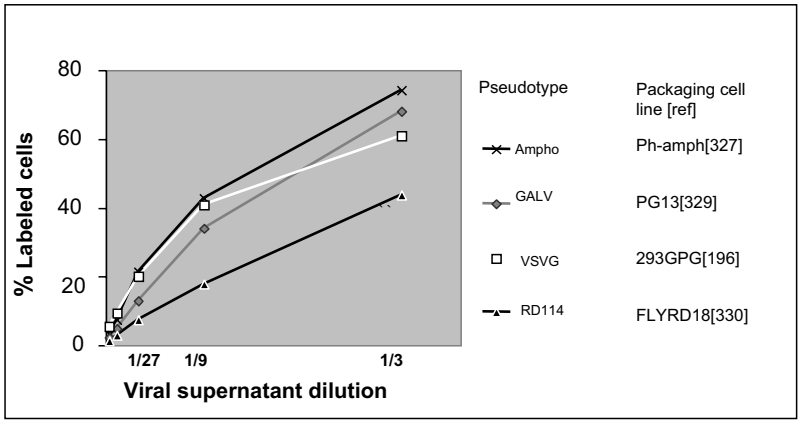
activity was blocked with 1.5% H_20_2 in phosphate-citrate buffer. To prevent nonspecific staining due to reactivity of the secondary anti-mouse IgG antibodies with the surrounding murine tissue, the DAKO ARK™ kit was applied according to manufacturers' recommendations (DAKO Corporation, Carpinteria, USA). For counterstaining we used hematoxylin and Giemsa (Merck, Darmstadt, Germany) which specifically colors the bone bright pink.[331] Samples were analyzed with light microscopy (E600 Nikon eclipse, Japan).

Results

Selection of vector envelope (Fig. 2)

Fluorescence microscopy showed effective EGFP transduction of both goat BMSC batches with all vector pseudotypes. Although these different pseudotyped viruses were used at identical MOI's, transduction with retroviral particles derived from the Phoenix-Ampho packaging cell line resulted in the highest transduction efficiency i.e. nearly eighty percent when using a 3-fold dilution of the virus. While the GALV and VSV-G pseudotyped viral vectors gave only slightly lower values, the efficiency with RD114 was substantially lower. Because of the ample experience with the Phoenix-Ampho system and the intention to transduce human BMSC's in future studies, the Phoenix-Ampho line was chosen for further experiments.

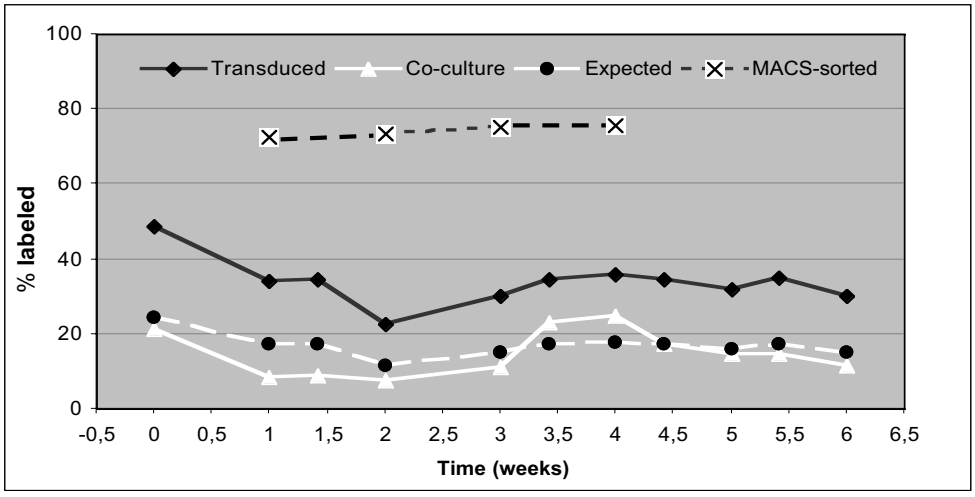
Figure 2 *Selection of optimal envelope pseudotype*
The transduction efficiency of the four different envelope pseudotypes as a function of the dilution of viral supernatant. With a three fold diluted supernatant about 80% of the goat BMSC's was transduced successful with the EGFP gene packaged with the amphotrope packaging cell line.



In-vitro analysis of transduced goat BMSC's (Fig. 3)

A single round of transduction of various batches of goat BMSC's with a 3-fold dilution of retroviral supernatant expressing NGFR, resulted in 40-60% transduction efficiency. In subsequent weeks, this percentage dropped to 30-40%. The long-term culture experiment also showed this decrease during the first period after the transduction, but thereafter stabilized around 35% for up to 6 weeks. The co-culture experiment showed the percentage of labeled cells to be less than expected the first week after transduction, indicating a relative decreased proliferation compared to the non transduced cell fraction. During the subsequent weeks, the percent $NGFR^+$ cells in the co-culture reached the expected percentage, indicating a minimal influence on cell proliferation at the long term. A single round of sorting with immunobeads yielded a stable 70-80% NGFR positive cells that were cultured for 4 weeks (Fig. 3).

Figure 3 *Long term label expression*
The transduction efficiency of this batch of BMSC's was 50% (squares). After an initial decrease and moderate increase, the percentage stabilizes around 35% for up to 6 weeks. The interrupted line (circles) shows the expected percentage labeled cells in the 50/50% mixture. The actual percentage labeled cells in the co-culture (triangles) showed to be initially lower, but finally close matched the expected percentage. One week after MACS sorting (X-X), the population remained 70-80% positive for four weeks.



Analysis of the retrieved in-vivo samples (Fig. 4)

All mice survived the planned implantation periods and all samples were retrieved without signs of infection. Immunistochemistry did not show aspecific binding of the 20.4 antibody (control samples with mock-tranduced cells and secondary antibody stainings were always negative). Analysis of devitalized constructs indicated some residual NGFR label, not associated with viable cells, up to 4 days after transplantation.

The samples implanted for 2 days showed NGFR positive cells on the scaffold that could be well distinguished from surrounding host tissue (Fig. 4a). At 4 and 7 days, small blood vessels were visible inside the scaffolds, NGFR positive cells were distributed evenly throughout the pores without a preference for the ceramic surface. Ten days after implantation, condensations of fibroblast-like cells on the scaffold surface appeared partially consisting of gene marked cells (Fig. 4b). By then, the samples were well vascularized (Fig. 4c). Comparable observations were made for the two weeks implanted samples. After four weeks, bone that harbored labeled cells was present inside all scaffolds that contained transduced cells. In some pores, also cartilage that contained NGFR positive cells was found (Fig. 4d,e). Six weeks' samples demonstrated lamellar bone in all vital samples. Besides inside the bone, labeled cells were also found within the osteoblast zones (Fig. 4f). No difference in bone forming capacity was observed between transduced and mock-transduced cell constructs (all samples gave bone).

Figure 4

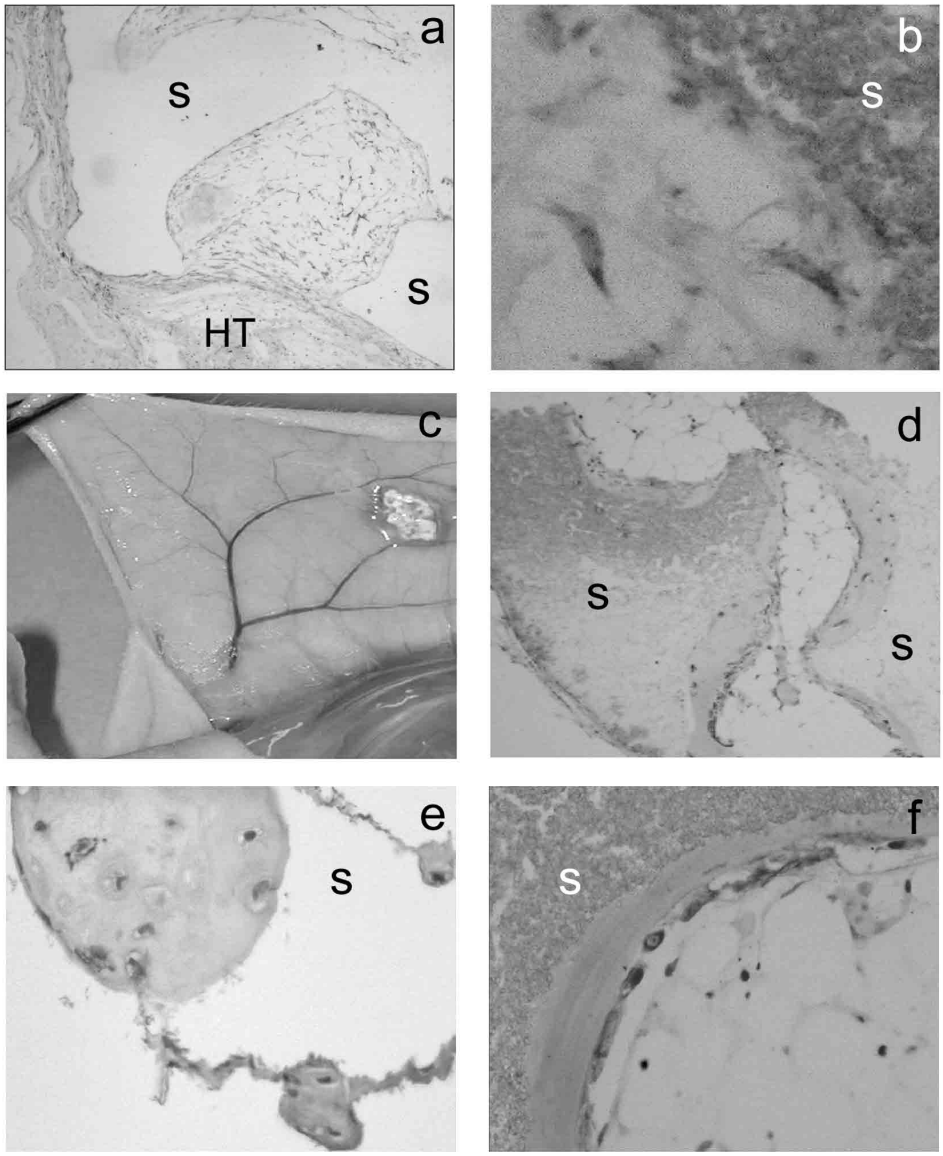


Figure 4 *Samples of in-vivo hybrid constructs with NGFR labeled cells* **(☞ p. 183)**
4a): Low magnification immunohistochemistry of 2 days implanted sample. The pores surrounded by the ghost of the scaffold (S) were filled with brown (labeled) goat BMSC's, distinctive from the surrounding host tissue (HT).
4b) High magnification immunohistochemistry of 10 days implanted sample. Condensations of fibroblast-like cells on the scaffold surface were found including labeled cells.
4c): Macroscopic image of sample in-situ at 10 days. Considerable neo vascularization of the hybrid construct was seen.
4d): Low magnification immunohistochemistry of 4 weeks implanted sample. New bone was lining the scaffold ghost. Many osteocyte lacunae were labeled (arrows).
4e): High magnification immunohistochemistry of 4 weeks implanted sample. Inside cartilage that occasionally formed, labeled cells were present (arrow).
4f): High magnification immunohistochemistry of 6 weeks implanted sample. Labeled cells were present within the osteoblast zone (arrow).

Discussion and Conclusions

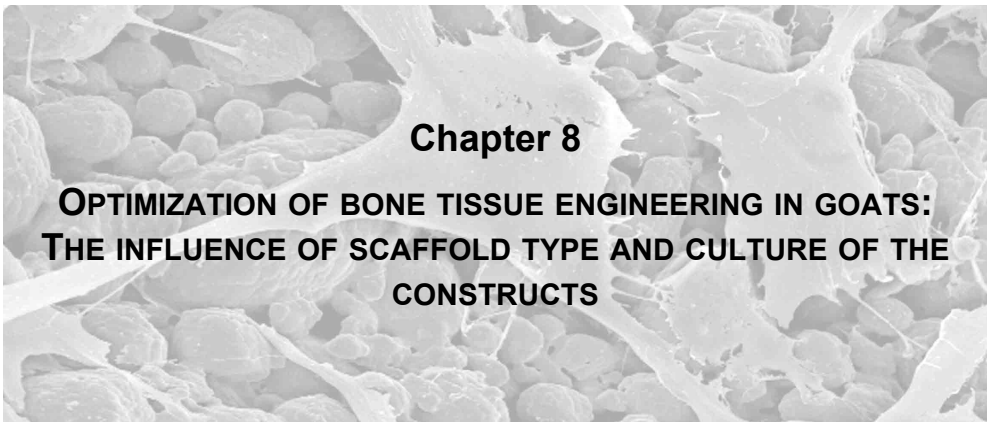
The aim of the present study was to retrovirally label goat BMSC's, and apply these for experimentation in bone tissue engineering. We found that 40-60% of the cells were labeled with the NGFR marker gene by means of a relatively simple retroviral transduction protocol, after a single round of transduction. When using retroviruses, the transduction is confined to replicating cells, and therefore a 100% transduction efficiency, was not expected.[197] In a large study, Mosca *et al.* investigated optimal parameters required to transduce BMSC's from eight different species, including the goat.[319] For goat BMSC's they reported a relatively low transduction efficiency with the amphotropic receptor binding envelope (<10%) compared to 50% when using their xenotropic (ProPak-X) packaging cell line. In our study the amphotropic envelope showed the best results. It is difficult to find an explanation for the discrepancy because different procedures of transduction were followed and the viral titers used in their study might have been different. Retroviral transduction of goat BMSC with xenotropic pseudotyped vector would provide the ultimate method to evaluate whether the results of our experiment would demonstrate the same.
For future studies, a labeled cell percentage close to 100% is preferred to allow quantitative interpretations of the contribution of host and donor cells to colonization of the scaffold and bone formation. We showed this may be accomplished by immunomagnetic sorting and expect even better results from FACS sorting, based on current investigations.
The results from the *in-vitro* culture study did not indicate a long-term effect of the marker gene on proliferation, because in the co-culture experiment the expected

percentage of labeled cells was matched. After an initial drop in the percentage of labeled cells during the first week from 60% to 35%, the percentage gene-marked cells stabilized at this level. Most likely, the untransduced cells have a slight growth advantage the first week after transduction.

We did not attempt to investigate the effect of the marker gene on in-vitro differentiation because the frequently used alkaline phosphatase assay has shown to be inadequate for goat and sheep BMSC's,[122,278] Furthermore, the most reliable information on differentiation potential is not provided by the *in-vitro* phenotype but by *in-vivo* behavior of the transduced cells.[7] The results from the *in-vivo* study indicated persistent gene expression following *in-vivo* growth and differentiation. The presence within osteocyte lacunae illustrated the bone forming capacity of the transduced cells. The presence in the osteoblast linings after 6 weeks suggests that transduced goat BMSC's cells were still actively forming bone. The observations related to *in-vivo* tissue engineered bone formation that we made in this study, correlated well with previous findings of CM-DiI labeled goat BMSC's.[121] In that study, we concluded that the CM-DiI label was not applicable for long-term investigation of the process of TE bone formation. With the retroviral NGFR label investigated in this study, long-term investigation of the process of bone TE in the goat model is now feasible.

Acknowledgements

The authors acknowledge the Netherlands Technology Foundation (STW; grant UGN.4966) for financial support. Furthermore we would like to acknowledge Tim Damen, the Immunohistochemistry Laboratory and the Laboratory of Experimental Cardiology of the University Medical Center Utrecht for assistance.



Chapter 8

OPTIMIZATION OF BONE TISSUE ENGINEERING IN GOATS: THE INFLUENCE OF SCAFFOLD TYPE AND CULTURE OF THE CONSTRUCTS

Summary

Introduction: Successful bone tissue engineering (TE) has been reported for various strategies to combine cells with a porous scaffold. Particularly the period after seeding until implantation of the constructs may vary between hours until several weeks. Differences between these strategies can be reduced to: (a) the presence of extracellular matrix; (b) the differentiation status of the cells; and (c) the presence of residual immunogenic serum proteins. We investigated these parameters in two types of scaffolds in a goat model of ectopic bone formation.

Methods: Culture expanded bone marrow stromal cells from eight goats were seeded onto two types of hydroxyapatite granules: HA60/400 (60% porosity, 400µm average pore size) and HA70/800. Scaffolds seeded with cells and control scaffolds were cultured for six days in medium containing autologous or semi-synthetic serum, in the presence or absence of dexamethasone. Other scaffolds were seeded with cells just before implantation in medium with or without serum. All conditions were implanted autologously in the paraspinal muscles for twelve weeks.

Results: Bone had formed in 87% of all TE constructs. Histomorphometry indicated significantly more bone in the HA70/800 scaffolds. Furthermore, a significant advantage in bone formation was found when the constructs had been cultured for 6 days.

Conclusion: Both scaffold characteristics (porosity) and TE strategy (culturing of the constructs) was demonstrated to be important for bone TE.

Introduction

Tissue engineering of autologous bone is a promising alternative for the surgically derived autologous bone graft. Since the early nineties, many investigators demonstrated the concept of combining osteoprogenitor cells with an appropriate scaffold to be osteogenic ectopically in rodents and larger mammals.[114,128,130,224] Recently, we demonstrated ectopic tissue engineered bone formation in a goat model.[131] The advantage of an ectopic model is that it allows fundamental research without the disturbing influence of host bone as present orthotopically. Furthermore, many experimental conditions can be evaluated in one animal for comparative studies.

The scaffolds most frequently used for bone TE are porous ceramics, pure hydroxyapatite (HA), or a composite with β-tricalcium phosphate (TCP).[68,128] The osteoconductivity and biocompatibility of these materials are advantageous for orthotopic application. The cells mostly used are Bone Marrow Stromal Cells (BMSC's).[63,76,128] These BMSC's can be administered by soaking the scaffold in fresh bone marrow,[228,234] or after culture expansion,[75,76,113] which was shown to be superior.[105,123,130,131]

Although many scaffold characteristics and the selection and expansion of BMSC's are quite similar between various studies, the strategy for building the final construct is remarkably different. For instance, some investigators seed undifferentiated "stem" cells under serum free conditions on the scaffold, followed by implantation within several hours.[118,123,130] Others culture the cells in the constructs one or more weeks prior to implantation, to allow extracellular matrix formation and to stimulate osteogenic differentiation by addition of specific differentiation factors such as dexamethasone.[103-105,131,224] Differences between these strategies may be reduced to the following parameters: (a) the presence of extracellular matrix in the scaffolds at time of implantation; (b) the differentiation status of the cells at time of implantation; and (c) the presence of potentially immunogenic serum proteins after seeding and culturing the constructs in the presence of commercial sera. In order to optimize our bone tissue engineering approach, we investigated these parameters in two different calcium phosphate scaffolds in a goat model of ectopic bone formation.

Materials and Methods

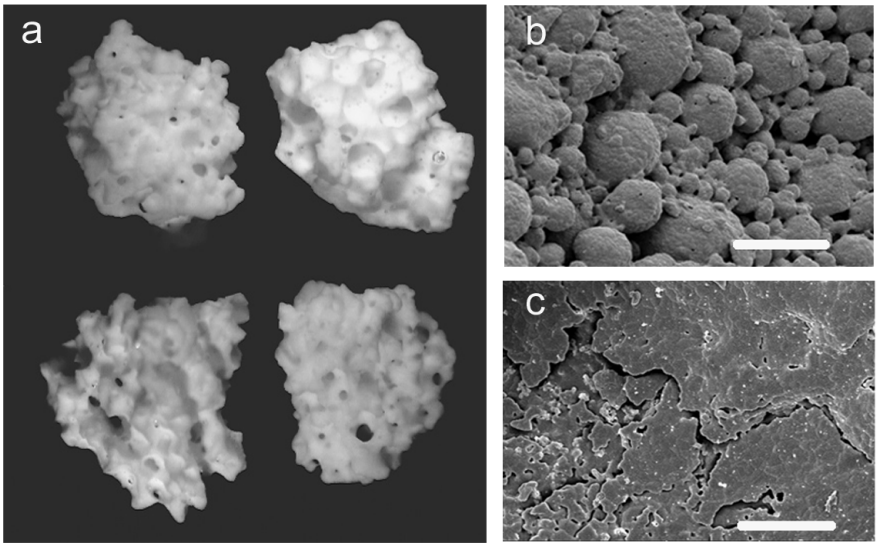
Experimental design

A total of eight adult goats were used for the experiment, for which approval was given by the local animal care committee. Forty-eight (8x6) units consisting of granules of each scaffold type (HA70/800 and HA60/400), were prepared and treated as single samples for all further treatment group related procedures (Table 1). One week before implantation, four treatment groups were created by seeding cultured BMSC's on the units under conditions mentioned in the paragraph *Culturing and seeding conditions*. These constructs were cultured for another 6 days in specific media. The night before implantation, another two treatment groups were created. Because these constructs were not subsequently cultured, a higher cell load was seeded to compensate for the cell increase on the cultured constructs. The units were implanted randomly in the paraspinal muscles of the goat from which the BMSC's were derived from (autologous implantation). This resulted in a sample-size of eight for each treatment group. To monitor bone formation in time, fluorochrome labels were administered after 5, 7, and 9 weeks and the animals were killed after 12 weeks. Bone formation in the scaffolds was investigated by histology and histomorphometry of non-decalcified sections.

Scaffolds

Two different scaffolds were used in the current experiment: (1) HA70/800, a 70% porous hydroxyapatite with an average pore size of 800µm (CAM Implants, The Netherlands). This scaffold was produced with spray dried HA powder and the interconnected pores were created using a H_2O_2 foaming method. According to the manufacturer the ceramic was sintered at a temperature above 1100°C. This resulted in a marked microporosity as shown in Fig.1. (2) HA60/400, a 60% porous hydroxyapatite with an average pore size of 400µm (IsoTis NV, The Netherlands). This scaffold was produced with commercial HA powder (Merck, The Netherlands) using a wax incorporation method to create an interconnected porous structure.[296] The sintering temperature was 1200ºC. Microporosity was less prominent in this scaffold (Fig.1). The chemical composition of the materials was analyzed by X-ray diffraction (XRD) and Fourier Transformed Infrared (FTIR) spectroscopy and indicated HA without additional phases or impurities. Granules of 30-50mm^3 of both scaffold types were selected by sieving, cleaned in an ultrasonic bath and then autoclaved. Two granules of each scaffold type (total 4 granules) were combined in a 10ml tube and processed as one unit for all further treatments. Eight units were impregnated with 100µg/ml fibronectin (Micronic, The Netherlands) to provide the units for the serum free seeding condition.[68]

Figure 1 *Scaffolds*
1a) Two granules (Ø=3-4mm) of both scaffold types that were combined in the treatment units. Macroscopically little difference can be seen.
1b) Scanning electron microscopy image of the HA70/800 surface. The material is composed of small HA beads resulting in high microporosity between the individual beads; (bar=20µm).
1c) SEM image of the HA60/400 surface. The material is more smooth resulting in less microporosity; (bar=20µm).



Culturing and seeding conditions (Table 1)

BMSC's were derived from both iliac wings and culture expanded as described in detail previously.[103] In brief, 30ml aspirates were plated in culture flasks ($5x10^5$ nucleated cells per cm^2) and cultured in a standard culture medium containing 15 vol% fetal bovine serum (FBS, Gibco, Scotland, lot# 3030960S). When colonies of adherent cells had formed, these were replated at 5000 cells/cm^2. At the 3^{rd} passage, one week before surgery, the cells were partially replated for culturing another week, or seeded on scaffold units. For seeding dynamically (on a roller bank) the cells were resuspended in different culture media at $5x10^5$ cells/ml and 3.2ml of this cell suspension was added to a tube containing one unit (= 10^7 cells per cm^3 scaffold). This procedure constituted the first four conditions:
(1) US-: The BMSC's were resuspended in medium containing 2 vol% Ultroser G (US. Invitrogen, The Netherlands) as a replacement of the 15 vol% FBS in the standard culture medium. US was expected to be less immunogenic as it is mainly synthetic. The exact constituents would not be given by the manufacturer but they did not exclude the presence of human and animal derived components.

(2) US+: as (1), with the addition of 10nM dexamethasone and 10mM β-glycerophosphate (DEX and BGP, Sigma, the Netherlands) to stimulate osteogenic differentiation.[106] This was the standard condition published before,[131] of which the other groups were deduced.
(3) AS+: the medium contained 15 vol% autologous serum (AS) instead of FBS plus DEX and BGP. AS was obtained from 200ml venous blood that was allowed to clot in a sterile glass jar overnight. Before application, the serum was heat inactivated for 30 minutes at 56-58ºC and filtered through a 0.2µm filter.
(4) Control: Units were maintained in US+ medium without cells seeded.
After seeding the units were taken to bacteriological 25-wells plates and the medium was refreshed with the same medium type as used for seeding. Then the units were cultured statically for another six days before implantation. This allowed cell expansion and extra cellular matrix formation.[131]
The cells that were not seeded were replated and maintained in the US- culture medium for another six days. Then the cells were resuspended at $5x10^5$ cells/ml and 17.6ml of this cell suspension was added to the units ($55x10^6$ cells per cm^3 scaffold) 14-16 hours before implantation according two protocols:
(5) USS-: Cells were resuspended and seeded in US- medium.
(6) SF-: Cells were resuspended in serum free medium and seeded on fibronectin-coated scaffolds.
In a previous study scanning electron microscopy (SEM) showed the 6-day culture period resulted in abundant ECM formation covering all of the scaffold surface.[131]

Table 1 *Treatment groups*
*Cells were loaded on units of four granules (2*HA60/400 + 2*HA70/800). The constructs were cultured six days before implantation in media with Ultroser G (US) or autologous serum (AS) with or without DEX and BGP administration. Other constructs were seeded short before implantation with Ultroser medium or serum free (USS, SF). Controls were maintained in the US+ condition without cells.*

Condition	Seeding load (cells/cm^3 scaffold)	Cultured	DEX and BGP	Serum type
1) US-	10^7	yes	no	Ultroser G
2) US+	10^7	yes	yes	Ultroser G
3) AS+	10^7	yes	yes	Autologous
4) Control	0	yes (no cells)	yes	Ultroser G
5) USS-	$5.5x10^7$	no	no	Ultroser G
6) SF-	$5.5x10^7$	no	no	no

Determination of cell seeding load for non-cultured constructs.

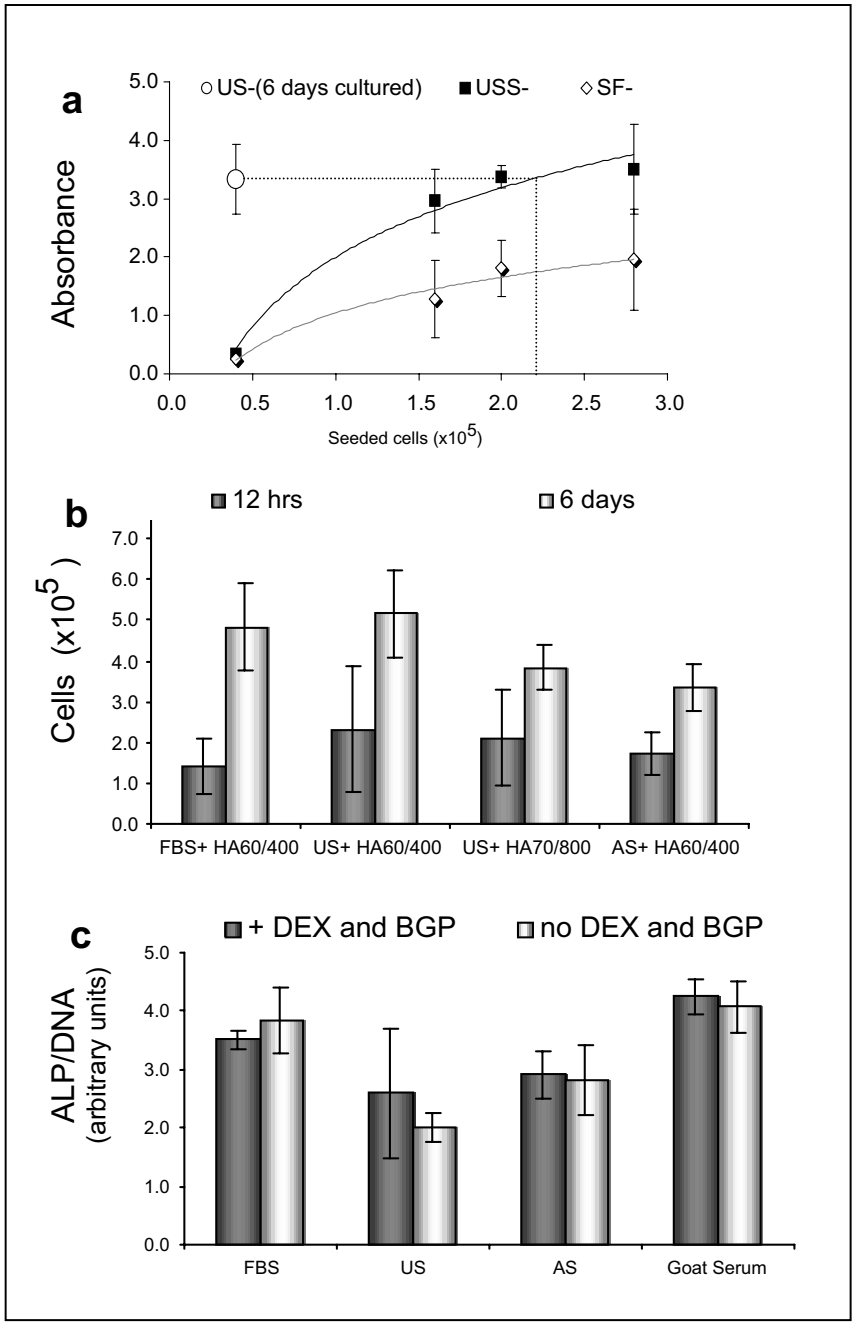
To standardize the cell number for all groups at implantation, we studied the required seeding load to normalize the cell number on the non-cultured constructs (USS- and SF-) in a separate study, prior to seeding of the *in-vivo* constructs. First cell numbers on 6 days cultured US- Constructs (HA60/400, *n*=6) were quantified by a tetrazolium salt assay, which measures mitochondrial metabolic activity (MTT, sigma).[332] The constructs were incubated in 200µl MTT solution for two hours and then lysed with 200µl DMSO. The light absorbance of the formazan product in the lysate was read at 570nm. Subsequently, constructs seeded with similar, 4, 5 and 7 times higher cell loads than the 6 days cultured constructs were analyzed (*n*=6) 14 hours after seeding in US- medium or serum free medium. The seeding load for an intensity equal to the cultured US- constructs was calculated from the function of the curve as shown in Fig. 2a.

Cell quantification

To determine the seeding efficiency and subsequent cell increase for the different scaffold materials and different medium conditions including the FBS medium, another cell quantification method that was described in detail before was applied in a separate *in-vitro* study (CyQUANT® kit Molecular Probes, Eugene, US).[131,297] Our experience was that absolute numbers (for seeding efficiency) can be calculated more accurately with this method as compared to the MTT assay. The constructs (*n*=5) were digested in a collagenase solution overnight on a shaking platform to retrieve all DNA. The cell number was calculated from a standard curve of identical cells.

Figure 2 *In-vitro measurements (error bars indicate the SD)*
2a) Relative cell quantities (by MTT assay) in HA60/40 constructs 14 hours after seeding with increasing cell loads compared to constructs seeded with $4x10^5$ cells and cultured for another 6 days. The open circle represents the cultured construct. Higher cell loads seeded in Ultroser medium (USS-) or in serum free medium (SF-) resulted in higher cell numbers in the non-cultured constructs. A seeding cell load of $2.2x10^6$ cells per granule was calculated from the USS- curve to normalize the cell number in the non-cultured constructs to the cultured construct. SF- could not be normalized.
2b) Estimated cell numbers in different combinations of scaffold and culture conditions by DNA quantification. The 6-day culture resulted in an average doubling of the DNA quantity. Culture of HA60/400 constructs in autologous serum (AS+) resulted in 35% less DNA as compared to identical constructs cultured in Ultroser medium (US+) ($p<0.02$).
2c) Relative alkaline phosphatase activity of goat cells cultured for 6 days in media containing FBS, Ultroser (US), autologous serum (AS) or allogenic goat serum. The response to DEX and BGP admission was ambiguous. No conditions were significantly different when all conditions were compared.

Figure 2



Alkaline phosphatase measurement

The alkaline phosphatase activity (ALP) in relation to the cell number (ALP/DNA) was measured to analyze cell response to DEX and BGP and to identify differences between the media. The standard culture medium with FBS, the US medium, the AS medium and medium containing 15% allogeneic goat serum were studied. Cells of one batch were plated in six-well plates ($5x10^4$ cells per well) and cultured for 6 days to allow formation of confluent sheets.[106] The cell sheet was resuspended in 1ml 0.05% Triton solution and homogenized by sonification. 500µl was used for the CyQUANT assay. Another 100µl was transferred to 100µl p-nitrohenyl phosphate (PNP, 104^R Sigma) in alkaline phosphatase buffer and incubated for 15 minutes to determine the rate of conversion into p-nitrophenol. The reaction was stopped with 500µl 0.1N NaOH. Absorbance was read at 405nm.

Animals and Implantation

Adult Dutch milk goats (19-26 months) were obtained at least four weeks prior to surgery. The surgical procedures were performed under general inhalation anesthesia, preceded by i.v. detomidine sedation (Pfizer, The Netherlands) and maintained by a halothane gas mixture (Sanofi, The Netherlands). After shaving and desinfection of the lumbar area, six separate skin incisions were made. By using blunt dissection, intramuscular pockets were created and filled with one unit according to a randomized scheme. Subsequently, the fascia was closed with a non-resorbable suture and the skin was closed in two layers. Postoperatively pain relief was given by buprenorphine (Shering-Plough, The Netherlands). In addition to the intramuscular implantations, the goats were subjected to a femur defect operation that will not be discussed in this paper.

Fluorochromes (all Sigma) were administered intravenously. At 5 weeks, Alizarin red (30mg/kg), at 7 weeks Calceine green (10mg/kg) and at 9 weeks Xylenol orange (100mg/kg).[300] After 12 weeks, the animals were killed by an overdose of pentobarbital (Organon, The Netherlands). As a control for osteogenicity, per goat, two HA70/800 granules of each condition and two granules of HA60/400 of the US+ condition were implanted subcutaneously for four weeks in nude mice (two mice per goat).[103] These implants were scored as bone or no bone in the 16 (8x2) granules that were implanted of each condition.

Post-mortem sample acquisition, histology and histomorphometry

After explantation, the units were fixated in 1.5% glutaraldehyde, dehydrated by graded ethanol series and embedded in polymethylmethacrylate. Semi-thin sections (10µm) were made with a Leica sawing microtome, stained with methylene blue and basic fuchsin for routine histology[302] and histomorphometry, or left unstained for epifluorescence microscopy. Per unit 4-10 sections were made to provide sections through the center of each individual granule. The sections were evaluated for general tissue response, bone formation and the fluorochrome labels using a fluorescence/light microscope (E600 Nikon, Japan) with a double filter block (dichroic mirror 505nm and 600nm). The mid-section through the single granules was chosen for histomorphometry. The percentage of bone occupying available space within the granules was measured using a VIDAS image analysis system (KS400, Zeiss, Munich, Germany), coupled to a light microscope. First, the area of interest was defined by outlining the specific granule. Pores that interrupted the exterior scaffold contour were crossed with straight lines. Then, after blinding the samples, the pore area and the bone area were identified and measured. Bone formation was expressed as percent bone in available space (bone area/pore area x100%).

Statistics

To analyze quantitative DNA and ALP/DNA data, ANOVA's with Bonferroni corrected post-hoc tests were performed. Histomorphometric results were analyzed by Friedman paired rank test and post-hoc Wilcoxon signed rank tests. All tests were performed with SPSS10 for Macintosh (significance level: $p<0.05$).

Results

In-vitro results (Fig. 2)

To normalize the cell numbers in the constructs that had not been cultured after seeding to cultured constructs, a 5.5 times higher seeding load was calculated for the USS- condition. The SF- condition could not be normalized as can be seen in Fig. 2a. The average seeding efficiency according the CyQUANT® assay was about 60%, without significant difference between the sera or scaffold materials. After the 6-day culture period, the total DNA reflecting cell numbers had doubled once on average, with about 35% less DNA in AS+ condition as compared to the US+ condition (p=0.018). This indicated a relatively low proliferation of the cells that, when cultured in flasks, have a doubling time of only one day.[131] Other comparisons did not show significant differences. (Fig. 2b). The ALP/DNA analysis as a reflection of cell differentiation demonstrated a comparable alkaline phosphatase activity for all study conditions. The effect of dexamethasone and BGP admission was ambiguous and not significant as can be seen in Fig. 2c.

In-vivo results

Although there were no surgical complications, two goats developed a painful nail disease for which they were terminated after 10 weeks instead of 12 weeks. Autopsy and cultures of blood and tissue did not indicate a cause related to the procedure. The data of these two goats were applied for qualitative analysis and paired comparisons within the animal. Histology showed that all granules were embedded in well vascularized soft tissue without signs of inflammation. Bone formation was found in all but the control conditions in amounts varying in and between the goats (Fig. 3). The bone was typically present on the HA surface of the smaller pores with an obvious preference for the HA70/800. In 35 of all 40 tissue engineered units (87%), bone had formed, and in 33 cases the HA70/800 implants yielded more bone than the HA60/400 implants (p<0.01). Fig.4 shows the result of histomorphometry on the 12-weeks implants. Although the standard deviation (between goats) was high, paired comparisons (within goats) revealed that the condition/material combination significantly influenced the percentage of bone area (p<0.01), also when considering only HA70/800 (p=0.01). The three conditions that were cultured for 6 days (US-, US+, AS+) had comparable bone percentages (9-13%) that were higher than the two conditions that were implanted directly after seeding (3-5%). Post-hoc analysis of the US- condition (no DEX and BGP admission) versus the USS- condition demonstrated a significant difference (p=0.02) in bone formation. Fluorochrome analysis showed the fluorochrome labels as distinct lines within the newly formed bone. The occasional presence of the 5

weeks label and the line pattern were comparable for all conditions. The fluorochrome line order indicated bone formation had started on the HA surface and was directed towards the pore center. The line shape often indicated bone was budding from the surface towards to opposite surface to bridge the pore (Fig.3). Since the distances between the different labels was very variable, no attempt was made to calculate the bone formation rate.

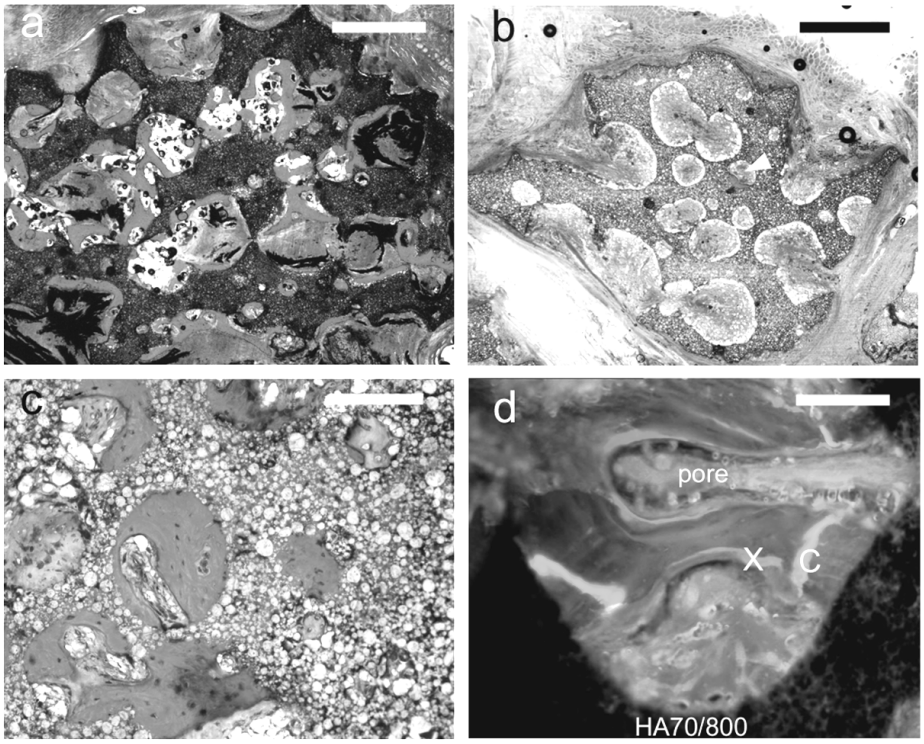
Figure 3 *Bone formation after 12 weeks implantation in goats* **(☞ p. 184)**

3a) HA70/800 scaffold of AS+ condition. Bone (red) is present in all pores in close contact to the scaffold surface; (bar=500µm).

1b) HA70/800 granule of USS- condition. Only little bone had formed (triangle); (bar=800µm).

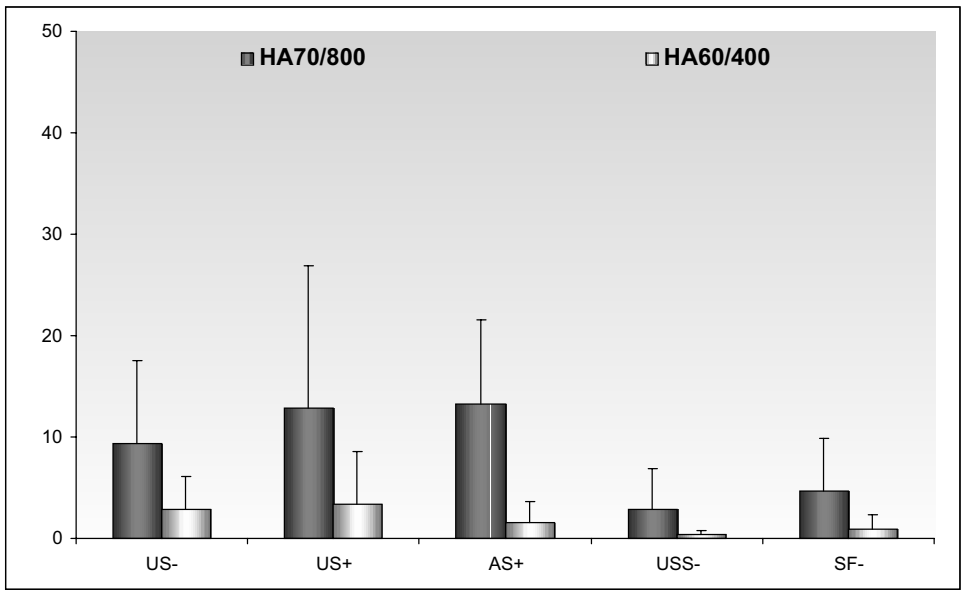
1c) HA70/800 of SF- condition, detail of bone. Note scaffold composition of small beads; (bar=100µm).

1d) HA70/800 of US- condition. Fluorescent microscopy shows the budding of bone from the scaffold surface to the pore center where two buds united, C = Calcein label (7 weeks) X = Xylenol orange label (9 weeks); (bar=50µm).



In the control granules implanted in mice, bone was also present in all cell containing groups ranging from 62.5% (10/16) in the US- constructs to 100% (16/16) in the SF- constructs. Remarkably HA60/400 and HA70/800 (both US+) showed similar bone formation, both 90%.

Figure 4 *Area % bone after 12 weeks implantation in goats (error bars indicate SD) HA70/800 yielded more bone than the HA60/400 (p< 0.01) Post-hoc paired comparisons indicated US- (cultured) had more bone than USS- (non-cultured) (p=0.02).*



Discussion and conclusions

In the present study, the effect of five different TE strategies was investigated ectopically in a goat model. Bone was found in all cell-based TE conditions with an obvious preference for the HA70/800 scaffolds and for constructs that were cultured before implantation. Neither the serum type nor dexamethasone admission influenced bone formation. An explanation for these findings could be that only the scaffold type and the presence of extracellular matrix is relevant for bone formation without any role for the cells *in-vivo*. This hypothesis can be supported by literature that reports a potential bone inductive capacity of porous ceramics and extracellular matrix.[152,294,333,334] However, the scaffolds without cells failed to show bone induction, and a previous study showed that identically cultured TE constructs that contained extracellular matrix and were devitalized before implantation, also failed to show any bone.[131] Therefore, the hypothesis of an osteoinductive mechanism without a contribution of the cells is not plausible. Different parameters for this cell-dependent bone TE were investigated: The presence of extra cellular matrix; the differentiation status of the cells; and the presence of potential immunogenic serum proteins.

The effect of extracellular matrix was investigated by comparing constructs cultured for 6 days to constructs that were not cultured after seeding that do not contain ECM.[131] In agreement with earlier observations in rats,[104,105] we found significantly more bone in the cultured constructs indicating an advantage for this situation. A remark that should be made is that the high cell load that was seeded on the non-cultured constructs resulted in a cell distribution that was less homogeneous (more clotting) as on the cultured constructs.

To analyze the effect of cell differentiation, we attempted to stimulate differentiation into the osteogenic lineage by adding DEX and BGP to the culture medium.[106] The *in-vitro* results of the ALP/DNA assay, however, showed a very moderate alkaline phosphatase activity in goat cells, with an ambiguous response to DEX and BGP admission. This is contrary to human or rodent cells frequently analyzed by the same method in our laboratory in which ALP/DNA is 10-20 times higher. This phenomenon was also observed by others with sheep BMSC's.[122] The absence of an ALP/DNA increase *in-vitro* might explain why there was no significant effect *in-vivo*.

The presence of serum proteins in US and especially in FBS that could elicit an immune reaction was a major concern. Therefore, the FBS-cultured constructs were not implanted. However, a sensitization test (on guinea pigs) of Ultroser G that was performed in a separate experiment, indicated an immune response against US that was even stronger than the response to FBS. Despite this finding, bone formation in

all cultured groups (US-, US+ and AS+) appeared similar and no advantage of serum free seeding was found in the non-cultured groups (USS- and SF-). This implies the absence of a profound inhibitory immune reaction and also shows the feasibility of using autologous serum.

The type of scaffold appeared to affect the outcome more than any of the other parameters. This was discussed earlier,[131] where we emphasized that a prominent feature of the HA70/800 was the higher microporosity. The larger surface area might result in increased dissolution and subsequent precipitation with the possible integration of inducing factors in the scaffold surface.[98,105] This phenomenon might be relevant only after prolonged implantation periods, which could explain the absence of a material related difference in the frequently applied nude mice model[121] (implanted for 4 weeks).

The MTT assay that we used for standardization of the cell numbers was not ideal because of conflicting data. The measured cell increase during the 6 days culture time was ten fold, whereas this quantity could be achieved with a 5.5 times higher seeding load (Fig. 2). Others reported a selective adherence of the MTT to plastics[335] which could have caused a relative under-estimation of low cell numbers. The cell loads applied for normalization were more likely too high than too low as, according to the DNA quantification method, the 6 day cell increase was only two fold. Another reason to assume that the cell numbers on the non-cultured constructs were sufficient, is by comparing our seeding load of $55x10^6$ cells/cm^3 to literature where only 10-$30x10^7$ cells per cm^3 scaffold are seeded.[119,122,123]

Finally, it should be realized that the area% bone we found in goats (on average 12% in the cultured constructs) is low when compared to rodents where 25% was reported,[336,337] This could be the result of a delayed vascularization related to an increased implant volume and lower rate of metabolism.[173,338] Further research on this topic will be necessary to upscale the technique for future clinical applications.

Acknowledgements

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Chapter 9

OPTIMIZATION OF BONE TISSUE ENGINEERING IN GOATS: A PEROPERATIVE SEEDING METHOD USING CRYOPRESERVED CELLS AND LOCALIZED BONE FORMATION IN CALCIUM PHOSPHATE SCAFFOLDS

Summary

Introduction: Bone tissue engineering by combining cultured bone marrow stromal cells (BMSC's) with a porous scaffold, is a promising alternative for the autologous bone graft. Drawbacks of the technique include the delay necessary for cell culture and the complicated logistics. We investigated methods to bypass these drawbacks. Furthermore, we investigated the localization of bone formation inside the scaffold.

Methods: Goat BMSC's were culture expanded and cryopreserved. Prior to surgery, part of the cells were thawed, cultured and seeded on porous calcium phosphate scaffolds. The constructs were cultured for another week until implantation. The remaining cryopreserved cells were thawed just before implantation and peroperatively resuspended in plasma before combining with the scaffold. Scaffolds impregnated with fresh bone marrow, devitalized cultured constructs and empty scaffolds, served as controls. All samples were implanted in the back muscles of the goats (n=7) for nine weeks.

Results: Histology showed little (<1%) bone in the empty and devitalized scaffolds, 4.2±5.1 area% bone in the bone marrow samples and significantly more bone in both the cultured and peroperatively seeded constructs (11.7±2.5 and 14.0±2.0%). The peripheral 350µm of the implants contained significantly less bone.

Conclusion: Peroperative preparation of osteogenic constructs with cryopreserved cells is feasible. These constructs yield substantially more bone than the scaffolds alone or scaffolds impregnated with fresh bone marrow. Bone deposition is much less on the scaffold periphery.

Introduction

At present, the autologous bone graft is the golden standard to reinforce or replace bone in many orthopaedic interventions. Unfortunately, harvesting is inherent to donor side complications and increased costs.[45] For that reason, a competent alternative is highly demanded. Tissue engineering (TE) of bone could be a solution. A concept of tissue engineering is to combine bone-forming cells with a porous scaffold to obtain a hybrid construct. Friedenstein and colleagues discovered and extensively investigated such cells in adult bone marrow and referred to these as colony forming unit fibroblasts (CFU-F's) or bone marrow derived stromal cells (BMSC's).[61] Since, the technique has been optimized, especially by applying porous ceramics as a scaffold material.[68,128] The proof of the concept of bone TE was shown with gene-marked cells in mice and rat.[128,191] Reports showing the feasibility in larger animals are fewer, but both ectopic osteogenicity of the constructs[130,131,278] and the successful application orthotopically was shown.[119,123]

Although the technique is promising, there are some important drawbacks. At first, there is a delay of at least 2 weeks necessary to culture expand the cells; secondly, because the cells are cultured before combination with the scaffold, the logistics are complicated to ensure the patient and the construct are prepared at the same time; thirdly, when the construct is cultured before implantation, animal serum is often required of which remnants in the final construct cannot be excluded. To overcome these drawbacks, methods have been developed to concentrate the osteogenic cells in fresh bone marrow by selective adhesion to the scaffold peroperatively.[6,47] The fraction of osteogenic cells in bone marrow however, is only 1-10 per 10^5 cells.[5,61] Therefore, expansion of the BMSC's is preferable when elective surgery is an option, especially in the case of large graft volumes (which need many progenitor cells). Cryopreservation allows long-term preservation of the expanded cells and can simplify planning considerably.[339] The main concern in planning then remains to thaw and replate the cells and, if applicable, culture the construct before application. When for some reason the operation cannot proceed after (construct) preparation, labor and cells are spilled. To overcome this drawback, we investigated the feasibility of combining cryopreserved cells and the scaffold peroperatively by using autologous plasma as a delivery vehicle. A potential advantage of this ex-vivo prepared clot, as compared to the haematoma that will normally occur after implantation, is the absence of erythrocytes, which are responsible for a detrimental increase in potassium concentration.[143] The peroperative method was compared to constructs that were cultured one week before implantation and to scaffolds seeded with and without fresh bone marrow.[233]

A more fundamental question that we investigated, concerned the distribution of newly formed bone. Although one would expect that new bone formation will preferably be close to the outside of the scaffolds, where cells can survive by diffusion and enhanced revascularization,[137,140,172] previous observations showed a preference for bone formation away from the scaffold periphery.[121,131,278] With regard to larger graft volumes consisting of separate granules, as for example used in posterior spinal fusions, peripheral bone formation will be a prerequisite for continuous bone bridging. Therefore, we separately analyzed bone formation in the outer 350µm of the constructs and compared this to the bone formation in the whole 7x7x7mm construct.

Materials and methods

Experimental design

A total of 7 adult goats were used after approval by the institutional animal care committee. Bone marrow aspirates were culture expanded and the BMSC's were cryopreserved. Ten days before the operation, aliquots of cryopreserved BMSC's were replated. After 3 days (one week before the operation), a proportion of these cells was seeded on ceramic scaffolds and the constructs were subsequently cultured, with or without the presence of dexamethasone. Cells that had not been seeded on scaffolds were maintained in culture to be detached just before the operation. At that time, also remaining aliquots of cryopreserved BMSC's were thawed. Peroperatively the cells were resuspended in autologous plasma and combined with the scaffolds. Fresh bone marrow was combined with the scaffold at the start of the operation. A total of eight conditions were created as discussed in more detail in the section *BMSC's culture and seeding conditions* and Table 1.

Scaffolds

Cubes of 7x7x7mm with rounded edges were prepared from OsSatura™ (IsoTis, Bilthoven, The Netherlands) cleaned in ultrasonic baths, steam autoclaved and dried before application. The ceramic consists of 80±5% hydroxyapatite (HA) and 20±5% tricalcium phosphate (TCP) as confirmed by X-ray diffraction (XRD) and Fourier Transformed Infrared (FTIR), no additional phases or impurities where detected. The 50% interconnected macroporosity was created by H_2O_2 foaming resulting in a 100-1000µm poresize. The material was sintered at 1200ºC allowing 15±5% microporosity (1-10µm). Previously, this ceramic showed to be osteoinductive ectopically in goats.[97]

Table 1 *Conditions*

Control ceramic cubes (7x7x7mm) were kept in autologous serum (AS) medium with DEX and BGP, but without cells, for one week (AS+NC). Others were impregnated with autologous plasma peroperatively (PlmNC). A third control was created by devitalizing cultured AS+ constructs before implantation. Scaffolds that were impregnated for 1 hour with fresh bone marrow (BM) provided a fourth control condition. Experimental conditions were obtained by 1-week culture of BMSC seeded scaffolds in AS medium with or without DEX and BGP (AS+ and AS-). Others were prepared peroperatively by using cultured or cryopreserved BMSC's resuspended in plasma (POcult. and POCP). Upon implantation the estimated cell load was identical for all groups.

Condition	Seeded cell load ($x10^6$)/cm^3 scaf.	Handling	Implanted cell load ($x10^6$)/cm^3 scaf.
1) AS+NC	none	7-day cult.	none
2) PlmNC	none	Peroperative plasma	none
3) AS+dev.	8	7-day cult. then frozen	8.7 (dead)
4) BM	3.5ml	1h attachment	n.a.
5) AS-	8	7-day cult.-DEX	8.7
6) AS+	8	7-day cult.+DEX	8.7
7) POcult.	8.7	Peroperative	8.7
8) POCP	8.7	Peroperative	8.7

BMSC's culture and seeding conditions

Autologous serum (AS) was derived from venous blood that was taken during the BM aspiration.[131] After the iliac wing BM aspirate, the nucleated cells were counted and plated in culture flasks, to obtain the BMSC's according to previously described methods.[131] When sufficient, cells were cryopreserved in medium containing 30% fetal bovine serum (FBS, Gibco, Paisly, Scotland, lot# 3030960S) and 10% dimethylsulfoxide (DMSO, Sigma, Zwijndrecht, The Netherlands). Ten days before the operation, aliquots of the cryopreserved cells were thawed with iced FBS, centrifuged for 10 minutes at 100G and replated in 30% FBS medium for 3 days. One week before operation, cells were detached, centrifuged and partially replated for another week in 15% AS medium. The remaining cell pellets were resuspended in AS medium at a concentration of $1x10^7$ cells/ml. Scaffolds were seeded with 275µl of droplets of this suspension ($8x10^6$ cells per cm^3 scaffold) and incubated at 37ºC. After two hours, AS medium was added with or without 10nM dexamethasone and 10mM β-glycerophosphate (DEX and BGP, both Sigma) which

have shown to stimulate osteogenic differentiation.[106] The constructs were cultured for one more week to generate an extracellular matrix.[131,278] One extra construct cultured in DEX and BGP medium was made and devitalized before implantation by repeated freezing in liquid nitrogen, to provide a control with extracellular matrix without viable cells.[131] Other control scaffolds were not seeded with cells but were maintained in medium containing DEX and BGP.
The day of surgery, cultured cells were detached, resuspended at 10^6 cells/ml in AS medium and 3.1ml was transported to the operation room. Cryopreserved cell aliquots were thawed with iced AS and resuspended like the cultured cells. During surgery, plasma was obtained by centrifuging 10ml venous blood at 1200G in a plastic tube. The transported cell suspensions were centrifuged at 300G, medium was decanted and the pellets were resuspended in 300µl plasma. Plasma with or without cells was combined with the dry scaffold and allowed to clot. The plasma penetrated the scaffold and the clot containing all cells ($8.7x10^6$ cells/cm^3 scaffold) was implanted. Bone marrow seeded scaffolds were prepared as previously described[131] by adding 3.5ml fresh bone marrow without anti-coagulant to a dry scaffold (10ml/cm^3 scaffold) at least one hour before implantation. The above procedures created the following groups (see also Table 1):

1 (AS+NC): scaffold without cells, maintained one week in AS+ medium;
2 (PlmNC): scaffold peroperatively impregnated with autologous plasma;
3 (AS+dev.): devitalized AS+ constructs (with extra cellular matrix);
4 (BM): scaffold impregnated with fresh bone marrow;
5 (AS-): constructs of scaffold and BMSC's cultured one week in AS medium without DEX and BGP;
6 (AS+): constructs cultured one week with DEX and BGP;
7 (POcult.): construct prepared peroperatively with the number of cultured BMSC's adjusted to the AS+ condition;
8 (POCP): peroperatively prepared with adjusted numbers of cryopreserved BMSC's.

Cell quantification

To determine seeding efficiency and cell increase after culture of the constructs in the AS+ condition, which was expected to be comparable to the AS- condition,[278] the DNA content of *in-vitro* constructs (*n*=6) was quantified with a CyQUANT® kit (Molecular Probes, Eugene, US).[131] In order to implant all conditions with equal cell numbers, the peroperatively seeded samples contained the amount of cells that was calculated for the 1 week cultured constructs (=$8.7x10^6$ cells/cm^3 scaffold).

Animals and Implantation

Adult female Dutch milk goats (24-36 months) were obtained at least four weeks prior to surgery. The surgical procedures were performed under general inhalation anesthesia, preceded by detomidine sedation (Pfizer, The Netherlands) and maintained by a halothane gas mixture (Sanofi, The Netherlands). After shaving and desinfection of the thoracolumbar area, a central skin section T8-L5 was made to expose the muscle fascia. Separate fascia incisions were made and by using blunt dissection, intramuscular pockets were created that were filled with one of the implants, according to a randomized scheme. The fascia was closed with a non-resorbable suture and the skin was closed in two layers. Postoperatively pain relief was given by buprenorphine (Shering-Plough, The Netherlands). In addition to the intramuscular implantations, the goats were subjected to a critical sized iliac defect that will not be discussed in the present paper. Sequential fluorochrome markers were administered; after 3 weeks Calcein Green (10mg/kg intravenously, Sigma); after 5 weeks Oxytetracyclin (32mg/kg intramuscular, Engemycine, Mycofarm, The Netherlands) and after 7 weeks Xylenol Orange (80mg/kg i.v. Sigma).[131] After 9 weeks, the animals were killed by an overdose of pentobarbital (Organon, The Netherlands).

Post-mortem sample acquisition, histology and histomorphometry

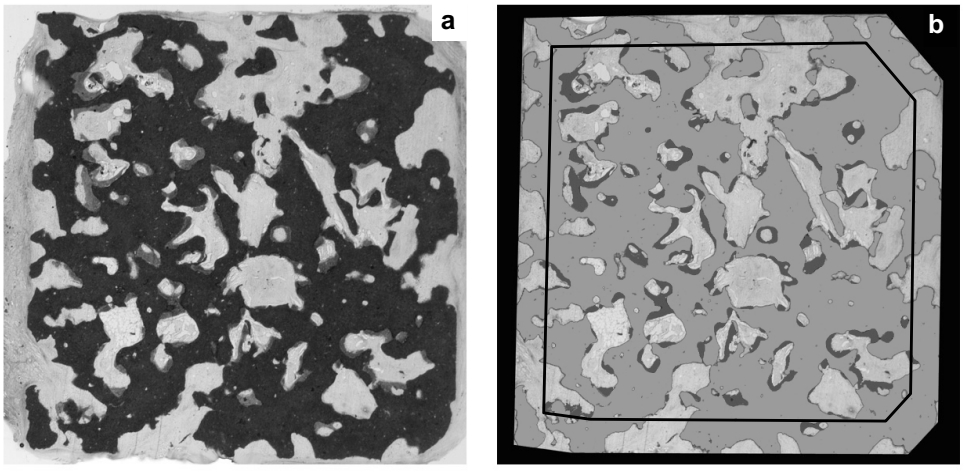
After explantation, the samples were fixated in 4%glutaraldehyde/5% paraformaldehyde, dehydrated by ethanol series and embedded in polymethylmethacrylate. Semi-thin sections (10µm) were made with a sawing microtome (Leica, Nussloch, Germany) through the center of the cubes. Sections were stained with methylene blue and basic fuchsin for routine histology and histomorphometry, or left unstained for epifluorescence microscopy. General tissue response, bone formation and the fluorochrome labels were evaluated by

fluorescence/light microscope (E600 Nikon, Japan) with a quatriple filter block (XF57, dichroic mirror 400, 485, 558 and 640nm, Omega filters, The Netherlands). For histomorphometry, high resolution, low magnification (10x) digital micrographs (300dpi) were made of blinded sections (Fig. 1a). With Adobe Photoshop 5.5, bone and scaffold were pseudocoloured red and green respectively. The area of interest was defined by outlining the scaffold, pores that interrupted the exterior contour were crossed with straight lines (Fig. 1b). Image analysis was carried out using a PC-based system equipped with the KS400 version 3.0 software (Zeiss, Oberkochen, Germany). A special program was developed, measuring the percentage of bone occupying available space (bone area%) and the percentage of available scaffold outline (which is the surface of the scaffold) in contact with bone (contact%). The contact% was expected to be more sensitive for newly formed bone, because previous work indicating this was strongly associated with the scaffold surface.[131] A peripheral area of interest was defined by drawing a line 40 pixels central from the outer contour (corresponded with approximately 350µm) (Fig.1b). Bone area% and contact% were also measured in the area between this artificial line and the outer contour.

Figure 1 *Image analysis procedures* **(☞ p. 185)**

1a): 10x magnification, high-resolution digital image of central section through a sample peroperatively seeded with cryopreserved cells (POCP).

1b): Pseudocoloured image of 1a, bone = red and scaffold = green. The area of interest was outlined with straight lines. After measurement of the whole sample, an imaginary line was drawn 40 pixels central to the outline, this corresponded with approximately 350µm. The area between this line and the outline was analyzed separately as the peripheral zone.



Statistics

To analyze quantitative DNA data, two-sided student t-tests were performed. Histomorphometrical data was analyzed with ANOVA according to a randomized block design. Bonferroni corrected post-hoc test were done to analyze differences between the groups. Differences between the peripheral and the total area were determined by repeated measurements. All tests were performed with SPSS10 for Macintosh (significance level p=0.05).

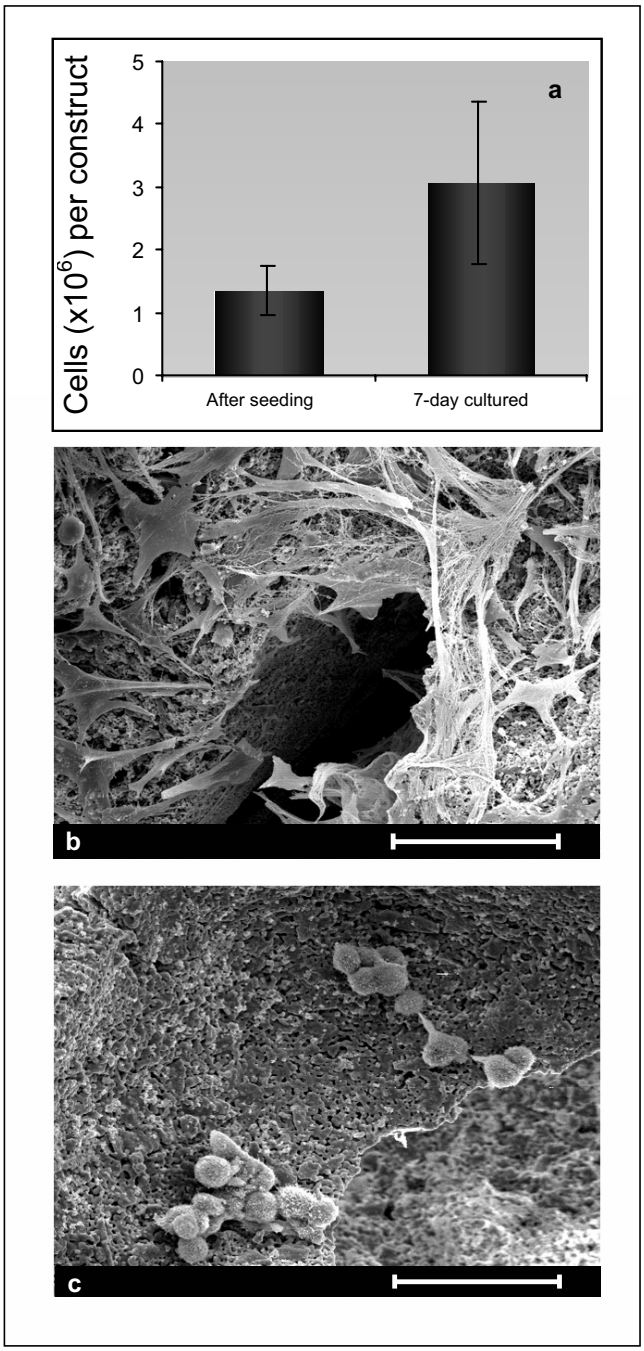
Results

In-vitro results

The BM aspirates contained 9.1±2.3x10^6 (mean±SD) nucleated cells/ml. Colonies formed after 5-7 days culture and the BMSC's were harvested when confluent, after 10-13 days. After the 2nd passage, 0.8-1.1x10^8 BMSC's were cryopreserved. Thawed cells showed minimal dead cells (<5% according trypan blue exclusion) and proliferated well before seeding on the scaffolds. Methylene blue staining of *in-vitro* constructs after seeding for two hours, indicated a homogeneous distribution of the cells in the scaffolds. The average seeding efficiency according the CyQUANT® assay was 49.1±14.7% (mean±SD) (Fig. 2a). After the 1-week culture, the DNA quantity had significantly increased (p=0.011), corresponding with 3.1±1.3x10^6 cells/scaffold (=8.7±3.7x10^6cells/cm^3). By then, scaffolds were fully covered with an extracellular matrix (Fig. 2b). After resuspension of cells in plasma peroperatively, the plasma remained in a liquid state, which allowed good penetration of the scaffolds. The plasma clotted within 10-20 minutes. SEM imaging of POCP constructs, showed deposition of aggregates of spherical cells throughout the scaffold (Fig. 2c).

Figure 2 *Analysis of in-vitro constructs*
*2a): Histogram of the calculated cell numbers derived from the CyQUANT" assay, error bars indicate the standard deviation. After seeding 2.75*10^6 cells (=8*10^6 cells/cm3), 1.4±0.4*10^6 adhered. After one week culture, the cell number significantly increased (p<0.01) to 3.1±1.3x10^6 (=8.7*10^6 cells/cm^3).*
2b): SEM image of 7-days cultured construct (AS+) Flattened cells with extra cellular matrix deposited around a pore orifice (bar = 50µm).
2c): SEM image of construct after seeding with cryopreserved cells in plasma (POCP). Aggregates of spherical cells adhered to the BCP ceramic of which the microporous structure is well visible (bar = 50µm).

Figure 2



In-vivo results

There were no surgical complications and all samples were retrieved. No macroscopic or microscopic signs of infection were found.

Histology showed consistent bone formation in all samples of the experimental conditions (AS+, AS-, POcult and POCP), occasionally with bone marrow inclusion. Less regularly (6/7 =86%), bone formation was found in the fresh BM condition. The conditions without viable cells occasionally showed spots of new bone (see Table 2 and Fig. 3). Bone was never present on the scaffold exterior and at first sight, bone formation in the peripheral zone (Fig. 1) seemed less. Fluorescence microscopy showed a line pattern and the presence of the first (3-week) label that was comparable for all conditions seeded with cells, including the BM group. No delayed bone formation was recognized in any of these groups, the 3-week label was always very close to the ceramic surface. In general, when bone had been formed in the conditions without viable cells, only the 7-week label was visible, except for one occasion (PlmNC), where the 5 week label was present. The fluorochrome label order indicated bone formation started from the scaffold surface and was directed centripetal, towards the pore center (Fig. 3).

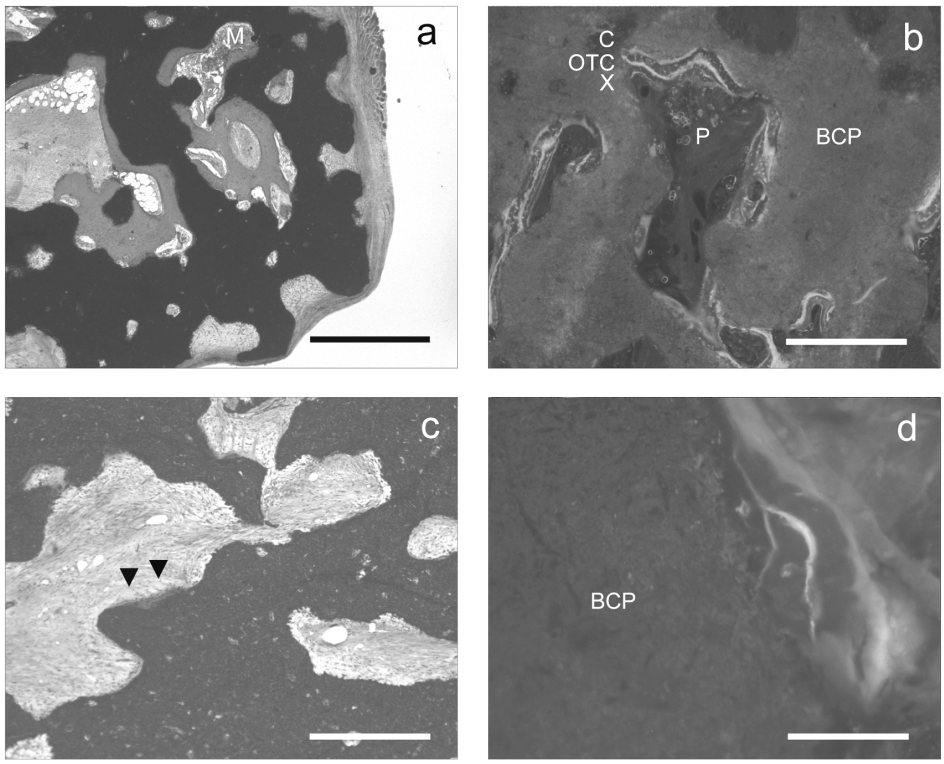
Histomorphometry indicated a scaffold macroporosity of 49.9±4.2%. Bone formation was comparable for all experimental (BMSC's seeded) conditions (Table 2 and Fig. 4). In these conditions, the area% bone was 11.2-14.0% and the contact% 25.8-27.3%. The BM group showed significantly less bone than the experimental conditions (area% 4.2%, contact% 8.8%, p<0.01). The conditions without viable cells showed minimal bone formation (<1%). Measurement of both the bone area% and the contact% in the peripheral 350µm of the experimental conditions indicated 5 to 6 fold lower values respectively as compared to the whole implant (p=<0.001).

Table 2 *Histomorphometry data of intramuscular samples in goat*
The percentage bone in available space (Bone area%) and the percentage scaffold-outline in contact with bone (Contact%). Both measurements where done for the whole sample and the peripheral 350µm. The bone-containing fraction of the samples is shown in the right column.

Condition	Bone area% (mean±SD)		Contact% (mean±SD)		% Positive
	Whole sample	Periphery	Whole sample	Periphery	samples
1) AS+NC	0.2±0.2	0.1+0.1	0.4+0.5	0.1+0.3	71%(5/7)
2) PlmNC	0.7±1.1	0.0±0.1	1.2±1.7	0.1±0.2	57%(4/7)
3) AS+dev	0.2±0.3	0.0±0.1	0.5±1.1	0.1±0.1	57%(4/7)
4) BM	4.2±5.1	0.5±0.7	8.8±10.5	0.9±1.3	86%(6/7)
5) AS-	11.2±2.8	2.7±2.2	26.2±10.2	5.2±4.6	100%(7/7)
6) AS+	11.7±2.5	1.9±1.2	22.8±9.1	3.9±2.5	100%(7/7)
7) POcult.	11.5±4.7	3.1±4.5	25.7±10.5	4.7±4.5	100%(7/7)
8) POCP	14.0±2.0	1.6±1.1	27.3±5.4	3.1±2.6	100%(7/7)

Figure 3 *Histology of 9 weeks intramuscularly implanted samples* **(☞ p. 185)**

3a): Undecalcified histology of 7-days cultured construct (AS+). Representative impression of bone formation, occasionally bone marrow (M) was seen (bar=1mm).
3b): Fluorescence microscopy of 7-days cultured construct (AS+). All labels are present: C = Calcein green 3-weeks; OTC = Oxytetracycline 5-weeks (yellow); X= Xylenol orange 7-weeks. Growth dynamics can be deduced from the line pattern showing growth from the BCP surface towards the pore (P) center (bar=400µm).
3c): Undecalcified histology of scaffold with plasma without cells (PlmNC). Discrete spots of bone formation (▼) in close relation to the scaffold surface can be observed (bar=400µm).
3d): Fluorescence microscopy detail of bone in scaffold without cells (PlmNC). Only the orange 7-weeks label was found in the spots of bone (bar=400µm).



Discussion

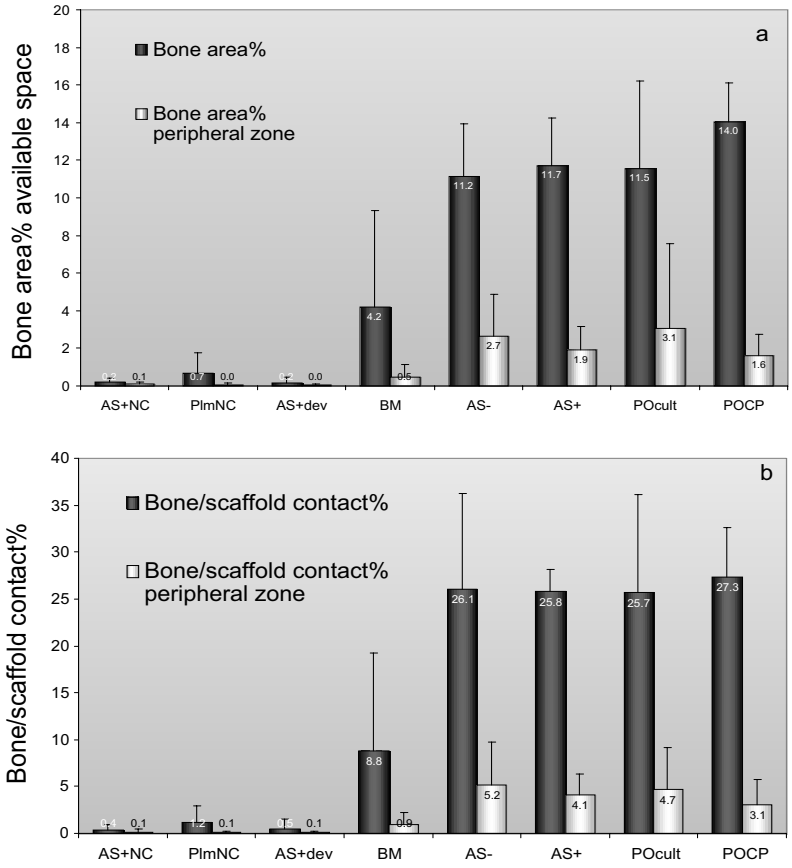
In the current study we demonstrated the feasibility of bone tissue engineering for several different methods that ranged from peroperative preparation of the construct with cryopreserved cells, to one week culture of constructs to stimulate osteogenic differentiation and matrix production. Furthermore we observed a remarkable decreased bone formation at the scaffold periphery. We did not observe differences between the experimental groups where BMSC's were used. Given the results of this study, to obtain a relevant difference of 25% with the AS+ condition with a power of 80%, the sample size of 7 was sufficient. Therefore, relevant differences between the BMSC's seeded conditions are unlikely. The peroperative seeding method with cryopreserved cells overcomes complicated logistics as is the case with cells and/or constructs that are prepared preoperatively. Furthermore, the need for culture medium containing (animal) serum is minimized, which may reduce the risk on residual animal serum proteins. Therefore, this peroperative method seems more appropriate.

The alternative of using fresh bone marrow showed to be successful as well, although this yielded only about a third as much bone, and not all samples showed bone (86%). Apparently, the very low number of osteoprogenitor cells in the bone marrow proliferated extensively in the 2-3 weeks after implantation, to generate sufficient cells for consistent bone formation.[6] Interestingly, a delay in bone formation, as can be expected to allow for this enormous cell expansion, was not found in the BM group, when compared to the BMSC's seeded groups. In both these conditions, the early 3-week fluorochrome label was found always very near to the ceramic surface. This may be explained by recent observations with labeled cells in mice concerning the cellular processes in 7-days cultured constructs.[121] After implantation, the BMSC's detached from the ceramic surface to loosely fill the pores for about 10 days, then part of the transplanted (labeled) cells condensed on the scaffold surface, where a morphological and functional differentiation towards an osteoblast phenotype occurred. If cell development would be similar in the goats, the situation after about two weeks implantation may have been quite comparable for all cell-seeded conditions including the BM condition: the pores occupied with a certain (reachable) amount of progenitor cells that condense on the scaffold surface where differentiation towards the osteogenic lineage starts. This implicates the importance of the scaffold surface in the success or failure of bone tissue engineering as was also shown in this and previous studies.[131,278]

The advantage of culturing the constructs before implantation, which we observed in the previous studies,[104,278] but not in this study, may be related to the scaffold used. Contrary to those studies, in the current study the scaffold itself showed to be

osteoinductive. It is conceivable that the osteoinductive capacity influenced the BMSC's. In that case, the more advanced osteogenic differentiation that existed in the cultured constructs may have become irrelevant. This could also explain the absence of the frequently observed stimulating effect of dexamethasone,[106,128] although this also can be explained by a possible resistance of goat BMSC's to dexamethasone.[122,278]

Figure 4 *Bone formation after 9 weeks intramuscular implantation in goats*
Black bars reflect the whole sample; white bars the peripheral 350µm. Error bars indicate the standard deviation.
4a): The percentage of the available space occupied by bone (Bone area%). BMSC's seeded samples (AS+, AS-, POcult, POCP) yielded significantly more bone than control samples with or without bone marrow (p<0.01). The peripheral zone contained far less bone (p<0.01).
4b): The percentage of available scaffold outline occupied by bone (Contact%). Black bars reflect the whole sample; white bars the peripheral 350µm. Error bars indicate the standard deviation.



The extracellular matrix, in combination with the osteoinductive scaffold was not sufficient for increased ectopic bone formation in the devitalized constructs. This supports the assumption that under normal circumstances, the cells survive implantation and are in some way responsible for new bone formation.[131] This assumption however, contrasts to the finding that there was far less bone formation in the scaffold periphery. Cell survival in this area can be expected to be much higher as a result of to revascularization and nutrient diffusion.[140] Actually, many studies suggest that cell survival is only possible up to 300µm inside grafts.[137,172] Therefore, another mechanism is likely to play an important role. As mentioned before, the osteoinductive scaffold could have stimulated osteogenic differentiation of the cells. Although the exact mechanism of this phenomenon remains a speculation,[152,340] osteoinduction is generally known to occur in well-confined cavities,[152] as was the case more central in our scaffolds. For example Ripamonti found bone induction only in block-shaped porous ceramics with cavities and not in granules. He referred to this morphological prerequisite as geometric induction.[152] It is conceivable that in such cavities the accumulation of factors that are needed for induction/differentiation of the cells is enabled.[341] Furthermore, such a microenvironment may favor calcium phosphate dissolution and reprecipitation, potentially together with important factors, a mechanism postulated before to be responsible for bone induction.[98,131] This induction theory may even be supported by the finding that osteogenesis inside arterosclerotic plaques only occurred as a final stage, inside calcified (hydroxyapatite) plaques.[342] It also implies an advantage of scaffolds with improved dissolution/reprecipitation potential, as is the case with an increased microporosity, shown before to be advantageous.[131,278] With regard to clinical application of the technique, the tendency for bone formation to occur only in confined areas remains a concern, because this will impede continuous bone formation that is for example obligatory for spinal fusion.[156] Further research to gain a more fundamental understanding of these phenomena will be needed.

Acknowledgements

The authors acknowledge the Netherlands Technology Foundation (STW; grant UGN.4966) for financial support. We also thank Mirella van den Doel for assistance with surgery and Maarten Terlou from the Image Analysis department of the University Utrecht for developing the software used for the histomorphometry.

Chapter 10

THE FEASIBILITY OF BONE TISSUE ENGINEERING IN A CRITICAL SIZE DEFECT IN THE GOAT

Summary

Introduction: Since the application of the autologous bone graft, the need for an alternative has been recognized. Tissue engineering (TE) of bone by combining bone marrow stromal cells (BMSC's) with a porous scaffold, is considered a promising technique. In this study we investigated the potential of tissue engineered bone to heal a critical sized defect in the goat. Orthotopic bone formation was compared to ectopic bone formation.

Methods: TE constructs were prepared from goat BMSC's and porous biphasic calcium phosphate ceramic scaffolds. These constructs and scaffolds without cells were implanted paired in critical sized iliac wing defects. Comparable samples were implanted intramuscularly. After 9 (n=7) and 12 (n=8) weeks implantation, the samples were analyzed histomorphometrically.

Results: After 9 weeks implantation in the iliac wing defect, significantly more bone apposition was found in the TE condition. After 12 weeks, the defects were almost completely filled with bone, but no significant advantage of tissue engineering was determined anymore. This contrasted with the intramuscular samples where TE implants showed significantly more bone at both time points.

Conclusion: Bone tissue engineering is feasible in critical sized defects. However, when appropriate osteoconductive/inductive materials are applied the effect of cell seeding may be temporary.

Introduction

Since the application of the autologous bone graft in orthopedic surgery, the need for an alternative has been recognized. Besides the well-known complications of the harvesting procedure,[45,46] other important disadvantages of the autologous bone graft include the extended surgical procedure and limited availability. Therefore, many substitutes have been developed, all of which have specific disadvantages and generally do not perform as well as the autologous graft. In order to be more successful, it is conceivable that substitutes will need one or more of the features that result in the superior functioning of the autologous bone graft. Although little is known about the exact mechanisms involved in the use of autologous bone grafts,[43] the osteoconductive and osteoinductive properties, in addition to a pool of viable osteoprogenitor cells, are likely to be such features.[42,232] Therefore, tissue-engineering (TE) of bone by combining osteoprogenitor cells (usually bone marrow derived stromal cells, BMSC's) with an appropriate synthetic scaffold, to create a so-called hybrid construct, is an interesting technique. The proof of the concept has been shown with genetically labeled cells in rodent studies both ectopically[66,116] and orthotopically.[118,191,213] Despite these successes in rodents, in larger animals only few studies have shown the feasibility of the technique orthotopically,[119,123] and even less studies address the question whether clinically sized constructs are osteogenic ectopically.[130,131,278] A comparison between ectopic and orthotopic functioning in larger animals has never been reported. It is conceivable that functioning of the technique in clinically sized grafts with a delayed vascularization will be more challenging due to the difficulties that can be anticipated for cell survival. Therefore, insight regarding the influence of the surrounding tissue will improve the knowledge on how and when bone tissue engineering will function. In the present study, we investigated tissue-engineered grafts in an established critically sized defect model that allows paired comparisons.[36] In the same animals, comparable constructs were evaluated ectopically in a model previously shown to be successful for bone tissue engineering.[131] To investigate the growth dynamics, sequential fluorochrome labels were administered. The samples were analyzed after 9 and 12 weeks implantation with different parameters focusing on the apposition and the amount of newly formed bone.

Materials and Methods

Scaffolds

Scaffolds were made of 50-60% macroporous, biphasic calcium phosphate (BCP, OsSatura™, IsoTis, The Netherlands). The ceramic consisted of 80±5% hydroxyapatite (HA) and 20±5% tricalcium phosphate (TCP) as confirmed by X-ray diffraction (XRD) and Fourier Transformed Infrared spectroscopy (FTIR), no additional phases or impurities where detected. The material was sintered at 1200ºC resulting in 15±5% microporosity (pores<10µm). Previously, this ceramic has been shown to be osteoinductive ectopically in goats.[97] Discs of Ø17x6mm (orthotopic implantation) and 7x7x7mm cubes (ectopic implantation) were cut, cleaned in ultrasonic baths and steam sterilized.

BMSC's culture and seeding conditions

Autologous serum (AS) was derived from 100ml venous blood that was taken at the time of BM aspiration.[131] The BMSC's were derived from 30ml iliac wing aspirates that were counted before plating in tissue culture flasks. The adherent cells were culture expanded according to previously described methods.[131] When sufficient numbers were achieved, the cells were cryopreserved in medium containing 30% fetal bovine serum (FBS, Gibco, Paisly, Scotland, lot# 3030960S) and 10% dimethylsulfoxide (DMSO, Sigma, The Netherlands). Ten days before surgery, the cells were thawed in pure FBS, centrifuged for 10 minutes at 100G and replated in medium containing 30% FBS. After 3 days, cells were detached, centrifuged and resuspended at a concentration of $1x10^7$ cells/ml in medium containing 15% AS. The discs were statically seeded by dropping 500µl of cell suspension on each side of the discs (=$8x10^6$ cells per cm^3 scaffold). The cubes were similarly seeded with 275µl of cell suspension per cube (=$8x10^6$ cells per cm^3). The constructs were incubated at 37ºC for two hours to allow cell attachment. Constructs and control scaffolds without cells were cultured for seven days in AS medium with 10nM dexamethasone and 10mM β-glycerophosphate (DEX and BGP, Sigma) that has been shown to stimulate osteogenic differentiation of rat and human BMSC's.[106,159] Seeding efficiency and cell proliferation during culture were determined on the 7x7x7 cubes (*n*=6) by a DNA quantification assay (CyQUANT® kit Molecular Probes, Eugene, US) as described before.[131] A Methylene blue staining was done on TE cubes and discs before and after the 7-day culture period to evaluate cell attachment and the distribution of cells and matrix after seeding.

Animals and Implantation

Adult female Dutch milk goats (24-36 months) were obtained at least four weeks prior to surgery. The surgical procedures were performed under standard conditions.[131] After shaving and disinfection of the dorsal thoracolumbar area, a central skin incision T8-L5 was made to expose the muscle fascia. Both iliac crests were identified and cleared of muscle tissue. Under constant saline cooling, central guide holes were drilled before Ø17mm trephine holes were made.[36] The implants were press fit placed into the defects according to a randomized scheme. The muscles were then sutured tight to the remaining fascia on the crests.
Intramuscular pockets were created by blunt dissection after separate fascia incisions in the paraspinal muscles (L1-3). After inserting the implant, the fascia was closed with a non-resorbable suture. The skin was closed in two layers. Postoperatively, pain relief was given by buprenorphine (Shering-Plough, The Netherlands). The goats received sequential fluorochrome labels at 3 weeks (Calceine green, 10mg/kg intravenously, Sigma), 5 weeks (Oxyteracycline, 32 mg/kg intramuscular, Engemycine, Mycofarm, The Netherlands) and 7 weeks (Xylenol orange, 80mg/kg i.v. Sigma).[131,300] Animals were killed by an overdose of pentobarbital (Organon, the Netherlands) after 9 weeks (*n*=7) and after 12 weeks (*n*=8).

Post-mortem sample acquisition, histology and histomorphometry

Explanted samples were fixated in 4%glutaraldehyde/5%paraformaldehyde, dehydrated by graded ethanol series, and embedded in polymethylmethacrylate. Semi-thin sections (10µm) were made with a sawing microtome (Leica, Nussloch, Germany). The mid-section through the samples was used for histomorphometry. To obtain the mid-section, the explanted iliac wing was ground in the plane parallel to the cortex, until the outer (circular) margin of the implant appeared. A central section, 3mm below the ground surface, was then cut from the 6mm thick discs. After cutting 3-4 central slides, additional sections were made perpendicular to the plane of the central section, to evaluate bone formation on the axial faces of the disc that had been exposed to the soft tissue. A similar method was used to obtain central sections from the ectopically implanted samples. Slides were cut 3.5mm below the outside of the 7x7x7mm cubes. Sections were either stained with methylene blue and basic fuchsin for routine histology and histomorphometry or left unstained for epifluorescence microscopy with a light microscope (E600 Nikon, Japan) equipped with a quadruple filter block (XF57, dichroic mirror 400, 485, 558 and 640nm, Omega filters, The Netherlands).

For histomorphometry, high resolution (300dpi), low magnification (10x) digital micrographs were made of blinded sections. Using Adobe Photoshop 5.5, bone and scaffold were pseudocoloured red and green respectively. The area of interest was defined by adjusting the radius of a circle to exactly fit the perimeter of the iliac implant. The area of interest of the cubic ectopic samples was outlined with straight lines bridging the pores that interrupted the exterior contour. Image analysis was performed using a PC-based system equipped with the KS400 version 3.0 software (Zeiss, Oberkochen, Germany). A special program was developed to measure the following parameters of bone: 1) the percentage bone in the entire region of interest (fill%); 2) the percentage bone in the available (pore) space (B/Parea% = bone area/ pore area x 100%); 3) the area of bone related to the area of scaffold (B/Sarea% = bone area/scaffold area x 100%) and 4) the percentage of available scaffold perimeter in contact to bone (contact% = scaffold perimeter length/bone-scaffold contact length x 100%). Fill% was measured to allow comparison with previous studies. This also accounts for the frequently used B/Parea% that is more informative on the filling of the available space.[131] In addition, the B/Sarea% was measured based on previous work indicating that tissue engineered bone formation is strongly associated with the scaffold surface.[131] It therefore seems more appropriate to relate the quantity of TE bone formation to available scaffold rather than available pore space. Similarly, contact% is more sensitive for early bone apposition which always occurs on the ceramic surface and has relatively little volume. Developmental differences between the above parameters indicate the growth dynamics of newly formed bone.

Statistics

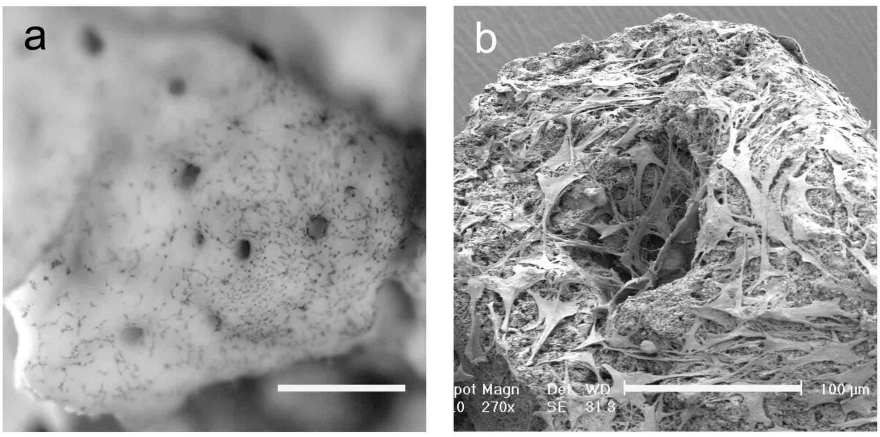
Data are shown as mean±standard deviation. Two-sided, student t-tests were performed to analyze differences between tissue engineered and control implants at each time point (paired) and between the time points (unpaired). The level of significance was set at p=0.05.

Results

In-vitro

The BM aspirates contained 9.7±2.6x10^6 (mean±SD) nucleated cells/ml. Colonies formed after 6-8 days culture and the BMSC's were harvested when confluent, after 10-14 days. After the 2nd passage, 0.8-1.5x10^8 BMSC's were cryopreserved. Thawed cells showed minimal dead cells (<5% according trypan blue exclusion) and proliferated well before seeding onto the scaffolds. The seeding efficiency of 8x10^6 cells/cm^3 cube, determined by DNA quantification, was 49.1±14.7% and, after 1-week of culture, cells doubled to 8.7±3.7x10^6 cells/cm^3. Stereomicroscopy after seeding showed homogeneous cell attachment throughout the entire scaffolds. The 1-week cultured constructs showed the cells were spread on the ceramic surface (Fig. 1a) with dense multilayers on the outside of the constructs. This was confirmed by SEM imaging that showed the scaffolds were covered with cells and extracellular matrix (Fig. 1b).

Figure 1 *In-vitro imaging of cultured constructs*
1a): Stereomicroscopy of central part of a 7-days cultured disc as used for iliac defect implantation. Methylene blue stained cells are spread and cover the concavity of the pores. The small orifices are the interconnections between the pores (bar = 500µm).
1b): SEM image of 7-days cultured cube. Flattened cells with extra cellular matrix positioned around an external pore interconnection orifice (bar = 100µm).



In-vivo results of orthotopic implants

There were no surgical complications and no macroscopic or microscopic signs of infection. The iliac wing implants were overgrown by tissue and could not be detected visually or manually (no motion).

Plain histology of the iliac wing implants revealed two cases (one for each condition in the 9-week group) of partial resorption of the inferior iliac wing border, resulting in only 80-90% of these implants being surrounded by bone. Besides these two implants, the scaffolds were totally integrated by the surrounding cancellous bone without interposition of fibrous tissue. No signs of scaffold resorption were present at both implantation periods. In the 9-week implants, bone formation appeared advanced in the TE group, although neither of the conditions showed bone formation in the center of the implanted discs. At the disc surfaces that were exposed to soft tissue, a similar distribution of new bone in close association with the surrounding bone was seen, while it was absent in the center. In the 12-week implants, considerable bone had formed and some defects were almost completely filled with bone in both conditions (Fig. 2 and 3a).

Fluorescent microscopy indicated bone was growing away from the scaffold surface and confirmed that no bone had formed in the middle of the defect in the first 9 weeks. No obvious differences in the presence or pattern of the labels was observed between TE and control samples (Fig. 3b,c).

Histomorphometry of the explanted samples indicated a porosity of 53.7±10.9% for the 9 week implants and 65.0±5.2% for the 12-week implants (mean±SD). After 9 weeks, the bone fill%, the B/Parea% (in available space) and the B/Sarea% (bone area related to scaffold area) were not significantly different between the TE and control samples (Table 1). However, at this follow-up, the contact% was significantly higher in the TE group as compared to the controls (24.7±9.3 vs. 17.9±8.4, $p<0.01$) (Fig. 4a). Because of the observed differences in porosity between the 9 and 12 weeks implanted samples, comparisons for the fill% and B/Parea% in time were considered not applicable and only the parameters that related bone directly to the scaffold were addressed. After 12-weeks, the B/Sarea% had increased significantly for both the TE and control condition when compared to the 9-week group. The contact% had increased less and did not show significantly more bone in the TE condition. Comparing the TE condition to the controls after 12 weeks did not reveal significant differences.

Figure 2 *Pseudocoloured images of the ∅17mm iliac wing implants*
2a): Control scaffold implanted for 9 weeks. Surrounding bone (dark grey) covers the periphery of the entire scaffold (light grey).
2b): TE sample implanted for 9 weeks in the same goat as 2a. No bone formation was observed in the middle of the 9-weeks implants.
2c): Control scaffold implanted for 12 weeks. The entire scaffold is covered with bone.
2d): TE sample implanted for 12 weeks contralateral to sample 2c. The scaffold is completely integrated in the surrounding cancellous bone.

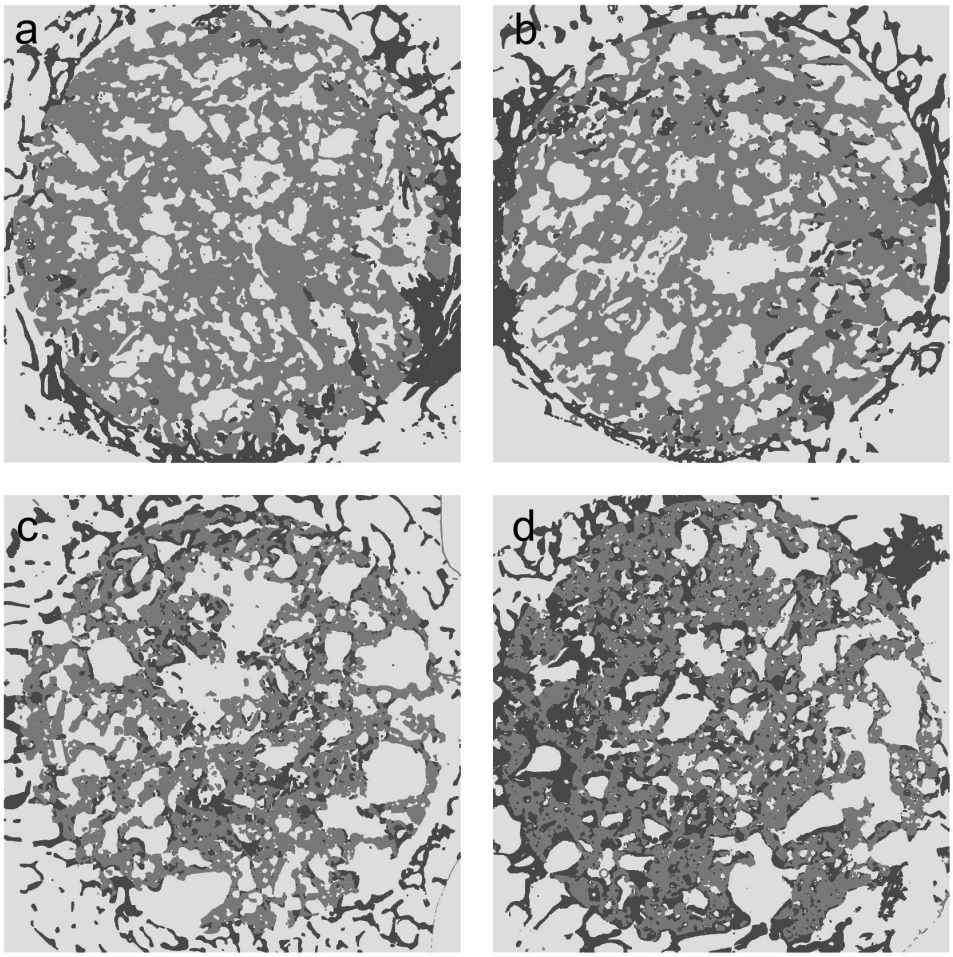


Figure 3 *Histology* **(☞ p. 186)**

3a): Low magnification micrograph of the ∅17mm iliac implant as shown in figure 2d (12 weeks TE). Squares indicate the source for the fluorescence microscopy images (3b, c).

3b): High magnification fluorescence microscopy of a peripheral area of the implant in 3a. Bone formation originated before 3 weeks on the outer side and inner side of the ceramic, as indicated by the Calcein green (C, 3-week) label. Fusion between surrounding bone and the scaffold was accomplished around 5 weeks as indicated by the yellow OTC label (bar = 50µm).

3c): High magnification fluorescence microscopy of a more central part. Only the Xylenol orange (X, 7-weeks) was present close to the BCP scaffold (bar = 50µm).

3d): Low magnification micrograph of 7x7x7mm TE construct implanted ectopically for 9 weeks. Bone formation was in close association with the scaffold surface, but never occurred on the exterior.

3e): Fluorescence microscopy of bone formed in the middle of 9 weeks implanted TE scaffold. All three fluorochrome labels were present. Growth dynamics can be deduced from the line pattern showing growth started from the BCP surface towards the pore (P) center (bar = 100µm).

3f): High magnification micrograph of TE construct implanted ectopically for 12 weeks. Typical trabecular bone formation lined with osteoblast zones (bar = 50µm).

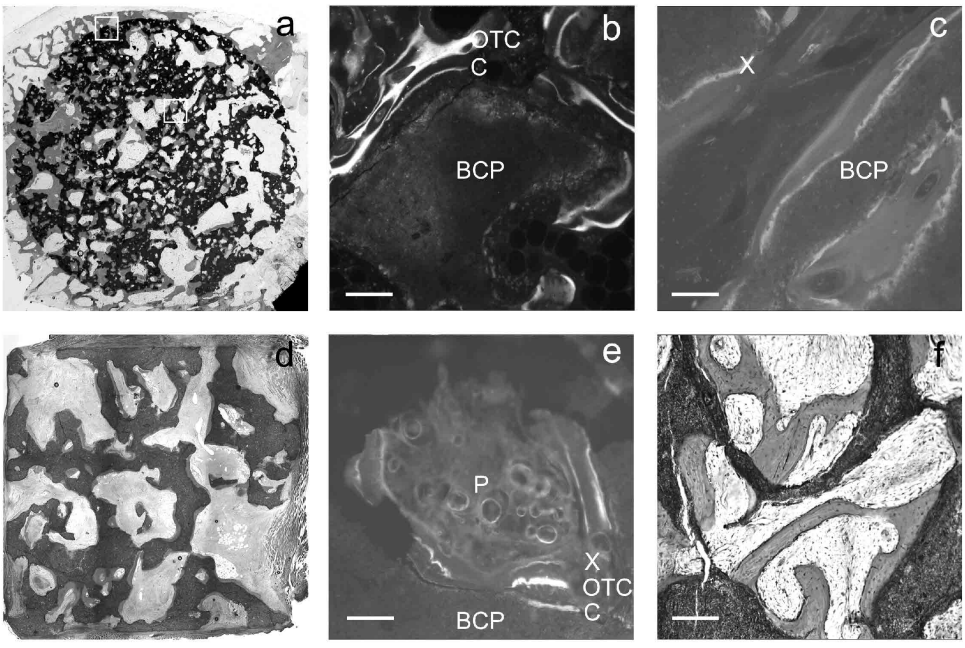


Table 1 *Histomorphometry of orthotopic and ectopic implants*
For both the orthotopic and ectopic locations, the area% bone in the whole sample (Total fill%); the area% bone in available pore space (B/Parea%); the area% bone related to scaffold area (B/Sarea%); and the percentage of the scaffold perimeter in contact to bone(Contact%) are shown. The effect of tissue engineering (TE) vs. the control with no cells (NC) and the effect of implantation time was analyzed. NA = Not Applicable (see text) and NS = Not Significant
Orthotopically an effect of TE existed only after 9 weeks for the contact% (^{*1}*) In time, the B/Sarea% increased in both TE and NC condition, the contact% increased only for the NC condition(*^{*2}*). Ectopically the effect of TE was obvious for all parameters. In time, the B/Sarea% increased (*^{*3}*) whereas the contact% slightly decreased in the TE condition(*^{*4}*).*

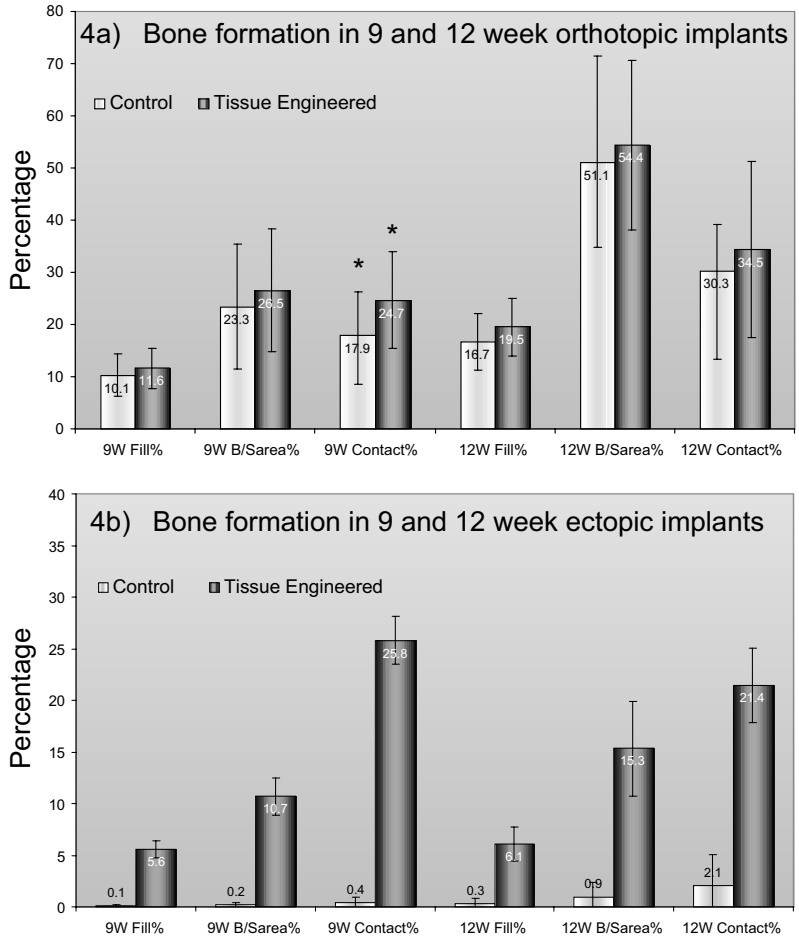
Location	Parameter	9 weeks		12 weeks		Level of significance	
		NC	TE	NC	TE	NCvs.TE	9vs.12W
Orthotopic (mean±SD)	Total fill%	10.1±4.3	11.6±3.9	16.7±5.4	19.5±5.5	NS	NA
	B/Parea%	18.9±9.3	22.1±8.3	25.1±7.1	29.0±10.0	NS	NA
	B/Sarea%	23.3±12.1	26.5±11.8	51.1.±20.5	54.4±16.2	NS	<0.01
	Contact%	17.9±8.4^{*1,2}	24.7±9.4^{*1}	30.3±8.8^{*2}	34.5±16.9	<0.01^{*1}	0.016^{*2}
Ectopic (mean±SD)	Total fill%	0.1±0.1	5.6±0.9	0.3±0.5	6.1±1.6	<0.01	NA
	B/Parea%	0.2±0.2	11.7±2.5	0.6±0.8	10.2±2.9	<0.01	NA
	B/Sarea%	0.2±0.2	10.7±1.8^{*3}	0.9±1.4	15.3±4.6^{*3}	<0.01	0.025^{*3}
	Contact%	0.4±0.5	25.8±2.4^{*4}	2.1±3.0	21.4±3.6^{*4}	<0.01	0.019^{*4}

In-vivo results of ectopic implants

All samples were retrieved without macroscopic or microscopic signs of infection.
Plain histology showed small foci of bone, lined by osteoblasts, in 5/7 controls (=71%) after 9 weeks, and in 6/8 (=75%) after 12 weeks. In all tissue engineered samples, ample bone was present. In these samples, bone lined by osteoblasts was closely related to the scaffold surface after 9 weeks and had a more trabecular appearance after 12 weeks. Bone was never found on the exterior of the scaffolds and seemed more abundant centrally in the scaffolds (Fig. 3d-f).
Fluorescence microscopy indicated that bone formation was always directed away from the scaffold surface and had started before 3 weeks implantation in the TE group (Fig. 3e) and only after 5 weeks implantation in the controls, which occasionally showed the 7-week label.
Histomorphometry of all slides indicated a scaffold porosity of 48.9±4.1 for the 9-week samples and 61.8±4.4 for the 12-week samples. After 9 weeks, all parameters indicated an about 50 times higher yield of bone inside the TE scaffolds (p<0.01) (Table 1 and Fig. 4b). To analyze an effect of time, again the fill% and B/Parea% where not considered. After 12 weeks, the B/Sarea% had significantly increased for

the TE condition, while the contact% had slightly decreased from 25.8 to 21.4%, which was significant (p=0.019). The substantial difference between TE and control groups remained for all parameters.

Figure 4 *Orthotopic and ectopic bone formation after 9 weeks (n=7) and 12 weeks (n=8)*
4a): Orthotopic implants in the iliac wing defect. The percentage of the total defect filled with bone (Fill%); the area of bone related to the scaffold area (B/Sarea%) and the percent bone apposition on available scaffold outline (Contact%) were measured. White bars reflect the controls; black bars the tissue engineered constructs. Error bars indicate the standard deviation. At 9 weeks, the contact% was significantly higher in the TE samples ([1]p<0.01).*
4b): In the ectopic samples the same parameters were measured. Always significantly more bone was found in the tissue engineered samples.



Discussion

In the current study in goats, we investigated bone formation in tissue engineered constructs both ectopically and orthotopically. Ectopically, the scaffolds without cells were shown to be osteoinductive in the majority of the goats for both implantation periods, as was expected based upon previous observations.[97] It is conceivable that this inductive capacity stimulated (part of) the seeded BMSC's because the BMSC population contains both determined and inducible osteoprogenitor cells (DOPC's and IOPC's).[78,170] Because of this inductive stimulus, the one-week predifferentiation of the cells as was done in this study may be irrelevant. Furthermore it appeared that at the moment of implantation only slightly more cells were present than the number of cells that was originally seeded (8.7 compared to $8x10^6/cm^3$) which also supports the frequently used alternative of combining the cells and scaffold just prior to surgery.[119,122,191] Ectopically the effect of tissue engineering was obvious, however, orthotopically the effect was less pronounced. Only at the early explantation period of 9 weeks, the tissue engineered samples demonstrated significantly more bone apposition on the available scaffold surface. This effect could not be detected after 12 weeks implantation, at which time the TE group showed only about 10% higher values for all parameters. It should be mentioned, that due to the unexpectedly large standard deviation, despite paired comparisons, the effect needed to be in the order of 40-50% to be detectable with a power of 80%.

Compared to other reports on orthotopic implantation for up to 5 months in large animal models,[119,123-125] the impact of TE in our study was modest. The most rational explanation for this lies in the use of different hybrid constructs. Both the osteogenicity of the TE constructs and the scaffold material itself must be considered. A lack of osteogenicity of the constructs at the orthotopic location is unlikely because the osteogenicity was shown clearly in the ectopic model. In previous studies with this model, it was demonstrated that viable cells were crucial to accomplish bone formation, indicating the osteogenic role of the cells.[131] Unfortunately, the osteogenicity of the constructs (ectopically) used by other researchers was not reported in their animal model. Another explanation concerns the scaffold material itself, which was shown to be osteoinductive. It is possible that bone conduction in combination with this inductive capacity, resulted in so much bone apposition, that the TE-related osteogenesis was overruled. This hypothesis is supported by the finding that bone formation was only found at the early (9 weeks) evaluation, and after 12 weeks bone was present also throughout the control implant with bone filling 16.7% of the entire defect area. For comparison, in previous studies with this model after the same time period, empty controls contained 13.5%

bone, a porous osteoconductive copolymer only 1.5%, allograft 13% and autograft 36%.[36] In that study also the grafted chips of the allo and autograft were counted as bone, whereas in the current study only new bone was counted.

A more discouraging explanation for the modest effect of TE may be proposed by the absence of early osteogenesis in the center of the iliac defects. This is not that surprising, considering the literature on cell survival in large unvascularized bone grafts, as cell survival is expected to be limited and not to exceed a distance of 300µm from a nutrient supply.[22,169,172] However, if this were true, ectopic bone formation should also be limited, since the diffusion depth in these samples was also much larger, up to 3.5mm. This appeared not to be the case, and on the contrary, bone seemed to be more abundant in the middle of the constructs. Although (micro)movement on the scaffold outside may account for absence of bone at the very exterior, the observation of extensive bone centrally does indicate that cells probably survived at relatively large diffusion depths, although this needs to be confirmed with techniques that can reliably identify the transplanted cells.[116,213] Cell survival in the orthotopic location, however, will be different from the ectopic location. The greater trauma with a subsequently larger heamatoma, results in more wound excudate and delayed revascularization. Another difference is the fracture healing response, which may interfere with the implanted cells. Typically, fracture healing does not rely on cells present inside the defect, but recruits the cells from the surrounding mesenchymal tissue.[147,148] It is possible that cells inside the defect were subject to phagocytosis during the immediate injury response.

The measured porosity of the 9 and 12 week implants were different (approximately 50 and 60%, respectively), resulting in different available spaces for bone formation. Although material degradation cannot be ruled out completely, this difference in porosity is likely the result of fluctuations in the normal production process. This complicated the investigation of growth dynamics by comparisons between the 9 and 12 week implants. Therefore, the fill% and the bone per available pore space (B/Parea%), which are directly related to the available pore space, were not statistically compared between this time points. By relating bone to the available scaffold material (B/Sarea%) and to the available scaffold perimeter (contact%), relevant comparisons could be made because bone apposition was always on the scaffold surface. Together with the fluorochrome markers, these parameters indicated bone formation was associated with the ceramic surface and grew cenripetally after initiation. In the ectopic samples, the percent scaffold outline occupied by bone decreased between 9 and 12 weeks, suggesting no new bone formed on the scaffold surface after 9 weeks. However, based on the increasing

B/Sarea% and the presence of osteoblast zones, bone formation itself continued (Fig. 3f).

In conclusion, we have shown the relative contribution of tissue engineering to bone formation ectopically and orthotopically. Although the constructs where osteogenic ectopically, orthotopic functioning was modest in comparison to the osteoconductive and osteoinductive capacity of the scaffold. In situations that may be considered to have and ectopic constituent or where the inherent conductive and inductive nature of the scaffold is likely to be limited, e.g. posterior spinal fusions, bone tissue engineering may be helpful.

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Chapter 11

GENERAL DISCUSSION

In this chapter, a brief overview of the findings that have been discussed in detail in the chapters 3-10 will be given.

In *Chapter 3*, the review article on spinal fusion models for bone tissue engineering, we discussed the most likely mechanism and the potential of bone tissue engineering. Although we could not elucidate the exact mechanism, we assumed that bone formation would be intramembranous. Furthermore we emphasized on the fact that little is known about the feasibility of the technique, which demands for a humble research attitude to primarily gain insight in the technique. The achievements of our research will be attended in the order of the specific aims of this thesis:

1) To investigate the role of cell viability in autologous bone grafts;
2) To investigate the role of cell viability in TE constructs;
3) To develop a labeling technique to trace cells used for TE;
4) To investigate methods to optimize bone tissue engineering;
5) To investigate the applicability of TE in clinically relevant models.

Ad. 1: To investigate the role of cell viability in autologous bone grafts

In *Chapter 4* we investigated the role of cell viability in autologous bone grafts. We observed that osteogenesis was far more advanced in viable transplanted grafts, especially in the ectopically implanted grafts. Furthermore, when viable bone chips were implanted, these were bridged with new bone forming a solid mass. Orthotopically, a less pronounced advantage of cell viability was determined after one month implantation in the condyle defect. This was not a critical size model and

host bone formation probably shaded the osteogenic activity of the grafted cells. Although it is tempting to conclude that cells do survive and make bone after transplantation, this study only permits the conclusion that viability at the moment of transplantation has an obvious effect on subsequent bone formation. What exactly happens to the cells after transplantation could not be determined with the current design. Therefore, several alternative explanations for the role of viability in autologous grafts should be kept in mind and further investigated. These include the following three:

1) Grafted cells do not participate in bone formation, but the extracellular matrix itself is osteoinductive (does not depend on viable cells) and is responsible for bone formation. We devitalized the control samples by freezing in liquid nitrogen. This procedure may have destroyed the extracellular osteoinductivity of the graft.

- This seems indeed a comprehensible explanation. However, literature indicated that freezing minimally effects bone induction,[48,292] as discussed in Chapter 4.

2) Grafted cells do not participate in bone formation, but prior to death, or in a senescent state, release a bone inducing substance that activates invading mesenchymal cells, a mechanism proposed by many authors.[42,57,170,172]

- Unfortunately, there is no literature available that directly investigates this possibility.

3) Donor cells do not participate in bone formation, but release prior to death or in a senescent state, factors which are responsible for increased vascularization. Since vascularization is closely related to bone formation,[343] as source of oxygen and nutrients, an orienting factor for bone apposition[344,345] and a potential source of stem cells which may be pericytes,[7] subsequent bone formation will be increased.

- Although the direct coupling remains to be proven, it was shown recently that increased neo-angiogenesis was present in constructs with viable BMSC's implanted in rats.[140] Furthermore, it was determined that BMSC's release stimulating levels of vascular endothelial growth factor (VEGF).[346,347]

Based on the studies we performed, as well as upon the available literature, these alternative explanations cannot be rejected. However, if viability of the autologous grafts would not have had any influence on bone formation and new bone would not be formed in either situation, it would have been unlikely that the cells play any role. Therefore, we conclude that with respect to the first aim of this thesis cells do play a role in autologous bone grafts. This provides a rationale for cell-based bone tissue engineering.

Ad. 2: To investigate the role of cell viability in TE constructs

In *Chapter 5* the rationale for cell-based bone tissue engineering was confirmed in the goat model of ectopic bone formation. The presence of extracellular matrix alone appeared to be insufficient for bone formation, provided that freezing did not affect bone induction negatively. To investigate the alternative hypothesis that cells do not participate in bone formation, but only release substances which promote bone formation, we conducted a simple test: Prior to ectopic implantation, TE constructs (*n*=4) were irradiated sub-lethally with 10 and 40 Gray.[75,226,230] The subsequent chromosomal damage prevented further proliferation (verified by *in-vitro* analysis). Despite the damage, the cells remained viable and metabolically active (possibly capable to release a substance that stimulates bone formation), as confirmed with an alamarBlue™ assay.[348] If the alternative hypothesis of substance release would have been true, bone formation should be present in these samples. However, we did not find any bone in those samples after 9 weeks implantation, whereas all non-irradiated constructs consistently showed bone as was described in Chapter 9. We realize that irradiation may have influenced the release of substances negatively and therefore cannot totally reject the alternative hypothesis. However, with respect to the second aim of this thesis, this finding increased the burden of indirect proof that cells do survive and are responsible for tissue engineered bone formation ectopically in the goat.

Ad. 3: To develop a labeling technique to trace cells used for TE

The only direct proof of cell osteogenicity and an ideal tool for further research of cell development would be the ability to identify these cells in the histological samples. In *Chapter 6* we investigated the “off the shelf” intramembranous CM-DiI label and unfortunately had to conclude this label was only applicable for evaluation of cells after short implantation periods (up to 10 days). Despite that many research groups did use the label for long-term evaluation,[120,186,316] in our experiment, transfer of the label was obvious. The consequence of that finding was that the label was inappropriate to determine the fate of the cells after longer implantation periods. As a next step, other fluorescent labels were investigated. When using intracellular labels instead of the intramembranous CM-DiI, again transfer was found and therefore these labels were also considered inappropriate to reliably show the cell fate after transplantation.

In collaboration with the department of Haematology of the UMC Utrecht, we developed a retroviral labeling method as described in *Chapter 7*. The membrane surface marker NGFR that we investigated, appeared to be stable and no influence on the bone forming capacity of the labeled cells was observed. We could proof the

direct coupling between the goat BMSC's and bone formation in our tissue engineered constructs (=osteogenicity) in the nude mice model. Furthermore, analyses of the sequential implantation times provided more insight in the process of bone formation. The cell layer on the constructs that were cultured for one week appeared not to continue after implantation with appositional bone formation. Instead, cells seemed to become detached and loosely filled the pores. Ten days after implantation, part of the cells condensed on the ceramic surfaces, were bone formation started. This observation is indicative of a local stimulus that probably is needed either to direct the relatively undifferentiated/dedifferentiated cells towards the osteogenic lineage or to provide a substrate for (bonding) osteogenesis. It is conceivable that this stimulus greatly depends upon the scaffold that is used as was discussed in more detail in chapters 8, 9 and 10. Additionally to the development of the retroviral cell surface marker (NGFR), we also transduced goat BMSC's with the luciferase gene. This gene encodes for the fire fly enzyme responsible for light emission. Cells that were labeled with this gene could be monitored in constructs for up to 6 weeks in mice, indicating their survival and maintenance. This pilot study has however, not yet been published. With respect to the third aim of this thesis, the most important merit of these studies is that we now have a tool to investigate cell behavior in clinically relevant goat models.

Ad. 4: To investigate methods to optimize bone tissue engineering

To optimize bone tissue engineering, the following issues are relevant:

The yield of bone;

Because this allows quantitative data it is most often applied. However, the optimal amount of bone is unknown, therefore the relevance of this parameter decreases with higher quantities, as it does not address other important aspects like distribution and remodelling.

The ease of the procedure;

An important optimization would be to overcome current disadvantages of the TE technique such as the delay necessary for cell culture and the complicated logistics to ensure the patient and the construct are prepared at the same time.

The functionality of the newly formed bone.

The ability of tissue engineered bone to generate a structural (fusion)mass and to participate in the physiologic (host) bone turnover would be an important issue to optimize.

In *Chapter 8* we found an advantage (in terms of *in-vivo* bone yield) for constructs that were cultured for one week after seeding of the cells. When the constructs were not cultured, which makes the procedure considerably less complicated, bone formation appeared to be feasible as well. Another important finding of that study was the feasibility of using autologous serum instead of FBS for cell culture. Because several granules were implanted together, we could investigate the tendency for bone bridging between these granules. This was observed to occur between ectopically transplanted autologous bone chips of comparable size, that fused to solid masses (*Chapter 4*). However, bone bridging was almost never observed between the tissue engineered granules, which is a concern for future clinical application that relies on the ability to form continuous masses.

In *Chapter 9* we further optimized the technique both with respect to the yield of bone and the ease of the procedure. We used another type of ceramic scaffold (biphasic calcium phosphate BCP). This ceramic was chosen because in a separate study on ectopic bone formation, it yielded about twice as much bone as the HA70/800 that we used before (n=8, p<0.01, not published). The ectopic bone yield of 14% of the available space after 9 weeks in the 7x7x7mm cubes (=0.35cm^3) is relatively high when compared to the limited literature that is available[130,131] and may be sufficient for functional application (as mentioned before, the optimal amount of bone is speculative). More importantly, compared to the study in *Chapter 8*, the bone yield was more predictable as reflected by the relatively low coefficient of variance and the Gaussian distribution of the data, which allowed for parametric statistical analysis. By using these scaffolds, culture of the constructs appeared not to be advantageous anymore in terms of bone yield. It was not investigated whether the enhancement of bone formation in time, as shown previously,[104] was (still) present. The method of peroperatively combining cryopreserved cells with the ceramic scaffold considerably reduced the logistics to the level of a standard elective procedure, and the labor of culturing cells and constructs was reduced. The functionality of the newly formed bone however remains a concern. The total absence of bone on the exterior of the implants and the dramatic decrease (>5 times less) of bone in the scaffold periphery indicated that bone formation is limited to a confined environment and may not participate in continuous mass formation. Besides this worrisome consequence of the typical distribution of newly formed bone, the similarity with the distribution of ectopically induced bone[11,101,157,333,349,350] also gave new insight in the potential mechanism of bone formation. Likely were the processes responsible for the scaffold to be osteoinductive, also essential for tissue engineered bone formation, either by inducing the cells to become osteoblast or by providing a suitable substrate for

osteogenesis. It is conceivable that this process starts with the formation of a biological carbonated apatite layer on the ceramic surface, with potentially the incorporation of inducing- or specific binding proteins, like integrins.[97,99,100,293,294,350-355] The hypothesis that an *in-vivo* ceramic surface modification preceded the actual osteogenesis was also strongly supported by the observations from the labeling studies (*Chapter 6 and 7*), which indicated that after implantation of cultured constructs (with cell multilayers on the scaffold surface), the cells detached and about a week later condensed again on the scaffold surface, where they started bone formation. Finally, the hypothesis is supported even more by the finding that the cells and the scaffold did not had to be linked at the moment of implantation in order to give bone, actually culturing the cells for a week *in-vitro* on the ceramic did not show more bone formation at all. With respect to the fourth aim of this thesis, we can conclude that the procedure can be optimized substantially, however, functionality of the bone remains a concern.

Ad. 5: To investigate the applicability of TE in clinically relevant models

This was first addressed in a critical size segmental femur defect that was developed for bone induction studies.[301] This pilot study, not further addressed in this thesis, with six different TE constructs of the HA70/800 scaffold (n=2 per condition) demonstrated a lack of relevant bone formation or fusion in these constructs, although all constructs showed to be osteogenic ectopically. We hypothesized this model was too challenging, due to the impossibility of rigid stabilization. Therefore, this model was discarded and replaced by the critical size defect model in the goat iliac wing, which is more stable and allows for paired comparisons. With this model, a moderate effect of tissue engineering could be observed as described in *Chapter 10*. This effect was only found at the early evaluation time of 9 weeks, for the parameter that is most sensitive for tissue engineered bone formation, namely, the percent of available scaffold surface where bone apposition was found. After 12 weeks implantation, no effect was observed any more. As mentioned in the discussion of this chapter, the effect of tissue engineering had to be over 40% to be determined with a power of 80%. Although we did not observe such an effect, it is unlikely this was related to a lack in osteogenicity. Namely, the ectopic implants showed perpetual bone formation as reflected by the increased bone yield between 9 and 12 weeks and the presence of osteoblast zones at 12 weeks. It is plausible that the scaffold itself was responsible for the absence of an obvious effect of tissue engineering. Not because the scaffold is unsuitable, but on the contrary, is that potent that the majority of bone is by

conduction/induction. This potency was reflected by the high bone yield in the control ceramics that where almost totally covered by bone after 12 weeks implantation.

Chapter 12

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

To draw conclusions on the applicability of cell-based strategies for bone tissue engineering, the most important tool is the available literature. For the work of this thesis, this was always the point of reference. Regarding the literature, efforts to back-up the feasibility of the concept of tissue engineering, or at least to provide indirect proof that cells play any role in clinically sized autologous bone grafts failed. The concept appeared unrealistic, because fundamental knowledge predicts cell death in constructs exceeding several mm^3 (chapter 2). Furthermore, there were no studies that showed cell survival and function in large animals. Based on the literature itself, but also on the failure of other well established research groups to report on relevant progress in terms of clinical successes, one should be skeptical. For comparison, numerous successful studies in large animals were reported for BMP-based tissue engineering and by now clinical successes of this technique are piling up. This skeptical viewpoint was the basis for the studies that were performed in this thesis. Despite the expectation of the principal author that making progress would be very challenging, many results of the studies were promising and can be summarized in the following list of conclusions supporting the feasibility of the technique.

Conclusions supporting the feasibility of tissue engineering

- Viability is an important factor for bone formation in autologous bone grafts of clinical relevant size;
- Viable cells are crucial for bone formation in tissue engineered constructs;
- Cells are directly involved in bone formation in small sized implants;
- Cell-based tissue engineering enhances bone formation orthotopically;
- The procedure of bone tissue engineering can be simplified considerably.

These conclusions are encouraging and provide a strong rationale to further develop the technique. However, this does not support clinical application of the technique at this moment. On the contrary, the list of worrisome conclusions indicates that both the applicability and the functioning of the technique needs further investigation.

Worrisome conclusions

- There is no necessity for viable cells in orthotopic grafting;
- Bridging between individual tissue engineered granules is exceptional;
- Transplanted cells are capable of fibrous tissue formation;
- Bone formation is restricted to confined areas;
- Orthotopically, the effect of tissue engineering is moderate and may be irrelevant at long-term implantation intervals.

Irrespective of the type of conclusion, the studies improved our knowledge on bone tissue engineering. Scientifically, the technique is intriguing and provides the opportunity to drastically improve our knowledge on many aspects of bone formation. Not only the role of putative stem cells and their differentiation is accessible for research, but also the role of the (micro) environment necessary for bone formation. This environmental aspect has merely been investigated in this thesis, but most likely plays a crucial role in providing a substrate for the adhesion of specific cells and their subsequent differentiation. The ultimate example of the power of this aspect is the ability of some scaffolds to induce bone formation without the addition of pre-selected cells or factors. We hypothesize that the processes involved in this phenomenon, more specifically, the dissolution of the ceramic surface and reprecipitation of a biological carbonated apatite layer are essential for the tissue engineered bone formation that we observed. With the current possibilities in biotechnology, these and many other aspects can be thoroughly investigated further.

If this thesis would be a departure for such studies, as a first step the role of the cells can be investigated in more detail. The retroviral labeling technique that we developed allows for histological identification, both in the ectopic and the orthotopic model. Luciferase labeling enables real time *in-vivo* evaluation of the cells, which is particularly interesting to determine a difference between orthotopic and ectopic implantation. Furthermore, strategies that improve the vascularization of the construct *in-vivo* can be accurately investigated by this method. Another opportunity lies in the finding that cryopreserved cells and scaffolds can be mixed peroperatively, which opens possibilities for less complicated elective procedures and the preparation of compositions that can be used as cement (e.g. CaP bone cement). Of course, scaffold induced bone formation should be investigated further as it is most likely that this phenomenon is enhanced by cell admission. The answer to improvement of functionality of the tissue engineered bone may be derived from such investigations or, as often is the case in medicine, found by serendipity. The final and most important conclusion that can be drawn at the end of this thesis is:

Cell-based bone tissue engineering has the potential to become an ideal autologous bone graft substitute

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Samenvatting in het Nederlands

Introductie

Binnen de orthopaedie wordt op grote schaal gebruik gemaakt van lichaamseigen bot. Dit wordt gebruikt om het genezingsproces van vaak gecompliceerde reparaties aan het bot te stimuleren. Een goed voorbeeld is de zogenaamde posterolaterale wervelfusie: hierbij wordt het lichaamseigen bot naast de wervellichamen gelegd. Nieuw bot dat vanuit de wervellichamen groeit kan het getransplanteerde bot als model/voorbeeld gebruiken en het in de loop van de tijd volledig vervangen. Zo kunnen de twee wervellichamen met nieuw bot aan elkaar vast groeien.

Door evolutionaire selectie heeft het menselijk lichaam zich gedurende minstens een miljoen jaar uiterst efficiënt ontwikkeld. Daarbij is geen rekening gehouden met de orthopaedische mogelijkheden van de laatste honderd jaar. Helaas is er dus geen (grote) voorraad reservebot aangemaakt. Het wegnemen van lichaamseigen bot (meestal uit de bekkenkam) is dus in principe een verminking die niet straffeloos kan plaats vinden. De nadelige gevolgen zijn veelomvattend en betreffen met name pijnklachten. Het is daarom een grote uitdaging een alternatief te ontwikkelen voor het lichaamseigen bot met wel alle voordelen, maar niet de nadelen. Daarvoor is het belangrijk de voordelen van het lichaamseigen bot te kennen en mee te nemen in de ontwikkeling van een alternatief: in de eerste plaats wordt het lichaamseigen bot per definitie niet als lichaamsvreemd ervaren en kan het probleemloos worden geïmplanteerd zonder afstotingsverschijnselen. Bij bot van andere (menselijke) donoren, dat veelvuldig als een alternatief wordt gebruikt, is acceptatie van het weefsel wel een probleem, naast het risico dat ziektekiemen kunnen worden overgebracht. In de tweede plaats is een belangrijk voordeel van met name het trabeculaire bot (uit de bekkenkam) dat dit een uitstekend voorbeeldmateriaal is voor het nieuwe reparatiebot. Ten derde, een omstreden maar zeer opvallend verschil tussen het lichaamseigen bot en de huidige alternatieven is dat het lichaamseigen bot vol met levende cellen zit op het moment van transplantatie. In hoeverre dit ook echt een voordeel is, is echter tot op heden onbekend. De belangrijkste reden om hieraan te twijfelen is dat de cellen gedurende de eerste periode na transplantatie afgesloten zijn van bloedvoorziening en daardoor moeilijk kunnen overleven. Vele fundamentele onderzoekers stellen daarom dat alleen in het allerbuitenste deel (zo'n 300µm), of in zeer kleine transplantaten (enige kubieke

millimeters) cellen kunnen overleven, terwijl de gangbare grootte van een klinisch toegepast transplantaat al gauw enige kubieke centimeters is (1000x zo groot). Desalniettemin wordt door vele (meer klinisch georiënteerde) onderzoekers verondersteld dat een aanzienlijk aantal van de cellen overleeft en bijdraagt aan nieuwe botvorming.

Op basis van de (omstreden) voordelen die het lichaamseigen bot heeft ten opzichte van de huidige alternatieven wordt daarom verondersteld dat een optimaal alternatief moet beschikken over de volgende drie eigenschappen:

1 Volledige acceptatie door de ontvanger;
2 Optimale voorbeeldsfunctie voor nieuw bot;
3 Voorzien van levende cellen die nieuw bot kunnen vormen.

Wanneer verschillende wetenschappelijke disciplines gaan samenwerken om vervangingsweefsel zoals een alternatief voor het lichaamseigen bot te ontwikkelen, noemen we dit *tissue engineering*. Een veelbelovende vorm van tissue engineering is ontstaan uit de combinatie van vindingen in de jaren zeventig van de vorige eeuw, door twee zeer verschillende disciplines.
Vanuit de technische chemie werd ontdekt dat het mogelijk is calciumfosfaten zo te mengen en te bakken dat een poreus keramiek ontstaat. Dit materiaal bleek volledig door het lichaam te worden geaccepteerd en, beter nog, een uitstekende geleider voor nieuwe botvorming. Eigenlijk is dat ook niet zo verwonderlijk omdat het mineraal waaruit bot voor het grootste deel bestaat, chemisch gezien, van dezelfde compositie is. Vanuit de haematologie (geneeskunde) was ontdekt dat om bloed-stamcellen, die werden gebruikt voor beenmergtransplantaties, buiten het lichaam te laten groeien, deze cellen op een matras van ondersteunende cellen gekweekt moesten worden. Deze cellen bleken in het beenmerg aanwezig en werden stromale cellen (= Latijn voor matrascellen) genoemd. Lang bleven deze cellen alleen verdienstelijk voor het kweken van de haematologische cellen, tot werd ontdekt dat deze stromale cellen zich in allerlei richtingen konden ontwikkelen. De celsoorten die zo ontstaan bevonden zich allemaal binnen een bepaalde embryonale kiemlaag, de zogenaamde mesodermale laag, waaruit het steun en bewegingsapparaat ontstaat, met onder andere bot en kraakbeencellen. Een andere naam voor deze cellen is ook wel mesenchymale stamcellen.
Het onderzoek kwam in een stroomversnelling toen bleek dat de combinatie van poreus keramiek als een dragermateriaal met deze cellen osteogeen was, dit wil zeggen uit zichzelf bot kon maken op plaatsen waar bot normaal niet voorkomt (bijvoorbeeld in de spieren). Sindsdien is er veel onderzoek gedaan naar tissue

engineered bot. Ook in een botomgeving (waar het uiteindelijk zal moeten functioneren) bleek het in staat de genezing te versnellen. Hoewel in het begin snel vooruitgang werd geboekt, met name in kleine proefdieren met relatief kleine implantaten, is de doorbraak naar de kliniek nog niet gekomen. Dit komt met name doordat grotere implantaten (met een veel slechtere overlevingskans) het een stuk minder goed blijken te doen. Zoals zo vaak bij nieuwe ontwikkelingen blijkt ook tissue engineering veel gecompliceerder dan gehoopt.

Het doel van ons onderzoek was verschillende aspecten van bot tissue engineering met betrekking tot de haalbaarheid in de klinisch situatie te onderzoeken:

1 Onderzoeken wat de rol is van levende cellen in lichaamseigen bot;
2 Onderzoeken wat de rol is van levende cellen in tissue engineered bot;
3 Ontwikkelen van een techniek om cellen te markeren en vervolgen;
4 Optimaliseren van de tissue engineering techniek;
5 Onderzoeken van de haalbaarheid van tissue engineering voor klinische toepassingen.

Daarvoor hebben we vooral gebruik gemaakt van een model waarbij we de werking onderzochten van relatief grote implantaten in de spieren van de geit. Het voordeel van dit model (in de spieromgeving) is dat veel verschillende condities tegelijkertijd kunnen worden onderzocht en dat het gevonden bot met zekerheid afkomstig is van het implantaat en niet deels van omringend bot, zoals in een botomgeving.

Bevindingen

Ad. 1: De rol van levende cellen in lichaamseigen bot hebben we onderzocht door bot zowel levend als dood te transplanteren in de spieren van geiten. Immers, als levende cellen een rol spelen, zou een verschil meetbaar moeten zijn. Na 12 weken implantatie was inderdaad een groot verschil zichtbaar. Een deel van de dode transplantaten was na 12 weken totaal verdwenen terwijl de levende transplantaten als stevige massa's van (nieuw) bot werden teruggevonden. In tegenstelling tot het dode bot had het levend getransplanteerde bot veel nieuw bot gevormd.

Ad. 2: De rol van levende cellen in tissue engineered bot hebben we op een vergelijkbare manier onderzocht. Ook hier bleek nieuwe botvorming in de spier alleen mogelijk met levend getransplanteerde cellen.

Ad. 3: Een andere methode om te onderzoeken wat de rol is van de cellen, is deze voordat ze worden getransplanteerd te markeren en later in het histologisch beeld te identificeren. Eerst hebben we een markering onderzocht met een fluorescerende stof. Deze kon de cellen goed markeren maar bleek helaas door niet-gemarkeerde gastheercellen cellen te kunnen worden overgenomen. Hierdoor kon de afkomst van gemarkeerde cellen niet meer met zekerheid worden vastgesteld. Een andere methode die we hebben ontwikkeld was met een zogenaamde retrovirale marker. Dit is een methode die met behulp van een virus een markering aanbrengt in het DNA van de cellen en daardoor zeer specifiek is en blijft. Deze methode bleek succesvol en in een studie naar tissue engineered bot in muizen konden we via een directe weg aantonen dat deze cellen inderdaad het nieuwe bot maakten.

Ad. 4: Voor het optimaliseren van de techniek hebben we verschillende dragermaterialen en procedures onderzocht. Wat betreft het dragermateriaal bleek dat naast de gewone (macro)porositeit ook de microporositeit van groot belang is. De door IsoTis ontwikkelde drager van biphasisch calciumphosphaat (BCP) met een hoge microporositeit bleek uiteindelijk optimaal. Daarnaast bleek het goed mogelijk om tijdens de operatie cellen die bewaard waren in vloeibare stikstof direct te gebruiken. Dit maakt de procedure een stuk eenvoudiger in vergelijking met de procedure die daarvoor werd gebruikt. Daarbij werd de combinatie van cellen en drager eerst nog een week doorgekweekt en werden de cellen door bepaalde stoffen toe te voegen gestimuleerd om botcel te worden. De operatie moest daarom al een week van te voren vast staan. Wanneer de operatie op het laatst toch niet door zou kunnen gaan (wat in een ziekenhuis niet ondenkbaar is) zou alle moeite voor niets zijn geweest.

Ad. 5: Om te onderzoeken of de tissue engineering techniek ook werkte in een klinisch relevant model, hebben we deze onderzocht in een groot botdefect in de geit. Het grote verschil met de andere studies in de spieren was: 1) de grootte van de transplantaten, waardoor overleven van cellen nog moeilijker was en 2) de omgeving. De botomgeving is toch heel anders dan in de spier. Het zou zelfs zo kunnen zijn dat het normale reparatieproces in bot, als reactie op een door ons aangebracht defect, helemaal niet gediend is van de grote hoeveelheid cellen die worden geïmplanteerd. Hoewel er op de korte termijn (9 weken) wel een positief effect van de cellen meetbaar was, bleek dit op de langere termijn (12 weken) niet meer aanwezig. Mogelijk komt dit doordat het reparatiemechanisme van het bot zo sterk is dat het effect van de cellen wordt overschaduwd.

Conclusie

De resultaten zijn tot nu toe zeer bemoedigend. Het levend zijn van zowel lichaamseigen bot als van tissue engineered bot bleek een absolute voorwaarde voor nieuwe botvorming in de spieren van de geit. Dit betekent dat het uitgangspunt van de noodzaak van levende cellen voor tissue engineering kan worden onderbouwd. Het onderzoek naar de gemarkeerde cellen heeft ook laten zien dat die cellen inderdaad in het nieuw gevormde bot aanwezig zijn. De manier waarop het nieuw gevormde bot zich gedraagt is echter nog onduidelijk, en met name of het echt een bijdrage levert in klinisch relevante situaties is nog niet aangetoond. De techniek staat dus nog in de kinderschoenen en van een volwaardig alternatief voor het lichaamseigen bot is nog geen sprake. Toch kunnen we op basis van het onderzoek concluderen dat tissue engineering de potentie heeft een ideaal alternatief voor het lichaamseigen bot te worden.

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De promotoren:

Prof. Dr. A.J. Verbout en Prof. Dr. C.A. van Blitterswijk

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De co-promotoren:

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Curriculum Vitae

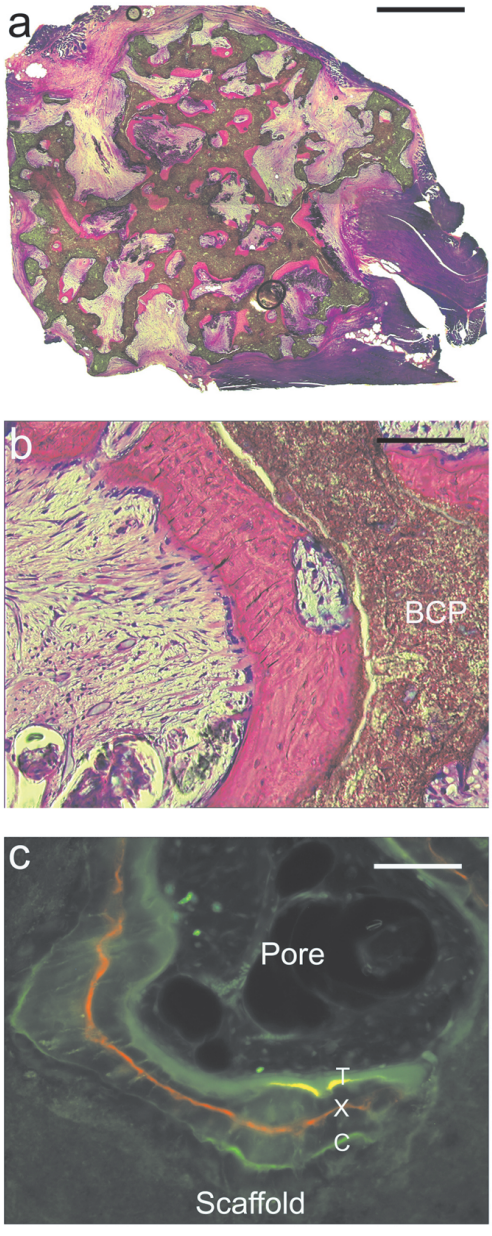
Moyo C Kruyt was born September 2nd 1973 in Nguludi, Malawi (Afrika). In 1992 he graduated from high school (VWO, NSG, Nijmegen) and started one year later to study medicine at the University of Utrecht. In 1998 his scientific research was on "The true smear status of defaulters in TBC treatment in Malawi" at the Queens University Hospital Blantyre, Malawi under supervision of Prof. Dr. Harries and Dr. M.J. Boeree, which resulted in the first authorship of a paper of the same name, published in the WHO bulletin 77(5), 386, 1999. In January 2000, he started as a PhD student on the STW funded project "Tissue engineered bone for spinal fusion", which was a collaboration between the department of orthopaedics at the University of Utrecht (Prof. A.J.Verbout) and IsoTis BV Bilthoven (Prof. C.A. van Blitterswijk). The research involved with this project, was the basis for the articles used in the current thesis and a number of presentations at (inter)national conferences. In September 2000 he obtained the degree of medical doctor at the University of Utrecht and in January 2004 his orthopaedic surgeon residency starts at the Rijnstate Hospital in Arnhem .

Color Figures

COLOR MICROGRAPHS OF RELEVANT FIGURES FROM CHAPTERS 3 THROUGH 10. FIGURE CAPTIONS MAY BE FOUND IN THEIR RESPECTIVE CHAPTERS.

Chapter 3

Figure 2 Micrographs of constructs implanted 9 weeks intramuscularly in goats



CF

Figure 1 Low magnification micrographs of etopic autologous implants in goats

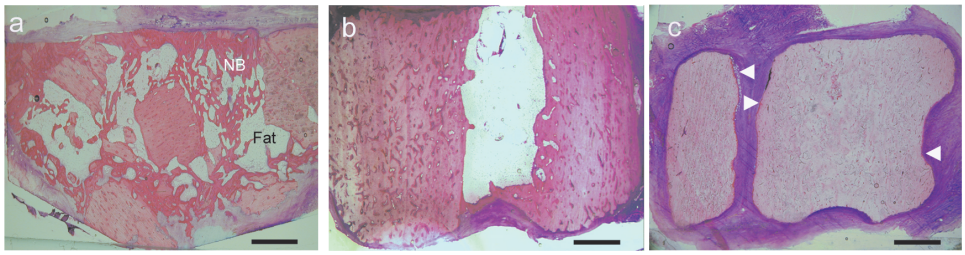
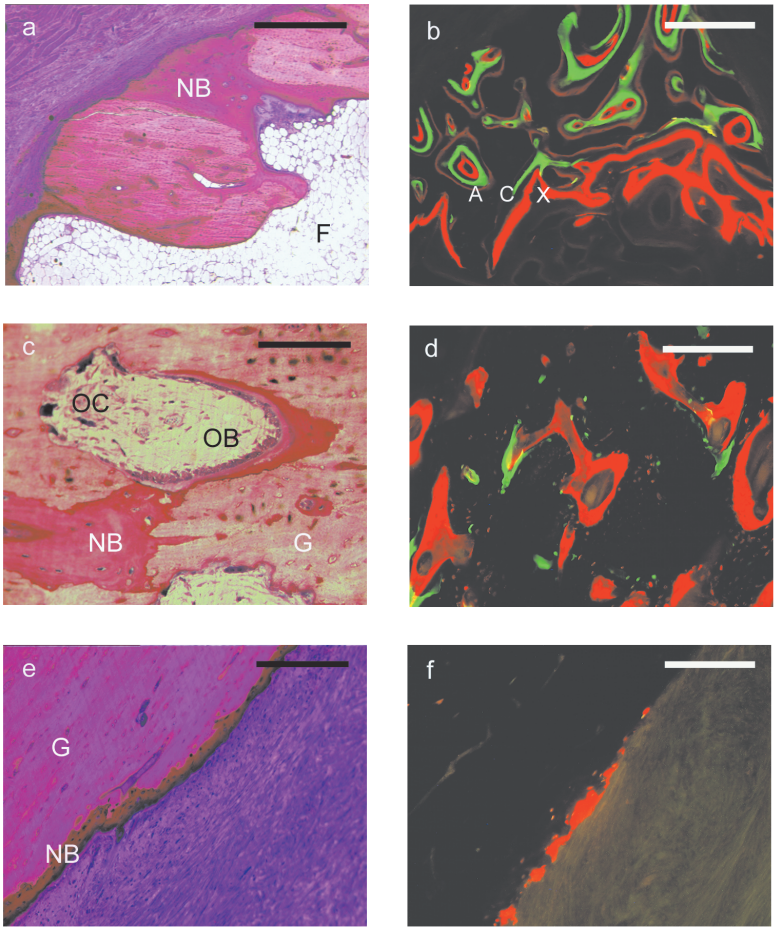


Figure 2 Histology and fluoroscopy of ectopic autologous implants in goats



Chapter 5

Figure 4 Histology of vital tissue engineered constructs implanted for 12 weeks

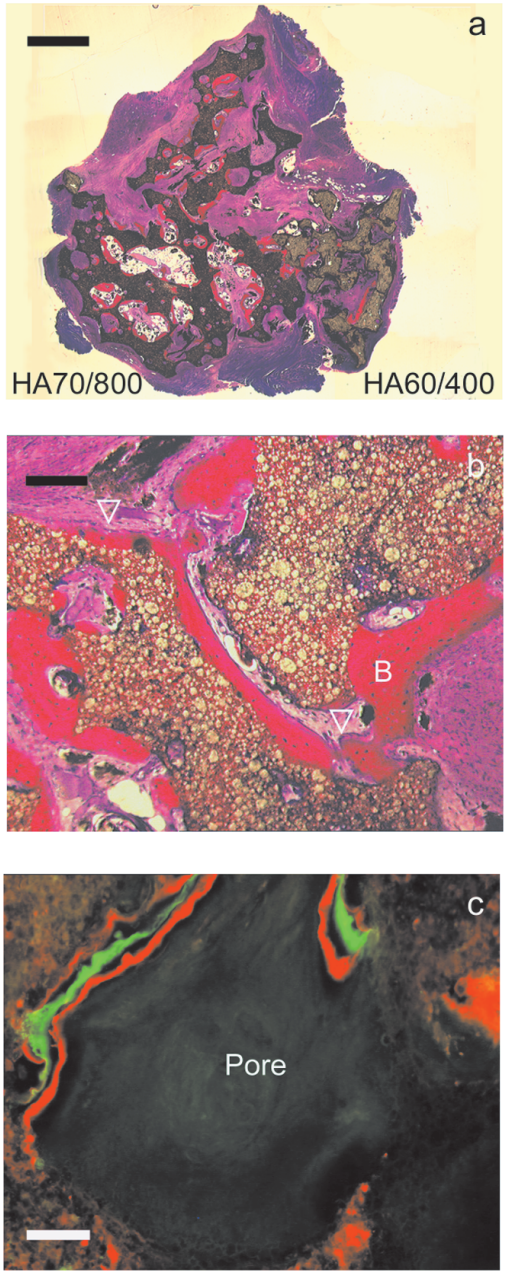


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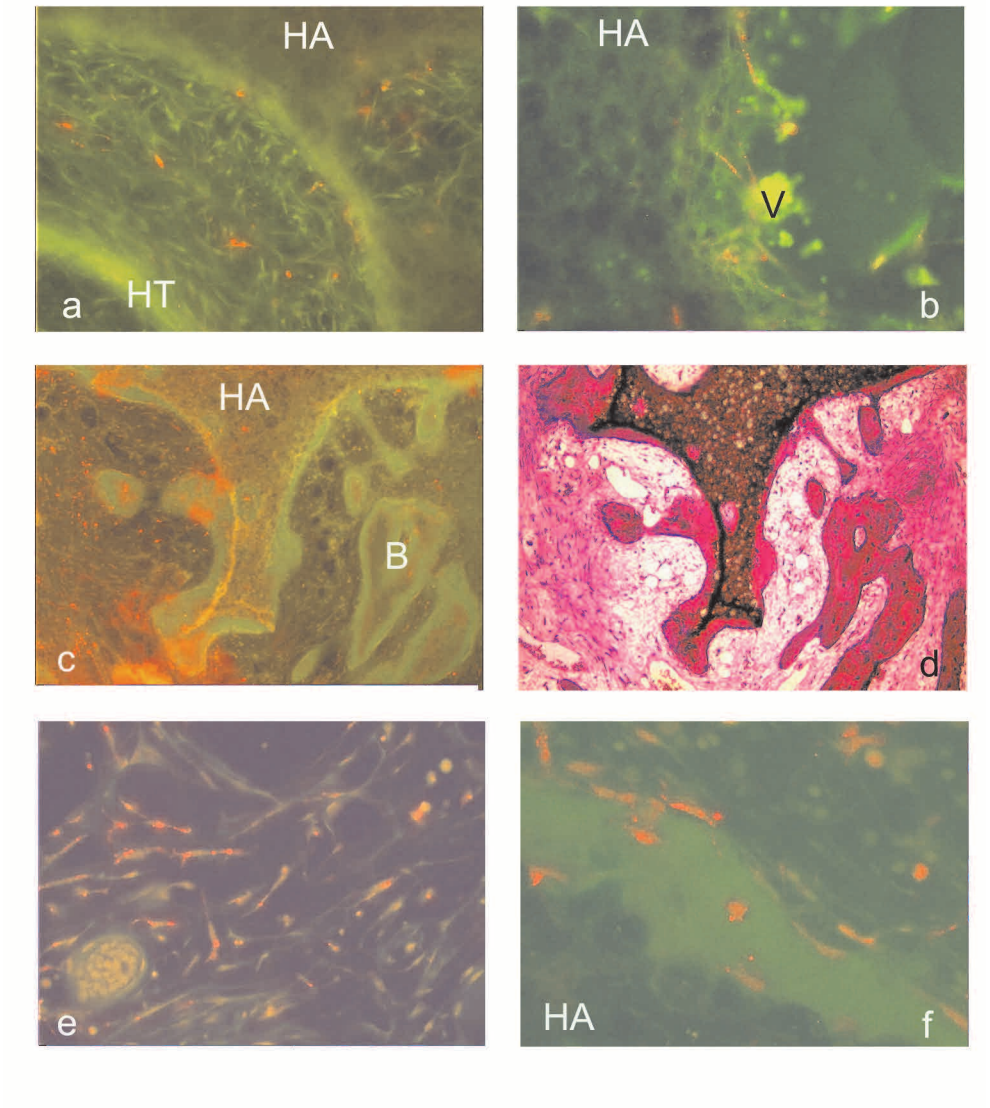
Figure 5 Micrographs of samples with CM-Dil labeled cells implanted in mice



Chapter 7

Figure 4 Samples of *in-vivo* hybrid constructs with NGFR labeled cells

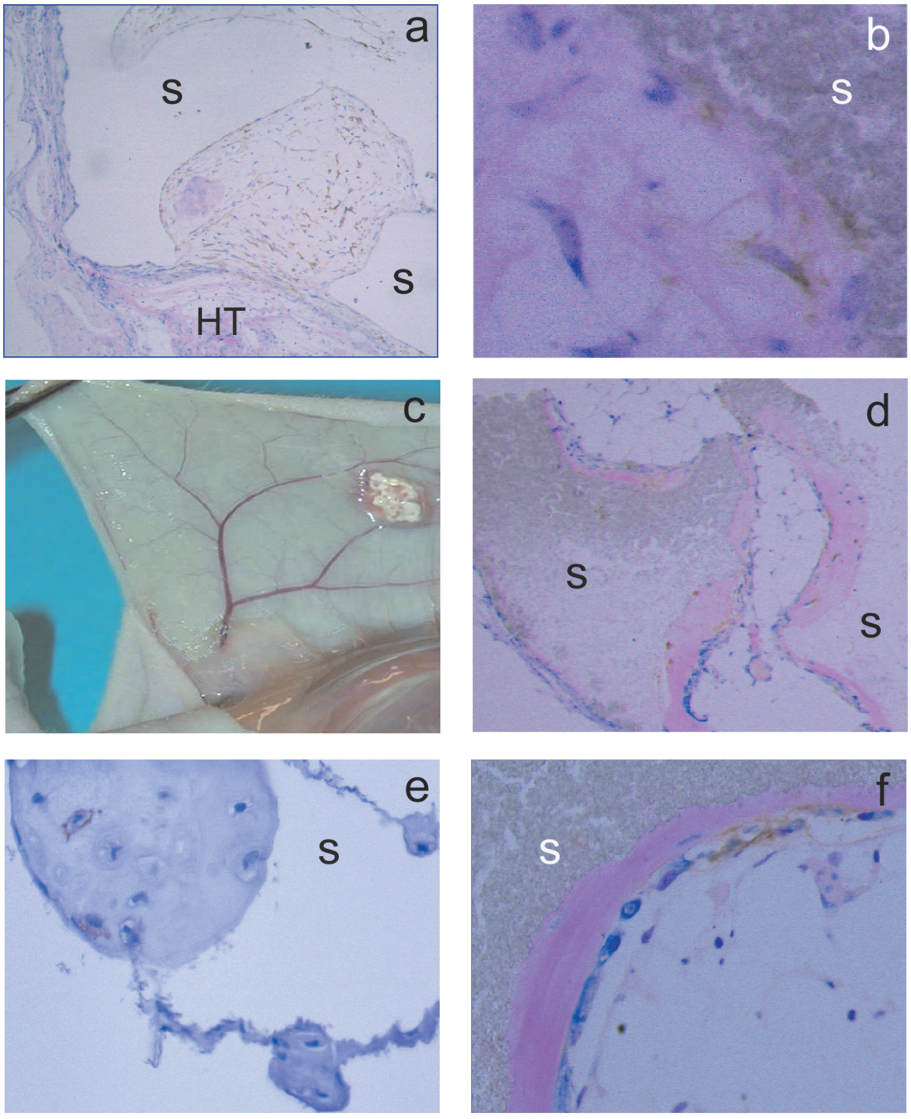
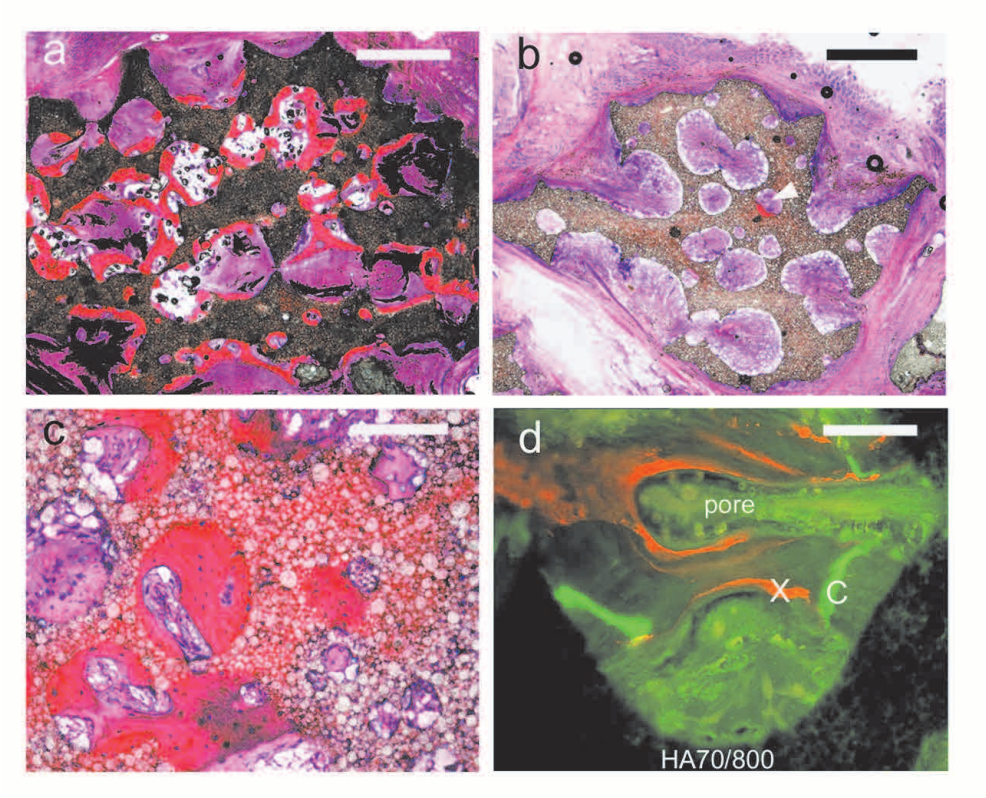


Figure 3 Bone formation after 12 weeks implantation in goats



Chapter 9

Figure 1 Image analysis procedure of ectopic samples

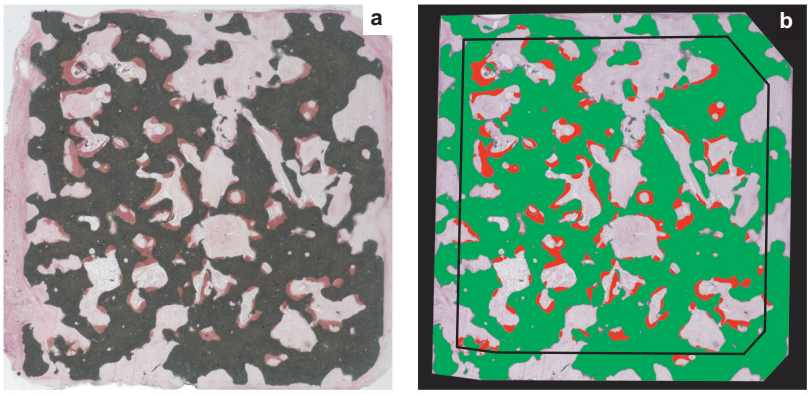


Figure 3 Histology of 9 weeks intramuscularly implanted samples

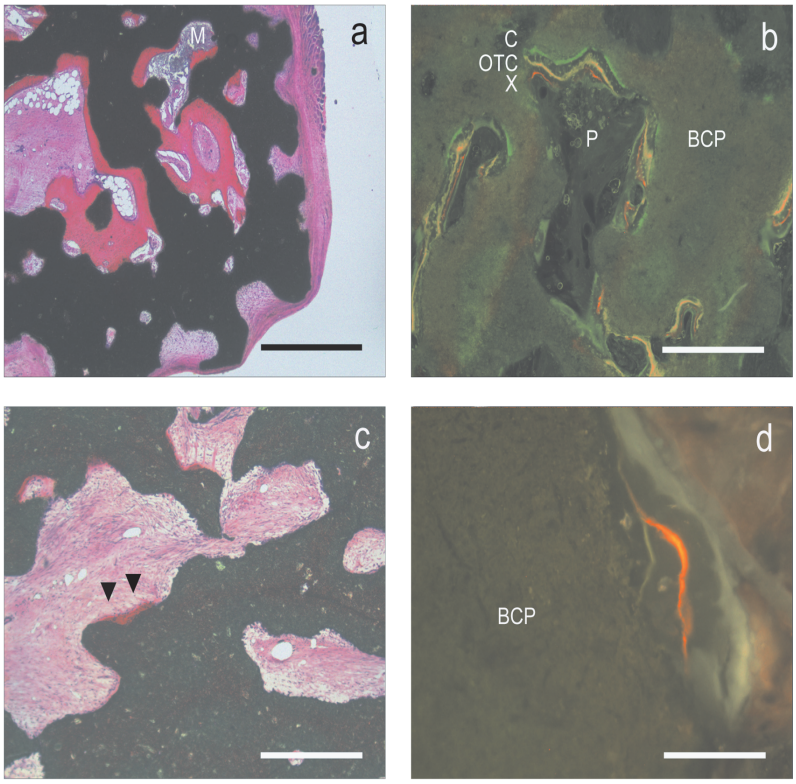


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Figure 3 Histology of tissue engineered samples orthotopically and ectopically