

Critical size bone defect reconstruction by an autologous 3D osteogenic-like tissue derived from differentiated adipose MSCs

Thomas Schubert^{a,b}, Sébastien Lafont^b, Gwen Beaurin^b, Guillaume Grisay^b, Catherine Behets^c, Pierre Gianello^b, Denis Dufrane^{a,b,*}

^a Endocrine Cell Therapy Unit, Center of Tissue and Cell Therapy, Cliniques Universitaires Saint-Luc, Université catholique de Louvain, 1200 Brussels, Belgium

^b Pôle de Chirurgie Expérimentale et Transplantation, Institut de Recherche Expérimentale et Clinique, Secteur des Sciences de la Santé, Université catholique de Louvain, 1200 Brussels, Belgium

^c Pôle de Morphologie Expérimentale, Institut de Recherche Expérimentale et Clinique, Secteur des Sciences de la Santé, Université catholique de Louvain, 1200 Brussels, Belgium

ARTICLE INFO

Article history:

Received 31 January 2013

Accepted 19 February 2013

Available online 16 March 2013

Keywords:

Bone

Critical-size defect

Pre-clinical

Adipose mesenchymal stem cell

Three-dimensional

Pigs

ABSTRACT

For critical size bone defects and bone non-unions, bone tissue engineering using osteoblastic differentiated adipose mesenchymal stem cells (AMSCs) is limited by the need for a biomaterial to support cell transplantation. An osteoblastic three-dimensional autologous graft made of AMSCs (3D AMSC) was developed to solve this issue. This autograft was obtained by supplementing the osteoblastic differentiation medium with demineralized bone matrix. Two surgical models were developed to assess the potential of this 3D osteogenic AMSC autograft. A four-level spinal fusion using polyetheretherketone cages was designed in six pigs to assess the early phase of ossification (8–12 weeks postimplantation). In each pig, four groups were compared: cancellous bone autograft, freeze-dried irradiated cancellous pig bone, 3D AMSC, and an empty cage. A critical size femoral defect ($n = 4$, bone non-union confirmed 6 months postoperatively) was used to assess the 3D AMSCs' ability to achieve bone fusion. Pigs were followed by CT scan and explanted specimens were analyzed for bone tissue remodeling by micro-CT scan, micro-radiography, and histology/histomorphometry. In the spine fusion model, bone formation with the 3D AMSC was demonstrated by a significant increase in bone content. In the critical-size femoral defect model, the 3D AMSC achieved new bone formation and fusion in a poorly vascularized fibrotic environment. This custom-made 3D osteogenic AMSC autograft is a therapeutic solution for bone non-unions and for critical-size defects.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Critical size bone defects following trauma, infection, congenital deficiency, or carcinologic resection remain a major healthcare issue because of the difficulties in reconstructing large bone segments [1–5]. The current gold standard remains bone grafting with autologous bone graft, but available quantities are limited and the harvesting procedure is burdened by comorbidities [6]. The use of massive bone allografts and large reconstruction with prosthetic implants is often combined with autologous cancellous bone grafting [6,7] or the use of osteoinductive materials such as demineralized bone matrix (DBM) [8,9] and bone morphogenetic

proteins [10–12] to overcome the lack of osteoinduction and osteogenic properties of synthetic/human materials.

Tissue engineering and cell therapy using mesenchymal stem cells (MSCs) have suggested the possibility of implanting a living tissue for bone reconstruction. Bone marrow was regarded as the gold standard for MSC isolation [13], but new sources such as adipose tissue were considered recently [14–17]. Adipose MSCs (AMSCs) demonstrated several advantages over those from bone marrow, including a less invasive harvesting procedure, an increased number of stem cell progenitors yielded from an equivalent amount of tissue harvested, increased proliferation and differentiation capacities, and better angiogenic/osteogenic properties [18–21]. Osteogenic differentiated AMSCs were shown to have a great healing potential in various pre-clinical models when seeded on various scaffolds, such as β -tricalcium phosphate, hydroxyapatite, type I collagen, poly lactic-co-glycolic acid, and alginate [22–27].

Despite encouraging results in small animal models, however, critical size bone reconstruction using scaffolds remains limited by

* Corresponding author. Pôle de Chirurgie Expérimentale et Transplantation, Institut de Recherche Expérimentale et Clinique, Secteur des Sciences de la Santé, Université catholique de Louvain, UCL/SSS/IREC/CHEX, Avenue Hippocrate 55, B11.55.04, 1200 Brussels, Belgium. Tel.: +32 2 764 55 75; fax: +32 2 764 55 89.
E-mail address: denis.dufrane@uclouvain.be (D. Dufrane).

the large size of the bone defect and consequently by the size of the implant to engineer. The cellular engraftment of the seeded cells is also limited by the poor diffusion of oxygen and nutrients. In addition, the cellular position within the scaffold is a major limitation for their *in vitro* and *in vivo* survival [28]. Bioreactors with flow perfusion of scaffolds were designed to improve cell migration within the implant for a more homogenous cellular distribution, cell survival by delivering oxygen and nutrients to the core of the implant, and osteogenic cell differentiation (by the fluid shear force) [29–31]. Although these techniques are promising, relevant pre- and clinical data in large animal models are limited [32].

To avoid the requirement of a scaffold for cellular transplantation, our laboratory developed a graft made of AMSCs differentiated in a three-dimensional (3D) osteogenic structure with DBM (patented) [33]. To assess the potential of this 3D osteogenic differentiated AMSC autograft, we developed two pre-clinical large animal models in pigs: (i) a multilevel spine fusion using polyetheretherketone (PEEK) interbody fusion cages; and (ii) a femoral non-union model in which the pseudarthrosis in the diaphysis of a femur was confirmed at six months. Subsequently, the defect was cured by implanting the 3D osteogenic differentiated AMSC autograft. The goal of the spine model was to assess the early phases of ossification and bone neoformation between 8 and 12 weeks postimplantation while the primary endpoint of the femoral non-union model was bone fusion between the two femur segments (up to 6 months postimplantation). Bone formation/fusion was assessed *in vivo* by CT scan and after sacrifice by micro-CT scan, micro-radiography, histology, and histomorphometry.

2. Materials and methods

All materials were obtained from Lonza (Basel, Switzerland), Sigma–Aldrich (St. Louis, MO, USA), or Invitrogen (Carlsbad, CA, USA), unless otherwise noted.

2.1. Source of the animals

Belgian landrace pigs ($n = 6$, <100 kg, <6 months) and MGH-miniature swine ($n = 4$, <50 kg, <6 months) were used as recipients. All procedures were undertaken after the approval of the local ethics committee for animal care of the Université catholique de Louvain (authorization number 2009b/UCL/MD/007). Animals were housed according to the guidelines of the Belgian Ministry of Agriculture and Animal Care.

2.2. The 3D osteogenic differentiated AMSC autograft

Inguinal subcutaneous adipose tissue was harvested under general anesthesia 3 months before each scheduled implantation. AMSCs were isolated by enzymatic digestion, expanded, and differentiated using previously described procedures [21,34,35]. Briefly, adipose tissues were washed in 0.9% NaCl, dissected in a Petri dish (to remove vessels and fibrous connective tissue), and transferred to a 50-ml Falcon tube containing collagenase. Tissues were digested at 37°C for 1 h. The collagenase was inactivated by adding proliferation medium [Dübelco's Modified Eagle Medium supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (2 mM), antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml) and fungicide (amphotericin B 1 µl/ml)]. After centrifugation, the supernatant was discarded, and the pellet was resuspended in proliferation medium and filtered through a 500-µm mesh screen. This filtrate was centrifuged to obtain the stromal vascular fraction, which was resuspended in proliferation medium. This initial passage of the primary cells was referred to as passage 0 (P0). The cultures were maintained in proliferation medium up to P4 before induction of differentiation by supplementing the proliferation medium with dexamethasone (1 µM), sodium ascorbate (50 µg/ml), and sodium dihydrophosphate (36 mg/ml) [21,36,37]. Osteogenic differentiation was confirmed by staining for calcium phosphate with Alizarin red and immunohistochemistry for osteocalcin.

The 3D structure (patented: PCT/EP2010/057847 [33]) was obtained by supplementing the differentiation medium with porcine DBM at 10 mg/ml. DBM was produced following the standard procedure of our University Tissue Bank (Unit of Tissue and Cellular Therapy of the Locomotor Apparatus, University Clinical Hospital St-Luc, Université catholique de Louvain, Brussels, Belgium). Briefly, porcine cortical bone grafts were harvested and chemically treated (see below). The grafts were ground to a powder (200 µm) that was decalcified by a 3-h immersion in 0.6 N HCl prior to freeze-drying. The osteogenic property of the resulting product was confirmed *in vivo*: freeze-dried cortical bone powder (as control) and DBM were

loaded into capsules and implanted in the left and right sides of the paravertebral musculature of nude rats, respectively. Implants were harvested at day 30 post-implantation and processed for histological analysis to confirm the resorption of the bone powder in the left side and the osteoinduction at the DBM implantation site. The supplementation of the differentiation medium with DBM induces the formation of the 3D osteogenic structure within the culture flasks. Differentiation, microstructure, and mineralization of each 3D osteogenic differentiated AMSC autograft were confirmed by Alizarin red staining, histology (hemalun-eosin, Masson's trichrome), and immunohistochemistry for osteocalcin (anti-osteocalcin mouse monoclonal antibody OC4-30, Abcam, Cambridge, MA, USA). The entire process to obtain this 3D osteogenic differentiated bone autograft required an average of 76.3 ± 24.6 days to complete.

The 3D osteogenic AMSC autografts designed to be implanted in the femur model were further assessed by histomorphometry and micro-CT scan using a peripheral quantitative computed tomography (pQCT) instrument (Model XCT Research SA, Stratec, Pforzheim, Germany). A sample of each graft was fixed in formalin, embedded in paraffin, and sectioned (5 µm). Hemalun–eosin and Masson's trichrome staining were performed. Histomorphometrical analysis was performed by point counting under a microscope at 20× magnification with a standard 100 cross-grid representing a surface of 0.16 mm² on the slide. Five regions of interest were analyzed per slide. Quantification of the cellular portion, the neosynthesized extracellular matrix (ECM), and the DBM fragments allowed us to define a ratio as [(DBM – ECM)/cell]. A high ratio means that the DBM content is high and the proportion of ECM and cells is low, thus indicating a poor quality of the graft. In contrast, a low index reflects a higher concentration of cells and ECM content for optimal osteogenesis from AMSCs.

2.3. Lumbar spine fusion model: short-term bone formation analysis

2.3.1. Interbody fusion cages and experimental groups

PEEK interbody fusion cages were used as support for the grafts (Plivios postero-lateral interbody fusion cages, DepuySynthes S.A., Brussels, Belgium).

Six Belgian landrace pigs were used as recipients. For each pig, four experimental groups were created and implanted randomly in the lumbar spine (Table 1): one cage was left empty (Empty group) and the three others were filled with freeze-dried irradiated cancellous pig bone graft (FD group), cancellous bone autograft (Auto group), or an engineered bone graft made of 3D osteogenic differentiated AMSCs (AMSC group). Four animals were sacrificed at 8 weeks postimplantation, and the remaining two at 12 weeks. Fig. 1 shows the experimental timeline followed for this surgical model.

2.3.2. Freeze-dried irradiated cancellous pig bone graft

Porcine bone grafts were harvested on freshly euthanatized pigs and preserved at -80°C . Grafts were processed according to our University Tissue Bank (Unit of Tissue and Cellular Therapy of the Locomotor Apparatus, University Clinical Hospital St-Luc, Université catholique de Louvain Brussels, Belgium) protocol for human bone allograft treatment, as previously described [38]. Briefly, allografts were cut to the required sizes and chemically treated. First, the pieces were defatted in baths of absolute acetone followed by NaOH immersion (for prion inactivation in clinical conditions) at room temperature. Reduction of immunogenicity was obtained by protein coagulation, nuclear acid precipitation, and cell membrane degradation following NaCl and oxygenated water treatment. After each step, allografts were intensively rinsed with a continuous distilled water flow. Allografts were freeze-dried (<5% of residual moisture) and stored at room temperature. Finally, treated grafts were sterilized by γ -irradiation at 25,000 Gy (IBA Mediris, Fleurus, Belgium). This treatment inactivates viruses such as HIV and hepatitis B and C and diminishes bacterial activity in clinical conditions. At the time of implantation, the bone graft was morselized using a rongeur and impacted into the PEEK cage.

2.3.3. Bone autograft

Cancellous bone autograft was harvested perioperatively from an adjacent vertebral body [39,40]. Taking care not to damage the lumbar pedicle, tissues were

Table 1

ID	Pig type	Intervention	Outcome
647	Landrace	Spine fusion	Euthanised at POW 8
500	Landrace	Spine fusion	Euthanised at POW 8
789	Landrace	Spine fusion	Euthanised at POW 12
761	Landrace	Spine fusion	Euthanised at POW 12
770	Landrace	Spine fusion	Euthanised at POW 8
827	Landrace	Spine fusion	Euthanised at POW 8
652	MGH-miniature	Femoral non-union	Bone fusion at POW 26
738	MGH-miniature	Femoral non-union	Bone growth, broken plate at POW 14
740	MGH-miniature	Femoral non-union	Bone fusion at POW 29
741	MGH-miniature	Femoral non-union	Euthanised at POW 29, no bone growth.

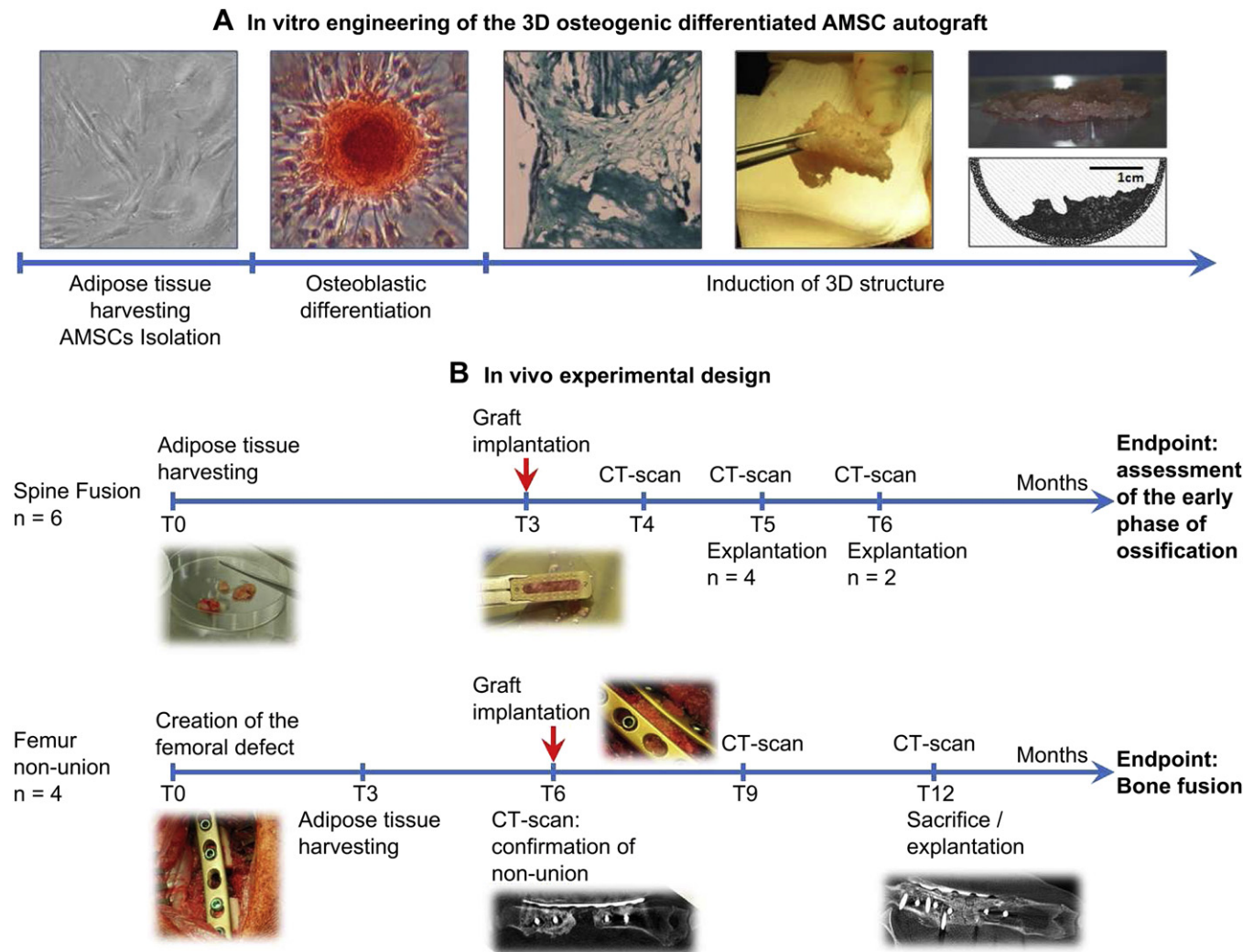


Fig. 1. Description of the experimental procedure. A. Steps involved in the in vitro engineering of the graft to achieve 3D structure. B. Spine fusion and femur non-union in vivo models.

dissected to the periosteum to expose the lateral side of the vertebral body. A 1×1 cm window was opened, and the bone cortex was lifted. A volume of ± 1 cm³ of cancellous bone graft was harvested using a curette. Bone wax (Bonewax, Ethicon, Auneau, France) was inserted in the defect to avoid unnecessary bleeding. The bone graft was then inserted into the PEEK cage and implanted.

2.3.4. Surgical procedure and follow-up

A lateral approach by a left lumbotomy was used to perform a four-level anterior lumbar interbody fusion procedure. Surgery was performed under strict aseptic conditions in an operating theater (Experimental Surgery Laboratory, Université catholique de Louvain). Pigs were anesthetized by intramuscular injection of a mix of tiletamine and zolazepam (Zoletil, Virbac, Glattbrugg, Switzerland) at 15 mg/kg and xylazine (Rompun, Bayer, Diegem, Belgium) at 2 mg/kg. General anesthesia was maintained by isoflurane inhalation (Forène, Abbott, Diegem, Belgium). Disc levels of interest (L2–L3, L3–L4, L4–L5, L5–L6) were identified by fluoroscopy. A left lumbotomy was performed and a trans-Psoas approach was used to expose the intervertebral discs. At each level, the annulus fibrosus was incised to expose the nucleus pulposus, which was removed. Adjacent cartilage endplates were reamed and the PEEK cages implanted.

Cefazolin (Cefazoline Sandoz 1 g, Sandoz, Vilvoorde, Belgium) was used for prophylactic antibiotherapy at a dose of 20 mg/kg. Local analgesia was achieved by subcutaneous infiltration of 20 ml of levobupivacaine at 2.5 mg/ml (Chirocaine, Abbott, Diegem, Belgium). General analgesics (Buprenorphine, Temgesic, Reckitt Benckiser Healthcare Ltd., Hull, UK) were administered routinely. Animals were housed separately with access to food and water ad libitum. Wound, pain, and general conditions were monitored daily. Postoperative radiological assessment of the implants was obtained by CT scan 2–3 days postoperatively. Pigs were then scanned every 4 weeks until sacrifice. Four pigs were euthanized at 8 weeks, the remaining two at 12 weeks postoperatively (Table 1). The spine specimens were

then explanted and processed for micro-CT scan, micro-radiography, non- and decalcified histology, and immunohistochemistry (see below).

2.3.5. Implant assessment

2.3.5.1. CT scan. Each animal was followed-up by CT scan (Siemens Somatom Definition AS, Brussels, Belgium) every 4 weeks. The content of each cage was analyzed sagittally by multiplanar reconstruction for which slice thickness was determined at 2 mm and the analyzed region of interest was a mean of ± 70 mm². Results are expressed in Hounsfield units (HU).

2.3.5.2. Micro-CT scan. Before histological processing, the microstructure and total bone density of the tissues within the PEEK cages were analyzed using a pQCT instrument (XCT Research SA, Stratec, Pforzheim, Germany). Results are expressed in mg/cm³.

2.3.5.3. Histology. For non-decalcified histology, explanted implants were fixed in methanol, embedded in methyl methacrylate (MMA) [41], and sectioned (200 μ m) using a diamond band saw (D-22581, EXAKT, Norderstedt, Germany). Slides were ground and polished up to 80 μ m between two sheets of ground glass to be microradiographed, stained with 1% methylene blue (85662, Merck, Hohenbrunn, Germany) and 1% Fuchsin (B-2340, Sigma–Aldrich), and finally mounted on glass slides for histomorphometry. For decalcified histology, explants were fixed in 4% formaldehyde overnight, decalcified, embedded in paraffin, and sectioned (5 μ m). Hemalun–eosin and Masson's trichrome staining were performed.

2.3.5.4. Microradiography. Microradiographs of each non-decalcified slide were produced for qualitative analysis purposes. Non-decalcified slides were placed on fine-grained radiological emulsifications (VRP-M, Slavich Geola, Vilnius, Lithuania) and exposed to X-rays (Machlett X-ray tube, Baltograph, Balteau, Liège, Belgium) for

1 h. X-ray films were revealed, rinsed, and fixed. The microradiographs were scanned before being mounted on histological slides.

2.3.5.5. Histomorphometry. Histomorphometrical analysis was performed on non-decalcified slides. Five slides of each four experimental groups in two pigs sacrificed at 8 weeks (nos. 770 and 827) and two pigs sacrificed at 12 weeks (nos. 761 and 789) were microphotographed at $2.5\times$ magnification (Nikon Camera Digital Sight DS5MC, powered by DS U2 unit and controlled by Nikon Element BR Ver. 3.00 SP5 software; Nikon, Brussels, Belgium). ImageJ 1.43i software (ImageJ, Image Processing and Analysis in JAVA, public domain, National Institutes of Health, Bethesda, MD, USA) was used to quantify the proportion of the area occupied by bone, chondroid tissue, fibrocartilage, necrosis, and fibrosis on each slide. The scale was established by matching computed pixels with a graticule picture. Results are expressed as a percentage of the area of the PEEK cage section.

2.4. Femoral non-union model: long term bone remodeling assessment

The objective of the femoral non-union model was to assess the 3D osteogenic AMSC autograft in the stringent conditions of a fibrotic poorly vascularized implantation site. MGH-miniature swine were used instead of Belgian landrace pigs because of their limited size/weight growth following implantation.

2.4.1. Surgical procedures

Four pigs underwent surgery to induce a femoral non-union. In each pig, a lateral paramuscular approach was used to create a critical bone defect of 1.5 times the diameter of the femoral shaft in the mid-diaphysis of one femur. The periosteum was removed from the diaphyseal shaft. The bone defect was stabilized by two 4.5-mm titanium locking compression plates positioned on the anterior and lateral sides of the femur. Postoperative care and follow-up were achieved as described in Section 2.3.4. Non-union was confirmed by the absence of bone healing on CT scan at 6 months postoperatively [2]. Three months after the non-union induction, adipose tissue was harvested; AMSCs were isolated, expanded up to P4, and differentiated into the 3D osteogenic AMSC bone autograft as described in Section 2.2.

After radiological non-union confirmation at >6 months, each animal was implanted with a 3D osteogenic AMSC autograft. Through the same surgical approach, one locking compression plate was temporarily removed to allow access to the non-union site. Fibrotic tissues were removed and the bone stumps were heightened. The 3D osteogenic AMSC autograft was then implanted, and the locking compression plate was replaced. Wound closure, prophylactic antibiotherapy, analgesia, and postoperative care were achieved as described in Section 2.3.4.

Follow-up by CT scan was performed at 3 and 6 months postimplantation of the 3D osteogenic differentiated AMSC autograft. At 6 months postimplantation, animals were sacrificed and the femurs were explanted. Fig. 1 shows the experimental timeline followed for this surgical model.

2.4.2. Implant assessment

Explants were micro-CT scanned, fixed, and embedded in MMA. They were subsequently cut at 200 μm , ground and polished for micro-radiography and histology (see Section 2.3.5.3). The endpoint was the cure of the non-union, defined as a bone bridging of the critical size bone defect on at least one cortical of the femoral shaft.

2.5. Statistics

All results are expressed as mean $\pm$ SD except for micro-CT scan measurements (mean $\pm$ SEM). The one-sample Kolmogorov–Smirnov test and Q–Q plots were used to assess the normality of values. Statistical differences between groups with a normal distribution were tested by Student's *t*-test or one-way analysis of variance with the Bonferroni post hoc test. When the distribution of variables was normal but without equality of variance, the Tamhane T2 post hoc test was used instead of the Bonferroni test. The Mann–Whitney and Kruskal–Wallis nonparametric tests were performed for non-normal distributions. Statistics were performed with PASW 18 (SPSS, IBM, New York, NY, USA). A *P* value <0.05 was considered as significant.

3. Results

3.1. Assessment of the 3D osteogenic AMSC autograft

All grafts demonstrated a 3D structure prior to implantation (Fig. 1). Non-differentiated cells were expanded up to P4 during a mean of 18.9 ± 4.4 days. AMSCs were osteogenically differentiated after 26.0 ± 6.4 days of culture. DBM was then added to the culture media after 36.6 ± 8.6 days, and the full 3D osteogenic differentiated AMSC autograft was obtained after a mean of 76.3 ± 24.6 days of culture. Immunohistochemistry for osteocalcin demonstrated osteoblastic differentiation and matrix deposition for each implant.

For grafts implanted in the critical size defects, a correlation was observed between the index defined by the equation $[(\text{DBM} - \text{ECM})/\text{cell}]$ and the fusion rate (Fig. 4A). A low index was associated with bone fusion (pigs 652 and 740). In contrast, a high index was correlated with a lack of in vivo bone fusion (pig 741).

3.2. Spine fusion model

3.2.1. CT scan analysis

The empty cages demonstrated a significant increase of density between 0, 8, and 12 weeks (86.0 ± 17.9 vs. 183.2 ± 69.1 vs. 276.5 ± 88.4 HU at 0 vs. 8 vs. 12 weeks, respectively, $P < 0.05$). In contrast, a significant decrease in radiological density was observed in the FD group (377.8 ± 67.9 vs. 154.5 ± 78.5 HU at 0 vs. 12 weeks, respectively, $P < 0.05$). A significant increase in radiological density was observed between 0 and 12 weeks postimplantation in the PEEK cages loaded with the 3D osteogenic AMSC autograft (90.2 ± 19.6 vs. 163.0 ± 38.2 HU at 0 vs. 12 weeks, respectively, $P < 0.05$, Fig. 2A).

Radiological density between different experimental groups at postoperative week 0 (just after implantation) demonstrated a significantly higher density in the FD (377.8 ± 67.9 HU) and Auto (284.5 ± 63.3 HU) groups in comparison with the empty cages (86.0 ± 17.9 HU) and the AMSC group (90.2 ± 19.6 HU; $P < 0.005$). In contrast, at 8 weeks postimplantation the FD group (155.0 ± 70.5 HU) demonstrated a decrease in radiological density and did not differ significantly from the empty cages (183.2 ± 69.1 HU) and the AMSC experimental group (112.0 ± 15.9 HU). The cages filled with autograft (292.5 ± 62.5 HU) demonstrated a significantly higher ($P < 0.005$) radiological density than the FD and the AMSC groups but not the Empty group. At 12 weeks postimplantation, no significant differences were found between groups.

3.2.2. Micro-CT scan density analysis

The empty cages demonstrated a significant increase in bone density between 8 and 12 weeks (202.9 ± 7.4 mg/cm³ vs. 262.1 ± 8.8 mg/cm³ at 8 vs. 12 weeks, respectively, $P < 0.001$). In contrast, a strong decrease in mineral content was observed in the FD group during the same interval (194.3 ± 7.5 mg/cm³ vs. 141.1 ± 7.6 mg/cm³ at 8 vs. 12 weeks, respectively, $P < 0.001$). In the Auto group, a decrease in bone density was observed (280.7 ± 7.9 mg/cm³ vs. 248.8 ± 10.4 mg/cm³ at 8 vs. 12 weeks, respectively, $P < 0.05$). The comparison of bone density between 8 and 12 weeks in the AMSC group demonstrated a significant increase of mineral content (149.5 ± 2.9 mg/cm³ vs. 169.8 ± 5.4 mg/cm³ at 8 vs. 12 weeks, respectively, $P < 0.005$, Fig. 2B). At 8 weeks, a significantly higher mineral density was found in the Auto group (280.7 ± 7.9 mg/cm³) as compared to all other experimental conditions ($P < 0.001$). The FD (194.3 ± 7.5 mg/cm³) and Empty (202.9 ± 7.4 mg/cm³) groups were not significantly different, but they had a significantly higher mineral content than the AMSC group (149.5 ± 2.9 mg/cm³, $P < 0.001$).

At 12 weeks postimplantation, the Empty (262.1 ± 8.8 mg/cm³) and Auto (248.8 ± 10.4 mg/cm³) groups were not statistically different but demonstrated a significantly higher mineral density than the AMSC (169.8 ± 5.4 mg/cm³, $P < 0.001$) and FD (141.1 ± 7.6 mg/cm³, $P < 0.001$) groups. A significantly denser mineral content was found for the AMSC experimental condition when compared to the FD group ($P < 0.05$).

3.2.3. Microradiographical and histomorphological analysis of the PEEK cages

Microradiographs demonstrated bone growth in the cages left empty (Fig. 2C). In contrast, massive bone resorption was confirmed in the FD group. In the Auto group, the presence of bone was

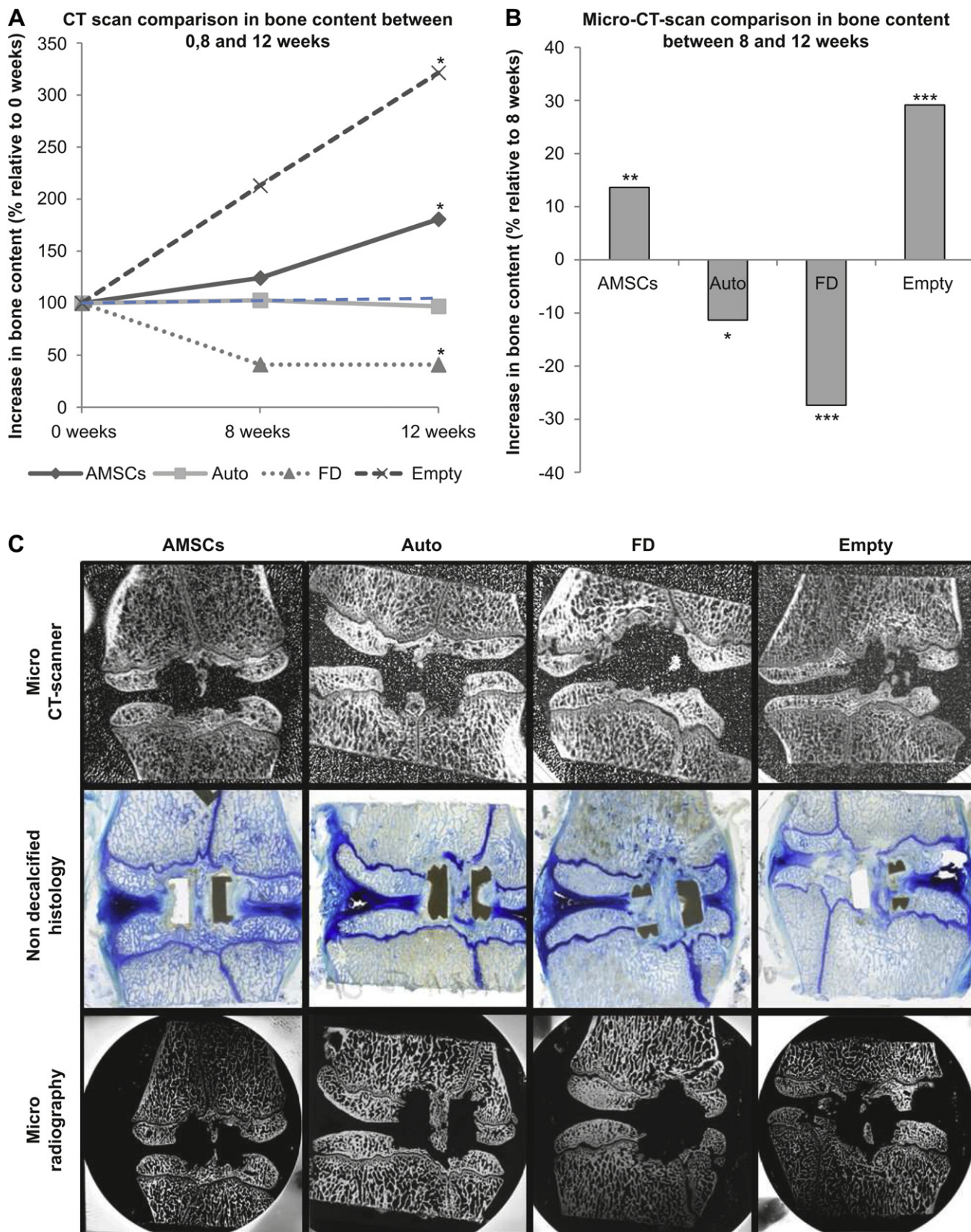


Fig. 2. Results of the radiological findings of the spine fusion model ($n = 6$ pigs, with four sacrificed at 8 weeks postimplantation and the remaining two at 12 weeks). A. CT scan results demonstrating the trend in mineral content relative to the immediate postoperative density (0 weeks), $*P < 0.05$. B. Micro-CT scan findings showing the modification of the bone content between 8 and 12 weeks, $*P < 0.05$, $**P < 0.005$, $***P < 0.001$. C. Pictures representative of the micro-CT scan, non-decalcified histology, and microradiographic observations between the four experimental conditions.

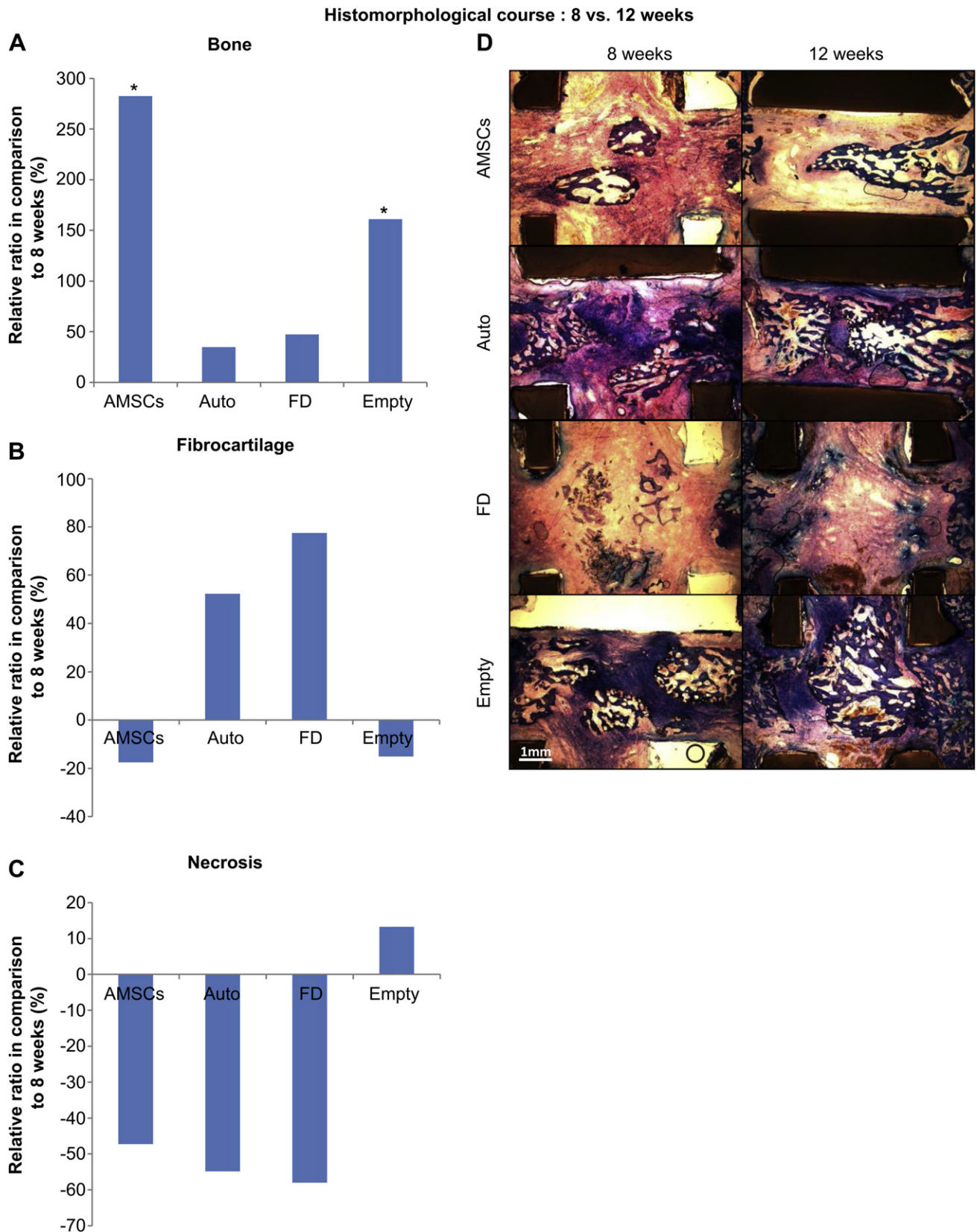


Fig. 3. Comparison of histomorphological findings between 8 and 12 weeks. Slides were assessed at 8 weeks ($n = 5$ for each experimental condition: AMSC, Auto, FD, Empty) and at 12 weeks ($n = 5$ for each experimental condition) by surface histomorphometry. A, B, and C. Results expressed as a ratio relative to 8 weeks for bone, fibrocartilage, and necrosis content, respectively, $*P < 0.05$. D. Representative microphotographs of each experimental group.

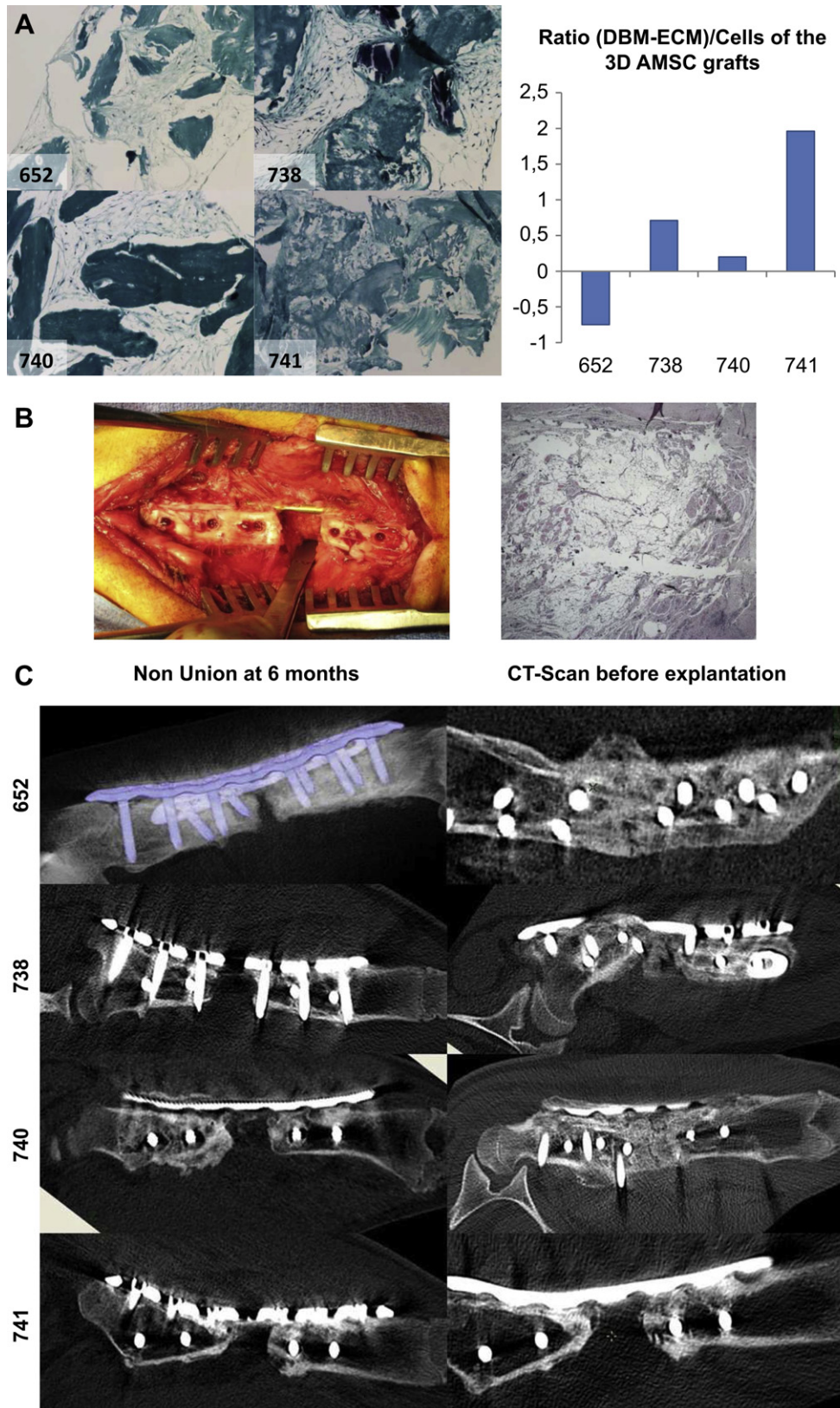


Fig. 4. Results of the femoral non-union model. A. Microphotographs of each engineered graft prior to implantation. Quantification of the demineralized bone matrix (DBM), extracellular matrix (ECM), and cell content is represented as a ratio: (DBM – ECM)/cell. A high ratio indicates poor quality and thus a poor osteogenic capacity. B. Demonstration of the gap with a poorly vascularized fibrotic content left between the bone stumps after 6 months of non-union. C. CT scan findings at 6 months of non-union and after implantation of the 3D osteogenic AMSC autograft.

confirmed at 8 and 12 weeks. In the AMSC group, bone neoformation was confirmed (Fig. 2C).

Surface histomorphometry demonstrated a significant increase in bone formation in the Empty group between 8 and 12 weeks ($8.3 \pm 5.2\%$ vs. $21.7 \pm 16.5\%$ of the total analyzed area in the PEEK cage at 8 vs. 12 weeks, respectively, $P < 0.05$; Fig. 3A). In the FD and Auto groups, no significant increase in bone content was observed. Bone neoformation quantified by surface histomorphometry in the AMSC group improved significantly between 8 and 12 weeks ($2.2 \pm 1.5\%$ vs. $8.5 \pm 5.5\%$ of the total analyzed area in the PEEK cage at 8 vs. 12 weeks, respectively, $P < 0.05$), as demonstrated by the ratio of bone quantification between 12 and 8 weeks (Fig. 3A, D). Histomorphometrical quantification demonstrated a decrease in fibrocartilage content between 8 and 12 weeks in the Empty group. In contrast, an increase of fibrocartilage tissue was observed in the FD and Auto groups. A decrease in fibrocartilaginous content was observed in the AMSC group. However, none of these results were statistically significant. Trends are depicted in Fig. 3B by the ratio in fibrocartilage quantification between 12 and 8 weeks.

The presence of necrotic tissue slightly increased in the Empty group and decreased in the FD, Auto, and AMSC groups as demonstrated by the ratio of necrotic tissue content between 12 and 8 weeks in Fig. 3C. None of these results were statistically significant.

3.3. Descriptive histology of the spine specimen

Bone neoformation was found in the Empty group. Histological analysis of the explanted specimen showed a succession of newly formed tissues with mainly fibrocartilage at the periphery of chondroid tissue around growing bone. The new bone appeared mainly as woven bone with fewer remodeling areas than in the AMSC group, especially at 8 weeks. Fibrotic tissues were very cellular. Necrotic tissues seemed to be fragments of bone that had fallen into the PEEK cage during implantation.

In contrast, in the FD group, mainly bone fragments and fibrotic tissues were observed. Fibrotic tissue was poorly cellularized. Little cartilage tissue was present at 8 weeks, and it started to appear at 12 weeks postimplantation. Bone fragments were weakly stained and pale and in the process of resorption. Necrotic tissues were mainly found at 8 weeks.

In the Auto group, bone fragments showed fewer remodeling processes when compared to the Empty and AMSC groups. Mineralized bone areas with osseous growth activity were contrasted with resorption zones with fewer new bone deposits. Fibrous tissue also looked less cellularized. Cartilage and chondroid tissue were observed.

Finally, in the AMSC group, structured tissue formation was observed in a succession of fibrotic well-cellularized tissue, fibrocartilage, chondroid tissue, and bone. Bone tissue was mainly woven bone with a lot of remodeling processes, particularly at 8 weeks. Lamellar bone with pre-osseous deposits was also observed in lower proportions (Fig. 3D).

3.4. Femoral non-union model

In all four pigs, a non-union of the femur diaphysis was confirmed by CT scan at >6 months by a macroscopic disruption between the bone segments (Fig. 4B, C). The pseudarthrosis was further demonstrated by the interposition of fibrotic tissue during re-intervention (Fig. 4B). In pigs 652 and 740, complete bone fusion was observed at 26 and 29 weeks postimplantation, respectively. Bone fusion was confirmed by CT scan (Fig. 4C), macroscopically at the explantation, by micro-CT scan, micro-radiography, and non-decalcified histology (Figs. 5 and 6). Pig 738 demonstrated bone

growth at the site of implantation but loss of stabilization due to plate breakage that occurred 14 weeks after graft implantation.

In pig 741, no bone neoformation occurred, as demonstrated by CT scan, micro-CT scan, and micro-radiography. A poorly vascularized fibrotic tissue was observed at the site of implantation.

Histological analysis of the harvested femur specimens of pigs 652, 738, and 740 demonstrated new bone formation in the initial 2-cm bone defect. In pig 738, a bone nodule was found at the site of non-union without any connection to the diaphyseal stumps (Fig. 5). When bone fusion was achieved, bone tissue found at the implantation site demonstrated remodeling with Haversian features (Fig. 6, pictures A and B) and denser bone formation at the periphery of the diaphysis (Fig. 6, picture C). Chondroid tissue was observed as well as unstructured cartilage, with evidence supporting the endochondral ossification pathway.

4. Discussion

Bone tissue reconstruction using MSCs has been contemplated for a long time. Studies have shown that adipose tissue is a reliable and efficient source for MSC isolation and osteogenic differentiation [21,42,43]. However, large bone defects remain problematic due to the necessity of a scaffold for cell delivery. In this regard, many advantages can be found in the 3D AMSC bone autograft. First, cell expansion can easily be matched to the size of the defect, and this technique allows large-scale production. AMSCs are fully differentiated prior to transplantation into osteoblasts by the addition of DBM, which in this case does not serve as a carrier alone. Moreover, the graft has intrinsic osteoinduction properties due to the differentiated AMSCs, as showed by Supronowicz et al., who demonstrated the increased bone induction potential of AMSCs cultured with DBM when compared to DBM alone [44]. The unique aspect of this concept is the induction of the 3D construct by the addition of DBM, helping the cells to synthesize ECM and start the mineralization process prior to in vivo implantation. Finally, the engineered product is easily handled during the surgical procedure, behaving as a sort of bone putty that can be molded into the bone defect.

To demonstrate the potential of this 3D osteogenic differentiated AMSC autograft, relevant pre-clinical data in large animal models are mandatory. Large animal experimental trials in orthopedic surgery were mostly developed in ovine and canine models. Pigs were less considered mainly because of handling difficulties. Previous studies have shown, however, that dogs and pigs are the best animal models for orthopedic studies because their bone mineral density, anatomy, morphology, and healing physiology are similar to those of humans [45–47]. The pig was already used for spine fusion models, and the differences between the human and porcine vertebrae have been characterized [48–51]. In models of large bone defects, the pig was previously used and demonstrated a strong advantage over sheep and goat in terms of bone structure and remodeling [45,52]. Therefore, we developed a spine fusion and a critical size bone defect model in pigs to assess the potential of our 3D AMSC autograft. Our spine fusion model focused on the early phase of bone neoformation and graft resorption between 0 and 12 weeks. In contrast, the femur model was designed as a critical size large defect model; we first proved the irreversibility of the defect and then tested the graft after 6 months of bone non-union.

In the spine fusion model, the empty cages showed the capacity of a blood clot to induce bone fusion between two vertebral bodies. When compared with other experimental groups, filling an interbody cage with any type of material has a lower fusion potential than the callus formed by blood components after implantation. Necrotic bone tissues observed in this experimental group were

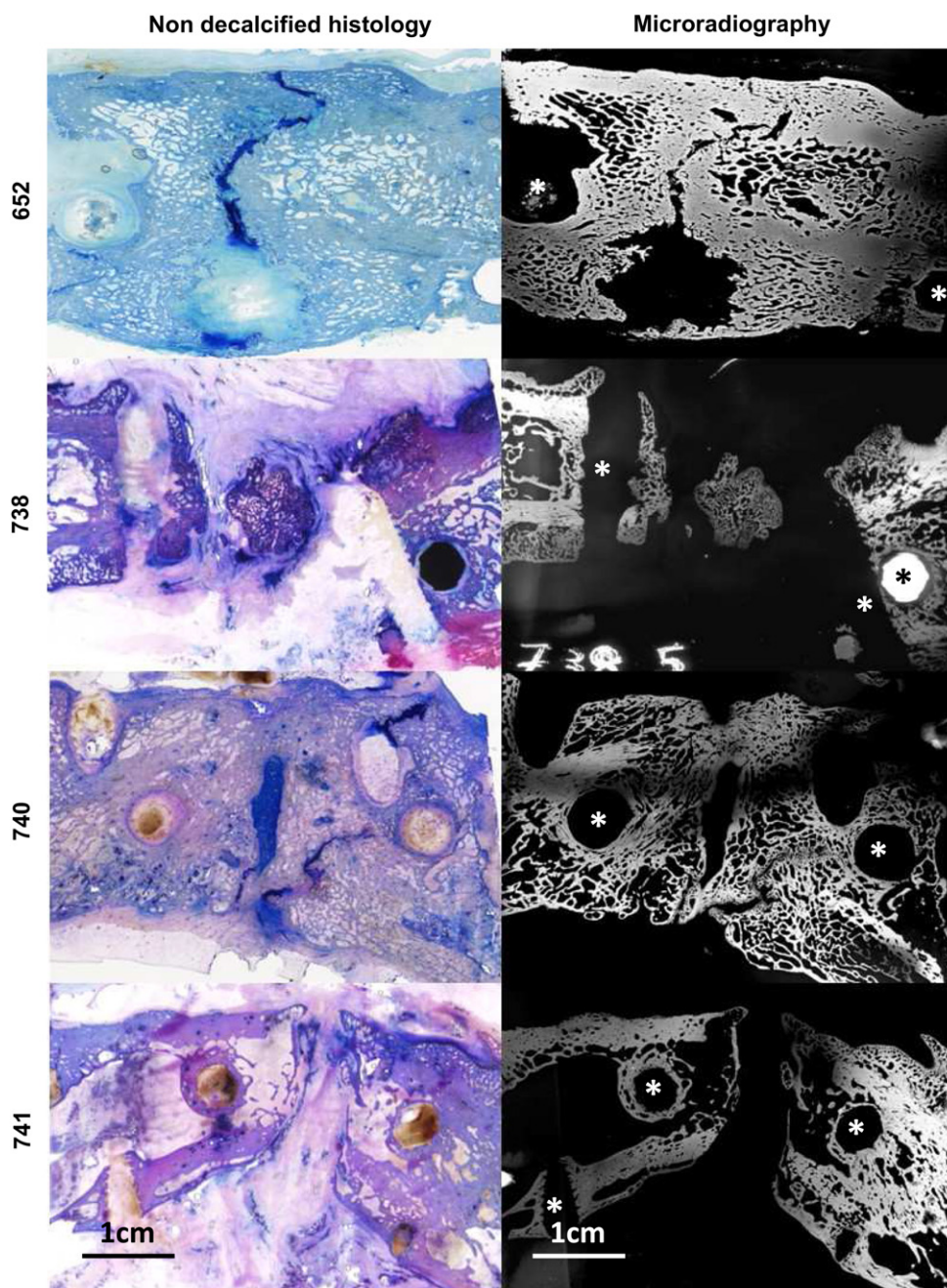


Fig. 5. Representative microphotographs and microradiographs of the explanted femur specimen of the four pigs implanted with the 3D osteogenic AMSC autograft (* = position of the screws).

probably bone fragments that fell into the interbody fusion cage during the implantation procedure. In contrast, none of the critical size defects left empty in the femur model had the ability to restore the excised bone tissue, demonstrating the limits of the physiological healing capacity of a postoperative blood clot.

In the spine fusion as well as in the femoral defect model, the auto-transplantation of 3D AMSCs proved feasible and easy to manage. Histology of the spine specimen showed a bone formation process similar to the Empty group with bone, fibrocartilage, and chondroid tissue. When compared to the FD and Auto groups, the healing process was closer to the bone healing physiology. In the femur model, the implantation demonstrated the potential of differentiated AMSCs to induce bone fusion in the poorly

vascularized fibrotic tissues found after 6 months of non-union. This demonstrated the osteogenic potential of osteoblastic differentiated AMSCs. These results are in accord with previous experimental studies that showed the ability of osteoblastic differentiated AMSCs to induce bone formation *in vivo* [42], in contrast with the poor bone consolidation capacity of non-differentiated AMSCs [53].

A major advantage of the 3D AMSC bone autograft is that it does not require a scaffold. In this aspect, a correlation was found between the number of living cells and ECM present in the graft prior to implantation and the fusion success. This finding revealed the importance of the cellular component of the construct and demonstrated the inability of DBM alone to induce new bone formation. However, in this particular application, DBM is used during

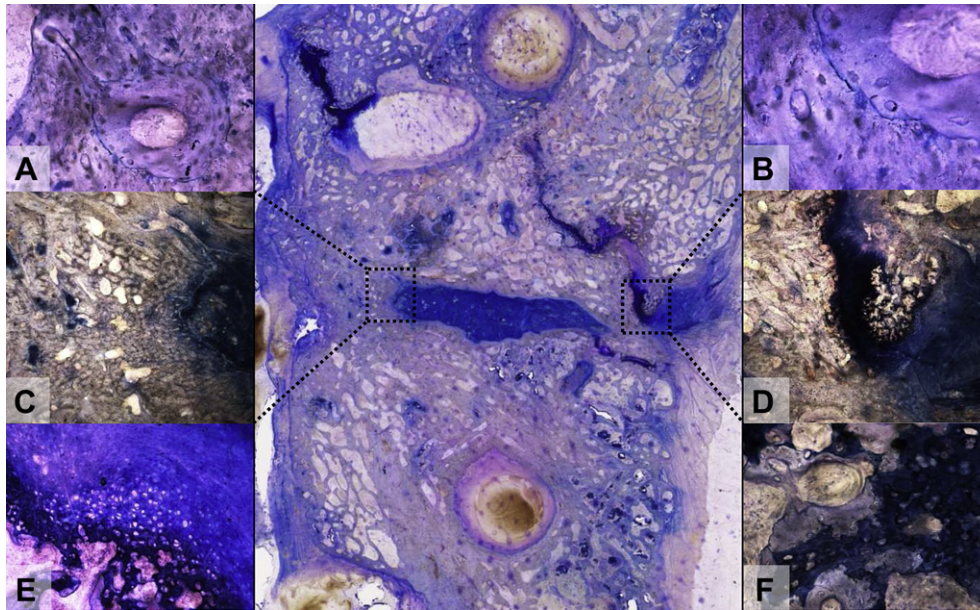


Fig. 6. Histological findings in the femoral non-union model after implantation of the 3D osteogenic AMSC autograft in pig 740. A and B. An osteon structure (at 20 $\times$ and 40 $\times$, respectively). C. Bone fusion zone (1.25 $\times$). D and E. Chondroid tissue and fibrocartilage (at 1.25 $\times$ and 10 $\times$, respectively). F. Bone remodeling with new bone deposits (20 $\times$).

culture to potentiate osteogenic differentiation and 3D formation. The osteoinduction capacity of the DBM was proved in an *in vivo* intramuscular nude rat model by the presence of woven bone, bone marrow, and mineralized tissue. Thus, bone induction factors such as bone morphogenetic proteins are probably exhausted during the long culture process, which remains the major drawback of this technique. Indeed, the time required for the full 3D differentiation amounts to 2.5 months, therefore prohibiting any use in emergency situations and requiring careful planning of surgical procedures.

In our spine model, remodeling and slight resorption were observed in the autograft experimental group. This behavior is compatible with the physiological healing of bone autograft. The main problem observed with autologous bone graft is that the bone is devascularized. Previous research demonstrated that the majority of osteocytes do not survive the harvesting and implantation procedure [54]. Although autologous bone remains the gold standard for bone grafting, the procedure is associated with harvesting-related comorbidities and is available in limited quantities [6,7]. In contrast, the 3D AMSC bone graft is expanded from a small amount of adipose tissue: harvesting-related morbidities are reduced to a minimum, and the expansion capacity means that quantity is not an issue.

In spine fusion surgery, freeze-dried irradiated bone allograft is often used for its ease of handling, despite its poor mechanical [55,56] and biological properties [38,57]. In our experimental model, this group demonstrated a massive resorption of the mineral content as assessed by CT scan, micro-CT scan, and micro-radiography. Small discrepancies between radiological and histomorphometrical findings can be explained by the partially resorbed residual bone allograft being stained on histological slides but sufficiently demineralized to not appear upon radiological examination. Such a massive resorption is compatible with previous experimental and clinical observations on the use of freeze-dried irradiated bone allograft [58,59]. In this experimental setup, the FD experimental condition was restricted to cancellous bone. Freeze-dried irradiated cortical and cortico-cancellous bone grafts have been used successfully in many surgical applications, such as spine fusion [60]. However, the chemical and physical treatment

applied to this type of grafts precludes their use for large bone defect reconstructions [59] in contrast with autologous bone and the 3D AMSC autograft.

5. Conclusions

This 3D osteogenic construct made of osteoblastic differentiated AMSCs was easily and reproducibly generated. It proved to be easily handled in operative conditions. In a model of spine fusion in pigs, the 3D AMSC graft demonstrated a potential to form new bone. The femoral non-union model showed the potential of our 3D engineered graft to induce bone fusion in the unfavorable fibrotic environment of a bone non-union. However, graft assessment of the cellular component prior to implantation is mandatory.

Acknowledgements

This work was supported by grants of the Salus Sanguinis and Saint-Luc Foundations. The authors thank DepuySynthes for their support and the Department of Radiology of the University Clinical Hospital of Saint-Luc, Brussels, for authorizing the CT scan access.

References

- [1] Tzioupis C, Giannoudis PV. Prevalence of long-bone non-unions. *Injury* 2007;38(Suppl. 2):S3–9.
- [2] Megas P, Panagiotis M. Classification of non-union. *Injury* 2005;36(Suppl. 4):S30–7.
- [3] Enneking WF, Campanacci DA. Retrieved human allografts: a clinicopathological study. *J Bone Jt Surg Am* 2001;83-A:971–A:986.
- [4] Enneking WF, Mindell ER. Observations on massive retrieved human allografts. *J Bone Jt Surg Am* 1991;73:1123–42.
- [5] Delloye C, Cornu O. Incorporation of massive bone allografts: can we achieve better performance? *Acta Orthop Belg* 2003;69:104–11.
- [6] Sen MK, Miclau T. Autologous iliac crest bone graft: should it still be the gold standard for treating nonunions? *Injury* 2007;38(Suppl. 1):S75–80.
- [7] Goulet JA, Senunas LE, DeSilva GL, Greenfield ML. Autogenous iliac crest bone graft: complications and functional assessment. *Clin Orthop Relat Res* 1997;339:76–81.
- [8] Wientroub S, Reddi AH. Influence of irradiation on the osteoinductive potential of demineralized bone matrix. *Calcif Tissue Int* 1988;42:255–60.
- [9] Urist MR. Bone: formation by autoinduction. *Science* 1965;150:893–9.

- [10] Dimitriou R, Giannoudis PV. Discovery and development of BMPs. *Injury* 2005;36(Suppl. 3):S28–33.
- [11] Urist M, Strates B. The classics: bone morphogenetic protein. *Clin Orthopaedics Relat Res* 2009;462:3051–62.
- [12] Donati D, Di Bella C, Lucarelli E, Dozza B, Frisoni T, Aldini NN, et al. OP-1 application in bone allograft integration: preliminary results in sheep experimental surgery. *Injury* 2008;39(Suppl. 2):S65–72.
- [13] Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV. Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol* 1966;16:381–90.
- [14] Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 2001;7:211–28.
- [15] Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, et al. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 2002;13:4279–95.
- [16] Zuk PA. The adipose-derived stem cell: looking back and looking ahead. *Mol Biol Cell* 2010;21:1783–7.
- [17] Gimble JM, Katz AJ, Bunnell BA. Adipose-derived stem cells for regenerative medicine. *Circ Res* 2007;100:1249–60.
- [18] Kubbier JW, Weyand B, Radtke C, Vogt PM, Kasper C, Reimers K. Isolation, characterization, differentiation, and application of adipose-derived stem cells. *Adv Biochem Eng Biotechnol* 2010;123:55–105.
- [19] Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE, et al. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation* 2004;109:1292–8.
- [20] Fraser JK, Wulur I, Alfonso Z, Hedrick MH. Fat tissue: an underappreciated source of stem cells for biotechnology. *Trends Biotechnol* 2006;24:150–4.
- [21] Schubert T, Xhema D, Vèriter S, Schubert M, Behets C, Delloye C, et al. The enhanced performance of bone allografts using osteogenic-differentiated adipose-derived mesenchymal stem cells. *Biomaterials* 2011;32:8880–91.
- [22] De Girolamo L, Arrigoni E, Stanco D, Lopa S, Di Giancamillo A, Addis A, et al. Role of autologous rabbit adipose-derived stem cells in the early phases of the repairing process of critical bone defects. *J Orthop Res* 2011;29:100–8.
- [23] Li H, Dai K, Tang T, Zhang X, Yan M, Lou J. Bone regeneration by implantation of adipose-derived stromal cells expressing BMP-2. *Biochem Biophys Res Commun* 2007;356:836–42.
- [24] Levi B, James AW, Nelson ER, Vistnes D, Wu B, Lee M, et al. Human adipose derived stromal cells heal critical size mouse calvarial defects. *PLoS One* 2010;5:e11177.
- [25] Peterson B, Zhang J, Iglesias R, Kabo M, Hedrick M, Benhaim P, et al. Healing of critically sized femoral defects, using genetically modified mesenchymal stem cells from human adipose tissue. *Tissue Eng* 2005;11:120–9.
- [26] Park D-J, Choi B-H, Zhu S-J, Huh J-Y, Kim B-Y, Lee S-H. Injectable bone using chitosan-alginate gel/mesenchymal stem cells/BMP-2 composites. *J Craniomaxillofac Surg* 2005;33:50–4.
- [27] Arrigoni E, Lopa S, De Girolamo L, Stanco D, Brini A. Isolation, characterization and osteogenic differentiation of adipose-derived stem cells: from small to large animal models. *Cell Tissue Res* 2009;338:401–11.
- [28] Yeatts AB, Geibel EM, Fears FF, Fisher JP. Human mesenchymal stem cell position within scaffolds influences cell fate during dynamic culture. *Bio-technol Bioeng* 2012;109:2381–91.
- [29] Gomes ME, Sikavitsas VI, Behravesh E, Reis RL, Mikos AG. Effect of flow perfusion on the osteogenic differentiation of bone marrow stromal cells cultured on starch-based three-dimensional scaffolds. *J Biomed Mater Res A* 2003;67:87–95.
- [30] Janssen FW, Van Dijkhuizen-Radersma R, Van Oorschot A, Oostra J, De Bruijn JD, Van Blitterswijk CA. Human tissue-engineered bone produced in clinically relevant amounts using a semi-automated perfusion bioreactor system: a preliminary study. *J Tissue Eng Regen Med* 2010;4:12–24.
- [31] Wang L, Hu Y-Y, Wang Z, Li X, Li D-C, Lu B-H, et al. Flow perfusion culture of human fetal bone cells in large beta-tricalcium phosphate scaffold with controlled architecture. *J Biomed Mater Res A* 2009;91:102–13.
- [32] Sørensen JR, Koroma KE, Ding M, Wendt D, Jespersen S, Juhl MV, et al. Effects of a perfusion bioreactor activated novel bone substitute in spine fusion in sheep. *Eur Spine J* 2012;21:1740–7.
- [33] Dufrane D, Delloye C. Multi-dimensional biomaterial and method for producing the same. Patent PCT/EP2010/057847; 2010.
- [34] Mitchell JB, McIntosh K, Zvonick S, Garrett S, Floyd ZE, Kloster A, et al. Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. *Stem Cells* 2006;24:376–85.
- [35] Cui L, Liu B, Liu G, Zhang W, Cen L, Sun J, et al. Repair of cranial bone defects with adipose-derived stem cells and coral scaffold in a canine model. *Biomaterials* 2007;28:5477–86.
- [36] Post S, Abdallah BM, Bentzon JF, Kassem M. Demonstration of the presence of independent pre-osteoblastic and pre-adipocytic cell populations in bone marrow-derived mesenchymal stem cells. *Bone* 2008;43:32–9.
- [37] Qu C, Zhang G, Zhang L, Yang G. Osteogenic and adipogenic potential of porcine adipose mesenchymal stem cells. *In Vitro Cell Dev Biol Anim* 2007;43:95–100.
- [38] Fawzi-Grancher S, Goebbels RM, Bigare E, Cornu O, Gianello P, Delloye C, et al. Human tissue allograft processing: impact on in vitro and in vivo biocompatibility. *J Mater Sci Mater Med* 2009;20:1709–20.
- [39] Arlet V, Jiang L, Steffen T, Ouellet J, Reindl R, Aebi M. Harvesting local cylinder autograft from adjacent vertebral body for anterior lumbar interbody fusion: surgical technique, operative feasibility and preliminary clinical results. *Eur Spine J* 2006;15:1352–9.
- [40] Steffen T, Downer P, Steiner B, Hehli M, Aebi M. Minimally invasive bone harvesting tools. *Eur Spine J* 2000;9(Suppl. 1):S114–8.
- [41] Erben RG. Embedding of bone samples in methylmethacrylate: an improved method suitable for bone histomorphometry, histochemistry, and immunohistochemistry. *J Histochem Cytochem* 1997;45:307–14.
- [42] Lee JA, Parrett BM, Conejero JA, Laser J, Chen J, Kogon AJ, et al. Biological alchemy: engineering bone and fat from fat-derived stem cells. *Ann Plast Surg* 2003;50:610–7.
- [43] Zhu Y, Liu T, Song K, Fan X, Ma X, Cui Z. Adipose-derived stem cell: a better stem cell than BMSC. *Cell Biochem Funct* 2008;26:664–75.
- [44] Supronowicz P, Gill E, Trujillo A, Thula T, Zhukauskas R, Ramos T, et al. Human adipose-derived side population stem cells cultured on demineralized bone matrix for bone tissue engineering. *Tissue Eng Part A* 2011;17:789–98.
- [45] Reichert JC, Saifzadeh S, Wullschlegler ME, Epari DR, Schütz MA, Duda GN, et al. The challenge of establishing preclinical models for segmental bone defect research. *Biomaterials* 2009;30:2149–63.
- [46] Pearce AI, Richards RG, Milz S, Schneider E, Pearce SG. Animal models for implant biomaterial research in bone: a review. *Eur Cell Mater* 2007;13:1–10.
- [47] Aerssens J, Boonen S, Lowet G, Dequeker J. Interspecies differences in bone composition, density, and quality: potential implications for in vivo bone research. *Endocrinology* 1998;139:663–70.
- [48] Foldager C, Bendtsen M, Zou X, Zou L, Olsen AK, Munk OL, et al. ISSLS prize winner: positron emission tomography and magnetic resonance imaging for monitoring interbody fusion with equine bone protein extract, recombinant human bone morphogenetic protein-2, and autograft. *Spine* 2008;33:2683–90.
- [49] Foldager C, Bendtsen M, Nygaard JV, Zou X, Büniger C. Differences in early osteogenesis and bone micro-architecture in anterior lumbar interbody fusion with rhBMP-2, equine bone protein extract, and autograft. *Bone* 2009;45:267–73.
- [50] Zou X, Zou L, Foldager C, Bendtsen M, Feng W, Büniger CE. Different mechanisms of spinal fusion using equine bone protein extract, rhBMP-2 and autograft during the process of anterior lumbar interbody fusion. *Biomaterials* 2009;30:991–1004.
- [51] Dath R, Ebinesan AD, Porter KM, Miles AW. Anatomical measurements of porcine lumbar vertebrae. *Clin Biomech* 2007;22:607–13.
- [52] Raschke M, Kolbeck S, Bail H, Schmidmaier G, Flyvbjerg A, Lindner T, et al. Homologous growth hormone accelerates healing of segmental bone defects. *Bone* 2001;29:368–73.
- [53] Niemeyer P, Fechner K, Milz S, Richter W, Suedkamp NP, Mehlhorn AT, et al. Comparison of mesenchymal stem cells from bone marrow and adipose tissue for bone regeneration in a critical size defect of the sheep tibia and the influence of platelet-rich plasma. *Biomaterials* 2010;31:3572–9.
- [54] Zerbo IR, De Lange GL, Joldersma M, Bronckers ALJJ, Burger EH. Fate of monocortical bone blocks grafted in the human maxilla: a histological and histomorphometric study. *Clin Oral Implants Res* 2003;14:759–66.
- [55] Cornu O, Banse X, Docquier PL, Luyckx S, Delloye C. Effect of freeze-drying and gamma irradiation on the mechanical properties of human cancellous bone. *J Orthop Res* 2000;18:426–31.
- [56] Cornu O, Schubert T, Libouton X, Manil O, Godts B, Van Tomme J, et al. Particle size influence in an impaction bone grafting model: comparison of fresh-frozen and freeze-dried allografts. *J Biomech* 2009;42:2238–42.
- [57] Delloye C, Cornu O, Druez V, Barbier O. Bone allografts: what they can offer and what they cannot. *J Bone Jt Surg Br* 2007;89-B:574–80.
- [58] Dziedzic-Gocławska A, Ostrowski K, Stachowicz W, Michalik J, Grzesik W. Effect of radiation sterilization on the osteoinductive properties and the rate of remodeling of bone implants preserved by lyophilization and deep-freezing. *Clin Orthop Relat Res* 1991;272:30–7.
- [59] Nather A, Thambyah A, Goh JCH. Biomechanical strength of deep-frozen versus lyophilized large cortical allografts. *Clin Biomech* 2004;19:526–33.
- [60] Munting E, Faundez A, Manche E. Vertebral reconstruction with cortical allograft: long-term evaluation. *Eur Spine J* 2001;10(Suppl. 2):S153–7.