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Scaffold-free Three-dimensional Graft From Autologous Adipose-derived Stem Cells for Large Bone Defect Reconstruction

Clinical Proof of Concept

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Abstract: Long bone nonunion in the context of congenital pseudarthrosis or carcinologic resection (with intercalary bone allograft implantation) is one of the most challenging pathologies in pediatric orthopedics. Autologous cancellous bone remains the gold standard in this context of long bone nonunion reconstruction, but with several clinical limitations. We then assessed the feasibility and safety of human autologous scaffold-free osteogenic 3-dimensional (3D) graft (derived from autologous adipose-derived stem cells [ASCs]) to cure a bone nonunion in extreme clinical and pathophysiological conditions.

Human ASCs (obtained from subcutaneous adipose tissue of 6 patients and expanded up to passage 4) were incubated in osteogenic media and supplemented with demineralized bone matrix to obtain the scaffold-free 3D osteogenic structure as confirmed in vitro by histomorphometry for osteogenesis and mineralization. The 3D ''bone-like'' structure was finally transplanted for 3 patients with bone tumor and 3 patients with bone pseudarthrosis (2 congenital, 1 acquired) to assess the clinical feasibility, safety, and efficacy. Although minor clones with structural aberrations (aneuploidies, such as tri or tetraploidies or clonal trisomy 7 in 6%–20% of cells) were detected in the undifferentiated ASCs at passage 4, the osteogenic differentiation significantly reduced these clonal anomalies. The final osteogenic product was stable, did not rupture with forceps manipulation, did not induce donor site morbidity, and was easily implanted directly into the bone defect. No acute (<3 mo) side effects, such as impaired wound healing, pain, inflammatory reaction, and infection, or long-term side effects, such as tumor development, were associated with the graft up to 4 years after transplantation.

We report for the first time that autologous ASC can be fully differentiated into a 3D osteogenic-like implant without any scaffold. We demonstrated that this engineered tissue can safely promote osteogenesis in extreme conditions of bone nonunions with minor donor site morbidity and no oncological side effects.

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Abbreviations: 3D = three-dimensional, ASCs = adipose-derived stem cells, ATMP = Advanced Therapy Medicinal Products, DBM = demineralized bone matrix, ECM = extracellular matrix, FACS = fluorescence-activated cell sorting, FISH = fluorescence in situ hybridization, GMP = good manufacturing practice, MSCs = mesenchymal stem cells, P4 = passage 4.

INTRODUCTION

Long bone nonunion in the context of congenital pseudarthrosis (1 in 140,000–250,000 births) or carcinologic resection (1% of all cancers, and an estimated incidence of 6/million per y, requiring intercalary allograft reconstruction) is one of the most challenging pathologies in pediatric orthopedics. Pathophysiological conditions and neo-adjuvant chemotherapy cause nonhealing bone in 15% to 55% of patients after allograft or prosthesis reconstruction.[1–6] The current gold standard for bone nonunion remains autologous cancellous bone graft from iliac crest (in most cases and in a small bone defect) containing bone marrow mesenchymal stem cells (MSCs), but available quantities are limited and the harvesting procedure is burdened by comorbidities.[7,8] The use of osteoinductive materials such as demineralized bone matrix (DBM) and bone morphogenetic proteins (BMPs) to overcome the lack of osteoinduction and osteogenic properties of synthetic or human materials remains relatively prohibitive in the pediatric context. The principle of caution is applied for derived bone growth factors because they have been implicated in the tumor process, and specific studies with long-term follow-up for safety are lacking.[6,9–15] Tissue engineering and cell therapy using MSCs have raised the possibility of implanting living tissue for bone reconstruction. Adipose-derived stem cells (ASCs) demonstrate several advantages over those from bone marrow (considered the gold standard), including a less invasive harvesting procedure, a higher number of stem cell progenitors from an equivalent amount of tissue harvested, increased proliferation and differentiation capacities, and better angiogenic and osteogenic properties in vivo.[16–24]

Critical size bone reconstruction using stem cells also remains limited by the large size of bone defects and consequently the size of the engineered implant requiring a scaffold. Tissue engineering can potentially provide treatment alternatives for conventional large bone defects. The application of different

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combinations of osteoconductive biomaterials, osteoprogenitor cells, and growth factors directly into the defect holds great potential for achieving bone healing in stringent and difficult conditions. Biomaterials should ideally possess properties such as mechanical strength, biodegradability, support. and stem cell differentiation with regard to mimicking bone-forming components to eliciting specific cellular responses and providing an ideal environment for bone formation. To date, no synthetic or biological scaffolds fulfil all these criteria since they can be influenced by the surrounding microenvironments or cause immunological problems.[25,26]

Several scaffold-free systems have been investigated, but creating sufficient thickness to fill a critical size bone defect is difficult.[27] We developed a graft made of scaffold-free autologous ASCs differentiated into a 3-dimensional (3D) osteogenic structure with DBM.[28] We previously demonstrated the safety and efficacy of this graft to cure a femoral critical size bone defect in a pig preclinical nonunion model at 6 months postimplantation.[28] Complete stem cell differentiation in an osteogenic 3D structure significantly improved the efficacy of bone reconstitution (by promoting angiogenesis and osteogenesis) and the safety through a lower risk of growth factor release.[29] After osteogenic differentiation, human and pig ASCs demonstrated similar in vitro (vascular endothelial growth factor release and viability in hypoxic conditions) and in vivo (angiogenicity and osteogenicity with cellular engraftment and graft mineralization, respectively) properties.[29,30]

Subsequent to the preclinical experiments, we then assessed the feasibility (ie, the reproducibility of manufacturing of 3D graft clinical batch) and safety (ie, the risk of MSCs within the tumor environment and pediatric context) of human autologous 3D osteogenic grafts to cure bone nonunion in extreme clinical and pathophysiological conditions. We also investigated the bone consolidation at a minimum of 1 year after reconstruction.

METHODS

This study was performed according to the Belgian Ministry of Health (AFMPS) guidelines for hospital exemption (and obtained the authorization by the national central authorities as the clinical number studies: ATMP-HE004) as per the Article 28 of European Regulation 1394/2007 on Advanced Therapy Medicinal Products. To qualify for this so-called hospital exemption (HE), the advanced therapy concerned should meet the following criteria: preparation on a nonroutine basis, preparation according to specific quality standards, use in a hospital, use under the exclusive responsibility of a medical practitioner, and comply with an individual medical prescription for a custom-made product for an individual patient As such, the legislator intends to provide patients the possibility to benefit from a custom-made, innovative individual treatment in the absence of valid therapeutic alternatives. All procedures for tissue procurement and clinical studies (for children patients) were approved by the Ethical Committee of the Medical Faculty (Universite Catholique de Louvain) as the national authorization number: B40320108542. All patients (parents of the children) signed the consent to participate to the study after verbal and written information by the principal investigator of the study. All consents were included and archived in the Case Report Form for each patient (and for a duration of 30 y).

All materials were obtained from Lonza (Verviers, Belgium), Sigma-Aldrich (St Louis, MO), or Invitrogen (Carlsbad, CA) unless otherwise noted.

Scaffold-free 3D Graft From Human ASCs and DBM Combination

Study Design and Patients

Between 2010 and 2012, 6 young patients were included in the study (Table 1). Three male patients with bone tumors (2 osteosarcomas for patients #1 and #2 and 1 Ewing sarcoma for patient #3 characterized by several clonal cytogenetic alterations). Preoperative chemotherapy was applied for a mean duration of 7 and 10 months before tumor resection. The anatomical reconstitution was performed with a metallic (Phenix) prosthesis and a human osteochondral allograft, respectively.

A 3D stem cell autograft was also proposed for 3 patients with bone nonunions due to congenital pseudarthrosis (n = 2 for patients #4 and 5) or acquired pseudarthrosis (in the context of erythroblastopenia, n = 1 for patient #6) that was untreatable with classical treatments such as surgery (eg, curettage, elongation, Fassier–Duval telescopic system, intramedullary fixation, and Ilizarov fixation), iliac crest autograft, and DBM alone. The autologous adipose stem cell transplant was

TABLE 1. Mesenchymal Stem Cell Harvesting and Processing for Clinical Application

Patient	Location	Sex	Age (y)	Adipose Volume (g)	Time of Proliferation to End of P3 (d)	No. cells at P3 ($\times 10^6$)	Time Between P4 and Transplantation of 3D Graft (d)
1	Left femur	M	10	2.10	29	12	52
2	Left tibia	M	11	0.32	38	9	42
3	Right tibia	M	12	1.12	42	18	101
4	Left ulna	M	6	0.67	42	19	88
5	Left tibia	F	6	0.24	47	18	80
6	Right tibia	M	13	7	45	20	66

The anatomical reconstitution was performed with a metallic prosthesis (Phenix, patient 1) and a human osteochondral allograft (patients 2 and 3). Tumors of patients 1 and 2 were classified as conventional high-grade osteoblastic osteosarcoma and included in the EURAMOS protocol (European and American Osteosarcoma Study Group: chemotherapy with methotrexate, doxorubicin, and cisplatin). Patient 3 had a high-grade Ewing sarcoma and was included in the Euro-Ewing protocol (chemotherapy with vincristine, ifosfamide, doxorubicin, VP-16). Adipose subcutaneous tissue was procured surgically (at the time of tumor biopsy for patients 1–3) or by aspiration with a syringe (for patients 4–6) at the leg for all patients except in the abdominal region for patient 4.

proposed after a mean of 35.3 months after diagnosis of bone nonunion without success by conventional therapies.

Clinical Grade Manufacturing of the 3D Graft

The Endocrine Cell Therapy Unit (Center of Tissue and Cell Therapy, Cliniques Universitaires Saint-Luc, Brussels, Belgium) is recognized as a clinical laboratory for isolating ASCs by the Belgian Federal Agency for Medicines and Health Products. All procedures for ASC isolation and expansion were performed in air-laminated flow grade A located in clean room grade B (validated annually by ICCE SA, Elsene, Belgium), in accordance with the Belgium Ministry of Health recommendations and European directives (regulation no. 1394/2007 for advanced cell therapy products). The environment for cellular culture was controlled by weekly particle counting (in static and dynamic conditions; Lasair II Particle Counter, Particle Measuring Systems Germany GmbH, Darmstadt, Germany) and microbiological testing at each manipulation, as recorded in the ''Graft Report.''

To isolate human ASCs, a mean of 1.9 ± 2.6 g of fatty tissue was harvested by a simple subcutaneous biopsy from 6 patients (Table 1) after informed consent and serologic screening.[31] ASC isolation (with GMP collagenase 0.075 g; 8000 PZ U/L; Serva Electrophoresis GmbH, Heidelberg, Germany), expansion, and differentiation were performed in line with good manufacturing practices (GMPs) and the ISO 9001–2008 quality management system. ASCs were then isolated and expanded in the proliferation medium (Dulbecco modified Eagle medium supplemented with 10% heat-inactivated and viral-tested fetal bovine serum certified by the US Department of Agriculture; Life Technologies, Grand Island, NY) up to passage 4 (P4) (after sequential trypsinizations).[28,29] At P4, ASCs were incubated (in 150-cm^2 culture flask) in osteogenic medium composed of the proliferation medium supplemented with dexamethasone (1 μM), sodium ascorbate (50 μg/mL), and sodium dihydrophosphate (36 mg/mL). After 15 to 18 days of ASC incubation, DBM was added (10 mg/mL) to create the 3D scaffold-free graft.[28]

Human DBM was provided by the University Tissue Bank (University Clinical Hospital, Saint-Luc, Brussels, Belgium) and was produced from multiorgan human donors. Diaphysis of femoral or tibial bone was cut and ground into particles between 200 and 700 μm for demineralization treatment. DBM was performed by grinding cortical bones from selected human donors (<45 y old; 7 donors were used for this experimental protocol). First, human bone tissue was defatted by acetone (99%) bath overnight, followed by washing in demineralized water for 2 hours. Decalcification was performed by immersion in 0.6 N HCl for 3 hours (20 mL solution per gram of bone) under agitation at room temperature. Then, demineralized bone powder was rinsed with demineralized water for 2 hours and the pH was controlled (normal ph between 7.00 and 7.84). If the pH was too acidic, DBM was buffered with phosphate solution at 0.1 M under agitation. Finally, DBM was freeze-dried and weighed. The DBM was sterilized with 25 kGy by gamma irradiation at −80°C. The osteogenic properties of DBM were assessed by the residual level of calcium concentration after the demineralization process (measured by calcium extraction contained in a mean of 1.3 g of DBM vs nondemineralized bone powder from each donor); and the osteoinduction by in vivo implantation in paravertebral musculature of nude rats (male, 6–8 wks old) to quantify the new bone formation (presence of bone marrow, osteoblast activity, and new bone formation) by histomorphometry (a standard 300 cross-grid for point counting under a microphotography at 10× magnification; 4 nonoverlapping areas per slide were studied) at 1 month postimplantation for demineralized versus non-DBM.

The 3D graft (was ready for implantation right off the plastic dish) was implanted after a mean of 71.5 ± 22.3 days of incubation in osteogenic medium (after P4) (Fig. 1). The day of implantation, the 3D graft was rinsed 3 times with transplantation medium (CMRL; Mediatec Inc., Manassas, VA) without phenol red and without antibiotics or sera. The graft was finally placed in a sterile culture flask enclosed in 3 sterile plastic bags. The graft was then transferred at room temperature, in less than 15 minutes, to the operating room for implantation.

Quality Control of Graft Release

The ASCs (at P4) were tested in specific medium to assess the capacity of differentiation towards the 3 main mesenchymal lineages. Confluent cultures of ASCs were induced to undergo adipogenesis, chondrogenesis, or osteogenesis by replacing the proliferation medium with specific induction media: for adipogenesis, Iscove modified Dulbecco medium with 20% fetal calf serum, 2 mM L-glutamine, bovine insulin (5 mg/mL), indomethacin (50 mM), 3-isobutyl-1-methyl-xanthine (0.5 mM), dexamethasone (1 mM), penicillin (100 U/mL), and streptomycin (100 mg/mL); for osteogenesis, proliferation medium supplemented with dexamethasone (1 μM), sodium ascorbate (50 μg/mL), and sodium dihydrophosphate (36 mg/mL); and for chondrogenesis, proliferation medium supplemented with 1 mM dexamethasone,10 ng transforming growth factor-β3, 40 mg/mL L-proline, 50 mg/mL sodium ascorbate, 36 mg/mL sodium dihydrophosphate, 100 mg/mL sodium pyruvate, and 100 mL/mL insulin-transferrin-selenium + premix. The medium was replaced every 2 days up to demonstrate the differentiation by oil red staining (for neutral lipids), alizarin red (for $CaPO_4$ deposition), and osteocalcin (for bone phenotype) and finally alcian blue (for proteoglycans), respectively.[28]

At P4, each batch of ASCs was also characterized by fluorescence-activated cell sorting (FACS). The specific cell surface marker profile was analyzed for CD44 (PE mouse antihuman CD44, BD Bioscience, Franklin Lakes, NJ), CD45 (FITC mouse antihuman CD45, BD Bioscience), CD73 (FITC mouse antihuman CD73, BD Bioscience), CD90 (PE mouse antihuman CD90, BD Bioscience), and CD105 (FITC mouse antihuman CD105, BD Bioscience). ASCs were stained with saturating amounts of monoclonal antibody. At least 10,000 events were analyzed by flow cytometry (FACScan, BD Biosciences) with CellquestPro software. Results were expressed in mean fluorescence intensity. In control, hematopoietic stem cells were analyzed.

When the final scaffold-free 3D graft was obtained with the optimal DBM concentration (ASCs + 10 mg/mL), the cellular viability and the graft integrity were determined by a histomorphological score including the cellular, extracellular matrix, and DBM contents: (DBM–ECM)/viable cells. A 20-mm^2 biopsy (day of the transplantation) was fixed in 4% paraformaldehyde overnight. We normalized the integrity of the 3D graft by the (DBM–ECM)/viable cells ratio between −1 and +1.[28]

Graft Safety

Cytogenetic stability was studied by karyotype and fluorescence in situ hybridization (FISH) analyses at P4 (undifferentiated and differentiated) of ASCs from the 6 patients to

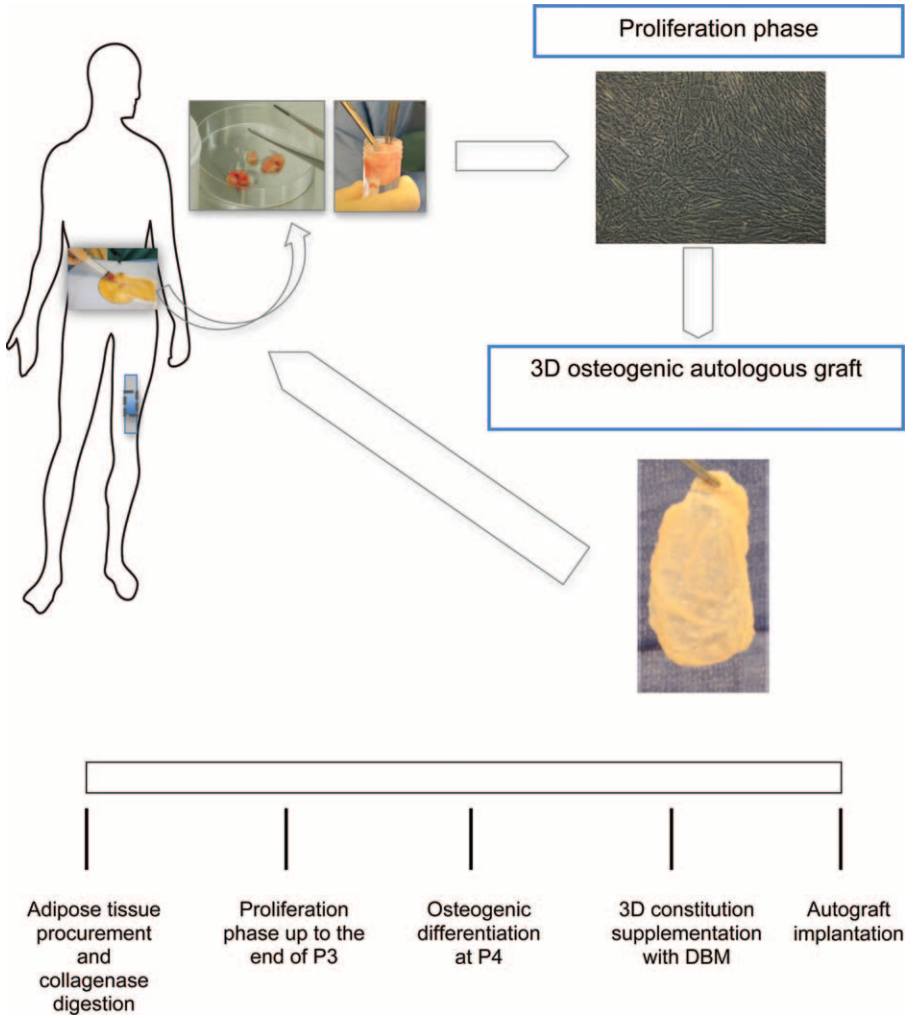


FIGURE 1. Protocol to obtain a 3-dimensional (3D) osteogenic graft made of adipose-derived stem cells (ASCs). Human ASCs were isolated following digestion of subcutaneous adipose tissues, expanded up to passage 4 (P4), and finally differentiated in 2 phases. The osteogenic differentiation was induced to create a 3D structure by the addition of demineralized bone matrix (DBM). The optimal concentration of DBM was adjusted with the anticipated function of the 3D construction to produce a sufficiently stable graft for manipulation by forceps.

assess the oncogenic safety of the cellular components of the 3D graft. Metaphase chromosomes were obtained according to standard protocols from cultured cells (ASCs) in the exponential growth phase after P4.[32] Twenty Giemsa-Trypsin-Wright banded metaphases were analyzed, and karyotypes were reported according to the last 2013 International System for Human Cytogenetics Nomenclature. The FISH experiment was performed according to standard protocols to detect aneuploidy of chromosomes 7 and 8 using CEP7/D7Z1 (SpectrumGreen or SpectrumOrange) or CEP8/D8Z2 (SpectrumOrange or SpectrumGreen) probes (Abbot Molecular, Ottignies/Louvain-la-Neuve, Belgium).[32] For the patient with tumors, specific probes according to the initial genomic alterations detected in the tumors were tested in the P4 ASCs (undifferentiated and differentiated): LSI 9p21/CEP 9 (Abbot SA, Wavre, Belgium), ON MDM2/SE 12 (Kreatech Diagnostics, Amsterdam, the Netherlands), LSI-RB1/13q1 42 (Abbot SA), LSI TP53/CEP 17 (Abbot SA), LSI EWSR1 (Abbot SA), and LSI-FLI1 and EWSR1 (Cytocell, Cambridge, UK). At least 100 nuclei were counted, and the thresholds were calculated following the inverse beta law, with a confidence interval of 99.9%.

Mycoplasma and endotoxin assays were also performed, as per current GMP guidelines, by TEXCELL SA (Evry, France) on cellular samples collected at P4 for undifferentiated and osteogenic cells (the last sample prior graft delivering). Microbiological testing was repeatedly performed at each media change (twice a week during the entire manufacturing of the graft) for aerobia, anaerobia, moisture, and yeast by BACTEC assays.

In-process controls (on cellular samples collected at P4 for undifferentiated and osteogenic cells up to the last sample before graft delivery) based on safety tests found no microbiological or mycoplasm contamination and no endotoxin contents for any manufacturing batch. Therefore, all manufactured 3D grafts fulfilled the release criteria for implantation.

The 3D Graft Implantation and Outcome

In case of tumor resection (patients 1–3), the 3D graft was placed directly at the junction between the native host bone and the bone allograft or the growing prosthesis. In case of bone nonunion, the 3D osteogenic graft was modeled to the ideal size of the bone defect and put directly into the hole without any fixation material.

The primary outcomes of the clinical trial were safety and feasibility of the procedure assessed at 12 months after implantation. The safety was studied in terms of adverse events (local or systemic) with clinical (inflammation, wound infection) and biological (C-reactive protein, fibrinogen, white blood cell count) assessments on a specific schedule: 2 times per week during the first month and once per month from month 1 to 12 postimplantation. The safety was also investigated by x-ray between 12 and 39 months posttransplantation to assess any secondary ectopic malignant tissue development.

The feasibility study was based on the ability to reproducibly obtain a 3D structure from autologous stem cells and allogenic DBM, the respect of the timing between the adipose tissue procurement and the surgical intervention (especially for tumor resection in combination with the chemotherapy presurgery), the capacity to produce enough 3D grafts to fill a large bone defect, and the surgical handling of the 3D graft being clinically relevant for large-scale application.

The secondary outcomes were the efficacy for bone tissue consolidation at the site of bone nonunion (radiologically assessed) and patient satisfaction based on quality of life (eg, walking, pain).

Statistical Analysis

Results are expressed as mean ± standard deviation. The 1-sample Kolmogorov–Smirnov test and Q-Q plots were used to assess the normal distribution of values. Statistically significant differences between groups were tested by 1-way analysis of variance with the Bonferroni post hoc test. Statistical tests were performed with PASW 18 (SPSS; IBM, Armonk, NY); $P < 0.05$ was considered significant.

RESULTS

At P4, human ASC characteristics were confirmed by differentiation in the adipose, osteogenic, and chondrogenic phenotypes, and by a significant shift in the mean fluorescence intensity curve (FACS) for CD44 (99.9 ± 0.2%), CD73 (96.5 ± 6.6%), CD90 (98.5 ± 1.6%), and CD105 (97.8 ± 2.0%). CD45 antigen expression was negative (5.2 ± 1.0%).

The quality of the human DBM was confirmed by a significant reduction of the calcium content (47 ± 116 vs 2615 ± 890 mg/dL of calcium corresponding to a mean demineralization of 98%; $P < 0.005$) and by significant higher in vivo osteogenesis (12 ± 5 vs 1 ± 3% of the explanted graft with osteoinductivity; $P < 0.05$) in comparison to non-DBM, respectively.

Clinical Safety and Efficacy

A mean of 16 ± 4 million ASCs per patient was available by the end of P3 (within 41 ± 6 d), which was sufficient for seeding into three 150-cm^2 culture flasks for P4 (Table 1). The osteogenic differentiation was then induced at P4 for 15 days (when ASCs were confluent) before supplementation of DBM at 10 mg/mL to create the 3D structure. All grafts demonstrated a 3D structure before implantation. The 3D graft was implanted at day 112 ± 26 after adipose tissue procurement, consistent with the preoperative chemotherapy course. Three grafts of 3×3 cm^2 (1 graft per 150-cm^2 flask) were produced per patient from ASCs supplemented with DBM 10 mg/mL (Fig. 1). The final product was stable and did not rupture with forceps manipulation.

No adverse event occurred at the site of adipose tissue biopsy.

The 3D structure was verified for each graft (preimplantation) by histomorphometric potency testing scored based on staining for osteogenic phenotype and mineralization of the matrix.

The complex clonal numerical and structural chromosomal aberrations detected in the bone tumor cells (Table 2) were not detected in the ASCs developed for each graft at P1 and P4 (both undifferentiated and differentiated status). Minor clones with structural aberrations detected in the undifferentiated ASCs at P4 were absent from the differentiated ASCs (Fig. 2).

No acute side effects such as inflammatory reaction, pain, or wound nonhealing were reported (Figs. 3 and 4).

No patient had complications during follow-up, but patient #2 underwent allograft removal because of intercalary allograft infection more than 10 months posttransplantation. No long-term complications were observed for patients 1 and 3 after a mean follow-up of 37 months. For both patients, ossification was rapidly initiated around the Phenix prosthesis and the bone allograft, respectively (Fig. 3). The junction of the native bone and the bone substitute began at 3 months postimplantation and remained consolidated up to 47 months. The stabilization of the allografts led to a normal quality of life more than 3 years postimplantation (Fig. 3B).

Although no acute complication was reported for patients with bone nonunion (patients 4–6), one case required material removal due to sepsis following screw and plate infection by *Staphylococcus aureus* at 10 months posttransplantation. No sign of bone consolidation was found. For patient #4, a surgical revision was performed at 9 months, due to incomplete or inefficient bone consolidation. In bone nonunion within an erythroblastopenia context, the bone consolidation was confirmed at 10 months postimplantation and maintained up to 29.8 months (Fig. 4).

No abnormal ectopic bone development was found radiologically after a mean of 32 months postimplantation for all patients (n = 6).

DISCUSSION

The combination of osteoprogenitor ASCs and growth factors included in osteoconductive DBM demonstrated the feasibility of manufacturing a clinical batch of 3D scaffold-free autologous and osteogenic graft; the safety of differentiated stem cells and growth factors in an oncological context; and the efficacy of surgical reconstruction of a large bone defect in a stringent clinical context, specifically bone pseudarthrosis and bone tumor resection.

The most important outcome of this study is the proof of concept in terms of feasibility for manufacturing a scaffold-free 3D implant from human autologous ASCs differentiated into an osteogenic phenotype with DBM. For clinical application of this advanced therapy of medicinal products, all procedures were validated using human ASCs (following GMPs) and DBM with the goal of being able to uniformly reproduce the manufacture of a structural and stable 3D implant in all patients despite clinical constraints, such as the timing of adjuvant chemotherapy, the

TABLE 2. Genetic Analysis of ASCs for Clinical Application—

Patient	Genetic Analysis of Native Disease	Undifferentiated ASCs	Osteogenic ASCs
1	Karyotype: 46, XY[20] —FISH: tri and tetrasomies 12cen/12q15 (31%), 17cen/17p13 (52%), 22q12 (37%), and 8cen (27%)—13q14/RB1 deletion (57%)	Nd	Karyotype: 46, XY[20] —FISH: tetrasomies (∼2%–3%)
2	Karyotype: 37∼38, X,-Y, add(1q), -1, -2, -3-, 3, -4, add(5q), add(6q), -8, -9, -10, -11, -11, add(12p), del(12p), -13, add(15p), -16, add(17p), -18, add(19p), -19, add(20q), -20, add(21p), -21, add(22p), -22, mar1∼mar11[cp5]/74–78<4n>, idem[2] —FISH: monosomies 9cen and 9p21/CDKN2A/CDKN2B, 13q14/RB1, 22q12/EWSR1 (28%–42%); polysomies (mainly tetrasomies) 8cen, 12cen, 12q15/MDM2, 17p13/TP53, and 22q12/EWSR1 (7%–14%)	Karyotype: 46, XY[50] with high number of chromosomal breakage at the same location cht(3)(q13) —FISH: tetrasomies 8cen, 9cen, 9p21/CDKN2A, and DCKN2B (2%–3%)	Nd
3	Karyotype: 48, XY,+8,t(11;22) (q24;q12),+12[7]/49,idem,+2[2]/49,idem,+20[1]/48, XY,idem,+add(12p)[2]/46,XY[6]—FISH: FLI1-EWSR1 fusion (77%) Trisomies 8cen (68%), 12cen, and 12q15/MDM2 (46%)	Karyotype: 47, XY,+7[10]/46, XY[26]—FISH: trisomy 7cen (5.5%) tetrasomies 7cen and 8cen (2%)	Karyotype: 46, XY,t(14;15)(q32;q11.2)[6]/46,t(X;15)(p11.2;q22),Y[2]/46, XY[92]—FISH: trisomy 7cen (12.5%); tetrasomies 7cen and 8cen (8%)
4	Karyotype: 46, XY[20]—FISH: normal pattern for 8cen, 9cen, 9p21/CDKN2A, and CDKN2B, 13q14/RB1, 17cen, 17p13/TP53, and 22q12/EWSR1	Karyotype: 47, XY,+7[2]—FISH: trisomy 7cen (6.5%) tetrasomies 7cen and 8cen (2.5%)	Karyotype: 46, XY[18]—FISH: tetrasomies 7cen and 8cen (10%), no trisomy 7cen
5	Nd	Karyotype: 46, XY[19]/92, XXYY[1] —FISH: no detectable tetrasomies 7cen (0.5%) and 8cen nor trisomy 7cen	Karyotype: 46, XY [20]—FISH: no detectable tetrasomies 7cen (1%) & 8cen nor trisomy 7cen
6	Karyotypes on bone marrow cells in 2002, 2003, 2007: normal	Karyotype: 46, XY,del(3) (p14)[1]/46, XY[38]/92, XXYY[1]—FISH: tetrasomies 7cen and 8cen (3%), no detectable trisomy 7cen	Karyotype: 46, XY,del(3)(p14)[2]/46, XY[38]—FISH: tetrasomies 7cen and 8cen (4%), trisomy 7cen (2.5%)

ASCs = adipose-derived stem cells, cen = centromere, FISH = fluorescence in situ hybridization.

Karyotypes and FISH of patients 1 to 3 were compared with individual control patients (6–13 y old with 2 congenital and 1 acquired bone pseudarthrosis). No native tumor abnormalities were found in undifferentiated and differentiated ASCs for patients 1– to 3. No difference was found between expanded and differentiated ASCs from patients with a bone tumor and bone pseudarthrosis.

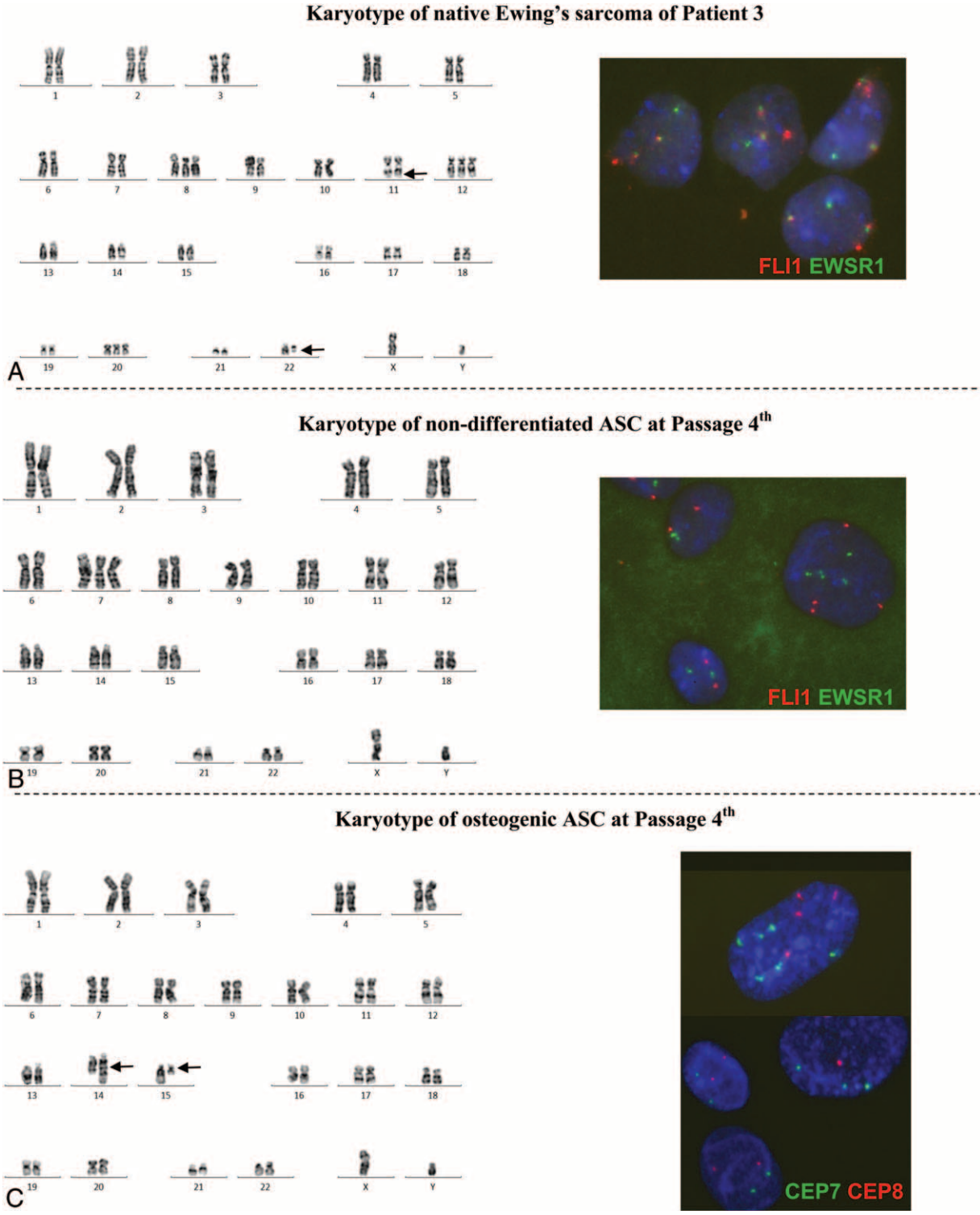


FIGURE 2. Genetic stability of ASCs in bone tumor context. Karyotype and FISH (FLI1-EWSR1 double fusion probe) analyses for the native tumor (A, arrow: t[11;22] [q24;q12]) and undifferentiated (B) and differentiated (C, arrow: t[14;15] [q32;q11.2]) ASC at P4 for patient 3: the FLI1-EWSR1 double fusion signals detected on the native tumour cells (A) were not detected in the ASCs at P4 (B, C), but minority tetrasomies of both probes by FISH suggesting tetraploidy were found for undifferentiated ASCs (B). Initial trisomy 8 was not detected, but minority tetraploidy (detected by the tetrasomies of both probes by FISH) was found after osteogenic differentiation (C) in comparison to the native tumor tissue. ASCs = adipose-derived stem cells, FISH = fluorescence in situ hybridization, P4 = passage 4.

	X-ray follow-up (months)	Early side effects	Late safety Oncogenicity	Efficacy – osteogenesis
Patient 1	27	No pain No wound problem	Wound healing problem (due to chemotherapy) needing surgical revision No bone tumour	Ossification surrounding knee growth prosthesis (at the junction host femur–prosthesis)
Patient 2	39	No pain Wound healing problem due to chemotherapy	Sepsis (*Enterococcus faecalis*) needing bone allograft removal (10 m later) No bone tumour	No complete consolidation at the time of bone allograft removal (at 10 months)
Patient 3	47	No pain No wound problem	No wound problem No bone tumour	Consolidation at 3 months (both host–graft junctions)

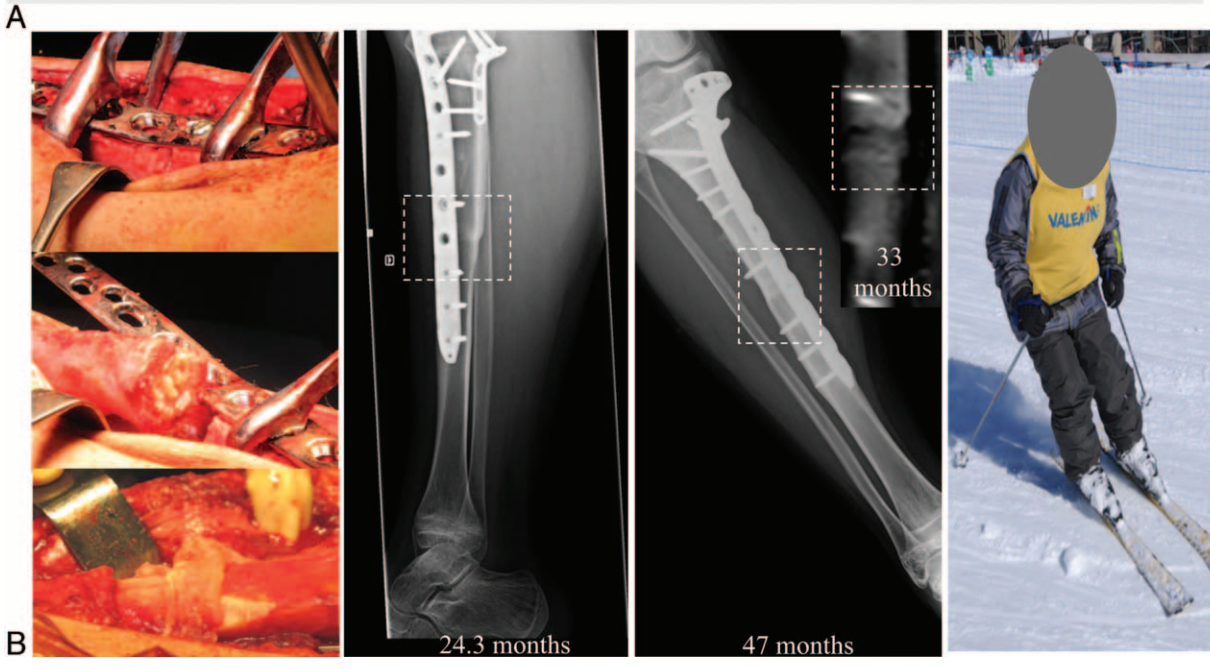


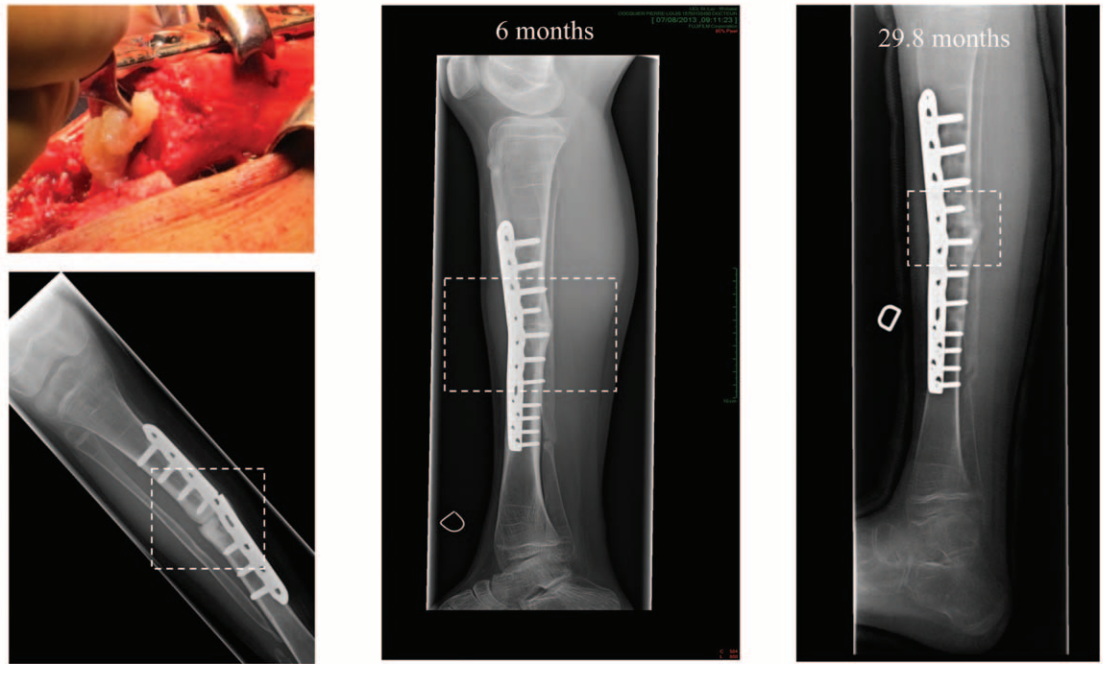
FIGURE 3. Bone defect at the junction between the native bone tissue and the intercalary allograft (after tumor resection at the right tibia). A, No patient had complications during follow-up, but patient #2 underwent allograft removal because of intercalary allograft infection more than 10 months posttransplantation. No long-term complications were observed for patients 1 and 3 after a mean follow-up of 37 months. B, The graft was placed at the direct junction between both tissues without any fixation material. The total consolidation was demonstrated at 24.3 months, the total bridge between the native tissue and the allograft was confirmed at 33 months postimplantation (as demonstrated by nuclear magnetic resonance), and the consolidation is currently maintained up to 47 months posttransplantation. A complete return to normal quality of life for patient #3 was confirmed after 3 years.

schedule of operating room access, and interdonor variability. A mean of 3.7 months for graft manufacture was compatible for clinical implantation, taking into consideration some delays due to patient state (minimum of 80 vs maximum of 143 d for patients 2 and 3, respectively). Another important issue for the clinical feasibility is the size of the implant generated to fill the bone defect. The size of generated 3D bone-like tissue (a mean of 12.6 cm^3 for the 3 grafts) was significantly increased by nearly 6 times (compared to 2 cm^3 of native adipose tissue), and it was always sufficient to fill the bone defect, which was a function of the clinical indication.

The primary issue for the clinical application of advanced cell therapy remains the safety of the patient. Although human MSC (deficient for p53 and/or Rb) failed to induce tumor formation in vivo, suggesting the safety of these cells in clinical application, Perrot et al[33] postulated a risk associated with autologous fat graft implantation in a postneoplasic context, especially for osteosarcoma. No tumor recurrence was observed

	X-ray follow-up (months)	Early side effects	Late safety oncogenicity	Efficacy – osteogenesis
Patient 4	38	No pain No wound problem	No wound problem No bone tumour	No efficient consolidation Pseudarthrosis recurrence needing surgical revision 9 months later
Patient 5	11	No pain No wound problem	Sepsis (*Staphylococcus aureus*) needing plate and screw removal 10m later No bone tumour	No consolidation at the time of plate removal (at 10 months) Necrosis due to infection
Patient 6	29.8	No pain No wound problem	No wound problem No bone tumour	Consolidation at 10 months

A



B

FIGURE 4. Bone nonunion in a case of congenital pseudarthrosis. A, No acute complication was reported for patients with bone nonunion (patients 4–6), 1 case required material removal due to sepsis following screw and plate infection by *Staphylococcus aureus* at 10 months posttransplantation. No sign of bone consolidation was found. For patient #4, a surgical revision was performed at 9 months, due to incomplete or inefficient bone consolidation. In bone nonunion within an erythroblastopenia context, the bone consolidation was confirmed at 10 months postimplantation and maintained up to 29.8 months. B, The 3D graft can easily be placed in the large bone defect of the left ulna of patient 4. Bone nonunion, found on the right tibia of patient 6, was initiated at 6 months postimplantation and consolidated up to 29.8 months. 3D = 3-dimensional.

in our series more than 3 years posttransplantation. Controversy exists concerning the potential for spontaneous transformation of MSCs after prolonged ex vivo culture, but several studies reported that MSCs have limited tendencies to develop tumors.[34–36] In the context of the pediatric population, especially in cases of bone tumor, all precautions were taken to guarantee ASC expansion and differentiation before transplantation. In contrast to native bone tumor, which harbored complex genomic alterations, FISH analysis revealed minor rates (near the detection threshold) of chromosomal aneuploidy, mainly tetrasomies, suggesting tetraploidy as classically observed in cultured cells (around the cut-off of 4.5% and different from the initial tumor clone) and known to be non-tumorigenic.[37] The clonal chromosomal and structural aberrations detected in the karyotypes of 2 patients were not associated with a selective growth advantage in vitro. In a context of chronic wound healing with undifferentiated human ASCs, we previously demonstrated minor rates (near the

detection threshold) of chromosomal aneuploidy up to passage 16 (cut-off ~4.5%) and the absence of adverse events in immunodeficient animal recipients (1 or 3 months postimplantation) and in patients (up to 22 months after implantation). In this study, the in vivo oncologic safety was confirmed by the absence of adverse events in patients up to 3 years after implantation; ASC delivery after a shorter in vitro culture (P4), thus avoiding the selection of tumor cell clones; and the stabilization of the genome by osteogenic differentiation.[38]

The implantation of a 3D osteogenic-like graft was sufficient to provide long-term safety as well as proof of efficacy in extreme clinical conditions. All manufactured 3D grafts were easily handled and implanted (without any additional fixation materials) in a defined and regular space (eg, the junction between the intercalary allograft and native bone) or in an irregular defect as found after tumor resection or in pseudarthrosis, respectively. Although the final 3D graft is not intended to directly restore the native bone mechanical properties, the low mineral content of the final 3D product (which is only a mean of 18% of that found in human adult trabecular bone: 277 vs 2876 mg/cm^3, respectively, data not shown) confers an optimal malleability for surgical implantation.[39] No side effects can be related to the 3Dgraft. The cases of reported infection were associated with the intercalary allograft and the fixation material contamination at the peri or postoperative time. The microbiological control of the 3Dgraft (at the time of implantation) and the culture media (used as vehicle for the transportation between clean room and operative room) did not reveal contamination. Bus et al[5] recently demonstrated that bone nonunion (between intercalary and native bone) remains the major cause (40% of patients) of second surgical intervention (with the use of cancellous bone allograft at 1 y after the initial surgery) to facilitate the union of allograft–host junctions after tumor resection. Although no junction (by x-ray) was found at 6 months after graft implantation (which can be associated to a lower degree of mineralization of the 3D graft compared to native bone), the 3D graft was found to promote irreversible bone consolidation up to 47 months, restoring normal quality of life, especially for patients #1 and #3. Although ASCs survived and promoted in vivo osteogenesis in bone nonunion (congenital or acquired) up to 29.8 months posttransplantation, which is characterized by low oxygen tension, fibrosis, and absence of a local favorable environment (low level of local and systemic growth factors, such as fibroblast growth factor 2), a lower rate of success was found in this category of patients (certainly due to the native pathophysiology of the pseudarthrosis).[40] Although in vivo histological analysis could not be performed for ethical reasons, x-ray analysis demonstrated total consolidation, which was initiated rapidly at 3 months postimplantation. This observation coincides with our previous preclinical results demonstrating in vivo consolidation of critical femoral size bone defect at 6 months postimplantation, which was characterized by endochondral and intramembranous ossification after the 3D graft implantation.[28]

CONCLUSIONS

We report for the first time that autologous ASCs can be fully differentiated into a 3D osteogenic-like implant without any scaffold. We demonstrated that this engineered tissue can safely promote osteogenesis in extreme conditions of bone nonunion, leading to restoration of bone anatomy and function, with minor donor site morbidity and no oncological side effects. Since bone nonunion incidence is increased due to comorbidity factors (eg, type 2 diabetes, smoking), this technology must demonstrate its potential in terms of clinical benefit in comparison to a gold-standard surgery (reduction of additional interventions, reduction of morbidity); improved cost effectiveness by the introduction of allogenic ASC source and automated/closed culture system to reduce the interbatch variability and manufacturing costs (eg, clean room, quality control testings, operator handling). A prospective controlled trial is needed for clinical relevant indications to clinically assess the 3D osteogenic-like implant.

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Author contributions: DD was the Principal Investigator of the clinical study in terms of study design, inclusion/exclusion patient criteria. DD developed the preclinical and clinical concept of the human autologous 3D graft from adipose stem cells. NA and DD validated the protocols of graft manufacturing, GMP production of the graft, and immunohistochemical analyses. NA produced the clinical batches of 3D grafts. CD and P-LD interpreted the clinical outcome in terms of safety and efficacy (x-ray analysis). HAP performed and interpreted the genetic analysis for graft release. WA coordinated the study design for clinical data management.

REFERENCES

1. Tzioupis C, Giannoudis PV. Prevalence of long-bone non-unions. *Injury.* 2007;38 (Supp. 2):S3–S9.
2. Enneking WF, Campanacci DA. Retrieved human allografts: a clinicopathological study. *J Bone Joint Surg Am.* 2001;83:971–986.
3. Enneking WF, Mindell ER. Observations on massive retrieved human allografts. *J Bone Joint Surg Am.* 1991;73:1123–1142.
4. Delloye C, Cornu O. Incorporation of massive bone allografts: can we achieve better performance? *Acta Orthop Belg.* 2003;69:104–111.
5. Bus MP, Dijkstra PD, van de Sande MA, et al. Intercalary allograft reconstructions following resection of primary bone tumors: a nationwide multicenter study. *J Bone Joint Surg Am.* 2014; 96:e26(1-11).
6. Dohin B, Kohler R. Masquelet's procedure and bone morphogenetic protein in congenital pseudarthrosis of the tibia in children: a case series and meta-analysis. *J Child Orthop.* 2012;6:297–306.
7. 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.
8. Goulet JA, Senunas LE, DeSilva GL, et al. Autogenous iliac crest bone graft: complications and functional assessment. *Clin Orthop Relat Res.* 1997;339:76–81.
9. Wientroub S, Reddi AH. Influence of irradiation on the osteoinductive potential of demineralized bone matrix. *Calcif Tissue Int.* 1988;42:255–260.
10. Urist MR. Bone: formation by autoinduction. *Science.* 1965;150:893–899.
11. Dimitriou R, Giannoudis PV. Discovery and development of BMPs. *Injury.* 2005;36(Suppl 3):S28–S33.
12. Urist M, Strates B. The classics: bone morphogenetic protein. *Clin Orthop Relat Res.* 2009;462:3051–3062.
13. Pountos I, Panteli M, Georgouli T, et al. Neoplasia following use of BMPs: is there an increased risk? *Expert Opin Drug Saf.* 2014;13:1525–1534.

14. Rocque BG, Kelly MP, Miller JH, et al. Bone morphogenetic protein-associated complications in pediatric spinal fusion in the early postoperative period: an analysis of 4658 patients and review of the literature. *J Neurosurg Pediatr.* 2014;14:635–643.
15. Kelly MP, Savage JW, Bentzen SM, et al. Cancer risk from bone morphogenetic protein exposure in spinal arthrodesis. *J Bone Joint Surg Am.* 2014;96:1417–1422.
16. Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV. Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol.* 1966;16:381–390.
17. Zuk PA, Zhu M, Mizuno H, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng.* 2001;7:211–228.
18. Zuk PA, Zhu M, Ashjian P, et al. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell.* 2002;13:4279–4295.
19. Zuk PA. The adipose-derived stem cell: looking back and looking ahead. *Mol Biol Cell.* 2010;21:1783–1787.
20. Gimble JM, Katz AJ, Bunnell BA. Adipose-derived stem cells for regenerative medicine. *Circ Res.* 2007;100:1249–1260.
21. Kuhbier JW, Weyand B, Radtke C, et al. Isolation, characterization, differentiation, and application of adipose-derived stem cells. *Adv Biochem Eng Biotechnol.* 2010;123:55–105.
22. Rehman J, Traktuev D, Li J, et al. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation.* 2004;109:1292–1298.
23. Fraser JK, Wulur I, Alfonso Z, et al. Fat tissue: an underappreciated source of stem cells for biotechnology. *Trends Biotechnol.* 2006;24:150–154.
24. Liao HT, Chen CT. Osteogenic potential: Comparison between bone marrow and adipose-derived mesenchymal stem cells. *World J Stem Cells.* 2014;6:288–295.
25. Roberts TT, Rosenbaum AJ. Bone grafts, bone substitutes and orthobiologics: the bridge between basic science and clinical advancements in fracture healing. *Organogenesis.* 2012;8:114–124.
26. Dimitriou R, Jones E, McGonagle D, et al. Bone regeneration: current concepts and future directions. *BMC Med.* 2011;9:66.
27. Langenbach F, Naujoks C, Smeets R, et al. Scaffold-free microtissues: differences from monolayer cultures and their potential in bone tissue engineering. *Clin Oral Investig.* 2013;17:9–17.
28. Schubert T, Lafont S, Beaurin G, et al. Critical size bone defect reconstruction by an autologous 3D osteogenic-like tissue derived from differentiated adipose MSCs. *Biomaterials.* 2013;34:4428–4438.
29. Schubert T, Xhema D, Ve?riter S, et al. The enhanced performance of bone allografts using osteogenic-differentiated adipose-derived mesenchymal stem cells. *Biomaterials.* 2011;32:8880–8891.
30. Schubert T, Poilvache H, Galli C, et al. Galactosyl-knock-out engineered pig as a xenogenic donor source of adipose MSCs for bone regeneration. *Biomaterials.* 2013;34:3279–3289.
31. Coleman SR. Structural fat grafts: the ideal filler? *Clin Plast Surg.* 2001;28:111–119.
32. Duhoux FP, Ameye G, Lambot V, et al. Refinement of 1p36 alterations not involving PRDM16 in myeloid and lymphoid malignancies. *PLoS One.* 2011;6:e26311.
33. Perrot P, Rousseau J, Bouffaut AL, et al. Safety concern between autologous fat graft, mesenchymal stem cell and osteosarcoma recurrence. *PLoS One.* 2010;5:e10999.
34. Trobaugh-Lotrario AD, Kletzel M, Quinones RR, et al. Monosomy 7 associated with pediatric acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS): successful management by allogeneic hematopoietic stem cell transplant (HSCT). *Bone Marrow Transplant.* 2005;35:143–149.
35. Meza-Zepeda LA, Noer A, Dahl JA, et al. High-resolution analysis of genetic stability of human adipose tissue stem cells cultured to senescence. *J Cell Mol Med.* 2008;12:553–563.
36. Bernardo ME, Zaffaroni N, Novara F, et al. Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. *Cancer Res.* 2007;67:9142–9149.
37. Roemeling-van Rhijn M, de Klein A, Douben H, et al. Culture expansion induces non-tumorigenic aneuploidy in adipose tissue-derived mesenchymal stromal cells. *Cytotherapy.* 2013;15:1352–1361.
38. Lafosse A, Desmet C, Aouassar N, et al. Autologous adipose stromal cells seeded on a human collagen matrix for dermal regeneration in chronic wounds: clinical proof of concept. *Plast Reconstr Surg.* 2015;136:195–279.
39. Rauch F, Schoenau E. Changes in bone density during childhood and adolescence: an approach based on bone's biological organization. *J Bone Miner Res.* 2001;16:597–604.
40. Granchi D, Devescovi V, Pratelli L, et al. Serum levels of fibroblast growth factor 2 in children with orthopedic diseases: potential role in predicting bone healing. *J Orthop Res.* 2013;31:249–256.