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Autologous bone marrow stromal cells are promising candidates for cell therapy approaches to treat bone degeneration in sickle cell disease☆

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ABSTRACT

Osteonecrosis of the femoral head is a frequent complication in adult patients with sickle cell disease (SCD). To delay hip arthroplasty, core decompression combined with concentrated total bone marrow (BM) treatment is currently performed in the early stages of the osteonecrosis. Cell therapy efficacy depends on the quantity of implanted BM stromal cells. For this reason, expanded bone marrow stromal cells (BMSCs, also known as bone marrow derived mesenchymal stem cells) can be used to improve osteonecrosis treatment in SCD patients. In this study, we quantitatively and qualitatively evaluated the function of BMSCs isolated from a large number of SCD patients with osteonecrosis (SCD-ON) compared with control groups (patients with osteonecrosis not related to SCD (ON) and normal donors (N)). BM total nuclear cells and colony-forming efficiency values (CFE) were significantly higher in SCD-ON patients than in age and sex-matched controls. The BMSCs from SCD-ON patients were similar to BMSCs from the control groups in terms of their phenotypic and functional properties.
SCD-ON patients have a higher frequency of BMSCs that retain their bone regeneration potential. Our findings suggest that BMSCs isolated from SCD-ON patients can be used clinically in cell therapy approaches. This work provides important preclinical data that is necessary for the clinical application of expanded BMSCs in advanced therapies and medical products.

1. Introduction

Sickle cell disease (SCD) is an autosomal recessive disorder characterized by the presence of abnormal hemoglobin S (Hb S). The polymerization of deoxygenated Hb S causes deformation of red blood cells (RBCs) into less pliable cells called sickled RBCs (Lonergan et al., 2001). These cells are prematurely destroyed at high rates, which lead to anemia. Furthermore, the sickled RBCs induce vascular occlusions that lead to tissue ischemia and infarction (Serjeant, 1997). Severe osteoarticular injuries often occur in patients with SCD. Osteonecrosis induced by a temporary or permanent loss of the blood supply to bone is a common complication that occurs in up to 50% of SCD patients and affects primarily the hip, but other joints and sites can also be affected (Hernigou et al., 2006; Vichinsky et al., 1999). The prevalence of osteoarticular injuries appears to be similar between homozygous patients (Hb SS), heterozygous patients (Hemoglobin S combined with C; Hb SC) and patients with various types of sickle-β-thalassemia (Akinyoola et al., 2009). Joint replacement is a controversial treatment option considering the young age of SCD patients (under 30 years of age) and is associated with several complications: intra-operative bleeding, infections, and loosening or early loss of the prosthesis (Marti-Carvajal et al., 2012). Autologous bone marrow (BM) grafting combined with core decompression is an effective strategy that preserves the native joint (Hernigou et al., 2008). However, SCD patients frequently have multifocal osteonecrosis (sometimes six to eight sites), and the number of osteoprogenitor cells present in BM harvested from the iliac crest is not sufficient to treat all of the lesions during the same procedure (Flouzat-Lachaniette et al., 2009; Hernigou et al., 2008). Given that anesthesia is risky in SCD patients, it would be useful

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to expand osteoprogenitor bone marrow stromal cells (BMSCs, also known as bone marrow-derived mesenchymal stem cells) in vitro, so that several lesions could be treated in the same procedure, thereby reducing the number of procedures requiring anesthesia (Stanley & Christian, 2013). BMSCs represent a promising therapeutic approach for bone tissue engineering because of their differentiation capacity and bone regenerative potential (Lee et al., 2003; Maumus et al., 2011). However, BMSCs also play a role in hematopoïesis; therefore, the changes occurring in SCD may reflect not only a hematopoietic disorder but also abnormalities in the activity of BMSCs (Bianco & Robey, 2004). As described by Kuznetsov and others, genetic defects or micro environmental changes in the BM can alter the number or biological activity of stromal cells (Bianco & Robey, 1999; Kuznetsov et al., 2009). The number of stem cells in the BMSC population can be approximated by measuring the colony-forming efficiency (CFE) (Kuznetsov et al., 2009), and appears to be higher in SCD patients than in normal individuals (Hernigou & Beaujean, 2002). However, the number of these cells has not been thoroughly characterized in a large series of patients, and the biological activity of BMSCs from SCD patients has not been studied in vitro. Furthermore, not all patients with SCD develop osteonecrosis, which suggests that the influence of BMSCs and their therapeutic potential is heterogeneous (Vichinsky et al., 1999). Indeed, BMSCs from some SCD patients seem to have a higher capacity for spontaneous bone repair than those from most SCD patients with bone disorders.

These data collectively suggest that the function of BMSCs is affected in SCD patients. Here, we quantitatively and qualitatively evaluated the function of BMSCs isolated from a large number of SCD patients with osteonecrosis (SCD-ON) compared with control groups (patients with osteonecrosis not related to SCD (ON) and normal donors (N)). A cell therapy approach involving ex vivo expanded BMSCs appears to be a useful method to limit the anesthesia risk to SCD patients and will increase the number of osteoprogenitor cells delivered to the site of osteonecrosis. To determine whether these cells are an innovative conservative treatment of SCD osteonecrosis, we characterized BMSCs from SCD patients and studied their osteogenic capacity in vitro and in vivo.

2. Materials and methods

2.1. BM collection

In the context of a validated cell therapy process in orthopedic surgery, we retrospectively reviewed 340 consecutive patients who underwent osteonecrosis treatment with autologous BM grafting (Hernigou & Beaujean, 2002). One hundred and seventy patients were affected by SCD and osteonecrosis (called in this study "SCD-ON patients"), comprising 85 female and 85 male patients (Age range of patients: 15–40 years; Average age of female patients = 29 +/− 6 years; Average age of male patients = 31 +/− 6 years; Average weight = 73 +/− 15 kg). BM was harvested from these patients outside of a sickle cell vaso-occlusive crisis. The remaining one hundred and seventy patients had osteonecrosis not related to SCD (called "ON patients") and consisted of 85 female and 85 male patients (Age range of patients: 14–40 years; Average age of female patients = 30 +/− 7 years; Average age of male patients = 32 +/− 6 years; Average weight = 65 +/− 12 kg).

BM was harvested under general anesthesia from the iliac crest of patients diagnosed with osteonecrosis. Then, a concentrated buffy-coat of approximately 50 ml was obtained after centrifugation on a Cobe 2991 cell separator (Terumo, Lakewood, Colorado) prior to the BM grafting procedure. The BM samples used in this study corresponded to the samples routinely used for product qualification in the quality control department for cell therapy between 2004 and 2012. Nucleated cells from the BM were counted automatically before buffy-coat concentration using an ABX Pentra 60 C+ (Horiba ABX, Montpellier, France). In addition, in the context of BM allografting in hematology, we retrospectively reviewed BM from 14 normal donors (called "N patients"). Informed consent was obtained from each patient (Approval number from the French research ministry: DC-2009-1049). All work presented was based on groups of patients who were matched in age and sex.

2.2. Platelet lysate (PL) preparation

Platelet apheresis collections performed at the "Etablissement Francais du Sang" (EFS, Rungis, France) were biologically qualified according to the French legislation. The platelet count in each product was measured automatically (with an ABX Pentra 60 C+, Horiba ABX, Montpellier, France). Only samples containing 1×10^9–2.5×10^9 platelets/ml were retained; they were frozen at −80 °C and subsequently used to obtain PL containing platelet-released growth factors. Different batches were obtained (from two to five apheresis collections) to adjust the concentration to 50×10^6 platelets/ml. This concentration of platelets was previously shown to facilitate three-fold faster expansion of BMSCs compared with fetal bovine serum (FBS) (StemCell Technologies, Grenoble, France) (Chevallier et al., 2010).

2.3. BM cell cultures

After automated counting, the nucleated cells from fresh BM were seeded at 2×10^5 cells/cm^2 in tissue culture flasks. A cell sample from fresh BM was used to confirm the BMSCs characteristics, as previously described (Chevallier et al., 2010). The BMSCs were expanded in α-modified Eagle's medium (αMEM) (PAA, Les Mureaux, France) supplemented with 5% PL and 0.5% ciprofloxacine (Bayer Pharma, Puteaux, France). Heparin (2UI/ml, Sanofi-Aventis, Paris, France) was added to avoid clot formation. The cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C, and the culture medium was changed twice a week. When the cells reached 80–90% confluence (passage 0; P0), they were detached using Trypsin/EDTA (PAA) and then replated at 1000 cells/cm^2 until 80–90% confluence (passage 1; P1). Figs. 3, 4, and 5 have been done with the same BMSCs samples from ON patients, normal donors (N) and SCD patients (SCD-ON).

2.4. Clonogenic potential

The colony forming efficiency (CFE) assays were performed to evaluate the clonal expansion of BMSCs. Nucleated cells from concentrated BM were seeded at 20 000 and 80 000 cells/cm^2 in duplicate into 25 cm^2 tissue culture flasks with αMEM containing 10% FBS (not heat inactivated; ref. 06,471 from STEMCELL™ Technologies, Grenoble, France) and supplemented with 0.5% ciprofloxacine. After 10 days of culture, the cells were washed with saline solution (NaCl 0.09%) (B. Braun, Melsungen, Allemagne), fixed in methanol (VWR, Fontenay Sous Bois, France) and stained with 7% Giemsa (RAL Technopolis, Bordeaux, France) at pH 7 for 15 min. The number of colonies was counted using an inverted microscope (magnification ×25).

2.5. Colony-forming units-alkaline phosphatase (CFU-ALP+) assay

We measured the number of ALP+ colonies in the BM preparations from three ON patients, three normal donors (N) and four SCD patients (SCD-ON). Nucleated cells from fresh BM were seeded at 20 000 and 80 000 cells/cm^2 in duplicate into 25 cm^2 tissue culture flasks with αMEM containing 10% FBS (not heat inactivated; ref. 06,471 from STEMCELL™ Technologies) and supplemented with 0.5% ciprofloxacine, 50 μM L-Ascorbic Acid-2-phosphate (AA), 10 mM β-glycerophosphate (β-gly) and 0.1 mM dexamethasone (Dex) (Sigma Aldrich, Saint Quentin Fallavier, France). After 10 days of culture, the formation of osteoblast progenitors was detected using an alkaline phosphatase assay, performed according to the manufacturer's specifications (Sigma Aldrich). The total number of colonies was then determined by counterstaining

with Mayer's hematoxylin solution. Alkaline phosphatase positive colonies were counted by two independent investigators.

2.6. Cell proliferation analysis

To compare cell proliferation at different passages between BMSCs isolated from SCD-ON patients, ON patients and normal donors (N), BMSCs (passage 1, P1) were plated at 1000 cells/cm^2 into 25 cm^2 tissue culture flasks. When the cells reached 80–90% confluence, the viable cells were detached, counted with trypan blue (in six replicates by two independent investigators) and replated at 1000 cells/cm^2 until 80–90% confluence (passage 2, P2). To estimate the duration of one mitosis event (i.e., the doubling time), we used the following formula: t/n, where t is the time for the BMSCs plated at 1000 cells/cm^2 to reach 80% confluence and n is the number of population doublings (the number of mitosis events to reach 80% of confluence). The number of population doubling was calculated by using the classical formula $n = log(y/x)/log\ 2$, where x is the number of cells originally plated and y is the number of cells at 80% of confluence (Chevallier et al., 2010). This experiment was repeated for multiple successive passages until t was higher than 15 days.

2.7. Flow cytometry

BMSCs (P1) from three ON and SCD-ON patients were resuspended in HBSS 1× (Invitrogen by Life Technologies, Villebon sur Yvette, France) with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC) conjugated antibodies against CD90, CD105, CD73 or CD34, or the corresponding mouse IgG1 isotype (all from Becton Dickinson and Company, Franklin Lakes, NJ, USA). The cells were washed and examined using a FACSCanto™ II (Becton Dickinson and Co). The expression of surface molecules was analyzed using BD FACS DIVA™ software (Becton Dickinson and Co.).

2.8. Osteogenic differentiation of BMSCs

For the in vitro osteoblast differentiation assay, BMSCs were seeded in 6-well plates. At 80% confluence, the media containing PL was supplemented with 50 μM AA, 10 mM β-gly and 0.1 mM Dex (Sigma Aldrich) for the treated wells (T). The controls wells (untreated: UT) were maintained with media containing only PL. On days 0, 7 and 14, cells were harvested, washed twice with HBSS 1× (Invitrogen by Life Technologies) and lysed for RNA extraction (Qiagen, Courtaboeuf, France). On day 21, the monolayers were fixed in 70% ethanol for 1 h at 4 °C and stained for 15 min with Alizarin Red-S (Sigma Aldrich). To quantitatively assess the relative amounts of calcium deposition, the Alizarin Red-S stain was extracted with acetic acid, neutralized with ammonium hydroxide, and then analyzed by colorimetric detection at 405 nm, as described by Gregory et al. (Gregory et al., 2004). Each condition was performed in duplicate.

2.9. Quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR)

Total mRNA was isolated from BMSCs cultures on days 0, 7 and 14 after P2 using an RNeasy mini-kit, according to the manufacturer's protocol (Qiagen). DNAse (Qiagen) – treated RNA was reverse transcribed with RT superscript III (Invitrogen). The cDNA was amplified using TaqMan-Polymerase chain reaction (Applied Biosystems) with specific primers for the genes of interest (Table 1) and monitored with an ABI Prism 7500 sequence detection system (Applied Biosystems, Rotkreuz, Switzerland). The amounts of cDNA of interest were normalized to GAPDH ($\Delta Ct = Ct_{\text{gene of interest}} - Ct_{\text{GAPDH}}$). The results are reported as relative gene expression ($2^{-\Delta Ct \times 100\,000}$). Quantitative PCR was performed in duplicate for each of the six different BMSCs samples analyzed for both conditions.

Table 1
TaqMan® Primers used for RT-qPCR of human genes.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Hs99999905_m1
Runt-related transcription factor 2 (Runx2)	Hs00231692_m1
Alkaline phosphatase (ALP)	Hs00758162_m1
Osteocalcin (OC)	Hs00609452_g1
Bone sialoprotein 2 (IBSP)	Hs00173720_m1

2.10. Bone-graft substitute

We used the processed human cancellous allograft Tutoplast® (Tutogen Medical). Tutoplast® processing involves delipidization, an osmotic cell destruction treatment, hydrogen peroxide treatment, and washing cycles to remove the noncollagen proteins, followed by a solvent dehydration step and, finally, a γ-irradiation procedure. The processed cancellous bone was cut manually into fragments that were 2–4 mm in size. Cancellous bone particles (porosity of 60%, macropores of 100–500 μm) of equivalent size, volume and weight (8 mg) were used to ensure a comparable surface area for analyses (80 m^2/g in average).

2.11. Cell seeding

Bone substitutes were dampened in αMEM at 37 °C for 2 h prior to cell seeding. Then, they were loaded with 3×10^5 BMSCs in 20 μl of culture medium in untreated 96-well culture plates for 3 h at 37 °C. The bone graft substitutes were subsequently cultured in 1 ml of PL medium at 37 °C in a 5% CO_2 atmosphere for seven days (Chevallier et al., 2010; Coquelin et al., 2012). Cell-free scaffolds were incubated under similar conditions and served as controls.

2.12. Evaluation of cell number by DNA quantification

Cell seeding was performed using an indirect method. The cells at the bottom of the untreated 96-well culture plate that were not attached to the scaffold were lysed with Tris-EDTA (TE) + 0.1% Triton X-100 and digested with 0.2 mg/ml of proteinase K (Invitrogen). The samples were incubated overnight at 52 °C, and then a succession of three heat shocks was performed, followed by sonication of lysates for 10 min. The DNA samples obtained were labeled with Picogreen® (Invitrogen), which only binds double-stranded DNA. After 10 min of incubation in the dark, the fluorescence was measured at 520 nm on a spectrofluorometer (Victor Wallac, PerkinElmer, Courtaboeuf, France). A standard curve was used to quantify the cell number.

2.13. Scanning electron microscopy (SEM)

Three hours and seven days after seeding, bone substitutes were fixed by immersion in 2.5% glutaraldehyde buffer (Sigma Aldrich) at 4 °C overnight. Then, they were dehydrated with increasing concentrations of ethanol (70–100%), and dehydration was completed with 60 min of incubation in hexamethyldisilazane (HMDS) (Sigma Aldrich). Finally, the samples were air-dried, sputter coated with gold nanolayer and analyzed with a scanning electron microscope (JEOL JSN-6301F, Croissy sur Seine, France) at LISA laboratories (Paris-Est University, Creteil, France).

2.14. Animal model and implantation procedure

All animal procedures were approved by a local ethics committee (approval n° 94–612) and conducted in accordance with the European guidelines for animal care (Directive 2010/63/EU). Nine CB17/Icr-Prkdc SCID (males, seven weeks old) purchased from Charles River

laboratories (Chatillon, France) were used in this experiment. The mice were anesthetized with isoflurane, and six subcutaneous dorsal pockets (0.5 cm incisions) were prepared on each mouse. In each pocket, one scaffold was implanted and the skin was closed by suture (Ethicon, San Lorenzo, Puerto Rico, USA). BMSCs from two ON patients, three normal donors (N) and four SCD-ON patients were tested in nine independent animals (n = 12, n = 18 and n = 24 scaffolds, respectively, per group). Cell-free scaffolds were implanted under similar conditions and served as controls. After seven weeks, the animals were killed by an overdose of pentobarbital. The samples were then excised and immediately fixed in ethanol 70%.

2.15. Histology

Specimens were decalcified for 3 h in 6.8% nitric acid (VWR) and then rinsed in tap water before being embedded in paraffin. Sections (3–5 μm) were stained with Masson's Trichrome, which is a three-color staining protocol comprising nuclear staining with hematoxylin, cytoplasmic staining with acid fuchsin/xylidine ponceau and collagen staining with Light Green SF (all from VWR), and visualized using standard light microscopy. Fifteen sections of each sample were analyzed (five from the beginning, five from the middle and five from the end). New bone formation was analyzed and quantified from 12, 13 and 19 sections for the ON, N and SCD-ON groups, respectively, using ImageJ software. New bone density was defined as the ratio of new bone area compared with the total implant area.

2.16. Statistical analysis

For cell proliferation analysis, all experiments were performed with BMSCs isolated from different patients. Quantitative PCR was performed at least in duplicate for each of 12 different BMSCs samples analyzed. Statistical analyses were performed using the unpaired nonparametric Mann–Whitney *U* test (GraphPad Prism5 software), and regression analysis was done using Microsoft Excel (Microsoft, Redmond, WA, USA). Differences between groups with a p-value of ≤0.05 were considered to be statistically significant ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). In all figures, each bar represents the mean ± Standard Deviation (SD).

3. Results

3.1. Colony forming efficiency and number of BM nucleated cells

The BM colony forming efficiency (CFE) was studied in 170 ON patients and 170 SCD-ON patients matched by age and sex. The number of nucleated cells per μl of BM was 13 262 ± 4 110 and 16 837 ± 8 018, respectively (mean ± SD; $p < 0.001$) (Fig. 1A). The CFE values per 10^6 nucleated cells were 17.2 ± 13.6 and 62.3 ± 49.9, respectively (mean ± SD; $p < 0.001$) (Fig. 1B). The CFE values and the number of nucleated cells were drastically elevated in SCD-ON patients. Based on these criteria, we previously showed similar results for BM from ON patients (n = 14 matched by age and sex) and normal donors (N) (n = 14 matched by age and sex) (Fig. 1C, D).

Linear regression analysis revealed that age-related CFE decline was more pronounced in SCD-ON than for ON samples. The inverse correlation between CFE and patient age was statistically significant ($r_{SCD-ON} = -0.22$; $^{**}p_{SCD-ON} = 0.004$ and $r_{ON} = -0.18$; $^{*}p_{ON} = 0.017$) (Fig. 1E). However, the determination coefficient (R^2) was close to 0, highlighting the fact that the ON and SCD-ON samples were not linear ($R^2_{ON} = 0.03$; $R^2_{SCD-ON} = 0.05$).

The number of ALP^+ progenitors from ON, N and SCD-ON BM was evaluated (Fig. 2 Ab). Counter staining showed negative colonies that were stained purple-blue with Mayer's hematoxylin (Fig. 2Aa). The percentage of ALP-positive colonies for each group was not significantly different. Our data for ON, N, and SCD-ON were 98.7 ± 2.3%, 84.7 ± 4.7% and 100.0 ± 0.1%, respectively (Fig. 2B).

3.2. Cell proliferation

BMSCs growth was evaluated by comparing the cell number and the doubling time of BMSCs during successive passages for BM isolated from six different patients for each condition (ON versus N versus SCD-ON). The doubling time (DT) of the BMSCs at P1 for each condition was not significantly different (Fig. 3A). In the subsequent passages, their proliferation was quite fast between P2 (ON DT P2 = 28.5 ± 3.5; N DT P2 = 31.5 ± 3.5 and SCD-ON DT P2 = 31.0 ± 4.2 h) and P5 (ON DT P5 = 41.1 ± 7.1; N DT P5 = 41.0 ± 4.9 and SCD-ON DT P5 = 43.6 ± 7.0 h), after which their doubling time gradually increased (Fig. 3B). The BMSCs doubling number for each condition increased similarly to reach around 38 doubling at P9. Our results showed no significant differences in growth kinetics from P1 to P9 between the BMSCs from each group.

3.3. Phenotypic characterization

Immunophenotypic characteristics of ON (n = 3) and SCD-ON (n = 3) BMSCs at P1 were compared by flow cytometry (Fig. 4). More than 94% of the expanded BMSCs for both conditions were strongly positive at P1 for CD90, CD105 and CD73, all of which are hallmarks of BMSCs (Dominici et al., 2006). The cultures did not contain hematopoietic lineage cells, as indicated by the absence of CD34-expressing cells.

3.4. In vitro mineralization

Osteoblastic differentiation was studied after 21 days of confluent culture in the presence of osteogenic inducers (AA, βGly and Dex) using BMSCs from ON patients (n = 2), N donors (n = 4) and six SCD-ON patients (n = 6). Calcium deposition was examined by Alizarin Red-S staining, which was quantified by optical density measurements. In the absence of osteoinductive factors in the medium (UT), no mineralization was observed (Fig. 5A). In the presence of osteoinductive agents (T), SCD-ON, ON and N BMSCs cultures developed equal amounts of alizarin-positive calcium deposition (Fig. 5A, B).

Osteoblastic gene expression was analyzed after zero, seven and 14 days culture in the presence of AA, βGly and Dex for BMSCs from six N donors and six SCD-ON patients (Fig. 5C). We observed up regulation of osteoblastic gene expression over time for both groups. At seven days, this up regulation was significant for the expression of the early osteoblastic gene Runx2, and at 14 days the late osteoblastic gene osteocalcin was also significant ($^{**}p < 0.01$). Although ALP gene expression was up-regulated in two SCD-ON patients, the difference between the BMSCs from SCD-ON and N samples was not significant, and we observed overall similar levels for osteoblastic gene expression for both groups throughout the time course experiment.

3.5. Cell adhesion, morphology and distribution on the scaffold

To determine whether the osteogenic potential of SCD-ON BMSCs can be translated to clinical cell therapy approaches, we assessed their adhesion capacity, morphology and distribution on bone substitutes that are currently used in orthopedics to bridging the gap in osseous defects compared with BMSCs from normal donors (Jager et al., 2011). To compare adhesion, we evaluated their seeding efficiency on scaffolds. As previously described by Coquelin et al., we quantified the cell number for both conditions by DNA quantification for 12 different BM samples (Fig. 6A) (Coquelin et al., 2012). After 3 h of static contact, the number of SCD-ON BMSCs (154 228 ± 81 150 cells) adhered to the scaffold was not significantly different from the number of adhered N BMSCs (179 892 ± 73 290 cells). Cell morphology and distribution were evaluated by Scanning Electron Microscopy (SEM) (Fig. 6B). Three hours after seeding, ball-shaped BMSCs were uniformly distributed on the biomaterial for both groups. After seven days of culture on the bone substitutes, no differences were observed between the two cell

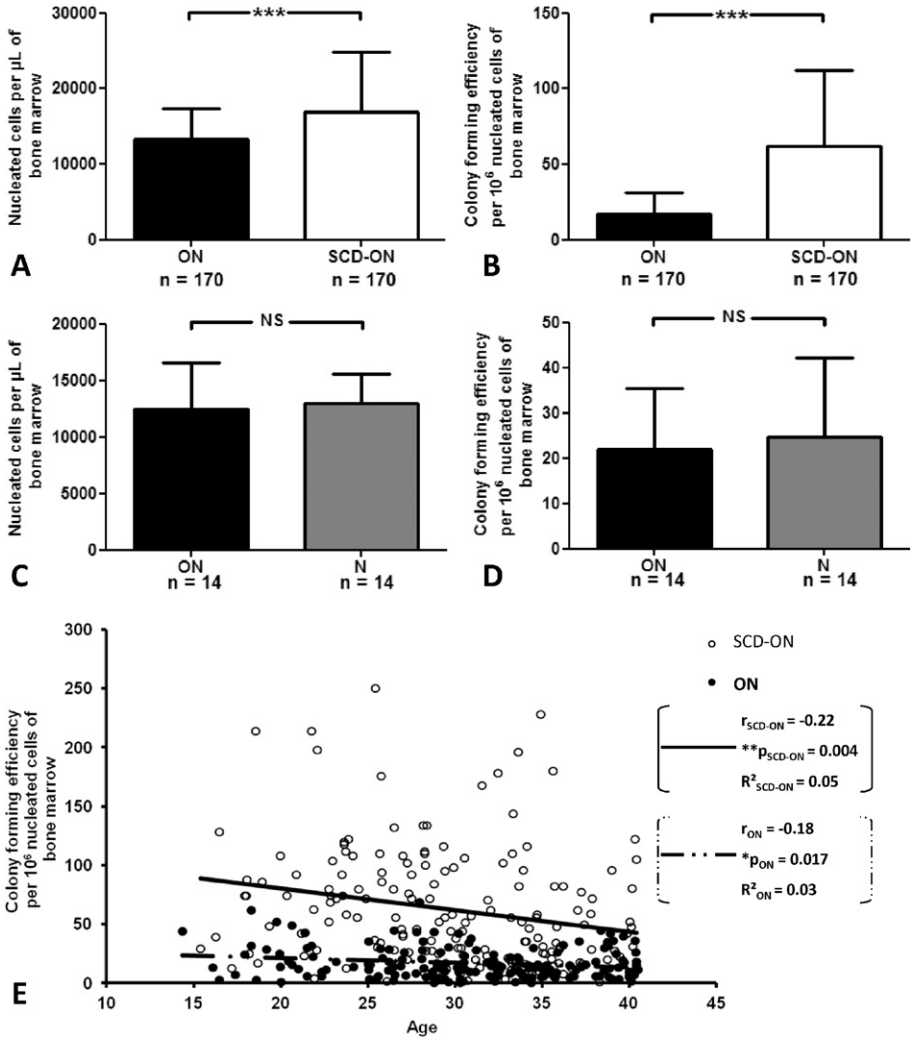

Fig. 1. Colony forming efficiency (CFE) and the number of bone marrow (BM) nucleated cells. BM from 170 patients with osteonecrosis not related to SCD (ON) and 170 SCD-ON patients, included in a protocol for orthopedic cell therapy and matched by age and gender, were analyzed for (A) the number of nucleated cells per µl of BM and (B) the CFE for 10^6 cells seeded and cultured for 10 days. BM from 14 ON patients included in a protocol for orthopedic cell therapy and BM from 14 healthy donors (N) included in a hematology protocol for BM allografting matched by age and gender were analyzed for (C) the number of nucleated cells per µl of BM and (D) the CFE for 10^6 cells seeded and cultured for 10 days. Statistical analysis (Mann–Whitney *U* test) were performed between values (NS: Not Significant; ***: $p < 0.001$). (E) Correlations between CFE and age were analyzed for ON (dotted and solid line; $n = 170$) and SCD-ON (solid line; $n = 170$) patients. Linear regression analyses were performed between values for each group. The regression values were $r_{ON} = -0.18$ for ON BMSCs ($^*p_{ON} < 0.05$) and $r_{SCD\text{-}ON} = -0.22$ for SCD-ON BMSCs ($^{**}p_{SCD\text{-}ON} < 0.01$). The determination coefficients were $R^2_{ON} = 0.03$ and $R^2_{SCD\text{-}ON} = 0.05$, respectively.

sources. SEM analysis showed that the cells from both sources fully covered the scaffold, forming several cell layers.

3.6. Capacity of SCD-ON BMSCs for ectopic bone formation in vivo

To evaluate the osteogenic capacity of SCD BMSCs for bone formation *in vivo*, we performed ectopic implantation of cellularized scaffolds in immunodeficient SCID mice ($n = 6$ scaffold per condition). BMSCs from ON patients ($n = 2$), N donors ($n = 3$) and from SCD-ON patients ($n = 4$) were tested in nine independent animals ($n = 12$, $n = 18$ and $n = 24$ scaffolds, respectively, per group). Cell-free scaffolds were implanted under similar conditions and served as controls; no bone was observed for this group (Data not shown). Neovascularization was observed on biomaterials seeded with BMSCs for all three groups. Analysis also revealed minimal scaffold resorption and no evidence of an inflammatory reaction (Data not shown). The scaffold was recognizable by longitudinally striated collagen highlighted in blue/green by Masson's Trichrome staining, with mineralized zones in red. In contrast, new bone tissue appeared blue (areas delimited with dotted lines), with osteocyte-like cells and osteoblast-like cells observed on scaffolds seeded with ON, N and SCD-ON BMSCs (Fig. 7Aa–f). BM-like elements were also observed for the three groups tested (Fig. 7Ab, d, f). Quantitative analysis was performed to compare the total new bone surface for the three groups. No significant differences were observed, with the averages being 10.43 ± 20.34%, 13.02 ± 17.86% and 9.55% ± 14.26% of new bone formation for the ON, N and SCD-ON BMSC groups, respectively (Fig. 7B).

4. Discussion

For several years, BMSCs have been largely studied and used as a new therapeutic tool for clinical applications because of their multipotent properties (Dimarino et al., 2013). Osteonecrosis is a serious complication of SCD (Milner et al., 1991). To treat this bone disease, we suggest a cell therapy approach with autologous BMSCs expanded *ex vivo*.

We evaluated the functional properties of BM stromal cells isolated from a large number of ON and SCD-ON patients ($n = 340$). Our CFE values for ON patients were consistent with those in the literature (Bernardo et al., 2007; Oreffo et al., 1998), although they were lower than CFE values reported in some publications (Doucet et al., 2005; Kuznetsov et al., 2000). This discrepancy may be due to the different

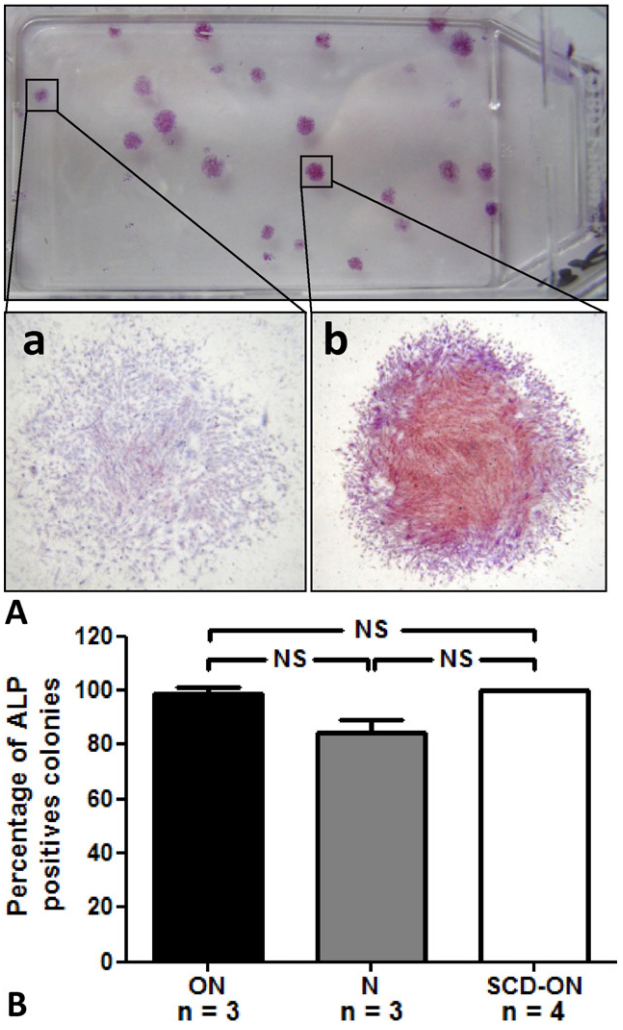

Fig. 2. Evaluation of ALP$^+$ progenitors (CFU-ALP$^+$). Bone marrow (BM) nucleated cells were seeded at low density and cultured for 10 days in osteogenic medium. (A) CFU-ALP$^+$ were detected by alkaline phosphatase (ALP) staining in red (a), and negative colonies were stained purple-blue with Mayer's hematoxylin (b). (B) The percentage of ALP positive colonies in each group. n = 3 in the ON group, n = 3 in the N group and n = 4 in the SCD-ON group. Representative images are shown for each group (magnification 2.5×). CFU-ALP$^+$ assay results are reported as the mean ± SD of duplicate cultures.

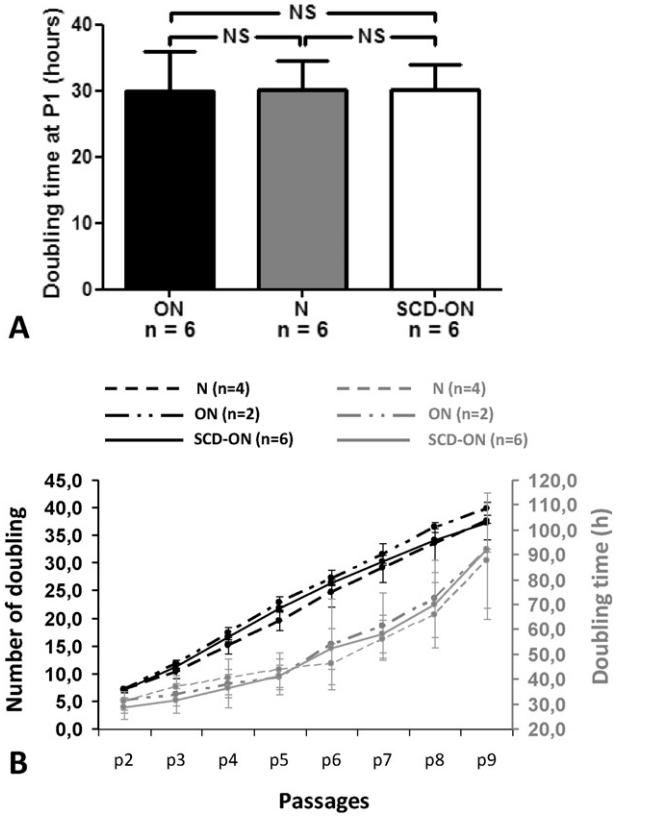

Fig. 3. ON, N and SCD-ON BMSCs cell proliferation in αMEM 5% Platelet Lysate (PL). (A) Doubling time in hours at passage 1 (P1) for the three sources of cells. (B) The number of population doublings (n), shown in black, and the doubling time in hours, indicated in gray, were compared over several culture passages from passage 2 (P2) to passage 9 (P9) for each condition (matched by age and gender). ON BMSCs are shown with dotted and solid lines, N BMSCs with dotted lines and SCD-ON with solid lines. Statistical analyses (Mann–Whitney *U* test) were performed between values and the data are reported as the mean ± SD.

sampling techniques used. In our case, a large volume of BM was collected before buffy coat concentration. We cannot exclude the possibility of a variable degree of peripheral blood contamination, which may explain the low CFE values per 1×10^6 nucleated marrow cells. Our source of BM collection cannot be compared with cell suspensions obtained after flushing fragments of human trabecular bone, as described in others studies (Doucet et al., 2005; Kuznetsov et al., 2009). Although their numbers are low, the BM stromal cells capable of forming osteoprogenitor colonies (CFU-ALP$^+$) represented more than 85% of the CFE values for ON, N and SCD-ON samples, reflecting their osteogenic potential.

We found that the total nuclear cells counts were significantly higher in SCD-ON patients than in the ON group. Among these nuclear cells, the CFE values were also significantly higher in SCD patients than in the age and sex-matched ON group. Consistent with previous reports, we observed that CFE value frequencies were inversely proportional to age for both conditions, with younger patients showing higher CFE counts than older patients (D'Ippolito et al., 1999; Galotto et al., 1999; Kuznetsov et al., 2009). Although the CFE values were higher in the BM of SCD-ON patients compared with the ON group, we observed a decrease with age. This inverse correlation with age may reflect an increase in the frequency of bone complications, such as delayed fracture healing in elderly individuals (with or without SCD).

The high CFE values in SCD patients were first described by Kuznestov et al. for seven pediatric patients (Kuznetsov et al., 2009). Our results confirm this finding in a homogeneous large population of adult patients. SCD is characterized by abnormal hemoglobin, which requires a high turnover of hematopoietic cells in the BM. This disturbance in the BM may explain the higher CFE values in SCD patients. The chronic hematopoietic hyperactivity in the BM enables sickled RBCs to be destroyed and renewed with RBCs to compensate for hemolytic anemia. BM stromal cells reside with hematopoietic stem cells. They cooperate through direct and indirect interactions through the release of cytokines and growth factors, thus forming the hematopoietic niche (Despars & St-Pierre, 2011). The high CFE values in SCD patients may sustain a high rate of hematopoiesis through these various interactions, which maintain bone metabolism and hematopoiesis in the BM cavity. Elevated BM activity is also observed in other hematological disorders, such as in some patients with myeloma (Takahira et al., 1994). However, despite the constitutive activation of the BM in SCD-ON patients, cytometric analysis based on MSCA1+/CD73+/CD90+/CD271+/CD45- expression did not detect BMSCs in the peripheral blood of these patients (data not shown). Because BMSCs do not or rarely circulate (Kuznetsov et al., 2009), the peripheral blood collected during erythrocyte exchange procedures is not a viable source of BMSCs, and the BM remains the main source of primary osteoprogenitor cells.

The BM of SCD-ON patients contained more stromal progenitors compared with the ON and N groups. Thus, we investigated whether SCD-ON BMSCs were functionally similar to ON and N BMSCs. Regarding

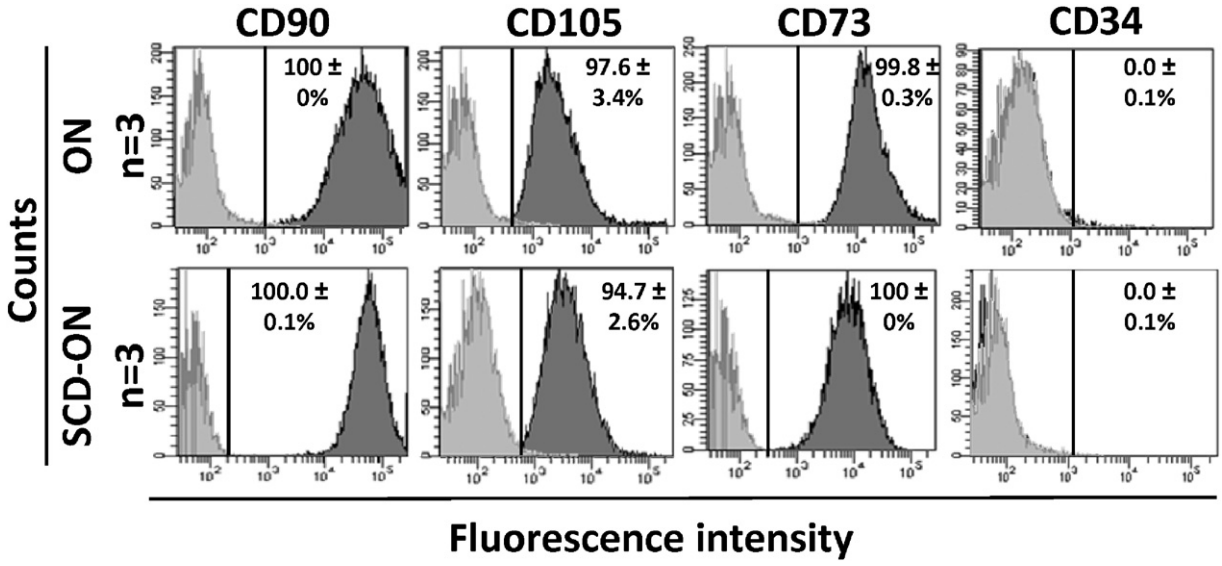

Fig. 4. Surface marker expression profiles of ON and SCD-ON BMSCs. Anti-CD90-FITC, anti-CD105-PE, anti-CD73-PE and anti-CD34-PE antibodies were used for phenotyping, shown in the dark gray histogram, and compared with their corresponding isotype (light gray) (n = 3 for both conditions).

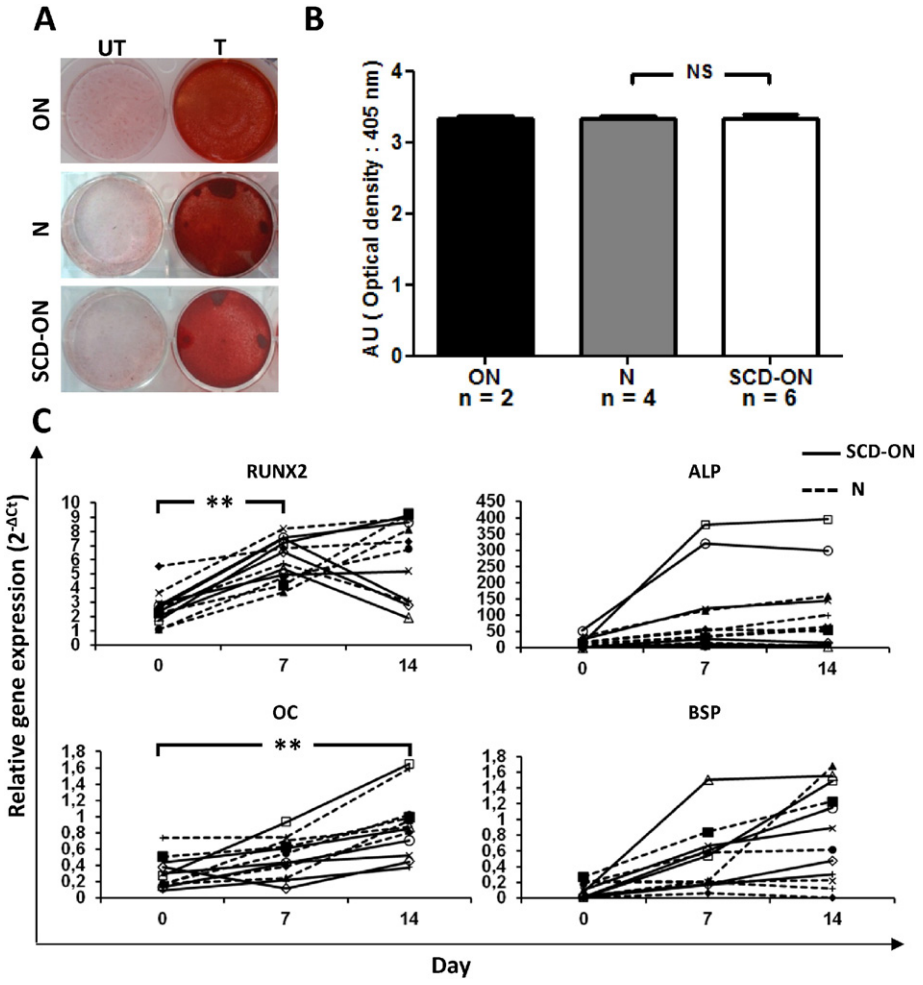

Fig. 5. Osteoinduction of BMSCs from six independent patients from every condition (ON vs N vs SCD-ON), matched by age and gender, using Ascorbic Acid (AA), β-Glycerophosphate (βGly) and Dexamethasone (Dex). BMSCs were cultured in Platelet Lysate (PL) medium at P2. (A) Alizarin red staining of untreated (UT) and AA + βGly + Dex treated (T) cells was performed after 21 days of culture. (B) Alizarin red staining intensity was measured for AA + βGly + Dex treated cells after 21 days of culture, and the optical density at 405 nm is reported as Arbitrary Units (AU). (C) The expression of osteoblastic genes was analyzed using quantitative RT-qPCR (TaqMan®) on days 0, 7 and 14 for six different BM-MSC samples from N and SCD-ON patients. Each experiment was done in duplicate, and the values for all genes were normalized to GAPDH expression. The results are presented as $2^{-\Delta Ct}$. SCD-ON is shown solid lines and N in dotted lines. Statistical analysis (Mann–Whitney *U* test) was performed to compare the SCD-ON and N values (NS: Not Significant) and to evaluate the changes between zero, seven and 14 days (**: $p < 0.01$ for SCD-ON and N BMSCs).

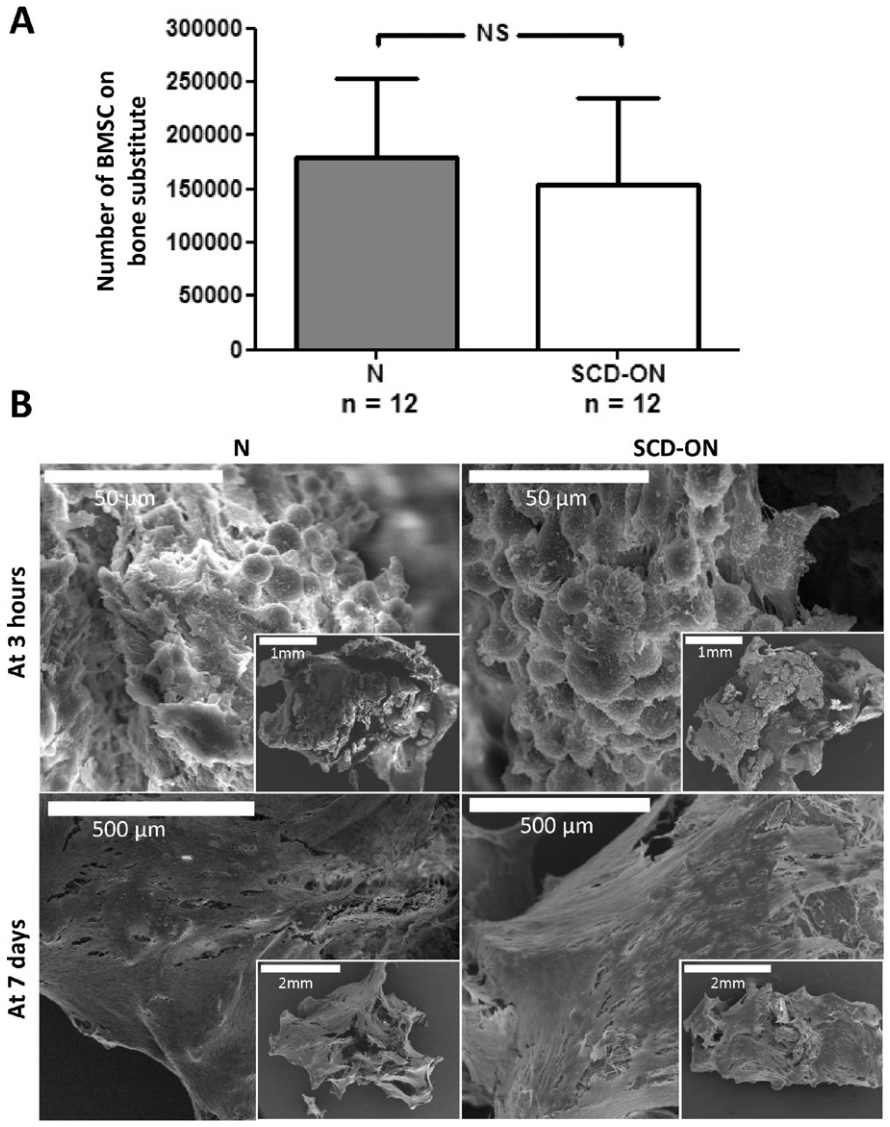

Fig. 6. Cell adhesion, morphology and distribution on a bone substitute. (A) Quantification of the number of BMSCs on scaffolds was performed for BM from 12 independent patients for both conditions, matched by age and gender (N vs SCD-ON BMSCs). Cell numbers were obtained by DNA quantification using Picogreen®. (B) N and SCD-ON BMSCs were observed with Scanning Electron Microscopy 3 h and seven days after seeding. Statistical analyses (Mann–Whitney *U* test) were performed to compare values (NS: Not Significant).

their proliferative capacity, BMSCs from SCD-ON patients adhered to plastic and could be expanded for several passages, similar to BMSCs from ON patients and N donors. The doubling time of each BMSCs population increased similarly with every passage. In addition to adherence to plastic, the expression of specific surface antigens is another criterion for defining BMSCs (Dominici et al., 2006). Cultured BMSCs from both SCD-ON and ON strongly expressed several antigens used to identify BMSCs (≤ 95% CD105, CD90 and CD73) and did not express hematopoietic antigens. We evaluated their osteogenic capacity in vitro and found that these cells produced calcium deposits to a similar extent in response to a classical osteoinduction cocktail. Furthermore, a semi-quantitative assay of mineralization and analysis of osteoblastic gene expression showed no significant differences between SCD-ON and N BMSCs. Several osteoblastic genes were up-regulated during differentiation, although the differences were not significant for ALP and BSP. This result was due to the use of human PL-supplemented culture medium, which has been shown to prime BMSCs cultures to undergo osteoblastic differentiation (Chevallier et al., 2010). We detected a higher level of ALP gene expression from two of six SCD-ON patients, but expression was not significantly upregulated compared with the normal donors; this result mainly reflected the cell heterogeneity between BMSCs from different BM donors in terms of their osteogenic activity. However, *in vitro* osteogenic differentiation assays cannot predict the *in vivo* osteogenic activity of BMSCs (Janicki et al., 2011; Mendes et al., 2004). Tissue regeneration is a complex process that requires the migration, adhesion and differentiation of BMSCs. To reach the site of an injury and initiate the healing process, cells must migrate to the target area and adhere. Cell number and cell morphology are directly linked to cell differentiation and bone formation (Mankani et al., 2007; Yang et al., 2012). For this reason, we investigated cellular adhesion and found that both SCD-ON and N BMSCs attached well to a scaffold and were uniformly distributed. Thus, SCD does not affect cellular morphology, adhesion or distribution. We then used an ectopic model to investigate the ability of SCD-ON BMSCs to contribute to bone formation in vivo. We found that the bone-forming capacity after in vivo ectopic implantation was similar for BMSCs obtained from SCD-ON patients and those derived from controls (ON patients and N donors). Disparities between our results and those of other studies are not related to the disease; rather, they are related to donor-to-donor heterogeneity and several other factors, including sampling bias during BM aspiration, age, sex and medication of the BM donor (Janicki et al., 2011; Phinney, 2012). Moreover, *in vivo* bone formation could lead to extramedullary

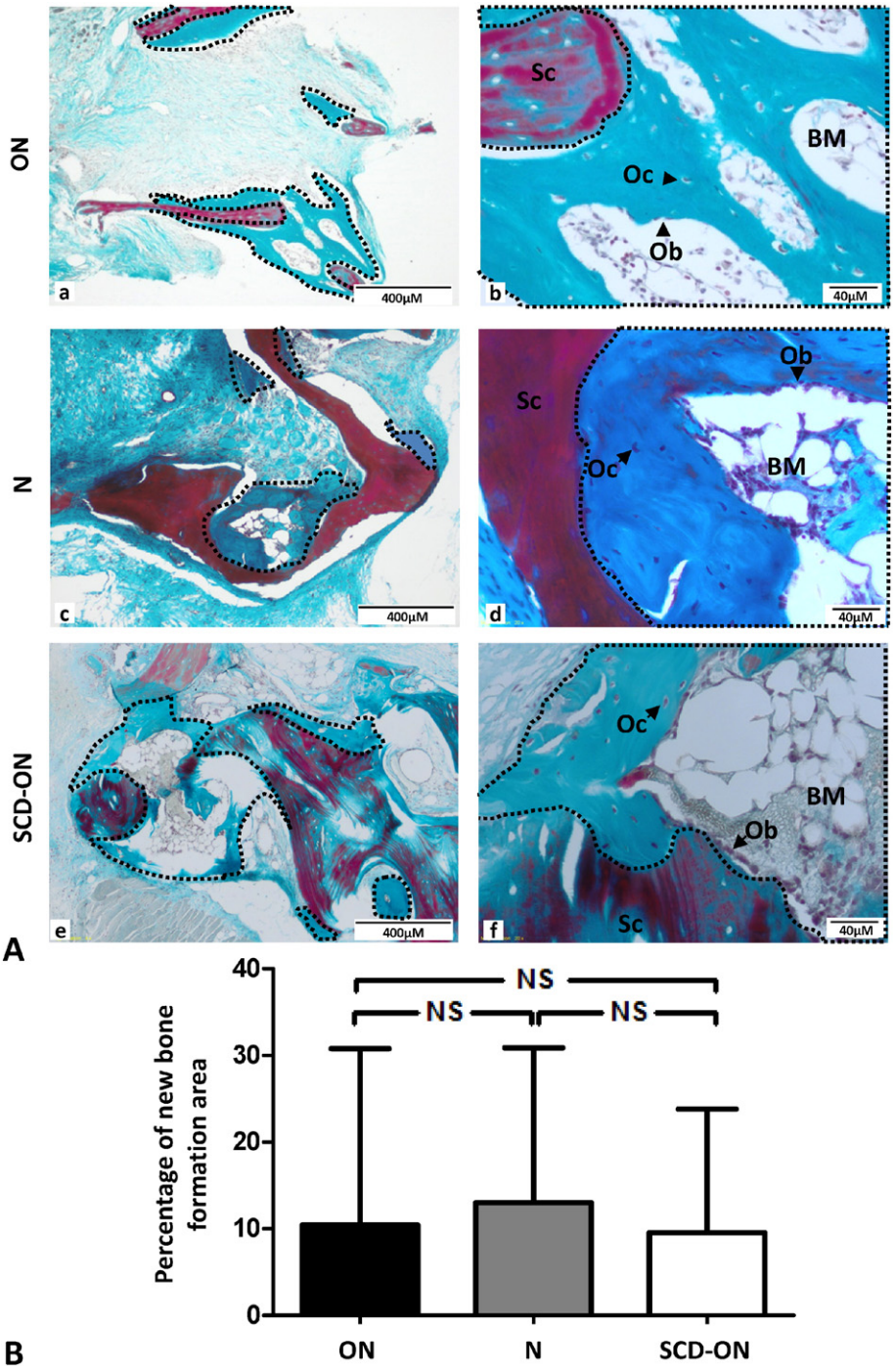

Fig. 7. New bone formation in vivo. (A) Histological analysis was performed after seven weeks of ectopic implantation. These analyses were performed for scaffolds with BMSCs from (a, b) two ON patients, (c, d) three N donors and (e, f) four SCD-ON patients, tested in nine independent animals. Decalcified implants (n = 12, n = 18 and n = 24 scaffolds, respectively, per group) were embedded in paraffin and stained with Masson's Trichrome (blue/green = collagen and bone; purple = nuclei; pink = cytoplasm). Sc: scaffold; FT: Fibrous Tissue; Oc: osteocytes; Ob: osteoblast; BM: bone marrow-like elements. Dotted lines: new bone formation. Magnification (a, c, e) 4×; (b, d, f) 20×. (B) The percentage of new bone formation area was quantified from 12, 13, and 19 sections for the ON, N and SCD-ON groups, respectively, using ImageJ software. New bone density was calculated as the ratio of new bone area compared with the total implant area.

hematopoiesis in each condition. In conclusion, despite a low number of sample sizes tested, these results suggest that there is no deficiency in the osteogenic potential of SCD patient's marrow stromal cell populations.

In patients with corticosteroid-induced osteonecrosis of the femoral head, Wang et al. found that bone repair is limited due to the low proliferation ability of BMSCs (Wang et al., 2008). The altered function of BMSCs may be responsible for the pathogenesis and progression of osteonecrosis. In contrast, the osteogenic abilities of BMSCs in SCD patients are not defective either *in vitro* or *in vivo*. However, BMSCs do not appear to prevent osteonecrosis in SCD patients, suggesting that BMSCs cannot migrate to the injury site (Hernigou et al., 2006; Poignard et al., 2012). Indeed, injured tissues express specific receptors or ligands that trigger the mobilization of BMSCs into circulation and facilitate the trafficking, adhesion and infiltration of BMSCs to damaged tissues through a mechanism that is similar to the recruitment of

leukocytes to sites of inflammation (Fong et al., 2011; Maumus et al., 2011). In SCD, these mechanisms seem to be defective because sickled RBCs block blood flow and, consequently, the supply of BMSCs.

Core decompression (either in combination with or without autologous BM grafting) is classically used to delay the progression of osteonecrosis. Core decompression reduces mechanical stress and enhances bone repair, but bone reconstruction remains incomplete (Gangji et al., 2004). One explanation is the small number of bone progenitor cells present in the femoral head and the trochanteric region, especially in patients with non-traumatic or corticosteroid-induced osteonecrosis (Hernigou et al., 1999). Decompression is more effective when combined with autologous BM grafting. Nevertheless, this approach is only successful during the early stages of the disease, probably due to the small number of BMSCs in the BM concentrate (0.001–0.01%) (Hernigou & Beaujean, 2002). The treatment of osteonecrosis is not standardized in SCD patients. Recently, after a mean follow-up of three years, decompression combined with physical therapy (*i.e.*, non-surgical treatment, such as electrical stimulation or physiotherapy) did not result in a better clinical outcome compared with physical therapy alone in patients with SCD (Marti-Carvajal et al., 2012). We show here that the BMSCs from SCD patients may be very valuable for the treatment of osteonecrosis (Kon et al., 2012). Their easy isolation and expansion could provide a large number of osteoblastic progenitors, which could limit the number of complications related to anesthesia and surgery in the SCD patients. Expanded BMSC therapy is a promising approach to treat bone disorders in SCD.

5. Conclusions

In SCD patients with osteonecrosis, the transplantation of a high number of osteoprogenitor cells into the hip is associated with a good outcome (Hernigou et al., 2009). According to our findings, SCD patients with osteonecrosis seem to be excellent candidates for surgery by core decompression combined with cell therapy involving autologous concentrated BM (Hernigou & Beaujean, 2002). We demonstrated here that SCD patients have a higher frequency of CFE values in the BM in a larger patient data based. Given that the BMSCs could be expanded *in vitro* and retained their functional osteogenic capacities *in vitro* and *in vivo*, we suggest that BMSCs isolated from SCD patients can be used clinically in cell therapy approaches. Such an approach has two key benefits: it limits the risk of anesthesia in this disease by facilitating the treatment of several lesions in the same procedure and increases the number of osteoprogenitor cells at the site of osteonecrosis. This work provides important preclinical data that is necessary for the clinical application of expanded stromal Cells for advanced therapies and medical products.

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