INTRAOPERATIVE ENGINEERING OF OSTEOGENIC GRAFTS COMBINING FRESHLY HARVESTED, HUMAN ADIPOSE-DERIVED CELLS AND PHYSIOLOGICAL DOSES OF BONE MORPHOGENETIC PROTEIN-2

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Abstract

Engineered osteogenic constructs for bone repair typically involve complex and costly processes for cell expansion. Adipose tissue includes mesenchymal precursors in large amounts, in principle allowing for an intraoperative production of osteogenic grafts and their immediate implantation. However, stromal vascular fraction (SVF) cells from adipose tissue were reported to require a molecular trigger to differentiate into functional osteoblasts. The present study tested whether physiological doses of recombinant human BMP-2 (rhBMP-2) could induce freshly harvested human SVF cells to generate ectopic bone tissue. Enzymatically dissociated SVF cells from 7 healthy donors (1×10^6 or 4×10^6) were immediately embedded in a fibrin gel with or without 250 ng rhBMP-2, mixed with porous silicated calcium-phosphate granules (Actifuse®, Apatech) (final construct size: $0.1 \ cm^3$) and implanted ectopically for eight weeks in nude mice. In the presence of rhBMP-2, SVF cells not only supported but directly contributed to the formation of bone ossicles, which were not observed in control cell-free, rhBMP-2 loaded implants. *In vitro* analysis indicated that rhBMP-2 did not involve an increase in the percentage of SVF cells recruited to the osteogenic lineage, but rather induced a stimulation of the osteoblastic differentiation of the committed progenitors. These findings confirm the feasibility of generating fully osteogenic grafts using an easily accessible autologous cell source and low amounts of rhBMP-2, in a timing compatible with an intraoperative schedule. The study warrants further investigation at an orthotopic site of implantation, where the delivery of rhBMP-2 could be bypassed thanks to the properties of the local milieu.

Keywords: Bone repair; stem cells; adipose tissue; osteogenesis; tissue engineering.

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Introduction

The standard of care in the treatment of bone defects in orthopaedic, trauma or reconstructive surgery is the transplantation of autologous bone grafts. Alternative options are the implantation of allografts or osteoconductive materials, the local treatment with osteoinductive growth factors such as BMP-2 or BMP-7, or combinations thereof (Berner *et al.*, 2011; De *et al.*, 2007; Saxer F *et al.*, 2010). The engineering of osteogenic bone graft substitutes based on osteoconductive scaffolds combined with autologous osteoprogenitors (mesenchymal stromal cells, MSC) as a biologically active component could provide an attractive alternative, but its translation into clinical practice has proven to be highly challenging (Berner *et al.*, 2011; Cuomo *et al.*, 2009; Evans *et al.*, 2007). Low MSC numbers found in the bone marrow generally require a step of cell expansion for graft manufacturing. This not only is known to be associated with a progressive loss of osteogenic differentiation capacity (Banfi *et al.*, 2000), but also requires processing under costly and tightly regulated Good Manufacturing Practice (GMP) conditions. Thus, cost-effectiveness of the classical bone tissue engineering paradigm still needs to be verified (Meijer *et al.*, 2007).

One possible solution proposed to overcome the limitations above is based on the 3D expansion of MSC directly within porous scaffolds (Braccini *et al.*, 2005). This was shown to reduce intra-individual differences, increase quality of grafts and streamline manufacturing in perfusion bioreactors, with the potential to introduce automation and thus reduce costs (Martin *et al.*, 2009). Another approach has more radically addressed the problem, by trying to eliminate the expansion phase, i.e. reducing the manufacturing process to a one-step surgical procedure. Such an intra-operative approach poses the essential requirements to identify an autologous source of cells that have (i) intrinsic osteogenic capacities *in vivo* without prior culture or osteoinduction and (ii) are available in sufficient numbers directly upon isolation. Freshly isolated bone marrow-derived cells, possibly harvested using a reamer-irrigator-aspirator (Cox *et al.*, 2011; Stafford and Norris, 2010), concentrated by immunoselection (Aslan *et al.*, 2006) or modified genetically (Evans *et al.*, 2007), have been proposed to be directly used for bone repair. Despite the promising data collected so far, the reproducible collection of a sufficient number of MSC across different patients remains to be demonstrated. The freshly-isolated stromal vascular fraction (SVF) of human

adipose tissue represents a possibly better cell source for a one-step surgical procedure, given its up to 500-fold larger number of clonogenic progenitors per volume of tissue sample compared to human bone marrow (Fraser *et al.*, 2006; Scherberich *et al.*, 2007). Two studies (Helder *et al.*, 2007; Vergroesen *et al.*, 2011) tested bone formation by autologous SVF cells, intraoperatively processed to generate grafts implanted in a goat spinal fusion model. Those studies demonstrated a superior bone healing when implants were loaded with SVF cells, but the model was not designed to assess the direct osteogenic properties of the SVF-based grafts. Our group recently demonstrated that ectopic implantation in nude mice of human SVF cells seeded on porous hydroxyapatite scaffolds results in the formation of human origin blood vessels and dense osteoid matrix, but no 'frank' bone formation (Müller *et al.*, 2010). These findings suggested that, in the absence of *in vitro* commitment, additional cues (e.g. osteoinductive factors) might be needed to support ectopic bone tissue generation *in vivo*.

In the present study, recombinant human bone morphogenetic protein-2 (rhBMP-2) was therefore used as an osteoinductive stimulus (Chen *et al.*, 2004; Jeon *et al.*, 2008) for the implanted SVF cells, at doses known to be insufficient to induce by themselves bone tissue formation (Fujimura *et al.*, 1995). RhBMP-2 was introduced in fibrin-ceramic-based constructs simultaneously with the freshly-isolated/SVF cells and immediately implanted ectopically in nude mice. Bone formation and the contribution of SVF cells to this process were studied 8 weeks after implantation. *In vitro* experiments were also performed to address whether rhBMP-2 enhances SVF cell osteogenic differentiation and/or the osteogenic recruitment of clonogenic SVF populations.

Material and Methods

Cell isolation
Adipose tissue, in the form of liposuction or excised fat samples, was obtained from 7 healthy female donors following informed consent and according to a protocol approved by the local ethical committee (EKBB, Ref. 78/07). Minced tissue from excised fat samples or lipoaspirates were processed as previously described (Güven *et al.*, 2011; Müller *et al.*, 2010) and the cell pellets resuspended in complete medium (CM), consisting of α-MEM supplemented with 10 % of foetal bovine serum (FBS), 1 % HEPES, 1 % sodium pyruvate and 1 % Penicillin-Streptomycin Glutamate (100x) solution (all from Gibco, www.invitrogen.com).

Cell characterisation
Fluorescence activated cell sorting (FACS)
SVF cells were analysed by cytofluorimetry with antibodies to CD105, CD90 and CD73 (mesenchymal markers), CD31 and CD34 (endothelial markers), the monocytic marker CD14 and the pan-haematopoietic marker CD45 (anti-CD105 antibody from AbD Serotec, www.abdserotec.com, all others from Becton Dickinson Bioscience, www.

bdbiosciences.com), as previously described (Gronthos *et al.*, 2001; Güven *et al.*, 2011).

### Frequency of clonogenic cells
The ratio of colony forming unit-osteoblasts (CFU-o) to the total number of formed colonies (colony forming unit-fibroblasts, CFU-f) (Friedenstein *et al.*, 1970; Baksh *et al.*, 2003) was determined by plating 100 SVF cells/well into six well plates. Cells were cultured with CM or osteogenic medium (OM), consisting of CM supplemented with 100 nM dexamethasone, 10 mM beta-glycerophosphate, and 0.05 mM ascorbic-acid-2-phosphate (Sigma-Aldrich, www.sigmaaldrich.com) for 14 d, in the presence or absence of the indicated concentration of rhBMP-2 (produced in CHO cells by R&D Systems, www.rndsystems.com). CFU-o were defined as colonies stained positive for alkaline phosphatase (ALP) activity, using a commercially available kit (104-LL kit, Sigma-Aldrich). The CFU-o/CFU-f ratio was determined following counter staining with buffered neutral red solution (N6264, Sigma-Aldrich), which allowed counting of the total number of CFU-f.

In vitro stimulation with rhBMP-2
SVF cells were plated on tissue culture plastic and grown to confluence in the presence of CM. Cells were then cultured for 14 d with either CM or OM, alone or further supplemented with either 50 or 500 ng/mL rhBMP-2 (produced in bacteria as previously described (Weber *et al.*, 2002), hereafter referred to as *own-produced*, or produced in mammalian CHO cells by R&D Systems) and analysed by reverse transcriptase real time polymerase chain reaction (RT-rt-PCR). Cells were then treated with lysis buffer (Qiagen, http://www.qiagen.com) enriched with 1/100 (V/V) β-mercaptoethanol (Sigma-Aldrich). RNA was extracted by using a NucleoSpin® RNA II kit (Macherey-Nagel, http://www.mn-net.com). The RNA was eluted in RNase-free water and transcription into cDNA was performed as previously described (Barbero *et al.*, 2003). The samples were analysed by using a GeneAmp® PCR System *9600 (Perkin Elmer, www.perkinelmer.com*) and the transcription levels of osteopontin (OP) and osteocalcin (OC) quantified, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as reference housekeeping gene (Frank *et al.*, 2002). SVF cells were similarly plated on tissue culture plastic, grown to confluence and cultured for 7 d with CM, alone or further supplemented with 500 ng/mL BMP-2 (R&D Systems). Cells were then detached with trypsin (Invitrogen) and analysed by cytofluorimetry with fluorochrome-conjugated antibodies to ALP and OC (both from R&D systems, www.rndsystems.com).

Generation and assessment of SVF cells-fibrin-ceramic constructs
One or four millions SVF cells were suspended in the fibrinogen phase (30 µL) of a polymerising fibrin gel (Tisseel®, Baxter, www.baxter.com), as described previously (Bensaid *et al.*, 2003; Müller *et al.*, 2010), with or without addition of 250 ng of rhBMP-2 (*own-produced* or from R&D Systems). Briefly, following mix with the
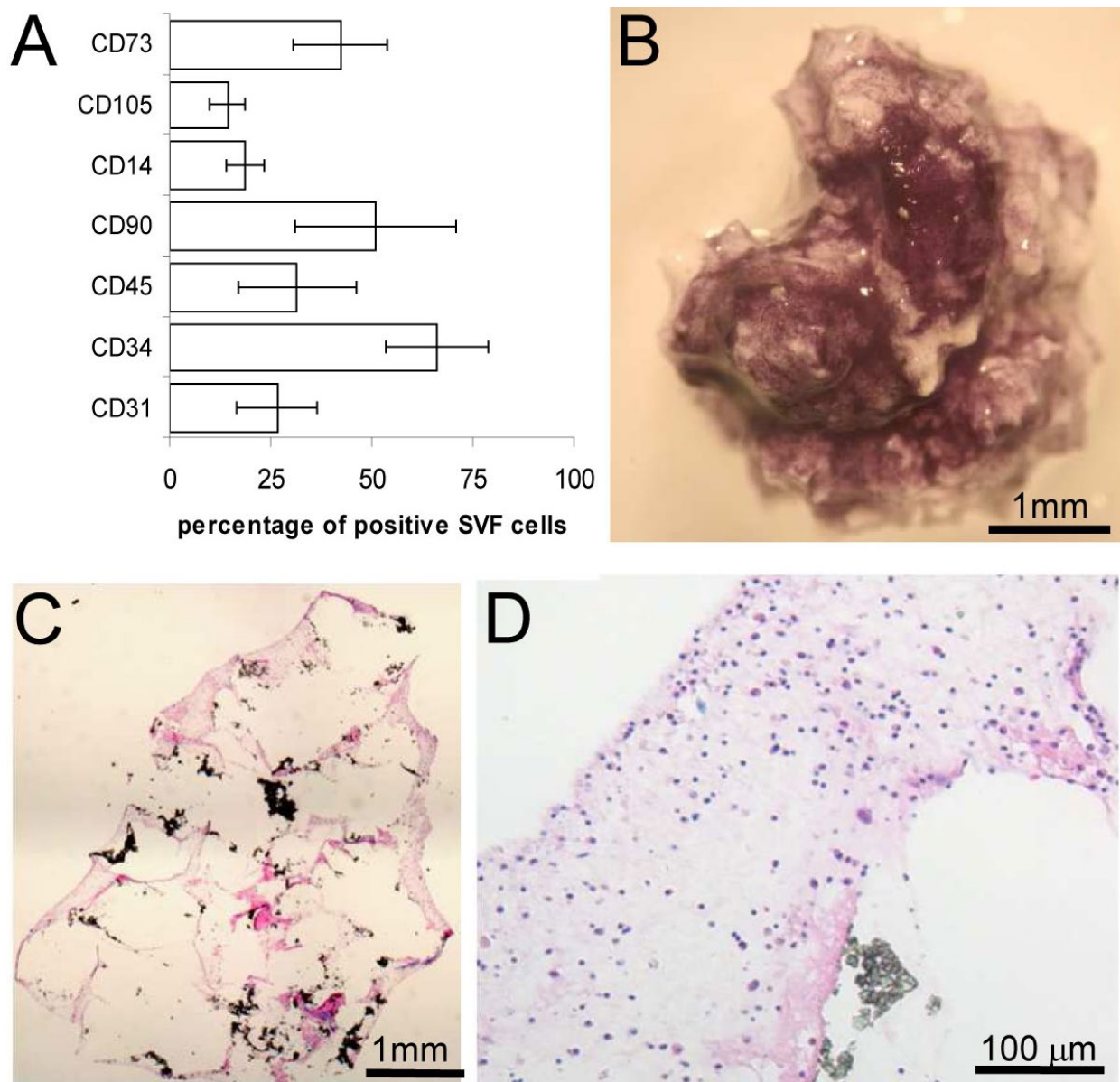
Fig. 1. Characterisation of cells and constructs *in vitro*. **(A)** Cytofluorimetric analysis of freshly-isolated SVF cells derived from 7 donors. For every CD marker, the average percentage of cells positive for the marker is plotted. Error bars represent standard deviations. **(B)** Representative picture of a tetrazolium-based metabolic staining (MTT assay) performed on SVF cells-fibrin gel-ceramic granules constructs to demonstrate the distribution of viable cells. **(C and D)** Macroscopic (C) and microscopic (D) pictures of haematoxylin/eosin staining performed on histological sections of decalcified, paraffin-embedded samples.

thrombin phase (30 µL), the solution was poured onto a volume of approx. 0.06 cm^3 of hydroxyapatite granulates of 1-2 mm size (Actifuse® ABX, ApaTech, www.apatech. com) pre-stacked in the wells of a 96-well plate. After 1-2 min, when the gels polymerised, the volume of the final constructs was 0.1 cm^3. Those constructs were covered with CM and transferred into a humidified incubator (37 °C, 5 % CO$_2$) for 10 min. Directly after fabrication, some constructs were incubated for 2 h at 37 °C in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) solution at a final concentration of 0.05 mg/ mL and the distribution of the blue/purple metabolised substrate of MTT was inspected macroscopically to assess cell viability. Other constructs were fixed overnight in 4 %

formalin, paraffin-embedded, sectioned and stained with haematoxylin/eosin (H&E) for qualitative assessment of the spatial distribution of the seeded cells. The remaining constructs were implanted in nude mice as described below.

***In vivo* implantation in nude mice and explant analysis**
The maintenance, surgical treatment and sacrifice of animals were performed in accordance with the guidelines of the local veterinary agency ("Kantonales Veterinäramt Basel-Stadt", permission #1797). Constructs were implanted in the subcutaneous tissue of nude athymic mice (CD1 nu/nu, Charles River, www.criver.com) and harvested after eight weeks following mouse sacrifice
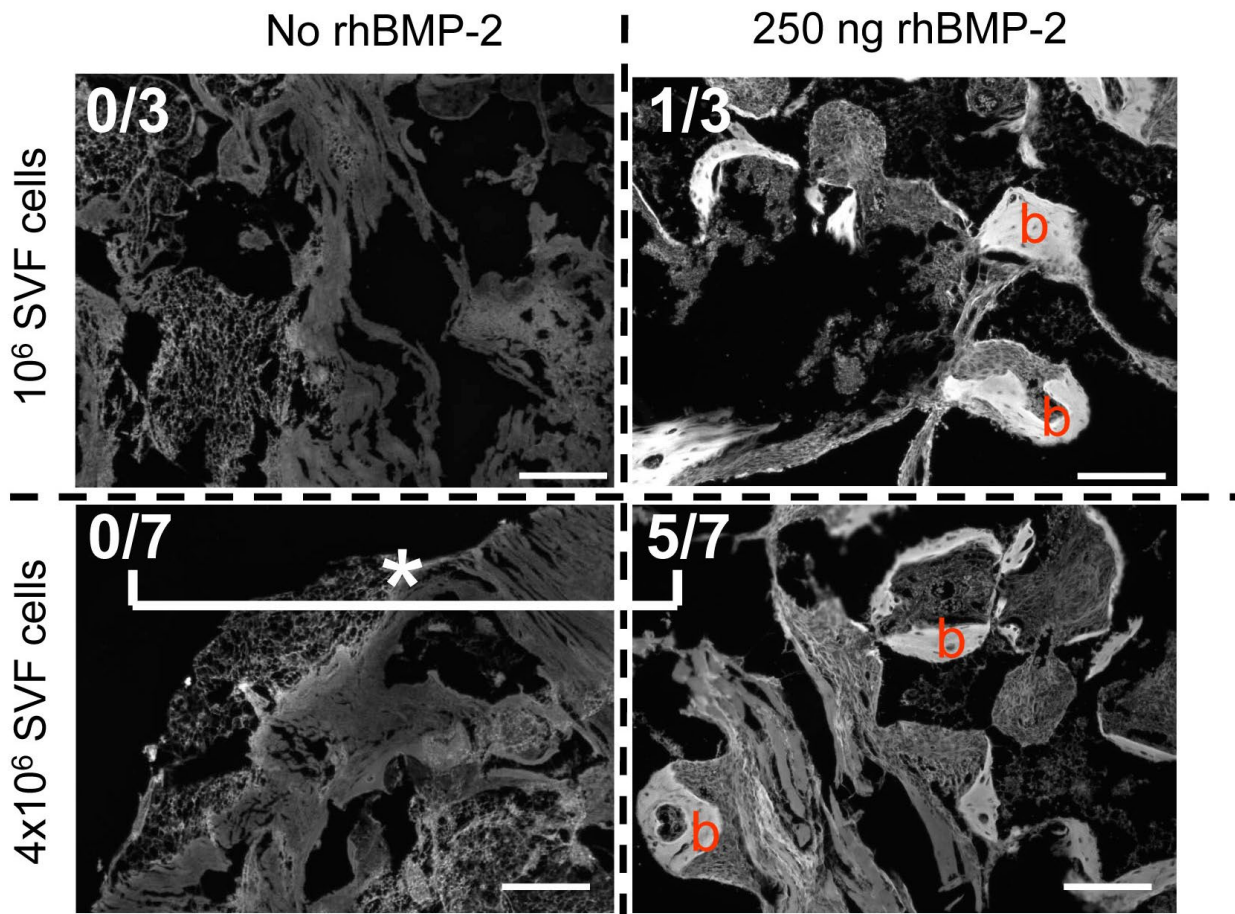
Fig. 2. Comparison of *in vivo* bone formation. Representative fluorescence microscopy pictures of histology sections of explanted, fixed and decalcified constructs. Experimental conditions are indicated in the figure. The values provided for each experimental condition is the ratio of donors exhibiting bone formation *in vivo* by the total number of donors tested. Scale bars represent 200 μm and (b) indicates bone tissue. * indicates a significant difference ($p < 0.05$) in bone formation as tested by Kruskal-Wallis test followed by *post-hoc* Dunn's tests.

by inhalation of CO_2. Tissues were fixed in 4 % formalin overnight, subjected to slow decalcification in 7 % w/v EDTA and 10 % w/v sucrose (both from Sigma-Aldrich) at 37 °C on an orbital shaker for 7-10 d and paraffin-embedded. Samples were then cross-sectioned (12 μm thickness) and processed for histological, histochemical and immunohistochemical stainings as follows. Standard H&E staining and Masson's trichrome staining (Kit Trichrome de Masson-Vert lumière, Réactifs RAL, www.ral-diagnostics.fr) were performed to identify bone tissue formation and maturation stage. Safranin-O staining allowed investigating the presence of sulphated proteoglycans inside the construct, characteristic of cartilaginous tissue. Tartrate resistant alkaline phosphatase (TRAP) staining (leukocyte acid phosphatase kit, Sigma-Aldrich) was used to identify osteoclasts, while the presence of osteoblastic cells and osteoid structures was assessed by immunostaining for human bone sialoprotein (BSP, Immundiagnostik AG, www.immundiagnostik. com) (Minkin, 1982; Papadimitropoulos *et al.*, 2011). The presence of donor-derived, human blood vessels was demonstrated by immunostaining with a biotin-conjugated antibody for human CD34 (Abcam, www.abcam.com), as previously described (Scherberich *et al.*, 2007). All human

cells in the explants were identified by chromogenic *in situ* hybridisation for the human-specific sequence ALU, using a biotin-conjugated DNA probe (ZytoVision, http://zytovision.com), as previously described (Müller *et al.*, 2010; Roy-Engel *et al.*, 2001).

Results

The percentage of CFU-f in the SVF preparations from different human adipose tissue samples averaged 14.7 ±6.8 % ($n = 4$). The fractions of different SVF subpopulations were highly variable across different donors, as assessed by the large standard deviations in the percentage of positive cells for different typical surface markers (Fig. 1A) and in accordance with previous reports (Müller *et al.*, 2009; Müller *et al.*, 2010). Once embedded in a fibrin gel around ceramic granules, SVF cells were viable and homogenously distributed throughout the construct, as evidenced by MTT metabolic staining (Fig. 1B). The structure of the construct was investigated by H&E staining of sections of decalcified samples (Fig. 1C and D). It allowed visualising the structural components of the constructs prior to implantation, including the fibrin
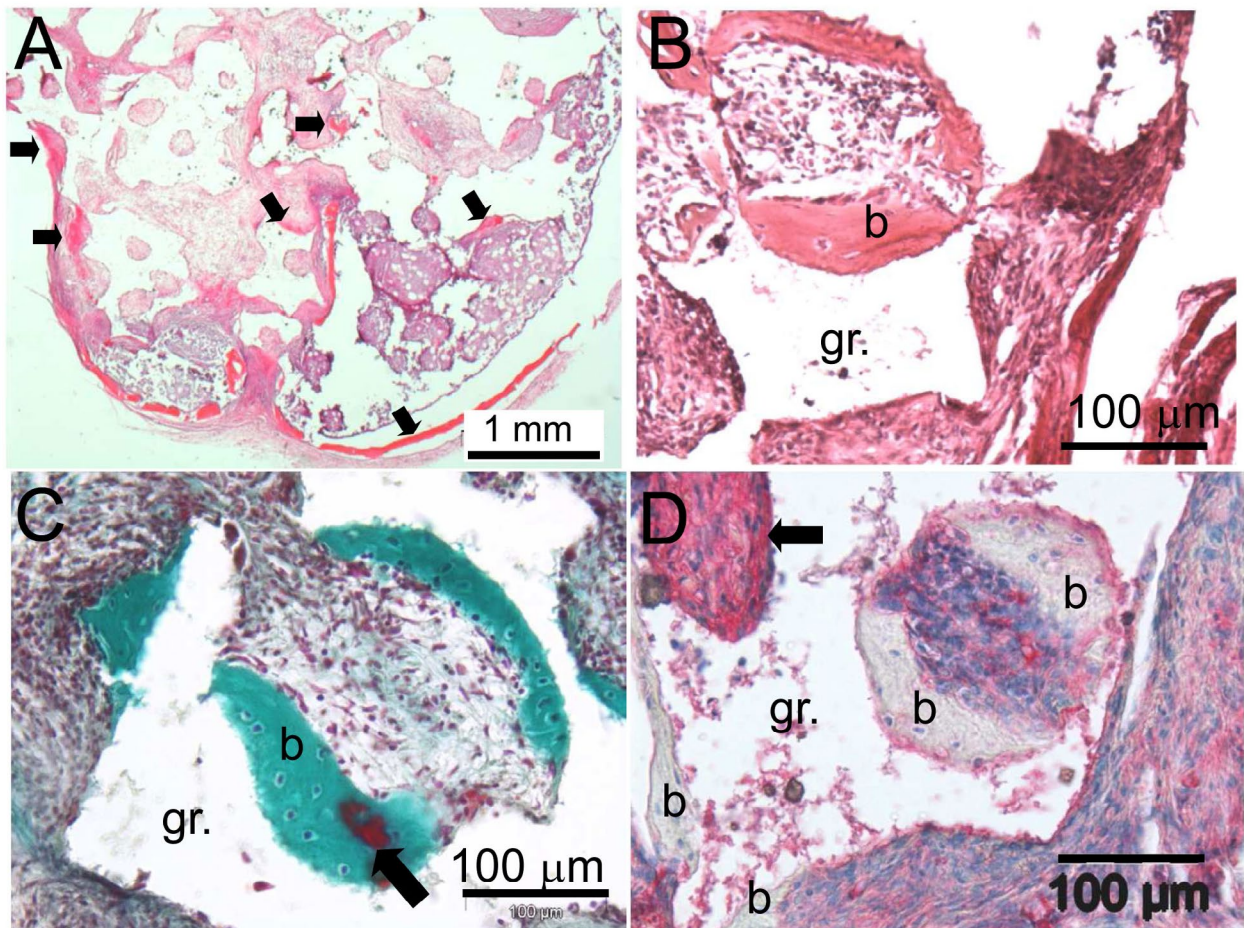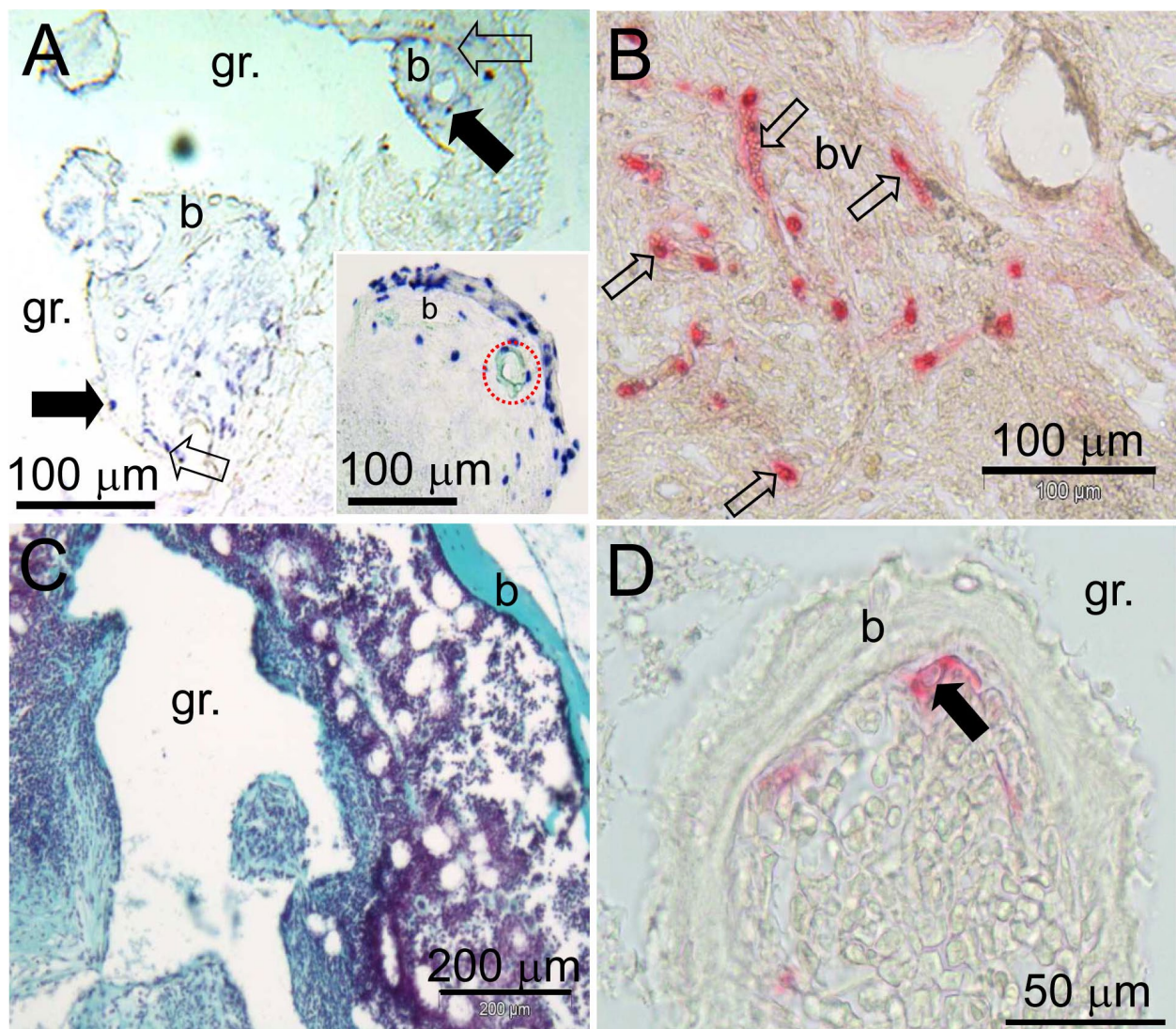
Fig. 3. Characterisation of newly-formed bone tissue. Histological analysis of sections of explanted, fixed and decalcified constructs seeded with 4 x 10^6 SVF cells and rhBMP-2. **(A)** Haematoxylin/eosin. Arrows show newly-formed bone tissue both in the pores of granules and in a shell around the construct. **(B)** Higher magnification of the same sections showing compact bone matrix (b) and osteocytes embedded therein. **(C)** Masson's trichrome staining. The green dye stains dense collagenous matrix identifying bone tissue (b) in contact with the ceramic granules (gr.), with various stages of maturation, in particular zones with red staining characterising elastic proteins (arrows). **(D)** Immunostaining for BSP. The arrow indicates a zone with BSP-positive osteoblastic cells, where initial bone formation is on-going. Areas with mature bone tissue (b) do not contain osteoblastic cells.

gel (pink stain in Fig. 1C and D), the embedded cells (blue stain in Fig. 1D) and the porous ceramic granules (void spaces in decalcified samples, Fig. 1C).

In the absence of incorporated BMP-2 and independent of the initial cell density (1 or 4 million SVF cells per 100 mm^3 construct), constructs explanted after subcutaneous implantation for 8 weeks in nude mice did not display evidence of frank bone tissue formation in any specimen, as assessed by H&E staining (data not shown) and by fluorescence microscopy (Fig. 2, left column) of sections taken at different depths. These findings are in accordance with our previously published results (Müller *et al.*, 2010). The addition of 250 ng of rhBMP-2 inside the fibrin gel in conjunction with 4 million SVF cells, resulted in the formation of ectopic bone tissue, at a frequency (5 out of 7 donors) which was significantly different from the corresponding condition in the absence of rhBMP-2 (0 out of 7 donors) (Kruskal-Wallis with *post-hoc* Dunn's test). The use of a lower number of cells (1 million SVF cells) in the presence of rhBMP-2 also resulted in bone tissue formation, at a frequency (1 out of 3 donors) which was

not significantly different from the one obtained with the higher cell number (Fig. 2).

Bone tissue formation was confirmed by H&E staining both around the entire construct, in between the ceramic granules and within their pores (Fig. 3A, black arrows). Bone tissue displayed the typical features of an 'ossicle' structure, including a dense collagenous matrix with embedded osteocytes and a rim of osteoblasts in contact with the osteoid tissue (Fig. 3B). Masson's trichrome staining further qualified that the bone tissues was at various stages of maturation, with local spots of red stained regions, indicating the presence of elastic proteins and characteristic of a more mature bone tissue (Fig. 3C, black arrow). Neighbouring already developed bone ossicles, areas of pre-osteoid tissue were also identified by positive immunostaining for bone sialoprotein (Fig. 3D, black arrow). As a control group, implantation of ceramic-fibrin-rhBMP-2 constructs without cells resulted in a merely fibrous tissue with no evidence of bone formation (data not shown).

Fig. 4. Characterisation of the contribution of SVF cells to tissue formation. Histological analysis of sections of explanted, fixed and decalcified constructs seeded with 4 x 10⁶ SVF cells and rhBMP-2. **(A)** *In situ* hybridisation for human-specific ALU sequences. Osteocytes (black arrows) and lining osteoblasts (open arrows) of human origin are identified by their nuclear ALU staining. Inset shows ALU nuclear staining of human endothelial cells at the level of human capillaries (red dashed line). **(B)** Immunostaining for human CD34 shows human blood vessels (bv, open arrows), filled with erythrocytes. **(C)** Safranin-O staining with no specific red staining indicative of sulphated proteoglycans and therefore of the generation of cartilaginous tissue inside the constructs. **(D)** TRAP staining showing the presence of multinucleated, osteoclastic cells in contact with newly formed bone (arrow). (b) indicates bone and (gr.) ceramic granules.

In order to study the contribution of implanted human SVF cells to the formation of tissue inside the construct, *in situ* hybridisation for human-specific ALU sequences was performed. Cells of human origin were identified both embedded within the bone matrix (putative osteocytes, black arrow in Fig. 4A) as well as at the bone matrix deposition front (putative osteoblasts, open arrow in Fig. 4A). ALU staining was also positive in the lumen of capillaries (putative endothelial cells, red circle in inset, Fig. 4A). The contribution of human vascular cells from the SVF to blood vessel formation was further confirmed by immunostaining for human CD34 (Fig. 4B, open arrows). The presence of erythrocytes in the lumen of the human capillary structures demonstrated functional connection with the host vasculature. Negative safranin-O staining indicated the absence of structures containing cartilage-specific glycosaminoglycans (Fig. 4C). TRAP staining identified the presence of multinucleated cells, likely of host origin, in contact with the newly formed bone (putative osteoclasts, arrow in Fig. 4D), suggesting an active remodelling process.

We next investigated the effect of rhBMP-2 on the percentage of osteoprogenitors recruited *in vitro* within the SVF cell population and the level of osteogenic induction of those osteoprogenitors *in vitro*. The CFU-o/CFU-f ratios, representing the fraction of clonogenic SVF cells displaying osteogenic properties, were higher in OM than in CM, but were not affected by rhBMP-2 at both tested concentrations
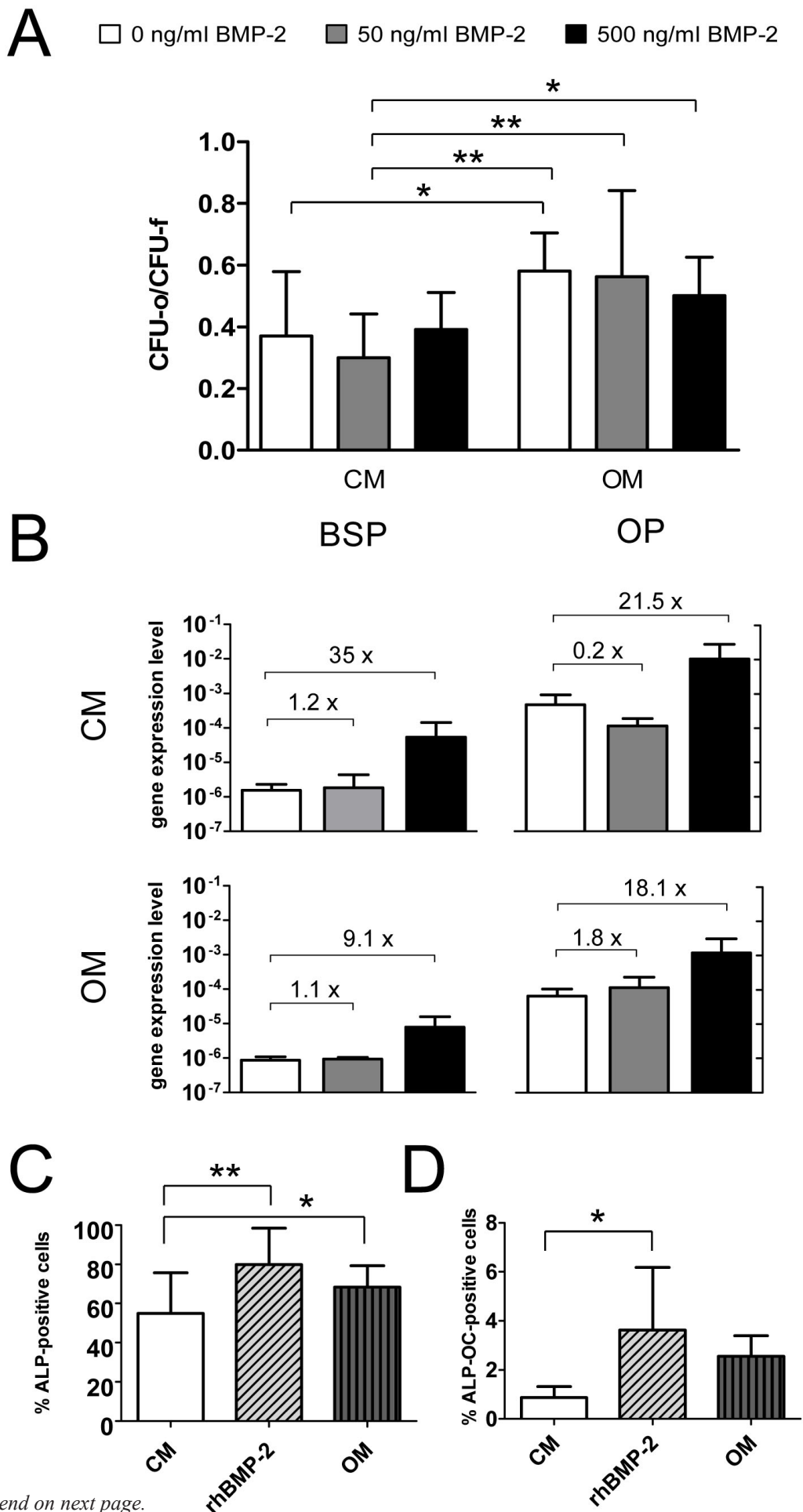
Fig 5. *Legend on next page.*

Fig. 5. (*on previous page*) *In vitro* effect of rhBMP-2 on human SVF cells. **(A)** Effect of 2 different doses of rhBMP-2 (50 and 500 ng/mL) on the CFU-o/CFU-f ratio of human SVF cells cultured with either complete medium (CM) or osteoblastic induction medium (OM). Experiments were performed in triplicates with cells from $n = 6$ independent donors. Average ±s.d. is represented. **(B)** Effect of 2 different doses of rhBMP-2 (50 and 500 ng/mL) on the expression levels of bone sialoprotein (BSP) or osteopontin (OP) mRNA. Results are represented as average ±s.d. of the ratio between marker expression levels and expression levels of GAPDH. Experiments were performed in duplicate with cells from $n = 3$ independent donors. **(C and D)** Effect of 500 ng/mL rhBMP-2 and OM on the percentage of cells expressing the osteoblastic markers alkaline phosphatase (ALP) **(C)** or ALP+osteocalcin (OC) **(D)**. Experiments were performed with cells from $n = 5$ independent donors. Average ±s.d. is represented. * and ** indicate statistically significant differences ($p < 0.05$ and $p < 0.01$, respectively) as tested by one-way ANOVA test followed by *post-hoc* Newman-Keuls tests.

(Fig. 5A). The level of osteogenic induction of SVF cells *in vitro* was assessed by the mRNA expression of osteoblastic markers, namely BSP and OP. The expression of these genes was not affected by medium supplementation with 50 ng/mL rhBMP-2, whereas it was consistently enhanced by the use of 500 ng/mL rhBMP-2, independently of the use of CM or OM (Fig. 5B). To confirm this trend in the effect of rhBMP-2 on the differentiation of the osteoprogenitors, the effect of 500 ng/mL rhBMP-2 on cells cultured with CM was tested by cytofluorimetry and compared to untreated cells (negative control) and cells cultured with OM (positive control). RhBMP-2 significantly increased the differentiation of osteoprogenitors (one-way ANOVA test followed by *post-hoc* Newman-Keuls tests), at levels similar to OM, based on the expression of ALP (Fig. 5C) and on cells co-expressing ALP and OC (Fig. 5D).

All results generated with own-produced or R&D-produced rhBMP-2, as specified in the Methods section, displayed consistent trends and no difference in efficiency at the same tested concentrations.

Discussion

This study validates an intraoperative manufacturing concept for the generation of grafts with osteogenic/vasculogenic potential derived from human adipose tissue. The formation of bone tissue was shown to require the delivery of a low dose of rhBMP-2, which could not induce ectopic ossification by itself. The reproducibility of bone tissue formation might well be improved by increasing the density of implanted SVF cells, which not only supported but directly contributed to bone tissue formation. The *in vitro* results suggest that the mode of action of rhBMP-2 did not involve an increase in the percentage of SVF cells recruited to the osteogenic lineage, but rather a stimulation of the osteoblastic differentiation of the committed progenitors.

Previous reports demonstrated that SVF cells, freshly isolated from adipose tissue and immediately implanted, can enhance bone healing in orthotopic experimental animal models (reviewed in Scherberich *et al.*, 2010). Autologous SVF cells have also been used in an intraoperative approach in a few clinical cases, demonstrating safety and a favourable clinical outcome (Lendeckel *et al.*, 2004; Pak, 2011). However, in all these studies, the direct contribution of the implanted cells

to bone formation was not addressed and therefore the intrinsic osteogenic capacity of freshly harvested SVF cells had not yet been demonstrated. The experimental setup used in the present work, namely an ectopic implantation site in a nude mouse model, allowed us to investigate the fate and mode of action of the implanted human SVF cells and therefore to conclude that SVF cells can directly form bone tissue, but only when stimulated *in situ* by rhBMP-2.

The induction of bone formation by BMPs *in vivo* was described for the first time in 1965 (Urist, 1965). Numerous *in vitro* and *in vivo* studies have later demonstrated enhanced bone repair by rhBMP-2, which is now FDA-approved in spinal, trauma and maxillo-facial surgery (Govender *et al.*, 2002; Hsu and Wang, 2008; Jones *et al.*, 2006; Smith *et al.*, 2008). The clinical use of rhBMP-2 is based on the principle of induction of osteogenesis by resident precursor cells and requires very high and non-physiological doses, which have been reported to be associated with aberrant bone formation (Deutsch, 2010), neurotoxicity (Smith *et al.*, 2008) or cancer development (Carragee *et al.*, 2011). As compared to commercially available products, which contain 1.5 mg/mL rhBMP-2, the concentration used in the present study (2.5 µg/mL construct) was about three orders of magnitude lower. The dose, which to the best of our knowledge is lower than the minimal one ever reported for stimulation of adipose-derived cells *in vivo* (Jeon *et al.*, 2008), was not intrinsically associated with osteoinductivity and supported bone formation only by acting in concert with the implanted cells. Moreover, it cannot be excluded that the combination of implanted human cells and rhBMP-2 could have recruited resident cells from the host which may have directly contributed to bone formation in conjunction with implanted bone-forming cells.

The actual osteogenic responsiveness of mesenchymal stromal cells to BMPs, in particular for osteoprogenitors derived from adipose and bone marrow tissues, is still controversial. Indeed, while some groups reported no significant difference of bone formation after addition of rhBMP-2 (Chou *et al.*, 2011; Diefenderfer *et al.*, 2003; Osyczka *et al.*, 2004; Zuk *et al.*, 2011) or transfection with hBMP-2 gene (Peterson *et al.*, 2005), some others reported induction of bone repair by BMP-2 stimulation of adipose-derived osteoprogenitors (Jeon *et al.*, 2008; Lee *et al.*, 2010). This discrepancy may result from factors such as the high inter-donor variability, the variety of animal models and experimental settings, as well as the doses of rhBMP-2

used (Zara *et al.*, 2011). Also age and sex of the donor seem to influence the osteogenic potential of osteoprogenitors (van Harmelen *et al.*, 2003; Zhu *et al.*, 2009), as well as their responsiveness to rhBMP-2 (Kim *et al.*, 2008). Our *in vitro* results indicate that rhBMP-2 specifically stimulated the osteoblastic differentiation of SVF cells. No effect of rhBMP-2 on adipose-derived cell differentiation was seen with 50 ng/mL, confirming a previous report (Zuk *et al.,* 2011) showing no effect at doses ranging 10-100 ng/mL. We however demonstrated in this study, both at the gene expression and protein expression level, that a 500 ng/mL concentration stimulated osteoblastic differentiation of adipose-derived cells. For the *in vivo* study, we used a cumulative dose of rhBMP-2 (2.5 µg/cm^3 construct) which was in the same range as the total amount of rhBMP-2 supplemented *in vitro* for the culture duration of 2 weeks (1.5 µg). However, considering the different release profiles (i.e., 3 repetitive doses for monolayer culture *in vitro, versus* burst release from the gel *in vivo*) and the different cell mixes (i.e., pure ASC *in vitro, versus* SVF cells containing mesenchymal, endothelial and haematopoietic populations *in vivo*), the effective concentration delivered to cells in the two models cannot be directly compared.

Both bacteria- and mammalian cell-derived rhBMP-2 were used in parallel in this study and demonstrated that the lack of response at 50 ng/mL was not due to the origin or glycosylation status of rhBMP-2. This confirmed our recent results showing that bacterial rhBMP-2 displays similar bioactivity for *in vitro* osteoblastic differentiation as compared to the medical grade, CHO cell-derived rhBMP-2 from Medtronic (Hanseler *et al.*, 2012). Interestingly, there have been promising results in enhancing bone formation by adipose tissue cells also by addition of vitamin D3 (Song *et al.*, 2011), alendronate (Wang *et al.*, 2010) or platelet-rich plasma (Liu *et al.*, 2008). Whether or not addition of these substrates, alone or in combination with rhBMP-2, could lead to a more reliable bone formation in the proposed setup will also have to be investigated. Finally, although rhBMP-2 was previously reported to have the potential to stimulate angiogenesis (Deckers *et al.*, 2002; Peng *et al.*, 2005), in our study the presence of human endothelial cells (positively stained for ALU sequences and for human CD34) in graft vascularisation did not appear to be increased in the presence of rhBMP-2 (data not shown).

Conclusion

This study reinforces the feasibility of an intraoperative use of autologous SVF cells for bone regeneration. The approach requires only one surgical procedure, similar to autologous bone grafting but clearly with reduced morbidity at the donor site. Moreover, it does not require extensive processing and culture of the isolated cells, thereby also reducing the costs and regulatory burdens otherwise associated with advanced cellular therapies. The use of low doses of rhBMP-2 was essential in the ectopic model to drive osteoblastic differentiation of SVF progenitors, but could be bypassed upon implantation at an orthotopic site, in the context of a bone environment where such physiological amounts of BMPs are likely already present. Recent work on the established interaction between the immune system and osteoprogenitor cell function (Liu *et al.*, 2011) also prompts for further studies in immunocompetent models with a proper onset of inflammatory processes. However, the introduction of alternative *in vivo* models requires the use of animal as opposed to human adipose-derived cells, which are known to have markedly different biological properties and osteogenic potential (Levi *et al.*, 2011) and thus would limit the potential clinical relevance of the generated findings. In this regard, one of the most compelling challenges in the routine clinical implementation of this approach is related to the large variability in phenotype and bone forming capacity of human adipose-derived cells from different donors (Scherberich *et al.*, 2007). Therefore, one additional effort will have to involve the identification of reliable quality control/potency markers of the implanted cells, in order to ultimately define the number of cells with a specific phenotype which should be introduced per unit of construct volume to ensure reproducible bone formation.

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Discussion with Reviewers

Reviewer I: These interesting findings need to be validated in bone defect models. In particular, the authors suggest that BMP-2 may not be required in orthotopic sites which could provide a favourable environment to promote osteoblastic maturation of MSCs. Likewise, we could assume that BMP-2 alone without cells in an environment rich in osteoblast progenitors may be sufficient to promote bone repair. These challenges could be addressed in bone lesions models in nude rats, which would allow the use of human MSCs.

Authors: This is a very good suggestion from the reviewer. We have indeed already initiated such a study in a femoral defect in nude rat to address this question. The study is currently in progress but preliminary data suggest that human SVF cells can generate bone tissue in the absence of BMP-2 in such a model. Comparison of the bone repair with SVF cells to acellular constructs loaded with only BMP-2 is also within the next series of experiments to be performed.