The possibility of using adipose tissue-derived stem cells (ATSC) as alternatives to bone marrow-derived stem cells (BMSC) for bone repair has garnered interest due to the accessibility, high cell yield, and rapid in vitro expansion of ATSC. For clinical relevance, their bone forming potential in comparison to BMSC must be proven. Distinct differences between ATSC and BMSC have been observed in vitro and comparison of osteogenic potential in vivo is not clear to date. The aim of the current study was to compare the osteogenesis of human xenofree-expanded ATSC and BMSC in vitro and in an ectopic nude mouse model of bone formation. Human MSC were implanted with biphasic calcium phosphate biomaterials in subcutis pockets for 8 weeks. Implant groups were: BMSC, ATSC, BMSC and ATSC mixed together in different ratios, as well as MSC primed with either osteogenic supplements (250 μM ascorbic acid, 10 mM β-glycerolphosphate, and 10 mM dexamethasone) or 50 ng/ml recombinant bone morphogenetic protein 4 prior to implantation. In vitro results show osteogenic gene expression and differentiation potentials of ATSC. Despite this, ATSC failed to form ectopic bone in vivo, in stark contrast to BMSC, although osteogenic priming did impart minor osteogenesis to ATSC. Neovascularization was enhanced by ATSC compared with BMSC; however, less ATSC engrafted into the implant compared with BMSC. Therefore, in the content of bone regeneration, the advantages of ATSC over BMSC including enhanced angiogenesis, may be negated by their lack of osteogenesis and prerequisite for osteogenic differentiation prior to transplantation.

**SIGNIFICANCE STATEMENT**

Adipose tissue-derived stem cells (ATSC) as a potential bone regenerative therapy has garnered widespread interest. In this study, significantly inferior osteogenesis and engraftment of ATSC were demonstrated in ectopic sites in nude mice compared with human bone marrow stem cells (BMSC). Conversely, ATSC showed enhanced angiogenesis compared with BMSC. These findings have implications for the use of ATSC in bone repair since their enhanced angiogenesis, high accessibility, high cell yield, and rapid in vitro expansion may be negated by their lack of osteogenesis and prerequisite of osteogenic differentiation prior to transplantation.

**INTRODUCTION**

Autologous bone grafts are compromised by the ensuing pain and morbidity associated with the bone harvest site. Tissue engineering strategies comprising synthetic biomaterial scaffolds with mesenchymal stromal stem cells (MSC) offer alternatives to overcome the limitations of autologous bone grafts. MSC isolated from the bone marrow (BMSC) [1] successfully initiate bone formation when transplanted into animals [2–4] and are being investigated in clinical practice for bone tissue repair [5, 6]. MSC derived from adipose tissue (ATSC) are receiving attention as a possible alternative to BMSC because they can be easily harvested by liposuction [7], the yield of ATSC from the same volume of adipose tissue is 500-fold greater [8], and they expand rapidly in vitro [9]. Many consider ATSC to have the same potential as BMSC since they bear all the hallmarks of stemness in vitro similar to BMSC, such as tri-lineage differentiation potential [10] and expression profiles for many surface markers [11, 12]. However, despite these similarities, further

**ABSTRACT**

Inferior In Vivo Osteogenesis and Superior Angiogenesis of Human Adipose-Derived Stem Cells Compared with Bone Marrow-Derived Stem Cells Cultured in Xeno-Free Conditions

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Key Words. Adipose stem cells • Bone marrow stromal cells • Bone • Tissue regeneration • Vascular development
Numerous studies have demonstrated that ATSC are capable of osteogenic differentiation in vitro [15, 16]. However, since bone formation encompasses multi-step coordinated participations from various cell types [4], a relevant assessment of bone forming capacity can only be gained by investigations in vivo. There are few clinical reports of bone reconstruction using ATSC. ATSC implanted ectopically with a ceramic scaffold coated with BMP-2 formed bone in a patient [17]; however, since BMP-2 alone induces bone formation [18], the bone formed in [17] cannot simply be attributed to ATSC. A second case reported ATSC mediated repair of skull defects in a 7-year-old [19]; however, it is not possible to attribute the bone healing to ATSC and not to the biomaterials alone, or indeed the rapid regenerative capacity of children. Finally, osteogenically differentiated ATSC showed healing in maxillary bone defects in patients [20].

Ectopic bone formation can be achieved by ATSC in ectopic sites when transplantation is preceded by either osteogenic pre-differentiation/priming [21–25] or by genetic manipulation with bone inducing genes [26]. However, while un-primed BMSC routinely form ectopic bone [2, 4], whether un-primed ATSC are capable of osteoinduction remains largely elusive, with only one study to date showing that unprimed ATSC can form bone in ectopic sites [21]. Others however showed that unprimed mice [27] and human [28, 29] ATSC could repair critical sized animal defects. Differences in passage number of MSC may contribute to dissimilar findings of bone forming potential since low passage numbers are vital in retaining the bone forming capabilities of MSC [30].

The lack of rapid vascularization within an engineered construct is a major hurdle in the healing of large bone defects and their translation into clinical therapies [31]. Therefore, strategies to enhance rapid angiogenesis within bone implants are crucial. Various studies investigated the potential of coculture systems, combining endothelial cells for their neovascularization potential with MSC for their osteogenic capacity [32]. A single source of human cells, having both osteogenic and angiogenic capabilities for clinical translation would be indispensable. Interestingly, ATSC have the ability to differentiate into endothelial cells, and improve neovascularization in vivo [33–35] to a larger extent than BMSC [36]. We therefore hypothesized that bone regeneration may be enhanced by the pro-angiogenic properties of ATSC.

Prior to the clinical use of ATSC for bone repair, it is necessary to show that their bone formation abilities are equivalent to BMSC. In this study, we characterized the in vitro osteogenic differentiation of BMSC and ATSC prepared in xeno-free conditions. The osteogenesis of expanded human BMSC and ATSC, in both passages 2 and 5, were then compared in an ectopic mouse model. Since no host osteogenic cells naturally reside in an ectopic site, the real osteoinductive capacity of implanted cells can be evaluated. This is the first study to evaluate bone formation from ATSC expanded in human platelet lysates (PL), which is used for the expansion of good manufacturing practice grade MSC for clinical use. We sought to quantify the degree of ectopic bone tissue formation, bone marrow presence, blood vessel density, and human cell engraftment. Finally, to evaluate the necessity of osteogenic priming of ATSC prior to implantation in order to achieve bone formation, we compared BMSC and ATSC that were primed with standard osteogenic supplements or bone morphogenetic protein 4 (BMP-4) prior to implantation.

**Biomaterial**

Granules of biphasic calcium phosphate (BCP), composed of hydroxyapatite and beta-tricalcium phosphate in a ratio of 20:80 in weight, ranging in size from 0.5 to 1 mm, were supplied by Biomatlante (Vigneux de Bretagne, France, http://biomatlante.com). The overall porosity (%vol) was 75 ± 5%, with a pore size distribution of 70% (0–10 μm), 20% (10–100 μm), and 10% (100 to 300 μm). Fifty milligrams aliquots of biomaterials were steam sterilized by autoclaving.

**Isolation and Characterization of MSC**

Bone marrow (BM) aspirates were obtained from the iliac crest of healthy donors, by standard puncture and aspiration in heparinized syringes. BMSC were isolated from mononuclear cells cultured at 1 × 10^5 cells per cm^2. ATSC were obtained by culturing the stromal vascular fraction (4 × 10^5 cells per cm^2) after collagenase digestion (NB4, ServaNordmark) of adipose tissue derived via liposuction as described previously [37]. Informed consent was obtained according to the Declaration of Helsinki and Ethical approval was received from the Ethical Committee of Toulouse University. In order to compensate for donor variability, BM of three human donors and AT of four human donors were used. Cells were expanded ex vivo in basal culture medium Alpha Minimum Essential Medium (α-MEM) supplemented with 100 U ml^-1 penicillin and 100 μg ml^-1 streptomycin, 8% pooled human PL, and 1 IU ml^-1 medical grade heparin. PL from several blood donors was prepared by IKT Ulm, as previously described [38]. PL was used as this permits a xenobiotic-free culture system, and we have previously shown that it can successfully maintain MSC expansion in vitro [2, 39, 40].

For phenotypic characterization of MSC, flow cytometry analysis was performed as previously described [39]. Briefly, cells were characterized by using the following antibodies: (a) CD105-FITC (clone 43A3), (b) CD90-FITC (clone SE10), (c) CD73-PE (clone AD2), (d) CD45, (e) CD34-PE (clone 4H11); (f) CD3-FITC (clone UCH1), CD73 was sourced from BD Biosciences (Le Pont de Claix, France, http://www.bd.com), CD3 was sourced from Beckman Coulter (Paris, France, https://www.beckman.com), while all others were purchased from BioLegend through Ozyme (Paris, France, https://www.ozyme.fr).

Tri-lineage differentiation capacity of expanded MSC was assessed. MSC differentiation was induced toward an osteogenic lineage by using standard osteogenic supplements (250 μM ascorbic acid, 10 mM β-glycerolphosphate, and 100 nM dexamethasone), while adipogenic or chondrogenic lineages were induced by using StemPro Adipogenesis Differentiation Kit and Chondrogenesis Differentiation Kit, respectively, (Gibco, Life Technologies, France, https://www.thermofisher.com). Mineralization was detected by alizarin red staining [39]; adipogenic differentiation was assessed by Oil Red O staining, while chondrogenic differentiation was detected by Alcian Blue staining.

**In Vitro Cell Growth and Osteogenic Potential of BMSC and ATSC Cultured with Standard Osteogenic Supplements**

MSC were plated at a seeding density of 5 × 10^3/cm^2 in 24 well plates. BMSC from three human donors and ATSC from four human donors were used in the current study for each experiment. Each donor was plated in triplicate for each measured
output. Seeded MSC were cultured for 24 hours in xeno-free basal media, after which they were cultured with or without the addition of standard osteogenic supplements for up to 21 days.

**Pico-Green Assay for DNA Content.** The quantification of cells was assessed at 1, 7, 14, and 21 days of culture. Cells were lysed by using a solution of 0.1% Triton X-100, 5 mM Tris-HCl, pH 8 and three freeze/thaw cycles. The quantity of double stranded DNA was measured in the supernatant of the solutions using a fluorescent Quant-IT PicoGreen dsDNA reagent assay kit (P7589, Invitrogen by Life Technologies, Saint Aubin, France, https://www.thermofisher.com) according to the manufacturer’s instructions. Fluorescent intensity was measured at 485 nm Excitation and 535 nm Emission on a microplate reader (Tristar LB 941; Berthold Technologies, Thoiry, France, https://www.berthold.com) and converted to ng of DNA by using a standard curve of known concentrations of a lambda DNA solution.

**Alkaline Phosphatase Measurement.** Extracellular alkaline phosphatase (ALP) was qualitatively evaluated by staining at days 7, 14, and 21 with a solution containing Fast Blue RR salt (Sigma Aldrich, Lyon, France, http://www.sigmaaldrich.com) and Naphthol AS-MX phosphates alkaline solution (Sigma Aldrich). Intracellular ALP was quantified at 1, 7, 14, and 21 days by using an ALP Colorimetric Assay Kit (Abcam, Cambridge, UK, http://www.abcam.com), on the same cell lysates that were used for DNA quantification. ALP enzyme converts the p-nitrophenyl phosphate (pNP) substrate to an equal amount of colored p-Nitrophenol (pNP). Colorimetric absorbance was measured at 405 nm using a microplate reader.

**Alizarin Red Staining for Detection of Mineralized Matrix.** Mineralization was quantified at 14 and 21 days by staining with 40 mM alizarin red solution (pH 4.1–4.3). The unbound stain was removed, and the bound stain was solubilized by using 10% (vol/vol) acetic acid. Ammonium hydroxide solution 10% (vol/vol) was added to neutralize the acid. Staining was enumerated by measuring samples in triplicate at an absorbance of 405 nm on a microplate reader and normalized to cell number using the measured DNA content per sample.

**Quantitative RT-PCR for Gene Expression of BMSC and ATSC Cultured with BMP-4.** P1-P2 passage BMSC and ATSC were cultured in 6 well-plates for 21 days in 50 ng/ml BMP-4, 10 mM β-glycerophosphate, and 50 mM ascorbic acid. Total RNA was extracted from MSC and purified by AllPrep DNA/RNA/Protein kit (Qiagen, Courtaboeuf, France, https://www.qiagen.com) and dosed by nanodrop (Thermo Fisher, Waltham, MA, https://www.thermofisher.com). RNA purity and integrity were checked using the Experion RNA Std Sens Reagents on the Experion automatized system (Biorad, Marnes-la-Coquette, France, http://www.bio-rad.com). All samples used for cDNA synthesis displayed a RNA integrity number (RIN) above 9.8. The reverse transcription (RT) was performed on 1 μg RNA by using high capacity cDNA reverse transcription kit (Applied Biosystem, https://www.thermofisher.com) and random hexamers in a final volume of 20 μl. The oligonucleotides for each target of interest, designed by using Primer Express software (PerkinElmer Life Sciences, Waltham, MA, http://www.perkinelmer.com) were used (forward and reverse) and listed in Supporting Information Table I. Quantitative PCR was performed on diluted cDNA (equivalent to 25 ng of starting purified RNA) using SSoFast EvaGreen Supermix (Biorad) with 500 nM of forward and reverse primers in a total volume of 20 μl on a CFX Real Time System (Biorad) following: 95°C, 3 minutes, and 40 cycles of denaturation (95°C, 10 seconds) and primers hybridization and amplification (60°C, 30 seconds). Each primer couple displayed interpretable PCR efficiencies (95%–105%). Melt curves and appropriate No RT control were used to validate amplification specificity. Data were analyzed on Bio-Rad CFX manager (threshold = 0.2), exported to DataAssist Software v3.0 (Applied Biosystems). Gene expression was calculated using the 2−ΔΔCT (or 2−ΔCT) method using Peptidylprolyl Isomerase A (PPIA) as appropriate reference gene which had the lesser M score.

**MSC/Biomaterial Implantation in the Subcutis of Nude Mice**

All animal experiments were performed according to Directive 2010/63/UE and after approval of protocols from the local ethical committee (CEEA, Pays-de-la-Loire, France). Cell groups, controls, and sample sizes are presented in Supporting Information Table II. In total, there were 6 to 8 implants per group, using cells from seven different human donors: three for BM and four for AT. These were the same donors as those used for in vitro experiments. Prior to in vivo implantations, BMSC and ATSC in passage 2 were cultured for 6 days in either basal media or media supplemented with osteogenic supplements (250 μM ascorbic acid, 10 mM β-glycerophosphate, and 10 mM dexamethasone) or 50 ng/ml rh BMP-4 (R&D systems, Minneapolis, MN, https://www.rndsystems.com). BMSC and ATSC were suspended 100 μl of basal media and mixed with BCP granules (2 × 106 cells/50 mg BCP for each implant) for 1 hour to allow cells to attach to the biomaterial. This cell/biomaterial ratio was previously determined to be optimal [2]. BMSC and ATSC were implanted separately or mixed together at the time of implantation in various ratios (9:1, 1:1, and 1:9). To evaluate the importance of passage number on bone formation and cell engraftment, a further group consisted of both BMSC and ATSC from the same donors as above, but in passage 5. Immuno-compromised nude female mice (RjOrl: NMRI-Foxn1nu/Foxn1nu), sourced from a professional breeder (Janvier Labs, Saint-Berthevin, France, https://www.janvier-labs.com) were placed under general anesthesia by inhalation of isoflurane, and two subcutaneous implants were placed on the dorsal side of each mouse. Animals were sacrificed at 8 weeks and following dissection, explants were fixed in 4% formal buffered solution.

**Histological Staining and Histomorphometry**

Explants were decalcified in a pH 7.4 solution containing 4.13% EDTA/0.2% PFA in PBS for 96 hours at 50°C using an automated microwave decalciﬁying apparatus (KOS Histostation, Milestone Med. Corp). Samples were dehydrated in ascending series of ethanol followed by butanol in an automated dehydration station (MicromMicrotech, Lyon, France). Samples were embedded in parafﬁn (Histowax; Histolab, Gottenburg, Sweden). Thin histology sections (3 μm thick) were stained by Masson trichrome technique. Slides were scanned (NanoZoomer; Hamamatsu, Photonics, Hamamatsu City, Shizuoka, Japan, http://www.hamamatsu.com) and observed by using the virtual microscope (NDP view; Hamamatsu). Histomorphometry of images was performed by using ImageJ software (National Institute of Health, Bethesda, MD). Four sections through each implant were analyzed and the percentages of biomaterial, bone, and BM were calculated per total area of explants.
Immunohistochemistry

Immunohistochemistry was performed on deparaffinized and rehydrated sections with the CD146 antibody (Rabbit monoclonal to CD146, ab75769, Abcam) which identifies pericyte cells in order to observe blood vessels. The section was pre-treated using heat mediated antigen retrieval with Tris-EDTA (pH 9) overnight at 60°C, followed by quenching of endogenous peroxidase activity by 3% hydrogen peroxide. Nonspecific binding sites were blocked with 2% goat serum, 1% bovine serum albumin (BSA). Sections were incubated with CD146 primary antibody overnight at 4°C, followed by incubation with goat anti-rabbit secondary antibody. The target antigen signal was amplified by using streptavidin peroxidase (DAKO, P0397). Diaminobenzidine (DAB) was used as the chromogen. All sections were dehydrated and counterstained using Gill’s hematoxylin and mounted using permanent mounting medium. The total number of blood vessels per implant was measured by using an automatic macro written in Image J and this was then normalized to the total cross-sectional area of the implants to give the number of blood vessels per mm². The automatic macro also calculated the average diameter of each blood vessel.

Cellular Engraftment at Implant Site

In Situ Hybridization. In situ hybridization using the human-specific repetitive Alu sequence, which encompasses approximately 5% of the total human genome was carried out to identify human cells. Sections were deparaffinized and rehydrated in graded series of ethanol and then treated with 20 µg/ml proteinase K (P2308, Sigma Aldrich, France), followed by tris buffered saline (TBS) tween 0.05% pH 7.6. Slides were treated with 3% H₂O₂ to block endogenous peroxidase activity. Sections were blocked with 1% BSA in TBS, followed by incubation with 2% BSA in TBS/tween for 25 minutes at RT. The hybridized probe was detected by immunohistochemistry using biotin-SP-conjugated IgG fraction monoclonal mouse anti-DIG (Jackson Immunoresearch, Baltimore, MD) diluted 1/200 for 1 hour at 37°C. Streptavidin peroxidase was added (1/800 in TBS tween 0.05%) for 1 hour at 37°C before DAB substrate addition (Dako). Sections were counterstained with Gill-2 hematoxylin (Thermo Shandon Ltd, Runcorn, U.K.) dehydrated, and mounted using Pertex (HistoLab Products AB, Sweden).

Quantitative Real-Time Polymerase Chain Reaction. Samples were lysed in DNA lysis buffer (Qiagen, France) and ground using the FastPrep-24 tissue and cell homogenizer (MP Biomedicals, Santa Ana, CA). Total DNA was isolated using QIAamp DNA Investigator Kit as described by the manufacturer (Qiagen, France). Human DNA was quantified by using human TaqMan Copy Number Reference Assay, RNase P (Applied Biosystems, France), and monitored with a 7500HT Fast Real-Time PCR System (Applied Biosystems). Results were normalized to that of time 0 as

\[ \Delta Ct = Ct_{time \_ t} - Ct_{time \_ T0} \]

and were expressed as the number of grafted hMSC per scaffold.

Statistical Analysis

MSC were plated in triplicate for each in vitro experiment and repeated for three different BM donors (n = 3) and four different AT donors (n = 4). A two-way analysis of ANOVA, with Sidak’s multiple comparison test was used to compare groups. For in vivo analysis, the same 3 BM donors and 4 AT donors were used. In vivo sample groups and sample sizes are presented in Supporting Information Table II. Kruskal-Wallis test with Dunn’s multiple comparison test was used to compare BM and AT groups. All statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc; La Jolla, CA) with statistical significance set as p < .05.

RESULTS

Phenotypic Characterization and Tri-Lineage Differentiation of ATSC and BMSC In Vitro

Characterization of BMSC and ATSC by flow cytometry is presented in Supporting Information Figure 1a. Both BMSC and ATSC displayed positive expression of stem cell surface markers CD 105, CD90, CD73, and negative for CD45, CD34, and CD3. Osteogenic, adipogenic and chondrogenic in vitro differentiation capacity of both BMSC and ATSC was confirmed by histochemical staining by alizarin red, Oil red O, or Alcian blue, respectively, (Supporting Information Figure 1b).

Superior Osteogenic Differentiation of ATSC Compared with BMSC In Vitro

As illustrated in Supporting Information Figure 2, proliferation of MSC progressed rapidly from days 1 to 7, and plateaued thereafter. It appeared that at all time points, the ATSC group had higher cell numbers compared with BMSC; however, this was not statistically significant.

Representative images of extracellular ALP production by BMSC and ATSC are shown in Figure 1A. In the BMSC group, ALP staining intensity increased from day 7 to 14 and it declined thereafter, and as expected there was also notably more staining in the osteogenic induced group compared with their undifferentiated control counterparts. ALP staining appears more intense and homogeneously spread in the ATSC compared with BMSC group. While osteogenic induction of ATSC did increase ALP staining, strikingly, the undifferentiated ATSC controls also showed significant ALP staining, and in particular more intense staining compared with their BMSC equivalents at all time points.

Intracellular ALP, quantified in cell lysates, and normalized to DNA quantity in the same sample, is presented in Figure 1B. As expected, osteogenic supplements enhanced ALP within cells. In keeping with the extracellular ALP results, intracellular ALP increased up to day 14 in the BMSC group and decreased thereafter. By day 21, intracellular ALP production by ATSC was significantly higher than BMSC, p < .05.

Representative images of alizarin red staining from each group are presented in Figure 1C, showing more intense mineralization in the ATSC group compared with the BMSC group. Quantitative data, obtained by solubilizing the stain, confirmed that ATSC had significantly more mineralization compared with BMSC at both days 14 (481 ± 143 vs. 181 ± 25 µg/well, p < .05) and 21 (739 ± 40 vs. 372 ± 24 µg per well, p < .05). Mineralization
normalized to DNA quantity in each group is presented in Figure 1D and similarly shows that ATSC had significantly higher mineralization compared with BMSC after 21 days of osteogenic induction (2.66 ± 0.95 vs. 1.32 ± 0.52 μg/ng DNA, p < .05).

Regarding osteoblastic gene markers, both BMSC and ATSC significantly increased their expression of bone ALP, RUNX2, OSTERIX (OSX), parathyroid hormone-receptor1 (PTHR1), Distal less homeobox 5 (DLX5), and Osteocalcin (OSC) when induced by BMP-4 (Fig. 2). Gene expression by osteogenically induced BMSC were significantly higher than those of ATSC however.

**Inferior Ectopic Bone Formation Potential of ATSC Compared with BMSC**

Eight weeks after implantation of passage 2 MSC, isolated from BM or AT, cross-sections through implants are shown in Figure 3A. BCP biomaterials alone showed no osteogenesis. In all BCP + BMSC implants (6/6 implants) there was abundant bone formation with mature BM present. Conversely, there was no bone formed in the BCP + ATSC group (0/8 implants). Figure 3B and 3C show that there was significantly more ectopic bone formation and BM presence in the BMSC group compared with BCP alone, BCP + ATSC, and the BCP + 1:9 BMSC/ATSC group (p < .05).

**Osteogenic Priming Initiates Ectopic Bone Formation by ATSC but It Remains Inferior to BMSC**

Figure 4A shows ectopic bone formation when BMSC and ATSC were either expanded in basal media or osteogenically primed prior to implantation and Figure 4B and 4C shows quantitative data of sections. Bone was formed in significantly higher quantities by BMSC compared with ATSC (p < .05) in undifferentiated, or those primed with osteogenic supplements or BMP-4 (Fig. 4B). Priming of BMSC with osteogenic supplements or BMP-4 showed no significant difference in bone quantity compared with undifferentiated BMSC. Priming ATSC with osteogenic supplements and BMP-4 yielded minute bone quantities in 1/8 implants and 2/8 implants, respectively.

**ATSC Enhance Neovascularization In Vivo**

All samples appeared well vascularized when analyzed after 8 weeks of implantation. Figure 5A shows CD146 immunostaining, showing blood vessels in brown. Figure 5B shows quantitative analysis of the number of blood vessels per mm² of implants. The addition of ATSC to BMSC in the mixed-cell type groups caused significantly increased neovascularization in these implants. Figure 6A shows CD146 immunostaining of groups with/without

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*Figure 1.* In vitro osteogenic differentiation of BMSC and ATSC when MSC were cultured with or without standard osteogenic induction media (250 μM ascorbic acid, 10 mM β-glycerolphosphate, and 100 nM dexamethasone) for up to 21 days. BMSC of three different human donors (n = 3) and ATSC of four different human donors (n = 4) were plated in triplicate for each time point. (A): Extracellular ALP secretion is shown qualitatively by Fast Blue RR salt and Naphthol AS-MX phosphates alkaline staining. (B): Intracellular ALP activity, data are presented as mean ± SD. (C): Mineralization was qualitatively assessed by alizarin red (AR) staining and representative images from each group are presented. (D): AR staining was solubilized and enumerated per well and normalized to DNA content, presented as mean ± SD. * indicates statistical differences (p < .05) between groups. Abbreviations: ALP, alkaline phosphatase; ATSC, adipose tissue-derived stem cells; BMSC, bone marrow-derived stem cells.
osteogenic priming. Figure 6B shows the quantitative analysis of this immunostaining, revealing that the unprimed ATSC group had significantly more \( p < .05 \) blood vessels present compared with the BMSC group. The average size of the blood vessels between groups did not differ (data not shown). With osteogenic priming, there was no significant difference in the number of vessels/area between the ATSC or BMSC groups.

Impact of MSC Passage Number on Ectopic Bone Formation and Cell Engraftment

While passage 2 BMSC formed ample bone in vivo (Figs. 3 and 4), we found that passage 5 cells from the exact same donors failed to form any ectopic bone in vivo (data not shown). There was no ectopic bone formed with either passage 2 or passage 5 ATSC. With regard to cellular survival and engraftment, when passage 2 BMSC and ATSC were implanted, in situ hybridization revealed that there were still human BMSC present at the implant site after 8 weeks, albeit a very minute portion compared with the quantity of host mouse cells; however, there were no human ATSC present after 8 weeks (Fig. 7A). With implanted passage 5 cells, rTPCR showed a major loss of human cells at the implant site, as soon as 24 hours, 70% of the cells had disappeared; however, no difference was seen between the BMSC and the ATSC. Then cells persisted on the biomaterial during the first week. At 4 weeks after implantation ATSC have all disappeared whereas a minute portion of BMSC are still present at the implant site (Fig. 7B).

**DISCUSSION**

Adipose tissue stromal cells are considered a favorable source of stem cells for tissue regeneration owing to their abundance and accessibility. This study shows that although ATSC achieve in vitro osteoblastic differentiation, and enhanced in vivo neovascularization compared with BMSC, passage 2 ATSC are significantly inferior in terms of their osteo-induction abilities in an ectopic environment in vivo. These findings may have implications for the use of ATSC in bone repair strategies as alternatives to BMSC.

BMSC and ATSC showed similar but not identical phenotypic characteristics by flow cytometry analysis. Although osteogenic gene expression was elevated by BMP-4 in BMSC compared with ATSC, ATSC showed increased ALP modulation and calcium deposition when induced by using standard osteogenic supplements. In spite of this no bone formation was observed with undifferentiated ATSC and it is important to note that the same human donors were used for both the in vitro and in vivo experiments. This supports the idea that in vitro osteogenic differentiation assays may not directly correlate with in vivo bone forming potential [41]. Whether the passage number of MSC affected their phenotypic characteristics or osteogenic potential in vitro was not evaluated in the current study.

In keeping with our previous studies, BMSC achieved ample bone formation with mature BM territories [2, 4]. This was cell passage dependent, with passage 2 cells forming abundant bone in vivo and passage 5 of the same donors completely losing this ability, which is in agreement with a previous study [42], but the cause is undetermined. The underlying exact mechanisms of bone formation remain to be elucidated.
formation by BMSC are unknown. However, based on our previous observations and those of others [2, 4, 43] we hypothesize that MSC release paracrine factors that recruit host cells to repair bone. The immune system and bone formation are inherently linked and we showed recently that implanted MSC recruit host monocytes to differentiate into macrophages and osteoclasts and then bone is formed by host MSC [4]. The exact mechanisms leading to the stark lower ability of passage 2 ATSC compared with BMSC in ectopic bone forming ability has not yet been investigated; however, it is in agreement with previous studies using cells of human [11, 44] and animal origin [45, 46]. There are differences in the secreted paracrine factors of BMSC and ATSC [47] and perhaps this plays a role.

To evaluate the bone-forming potential of harnessing the osteogenic capabilities of BMSC with the pro-angiogenic advantages of ATSC, BMSC, and ATSC were mixed together at the time

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**Figure 3.** Ectopic bone formation by BCP biomaterial with MSC in the subcutis of nude mice after 8 weeks. (A): Masson trichrome staining on sections through implants with BCP (gray) and MSC from three bone marrow donors (BMSC) and four adipose tissue donors (ATSC), or a mixture of both. Newly formed bone is evidenced in green. Scale bars: 1 mm for images (40 μm for image inserts). The bone incidence score on each group represents the number of implants with newly formed ectopic bone over the total number of implants in that group. (B): Histomorphometry quantification of bone and (C) of mature bone marrow was performed on four sections of every implant. * indicates statistical differences between groups (p < .05). Data are presented as mean ± SE of the mean. Abbreviations: ATSC, adipose tissue-derived stem cells; BCP, biphasic calcium phosphate; BMSC, bone marrow-derived stem cells.
of implantation in various different ratios. Although the addition of ATSC to BMSC gave rise to more vessels, no osteo-inductive advantage was garnered from mixing MSC of these different tissue origins.

Priming cells with standard osteogenic supplements prior to implantation made no difference to the in vivo bone formation by BMSC. However, the duration of osteogenic priming is paramount [48]; therefore, it is possible that the 6-day duration in the present study has surpassed the timing whereby osteogenesis is enhanced. Alternatively, the 8-week end time point may be too late to observe such a difference. Osteogenic priming of ATSC either with osteogenic supplements or BMP-4 prior to implantation imparted a minor bone forming ability to ATSC, in agreement with previous studies [22, 49]. The quantity of bone formed by osteogenic primed ATSC remained significantly lower than BMSC in all groups however. To the best of our knowledge there are only two other studies which compared the bone forming potential of unprimed and osteogenically primed ATSC and these produced conflicting findings [21, 50], whereby [21] found that unprimed hATSC were capable of osteoinduction in ectopic sites [21], in contrast to our findings here, and those of [50]. Importantly, no study to date compared ATSC to their BMSC counterparts, with or without osteogenic induction, as was the case in the current study.

While not exhaustive, there have been several studies which have evaluated the capacity of ATSC for bone regeneration in critical sized defects; however, a clear lack of consensus regarding the

Figure 4. Ectopic bone formation by osteogenically primed MSC in nude mice after 8 weeks. (A): Masson trichrome staining on sections through implants with BCP biomaterial (gray) and MSC from three bone marrow donors (BMSC) and four adipose tissue donors (ATSC) with or without 6 days of priming prior to implantation with either basal media or the addition of osteogenic supplements (250 μM ascorbic acid, 10 mM β-glycerolphosphate, and 10 nM dexamethasone) or 50 ng/ml rh BMP-4. Newly formed bone is evidenced in green, while bone marrow is observed in brown. Highlighted regions in red are magnified and shown to the right of each image of whole implants. Scale bars represent 1 mm and 50 μm for magnified images. The bone incidence score on each group represents the number of implants with newly formed ectopic bone over the total number of implants in that group. (B): Histomorphometry quantification of bone and (C) of mature bone marrow were performed on four sections of every implant with * indicating statistical differences between groups (p < .05). Data are presented as mean ± SE of the mean. Abbreviations: ATSC, adipose tissue-derived stem cells; BCP, biphasic calcium phosphate; BMP-4, bone morphogenetic protein 4; BMSC, bone marrow-derived stem cells.
osteoinductive potential of ATSC exists. Some showed bone healing by osteogenic primed animal ATSC [51–53] and BMP-2 transfected ATSC [54]. Studies that directly compare the potential of unprimed or osteogenically primed ATSC in skull bones are in agreement with the current study, whereby osteogenic priming is a prerequisite for achieving bone healing using either animal [55, 56] or human [57] ATSC. Furthermore, the need for osteogenic manipulation of ATSC prior to implantation seems to also extend to orthopedic sites, whereby osteogenically primed ATSC and genetically modified ATSC transfected with BMP-2 and RunX2 healed critical-sized defects in animals [60–62], and unprimed ATSC failed to heal critical-sized tibia defects in rabbits and sheep [58, 59]. Conversely, others show that unprimed animal [27] or human [28, 29] ATSC can induce bone formation in skull bones, and while the discrepancies between the latter studies and the current study remains undetermined, several factors may play a role. First, they are defect models, unlike ectopic sites, and perhaps the interplay of ATSC with resident cells in the defect somehow augmented their osteoinductive capacities; however, this does not explain the lack of osteoinduction by unprimed ATSC in other defect model studies [55–57]. Furthermore, although these studies use unprimed ATSC, they were cultured on apatite coated polymers prior to implantation [27, 28]. Apatite scaffolds have been shown to induce osteogenic differentiation of MSC without the addition of osteogenic supplements [63–66], although the 24-hour incubation [27, 28] may be too short to trigger any differentiation in this case. Finally, these studies culture ATSC in DMEM with FBS [27, 28], in contrast to our α-MEM with PL media, and were lower passage number (from passage 1) [27], compared with the present study. It may be possible that ATSC lose their bone induction abilities with culture in different serums or after passage 1 and explain the lack of osteoinductivity in the current study. Indeed, it has been shown that adipose floating spheroids cultured in serum-free medium contain cells that show

Figure 5. Neovascularization within implants of BCP with MSC in the subcutis of nude mice after 8 weeks. (A): CD146 immunostaining for pericytes on sections through implants with BCP biomaterial (gray) and MSC from three bone marrow donors (BMSC) and four adipose tissue donors (ATSC) or a mixture of both. Blood vessels are stained in brown. Scale bars represent 50 μm. (B): Histomorphometry quantification of the number of blood vessels/mm² with * indicating statistical differences between groups (p < .05). Data are presented as mean ± SE of the mean. Abbreviations: ATSC, adipose tissue-derived stem cells; BCP, biphasic calcium phosphate; BMSC, bone marrow-derived stem cells.
high in vitro osteogenic differentiation and in vivo bone formation capacity which is lost by expanding plastically adhered ATSC [67]. Whether any of these parameters are critical factors controlling the bone forming potential of ATSC warrants further investigation and there remains a shortage of studies using human ATSC. Very recently, a rare, long-term patient follow-up (6 years) of cranial defect reconstruction using autologous ATSC found all five patient outcomes unsatisfactory, and that no clear evidence that this technique induced bone healing, or that ATSC or their progeny formed new bone [68].

In this study, ATSC significantly increased neovascularization compared with BMSC. This is in agreement with findings from vascular ischemia studies [36] and may be due to their greater production of vascular endothelial cell growth factor compared with BMSC [69]. Since lack of vascularization is an important limitation of tissue engineered constructs, we hypothesized that increased angiogenesis would enhance bone formation. However, the addition of ATSC to BMSC in the mixed-cell groups increased angiogenesis compared with BMSC alone, but this enhanced angiogenesis did not translate to more bone formation. This indicates that ATSC may be more suitable as a cell therapy for ischemic diseases than for bone repair strategies.

The difference in the survival ability of implanted BMSC and ATSC found in the current study is interesting, and the cause is not known. Both ATSC and BMSC possess immunoregulatory properties [70–73]. The nude NMRI mice used in this study lack a thymus and therefore have a hugely reduced number of T cells, thereby
permitting the evaluation of human cells in this model. However, the natural killer cells (NK) are still present, and it was demonstrated previously that only BMSC inhibit NK cells cytotoxicity, not ATSC [74], which may permit the more sustained survival and activity of BMSC compared with ATSC found in this study. Whether this contributes to the striking difference in bone formation is unlikely however due to the very small number of remaining MSC and also since we showed that passage 5 BMSC also persist at the implant site but do not initiate bone formation. This would suggest that BMSC lose their capacity to release key bone initiating paracrine factors with passaging, a hypothesis that is currently under investigation.

CONCLUSION

This study has shown consistent and robust osteogenic differentiation of ATSC in vitro; however, this did not translate to in vivo findings which showed that ATSC are not osteoinductive, in contrast to BMSC. Osteogenic priming is a prerequisite for achieving ectopic bone formation by ATSC. Together, this data suggests that ATSC are not as suitable for bone regeneration applications as BMSC, particularly in terms of the isolation and culture procedures used in the current study. Nevertheless, ATSC do still hold the advantage of ease of harvesting, high yield, and improved neovascularization over BMSC and may therefore be advantageous for alternative cell therapy applications.

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AUTHOR CONTRIBUTIONS

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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