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***In vivo* formation of bone and hematopoietic territories by transplanted human bone marrow stromal cells generated in medium with and without osteogenic supplements**

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Abstract

Autologous transplantation of human bone marrow stromal cells (BMSCs) has been successfully used for bone reconstruction. However, in order to advance this approach into the mainstream of bone tissue engineering, the conditions for BMSC cultivation and transplantation must be optimized. In a recent report, cultivation with dexamethasone (Dex) significantly increased bone formation by human BMSCs *in vivo*. Based on this important conclusion, we analyzed the data accumulated by our laboratory where human BMSCs have been routinely generated using media both with and without a combination of two osteogenic supplements: Dex at 10⁻⁸M and ascorbic acid phosphate (AsCP) at 10⁻⁴M. Our data demonstrate that for 22 out of 24 donors, BMSC strains propagated with and without Dex/AsCP formed similar amounts of bone *in vivo*. Thus, human BMSCs do not appear to need to be induced to osteogenic differentiation *ex vivo* prior to transplantation. Similarly, for 12 of 14 donors, BMSC strains cultured with and without Dex/AsCP formed hematopoietic territories to a comparable extent. While Dex/AsCP did not increase bone formation, they significantly stimulated BMSC *in vitro* proliferation without affecting the number of BMSC colonies formed by the Colony Forming Units-Fibroblast. We conclude that for the substantial majority of donors, Dex/AsCP have no effect on the ability of BMSCs to form bone and myelosupportive stroma *in vivo*. However, due to increased BMSC proliferation, the total osteogenic population obtained from a single marrow sample is larger after cultivation with Dex/AsCP than without them. Secondary to increased BMSC proliferation, Dex/AsCP may stimulate bone formation if BMSCs and/or the transplantation system are less than optimal.

Keywords

human bone marrow stromal cells; *in vitro* cultivation; dexamethasone; ascorbic acid phosphate; *in vivo* transplantation; bone formation; hematopoietic territories

1. Introduction

Treatment of bone loss remains an area of concern for reconstructive surgeons. A potential alternative to the mainstay of therapy, autogenous bone graft, would be the use of

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Effect of osteogenic supplements on *in vivo* bone formation by human BMSCs

autologous culture-expanded osteoprogenitor cells, such as bone marrow stromal cells (BMSCs). BMSC populations include multipotential skeletal stem cells capable of differentiating into a repertoire of skeletal tissues, including bone, cartilage, hematopoiesis-supporting stroma, and adipocytes (Friedenstein et al., 1987, Friedenstein et al., 1974, Friedenstein et al., 1982, Owen, 1988, Owen and Friedenstein, 1988). BMSCs have been used to close defects in the calvarium and the long bones of experimental animals, and to augment bone deficiencies, such as those associated with mandibular atrophy (Bruder et al., 1998, Krebsbach et al., 1998, Kuznetsov et al., 2008, Mankani et al., 2006a, Mankani et al., 2006b). Autologous BMSCs were successfully used to treat bone defects in human patients (Kitoh et al., 2007, Krzymanski and Wiktor-Jedrzejczak, 1996, Marcacci et al., 2007, Osepjan et al., 1987, Quarto et al., 2001, Ueda et al., 2005), a novel bone engineering approach that has been steadily expanding.

For autologous human BMSC (hBMSC) generation, the starting material is often limited to a single bone marrow aspirate. In order to avoid additional, more extensive interventions, the techniques for BMSC *in vitro* propagation and *in vivo* transplantation need to be optimized. Recently, this approach seemed to receive an important boost by demonstration that hBMSCs pre-cultured in medium with an osteogenic supplement, dexamethasone (Dex), formed significantly more bone *in vivo* than BMSCs cultured without the supplement (Song et al., 2009).

For a number of years, our group has routinely generated hBMSC strains employing media both with and without a combination of two osteogenic supplements, Dex and ascorbic acid phosphate (AscP), and used these strains for *in vivo* transplantation. In the transplants, the amount of new bone was analyzed. In addition, the extent of hematopoietic territories was examined because skeletal stem cells, needed for bone maintenance during bone turnover, reside in the stroma that supports hematopoiesis (Owen and Friedenstein, 1988). Thus, the establishment of hematopoiesis-supporting stroma in conjunction with the new bone signifies the presence of multipotential skeletal stem cells in the transplanted BMSC populations (Bianco and Robey, 2004) and, consequently, the ability of the new bone to maintain itself. In this report, we formally compared *in vitro* proliferation and *in vivo* differentiation of hBMSC strains generated with and without Dex/AscP.

2. Materials and Methods

2.1. Bone Marrow Specimens

Surgical waste specimens of human bone, as well as iliac crest bone marrow aspirates and core biopsies, were obtained from donors ranging in age from 6 months to 76 years, in accordance with the NIH regulations governing the use of human subjects under protocol 94-D-0188 or OHRS Assurance #4165. In two cases, bones were received by necropsy. Age, gender, diagnosis, and site of harvest for donors used for comparative transplantation assay are shown in Table 1. For other experiments, such as *in vitro* proliferation studies, iliac crest biopsies from juvenile donors with scoliosis, and bone from the neck of the femur of adult donors undergoing degenerative hip replacement, were used.

2.2. Cell Suspensions

Single cell suspensions of bone marrow were prepared as described previously (Kuznetsov and Gehron Robey, 1996, Kuznetsov et al., 1997a, Kuznetsov et al., 1997b). Briefly, fragments of trabecular bone and marrow were scraped into α -modified Minimum Essential Medium (α MEM, Invitrogen, Grand Island, NY) with a steel blade. These marrow preparations, as well as bone marrow aspirates, were pipetted and consecutively passed through 16 and 19 gauge needles to break up cell aggregates. The resulting cell suspensions

were filtered through a nylon cell strainer (352350, Becton Dickinson, Franklin Lakes, NJ) to remove remaining cell aggregates.

2.3. Primary BMSC colony forming efficiency assay

Bone marrow single cell suspensions were plated in either triplicate or quadruplicate into 25 cm² filter cap tissue culture flasks (Nalge Nunc Intl, Naperville, IL) containing 5 ml of culture medium, at a clonal density of $4-8 \times 10^3$ cells/cm². Standard culture medium consisted of α MEM, 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin sulfate (all Invitrogen), and 20% fetal bovine serum of a pre-selected lot (Kuznetsov et al., 2009) (Atlanta Biologicals, Inc., Norcross, GA; Equitech-Bio, Kerville, TX; Valley Biomedical, Inc., Winchester, VA; HyClone, Logan, UT). The culture medium contained either no osteogenic supplements, or various concentrations of dexamethasone (Dex), ascorbic acid, or sodium- β -glycerophosphate (β -GP, all from Sigma, St. Louis, MO), or a combination of Dex at 1×10^{-8} M and L-ascorbic acid phosphate magnesium salt n-hydrate (AscP, Wako, Osaka, Japan) at 10^{-4} M. Cultivation was performed at 37 °C in a humidified atmosphere of 5% CO₂ in air, with no medium replacements. Cultures were harvested 11 to 13 days after plating, fixed with absolute methanol and stained with a saturated aqueous solution of methyl violet (Sigma). Colonies, initiated by the Colony Forming Units-Fibroblast (CFU-Fs) and containing 50 or more cells of fibroblastic morphology, were counted, and colony-forming efficiency (CFE, number of BMSC colonies per 1×10^5 marrow nucleated cells) was calculated.

2.4. BMSC proliferation assay

Single cell suspensions of bone marrow were plated into culture medium with or without Dex/AscP, either in triplicate or in quadruplicate, at $2.7 - 4 \times 10^4$ cells/cm², a density that considerably exceeds clonal densities used for the primary CFE assay. Full medium replacements were performed two days after plating and twice a week thereafter. Fourteen to 16 days after plating, the cells were detached with 0.05% Trypsin-EDTA (Invitrogen), and total cell numbers were calculated for each flask separately using the Vi-Cell™ XR Cell Viability Analyzer (Beckman Coulter, Inc., Fullerton, CA).

2.5. Generation of hBMSC strains

Marrow single cell suspensions from surgical waste were plated into 75 cm² filter cap tissue culture flasks (Corning, Inc., Corning, NY) at $2-6.7 \times 10^4$ cells/cm². Bone marrow aspirates were plated at $6.7 \times 10^4 - 6.7 \times 10^5$ cells/cm². On day 13 to 16, when large BMSC colonies were approaching confluence, the 1st passage was performed as described above. The subsequent passages, from 2nd to 4th, were performed in the same manner, 4 to 6 days apart. Starting with the initial plating of the original cell suspension and until the last passage, each individual BMSC strain was cultured in the same medium, either with or without a combination of two osteogenic supplements: Dex at 1×10^{-8} M and AscP at 10^{-4} M; this Dex concentration was 10 times lower than that used by Song and co-authors (Song et al., 2009).

2.6. Secondary BMSC colony formation assay

BMSC strains of passages 3 or 4 generated in medium with and without Dex/AscP were compared. The cells were plated into T-25 flasks in quadruplicate, at 40 cells/cm². After 11 days of cultivation in standard conditions, without medium replacements, the cultures were harvested and treated as described for the primary CFE assay. BMSC colonies containing 50 or more cells were counted, and secondary CFE was calculated as percentage of colony number from the number of cells plated.

2.7. In vivo transplantation assay

The differentiation potential of hBMSC strains generated in medium with and without Dex/AscP was compared by an *in vivo* transplantation assay (Krebsbach et al., 1997, Kuznetsov et al., 1997b). Briefly, $1.2 - 3.0 \times 10^6$ BMSCs of passage 2 to 4 were mixed with 40 mg of sterile hydroxyapatite/tricalcium phosphate (HA/TCP) powder (Zimmer, Warsaw, IN, particle size 0.5 – 1.0 mm) in 1.8 ml CryoTube tubes (Nunc, Roskilde, Denmark) containing 1 ml of culture medium. Previously, it was shown that for 40 mg HA/TCP transplants, the use of more than 1×10^6 hBMSCs did not result in any further increase in bone or hematopoiesis formation, indicating saturation of the HA/TCP surface (Mankani et al., 2007). In the current study, all transplants received cell numbers exceeding the 1×10^6 threshold. Cells were allowed to attach to the particles for 90 min at 37°C with slow rotation (25 rpm); the particles with attached cells were then collected by brief centrifugation (1,000 rpm, 1 min). Following this procedure, greater than 80% of BMSCs were shown to be attached to the HA/TCP particles (Mankani et al., 2001). The cell-particle mixtures were either implanted immediately, or they were first incorporated into a secondary matrix composed of a fibrin clot (Kuznetsov et al., 2000).

Each transplant was placed into a subcutaneous pocket in the back of a 8-15-wk-old immunodeficient female beige homozygous mouse (bg-nu/nu-xid, Harlan Sprague Dawley, Indianapolis, IN), and the incision was closed with 9-mm steel wound clips (Roboz Surgical Instrument Co., Inc., Rockville, MD). Surgeries were performed under isoflurane anesthesia in accordance to the specifications of an institutionally approved small animal protocol. The transplants were harvested at different time points post-surgery, usually at 8 weeks but in some experiments, from 3 to 16 weeks. They were fixed with fresh 4% phosphate-buffered formalin (Sigma), demineralized with 10% EDTA, pH 8.0 (Quality Biologicals, Inc., Gaithersburg, MD), cut into two halves along the longest axes, and embedded in paraffin with the cut surfaces towards the knife. Serial, 6 μ m thick sections were prepared in such a way that most areas of the transplant were represented on the slides. The hematoxylin and eosin-stained sections were examined histologically. The extent of bone within each transplant was scored on a semiquantitative, exponential scale from 0 to 4 in a manner described previously (Kuznetsov et al., 1997b):

score 0, no bone formation;

score 1, minimal bone formation, just a single or a few bone trabeculae in one or a few sections;

score 2, low bone formation, multiple bone trabeculae are in several parts of some sections but bone occupies only a small portion of the sections;

score 3, moderate bone formation, bone occupies a significant portion but less than one half of most sections;

score 4, abundant bone formation, bone occupies greater than one half of each section.

When the bone scores reported on this scale were directly compared to histomorphometric measurements of tissue sections, a high correlation was previously observed between the bone score and the square root of the fraction of bone area to total transplant area ($r = 0.973$) (Mankani et al., 2001). Using this scoring system, multiple whole sections of each transplant were visualized, rather than just a few isolated microscopic fields that, due to the irregular shapes of the particles, can be highly variable in terms of bone formation.

Similarly, the extent of hematopoiesis within each transplant was scored on a semiquantitative, exponential scale from 0 to 4:

score 0, no hematopoiesis;

score 1, minimal hematopoiesis, just a single or a few small areas in one or a few sections;

score 2, low hematopoiesis, hematopoietic areas are in several parts of some sections but they occupy only a small portion of the sections;

score 3, moderate hematopoiesis, areas of hematopoiesis occupy a significant portion but less than one half of most sections;

score 4, abundant hematopoiesis, fields of hematopoiesis occupy greater than one half of each section.

2.8. Determination of the origin of new bone

To determine the origin of the cells forming new bone, *in situ* hybridization for *alu*, human-specific repetitive DNA sequences, was performed as previously described (Kuznetsov et al., 2011).

2.9. Statistical analysis

Analysis of variance was performed and post-test comparison was done using either the Bonferroni multiple comparison test or unpaired t test (InStat, GraphPad, San Diego, CA). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. BMSC in vitro proliferation

For eight out of nine donors, primary CFE values lay within the normal human CFE limits shown earlier to range from 11.3 to 101.3 per 1×10^5 nucleated marrow cells (Kuznetsov et al., 2009) (Fig. 1 A-D). The osteogenic supplements, Dex, ascorbate, and β -GP, did not, at any concentration, raise CFE, but rather decreased, or even completely suppressed, hBMSC colony formation at higher concentrations (1×10^{-5} M for Dex, 50 to 250 mg/ml for ascorbate, and 100 mM for β -GP (Fig. 1 A, B)). A combination of Dex at 10^{-8} M and AsCP at 10^{-4} M caused no significant CFE changes in any of seven donors tested (Fig. 1 C, D). However, Dex/AsCP significantly increased total BMSC numbers in primary marrow cell cultures (Fig. 1 E). Because BMSC colony numbers were unaffected, this indicates that in medium with Dex/AsCP, individual colonies consisted of more cells than in medium without the supplements. Thus, Dex/AsCP increased the BMSC proliferation rate. Additionally, BMSC strains continuously cultured with Dex/AsCP retained more cells capable of secondary colony formation than strains grown without the supplements (Fig. 1 F).

3.2. In vivo formation of bone and hematopoietic territories

New bone was first observed 3 weeks post transplantation as thin, eosin-positive rims covering the vehicle particles. Seven- to eight week-old BMSC transplants from the majority of donors revealed either moderate (score 3) or abundant (score 4) bone (Fig. 2, Fig. 3 A, C). The new bone had a lamellar structure and was formed on the surfaces of HA/TCP particles. Osteocytes were seen embedded in bone matrix and osteoblasts lined the luminal surfaces of the bone (Fig. 3 A). Both osteocytes and osteoblasts stained positively for *alu*, human-specific repetitive DNA sequences (Fig. 3 D - F), demonstrating that the new bone had human origin and was, indeed, formed by implanted hBMSCs.

Tiny areas of hematopoiesis first appeared in 4.5-week-old transplants and grew rapidly in most experiments, such that by 7 to 8 weeks post-transplantation, hematopoietic areas of varying dimensions were seen in the majority of transplants, accompanying bone and always adjacent to it (Fig. 3 A). Areas of hematopoiesis were formed mostly in the transplants

featuring either abundant (score 4) or moderate (score 3) bone, rarely - in the transplants with low (score 2) bone, and never - in the transplants with minimal (score 1) or no bone. Hematopoietic areas displayed cells of all lineages of erythroid and myeloid differentiation, including megakaryocytes. The latter, together with the distinctive, immature myeloid cells of mouse origin, proved that it was, indeed, genuine *in situ* hematopoiesis, rather than a mere accumulation of hematopoietic cells. The network of stromal cells underlying the hematopoietic areas was barely distinguishable, with the exception of numerous adipocytes. The hematopoietic areas were penetrated by large, sinusoid-like blood vessels (Fig. 3 A). In addition to vehicle particles and areas of bone and hematopoiesis, the transplants featured variable amounts of fibrous tissue (Fig. 3 A – C). By 16 weeks post-transplantation, the latest time point of this study, no further increase in bone formation was observed, in agreement with previous observations (Mankani et al., 2007), while hematopoietic territories could grow slightly (Fig. 2A).

Neither the amount nor the morphology of new bone showed any noticeable differences based on BMSC passage number (from 2 to 4), the use of a secondary vehicle, fibrin clot, or the numbers of BMSCs per transplant (from 1.2 to 3 million). The latter result confirmed our earlier observation that for 40 mg HA/TCP transplants, 1×10^6 BMSCs represented a threshold, beyond which any further increase in cell number was not matched by increased bone formation (Mankani et al., 2007). Transplants of BMSCs from elderly donors (H-193, H-294) demonstrated abundant bone, as well as extensive hematopoiesis (Fig. 2 A), and were morphologically indistinguishable from the transplants from the majority of younger donors.

All donors were divided into 3 groups. The first, unaffected group consisted of 17 patients with no obvious marrow or bone pathology (Table 1 A, Fig. 2 A). The second group included four patients with aplastic anemia (Koh et al., 2010) (Table 1 B, Fig. 2 B), and the third group, three patients with two forms of lipodystrophy (Garg et al., 1999, Hegele, 2000) (Table 1 C, Fig. 2 C). Contrary to the majority of 7- to 16-week-old transplants from unaffected donors that featured moderate or abundant bone and good hematopoiesis (Fig. 2 A), the transplants from aplastic anemia and lipodystrophy patients lacked hematopoiesis and mostly presented low, minimal, or no bone (Fig. 2 B, C). Mean bone scores, both with and without Dex/AscP (Fig. 2), were significantly higher for the unaffected group than for two other groups ($p < 0.001$).

For 22 out of 24 donors, BMSC strains grown with and without Dex/AscP demonstrated comparable levels of bone formation (Fig. 2). BMSCs generated with Dex/AscP formed significantly more bone than those cultured without the supplements only for two aplastic anemia donors, H-103, and H-106 (Fig. 2 B). Similarly, for 12 out of 14 donors whose transplants featured hematopoiesis, BMSC strains generated with and without Dex/AscP formed hematopoietic areas of comparable extent (Fig. 2 A). BMSCs grown with Dex/AscP formed larger hematopoietic territories than those cultured without the supplements only for donors H-91 and H-95 (Fig. 2 A).

4. Discussion

While several clinical studies have demonstrated that new bone can be successfully created, and bone-related conditions successfully treated, by orthotopic autologous BMSC transplantation (Kitoh et al., 2007, Krzymanski and Wiktor-Jedrzejczak, 1996, Marcacci et al., 2007, Osepjan et al., 1987, Quarto et al., 2001, Ueda et al., 2005), this novel tissue engineering approach is far from being fully developed and broadly accepted. Both cultivation and transplantation of BMSCs have yet to be substantially optimized in order to ensure that the newly formed bone is of the best quality, clinically relevant, and robust to

meet therapeutic needs. These requirements can be particularly crucial if the starting material for BMSC cultures is limited, as is usually the case with autologous marrow aspirates. Any improvements leading to increased BMSC *in vitro* proliferation or subsequent *in vivo* bone formation would therefore be of considerable value.

Recently, hBMSCs seeded onto fibronectin-coated HA/TCP cubes and cultured for 4 weeks in medium with Dex (10^{-7} M) were shown to form significantly more bone 6 weeks post-transplantation than BMSCs cultured without Dex (Song et al., 2009). This finding, if extended beyond the isolated study, would constitute an important improvement of hBMSC *ex vivo* expansion and transplantation techniques. This result comes as no surprise to many researchers, and may even have been anticipated. Indeed, after it had been realized that glucocorticoid excess results in osteopenia (Chen and Feldman, 1979, Peck et al., 1969), *in vitro* studies unexpectedly revealed that Dex, a potent synthetic glucocorticoid, stimulated osteogenic differentiation of many different cell preparations derived from bone (Beresford et al., 1994, Cheng et al., 1994, Herbertson and Aubin, 1995, Kasugai et al., 1991). Dex was, therefore, termed an “osteogenic supplement”, along with a few other additives, such as ascorbic acid and β -GP. The importance of ascorbic acid for connective tissues was realized as early as the sixteen century (Pinnell, 1985), an observation scientifically corroborated four hundred years later (Jeffrey and Martin, 1966). Ascorbic acid is crucial for collagen synthesis (Hata and Senoo, 1989, Pinnell, 1985), and, consequently, for osteogenesis (Jeffrey and Martin, 1966). AscP used in this study is a long-acting derivative of ascorbic acid that retains its biological properties (Hata and Senoo, 1989). *In vitro* osteogenic differentiation caused by osteogenic supplements involves both the expression of osteogenic markers and the formation of alizarin-positive, von Kossa-positive “bone nodules” (Beresford et al., 1994, Yamanouchi et al., 2001, Cheng et al., 1994, Cheng et al., 1996, Fromigue et al., 1997, Xiao et al.). It would seem logical to surmise that osteogenic supplements could increase subsequent *in vivo* bone formation by cells exposed to them, or else that strong osteogenic differentiation of particular cells *in vitro* could predict good bone formation by these cells *in vivo*. However, such correlations have remained controversial. On the one hand, only those hBMSC strains that demonstrated high levels of osteogenic markers *in vitro* formed bone *in vivo* (Chen et al., 2005, Jaquiere et al., 2005). Conversely, numerous studies using both BMSCs and fibroblast-like strains of different origin demonstrated that patterns of *in vitro* osteogenic differentiation did not correlate with, and could not foretell, either the ability or the extent of bone formation by these cells *in vivo* (Derubeis et al., 2003, Mendes et al., 2004, Castano-Izquierdo et al., 2007, Bennett et al., 1991, Sacchetti et al., 2007, Sabatini et al., 2005, Sudo et al., 2007). Apparently, phenotypes and markers forced upon cells by artificial *in vitro* stimuli have little relevance to the genuine differentiation potential of these cells revealed in physiological conditions *in vivo*.

The intention of this paper was not to replicate the experiments of Song and co-authors, but rather, to compare their results with the data accumulated by our laboratory for the last 15 years. We demonstrated that for the substantial majority of hBMSC strains, continuous exposure to Dex and AscP did not increase subsequent bone formation, implying that hBMSCs need no *ex vivo* osteogenic induction in order to form optimal bone *in vivo*, a conclusion that differ from the one reached by Song et al. (Song et al., 2009). The discrepancy may have several explanations based on multiple differences between our experimental designs. Song and co-authors employed 10 times higher Dex concentration (10^{-7} M) than we did, and used Dex alone, while we used Dex plus AscP. They co-cultured BMSCs with the vehicles for 4 weeks prior to transplantation, while we implanted cell/vehicle mixtures following mere 90 minutes of adhesion. Most importantly, we used HA/TCP ceramic powder that ensured the attachment of greater than 80% of BMSCs following a 90 minute adhesion (Mankani et al., 2001). Song and co-authors employed solid ceramic cubes that are notoriously hard targets for cell penetration, even when supplementary

techniques, such as negative pressure or fibronectin covering, are used. As a result of limited cell attachment/dissemination, little bone (score 1), restricted to the periphery of the transplants, was formed in ceramic cubes by hBMSCs cultured without Dex (Song et al., 2009). Similarly cultured hBMSCs implanted with HA/TCP powders formed more bone (scores 3 to 4), evenly distributed throughout the transplants. Based on this difference, we believe that the transplantation technique employing solid ceramic blocks is not as efficacious as the particle-based approach. The differences between the two methods may grow even further with the advent of larger, clinically relevant transplants in need of rapid vascularization.

It is possible that the exuberant bone formation by unstimulated BMSCs implanted with HA/TCP particles left no room for additional bone formation by “induction”. On the other hand, BMSCs transplanted with HA/TCP cubes formed significantly more bone following Dex exposure (Song et al., 2009), an effect that does not currently have an explanation. As was shown in this paper, Dex, together with AscP, stimulated BMSC *in vitro* proliferation. It is possible that, during the 4-week co-cultivation with ceramic cubes (Song et al., 2009), Dex accelerated proliferation of BMSCs that managed to reach higher local cell densities and to migrate into the central parts of the cubes, resulting in an increased osteogenesis throughout the transplants. Hence, Dex could have stimulated bone formation (Song et al., 2009) by promoting BMSC *in vitro* proliferation, or else by stimulating BMSC attachment (Cicconetti et al., 2007), rather than by increasing their inherent osteogenic capacity. Indeed, the total surface available for adhesion is significantly higher in HA/TCP particles than in solid cubes, which may make the contribution of Dex as an adhesion promoter dispensable for the particle protocol but not for other types of constructs. Be it based on increased proliferation or attachment, Dex/AscP may stimulate osteogenesis only with vehicles less optimal for *in vivo* bone formation, and only following BMSC co-cultivation with such vehicles. This may explain why, following a 10-day cultivation within collagen sponges, another vehicle found not to be suitable for osteogenesis by hBMSCs (Krebsbach et al., 1997), bone was formed only by cells cultured with Dex (Yamanouchi et al., 2001).

Contrary to the majority of 22 donors, BMSCs from the remaining 2 donors, H-103 and H-106, formed more bone following Dex/AscP exposure. Both of these donors had profound bone marrow pathology, with their BMSCs displaying a substantially diminished osteogenic potential. Perhaps these BMSCs have an abnormality in maintaining cell viability or in achieving attachment to HA/TCP particles *in vitro*, that was overcome by the beneficial effects of Dex/AscP-stimulated BMSC proliferation (assuming that this effect continued *in vivo*) and/or attachment (Cicconetti et al., 2007). It should be emphasized that for other pathological BMSC strains with similarly compromised osteogenic potential, the exposure to Dex/AscP did not affect bone formation. It was, thus, not the low osteogenic potential by itself that made BMSCs susceptible to Dex/AscP stimulation. In this study, BMSC strains from elderly donors formed normal amounts of bone, making age-related changes in BMSC osteogenic potential a less probable contributor to the pathogenesis of senile osteoporosis. We reported previously that Dex/AscP increased *in vivo* bone formation by a majority of hBMSC clonal strains (Kuznetsov et al., 1997b). This result can also be explained by the challenged proliferative status of strains initiated by a single CFU-F that has to progress through at least 21-22 population doublings *in vitro* in order to achieve sufficient numbers of cells for *in vivo* transplantation. On the contrary, polyclonal BMSC strains used in the current study, and elsewhere, are usually initiated by thousands of CFU-Fs each, and are, therefore, much less challenged, proliferation-wise. Taken together, Dex may increase bone formation by hBMSCs when the cells and/or the transplantation vehicles are less than optimal.

In addition to bone formation, we focused our attention on the creation of hematopoietic territories by transplanted hBMSCs. Clonal analysis of BMSC populations demonstrated that only some CFU-Fs are multipotential, being able to differentiate into bone, cartilage, and create the hematopoietic microenvironment, including stroma and adipocytes, while others are unipotent and form just bone (Chailakhian et al., 1978, Gerasimov Iu et al., 1986, Kuznetsov et al., 1997b, Muraglia et al., 2000). The establishment of stroma and marrow alongside the new bone could, therefore, be considered as a surrogate marker for the presence of multipotential skeletal stem cells among the transplanted BMSC populations (Bianco and Robey, 2004). This concept has an important relevance to tissue engineering implying that BMSC strains that form bone but not hematopoietic territories include committed osteogenic progenitors, but lack multipotential skeletal stem cells; the new bone formed by such strains may not have the ability to maintain itself and may vanish with time.

The formation of hematopoietic territories, always in conjunction with good bone, was demonstrated by BMSCs from 14 donors. For 12 of them, BMSCs cultured with and without Dex/AscP developed hematopoietic territories of comparable extent. BMSCs exposed to Dex/AscP formed more extensive hematopoietic areas (albeit similar amounts of bone) than BMSCs cultured without the supplements, only for donors H-91 and H-95. It is unclear why BMSCs from just these two donors who presented with misplacement or bowing of otherwise normal bones (Tsirikos and Garrido, 2010, Sabharwal, 2009) required Dex and AscP in order to form hematopoietic microenvironment.

In this study, Dex/AscP stimulated BMSC *in vitro* proliferation without increasing CFE, or colony numbers, in primary marrow cell cultures. Apparently, the adhesion of CFU-Fs to the plastic and their entry into proliferation were not affected, while the duration of BMSC proliferation cycle was shortened, by Dex/AscP. Previously, stimulation of hBMSC proliferation by both AscP (Choi et al., 2008) and Dex (Juneja et al., 1984, Pri-Chen et al., 1998, Xiao et al., 2010) was reported. While glucocorticoid excess is the third most common cause of osteoporosis, no exact mechanisms have been identified (Canalis, 1983, Kim et al., 1999, Weinstein et al., 1998). If the stimulating effect of Dex on BMSC proliferation applies to their *in situ* precursors, adventitial cells (Sacchetti et al., 2007, Kuznetsov et al., 2009), then the excessive, glucocorticoid-induced, *in vivo* proliferation may exhaust the proliferation potential of these cells causing their diminished self-renewal capacity, decreased osteoblast production, and osteoporosis.

In summary, hBMSCs from multiple donors were transplanted *in vivo* in conjunction with HA/TCP particles. For the substantial majority of donors, BMSC strains generated and continuously propagated in medium with and without osteogenic supplements, Dex and AscP, formed similar amounts of bone and of hematopoiesis-supporting stroma, demonstrating that hBMSCs do not need to be induced to osteogenic differentiation prior to transplantation. Without directly influencing BMSC ability to form bone and myelosupportive stroma, Dex/AscP were shown to stimulate BMSC proliferation *in vitro*. The increased BMSC proliferation and/or adhesion may serve as a compensatory mechanism improving bone formation when either osteogenic cells are compromised or vehicles less than optimal for osteogenesis than HA/TCP particles are used. Due to the enhanced proliferation but unaltered osteogenic potential, the total osteogenic cell population generated from a single marrow sample is larger after cultivation with Dex/AscP than without them. Supplementing medium with Dex/AscP can be, therefore, recommended for generation and cultivation of autologous hBMSCs for bone tissue engineering.

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Fig. 1.

hBMSC proliferation in medium with and without osteogenic supplements. (A, B) BMSC colony formation in primary bone marrow cell cultures in medium with various concentrations of dexamethasone (Dex), ascorbate (Asc), and sodium- β -glycerophosphate (β -GP). **, $p < 0.01$ in comparison to the control groups (no medium supplements). (C,D) BMSC colony formation in primary bone marrow cell cultures in medium either with or without a mixture of Dex at 1×10^{-8} M and ascorbic acid phosphate (AscP) at 1×10^{-4} M. For each donor, differences between the colony numbers with and without Dex/AscP are not statistically significant.

(E) Total BMSC numbers harvested from primary bone marrow cell cultures in medium either with or without Dex/AscP.

(F) BMSC secondary colony formation in cultures of BMSC strains from several donors in medium either with or without Dex/AscP. For both E and F, differences between the groups with and without Dex/AscP: *, $p < 0.05$; ***, $p < 0.001$.

Fig. 2.

Bone and hematopoiesis formed in the transplants of hBMSCs generated with and without Dex/ascP. Y-axis: scores of bone and hematopoiesis, from 0 to 4. Each bar represents a single transplant. White bars, bone formed by BMSCs generated with Dex/AscP; grey bars, bone formed by BMSCs generated without Dex/AscP; black bars, hematopoiesis. The majority of the transplants were harvested 7 to 10 weeks post-transplantation (unmarked). #, ##, and ###: the transplants harvested, respectively, 3 to 4, 6, and 16 weeks post-transplantation. Means + SEM are for 7 to 10-week-old transplants. * p < 0.05; ** p < 0.01. For patient information, see Table 1.

Fig 3.

Eight-week-old transplants of hBMSCs into immunocompromised mice.

(A) medium without Dex/AscP, bone score 4, hematopoiesis score 2.

(B) medium with Dex/AscP, bone score 2, no hematopoiesis.

(C) medium without Dex/AscP, bone score 4, hematopoiesis score 3.

(D) medium without Dex/AscP, bone score 4.

(E) human bone (positive control).

(F) mouse bone (negative control).

A – C, H&E; D – F, staining for *alu*; A, 20x; B – F, 10x.

b, bone; v, vehicle; ft, fibrous tissue; h, hematopoiesis; a, adipocyte; green arrows, osteocytes; blue arrows, osteoblasts; yellow arrow, megakaryocyte; black arrow, blood vessel.

Table 1

Donors used for the transplantation experiments

Donor's ID	Donor's age, gender	Specimen location	Diagnosis
A.		Unaffected bone /marrow (17)	
H-37	18 yo F	Iliac crest	Scoliosis
H-40	14 yo F	Iliac crest	Scoliosis
H-57	14 yo F	Iliac crest	Scoliosis
H-60	16 yo F	Iliac crest	Scoliosis
H-70	0.5 yo M	Hip	Bladder extrophy
H-76	13 yo F	Iliac crest	Scoliosis
H-80	16 yo F	Spinal fusion	Scoliosis
H-87	8 yo M	Iliac crest	Neurofibroma
H-90	38 yo F	Femoral head	Avascular necrosis
H-91	14 yo F	Iliac crest	Spondylolisthesis
H-92	27 yo M	Iliac crest	Cerebral palsy
H-95	14 yo M	Femur	Blount disease
H-108	42 yo M	Sternum	Accident, alcoholism (necropsy)
H-120	19 yo M	Foot	Flat foot
H-153	1 yo M	Foot	Polydactily
H-193	76 yo F	Hip	Degenerative hip replacement
H-294	77 yo F	Femur	Alzheimer disease (necropsy)
B.		Aplastic anemia (4)	
H-103	63 yo F	Iliac crest	Aplastic anemia
H-104	40 yo F	Iliac crest	Aplastic anemia
H-105	35 yo M	Iliac crest	Aplastic anemia
H-106	27 yo F	Iliac crest	Aplastic anemia
C.		Lipodystrophy (3)	
HBMA-31	48 yo F	Hip	Familial partial lipodysrophy
HBMA-32	27 yo F	Hip	Familial partial lipodysrophy
H-130	29 yo F	Iliac crest	Congenital generalized lipodysrophy

HBMA, human bone marrow aspirate.