Systemic transplantation of human adipose-derived stem cells stimulates bone repair by promoting osteoblast and osteoclast function

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Abstract

Systemic transplantation of adipose-derived stem cells (ASCs) is emerging as a novel therapeutic option for functional recovery of diverse damaged tissues. This study investigated the effects of systemic transplantation of human ASCs (hASCs) on bone repair. We found that hASCs secrete various bone cell-activating factors, including hepatocyte growth factor and extracellular matrix proteins. Systemic transplantation of hASCs into ovariectomized mice induced an increased number of both osteoblasts and osteoclasts in bone tissue and thereby prevented bone loss. We also observed that conditioned medium from hASCs is capable of stimulating proliferation and differentiation of osteoblasts via Smad/extracellular signal-regulated kinase (ERK)/JNK (c-jun NH2-terminal kinase) activation as well as survival and differentiation of osteoclasts via ERK/JNK/p38 activation in vitro. Overall, our findings suggest that paracrine factors secreted from hASCs improve bone repair and that hASCs can be a valuable tool for use in osteoporosis therapy.

Keywords: adipose-derived stem cell • osteoblast • osteoclast • osteoporosis • systemic transplantation

Introduction

Osteoporosis is a prevalent bone disease that is characterized by loss of bone mass and strength, leading to fragility fracture [1]. Osteoblasts, which are derived from mesenchymal stem cells (MSCs), are ultimately responsible for bone formation; osteoclasts are derived from pluripotent hematopoietic cells and are capable of resorbing bone. During adult life, bone is continuously remodelled by orchestrated cross-talk between osteoblasts and osteoclasts [2], and an imbalance in their function results in decreased bone quality, most commonly represented by the osteoporotic phenotype. A relatively higher bone resorption activity by osteoclasts than bone formation activity by osteoblasts leads to bone loss. High- and low-turnover osteoporotic
Recent progress in stem cell biology has provided a promising strategy for treatment of multiple degenerative disorders [3]. In particular, adult stem cells have emerged as an important issue due to the potential for use of their ex vivo expanded progenies in cell-based regenerative medicine, tissue engineering and cancer therapy [4]. Adult stem cells participate in replenishment of cells that are lost during regeneration of damaged tissue, as well as in normal tissue development. MSCs, which possess unique immunosuppressive and anti-inflammatory properties and a capacity for homing to injured tissues, have been isolated from various tissues, including bone marrow, adipose, hair follicles, spleen, placenta, umbilical cord blood, foetal liver and lung [4]. MSCs obtained from adipose tissues could constitute a promising source of cells for use in cell-based therapy and tissue engineering [5]. Therapeutic potential for bone regeneration by systemic transplantation of genetically manipulated MSCs engineering for tissue regeneration [4]. Additive support of ASCs in cardiovascular disorders and diabetes mellitus, and in bioengineering [5]. Therapeutic potential for bone regeneration by systemic transplantation of genetically manipulated MSCs [3].

The main benefits of adipose-derived stem cells (ASCs) in therapeutic applications, as compared with bone marrow-derived MSCs, are that adipose tissue is readily accessible and relatively abundant, and the stem cell population can be easily harvested by simple methods, such as liposuction or surgical resection, and can be rapidly expanded ex vivo [7]. ASCs have also been shown to support differentiation of haematopoietic progenitors into myeloid and B lymphoid cells [8]. ASC-derived cellular therapy has been investigated with respect to a wide variety of human diseases, such as skeletal muscle disorders, cardiovascular disorders and diabetes mellitus, as well as in normal tissue development. MSCs, which possess unique immunosuppressive and anti-inflammatory properties and a capacity for homing to injured tissues, have been isolated from various tissues, including bone marrow, adipose, hair follicles, spleen, placenta, umbilical cord blood, foetal liver and lung [4]. MSCs obtained from adipose tissues could constitute a promising source of cells for use in cell-based therapy and tissue engineering [5]. Therapeutic potential for bone regeneration by systemic transplantation of genetically manipulated MSCs [3].

Materials and methods

Isolation and culture of hASCs

Human subcutaneous adipose tissues were obtained under approval from the institutional review board of Yungnam University Medical Center. The lipoaspirate was incubated with collagenase type I solution (Worthington Biochemical, Lakewood, NJ, USA) for 1 hr at 37°C, and filtered through 500 and 250 μm filters. Following centrifugation, the stromal vascular fraction was resuspended in DMEM (HyClone, Logan, UT, USA) supplemented with 10% foetal bovine serum (FBS) (HyClone), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured under a humidified atmosphere of 5% CO2 at 37°C and used for experiments at passages 3 to 5.

Preparation of conditioned media

hASCs and human embryonic kidney (HEK)293T fibroblast cells were seeded on a 100 mm dish in DMEM supplemented with 10% foetal bovine serum. At 90% confluence, cells were washed three times with phosphate-buffered saline and the medium was replaced with serum-free medium. After 1 hr, the medium was removed and fresh serum-free medium was added. As a control, conditioned media from hASCs (hASC-CM) and HEK293T cells (HEK293T-CM) were collected at 60 and 24 hrs after culture, respectively, which did not cause cell death for the indicated times (data not shown). Media were centrifuged at 300 × g for 5 min., and filtered through a 0.22 μm syringe filter. For in vitro cultures of osteoblasts and osteoclasts in hASC-CM, equal volumes of hASC-CM and α-MEM (HyClone) with FBS adjusted to 10% were used without concentration (50% hASC-CM). HK293T-CM/α-MEM (1:1) or α-MEM (1:1) was used as a control medium. For Western blot analysis and mass spectrometric identification of secreted proteins in hASCs, hASC-CM were concentrated 50-fold using an Amicon Ultra-15 (Millipore, Bedford, MA, USA) with a 10,000 molecular weight cutoff.

RT-PCR, Western blotting and ELISA assay

For detection of mRNA level, total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. About 2 μg of total RNA was reverse transcribed using Moloney murine leukaemia virus reverse transcriptase (Promega, Madison, WI, USA) with oligo dt at 42°C for 1 hr. Detailed information on PCR, including primer sequences and cycles, is provided in Table S1. For Western blotting, cells were lysed by addition of lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% SDS, protease inhibitors (Complete tablets, Roche Molecular Biochemicals, Mannheim, Germany), 1 mM Na3VO4 and 1 mM NaF. Proteins were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane and probed with specific antibodies. All antibodies used in this study are described in the Supporting Information. Protein levels of HGF in serum were measured using the Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis, MN, USA), Urine deoxypyridinoline (DPD) was assayed by competitive enzyme immunoassay using the MicroVue DPD EIA kit (Quidel Corporation, Santa Clara, CA, USA).
Protein identification using mass spectrometry

As previously described, proteins obtained from SDS-PAGE gels or from concentrated hASC-CM were identified using mass spectrometry (Gwangju Center, Korea Basic Science Institute, Korea) [13]. Detailed methods are described in the Supporting Information.

Microcomputed tomography and histological analysis of bone

A total of 2 × 10⁵ cells were injected into sham-operated or OVX female ddY mice (8 weeks old, Central lab animal, Korea) via tail vein on post-operative day 4 and killed at day 24 after injection (n = 6 per group). Microcomputed tomography (μCT) and histological analysis were performed, as reported previously [14, 15]. Trabecular morphometry within the proximal tibia was quantified using high resolution μCT (Skyscan 1076 μCT, Aartselaar, Belgium). From μCT data, bone loss indices, including bone volume/total volume (BV/TV), trabecular number (Tb.N) and bone mineral density (BMD) were assessed. For analysis of bone formation, mice were injected with calcine (10 mg/kg) on post-operative day 14 and day 21 and were killed at post-operative day 24. For histological evaluation, serial 5-μm-thick sagittal sections were made using a microtome. Haematoxylin and eosin staining was used for detection of osteoblasts and tartrate resistant acid phosphatase (TRAP) staining was used for visualization of osteoclasts. Histological images were analysed by an Aperio ScanScope Model T3 and ImageScope software (Aperio Technologies, Inc., Vista, CA, USA). All animal procedures were approved by the institutional review board of Yeungnam University Medical Center and were in accordance with the Guide for the Care and Use of Laboratory Animals.

Detection of hASCs after intravenous injection

After 8-week-old female ddY mice had been OVX, hASCs (2 × 10⁵ cells/mouse) labelled with iron oxide (Ferridex, Berlex Laboratories, Inc., Wayne, NJ, USA) were injected into OVX mice via tail vein on post-operative day 4 and killed at day 24 after injection (n = 6 per group). Bone tissues were fixed and decalcified with 0.6 M HCl and calcium ion concentration was determined using a QuantiChrom™ calcium assay kit (DICA-500, BioAssay Systems, Hayward, CA, USA), according to the manufacturer’s procedures. In order to normalize this assay, the remaining cells were lysed in 0.1 M NaOH/0.1% SDS, and protein concentration was determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). Detailed methods for cell adhesion and spreading assays are described in Supporting Information.

In vitro assays of osteoblast differentiation and function

Bone marrow-derived monocytes were isolated from the tibia and femur of 6-week-old C57BL/6J mice (Central lab animal) by flushing the bone marrow cavity. Osteoclast precursors were prepared as described [16] and detailed methods are described in the Supporting Information. To induce osteoclast differentiation, osteoclast precursors were cultured with 5 ng/ml of macrophage colony-stimulating factor (M-CSF) for 12 hrs, and the medium was then replaced with either control medium (50% HEK293T-CM in α-MEM or 50% DMEM in α-MEM) or 50% hASC-CM (50% hASC-CM in α-MEM) in the absence or presence of M-CSF (30 ng/ml) or receptor activator for nuclear factor κB ligand (RANKL, 50 ng/ml) for the indicated time periods. When cultured in 50% hASC-CM in α-MEM, the final concentration of FBS was adjusted to 10%. For positive controls, osteoclast precursor cells were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 3 to 4 days. For identification of osteoclasts, TRAP staining was performed with an Acid Phosphatase Kit (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer’s instructions. A TRAP solution assay was performed by addition of 5.5 mM P-nitrophenyl phosphate, a colorimetric substrate, in the presence of 10 mM sodium tartrate at pH 5.2. The reaction product was quantified by measurement of optical absorbance at 405 nm.

Statistical analysis

Data are presented as means ± S.D. from at least three independent experiments. Differences were considered statistically significant if P-value was less than 0.05. Statistical analyses were performed with the two-tailed Student’s t-test for analysis of differences among groups.

Results

Characterization of hASCs

Cell surface marker expression by hASCs isolated from human adipose tissue was characterized. Flow cytometric analysis of hASCs revealed that high levels of MSC-related antigens CD13, CD29, CD44, CD90 and CD166 were expressed; however, haematopoesis-related antigens CD31, CD34, CD117 and human leukocyte antigen CD45 were not expressed (Fig. S1). In additional, the ability of hASCs to differentiate into osteoblast, chondrocyte and adipocyte lineages at passage 3 was experimentally confirmed by von Kossa staining, Alcian blue staining and Oil red O staining, respectively (Fig. S2). These results showed that hASCs have multilineage potential and characteristics of MSCs.
Gene and protein expression of bone-related factors in hASCs and mass spectrometric identification of proteins secreted from hASCs

It is well established that hASCs have the ability to secrete a variety of cytokines, including angiogenic, anti-apoptotic and pro-inflammatory factors [11]. In this study, we examined expression and secretion of bone-related growth factors and cytokines in hASCs. Using RT-PCR, we observed expression of genes encoding HGF, M-CSF, bone morphogenetic protein 2 (BMP-2), BMP-4, osteopontin, RANKL and tumour necrosis factor-α (TNF-α) (Fig. 1A). Secretion of HGF, M-CSF, bone morphogenetic protein 2 (BMP-2), BMP-4, osteopontin, RANKL and tumour necrosis factor-α (TNF-α) were confirmed in hASC-CM using Western blotting and ELISA (Fig. 1B and C).

To determine whether other proteins that affect bone function were present in hASC-CM, the secretome of primary hASC cultures was analyzed using both gel-based and non-gel-based approaches. First, SDS-PAGE combined with matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry was used for protein identification in hASC-CM. Tryptic peptides from each band were identified using peptide mass fingerprinting and peptide sequencing, identifying 33 proteins in hASC-CM (Table S2). Second, we subjected the proteins of hASC-CM to in-solution tryptic digestion and nanoLC-tandem mass spectrometry, and identified 23 proteins in hASC-CM (Table S3). Table 1 shows a list of a total of 43 proteins identified by gel- and non-gel-based methods. Thirty-three proteins (77%) contained a predicted secretion signal peptide, and several proteins were reported to be involved in bone function. For example, secreted protein acidic and rich in cysteine (SPARC), peristin and fibronectin are known to stimulate proliferation and differentiation of osteoblasts [18–20]; β2-microglobulin and thrombospondin-1 are involved in stimulation of osteoclast formation and function [21, 22]. The presence of some proteins identified in mass spectrometric analysis, such as peristin, plasminogen activator inhibitor 1 and SPARC was validated using Western blotting (Fig. 1B). Combined results from RT-PCR, Western blotting, ELISA and mass spectrometric protein identification demonstrated the release by hASCs of a variety of proteins and cytokines related to bone function, suggesting that paracrine factors produced by hASCs might possibly play an important role in bone remodelling and repair.

Effects of systemic transplantation of hASCs on OVX-induced bone loss

We performed μCT analysis to determine the impact of hASCs on OVX-induced osteoporotic mice (Fig. 2A). Systemic transplantation of hASCs into OVX mice prevented OVX-induced bone loss in mice. When compared with OVX mice, bone loss indices, including bone volume fraction, trabecular number and BMD in hASC-transplanted OVX mice were restored to normal. The recovery effect of hASCs on bone loss was confirmed by measurement of the concentration of urinary DPD, a useful marker of bone resorption. Compared with OVX mice, a significantly lower level of urinary DPD was exhibited by hASC-transplanted OVX mice (Fig. 2B). Mineral apposition rate and bone formation rate, as measured by calcein label-based analysis, were restored to the level of sham-operated mice in hASC-transplanted OVX mice (Fig. 2C). Of particular interest, histological analysis showed that the numbers of both osteoblasts and TRAP+ osteoclasts adhering to trabecular bone surfaces were increased in hASC-transplanted OVX mice compared with sham and control OVX mice (Fig. 2D), suggesting that systemic transplantation of hASCs in OVX mice might affect proliferation or differentiation of osteoblasts and osteoclasts.

HGF that play important roles in bone formation and remodeling [17, 23] were expressed by hASCs (Fig. 1). Consistently, serum level of human HGF was markedly higher in hASC-transplanted OVX mice than in control OVX mice (Fig. 2E). Because oestrogen plays a critical role in OVX-induced bone loss [24], we explored the ovarian and uterine status of recipient mice (Fig. S3). Oestrogen-deficient OVX mice showed decreases in uterine weight and diameter. No significant differences in ovarian and uterine...
status were observed in hASC-transplanted OVX mice and control OVX mice. From these data, we confirmed the effectiveness of our OVX surgeries and the oestrogen status in the OVX mice.

For detection of hASCs in OVX mice after systemic transplantation, hASCs labelled with iron oxide were transplanted into OVX-induced osteoporotic mice via tail vein injection on post-operative day 4 and mice were killed on day 24 after transplantation. Distribution of hASCs was assessed by Prussian blue staining. Iron-labelled transplanted cells were detected in the periosteum of recipient OVX mice (Fig. 2F).

Table 1 Mass spectrometric identification of proteins secreted by hASCs

| Protein (accession number) | SignalP* | Bone-related function                           | Protein (accession number) | SignalP | Bone-related function                           |
|----------------------------|----------|-----------------------------------------------|----------------------------|----------|-----------------------------------------------|
| Fibronectin (Q9UMK2)       | 0.997    | Osteoblast survival and differentiation [20]  | Collagen α1 (I) chain (Q9UML6) | 0.999    | Osteoblast maturation and differentiation [42] |
| Collagen α2 (I) chain (P08123) | 0.997    | Osteoblast maturation and differentiation [42] | Thrombospondin-2 (P35442)   | 1.000    | Osteoblast differentiation [43]               |
| Collagen α1 (III) chain (P02461) | 0.999    |                                                | Collagen α1 (VI) chain (P12109) | 1.000    |                                                |
| SR calcium ATPase 1 (Q14983) |          |                                                | Thrombospondin-1 (P07996)    | 0.994    | Osteoclast function [22]                      |
| α-actinin-1 (P12814)       |          | Protein FAM40A (Q5VSL9)                        |                            |          |                                               |
| Periostin (Q15063)         | 0.999    | Osteoblast proliferation and differentiation [19] | β ig-h3 (Q15582)            | 1.000    | Osteoblast adhesion and differentiation [44]  |
| Thrombospondin-5 (P49747)  | 1.000    | Chondrocyte proliferation [45]                 | Matrix metalloproteinase 1 (P03956) | 1.000    | Osteoblast differentiation [46]               |
| 72 kD type IV collagenase (P08253) | 1.000    | Bone cell growth and proliferation [47]       | Plasminogen activator inhibitor 1 (P05121) | 0.999    | Bone mineralization and bone growth [48]      |
| Albumin (P02768)           | 1.000    |                                               | Moesin (P26038)             |          |                                               |
| Galectin-3-binding protein (Q08380) | 1.000    | Protein disulfide-isomerase (P07237)           |                            |          |                                               |
| Fibulin-3 (Q12805)         | 0.999    |                                               | Vimentin (P08670)           |          |                                               |
| EF-1-alpha 1 (P68104)      |          |                                               | Serpin A12 (Q8IWF75)        |          |                                               |
| Cathepsin L1 (P07711)      | 0.999    | Bone resorption [49]                          | Cathepsin D (P07339)        | 1.000    | Osteoblast calcification [50]                 |
| Glia-derived nexin (P07093) | 0.995    |                                               | Pentraxin-related protein PTX3 (P26022) | 1.000    |                                               |
| β-actin (P60709)            |          |                                               | Decorin (P07585)            | 1.000    |                                               |
| Follistatin-related protein 1 (Q12841) | 1.000    | Sulphydryl oxidase 1 (O00391)                  |                            |          |                                               |
| IGF-binding protein 7 (Q16270) | 0.998    | Cathepsin B (P07858)                          |                            |          |                                               |
| Peptidyl-prolyl cis-trans isomerase B (P23284) | 0.863    | SPARC (P09486)                               |                            |          |                                               |
| Lumican (P51884)           | 1.000    | Bone formation [51]                           | Metalloproteinase inhibitor 2 (P16035) | 1.000    | Osteoblast differentiation [52]               |
| Transgelin (Q01995)        |          |                                               | Peroxiredoxin-1 (Q06830)    |          |                                               |
| Metalloproteinase inhibitor 1 (P01033) |          | Bone turnover [53]                           | Protein S100-A6 (P06703)    |          |                                               |
| β2-microglobulin (P61769)  | 1.000    | Osteoclast formation [21]                     |                            |          |                                               |

*SignalP indicates the probability score calculated with SignalP software.
CM can be substituted for M-CSF. For prostaglandin E2, and that osteoclastogenic factors in hASC-osteoblastogenic factors present in hASC-CM can be substituted to induce their differentiation (Fig. 3B). These results indicate that cells (MNCs), whereas HEK293T-CM and RANKL treatment failed differentiation of osteoclast precursors into TRAP deposition, but did not with HEK293T-CM and VitD3 (Fig. 3A). In addition, combined treatment of hASC-CM- and RANKL-induced differentiation of osteoclast precursors into TRAP+ multinucleated cells (MNCs), whereas HEK293T-CM and RANKL treatment failed to induce their differentiation (Fig. 3B). These results indicate that osteoblastogenic factors present in hASC-CM can be substituted for prostaglandin E2, and that osteoclastogenic factors in hASC-CM can be substituted for M-CSF.

**Stimulatory effect of hASC-CM on osteoblast proliferation and differentiation**

Compared with sham-operated and OVX mice, systemic transplantation of hASCs into OVX mice resulted in an increase in the number of osteoblasts and osteoclasts on bone surfaces (Fig. 2D). To elucidate the molecular mechanisms responsible for in vivo results, we investigated the question of whether hASC-CM could affect the physiological properties of osteoblasts, including proliferation, adhesion, spreading and differentiation. We found that hASC-CM induced an increase in the osteoblast population, compared with the control medium (Fig. 4B and C). These enhanced activities of cell attachment and spreading were completely inhibited by EDTA, but not by heparin, suggesting that cell attachment and spreading in osteoblasts might be promoted by hASC-CM through a divalent cation-dependent mechanism. Whereas cultures supplemented with either hASC-CM or VitD3 alone did not induce matrix mineralization of osteoblasts, addition of both hASC-CM and VitD3 to cultures accelerated the mineralization of osteoblasts (Fig. 4D).

We next examined the effects of hASC-CM on signalling events in osteoblasts. As shown in Figure 4E, short-term treatment of osteoblasts with hASC-CM resulted in increased phosphorylation of receptor-regulated Smad 1/5/8, which is known to function as an intracellular mediator of osteoblast differentiation signalling [27], and to play an important role in early osteoblastogenesis [2]. However, neither phosphorylation nor expression levels of β-catenin in osteoblasts were altered by treatment with hASC-CM. In addition, we measured the activities of three MAPKs, ERK, c-jun NH2-terminal kinase (JNK) and p38 in osteoblasts after long-term treatment with hASC-CM. Maximal activation of both ERK and JNK, which can, in part, stimulate osteoblast differentiation [28] was induced by day 3 after treatment with hASC-CM however, p38 was not affected (Fig. 4F).

**Stimulatory effect of hASC-CM on osteoclast precursor survival and osteoclast differentiation**

The combination of M-CSF and RANKL is indispensable for both survival and differentiation of osteoclast precursors [14]. To evaluate the effect of hASC-CM on survival and proliferation of osteoclast precursors, we counted the numbers of osteoclast precursors after exposure to hASC-CM. The number of osteoclast precursors cultured with hASC-CM was considerably higher than that of osteoclast precursors cultured with control medium (Fig. 5A). To determine whether hASC-CM could stimulate osteoclast differentiation, osteoclast precursors were cultured under the control medium or hASC-CM in the presence or absence of M-CSF or RANKL and stained with TRAP to detect mature osteoclasts. TRAP+ MNCs were not detected in osteoclast precursors cultured...
in hASC-CM in the presence or absence of M-CSF. However, osteoclast precursors cultured in hASC-CM in the presence of RANKL formed much higher numbers of TRAP<sup>+</sup> MNCs at day 9 of culture (Fig. 5B), consistent with the result shown in Figure 3B, suggesting that hASC-CM could efficiently support osteoclast formation in a manner similar to that of M-CSF. TRAP activity was also higher in osteoclast precursors cultured with hASC-CM than in osteoclast precursors cultured with control medium (Fig. 5C). mRNA expression of osteoclastic markers, such as cathepsin K, TRAP, NFATc1 and matrix metalloproteinase-9 were induced when osteoclast precursors were cultured in hASC-CM (Fig. 5D). All three major subfamilies of MAPKs, ERK, JNK and p38, which are known to play an important role in osteoclastogenesis, were activated immediately after hASC-CM treatment in osteoclast precursors (Fig. 5E). In contrast to the stimulatory action of hASC-CM in osteoblast adhesion and spreading (Fig. 4B and C), hASC-CM did not affect adhesion and spreading of osteoclast precursors (data not shown).

### Discussion

Bioactive trophic factors secreted by ASCs have been reported to directly contribute to angiogenic and anti-apoptotic effects in ischemic limb disease, restoration of heart function and nerve sprouting following myocardial infarction, and skin wound healing [9, 10, 29, 30]. In this study, we showed that hASC-based therapy via systemic transplantation could be effective in bone repair by a mechanism predominantly mediated through secretion of paracrine factors by hASCs.

Osteoporosis is a chronic and complex disease involving an uncoupling between bone formation and bone resorption. Multiple pathogenetic mechanisms are involved in loss of bone mass and microarchitectural reduction of bone tissue in osteoporosis [1]. Bone repair is known to be a complex physiologic process that is regulated by several cell types, as well as the extracellular matrix and growth factors [31]. Secretion of important growth factors with potential for mediation of bone repair, including vascular endothelial growth factor, IGF-1 and TGF-β [32] from ASCs has been demonstrated [11]. In addition, we found that hASCs could express and secrete various cytokines, growth factors and proteins that are required for bone function and remodelling. These included M-CSF, RANKL, BMP-2, BMP-4, HGF and bone-related extracellular matrix proteins. Our in vivo results revealed that OVX-induced bone loss was restored by systemic transplantation of hASCs into recipient OVX mice. We further showed that hASC-injected OVX mice exhibited an increase in the number of both osteoblasts and osteoclasts. This could be explained by the balance between bone resorption and bone formation. The levels of bone resorption by osteoclasts did not exceed those of bone formation by osteoblasts, resulting in a net increase of bone mass. These findings indicate that hASCs can rescue oestrogen...
deficiency induced bone loss by simultaneous stimulation of osteoblast-mediated bone formation and osteoclast-mediated bone resorption in recipient OVX mice.

Stem cell homing capacity to the site of tissue injury is important for effective stem cell therapy. When hASCs labelled with Qtracker-delivered quantum dots were injected into OVX mice on post-operative day 28, a quantum dot signal could not be detected in bone at 60 min. after injection (Fig. S4A). A control experiment was performed with an experimental ischemia model in rat kidney to evaluate our system for assessment of the distribution of hASCs. Intense fluorescence from Qtracker labels and iron-labelled cells could be clearly seen at the ischemic site after injection of quantum-dot labelled or iron-labelled hASCs into ischemic kidney rats via tail vein (Fig. S4B and C). These results demonstrate that hASCs can move promptly into the damaged site in an acute ischemia-induced kidney model, but were not able to move into bone of OVX-induced osteoporotic mice. We made a profound attempt to identify localization of transplanted hASCs in a whole tissue. Among various tissues, tissue sections made from tibias of OVX mice following intravenous administration of hASCs showed that the transplanted cells were localized in the periosteum, but not in cortical bone. Periosteum is located at the outer bone surface along the periosteal cortex of cortical bone, and periosteal bone apposition plays a critical role in skeletal development [33]. Periosteum has been reported to show a dramatic response to bone growth factors with a significant increase in new bone formation. However, the question of whether or not the position of hASCs on periosteum specifically targeted a nearby part of the damaged bone is obscure. Our findings suggest that hASCs might not be directly involved in transdifferentiation into osteoblasts in trabecular and cortical bones. Instead, secreted factors from hASCs that are located sporadically all over the body and/or located in periosteum of the damaged bone tissue might be implicated in bone regeneration.

Synthesis of VitD₃ has been reported to occur in bone cells [34], and stimulation of osteoblast matrix mineralization by HGF in concert with VitD₃ has also been reported [35]. Our study showed that hASC-CM together with VitD₃-induced mineralization of
osteoblast cells, indicating the possibility that the combination of HGF secreted from hASCs and VitD3 produced by bone cells plays an important role in osteoblast differentiation in vivo. Cellular adhesion to extracellular matrix is known to be essential to osteoblast survival, proliferation and differentiation [20]. In our experiments, cell attachment and spreading in osteoblasts were markedly increased by treatment with hASC-CM, and those increased activities were attenuated in the presence of EDTA, demonstrating that hASC-CM promotes adhesion and spreading of osteoblasts in a divalent cation-dependent manner. Integrins expressed on the plasma membrane of osteoblasts are known to require extracellular divalent cations in order to bind to their ligands [36], and interaction of integrin with extracellular matrix components activates osteoblast survival and matrix mineralization [20]. Therefore, various extracellular matrix proteins in hASC-CM, including fibronectin and type I collagen are likely to participate not only in cell adhesion, but also in proliferation and differentiation of osteoblast cells via integrin signalling.

Because M-CSF and RANKL are known to be critical for survival and differentiation of osteoclasts, and were detected in hASC-CM, we expected that survival and differentiation of osteoclast precursors might be induced by hASC-CM. Indeed, we found that hASC-CM was able to maintain the survival of osteoclast precursors. However, hASC-CM failed to direct the differentiation of osteoclast precursors into TRAP$^{+}$ MNCs. Compared with precursors cultured in control medium and RANKL, osteoclast precursors cultured in hASC-CM and RANKL formed much higher numbers of TRAP$^{+}$ MNCs. In the presence of RANKL, HGF has been shown to support osteoclast differentiation in a manner similar to that of M-CSF [37]. In addition, HGF receptor was expressed by both osteoclast and osteoblast cells, indicating the possibility that the combination of HGF secreted from hASCs and VitD3 produced by bone cells plays an important role in osteoblast differentiation in vivo.

Figure 5: Stimulatory action of hASC-CM in survival and differentiation of osteoclast precursor cells. (A) Osteoclast precursor survival. Osteoclast precursors ($2 \times 10^5$ cells per well in 6-well plates) were cultured in the presence of 50% hASC-CM for the indicated times and trypan blue-excluded viable cells were then counted using a haemocytometer. Data represent mean ± S.D. ($n = 3$). (B) Osteoclast differentiation. Osteoclast precursors ($5 \times 10^5$ cells per well in 48-well plates) were cultured for 8 days in control medium or in 50% hASC-CM in the presence or absence of M-CSF (30 ng/ml) or RANKL (50 ng/ml), as indicated. Media were replenished on day 2. After 8 days, cells were stained with TRAP and the number of TRAP$^{+}$ MNCs (≥3 nuclei) was counted. Data represent mean ± S.D. ($n = 3$). Representative images of three independent experiments are presented. Scale bar: 100 μm. (C) TRAP activity. Osteoclast precursors ($2 \times 10^5$ cells per well in 6-well plates) were incubated for the indicated times in control medium or in 50% hASC-CM and subjected to the TRAP assay. Data are representative of three independent experiments and expressed as mean ± S.D. ($n = 3$). (D) Osteoclast-specific gene expression. Osteoclast precursors were cultured in 50% hASC-CM for 2 days and the mRNA level for osteoclast marker genes was then determined using RT-PCR. Data are representative of three independent experiments. (E) Osteoclast-stimulating signals. Following adaption of osteoclast precursors for 12 hrs in the presence of M-CSF (30 ng/ml) and further incubation without M-CSF for 6 hrs, cells were stimulated with control medium or 50% hASC-CM for the indicated times and immediate responding signals in osteoclast precursors were analysed by Western blotting with specific antibodies to p-ERK, p-JNK and p-p38. p-actin was used as a loading control. The gel is representative of three independent experiments. b: $P < 0.01$; c: $P < 0.001$. © 2011 The Authors
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hASCs transplanted via the circulatory system could function as antiresorptive and anabolic agents, and could be a valuable therapeutic option for treatment of both high- and low-turnover osteoporosis. These results are consistent with those of a previous report showing that ASCs could support differentiation of haematopoietic progenitor cells [8]. Involvement of ERK in osteoclast survival has been reported [38], and involvement of p38 and JNK in osteoclast differentiation has also been reported [39, 40]. ERK, JNK and p38 were strongly activated in response to hASC-CM in osteoclast precursors, implying that paracrine factors released by hASC could transiently activate all three major MAPKs (ERK, JNK and p38) that contribute to induction of osteoclastogenesis. In addition, hASC-CM induced maximal activation of ERK and JNK at day 3 after osteoblast culture. Taken together, these results suggest that enhanced survival and differentiation of osteoblasts and osteoclasts in cultures containing hASC-CM were mediated, at least in part, by activation of MAPKs.

Key mechanisms by which ASCs might repair and regenerate damaged tissues have been suggested to include paracrine effects of secreted cytokines and growth factors, and direct differentiation to a desired cell lineage [5]. In a developing mouse model, differentiation into osteoblasts appears to be especially important for treatment of osteogenesis imperfecta [41]. However, hASC-induced improvement of bone mass could not be fully explained by direct differentiation into osteoblasts because hASC-injected OVX mice exhibit increased numbers of both osteoblasts and mature osteoclasts, and hASC-CM simultaneously stimulates proliferation and differentiation of both osteoblasts and osteoclasts in vitro. Based on in vivo and in vitro results from our adult mouse model, we suggest that paracrine effects induced by systemic transplantation of hASCs are the predominant mechanism mediating the therapeutic effects of hASCs in treatment of osteoporosis. Collectively, hASCs transplanted via the circulatory system could function as antiresorptive and anabolic agents, and could be a valuable therapeutic option for treatment of both high- and low-turnover osteoporosis.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Flow cytometric analysis for cell surface marker expression in hASCs.

Fig. S2 Multilineage differentiation capacity of hASCs.

Fig. S3 Measurement of uterus thickness in sham-operated (Sham) and OVX mice systemically transplanted with or without hASCs.

Fig. S4 Localization of hASCs after systemic transplantation.

Table S1 Primers used in this study.

Table S2 Mass spectrometric identification of proteins secreted by hASCs using a gel-based approach.

Table S3 Mass spectrometric identification of proteins secreted by hASCs using a non-gel based approach.

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