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A novel rich source of human mesenchymal stem cells from the debris of bone marrow samples

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ABSTRACT

The debris from human bone marrow (BM) samples is generally filtered out and discarded prior to isolation of mesenchymal stem cells (MSCs). The purpose of this study is to develop a method to harvest MSCs from the debris and investigate their biological characteristics compared with the marrow counterparts. The BM tissue fragments were digested with collagenase and this treatment yielded mononuclear cells half to those from the corresponding filtered BM. The frequencies of colony-forming unit-fibroblast in these two cell populations were not significantly different. MSCs of two origins exhibited similar morphological and phenotypic features. Fluorescent dye-dilution assay showed that they grew at comparable rates both in the primary and passaging cultures. Further, they could be induced into osteoblasts, chondroblasts and adipocytes, as revealed by histological and molecular examinations. Thus, BM tissue fragments may serve as a new source of MSCs in the settings of bench experiments and clinical trials.

Mesenchymal stem cells (MSCs), sometimes referred to as marrow stromal cells or multipotential stromal cells, represent a class of adult progenitors capable of differentiating into several mesenchymal lineages including osteoblasts, chondroblasts, and adipocytes. Increasing data have endowed MSCs as a promising candidate of seed cells for repair and regeneration of a variety of mesenchymal tissues such as bone, cartilage and muscle [1–3]. Furthermore, they are able to produce numbers of functional growth factors and cytokines that might be clinically useful for the treatment of diabetes mellitus [4], graft-versus-host disease [5,6], neurological disorders [3,7], liver and renal failures [8,9].

MSCs can be isolated from many tissues though bone marrow (BM) is the commonest origin. Ordinarily, human marrow MSCs culture is developed from mononuclear cells harvested by gradient centrifugation, prior to which, the tissue fragments in the bone marrow samples are generally filtered out and discarded. These fragments contain adipose tissue, compact bone spicules and blood vessel networks, all of which may serve as sources for MSCs [10–13]. Therefore, we assumed that the debris filtered out from human BM might abound in MSCs. To prove this assumption, we collected BM debris by filtering marrow samples through a mesh and developed MSCs culture with them. Our results showed that BM spicules contained abundant MSCs with similar biological features compared with their marrow counterparts.

Materials and methods

Isolation and culture of MSC form BM debris and BM. BM was harvested from 7 donors (three women and four men at the age ranging from 28 to 58 years,) following informed consent according to the guideline from Beijing Institute of Radiation Medicine Committee on the Use of Human Subjects in Research. Collected marrow was passed through a cell-strainer with the pore size of 80 μm in diameter (BD, USA). Mononuclear cells were isolated from the filtered BM by the routine density gradient centrifugation method. The debris on the filter was washed three times with PBS, removed into a culture dish by flushing repeatedly to the back-side with 10 ml of the mixture of collagenase I and II (1 mg/ml, Gibco), and then digested at 37 °C for 6 h. The digested mononuclear cells were isolated also by gradient centrifugation. Mononuclear cells of both origins were suspended in α-MEM supplemented with 10% fetal bovine serum screened for culture-expansion of human MSCs (Stem Cell Co.) and incubated in the atmosphere of 5% CO_2 and 95% humidity at 37 °C. The non-attached cells were washed away after 48 h and attached cells were passaged by trypsinization after cell density reached nearly 80%.

Abbreviations: MSCs, mesenchymal stem cells; BM, bone marrow; ALP, alkaline phosphatase; CFSE, carboxyfluorescein diacetate succinmidyl ester; Runx2, runt-related transcription factor 2; Col II, type II collagen; Sox9, SRY (sex determining region Y)-box 9; PPAR-γ, peroxisome proliferator-activated receptor gamma; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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Proliferation and colony-forming ability analysis. 1×10^7 mononuclear cells were labeled with 10 μl of 5 mM carboxyfluorescein diacetate succinmidyl ester (CFSE, Sigma–Aldrich) as described in our previous work [14]. Aliquot of 1×10^6 labeled cells were fixed with 2% PBS buffered paraformaldehyde as positive control for flow cytometer analysis and typically, the positivity could reach up to 98%. The remaining cells were seeded into 2 culture dishes of 50 cm^2 for 8 days, followed by cell harvesting for flow cytometric analysis. Moreover, the proliferation rates of passaged MSCs from both sources were also examined with CFSE dilution assay after 3 days of culture.

For colony forming unit-fibroblast (CFU-F) assay, 2×10^7 mononuclear cells were seeded into 4 culture dishes of 50 cm^2 and cultured in the medium described above for 8 consecutive days. The attached cells were then stained with Wright–Giemsa solution and the number of colonies was calculated with the software of Quantity One 4.4.

Analysis on phenotypic features. After trypsinization, aliquots of 1×10^6 MSCs were rinsed twice with a cold PBS, suspended in 0.5 ml PBS, and labeled with the PE or FITC-conjugated primary antibodies at 4 °C for 20 min. Monoclonal antibodies (mAbs) including $CD14^{FITC}$, $CD31^{FITC}$, $CD29^{PE}$, $CD34^{PE}$, $CD45^{PE}$, $CD105^{FITC}$, $CD73^{PE}$, $CD166^{PE}$, HLA-ABC^{FITC}, mouse IgG-γ^{PE}, and IgG-γ^{FITC} were purchased from BD Biosciences (San Jose, CA). Cells were then washed again with PBS and fixed with 2% paraformaldehyde. Events were collected with FACScan (BD Bioscience) and the data were analyzed with WinMdi software 2.9.

Induction of osteogenic and chondrogenic differentiation. MSCs at passage 2 were seeded into a 24-well culture plates at density of 5000 cells per well and osteogenic differentiation was induced by the addition of 0.1 μM dexamethasone, 50 μM ascorbic acid 2-phosphate, and 10 mM β-glycerophosphate as previously described with a little modification [11,15,16]. After 14 days, cellular activity of alkaline phosphatase (ALP) and extracellular mineralization deposition were determined with ALP immunohistochemistry kit (Sigma–Aldrich) following the manufacture's protocol, or by silver staining [15], respectively.

For adipogenic differentiation, MSCs were seeded a 24-well plate at 10^4 cells per well and induction was elicited by the supplement of agents containing 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX, Sigma–Aldrich), and 100 μM indomethacin (IDM, Sigma–Aldrich). Cells were incubated for 2 weeks and intracellular lipid droplets were revealed by Oil-Red staining [15].

RNA isolation and reverse transcription analysis. Aliquots of MSCs (5×10^4) at passage 2 were seeded into a 25 cm^2 culture flask in growth medium with 100 nM dexamethasone for 7 days as previously described [11,17]. Total cellular RNA was extracted with Trizol reagent (Gibco) and reverse-transcribed with Moloney murine leukemia virus reverse transcriptase. Transcripts were amplified using intron-spanning primers specific for the analyzed human genes (Table 1). Amplifications were performed in a Bio-Rad thermal cycler (Perkin-Elmer) for 25–32 cycles (typically: 94 °C/30 s; 40–55 °C/45 s; 72 °C/60 s) after an initial denaturation at 94 °C of 2 min. PCR products in a 5 μl aliquot were size-separated by electrophoresis in 1% agarose gel.

Statistical analysis. Data were expressed as mean values incorporating the standard deviation. Statistical significance was analyzed by Student's *t* test. A *P* value less than 0.05 was considered significant.

Results

To observe if MSCs existed in the debris of human BM samples, the tissue fragments were digested with collagenase and mononuclear cells were harvested by gradient centrifugation. The proportion of mononuclear cells was about 80% in the cell suspension from the digested BM debris, which was obviously higher than that in the BM analogue. Moreover, typically, the number of mononuclear cells in the digested debris could reach up to half of that from the filtered marrow.

When the digested cells were cultured in the standard medium for *ex vivo* expansion of human MSCs, the attached cells were consistent in morphology with the routinely described MSCs that are isolated from BM (Supplementary Figure 1A and B). CFU-F, an

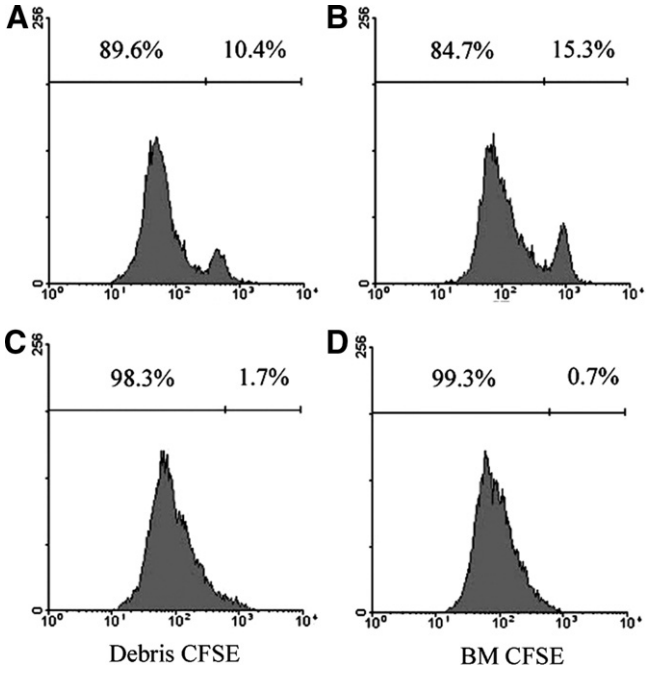

Fig. 1. MSCs from digested BM debris and BM exhibit similar proliferation activities. Typical flow cytometric analysis shows that over 80% of MSCs from both debris (A) and BM (B) experienced cell division in the primary culture and over 98% of MSC from both sources entered into cell division cycles at second passage (C from debris and D from BM). Data are representative of 5 individual experiments. *X*-axis: relative fluorescence intensity; *Y*-axis: events.

Table 1
The primer sequences for PCR

Genes	Forward primers	Reverse primers	Products size (bp)	Accession number
Osteocalcin	gcggtgcagagtccagcaaa	cctcctgaaagccgatgtggt	198	NM_199173
Col II	agcagcaagagcaagga	gacagcaggcgtaggaa	133	NM_001844
Sox9	caggctttgcgattt	cagcagcaccgtttt	148	NM_000346
Runx2	gtcttacccctcctacctga	attcgtgggttggagaagc	206/272	NM_001024630
PPAR-γ	ccatccgcatctttca	ggactcagggtggttca	453	NM_005037
GAPDH	agaaggctggggctcatttg	aggggccatccacagtcttc	258	NM_002046

Col II, indicates type II collagen; Sox9, SRY (sex determining region Y)-box 9; Runx2, runt-related transcription factor 2; PPAR-γ, peroxisome proliferator-activated receptor gamma; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

in vitro correlate to MSCs *per se*, was evident in both kinds of culture (Supplementary Figure 1C and D). The average numbers of colonies were not statistically different, 2.2 ± 1.4 colonies/cm^2 versus 1.5 ± 0.3 colonies/cm^2 (Supplementary Figure 1E).

To further investigate if these two kinds of cells occupied similar proliferation ability, CFSE dilution assay was performed. The results showed that the proportions of MSCs from both sources that experienced cell divisions in the primary culture, i.e. passage 0, were 91.70 ± 2.50% and 88.08 ± 5.39% (Fig. 1A and B) and that, cells at passage 2 from two origins proliferated at a similar rate (Fig. 1C and D).

Marrow MSCs exhibit characteristic phenotypic profiles [14]. It was also the case for MSCs from digested BM debris in this study. FACS technique showed that the cultured cells were homogenously positive for CD29, CD73, CD105, CD166, HLA-ABC, and negative for hematopoietic and endothelial cell surface markers including CD14, CD31, CD34, and CD45 (Fig. 2).

Further differentiation assays showed that these cells could be induced into osteoblasts and adipocytes, as revealed by ALP (Fig. 3A and B), Von Kossa (Fig. 3C and D) and Oil red O staining (Fig. 3E and F). To confirm their differentiation potentials, MSCs were cultured with a non-specific inductive agent dexamethasone for 7 days, followed by the evaluation of lineage-specific gene expression by RT-PCR. The results showed that dexamethasone treatment promoted debris-derived MSCs to express elevated mRNA levels of osteogenesis-related genes Runx2 and osteocalcin, adipogenesis-specific gene PPAR-γ, and chondrogenesis associated genes Sox9 and Col II (Fig. 4).

Discussion

Although MSCs can be isolated from many tissues, bone marrow is a reproducible and convenient source of these cells from nearly all species tested except mice [11]. In this study, we tried to isolate MSCs from the tissue fragments in the human marrow samples, which are mainly composed of adipose tissue, compact bone fragments and vessel networks. Our results here suggest that these

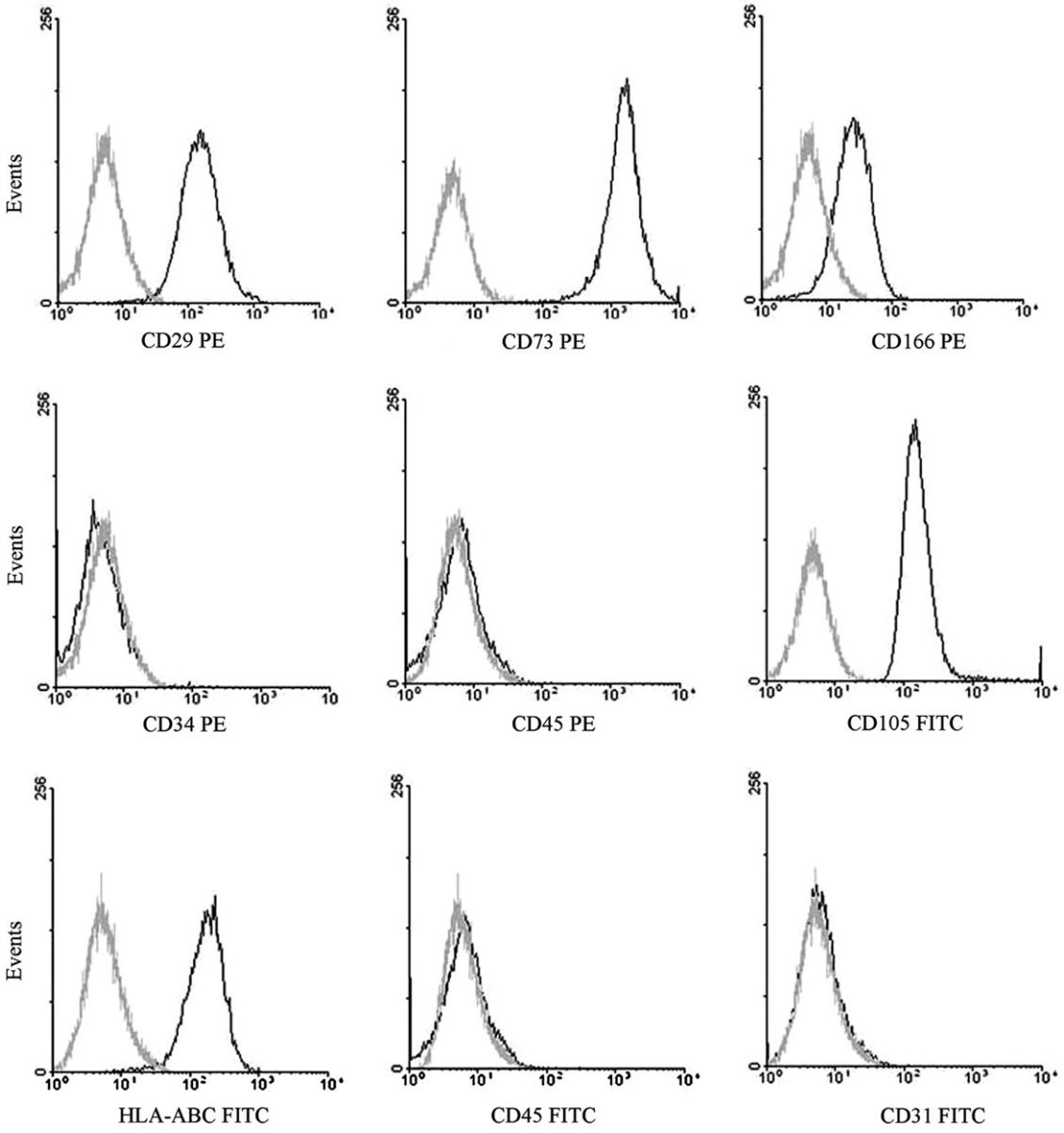

Fig. 2. Immunophenotypic characteristics of MSCs. MSCs were stained with surface antibodies and analyzed by FACS. FITC, fluorescein isothiocyanate; PE, phycoerythrin. Gray lines represent isotype control. *X*-axis: relative fluorescence intensity; *Y*-axis: events.

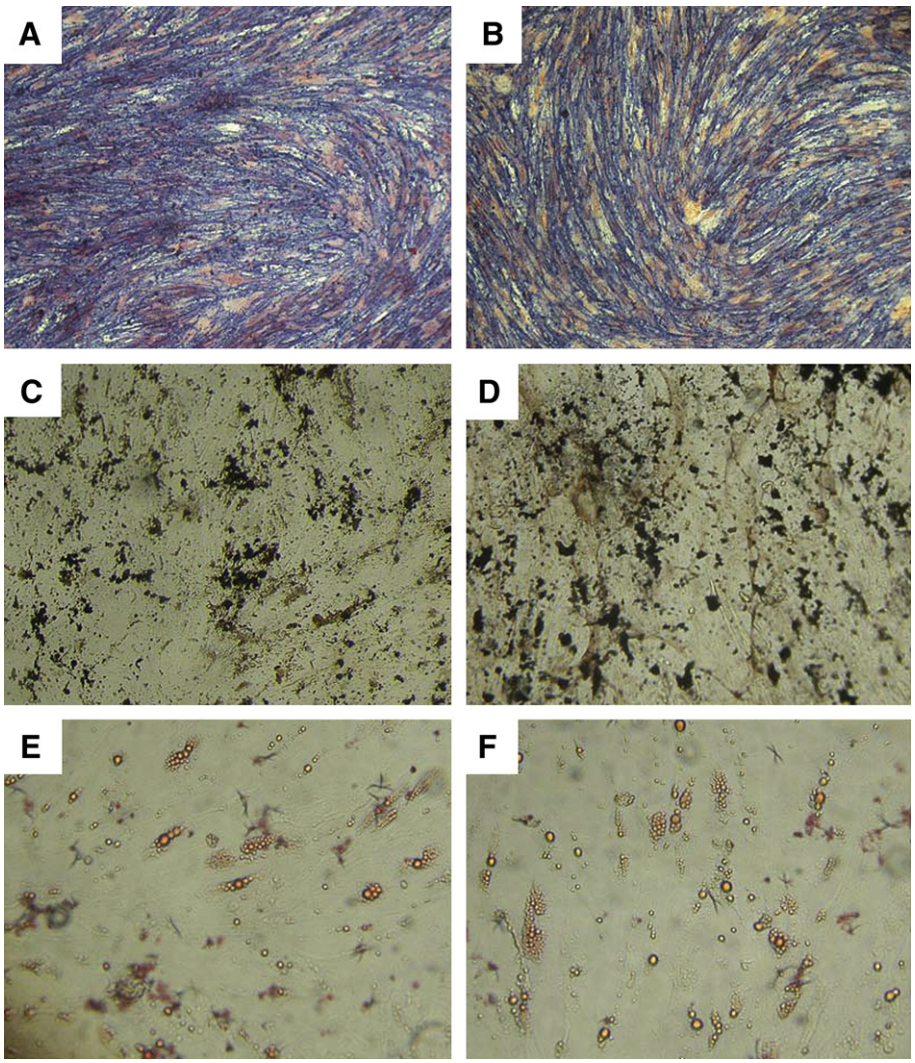

Fig. 3. Osteogenic and adipogenic differentiation of MSCs. Mineralization deposition of osteogenesis was stained with silver nitrate (A,B). The ALP activity of osteocytes was stained with ALP immunohistochemistry kit (C,D). Adipogenesis of MSC was stained with Oil Red (E,F). The left panel (A, C, and E) represents MSCs derived from debris and the right represents BM MSCs (B, D, and F).

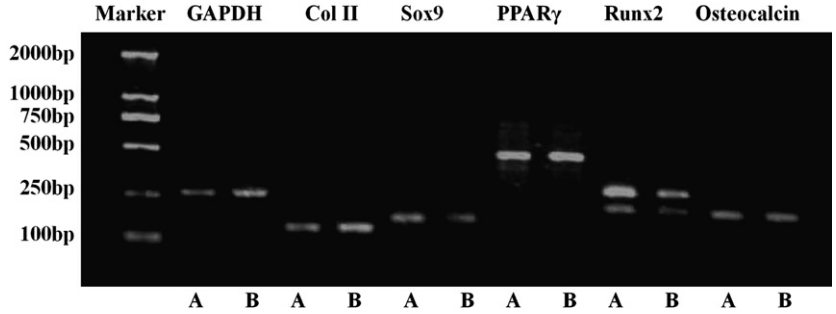

Fig. 4. A typical result of gel-electrophoresis of PCR products to evaluate the expression of differentiation-related genes in dexamethasone-treated MSCs. The amount of total RNA was standardized by the expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Lanes A represent MSCs derived from BM, while lanes B represent those from the digested debris. The RT-PCR product size of Runx 2 isoform b is 206 bp and that of isoforms a and c is 272 bp.

cells meet the generally accepted standard criteria including the fibroblast-like morphology, the expression of some cell-surface markers, and the capacity of differentiation into osteoblasts, chondroblasts, and adipoblasts [16,18,19]. Furthermore, they grew at a rate comparable to that of their marrow counterparts. The results here support the assumption that MSCs are distributed widely both in human bone marrow and other tissues [10–13,18–21].

In the setting of bone marrow transplantation, aspirated BM grafts are usually filtered through graded meshes to remove the debris to reduce the risk of thrombosis. Our results here suggest that these filtered-out tissue fragments, generally discarded by the physicians, may serve as one of the sources for human MSCs isolation. Furthermore, in the setting of MSCs-based cell therapy, the use of mononuclear cells from

digested marrow spicules can reduce the demand of marrow sample volumes.

In conclusion, we have developed an easy-handling method to isolate human MSCs from marrow tissue fragments, and our results here might provide some useful information to the understanding of MSCs niches *in vivo*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.08.131.

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