

Simultaneous isolation of mesenchymal stem cells and endothelial progenitor cells derived from murine bone marrow

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Abstract. Mesenchymal stem or stromal cells (MSCs) are identified as sources of pluripotent stem cells with varying degrees of plasticity. Endothelial progenitor cells (EPCs) originate from either bone marrow (BM) or peripheral blood and can mature into cells that line the lumen of blood vessels. MSC and EPC therapies exhibit promising results in a variety of diseases. The current study described the simultaneous isolation of EPCs and MSCs from murine BM using a straightforward approach. The method is based on differences in attachment time and trypsin sensitivity of MSCs and EPCs. The proposed method revealed characteristics of isolated cells. Isolated MSCs were positive for cell surface markers, cluster of differentiation (CD)29, CD44 and stem cell antigen-1 (Sca-1), and negative for hematopoietic surface markers, CD45 and CD11b. Isolated EPCs were positive for Sca-1 and vascular endothelial growth factor receptor 2 and CD133. The results indicate that the proposed method ensured simultaneous isolation of homogenous populations of MSCs and EPCs from murine BM.

Introduction

Recently, an increasing number of studies have focused on stem cells for cell therapy (1-3). The application of stem cell to

the field of regenerative medicine provides novel strategies to induce tissue repair (4,5). Studies have documented the effect of mesenchymal stem or stromal cells (MSCs) and endothelial progenitor cells (EPCs) on postnatal vasculogenesis (6,7). MSCs act as precursors of mesenchymal tissue cells. Friedenstein *et al* (8) first described MSCs as fibroblast precursors from bone marrow (BM) and Caplan (9) first proposed the term 'mesenchymal stem cell'. MSCs exhibit a self-renewing capacity, ability to differentiate into multiple lineages and immunomodulatory potential (10). EPCs are precursors of endothelial cells and can mature into cells that line the lumen of blood vessels. Since Asahara *et al* (11) first detected EPCs in adult peripheral blood, more findings have indicated that EPCs serve an important role in endothelium maintenance and thus are involved in re-endothelialisation and neovascularisation (12,13). A previous study by our group demonstrated that EPCs were possible biological components of stem-cell niches and affected biological processes of MSCs (14). BMs are major sources of MSCs and EPCs in mice. Therefore, the current study aims to obtain the two types of cells from murine BM.

Methods for isolation of MSCs and EPCs include plastic adherence (15,16), density gradient centrifugation (17,18), immunomagnetic selection (11,19,20) and flow cytometry sorting (21). However, no optimal method is available for retrieval of such cells (22). In addition to fibroblastic cells, primary cultures derived from BM contain fibroblasts, macrophages, endothelial cells, adipocytes, hematopoietic stem cells (HSCs), EPCs and red cells. These cells in BM exhibit different adherent capacities; in particular macrophages and mature endothelial cells easily attach to dish wall, followed by fibroblasts and fibroblastic cells, finally adipocytes, HSCs and EPCs adhere poorly to dish walls (23,24). Based on the plastic adherent property, MSCs and EPCs were isolated simultaneously. Purification of MSCs and EPCs was also conducted since MSCs differentiate into a trypsin-sensitive population, whereas EPCs differentiate into a trypsin-resistant population (25).

The present study aimed to demonstrate an improved method of plastic adherence to isolate homogenous populations of MSCs with good proliferation and differentiation capacities. Furthermore, it was explored whether EPCs could

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also be obtained while avoiding the sacrifice of numerous mice.

Materials and methods

Isolation and culture of MSCs and EPCs derived from BM. A total of 20 male C57BL/6 mice (6-8 weeks old, 25-35 g) were purchased from the Laboratory Animal Center of Xinjiang Medical University (Urumqi, China). Mice were maintained under a 12 h light/dark cycle at 25±2°C with 50±5% humidity. Food and water were available *ad libitum*. The experimental animal protocol used in the present study was approved by the Animal Experimental Ethics Committee of Shihezi University (Shihezi, China). The mice were euthanized by trained personnel using CO₂ inhalation. Femurs and tibias were separated, and muscles and connective tissues were manually removed (Fig. 1). Then, a 5 ml syringe was filled with complete Dulbecco's modified Eagle's medium (DMEM), which consisted of low-glucose DMEM (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 10% foetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin and 100 U/ml streptomycin (both Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The ends of the tibias and femurs, inferior to the medullary cavity, were cut with scissors. The marrow in the medullary cavity was flushed out by inserting a syringe needle (27-gauge) attached to the 5 ml syringe into one end of the bones. Finally, BM cells were filtered into 60 mm plastic culture dishes through a 200-mesh filter. The culture was maintained at 37°C in a humidified incubator containing 95% air and 5% CO₂. After 4 h, non-adherent cells that accumulated on the surface of culture dish were pipetted into a new culture dish, as described previously (26). Cells in the original dish were cultured for an additional 8 h, followed by gradual replacement of the medium with 1.5 ml fresh complete DMEM to obtain MSCs. Thereafter, this step was repeated every 8 h for 72 h for culture initiation. Cells in the new culture dish were cultured for 48 h to obtain EPCs, as described previously (18). Then, non-adherent cells from 2 mice were collected, plated in a 60 mm culture dish coated with human fibronectin (Gibco; Thermo Fisher Scientific, Inc.) and maintained in endothelial growth medium (EGM), which contained endothelial cell 'basal medium-2, EGM™-2 MV SingleQuots™' (both Lonza Group, Ltd., Basel, Switzerland), 100 U/ml penicillin and 100 U/ml streptomycin (27). Following 72 h of culturing, non-adherent cells were removed. The medium for MSCs and EPCs was replaced every 3-4 days. After 4, 7 and 14 days, cells were visualised under an inverted microscope (Zeiss Axio Observer; Zeiss AG, Oberkochen, Germany) at magnification, x50.

To obtain homogenous populations of MSCs, the MSC culture was treated with 1 ml StemPro Accutase (Gibco; Thermo Fisher Scientific, Inc.) for 2 min when 90-100% confluence was reached. Cells detached within 2 min, then were harvested and sub-cultured. At 90-100% confluence, the EPC culture was similarly treated with 1 ml StemPro Accutase to homogenise EPCs. In contrast to MSCs, a number of cells detached after 2 min, whereas the cells that had not detached were treated for another 3 min, harvested and sub-cultured.

Fluorescence-activated cell sorting (FACS) analysis. Passage 3 cells in complete DMEM were detached using StemPro Accutase and counted. Then, 2×10⁵ cells in 100 µl buffer containing PBS (Hyclone; GE Healthcare Life Sciences) and 2% FBS (Hyclone; GE Healthcare Life Sciences) were divided into aliquots in 1.5 ml centrifuge tubes. Cells were stained with fluorescent isothiocyanate (FITC)-conjugated, phycoerythrin (PE)-conjugated or PE-Cyanine7-conjugated anti-mouse cluster of differentiation CD29 (cat. no. 11-0291; dilution, 1:50), CD44 (cat. no. 12-0441; dilution, 1:160), stem cell antigen-1 (Sca-1; cat. no. 11-5981-82; dilution, 1:100), CD45 (cat. no. 25-0451; dilution, 1:160), CD11b (cat. no. 11-0112-82; dilution, 1:100), CD133 (cat. no. 11-1331-80; dilution, 1:100) and vascular endothelial growth factor receptor 2 (VEGFR-2; cat. no. 12-5821-82; dilution, 1:40; all eBioscience, Thermo Fisher Scientific, Inc.) antibodies in the dark at 4°C for 30 min. Cells stained with FITC-conjugated (cat. no. 11-4714-81; dilution, 1:100) or PE-conjugated (cat. no. 12-4724-42; dilution, 1:100) anti-mouse immunoglobulin (Ig)G (both eBioscience, Thermo Fisher Scientific, Inc.) in the dark at 4°C for 30 min served as controls. Thereafter, cells were pelleted by centrifugation at 400 x g at 4°C for 5 min. Following two washes with PBS, cells were examined using the FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and data were analysed using FlowJo 7.6 software program (Tree Star, Inc., Ashland, OR, USA).

After 14 days, cells in EGM were detached, counted and separate using the aforementioned protocol. The cells were stained with FITC-conjugated or PE-conjugated anti-mouse Sca-1 (dilution, 1:100), VEGFR-2 (dilution, 1:40) and CD133 (dilution, 1:100; all eBioscience, Thermo Fisher Scientific, Inc.) in the dark at 4°C for 30 min. Cells stained with FITC-labelled or PE-labelled anti-mouse IgG served as controls. Cells were pelleted and analysed using the aforementioned protocol.

Differentiation of MSC assays. Passage 3 cells in complete DMEM were seeded in 6-well plates at 1×10⁵ cells/well. When the cells reached 60-70% confluence, DMEM complete medium was carefully aspirated from each well, and 2 ml osteogenic differentiation medium was added. Osteogenic differentiation medium consisted of DMEM complete medium, 10 mM β-glycerol phosphate, 50 µM ascorbate and 10⁻⁷ M dexamethasone (all Cyagen Biosciences Inc., Guangzhou, China) as described previously (27,28). The medium was changed twice per week for 3 weeks. Thereafter, cells were rinsed twice with PBS, fixed with 4% paraformaldehyde for 30 min and stained with alizarin red S for 5 min. Cells were visualised under an inverted microscope at magnification, x100.

For adipogenic differentiation, passage 3 cells in complete DMEM were seeded in 6-well plates at 1×10⁵ cells/well. When cells reached 100% confluence or during the post-confluent stage, complete DMEM was carefully aspirated from each well and 2 ml adipogenic differentiation medium was added. The medium consisted of complete DMEM, 200 µM indomethacin, 10⁻⁷ M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine and 10 µM insulin (all Cyagen Biosciences Inc.) as previously described (27,28). After 3 days, the medium was replaced with complete DMEM. After 24 h, the medium was changed back to adipogenic differentiation medium. Following 3-5 cycles of induction and maintenance, the cells were cultured in

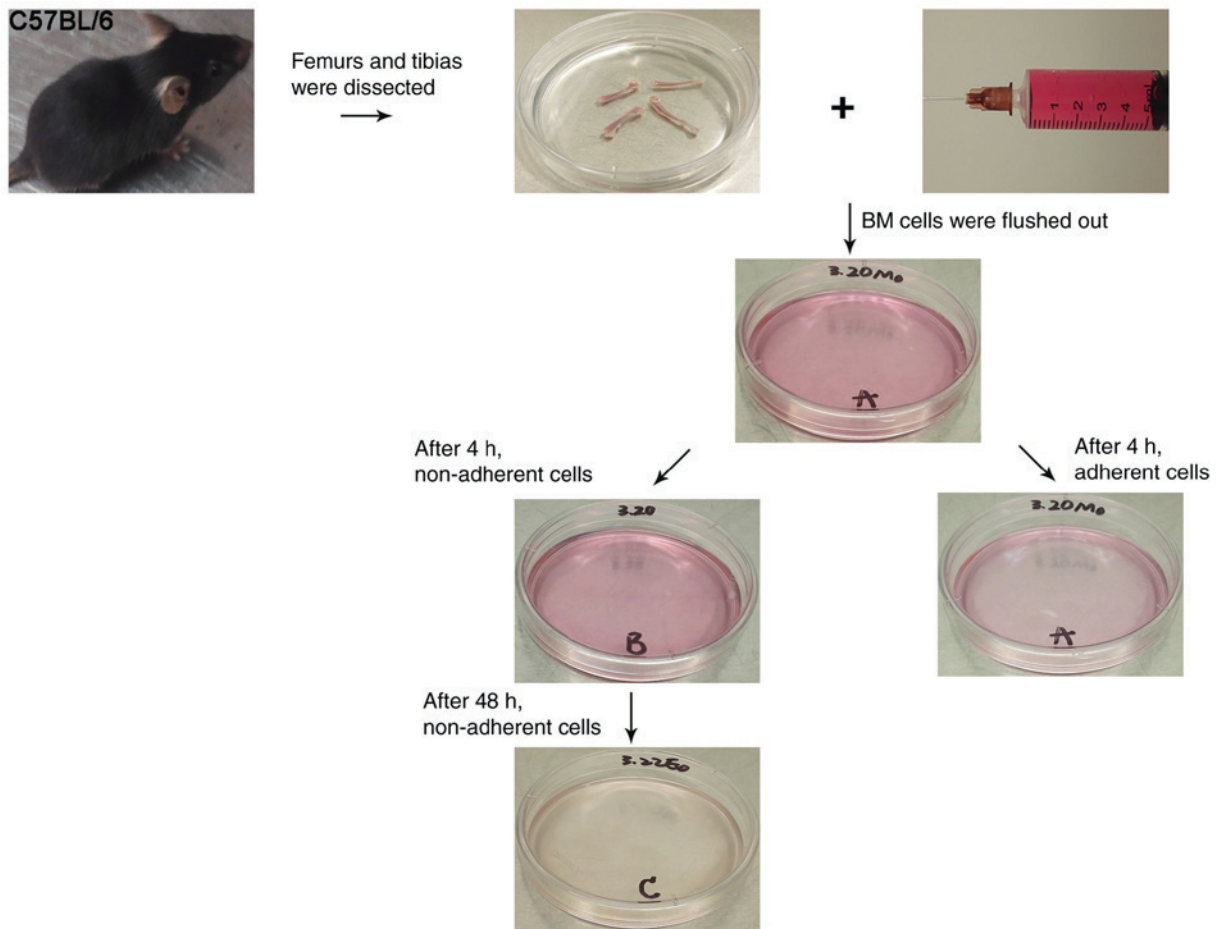


Figure 1. Flow chart for the protocol of simultaneous isolation of MSCs and EPCs derived from murine BM. BM cells were flushed out of femurs and tibias with DMEM complete medium. To obtain MSCs, the medium in culture dish A was gradually replaced with 1.5 ml fresh DMEM complete medium was performed every 8 h for 72 h. To obtain EPCs, non-adherent cells were plated in culture dish C, which was coated with human fibronectin, and maintained in endothelial growth medium. MSCs, mesenchymal stem or stromal cells; EPCs, endothelial progenitor cells; BM, bone marrow; DMEM, Dulbecco's modified Eagle's medium.

adipogenic differentiation medium until lipid droplets were sufficiently large and round. Then, the cells were fixed with 4% paraformaldehyde for 30 min and stained with oil red O for 30 min at room temperature. Cells were visualised under an inverted microscope at magnification, x50.

For chondrocytic differentiation, passage 3 cells in complete DMEM were seeded in 6-well plates at 1×10^5 cells/well. When cells reached 60-70% confluence, complete DMEM was carefully aspirated from each well and 2 ml chondrocytic differentiation medium was added. Chondrocytic differentiation medium consisted of DMEM with 1% FBS (Hyclone; GE Healthcare Life Sciences), 1 mM sodium pyruvate, 50 μ M ascorbate, 50 mg/ml proline, 20 ng/ml TGF- β_3 , 1% ITS supplement and 10^{-7} M dexamethasone (all Cyagen Biosciences, Inc.) as previously described (2,28). The medium was changed twice per week for 3 weeks. Thereafter, cells were rinsed twice with PBS, fixed with 4% paraformaldehyde for 30 min and stained with alcian blue for 30 min at room temperature. Cells were visualised under an inverted microscope at magnification, x100.

Fluorescent-co-staining assay. After 7 days in culture, to examine the presence of specific scavenger receptors for acetylated low-density lipoprotein (acLDL) and murine endothelial

cell markers, attached cells in EGM underwent dual binding with 1,1'-dioctadecyl-3,3',3'-tetramethylidocarbocyanine perchlorate (DiI)-labelled acLDL (Molecular Probes; Thermo Fisher Scientific, Inc.) and FITC-labelled *Bandeiraea simplicifolia* lectin I (BS I; Sigma-Aldrich; Merck KGaA). Cells were initially incubated in EGM containing 5 μ g/ml DiI-acLDL for 4 h at 37°C and then fixed with 4% paraformaldehyde for 10 min at room temperature. Following washing with PBS, cells were stained with 10 μ g/ml FITC-labelled BS-I lectin for 1 h at 37°C. Samples were viewed by confocal laser scanning microscopy (Zeiss LSM 510 Meta; Zeiss AG) at magnification, x100. Double-labelled fluorescent cells were identified as differentiating EPCs.

Tube-like structure formation assay. A 24-well plate was coated with Matrigel (BD Biosciences), which was melted into liquid at 4°C overnight. Subsequently, the plate was placed on ice and incubated for 30 min at 37°C in a 5% CO₂ humidified incubator to allow solidification of Matrigel. Following 14 days in culture, 6×10^4 EPCs without any staining were seeded in the plate and cultured for 6-8 h at 37°C in a 5% CO₂ humidified incubator. Finally, images were randomly captured using an inverted microscope at magnification, x200.

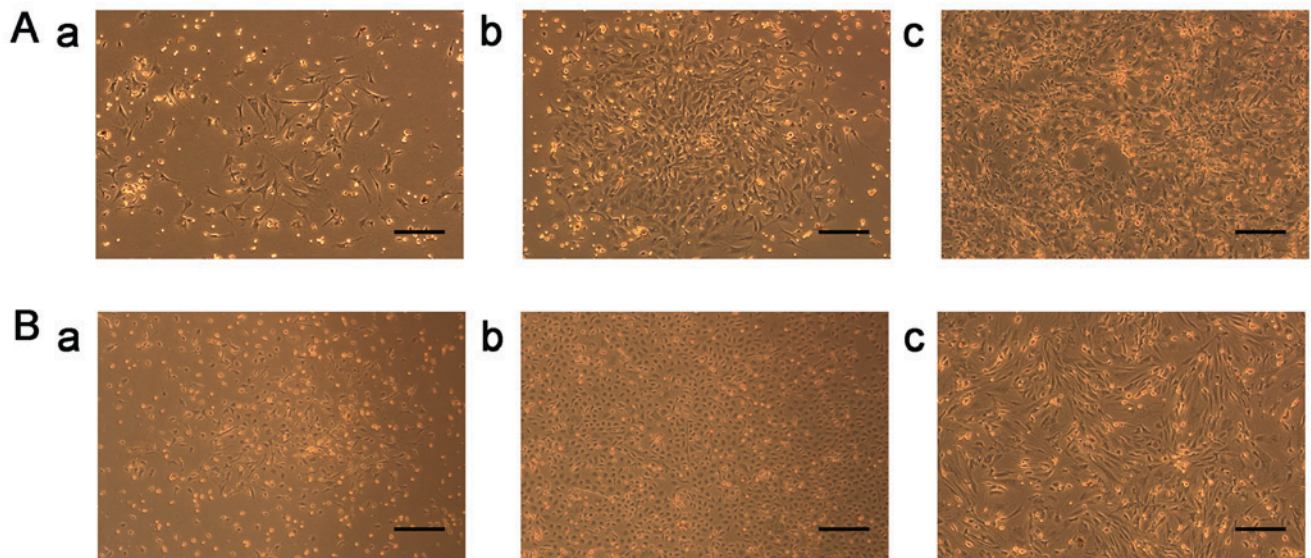


Figure 2. Culturing BM cells produce typical MSCs and EPC-derived endothelial cells. (A) Cell cultures in Dulbecco's modified Eagle's medium complete medium (a) 4 days, (b) 7 days and (c) 2 weeks after the cessation of frequent medium changes. (B) Cell cultures in endothelial growth medium (a) 3 days, (b) 2 weeks and (c) 3 weeks after isolation under endothelial-specific conditions. Scale bar, 200 μ m. MSCs, mesenchymal stem or stromal cells; EPCs, endothelial progenitor cells; BM, bone marrow.

Results

Culturing BM cells produce typical MSCs and EPC-derived endothelial cells. Following 72 h of culture initiation, non-adherent hematopoietic cells were removed with frequent medium changes. Adherent cells appeared as individual cells; they proliferated and gradually formed small colonies at approximately day 4 (Fig. 2Aa). During the 7-day culture, typical colonies of fibroblastic cells appeared (Fig. 2Ab), as described by Ji *et al* (29). The number of cellular colonies with different sizes markedly increased, cells reached near 100% confluence within 14 days and were triangle in shape (Fig. 2Ac). After 1 min of enzyme digestion, numerous cells with triangle-like morphology were detached (data not shown). With further passages, colonies became more homogeneous (data not known).

Following 3 days of isolation under endothelial-specific conditions, cells formed colonies, which were composed of a centre of round cells with elongated spindle-shaped cells sprouting at the periphery (Fig. 2Ba). A total of 2 weeks of isolation yielded colonies of outgrowth cells (late EPCs), which exhibited a 'cobblestone' morphology and a monolayer growth pattern at confluence (Fig. 2Bb). Following culture for 21 days, cells were fusiform, which is typical of EPC-derived endothelial cells (Fig. 2Bc), as described by Ingram *et al* (30).

MSCs and EPCs exhibit typical MSC- and EPC-markers, respectively, following the novel protocol. Cells were analysed for cell surface antigens by FACS. The results revealed that cells in complete DMEM were homogeneously positive for MSC markers CD29 (92.8%), CD44 (97.6%) and Sca-1 (90.4%), but negative for hematopoietic markers CD11b (0.8%) and CD45 (1.9%) (Fig. 3A). The majority of the cells in complete DMEM did not express CD133 (4.7%) and VEGFR-2 (2.0%).

Following 14 days in the EGM culture, FACS demonstrated that the cells expressed endothelial cell lineage antigens

VEGFR-2 (50.3%), Sca-1 (64.9%) and CD133 (86.6%) (Fig 3B). These findings confirmed that cells possessed typical characteristics of EPCs.

MSCs can differentiate into osteoblasts, adipocytes and chondrocytes. Following 3 weeks of osteogenic induction, calcium deposits were stained using alizarin red S, demonstrating that MSCs were undergoing osteogenesis (Fig. 4A). Similarly, following supplementation with adipogenic differentiation medium for 3 weeks, MSCs stained with oil red O were positive for adipocyte globules, indicating that they expressed an adipocyte phenotype (Fig. 4B). The MSCs stained with alcian blue demonstrated an accumulation of cartilaginous proteoglycans, which suggested that the cells had differentiated into chondrocytes (Fig. 4C). MSCs cultured in complete DMEM retained their osteoblastic, adipocytic and chondrocytic differentiation potentials until passage 10 (data not shown).

EPCs demonstrate endothelial cell characteristics and are able to form tube-like structures. Following supplementation with EGM for 7 days, uptake of acLDLs and binding of BS-1 lectin was exhibited in attached EPCs, demonstrating their endothelial cell characteristics (Fig. 5A). Tube-like structures were observed following the culturing of EPCs on Matrigel (Fig. 5B).

Discussion

Previous studies have revealed that highly purified MSCs and EPCs can be obtained by employing three methods: Density gradient centrifugation (17,18), immunomagnetic selection (11,19,20) and flow cytometry sorting (21). However, these three types of experimental process significantly affect cellular activity. There are a small number of BM cells in small animals, such as small, 1-week-old mice, and fibroblastic cells comprise 0.001-0.01% BM cells (22). Thus, technical difficulties may arise during isolation of fibroblastic cells from mice.

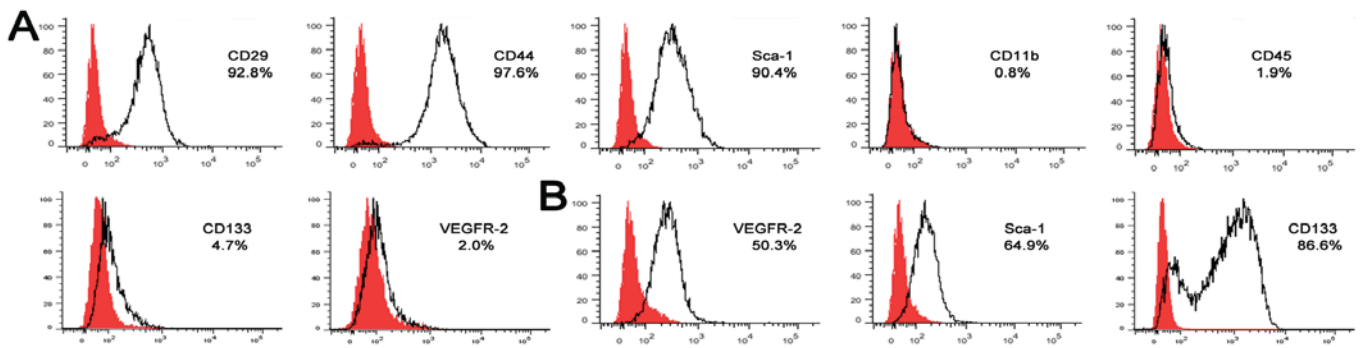


Figure 3. MSCs and EPCs exhibit typical MSC- and EPC-markers, respectively, following the novel protocol. Histograms of fluorescence-activated cell sorting analyses cell surface markers in (A) MSCs and (B) EPCs. Respective isotype controls are shown in red. X-axes, fluorescence intensity; Y-axes, cell counts; CD, cluster of differentiation; Sca-1, stem cell antigen-1; VEGFR-2, vascular endothelial growth factor receptor 2; MSCs, mesenchymal stem or stromal cells; EPCs, endothelial progenitor cells.

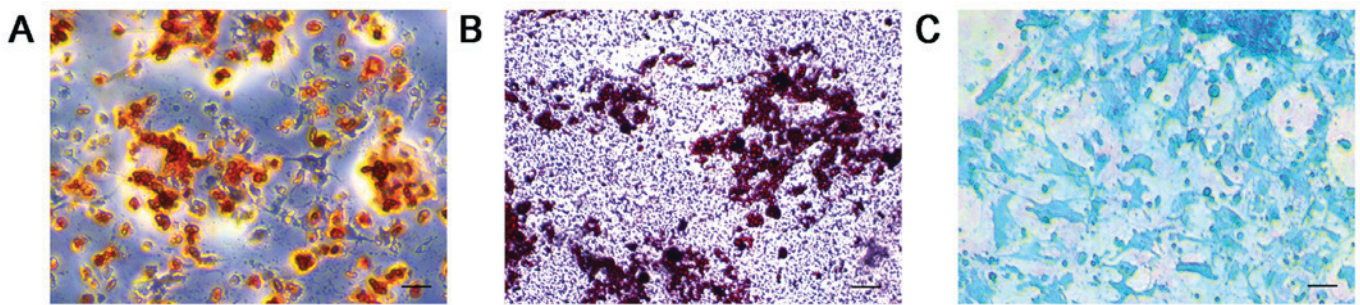


Figure 4. MSCs can differentiate into osteoblasts, adipocytes and chondrocytes. (A) Alizarin red staining revealed calcium deposits (bright orange-red) in MSCs induced by osteogenic differentiation medium. Scale bar, 100 μ m. (B) Oil red O staining revealed adipose globules (purple) in MSCs induced by adipogenic differentiation medium. Scale bar, 200 μ m. (C) Alcian blue staining revealed cartilaginous proteoglycans (blue) in MSCs induced by chondrocytic differentiation medium. Scale bar, 100 μ m. MSCs, mesenchymal stem or stromal cells.

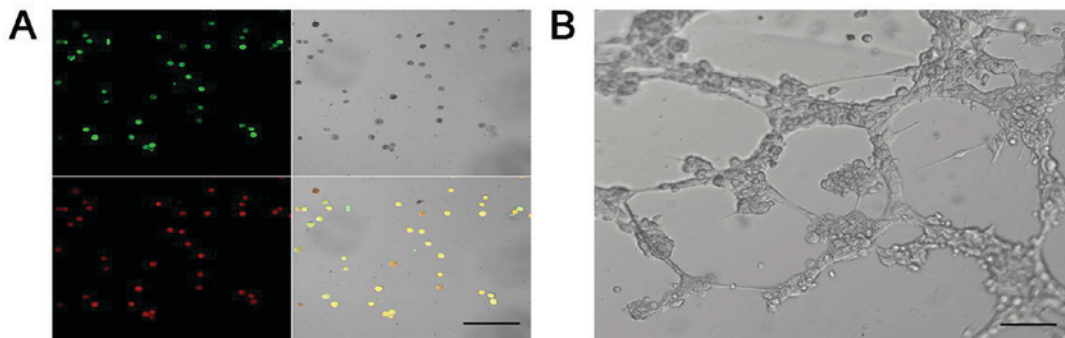


Figure 5. EPCs demonstrate endothelial cell characteristics and are able to form tube-like structures. (A) Fluorescence confocal microscopy illustrates the uptake of DiI-acLDL (red), bonding of FITC BS-I lectin (green), double staining with DiI-acLDL, FITC BS-I (yellow), and the cellular morphology in bright field (grey) after 7 days of culturing. Scale bar, 100 μ m. (B) EPCs after culturing on Matrigel for 8 h. Scale bar, 50 μ m. EPCs, endothelial progenitor cells; DiI-acLDL, 1,1'-dioctadecyl-3,3,3',3'-tetramethylidocarbocyanine perchlorate-labelled acetylated low-density lipoprotein; FITC-labelled BS-I lectin, fluorescent isothiocyanate-labelled *Bandeiraea simplicifolia*-I lectin.

By contrast, cells can easily be obtained based on their plastic adherence characteristics, but this method cannot be used to retrieve pure cells (31). In the present study, pure MSCs and EPCs were simultaneously isolated and obtained from murine BM. The protocol employed in the current study featured three important points: Firstly, the medium was frequently changed to prevent the adherence of non-MSCs to the culture dish; secondly, the attachment time for MSCs and EPCs differed; thirdly, the duration of trypsinisation was well controlled.

MSCs resembled fibroblasts in terms of their morphology and due to their colony formation; these characteristics were identified in MSCs from numerous species, including humans (32), rats (33), mice (34) and rabbits (35). However, the expandability of MSCs *in vitro* varied significantly among different species, and different methodologies for isolation and plating of cells. In the present study, isolated cells were identified as MSCs and EPCs on the two bases: i) MSCs are fibroblast-like clonogenic cells (colony-forming unit-fibroblast)

with high replicative capacity *in vitro* (36) and ii) EPCs exhibit the ability to form colonies or small clusters of cells based on differences in proliferative and/or differentiation potentials (37). MSCs and EPCs formed colonies, demonstrating their stem and/or progenitor cell characteristics.

These observations were further supported by detecting expression levels of cell surface antigens. Surface antigens for murine-derived MSCs are not well defined (38). Generally, MSCs are characterized by an immunophenotype depicted as positive for Sca-1, CD29 and CD44, and negative for CD11b and CD45 because CD11b is expressed by monocytes, granulocytes and natural killer cells, and CD45 is expressed in all lymphohematopoietic cell lineages (34). The results of FACS analysis revealed that the cells scarcely expressed surface markers CD11b and CD45, but expressed high levels of CD29, CD44 and Sca-1. This finding is consistent with previously published data on MSC surface markers and implies that the proposed method obtains pure MSCs. Surface markers for murine-derived EPCs also remain unclear (39). It was hypothesised that CD133 is a reliable phenotypic marker that can be used to isolate bona fide EPCs (40). In addition, VEGFR-2 is an endothelial-specific marker and Sca-1 is expressed only in murine species (27,41). Thus, VEGFR-2, Sca-1 and CD133 are considered surface markers of murine-derived EPCs. In the present study, CD133 and VEGFR-2 expression was also evaluated in MSCs in the current study to determine if EPCs differentiated from MSCs. The results revealed that CD133 and VEGFR-2 expression was low in MSCs, which may disprove the EPC differentiation from MSCs. FACS analysis indicated that EPCs expressed VEGFR-2, Sca-1 and CD133.

Finally, functional analysis was performed for prospective identification and further characterisation of progenitors. Multilineage differentiation potential is considered an important quality of MSCs (42). In the present study, cells cultured in complete DMEM successfully differentiated into osteogenic, adipogenic and chondrogenic lineages in the presence of tissue-specific induction media. EPCs cultured in EGM medium incorporated acLDL and bound BS-1 lectin, which characterise endothelial function.

In summary, compared with other approaches for separating MSCs and EPCs, plastic adherence is a simple and efficient method. Through the principle of adhesion, homogeneous MSCs and EPCs can be simultaneously obtained as potential resources for the basic study of stem cell therapy and regenerative medicine.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XW isolated cells, and was a major contributor in writing the manuscript. ZZ analyzed and interpreted the results of FACS. HZ performed differentiation of MSC assays. JH conducted the fluorescent co-staining assay. WF performed the tube-like structure formation assay. MZ fed the mice and was involved in the isolation of MSCs. JG participated in isolating EPCs. JX assisted with the differentiation of MSC assays. QG prepared test reagents and was involved in writing the Materials and methods section. XC made substantial contributions to the acquisition of FACS data, including the selection of fluorescent antibodies and operation of the test. XWu conceived and designed the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experimental animal protocol used in the present study was approved by the Animal Experimental Ethics Committee of Shihezi University (Shihezi, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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