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ORIGINAL ARTICLE

Characterisation of multipotent stem cells from human peripheral blood using an improved protocol

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Abstract *Background:* A promising approach of bone repair is to use stem cells, such as mesenchymal stem cells (MSCs). Seeking available source of MSCs still remains a great challenge in tissue engineering and cell therapy. Peripheral blood (PB) emerges as an alternative source of MSCs which can be easily acquired with minimal invasiveness. This study was undertaken to evaluate the multipotency of PB-MSCs and effects of human PB-MSCs transplantation on ectopic bone regeneration in nude mice.
Methods: Human venous blood collected was mixed with heparin and then red blood cells were removed using red blood cell lysis buffer. Cell suspension was cultured in normoxia-culture and hypoxia-culture conditions, respectively. The non-adherent cells were removed by half changing culture media every three days. Cells were selected due to plastic adherence. The adherent cells were then passaged and subjected to multi-differentiation induction assays in vitro and in vivo ectopic bone formation assay.
Results: Characterization assays indicated that cells cultured under hypoxia possessed potent multi-lineage differentiation capacity and expressed Nanog and Lgr5, as well as a series of MSC surface antigens (including CD29, CD90, CD105, and CD73). Additionally, regenerated bone

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tissues by transplantation of human PB-MSCs in vivo were confirmed by histological examinations of ectopic osteogenesis assay. A purified population of MSCs can be obtained within a short period of time using this protocol with a successful rate of 60%.
Conclusion: We reported an effective and reliable method to harvest highly purified MSCs with potent multi-differentiation potential from human peripheral blood. Lgr5 may be a potential biomarker for identification of a subpopulation of PB-MSCs.
The translational potential of this article: PB-MSCs is an alternative cell source for cell therapy, which may be harvested, culture expanded and PB-MSCs loaded with β-tricalcium phosphate (β-TCP) may be used to promote bone repair.
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Introduction

Cell therapy is a novel approach for curing otherwise difficult diseases [1]. Mesenchymal stem cell (MSC)—based therapy exerts profound therapeutic potential for treating a variety of diseases, such as neural diseases [2], wounds [3], myocardial infarction [4] and various musculoskeletal diseases [5,6]. MSCs are multipotent stromal cells which are capable of self-renewal and differentiation into lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, muscle and marrow stroma [7]. Studies have demonstrated that bone marrow MSCs (BM-MSCs) in combination with scaffolds improved bone formation after transplantation into heterotopic sites or repaired bone defects in animal models [8,9].

As a kind of adult stem cells, various populations of multipotent postnatal MSCs share the expression of embryonic transcription factors, such as Nanog, which is essential for the maintenance of self-renewal and undifferentiated state in MSCs [10]. Leucine-rich repeat-containing G protein—coupled receptor 5 (Lgr5), a Wnt target gene, modulates Wnt signalling strength through binding to its ligand R-spondin [11]. Lgr5 is widely used as a molecular marker for self-renewing multipotent adult stem cell populations in multiple organs, including the gut, stomach, hair follicle, mammary gland, kidney and ovary [11—15]. Although BM is the most abundant source of MSCs, it may not be the optimal source because the acquisition of BM aspirate is an invasive and painful procedure [16]. Thus, seeking an alternative source of MSCs remains a major challenge for cell therapy and tissue engineering.

Increasing evidence suggested that peripheral blood (PB) may be an alternative source of stem/progenitor cells for clinical application besides BM, including haematopoietic stem cells, endothelial progenitor cells and MSCs [17—19]. Studies have shown that adult stem cells patrol in the bloodstream and circulate through peripheral organs physiologically and pathologically, which may be required for tissue homoeostasis and tissue repair. In general, MSCs are present in the peripheral circulation at a very low frequency at a steady state. In response to injury signals, however, the number of MSCs in the bloodstream would increase dramatically [17,18,20,21]. Thus, PB, which can be collected with minimal invasion, has been identified as an important alternative source of MSCs for cell therapy during recent years.

PB-derived MSCs (PB-MSCs, also termed as circulating MSCs) have been demonstrated to possess trilineage differentiation capacity (osteogenic, adipogenic and chondrogenic) and express a series of surface antigens (e.g., CD29, CD73, CD105 and CD90), which is very similar to that of BM-derived MSCs [21]. However, until now, the optimized culture protocol of PB-MSCs has not been well defined, and their biologic properties are not fully understood. Therefore, this study was to optimize in vitro culture and expansion conditions of PB-MSCs and evaluate their multipotency and potential implications in bone repair.

Materials and methods

Collection of human blood samples

Venous blood samples (10 mL each donor) were collected from healthy adult donors (age ranges from 25 to 50 years, $n = 5$). The collection process was approved by the Clinical Research and Ethics Committee of the Chinese University of Hong Kong or Guangzhou University of Chinese Medicine. The experimental procedures for blood samples and PB-MSCs were carried out according to the approved guidelines. All PB-MSCs used in this study were harvested with the informed consent of the donors for the use in scientific research.

Human PB—derived MSC culturing

The blood samples mixed with heparin were subjected to red blood cell lysis buffer (420301; BioLegend, USA). The cells collected were then resuspended in complete culture media containing α—minimum essential medium (α-MEM; Gibco/Invitrogen, Thermo Fisher Scientific, USA) supplemented with 17% foetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin/neomycin. The cell suspension was seeded at a density of 0.5×10^6 cells/cm^2 and then incubated in a humidified atmosphere of 5% CO_2 at 37°C in two groups: (1) normoxia culture of 21% O_2 and

(2) hypoxia culture of 5% O_2 (a hypoxic chamber: Galaxy 48R, C0-48-230; New Brunswick, Edison, NJ, USA). Half culture media were replaced with fresh complete culture media every 3 days. Once the primary cultured cells reached a confluence of over 60%, the cells were passaged regularly and cultured in complete culture medium of 10% FBS. The culture medium was refreshed every 3 days.

Human BM—derived MSC culturing

BM aspirates were obtained from the fracture patients during surgery with the patients' full consent. The marrow aspirate was diluted with the same volume of normal saline and loaded onto the Ficoll-Paque media solution (GE Healthcare, Piscataway, NJ, USA). After centrifugation at 400 g for 30 min, MSCs were collected from the interface and washed twice with phosphate-buffered solution (PBS). The cells collected were cultured at 1×10^6 cells/cm^2 in T-75 cell culture flasks (Corning, USA) in α-MEM supplemented with 10% FBS, 100 units/mL penicillin and 100 ug/mL streptomycin and incubated at 37°C under hypoxia or normoxia conditions. The nonadherent cells were discarded 3 days later during media exchange, and the adherent MSCs were cultured to 80% confluence before passaging with 0.25% trypsin—EDTA (Gibco).

Crystal violet staining assay

The cells were washed three times with 1× PBS. The cells were fixed with 4% (wt/vol) paraformaldehyde (PFA) and stained with 0.05% (wt/vol) solution of crystal violet. The dishes were washed with running water and air-dried. The results of staining were captured under a microscope.

In vitro multilineage differentiation assays

Adipogenic differentiation

Human PB—derived MSCs (hPB-MSCs) were trypsinized and seeded in a 6-well plate with growth media at a concentration of 1×10^5 cells per well. The cells were incubated in α-MEM complete medium until a confluence of 90% was reached. The medium was then replaced by an adipogenic induction medium containing 10% FBS, 1 μM dexamethasone, 10 μg/mL insulin, 50 μM indomethacin and 0.5 mM isobutylmethylxanthine. The cells cultured in a basal medium of α-MEM supplemented with 10% FBS served as a negative control. Adipogenic induction lasted for 2 weeks, and the medium was changed every 3 days. The cells were fixed with 4% PFA for 30 min. Oil Red O staining was applied, and the results were observed under a microscope.

Osteogenic differentiation

The cells were seeded and cultured until a confluence of about 80% was reached. The medium was replaced by an osteogenic induction medium containing 100 nM dexamethasone, 10 mM β-glycerophosphate and 0.05 mM L-ascorbic acid 2-phosphate. The cells cultured in a basal medium of α-MEM supplemented with 10% FBS served as a negative control. After 2 weeks of induction, Alizarin Red staining was applied to assess mineralization. The cell/matrix layer was stained with 0.5% Alizarin Red S (pH 4.1) for 5 min. The positive stain was viewed under a microscope.

Chondrogenic differentiation

The cells were seeded and cultured until a confluence of over 90% was reached. The cells were incubated in a chemically defined chondrogenic induction medium consisting of high-glucose DMEM supplemented with 10 ng/mL recombinant human transforming growth factor-β1, 100 nM dexamethasone, 1 mM sodium pyruvate, 0.2 mM ascorbic acid 2-phosphate (Sigma-Aldrich, MO, USA) and ITS + Premix (1:100; BD Biosciences, CA, USA). The medium was changed every 3 days. The induction process lasted 2 weeks. Alcian blue staining was applied. About 4×10^5 MSCs were centrifuged in a 15-ml polypropylene tube, and the pellets were cultured in chondrogenic induction media for 3 weeks. The cells cultured in a basal medium of α-MEM served as a negative control, with the media changed every 2 days. After 3 weeks, the cell pellets were fixed in 4% PFA and embedded in paraffin. Safranin O staining was applied. The staining results were viewed under a phase contrast microscope (Leica Microsystems Wetzlar Gesellschaft mit beschrankter Haftung, Wetzlar, Germany).

Neurogenic induction assay

The hPB-MSCs were seeded and incubated in the α-MEM complete medium until a confluence of about 30% was reached. The cells were exposed to neurogenic induction media (5752; NeuroCult™ NS-A Differentiation Kit, Stemcell Technologies, Canada), and the induction medium was refreshed every 2 days. The cells cultured in a basal medium of α-MEM supplemented with 10% FBS served as a negative control. The induction process lasted 3 weeks. Neurogenic differentiation was confirmed by immunofluorescence assessment of Glial Fibrillary Acidic Protein (GFAP; G3893; Sigma-Aldrich).

Immunocytochemistry

The cells were seeded onto slides in a 12-well plate (3000 cells per well). The cells were incubated in α-MEM complete culture media for 24 h. The cells were fixed with 4% PFA for 30 min after washing with PBS. Then, the cells were blocked with PBS containing 1% bovine serum albumin (BSA; Sigma-Aldrich, MO, USA) for 30 min at 37°C followed by permeabilisation with 0.5% Triton X-100. Anti-GFAP (1:300; Sigma-Aldrich, MO, USA), anti-CXCR4 (1:300; R&D Systems, USA), anti-Nestin (1:300; Sigma-Aldrich, MO, USA), anti-Nanog (1:300; BD Biosciences, CA, USA) and anti-Lgr5 (1:300; Abcam, USA) antibody were separately added and incubated at 4°C overnight. Alexa Fluor 488-conjugated goat anti-rabbit or fluorescein isothiocyanate (FITC)-conjugated anti—mouse secondary antibodies (Life Technologies, USA) were incubated with the cells at 37°C for 40 min. The cells were sealed using glycerin containing 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies) after washing with PBS and then observed under a confocal microscope.

Flow cytometry

The hPB-MSCs were enzymatically digested with 0.25% trypsin—EDTA for 30 s at room temperature. The cells were resuspended in resuspension buffer containing 1% BSA (Sigma-Aldrich, MO, USA) at 10^6 cells/mL. A volume of 300 μL cells was transferred into a 5-mL flow cytometry tube. The cell aliquots were incubated with purified anti-phycoerythrin (PE)-conjugated CD29, CD90, CD105, CD44 or CD73 or FITC-conjugated CD34, or CD45 or each corresponding isotype control antibody (BD Biosciences, CA, USA) for 30 min at room temperature in the dark. The cells were resuspended in 1000 μL of resuspension buffer after washing with PBS. Then, cell suspension was analysed by flow cytometric analysis using Cell Quest software (BD Biosciences, CA, USA). BD FACS Canto II was used for data acquisition by adjusting voltage and compensation using appropriate excitation and detection channels. Data analysis was performed using FlowJo software (BD Biosciences, CA, USA).

Real-time quantitative polymerase chain reaction analysis

Total RNA of the cultured cells was extracted using TRIzol (Invitrogen). cDNAs were reverse transcribed from RNA samples by PrimeScript RT Master (Takara, Japan). The Power SYBR Green PCR Master Mix (Thermo Fisher, USA) was applied for the detection of target mRNAs using ABI 7300 Fast Real-Time PCR Systems (Applied Biosystems, USA). The relative fold changes of candidate genes were analysed by using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Total protein of the cells was extracted using radioimmunoprecipitation assay lysis buffer (25 mM Tris-Cl [pH 8.0], 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 0.1% Nonidet P-40) supplemented with complete mini protease inhibitor cocktail (Roche, USA). The supernatant of cell lysates collected after centrifugation at 15,000 rpm for 15 min at 4°C was mixed with 5x loading buffer and boiled for 5 min. The protein samples were subjected to electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Then, proteins were transferred onto a polyvinylidene fluoride membrane at 100 V for 60 min and incubated in blocking solution (5% nonfat dry milk in tris-buffered saline (TBS) containing 0.1% Tween 20) for 60 min to reduce nonspecific binding. The membranes were then incubated overnight at 4°C with the following primary antibodies: Lgr5 (1:2000; Abcam) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:3000; Abcam). The membranes were washed extensively and visualized on an X-ray film using a Kodak film developer (Fujifilm, Japan) after incubation with appropriate secondary antibodies for 1 h.

In vivo ectopic bone formation assay

The harvested PB-MSCs were individually cultured and assayed. A total of 2×10^5 hPB-MSCs of Passage 3 in suspension were slowly injected into sterilized, porous and resorbable β-tricalcium phosphate (β-TCP) scaffolds (Shanghai Bio-lu Biomaterials Co., Ltd., Shanghai, China) with diameters of 1—2.5 mm. Empty scaffolds with culture media served as the empty controls. After being incubated at 37°C for 3 h to allow cell attachment, the hPB-MSCs/β-TCP constructs were subsequently cultured for 3 days in vitro before implantation. The grafts with the cells were implanted subcutaneously into the dorsal and subcutaneous spaces of three nude mice under general anaesthesia (0.2% xylazine and 1% ketamine in PBS). The transplants were harvested 8 weeks after implantation. The samples were fixed with 4% PFA, dehydrated and embedded in paraffin and then subjected to histological examination. Haematoxylin and eosin staining and immunohistochemical staining of osteocalcin (sc30045; 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were applied. Antigen retrieval was performed by incubating in 10 mM warm citrate buffer (pH 6.0) for 20 min at 60°C. The sections were incubated with primary antibodies overnight at 4°C after blocking with blocking solution (5% animal serum in PBS/1% BSA). The osteoid matrix areas were measured using ImageJ software (NIH, Bethesda, Maryland, USA; https://imagej.nih.gov/ij/); five random microscopic fields were chosen from each sample and measured as reported previously [22—24]. All procedures of animal studies were performed in accordance with the guidelines approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong.

Statistical analysis

At least three sets of independent experiments were performed for each assay. All quantitative data were transferred to statistical spreadsheets and analysed by a commercially available statistical program SPSS version 16.0 (IBM SPSS Inc., Chicago, IL, USA). The student t test was used for comparison of mean values between the two groups with $p < 0.05$ considered as statistically significant.

Results

Cell morphology observation

Under general culture (normoxia culture) conditions (21% O_2 and 5% CO_2), adherent cells of several shapes were observed 7 days after seeding (Fig. 1A). However, the cells of the normoxia culture group exhibited several different shapes and proliferated slowly and reached a confluence of around 60% on Day 21 (Fig. 1B). In contrast, for cells under hypoxic culture (5% O_2 and 5% CO_2), adherent cells exhibiting a homogeneous cell shape (spindle shape) appeared 5 days after seeding (Fig. 2A—B). Colonies appeared 7 days later (Fig. 2C—D) and reached a confluence of about 90% 2 weeks later (Fig. 2E). After passaging, the cells retained spindle shape and fibroblast-like morphology (Fig. 2F). In addition, the results of crystal violet staining (Fig. 2F) showed that hPB-MSCs still possessed colony-forming ability after passaging. Hypoxia culture may be instrumental for cell viability and stemness maintenance by mimicking natural stem cell niche in vivo and the plastic adherence ability of hPB-MSCs [25].

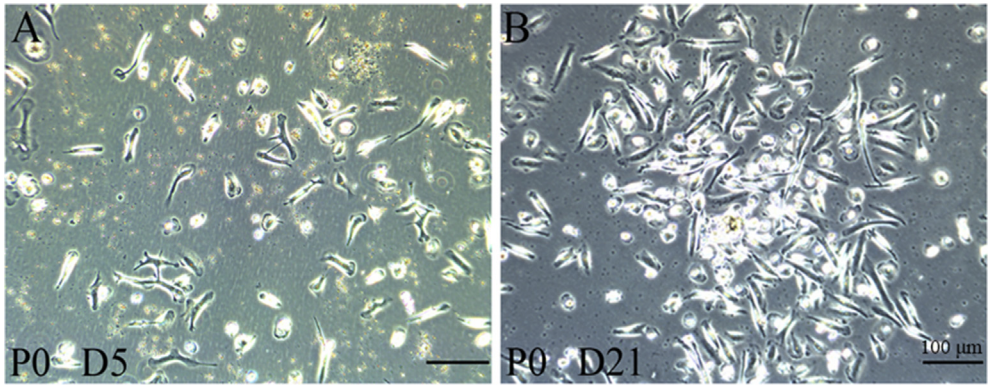

Figure 1 Morphology of adherent cells under general culture conditions (21% O_2 and 5% CO_2). (A) Adherent cells exhibiting several different slim bar shapes were observed 5 days later after seeding. (B) Colonies appeared 21 days later; however, cell number did not reach enough confluence for passaging (less than 70% confluence). Scale bar: 100 μm.

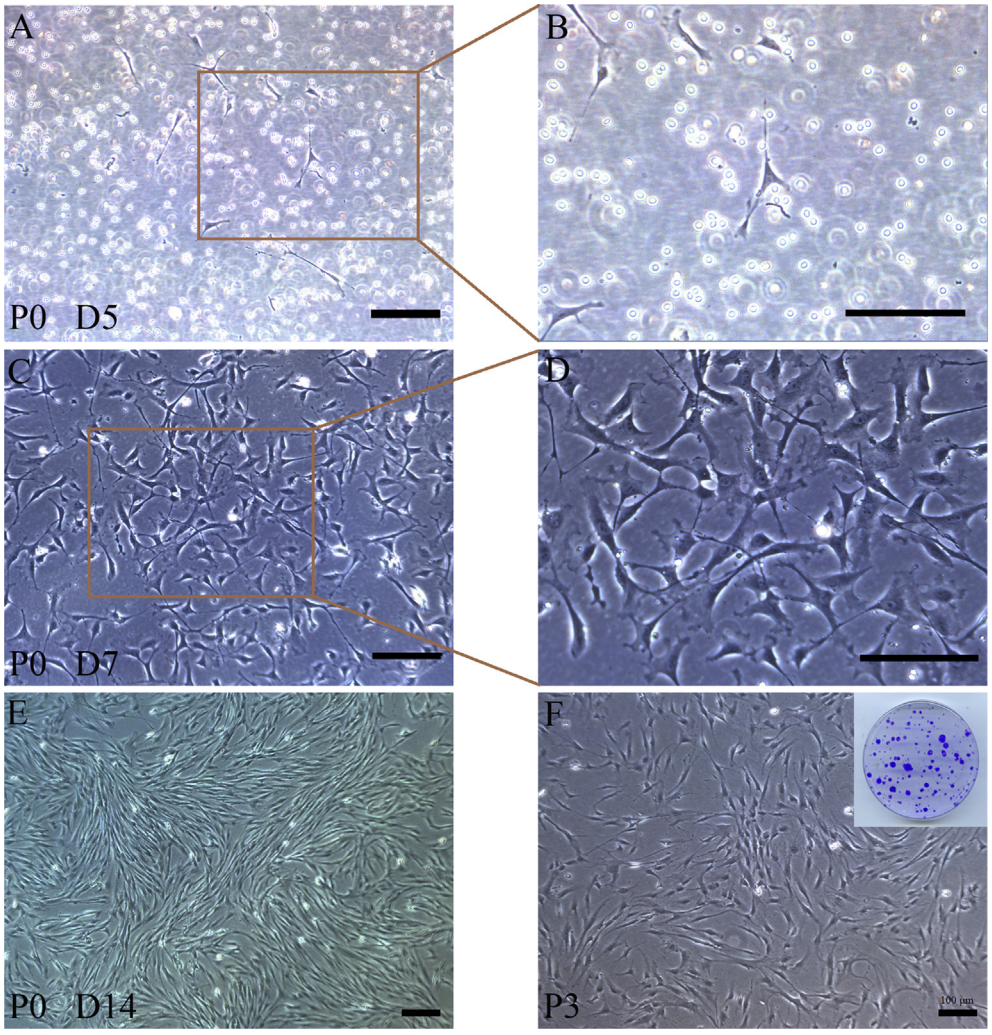

Figure 2 Morphology of adherent cells under hypoxic culture conditions (5% O_2 and 5% CO_2). (A and B) Adherent cells displaying spindle-shaped morphology were observed 5 days after seeding. (C and D) Colonies appeared 7 days later. (E) The cells reached a confluence of about 90% 2 weeks later. (F) The cells remained in spindle shape after passaging. In addition, cell colonies were stained with crystal violet, suggesting that cells at Passage 3 still possessed colony-forming ability. Scale bar: 100 μm.

Characterisation of hPB-MSCs by multidifferentiation induction and immunophenotyping assays

To test the multipotency of the cultured hPB-MSCs, the cells were exposed to various types of differentiation induction media. The results of multidifferentiation induction assays (Fig. 3 and Supplementary Fig. 1C) indicated that hPB-MSCs were capable of osteogenic, adipogenic, chondrogenic and neurogenic differentiation under appropriate induction conditions. mRNA levels of chondrogenic-associated markers were upregulated dramatically compared with those of the noninduction group after 10-day chondrogenic induction (Supplementary Fig. 1B). Simultaneously, the immunostaining results showed that the cells isolated from hPB were positive for CXCR4, Nestin, Nanog and Lgr5 (Fig. 4). Lgr5, a cell surface protein, was mainly expressed in the cytoplasm of PB-MSCs.

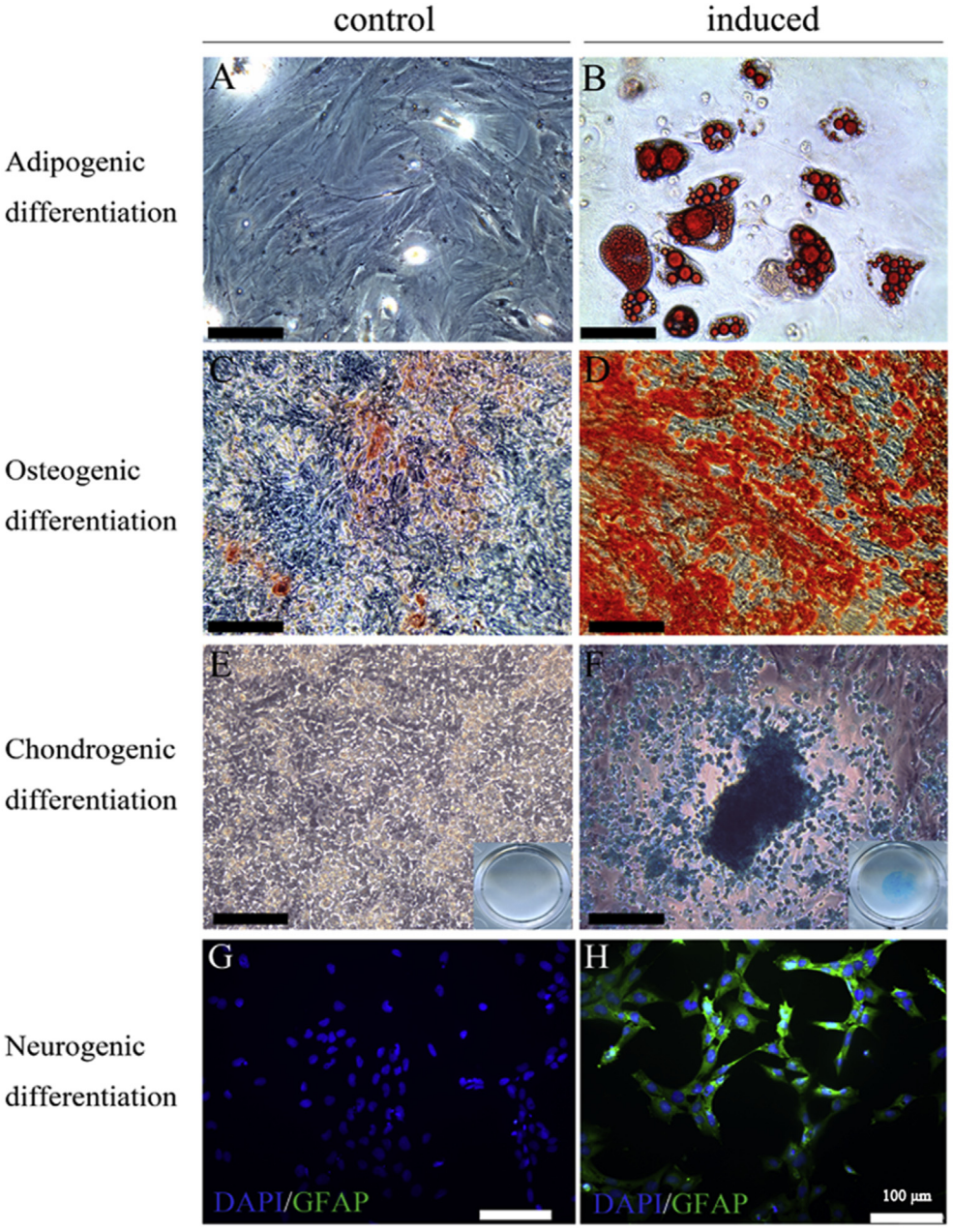

Figure 3 Characterization by multidifferentiation induction assays. Peripheral blood—derived adherent cells were induced to differentiate into adipocytes (B), osteoblasts (D), chondrocytes (F) and neurons (H). (B) Adipogenesis was indicated by the presence of neutral lipid droplets that stained with Oil Red O staining. (D) Osteogenesis potential was identified with positive Alizarin Red staining. (F) Chondrogenic differentiation potential was indicated by positive alcian blue staining. (H) Neurogenic differentiation was demonstrated by positive neurogenic marker (GFAP; the glial fibrillary acidic protein) using immunocytochemistry. (A, C and E & G) Noninduction groups served as the control. Cell nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole). Scale bar: 100 μm.

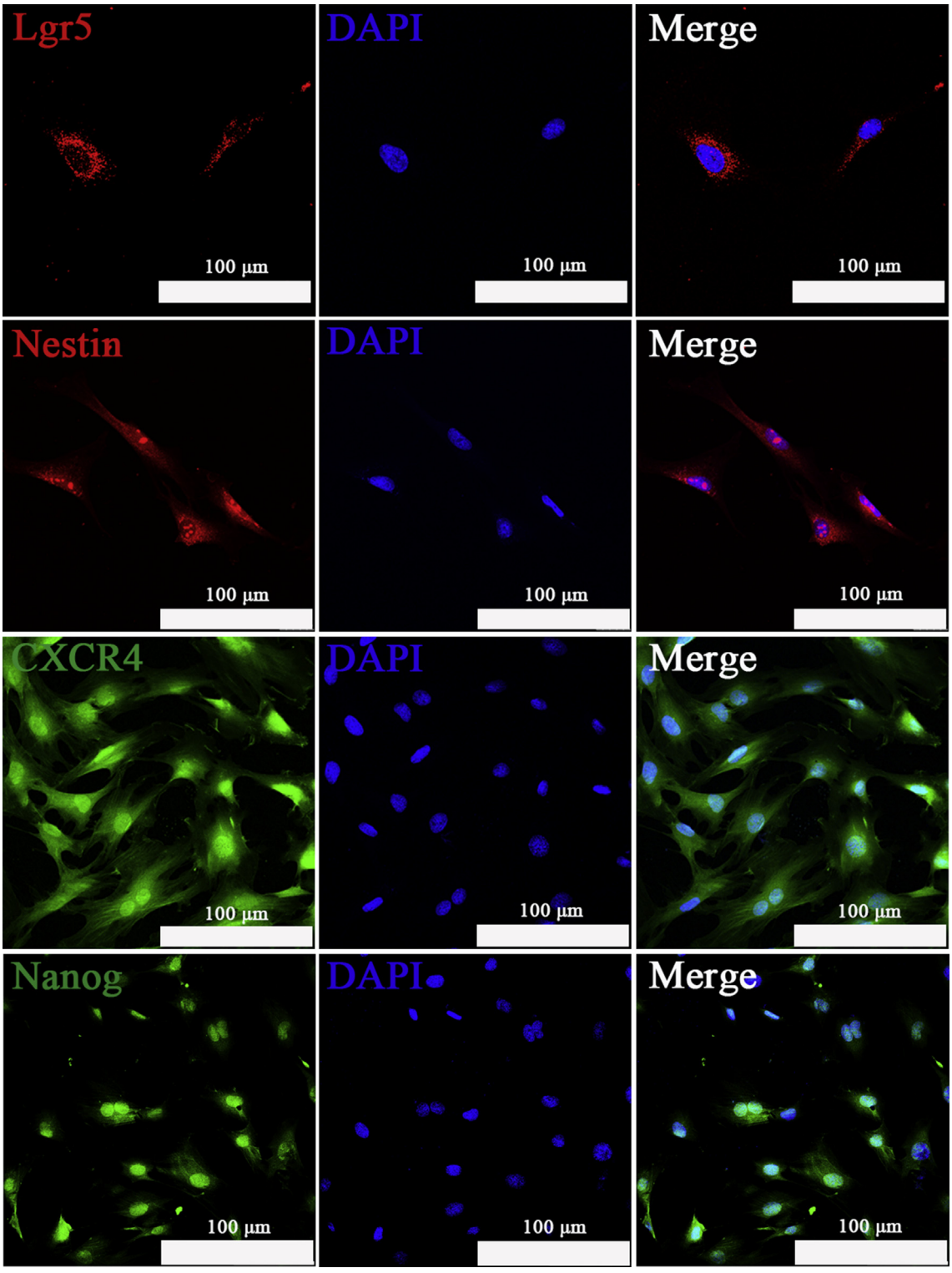

Figure 4 Immunofluorescence staining evaluation of Lgr5, Nestin, CXCR4 and Nanog. Fluorescence microscopy indicates Lgr5-positive (red), Nestin-positive (red), CXCR4-positive (green) and Nanog-positive (green) human peripheral blood—derived mesenchymal stem cells (hPB-MSCs). The expression of Lgr5 was mainly localized in the cytoplasm of hPB-MSCs. The expression and localization of Nestin and CXCR4 were both in the nuclei and cytoplasm of hPB-MSCs. The expression of Nanog was mainly localized in the nuclei. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole; blue). Scale bar: 100 μm.

Simultaneously, the results of Western blot further indicated the increased expression of Lgr5 in hPB-MSCs compared with hBM-MSCs (Supplementary Fig. 1A), suggesting that Lgr5 may become a potential biomarker to identify PB-MSCs. In addition, flow cytometric analysis showed that hPB-MSCs were strongly positive for CD90, CD29, CD105, CD44 and CD73 (more than 95% expression rate), but lacked the expression of haematopoietic markers (less than 2% expression rate for CD45 and CD34) (Fig. 5).

Potential clinical implications of hPB-MSCs in ectopic bone formation in vivo

We then further evaluated whether hPB-MSCs possessed bone-forming capacity in vivo using a well-established mouse transplantation model in which sterilized porous β-TCP scaffolds were used as carrier vehicles. The implants were harvested 8 weeks after transplant, containing all cellular components and matrix structures. The formation of osteoid matrix in bone-like tissue was demonstrated by positive haematoxylin and eosin and osteocalcin staining of the osteoid (Fig. 6), confirming the ectopic bone formation capacity of PB-MSCs in vivo.

Discussion

Cell therapy provides a novel approach for treating a broad spectrum of diseases. MSCs, one of the most intensely studied candidates for cell therapy, are diverse subsets of multipotent precursors present in the stromal fraction of

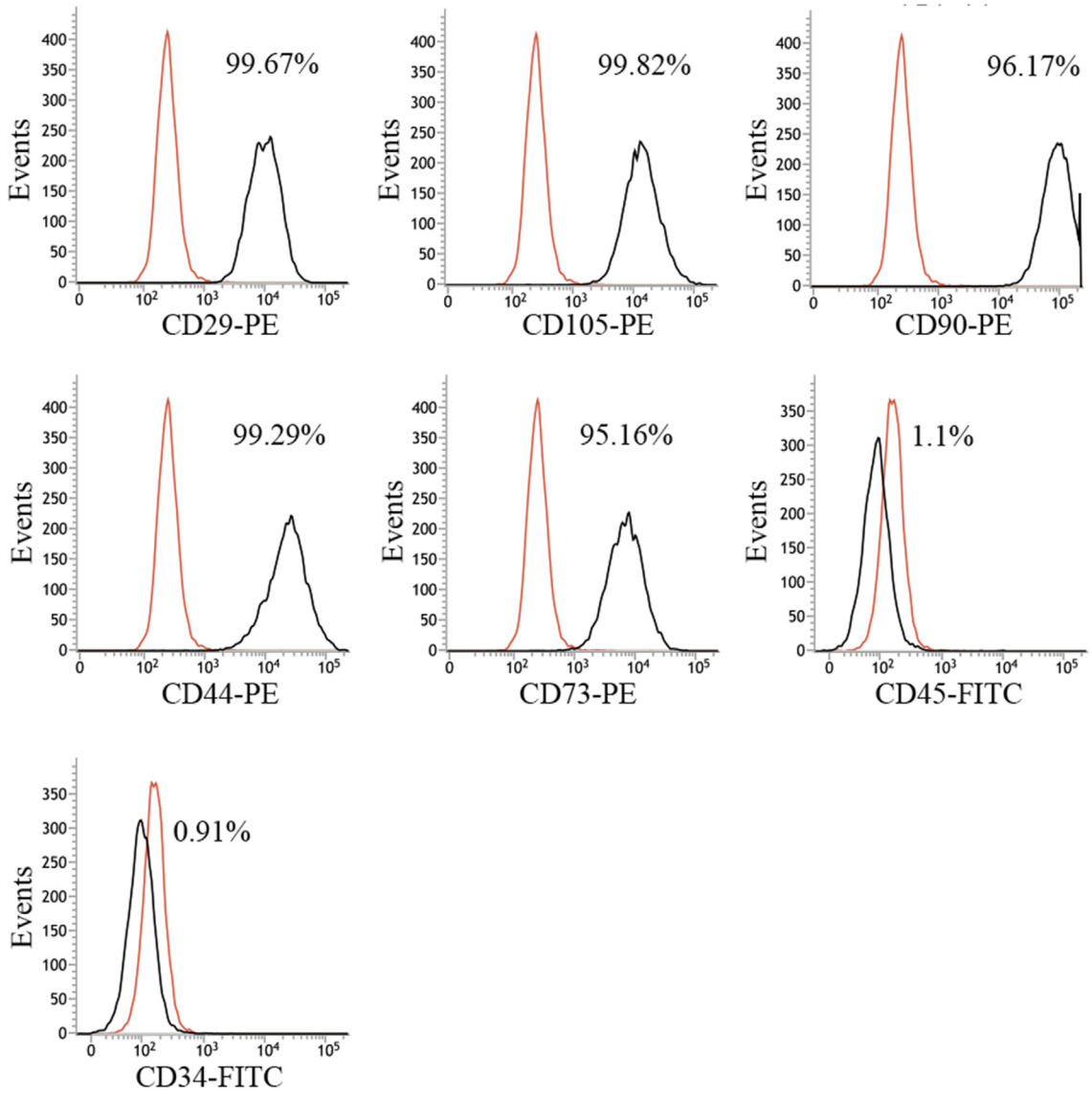

Figure 5 Immunophenotypic characterisation of human peripheral blood—derived mesenchymal stem cells (hPB-MSCs). The results of flow cytometric analysis showed that hPB-MSCs of Passage 3 were strongly positive for MSC-specific surface markers (more than 95% expression rate), including CD29, CD90, CD105, CD44 and CD73. However, cells cultured were negative for CD34 and CD45 (less than 2% expression rate). The expression of every surface antigen is shown together with their corresponding isotype control. FITC, fluorescein isothiocyanate; PE, phycoerythrin.

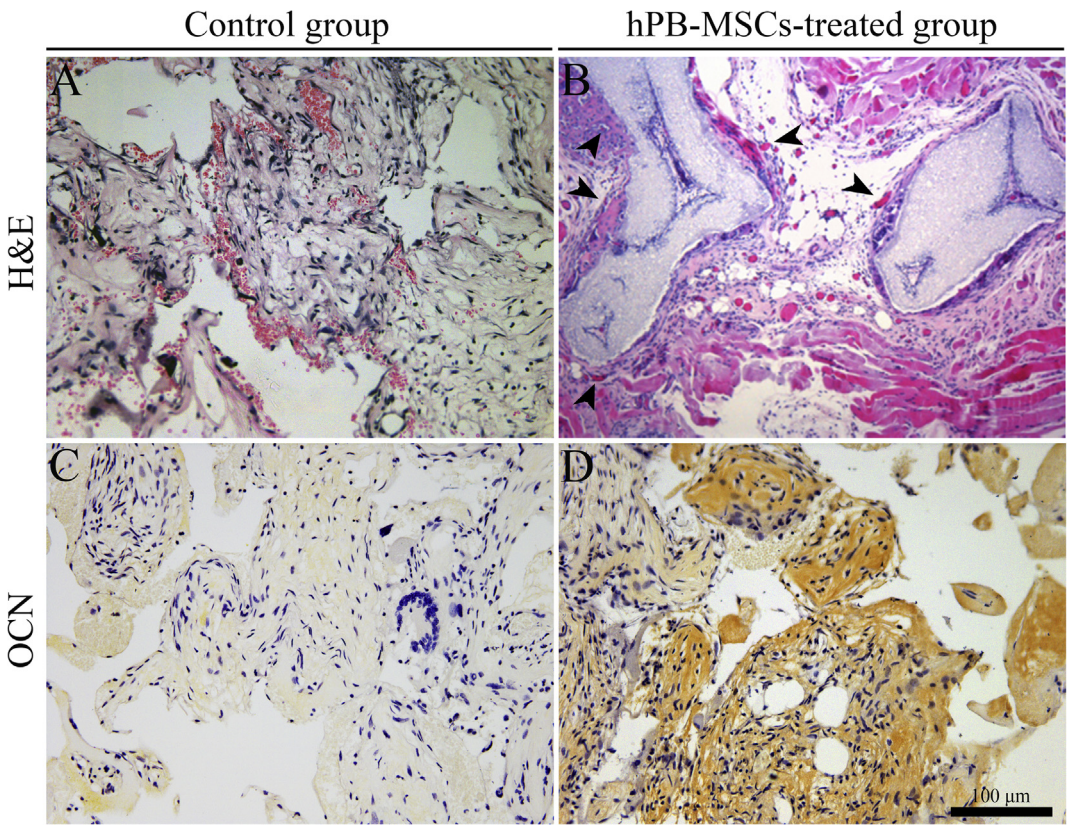

Figure 6 Histological examination of regenerated bone tissues by transplantation of human peripheral blood—derived mesenchymal stem cells (hPB-MSCs) in vivo. The hPB-MSCs were seeded onto sterilized porous β-tricalcium phosphate (β-TCP) granules and then implanted subcutaneously into the dorsal surfaces of nude mice. Transplantation of empty scaffolds served as the controls. The transplants were harvested 8 weeks later for histologic examination. Five microscopic fields from each sample were chosen for quantification of new bone tissue areas (n = 3). (A) Representatives images of transplantation of empty scaffolds stained with H&E. (B) Representative images of generated bone tissues in hPB-MSC-loaded transplants stained with H&E. (C) Results of immunohistochemical staining of osteocalcin (OCN) of the control group as the negative control. (D) Images of immunohistochemical staining of OCN, confirming the ectopic osteogenic capacity of hPB-MSCs in vivo. Amorphous osteoid matrix could be observed in pores of transplants. Scale bar: 100 μm. H&E, haematoxylin and eosin.

many adult tissues [26]. PB has been considered as a potentially alternative cell source for various therapeutic applications because of the ease of cell retrieval and blood bank storage during recent decades [17,21,27—29]. However, the existence and origin of MSCs in PB remain controversial.

To date, the isolation and culture of MSCs from adult PB have been reported to have met with successes [28,30—32] and failures [33,34], which may be associated with the low percentage of PB-MSCs in blood at a steady state and different isolation protocols applied. Some researchers even proposed that MSCs may lose plastic adherent property after mobilization into the PB. In addition, the standard protocol for the isolation and expansion of hPB-MSCs has not been well defined. Furthermore, the biological characteristics of PB-MSCs are not fully understood. Therefore, it is necessary to develop an optimized method for the isolation and expansion of hPB-MSCs.

It is generally believed that serum is essential for cell survival and proliferation. Elevated serum levels may stimulate and enhance the ability of cell adhesion; in the meanwhile, however, elevated serum concentrations may lead to spontaneous differentiation and ageing of the stem cells [35—37]. Thus, it is difficult to define an optimal concentration of serum for hPB-MSC culture. In this protocol, we cultured primary cells after seeding with a complete culture medium supplemented with 17% FBS to stimulate cell adhesion and enrichment. Once the primary cells reached a confluence of over 60%, the cells were passaged regularly and cultured in a culture medium with 10% FBS to prevent spontaneous differentiation and cell ageing.

In the present study, we have improved the cell yields of PB-MSCs using hypoxia culture condition, despite the low starting cell numbers of mononuclear cells. We have achieved a 60% successful rate for culture and expansion of PB-MSCs from healthy individuals. We believe that further improvement is still needed to increase the successful rate of isolating and culturing of the PB-MSCs. Further optimization in culture protocol (e.g., growth factors supplementation and 3D culture system) is needed to better mimic the in vivo stem cell niches to increase the yields of PB-MSCs. No additional growth factors were added in this protocol, making it simple and easy for potential clinical

applications. Studies have reported the beneficial effects of various growth factors and cytokines for the enrichment and multipotency maintenance of MSCs [38,39]. Therefore, it may be instrumental for rapid expansion and maintenance of stemness of PB-MSCs through supplementing some well-defined growth factors or cytokines (such as fibroblast growth factor 2 or basic fibroblast growth factor) in culture media [40—42].

The results of this study indicated the in vivo ectopic bone formation capacity of circulating PB-MSCs, suggesting the potential use of hPB-MSCs in bone repair. We recommend hypoxic condition for culturing primary PB-MSCs. Furthermore, we found that, a subset of PB-MSCs expressed a higher level of Lgr5, a novel adult stem cell marker which has been identified and become a research focus in recent years [13,43], and the $Lgr5^{+}$ PB-MSCs possessed higher osteogenic potential. Hence, Lgr5 may be used as a biomarker to identify PB-MSCs.

In conclusion, our results indicate that the population of MSC-like cells do exist within the peripheral circulation of humans, and these cells can be expanded rapidly in culture under hypoxia conditions. PB-MSCs display a fibroblast-like morphology, exhibit adherence and colony-forming ability, express mesenchymal surface markers and embryonic markers, especially a higher level of Lgr5, and have potent multidifferentiation potential into osteoblasts, adipocytes, chondrocytes and neurons *ex vivo* under appropriate induction conditions, as well as in vivo ectopic bone formation ability. PB-MSCs may become an ideal alternative cell source for tissue engineering and cell therapy.

Conflicts of interest

No competing financial interests exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2019.02.003.

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