Introduction

Stem cells have enormous utility in life science research. Specifically, bone marrow mesenchymal stem cells (BMSCs) which are adult stem cells derived from mesodermal cell lineages with self-renewable capacities and multi-directional differentiation potentials, are especially important [1]. Given appropriate culture conditions, BMSCs have been reported to differentiate into chondrocytes [2–3], osteocytes [4], adipocytes [5], endothelial cells [6], myocytes, cardiomyocytes [7], and even hepatocytes [8–9] and neurons [10], both of which are non-mesodermal in origin. BMSCs, as stem cells, have advantages of availability, culture expansion, low immunogenic properties [11–13], and ease of genetic manipulation, so they have wide use in numerous clinical applications, including tissue engineering [2–3], autoimmune disease [14] and myocardial infarction treatment [15], wound repair [16] and tissue regeneration [17].

Several methods are currently available for BMSC isolation and cultivation. Although separation methods that include immunomagnetic beads or flow cytometry generate BMSCs with higher purity, the expense, procedural complications, and the cell damage that occurs restricts their usage. At present, untreated whole BM adherent cultures, RBC lysis with ammonium chloride, and Ficoll density gradient centrifugation are the most common methods for obtaining BMSCs with acceptable purity, viability, and cost. However, the best method for isolating large numbers of BMSCs is uncertain. Peterbauer [18] reported that the highest BMSCs yields were obtained with RBC lysis, but this method was only compared to density gradient centrifugation. Horn and colleagues [19] compared RBC lysis with Ficoll density fractionation and untreated whole BM adherent cultures, reporting that BMSCs can be efficiently isolated by RBC lysis. Also, their technique was faster and could be standardized more easily for clinical applications. However, we found that untreated whole BM adherent cultures are more efficient than RBC lysis for isolating and purifying BMSCs. According to the literature [19,24], 6 volumes of RBC lysis completely lysed erythrocytes and platelets in BM. However, 3 volumes of RBC lysis retained few erythrocytes, platelets, and some cell fragments. Through comparative analysis of different quantities of RBC lysis buffer, we further evaluated the effects of erythrocytes and platelets on proliferation of rabbit BMSCs in vitro. With this in mind, we compared untreated whole BM adherent cultures, 3 volumes of RBC lysis, 6 volumes of RBC lysis, and Ficoll density gradient centrifugation under the same conditions to find the best method for isolation and purification of rabbit BMSCs in vitro.

Methods

Bone marrow aspiration

All animal procedures were approved by the Yangzhou University Laboratory Animal Care (Yangzhou, China) and this
study was carried out in strict accordance with the Guidelines on the Care and Use of Laboratory Animals issued by the Chinese Council on Animal Research and the Guidelines of Animal Care. BM was harvested under aseptic conditions from the tibia and femur condyle of 6 anaesthetized New Zealand rabbits (0.75 kg, 1 month old, male). Then, a 32 ml mixture of BM aspirates and PBS partitioned in four equal fractions for simultaneous BMSCs isolation using 1) untreated whole BM aspirate, 2) 3 volumes of RBC lysis with ammonium chloride, 3) 6 volumes of RBC lysis with ammonium chloride, or 4) Ficoll density-gradient centrifugation (Figure 1).

1) Isolation of BMSCs from untreated whole BM blood. The BM-PBS mixture (8 ml) was centrifuged for 5 min at 1,000 rpm (Labotuge 400R, ThermoFisher, Germany) and supernatant was removed. Pellets were washed with PBS and centrifuged again. To whole cell sediment, 8 ml culture medium was added consisting of 89% DMEM/F12 (HyClone, Utah, USA), 10% fetal calf serum (FCS, HyClone), and 1% penicillin/streptomycin/amphotericin B (Sangon Biotech, Shanghai, China).

2) Isolation of BMSCs via 3 volumes of RBC lysis. The BM-PBS mixture (8 ml), was centrifuged for 5 min at 1,000 rpm and the supernatant was removed. The pellet was mixed with 3 volumes of RBS lysis buffer (Beyotime, Nantong, China) and incubated for 5 min at room temperature (RT) on a horizontal shaker. When the mixed liquid was light pink, five volumes of PBS was added to the mixture, and the mixture was centrifuged again. Subsequently cells were washed two more times with PBS and medium was added (up to 8 ml) for cell culture.

3) Isolation of BMSCs via 6 volumes of RBC lysis. The BM-PBS mixture (8 ml) was centrifuged and the pellet was mixed with 6 volumes RBS lysis buffer and treated as previously described above.

4) Isolation of BMSCs by Ficoll density-gradient centrifugation. BM-PBS mixture (8 ml) was allowed to stand for 15 min, and the supernatant was slowly transferred to a 15 ml centrifuge tube containing an equal amount of Ficoll-Paque® (Sigma, St Louis MO, USA). After 20 min of centrifugation (2,000 rpm) without braking, mononuclear cells (MNCs) were collected from the interphase (cloud-like cell layer), and filled to 8 ml with culture medium.

Four different cell suspensions as described above were also seeded in 96-well cell culture plates (Corning, New York, USA) and each cell suspension had 8-well replicates with 0.2 ml/well, and blank control wells were used. Four plates were replicated (1-IV). The cell suspensions were transferred to four 25 cm² cell culture flasks (Corning) with 1.6 ml medium per flask. All cells were cultured at 37°C, 5% CO₂ and 95% humidity. Non-adherent cells were carefully washed and the culture medium was changed after 48 h. Thereafter, the culture medium was replaced every 2-3 days, and the cells were subcultured at 90% confluence. The above experiment was repeated 6 times.

Identification of BMSCs
Morphologic analysis. Cell shape and proliferation was analyzed daily with inverted phase contrast microscopy (CKX41SF, Olympus, Japan).

CD antigen analysis. Immunofluorescence analysis was performed as previously described [20]. Briefly, cells from passage 4 were fixed with 4% paraformaldehyde (CP, Shanghai, China) for 90 min, then, cells were washed with PBS for 5 min, incubated with 0.5% Triton X-100 (Sigma) at 37°C for 20 min, washed twice with PBS for 5 min each, blocked with 1% BSA at 37°C for 30 min, incubated with anti- rabbit antibodies: CD34-PE and CD44-PE (Bioss, Beijing, China), overnight at 4°C, and finally washed five times with PBS for 2 min each. Antibodies were replaced with PBS for negative control staining. CD34-PE and CD44-PE antibody distribution was observed with fluorescent microscopy (BX41TF, Olympus).

Flow cytometry was used to confirm surface antigen marker CD34 and CD44 expression. First, 1×10⁶ BMSCs were incubated with anti-CD34-PE, anti-CD44-PE for 30 min at 4°C in the dark. Labeled cells were washed, collected, and analyzed using the FACScan flow cytometry system (BD, Franklin Lakes, USA). Antibody was replaced with PBS for negative control staining.

Chondrogenic differentiation assays
Chondrogenic differentiation experiments were performed with passage 4 cells from the four BMSCs isolation methods. First, 1×10⁶ cultured cells were plated in 6-well plates (Corning) and covered with chondrogenic medium, consisting of BMSC medium supplemented with 10 ng/ml transforming growth factor-β1 (TGF-β1, PeproTech, Princeton, USA), 100 ng/ml insulin-like growth factor-1 (IGF-1, PeproTech), 50 μg/ml ascorbic acid (Sigma) and 10 mmol/L dexamethasone (Sigma). The medium was replaced every 2-3 days. After 21 days, the four cell groups were analyzed by anti-rabbit Collagen II (Boster, Wuhan, China) staining overnight at 4°C, as described previously [18,21].

Analysis of four primary cells’ proliferative activity by MTT assay
On day 4, 7, 10 and 13 after plating, an MTT assay was used to detect the proliferation activity as previously described [22]. In brief, cells were incubated with 20 μl 5 mg/ml MTT (Sigma) for 4 h. Then medium was removed and formazan salts were dissolved with 150 μl of dimethyl sulfoxide (DMSO, Sigma), and the absorbance was read at 570 nm with an automated microplate...
reader (BioTek, Highland Park, USA). All procedures were repeated 6 times and cell proliferation for all four cell culture types was analyzed.

**Proliferation assays for passaged cells**

MTT (Sigma) assay was used to measure cell proliferation from different cell passages. Confluent 2nd, 3rd, 4th, and 5th passage cells were trypsinized and re-suspended separately. All cell passages were counted and seeded at a density of 3,000 cells/well in 10 96-wells plates, with 8 wells for each passage. Blank controls were used. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO2. The MTT assay was conducted at day 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10, prior to treatment to establish a growth curve for differently passaged cells. Each experiment was repeated 3 times (N = 8/group).

**Statistical analysis**

Data from the four primary cell cultures and the number of different cell passages are reported as means and standard deviations (SD). *p* < 0.05 was considered statistically significant.

**Results**

**Identification of BMSCs**

**Morphologic analysis.** RBCs were observed in all four groups after cells were seeded in cell culture flasks. The whole BM blood group had the most RBCs, followed by the group treated with 3 volumes of RBC lysis, the group treated with 6 volumes of RBC lysis, and the density gradient centrifugation group. Fusiform adherent cells were obvious when the four groups of BMSCs were cultured for 24 h. After two replacements of culture medium, many impure cells including RBCs, platelets and leucocytes in the culture were cleared. Cells also formed many CFU-F colonies after the 24-h incubation in all four groups. However, more CFU-F colonies were observed from untreated whole BM blood cultures compared with other culture methods. CFU-F colonies from untreated whole BM blood cultures formed earlier than cells isolated from other methods. Cell morphology was varied: spindle-shaped cells, and triangular, irregular, and unorganized cell arrangements were observed. At day 10, cells reached 80% confluence. At day 13, the cells had a uniform spindle shape, and reached ~100% confluence (Figure 2). When cells were incubated for 15 days, they became senescent, with an irregular shape, cell atrophy, and more cell gaps. Six hours after confluence adherent cells were detached with 0.25% trypsin (Gibco, Carlsbad, USA) and re-seeded, some cells began to grow adherently. After 24 h, almost all cells adhered to cell culture flask, and after 10 days, cells reached 100% confluence.

**Analysis of immunophenotype.** Immunofluorescent staining indicated that the CD44-FITC cell marker fluoresced green on the cell surface under a fluorescent microscope. CD34-PE was fluorescent brown (not green). So, cultured cells were positive for CD44, but negative for CD34 (Figure 3). Flow cytometry was used to quantify BMSCs percentages, and CD44-positive cells were 98.12, 98.85, 93.32, and 94.32%, and the CD34-positive cells were 3.25, 2.37, 5.31, and 3.50%. These data confirmed that cells obtained from all four methods were BMSCs. There were no obvious differences among the four groups of BMSCs (Figure 4).

**Analysis of differentiation potential**

Collagen type II immunostaining confirmed that BMSCs isolated by four unique methods were equally suited for synthesis of high levels of type II collagen in chondrogenic differentiation cultures. As shown in Figure 5, no differences in differentiating potential were observed among the four uniquely isolated groups of BMSCs.

**Proliferative activity of primary cells**

Analysis of average absorbances of cells isolated from four methods. All four groups of primary cells began to grow adherently at about 24 h, and the log growth phase of cells was stable around the 4th-9th day. Growth curves of all four cell groups were “S”-type curves. Primary cells reached 80% confluence at day 10 and at day 13 cells were ~100% confluent (lag phase). Statistically significant differences among the four primary BMSC growth curves were observed (Figure 6). Data show that untreated whole BM blood isolates were superior to the other 3 groups at the 4th, 7th, 10th, and 13th day (*p* < 0.05) (Figure 7).

**Passage cell proliferation assays**

The proliferation of the 2nd, 3rd, 4th, and 5th cell passages were analyzed with an automated microplate reader (570 nm absorbance) at day 1-10. As shown in Figure 8, growth curves of passaged cells had an “S” shape and growing latent phases of passaged cells became stable at the 1st to 2nd day. The log phase of growth appeared about the 3rd to the 8th day and then cells reached the plateau. The fourth and fifth passages of cells were ~100% confluent at 7 days, and entered a lag phase. However, the second and third passages of cells entered the lag phase 2 days later. Thus, cells from the 4th passage had the greatest proliferation ability (*p* < 0.05), with a greater growth speed and cell quantity than other passages.

**Discussion**

The process of purification and separation of BMSCs is crucial for further clinical trials, because untreated whole BM blood included erythrocytes, leukocytes, and platelets, but, the proportion of mononuclear cells was minimal. Isolation and purification of BMSCs from untreated whole BM blood is a common technique, growing cells in cell culture flasks until harvesting of adherent cells [23]. The RBC lysis method depends on the passage of NH3 and CO2 through the membrane, which are spontaneously converted to NH4+ in the cell and HCO3- by carbonic anhydrase in the erythrocyte and platelet, respectively and thus could be the force behind a continuous flow of NH3 and CO2 entering the cell. This will change the osmotic fragility of erythrocytes and disrupt the membrane structure [24–25]. Lysis frequencies affect BMSC viability. Here, we compared 3 and 6 volumes of RBC lysis. Density-gradient centrifugation methods are based on the suspension density of BM, and the harvesting of mononuclear cell layers after centrifugation [26–28].

Dynamic cell morphology observations revealed that BMSCs grew in clusters with various morphologies. BMSCs had a uniform morphology at day 10–13, and were senescent at day 15. Therefore, subculturing is best when primary cells are cultured within 2 weeks.

Cell surface markers are key to identifying BMSCs [29] and our BMSCs preparations were positive for CD29, CD44, CD90, CD105, and negative for hematopoietic markers and endothelial markers such as CD14, CD34, CD45 and CD184. Immunofluorescence and flow cytometry confirmed that all cells from the four methods were positive for expressions of CD44 and negative for CD34, thus this confirmed BMSCs [30–32]. Data show that the four methods employed in the current investigation were not different with respect to cell purity.

Collagen type II is a marker specifically produced in cartilage cells, the expression of which increases during chondrogenic
Figure 2. Morphologic analysis of BMSCs. Many RBCs were observed under phase-microscopy, after cells were seeded in cell culture flasks. RBCs in: untreated samples > after 3 volumes of RBC lysis > after 6 volumes of RBC lysis—Ficoll samples. Fusiform adherent cells of four groups of BMSCs were observed after 24 h cultivation. Cells from four groups yielded many CFU-F colonies, when cells were cultured for 4–10 days, but the CFU-F count of the untreated sample was the greatest. At day 13, the four groups of BMSCs all had uniform spindle morphology, and reached about 100% confluence. Scale bar = 100 μm, applies to all images.

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Figure 3. Immunofluorescence staining of BMSCs. CD44-PE was fluorescent green on the cell surface. CD34-PE was fluorescent brown, and this corresponded to BMSCs stained with antibody against CD44 and CD34, Scale bar = 100 μm.

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Methods of Isolation and Cultivation BMSCs

**Diagram:**

### CD44-PE

- **Untreated**
- **3 volumes of RBC lysis**
- **6 volumes of RBC lysis**

### CD34-PE

- **Untreated**
- **3 volumes of RBC lysis**
- **6 volumes of RBC lysis**

**Legend:**

- M1: Represents the marker for analysis.

**Axis:**

- x-axis: Event counts (10^4 to 10^6)
- y-axis: FL2-H fluorescence intensity (0 to 100)

**Colors:**

- Black line: Control
- Red line: Sample

**Notes:**

- The diagrams illustrate the expression levels of CD44 and CD34 under different conditions.

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*Image Source:* PLOS ONE, February 2014, Volume 9, Issue 2, e88794
differentiation [33]. Here, we showed that the chondrogenic differentiation potential of expanded cells, as assessed by microscopic evaluation of the appearance collagen type II, did not differ among the four isolation methods.

We compared four isolation strategies of BMSCs from untreated whole BM, 3 and 6 volumes of RBC lysis, and Ficoll density-gradient centrifugation. We observed that untreated whole BM cultures were the most suitable and reliable methods of isolating rabbit BMSCs. BMSCs from untreated whole BM grew stably in vitro, proliferated rapidly, harvested large yields, and were not different with respect to cellular proliferation, morphology, immunophenotype, and differentiation potential. Advantages of untreated whole BM methods are convenience, low probability of microbial contamination, and a high ratio of cultivated cells. Therefore, this method is effective for large-scale experimental research. Our data also suggest that abundant erythrocytes and platelets in BM-PBS mixtures could affect BMSCs adherent growth, but that small amounts of erythrocytes and platelets promoted adherence. Evidence suggests that erythrocytes and platelets promote initial growth of BMSCs colonies, which might be attributed to various growth factors and an intact original BMSCs microenvironment [19,34–35]. With RBC lysis and Ficoll

Figure 4. Flow cytometry. CD44 positive BMSCs were 98.12, 98.85, 93.32, and 94.32%, and CD34 positive cells were 3.25, 2.37, 5.31, and 3.50%, as shown by flow cytometry (autofluorescence is marked as a white filled histograms). There were no obvious differences among the four groups (p<0.05). Cells that expressed CD44 markers, but not CD34, correspond to BMSCs.

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Figure 5. Chondrogenic differentiation of rabbit BMSCs isolated by four methods. Collagen type II immunostaining showed that the majority of BMSCs were differentiated into a chondrogenic lineage for 21 days (E–H), and were simultaneously cultured in control medium (A–D). Scale bar = 50 μm, applies to A–D, and scale bar = 100 μm, applies to E–H.

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density-gradient centrifugation methods, erythrocytes and platelets are completely removed, as well as growth factors that may affect BMSC proliferation. Also, frequent manual manipulation of cells may cause contamination and mechanical damage [27]. Analysis of two groups treated with RBC lysis confirm that 3 volumes of RBC lysis yielded greater proliferative BMSCs compared to 6 volumes of RBC lysis.

Analysis of second, third, fourth, and fifth cell passages revealed that the growth curve of all cells passages had an “S” shape and that the logarithmic growth phase occurred at day 3 to 7. At 9 days, most cells were spindle-shaped and regular, and were approximately 100% confluent. Fourth generation BMSCs has a greater proliferation capacity than the other 3 generations, and the second generation was the weakest with respect to proliferation capacity.

In conclusion, untreated whole BM blood adherent culture methods offer a reliable, simple, and efficient method for isolation and purification of BMSCs. With this method the average colony size is larger compared with RBC lysis and Ficoll density-gradient centrifugation procedures. The BM blood adherent culture technique did not affect BMSC heterogeneity, differentiation

Figure 6. Comparison of four primary BMSC growth curves. All growth curves form four ell groups had an “S” form and no isolation method was shown to change cell growth. doi:10.1371/journal.pone.0088794.g006

Figure 7. Cell proliferative activity among four primary cells. Cells from untreated whole BM blood proliferated better than the other 3 groups as shown by MTT assay. The MTT assay was conducted at the fourth, seventh, tenth and thirteenth day (p<0.05). doi:10.1371/journal.pone.0088794.g007

Figure 8. Cell proliferation assays for different cell passages. Cells from the fourth passage had the greatest proliferation, and second passage cells had the least proliferative ability (p<0.05). doi:10.1371/journal.pone.0088794.g008
potential, or biological activity compared to the other methods. So, this technique is most appropriate for experimental research and clinical applications of BMSCs.

Author Contributions
Conceived and designed the experiments: WDZ FBZ HCS. Performed the experiments: WDZ GY SP FS XCL. Analyzed the data: WDZ FBZ. Contributed reagents/materials/analysis tools: RBT SH. Wrote the paper: WDZ.

References

1. Jones E, McGonagle D (2008) Human bone marrow mesenchymal stem cells in vivo. Rheumatology (Oxford) 47:126–131.
2. Macchiarini P, Jungebluth P, Ge T, Anaght A, Rees LE, et al. (2008) Clinical transplantation of a tissue-engineered airway. Lancet 372:2023–2030.
3. Jungebluth P, Alici E, Baiguer S, Le Blanc K, Blomberg P, et al. (2011) Tracheoebronchial transplantation with a stem-cell-seeded biartificial nanocomposite: a proof-of-concept study. Lancet 378:1997–2004.
4. Heino TJ, Hentunen TA (2008) Differentiation of osteoblasts and osteocytes from mesenchymal stem cells. Curr Stem Cell Res Ther 3:131–145.
5. Karagiani M, Brinkmann I, Kinzebach S, Grasal M, Weiss C, et al. (2013) A comparative analysis of the adipogenic potential in human mesenchymal stromal cells from cord blood and other sources. Cytotherapy 15:75–88.
6. Crisan M (2013) Transition of mesenchymal stem/stromal cells to endothelial cells. Stem Cell Res Ther 4:95.
7. Zhao JW, Zhang MR, Ji QY, Xing FJ, Meng JJ, et al. (2012) The role of slingshot-1, SSH1L1 in the differentiation of human bone marrow mesenchymal stem cells into cardiomyocyte-like cells. Molecules 17:14975–14994.
8. Pulavendran S, Rajam M, Rose C, Mandal AB (2010) Hepatocyte growth factor incorporated chitosan nanoparticles differentiate murine bone marrow mesenchymal stem cells into hepatocytes in vitro. JET Nanobiotechnol 4:51–60.
9. Feng Z, Li C, Jiao S, Hu B, Zhao L (2011) In vitro differentiation of rat bone marrow mesenchymal stem cells into hepatocytes. Hepatogastroenterology 58:2081–2086.
10. Mohammad-Gharibani P, Pirahali T, Mesebah-Namin SA, Arabkhariaj D, Kazemi H (2012) Induction of bone marrow stromal cells into GABAergic neuronal phenotype using creatine as inducer. Restor Neur Res Neurosci 30:511–525.
11. Smit AM, van Vliet P, Hassink RJ, Goumans MJ, Doevendaal PA (2005) The role of stem cells in cardiac regeneration. J Cell Med 9:25–36.
12. Bobin S, Jarocha D, Majka M (2006) Mesenchymal stem cell characteristics and clinical applications. Folia Histochim Cytobiol 44:215–230.
13. Rastegar F, Shenaq D, Huang J, Zhang W, Zhang BQ, et al. (2010) Mesenchymal stem cells: Molecular characteristics and clinical applications. World J Stem Cells 2:67–80.
14. Reinolds ME, de Fijter JW, Rotsloh H, Bajema IM, de Vries DK, et al. (2013) Autologous bone marrow-derived mesenchymal stromal cells for the treatment of allograft rejection after renal transplantation: results of a phase I study. Stem Cells Trans Med 2:107–111.
15. Williams AR, Hatzistergos KE, Addicot B, McCall F, Carvalho D, et al. (2013) Enhanced effect of combining human cardiac stem cells and bone marrow mesenchymal stem cells to reduce infarct size and to restore cardiac function after myocardial infarction. Circulation 127:213–223.
16. Zhao LP, Yuan F, He B, Wang MG, Zhong XL (2012) Isolation of pig bone marrow mesenchymal stem cells suitable for one-step procedures in chondrogenic regeneration. J Tissue Eng Regen Med 4:485–490.
17. Horn P, Bork S, Diehlmann A, Walenda T, Eckstein V, et al. (2008) Isolation of human mesenchymal stromal cells is more efficient by red blood cell lysis. Cytotherapy 10:676–685.
18. Peterbauer-Scherb A, van Grienven M, Meini A, Gabriel C, Redl H, et al. (2010) Isolation of pig bone marrow mesenchymal stem cells suitable for one-step procedures in chondrogenic regeneration. J Tissue Eng Regen Med 4:485–490.
19. Horn P, Bork S, Diehlmann A, Walenda T, Eckstein V, et al. (2008) Isolation of human mesenchymal stromal cells is more efficient by red blood cell lysis. Cytotherapy 10:676–685.
20. Berndt-Weis ML, Kauri LM, Williams A, White P, Douglas G, et al. (2009) Global transcriptional characterization of a mouse pulmonary epithelial cell line for use in genetic toxicology. Toxicol In Vitro 23:616–633.
21. Fukunou T, Sperling JW, Sanyal A, Frazzimmons J, Reinhold G, et al. (2003) Combined effects of insulin-like growth factor-1 and transforming growth factor-beta1 on periskeletal mesenchymal cells during chondrogenesis in vitro. Osteoarthritis Cartilage 11:53–64.
22. Horn P, Qiang WD, Wei T, Zhang S, Hu YL, et al. (2008) Preliminary separation of the growth factors in platelet-rich plasma: effects on the proliferation of human marrow-derived mesenchymal stem cells. Chin Med J 122:83–87.
23. Jarocha D, Lokisiewicz E, Majka M (2008) Advantage of mesenchymal stem cells (MSC) expansion directly from purified bone marrow CD105+ and CD271+ cells. Folia Histochim Cytobiol 46:307–314.
24. Al Rattat F, De Kock J, Rambor E, Heymans A, Vanhaecke T, et al. (2011) Evaluation of the multipotent character of human adipose tissue-derived stem cells isolated by Ficoll gradient centrifugation and red blood cell lysis treatment. Toxicol In Vitro 25:1224–1230.
25. Horn P, Bork S, Wagner W (2011) Standardized isolation of human mesenchymal stromal cells with red blood cell lysis. Methods Mol Bio 690:23–35.
26. Pierini M, Duza B, Lucarelli E, Tazzari PL, Ricci F, et al. (2012) Efficient isolation and enrichment of mesenchymal stem cells from bone marrow. Cytotherapy 14:686–693.
27. Poel G, Moller K, Frölich W, Schula I, Bolze J, et al. (2012) Density gradient centrifugation compromises bone marrow mononuclear cell yield. PLoS One 7:e50293.
28. Schallmoser K, Rohde E, Renisch A, Bartmann G, Thaler D, et al. (2008) Rapid large-scale expansion of functional mesenchymal stem cells from unmanipulated bone marrow without animal serum. Tissue Eng Part C Methods 14:185–196.
29. Dominini M, Le Blanc K, Illmer L, Slaper-Cortenbach I, Marin F, et al. (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315–317.
30. Conget PA, Minguez JL (1999) Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. J Cell Physiol 181:67–73.
31. Foster JJ, Zeemann PA, Li C, Mann M, Jensen ON, et al. (2005) Differential expression profiling of membrane proteins by quantitative proteomics in a human mesenchymal stem cell line undergoing osteoblast differentiation. Stem Cells 23:1367–1377.
32. Lee JW, Gupta N, Serkov V, Mathay MA (2009) Potential application of mesenchymal stem cells in acute lung injury. Expert Opin Biol Ther 9:1259–1270.
33. Rinse J, Kaps C, Schmitt B, Büsser K, Bartel J, et al. (2002) Porcine mesenchymal stem cells. Induction of distinct mesenchymal cell lineages. Cell Tissue Res 307:321–327.
34. Muller I, Kordisch S, Holzwarth G, Spano C, Jensen G, et al. (2006) Animal serum-free culture conditions for isolation and expansion of multipotent mesenchymal stromal cells from human BM. Cytotherapy 8:437–444.
35. Schallmoser K, Bartmann G, Rohde E, Renisch A, Kaschofer K, et al. (2007) Human platelet lysate can replace fetal bovine serum for clinical-scale expansion of functional mesenchymal stromal cells. Transfusion 47:1436–1446.