Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/jobcr

Original Article

Comparative study of different centrifugation protocols for a density gradient separation media in isolation of osteoprogenitors from bone marrow aspirate

Noel Ye Naung^{a,*}, Srisurang Suttapreyasri^b, Suttatip Kamolmatyakul^c,
Thongchai Nuntanaranont^b

^a Department of Oral Sciences, School of Dentistry, University of Otago, Dunedin, New Zealand

^b Department of Surgery, Faculty of Dentistry, Prince of Songkla University, Hatyai, Thailand

^c Department of Preventive Dentistry, Prince of Songkla University, Hatyai, Thailand

ARTICLE INFO

Article history:

Received 24 October 2014

Accepted 11 November 2014

Available online 6 December 2014

Keywords:

Bone marrow stromal cells

Osteoprogenitor cells

Density gradient centrifugation

Ficoll

STRO-1

ABSTRACT

Introduction: Human bone marrow contains osteoprogenitors capable of differentiating into osteoblasts. Density gradient centrifugation (DGC) is a commonly used method to isolate osteoprogenitors from bone marrow. Numerous studies used different dilution and centrifugation protocols, which might affect cell yields and quality. Moreover, the relative isolation efficiencies of the different separation protocols have not been investigated. This study compares the enrichment efficacy of the two different centrifugation protocols for a commonly used DGC media in isolation of osteoprogenitors.

Material and method: Bone marrow was aspirated from human anterior iliac crests. Osteoprogenitors are isolated with Ficoll DGC media. A centrifugal force of 400 g and 1:1 dilution was compared with the centrifugal force of 1000 g after three dilution times with a buffer. **Results:** The average numbers of isolated cells were significantly higher when using lower centrifugal force with 1:1 dilution, however, there was no detectable difference between Colony-forming unit-fibroblast (CFU-F) forming capacity, STRO-1 positivity, osteogenic differentiation or mineralization abilities between protocols.

Conclusion: Both protocols could isolate competent and functional osteoprogenitors, while a lower centrifugal force (400 g) with 1:1 dilution produced recovery of more osteoprogenitors.

Copyright © 2014, Craniofacial Research Foundation. All rights reserved.

* Corresponding author.

E-mail address: noel.naung@otago.ac.nz (N.Y. Naung).

<http://dx.doi.org/10.1016/j.jobcr.2014.11.004>

2212-4268/Copyright © 2014, Craniofacial Research Foundation. All rights reserved.

1. Introduction

Autogenous bone is the gold standard grafting material and its osteogenic capacity resides in the bone marrow.¹ The bone marrow stromal system contains multipotent mesenchymal stem cells and osteoprogenitor cells.² Bone marrow aspirate was considered the richest and most readily available source of osteoprogenitors, which can be a valuable alternative bone graft substitute to prevent grafting related morbidity and complications.¹

The osteogenic potential of bone marrow aspirate was first reported in 1869 by Goujon.³ In 1970, Friedenstein and colleagues cultured adult bone marrow cells and demonstrated the ability to form fibroblastic colonies known as colony-forming units – fibroblasts (CFU–F).⁴ These cells are capable of differentiating into bone, cartilage, fat and other mesenchymal lineage cells.^{5,6} Each CFU–F is derived from a single cell, therefore the number of CFU–F colonies reflects the number of stem or progenitor cells.⁴ The early preosteogenic stem cell surface marker, monoclonal antibody STRO-1, can also be used to characterize the osteogenic precursor cells from aspirates of human bone marrow.⁷

The osteoprogenitor cells are limited in number, containing only approximately 0.005% of the nucleated cells in the fresh bone marrow aspirates.¹ Hernigou treated 60 non-union or osteonecrosis patients with bone marrow aspirates and seven atrophic non-unions failed to heal as a result of significantly lower numbers and concentration of osteoprogenitors injected.⁸ Therefore, the ability to isolate the maximum number of marrow stromal mesenchymal stem and osteoprogenitor cells with the highest replication and differentiation potential is crucial for the success of bone tissue engineering applications. In addition, contamination of the osteoprogenitors with red blood cells impairs the efficacy of the cell therapy.⁹

Bone marrow stromal cells can be isolated and purified by removing unwanted cells using different techniques including density gradient centrifugation. Density gradient centrifugation is a technique that allows the separation of cells depending on their density. Ficoll, a high molecular weight sucrose polymer, has been widely used for isolation of mesenchymal stem and osteoprogenitor cells from bone marrow.¹⁰ Ficoll–Paque PREMIUM 1.073 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was chosen in this study because, when compared to standard Ficoll (1.077 g/ml), a lower density (1.073 g/ml) has proven benefits in isolating the lower density mononuclear cells and mesenchymal stromal cells with higher proliferation potential, which can ultimately benefit all clinical applications.¹¹

A number of studies using Ficoll to remove the mononuclear cells from the bone marrow used different centrifugation forces (from 2600 g to 2500 g) as well as different ratios of dilution (1:1 to 1:3).^{12–14} A centrifugal force of 400 g and 1:1 dilution is the general protocol recommended by the manufacturer, which has been used with success to isolate the mononuclear cells from the blood, however, the optimum result may not be obtained due to the difference in nature of the bone marrow and the blood such as cellularity, viscosity and weight. According to the basic principles of

centrifugation, differences in the centrifugal force could affect the sedimentation rate and the viability of cells.¹⁵ Accordingly, the ratio of dilution, in other words, the viscosity of the bone marrow, could also affect the efficacy of separation.¹⁵ Therefore, we assumed that different centrifugation forces and the ratio of dilution might affect the quantity and quality of the isolated bone marrow stromal cells and osteoprogenitor cells.

Currently, to our extent of knowledge, the relative isolation efficiencies of the different separation protocols have not yet been investigated. The aim of the study was to compare the enrichment efficacy of the two different centrifugation protocols for the Ficoll density gradient separation media on bone marrow osteoprogenitor cell separation.

2. Materials and methods

2.1. Bone marrow harvesting

Bone marrow samples were obtained by aspiration from the anterior iliac crests of ten patients undergoing alveolar bone grafting surgery at the dental hospital, Prince of Songkla University after informed consent. Ethical approval was obtained from the Ethical Board of the Faculty of Dentistry.

2.2. Bone marrow aspiration technique

Bone marrow was aspirated from the anterior iliac crests under general anesthesia. A 2 mm stab incision was made through skin and subcutaneous tissue on the anterior iliac crest about 2 cm posterior from the anterior superior iliac spine. A Klima-Rosegger bone marrow aspiration needle (diameter 14 G, 1.5 inches long) was inserted into the cancellous bone of the iliac crest between the inner and outer tables of the iliac crest. After removing the obturator, the marrow was aspirated in small fractions (<4 ml) and continuous aspiration (more than 6 s) was avoided to reduce the degree of dilution by peripheral blood¹⁶ (Fig. 1). Perforations were made 1 cm apart from each site to avoid dilution by bleeding from the previous area.

Twenty milliliters of aspirated marrow was collected into the 50 ml sterile Falcon tube containing 1 ml of anticoagulant solution (1000 units of heparin in sterile normal saline solution).

2.3. Grouping

The experiment was performed to compare between 2 different centrifugation forces and dilution protocols as shown in Table 1. A total of 20 ml aspirated bone marrow was equally divided and put into two 50 ml plastic tubes.

2.4. Bone marrow processing

The bone marrow was first filtered through a 100 µm cell strainer to remove bone fragments, cell clumps and fat. The bone marrow in each tube was diluted with a buffer in accordance with each protocol.

Fig. 1 – Aspiration of bone marrow from anterior iliac crest with a Klima-Rosegger bone marrow aspiration needle.

2.5. Isolation of mononuclear cells

The diluted bone marrow samples were layered on Ficoll–Paque PREMIUM 1.073 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) slowly and carefully to prevent mixing of the centrifugation medium and the diluted bone marrow samples. Then each tube was centrifuged at a given g force according to each protocol for 40 min at 18 °C in a swing bucket rotor with the brake off. After centrifugation, the upper layer containing plasma and platelets was removed using a sterile pipette, leaving the mononuclear layer undisturbed at the interface (Fig. 2). The mononuclear cell layer was transferred to a sterile centrifuge tube using a sterile pipette and then diluted with 20 ml of buffer. The cells were then washed by centrifugation at 400 g for 10 min at 18 °C in a swing bucket rotor with the brake off. After removing the supernatant, the isolated mononuclear cells were suspended in the standard cell culture medium (α -MEM supplemented with 10% FBS, 100 units/ml penicillin, 100 μ g/ml streptomycin and 1% Fungizone).

2.6. Counting the mononuclear cells

The isolated mononuclear cells were counted with a standard Malassez hemocytometer.

2.7. Cell culture

The 10^6 mononuclear cells were plated in each well in the 6-well culture plates in standard cell culture medium and

incubated at 37 °C in a humidified 5% CO₂ environment. On day 7, the culture medium was replaced with osteogenic medium containing α -MEM, 10% FBS, penicillin (100 units/ml), streptomycin (100 μ g/ml), ascorbic acid (50 μ g/ml), β -glycerophosphate (4 mM) and dexamethasone (100 nM). The medium was changed every three days for the entire duration of culture.

2.8. Characterization of osteoprogenitors

2.8.1. Number of CFU–F colonies

After 10 days, cultured cells with high proliferative activity become colonies. Cell aggregate of more than 50 cells were counted as one CFU–F colony by using a Nikon Eclipse Ti–S inverted microscope and captured by the attached DS–Qi1 camera by using NIS–Elements Imaging Software. Results were expressed as the mean number of colony-forming units per 10^6 mononuclear cells.

2.8.2. STRO-1 flow cytometry analysis

On day 10, the cultured bone marrow stromal cells were analyzed for expression of osteoprogenitor cell marker Stro-1 by flow cytometry. Adherent cells were released with trypsin/EDTA and washed. 10^6 cells were suspended in 90 μ l of PBS with 12 μ l of mouse anti stro-1 IgM antibody (Invitrogen, Carlsbad, CA) and incubated for 1 h at 4 °C. The cells were then washed and incubated with 10 μ l of the labeled secondary antibody, AlexaFluor 488 conjugated Goat anti-mouse IgM antibody (Invitrogen, concentration 1 μ g), for 1 h at 4 °C. The cells were then washed and fixed with cold buffered 2% paraformaldehyde. For negative controls, samples with omission of both antibodies, omission of the STRO-1 antibody and omission of the secondary antibody were also analyzed by flow cytometry. Analysis of 10,000 events was performed in a Cytomics FC 500 Flow Cytometer (Beckman Coulter, Pasadena, CA).

2.8.3. Osteogenic differentiation

2.8.3.1. Characterization of osteoblastic phenotype. After being cultured in osteogenic induction medium for 14 days, cells were fixed with 10% neutral-buffered formalin for 5 min, then assayed for alkaline phosphatase (ALP) activity. Briefly, the substrate solution was prepared by dissolving 8 mg of naphthol AS-TR phosphate in 0.3 ml of N, N'-dimethylformamide, while a separate solution of fast blue BB was prepared by dissolving 24 mg in 30 ml of 100 mM Tris (pH 9.6). The above solutions were mixed and then 10 mg of MgCl₂ was added and dissolved, and the pH was adjusted to 9.0 with 1 N HCl. The cells were incubated with fresh substrate at 37 °C for 30 min, then rinsed extensively with distilled water and photographed.¹⁰

2.8.3.2. Alkaline phosphatase quantitative assay. Alkaline Phosphatase (ALP) enzyme activity of the cell layer was measured in triplicate manner at day 7, 10 and 14. At the end of the prescribed time periods, the cell layers were rinsed twice with PBS and osteoblasts were lysed with 0.2% Triton-X in PBS for measurement of enzymatic activity.

The quantitative measurement of ALP activity was determined by formation of yellowish p-nitrophenol (p-NP), using

Table 1 – Grouping.

Protocol	Dilution	Force
A	1:1	400 g
B	1:3	1000 g

Fig. 2 – Ficoll density gradient centrifugation.

colourless *p*-nitrophenolphosphate (*p*-NPP) as the substrate. 50 μ l of supernatant was added to 50 μ l *p*-nitrophenolphosphate (4.34 mM), 100 mM glycine (pH 10.3), and 1 mM $MgCl_2$, mixed well and incubated at 25 °C for 60 min, and protected from light. The reaction was then stopped by adding 20 μ l of 1 M NaOH solution and incubated at RT for 30 s. The enzymatic activity was quantified by absorbance measurements at 405 nm in a micro plate reader and calculated according to a series of *p*-nitrophenol standards.

Enzymatic activity was normalized to total protein concentration by the Pierce® BCA Protein Assay Kit (Thermo Scientific). Protein concentration of samples were calculated from a standard curve and ALP activity was standardized as nano-moles of *p*-nitrophenol liberated per milligram of total cellular protein.¹⁷

2.8.3.3. Detection of bone mineralization. The presence of mineralized nodules (phosphate and calcium deposits) was confirmed cytochemically by using von Kossa and Alizarin Red S staining at day 28.

2.8.3.3.1. von Kossa (phosphate staining). Calcium phosphate deposits were detected by von Kossa technique to reveal back stained phosphate deposits. The cultured plates were rinsed twice with ice cold PBS solution after removing the osteogenic medium and fixed with 10% formaldehyde for 15 min, then rinsed with distilled water. 5% silver nitrate solution was added and the plates were incubated for 30 min in a dark room, and then the plate was exposed to bright sunlight until mineralized nodules were seen as dark brown to black spots.¹⁸

2.8.3.3.2. Alizarin Red S (calcium staining). Alizarin Red S (Sodium alizarin sulfonate) staining was used to reveal the presence of calcium deposits. 2% alizarin red S solution was prepared in distilled water and the pH was adjusted to 4.1–4.3. Cultures were fixed with 10% formaldehyde for 15 min, washed with distilled water and stained with alizarin red S for 15 min. After removing excess incorporated dye with distilled water, red mineralized nodules became visible.¹⁹

2.9. Statistical analysis

Statistical Analysis was carried out using SPSS 14.0 software (SPSS, Chicago, IL). The data were presented as the mean and standard deviation (SD). The paired *t* test was used to compare the differences between the two centrifugation protocols and *p* < 0.05 was considered statistically significant.

3. Results

Ten patients undergoing secondary alveolar bone grafting were volunteered to participate in this study. There was no intra operative and postoperative morbidity or complications related to bone marrow aspiration, and the surgery was uneventful. The mean age of the patients was 9.5 years. The youngest patient was 4.6 years old and the oldest patient was 23 years old.

Fig. 3 – Isolated mononuclear cell count.

Fig. 4 – Proliferation of bone marrow mononuclear cells on different days: Day 3 (left), Day 10 (right) (10x magnification).

3.1. Mononuclear cell count

The average numbers of isolated bone marrow mononuclear cells are shown in Fig. 3.

There was a statistically significant difference here between protocols ($p = 0.037$), and Protocol A produced recovery of more bone marrow derived mononuclear cells.

3.2. Cell culture

Within 24 h of culture, the cells with adherence capacity from both protocols began attaching on the plates in both protocols. Most of the cells that attached to the plastic surface exhibited a fibroblast-like spindle shape. Most cells attached to the surface within the first three days and non-adherent cells were reduced with subsequent medium change. The attached cells continued to proliferate and began differentiating into osteoblastic phenotypes after changing to osteogenic media (Fig. 4).

3.3. Characterization of osteoprogenitors

3.3.1. Formation of CFU–F

Fibroblastic colonies appeared and became clearer as the incubation period prolonged (Fig. 5).

Fig. 5 – CFU–F colony under microscope (4x magnification).

3.3.2. Number of CFU–F colonies

Approximately 43–64 CFU–Fs were produced from 10^6 mononuclear cells in each well. The mean $\pm$ SD number of alkaline phosphatase positive CFU–Fs in Protocol A was 53 ± 6 , which was not significantly different from Protocol B (51 ± 8) (Fig. 6).

3.3.3. STRO-1 flow cytometry analysis

Cells incubated with an unlabeled pure stro-1 antibody (Fig. 7C, G) showed no fluorescence expression, which was similar to the cells with no antibody staining (Fig. 7D, H). Small amounts of non-specific fluorescence expression was seen in the cells incubated with AlexaFluor 488 conjugated goat anti-mouse antibody (Fig. 7B, F), and their expression was added in the range of the negative control groups in order to get truly positive STRO-1 positive cells (Fig. 7A, E). Again, no difference was detected statistically between both protocols.

3.4. Osteogenic differentiation

3.4.1. Characterization of osteoblastic phenotype by ALP staining

Osteoprogenitor cells cultured in osteogenic medium under the influence of dexamethasone, β -glycerophosphatase and

Fig. 6 – Numbers of CFU–F from each protocol.

Protocol A

Protocol B

Fig. 7 – Fluorescence activated cell analysis of adherent cells 10 days after seeding showing STRO-1 positivity (A, E), compared with negative controls (B – D, F – H): cells with AlexaFluor 488 conjugate Goat anti-mouse IgM antibody (B, F), cells with pure stro-1 (C, G), and cells only without any antibody (D, H).

Fig. 8 – ALP positive osteoblasts (Above), and ALP positive CFU–Fs produced from single osteoprogenitor cells (Below).

ascorbic acid progressed to osteoblastic differentiation, which is visualized by ALP cytochemical staining (Fig. 8) and quantified by colorimetric measurement (Fig. 9).

The quantitative study of alkaline phosphatase revealed ALP activity by osteoblasts on day 7 and increased within a few days with its peak on day 10, and then it reduced in values. ALP activity did not significantly differ between protocols for

each time interval; however, a significant increase in enzyme activity was detected when comparing between day 7 and 10 for both protocols (Fig. 9).

3.5. Detection of in-vitro mineralization

Formation of mineralized nodules in osteogenic cell culture was confirmed by positive Alizarin Red S and von Kossa staining in both protocols. The mineralized nodules appeared bright red when stained with alizarin red S and stained black when treated with silver nitrate in the von Kossa method (Fig. 10).

Fig. 9 – Osteoblastic activity of bone marrow stromal osteoprogenitor cells.

4. Discussion

A sufficient number of competent osteoprogenitor cells in the graft or healing site is essential for bone healing or regeneration.⁸ Aspirated bone marrow serves as the richest supply of osteoprogenitor cells.¹ Ability to isolate the highest possible number of purified stromal stem and osteoprogenitor cells from the least possible amount of bone marrow is crucial for bone tissue engineering applications.

This study was done with the assumption that different centrifugation forces and different dilutions might have different enrichment efficacy upon osteoprogenitor cells. In

Fig. 10 – Phase contrast micrograph of 28-day-old mineralized nodules: Alizarin Red S (left), von Kossa (Right) (4× mineralization).

this study, by using Ficoll lower density gradient separation medium (1.073 g/ml), relative enrichment efficacies of the two centrifugation protocols were compared.

The same amount of bone marrow was aspirated from each volunteer by using a standard proven technique for consistency in quality of aspirated bone marrow. This technique ensured that bone marrow was aspirated, not blood.¹⁶

Bone marrow was aspirated from the anterior iliac crest because it was also the donor site for secondary alveolar bone grafting. Our aim was to avoid any added morbidity for the patients from bone marrow aspiration. Moreover, the area of iliac crest is broad and easily assessable, and the pain associated with needle aspiration is minimal, which provides considerable benefits for patients compared to open harvesting.

In our study, a lower centrifugal force (400 g in Protocol A) provided a higher number of isolated mononuclear cells. However, it was questionable whether the higher number came from an increase in the number of osteoprogenitors or that of other unwanted cells. In contrast, a higher centrifugal force (1000 g, Protocol B) isolated a lower number of mononuclear cells. Again, whether or not the protocol could separate more purified osteoprogenitors and less number of other cells was not clear; this study aimed to find the answer.

The mean number $\pm$ SD of mononuclear cells isolated from both protocols was $6.87 \times 10^7 \pm 4.84 \times 10^7$ and $4.70 \times 10^7 \pm 3.93 \times 10^7$ respectively. Bone marrow cellularity and number of stem and progenitor cells greatly differ individually according to age, sex and physiological requirement of the body, which leads to wide standard deviation when compared statistically. Therefore, a paired *t* test was used to compare the differences between the individual patients separately.

In the present study, lower degree of dilution provided higher numbers of isolated mononuclear cells. Higher viscosity of bone marrow (Protocol A) produced higher numbers of mononuclear cells; however, again, whether or not it produced a higher number of osteoprogenitors still needs to be investigated further.

Therefore, CFU-F forming efficacy and STRO-1 positivity of isolated mononuclear cells were compared in order to estimate the numbers of osteoprogenitors present in each

protocol. Osteogenic differentiation and mineralization efficacy of these isolated osteoprogenitors were also compared.

It was found that both protocols could produce high numbers of CFU-Fs (no significant differences). In this study, 43–64 CFU-Fs per 1 million cells (around 0.0043%–0.0064%) were isolated from bone marrow, which was similar to the estimates of other studies (>0.005%), therefore the techniques of aspiration, centrifugation and cell culture used in this study were comparable to other studies and thus considered reliable.²⁰

There were various agents in the literature used to isolate bone marrow stromal stem cells such as STRO-1, CD 18, CD 106, CD 146, etc. Among these, STRO-1 antibody has the highest affinity and efficiency for isolating all colony-forming osteogenic precursor cells isolated from aspirates of adult bone marrow as a standalone agent. It has no reactivity to hematopoietic stem and progenitors cells, and its specificity is strictly restricted to early preosteogenic stem cells.^{7,21,22}

Flow cytometry analysis confirmed the presence of equal number of osteoprogenitors by an STRO-1 osteoprogenitor cell marker (around $10 \pm 3\%$) in each protocol. Our results were also comparable to other studies done by Grothos (9.2%) and Simmons (14.7%), meaning both methods of separation could yield an optimum number of STRO-1 positive osteoprogenitors.^{23,24}

Isolated osteoprogenitors from both protocols showed alkaline phosphatase activity, which is an early osteoblastic lineage marker. No significant difference was detected in each protocol proving that competent and functional osteoprogenitors were able to recover from both protocols and the centrifugation process didn't alter their function.

In addition to this, mineralization was also detected in both protocols by Alizarin Red S and von Kossa staining as a proof of their ability to secrete calcium and phosphate minerals, reflecting the terminal differentiation of osteoprogenitors into osteoblasts.

5. Conclusion

This study revealed that the total number of bone marrow derived osteoprogenitor cells were significantly higher in

Protocol A than in Protocol B, suggesting that a lower centrifugation force (400 g) with 1:1 was preferable in terms of recovery of more osteoprogenitors.

However, the present work showed that there was no detectable difference between CFU-F forming capacity, STRO-1 positivity, osteogenic differentiation or mineralization abilities between protocols.

Further studies with a higher number of patients are necessary to assess overall efficacy and function of these osteoprogenitor cells, and to find the optimum dilutional and centrifugation factors customized for isolation of stromal osteoprogenitor cells from human bone marrow aspirations.

Conflicts of interest

All authors have none to declare.

Acknowledgements

Our sincere gratitude goes to Associate Professor Premjit Arpornmaeklong and Associate Professor Prisana Pripatnont for their expert advice and help. We would like to thank Mr. Jakchai Jantaramano and Mrs. Siriwan Junrounussit for technical assistance, Mrs. Somporn Sretrirutchai for assistance in flow cytometry and Mr. Mitch Atkins for proof-reading. We are also grateful for the financial support provided by the Graduate School and Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Prince of Songkla University (D2111/53).

REFERENCES

- Dawson JI, Smith JO, Aarvold A, et al. Enhancing the osteogenic efficacy of human bone marrow aspirate: concentrating osteoprogenitors using wave-assisted filtration. *Cytotherapy*. 2013;15:242–252.
- Krebsbach PH, Kuznetsov SA, Bianco P, Robey PG. Bone marrow stromal cells: characterization and clinical application. *Crit Rev Oral Biol Med*. 1999;10:165–181.
- Goujon E. Recherches expérimentales sur les propriétés du tissu osseux. *J Anat*. 1869;6:399–421.
- Friedenstein AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet*. 1970;3:393–403.
- Caplan AI. Mesenchymal stem cells. *J Orthop Res*. 1991;9:641–650.
- Haynesworth SE, Goshima J, Goldberg VM, Caplan AI. Characterization of cells with osteogenic potential from human marrow. *Bone*. 1992;13:81–88.
- Gronthos S, Graves SE, Ohta S, Simmons PJ. The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors. *Blood*. 1994;84:4164–4173.
- Hernigou P, Poignard A, Beaujean F, Rouard H. Percutaneous autologous bone-marrow grafting for nonunions. Influence of the number and concentration of progenitor cells. *J Bone Joint Surg Am*. 2005;87:1430–1437.
- Assmus B, Tonn T, Seeger FH, et al. Red blood cell contamination of the final cell product impairs the efficacy of autologous bone marrow mononuclear cell therapy. *J Am Coll Cardiol*. 2010;55:1385–1394.
- Chang Y, Hsieh PH, Chao CC. The efficiency of Percoll and Ficoll density gradient media in the isolation of marrow derived human mesenchymal stem cells with osteogenic potential. *Chang Gung Med J*. 2009;32:264–275.
- Grisendi G, Anneren C, Cafarelli L, et al. GMP-manufactured density gradient media for optimized mesenchymal stromal/stem cell isolation and expansion. *Cytotherapy*. 2010;12:466–477.
- Colter DC, Class R, DiGirolamo CM, Prockop DJ. Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. *Proc Natl Acad Sci USA*. 2000;97:3213–3218.
- DiGirolamo CM, Stokes D, Colter D, Phinney DG, Class R, Prockop DJ. Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br J Haematol*. 1999;107:275–281.
- Mareschi K, Rustichelli D, Calabrese R, et al. Multipotent mesenchymal stromal stem cell expansion by plating whole bone marrow at a low cellular density: a more advantageous method for clinical use. *Stem Cells Int*. 2012;2012:920581.
- Sharpe PT. Laboratory Techniques in Biochemistry and Molecular Biology: Methods of Cell Separation.
- Muschler GF, Boehm C, Easley K. Aspiration to obtain osteoblast progenitor cells from human bone marrow: the influence of aspiration volume. *J Bone Joint Surg Am*. 1997;79:1699–1709.
- Smith PK, Krohn RI, Hermanson GT, et al. Measurement of protein using bicinchoninic acid. *Anal Biochem*. 1985;150:76–85.
- Declercq H, Van den Vreken N, De Maeyer E, et al. Isolation, proliferation and differentiation of osteoblastic cells to study cell/biomaterial interactions: comparison of different isolation techniques and source. *Biomaterials*. 2004;25:757–768.
- Wang YH, Liu YL, Maye P, Rowe DW. Examination of mineralized nodule formation in living osteoblastic cultures using fluorescent dyes. *Biotechnol Prog*. 2006;22:1697–1701.
- Block JE. The role and effectiveness of bone marrow in osseous regeneration. *Med Hypotheses*. 2005;65:740–747.
- Gronthos S, Simmons PJ. The growth-factor requirements of STRO-1-positive human bone-marrow stromal precursors under serum-deprived conditions in-vitro. *Blood*. 1995;85:929–940.
- Gronthos S, Zannettino ACW, Graves SE, Ohta S, Hay SJ, Simmons PJ. Differential cell surface expression of the STRO-1 and alkaline phosphatase antigens on discrete developmental stages in primary cultures of human bone cells. *J Bone Miner Res*. 1999;14:47–56.
- Gronthos S, Zannettino AC. A method to isolate and purify human bone marrow stromal stem cells. *Methods Mol Biol*. 2008;449:45–57.
- Simmons PJ, Torok-Storb B. Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. *Blood*. 1991;78:55–62.