Cellular properties and multilineage potential of bone marrow mesenchymal stem cells for cartilage regeneration

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Abstract
Bone marrow-derived mesenchymal stem cells (BMSCs) have high self-renewal and trilineage differentiation capacity which makes them a potential source for the regeneration of cartilage tissue. Hence, the present study was aimed to evaluate human BMSCs on their cellular and biological properties such as morphology, viability, proliferation and population doubling time (PDT), colony-forming-unit assay, senescence activity, cytogenetic stability, alkaline phosphatase (ALP) activity, surface marker expression, and multilineage potential towards osteocytes, adipocytes and chondrocytes. Established BMSCs (n=3) exhibited spindle-shaped morphology during primary culture and early passages but acquired characteristic fibroblast-like features later. Viability was >95% at all passages; however, BMSC1 showed decreased viability during later passages (P3-P5). PDT values were slightly higher in BMSC2 and BMSC3 when compared to BMSC1. BMSCs showed colony formation ability and positive staining pattern for ALP activity.

Further, BMSCs had a positive expression for CD29, CD90 and CD73 and negative expression for CD34 and CD45. All BMSCs were successfully differentiated towards osteocytes, adipocytes and chondrocytes upon specific induction. BMSCs were shown to be proliferative with expression of MSC-specific phenotypic markers and ability for multilineage differentiation potential. These BMSCs could possibly be used in prospective therapeutic applications in cartilage regeneration.

Keywords: Bone marrow mesenchymal stem cells, cellular properties, differentiation, human.

Introduction
Among tissue specific adult stem cells, mesenchymal stem cells (MSCs) offer a great promise for the tissue engineering of musculo-skeletal structures. MSCs were first isolated from the bone marrow as stromal cells by Friedenstein et al.⁷ Subsequently, MSCs have successfully been harvested from several tissues including bone marrow, muscle, fat, skin, peripheral blood, umbilical cord blood and Wharton’s jelly⁹. Bone marrow stoma proves to be major source of MSCs with their high proliferation potential and ability to differentiate into progenitor cells of distinct mesenchymal lineages.

Bone marrow-derived MSCs (BMSCs) represent only 2-3% of total mononuclear cells, but can be easily isolated and expanded for several passages without the loss of biological characteristics and differentiation potential. As defined by International Society for Cell Therapy (ISCT), MSCs in culture exhibit plastic adherent fibroblast-like morphology, express mesenchymal surface markers, CD29, CD73, CD44, CD90, and CD105, CD166 but not express hematopoietic cell markers, CD14, CD34 and CD45 and are capable of differentiating in vitro into osteoblasts, adipocytes and chondrocytes⁸.

Cartilage defects in the weight-bearing joints pose a considerable restriction to the patient and are a major cause of short- and long-term disability. A significant number of adolescents and younger adults are exposed to chondral defects secondary to trauma, sports-related injuries, and osteochondritis dissecans (OCD). It is well proven that cartilage defects require early treatment because they have a poor intrinsic healing ability due to lack of vascular supply and less proliferative capacity of chondrocytes.⁴,¹⁹, twenty-one.

In the past three decades, various treatment options for chondral defects have evolved aiming towards better cartilage regeneration. These include bone marrow stimulation techniques; microfracture technique that popularized in 1980’s had significant results but it was limited due to its short-lived cartilage. Osteochondral grafting with both autologous and allogenic source repairs native hyaline cartilage, but it is limited by the size and number of lesions and donor site morbidity. Cell-based therapy by autologous chondrocyte implantation (ACI) is already in use clinically for treatment of cartilage defects. ACI produces hyaline cartilage and can be used for large lesions. However, it is unaffordable and two-stage procedure with additional complications of an arthotomy.²²

For cell-based treatment strategies, MSCs are an attractive cell population to possibly minimize the time period for cartilage repair in vivo, as these cells possess higher proliferation rate and multipotency compared to chondrocytes and, therefore, more cells could take part in cartilage repair⁹. Transplantation of MSCs may have the
potential to resurface the cartilage defects to overcome the disadvantages of ACI. Although MSCs could be harvested from several adult tissues, bone marrow aspirate is widely accepted as a cell source for cartilage regeneration and holds great potential for the development of new treatment strategies for cartilage repair.

Therefore, in the present study, in vitro established BMSCs were evaluated for cellular and biological properties in terms of morphology, viability, proliferation rate, colony-forming ability, senescence activity, alkaline phosphatase activity, expression of cell surface markers, cyogenetic stability and multilineage potential and assessed their option as a clinically feasible source for cartilage regeneration.

Material and methods

Chemicals and media: All chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) and media from Gibco (Invitrogen, Thermofisher Scientific, Grand Island, NY, USA) unless otherwise specified.

Collection of bone marrow, isolation and expansion of BMSCs: The study was approved by Institutional Ethics Committee and Institutional Committee for Stem Cell Research (IC-SCR), Nitte (Deemed to be University). Bone marrow aspirates were collected and handled after obtaining informed consents from 3 patients aged 19-23 years who were undergoing knee arthroscopy procedure for ligament injury with cartilage damage. Under local anaesthesia, about 59 ml of bone marrow suspension was harvested from posterior iliac crest of donor and collected in a 50 ml tube containing the same volume of heparinized (10 U/mL) phosphate-buffered saline (PBS) to prevent clotting.

Mononuclear cells from bone marrow aspirate were isolated by density gradient solution (Ficoll-Paque PLUS; 1.077 g/mL, GE Healthcare Life Sciences, Uppsala, Sweden). After centrifugation at 1200 rpm for 40 minutes at 20°C, the mononuclear cell layer (buffy coat) was removed from the interphase and washed twice in PBS. Nucleated cells were cultured in advanced Dulbecco’s modified eagle’s medium (ADMEM) supplemented with 10-20% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2 in air by changing culture medium twice a week until the cells reached 80-90% confluency. Once confluent, all BMSCs were dissociated using a 0.25% (w/v) trypsin and made into pellets by centrifugation at 1000 rpm for 5 min. Cells were then re-grown in ADMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2 in air by changing culture medium twice a week and passed five times for further analysis. Analyses were conducted in triplicate unless otherwise specified. Cell lines were designated as BMSC1, BMSC2, and BMSC3.

Morphology and viability assay: Cell morphology was assessed by phase-contrast microscope and changes in morphology during different time points of culture and also at various passages were recorded. Cell viability was performed by trypan blue exclusion test with a hemocytometer. Cell viability was assessed at every sub-passage of BMSCs.

Proliferation rate and population doubling time (PDT): To determine the proliferation rate of BMSCs, cells were plated at 500-2000 cells/cm² in each tissue culture plate and cultured for 12 days. Cells in every three wells were detached with 0.25% trypsin-EDTA on day 3, 6, 9 and 12 and counted using a hemocytometer. The culture medium was changed every 3 days. The average cell numbers for each three wells in every replicate were determined. The experiment was performed in triplicate and the proliferation rate was calculated. Population Doubling Time (PDT) was assessed by counting BMSCs using a hemocytometer under phase-contrast microscope. PDT was calculated as, PDT= t(log2)/ (log Nt-log No) where t represents culture time, and No and Nt are the cell numbers before and after seeding respectively.

Colony-forming-unit (CFU) ability: Colony-forming-unit (CFU) ability of BMSCs was determined by plating cells at a seeding density of 10000 per well of 12-well culture dish and assay was performed using crystal violet staining. Cells were grown for 2 weeks with a change of culture medium at every 3 days interval. The cells were subsequently fixed with ice-cold ethanol for 5 min. Positively stained colonies were then observed under a light microscope.

Alkaline phosphatase (ALP) activity: To assess alkaline phosphatase activity (ALP), cells were seeded at a density of 10000 per well of 12-well culture plate, and assay was performed by following the manufacturers’ instructions of ALP staining BCIP/NBT kit (Promega, Madison, WI, USA). Cells were grown for 2 weeks with regular change of a fresh media twice a week. The cells were fixed with 4% paraformaldehyde for 20 min. After staining, cells were observed under a light microscope.

Cytogenetic stability: Cytogenetic stability was assessed by GTG banding on metaphase spreads from cultured BMSCs. GTG banding was carried out using trypsin (0.05%) and 1% Giemsa stain. Well banded metaphase spreads were analyzed using fluorescence microscope (Olympus, Tokyo, Japan).

Senescence activity: Evaluation of senescence activity in BMSCs was performed by following the manufacturers’ instructions of Senescence associated (SA)-β-Gal staining kit (Cell Signaling Technology, MA, USA). Briefly, cells were washed with PBS and incubated for 15 min with 3.7% formaldehyde, and again washed twice with PBS. Colour development was observed by incubation overnight at 37°C with the provided staining solution (40mM citric acid/sodium phosphate pH 6.0, 150 mM NaCl, 2 mM MgCl2, 5 mM potassium ferricyanide, and 1 mg/ml X-gal in DMSO).
Plates were then observed for the development of blue colour by microscopy.

**Analysis of MSC-specific markers by flow cytometry analysis:** BMSCs at ~80% confluence were washed twice in PBS and analyzed for the expression of stem cell markers (CD29, CD73, CD90 and CD105) and the absence of CD34 and CD45 using flow cytometry (BD FACSCalibur, Becton Dickinson, NJ, USA). Primary antibodies used were Alexa fluor-488 conjugated anti-mouse CD105 (Biolegend, CA, USA, 1:50) labeled directly at 37°C for 1 hr, and unconjugated CD29 (eBioscience, CA, USA, 1:50), CD73 (Biolegend, 1:100), CD90 (eBioscience, 1:100), CD34 (Biolegend, 1:100) and CD45 (eBioscience, 1:100) were incubated for 2 hrs at 37°C.

Following washes with cell staining buffer (Biolegend), fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (eBioscience, 1:100) used as a secondary antibody was labeled for 1 hr at room temperature. The standard was established by isotype-matched control (eBioscience). A total of 10,000 FITC-labeled or Alexafluor labeled cells were acquired and analyzed by a BD FACS Calibur with Cell Quest software (Becton Dickinson).

**Osteogenic differentiation:** BMSCs were cultured at a density of 10,000 cells per well in a basal growth medium containing DMEM with 10% FBS. Once cells reached 70% confluent, they were induced for 21 days in osteogenic media consisting of 1 μM dexamethasone, 10 mM β-glycerophosphate and 100 μM ascorbic acid in growth media. After induction, mineralization was detected by Alizarin red S staining.

**Adipogenic differentiation:** For adipogenesis, BMSCs were cultured at a density of 10,000 cells per well in basal growth medium containing DMEM with 10% FBS. Once cells reached 70% confluence, medium was changed to adipogenic media containing 1 μM dexamethasone, 100 μM indomethacin, 10 μg/ml insulin, 500 μM 3-isobutylmethylxanthine in growth media. To confirm adipogenesis, the presence of oil droplets in differentiated adipocytes was evaluated by Oil red O staining.

**Chondrogenic differentiation:** For chondrogenic induction, BMSCs were cultured at a density of 10,000 cells per well in basal growth medium containing DMEM with 10% FBS. After 80-90% confluence, cells were grown in chondrogenic induction media consisting of 1 μM dexamethasone, 1% insulin-transferrin-selenium (ITS), 500 μM ascorbic acid, 10 ng/ml TGF-β3 (Peprotech, NJ, USA) for 28 days. Alcian blue staining was performed for assessing the presence of chondrocytes through proteoglycans deposition.

**Statistical analysis:** All data are expressed as the mean ± standard deviation (SD) from at least three independent experiments and one-way ANOVA was performed by GraphPad Prism software (GraphPad, CA, USA). Significance was set at p <0.05.

**Results and Discussion**

The present *in vitro* study on BMSCs evaluated their cellular and biological properties based on morphology, viability, proliferation rate, colony-forming ability, senescence activity, alkaline phosphatase activity, expression of cell surface markers, cytogenetic stability and mesenchymal lineage potential.

**Morphological features:** Isolated and cultured cells from three donors showed small, spindle-shaped heterogeneous morphology during the primary culture and early passages of 1 (Passage 1) as depicted in figure 1. However, during the subsequent passages (Passage 3), the cells acquired fibroblast-like morphology, which is typical characteristic of BMSCs and grown as a homogeneous population in culture dishes.

In this study, in order to isolate a more homogeneous MSCs population, bone marrow mononuclear cells were separated by density gradient centrifugation using Ficoll-Paque solution, and eliminated the heterogenous mixture of cells as observed in studies establishing bone marrow cells without separation. After primary culture, BMSCs were passaged through four or five propagations and they retained the characteristic morphology of spindle-shaped cells.

**Viability, proliferative potential and senescence activity:** Viability of BMSCs from all the cell lines at different passages was determined and any changes in viability during culture expansion were recorded (Figure 2A). No significant differences in the mean values of viability were observed between BMSC2 (99.61±0.36) and BMSC3 (98.54±1.65) (p>0.05) during passage 1-4. However, during passage 5, a significant difference (p<0.05) in viable cell number was observed between BMSC1 and BMSC2, and BMSC1 and BMSC3. Despite this, the viable BMSCs were still in high number throughout the *in vitro* expansion period.

The proliferation of BMSCs in the initial days of culture (day 0 to day 6) was comparatively slow, but remarkably increased from day 6 to day 12 as represented graphically in figure 2B. There was a significant difference (p<0.05) in the cell numbers between the BMSC samples on day 9 and day 12. These cell numbers corresponded to the mean PDT values which were found to be least in BMSC3 followed by BMSC2 and BMSC1 (Figure 2C). However, when the log PDT values were analyzed by one way-ANOVA, no significant difference (p<0.05) was observed between the samples. Collectively, except for BMSC1, other BMSCs showed a greater proliferative capacity especially after 6 days of culture. Differences in growth kinetics of BMSCs in this study are most likely attributed to the variations in collection of bone marrow aspirate and also the number of proliferative cells established initially in the primary culture.
Senescence in MSCs is considered as a critical aspect for cellular therapy and safety assessment. The detection of senescence-associated (SA)-β-galactosidase expression by senescent cells is the most routine assay followed for its evaluation. β-galactosidase staining of BMSCs in this study revealed the varying presence of cells generating a blue precipitate as presented in the percentage expression of cells (Figure 2D) and by a representative image (Figure 3B).

BMSCs2 and BMSC3 cell lines showed a very low number of cells with β-galactosidase detection with no significant difference in percentage expression. But, as observed with low proliferation ability and high PDT value, BMSC1 exhibited slightly higher senescence induced by culture expansion. It is suggested that in vitro expansion times of MSCs must be reduced as much as possible, as the replicative senescence may have a detrimental effect on cellular therapy efficacy by altering their biological properties. Replicative senescence is a common phenomenon observed in all healthy somatic cells and it seems to be applicable for in vitro cultured BMSCs.

**Colony-forming-unit (CFU) assay and alkaline phosphatase (ALP) activity:** CFU ability of BMSCs was determined at passage 3, and the colonies/clusters were recorded by positively stained colonies after 2 weeks of culture (Figure 3A). Crystal violet stain was used to recognize the colonies formed under in vitro culture. Positively stained colonies were observed in all the cell lines of BMSCs. When cultured at a low density, they facilitate individual cell to attach and proliferate in colonies/clusters. This suggests that more naïve BMSCs with clonogenic potential were present in a population with CFU-efficiency. However, the possibility of obtaining these cells in a large number with CFU-efficiency during long-term culture needs to be determined.

ALP assay was performed for BMSCs at passage 4, and the results clearly showed a positive dark purple staining in 12-well dishes cultured with an initial seeding density of 10,000 cells/well (Figure 3C and D). The level of ALP activity correlates well with the potency and undifferentiated status of stem cells. Further, it has been demonstrated that in vivo bone forming capacity of the BMSCs could be projected by the magnitude of stimulation of ALP during the in vitro osteogenic differentiation process. Higher levels of ALP expression indicate the presence of proosteogenic MSCs in the established cell cultures.

**Cell surface marker analysis:** Flow cytometry analysis was performed to assess the expression of multipotent stem cell markers in BMSCs. A total of 10,000 cells were analyzed in duplicate. Overall, the data showed that the BMSCs had positive expression of MSC markers such as CD29, CD73 and CD90 (all with >85% positive expression) in contrast to hematopoietic cells markers, CD34 and CD45 which showed <5% expression (Figure 4A-F). The results of the membrane marker expression analysis demonstrated that there is substantially more homogeneity in the established BMSC cultures in this study. Examination of additional markers is required for identifying BMSC subpopulations with variable expressions, and that could be the most promising way to determine cells with cartilage forming capacity.

**Cytogenetic stability:** Cytogenetic stability of BMSCs was determined by GTG banding and the results showed normal ploidy indicating that no chromosomal abnormalities were induced during the culture expansion (Figure 5A-C).

Our results revealed that expanded BMSCs did not display any unbalanced chromosomal rearrangements such as deletion or duplication. Earlier studies have reported stable chromosomes in culture for MSCs derived from different tissue origins. In contrast, a few studies have documented that human MSCs acquire cytogenetic alterations in culture both in autosomes and sex chromosomes. Thus, it is highly imperative to consider the factors such as tissue source, culture conditions and culture duration that influence the genetic stability before clinical application.

**In vitro differentiation into osteocytes, adipocytes and chondrocytes:** In vitro differentiation potential of BMSCs into osteocytes, adipocytes and chondrocytes was evaluated by following standard protocols, and the cytochemical staining was performed to confirm the presence of induced cells (Figure 6A-D). BMSCs in non-inductive basal medium demonstrated a fibroblast-like morphology throughout the culture period of 21 days (Figure 6A, C and E). Under osteogenic conditions, BMSCs formed polygonal structures with deposition of calcium mineralized nodules in the cultures (Figure 6B). This was evidenced by positive staining with Alizarin red S solution.

Following induction in adipogenic medium, BMSCs were capable of forming neutral lipid vacuoles after 21 days of culture and it was confirmed by Oil red O staining (Figure 6D). Under chondrogenic differentiation conditions for 21 days, BMSCs were able to synthesize glycosaminoglycans (GAGs) as an indication of presence of cartilage cells and it was subsequently confirmed by Alcian blue staining (Figure 6F). It is suggested that the general heterogeneity of bone marrow cell population can lead to variable results of differentiation along osteogenic, adipogenic and chondrogenic pathways. However, in this study, all the BMSCs could go through mesenchymal lineage differentiation including osteogenesis, adipogenesis and chondrogenesis. This multipotency feature is particularly valid for cartilage-based therapies in which BMSCs are expected to have highly active role such as a large amount of GAGs accumulation. Thus, for the purpose of therapeutic approaches, a minimum requirement for BMSCs is employing standardized cell culture conditions and potency evaluation methods in the lines of growth kinetics, genetic stability and lineage differentiation ability.
However, varying composition of cell types in bone marrow could impact the changes in rate of proliferation and phenotypic markers expression as observed in this study. It has been shown that even various donor-derived samples that did not have great proliferation activity at the early stage of culture may be enriched in the course of in vitro expansion resulting in higher differentiation potential\textsuperscript{3,10}.

Figure 1: Morphology of BMSCs. Representative images showing adhered cells with a small, spindle-shaped morphology during the primary culture establishment on day 6 (A). At passage 1, relatively heterogeneous population of BMSCs was observed (B). However, by passage 3, BMSCs displayed a characteristic fibroblast-like morphology.

Images: 4x

Figure 2: Viability, proliferative potential and senescence activity of BMSCs. Percentage cell viability of BMSCs from passage 1 to 5 (A). Proliferative potential of BMSCs determined by counting the cell number at days 3, 6, 9 and 12 after plating (B). Average population doubling time (PDT) was calculated by using the cell number plated initially and after harvesting on day 12 (C). Percentage of cells expressing β-galactosidase as an indicator of senescence (D). All values represent Mean ±SD; n=3, triplicates; a, b, c represent significant difference (p<0.05) between the cell lines.
Figure 3: Colony-forming-unit (CFU) assay, senescence and alkaline phosphatase activity in BMSCs. Representative images showing the CFU ability of BMSCs by Crystal violet staining (A) and the detection of cells expressing β-galactosidase as a measurement of senescence (B) Macroscopic and microscopic views of BMSCs demonstrating the alkaline phosphatase activity (C and D respectively). Images-4x and 10x.

Figure 4: Expression of cell surface markers of BMSCs. Representative images showing the flow cytometry analysis for characteristic markers of MSCs such as (A) CD29, (B) CD73, (C) CD90, (D) CD34 and (E) CD45. BMSCs were positive for CD29, CD73 and CD90, but negative for CD34 and CD45. Lined and filled histograms show isotype control and expression of indicated markers, respectively. Percentage expression of markers in BMSCs is presented graphically (F).
Figure 5: Cytogenetic stability of BMSCs. Cytogenetic stability was determined by GTG banding and the results showed normal ploidy during the culture expansion of (A) BMSC1, (B) BMSC2 and (C) BMSC3.

Figure 6: *In vitro* differentiation of BMSCs into osteocytes, adipocytes and chondrocytes. Mesenchymal lineage potential of BMSCs is confirmed by tri-lineage differentiation process for 21 days. BMSCs in non-inductive basal medium demonstrated a fibroblastic morphology throughout the culture period (A, C and F). Under osteogenic conditions, BMSCs showed with deposition of calcium mineralized nodules in the cultures as indicated by Alizarin red S (B, arrows). Upon adipogenic induction, BMSCs were capable of forming neutral lipid vacuoles as confirmed by Oil red O (D, arrows). Under chondrogenic conditions, BMSCs were able to synthesize glycosaminoglycans (GAGs) as an indication of presence of cartilage cells as evidenced by Alcian blue staining. (F, arrow).

Images: 4x, 10x
Conclusion
In conclusion, our results indicate that BMSCs were highly proliferative with expression of MSC-specific phenotypic markers and ability for multilineage differentiation potential. These cellular and biological findings of BMSCs are intended to facilitate in validation using more number of donors with a clinical-grade process for prospective therapeutic applications in cartilage regeneration.

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