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RESEARCH ARTICLE

In vitro assessment of osteogenic and chondrogenic differentiation potential of human bone marrow derived mesenchymal stem cells

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Abstract

Background
Damaged or diseased bone and cartilage frequently leads to progressive debilitation resulting in a marked decrease in quality of life. Cell and tissue engineering has emerged as promising field to assure establishment of viable substitutes for failing organs or tissues by *in vitro* generation and identification of desired cell types.

Methods
In present study, adult human bone marrow blood was used to isolate mesenchymal stem cells (MSCs) using Ficoll-paque density gradient centrifugation. MSCs were cultured *in vitro* and characterized using specific markers. Differentiation potential of MSCs was observed for osteogenic and chondrogenic lineages in different culture conditions. MSCs assumed a polymorphic, fibroblast-like morphology after 2-3 days in culture and showed positive expression for CD90, CD105 and negative for CD34.

Results
MSCs in osteogenic induction medium changed their morphology from a fibroblastic appearance to a more polygonal appearance and showed positive staining with von kossa whereas in chondrogenic induction medium assumed a round, nodule like morphology after 2-3 day in culture. Gene expression analysis showed positive expression for osteoblast markers, osteocyte markers and chondrogenic markers.

Conclusion
The present study provides a significant clue towards the generation of bone and cartilage tissues.

1. Introduction

Tissue engineering using cell biology, engineering, material sciences, and surgical approaches provide a new possibility to find functional tissues using living cells, biomatrices, and signaling molecules (Langer et al. 1993; Vacanti et al. 1999; Vacanti et al. 2000). Bone marrow derived mesenchymal stem cells (MSCs) has been suggested as a suitable option for cell-based tissue engineering therapies. This is due to their capacity for self-renewal and their potential to differentiate into numerous different tissue types, such as bone, cartilage and fat (Dominici et al. 2006). While MSCs strategies have proved somewhat successful for regeneration of certain tissues, such as tendons and cartilage (Butler et al. 2010; Awad et al. 2003; Haleem et al. 2010), it is not yet clear precisely how osteogenic and chondrogenic differentiation can be optimized.

Bone tissue regeneration has faced with various challenges to produce large bone constructs for clinical applications. Various studies have demonstrated the osteogenic differentiation of MSCs *in vivo* is regulated by osteoblasts and osteocytes. The role of osteoblasts and osteocytes as bone regulatory cells is examined, focusing on the osteogenic response induced in MSCs when exposed to factors from osteoblast and osteocyte cells (Birmingham et al. 2012).
Articular cartilage is another prominent target for tissue engineering strategies because injuries of articular cartilage do not heal spontaneously without direct access to a significant source of reparative cells (Hunter et al. 1743; Buckwalter et al. 1998a; Buckwalter et al. 1998b; Mankin et al. 1974; Mankin et al. 1996). Majority of approaches to repair or regenerate articular cartilage are cell-based, aiming to provide a population of reparative cells to the injured site. Cells used to develop these strategies can be either differentiated chondrocytes isolated from unaffected areas of the joint surface (Risbud et al. 2002; Vacanti et al. 1994; Freed et al. 1994; Frenkel et al. 1997; Minas et al. 1998; Giannini et al. 2001; Breinan et al. 2001) or progenitor cells capable of differentiating into chondrocytes and can be isolated from a variety of tissues (Hunziker et al. 1996; Luis et al. 2011). As harvesting a tissue biopsy from valuable healthy articular cartilage will result in an additional injury, which ultimately cannot repair itself, this cell source does not seem to be a good choice.
Therefore, a number of research efforts are directed to the isolation and characterization of MSCs for understanding the mechanisms involved in their differentiation into osteogenic and chondrogenic lineages. Bone marrow is the most common source for the isolation of adult human MSCs (Minguell et al. 2001) and considered as attractive candidates for tissue repair due to their chondrogenic and osteogenic potential, ease of harvest, and expansion in culture. To improve the quality of regenerated tissue by autologous chondrocyte implantation (ACI) or cartilage regeneration, it is essential to identify certain more specific marker expression correlated with the differentiation status of monolayer expanded human articular chondrocytes and to define the index for discriminating dedifferentiated cells from monolayer expanded human MSCs.
The present study has focused on the isolation, *in vitro* proliferation and differentiation potential of MSCs into chondrogenic and osteogenic lineages under controlled conditions. That was further assessed by microscopic and molecular analysis for their specific markers. The study will emphasize a synergistic relationship between MSCs and their osteogenic and chondrogenic lineages supporting the hypothesis that the syncytium of osteocytes and osteoblasts regulates bone formation and MSCs produces chondrocytes with cartilage regeneration potential by means of active differentiation in controlled environment.

2 Materials and Methods

2.1 Mononuclear cells (MNCs) isolation from human bone marrow blood

Bone marrow blood samples were obtained from the patients (mean age: 55 years, range: 45–65 years) undergoing hemiarthroplasty due to femoral neck fracture or its sequelae. The Ethics Committee of Deccan College of Medical Sciences approved the study. Informed consent was obtained prior to the sample collection from all individuals included in the study.
10 mL of bone marrow blood was collected in a heparinized tube (0.3mL, 5000 U/mL) to prevent coagulation, and diluted with 10 mL of phosphate-buffered saline (1X PBS). Diluted blood sample was carefully layered on top of 10mL of Ficoll-paque plus density gradient medium (density of 1.077 g/ml, GE Healthcare) to minimize mixing of blood with Ficoll. Centrifuged at room temperature (15 - 25°C) for 30 minutes at 400 x g with brake off and discarded the upper plasma layer without disturbing the plasma-Ficoll interface. Retaining mononuclear cells (MNCs) layer at the plasma-Ficoll interface was transferred to a fresh sterile tube without disturbing the erythrocyte/granulocyte pellet. MNCs were washed once with Dulbecco's Modified Eagles Medium (DMEM, Invitrogen). Cells were titrated by gentle pipetting to remove cell clumps and subjected to cell viability testing and counting.

2.2 Cell counting and viability testing

Cell viability of the cells was determined by Trypan-blue exclusion method and MTT cell viability assay. Cells were counted using a hemocytometer and percentage cell viability was calculated.

2.3 In vitro proliferation of MNCs for MSCs isolation

Cells were cultured as monolayer at a density of 1.2 X 10^4 cells/cm^2 in DMEM-F12 medium, supplemented with 10% Fetal calf serum (FCS, Invitrogen), 100U/mL Penicillin (Invitrogen), 100µg/mL streptomycin (Invitrogen), and 0.25 mg/mL amphotericin B (Invitrogen). Culture plates including controls were incubated at 37°C in a humidified environment with 5% CO_2. Culture media was changed every after 48h. Cells were subjected to next passage after 10days of initial proliferation. Nonadherent cells were removed from the dish during medium changes and

subsequent passaging. Typically 80–90% of confluence was reached by day 10. When the cells reached 80% of confluence, they were detached from the culture dish using 0.25% trypsin containing 1mM EDTA (Gibco), washed with 1X PBS, counted, and plated again. After culturing 3 passages, cells were suspended in cryo-preservation medium containing 90% Fetal calf serum (FCS) and 10% dimethylsulfoxide (DMSO). Cells on the 2nd passage were used for further studies.

2.4 Immunophenotypying of MSCs

Immunophenotyping of cultured cells was done using flow cytometry (BD Biosciences, CA, USA). 1 X 10^6 cells/mL were used for cell surface antigen staining. Briefly, the cells were incubated with CD34-FITC (BD biosciences, USA) and CD105-PE (BD Biosciences, USA) antibodies for 30 min in dark. Goat anti-mouse FITC (BD biosciences, USA) was used as isotype control. Approximately 10,000 events were acquired for each sample on a FACS Calibur flow cytometer and analyzed using the Cell Quest software (Becton Dickinson).

2.5 Differentiation potential

2.5.1 In vitro chondrogenesis of MSCs

MSCs from 2nd passage were harvested from the culture plates using 0.25% trypsin-EDTA and centrifuged at 500x g for 5 min at 4°C. Cells were resuspended at a density of 1 x 10^6 cell/mL in DMEM/F-12 supplemented with 10^{-7} M dexamethasone (Sigma), 50 µM ascorbate-2-phosphate (Sigma), 1% ITS cell culture supplement (Sigma), 1 mM sodium pyruvate (Sigma), 50 µM L-proline (Sigma) and 5ng/mL of TGF-β2 (Sigma). Plates were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2 for up to 4 weeks. Fresh medium was added every third day. After 4th week cells were harcested and subjected to RT-PCR analysis to see the expression of chondrocyte specific markers.

2.5.2 In vitro induction of MSCs into osteogenesis

1 X 10^6 MSCs/mL were cultured in DMEM/F-12 supplemented with 10% FCS (Sigma), penicillin/streptomycin solution (10,000 IU/100 ml), 10^{-7}M dexamethasone (Sigma), 10 mM-glycerophosphate (Sigma) and 50 µM ascorbate-2-phosphate (Sigma). Cells were maintained at 37°C and 5% CO_2 for 21 days. Medium changes were made every after 3 days. Negative control cells were maintained in cell culture medium containing 10% FCS. Differentiated cells after 3 weeks were analyzed for osteogenic markers.

2.6 Microscopic assessment of chondrogenesis and osteogenesis under different culture conditions

Monolayer cultures induced with osteogenic differentiation medium were stained with von Kossa for mineralized matrix deposition. Briefly, cultures were first fixed 10 min in methanol, washed three times with aqua dest and incubated for 1 h with silver nitrate solution in the dark. Cells were washed and fixed with 5% sodium thiosulfate solution and counterstained with 0.1% nuclear fast red-solution to visualize the cell nuclei.

High density cultures treated with chondrogenic differentiation medium were mounted on Superfrost slides, air dried and fixed in acetone for 20 s before staining with alcian blue (0.05% alcian blue in 3% acetic acid (pH 1.5) plus 0.3 M $MgCl_2$) for 2 h to detect extra-cellularly deposited cartilage specific proteoglycans (CSPG).

Both the differentiated cultures were evaluated under a microscope (Carl Zeiss, Germany) and the photomicrographs were digitally acquired.

2.7 RNA Isolation and RT-PCR Analysis

For gene expression analysis samples were categorized into 3 groups: 1) *in vitro* proliferated MSCs, 2) MSCs cultured in chondrogenic induction medium 3) MSCs cultured in osteogenic induction medium. Total RNA was extracted from all three groups of cells using Trizol (Invitrogen, USA), according to the manufacturer's instructions. Reverse transcription reaction was performed using a PCR thermal cycler (BIORAD). 500ng of total RNA was used for DNA synthesis using M-MLV reverse transcriptase (Fermentas). Later, PCR was performed on the Thermocycler S100 (BIORAD) using Taq DNA polymerase (Fermentas). The amplicons were amplified at 94°C for 5min, for 40 cycles of 94°C for 30sec, 55-58°C for 30sec and 72°C for 30sec, and finally extended at 72°C for 5min. GAPDH was used as an internal control to normalize the amount of cDNA in each sample. Specificity of primers was checked using variable concentrations of cDNA (25ng, 2.5ng and 0.25ng) with each gene compared to the GAPDH. The primers used in the study are listed in Table 1.

2.8 Statistical analysis

Descriptive statistics were used to determine group means and standard deviations in numerical data. Statistical analysis was performed with analysis of variance (ANOVA). Significance was set at $p < 0.05$.

3 Results

3.1 Monolayer cell culture of isolated MSCs

In monolayer culture the isolated bone marrow MSCs assumed a polymorphic, fibroblast-like morphology. Colony formation was observed after 3–5 days in culture. After 7-10 days in culture, the colonies became confluent (Fig. 1).

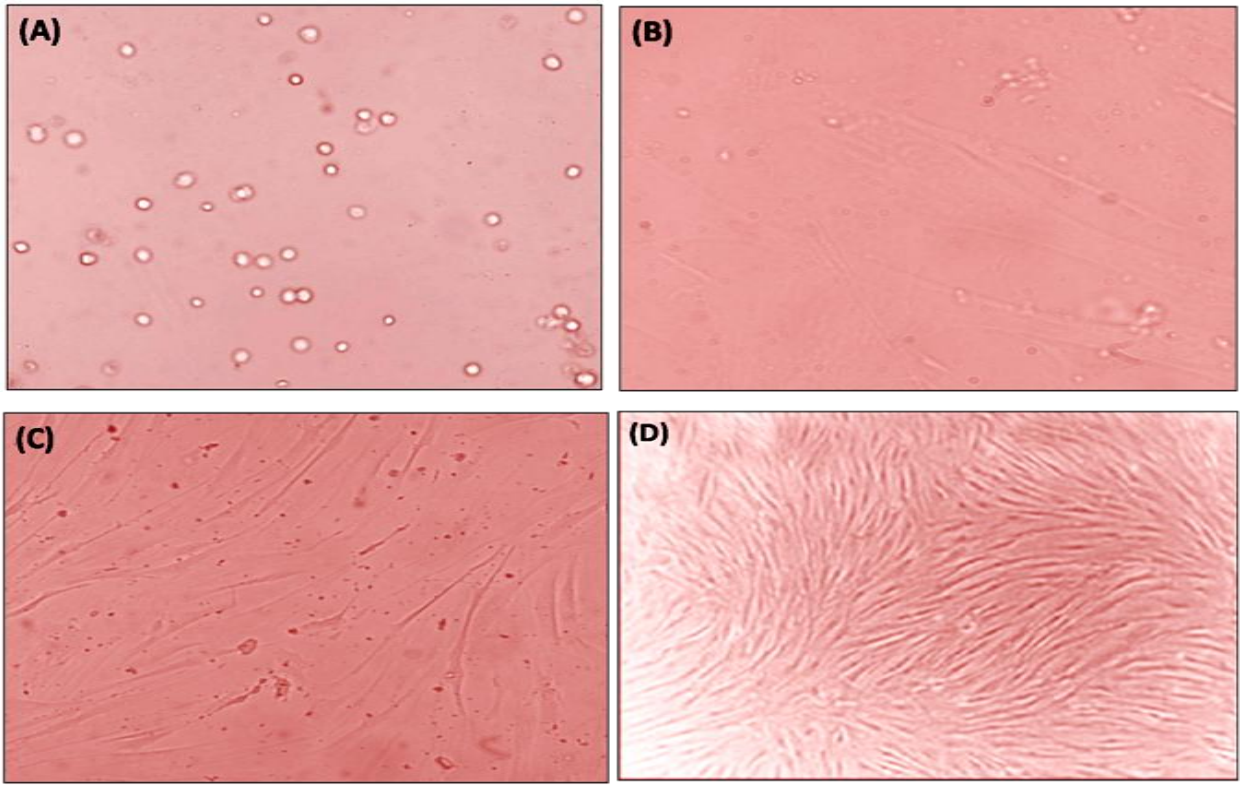

Fig. 1 *In vitro* expansion of Mesenchymal stem cells on monolayer (A-B) Microscopic observation showed polymorphic, fibroblast-like morphology of cultured MSCs in monolayer culture after 2-3 days of initial seeding. (C) Colony formation was observed after 5 days in culture. Cells actively searched for cell-to-cell contacts. (D) After 7-10 days in culture, the colonies became 70-80% confluent. (Magnification: 20x)

3.2 Immunophenotypying

Flow cytometric analysis demonstrated the expression of distinguishing MSCs antigens (CD105, CD90), and the absence of hematopoietic antigen (CD34). ≥98% of cells was found positive for CD105-PE and ≥97% for CD90-PE. However, they were negative for hematopoietic marker CD34-FITC. (Fig. 2).

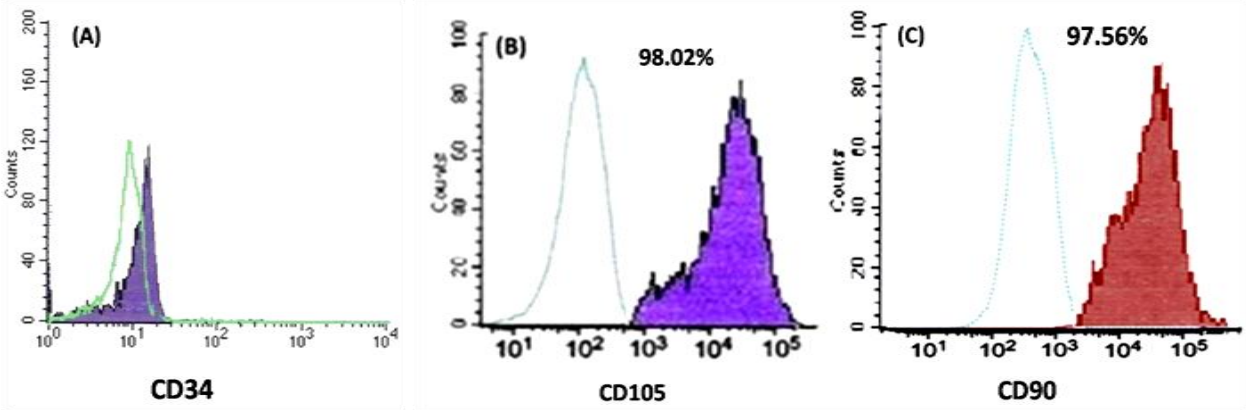

Fig. 2 Immunophenotypic analysis of mesenchymal stem cells (A) cells showed negative expression for CD34 (B) 98.02% cells expressed CD105 and (C) 97.56% cells showed positivity for CD90

3.3 RT-PCR analysis of in vitro proliferated MSCs cells

In vitro proliferated cells were characterized using MSCs specific markers CD105, CD90 and hematopoietic marker CD34. Gene expression analysis showed positive expression for MSCs specific markers, CD90 and CD105 whereas CD34 represented negative expression (Fig. 3).

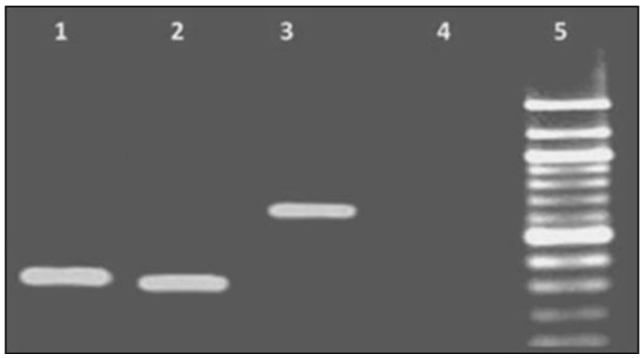

Fig. 3 RT-PCR analysis image representing gene expression pattern for human MSCs specific markers (Lane 1: GAPDH (150bp), Lane 2: CD90 (142bp), Lane 3: CD105 (325bp), Lane 4: CD34 (202bp) and Lane 5: DNA Ladder)

3.4 Chondrogenic and Osteogenic differentiation potential of hMSCs

The differentiation potential of the isolated human bone marrow MSCs was tested by culturing under different culture conditions.

3.4.1 Osteogenic differentiation capacity

3.4.1.1 Light microscopy

After 3 weeks in osteogenic induction medium cells changed their morphology from a fibroblastic appearance to a more polygonal appearance and formed nodules, they also showed positive staining with von Kossa stain. Whereas, control cultures (uninduced cells), became over-confluent and retained their fibroblast like appearance and were stained negative for mineral deposition (Fig. 4).

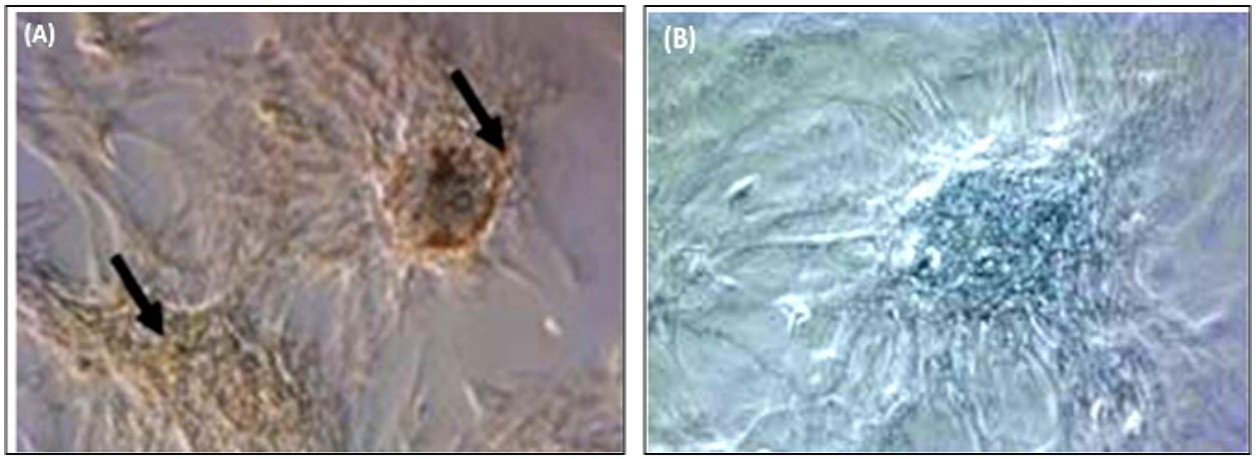

Fig. 4 Light microscopic demonstration of osteogenic differentiation with Von Kossa staining. (A) After 3 weeks in osteogenic induction medium MSCs changed from a fibroblastic appearance to a more cuboidal appearance, were surrounded with an abundant matrix and formed mineralised nodule. (B) Osteoid tissues were stained positive with von Kossa stain for mineral deposition in their newly formed matrix. (Magnification: 40x)

3.4.1.2 RT-PCR analysis

After 3 weeks of induction cells showed positivity for osteoblast markers (RUN-X2, ALP and COL1A1) and osteocyte markers (DMP1, Phex, CX43 and MEPE). Control (uninduced cells) cell did not show any expression for any of these markers (Fig. 5).

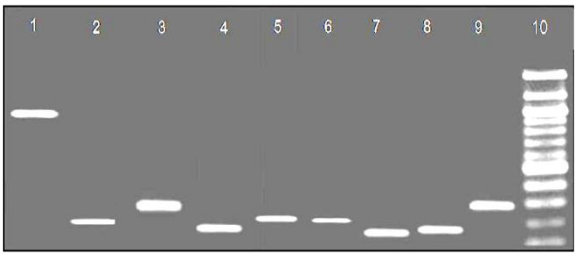

Fig. 5 RT-PCR gel image representing gene expression pattern for human MSCs derived Osteoblasts and Osteocytes specific markers (Lane 1: BMP2 (672bp), Lane 2: RUNX2 (102bp), Lane 3: ALP (151bp), Lane 4: COL1A1 (80bp), Lane 5: DMP1 (106bp), Lane 6: PHEX (101bp), Lane 7: CX43 (69bp), Lane 8: MEPE (81bp), Lane 9: GAPDH (150bp) and Lane 10: DNA Ladder)

3.4.2 Chondrogenic differentiation capacity

3.4.2.1 Light microscopy

Human MSCs when cultured in chondrogenic induction medium assumed a round, nodule like morphology already after 2-3 day in culture. Alcian blue staining after 4 weeks in culture revealed high content of cartilage specific proteoglycans that was more intense and uniform in the center of the culture and decreased and became irregular towards the outer rim, suggesting a higher content of cartilage specific proteoglycans in the inner part of the cultures. In contrast to this, control cultures showed little or no alcian blue staining (Fig. 6).

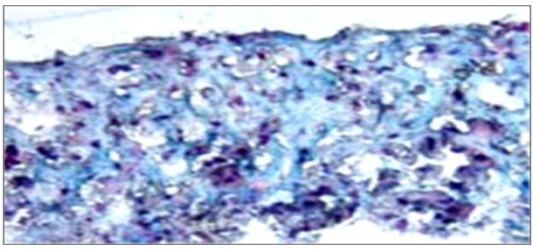

Fig. 6 Light microscopic demonstration of cartilage tissue formation with Alcian Blue staining. After 4weeks the high density cultures treated with chondrogenic induction medium high content of cartilage specific proteoglycans were observed. (Magnification: 40x)

3.4.2.2 RT-PCR analysis

After 4 weeks induced and uninduced cells were analyzed for chondrocyte specific gene expression using RT-PCR. Only induced cells were found to be positive for chondrocyte specific markers Collagen type I, Collagen type II and Aggrecan. Control (uninduced) cells were found to be negative for these markers (Fig. 7).

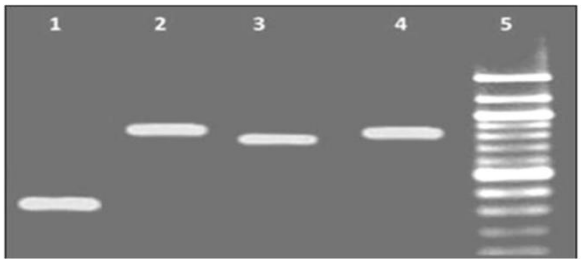

Fig. 7 RT-PCR analysis image representing gene expression pattern for human MSCs derived chondrocyte specific markers (Lane 1: GAPDH (150bp), Lane 2: Collagen Type I (599bp), Lane 3: Collagen Type II (400bp), Lane 4: Aggrecan (509bp) and Lane 5: DNA Ladder)

4 Discussions

The results of this study show that culturing human bone marrow derived MSCs as monolayer have capability to proliferate *in vitro* that can further redirected into osteocytes and chondrocytes by culturing in their respective induction medium. Moreover, these studies suggest that osteogenic and chondrogenic differentiation of MSCS are influencing after characterization by using their key markers from immature to mature cell phenotypes.

Most interestingly, it was found that when MSCs were exposed to factors from both osteoblasts and osteocytes simultaneously, the osteogenic effect was higher than exposure to either cell type alone. This data confirms that a functional relationship exists in the osteocyte-osteoblast network. This study gives an important insight into the natural cues for osteogenic differentiation within the stem niche *in vivo*. Typically, *in vitro* osteogenic factors are introduced directly into the culture medium of MSCs to drive the MSCs towards the osteogenic differentiation.

The identification of more specific markers to characterize MSCs and their specific lineage differentiation status based on cell surface marker expression would contribute to bone and cartilage tissue engineering, from quality control for cell expansion to the optimization of culture conditions. Further validation of these cell molecules as markers of the differentiation status for osteocytes and chondrocytes will require determination of whether the profile of cell surface proteins induced by monolayer culture can be reversed using redifferentiation procedures. Therefore, in this study, we investigated gene expression profile for the correlation with the differentiation of monolayer expanded human MSCs to define the index for cell proliferation and differentiation related to the defined markers.

We observed phenotypic and morphological changes in MSCs when cultured in vitro as monolayer. Spherical cells turned completely into fibroblast like appearance after 10 days in proliferation conditioned medium and expressed hMSCs specific markers such as CD90 and CD105 whereas, they lacked the expression of hematopoietic cell marker i. e. CD34. Immunophenotypic analysis showed ≥98% cells were found positive for CD105 and ≥97% for CD90 markers which clearly indicates the high density proliferation of MSCs *in vitro*. Microscopic observation in different culture conditions showed their differentiation potential and morphological changes in different cell phenotypes. Molecular markers for chondrocyte, osteoblasts and osteocytes showed high degree of differentiation potential measurement and characteristics at a definite time period.

In summary, the present study demonstrates that mRNA expression, immunophenotypic analysis and microscopic characterization of MSCs in different culture conditions provide a significant tool to understand the basic biology to determine its proliferation and differentiation potential *in vitro*. The study also gives a significant clue towards the *in vitro* generation of bone and cartilage tissues that can further be applied in regeneration of these tissues and can be identified for their enumeration *in vivo* using these defined markers.

5 Acknowledgements: None

6 References

1. Langer R and Vacanti JP. (1993) Tissue engineering. Sci., 260(5110): 920–926.
2. Vacanti CA and Vacanti JP. (2000) The science of tissue engineering. Orthop. Clin. North Amer. 31(3): 351–356.
3. Vacanti J and Langer R. (1999) Tissue engineering: the design and fabrication of living replacement devices for surgical re-construction and transplantation. Lancet. 354: SI32–SI4.
4. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D and Horwitz E. (2006) Minimal criteria for defi ning multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytoth. 8: 315-317.
5. Butler DL, Gooch C, Kinneberg KRC, Boivin GP, Galloway MT, Nirmalanandhan VS, Shearn JT, Dyment NA and Juncosa-Melvin N. (2010)The use of Mesenchymal stem cells in collagen-based scaffolds for tissue-engineered repair of tendons. Nature Protoc. 5: 849-863.
6. Awad HA, Boivin GP, Dressler MR, Smith FNL, Young RG and Butler DL. (2003) Repair of patellar tendon injuries using a cell-collagen composite. *J.* Orthop. Res. 21: 420-431.
7. Haleem AM, Singergy AAE, Sabry D, Atta HM, Rashed LA, Chu CR, El Shewy MT, Azzam A and Abdel Aziz MT. (2010) The clinical use of human culture-expanded autologous bone marrow mesenchymal stem cells transplanted on platelet-rich fibrin glue in the treatment of articular cartilage defects: A pilot study and preliminary results. Cartil. 1: 253-261.

8. Birmingham E, Niebur GL, McHugh PE, Shaw G, Barry FP and McNamara LM. Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche. European Cells and Mat. 23: 13-27.

9. Hunter W. (1743) Of the structure and diseases of articulating cartilages. Philosoph. Trans. 42: 514–521.

10. Buckwalter J. (1998a) Articular cartilage: injuries and potential for healing. J. Orthop. Sports Phys. Ther. 28: 192–202.

11. Buckwalter JA and Mankin HJ. (1998b) Articular cartilage repair and transplantation. Arth. and Rheum. 41(8): 1331–1342.

12. Mankin HJ and Buckwalter JA. (1996) Restoration of the osteoarthrotic joint [editorial]. The J. Bone & Joint Surg. 78(1): 1–2.

13. Mankin HJ. (1974) The reaction of articular cartilage to injury and osteoarthritis (first of two parts). New Eng. J. Med. 291(24): 1285–1292.

14. Risbud MV and Sittinger M. (2002) Tissue engineering: advances in in vitro cartilage generation. Trends in Biotechnol. 20(8): 351–356.

15. Vacanti CA, Kim W, Schloo B, Upton J and Vacanti JP. (1994) Joint resurfacing with cartilage grown in situ from cell-polymer structures. Ame. J. Sports Med. 22(4): 485–488.

16. Freed LE, Grande DA, Lingbin Z, Emmanual J, Marquis JC and Langer R. (1994) Joint resurfacing using allograft chondrocytes and synthetic biodegradable polymer scaffolds. J. Biom. Mat. Res. 28(8): 891–899.

17. Frenkel SR, Toolan B, Menche D, Pitman MI and Pachence JM. (1997) Chondrocyte transplantation using a collagen bilayer matrix for cartilage repair. The J. Bone & Joint Surge. 79(5): 831–836.

18. Minas T. (1998) Chondrocyte implantation in the repair of chondral lesions of the knee: economics and quality of life. The Ame. J. Orthop. 27(11): 739–744.

19. Giannini S, Buda R, Grigolo B and Vannini F. (2001) Autologous chondrocyte transplantation in osteochondral lesions of the ankle joint. Foot & Ankle Int. 22(6): 513–517.

20. Breinan HA, Minas T, Hsu HP, Nehrer S, Shortkroff S and Spector M. (2001) Autologous chondrocyte implantation in a canine model: change in composition of reparative tissue with time. J. Orthop. Res. 19(3): 482–492.

21. Hunziker EB and Rosenberg LC. (1996) Repair of partial-thickness defects in articular cartilage: cell recruitment from the synovial membrane. The J. Bone & Joint Surg. 78(5): 721–733.

22. Luis A. Solchaga, Kitsie J. Penick, and Jean F. Welter. (2011) Chondrogenic Differentiation of Bone Marrow-Derived Mesenchymal Stem Cells: Tips and Tricks. Meth. Mol. Biol. 698: 253–278.

23. Minguell JJ, Erices A and Conget P. (2001) Mesenchymal stem cells. Exp. Biol. Med. 226(6): 507–520.