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A Simple Method for Isolation, Propagation, Characterization, and Differentiation of Adult Mouse Bone Marrow-Derived Multipotent Mesenchymal Stem Cells

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Abstract

Mesenchymal stem cells (MSCs) are spindle-shaped, adherent, clonogenic, non-phagocytic, fibroblastic and multipotent in nature with the intrinsic ability of self-renewal and proliferation. Bone marrow is the richest source of MSCs for studying the underlying processes of proliferation, self-renewal, and multiple-lineage differentiation. There have been several studies to improve the method of isolation, propagation, characterization and differentiation of mesenchymal stem cells from the mouse bone marrow, but none are widely acceptable. Owing to unavailability of universally acceptable method of MSCs culture continuous efforts are being made in this direction. Here, we report a simple method with some subtle modifications aiming to improve the overall method of isolation, culture, propagation and differentiation of MSCs *in vitro*. Following this protocol, we have isolated MSCs with typical spindle-shaped morphology as shown by scanning electron microscopy. These cells also showed expression of MSC-specific markers, CD29 (98.94% ± 0.67%), CD44 (84.27% ± 7.77%), Sca-1 (92.70% ± 3.81%) with negligible expression of HSC-specific markers such as CD45 (0.40% ± 0.10%), CD34 (0.15% ± 0.05%) and CD11b (0.45% ± 0.15%). MSCs were also found to differentiate into mesodermal lineages such as adipocytes, osteocytes and chondrocytes as well as ectodermal neuron-like cells. Moreover, MSCs showed differential basal expression of pluripotency-associated transcription factors such as Oct4, Nanog, Sox2 and Myc. Based on the above findings, we propose a simple protocol that can be used to isolate, culture, propagate and characterize multipotent MSCs from mouse bone marrow for experimental and application purposes.

Keywords: Bone marrow; Mesenchymal stem cells; Propagation; Differentiation; Pluripotency

Introduction

Mouse bone marrow-derived mesenchymal stem cells (BM-MSCs) are multipotent, heterogeneous cell populations, isolated from various organs and tissues, including bone marrow [1,2]. MSC comprises of 0.01% to 0.1% of total adult bone marrow mononuclear cells, and its culture was first established by its intrinsic surface adherence propensity [3]. MSCs are of immense interest as they can be easily isolated and cultured, with exception of c57bl6, and propagated in high number even from a very small amount of sample for experimental and therapeutic purposes. During culture, they generate colonies of various shapes and sizes, containing morphologically three distinct cell types: spindle-shaped, flattened, and extremely small rapidly proliferating cells (RS cells). Single cell-derived colonies have been expanded till 50 population doublings in a period of around 10 days [4]. Similarly, alkaline phosphatase staining shows three distinct types of colonies with some colonies appearing highly positive, some showing positive in centre and negative in periphery and others are completely negative [5]. Along with MSCs, several other types of cells, including osteoblasts, adipocytes, endothelial cells, vascular pericytes, stromal cells constitute the bone marrow microenvironment and thereby precisely regulate self-renewal and differentiation of haematopoietic stem cells (HSCs), another major population of bone marrow resident stem cells [6]. HSCs are predominantly quiescent and express distinctive markers, such as c-kit (Stem Cell Growth Factor Receptor/CD117) and Sca-1 (Stem Cell Antigen-1/Ly6a) [7]. Phenotypically, MSCs from mouse and human bone marrow express a number of markers, e.g. CD29 (integrin β1), CD44 (Ly-24), CD73 (SRC homology 3 domain; SH3/4), CD90 (Thy-1), CD71 (transferrin receptor), CD166 (Activated Leukocyte Cell Adhesion Molecule; ALCAM), CD105 (SH2), Stro-1 along with certain cell adhesion molecules, including CD106 (VCAM-1), CD146 (MCAM). However, these MSCs generally do not express hematopoietic markers: CD45 (LCA, Ly-5), CD34, CD14, CD11; the co-stimulatory molecule: CD80 (B7-1), CD86 (B7-2), CD40 and the cell adhesion molecules: CD31 (PECAM-1), CD56 (NCAM-1), MHC-II [2].

Adult mesenchymal stem cells tend to differentiate and generate cells of mesodermal origin like osteocytes, adipocytes and chondrocytes [8], as well as ectodermal and endodermal lineages both *in vitro* and *in vivo* [9]. The differentiation of MSCs into various lineages is precisely regulated at multiple sequential steps, and represented by distinctive morphological and molecular characteristics. For instance, during initial stage of adipocytic differention, MSCs starts showing accumulation of several small cytoplasmic lipid droplets, which grow with the passage of time and occupy almost entire cytoplasmic space. Similarly, during *in vitro* osteogenic and chondrogenic differentiation, remarkable morphological changes, including retraction of elongated cellular processes, appearance of polygonal shape, well-developed endoplasmic reticulum and Golgi complex are observed by the day 7, which become most pronounced by the end of two weeks. In addition, several extracellular matrix proteins, including collagen and chondroitin

sulphate-proteoglycan are secreted in the extracellular spaces, cementing differentiating cells together [10].

Besides, bone marrow-derived mesenchymal stem cells have also been differentiated and studied, with the purpose of checking their multipotency as well as for being used as *in vitro* model for studying neurodegenerative disease [11]. The *in vitro* neuronal differentiation of bone marrow-derived MSCs is accomplished using various combinations of neurogenic inducers like retinoic acid, b-FGF and, β-ME [12]. Retinoic acid (RA), a vitamin A-derivative, facilitates neuronal differentiation of MSCs under appropriate cellular conditions [13]. In addition, RA plays very important role in neuronal development including anterior-posterior patterning in the nerve cord, formation of tubus medullaris, and cortical and motor neuronal regeneration [14]. RA-induced and/or pre-treated MSC-derived neurons have shown tangible benefits on neuronal system. For instance, pre-treatment of MSCs with RA induced better neuronal differentiation following transplantation in spinal cord injury (SCI) animal model [15]. Therefore, *in vitro* studies on MSC-derived neuronal cells may have potential for clinical applications of cell-based therapy.

Materials and Methods

Animals

Inbred male C57BL/6 (H2b) mice, (4-6 weeks old) were used for all experiments. Animals were bred and maintained in the animal house facility (25°C, 50% relative humidity) of Jawaharlal Nehru University. All experiments were performed as per the institutional guidelines on animal care and ethics set by Jawaharlal Nehru University.

Reagents, media, serum, culture plate and flask, antibodies, kit

DMEM (Dulbecco's Modified Eagle Medium)-High Glucose (DMEM-HG 4500 mg/500 ml) (cat. no. D 5796), DMEM-low glucose (DMEM-LG 1000 mg/500 ml) (cat. no. D 6046), fetal bovine serum heat-inactivated (FBS; cat. no. F4135), Penicillin/Streptomycin/Amphotericin B solution (cat. no. A5955), 0.25% Trypsin/EDTA solution (cat. no. T4049), Dexamethasone (Dex.; cat. no. D2915), Insulin solution (10 mg/ml; cat. no. I 9278), Isobutyl methyl xanthine (IBMX; cat. no. I 7018), Linoleic Acid (cat. no. L5900), Indomethacin (cat. no. I7378), Sodium Phosphate dibasic (Na2HPO4, cat. no. S7907), Sodium Phosphate monobasic (NaH_2PO_4; cat. no. S8282), Sodium Selenite (cat. no. S5261), Transferrin (cat. no. T8158), Albumin Bovine Serum (cat. no. A9647), L-Ascrbic acid-2-phosphate sesquimagnesium salt (cat. no. A8960), Weigert's Iron Hematoxylin solution (cat. no. HT1079), Oil red-O (cat. no. O0625), Safranin O (cat. no. 84120), Trypanblue (cat. no. T8154), Alkaline Phosphatase detection kit (Kit 86 R), β-Mercaptoethanol (cat. no. M7522), Retinoic acid (cat. no. R2625), TRI Reagent (cat. no. T9424), DEPC (cat. no. D5758), Poly-L-lysine hydrobromide (cat. no. P2636), EtBr (cat. no. E8751), primary Oct3/4 antibody (cat. no. O8389) and agarose (cat. no. A9539) were purchased from Sigma-Aldrich (USA). TGF-β3 (Transforming Growth Factor-β3; cat. no. 100-36) was from Peprotech. Serological pipettes, 5 ml and 10 ml, sterile and individually wrapped (cat. no. 4051 and 4101, respectively), 15 ml and 50 ml polypropylene centrifuge tubes, sterile (cat. no. 430052 and 430291, respectively), 25 cm^2 cell culture flask, polystyrene (cat. no. 430639), six-well cell culture plate (cat. no. 3506), 0.20 µm low protein binding sterilization syringe filter, Poly Ether Sulfonate (cat. no. 431229) were from Corning, USA. Cell strainer, 70 µm, nylon (cat. no. 352350), and FITC-labelled: anti-CD44 (cat. no. 553133), anti-CD45 (cat. no. 553079), Rat IgG2b k Isotype control (cat. no. 553988), Sheath fluid (cat. no. 342003) were from BD Bioscience, and anti-Sca-1 (cat. no. 11-5981), ant-CD34 (cat. no. 11-0341), anti-CD11b (cat. no.11-0112), Rat IgG2a k Isotype control (cat. no. 11-4321), and PE-labelled anti-CD29 (cat. no. 12-0291), Armenian Hamster IgG isotype control (cat. no. 12-4888) from eBioscience. The goat anti rabbit IgG-TRITC labelled (cat no. RTC2) was from Genei. Biochemical and molecular biology reagents, such as ethanol, formaldehyde solution (37% to 41% w/v), xylene, isopropanol, methanol, glacial acetic acid, Giemsa, Crystal Violet and paraffin wax were from Merck (Germany). The enzymes and markers were MMLV-RT (Promega; cat. no. M-1701), Taq DNA polymerase (NEB; cat. no. M0273L), dNTPs (Promega; cat. no. U1330), DNA markers 1.0 kb (MBI Fermentas; cat. no. SM1163), 100 bp (MBI Fermentas; cat. no. SM1143).

Isolation, culture, and propagation of primary mesenchymal stem cells

To isolate primary mesenchymal stem cells, marrow cells were collected from bone marrow of femurs, tibiae and humeri of 4-6 weeks old male mice. In brief, mice were sacrificed by cervical dislocation; the external body surface was sterilized by wiping with 80% (vol/vol) ethanol-soaked cotton swab. The femurs, tibiae and humeri were dissected out, and cleaned off all associated connective tissue. The epiphysis of each bone was cut open with sterile scissors, and then bone marrow cells were harvested by flushing with pre-warm solution containing 2% heat-inactivated fetal bovine serum, 1 × antibiotic/antimycotic solution in 1 × PBS, and then collected in a 15 ml polypropylene tube. Cells were dislodged, filtered through the cell strainer to remove any cell clumps, muscles, and bone debris, and centrifuged at 268 × g for 5 min at room temperature. The cell pellet was washed and resuspended in DMEM supplemented with 15% FBS and 1 × antibiotic/antimycotic solution. Cells were counted on haemocytometer at 100 × magnification after staining with 0.4% trypan blue solution to assess cell number and viability. In T-25 cm^2 canted cell culture flasks, 25 × 10^6 cells were seeded at the cell density of 1 × 10^6 cells/cm^2 area, and then placed at 37°C in 5% CO_2-humidified incubator. Non-adherent, dead, and floating cells were removed after 48 hrs and subsequently at every three to four days till the adherent cells reached 70% to 80% confluency. Thereafter, adherent cells were washed twice with pre-warmed (37°C) 1 × PBS, and trypsinized with pre-warmed 1.0 ml to 2.0 ml of 0.25% trypsin/1 mM EDTA at 37°C for 2-3 min. Trypsinzation was inhibited by adding 2 ml to 3 ml complete medium, cells were collected and centrifuged in a 15 ml polypropylene tube at 268×g for 5 min. Cell pellet was resuspended in complete medium and was split into 1:2 ratio at first passage (P0) and 1:3 ratio at second (P1) and subsequent passages (P2 and P3). All the experiments were carried out with MSCs P3 until and unless specified otherwise.

Study of morphological features of bone marrow cells and mesenchymal stem cells

Bone marrow cells were flushed with 1 × PBS and vortexed gently to dislodge the cells. This single cell suspension was attached on poly-D-lysine coated coverslip, and then fixed in 2.5% glutaraldehyde solution in 0.1 M sodium phosphate buffer (pH 7.2) overnight at 4°C. On the next day morning, cells were washed with cold PBS, and then

dehydrated in a series of increasing ethanol gradients (30%, 50%, 70%, 90% and 100%), 5-10 min in each. On the other hand, MSCs P3 were trypsinized, harvested and further grown on sterile cover slips in 35 mm tissue culture dishes for 10-12 days in complete DMEM (15% FBS and 1 × antibiotic/antimycotic solution). The medium was changed after every 3 to 4 days. For scanning electron microscopy (SEM) analysis, cells were washed twice with 1 × PBS following removal of the medium and immediately fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) overnight at 4°C. Thereafter, fixed cells were washed with 1 × PBS, and then serially dehydrated in up-grade ethanol (30%, 50%, 70%, 90% and 100%) for 15 to 20 min each. BMCs and MSCs P3 were briefly coated with gold in the sputtering machine and eventually observed with the scanning electron microscope (Zeiss EVO40).

Gene expression analysis for various markers and pluripotency-associated factors

Total RNA was isolated from bone marrow cells and MSCs P3 using TRI-Reagent. Complementary (c) DNA was synthesized from 1.0 µg of total RNA in a 25 µl reaction mixture containing 0.5 ng of oligo (dT) primer, 0.5 mM dNTPs, 20 U RNasin, 100 U of M-MLV reverse transcriptase and 5 µl of 5 × M-MLV RT reaction buffer. The primers and the annealing temperatures were used as follows: CD29 (68°C), 5'CCCAGCCAGTCCCAAGTGCC3' (forward) and 5'CACACGCCATTGCCTCCAC3' (reverse); CD73 (61°C), 5'TGCCTCAACGCCAGCCTGTG3' (forward) and 5'TCCCCACAACCTCACCGCCA3' (reverse); CD45 (50°C), 5'CTCCCAAGGAGTATGAGTCCAT3' (forward) and 5'GGCCAATACTGATCACACTCCA3' (reverse); Rex-1 (61°C), 5'CACCATCCGGGATGAAAGTGAGAT3' (forward) and 5'ACCAGAAAATGTCGCTTTAGTTTC3' (reverse); Nestin, (63°C) 5'GAGAAGACAGTGAGGCAGATGAGTTA3' (forward) and 5'GCCTCTGTTCTCCAGCTTGCT3' (reverse); Oct 4 (76°C), 5'TCTTTCCACCAGGCCCCCGGCTC3' (forward) and 5'TGCGGGCGGACATGGGGAGATCC3' (reverse); Sox-2 (62°C), 5'AGCTAGACTCCGGGCGATGA3' (forward) and 5'TTGCCTTAAACAAGACCACGAAA3'(reverse); Nanog (57°C) 5'CAGGTGTTTGAGGGTAGCTC3' (forward) and 5'CGGTTCATCATGGTACAGTC3' (reverse); Myc (83°C) 5'TGACCTAACTCGAGGAGGAGCTGGAATC3' (forward) and 5'AAGTTTGAGGCAGTTAAAATTATGGCTGAAGC3'(reverse). To check expression of each gene, 2.5 µl of cDNA was used with their respective primers in 25 µl total reaction mixture. The amplicons were resolved on 1.5% agarose gel and the images were acquired using a gel-documentation system (Alpha Imager).

Immunofluorescence of Oct 4

Mouse bone marrow cells (BMCs) were isolated from the 5-6 weeks old C57Bl6/J mouse, counted and resuspended in serum-free Dulbecco's Modified Eagle Medium-High glucose (DMEM-HG) media at density 2×10^7 cells/ml. Thereafter, 10 µl of cell suspension was put in the form of a drop right in the centre of 4 cm^2 microscopic coverslip, already coated with 0.1 mg/ml poly-L-lysine solution prepared in double-distilled-autoclaved-0.2 µm filtered water. The coverslip was transferred inside a moist chamber for 30 min to allow the cells to properly stick on the coated-surface. On the other hand, MSCs P3 grown in 15% fetal bovine serum (FBS) and 1 × antibiotic/antimycotic in DMEM-HG medium on 4 cm^2 sterile coverslip in 6 well plates were washed twice with cold PBS, fixed with 4% buffered paraformaldehyde for 20-30 min at RT. Paraformaldehyde solution was drained off and cells were washed first with 3 × PBS, and then with 1 × PBS, twice in each, and then permeabilized with 0.1% triton X-100 in 1 × PBS [16]. To minimize non-specific bindings, blocking was done with 1%-2% BSA in 1 × PBS for 30 min at room temperature. The cells were washed twice with PBS and incubated with primary Oct3/4 (Sigma) antibodies at 1:200 dilutions at 40C overnight. Following overnight incubation, cells were washed thrice with 0.1% Tween-20 (PBST) in 1 × PBS, 10 min each at RT. The secondary antibody, TRITC-labelled goat anti-rabbit IgG was added at 1:400 dilutions, and incubated for 30 min in dark at room temperature. The cells were washed thrice with PBST and eventually mounted in 0.5 µg DAPI containing Vectasheild mounting medium (Vector Labs H-1200). Fluorecence-based signal was analysed by laser confocal microscope with Fluorescence Correction Spectroscopy (FCS) Olympus Fluo View^{TM} FV1000.

Immunophenotyping

Bone marrow cells were isolated from 4-6 weeks old mouse, counted, and then aliquoted in staining tubes at around 1×10^6 cells/ml. Eventually, cells in each tube were centrifuged, washed and finally suspended in 50 µl staining buffer (2% FBS in 1 × PBS). Similarly, MSCs P3 were washed with 1 × PBS, harvested following 0.25% trypsin/EDTA treatment and resuspended in 1 × PBS containing 2% FBS. Cells were counted by a haemocytometer under light microscope, and incubated with PE-conjugated anti-mouse CD29, FITC-conjugated anti-mouse CD44, Sca-1, CD34, CD44 and CD11b along with the isotype control FITC-conjugated Rat IgG2a K, FITC-conjugated Rat IgG2b k, and PE-conjugated Armenian Hamster IgG for 30 min on ice in dark, washed twice with 2% FBS in 1 × PBS, resuspended and analyzed using FACS calibur (Beckton Dickinson, USA).

Mesodermal tri-lineage differentiation

A. Adipocyte differentiation: To test the differentiation potential of MSCs P3 into adipocytes, the cells were washed, trypsinized, centrifuged at 268×g, resuspended in complete medium and plated at around 3000 cells/cm2 in 6-well tissue culture plate. After 36 hours of incubation, the medium was replaced with differentiation/induction medium prepared in DMEM-LG, supplemented with 2% FBS, 1 × antibiotic/antimycotic solution, 5 µg/ml insulin, 1 mM dexamethasone, 50 µM indomethacin and 500 nM isobutyl methyl xanthine. The medium was changed twice a week for three consecutive weeks. The formation of adipocytes was evaluated by fixing the cells in neutral-buffered formalin (NBF) and staining by oil-red O dye (HiMedia) for 20 min at room temperature. The photographs were taken by a bright field microscope attached with a camera.

B. Osteocyte differentiation: For osteocyte differentiation, MSCs P3 were plated at 3000 cells/cm^2 in six-well plate in complete medium and incubated for 36 h. The complete medium was replaced with differentiation medium at 3-4 days interval for around 3 weeks. Osteocyte differentiation medium comprised of DMEM-LG, supplemented with 2% FBS, 1 × antibiotic/antimycotic solution, 50 µM L-ascorbic acid-2-phosphate sesquimagnesium salt, 10 nM dexamethasone and 10 mM β-glycerol phosphate. After 21 days in induction medium, cells were washed with 1 × PBS, fixed with citrate-acetone-formaldehyde fixative and stained as per the instructions provided in the sigma kit 86 R. In short, after washing and fixation, the mixture of diazonium salt solution (sodium nitrate solution and FRV-alkaline solution) and Naphthal AS-BI alkaline solution was added in

each well, and incubated for 15-20 min in complete dark. The cells were rinsed with tap water and counter-stained with haematoxylin for 1-2 min, and eventually cover slip-mounted in 50% glycerol in PBS. Photographs of different regions of each well were taken under a bright field microscope.

C. Chondrocyte differentiation: To induce differentiation of mesenchymal stem cells into chondrocyte lineage, 3×10^5 MSCs P3 were pelleted down at 603 × g, as a three dimensional cell aggregate or deposit in a 15 ml polypropylene tube, and incubated in a 1.5 ml of chondrogenic differentiation medium in cell culture incubator. The medium was replaced at every three days for 24 days. Chondrogenic induction medium consisted of DMEM-HG, 1 × antibiotic/antimycotic solution, 6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 µg/ml sodium selenite, 5.33 µg/ml linoleic acid, 1.25 mg/ml bovine serum albumin, 100 nM dexamethasone, 50 µM ascorbic acid and 10 ng/ml transforming growth factor β3 (TGF-β3). Following completion of chondrogenic induction, the resulting cell pellet was fixed with neutral buffered formalin for 24 h. Thereafter, pellets were dehydrated, embedded in paraffin and cut into 5 µm thick sections on the microtome. Sections were deparaffinised, hydrated, and stained in safranin-O, and thereafter, counterstained with haematoxylin, and eventually mounted in aqueous glycerol gelly. Photographs of different regions of the section were taken under a bright field microscope.

Neuro-ectodermal differentiation

The potential of MSCs to differentiate into ectodermal lineage was tested by differentiating them into the neuron-like cells. This was carried out by following two different neuronal differentiation protocols with some modifications [17]. MSCs P3 were trypsinized, harvested and seeded in six well plate on glass cover slips in the wells at a density of 0.1×10^6 cells per well and grown in complete medium (DMEM-HG+15% FBS and 1 × antibiotic/antimycotic solution) for 48 h. Thereafter, the cells were given two different treatments as follows; treatment 1:100 µM β-mercaptoethanol (β-ME)+1 µM Retinoic Acid (RA) in the medium (DMEM-HG+10% FBS and 1 × antibiotic/antimycotic solution), and treatment 2: 30 µM RA alone in the medium. Cells were further incubated with induction media for 10-12 days with the change of medium at an interval of 3-4 days. Following completion of neuronal induction, the resultant neuron-like cells were washed with 1 × PBS, fixed in 95% alcohol and stained with 10% Giemsa prepared in PBS (pH 7.2). Morphology of the untreated control cells and β-ME+RA as well as RA-treated cells were recorded with bright field microscope and scanning electron microsope.

Statistical analysis

The student's t test was used to determine significant difference between the groups. The difference was considered statistically significant at $P<0.05$ or less, and data were illustrated as mean ± SEM from three independent experiments.

Results

Isolation, culture, and propagation of mesenchymal stem cells

Bone marrow cells, seeded at the density of 1×10^6 cells/cm^2 area in T-25 tissue culture flasks, settled down within 10 minutes of seeding and appeared round-shaped with various sizes (Figure 1A). Thereafter, cells were incubated at 37°C in 5% CO_2+95% air and humidified cell culture incubator for 48 hours. During incubation, 10% to 20% cells adhered on the surface, of which nearly 4% to 5% cells showed elongated and oval-shaped morphologies, while others remained round-shaped (Figures 1B and 1C). The non-adherent and floating cells were collected and assayed for viability by trypan blue staining. Around 70% cells were trypan-blue positive (dead cells), while 30% remained unstained, indicating live cells among the non-adherent and floating cell population (Figure 1M). The adherent cells were further cultured and propagated by replenishing the medium (4 ml fresh medium+1 ml recently used conditioned medium), 5 ml each time at the interval of 3-4 days till the cells became sub-confluent (70% to 80%), and the cell density and morphology were recorded by light microscopy (Figures 1D and 1E). This sub-confluent population of cells was called as passage 0 (P0) cells. Till this stage, most of the cells acquired spindle-shaped morphology except only few cells, which were still oval or round in shape (Figure 1F). The P0 cells were harvested by trypsinization for 2-3 minutes at 37°C, and reseeded in 1:3 ratio and subsequently cultured to obtain passage 1 (Figure 1G), passage 2 (Figure 1H) and passage 3 (Figure 1I) cells. Majority of cell population showed spindle-shaped morphology with distinct nucleus and well-defined cell boundary observed under light microscope following various staining (Figures 1J-1L).

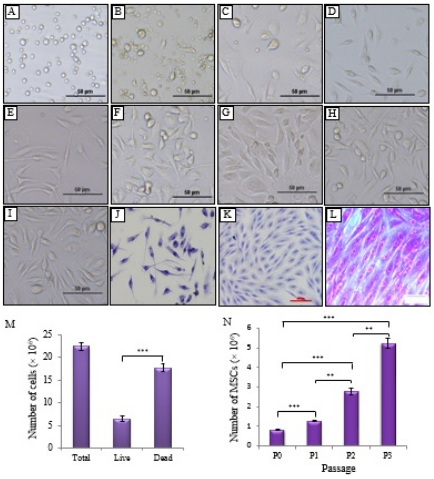

Figure 1: Culture and propagation of mouse bone marrow-derived mesenchymal stem cells (MSCs). (A): Morphology of bone marrow cells (BMCs) under bright field microscope on day 0 (10 min post-seeding). (B): Passage 0, 48 h after initial cell seeding (P0). (C): Passage 0 (P0) cells, 48 h after first time change of medium. (D): Passage 0 (P0) cells after 72 h. (E): Passage 0 (P0) cells after day 5. (F): Passage 0 (P0) cells on day 12. (G): Passage 1 (P1) cells on day 11. (H): Passage 2 (P2) cells. (I): Passage 3 (P3) cells. (J, K and L): P3 cells stained with Hematoxylin & Eosin, Giemsa and Crystal Violet, respectively. (M): Viability assay of floating cells after 48 h culture. (N): Cell-yield of cultured bone marrow-derived mesenchymal stem cells through P0-P3. Scale bars=50 µm (A-J), 200 µm (K), 100 µm (L). Results shown are mean ± SEM of three independent experiments. *, $P \leq 0.05$, **, $P \leq 0.01$ and ***, $P \leq 0.001$.

Morphological features of bone marrow cells and mesenchymal stem cells

The scanning electron microscopy of intact bone marrow showed entangled mass of various fibrous tissues impregnated with RBCs, mononuclear cells and few very small cells. These small cells could be the homogenous population of rare pluripotent stem cells; referred as very small embryonic-like (VSELs) cells (Figure 2A) among the total bone marrow cell population. The single cell suspension of bone marrow cells showed somewhat round morphology but varied in size, ranging from 2 µm to 3 µm in diameter. RBCs, with distinctive concave appearance, were also present along with mononuclear bone marrow cells (Figures 2B and 2C). The bone marrow mononuclear cells showed numerous distinct microvilli-like structures all over the cell surface known to harbour several functional receptors and immunoglobulins (Figure 2D) as previously reported in freshly isolated bone marrow cells. On the other hand, the surface of RBCs appeared smooth owing to lack of numerous microvilli. Similarly, MSCs P3 ranged from 50 µm to 70 µm in length, showed different shapes and sizes, including spindle-shape, flattened, oval/round shape and even few cells appeared as star-shaped, and this morphological heterogeneity was maintained through various passages to differing extent (Figure 2E). However, majority of MSCs P3 population showed typical spindle-shape morphology (Figure 2F), which was found consistent with the ISCT (International Society for Cellular Therapy)-2006 recommendations.

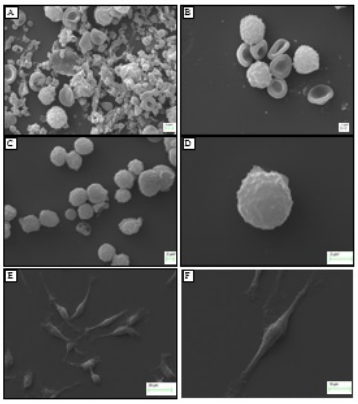

Figure 2: Morphology and heterogeneity of bone marrow cells (BMCs) and MSCs (P3) by scanning electron microscopy (SEM). (A): Intact bone marrow containing bone marrow cells, RBCs and fibrous tissue (×7410 magnification). (B): BMCs along with red blood cells (×8560 magnification). (C): BMCs showing round-shape morphology with size ranging from 1 µm to 3 µm (×5540 magnification). (D): BMCs showing microvilli on the cell surface (×16550 magnification). (E): MSCs (P3) showing three distinct cell populations in the culture: spindle-shaped, cuboidal or flattened and extremely small rapidly proliferating cells (RS cells) (×1960 magnification). (F): SEM of MSCs (P3) showing a typical spindle-shape morphology with oval-shaped nucleus in the middle (×3760 magnification). Scale bars=2 µm (A, C, D), 1 µm (B), 20 µm (E), 10 µm (F).

Immunophenotypic characterization of bone marrow cells and mesenchymal stem cells

Bone marrow cells (BMCs), the initial source for primary mesenchymal stem cells, as well as P3 mesenchymal stem cells (MSCs P3) were immunophenotyped for widely used MSC-markers, such as CD29, CD44 and Sca-1, as well as HSC-markers, CD34, CD45 and CD11b. Both BMCs and MSCs showed differential expression patterns with respect to the above markers. The BMC showed positive expression for all the markers as follows: CD29 (82.86% ± 6.29%), CD44 (78.43% ± 3.06%), Sca-1 (27.55% ± 4.50%), CD45 (70.44% ± 2.39%), CD11b (30.18% ± 1.71%) and CD34 (26.36% ± 4.41%), while MSCs P3 were positive for CD29 (99.43% ± 0.671%), CD44 (84.96% ± 7.778%), Sca-1 (91.70% ± 3.811%) and negative for CD34 (0.16% ± 0.052%), CD45 (0.54% ± 0.103%) and CD11b (0.27% ± 0.153%), respectively (Figures 3A-3C).

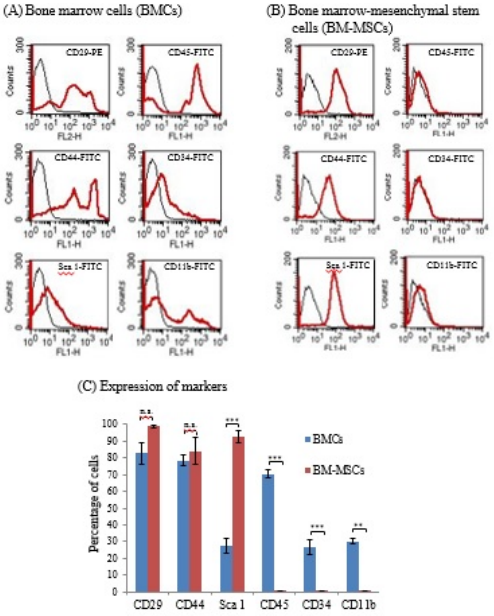

Figure 3: Immunophenotypic characterization of mouse bone marrow cells and marrow-derived mesenchymal stem cells (A): Immunophenotyping of freshly isolated mouse bone marrow cells with respect to the markers CD29, CD44, Sca-1, CD45, CD34 and CD11b. (B): MSCs P3 were harvested following trypsin-digestion and stained with phycoerythrin (PE)-conjugated anti-mouse CD29 and fluorescein isothiocyanate (FITC)-conjugated ant-mouse CD44, Sca-1, CD34, CD45 and CD11b antibodies. Expression of each marker is shown along with their respective isotype control. (C): Quantitation of cell surface markers on BMCs and MSCs P3. Results are shown as mean ± SEM from three independent experiments. *, $P \leq 0.05$ **, $P \leq 0.01$ and ***, $P \leq 0.001$.

Mesodermal tri-lineage differentiation of mesenchymal stem cells

To examine whether the MSCs P3 are capable of differentiating into mesodermal lineages, they were cultured under adipogenic, osteogenic and chondrogenic conditions, as described in the method section, and compared with cells grown under control condition. After staining

with lipophilic oil red O, the control did not show any morphological and cytoplasmic changes and were negative for oil red O staining (Figure 4A), while distinct cytoplasmic lipid droplets were observed in MSCs grown in adipogenic medium (Figures 4B and 4C). Upon quantitation, around 80% cells were found to be differentiated into adipocytes (Figure 4D). Similarly, MSCs P3 were cultured in osteocyte-induction medium for 16-18 days and checked for expression/activity of alkaline phosphatase, a marker for osteocytes. The control cells neither changed in morphology nor showed alkaline phosphatase activity (Figure 4E), while differentiated cells retracted their cellular processes, became oval and rounded with the passage of time and stained highly positive for alkaline phosphatase activity (Figure 4F). Besides, there were several concentric layers of extracellular depositions around the MSC-differentiated osteocytes (Figure 4G). Around 80% cells were found to be alkaline phosphatase positive (Figure 4H).

To check the chondrocyte differentiation potential, MSCs-pellets were separately incubated in chondrocyte-induction medium in CO_2 incubator at 37°C. During incubation in chondrogenic induction medium the pellet grew in size and density and by three weeks, the pellet became considerably compact and bigger in size compared to the control pellet. For chondrogenic evaluations, these pellets (control and differentiation sets) were washed, fixed, paraffin-embedded and cut into 5 µm to 7 µm thin sections. These sections were stained with safranin O, an extracellular matrix staining dye. The control pellet-derived section showed less compaction and also lighter staining (Figure 4I). Conversely, the chondrogenic induction pellet-derived sections were very compact and stained darkly by safranin O staining (Figure 4J). Furthermore, we also observed compact and darkly stained nodule of chondrocytes representing the cartilage-like tissue (Figure 4K).

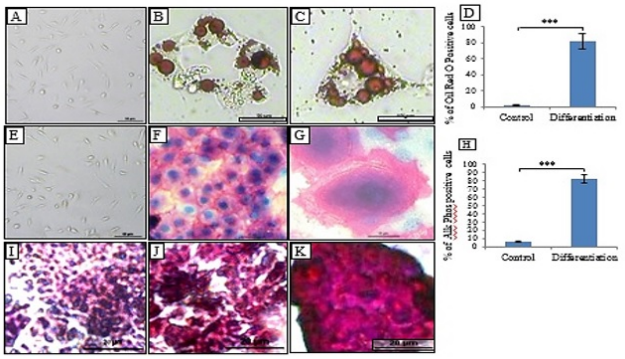

Figure 4: Mesodermal differentiation of MSCs (P3) in culture. (A): Control (normal medium). (B): Oil red O staining of adipocytes differentiated from MSCs in adipocytes medium. (C): A single adipocyte showing arrangement of lipid droplets. (D): Quantitation of adipocytes differentiated from MSCs. (E): Control. (F): Alkaline phosphatase activity staining along with Hematoxylin counter staining of osteocytes differentiated from MSCs. (G): A single osteocyte showing expression of alkaline phosphatase activity, the nucleus is stained as dark blue by Haematoxylin. (H): Quantitation of osteocytes differentiated from MSCs. (I): Control. (J): Safranin O +Haematoxylin-stained chondrocytes. (K): Chondrocytic nodule as a mass of tissue. Data are presented as mean ± SEM. *, $P \leq 0.05$, **, $P \leq 0.01$ and ***, $P \leq 0.001$. (D&H) (n=3). Scale bars=50 µm (A, B, C, D), 20 µm (E), 10 µm (F), 20 µm (G, H, I).

Simultaneous and various stages of osteogenic differentiation

During osteogenic differentiation, both morphological and cellular heterogeneities are retained and maintained, which could be observed along with and even till the end of the differentiation process. Following haematoxylin and alkaline phosphatase double-staining, MSCs in the control well stained only by haematoxylin (Figure 5A), while the differentiated cells (osteocytes) showed strong staining for both haematoxylin and alkaline phosphatase (Figure 5B). We observed some concomitant heterogeneity at the level of cellular morphology, differential intercellular and intracellular alkaline phosphatase activities. Following induction by osteocyte differentiation medium, MSCs acquired round, oval and star-shaped morphologies, and the nucleus displayed numerous punctuated distribution of chromosomal DNA (chromatin) (Figure 5B). Cellular heterogeneity was also clearly observed in terms of the alkaline phosphatase activities among the differentiated cells due to differential colour intensity (Figure 5C). Furthermore, these alkaline phosphatase positive cells showed various morphologies, including star-shaped, rounded and oval, and some globular shape, suggesting the morphological heterogeneity among the differentiated osteocytes (Figures 5C and 5D). Cells at late stages of osteogenic differentiation showed completely oval/round shape and morphology, became non-adherent, and started secreting substantial amount of extracellular matrix (Figure 5E). Extracellular matrix deposition with distinct pattern was also observed prominently (Figure 5F).

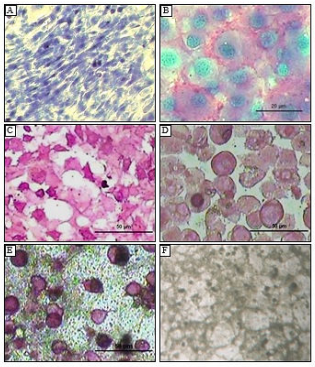

Figure 5: Heterogeneity and different stages of osteocytic differentiation of MSCs (P3). (A): Control cells showing darkly-stained nuclei, but no alkaline phosphate activity. (B): Alkaline Phosphatase activity of osteocytes counter-stained with Hematoxylin showing condensed, punctated chromosomes in the nucleus. (C): MSC-derived osteocytes showing star-shape morphology with differential alkaline phosphatase activity. (D): Osteocytes showing oval/round-shaped morphology and alkaline phosphatase activity. (E): Few osteocytes are still attached with extracellular matrix secreted by them after differentiation. (F): Extracellular matrix is deposited in polygonal and hexagonal patterns. Scale bars=50 µm.

Expression of marker genes and pluripotency-associated factors

Differential expression of the surface markers, such as CD29, CD73, Rex-1 and nestin, neuronal cell marker at RNA level was observed both in BMCs and MSCs P3 by RT-PCR. MSCs P3 showed differential and subtle enrichment for CD29, CD73, Rex-1 and nestin compared to the bone marrow cells (Figures 6A and 6B). Besides, we also checked and found the basal expression of pluripotency-associated markers/transcription factors, such as Oct4, Nanog, Sox2 and Myc. The level of basal mRNA expression of Sox_2, Nanog and Myc was found to be significantly higher in MSCs P3 compared to bone marrow cells, while surprisingly, no significant difference was observed with respect to the Oct4 expression (Figures 6C and 6D). Furthermore, the expression of Oct4 protein was observed by immunofluorescence in both BMCs and MSCs, in a circular form in the BMCs (Figure 6E), but mostly in the cytoplasm with higher concentrations at terminal ends of the spindle-shaped cells (Figure 6F) in the MSCs.

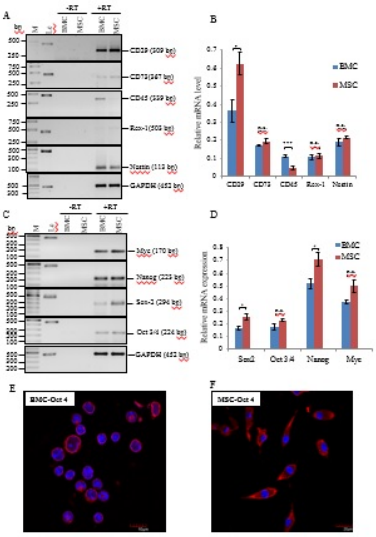

Figure 6: Expression of surface markers and pluripotency-associated transcription factors. (A, B): Reverse transcription polymerase chain reaction (RT-PCR) analysis of bone marrow cells and MSCs (P3) showing enrichment of MSC-positive markers like CD29, CD73, Rex-1 and Nestin, and lower expression of HSC marker, CD45 compared to the bone marrow cells. (C, D): The expression of pluripotency-associated transcription factors, such as Oct3/4, Sox^{-2}, Nanog, and Myc was found to be significantly upregulated in MSCs as compared to the bone marrow cells. (E, F): Cellular localization of Oct4 protein (red) and DNA (blue) by merged immunofluorescence in BMCs and MSCs P3, respectively. Results shown are mean ± SEM of three independent experiments. *, $P \leq 0.05$, **, $P \leq 0.01$, ***, $P \leq 0.001$, $P \geq 0.05$ and n. s., not significant. bp, base pair; M, 100 bp and 1.0 Kb DNA ladder; Lc, loading control (452 bp); RT, reverse transcriptase.

Ectodermal (neuron-like) differentiation of mesenchymal stem cells

MSCs P3, cultured for 12 days at the cellular density of 0.1×10^6 cells per well/2 ml complete medium containing the two inducers, showed different types of neuron-like morphologies. Control cells, cultured in DMEM-HG-15% FBS showed typical spindle-shaped morphology (Figures 7A and 7B), while cells incubated in the medium containing both 1.0 µM β-mercaptoethanol and 1.0 µM retinoic acid (RA), showed less cellular elongation, higher interneuronal connectivity and less number of bipolar neurons with multiple projections of neurites per cell. The neurite projections radiated outwardly and morphologically resembled like oligodendrocytes (Figures 7C and 7D). On the other hand, cells incubated in medium containing 30 µM RA alone, showed considerable lengthwise cellular elongation with less neurite projections, few inter-neuronal connections and higher number of bipolar neuronal morphology (Figures 7E and 7F). Following SEM imaging, MSC-derived neuron-like cells (Figure 8A) appeared to possess several branched dendrite-like cellular projections on one end of the cells (dendritic end) (Figure 8B), and clustered globular vesicles at the axonal end (Figure 8C).

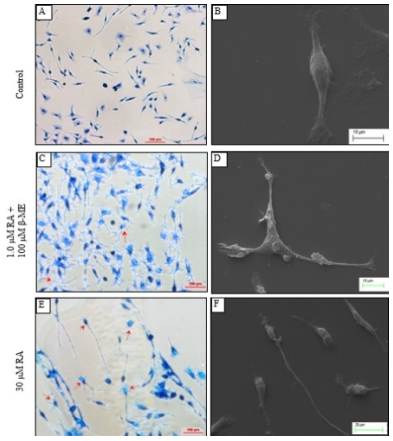

Figure 7: Neuro-ectodermal differentiation of MSCs (P3). Cells were cultured on coverslips in 6-well plates. Two types of treatments were used to obtain MSCs-derived neuron-like cells. (A): MSCs grown on coverslip for 10-12 days in normal medium (control cells) following 0.5% Giemsa-staining. (B): SEM image of control cells. (C): MSCs cultured in presence of 1.0 µM Retinoic acid (RA)+100 µM β-mercaptoethanol (β-ME) for 10-12 days showing morphological features typical of neurons, such as spherical cell body with extending processes (neuritis). (D): SEM of MSC-derived neuronal cells after treatment (RA+β-ME). (E): MSCs following 30 µM RA-treatment for 10-12 days showing different types of neuronal morphology and, (F): SEM of 30 µM RA-treated cells. Scale bars=100 µm (A, C, E), 10 µm (B, D), 20 µm (F).

Discussion

Mammalian bone marrow harbours a myriad of stem cells with distinctive morphological and functional features such as hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), multipotent adult progenitor cells (MAPCs) and very small embryonic-like stem cells (VSELs) [18,19]. Though MSCs have been isolated, propagated and characterized from virtually all tissues and organs, but bone marrow still remains the most common and consistently reliable source for *in vitro* studies.

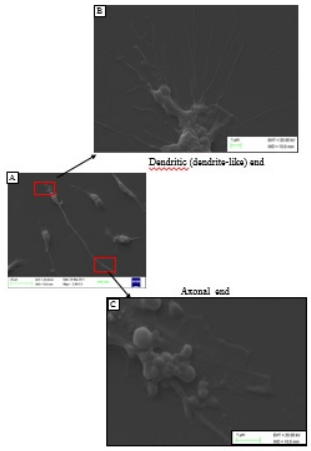

Figure 8: Neuronal differentiation of MSCs. SEM of axonic and dendritic ends of a neuron-like cell. (A): Neuron-like cells. (B): The dendritic-end of a neuron with several branched and unbranched dendrite-like processes emanating from cyton. (C): The axonal-end showing several globular vesicles clustered at the end of the axon terminal. Scale bars=20 µm (A), 1 µm (B, C).

Although *in vitro* culture systems provide sufficient cells to study and characterize cellular and biochemical properties, reports have shown that these cells do not remain static and their properties, including expression of the surface markers, change as a function of passage, medium/growth factors and other physicochemical factors [20,21]. Hence, during culture, immense caution needs to be taken into consideration so as to minimize the effects of extraneous factors. The problem is further compounded during the culture and study of mouse BM-MSCs as they are relatively difficult to expand and maintain [22,23]. During culture through various passages, MSCs grew and proliferated predominantly in colonies all over the surface, and consistently showed three distinct cell populations: spindle-shaped, large cuboidal or flattened cells and small round cells as reported earlier [4]. Besides, few cells showed trigonal and polygonal morphological appearances which may represent bone marrow neuronal cell population as reported earlier [24]. As per the international society for cellular therapy (ISCT)-2006 rules, morphological appearance of MSCs is one of the few typical features to be looked into during culture and, in fact, is the first parameter of their tripartite characterization. This says that MSCs ought to be of typical spindle-shape in morphological appearance [25]. Therefore, during characterization, we decided to look at morphological features of bone marrow cells, which served as initial cell source for primary MSC culture, and then compared them with the MSCs P3. The majority of cell population showed spindle-shape morphology with centrally located distinct nucleus and well-defined cell boundary observed following various staining (Figures 1J-1L and Figures 2E and 2F).

Contrary to BMCs, mesenchymal stem cells clearly showed higher positive percentage with respect to CD29 (99.43% ± 0.671%), CD44 (84.96% ± 7.778%) and Sca-1 (91.70% ± 3.811%), the markers attributed to bone marrow-derived mesenchymal stem cells (BM-MSCs), indicating enrichment and homogeneity among cells as a result of selective *in vitro* expansion of the culture. On the other hand, haematopoietic markers like CD34 (0.16% ± 0.052%), CD45 (0.54% ± 0.103%) and macrophage-marker, CD11b (0.27% ± 0.153%) were almost negligible on the cultured MSC P3 cells, showing absence of hematopoietic contamination (Figure 3B) owing to loss of hematopoietic cell lineages during the culture. More importantly, the immunophenotypic characteristics of our MSCs were comparable to previously reported studies [26,27].

MSCs under adipogenic induction medium slowly undergo morphological transformation and became oval-shaped, with cytoplasmic deposition of numerous lipid droplets by 7th day. By 18th day, lipid droplets increased to their maximum size and covered almost entire cytoplasm. Upon staining, lipid droplets in differentiated cells became dark red due to their affinity and binding with lipophilic oil red O dye (Figures 4B and 4C). We found 81.66% ± 10.03% of mesenchymal cell-derived adipocytes positive for oil red O stain following 18 days of culture in adipocyte induction medium (Figure 4D), indicating strong adipogenic potential of these cells. Similarly, cells under osteocytic differentiation showed retraction of cellular processes and stained darkly for alkaline phosphatase activity (Figure 4F). Furthermore, close-up view showed broken concentric layers of extracellular deposition around the MSC-differentiated osteocytes which, to some extent, reflected the process and pattern of bone formation *in vivo* (Figure 4G). Upon quantitation, 82.33% ± 5.23% alkaline phosphatase positive MSC-derived osteocytes were observed following 16-18 days of induction in osteocyte differentiation medium (Figure 4H). The chondrogenic induction pellet-derived sections were very compact, due to secretion of extracellular matrix as well as it stained darkly by safranin O staining (Figure 4J), suggesting higher synthesis and increased secretion of chondrocyte extracellular matrix. The deposition of extracellular matrix components, especially collagen II and chondroitin sulphate, between the intercellular spaces, caused increase in the pellet size and formation of the chondrocyte nodule (Figure 4K). The results of the mesodermal differentiation assays of MSCs *in vitro* suggested that MSCs possessed multipotential characteristics, and they could differentiate into adipocytes, osteocytes, and chondrocytes, which is consistent with previous studies [28] and, therefore, these MSCs may be used for various experimental and application purposes.

Mouse bone marrow MSCs contain subsets of cells with distinct differentiation potentials such as monopotential osteogenic, as well as bipotential osteo- and adipogenic clones, and thus suggest heterogeneity among MSCs [29]. During osteogenic differentiation, concomitant heterogeneity, at the level of cellular morphology, differential intercellular and intracellular alkaline phosphatase activities were also clearly visible. The MSCs acquired round, oval and star-shaped morphologies and the nuclei displayed numerous punctated arrangements of chromosomal DNA (chromatin) (Figure 5B). The heterogeneity was also distinctly observed in terms of the alkaline phosphatase activities among the differentiated cells displayed by differential colour intensity (Figure 5C). Furthermore, these alkaline phosphatase positive cells showed various morphologies, including star-shaped, rounded, and oval and some globular shape, suggesting the morphological heterogeneity among the differentiated osteocytes, and may be representing either different stages or differential degree of osteocytic differentiation (Figures 5C and 5D). The extracellular matrix secreted by the osteocytes was deposited in a specific pattern, i.e., around the cells and these cells were eventually detached leaving behind hexagonally patterned-matrix depositions (Figure 5F).

Bone marrow cells and mesenchymal stem cells showed expression of several markers like CD29, CD73, Rex-1 and nestin, a neuronal cell

marker. MSCs P3 showed differential enrichment for CD29, CD73, Rex-1 and nestin compared to the bone marrow cells, suggesting increased homogeneity of mesenchymal stem cells with respect to the gene expression profile as a result of *in vitro* expansion (Figures 6A and 6B). Stem cell self-renewal and pluripotency are regulated by several factors concertedly working in a molecular circuitry. There are four transcription factors viz. Oct3/4, Nanog, Klf4 and Myc, which were thought to be important for the regulation of cell proliferation and stemness genes, and hence for self-renewal and pluripotency [30,31]. However, with the progress in understanding of molecular mechanisms underlying stemness, only Oct3/4 alone has been found sufficient for pluripotency and/or induction of pluripotency [32]. We found the expression of Oct3/4 in bone marrow cells (Figure 6E) and MSCs P3 cells (Figure 6F). Contrary to the well-established nuclear localization, we found Oct3/4 mainly localized in the cytoplasm with higher concentration in the elongated processes of MSCs P3. These different aspects might be looked from the perspective other than stemness maintenance as also seen in a previous report [33].

The concept of pluripotency of MSCs has been expanded owing to its trans-differentiation potential, with a few recent reports showing functional ectodermal differentiation like neurons [34] and endodermal differentiation into endothelial cells [35] both *in vitro* and *in vivo* following transplantation and functional integration. Following SEM imaging, the MSC-derived neuron-like cells (Figure 8A) appeared to have several branched dendrite-like cellular projections on one end of the cells (dendritic end) (Figure 8B), and several globular vesicles clustered at the axonal end (Figure 8C), which could be neurotransmitter containing vesicles. Furthermore, around 71.5% ± 3.5% and 73.5% ± 9.5% cells were bearing neurite-like projections following 1.0 μM β-mercaptoethanol+1.0 μM retinoic acid and 30 μM RA treatments, respectively. These methods gave us very high number of neuron-like cells with lots of interconnections similar to published finding [36]. This could be further characterized and used for screening of neurogenic agents, as well as can be tested for effects of some neurotoxic drugs to show their *in vitro* response. Since these MSC-derived neuron-like cells are differentiated on a glass coverslip representing a neuronal network, possibly it could also be used for cellular neurophysiological experiments. Furthermore, these biological processes depict that the bone marrow-derived mesenchymal stem cells are multipotent and share characteristics of well established multipotent stem cells unrestricted to mesodermal differentiation pathways.

Author Contributions

J.K.C. and P.C.R. were involved in the conception, design, data analysis, interpretation and writing of the paper. J.K.C. carried out all experiments and assembled the data.

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