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Comparative Cellular and Molecular Analyses of Pooled Bone Marrow Multipotent Mesenchymal Stromal Cells During Continuous Passaging and After Successive Cryopreservation

Murali Krishna Mamidi,[1,2] Kavitha Ganesan Nathan,[1] Gurbind Singh,[1] Saratha Thevi Thrichelvam,[1] Nurul Ain Nasim Mohd Yusof,[3] Noor Atiqah Fakharuzi,[3] Zubaidah Zakaria,[3] Ramesh Bhonde,[2] Anjan Kumar Das,[1*] and Anish Sen Majumdar[4**]

[1] *Stempeutics Research Malaysia Sdn. Bhd., Technology Park Malaysia, 57000 Kuala Lumpur, Malaysia*

[2] *Manipal Institute of Regenerative Medicine, Manipal University Branch Campus, # 10 Service Road, Domlur Layout, Bangalore 560071, Karnataka, India*

[3] *Hematology Unit, Cancer Research Centre, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia*

[4] *Stempeutics Research Pvt Ltd, Akshay Tech Park, Whitefield, Bangalore 560066, Karnataka, India*

ABSTRACT

The clinical application of human bone marrow derived multipotent mesenchymal stromal cells (MSC) requires expansion, cryopreservation, and transportation from the laboratory to the site of cell implantation. The cryopreservation and thawing process of MSCs may have important effects on the viability, growth characteristics and functionality of these cells both in vitro and in vivo. More importantly, MSCs after two rounds of cryopreservation have not been as well characterized as fresh MSCs from the transplantation perspective. The objective of this study was to determine if the effect of successive cryopreservation of pooled MSCs during the exponential growth phase could impair their morphology, phenotype, gene expression, and differentiation capabilities. MSCs cryopreserved at passage 3 (cell bank) were thawed and expanded up to passage 4 and cryopreserved for the second time. These cells (passive) were then thawed and cultured up to passage 6, and, at each passage MSCs were characterized. As control, pooled passage 3 cells (active) after one round of cryopreservation were taken all the way to passage 6 without cryopreservation. We determined the growth rate of MSCs for both culture conditions in terms of population doubling number (PDN) and population doubling time (PDT). Gene expression profiles for pluripotency markers and tissue specific markers corresponding to neuroectoderm, mesoderm and endoderm lineages were also analyzed for active and passive cultures of MSC. The results show that in both culture conditions, MSCs exhibited similar growth properties, phenotypes and gene expression patterns as well as similar differentiation potential to osteo-, chondro-, and adipo-lineages in vitro. To conclude, it appears that successive or multiple rounds of cryopreservation of MSCs did not alter the fundamental characteristics of these cells and may be used for clinical therapy. J. Cell. Biochem. 113: 3153–3164, 2012.

KEY WORDS: HUMAN BONE MARROW DERIVED MULTIPOTENT MESENCHYMAL STROMAL CELLS (MSC); EXPANSION; CRYOPRESERVATION; DIFFERENTIATION; CHARACTERIZATION AND MSC TRANSPLANTATION/INFUSION

Multipotent mesenchymal stromal cells (MSCs) were first identified by Friedenstein et al. [1976] as a separate non-hematopoietic stem cell population residing in the bone marrow. Later MSCs were isolated and expanded from individual bone marrow aspirates by many researchers [Lazarus et al., 1997; Hernigou et al., 2006]. These stromal cells are popularly known as

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*Correspondence to: Anjan Kumar Das, Stempeutics Research Malaysia Sdn. Bhd., Technology Park Malaysia, 57000 Kuala Lumpur, Malaysia. E-mail: anjan.das@stempeutics.com.my

**Correspondence to: Anish Sen Majumdar, Stempeutics Research Pvt Ltd, Akshay Tech Park, Whitefield, Bangalore 560066, Karnataka, India. E-mail: anish.majumdar@stempeutics.com

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marrow stromal stem cells or mesenchymal stromal cells or multipotent mesenchymal stromal cells (MSCs). In addition to bone marrow, MSCs have been shown to originate from the mesenchymal and connective tissues including peripheral blood [Zvaifler et al., 2000], umbilical cord blood [Lee et al., 2004], placenta [Igura et al., 2004], amniotic fluid and membrane [Tsai et al., 2004], dental pulp [Gronthos et al., 2000], deciduous teeth [Miura et al., 2003], adipose tissue [Zuk et al., 2002], and fore skin [Mamidi et al., 2011]. MSCs obtained from these tissues also express similar phenotypic and functional characteristics as those obtained from the bone marrow.

MSCs are characterized by a set of minimum criteria that includes the presence of certain cell surface markers and in vitro differentiation potential in response to specific stimuli [Dominici et al., 2006]. MSCs are plastic-adherent and expanded in culture without the loss of their differentiation capacity. More importantly, large-scale expansion capability of MSCs in the undifferentiated state permits the development of a number of MSC-based therapies [Barry and Murphy, 2004]. By definition, MSCs are capable of differentiating into osteogenic, chondrogenic, and adipogenic tissues and many studies have confirmed this [Lindner et al., 2010]. Although MSCs were initially defined by their ability to differentiate into cells of mesodermal origin, recent studies have provided support for their capacity to differentiate into cells from all three germ layers [D'Ippolito et al., 2004]. Moreover, their plasticity has been well documented and they have been successfully differentiated into a wide variety of lineages including neuron like cells [Bae et al., 2011], cardiomyocytes [Zhao et al., 2011], endothelium [Bai et al., 2010], and hepatocytes [Pournasr et al., 2011].

MSCs modulate the function of the immune cell populations involved in alloantigen recognition and elimination, including antigen presenting cells, T cells, B cells, and natural killer (NK) cells. MSCs exert powerful anti-inflammatory and immunosuppressive effects targeting the main immune cell subsets by producing various soluble factors. These immune regulatory properties are inducible by inflammatory mediators such as interferon-γ (INF-γ). Upon transplantation, MSCs promote endogenous growth, decrease apoptosis, reduce levels of free radicals and regulate inflammation, primarily through paracrine actions, thus enhancing the functionality of the damaged tissues or cells [Joyce et al., 2010].

Collectively this wide range of biological properties of MSCs attracted the attention of many researchers hoping to utilize these cells for diverse therapeutic applications. They have been extensively tested and proven effective in preclinical studies for various diseases where the cells are being delivered via different routes of administration [Arinzeh, 2005; Chugh et al., 2009]. MSC are concurrently being tested in a large number of clinical trials worldwide for diseases like myocardial infarction, stroke, meniscus injury, limb ischemia, graft-versus-host disease and different types of autoimmune disorders (www.clinicaltrials.gov). These findings indicate that the possibilities of MSCs in clinical applications are evident and exciting. However, MSCs are required to be cryopreserved for long-term storage and transportation for clinical applications. The use of cryopreserved MSCs in cell therapy requires preservation of their stem cell like properties such as proliferation and differentiation capabilities. Therefore, it is necessary to determine whether the process of cryopreservation affects either the proliferative capacity of MSCs, or their developmental potential. If MSCs are to be used as a drug, it is essential that they should be preserved at the hospital or clinic for use as necessary. Active cultures are not suitable for this purpose. In order to examine that the multiple crypopreserved and active cultures do not differ substantially, we compared fresh (active) ongoing cultures with multiple times cryopreserved and resuscitated MSCs (passive) and demonstrated that the successive cryopreserved MSCs shows normal characteristics as active cultures.

MATERIAL AND METHODS

MSC ISOLATION AND LARGE-SCALE EXPANSION

MSCs were obtained from bone marrow samples of healthy donors aged between 20 and 35 years after obtaining informed consent. The protocol was approved by the institutional ethics committee (Manipal Hospital, Bangalore, India). Briefly, bone marrow aspirate was diluted (1:1) with knockout Dulbecco's modified Eagle's medium (KO-DMEM; Gibco–Invitrogen), and centrifuged at 1,800 rpm for 10 min to remove the anti-coagulant. The supernatant was removed and the bone marrow was again diluted with KO-DMEM. The bone marrow mononuclear cells (MNCs) were separated by the Ficoll density gradient method (1.077 g/ml density) in 50 ml centrifuge tubes (Falcon, Becton-Dickinson). Bone marrow MNCs accumulated on the Ficoll–plasma interface were isolated and washed again with KO-DMEM. Isolated cells were plated into three T-75 cm^2 culture flasks (Falcon, Becton-Dickinson) and cultured in KO-DMEM supplemented with 10% fetal bovine serum (FBS; HyClone), 2 mM glutamax, pen-strep (Gibco–Invitrogen) and incubated at 37°C, 5% humidified CO_2. The non-adherent cells were removed after 48 h by replacing with fresh media. Subsequently the medium was replenished every 48 h. Upon confluency, the cells were harvested with 0.25% trypsin–EDTA (Gibco–Invitrogen) and replated in a single one-cell stack at a density of 1,000 cells/cm^2 (Corning Life Sciences; cell stacks are large cell culture containers used for bio-production) as published by us earlier [Pal et al., 2008]. For the work described in this manuscript, MSCs from three different individual donors were cultured separately and pooled at passage 2. Pooled MSCs were expanded to one more passage (passage 3) in similar culture conditions as described above.

CRYOPRESERVATION AND RESUSCITATION OF MSCs

Pooled MSCs at passage 3 were harvested using 0.25% trypsin–EDTA and centrifuged at 1,800 rpm for 10 min. The cells were resuspended in freezing solution containing 90% (v/v) sterile FBS and 10% (v/v) dimethylsulfoxide (DMSO; Sigma). Cells were loaded in 2 ml cryovials (Nunc) at a concentration of 3×10^6 cells/vial and frozen using a programmable slow freezing unit (Planar Kryo 560-16). After freezing, the cryovials were transferred in a liquid nitrogen vapor-phased cryo-container (Statebourne Cryogenics; BioSystem 36) for long-term storage. Prior to cryopreservation, fresh MSCs were characterized both phenotypically and functionally

and the data corroborated with those reported by us earlier [Pal et al., 2008].

The frozen stocks of MSCs at passage 3 were thawed in a constant-temperature water bath at 37°C by shaking lightly. After 1 or 2 min, cells were resuspended in complete medium and centrifuged at 1,800 rpm for 10 min. Cell number and viability were determined using trypan blue stain and 7-amino actinomycin D (7-AAD) respectively. Then the cells were cultured at a density of 1,000 cells/cm^2 in cell stacks under 37°C and 5% CO_2. At 70–90% confluence over about 10–12 days, cells were passaged as described above. For analysis of growth characteristics, MSCs were expanded and characterized at every passage. We again cryopreserved the MSCs at passage 4 and thawed the cells to check whether the introduction of this second step of cryopreservation has any impact on the MSCs. The resuscitated MSCs at passage 4 were cultured, expanded, and characterized as described above and compared with freshly harvested MSCs at their respective passages.

DETERMINATION OF TOTAL COUNT, VIABILITY, POPULATION DOUBLING NUMBERS (PDN), AND POPULATION DOUBLING TIME (PDT)

To determine total yield, cells were plated at a density of 1,000 cells/cm^2 in single one-cell stack and cultures were allowed to grow until they reach about 90% confluence. Next, the cells were detached by trypsinization, neutralized with culture medium containing 10% FBS and centrifuged at 1,800 rpm for 10 min. The cell pellet was gently tapped, resuspended in medium; assessed for total count and viability by trypan blue dye exclusion method before plating. Further, the cell viability was confirmed by 7-AAD staining. To examine cell growth rate, the number of population doubling (PDN) as well as the time required by cells for each population doubling (PDT) were determined during successive subcultures. These were calculated from hemocytometer counts ($n = 3$) for each passage according to the following formula: $PDN = [\log10(NH) - \log10(NI)]/\log10(2)$ where NI is the inoculum cell number and NH is the cell harvest number [Nekanti et al., 2010]. To obtain the cumulated doubling number, the population doubling for each passage was calculated and then added to the population doubling number of the previous passage. PDT was calculated using the formula: $PDT = tplg2/(lgNH - lgNI)$, where NI is the inoculums cell number; NH is the cell harvest number; and "t" is the time of the culture (in hours) [Nekanti et al., 2010].

MONOCLONAL ANTIBODIES AND IMMUNOPHENOTYPING BY FLOW CYTOMETRY ANALYSIS

MSCs were harvested upon reaching 90% confluency and resuspended in DPBS at a cell density of 1.0×10^6 cells/ml. Two hundred microliters of the cell suspension (approximately 1×10^5 cells) was incubated with the labelled antibodies in dark for 30 min at 4°C. The following antibodies were used to mark the cell surface epitopes-CD90-phycoerythrin (PE), CD44-PE, CD73-PE, CD166-PE and CD34-PE, CD45-fluoroisothiocyanate (FITC), and HLA-DR-FITC (all from BD Pharmingen, San Diego, CA; Table I). All analyses were standardized against negative control cells incubated with isotype specific IgG1-PE and IgG1-FITC (BD Pharmingen). At least 10,000 events were acquired on Guava Technologies flow cytometer, and the results were analyzed using Cytosoft, Version 5.2 (Guava Technologies, Hayward, CA).

DETERMINATION OF CELL SENESCENCE BY β-GALACTOSIDASE ASSAY

MSCs were stained using senescence β-galactosidase staining kit (Cell Signaling, Danvers, MA) according to the manufacturer's protocol. Briefly, the cells from passages 4 to 6 grown on 6-well culture plates were washed twice with DPBS and fixed with 4% paraformaldehyde for 10 min. Cells were washed again with DPBS and incubated with β-galactosidase substrate staining solution (150 mM NaCl, 2 mM $MgCl_2$, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 40 mM citric acid, and 40 mM sodium phosphate at pH 6.0 containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-D-β-galactosidase) for overnight at 37°C. Senescence cells were identified as blue-stained cells by standard light microscopy, and a minimum of 100 cells were counted in 10 random fields to determine the percentage of β-galactosidase-positive cells [Nekanti et al., 2010].

PROPIDIUM IODIDE (PI) UPTAKE

The cell cycle distribution of MSCs was determined by PI staining assay [Jing et al., 2010]. Single cell suspension of MSCs was fixed overnight using ice-cold 70% ethanol at −20°C (cells may be stored in 70% ethanol at −20°C for several weeks prior to PI staining and analysis). Cells were centrifuged at 1,800 rpm for 10 min, ethanol decanted gently and washed with 0.1% BSA dissolved in 1× DPBS. MSCs were stained with PI/RNAse staining solution (50 μg/ml of PI and 0.1 mg/ml RNAse in DPBS) for 1.5 h at 42°C in darkness. Cell

TABLE I. Represents the List of Conjugated and Unconjugated Antibodies Used in the Study

Antigen	Antibody	Dilution	Brand	Application
CD-44	Anti-human CD44 (550989)	1:50	BD Biosciences, CA, USA	Immunophenotyping by flow cytometry
CD-73	Anti-human CD73 (550257)	1:50	BD Biosciences, CA, USA	Immunophenotyping by flow cytometry
CD-90	Anti-human CD90 (555596)	1:50	BD Biosciences, CA, USA	Immunophenotyping by flow cytometry
CD-166	Anti-human CD166 (559263)	1:50	BD Biosciences, CA, USA	Immunophenotyping by flow cytometry
CD-45	Anti-human CD45 (555482)	1:50	BD Biosciences, CA, USA	Immunophenotyping by flow cytometry
CD-34	Anti-human CD34 (555821)	1:50	BD Biosciences, CA, USA	Immunophenotyping by flow cytometry
HLA-DR	Anti-human HLA-DR (347363)	1:50	BD Biosciences, CA, USA	Immunophenotyping by flow cytometry
STRO-1	Anti-human STRO-1 (sc-47733)	1:20	Santa Cruz Biotechnology	Immunofluorescence and flow cytometry

cycle distribution was analyzed by flow cytometry using Cytosoft, Version 5.2 software (Guava Technologies).

IMMUNOFLUORESCENT STAINING AND IMAGE ANALYSIS

MSCs were plated on two-well chamber slides (BD Biosciences) and allowed to grow for uniform confluence. Staining procedure was performed as described previously by us [Mamidi et al., 2011] using the primary antibody STRO-1 (Santa Cruz; Table I).

TOTAL RNA ISOLATION, cDNA SYNTHESIS, AND SEMI-QUANTITATIVE RT-PCR

Total RNA was isolated using TRIZOL reagent (Invitrogen) as per the manufacturer's protocol. One microgram of RNA was used to synthesize the cDNA using Superscript II First Strand Synthesis System (Invitrogen) as per the manufacturer's instructions. Then, 1 μl of cDNA was amplified by PCR using the Abgene 2× PCR master mix (Abgene, Epsom, UK) and appropriate primers (Table II). PCR reaction was performed using the published protocol [Mamidi et al., 2010].

QUANTITATIVE CONFIRMATION OF SEMI-QUANTITATIVE RT-PCR

Quantitative amplifications were carried out in duplicate using SYBR green master mix (Applied Biosystems, Foster City, CA) as described earlier [Govindasamy et al., 2010]. Quantitative PCR reactions were run on an ABI 7900HT RT-PCR system (Applied Biosystems) using the primers listed in Table II and the results were analyzed by SDS v2.1 software. All values of the target genes were normalized against endogenous control GAPDH.

DIFFERENTIATION POTENTIAL OF MSC

To assess the mesodermal differentiation potential, MSCs were cultured at a density of 1,000 cells/cm^2 in six-well plates (Nunc) and were allowed to reach confluence. Differentiation potential of MSCs towards osteogenic, chondrogenic, and adipogenic lineages was assessed using published protocols [Mamidi et al., 2011]. For osteogenesis, cells were grown in KO-DMEM (Gibco–Invitrogen) supplemented with 10% FBS (Hyclone), 2 mM glutamax (Invitrogen), 30 mg/ml ascorbic acid, 10^{-8} M dexamethasone and 10 mM β-glycerophosphate (Sigma) for 3 weeks. Osteogenic medium-treated cells were applied for Alizarin red staining to observe calcium deposits. For chondrogenesis, cells were cultured in KO-DMEM media supplemented with 1× insulin–transferrin–selenium (ITS; Sigma), 50 mM L-ascorbic acid (Sigma), 25 mM L-proline (Sigma), 10 ng/ml of transformation growth factor-β (TGF-β; Sigma) and 55 mM sodium pyruvate (Invitrogen). Proteoglycan accumulation was evaluated by Alcian blue staining (Sigma) after 3 weeks. To induce adipogenic differentiation, cells were cultured up to 3 weeks in KO-DMEM supplemented with 10% FBS (Hyclone), 2 mM glutamax (Invitrogen), 1 mM dexamethasone, 0.5 mM isobutyl-methylxanthine, 1 mg/ml insulin, and 100 mM indomethacin (Sigma). Formation of lipid droplets in the generated adipocytes was visualized by Oil Red O staining (Sigma). Osteo-, chondro-, and adipogenic differentiation images were captured using Olympus

TABLE II. Represents the List of Semi-Quantitative, Quantitative Real-Time RT-PCR Primers and Reaction Conditions Analyzed in the Study

Serial no.	Gene symbol	Primer sequences (5′–3′)	Tm (°C)	Product size (bp)	Gene bank accession number
1	GAPDH	5′-TGAAGGTCGGAGTCAACGGATT-3′ 5′-CATGTGGGCCATGAGGTCCACCAC-3′	60	983	NM_002046.3
2	Oct-4	5′-CGACCATCTGCCGCTTTGAG-3′ 5′-CGACCATCTGCCGCTTTGAG-3′	57	572	NM_203289.3
3	Nanog	5′-TCCTCCATGGATCTGCTTATTCA-3′ 5′-CAGGTCTTCACCTGTTTGTAGCTGAG-3′	58	259	NM_024865.2
4	Sox-2	5′-CCCCCGGCGGCAATAGCA-3′ 5′-TCGGCGCCGGGGAGATACAT-3′	58	447	NM_003106.2
5	Rex1	5′-GCGTACGCAAATTAAAGTCCAGA-3′ 5′-ATCCTAAACAGCTCGCAGAAT-3′	55	302	NM_174900.3
6	hTERT	5′-AGCTATGCCCGGACCTCCAT-3′ 5′-GCCTGCAGCAGGAGGATCTT-3′	59	184	NM_198253.2
7	Nestin	5′-CAGCGTTGGAACAGAGGTTGG-3′ 5′-TGGCACAGGTGTCTCAAGGGTAG-3′	58	388	NM_006617.1
8	NEFH	5′-ACGCTGAGGAATGGTTCAAG-3′ 5′-GCCTCAATGGTTTCC-3′	58	555	NM_006158.2
9	MEF-2C	5′-GATGCGGACGATTCCGTAGG-3′ 5′-TGGTGCCTGCACCAGACGTG-3′	59	327	NM_002397.3
10	C-actin	5′-TCTATGAGGGCTACGCTTTG-3′ 5′-CCTGACTGGAAGGTAGATGG-3′	59	668	NM_005159.4
11	HNF-3β	5′-GACAAGTGAGAGAGCAAGTG-3′ 5′-ACAGTAGTGGAAACCGGAG-3′	56	234	NM_153675.1
12	Albumin	5′-CCTTTGGCACAATGAAGTGGGTAACC-3′ 5′-CAGCAGTCAGCCATTTCACCATAGG-3′	55	354	NM_000477.3
13	Osteocalcin (BGLAP)	5′-AAACCCAGCGGTGCAGAGT-3′ 5′-GGCTCCCAGCCATTGATACA-3′	50	108	NM_199173.4
14	Sox-9	5′-GTGCTCAAAGGCTACGACTG-3′ 5′-CGT TCT TCACCGACTTCCTC-3′	48	316	NM_000346.3
15	Lipoprotein lipase (LPL)	5′-GCGCCCCGAGATGGA-3′ 5′-TTAGGGCAAATTTACTTTCGATGTC-3′	45	140	NM_000237.2

advanced research microscope (Olympus IX 71) and Image-Pro Express software (Media Cybernetics).

STATISTICAL ANALYSIS

All the experiments were replicated three times (n = 3). Error bars on the graphs show standard deviation of three replicates. Values were considered statistically significant only when error is <5%.

RESULTS

IMPACT ON VIABILITY, GROWTH CHARACTERISTICS, AND ADHERENCE SURFACE QUALITY OF ACTIVE AND PASSIVE MSCs

In order to address the quality issues of MSCs after successive cryopreservation, we used cryopreserved pooled allogenenic MSCs that were frozen at P3. Upon thawing of the cells, MSCs were plated and characterized at P4. The harvested MSCs obtained at P4 were divided into three groups: (1) MSCs used for characterization; (2) MSCs were plated for P5 active culture; and (3) MSCs were cryopreserved for a period of time and subsequently thawed and plated for passive culture (Fig. 1A).

The viability of MSCs was observed close to 80% when the cells were analyzed immediately after revival (before plating) at passage 3 and 4 (Fig. 1B,C). Increase in the number of viable cells at P4 has been witnessed to more than 95% as they were harvested after plating and this high percentage of cell viability was maintained up to passage 6 in both group of cells obtained from active cultures (Fig. 1: E1–E3) as well as from the passive cultures (Fig. 1: E4–E5). We observed spindle shaped MSC morphology for both passive culture (Fig. 1: D4) and active cultures (Fig. 1: D2). The cells expanded from both the groups showed typical MSCs spindle shape morphology up to passage 6 (Fig. 1: D3 and D5). Further, the quality of MSCs was demonstrated by the expression of STRO-1 at every passage (Fig. 1: D1–D5; figure inserts).

To analyze the growth rate of both active and passive cultures we have calculated the total cell yield using trypan blue exclusion method. We observed that the total yield was 46.5×10^6 at passage 4, where as this total yield was decreased to 30×10^6 at passage 5 active culture; when compared to passage 5 passive cultures the total yield was 29.5×10^6 suggests no differences for total yield at active and passive cultures (Table III). Further, we noticed that the total cell count was decreased to half at passage 6 expanded from

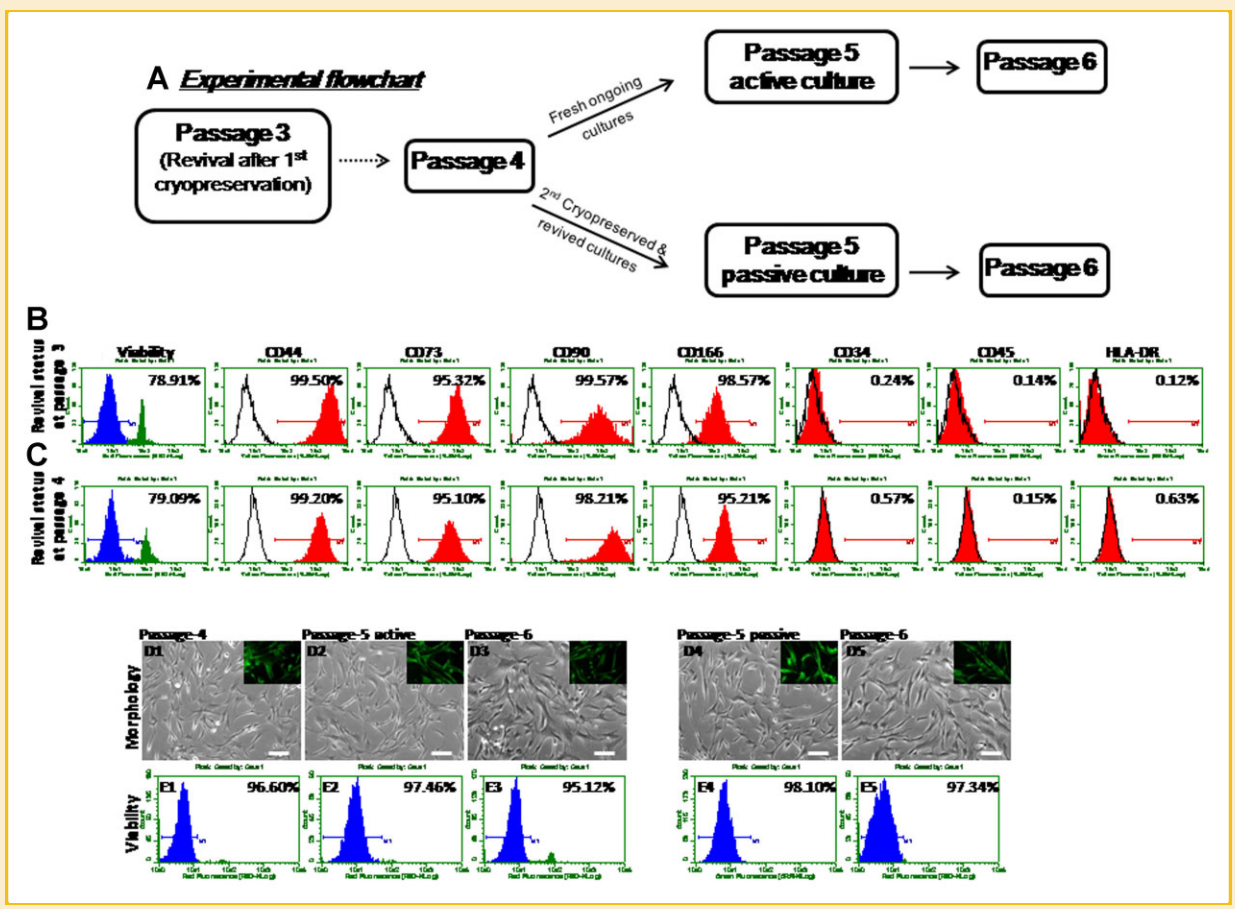

Fig. 1. Growth characteristics. A: Experimental flow chart of the study. B,C: Viability and stromal marker expression of resuscitated MSCs at passage 3 and 4, respectively. D1–D5: Morphology and the figure inserts show the expression of STRO-1 by MSCs at every passage. Scale bars represent 100 μm. E1–E5: Viability of MSCs observed at different passages. Abbreviation: MSC: human bone marrow multipotent mesenchymal stromal cells.

TABLE III. Comparative Growth Kinetics of MSCs for Active Versus Passive Cultures at Different Passages

Growth kinetics	Passage 4	Passage 5—active	Passage 6	Passage 5—passive	Passage 6
Total yield	46.5×10^6 cells	31×10^6 cells	11×10^6 cells	29.5×10^6 cells	12×10^6 cells
PDN	6.2	5.6	4.1	5.5	4.2
PDT	50.39	59.92	81.71	56.12	79.29

PDN, population doubling numbers; PDT, population doubling time in hours.

both active (12×10^6) and passive (11×10^6) cultures (Table III). This gradual decline in the proliferative ability of MSCs with concomitant raise in the passage number was further confirmed by calculating PDN and PDT (Table III).

CORRELATION OF PHENOTYPIC EXPRESSION BETWEEN ACTIVE AND PASSIVE MSCs

First we analyzed the stromal cell marker expression of thawed cells at passage 3 and 4. We observed more than 95% positivity for CD44, CD73, CD90, and CD166 while the expression of CD34, CD45, and HLA-DR was found to be negative (Fig. 1B,C). Next, comparison of stromal marker expression of passive culture cells showed no significant differences with those grown in active cultures. We observed more than 95% of stromal marker expression in both passive and active MSC cultures (Fig. 2). Cells expanded from both the groups retained high percentage of stromal marker expression at passage 6 (Fig. 2). As expected, the expression of CD34, CD45, and HLA-DR was found to be negative in all cell groups which confirmed their mesenchymal nature.

CELL CYCLE DISTRIBUTION

The DNA content was analyzed from both active and passive cultures of MSCs. We observed a large percentage (close to 80%) of cells were in the G0/G1 phase in all groups of MSCs (Fig. 2A–E). This suggests similar proliferation rate of MSCs in both active and passive cultures. Although there were minor differences in the S-phase, there was no alterations of cells in G0/G1 phase, suggesting that both active and passive group of cells are proliferating in same manner (Fig. 2A–E). These data collectively suggest that the successive cryopreservations of MSCs do not significantly alter their rate of proliferation compared to actively passaged cells.

PROLIFERATION CAPACITY OF MSC WAS CONFIRMED BY CELLULAR SENESCENCE

MSCs possess a limited lifespan during in vitro culture since they are known to undergo senescence [Wagner et al., 2008]. To look at the percentage of cells undergoing senescence, we have employed the enzyme lysosomal pH 6.0 β-galactosidase (β-gal) as a senescence marker. At passage 4, the β-gal positive cells were found to be $1.5 \pm 0.08\%$ (Fig. 3A), suggesting the presence of a very small fraction of aged cells at this stage. We observed similar percentages of β-gal positivity for both active ($4.6 \pm 0.23\%$; Fig. 3B) and passive ($4.3 \pm 0.21\%$; Fig. 3C) stages of these cells. This percentage β-gal positivity was increased at passage 6 when the cells were expanded from both active ($18 \pm 0.9\%$; Fig. 3D) and passive ($16 \pm 0.8\%$; Fig. 3E) cultures. These results indicate that there were no significant differences observed for aged cells in both active and passive cultures. The percentage of senescence positive population is depicted in a bar chart (Fig. 3F,G).

GENE EXPRESSION PROFILE OF MSCs AT DIFFERENT PASSAGES

MSCs have been shown to express certain transcription factors that are important for maintaining pluripotency of the stem cells in early passages [Ock et al., 2010; Pierantozzi et al., 2011]. We intended to determine the mRNA expression levels of certain key pluripotency markers in our active and passive cultures of MSC population. We did not observe any significant differences between the two groups of cells with respect to Oct4, Nanog, Sox-2, and Rex-1 (Fig. 3H). The expression of these pluripotent markers was observed to demonstrate a modest down regulation at passage 5 and a significant decrease at passage 6 in both the groups. Weak hTERT expression was observed at P4, and this expression was lost at subsequent passages. Further, to confirm this passage related loss of pluripotency, we have quantified the expression levels of pluripotent markers using quantitative real-time PCR and observed a significant decrease in the fold expression of Oct4, Nanog, Sox-2, and Rex-1 at passage 6 (Fig. 4A–D).

Spontaneous differentiation of stem cells is known to be associated with the expression of lineage specific markers. Spontaneous differentiation during regular passaging of MSCs was analyzed by using lineage-specific markers corresponding to ectoderm, mesoderm and endoderm by semi-quantitative RT-PCR. First we checked the expression of Nestin (a primitive neural stem cell marker) and neurofilament heavy polypeptide (NEFH) expressed in neuroectodermal cells and also in neuronal progenitors. The expression level of Nestin was observed to be considerably upregulated from passage 4 to 6; whereas the expression of NEFH was not observed at passage 4 and 5, but modest expression of NEFH was observed at passage 6 (Fig. 3I). Next, myocyte enhancer factor 2C (MEF-2C) and α-cardiac actin (C-actin) were analyzed to monitor mesoderm differentiation. Similarly, enhanced expression of MEF-2C was observed from passage 4 to 6 and C-actin was not observed at passage 4 and 5. Interestingly C-actin was expressed prominently at passage 6. Finally, to analyze the endodermal lineage differentiation, we checked the expression of hepatocyte nuclear factor-3-β (HNF-3-β) and albumin (hepatic marker). The expression levels of HNF-3β showed a uniform upregulation from passage 4 to 6 and we have not noticed any expression of albumin except a faint expression at passage 6. In addition, quantitative real-time PCR analysis supported our semi-quantitative RT-PCR results confirming the upregulation of genes associated with ecto-, meso-, and endoderm lineages including NEFH, C-actin, and HNF-3β from passage 4 to 6 in both groups (Fig. 4E–G). We reasoned that the decrease in the self renewal capacity of MSCs shown by the down

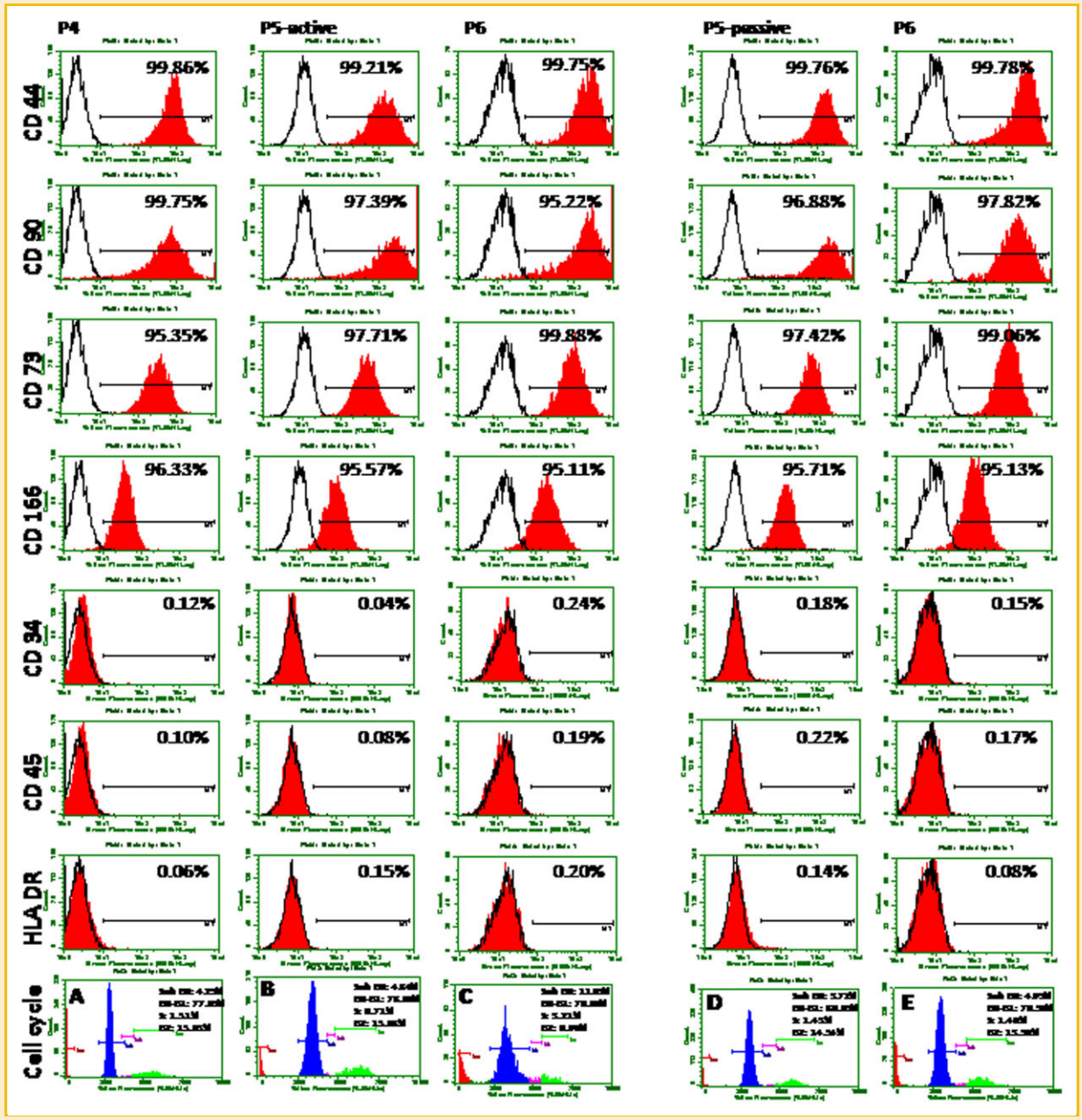

Fig. 2. Phenotypic expression. Detection of MSC surface marker expression and cell cycle distribution. MSCs were cultured for different passages, labeled with the indicated antibodies and analyzed by flow cytometry. An open area represents an antibody isotype control for background fluorescence and a shaded area shows signal from MSC surface marker antibodies. Representative histograms are depicted. A–E: Cell cycle analysis of MSC at different passages.

regulation of pluripotent markers leads to the spontaneous differentiation of MSCs at passage 6 as shown by the fold increase of lineage specific markers (Fig. 4H).

DIFFERENTIATION POTENTIAL OF MSCs AT ACTIVE AND PASSIVE STAGES

The hallmark of MSC is its ability to differentiate into mesodermal lineage. We therefore undertook studies to induce differentiation of MSC into osteogenic, chondrogenic, and adipogenic lineages by using standard MSC differentiation protocols [Mamidi et al., 2011]. The functional capacity of passive MSCs was observed to be similar to that of the active MSCs, showed by their evenly balanced differentiation potential towards mesodermal lineage under appropriate stimuli. Uniform osteogenic induction was observed after 3 weeks of MSC culture from passage 4 to 6 including active and passive MSC cultures. Cells underwent morphological changes and formed cell piling up eventually demonstrating abundant amounts of calcium deposits typical of bone formation detected by Alizarin red staining (Fig. 5A–E). The chondrogenic potential was confirmed by formation of sulfated proteoglycans verified by Alcian blue

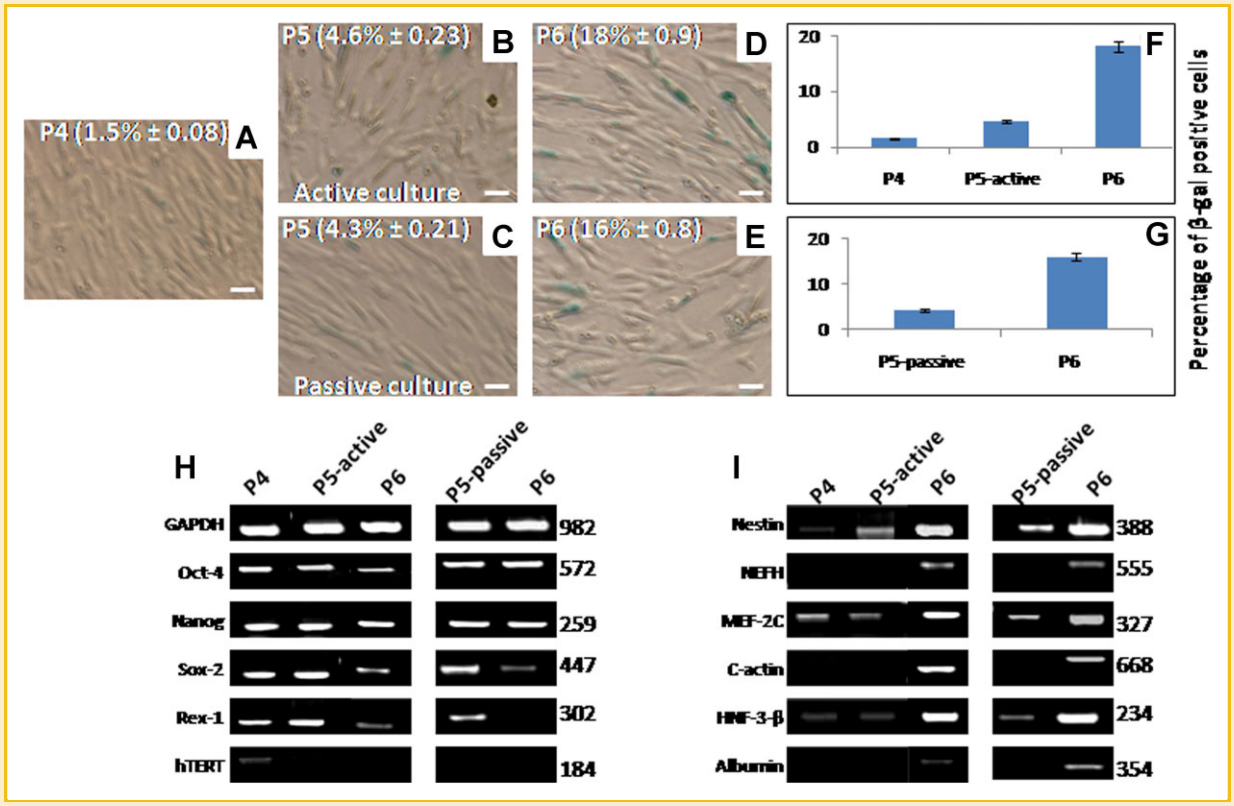

Fig. 3. Replicative senescence and gene expression profile of MSCs. A–E: Senescence associated β-galactosidase staining of MSCs at different passages. F,G: Quantification of percentage (%) of β-gal positive cells cultured for various passages. Results represent the average of at least three culture replicates (n = 3) with SEM. Scale bar = 100 μm. Abbreviation: SEM, standard error mean. H: Reverse transcription polymerase chain reaction (RT-PCR) analysis of stemness associated markers in MSCs cultured for different passages. I: Lineage- or tissue-specific markers expressed in MSCs cultures at various passages.

staining (Fig. 5F–J). Adipocytes displayed intracellular lipid accumulation verified by Oil Red O staining (Fig. 5K–O). Figure inserts refer to non-induced controls. Furthermore, we analyzed the expression level of MSC differentiation specific markers for osteogenesis (Osteocalcin or BGLAP); chondrogenesis (Sox-9); and adipogenesis (Lipoprotein lipase or LPL) by semi-quantitative RT-PCR (Fig. 5P). These data clearly demonstrate that passive MSCs exhibited the differentiation potential into all three mesodermal lineages, thereby confirming the functional ability of differentiation in vitro. Further, MSCs cultured from both active and passive cultures showed similar extent of differentiation to osteo-, chondro-, and adipogenic lineages.

DISCUSSION

Cryopreservation is an important method to keep cells as seeds. Earlier researchers described that cryopreservation for a single time using 10% DMSO and slow cooling does not affect the viability of BM as well as adipose-derived MSCs [Kotobuki et al., 2005; Liu et al., 2008]. Similarly many other studies showed that the onetime cryopreservation and thawing of MSCs does not affect their biological and functional characteristics [Pittenger et al., 1999; Yamaguchi et al., 2001; Thirumala et al., 2009]. We undertook the present study to clarify whether successive cryopreservations of MSC have any influence on their properties. Our experiments revealed that the MSCs even after successive cryopreservations showed the typical features of spindle-shaped cell bodies, confluence after a lag phase of 10–12 days and retained high percentage of viability (Fig. 1B–E). These cells expressed several surface epitopes such as CD44, CD73, CD90, and CD166, were negative for CD34 and CD45 (demonstrating their non-hematopoietic nature) and for HLA-DR with the majority of the cells were being in the G0/G1 phase, confirming the retention of potential MSC characteristics even after successive cryopreservation and resuscitation of MSCs.

Biological properties of MSCs are not everlasting and their proliferation and differentiation properties are gradually lost with passaging process or during sub-culture [Kretlow et al., 2008]. We speculate that the long-term cultivation of MSCs may fail because of replicative senescence. Unlike apoptotic cells, senescent cells remain alive despite a derangement of function [Itahana et al., 2001]. There are many factors that can cause aging or senescence such as irreversible DNA damage, reactive oxygen species (ROS) and shortening of telomeres. One theory suggests that β-gal activity is associated with the Ras pathway [Minamino et al., 2003] and with lysosomal dysfunction [Kurz et al., 2000]. Liu et al. [2004] observed that MSC of telomerase-knockout mice showed premature senescence. Another report showed that the prolonged MSC cultivation

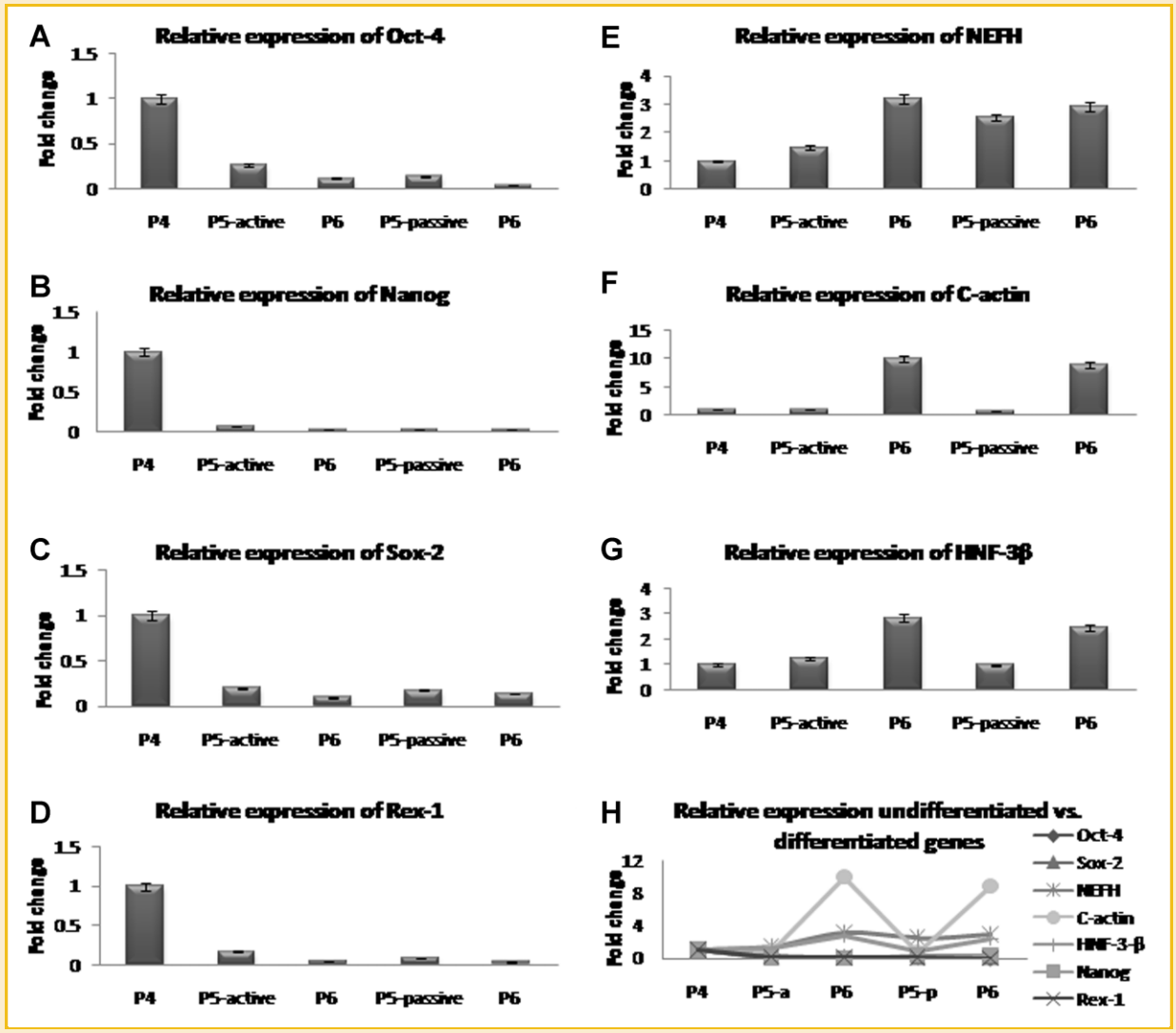

Fig. 4. Validation of quantitative real-time RT-PCR using SYBR green. Values are normalized against the endogenous control GAPDH. A–D: Reveals relative fold expression of stemness associated markers such as Oct-4, Nanog, Sox-2, and Rex-1. E–G: Depicts the relative fold expression of NEFH, C-actin, and HNF-3β corresponds to ecto-, meso-, and endoderm lineages. H: Sigmoid and bar graphs indicates the down regulation of stemness markers and up-regulation of lineage- or tissue-specific markers.

increases β-gal activity in pig MSCs [Vacanti et al., 2005]. These observations were corroborated to human MSCs as well, where Stenderup et al. [2003] demonstrated increased β-gal activity in late passage MSCs. In this context our data is of significance wherein we also noticed increased β-gal activity in the later passages of MSCs (Fig. 3A–G). As we observed here, the increase in the percentage of senescence positivity is associated with a gradual decrease in their proliferative capacity. A recent report showed that this replicative senescence in MSCs seems to be controlled epigenetically [Schellenberg et al., 2011]. In contrast to embryonic stem cells, adult MSCs undergo senescence since they have a role in maintaining tissues and organs involved with homeostasis. Hence, undue senescence of MSCs can disrupt the integrity of tissues and organs. It is therefore extremely important to identify the stage or passage of MSCs where the cells can be expanded to maximum numbers with minimum β-gal positive cells for therapeutic/clinical applications.

In addition to the above described characteristics, another defining feature for therapeutic applications of resuscitated MSCs is to successfully maintain their self-renewal along with their differentiation potential. It has been shown that the resuscitated MSCs retained stemness associated marker such as Oct-4 expression [Tondreau et al., 2005; Ren et al., 2006]. Riekstina et al. [2009] reported that MSCs expressed Oct4 as well as Nanog. There was another report which showed the positive expression of Oct-4, Rex-1, and Sox-2 by MSCs [Izadpanah et al., 2006]. Our results are in agreement with these studies where we have demonstrated the positive expression of stemness markers such as Oct-4, Nanog, Sox-2, and Rex-1 for successive post-cryopreserved MSCs, suggesting their possible multipotentiality. However, the expression levels of these markers were down regulated during later passages of expansion (Fig. 3H) similar to the previous results [Pierantozzi et al., 2011], where the number of Nanog-positive cells were reduced in late passages of adipose and cardiac MSCs. Several reports have

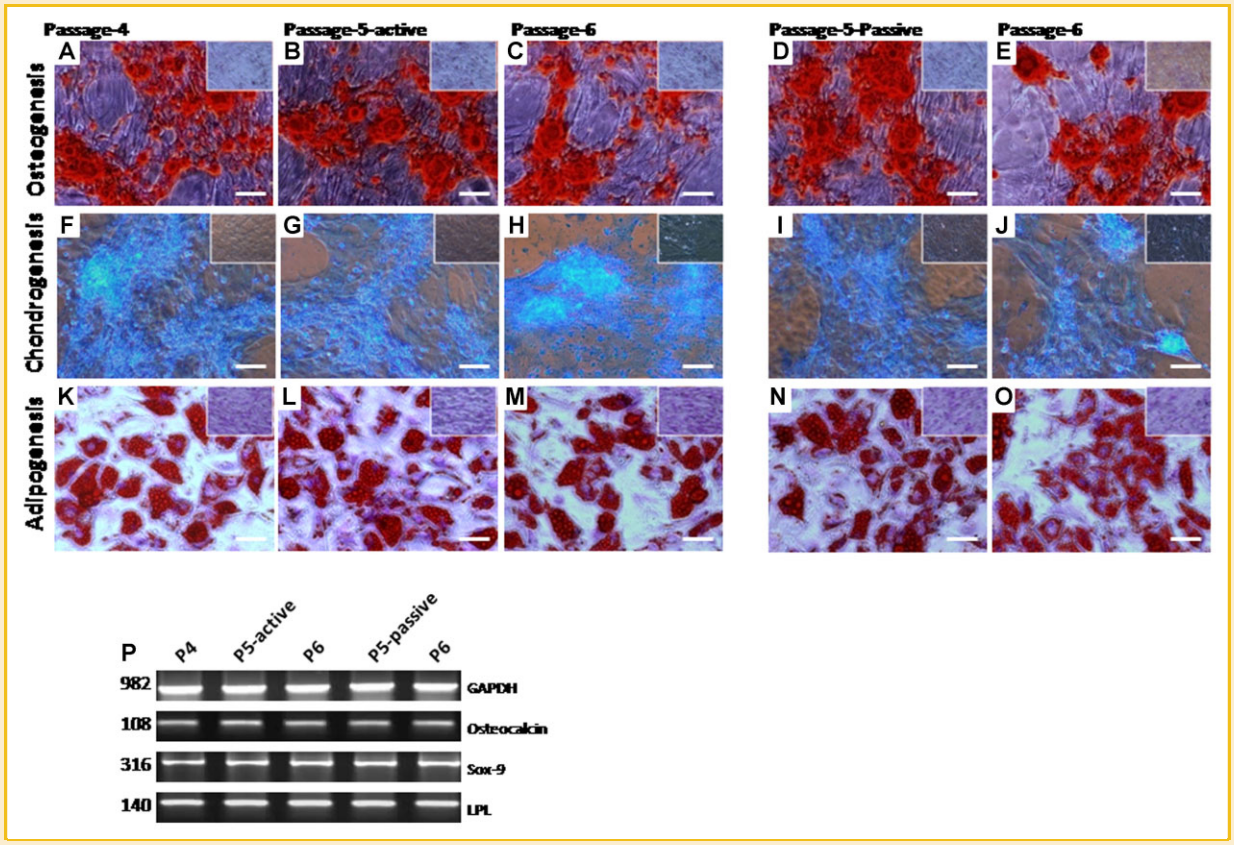

Fig. 5. Multilineage differentiation potential of MSCs cultured for different passages. Active passive cultures of MSCs were investigated for in vitro multilineage differentiation capacity. A–E: Formation of mineralized matrix was detected by Alizarin red stain confirming the osteogenic differentiation. F–J: Chondrogenic differentiation was demonstrated by Alcian blue staining. K–O: Adipogenesis was confirmed by neutral oil droplet formation stained with Oil Red O. Scale bars represent 200 μm. Non-induced control cultures without osteogenic (A–E), chondrogenic (F–J), or adipogenic (K–O) differentiation stimuli are shown in the figure inserts. Representative results of three independent experiments are shown. P: RT-PCR results showing equivalent expression levels of genes for osteogenesis (Osteocalcin), chondrogenesis (Sox-9), and adipogenesis (LPL) in all cultures.

shown that pluripotency of stem cells has been strongly regulated by Oct-4 functioning together with Sox-2 and Nanog at the core of a transcription factor network [Ralston and Rossant, 2010]. Although positive reactivity of MSCs to primitive stem cells markers alone may not be sufficient to define their pluripotency. However, it certainly throws up new perspectives regarding the function and regulation of these marker transcripts in adult MSCs.

At the same time we observed a substantial upregulation of lineage- or tissue-specific markers at passage 6 (Fig. 3I), suggesting the decrease of stem cell like properties with concomitant rise of differentiated phenotypes due to spontaneous differentiation. These observations were supported by the previous results where researchers showed the expression of ecto-, meso-, and endodermal lineage specific markers by human adult bone marrow derived cells [D'Ippolito et al., 2004]. Researchers have further explained that the expression of Oct-4 and Nanog in stem cells derived from different adult tissues, may help them to retain the in vitro multilineage differentiation potential [Pochampally et al., 2004].

In depth characterization of cryopreserved MSCs is critical since majority of clinical trials are using cryopreserved cells in clinical settings. The use of cryopreserved MSCs in cell therapy also requires preservation of their mesodermal lineage differentiation along with their self-renewal properties. Several studies showed the differentiation ability of single time cryopreserved MSCs. Bruder et al. [1997] showed that the resuscitated MSCs could be sub cultured for many passages without noticeable loss of osteogenic differentiation potential. Next, these results were validated by showing that the MSCs do not lose their osteogenic differentiation capacity after cryopreservation [Kotobuki et al., 2005]. In a recent report, researchers demonstrated that the adult stem cells derived from human dental pulp retained multilineage differentiation ability after slow cooling in 7.5–10% DMSO [Woods et al., 2009]. In the present study, MSCs were frozen first at P3 and subsequently at P4 and showed similar mesodermal differentiation potential after successively cryopreserved and resuscitated MSCs (Fig. 5A–O). Thus the introduction of a second cryopreservation step has not resulted in any type of aberrance in MSC characteristics and in vitro differentiation potential.

There are several advantages of using successively cryopreserved cultures as opposed to fresh cultures for therapeutic/clinical use of stem cells. These are enumerated as follows: (a) successively cryopreserved MSCs can be given as and when the cells are required

for transplantation by simple wash either with media or DPBS. This will avoid the need for cell culture just before transplantation, which takes an enormous amount of time and is a major hindrance when the patient requires cells in an emergency setting. Thus, successive or multiple cryopreservation of MSCs makes it available on the shelf for treatment. (b) Transportation of cryopreserved MSCs is logistically easier than fresh cultures. (c) Applicability of cryopreserved MSCs in the clinical set up could be easier, if the operation theaters (OT) can be attached with a small cell processing facility where the cells can be processed and passed to OT via sterile pass box. (d) This simple procedure will offer potentially low bioburden because they are not exposed to the external environment while processing and transplantation thereby eliminating the contact with potential contagious agents. (e) Use of passive culture is simple and faster compared to active cultures and makes the clinical trials much more controlled and effective. (f) Clinical use of cryopreservation of MSCs may be more practical to save time, culture materials and is cost effective. Cells can be prepared in batches and stored for future applications. Potential limitation of this method is that the cells are contaminated with DMSO and one can effectively overcome this limitation with proper washing of cells immediately after thawing or while processing the cells for therapeutic/clinical use.

In summary, the present study demonstrated that the MSCs after successive cryopreservations retained their multipotency and that the resuscitated MSCs indeed exhibited all essential multipotent characteristics. These observations suggest that the ''memory'' of proliferation and differentiation in MSCs is not affected by multiple rounds of cryopreservation. Further, we believe that this core set of characterization data will be extremely useful in clinical settings and allow the rapid progress of MSCs for therapeutic/clinical use.

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