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Complete in vitro Characterization of Human Umbilical Cord Wharton's Jelly derived Mesenchymal Stem Cells

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Abstract

During the last decade, research interests in the scientific community has focused on a particular group of tissues called as "perinatal tissues" which include fetal annexes such as placenta, amniotic membranes and umbilical cord. As large numbers of cells are required for MSCs clinical applications (cells doses up to 5 million MSC/kg body weight), together with the low frequency of MSCs in tissues, we require an efficient and Good Manufacturing Practices (GMP)-compliant ex-vivo expansion process. The field, nevertheless, faces several challenges. Persistent issues include the definition of a MSC and comparability between MSC preparations. This is because of inherent cell heterogeneity, the absence of markers that are unique to MSCs, and the difficulty in precisely defining them by developmental origin. Differences in the properties of MSCs also depend on the site of tissue harvest, phenotypic and genotypic characteristics of the donor and the isolation, expansion and storage methods used. These differences may be sufficient to ensure that attributes of the final MSC product could differ in potentially significant ways.

Keywords: Wharton's Jelly, Master Stock, Single cell clones, Proliferation Kinetics, CFU-F Colonies.

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INTRODUCTION

Cell based therapy is a procedure in which cellular material is injected into a patient; this generally means intact, living cells. For example, T cells capable of fighting cancer cells via cell-mediated immunity may be injected in the course of immunotherapy [1].

The capacity of most tissues to regenerate derives from stem cells, but there are many barriers to the use of stem-cell-based therapies in the clinic. Such therapies, however, have the potential to improve human health enormously, and knowledge gained from studying cells in culture and in model organisms is now laying the groundwork for a new era of regenerative medicine [2].

The presence of non-hematopoietic cells within the bone marrow was first demonstrated by Friedenstein et al in 1970's (Friedenstein, 1974; Friedenstein, 1976). Fibroblast-like cells in the bone marrow were already suggested by observations made by Cohnheim, a German pathologist, in 1867, more than 140 years ago, where he described wound invasion by contractile cellular elements derived from leukocytes (Prockop, 1997). Friedenstein et al used a protocol that is still in use today to isolate multipotent marrow stromal cells from bone marrow. This protocol uses the ability of these cells to adhere to plastic, whereas hematopoietic stem cells were washed away after 4 hours. Heterogeneous cells from small colonies of 2 to 4 cells and remain dormant for some days. After this initial phase, colonies start to grow and cells proliferate and become homogeneously fibroblastic (Pittenger, 1999). *In vitro*, these cells can differentiate into osteoblasts, chondrocytes, adipocytes and myoblasts (Friedenstein, 1987; Howlett, 1986; Keating, 1990; Wakitani, 1995). Due to their expansion and multilineage differentiation *ex vivo*, these cells were called mesenchymal stem cells. They were more recently renamed multipotent mesenchymal stromal cells, as of a consensus stated by the International Society for Cellular Therapy (Horwitz, 2005). The terminology "mesenchymal stem cells" should be used only for cells from primary tissues that can generate colony forming units-fibroblasts (CFU-F), and tissue repopulation with multilineage differentiation *in vivo* (Horwitz, 2005).

During the last decade, research interests in the scientific community has focused on a particular group of tissues called as "perinatal tissues" which include fetal annexes such as placenta, amniotic membranes and umbilical cord [3, 4, 5]. Several reports have demonstrated many advantages regarding the use of these cells over embryonic and adult stem cells, suggesting that perinatal stem cells may represent the most promising stem cell source for regenerative medicine and for the treatment of various diseases.

The potential utility of the cells lies in their multifunctional properties: They modulate the immune system, enhance engraftment of hematopoietic stem cells, promote tissue

healing and contribute to structures such as bone, cartilage and fat. In addition, as culture-expanded cells, they may provide critical trophic support for normal tissue maintenance and protection and recovery from tissue injury [6].

The introduction of a newer, non controversial source of stem cells from the Wharton's jelly by Dr Mark Weiss and Colleagues way back in 2006 marked the advent of several investigations in this domain [7].

As large numbers of cells are required for MSCs clinical applications (cells doses up to 5 million MSC/kg body weight) [8, 9], together with the low frequency of MSCs in tissues, we require an efficient and Good Manufacturing Practices (GMP)-compliant ex-vivo expansion process. The field, nevertheless, faces several challenges. Persistent issues include the definition of a MSC and comparability between MSC preparations. This is because of inherent cell heterogeneity, the absence of markers that are unique to MSCs, and the difficulty in precisely defining them by developmental origin. Differences in the properties of MSCs also depend on the site of tissue harvest, phenotypic and genotypic characteristics of the donor and the isolation, expansion and storage methods used. These differences may be sufficient to ensure that attributes of the final MSC product could differ in potentially significant ways [10].

The International Society for Cellular Therapy (ISCT) established the minimal requirements of the MSC: (1) plastic adherence, (2) positive expression of the cell surface markers CD73, CD90 and CD105, and negative expression for the hematopoietic markers CD34 and CD45, (3) MSCs need to demonstrate the ability to differentiate into mesenchymal cell types including adipocytes, chondrocytes and osteoblasts [11].

In this study we are focusing on the expansion and characterization of MSCs isolated from Human Umbilical Cord Wharton's Jelly using our patented protocol and create a master cell bank. Derive single cell clones from the master cell bank established employing limiting dilution trying to establish cells committed to a single lineage and better understand the proliferation capacity of the cells.

MATERIALS AND METHODS

Cell Isolation:

The Umbilical Cords (UCs) from consenting full-term caesarian section patients were provided immediately upon delivery in a previously supplied tube containing Ringer Lactate containing gentamicin (50 µg/ml; Abbott). The collected UCs was processed and cultured using our patented protocol (132/CHE/2010 A – Number 272353).

Cell Culture:

The cells were plated in 6 well tissue culture polystyrene plate (Falcon) in medium (DMEM-F12 Low glucose with 20% Fetal Bovine Serum - Gibco), which was changed twice weekly. At day 7, adherent cells, judged 80%–90% confluent by phase contrast microscopy, were passaged using 0.25% trypsin -1mM EDTA solution (Gibco). They were then plated in 6 well tissue culture polystyrene plates at 1000cells/cm^2 in medium. The growth kinetics (Population doubling time) was noted.

$$\mathbf{PD = \frac{log(harvest\text{-}seeded)}{log2}}$$

Passaging cell and cryopreservation:

During UCMSCs expansion, cells were subculture every 7^{th} day irrespective of confluency. They were passaged and re-seeded or frozen down. Briefly, the adherent monolayer was washed with normal saline, detached with 0.25 % trypsin 1mM EDTA enzyme treatment for 2-3 min at 37 °C and the trypsin inactivated with 20 % FBS-containing media. The cells were then centrifuged at 1500rpm for 5 min and the cell pellet suspended in the appropriate volume of media and seeded at 1000cells/cm2 or 20 % FBS-containing media containing 10 % v/v dimethyl sulfoxide (Origen) for storage in liquid nitrogen to create a backup.

Limiting Dilution:

The P3 (D22) cells from the master cell stock was taken and was serially diluted till we get 1cell/100µL with medium and was plates in a 96 well tissue culture polystyrene plate (Falcon). Medium was changed every third day. Upon confluency the single cell clones was harvested and sub cultured at a density of 1000cells/cm^2 as the master cell stock and the growth kinetics was noted.

Surface marker analysis:

For flowcytometric analysis of cultured cell phenotypes, cells were stained with antibodies according to BD Stemflow hMSC Analysis Kit and the percentage of expressed cell surface antigen was calculated for 10,000 gated-cell events.

In Vitro Multi-Lineage Differentiation:

Multi-lineage potential was assessed by incubating cells under specific conditions to

induce differentiation into osteoblasts, chondrocytes, and adipocytes. After differentiation, multi-lineage potential was evaluated. Briefly, osteoblast formation was assessed by measuring the level of alizarine red staining (Sigma); chondrocyte formation was determined by alcian blue (Sigma); adipocyte formation was assessed based on staining of accumulated lipid vacuoles with oil Red O (Sigma).

Senescence-Associated β-Gal Staining (Gal Staining):

Senescence β-Galactosidase Staining Kit (Cell Signaling) was used as a biomarker of senescence in MSCs. Gal activity was qualitatively assessed as per the manufacturer's instructions, followed by microscopy. The percentage of senescent cells was represented by the number of stained cells in the total population.

Cell cycle analysis:

Ethanol-fixed 1×10^6 cells were suspended in 1 ml of staining solution (PI - propidium iodide, 5 μg/ml in PBS). Then cells were incubated for 30 min at 37°C in the dark and the fluorescence was analyzed using a flow cytometer. 10000-20000 events were scored from each stained sample and analyzed. Cells in S and G2/M phases of cell cycle were analyzed together and used as an indirect measure of proliferative activity of cells.

CFU-F Assays:

The cells are plated in a 35mm culture dish at a concentration of 150 cells per plate in 2mL medium. The cells are incubated at standard condition for about 14 days with media change every other day. On day 14, the culture was stained with geimsa and colonies with 25cells were enumerated.

Karyotyping:

The cells were treated with Colchicine and was harvested using trypsin-EDTA. The cells were treated with 0.075M solution of KCl and fixed with 3:1 mixture of methanol and glacial acetic acid. The fixed cell suspension was dropped on to the slide and slides were differentially stained for karyotyping with Giemsa. For each MSC culture at least 5 metaphases were analyzed.

Gene expression analysis:

RNA is isolated using trizol reagent (Bangalore Genei M-MuLV RT PCR KIT). After

purification of RNA, conversion to cDNA is necessary in order to proceed to q-PCR and was done according to manufacturer's protocol (Bangalore Genei). cDNA amplification for Sox 2, Oct 4, Nanog and beta-Actin is performed by using Ependorf Thermo Cycler Machine. Primer sequence of the genes are shown in Table: 1, and PCR conditions for these genes are listed in Table: 2. The amplified product was was run on 1% agarose gel.

Table 1: Primer sequence and their product size.

Gene	Forward primer	Reverse primer	size (bp)
Sox2	GCCCAGGAGAACCCCAAGAT	GGGTGCCCTGCTGCGAGTA	520
Oct4	GAAGCTGGACAAGGAGAAGCT	CATGCTCTCCAGGTTGCCTC	379
Nanog	GCGAATCTTCACCAATG	TTTCTGCCACCTCTTAC	407
Beta-ACTIN	GCGGCATCCACGAAACTAC	TGATCTCCTTCTGCATCCTGTC	138

Table 2: PCR conditions for amplification of cDNA.

Gene	Initial Denaturation	Denaturation	Annealing	Extension	Cycles	Final extension
Sox2	95^{o}C, 5min	94^{o}C, 30sec	58^{o}C, 30sec	72^{o}C, 30sec	30	72^{o}C, 5mins
Oct4	95^{o}C, 5min	94^{o}C, 30sec	58^{o}C, 30sec	72^{o}C, 30sec	30	72^{o}C, 5mins
Nanog	95^{o}C, 5min	94^{o}C, 30sec	54^{o}C, 30sec	72^{o}C, 30sec	30	72^{o}C, 5mins
Beta-ACTIN	95^{o}C, 5min	94^{o}C, 30sec	58^{o}C, 30sec	72^{o}C, 30sec	30	72^{o}C, 5mins

Real-time quantitative PCR:

The real-time PCR amplification reactions were conducted using a Real-time PCR System instrument (Applied Biosystems) to determine the differentiation gene expression of the differentiated cells to confirm differentiation into adipocyte, osteocyte and chnondrocyte. Undifferentiated Bone Marrow mesenchymal stem cells were used as a base line for gene expression. We selected one marker for each lineage i.e., Col 10 for chondrogenic lineage, FABP 4 for adipogenic lineage, BGLAP for osteogenic lineage 18s as an endogenous internal control.

RESULTS

WJ-MSCs isolated using our patented method and cultured in DMEM-F12 supplemented with FBS under microscopic observation showed that WJ-MSCs cells were plastic adherent (figure: 1A) and had fibroblast-like morphology (figure: 1C),

which is one of the prime property of MSCs and this property was retained till passage 18 (figure: 2-A to D). Population doubling time was calculated from passage 1 through 18 to measure the proliferative capacity of WJ-MSCs master cell bank and were able to maintain stable population doubling time of 33 hours until passage 18 (figure: 3). Since there was no significant increase or decrease in doubling and the cells had undergone 105 doubling which was more than sufficient to make them a viable source when compared to bone marrow MSC could only undergo about 50 doublings.

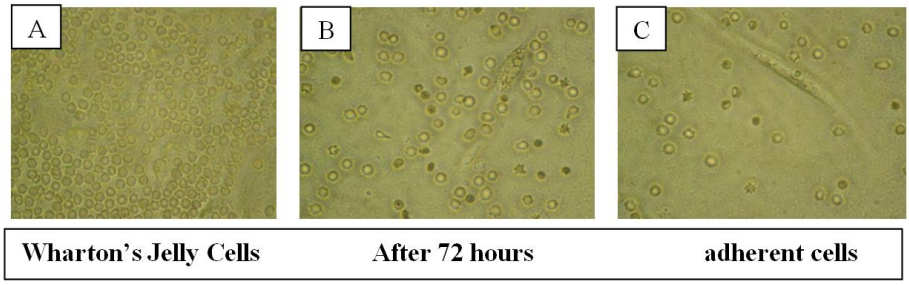

Fig.1: (A) Wharton's Jelly derived non adherent cell on Day 1 (B) Cells in culture plate on day 3, (C) Adherent mesenchymal cell after washing on day 3.

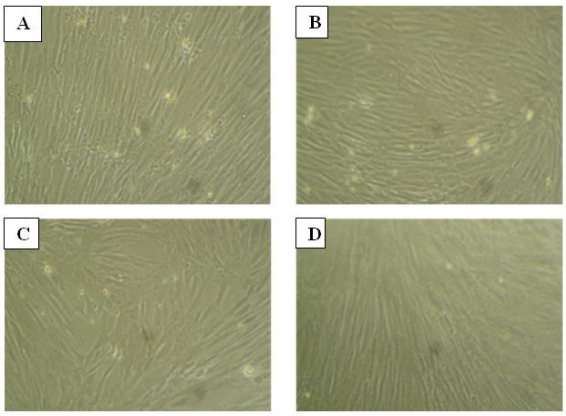

Figure 2: (A) confluent culture at the time of trypsinization master cell bank early passage, (B) confluent culture at the time of trypsinization master cell bank mid passage, (C) confluent culture at the time of trypsinization master cell bank late passage, (D) confluent culture at the time of trypsinization Bone marrow.

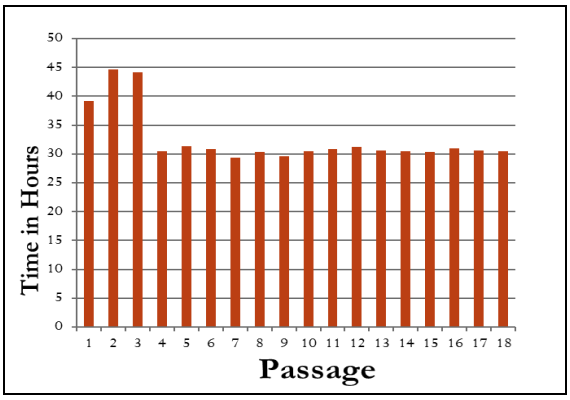

Figure 3: Graph showing doubling time in hours of the Master cell bank.

The cells from the master stock at P3 which were subjected for limiting dilution had a doubling time ranging from 100hours to 300hours initially (figure: 4A) but after passage 7 and passage 9 they stabilized and displayed a doubling time of 33hour as comparable to master cell bank (figure: 4B). Also the single cell clones indicated that there are two population of cells with different doubling time, based on which they were classified into fast clone (Clone F) and slow clone (Clone S).

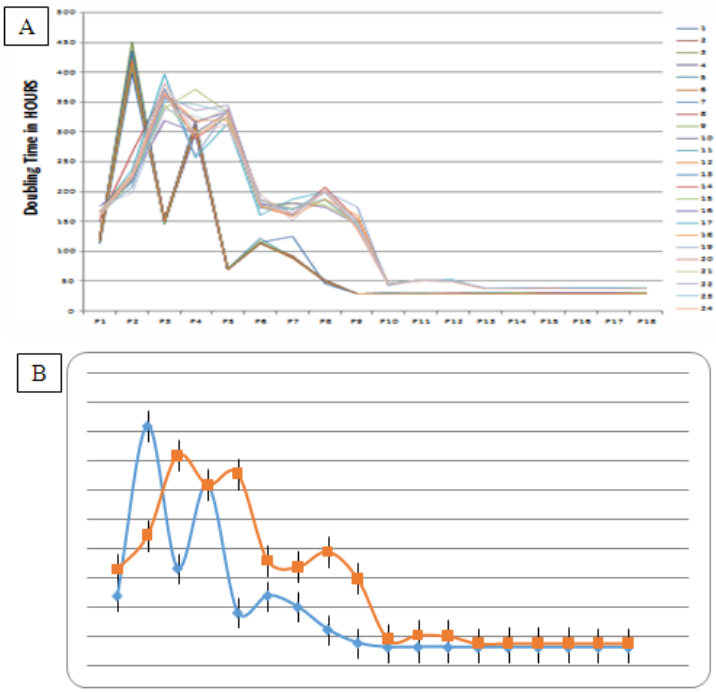

Figure:4: (A) Proliferation Kinetics of all the clones showing two distinct populations (Clone F as fast growing and Clone S as slow growing), (B) Graph showing the proliferation kinetics of Clones F and Clone S.

BETA GAL SENESCENCE ASSOCIATED ASSAY

The activity of the enzyme β-Gal was assessed at pH 6 using the Senescent Cells Staining Kit and calculating the percentage of MSC's staining bluish green (Figure: 5 C-G). The mean percentage of β-Gal positive cells of master cell bank and clones are shown in table: 3. Mesenchymal Stem Cells was also subjected for heat shock and were analyzed for percentage senescent cells which served as positive control (Figure: 5B). HeLa Cells subjected for heat shock were also analyzed as a control (Figure: 5 H-I).

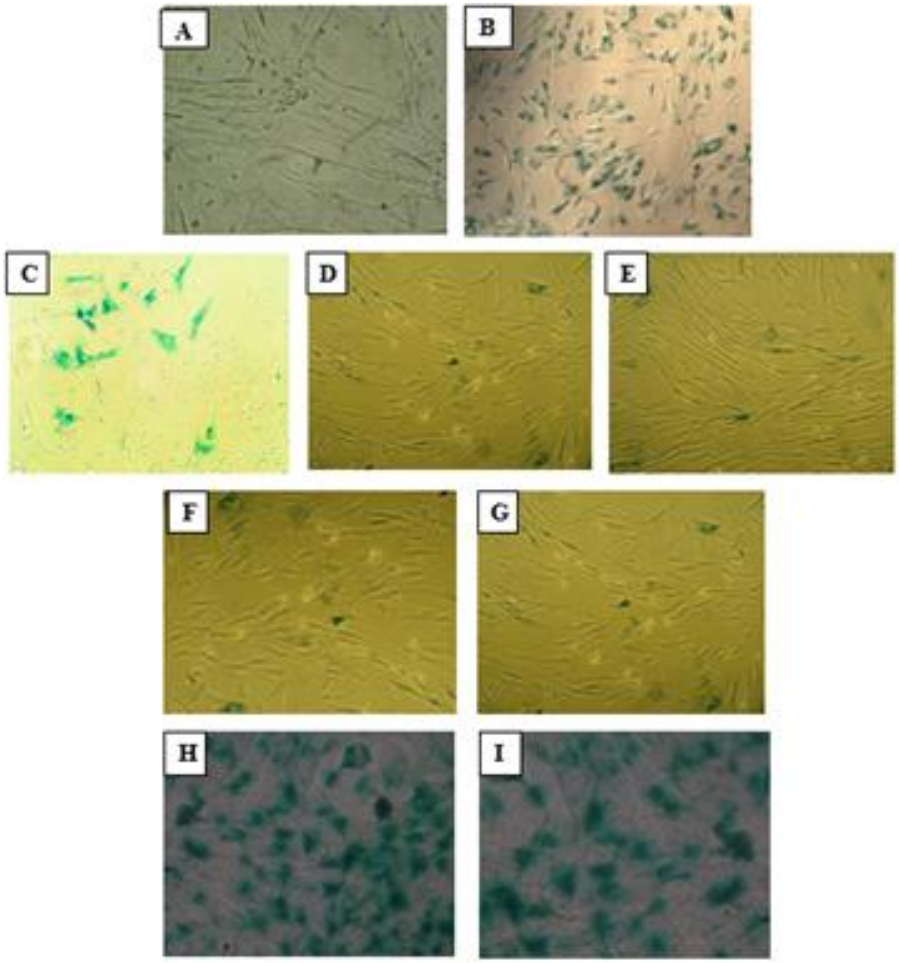

Figure 5: Collated data of 3 independent experiments (A-I) representative of 3 independent experiments, (A) MSC control sample showing negative for senescence, (B) MSC heat shock (positive control), (C) Master cell bank early passage, (D) Master cell bank mid passage, (E) Master cell bank late passage, (G) Fast clone, (H) Slow clone, (H-I) HeLa heat shock (positive control).

Table 3: Collated data of 3 independent experiments (A) table showing % positive cells.

Passage No.	%Senescent cells
Master Stock Early Passage (P3/D22)	8.10±1.5%
Master Stock Mid Passage (P7/D44)	8.38±1.4%
Master Stock Late Passage (P18/D105)	9.29±0.94%
Fast Clone (P7/D16)	7.98±1.65%
Slow Clone (P9/D16)	8.41±1.46%

DIFFERENTIATION

The differentiation potential of cells from master cell bank early passage cells (P3, D22), mid passage cells (P7, D44), late passage cells (P18, D105), (Figure: 6), fast clone cells (P7, D16) and slow clone cells (P9, D16) (figure: 7) was determined by culturing in either adipogenic, osteogenic or chondrogenic medium for 21 days. Adipogenic differentiation was confirmed by the appearance of large cytoplasmic lipid vacuoles that stained red using Oil Red O. MSC's cultured in control (only DMEM-F12 media) did not stain. Osteogenic differentiation was confirmed by the accumulation of cell aggregates and calcium deposits that stained red with Alizarin Red S. MSC's cultured in control media did not stain. Chondrogenic differentiation was confirmed by accumulation of proteogylcans that stained with Alcian blue. MSC's cultured in control media did not stain. W138 fibroblast cells were also subjected for differentiation and stained accordingly which did not stain and indicated that they lack the potency to differentiate. Bone Marrow Mesenchymal Stem Cells (BM-MSCs) were also subjected for differentiation and stained accordingly which stained and indicated their differentiation potency and severed as a control (figure: 6).

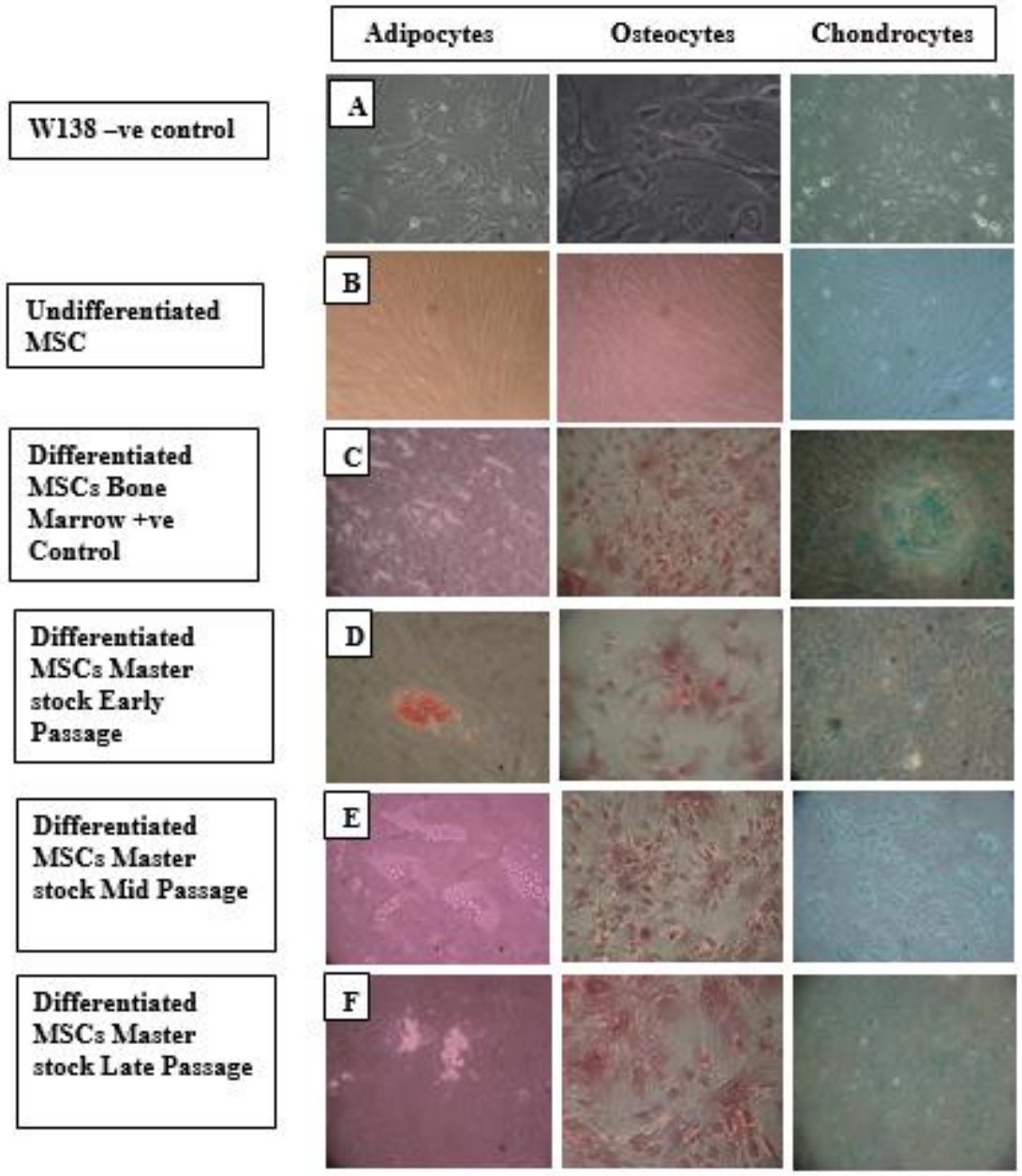

Figure 6: Collated data of 3 independent experiments. (A) W138 –ve control, (B) Undifferentiated Cells, (D) differentiated cells of bone marrow +ve control, (D-F) representing one of 3 independent experiments for the master stock. (D) differentiated cells of master stock early passage, (E) differentiated cells of master stock mid passage, (F) differentiated cells of master stock late passage.

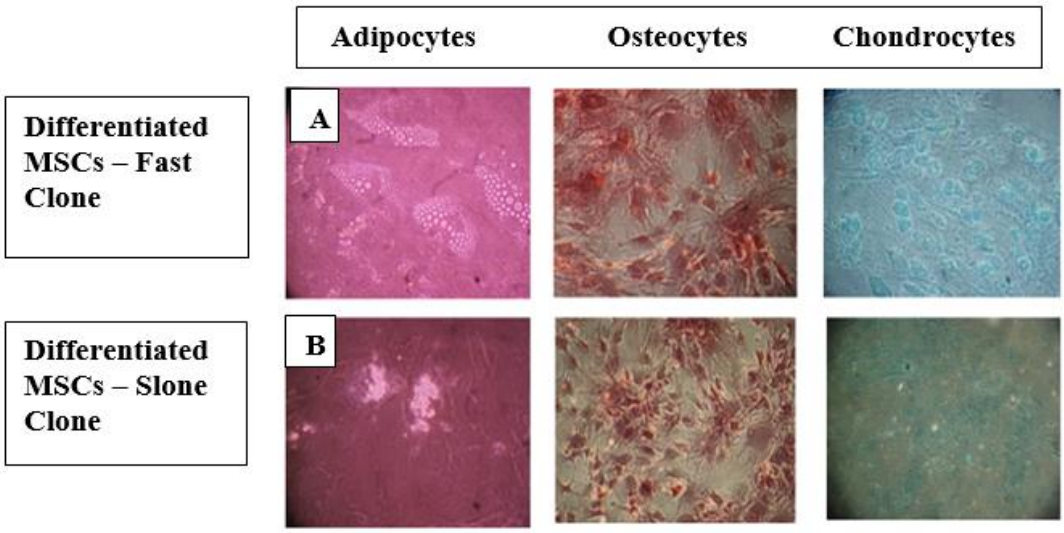

Figure 7: (A-B) representing one of 3 independent experiments for theSingle cell clones. (A) differentiated cells of Fast clone, (B) differentiated cells of Slow clone.

Table 4: Collated data of 3 independent experiments showing the different percentage of differentiated MSCs of master stock cells, single cell clones, bone marrow cells, and W138.

	Adipocytes	**Osteocytes**	**Chondrocytes**
Master Stock Early Passage (P3/D22)	25.2±10.89%	99.9±0.1%	20.57±10.84%
Master Stock Mid Passage (P7/D44)	25.37±9.51%	99.9±0.1%	27.69±10.03%
Master Stock Late Passage (P18/D105)	27.64±11.21%	99.9±0.1%	26.84±11.62%
Fast Clone (P7/D16)	26.18±9.36%	99.9±0.1%	23.67±10.64%
Slow Clone (P9/D16)	24.64±10.31%	99.9 ±0.1%	27.87±11.26%
Bone Marrow Control (P3/D22)	25.68%±8.65%	99.9%±0.1%	23.41%±9.62%
W138	Nil	Nil	Nil

SURFACE MARKER ANALYSIS

WJ-MSCs were analyzed for expression of mesenchymal stem cell markers. The cells staining negative for hematopoietic surface markers were gated to estimate the percentage of mesenchymal stem cells. The gated MSC population was heterogeneous in size and granularity as demonstrated by forward and side scatter profiles (Figure: 8). Bone Marrow mesenchymal stem cells were used as a standard positive control. The cells of master stock early passage (P3, D22), master stock mid passage (P7, D44), master stock late passage (P18, D105), fast clone (P7, D16) and slow clone (P9, D16) were analyzed and the percentage expression of each surface marker and the overall mean are shown in Table: 5.

A panel of markers was tested. All WJ-MSCs preparations were negative for hematopoietic marker CD34 and were positive for CD44, CD73, CD90 and CD105, which are known to be expressed on MSCs (Table: 5) and their respective histograms are shown in figure: 10. It was found that as the number of doubling increased CD90 expression increased the expression of other MSC markers like CD44, CD73 and CD105 decreased drastically.

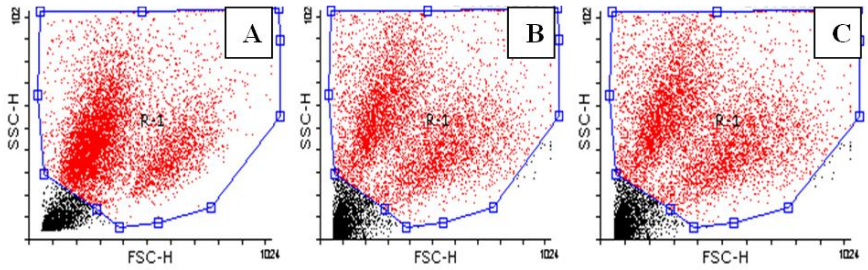

Figure 8: Scatter profiles of MSCs. (A) representative scatter profiles of Master Stock, (B) representative scatter profiles of fast clone (C) representative scatter profiles of slow clone.

Table 5: Table showing the percentage population of each marker at different passages. representing one of 3 independent experiments for the master cell bank, single cell clones and bone marrow.

Markers	Master Stock Early Passage (P3/D22)	Master Stock Mid Passage (P7/D44)	Master Stock Late Passage (P18/D105)	Fast Clone (P7/D16)	Slow Clone (P9/D16)	Bone Marrow Passage (P3/D18)
CD44	96.63	96	38.47	24.32	31.61	45.63
CD73	73.98	63.94	35.53	15.14	18.04	36.54
CD90	0.77	0.98	25.21	13.74	16.80	28.39
CD105	90.02	70.57	13.48	1.02	1.72	16.94

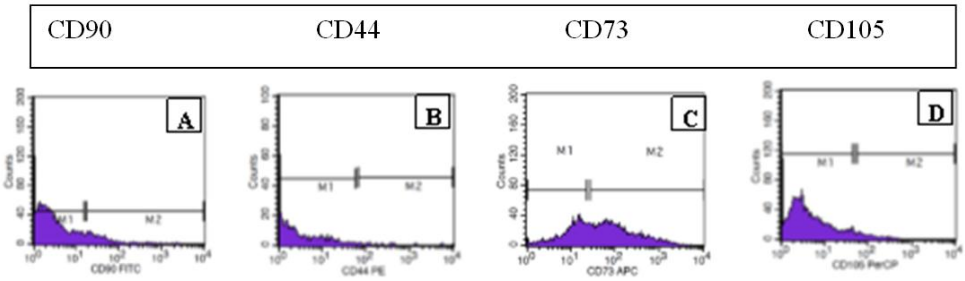

Figure 9: (A-D) Histogram showing the expression levels of the markers of bone marrow control, representing one of 3 independent experiments of bone marrow.

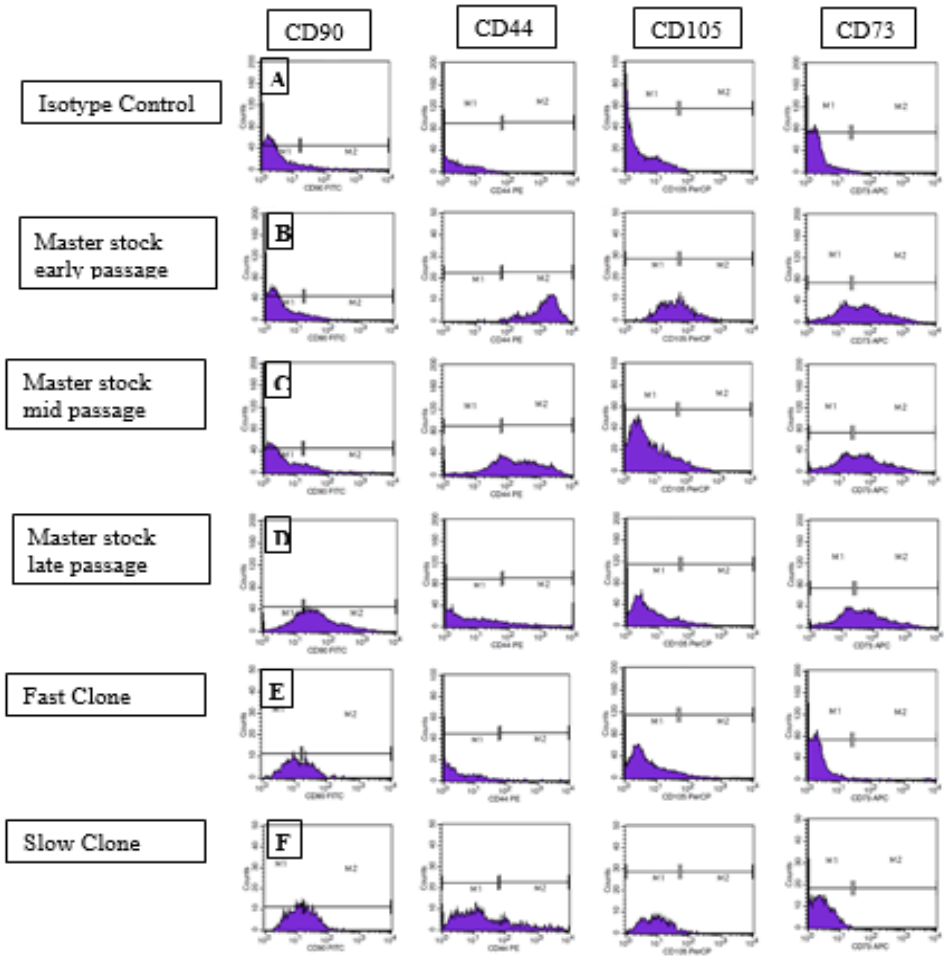

Figure 10: Histogram showing the expression levels of the markers, (A) Isotype controls, (B) master stock early passage, (C) master stock mid passage, (D) master stock late passage, (E) fast clone, (F) slow clone, representing one of 3 independent experiments for the master stock.

CELL CYCLE ANALYSIS

The cell cycle distribution of MSCs at various passages was determined by FACS analysis after the cellular DNA was stained with propidium iodide. Single-variable histograms of DNA provided data describing the percentages of cells in G0-G1, S, and G2-M. The histogram profile of cell cycle analysis master stock at early, mid and late passage along with fast and slow clone is shown in figure: 11. The percentage of cells distributed in various phases of cell cycle is shown in table: 6.

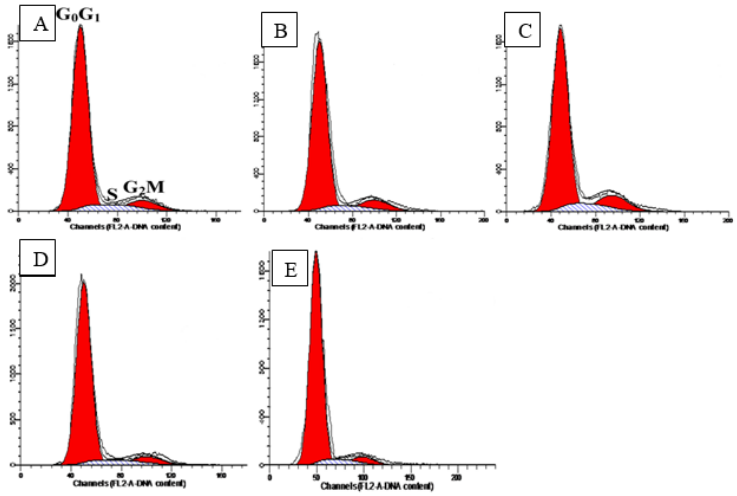

Figure 11: Showing cell cycle profile of (A) the master stock early passage, (B) master stock mid passage, (C) master stock late passage, (D) fast clone, (E) slow clone, representing one of 3 independent experiments.

Table 6: showing percentage of cells distributed in various phased of a cell cycle of master stock early passage, master stock mid passage, master stock late passage, fast clone and slow clone, representing one of 3 independent experiments.

	G0/G1	**S**	**G2/M**
Master Stock Early Passage (P3/D22)	54.28%±3.21%	27.7%±1.72%	12.44%±1.85%
Master Stock Mid Passage (P7/D44)	60.81%±1.96%	25.56%±2.05%	8.45%±2.16%
Master stock Late Passage(P18/D105)	68.64%±2.45%	23.95%±3.46%	7.39%±1.67%
Fast Clone (P7/D16)	74.50%±1.54%	10.66%±2.41%	8.61%±1.59%
Slow Clone (P9/D16)	71.98%±1.68%	13.85%±2.22%	8.9%±1.98%

COLONY FORMING UNIT-FIBROBLAST ASSAY (CFU-F)

CFU-F cultures on day 14 were stained with giemsa and colonies with 25cells were counted. Mesenchymal stem cells had the ability to form fibroblast like colonies (Figure: 12 A-E), were as W138 cells lacked the ability to form a colony (figure: 12 F). The percentages of cells with ability to form colonies form master stock early, mid and late passage along with fast and slow clone are shown in table: 7.

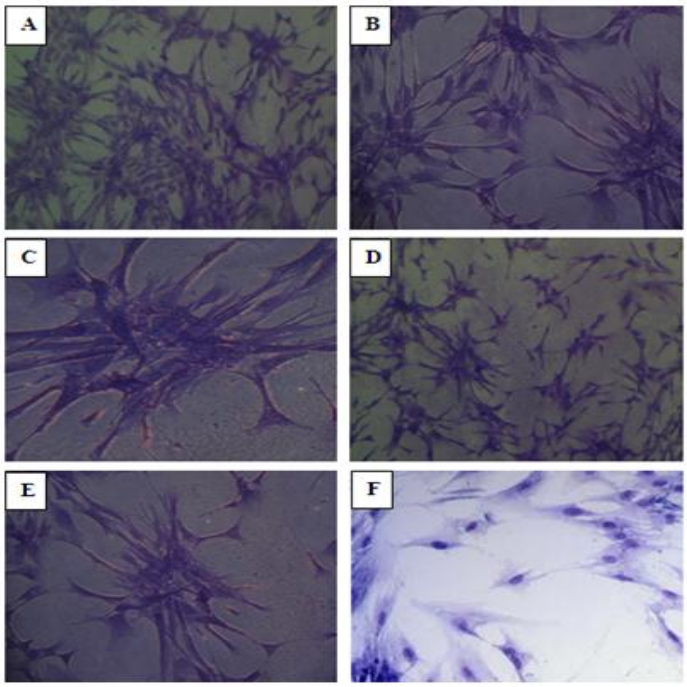

Figure 12: Collated data of 3 independent experiments (A) CFU-F colony of master stock early passage, (B) CFU-F colony of master stock mid passage, (C) master stock late passage, (D) CFU-F colony of fast clone, (E)CFU-F colony of slow clone, (F) W138 showing no colonies of CFU-F, representing one of 3 independent experiments.

Table 7: Table showing % of colonies formed from collated data of 3 independent experiments.

Groups	% of colonies Formed
Master Stock Early passage	39.87±1.06%
Master Stock Mid passage	32.85±1.02%
Master Stock Late passage	35.48±2.12%
Clone F	34.65±1.14%
Clone s	36.16±1.08%

CFU COLONY DIFFERENTIATION

CFU colonies from master stock early passage, mid passage and late passage along with fast and slow clones were subjected for differentiation into chondrocytes, adipocytes and osteocytes. It was found that the cells in the core of the colony was able to differentiate into chondrocytes and osteocytes (figure: 13 C), the cells in the periphery of the colony was able differentiate into adipocyte and osteocyte (figure: 13 A), and the cells outside the colony without the ability to form colony only differentiated into osteocytes (figure: 13 B).

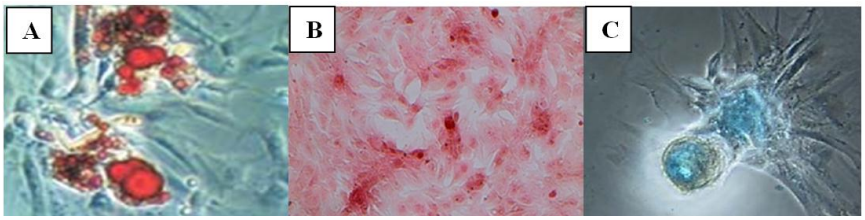

Figure 13: CFU colony differentiated into (A) adipocyte, (B) Osteocyte and (C) Chondrocyte.

Karyotyping:

The metaphase spreads of cells from master cell bank early passage (P3, D22), mid passage (P7, D44), late passage (P18, D105), fast clone (P7, D16) and slow clone (P9, D16) were subjected for G banding and it was found that they had a normal karyotype without any structural and numerical aberrations displayed genetic stability of the cells (Figure: 14).

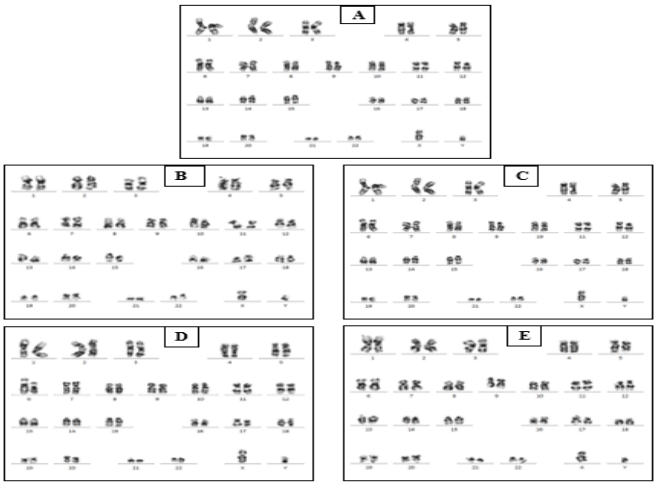

Figure 14: (A) Karyogram showing normal karyotype of master stock early passage, (B) Karyogram showing normal karyotype of master stock mid passage, (C) Karyogram showing normal karyotype of master stock late passage, (D) Karyogram showing normal karyotype of fast clone, (E) Karyogram showing normal karyotype of slow clone.

Pluripotency Gene Expression Analysis:

After the initial characterization of the properties of MSC populations, we verified expression of OCT4, SOX2, and NANOG in these cells. Expression of Nanog, OCT4 and SOX2 mRNAs was detected in MSC preparations analyzed. Expression of Beta-Actin was also analyzed as a control which is a house keeping gene. W138, MCF-7 and HeLa cells were also analyzed as a control for the expression of the four markers i.e., Nanog, OCT4, SOX2 and Beta-Actin. Except HeLa cells failing to express SOX2 and W138 cells failing to express OCT4, all the other cells expressed all the four gene (Figure: 15) indicating the cells retained the pluripotency property.

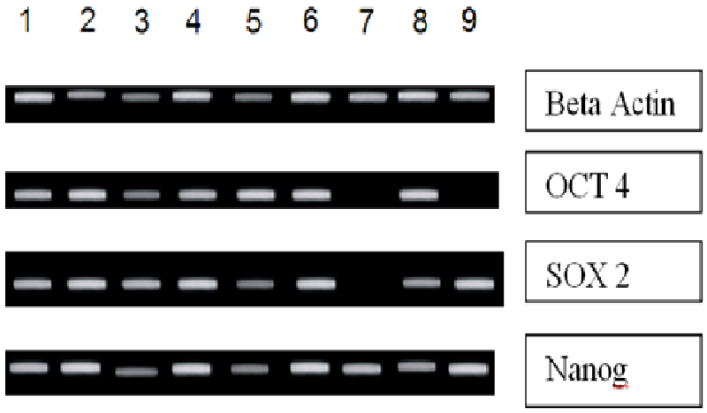

Figure 15: Consolidated gel picture showing expression of pluripotent genes from 3 independent experiments. Lane (1) Bone marrow +ve control, (2) Master stock early passage, (3) Master stock mid passage, (4) Master stock late passage, (5) Fast clone, (6) slow clone, (7) HeLa cells, (8) MCF 7 cells, (9) W138 cells.

REAL TIME PCR

The differentiated cells were analyzed for expression of lineage specific cell markers like Col 10 for chondrocytes, FABP 4 for adipocytes and BGLAP for osteocytes by Real Time PCR. It was found that the differentiated cells from master stock early, mid and late passage along with fast and slow clone had the property to differentiate into the mature bone, cartilage and fat forming cells. It is found that about 2.5-fold expression of BGLAP in osteocytes indicating that majority of cells differentiating into osteocytes and only a marginal increase in FABP4 (adipocyte gene) and COL10 (chondrocyte gene) of about 1.2 to 1.7 as normalized to undifferentiated cells (Figure: 16).

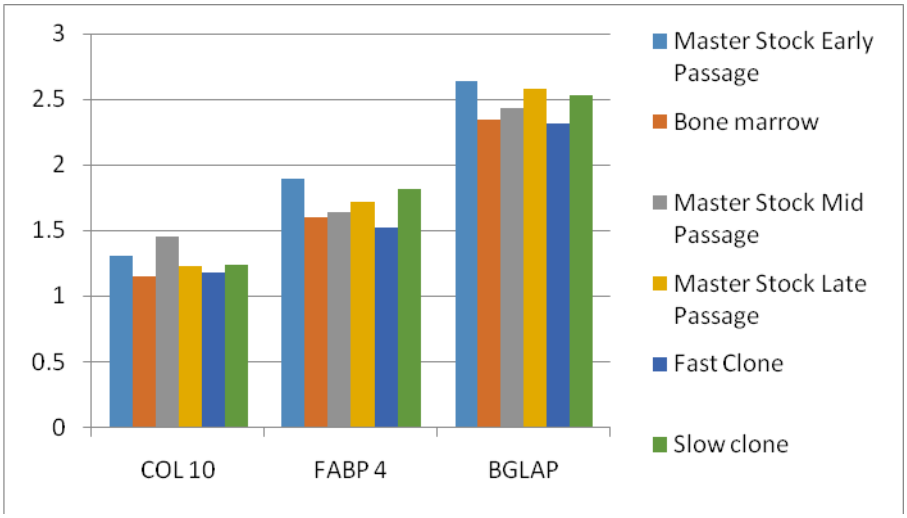

Figure 16: Collated graph of 3 independent experiments showing the fold change in gene expression of differentiated cells by Real time PCR.

DISCUSSION

The mesenchymal stem cell (MSC) field continues to rapidly progress with a number of clinical trials initiated and completed, with some reported successes in multiple clinical indications and a growing number of companies established. The field, nevertheless, faces several challenges. Persistent issues include the definition of a MSC and comparability between MSC preparations. This is because of inherent cell heterogeneity, the absence of markers that are unique to MSCs and the difficulty in precisely defining them by developmental origin. Differences in the properties of MSCs also depend on the site of tissue harvest, phenotypic and genotypic characteristics of the donor and the isolation and storage and expansion methods used. These differences may be sufficient to ensure that attributes of the final MSC product could differ in potentially significant ways. Since there are currently no gold standards, there is a need for well characterized MSCs.

In our study, cells showed adherence to the plastic dish surface and spindle-shaped fibroblast like morphology fulfilling the criteria laid by ISCT. Other defining characteristic of stem cells is their self-renewal potential, the ability to generate identical copies of themselves through mitotic division over extended time periods (even the entire lifetime of an organism). In this study we have found that WJ-MSCs had a doubling time of 33hours and this was constant till passage 18 (P18) by then the cells had undergone 105 doubling, retaining their self-renewal property making them ideal candidate for stem cell-based therapy as they could be expanded to get a higher number of cells. Because of their higher proliferation potential and these cells are expanded in vitro, it is essential to characterize these cells for their phenotypic and

functional characteristics. In our study we have subjected the cells for extensive characterization at three time points viz. Early passage (P3/D22), Mid passage (P7/D44) and Late passage (P18/D105).

Further to this, the cells were subjected for flow cytometry analysis to understand their surface phenotype for presence of MSC-specific markers (CD44, CD73, CD90, and CD105) and for the absence of the hematopoietic markers (CD34 and CD45) and were induced for differentiation to understand its ability to differentiate ability into adipocytes, chondrocytes and osteocytes.

We analyzed MSCs from master cell bank (early, mid and late passage) for expression of surface markers like CD44, CD90 and CD 105. Bone marrow cells were also analyzed as a standard. The flow cytometry forward and side scatter profiles of master cell bank indicated that there are two different populations of cells with different size and granularity. About 90% of the populations were positive for surface marker like CD44, CD73 and CD105 but showed very low expression of CD90 of about 1%. It was found that CD90 expression increased up to 25% as the number of doubling increased and indicating the creeping in of heterogeneity.

Master stock cells maintained their doubling time of 33 hours in culture with around 25% (average) of cells in S phase in sync with the literature [12, 13, 14] and could be kept in culture for 105 doublings. It is told that the G0/G1 and G2/M ratio is about 3:1 which is in sync with our data. It is found that as Go/G1 phase cells increased with the number of doubling it is also found that cells in S phase decrease [15, 16, 17]. Thus these cells were divided into early, mid and late passage and put to test. Cells did not display senescence as assessed by the beta galactosidase assay, the fact that we observed a gradual slow down as seen in less percentage of cells in S phase in the late passages (doubling 105). Cells at 105 doublings (late passage) also displayed a normal karyotype.

In our studies with master stock cells, functional assay of differentiation indicated that only 30% of cells responded to chondrogenic cues and adipogenic cues. Nearly all cells responded to osteogenic cues [18, 19, 20]. Although the CFU-F colony assay confirmed the number of stem cells capable of forming colonies were in the range of 30%. This could be a reason for observing around 30% cells capable of differentiation into adipose and chondrocyte lineage. Cells which were not in the core of the CFU colony were more amenable to osteocyte differentiation. This could infer that unipotent cells in culture chose to respond to osteocyte differentiation as a default pathway. Non responder cells (i.e. 70% cells in adipose and chondrocyte differentiation plates) maintained a status quo. These cells could have become committed fibroblasts, but needs validation.

Therefore, these cells were assayed for CFU-F assay which is considered as a gold standard test for “stemness” were only a true stem cells could form a colony but not

the committed cells. Interestingly it was found that only about 30% of the cells were able to form colonies but all in master cell bank (Early, mid and late passage), fast clones and slow clones. Thus, indicating that heterogeneity is an intrinsic problem in MSC population.

Further to the above data, we had to answer if these cells are truly pluripotent retaining their pluripotency gene expressions such as OCT4, SOX2 and Nanog. Global gene expression profiling is a useful tool to identify genes involved in stem cell commitment and differentiation as a function of different inductive microenvironments. In fact, this approach has already been used successfully to identify genes that regulate osteogenic, adipogenic, and chondrogenic differentiation of MSCs [21, 22]. It was found that the master cell stock (Early, Mid and Late passage), Fast clone and slow clone retained their pluripotency gene expression indicating pluripotency of these cells.

An important finding is that a large percentage of the cultured cells, mainly in Wharton's jelly, remained in the quiescent state (G0/G1). Quiescence is a peculiar feature of stem cells *in vivo* although they are characterized by a high proliferative capability and show a huge self-renewal potential *in vitro*. To confirm this hypothesis, the MSCs were subjected for cell cycle analysis by flow cytometry and was found that the percentage of G0/G1 cells in master cell stock is about 50%-60%, were as in single cell clone it was increase to about 75%. Therefore, it is important to analyze that are these quiescent G0/G1 cells are senescent [23, 24, 25, 26, 27].

Senescence is an underestimated problem in MSC production. MSCs have only limited number of population doublings, known as Hayflick limit, before senescence growth arrest occurs (Hayflick, 1963). In MSC, this is typically from 20 to 50 doublings, depending on cell source and culture conditions (Izadpanah et al, 2006; Suchánek et al, 2007, Cholewa et al, 2011). In our study, we subjected MSCs cells from master cell bank and single cell clones for beta-galactosidase assay to check the number of cell senescent. It is found that only about 8% to 10% of the population was senescing [28, 29].

Previous results confirmed that modifications in the cell phenotype as well as in their behavior, or genetic changes in karyotype might occur depending on long-term cultures *in vitro*, reducing the feasibility of the cultured cells for therapeutic purposes. In our study we found cells from master cell bank (Early, Mid and Late passage) and single cell clones (Fast and Slow) had a normal karyotype without any structural and numerical aberrations [30, 31].

To further confirm the differentiation potential of these cells, the differentiated cells were analyzed for expression of lineage specific gene such as BGLAP for Osteocytes, FABP4 for adipocytes and COL10 for chondrocyte by Real Time PCR. It was found that the osteocyte cultures had a significant increase of about 2 folds indicating that

most of the cells were differentiating into osteocytes. Whereas, the adipocyte and chondrocyte cultures had slightly higher level as that of base of about 1.2 fold to 1.4 fold in indicating only a subset population in the culture is able to differentiate into adipogenic and chondrogeneic lineage [32, 33, 34].

CONCLUSION

In conclusion the study demonstrates the possibility of large scale production of Wharton's jelly derived stem cells. The fact that they retain their properties till late passage shows that expansion does not improve on their stemness. Clinical trials have clearly shown that allogeneic mesenchymal stem cells can be utilized due to their hypo immunogenic properties. This provides new avenues for clinical applications wherein off the shelf mesenchymal stem cells could be made available for clinical conditions. Optimal methods have to be incorporated to deliver cells in targeted areas. With stem cells, we are presented with a versatile material with which we may be able to rebuild many structures found in the body. But the challenge of how to construct three-dimensional tissues from them still remains. Recently, biologists and materials scientists have realized that scaffolds can and should be designed that direct and enhance cell function and differentiation.

Composites like collagen membranes on which mesenchymal stem cells can be grown is to be thought off. The type of material and the cues that are incorporated in the scaffold play a large role in directing the fate of the stem cells seeded inside. Different scaffold materials can be blended together to achieve additional properties and benefits. Also, many different biomaterials exist that have not yet been adapted for use with stem cell culture, which could be explored in future studies. Another consideration for developing engineered scaffolds containing stem cells is how to incorporate or produce of a vascular network inside of the scaffold that would allow it to integrate with actual tissue and restore function lost after injury or disease. It can be achieved through a variety of ways including through co-culture with endothelial cells or by adding cues to promote angiogenesis into the scaffolds. Overall, this can provide a starting point for further development of biomaterial scaffolds combined with stem cells for tissue engineering applications.

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