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ISOLATION, CHARACTERIZATION AND SCALE-UP OF FOETAL AMNIOTIC MEMBRANE DERIVED MULTIPOTENT STROMAL CELLS FOR THERAPEUTIC APPLICATIONS

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ABSTRACT

Stem cells are the future frontier in curing diseases and preventing ailments. Recent studies have shown that Multipotent Stromal Cells are promising candidates for stem cell therapy and regenerative medicine. Amniotic membrane is a good source of MSCs and potentially be used for regenerative medicine. However, the use of MSCs for therapeutic application is based on their subsequent large-scale *in-vitro* expansion. A fast and efficient protocol for generation of large quantities of MSCs is required to meet the clinical demand and biomedical research needs. In the present study, we successfully isolated, characterized and up-scaled up to P2 to get as many as $5.57x10^9$ cells from a single placenta and also observed up to P10 with no significant change in viability, stemness and karyotyping. To confirm differentiation potential, a multi-lineage differentiation assay was conducted for their exploitation and proliferation was measured based on CPDL. Immuno-phenotyping showed that major population was of mesenchymal origin. We concluded that AM-MSCs is a promising candidate for stem cell therapy.

KEYWORDS: Multipotent Stromal Cells (MSCs), Amniotic Membrane (AM), Cumulative Population Doubling Level (CPDL), Passage (P), Cluster of Differentiation (CD), Umbilical Cord (UC).

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INTRODUCTION

Recent progresses in the study of stem cells have opened new perspectives for their use in cell therapy. The current sources of stem cells are embryonic stem cells and adult stem cells; however, these pose many problems, both ethically and technically. The find other stem cell sources that do not raise ethical problems, easily accessible and sufficiently numerous to be used for therapeutic purposes has to be taken in approaching a new strategy in stem cell isolation. The new source that meets all these requirements is human placenta. Hassle free isolation, lack of any ethical issues, expansion, multilineage differentiation capacity are other added functional advantages making them a more rewarding alternative for existing stem cells therapy and regenerative medicines. So far bone marrow is the most widely accepted, classic source of MSCs. However, for clinical use bone marrow may not be an ideal candidate due to its highly invasive procedure, rapid decline in cell number and differentiation potential with the increasing age and limited potency[1].

Currently, many efforts are being performed for the development of different cell-based systems as test objects for determination of drug related effects in pharmacological screening using MSCs. Still further study is necessary to determine standardization of clinical protocols and its limitations in stem cell culturing. It is unknown till date, whether MSCs engraft in the targeted area for a longer period of time and whether display self-renewal as well as multilineage potency in vivo[2,3]. Since the amount of MSCs needed in the tissue regeneration is very high; scaling up is necessary for the successful clinical application. For this reason only establishing parameters for GMP related expansion and routine clinical application of expanded cells as well as minimum doses required to avoid thrombo-embolic complication and infection are critical aspects to be determined.

The aim of this study was to isolate, characterize and scale-up of Multipotent Stromal Cells from foetal amniotic membrane in order to prove their stem cell properties, because these are less studied and are supposed to have an increased differentiation potential toward an important number of cell types, which is significantly higher than that of adult type stem cells. We here in the current investigation have tried to expand the amniotic membrane derived stem cells at minimum passage level for the successful therapeutic application. We successfully isolated a large number of AM-MSCs at Passage 2 (P2); sufficient for clinical application. We carried out different quality analysis to check the efficacy of isolated cells at various stages of expansion.

MATERIALS AND METHODS

(i) Isolation of Amniotic membrane Multipotent Stromal Cells

Amniotic membrane (n = 5) was obtained from a Caesarian delivery process with parental permission. In sterile condition, entire amniotic membrane transferred into a labeled tube containing collection medium with antibiotics. Collection medium used in our study was Dulbecco's modified Eagle medium - Nutrient mixture Ham's F-12 (1:1) with L-Glutamine (1X); 2.438g/L Sodium Bicarbonate (DMEM/F12; Gibco, USA) and 0.125 mg/ml Cefoperazone sodium[4] (MP Biomedicals, LLC), The collected amniotic membranes were washed with 0.9% normal saline three times under sterile conditions to remove unwanted blood clots and debris. To detach and remove the epithelial layer from the amniotic membranes, the membranes were treated with 0.125% trypsin for 30 mints at 37^0C. After trypsinization, the membranes were washed with 0.9% normal saline three times. After washing, the amniotic membrane was minced 20mm^3 in size with a surgical blade. The minced tissue was digested at 40^0C for approximately 1h with collagenase type I (2 mg/mL; Worthington Biochemical, USA). The digested samples were washed in phosphate-buffered saline (PBS; Corning, USA) and centrifuged at 350 × g for 10 min. The cell pellet was resuspended in basal culture medium for future use.

(ii) Primary expansion

The cell pellet was plated in tissue-culture-grade T-75 flask (Corning, NY, USA) containing Dulbecco's modified Eagle medium – Nutrient mixture Ham's F-12 (1:1) with Glutamax (1X); 2.438g/L Sodium Bicarbonate; Sodium Pyruvate (DMEM/F12+; Gibco, USA) and 10% fetal bovine serum (FBS; Gibco, US) supplemented with 3ng/mL bFGF (Sigma, USA), 0.0625 mg/ml Cefoperazone sodium[4] (MP Biomedicals, LLC) was used for all the experiments. All flasks were left undisturbed in a 5% CO_2 incubator at 37°C for 4-5 days and later fresh culture medium was added to the flasks. Adherent cells were allowed to expand for 8–12 days by changing the media at an interval of 4-5 days. Cells were harvested and counted at 70–80% confluency using 0.25% trypsin (Gibco, USA).

(iii) Seeding density for culture flasks

For up- scaling experiments, harvested cells at P0 were seeded at density of 3000 cells/ cm^2 for passage1 in T-175 tissue culture flasks (Corning, NY, USA) and 3000 cells/cm^2 were plated at passage2 in hyper flasks (Corning, NY, USA). The harvested cells were suspended in a cryoprotectant solution composed of 90% complete media and 10% dimethyl sulfoxide (DMSO) (Origen Biomedical, USA) and stored in the vapor phase of a liquid nitrogen tank until further use.

(iv) Cumulative Population Doubling Level (CPDL) analysis

A cell proliferation assay was performed as described with some modifications[5]. Estimated growth efficiency and proliferation potential of the amniotic membrane MSCs were determined based on the total CPDL using the formula CPDL = ln (Nf / Ni) ln2, where Ni is the initial number of cells seeded, Nf is the final number of harvested cells, and ln is the natural log. Cells (3000 cells/ cm^2) were plated in culture flask and subcultured for 4-5 days. The cells were counted and subculture in a culture flask with 3000 cells/cm^2 as seeding capacity. To determine the CPDL, the population doubling for each passage was calculated and then added to the population doubling levels of the previous passage levels.

(v) Immunophenotyping

Harvested cells were analyzed for immuno phenotypic characterization with $1x10^5$ cells were incubated with specific mouse anti-human primary antibodies conjugated to flurochromes. Cells were stained with antibodies CD90-FITC (2µl), CD73-APC (5µl), CD105-PE (20µl), CD45-FITC (20µl), CD34-PE (20µl), CD79a-APC (20µl) and HLA-DR (5µl). All antibodies were procured from (BD Pharmingen, NJ). Cells incubated with identical concentrations of FITC- (5µl), PE- (5µl), PercpCyc.5.5 (5µl), APC- (5µl) conjugated mouse IgG isotype secondary antibodies (BD Pharmingen, NJ) served as isotype controls and cell viability was measured using 7AAD. After incubation for 10mins at 37°C cells were acquired by flowcytometer (FACSCalibur, BD Biosciences, USA). Approximately 10,000 events were detected and data analysis was done using the CellQuest Prosoftware (BD Biosciences, USA).

(vi) Adipogenesis

AM-MSCs were treated with adipogenic differentiation medium composed of dexamethasone (1 µM), indomethacin (60 µM), 3-isobutyl-1-metylxanthine (500 µM), insulin (5 µg/mL; Sigma-Aldrich, USA), and 10% FBS in LG-DMEM for 18 days after reaching 80% confluency. The medium was replaced twice weekly. After differentiation, Oil Red O staining was conducted to confirm the formation of lipid inclusions. The cells were fixed in 10% formalin for at least 1 h and rinsed with 60% isopropanol prior to incubation in freshly diluted Oil Red O for 10 min. Stains were solubilized with 100% isopropanol, and Hemotoxylin was used as a counter stain.

(vii) Chondrogenesis

AM-MSCs were treated with chondrogenic differentiation medium. The cells (0.5×10^6) were transferred to a 15-mL polypropylene tube and pelleted. The pellets were cultured in 1 mL of chondrogenic differentiation medium (Millipore, US) for 14 days. The medium was changed three times weekly. After differentiation, the pellets were were fixed with 4% formaldehyde for 30 mins and stained with 1% Alcian blue (Sigma, USA) in 0.1N HCL solution for 30 minutes

(viii) Osteogenesis

AM-MSCs were treated with osteogenic differentiation medium containing ascorbic acid 2-phosphate (50µM), dexamethasone (100nM), β-glycerophosphate (10mM; Sigma-Aldrich, USA), and 10% FBS in DMEM F12. The cells (0.1×10^6) were plated in triplicate on 6-well plates. When the cells were 80% confluent, the culture medium was changed to the osteogenic differentiation medium and the cells were incubated for 21 days. The differentiation medium was changed twice weekly. After differentiation, the cells were stained with Alizarin Red S and the cells were washed with PBS and fixed with ice-cold 70% ethanol for 1 h at 4°C. Next, the cells were rinsed three to four times with distilled water and stained with Alizarin Red S (40 mM, pH 4.2; Sigma-Aldrich, USA) for 10 min at room temperature. To remove excess dye, the cells were rinsed with distilled water. Cells stained with Alizarin Red S were solubilized and exposed to 1% silver nitrate (Sigma Aldrich, USA) under bright light for 60 min. Images were captured using CKX41 Olympus inverted microscope (Olympus, Japan).

(ix) Karyotype Analysis

Karyotyping of final harvested cells was carried out using standard Q-band analysis at passage10. Briefly, mitosis was inhibited in AM-MSCs with 0.1 µg/mL colchicinegma for 20 min. The cells were then suspended in a hypotonic solution (0.075M KCl) and incubated for 20 min at 37°C. Next, the cells were pelleted at 1,000 rpm for 10 min and fixed by washing three times in methanol: glacial acetic acid (3: 1). Chromosome spreads were obtained by pipetting the cell suspension onto clean glass slides and air-dried. Metaphases were captured with Olympus BX51 Bright field microscope using karyovision software (Olympus, Japan), the chromosomes were counted, and banding patterns were analyzed.

(x) Endotoxin Assay

Endotoxin levels were determined by the gel clot Limulus Amebocyte Lysate test method (LAL), endotoxin produces a gelation reaction with amebocytes or circulating blood of *Limulus polyphemus* (American Horseshoe crab). Limulus Amebocyte Lysate was reconstituted with reagent water and mixed in equal parts of the sample (Lonza, USA). After incubation in the presence of endotoxin; gelation occurs but in the absence of endotoxin, gelation does not occur. The LAL test requires serial dilutions with neutral pH. LAL is limited to aqueous solutions or extracts of test specimen hence endotoxin testing was performed on the cell supernatant obtained at the time of P2. The lysate sensitivity used in the assay was 0.125EU/ml.

(xi) Cell Counts and Viability

Cell numbers were determined by using a hemocytometer and viability was assessed by 0.4% trypan blue dye (Gibco, USA) exclusion method. AM-MSCS were determined using CKX41 Olympus inverted microscope (Olympus, Japan).

(xii) Sterility and Mycoplasma Assay

Sterility (aerobic, anaerobic, fungal) and mycoplasma assays were performed before cryopreservation. 3ml of sample was injected into aerobic and anaerobic BacT Alert bottle (Biomerieux, INC, Derham) and incubated for 7 days. Mycoplasma detection assay was done by Lucetta Luminometer method; the commercially available kit, MycoAlert (Lonza, USA) was used for the detection of mycoplasma in the cell culture supernatant.

RESULTS

Expansion of MSCs

Approximately 9.8 to 14.8 $x10^6$ cells were isolated primarily from the tissue (n=5) at the end of passage 0 after 8-12 days. Cells were trypsinized and were seeded into 3000cells/cm^2 in T-175 flasks. Once confluency achieved 1.12 $x10^8$-2.82 $x10^8$ adherent cells were harvested. Adherent cells with fibroblastic morphology could be observed as early as 24h after seeding (Figure1). After successive passages with a 1:3 split ratio, 1.52 $x10^9$- 5.57 $x10^9$ P2 MSCs were harvested in 4-5 days and cryopreserved at the end of first expansion period. Viability of the freshly harvested cells was greater than 95% in all cases. The harvested cells from one amniotic membrane using current method yields 1.63-2.47 x 10^4cells/cm^2 at P0, 3.43- 5.71 $x10^4$ cells/cm^2 at P1, 4.07-5.93 $x10^4$ cells/cm^2 at P2 and 3.31- 5.66 $x10^4$ cells/cm^2 at P10 accordingly (Table1).

Table 1
Basic Data of Amniotic Membrane Expansion MSCs

Amniotic Membrane		AM 1	AM 2	AM 3	AM 4	AM 5
P0	Total Nucleated Cells ($X10^6$)	9.8	11.7	12.6	14.8	11.2
	Duration of P0 Culture (Days)	12	10	8	9	10
	Harvested Cells ($X10^4/cm^2$)	1.63	1.95	2.1	2.47	1.87
P1	No of T175 flasks Used at P1	19	22	24	28	21
	Total Yield of Cells at P1 ($X10^8$)	1.12	1.89	2.20	2.82	1.69
	Harvested Cells ($X10^4/cm^2$)	3.43	4.86	5.26	5.71	4.51
	Duration of P1 Culture (Days)	5	4	4	4	4
P2	No of Hyper flasks Used at P2	21	36	42	55	32
	Total Yield of Cells at P2 ($X10^9$)	1.52	3.12	3.85	5.57	2.65
	Harvested cells ($X10^4/cm^2$)	4.07	4.94	5.23	5.93	4.71
	Duration of P2 Culture (Days)	4	4	4	5	4
P10	No of T175 flasks Used at P10	2	2	2	2	2
	Total Yield of cells at P10 ($X10^6$)	11.6	16.2	18	19.8	15
	Harvested cells ($X10^4/cm^2$)	3.31	4.63	5.14	5.66	4.29
	Duration of P2 Culture (Days)	4	4	4	4	4

Morphology of Amniotic Membrane MSCs from Phase-Contrast Microscope

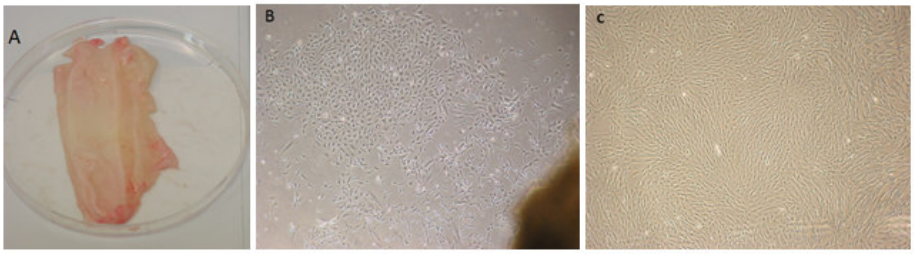
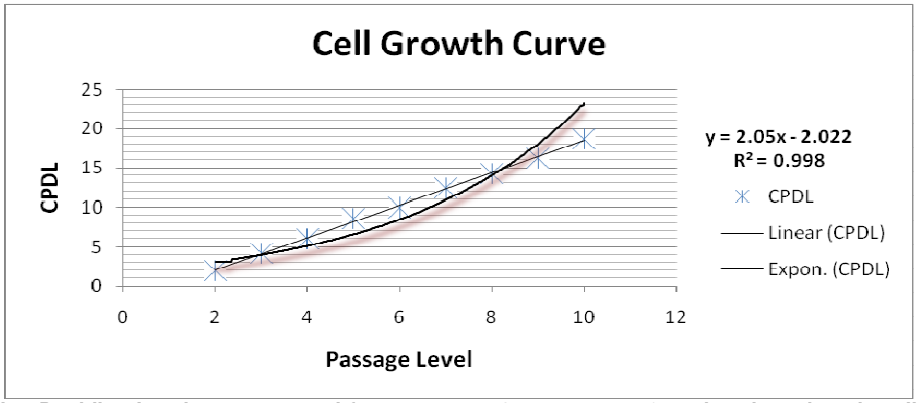


Figure 1
(A)Harvesting of Amniotic membrane, (B) Migration of AM-MSCs from the explants after 10-12 days, (B) AM-MSCs form a monolayer of adherent fibroblat-like cells by day 19. Scale bar = 50µm.

Cumulative Population Doubling Level (CPDL) Analysis

To measure the proliferation potential of the isolated amniotic membrane derived multi potent stromal cells, we calculated the cumulative population doubling level. Amniotic membrane derived multi potent stromal cells (3000 cells/cm^2) were seeded in a culture plate and sub cultured 4-5 days later. The procedure was repeated until passage 10 to measuring the CPDL. The stable increasing graph of cell growth was observed (Graph 1).

Graph 1
Cell Growth Curve of Amniotic Membrane MSCs.



The Cumulative Population Doubling Level was measured from passage 2 to passage 10, and evaluated as described in the materials and methods section. Cells grew consistently until passage 10.

Phenotype and Purity

Our analysis of a variety of cell surface markers on amniotic membrabe derived multi potent stromal cells confirmed the lack of haematopoietic and endothelial markers, such as CD34, CD45 and CD79a, and revealed a similar epitope profile to bone marrow MSCs, such as CD73, CD90 and CD105. Moreover, the self-renewal capacity of these cells is remarkable, which could be readily expanded in vitro by serial passage every 4–5 days for over 10 passages, without visible changes in either growth patterns or morphology. Flowcytometry studies showed that more than 90% of the amniotic membrabe derived multi potent stromal cells displayed uniform mesenchymal stem cell markers such as CD90, CD105 and CD73. Amniotic membrabe derived multi potent stromal cells were negative for markers such as CD45, CD34 and CD79a. Expression of HLA-DR antigen was not expressed although the cells expressed HLA-ABC antigen. Viability of the cells was determined to be more than 90% by 7-AAD dye exclusion method using flow cytometry (Figure 2).

Immunophenotype of Amniotic Membrane derived MSCs

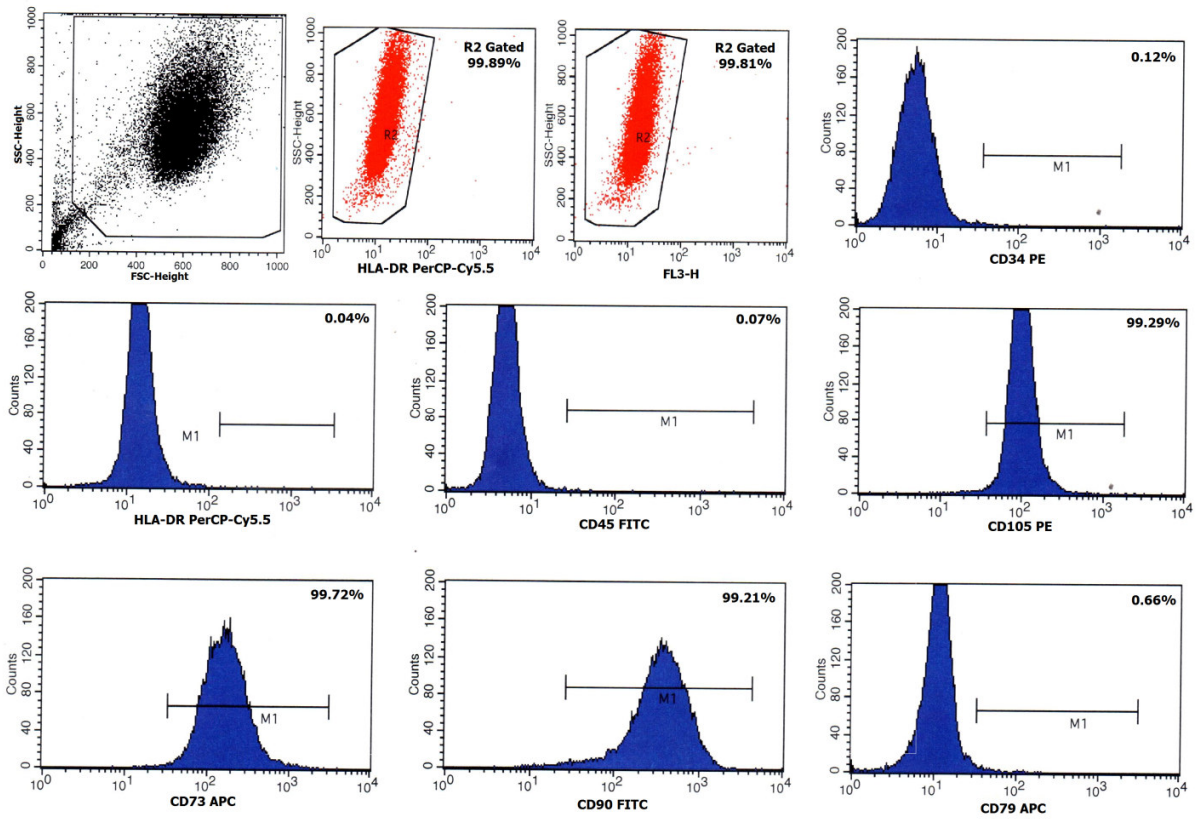


Figure 2

Representative flow cytometry analysis of AM-MSCs after expansion when labeled with antibodies against human antigens CD34(PE), HLA-DR(PerCP-Cy5.5), CD45(FITC), CD79(APC) as negative markers and CD105(PE), CD73(APC), CD90(FITC) as Mesenchymal specific markers; Color shaded histogram represents positive reactivity with the indicated antibody.

Tri-lineage Differentiation

The expanded amniotic membrane MSCs were subjected to Adipogenic, Osteogenic and Chondrogenic differentiation. To demonstrate the isolation of MSCs and to investigate their differentiation potential, the fibroblastoid cells from P10 were cultured under conditions that are favourable for osteogenic, adipogenic and chondrogenic differentiation, respectively. Under osteogenic conditions, the spindle shape

of the fibroblastoid cells flattened and broadened with increasing time of induction and formed extra cellular matrix are confirmed by Alizarin red staining. These results were strongly suggest that the fibroblastoid cells are differentiated into osteogenic cells (Fig. 3E, F).
After 3 weeks of differentiation, chondrogenic differentiation was confirmed by, Positive staining of collagen fibers along with Alcian blue indicated that MSCs were successfully differentiated to chondrocytes. No changes were observed in undifferentiated or chondrogenic control cells (Figure 3C, D). We found that most of the cell populations in this assay appear to differentiate into chondrogenic cells. Under adipogenic induction, morphological changes in the fibroblastoid cells as well as the formation of accumulated neutral lipid vacuoles were observed and visualized by Oil Red O staining. No changes were observed in undifferentiated adipogenic control (Figure 3A, B).

Trilineage differentiation of Amniotic Membrane derived MSCs

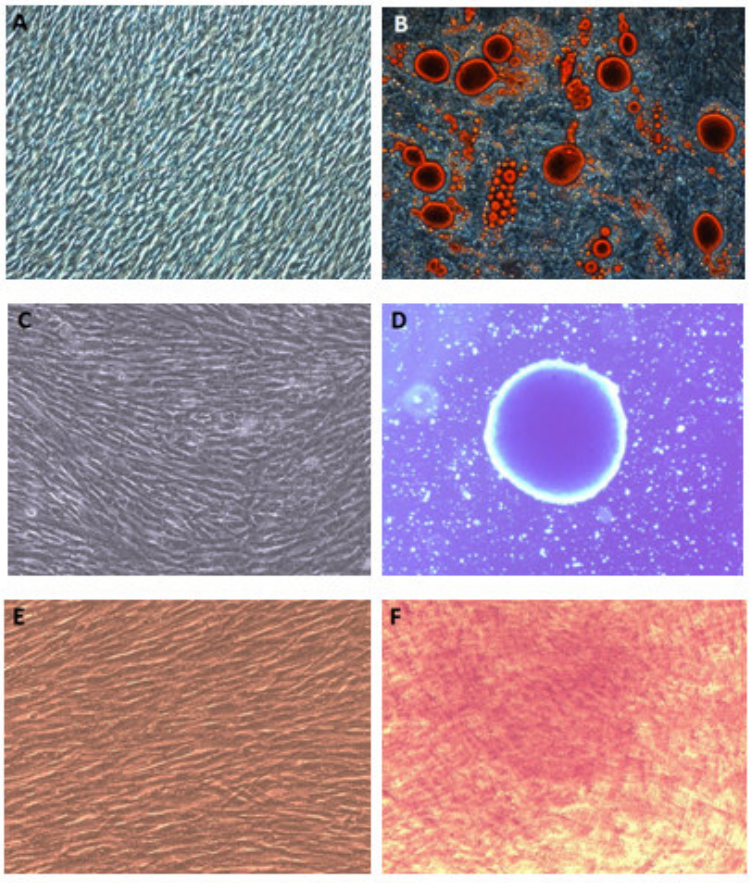


Figure 3

Amniotic membrane - MSCs derived after expansion were induced to differentiate along (B) Adipogenic (oil red O staining), (D) Chondrogenic proteoglucans formation (alcian blue), (F) Osteogenic (alizarin red S) lineages. (A,C,E) corresponding uninduced control cultures were stained for comparision. Scale bar = 50µm.

Karyotype analysis

The Metaphase chromosome spreads were analyzed in different batches of passaged 10 MSCs wherein their karyotyping pattern was observed to be normal. The Genetic stability was analyzed for chromosome after conventional giemsa banding for expanded MSCs was also observed to be normal (Figure 4).

G-banded karyotype of expanded Amniotic Membrane derived MSCs

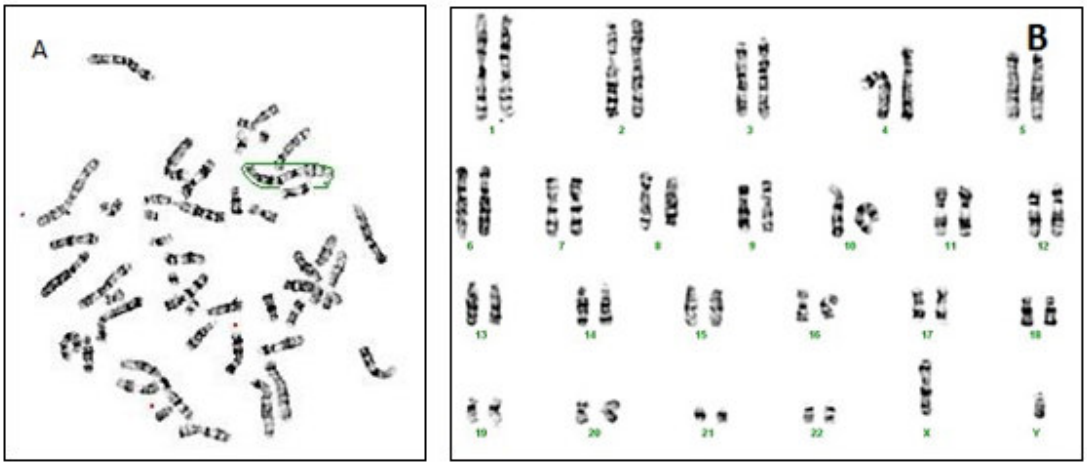


Figure 4
(A) The metaphase chromosome spreads were analyzed of P10 MSCs. (B) Representative result of independent experiment is shown.

Microbiological tests
Cultures were observed to be absolutely sterile and free of any aerobic, anaerobic and fungal contamination when checked periodically on the supernatants. Endotoxin levels of the final products were lower than 0.2EU/ml.

DISCUSSION

Amniotic membrane of the human placenta is a source of abundant stem cells, which consist of epithelial and stromal cell. Recent studies have shown that stromal cells of the human amniotic membrane, also called human amniotic Mesenchymal stem cells (hAMSC), possess the similar characteristics as those of bone marrow mesenchymal stem cells[6,7]. After parturition, the placenta is classified as medical waste and usually discarded. There are, however, clinical studies showing that the amniotic membrane can potentially be used to promote wound healing and corneal reconstruction[8,9]. These stem cells have tri-lineage differentiation and self-renewal capabilities, which are characteristic MSC features. MSCs has many advantages for use in cell-based therapy including immune privilege, an absence of associated ethical issues, and no requirement of invasive procedures for harvesting the amnion. Therefore, a greater diversity of stem cell sources is required. In the present study, we successfully isolated and characterized MSCs from amniotic membrane. The collected amniotic membrane was enzymatically digested to recover the cells[10]. After digestion, the MSCs was seeded in basal culture medium until passage 10; with an initial seeding density of 3000 cells/cm^2. We could expand these cells to a clinical quantity of 5.57x10^9 cells at passage 2 within 21 days. Apart from these we also observed that this source allowed rapid initial isolation of large number of cells, avoiding the necessity of extensive multiplication and potential epigenetic changes[11,12]. Using this procedure, we obtained cells from five amniotic tissue samples showed similar morphologies. The cells were sub cultured on the surface of plastic culture flasks. A single cell line was randomly selected. All experiments including CDPL determination, flow cytometric analysis, and differentiation studies were conducted using only the selected cell line (in triplicate). A consistent level of cell proliferation is a distinct characteristic of stem cells. These cells have a self-renewal ability related to continuous and steady proliferation.

Our results demonstrated that the isolated MSCs are capable of self-renewal, a typical feature of mesenchymal cells. Furthermore, the cells were fibroblast-like, adhered to the plastic

culture dish surface, and possessed a normal karyotype. Due to their robust proliferation, the MSCs may be sufficient for in vivo applications. Immunophenotype characterization indicated that the MSCs were positive for MSCs markers such as CD73 (99.72%), CD90 (99.21%), and CD105 (99.29%). MSCs display a distinctive pattern of cell surface antigens such as CD73, CD90, and CD105. Antigens that are not typically found on MSCs include CD79, CD34, CD45, and HLA-DR[13]. These cells were found to have an immunophenotype characteristic of MSCs and are capable of self-renewal[14]. Moreover, the rate of AM-MSC proliferation was stable until passage 10. Taken together, these findings suggest that AM-MSCs are a potential source of stem cells and could be useful for regenerative medicine.

In addition, we demonstrated the multipotent differentiation capability of AM-MSCs. We found that AM-MSCs differentiated into osteocytes, adipocytes and chondrocytes using specific culture media. AM-MSCs grown under osteogenic differentiation conditions acquired calcium deposits identified with Alizarin Red S. Calcium accumulation was not observed in cells cultured under control conditions. In the adipogenic differentiation study, lipid droplets were observed in the cells after 18 days of differentiation. To confirm adipogenic differentiation Oil Red O staining was performed. Differentiated cells were positive for Oil Red O staining, although only a few cells were stained and contained fat droplets. In the chondrogenic differentiation study, cells that were cultured in chondrogenic differentiation medium aggregated and formed pellets unlike cells that were grown in the control medium. The differentiated cells were also positive for Alcian blue staining.

In through our work we would summarize that, amniotic membrane represent an appealing source of MSCs for cell therapy. We studied a standardized process for expansion of AM-MSCs for clinical use. The data obtained indicate that the expanded AM-MSCs are homologous, genetically stable, safe, and biologically active. Moreover, the rate of amniotic membrane multipotent stromal cells proliferation was stable until passage 10. Taken together, these findings suggest that up scaled AM-MSCs are a potential source of stem cells and could be useful for therapeutic applications.

CONCLUSION

In conclusion, we demonstrate the capacity of amniotic membrane multipotent stromal cells as a suitable clinical therapy source for ailment of various complications and diseases. Though the efficiency of multipotent stromal cells is well understood from conglomerated clinical and preclinical data, it is still very crucial to understand whether these cells are functionally important in homing capacities. Furthermore, an animal toxicity study would determine the clinical toxicity associated with the therapeutic product, adequate safety margin, dosage limitations and route of administration to ensure systemic dissemination of MSCs for intend therapeutic usage warrants further investigation.

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