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GENERATION AND CHARACTERISATION OF HUMAN MESENCHYMAL STEM CELLS DERIVED FROM UMBILICAL CORD AND PLACENTA

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ABSTRACT

Mesenchymal stem cells (MSC) have emerged as a great therapeutic potential in regenerative medicine and tissue engineering, hence created a vast demand for its large clinical scale production. In current study, we have generated MSC from human umbilical cord and placenta tissues that are easily accessible and direct comparisons were made in opting for a better alternate source of MSC in replacement of bone marrow.

MSC were successfully generated, assessed for the morphological changes; surface protein expression via immunophenotyping; early embryonic stem cell (ESC) transcriptional factor expression via RT-PCR and mesodermal differentiation ability.

UC-MSC and PLC-MSC appeared fibroblastic-like cells and expressed the common mesenchymal surface markers. Both MSC expressed the ESC markers and were able to differentiate into adipocytes, osteocytes and chondrocytes upon induction. In comparison, UC-MSC showed a significantly rapid growth kinetic with higher cell yield and shorter doubling time as compared to PLC-MSC.

In our findings, both UC-MSC and PLC-MSC shared similar mesenchymal markers and properties however; UC-MSC appears as a better source of MSC as they display superior differentiation potential and growth kinetics than PLC-MSC.

1.0 Introduction

Mesenchymal stem cells (MSC) are unspecialised and non-haematopoietic stem cells that have a capacity for self-renewal and multi-potential differentiations. These plastic-adherent cells appear as spindle-like cells in *in vitro* culture. When given or in the presence of appropriate stimuli, MSC are able to undergo multi-lineage differentiation especially towards mesodermal lineage and have the ability to home at sites of injured tissues where they can promote repair which made them feasible for targeted regenerative medicine [1-5].

The current definition of MSC is an ongoing challenge because the factors that influence *in vitro* culture of MSC such as passage, cell density and cell culture media affect the characteristics of the MSC in term of surface marker expression, proliferation rate and differentiation potential. However, the conventional method of describing MSC is based on expression of surface molecules such as CD105,

CD73, CD90, CD29, MHC class I and negative expression for haematopoietic markers (CD45, CD34, CD14) and endothelial markers (CD34 and CD31) [2, 6-9]. Interestingly, MSC are hypo-immunogenic; lacking immunological markers such as MHC II and co-stimulatory molecules (CD80 and CD86). This may grant tolerance whereby MSC may escape immuno-recognition in transplantation settings [10-14]. Taken together all these properties of MSC, studies have shown that MSC can serve as a therapeutic tool for tissue engineering and cell-based therapy for they can regenerate damaged tissues and suppress the inflammatory diseases such as autoimmune diseases and Graft-versus-host disease (GVHD) [15-22].

Traditionally bone marrow (BM) is the common source of MSC as they are being well studied and used widely in recent clinical studies. However, acquiring BM-MSC requires an invasive procedure and they are inaccessible in large volume resulting in significant decrease in cell number (0.01%-0.001% of nucleated cells) and proliferation capacity that is reciprocal with age [23]. Along with this, MSC were isolated from various sources of tissues such as bone, palatine tonsil, dental pulp and cartilage apart from bone marrow [24-29]. MSC have also been isolated from pathological tissues of patients with rheumatoid arthritis and malignancies. This indeed suggests that mesenchymal stem cells reside in virtually all postnatal organs and tissues [30, 31]. The frequency of MSC in newly formed or preliminary sources of tissues is expected to be higher than the adult tissues as they may play important role in early tissue formation. In line with this, MSC are also highly attainable from extra-embryonic tissues such as umbilical cord [32], amniotic fluid [33] and placenta [1] as well as foetal tissues (blood, liver, bone marrow and lung) and embryonic stem cells (ESC) [34-36]. While foetal tissues and ESC stem cells faces ethical issues, extra-embryonic tissues such as umbilical cord and placenta that are highly disregarded, were discarded upon delivery of the newborns and may serve as an ideal source of alternative, non-invasive and non-controversial stem cell population. Realistically, this explains the growing popularity among researchers to utilise these tissues.

Therefore in this study, we performed a comparison study on the generation and expansion of MSC derived from human umbilical cord (UC-MSC) and chorion placenta (PLC-MSC) and further comparisons were made for their important characteristics such as mesenchymal properties and their differentiation ability in order to obtain an alternative source of MSC for BM-MSC for therapeutic scale manufacture.

2.0 Materials and Methods

2.1 Generation and Culture of Mesenchymal Stem Cell

Human delivery waste such as umbilical cord (n=10) and placenta (n=5) were obtained from the Obstetrics and Gynaecology Britannia Women & Children Specialist Centre. All studies were approved by the ethical committee requirements of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. Samples were obtained with written and informed consent from the patients. Samples were collected upon delivery from normal full term pregnancies with the assistance of gynaecologists. One cm^3 of samples were excised from the umbilical cord and placenta (chorion) and the blood vessels in the umbilical cord samples were removed. Samples were disinfected with 70% alcohol and rinsed in 1X PBS before mincing. The minced tissues were digested in 0.4% type II collagenase (Worthington, New Jersey, USA) and 0.01% DNAse (Worthington, New Jersey, USA) followed by mechanical dissociation using hand held cell homogenizer (Hassen Wagger) at 9000rpm for 10-15minutes. The suspensions were filtered, washed and cell pellets were resuspended and cultured in MSC complete media containing Dulbecco's Modified Eagle's medium with nutrient mixtures F-12 (HAM) [1:1] with GLUTAMAX-I (Gibco, Invitrogen, USA), 10% foetal bovine serum (Stem Cell Technology Inc., London, UK), 1% Penicillin and Streptomycin (Gibco, Invitrogen), 0.5% Fungizone (Gibco, Invitrogen), 0.1% Gentamicin (Gibco, Invitrogen) and 40ng/ml basic fibroblastic growth factor (bFGF) (Promega). Primary cell cultures were incubated in 37˚C humidified 5% CO_2 incubator and non-adherent cells were removed by changing the media. Adherent MSC were harvested via trypsinisation (0.05% trypsin-EDTA, Invitrogen, BRL, Canada) to perform other downstream experiments.

2.2 Immunophenotyping of MSC

Upon reaching 90% confluence, MSC were harvested and analysed to detect cell surface markers by incubating them with flourochrome conjugated anti human antibodies against CD29, CD90, CD73, MHC class I, MHC class II, CD45, CD34, CD80, CD86 (Becton Dickinson, Biosciences Pharmingen) and CD105, STRO-1 (R&D System). Stained cells were re-suspended in PBS and were acquired by using a FACSCalibur flow cytometer (Becton Dickinson). Flow cytometry data were analysed using Cell Quest Pro software provided by the manufacturer.

2.3 Growth Kinetics and Doubling Time of MSC

Four thousand cells of UC-MSC or PLC-MSC per well were cultured in 6-well plates and media was changed twice weekly. Cultures were maintained until till day 14^{th} and triplicates of MSC were harvested for every 2 days and subjected to Trypan blue dye exclusion cell count method to evaluate the growth kinetics of MSC. To determine the doubling time, 0.3×10^6 of UC-MSC or PLC-MSC were cultured at every passage in 100mm petri dish. Media was changed every 3 days until cells reach confluence. Cells were then harvested using 0.05% trypsin-EDTA (Invitrogen,

Canada), and trypan blue cell count was performed. The cell yield, initial seeding numbers, and days in culture were recorded to determine the doubling time using the Patterson Formula*

*Td=Tlg2/lg(Nt/N0); Td is the doubling time (h), T is the time taken for cells to proliferate from N0 to Nt (hour), and N is the cell count.

2.6 Statistical Analysis

Student T-test was performed to compare the values of two means. The data were expressed as mean ± SEM at significance level of $p \leq$ of 0.05.

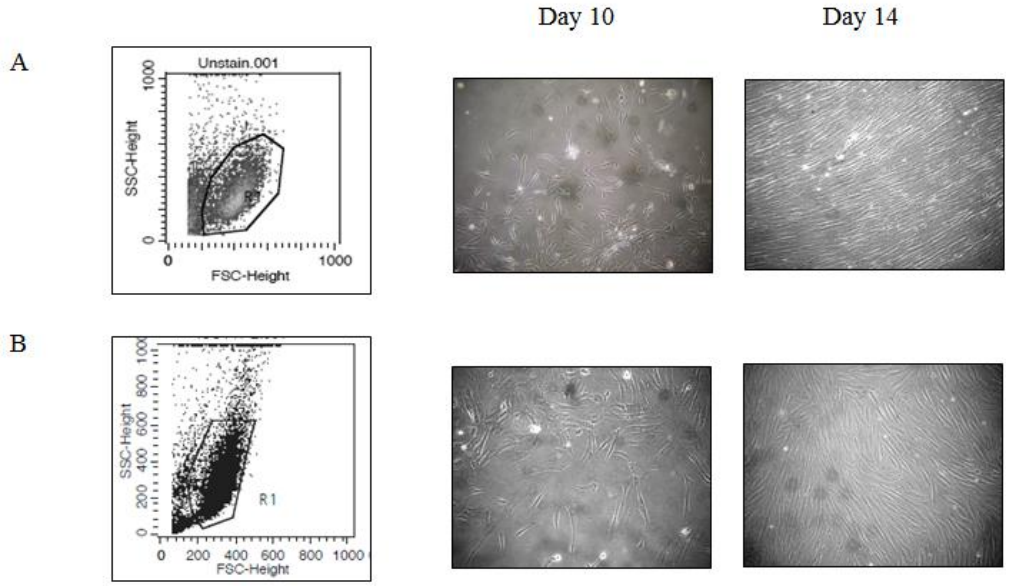

Fig.1 Features of MSC from different sources: The display of FACS density plot and photomicrographs of MSC from UC-MSC (A) and PLC-MSC (chorion) (B). The photomicrographs were taken on day 10 and day 14 of MSC in culture and upon reaching confluence, MSC were harvested and flow cytometer analysis was conducted. Size scatter (SSC) and forward scatter (FSC) indicates the granularity and size respectively. All photo micrographs were taken using phase contrast microscope with 100x magnification.

2.4 Differentiation Assay

Upon reaching 100% confluence, MSC were induced for adipogenic (CHEMICON, USA), osteogenic (CHEMICON, USA) and chondrogenic differentiation (STEMPRO®, US). The assays were carried out according to the manufacturer's protocol. Upon completing the differentiation induction period, the adipocytes, osteocytes and chondrocytes were fixed and stained with Oil Red O Solution, Alizarin Red Solution and Alcian blue solution respectively. Photomicrographs were captured using phase contrast microscope at 100x magnification.

2.5 RT-PCR of MSC

Total RNA was extracted by lysing cells with TRIzol® Reagent (Invitrogen, USA). RNA was transcribed using The ImPromII™ Reverse Transcription System (Promega, USA) kit followed by PCR using Taq DNA Polymerase kit (Qiagen). RT-PCR was performed with oligonucleotide primers as in Table.1. PCR products were resolved in 1.5% agarose gel and photographed using FluorChem 5500 (Alpha Innotech).

3.0 Results

3.1 Morphological Study of MSC

MSC were successfully generated from umbilical cord and placenta (chorion). The morphological features of both sources of MSC at early passages were compared by flow cytometry and images were captured using phase contrast microscope. Figure 1 shows formations of homogenous monolayer of adherent, spindle shaped fibroblastic-like cells of primary cultures of UC-MSC and PLC-MSC (chorion) on day 10th and day 14th at P0. The scatter plot shows that both sources of MSC were smaller and has higher granularity (Figure 1). Early passages of UC-MSC and PLC-MSC cultures were proliferating rapidly and were well defined spindle shaped cells. However these features gradually changed at later passages, (P15 onwards) whereby UC-MSC and PLC-MSC (from chorion) showed some morphological changes; they appeared unhealthy and eventually died (Figure 2). There were no significant differences between UC-MSC and PLC- MSC throughout the passages.

3.2 Immunophenotyping of MSC

At passage 2, the expanded MSC cultures were harvested and were subjected for immunophenotyping using flow cytometer.Immunophenotyping was performed using a panel of anti human antibodies (Figure 3A and 3B).

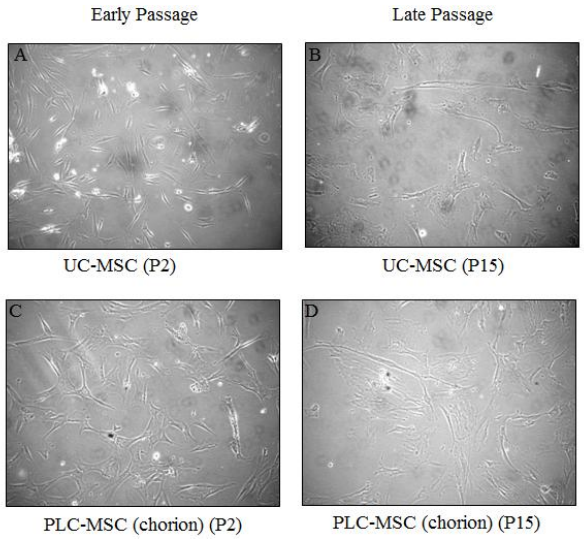

Fig.2 Comparison of early and late passages of primary MSC cultures: MSC were cultured in MSC complete media and the appearance of MSC at early passage (P2); smaller in size, fibroblastic-like cell, well defined shape and strongly adherent to plastic surface (A and C). The appearance of MSC at late passage; larger in size, polyglonal shaped, less defined and less defined to plastic surface (B and D). Images were captured using phase contrast microscope at magnification 100x.

More than 95% of UC-MSC and PLC-MSC (chorion) were positive for CD105, CD29, MHCI, CD73, and CD90. All samples showed negative expression for CD45, CD34, CD80, CD86, MHCII and bone marrow stromal cell progenitors (Stro-1).

3.3 Growth Kinetic Analysis and Doubling Time of MSC

Our observation suggest that both UC-MSC and PLC-MSC depicted an initial lag phase of 4 days and an exponential log-phase from day 4-10 before plateau was reached. However, UC-MSC had progressed rapidly with an aggressive exponential log-phase and a significantly higher cell number as compared PLC-MSC (Figure 4A). Doubling time of UC-MSC falls within (20-50 hours) which is approximately 2-3 folds shorter than PLC-MSC (30-60 hours). The average doubling time of UC-MSC (32 hours) were measured to be significantly shorter than PLC-MSC (41 hours).

3.4 Embryonic transcriptional factors (ESC) expression by MSC

Reverse transcription polymerase chain reaction was performed and the PCR products were assessed for embryonic stem cell markers. UC-MSC and PLC-MSC (from chorion) expressed transcription factors that regulate maintenance of pluripotent state in ESC; Nanog, Sox2, Rex-1, and Oct4 (Figure 5). In comparison, expression of Sox2 was higher in UC-MSC than PLC-MSC.

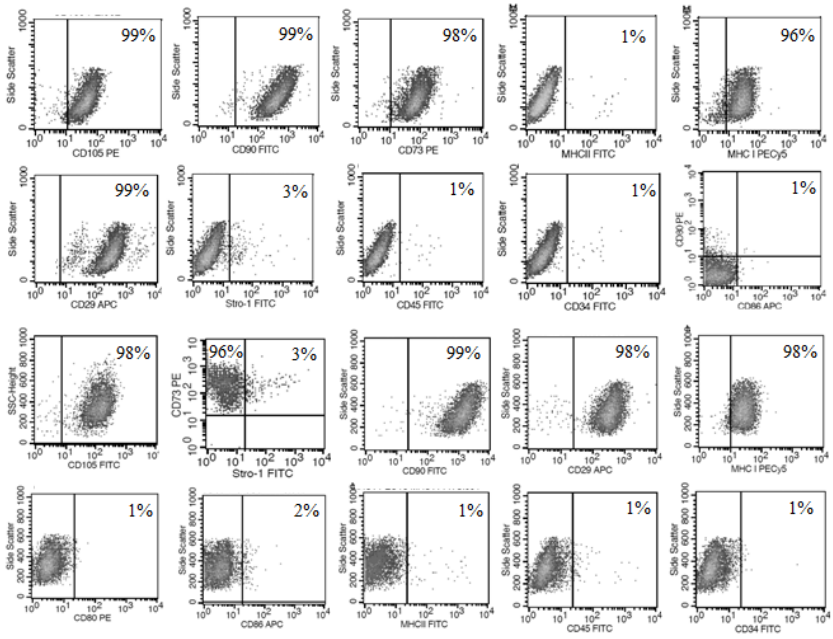

Fig.3 Immunophenotyping of UC-MSC and PLC-MSC (chorion): MSC at P2 were stained for mesenchymal cell surface markers using a range of anti-human antibodies. Results are representative of 3 repeated experiments.

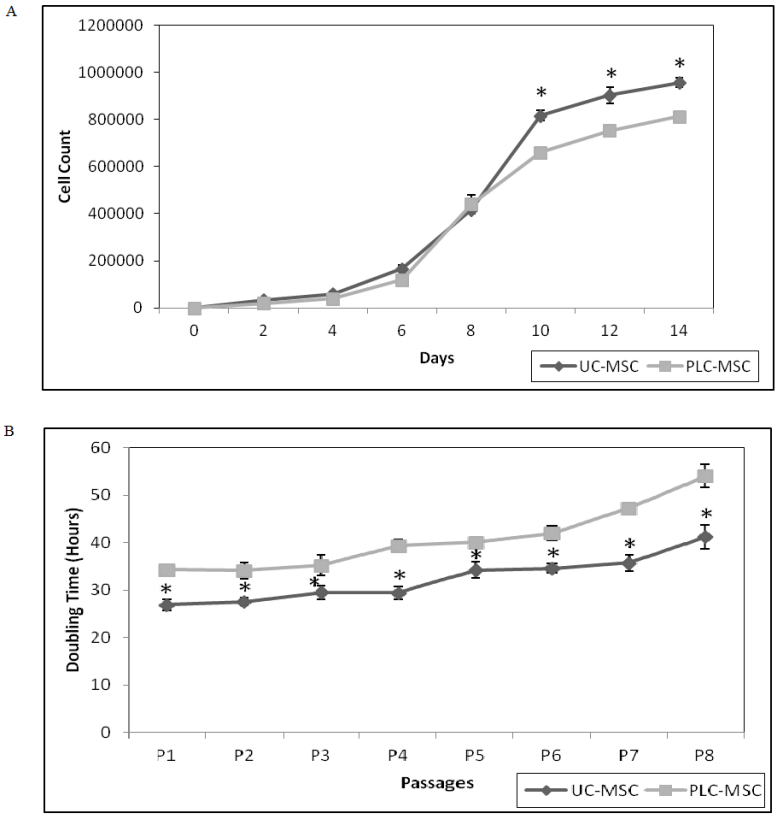

Fig. 4 Comparison of Growth Kinetics of UC-MSC and PLC-MSC: Average growth kinetics of MSC were measured by determining triplicates cell count number every 2 days in culture (A). Doubling time of MSC at every passage werealso calculated (B). The average doubling time of UC-MSC and PLC-MSC are 32 and 41 hours respectively. The results are the representative of 3 repeated experiments with ± standard deviation.

3.5 Mesodermal Differentiation Potential of MSC

Both UC-MSC and PLC-MSC cultures were able to differentiate into adipocytes (formation of lipid vacuoles and were stained red with Oil-Red-O), osteocytes (deposition of calcium minerals when stained orangy-red with Alizarin Red) and chondrocytes (formation of proteoglycan stained blue

with Alcian Blue solution) in comparison with the controls (Figure 6). Images were captured using phase contrast microscope at 100x magnification. Both the differentiated MSC also expressed adipogenic (Lipoprotein Lipase), osteogenic (Osteopontin and Osteocalcin) and chondrogenic(Aggrecan) gene markers while expression of these markers were absent in the controls (undifferentiated MSC).

4.0 Discussion

Our study has shown that MSC were successfully generated from human umbilical cord and placenta tissues. The generation of MSC resulted in high yield of nucleated cells and upon expansion, a homogenous plastic adherence spindle fibroblastic-like cells were obtained. Both sources of MSC were easy to obtain, cultured at low cost and consistently harboured similar results with BM-MSC for morphology and cell surface markers with no significant differences in relative to their sources (Figure 1) [37]. The successful generation of MSC cultures from these sources were also dependent on the quality of the samples received upon delivery. The generated MSC demonstrated rapid growth kinetics with a shorter lag phase and a prolonged log phase before reaching a plateau. In comparison, UC-MSC showed rather a significant higher rate of proliferation with higher number of proliferating cells and shorter doubling time of 32 hours as compared to PLC-MSC. This finding is beneficial in utilising rapidly proliferating UC-MSC to aid in large scale production of MSC for future research and clinical studies.

On average, both MSC were successfully cultured until passage 15 and beyond this passage, MSC underwent morphological changes, formation of debris and granules in supernatant and eventually senesced. This is in line with

Table 1: PCR Primer

Gene	Forward Primer	Reverse Primer
Nanog	AGTCCCAAAGGCAAACAACCCACTTC	ATCTGCTGGAGGCTGAGGTATTTCTGTCTC
Sox2	ATGCACCGCTACGACGTGA	CTTTTGCACCCCTCCCATTT
Rex-1	CAGATCCTAAACAGCTCGCAGAAT	GCGTACGCAAATTAAAGTCCAGA
Oct4	CGACCATCTGCCGCTTTGAG	CCCCCTGTCCCCCATTCCTA
Osteopontin	GAAGGACAGTTATGAAACGAGT	AACATAGACATAACCCTGAAGC
Osteocalcin	ATGAGAGCCCTCACACTCCT	CAAGGGGAAGAGGAAAGAAG
Lipoprotein	CTTCTGTTCTAGGGAGAAAGTG	TGCTGTGTAGATGAGTCTGATT
Aggrecan	GCCTTGAGCAGTTCACCT	CTCTTCTACGGGACAGC
GAPDH	TTGCAACTGTTTTAGGACTTT	AGCATTGGGAAATGTTCAAGG

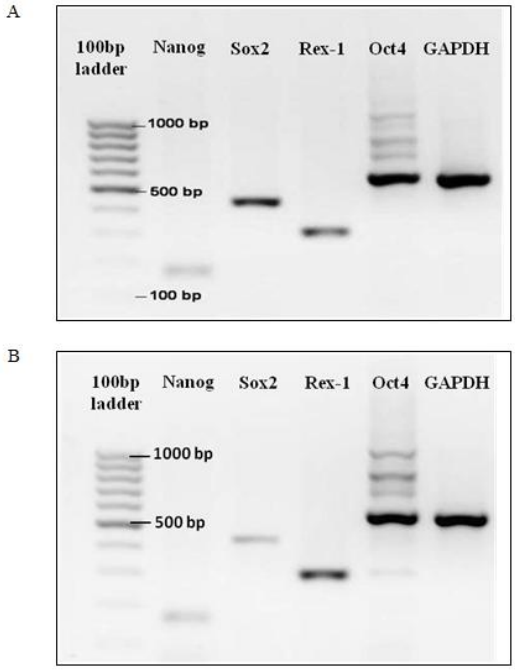

Fig. 5 Expression of embryonic stem cell transcriptional factors in MSC: Both UC-MSC (A) and PLC-MSC (B) expressed ESC transcriptional factors that are required to sustain the self renewal and pluripotency. GAPDH was used as a housekeeping gene.

other studies where the self-renewal potency of MSC decreases in long-term in vitro culture [38]. MSC senescence causes changes in its morphology and appeared ore spread out with visible intracellular fibrous structures described by [39]. According to Mareddy et al., MSC cultures undergo senescence upon expansion and this is indicated with the slow growth and reduced differentiation ability of MSC even though still expressing normal levels of MSC surface markers [40]. For this reason, MSC at early passage where structure & morphological changes due to prolonged in vitro culture can be avoided [41].

UC-MSC and PLC-MSC expressed neither haematopoietic markers (CD34 and CD45) nor immunological markers (MHC II, CD80 and CD86). They also negatively expressed bone marrow stromal cell progenitors (Stro-1). Stro-1 expression is important as it may expedite sub-classification of MSC to be either used in tissues for gene delivery or to support haematopoietic engraftment [42, 43]. Both MSC were consistently positive for endothelial progenitor (CD105), integrin markers (CD29), and major histocompatibility class I antigen (MHCI), CD73 and CD90. The positive expression of MHC class I by both MSC is important to protect MSC from certain NK cell mechanism of deletion. Overall, there were no significant difference in the surface markers profile among UC-MSC and PLC-MSC and remained the same throughout the passages till P15 for both MSC (data not shown).

Nonetheless, MSCs generated from umbilical cord and placentas were further confirmed for their differentiation potential into mesodermal lineages upon induction. Both UC-MSC and PLC-MSC successfully differentiated into adipocytes, osteocytes and chondrocytes when induced. Our observations are similar to other sources of MSC [44-46]. In addition, we also looked at the molecular level gene expression for lipoprotein lipase, osteopointin, osteocalcin and aggrecan in differentiated MSC. Both differentiated MSC exhibited profound expression of these markers as compared to the non-induced MSC. RT-PCR analysis showed that UC-MSC and PLC-MSC expressed Nanog, Sox2, Rex-1 and Oct4 that are essential in sustaining the "stemness" of a cell. These transcriptional factors are highly expressed in the pluripotent embryonic stem cells [47]. Since UC-MSC and PLC-MSC also express these markers, it could be these genes that regulate the self renewal, pluripotency, and differentiation capacity of stem cells [43, 48, 49]. Among the ESC transcriptional factors, UC-MSC expressed profoundly higher expression of Sox2 gene than PLC-MSC while other ESC markers remained similar. Previously we mentioned that UC-MSC has higher proliferating capacity with a shorter doubling time to that of PLC-MSC.

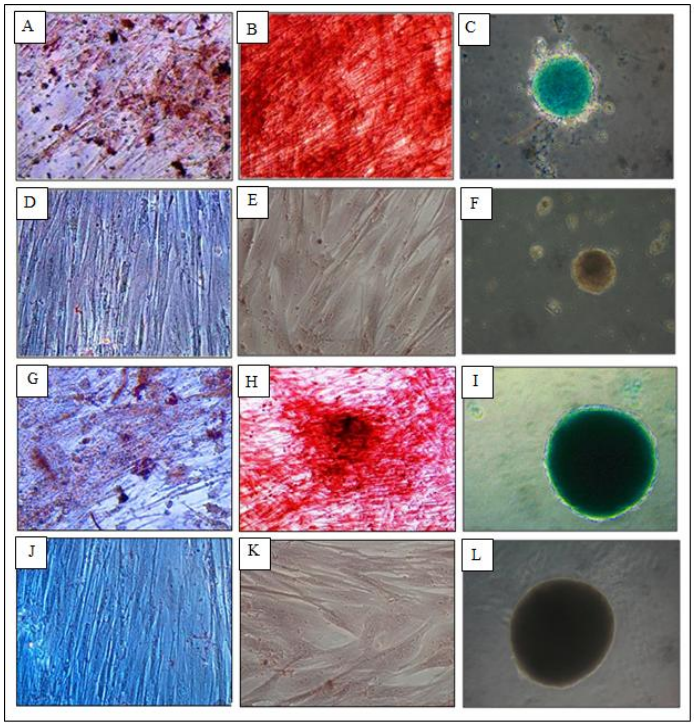

Fig. 6 Differentiation potential of UC-MSC and PLC-MSC into adipocytes, osteocytes and chondrocytes: UC-MSC and PLC-MSC in adipogenic, osteogenic, chondrogenic or normal cell culture medium after 3 weeks: Formation of lipid droplets; stained red in Oil-Red-O (A and G), calcium deposition; stained orangy-red in Alizarin Red (B and H) and formation of proteoglycans; stained blue with Alcian blue solution (C and I) confirms the ability of to differentiate into mesodermal lineages. D, E, F, J, K and L are the controls for UC-MSC and PLC-MSC differentiation. Picture was taken using phase contrast microscope.

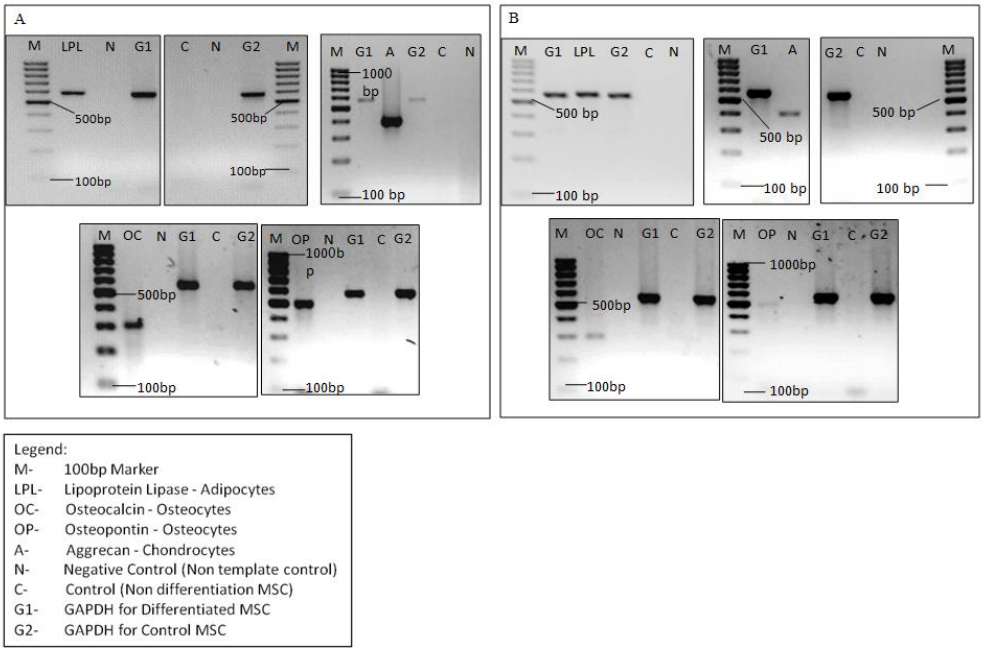

Fig.7 MSC differentiates into mesodermal lineages: Upon completing adipogenic, osteogenic and chondrogenic induction, UC-MSC (A) and PLC-MSC (B) were harvested and RT-PCR was performed. PLC-MSC differentiated into adipocytes; expressing Lipoprotein Lipase (LPL), chondrocytes; expressing Aggrecan (A) and osteocytes; expressesing Osteocalcin (OC) and Osteopontin (OP); and. UC-MSC were more prone to osteogenic and chondrogenic differentiation for they express higher of Osteopontin, Osteocalcin and Aggrecan gene expression than PLC-MSC in relative with GAPDH. PLC-MSC cultured in MSC complete media were used as control for this assay. GAPDH were used as the reference gene.

We believe Sox2 may play an important role in controlling the proliferation and multipotentiality of MSC as previous report have demonstrated that knockdown of Sox2 had significantly inhibited the multipotentiality and proliferation of human bone marrow MSC [50]. Furthermore, we also show that UC-MSC has a higher differentiation capacity towards osteocytes and chondrocytes for they express higher expression of osteopontin, osteocalcin and aggrecan gene in comparison to PLC-MSC.

5.0 Conclusion

MSC generated from human umbilical cord and placenta share similar mesenchymal features to that of others sources of MSC. UC-MSC would be a preferred alternative source of MSC for future use in replacement of BM-MSC as they show a higher rate of proliferation and multipotential capacity than that of PLC-MSC.

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