# Generation and characterisation of human umbilical cord derived mesenchymal stem cells by explant method

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## ABSTRACT

**Mesenchymal stem cells (MSCs) derived from human umbilical cord (UC) have been considered as an important tool for treating various malignancies, tissue repair and organ regeneration. Umbilical cord-derived mesenchymal stem cells (UC-MSCs) are better alternative to MSCs that derived from bone marrow (BM-MSCs) as they are regarded as medical waste with little ethical concern for research and easily culture-expanded. In this present study, the foetal distal end of human UC was utilised to generate MSC by explant method. Upon *in vitro* culture, adherent cells with fibroblastic morphology were generated with rapid growth kinetics. Under the respective inductive conditions, these cells were capable of differentiating into adipocytes and osteocytes; express an array of standard MSC's surface markers CD29, CD73, CD90, CD106 and MHC-class I. Further assessment of immunosuppression activity revealed that MSCs generated from UC had profoundly inhibited the proliferation of mitogen-activated T lymphocytes in a dose-dependent manner. The current laboratory findings have reinforced the application of explant method to generate UC-MSCs thus, exploring an ideal platform to fulfil the increasing demand of MSCs for research and potential clinical use.**

**KEY WORDS:**
*Mesenchymal stem cell, Explant method, Human umbilical cord, Immunophenotyping, Immunosuppression*

## INTRODUCTION

It has been shown that mesenchymal stem cells (MSCs) can give rise to cells of mesodermal lineages such as bone, adipose tissue, cartilage, tendon and skeletal muscle.[1-3] Beside lineage-specific differentiation, MSC also potentially trans-differentiate into various non-mesodermal lineage tissues including pancreatic islet cells,[4] cardiac muscles,[5] hepatocytes[6] and neural cells.[7] Unlike, other tissue-specific adult stem cells, MSCs hold a high potential to be considered as a therapeutic agent due to its targeted homing capacity to the site of injuries and ability to differentiate into many different mesenchymal and parenchymal cell types.[8] Thus, MSCs are considered as a 'magic healing cells' that secrete large amounts of pro-angiogenic, anti-inflammatory and anti-apoptotic cytokines/factors that exert unique reparative and immunosuppressive properties, which may play a role in the induction of tissue regeneration, transplantation tolerance and control of autoimmunity.

Historically, bone marrow (BM) was considered as the most common source of MSCs and designated as a gold standard as they have been broadly used in research and development of clinical therapies. However, the use of BM-MSCs is not always feasible due to many reasons. The incidence of a high degree of microbial exposures during BM aspiration, the low yield of cell numbers and reduction of proliferative and differentiation capacity with increasing age of the donor have hindered an extensive use of BM. Furthermore, many disease conditions limit the accessibility of BM-MSC as such BM marrow failure, aplastic anaemia, leukaemia and post-myeloablative irradiation or chemotherapy which could lead to the low cellular/stromal fraction in BM. In such circumstances, a third person's stem cells would be an excellent alternative option but it could be stalled by the availability of a suitable donor. These technical hitches can delay the treatment process and further affect the outcome of the treatment. This predicament has initiated an urge to find an alternative source of MSCs and certainly, UC-MSCs could serve as an ideal candidate.

Mesenchymal stem cells can be procured from birth-associated waste tissues such as placenta,[9] amniotic membranes,[10] umbilical cord blood (UCB)[11-12] and umbilical cord tissues.[13,14] Ontologically, stem cells that derived from earlier phases of life possess a higher proliferative capacity as foetal MSCs replicate much faster than adult BM-MSCs. The umbilical cord can be considered as the best alternative to BM as they are regarded as medical waste with little ethical concern for research.[15] Several reports have indicated that MSC generated from various anatomical parts of UC vary in term of initial stem cell composition and frequency; the ability to form colony forming units (CFU).[14,16-17] Several research articles have illustrated divergences in the characteristics of MSC that have been isolated using different methods and anatomical parts of the UC.[18-19]

The present study has employed a simple method namely 'explant technique' to generate MSCs from the Wharton's Jelly of UC. Unlike other isolation methods such as enzymatic digestion, positive and negative selection by magnetic or fluorescent activated cell sorting, explant method is relatively economical, less laborious and time-saving.

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## MATERIALS AND METHODS

*Sample*

Human UC samples of full-term delivery were collected from the Britannia Women and Children Specialist Centre, Kajang, Selangor with the assistance of gynaecologists. All samples were collected with a written informed consent from respective parents. This research study was approved by the institutional reviewing board for ethical clearance of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia.

*Generation of UC-MSC by explant method*

Firstly, umbilical cord blood was discarded and 1-2 inches of foetal distal end of umbilical cord that connected to the newborn was snipped and immersed in transportation media consisting of Dulbecco's Modified Eagle's medium with Glutamax-1 and nutrient mixture F-12 (HAM) [1:1] (DMEMF12) (Gibco, Invitrogen) supplemented with 2% penicillin and streptomycin (Gibco, Invitrogen), 1% Fungizone (Gibco, Invitrogen), 0.2% Gentamicin (Gibco, Invitrogen). Samples were stored in ice box during transportation and processed within 3 hours of collection to ensure high quality of stem cell retrieval. At the laboratory, UC was disinfected in 70% ethanol for 30 seconds and soaked in 1x PBS to remove the excess blood. The blood vessels were then removed; Wharton's jelly was retrieved and minced into a paste-like consistency. The paste-like tissue was then suspended in MSC complete media and cultured in T25 cell culture flask. The complete culture media for MSC consist of DMEMF12, supplemented with 10% optimised bovine serum for MSC's expansion (Stem Cell Technology Inc.), 1% of penicillin/streptomycin, 0.5% fungizone, 0.1% gentamicin with 40ng/ml basic fibroblast growth factor-2 (bFGF-2) (Peprotech). Primary cultures were incubated for at least 2-3 weeks at 37°C humidified atmosphere with 5% $CO_2$ incubator. The first media change was performed once the outgrowth of an adherent cell was observed and the paste-like tissues (explants) were removed during the first media change.

*Expansion and Cryopreservation*

Upon reaching 70-80% confluency, adherent cells were harvested via trypsinization using 0.05% trypsin-EDTA (Gibco, Invitrogen) at 37°C for 3-5 minutes. The detached cells were centrifuged at 1,800 rpm for 10 min. Cells were sub-cultured further at the density of 12,000 cells/$cm^2$ in the T25 culture flask. Adherent cells that were culture expanded up to passage 20, however, only passage 3-7 were utilised for the following experiments or cryopreserved in freezing media that contains 10% DMSO (Sigma-Aldrich) and 90% foetal bovine serum (Gibco, Invitrogen).

*Analysis of cell surface markers*

Upon confluency, culture-derived adherent cells were labelled with mouse anti-human monoclonal antibodies. The panel of specific monoclonal antibodies used are CD29, CD73, CD90, CD105, CD14, CD34, CD45, CD80, CD86, HLA-ABC and HLA-DR. Antibody labelling for negative control was carried out with matched isotype controls. Stained cells were analysed using Fortessa flow cytometer (Becton Dickinson). Approximately, 1-10x$10^4$ events/cells were acquired and data were analysed using FACS Diva software provided by the manufacturer. All antibodies were purchased from Becton Dickinson and were used at volume of 5µl/$10^5$ cells in 100µl of total staining volume.

*Differentiation Assays*

Differentiation into osteogenic and adipogenic lineages was conducted by plating the adherent cells from passage 3 in a 6-well plate and incubated at 37°C in 5% $CO_2$ humidified air. The adipogenic or osteogenic induction media (StemPro Adipogenesis Differentiation Kit or Osteogenesis Differentiation Kit, Gibco, Invitrogen) were added once cells achieved 100% confluency. Media change was pursued every 2-3 days until 21 days of the induction period. Upon completion of differentiation, 4% of paraformaldehyde was used to fix the adipocytes for 30 minutes followed by staining with Oil Red O solution. As for osteogenic differentiation, differentiated osteocytes were fixed in ice-cold 70% ethanol and osteogenic differentiation was detected by Alizarin Red S staining.

*Quantitative Reverse Transcription Polymerase Chain Reaction (qPCR)*

Differentiation of the adherent cells towards osteogenic and adipogenic was also further confirmed by assessing the gene expression level. The total RNA extraction was performed from differentiated cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The extracted mRNA product was converted to cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche). PCR amplification and quantification protocol were in accordance to the Light Cycler 480 DNA SYBR Green Master (Roche). The amount of cDNA were used in every experiment regardless of the mRNA transcript primer as the concentration of mRNA were predetermined prior to reverse transcription. GAPDH mRNA transcript primers were used as a housekeeping gene. Primer sequence for adipocytes and osteocytes is shown in Table I.

*Proliferation assay*

The proliferation of T cells was measured using tritiated thymidine incorporation assay. Briefly, UC-MSCs were co-cultured with a fixed number (5x104) peripheral blood mononuclear cells as the source of T cells. T cells stimulated with phytohemagglutinin-L (PHA-L) at 5 µg/ml (Roche), at various ratios (1:5, 1:10, 1:50 and 1:100) with UC-MSCs in a 96-well plate and incubated for 48h and 72h periods. At the final 18 hours of the incubation period, cells were pulsed with 10µl of $^3$H-TdR (0.037 MBq/well [0.5µCi/well]). At the end of the assay, cells were harvested onto glass fibre filter mats (Perkin Elmer) using an automatic cell harvester (Harvester Mach III M, TOMTEC, USA) and read by scintillation beta counter (MicroBeta Trilux).

*Statistical Analysis*

All value measurements were presented as mean ± SD unless stated otherwise and comparisons for all pairs were performed by student's t-test using Microsoft Office 2007 (Excel) with significance levels at $p<0.05$.

## RESULTS

*Explant method yields adherent cells with MSC's morphology*

A total of eight UC samples were utilised to generate MSC and

**Table I: Primer sequence for adipocytes and osteocytes**

| Primer | 5'-3' | Sequence | Annealing temp (°C) |
|---|---|---|---|
| GAPDH | Forward | TGAACGGGAAGCTCACTGG | |
| | Reverse | TCCACCACCCTGTTGCTGTA | 54 |
| Adipoq | Forward | GCAGGGGAAGCAGGACT | |
| | Reverse | TGCAGTCTGTGGTTCTGATTC | 60 |
| FABP4 | Forward | ATCACATCCCCATTCACACT | |
| | Reverse | ACTTGTCTCCAGTGAAAACTTTG | 54 |
| Osteocalcin | Forward | CGCCTGGGTCTCTTCACT | |
| | Reverse | CTCACACTCCTCGCCCTAT | 60 |
| Osteonectin | Forward | CCAGGCAAAGGAGAAAGAAGA | |
| | Reverse | CTCTAAACCCCTCCACATTCC | 54 |

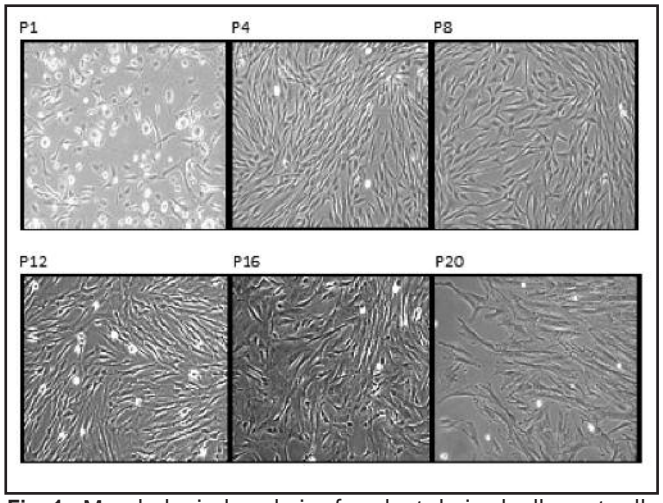


**Fig. 1:** Morphological analysis of explant-derived adherent cells culture.

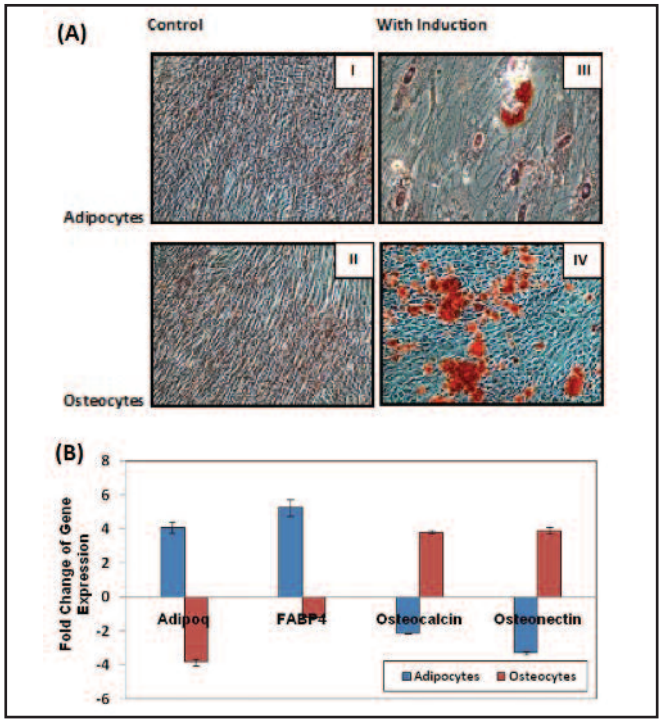


**Fig. 3:** Mesodermal differentiation of explant-derived adherent cells.

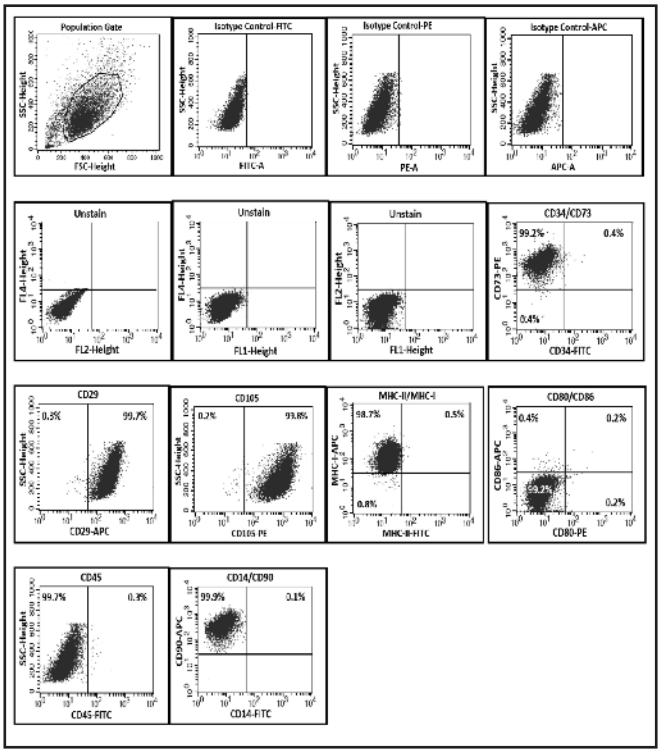


**Fig. 2:** Immunophenotyping of explant-derived UC adherent cells.

four of them were succeeded by MSC generation and characterisation which is accounted for 50% of success rate. Upon *in vitro* culture, the explanted UC gave rise to colonies of adherent cells at 5-7 days of primary culture. The primary culture achieved confluency at day 20-23 and sub-cultured till passage 20. The primary culture (P0) and passage 1 adherent cells comprised of heterogeneous populations where a substantial fraction of cells was haematopoietic origin such as macrophages. However, upon subsequent passages, the adherent cells has acquired a homogeneous morphology that resembles spindle-shaped fibroblastic cells with scanty cytoplasm and prominent nucleus. It was noticed that the time taken to reach confluency was reduced with a number of passages. Although a uniformity in morphological appearance was noted from P3-P20, however, gradual changes in cell's size between early and late passages was recorded. The explant-derived adherent cells at earlier

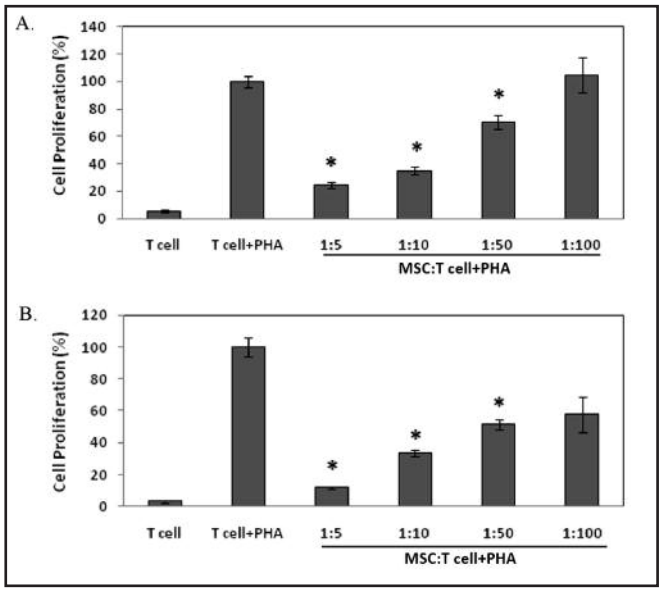


**Fig. 4:** Explant-derived adherent cells inhibit T cell Proliferation.

passage were smaller and well defined with fibroblast-like appearances whilst cells at later passage were relatively larger and flattened despite the uniformity within the same culture.

Minced umbilical cord Wharton's jelly was explanted in MSC complete media and allowed to confluence. Cells were trypsinized upon confluency and sub-cultured till passage 20 (P20). Photomicrograph of adherent cells at different passages (P1-P20) indicates changes in cell's size and homogeneity traits at subsequent passages. Photomicrographs were taken using phase contrast microscopic with 200 x magnification.

*Explant-derived adherent cells display MSC's immunophenotype*
Adherent cells of explanted UC were harvested at passage 3 and subjected for the immunophenotyping. Based flow cytometer analysis, the adherent cells were, at least, >95% positive for the standard MSC-positive markers namely CD29, CD73, CD90, CD105 and MHC-I. As expected, they were negative (<5%) for haematopoietic markers CD14, CD34, CD45, CD80, CD86 and MHC-II. The expression of positive and negative surface markers is in accordance with a globally accepted minimal definition for MSCs.

Approximately, $2x10^6$ adherent cells from passage 3 were harvested and subjected to the fluorochrome-conjugated monoclonal antibodies staining. Unlabeled and isotype controls were used as negative controls and a minimum of 10,000 cells/events were recorded by flow cytometer. Antibodies that stained the specific surface markers > 95% considered as positive and <5% as negative. This is a representative data of 3 independent experiments.

*Explant-derived adherent cells differentiate into adipocytes and osteocytes*
The ability of the explant-derived adherent cells to differentiate into mesodermal lineage was determined by a conventional biochemistry staining and further confirmed by gene expression assay. Adipogenic differentiation was measured on day 23 where the formation of lipid vacuoles in differentiated cells was detected by positive staining of Oil red O. Meanwhile; the osteogenic differentiation was detected by the deposition of calcium which is stained red by Alizarin Red Solution as shown in Figure 2A. Under the respective inductive media, explant-derived adherent cells had showed a respective mesodermal differentiation. The qualitative differentiation assay was further confirmed by the quantitative PCR analysis. Among four genes chosen, two of adipocytes (Adipoq & FABP4) and two of osteocytes (osteocalcin & osteonectin), differentiated explant-derived adherent cells showed the up-regulation of adipocytes genes and down-regulation of osteocytes genes for adipogenic differentiation. Whereas up-regulation of osteocyte genes and down-regulation of adipocyte genes were observed in osteogenic differentiation (Figure 2B). All positively upregulated genes showed 4 times fold change whereas the negatively downregulated genes showed -2 times fold change.

Confluenced adherent cells were induced to differentiate into relevant mesodermal lineages in respective inductive media. Cells were cultured in normal media (A.I & A.II) as controls. Upon adipogenic induction, the accumulated intracellular lipid granules were stained with Oil Red O (A.III) and further verified with qPCR where the expression of adipogenic genes was escalated while suppressing the osteogenic genes (B). The osteogenic differentiation was witnessed by calcium deposits stained with Alizarin Red Solution (A. IV) and verified by upregulation of osteogenic genes and downregulation of adipogenic genes (B). Photomicrographs were taken using phase contrast microscopic with 200x magnification

*Explant-derived adherent cells display a dose-dependent inhibitory activity on T cell's proliferation*
The immunosuppressive activity of explant-derived adherent cells was tested on mitogen-activated T cell's proliferation. The proliferation of T cell was determined by 3H-TdR incorporation assay after 48h and 72h cultivation period. The net proliferation of T cells was deduced after subtraction of the relevant proliferation of individual MSC culture. Explant-derived adherent cells had significantly inhibited the PHA-stimulated T cell's proliferation in a dose-depended manner where the highest inhibition was recorded when the number of added adherent cells was greater. The anti-proliferative activity exerted by the explant-derived adherent cells was much prominent when the incubation period of co-culture was extended to 72 hrs (Figure 4).

Explant-derived adherent cells at passage 3 were co-cultured with PHA-stimulated PBMC at various ratios where the number of PBMC were fixed as $5x10^4$ cells for 48 hrs (A) and 72 hrs (B). Adherent cells alone and co-cultured with unstimulated PBMC were served as controls. At the end of the assay, cells were harvested onto glass fibre filter mats using an automatic cell harvester and the incorporated tritiated thymidine was measured using scintillating counter. The explant-derived adherent cells inhibited T cells proliferation in a dose-dependent manner. Results were presented as a percentage of cell proliferation ± SD of 3 independent experiments. *P < 0.05 were compared to positive control (T+PHA).

## DISCUSSION

Stem cell therapy has become an inevitable arm of modern medicine as many diseases that were once considered as terminal now could be treated with various stem cells. Unlike other stem cells, MSCs are often generated or retrieved from different sources as almost all human organs are considerably populated with MSCs. Among all these tissue sources, human UC is considered as one of the highly potential sources of MSCs.[20-21] Although, UC serves as superior source as compared to the UC blood, yet the nature of MSCs generated from UC is often influenced by many factors such as the term of pregnancy, transportation of sample, the anatomical region of cord that been used for stem cell generation and culture condition.

Several methods have been commonly employed to generate MSCs from UC such as enzymatic digestion,[22] enzymatic-mechanical dissociation [23] and explants method.[24] In this present study, MSCs were generated from explant method where the human umbilical cord Wharton's jelly is minced and cultured in the optimised media. In comparison to the other methods, the explant method relies on the ability of MSCs to migrate and adhere to the plastic surface when they are cultured at *in vitro*. The explant method has certainly possessed some advantages over the conventional enzymatic digestion method. The mechanical mincing process during the preparation phase allows the release of intracellular compounds and cytokines that would promote the migration and establishment of MSC growth at *in vitro*. Moreover, the explanted minced umbilical cord tissues is a mini 3 dimension that mimics the real stem cell niche and permits the MSC's precursors to migrate towards the plastic surface. Unlikely, the chemical/enzymatic digestion extracts the stem cells from the existent stem cell niche which may affect the nature or propagation of the stem cells. Economically, explant method is also considered as cost-saving and less laborious as compared to the other existing methods. Comparatively, the time duration needed to process sample and requirement of research reagents are much lesser in explant method whereas the conventional enzymatic digestion requires at least 3-4 hours and demands specific reagents such as costly enzyme cocktails.[23] Although only half of the total tested samples had successfully yielded MSC population, yet the recorded 50% of successful rate is considerably higher when compared with the previous finding (25%) which had employed the explant method as well.[23]

Although, the generation of UC-MSCs via explant method is not a new finding as it has been formerly reported by others, yet the current study further advanced the existing protocol/procedures in term of sample processing and culture condition that were enhanced the successful rate of MSC's generation. The current protocol has utilised an optimal volume of culture media and also an additional replating step (when needed) to maximise the yield of adherent cell colonies. Since the formation and number of colony forming units are vital for the successive expansion of MSCs, thus, the primary culture of minced tissue was prolonged up to 2-3 weeks to allow a sufficient time for MSC precursors to mobilise and form a colony. The adherent cells generated by explanted UC comply all required characteristics that delineated by International Society for Cellular Therapy (ISCT) to minimally define MSCs. Morphologically, explant-derived adherent cells adhered to the plastic surface and resemble a spindle-shaped fibroblastic morphology which is consistent with a typical MSC's appearance at *in vitro* culture.[23,25] When explant-derived adherent cells were grown in respective adipogenic and osteogenic induction media, they are able to differentiate into relevant mesodermal cell types by displaying the lipid deposition and accumulation of calcium.

A gradual change in size was also noticed especially at late passages (P12-20) despite the uniformity of morphological appearance of MSCs after passage 3-4. It has been known the extensive *in vitro* expansion is often accompanied with replicative senescence.[26] Nevertheless, the repercussion of replicative senescence of MSCs can be reduced by supplementing growth factors that harness the expansion-induced senescence such as basic fibroblast growth factor 2 (bFGF-2).[27] Hence, the culture condition of current study has consumed bFGF-2 to generate and expand UC-MSCs till passage 20. Although the external factors such as differences in laboratory protocols, selection of culture media and the processing techniques variably contribute to the cellular senescence, yet confounding internal factor such different tissue sources [28-29] or event various anatomic locations within same tissue as well affect the growth kinetics of MSCs.[30]

The characterisation of MSCs was also conducted with immunophenotyping, where the expression of specific surface markers was assessed using fluorochrome-conjugated monoclonal antibodies staining. The explant-derived adherent cells at passage 3 onward were positive for the typical MSCs surface markers such as CD29, CD73, CD90 & CD105 and they lack haematopoietic markers namely CD14, CD34, CD45, CD80 & CD86. Although, the immunophenotype profile of MSCs could vary due to different laboratory methods and protocols, yet the current study reports that the acquired immunophenotyping of the explant-derived UC cells complies the universally accepted standard, thus confer the explant-derived adherent cells are MSCs.[31,32,33] One of the inherent nature of MSCs is to suppress the immune response of various immune cells.[35,36, 37] Thus, the generated MSCs from explanted UC were further tested for their ability to suppress the proliferation of T cells. As expected, adding UC-MSC to PHA-L activated T cells culture had profoundly suppressed the proliferation of activated T cells in a dose-dependent fashion.

Based on the current research findings, the explant-derived adherent cell from the foetal end of human umbilical cord possessed similar characteristics and properties of MSCs. When a potential clinical application of UC-MSCs is considered, the explant method will serve superior choice as this technique involves a minimal manipulation of samples that was not subjected to any chemical/enzymatic treatment or sorting. Thus, the explant method could be further developed to generate MSCs for clinical use and research purposes.

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