Phenotypic and functional characterization of mesenchymal stem cells from chorionic villi of human term placenta

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Running Title: the characteristics of placental mesenchymal stem cells

The authors declare no potential conflicts of interest

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Abstract

Background

Bone marrow derived mesenchymal stem cells (BM-MSCs) are used extensively in transplantation but their use is associated with many problems including low abundance in BM, low overall number, decreased differentiation potential with age and the invasive isolation procedures needed to obtain BM. We report a novel method of isolating placental MSCs (pMSCs) from chorionic villi, which exhibit the phenotypic and functional characteristics that will make them an attractive source of MSCs for cell-based therapy.

Methods

A novel explant approach was used to isolate pMSCs from chorionic villi of human placentae. These pMSCs were characterized by flow cytometry and were differentiated into adipocytes, osteocytes and chondrocytes using differentiation medium as demonstrated by cytochemical staining. The gene and protein expression profiles of pMSCs were also characterized using real time polymerase chain reaction (PCR) and flow cytometry, respectively. In addition, cytokine secretion by pMSCs was also analysed using sandwich enzyme-linked immunosorbent assay (ELISA) technique. Moreover, the migration and proliferation potentials of pMSCs were also determined.

Results

pMSCs were isolated from fetal part of the chorionic villi and these pMSCs expressed CD44, CD90, CD105, CD146, CD166 and HLA-ABC but not CD14, CD19, CD40, CD45, CD80, CD83, CD86 and HLA-DR. In addition, these pMSCs differentiated into osteocytes, chondrocytes and adipocytes and they also expressed several adhesion molecules, chemokines/receptors, growth factor receptors and cytokines/receptors. Moreover, they

secreted many cytokines (IL-1Ra, IL6, IL8, IL10, IL11 and IL15) and they were able to proliferate. Furthermore, they migrated in response to chemotactic factors including stromal cell-derived factor-1 (SDF-1), platelet derived growth factor (PDGF), hepatocyte growth factor (HGF), and monocyte chemotactic protein-1 (MCP-1).

Conclusions

We devised a novel explant method of isolating pMSCs that expressed many biological factors responsible for mediating cellular processes such as migration/homing, immune modulation and angiogenesis. Therefore, we suggest that pMSCs prepared from human term placental chorionic villous explants are an attractive source of MSCs for cell therapy.

Keywords

Chorionic villi, placental mesenchymal stem cells, characterization, migration, proliferation

Introduction

Stem cells are broadly classified as embryonic stem cells (ESCs) or adult stem cells (ASCs). ESCs can be obtained from the blastocyst of the early embryo. ESCs are pluripotent and differentiate into cell derivatives of the three germ layers: endoderm, ectoderm and mesoderm. Adult stem cells can be derived from a variety of tissues. However, the most characterized source of ASCs is the adult bone marrow (BM). One main subset of ASCs is the mesenchymal stem cells (MSCs). MSCs were firstly described as spindle shaped cells derived from BM that adhered to plastic and formed fibroblast colonies, which were called colony-forming unit fibroblasts (CFU-F) [1]. MSCs are multipotent cells can differentiate into cell derivatives of the mesenchymal lineage, including adipocytes, osteocytes, chondrocytes and myocytes [2,3]. In addition, there is some evidence that MSCs can "transdifferentiate" into hepatocytes, neurons and astrocytes [4]. MSCs have been isolated from a variety of adult tissues including liver, bone marrow, dental pulp, adipose-tissue, endometrium, muscle, amniotic fluid, placenta and umbilical cord blood[5-11].

There is much discussion in the literature about the real nature of cells called MSCs since heterogeneous populations of cells isolated using a range of procedures have been called MSCs. Currently, the International Society for Cellular Therapy has provided minimum criteria to identify MSCs; MSCs must be plastic-adherent, more than 95% of the population must express CD105, CD73 and CD90, and lack the expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II. MSCs must differentiate to osteocytes, adipocytes and chondrocytes *in vitro* [12].

Regardless of the absence of decisive markers for MSCs, the heterogeneous nature of MSC populations and variations in their differentiation potential, MSCs remain an attractive source of stem cells for the treatment of human diseases [13,14].

Clinically, human adult BM is the usually used as a source of MSCs. However, the use of adult BM has limitations. First, the abundance of MSCs in adult BM is low (approximately 0.001–0.01%)[15]. In addition, the overall number and differentiation potential of BM-MSCs decreases with age[16]. Moreover, BM harvesting is an invasive procedure. Consequently, the search for alternative sources of MSCs that could be used clinically is essential. Placenta is abundantly available and easily accessible because it is discarded after normal delivery. The placenta has become increasingly attractive as a source of MSCs. Several researchers isolated pMSCs and characterized their growth kinetics, cell marker expression, and multilineage differentiation into osteocytes, chondrocytes, adipocytes, and neuron-like cells[8,17-19]. However, in contrast to BM-MSCs, the biological characteristics of pMSCs are poorly understood. For stem cell-based therapy, it is essential to isolate highly enriched populations of MSCs and to identify the spectrum of molecules they express, which mediate important cellular functions including homing/migration, proliferation, differentiation, immune modulation and angiogenesis.

In this study, we developed a novel method to isolate highly enriched MSCs, with increased yields, from the chorionic villi of human placentae by treating tissues with Trypsin. MSCs were characterized for their expression of a broad spectrum of adhesion molecules, chemokines/receptors, cytokines/receptors and growth factors. Also, we examined the ability of these pMSCs to secrete cytokines, to proliferate and also to migrate ***in vitro***. Our study

demonstrated the potential of pMSCs from chorionic villous explants as a source of MSCs for cell based therapy.

Materials and Methods

Ethics of Experimentation

This study was approved by the institutional research board at King Abdulla International Medical Research Centre/ King Abdulaziz Medical City, Riyadh, Saudi Arabia. All placentae were obtained with informed consent.

Placentae

Human placentae were obtained from uncomplicated pregnancies following normal vaginal delivery (38- 40 weeks of gestation). The gestational age and fetal viability of all pregnancies were confirmed by early ultrasound examination before 20 weeks gestation. The placentae were used within 2 h of delivery.

Isolation of Mesenchymal Stem Cells from Placental Explants *in vitro*

To isolate pMSCs from chorionic villi, the explant approach was used. Tissues from 20 placentae were dissected and washed with ice-cold phosphate buffered saline (PBS), pH 7.4. After separating and discarding the maternal decidua that remains attached to the surface of the cotyledons, the chorionic villi from the fetal portion were cut into pieces of approximately 40 mg wet weight and washed with PBS and then incubated with 2.5% trypsin (Invitrogen,

Saudi Arabia) diluted in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM-F12) (Invitrogen, Saudi Arabia) containing (271 unit/ml) DNase (Invitrogen, Saudi Arabia), 100 μg/ml streptomycin and 100 U/l penicillin with gentle rotation at 4°C overnight. Trypsin untreated tissues were cultured immediately as described below. Following washing of untreated or trypsin-treated tissues with PBS for three times, the tissues were allowed to adhere to the plastic in 6 well plates (BD, Saudi Arabia) at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air for 60 min. Then, DMEM-F12 medium containing 10% Mesenchymal Stem Cell Certified fetal bovine serum (MSCFBS) (Invitrogen, Saudi Arabia), 100 μg/ml of L-glutamate, 100 μg/ml streptomycin and 100 U/l penicillin was gently added to the tissues and cultured at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Every two days, the medium was removed and replaced with fresh medium. On day 14, the tissues were removed and cells migrated out from the cut ends of the tissues were then harvested with TrypLE™ Express detachment solution (Invitrogen, Saudi Arabia) and counted using Trypan blue on a haemocytometer chamber. The number of cells was expressed as the total number of cells per cultured 40 mg of tissue. Cells at a density of 1 x 10^5 cells in 75 cm^2 flask (Becton Dickinson, Saudi Arabia) were re-cultured until they reached 75% confluency and then were used in subsequent experiments. Cells were visualized under an inverted Nikon ECLIPSE Ti U microscope (Nikon, Saudi Arabia) and photomicrographs were recorded using Nikon DS-Qi1 camera and Software (Nikon, Saudi Arabia). Twenty placentae were used in this study. Cells were isolated from passage zero to passage five. Untreated and Trypsin-treated tissues were analyzed in subsequent experiments. Passage zero is defined as the cells which were harvested immediately after the removal of tissues.

Flow Cytometry

Cells were harvested using TrypLE™ Express detachment solution. For analysis, 1 x 10^5 of cells were stained with antibodies listed in Table 1for 30 min and then were washed twice with cold PBS by centrifugation at 150xg for 5 min at 8°C. The expression of the corresponding cell surface proteins was assayed by an FC500 (Beckman Coulter, Saudi Arabia) flow cytometer.

Colony Forming Unit (CFU) Assay

Colony forming efficiency of pMSCs was assessed using CFU assay in which cells were seeded into six well plates at a density of 100 cells/well in DMEM-F12 medium containing 10% MSCFBS, 100 μg/ml of L-glutamate, 100 μg/ml streptomycin and 100 U/l penicillin. The medium was replaced with fresh medium every 3 days. After 14 days incubation at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air, the medium was removed and the cells were then washed with PBS for three times. Cells were then fixed with 4% paraformaldehyde in PBS, pH 7.4 for 30 min at room temperature (RT). After washing cells twice with PBS, they were stained with haematoxylin (Invitrogen, Saudi Arabia) for 5 min at RT, rinsed with distilled water, visualized under Micros ASTRIA MCX100LCD light microscope (MICROS, Saudi Arabia), and images were then captured for analysis. Aggregates of ≥50 cells were scored as colonies. Each experiment was performed on twenty separate occasions and samples were in triplicate unless otherwise specified. The numbers of passages and placentae were as above.

Osteogenic Induction

Cells at a density of 7.4 x 10^3 were seeded in 8-well culture slide (Becton Dickinson, Saudi Arabia) in DMEM-F12 medium containing 10% MSCFBS 100 μg/ml of L-glutamate, 100

μg/ml streptomycin and 100 U/l penicillin and then cultured at 37°C in a humidified atmosphere containing 5% CO2 and 95% air. When cells reached 70% confluency, the medium was removed and the cells were washed twice with PBS and then cultured in osteogenic differentiation medium containing osteogenic supplement (Part # 390416, R & D systems, Saudi Arabia), 10% MSCFBS, 100 μg/ml of L-glutamate, 100 μg/ml streptomycin and 100 U/l penicillin. The medium was replaced with fresh medium every three days. After 21 days, osteocytes were washed twice with PBS and fixed with 4% paraformaldehyde in PBS, pH 7.4 for 20 min at RT. After washing the cells twice with PBS, they were stained with 2% Alizarin Red S solution, pH 4.2 for 3 min at RT, rinsed three times with distilled water and then visualized under Micros ASTRIA MCX100LCD light microscope. Images were then captured. Each experiment was performed in duplicate. Cells isolated from passage zero to passage five from 10 placentae were randomly selected. Cells without addition of differentiation medium were used as a negative control.

Chondrogenic Induction

Cells at density of 2.5 x 10^5 in a 15 ml conical tube were centrifuged at 200 x g for 5 min at RT. After removing the culture medium, cells were resuspended in DMEM-F12 medium and centrifuged at 200 x g for 5 min at RT. After aspirating the medium, cells were resuspended in chondrogenic differentiation medium containing chondrogenic supplement (Part # 390417, R & D systems, Saudi Arabia), 10% MSCFBS, 100 μg/ml of L-glutamate, 100 μg/ml streptomycin and 100 U/l penicillin and then centrifuged at 200 x g for 5 min at RT. After centrifugation, the medium was not removed from the tube in order not to disturb the cell pellet. The cap was then loosened to allow gas exchange and the tube was incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. The medium was replaced with

fresh medium every three days without disturbing the cell pellet. After 21 days, the chondrocyte pellet was gently washed twice with PBS and liquid nitrogen was then used to freeze cell pellet in cryo-embedding medium (Leica Microsystems, Saudi Arabia). The frozen cell blocks were cut into 10 µm serial sections using a cryostat (Thermo Scientific, Saudi Arabia) and collected on glass slides (Leica Microsystems, Saudi Arabia). Slides were then fixed in 4% (w/v) paraformaldehyde in PBS, pH 7.4 for 20 min at RT. After washing slides twice with PBS, they were stained with 1% Alcian Blue solution prepared in 0.1 N HCL for 30 min at RT. After rinsing slides with 0.1 N HCl for three times, distilled water was added to neutralize the acidity. Then, the slides were visualized under the light microscope and images were captured. The number of replicates, passages, numbers of placentae and controls were as above.

Adipogenic Induction

Cells at a density of 3.7 x 10^4 were seeded in 8-well culture slide in DMEM-F12 culture medium containing 10% MSCFBS, 100 µg/ml of L-glutamate, 100 µg/ml streptomycin and 100 U/l penicillin and then incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. When cells were 100% confluent, the medium was removed and cells were then washed twice with PBS and cultured in adipogenic differentiation medium containing adipogenic supplement (Part # 390415, R & D systems, Saudi Arabia), 10% MSCFBS, 100 µg/ml of L-glutamate, 100 µg/ml streptomycin and 100 U/l penicillin. Then, cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. The medium was replaced with fresh Medium every three days. After 21 days, adipocytes were washed twice with PBS and fixed with 4% paraformaldehyde in PBS, pH 7.4 for 20 min at RT. After washing cells twice with PBS, they were incubated with 100 fold dilution LipidTOX™ Green

Neutral Lipid Stain for 30 min in the dark at RT and then were rinsed twice with PBS. Finally, SlowFade® Gold (Invitrogen, Saudi Arabia) was applied and cells were visualized under Nikon ECLIPSE 80i fluorescent microscope and images were captured using Nikon DS-Qi1 camera and Software. The number of replicates, passages, numbers of placentae and controls were as above.

Gene Expression Profile of Human Placental Mesenchymal Stem Cells (pMSCs)

To determine the expression of genes listed in Table 2, real time polymerase chain reaction (RT-PCR) was performed in which QuantiTect Primer Assay (Qiagen, Saudi Arabia) was used according to the manufacturer's instructions. Following extraction of total RNA from cells using the FastLane Cell cDNA kit (Qiagen, Saudi Arabia) according to the manufacturer's instructions, cDNA was synthesised using the Fast Lane Cell cDNA kit and RT Primer Mix (Qiagen, Saudi Arabia) at 42°C for 30 min. Then, mRNA was quantified by real-time PCR using QuantiTect SYBR Green PCR Kit (Qiagen, Saudi Arabia), according to the manufacturer's instructions and the reaction was then carried out in triplicate on the CFX96 real-time PCR detection system (BIO-RAD, Saudi Arabia). The data were then analysed using CFX manager software (BIO-RAD, Saudi Arabia). The assessment of gene expression was based on the following CT (cycle threshold) values: negative expression (-) if CT value is zero, weak expression (+) if CT> 35, moderate expression (++) if CT between 29 and 35 and strong expression (+++) if CT< 29. The relative expression level of the housekeeping gene β-actin or GAPDH or 18S rRNA was used to normalize target gene expression. Also, relative quantitation of target genes was expressed as a percentage of the housekeeping gene product as previously described[20]. Cells isolated from passage one of twenty placentae were analyzed in this study.

Enzyme- Linked Immunosorbent Assay (ELISA) for Detection of Cytokines

Secretion of IL1Ra, IL4, IL6, IL8, IL10, IL11, IL13, IL16, IL4 and IL20 was assayed by the Quantikine Human Immunoassay kits (R & D Systems), according to the manufacturer's instructions. Cells were washed three times with PBS and then cultured for 48h at a density of 2 x 10^6 in 6 well plate containing DMEM-F12 medium composed of 10% MSCFBS, 100 µg/ml of L-glutamate, 100 µg/ml streptomycin and 100 U/l penicillin at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Conditioned media derived from the cultured cells were analysed by the quantitative sandwich immunoassay. Culture medium not incubated with cells was used as the negative control. Each experiment was performed in duplicate. Cells isolated from passage one of 10 placentae were analyzed.

Proliferation of pMSCs

The effect of selected cytokines on the proliferation of pMSCs was studied by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay using Vybrant® MTT Cell Proliferation Assay Kit (Invitrogen, Saudi Arabia). In brief, cells were seeded at a density of 2 x 10^3 per well in 96-well tissue culture plates containing DMEM-F12 medium with 10% MSCFBS, 100 µg/ml of L-glutamate, 100 µg/ml streptomycin and 100 U/l penicillin and then cultured at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. We examined nine human recombinant cytokines (3ng/ml IL-4, 400ng/ml hepatocyte growth factor (HGF), 10 ng/ml IL-1, 10 ng/ml IL-6, 150 ng/ml IL8, 40 ng/ml Rantes and 40 ng/ml IL7A) [21]. These cytokines were dissolved in culture medium and then added to the cell culture and incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air for 48 h. The used

concentrations gave an optimal effect as shown in our dose response experiment and also as previously shown [21]. After replacing the medium with fresh medium, cells were treated with 0.5 mg/ml MTT solution and then were incubated at 37 °C for 4 h. Then, the dark blue formazan product of MTT was lysed with 40 mM HCl in isopropyl alcohol and measured at 570 nm using an ELISA plate reader (Spectra MR, Dynex Technologies, Saudi Arabia). 0.5 mg/ml MTT solution in medium not exposed to cells was used as the negative control. The number of replicates, cell passage and number of placentae were as above.

Migration of pMSCs

Migration assays were performed in transwell inserts (BD, Saudi Arabia) with 8μm pore filters in 12 well plates. Cells at a density of 5 x 10^5 were added to the upper side of the transwell filter chamber in a 12 well plate and DMEM-F12 migration medium containing 100 μg/ml of L-glutamate, 100 μg/ml streptomycin and 100 U/l penicillin with chemotactic factors was added to the bottom chamber. Migration observed in the presence of 30% FBS and with medium alone served as positive and negative controls, respectively. After overnight incubation at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air, the upper side of the filters was carefully washed with PBS and cells remaining on the upper face of the filters were gently removed with a cotton wool swab. Transwell filters were stained using haematoxylin for 1 min, and then washed with tap water, cut out with a scalpel, and mounted with Aquamount onto glass slides, putting the lower face on the top and then covered with coverslips. The total number of cells that had migrated was counted using light microscopy at 100X magnification. To avoid a bias related to microscopic counting, cells were blind counted. Data were expressed as numbers of total migrated cells per insert related to that of the negative control. We examined the chemotactic activity of stromal derived factor, SDF

(150 ng/ml), platelet derived growth factor, PDGF (10 ng/ml), hepatocyte growth factor, HGF (50 ng/ml), and MCP (100 ng/ml). All chemotactic factors used were recombinant factors of human origin (R & D Systems, Saudi Arabia). The used concentrations gave an optimal migratory effect as previously published [22]. The number of replicates, cell passage and number of placentae were as above.

Statistical Analysis

Data were analysed using the *t*-test. These analyses were performed using GraphPad Prism 5. Results were considered to be statistically significant if $P < 0.05$.

Results

Isolation, Flow Cytometry Analysis and Differentiation of pMSCs

Chorionic villi (approximately 40 mg wet weight) were treated with 2.5% Trypsin and then were attached to the plastic surface in 6 well plates and cultured in DMEMF-12 medium containing 10% MSCFBS for 14 days. The pMSCs migrated out of untreated and trypsin treated tissues exhibited a fibroblast like cell morphology (Fig. 1). Contamination of maternal derived cells in the pMSC populations was assessed by detecting SRY gene using real time PCR. The real time PCR of all samples (pMSCs isolated from passage zero to passage five of untreated and trypsin-treated tissues of 20 placentae) resulted in amplification products with SRY primers, which were detected at a CT value of 23 (±2) of 40 cycles. The GAPDH housekeeping gene was detected at CT value of 15 (± 3) of 40 cycles in all samples. Medical records revealed all placental stem cell preparations that gave SRY signals corresponded to pregnancies with male births, whereas those preparations that did not amplify with SRY primers were from female births. This confirms that pMSC preparations contained fetal-derived cells. The number of cells migrated out from the cut ends of the tissues after 14 days in culture was counted using Trypan blue on a haemocytometer chamber. The total number of migrated cells was then expressed per 40 mg of cultured tissue. The result showed that Trypsin significantly increased ($P<0.0001$) the number of cells produced by the explanted chronic villi as compared to Trypsin untreated villi (Fig. 1). There were 11.55×10^3 ($\pm 1.23 \times$

10^3) and 24.660 x 10^3 (±2.67 x10^3) cells produced per 40 mg of untreated and trypsin treated tissues, respectively (Fig. 1).

We determined the phenotype of MSCs isolated from both untreated and Trypsin- treated tissues by flow cytometry. Cells at passage zero of Trypsin- treated tissues were positive for MSC markers including CD44 (97% ±3%), CD90 (94% ±6%), CD146 (93% ±7%), CD166 (98% ±2%) and CD105 (99% ±1%). The expression of MSC markers by pMSCs increased at passage one (Fig. 2) but then did not change significantly through serial passages (passage two to passage five) (data not shown). Also, these cells isolated from passage zero to passage five expressed HLA-ABC (99% ±1%). In addition, they were negative for CD19, CD45, HLA-DR, CD80, CD86, and CD40 (Fig. 2). Similarly, cells isolated from untreated tissues were positive for all MSC markers and HLA-ABC and they were also negative for CD19, CD45, HLA-DR, CD80, CD86, and CD40 (data not shown). This expression of molecules by cells isolated from untreated tissues did not decrease/change through serial passages (data not shown). In addition, the expression profile of these markers by cells isolated from untreated and Trypsin- treated tissues were not significantly different (data not shown).

To estimate the potential of pMSC differentiation into several cell lineages, pMSCs isolated from Trypsin-treated chorionic villi were only studied since the phenotype markers expressed by pMSCs and the chondrogenic efficiency (below) of pMSCs isolated from untreated and Trypsin-treated tissues were not significantly different or changed through serial passages (data not shown). Therefore, cells isolated from Trypsin-treated chorionic villi were cultured in adipogenic, osteogenic and chondrogenic medium. At the end of the induction period, cells were differentiated into their respective cells. The qualitative confirmation of differentiation was made by Alizarin Red S solution for osteogenic differentiation, Alcian Blue solution for

chondrogenic differentiation and LipidTOX™ Green Neutral Lipid Stain for adipogenic differentiation (Fig. 3). In addition, the qualitative differentiation potential of pMSCs through serial passages (zero to five) was the same for the 10 placentae studied.

Colony Forming Unit Assay

Cells were clonogenic as shown by colony forming unit assay (Fig. 3). Clusters of ≥50 cells were counted as colonies. Seeding cells at density of 100 cells per well resulted in 19.1 ±2.2% and 21.5 ±3.7 of the total cells isolated from untreated and trypsin treated tissues, respectively per well forming colonies, with an average of 51.1 ± 7.2 cells/colony and 53.6 ±10.3 cells/colony for cells isolated from untreated and Trypsin- treated tissues, respectively (26 colonies counted for each sample). The clonogenic ability of MSCs isolated from untreated and Trypsin-treated tissues did not differ significantly or change through serial passages (data not shown).

Characterization of pMSCs by Real Time PCR and Flow Cytometry Techniques

The pMSCs from passage one of Trypsin-treated tissues were examined for the expression of genes encoding adhesion molecules, chemokines/receptors, cytokines/receptors and growth factors using PCR. The level of mRNA expression was assessed based on the CT value. We analysed 47 cytokines, 55 chemokines, 35 adhesion molecules, 30 growth factors (Table 2). In addition, the expression of some of these proteins by pMSCs was also examined using flow cytometry (Table 1). pMSCs expressed a broad spectrum of adhesion molecules, chemokines/receptors, cytokines/receptors and growth factors as shown in Tables 3-6.

Human pMSCs express a Distinct Set of Adhesion Molecules

Human pMSCs expressed a broad spectrum of adhesion molecules including integrin (α1, α2, α2b, α3, α4, α5, α6, α7, α8, α10, α11, αE, α M, αV, ß1, ß3, ß4, ß5, ß6, ß7, ß8), selectin P ligand and immunoglobulin superfamily (ICAM1, ICAM2, PECAM, VCAM and neuroplastin) as shown in Table 3. In addition, the flow cytometry analysis of surface protein expression by pMSCs revealed that pMSCs expressed ICAM-1 (CD54) (74.6 ± 13%).

Human pMSCs express a Distinct Set of Chemokines/Receptors

Human pMSCs expressed a broad spectrum of CXC Chemokine/Receptor family (CXCL3, CXCL5, CXCL6, CXCL10, CXCL11, CXCL12, CXCL14, CXCL16, CXCR1, CXCR3, CXCR4, CXCR5, and CXCR6), C Chemokine/Receptor family (XCL1 and XCL2), CX3C Chemokine/Receptor family (CX3CL1 and CX3CR1), CC Chemokine/Receptor family (CCL1, CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL18, CCL19, CCL21, CCL24, CCL25, CCL26, CCR1, CCR3, CCR4, CCR6 and CCR7) as shown in Table 4. At the protein level, pMSCs expressed CXCR1 (27.1% ±4%), CXCR2 (6.7% ±3%), CXCR3 (22.5% ±5%), CXCR4 (9.4% ±1.5%), CXCR7 (10.3% ±2%), CX3CR1 (10.6% ±1%), XCR1 (5.3% ±0.5%) and CXCL4 (8.7% ±1%) as shown in Fig. 4. In addition, pMSCs lacked the expression of CCR1- CCR10, CXCL1, CXCL5, CXCL9, CXCL10 (IP-10), CXCL12, CX3CL1, CCL2 (monocyte chemotactic protein-1: MCP-1) and CCL5 (RANTES), Fig. 4.

Human pMSCs express a Distinct Set of Cytokines

Human pMSCs expressed a broad spectrum of cytokines/receptors including inflammatory IL1A, IL1B, IL6, IL8, IL12A, IL15, IL16, IL18, IL20, IL23A, IL26, IL27, IL28A, IL29,

IL31, IL32, IL33, IL34, IL1R1, IL6R, IL7R, IL17RC and tumor necrosis factor receptor superfamily, member 1A and 1B and anti-inflammatory cytokines/receptors IL4, IL5, IL6, IL10, IL19, IL24, IL25, IL27, IL32, IL4, IL6 and IL10RA as shown in Table 5. In addition, the FACS analysis of surface protein expression by pMSCs revealed that pMSCs expressed CD130 (31.4% ±17%), the signal-transducing receptor for cytokines of the IL-6 family but lacked the expression of tumor necrosis factor receptor superfamily, member 1A (CD120a) and 1B (CD120b) and interleukin-7 receptor-α (CD127) as well as IL8.

Human pMSCs express a Distinct Set of Growth Factor Receptors

Human pMSCs expressed a broad spectrum of growth factor receptors including platelet-derived growth factor receptor, interferon gamma receptor 1 and 2, interferon (alpha, beta and omega) receptor 1 and 2, fibroblast growth factor receptor 1 and 3, transforming growth factor, beta receptor 1 and II, epidermal growth factor receptor, insulin-like growth factor 1 receptor , colony stimulating factor 2 receptor, beta, formyl peptide receptor 2, opioid growth factor receptor, vascular endothelial growth factor, vascular endothelial growth factor receptor 2, hepatocyte growth factor receptor, epidermal growth factor as shown in Table 6.

Human pMSCs express Immune Suppressive Genes and Negative Co-Signalling Proteins

The analysis of mRNA expression of pMSCs isolated from passage one of Trypsin- treated tissues revealed that these cells expressed the immunosuppressive enzyme indoleamine-2,3-dioxygenase (IDO). In addition, the flow cytometry analysis showed that pMSCs expressed the negative co-signalling proteins B7H-3 (99.4% ± 1%), CD273 (55.5% ±10%) and CD274 (53.3% ± 15%). In contrast, pMSCs lacked the expression of the positive co-signalling protein, B7H2 and the program death receptor (CD279).

Human pMSCs Secrete Various Cytokines

Stem cells are known to regulate the functions of many cells through the secretion of cytokines. Therefore, we explored whether the pMSCs isolated from passage one of Trypsin-treated tissues secreted cytokines or not. Several cytokines was detected in the conditioned medium of pMSCs by ELISA. This includes IL1Ra (915 pg/ml ± 77), IL6 (294 pg/ml ± 54), IL-8 (254 pg/ml ± 43), IL-10 (244 pg/ml ± 34), IL-11 (451 pg/ml ± 62) and IL-15 (176 pg/ml ± 92).

The Proliferation Potential of pMSCs

In attempt to determine whether pMSCs isolated from passage one of Trypsin- treated tissues can proliferate in response to cytokines, we studied the effect of different cytokines on the proliferation of these pMSCs using the MTT assay. The results showed that IL-4 and HGF significantly inhibited ($P<0.01$) the proliferation of pMSCs as shown in Fig.5. In contrast, pMSC proliferation was significantly increased in the presence of IL-1 ($P <0.01$), IL-6 ($P <0.01$), IL-8 ($P< 0.05$), Rantes ($P <0.01$) and IL-17A ($P <0.01$), Fig. 5.

The Migration Potential of pMSCs

As a first evaluation of the migration potential of pMSCs isolated from passage one of Trypsin- treated tissues ***in vitro***, we examined the effects of different chemotactic factors on the migration of pMSCs using a transwell migration assay. The result showed that these chemotactic factors stimulated the migration of pMSCs. The number of migrated pMSCs increased significantly ($P<0.0001$) in the presence of SDF (530 ±30.13), PDGF (745.2

±29.82), HGF (597.2 ±41.51) and MCP-1 (557.7 ±32.29) as compared to negative control (306.2 ±16.44) (Fig. 5).

Discussion

A novel *in vitro* model to isolate pMSCs from chorionic villous explants

In this study, we developed a novel method to isolate MSCs from fetal portion of the chorionic villi of human placentae. We have modified the previously published explant approach which was shown to be a better choice than the enzyme digestion methods since it can isolate MSCs from the chorionic villi without generation of extracellular matrix, damaged cells, endothelial cells and blood cells[23,24]. We treated chorionic villi with Trypsin, which increased the number of isolated pMSCs by approximately two fold (Fig. 1). The mechanism by which Trypsin mediates this effect needs to be further investigated, but it is possible that this digestive enzyme [25] disrupted the structure of the placenta that facilitated the movement of MSCs out of their niche[26]. In addition, Trypsin did not change the morphology of pMSCs. pMSCs isolated from both untreated and Trypsin- treated tissues exhibited a fibroblast like cell morphology (Fig. 1).

We cultured pMSCs, which were isolated from untreated and Trypsin- treated tissues, for five passages. Interestingly, the hematopoietic cells in pMSC populations were absent from passage zero to passage five (Fig. 2). This result is novel since previous studies have reported that the frequency of hematopoietic cells diminished after serial passages in culture[23,24,27]. In addition, pMSCs isolated from passage zero of Trypsin treated- tissues were positive for

MSC markers, HLA-ABC, and were negative for HLA-DR and the co-stimulatory molecules (Fig. 2). The expression of these molecules by pMSCs isolated from untreated and Trypsin treated- tissues was similar and also did not decrease through serial passages in culture. In addition, these pMSCs exhibited differentiation and clonogenic abilities (Fig. 3); thus further confirmation that these cells have stem cell-like properties.

We are the first to perform this extensive phenotypic, differentiation and self-renewal analysis on pMSCs isolated from serial culture passages. Our method can yield highly enriched pMSC populations in a time period (~two weeks) less than the previously published time[23,24]. In addition, Trypsin treatment can increase the number of pMSCs produced by the explants without affecting the phenotype or differentiation or self-renewal abilities of pMSC. Consequently, our method can be a better choice in the field of stem cell research to isolate and enrich pMSC populations in early passages.

pMSCs express a specific combination of adhesion molecules

Adhesion molecules play major roles in several cellular processes, such as cell growth, differentiation and homing. Adhesion molecules are classified into immunoglobulin superfamily cell adhesion molecules (CAMs), integrins, cadherins, and selectins. Our study is the first to show the expression of a comprehensive range of adhesion molecules by pMSCs (Table 3). In addition, we showed for the first time that pMSC populations expressed these adhesion molecules including α7, α8, α10, αE, αM, β3, ICAM2, PECAM and selectin P ligand. Moreover, similar to other study[17], we showed that pMSCs expressed these molecules α1, α2, α2b, α3, α4, α5, α6, α7, α8, α11, αV, β1, β4, β5, ICAM1, VCAM, CD166 and CD44. However, the significance of these molecules in pMSC biology should be further investigated, but it is likely that pMSCs express adhesion molecules to mediate their adhesion to endothelial cells and migration to tissues through a homing process similar to leukocytes.

pMSCs express a specific combination of chemokines/receptors

Chemotactic molecules such as chemokines are essential to attract MSCs to the damaged tissue[28]. Chemokines are a family of proteins that regulate many functions such as chemotaxis. The chemokine family is divided into four sub-families: CXC, CC, C and CX_3C chemokines. Chemokine receptors are classified as G protein coupled receptors for CXC, CC, C or CX_3C chemokines[29]. Our knowledge regarding the expression profile of chemokines/receptors by pMSCs is limited; thus we have performed this first comprehensive study to demonstrate that pMSCs express several chemokine receptors including CCR1, CCR3, CCR4, CCR6, CCR7, CXCR1, CXCR3, CXCR4, CXCR5, CXCR6 and CX3CR1 as it has been previously reported [17,20]. In addition, we report for the first time the expression of many chemokine ligands by pMSCs (Table 4). However, some of these ligands and receptors were either absent or expressed at low level by pMSCs (Fig. 4). This is may be because we used unstimulated cells in this study since the pre-treatment of MSCs with inflammatory cytokines such as TNF-α was shown to increase the expression of chemokine receptors[22]. In addition, the use of Western blot is a better technique than FACS since it can detect low level of protein[22]. The expression of chemokines by pMSCs may account for their migration ability as we showed in this study. However, more studies are essential to determine the precise functional roles for these molecules in mediating the pMSC repair response.

pMSCs express a specific combination of cytokines, growth factors and immune molecules

Cytokines are signaling molecules that mediate many of cell functions, such as immune modulation and migration. BM-MSCs express many cytokines[15,30], but our knowledge regarding the expression of cytokines by pMSCs is limited. Therefore, we performed this

study to show that pMSCs express and secrete several inflammatory/ anti-inflammatory cytokines (Table 5). However, the biological significance of these cytokines in pMSC function should be further examined. However, these cytokines may mediate pMSC migration as it has been reported for IL-1 and IL-6[31,32]. In addition, pMSCs may use these cytokines to mediate their immune functions through different mechanisms such as induction of regulatory T cells by IL-15[33], IL-10 and IL1Ra[34] or inhibition of T cell functions by IL-27 and IL-31[35,36] or antagonizing HIV infection by IL-32[37] or reducing inflammation and killing cancerous cells by IL-24[38,39].

This is the first study which demonstrates that pMSCs isolated from chorionic villi by the explant method express a broad spectrum of cytokines, growth factor receptors (Tables 5 and 6) and costimulatory molecules (B7H3, CD273 and CD274) as well as the immune suppressive enzyme IDO, which can induce T cell death[40-43]. These molecules can potentially mediate pMSC functions including proliferation, differentiation, angiogenesis, migration, program cell death (apoptosis), antitumor activity, antiviral function and immune modulatory functions. Therefore, our study demonstrates that our pMSC populations may have a significant potential in treating different types of human diseases including immune disorders, cancers and infectious diseases. However, we need to demonstrate the potential therapeutic ability of these pMSCs by performing functional studies ***in vitro*** and ***in vivo***. Comprehensive analysis of pMSC properties may aid in deciding which clinical applications will benefit most from the use of pMSCs. For example, our data suggest pMSC populations are attractive candidates for the treatment of disorders involving T cells where immunosuppression of T cell activity by pMSCs may be advantageous. Multiple sclerosis, where autoreactive T cells mediate inflammation of the central nervous system (CNS), may benefit from the immunosuppressive molecules expressed by pMSCs.

Conclusion

We have devised a novel method using a tissue explant approach to isolate pMSCs from chorionic villous tissue of the normal human placenta. Our study is novel and comprehensive. We showed that pMSCs exhibited important features of the MSC lineage and expressed specific combinations of adhesion molecules, chemokines/receptors, cytokines/receptors and growth factors; many of which are known to be involved in cell proliferation, differentiation, migration and repair.

Acknowledgment

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Fig. 1: Photomicrographs showing representative examples of pMSCs migrated out of untreated chorionic villous tissue during culture at day 5 (A) and day 14 (B) and pMSCs migrated of trypsin treated chorionic villous tissue during culture at day 5 (C) and day 14 (D). Total number of pMSCs migrated out from the chronic villi after 14 days in culture (E). Trypsin significantly increased the number of pMSCs migrated per 40 mg villous explant (P> 0.0001). Twenty placentae were used in this study. Scale bars represent 50 µm and Bars represent standard errors.

Fig. 2: Histograms showing representative examples of placental mesenchymal stem cells (pMSCs) isolated from passage one stained with cell surface markers. pMSCs were negative for the hematopoietic markers (CD45, CD14 and CD19), costimulatory molecules (CD80, CD86, CD40, CD83 and B7H2) and HLADR while pMSCs were positive for CD44, CD90, CD105, CD146, CD166 and HLA-ABC. Negative controls (IgG or IgM) were used (Data now shown). pMSCs from passage zero to passage five of 20 placentae were analysed.

Fig. 3: Photomicrographs showing representative examples of colony forming unit of pMSCs isolated from passage one (A), the differentiation of pMSC isolated from passage 1 into osteocyte as demonstrated by Alizarin Red S staining of osteocytes after 21 days (B), chondrocyte as demonstrated by Alcian Blue staining of cross-section of chondrogenic pellet after 21 days (C) and adipocyte as demonstrated by HCS LipidTOX™ Green neutral lipid staining of adipocytes after 21 days (D). Scale bars represent 50 µm

Fig. 4: Histograms showing representative examples of placental mesenchymal stem cells (pMSCs) from passage one stained with antibodies against chemokines/receptors. Negative controls (IgG or IgM) were used (Data now shown). Twenty placentae were used in this analysis.

Fig. 5: The effect of different cytokines on the proliferation of pMSCs (A) and the effect of different chemotactic factors (SDF, PDGF, HGF and MCP-1) on the migration of pMSCs (B). The proliferation of pMSCs was significantly suppressed in the presence of IL-4 (*$P < 0.01$) and HGF (*$P < 0.01$) whereas significantly increased in the presence of IL-1 (*$P < 0.01$), IL-6 (*$P < 0.01$), IL-8 (**$P < 0.05$), RANTES (*$P < 0.01$) and IL17A (*$P < 0.01$). The chemotactic factors significantly (*$P< 0.0001$) stimulated the migration of pMSCs in transwell migration plates. pMSCs isolated from Trypsin- treated tissues at passage one of 10 placentae were analyzed. Bars represent standard errors.

Table 1: Antibodies used in this study to characterize the isolated placental mesenchymal stem cells (pMSCs)

Antibody	Conjugate	Dilution	Antibody	Conjugate	Dilution	Antibody	Conjugate	Dilution
CD45	FITC	1:10	CD279	PC5	1:10	CCR8	PE	1:10
CD19	FITC	1:10	CD44	FITC	1:10	CCR9	PE	1:10
CD90	FITC	1:10	CD120a	PE	1:10	CCR10	PE	1:10
HLA-ABC	FITC	1:10	CD120b	PE	1:10	CXCR1	PE	1:10
HLA-DR	FITC	1:10	CD127	PE	1:10	CXCR2	PE	1:10
CD105	PE	1:10	CD130 (IL-6RB)	PE	1:10	CXCR3	PE	1:10
CD146	PE	1:10	CD134	PE	1:10	CXCR4	PE	1:10
CD166	PE	1:10	CCR1	PE	1:10	CXCR7	PE	1:10
CD80	PE	1:10	CCR2	PE	1:10	CX3CR1	PE	1:10
CD86	PE	1:10	CCR3	PE	1:10	XCR1	PE	1:10
CD40	PE	1:10	CCR4	PE	1:10	CCL11	PE	1:10
CD54	FITC	1:10	CCR5	PE	1:10	CX3CL1	PE	1:10
CD273	PC5	1:10	CCR6	PE	1:10	CXCL1	PE	1:10
CD274	PC5	1:10	CCR7	PE	1:10	CXCR7	PE	1:10

Table 2: Genes used in this study to characterize human placental mesenchymal stem cells (pMSCs). Validated real-time PCR primer sets for the genes above were obtained (Qiagen, Saudi Arabia)

	Cytokine Receptors		Chemokines				Chemokine Receptors		Growth Fctors			Adhesion M	
IL26	IL1R1	IL22RA1	CCL1	CCL17	CX3CL1	XCL2	CCR1	CXCR3	PDGFRA	FGFR3	TNFRSF1A	Integrin α1	Integrin β
IL27	IL2RA	IL22RA2	CCL2	CCL18	CXCL3		CCR2	CXCR4	PDGFRB	TGFBR1	TNFRSF1B	integrin α2	integrin α
IL28A	IL2RB	IL3RA	CCL3	CCL19	CXCL5		CCR3	CXCR5	FGFR4	TGFBR2	MET	integrin α2b	integrin α
IL29	IL2RG		CCL4	CCL20	CXCL6		CCR4	CXCR6	FGFR2	FPR2	EGF	integrin α 3	Integrin β
IL31	IL4R		CCL5	CCL21	CXCL9		CCR5	CX3CR1	IFNGR1	OGFR	IDO	integrin α 4	Integrin β
IL32	IL6R		CCL7	CCL22	CXCL10		CCR6	XCR1	IFNGR2	IGF1R		integrin, α 5	integrin β
IL33	IL7R		CCL8	CCL23	CXCL11		CCR7		IFNAR1	CSF2RB		integrin α6	integrin β
IL34	IL9R		CCL11	CCL24	CXCL12		CCR8		IFNAR2	EGFR		integrin, α 7	integrin β
	IL10RA		CCL13	CCL25	CXCL13		CCR9		NGFR	NPTN		integrin, α 9	ICAM1
	IL10RB		CCL14	CCL26	CXCL14		CCR10		KDR	HRH4		integrin, α D	ICAM2
	IL17RC		CCL15	CCL27	CXCL16		CXCR1		IFNK	FLT4		integrin, β 6	PECAM
	IL18R1		CCL16	CCL28	XCL1		CXCR2		FGFR1	FLT1		integrin, β7	VCAM

Table 3: The expression of adhesion molecules in human placental mesenchymal stem cells (pMSCs) as determined by real time PCR: (-) no expression: (+) weak expression: (++) moderate expression: (+++) strong expression of mRNA

hesion Molecules	**Results**	**Adhesion Molecules**	**Results**	**Adhesion Molecules**
ntegrin, alpha 1	++	integrin, alpha L (CD11A)	-	PECAM (CD31)
n, alpha 2 (CD49B)	+++	integrin, alpha M (CD11b)	+	VCAM (CD106)
n, alpha 2b (CD41)	++	integrin, alpha V (CD51)	+++	MEDCAM
n, alpha 3 (CD49C)	+++	integrin, alpha X (CD11c)	-	SELE: selectin E
n, alpha 4 (CD49D)	+++	integrin, beta 1 (CD29)	+++	SELL: selectin L
n, alpha 5 (CD49e)	+++	integrin, beta 2 (CD18)	-	SELP: Selectin P (CD62)
in, alpha 6 (CD49f)	+++	integrin, beta 3 (CD61)	++	SELPLG: Selectin P ligand
ntegrin, alpha 7	++	integrin, beta 4 (CD104)	++	
ntegrin, alpha 8	++	integrin, beta 5	++	
ntegrin, alpha 9	-	integrin, beta 6	++	
tegrin, alpha 10	++	integrin, beta 7	++	
tegrin, alpha 11	+++	integrin, beta 8	++	
tegrin, alpha D	-	ICAM1 (CD54)	++	
n, alpha E (CD103)	++	ICAM2 (CD102)	++	

Table 4: The expression of chemokine receptors and chemokine ligands in human placental mesenchymal stem cells (pMSCs) as determined by real time PCR: (-) no expression: (+) weak expression: (++) moderate expression: (+++) strong expression of mRNA

Chemokines	**Results**	**Chemokines**	**Results**	**Chemokines**	**Results**	**Chemokines Receptors**	**Results**	**Chemokines Receptors**	**Results**
CCL1	++	CCL19	++	CXCL9	-	CCR1	++	CXCR5	++
CCL2	++	CCL20	-	CXCL10	++	CCR2	-	CXCR6	++
CCL3	++	CCL21	+++	CXCL11	++	CCR3	++	CX3CR1	++
CCL4	+++	CCL22	-	CXCL12	++	CCR4	++	XCR1	-
CCL5	++	CCL23		CXCL13	-	CCR5	-		
CCL7	++	CCL24	++	CXCL14	++	CCR6	++		
CCL8	++	CCL25	++	CXCL16	++	CCR7	++		
CCL11	-	CCL26	++	XCL1	+++	CCR8	-		
CCL13	-	CCL27	-	XCL2	++	CCR9	-		
CCL14	-	CCL28	-			CCR10	-		
CCL15	-	CX3CL1	++			CXCR1	++		
CCL16	-	CXCL3	+++			CXCR2	-		
CCL17	-	CXCL5	++			CXCR3	++		
CCL18	+	CXCL6	+++			CXCR4	++		

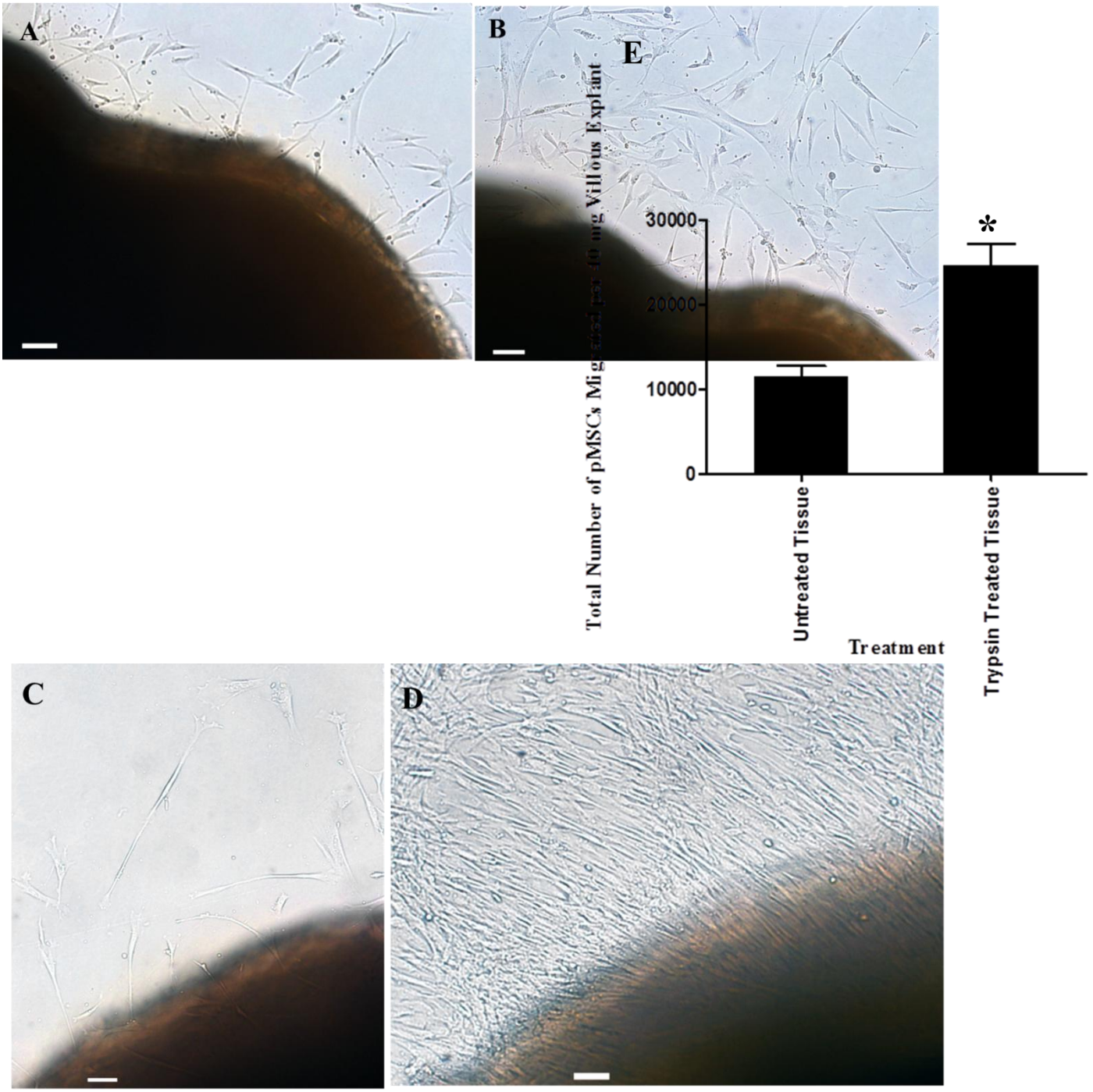
Table 5: The expression of cytokine mRNA in human placental mesenchymal stem cells (pMSCs) as determined by real time PCR: (-) no expression: (+) weak expression: (++) moderate expression: (+++) strong expression of mRNA

Cytokines	Results	Cytokines	Results	Cytokines	Results	Cytokine Receptors	Results	Cytokine Receptors	Results
IL1A	+	IL13	-	IL26	+	IL1R1	+	IL18R1	-
IL1B	+	IL15	+	IL27	+	IL2RA	-	IL22RA1	-
IL2	-	IL16	+	IL28A	+	IL2RB	-	IL22RA2	-
IL3	-	IL17A	-	IL29	+	IL2RG	-	IL18R1	-
IL4	+	IL18	+	IL31	+	IL3RA	-		
IL5	+	IL19	+	IL32	+++	IL4R	+		
IL6	+++	IL20	+	IL33	+	IL6R	+		
IL8	+++	IL21	-	IL34	+	IL7R	+		
IL9	-	IL22	-			IL9R	-		
IL10	+	IL23A	+			IL10RA	-		
IL12A	+	IL24	+			IL10RB	+		
IL12B	-	IL25	+			IL17RC	+		

Table 6: The expression of growth factors in human placental mesenchymal stem cells (pMSCs) as determined by real time PCR: (-) no expression: (+) weak expression: (++) moderate expression: (+++) strong expression of mRNA

Growth Factor Receptors	Results	Growth Factor/ Receptors and immune molecule	Results
PDGFRA: platelet-derived growth factor receptor, alpha	+++	**FPR2:** formyl peptide receptor 2	++
PDGFRB: Platelet-derived growth factor receptor, beta	+++	**OGFR:** Opioid growth factor receptor (OGFR)	++
FGFR4: Fibroblast growth factor receptor 4	-	**IGF1R:** Insulin-like Growth Factor 1	+++
FGFR2: Fibroblast growth factor receptor 2	-	**CSF2RB:** Colony stimulating factor 2 receptor, beta	++
IFNGR1: Interferon gamma receptor 1	+++	**EGFR:** Epidermal Growth Factor Receptor	+++
IFNGR2: Interferon gamma receptor 2	+++	**NPTN:** Neuroplastin	+++
IFNAR1: Interferon (alpha, beta and omega) receptor 1	+++	**HRH4:** Histamine receptor H4	++
IFNAR2: Interferon (alpha, beta and omega) receptor 2	++	**FLT4:** fms-related tyrosine kinase 4	-
NGFR: Nerve growth factor receptor	-	**FLT1:** fms-related tyrosine kinase 1 (vascular endothelial growth factor)	++
KDR: kinase insert domain receptor (type III receptor tyrosine kinase)	++	**TNFRSF1A:** tumor necrosis factor receptor superfamily, member 1A	+++
IFNK: interferon, kappa	-	**TNFRSF1B:** tumor necrosis factor receptor superfamily, member 1B	++
FGFR1: fibroblast growth factor receptor 1	+++	**MET:** met proto-oncogene (hepatocyte growth factor receptor)	+++
FGFR3: fibroblast growth factor receptor 3	+++	**EGF:** Epidermal Growth Factor	++

TGFBR1: transforming growth factor, beta receptor 1	++	Indoleamine 2,3-dioxygenase	+
TGFBR2: transforming growth factor, beta receptor II	+++		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Untreated Tissue
Trypsin Treated Tissue
Treatment
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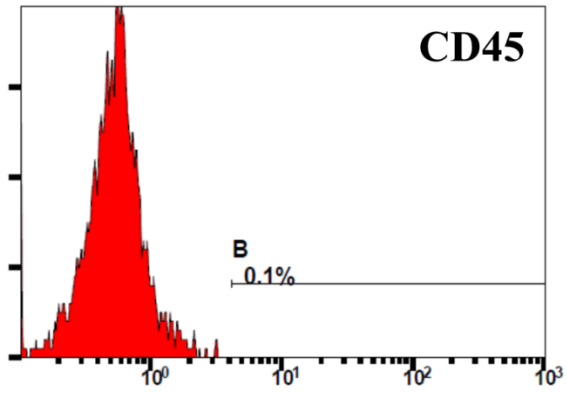
Fig. 1: Photomicrographs showing representative examples of pMSCs migrated out of untreated chorionic villous tissue during culture at day 5 (A) and day 14 (B) and pMSCs migrated of trypsin treated chorionic villous tissue during culture at day 5 (C) and day 14 (D). Total number of pMSCs migrated out from the chronic villi after 14 days in culture (E). Trypsin significantly increased the number of pMSCs migrated per 40 mg villous explant (P> 0.0001). Twenty placentae were used in this study. Scale bars represent 50 µm and Bars represent standard errors.


CD45
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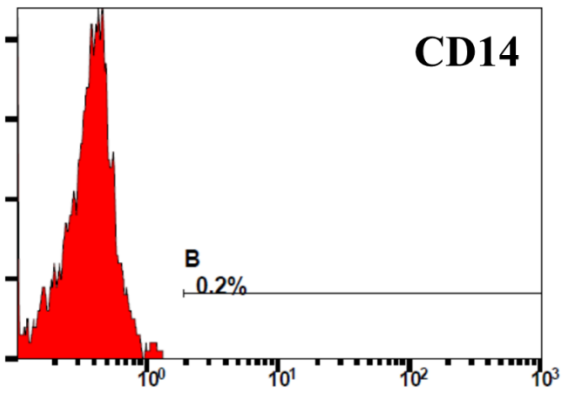
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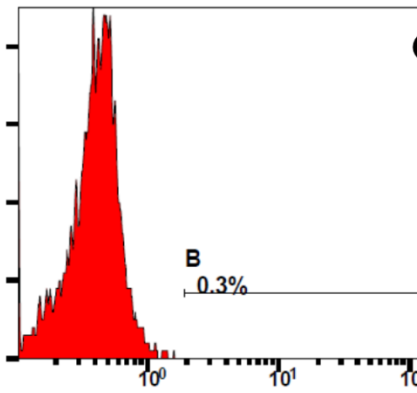
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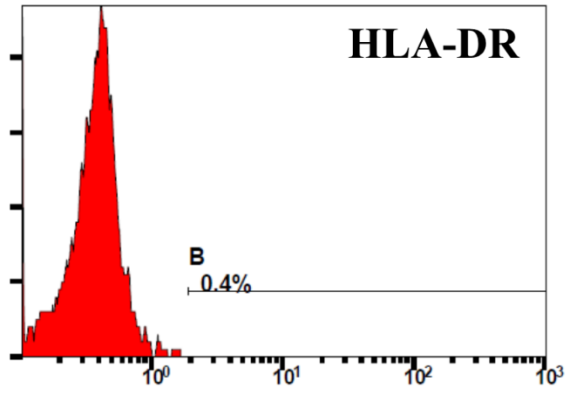
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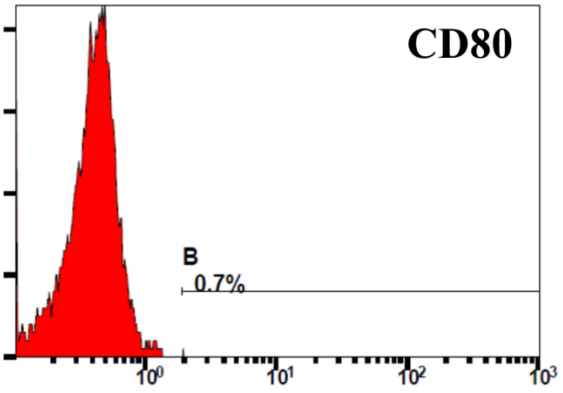
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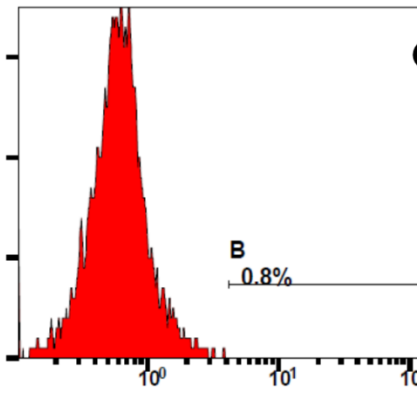
B
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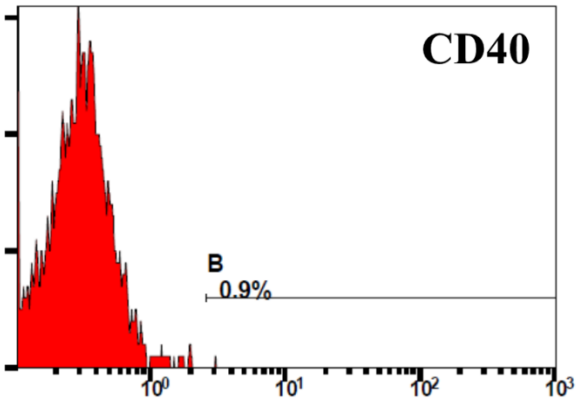
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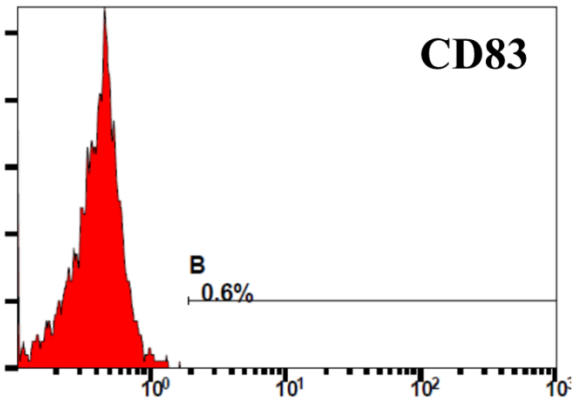
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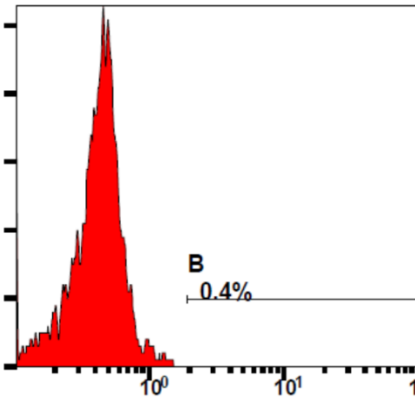
B
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10^0
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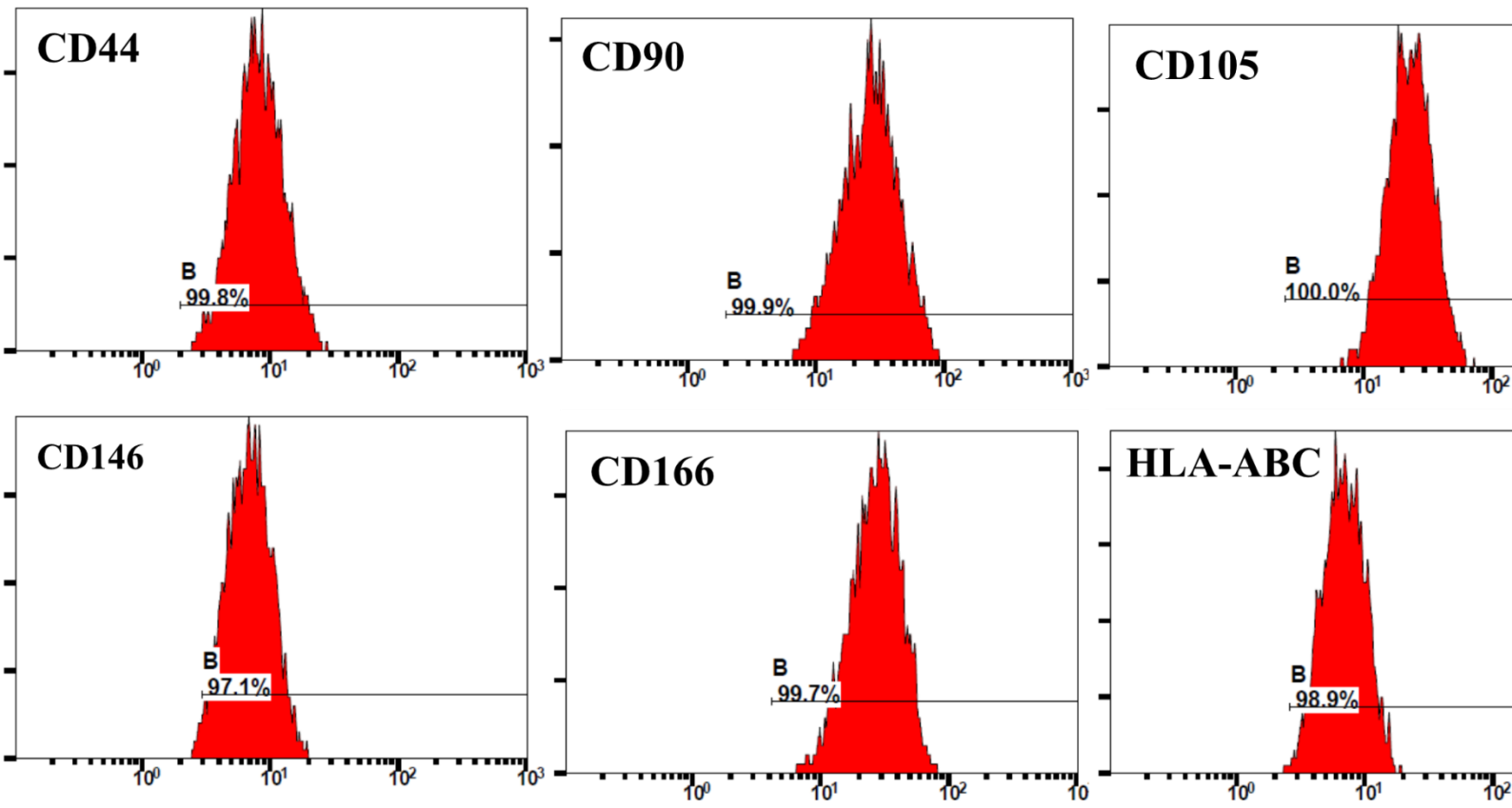


Fig. 2: Histograms showing representative examples of placental mesenchymal stem cells (pMSCs) isolated from passage one stained with cell surface markers. pMSCs were negative for the hematopoietic markers (CD45, CD14 and CD19), costimulatory molecules (CD80,

CD86, CD40, CD83 and B7H2) and HLADR while pMSCs were positive for CD44, CD90, CD105, CD146, CD166 and HLA-ABC. Negative controls (IgG or IgM) were used (Data now shown). pMSCs from passage zero to passage five of 20 placentae were analysed.

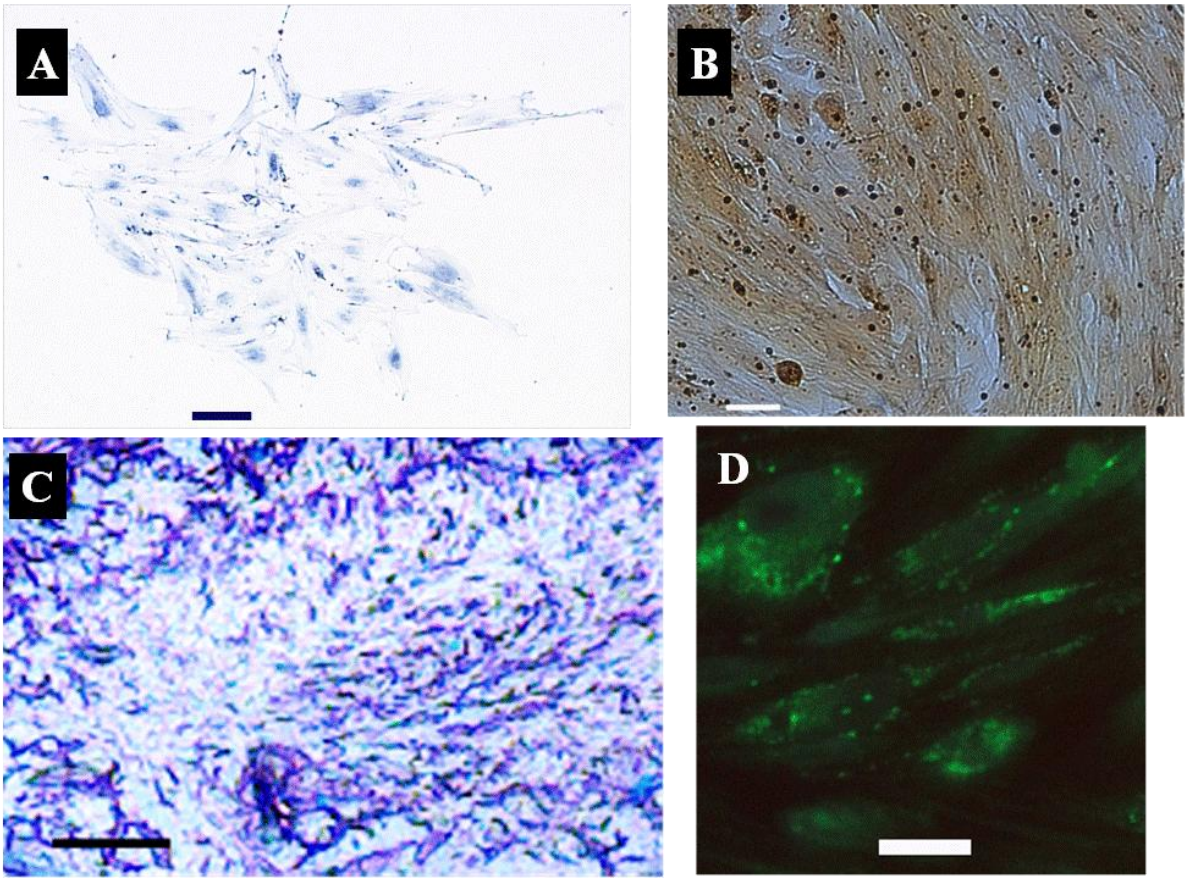
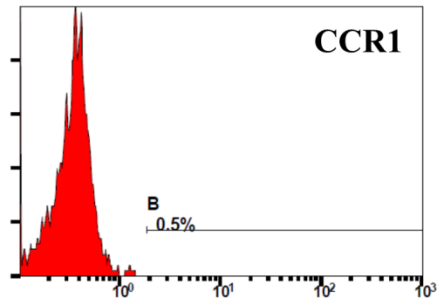


Fig. 3: Photomicrographs showing representative examples of colony forming unit of pMSCs isolated from passage one (A), the differentiation of pMSC isolated from passage 1 into osteocyte as demonstrated by Alizarin Red S staining of osteocytes after 21 days (B), chondrocyte as demonstrated by Alcian Blue staining of cross-section of chondrogenic pellet after 21 days (C) and adipocyte as demonstrated by HCS LipidTOX™ Green neutral lipid staining of adipocytes after 21 days (D). Scale bars represent 50 µm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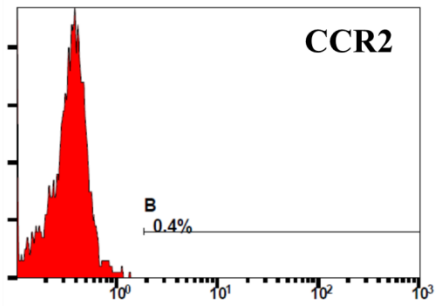
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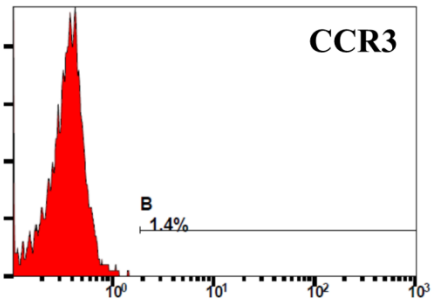
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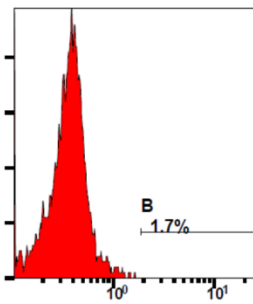
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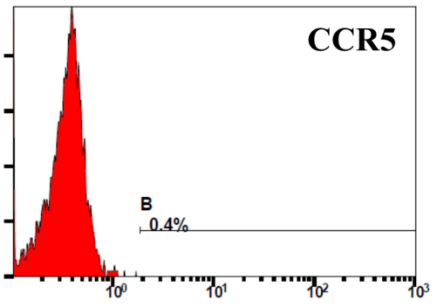
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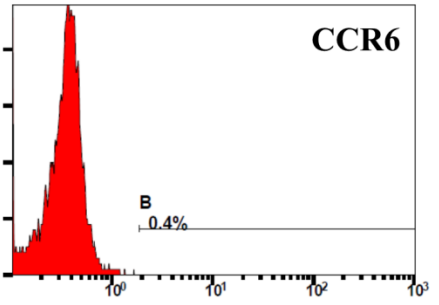
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B
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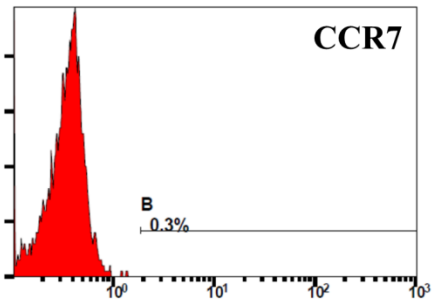
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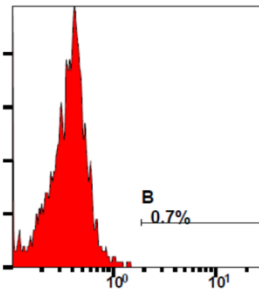
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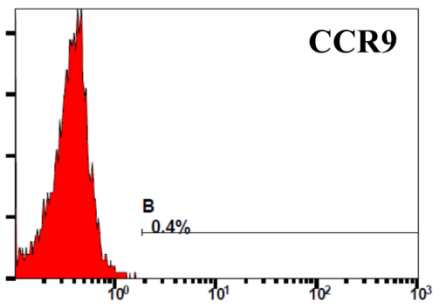
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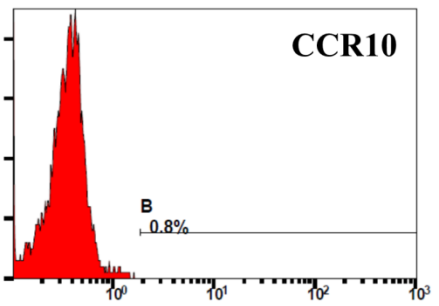
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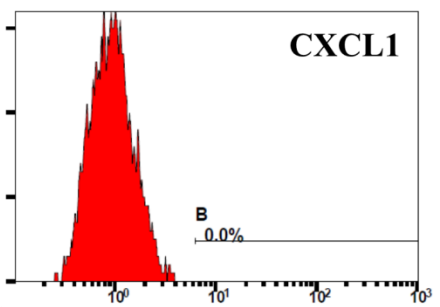
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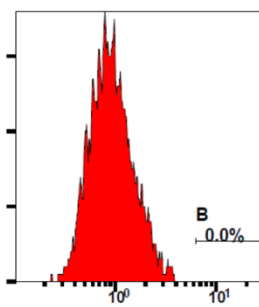
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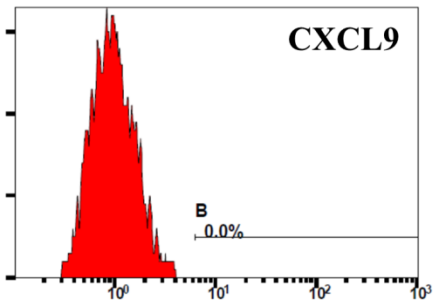
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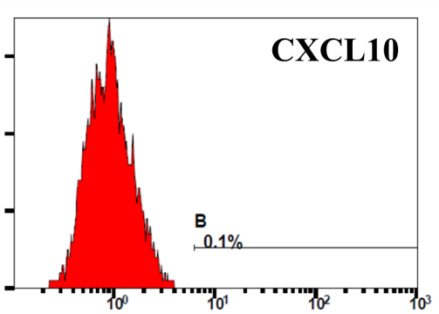
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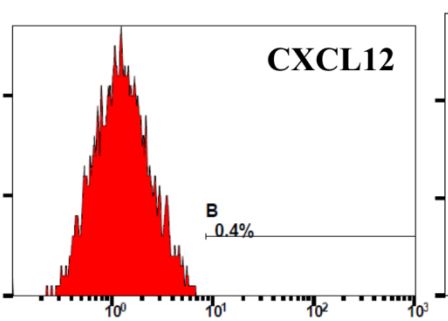
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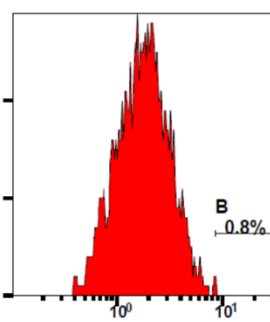
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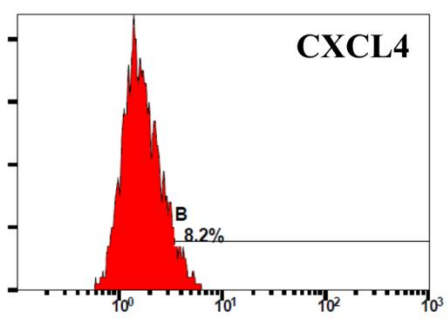
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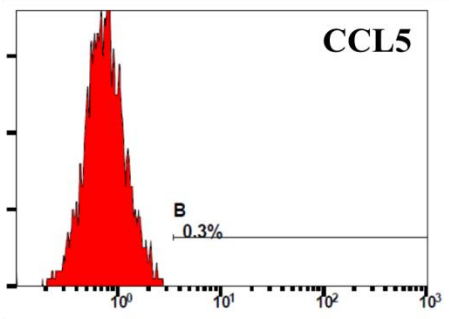
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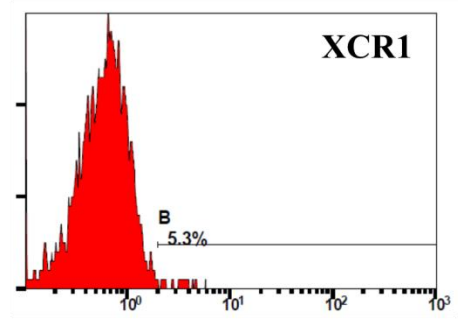
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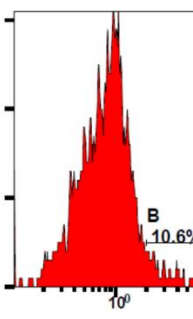
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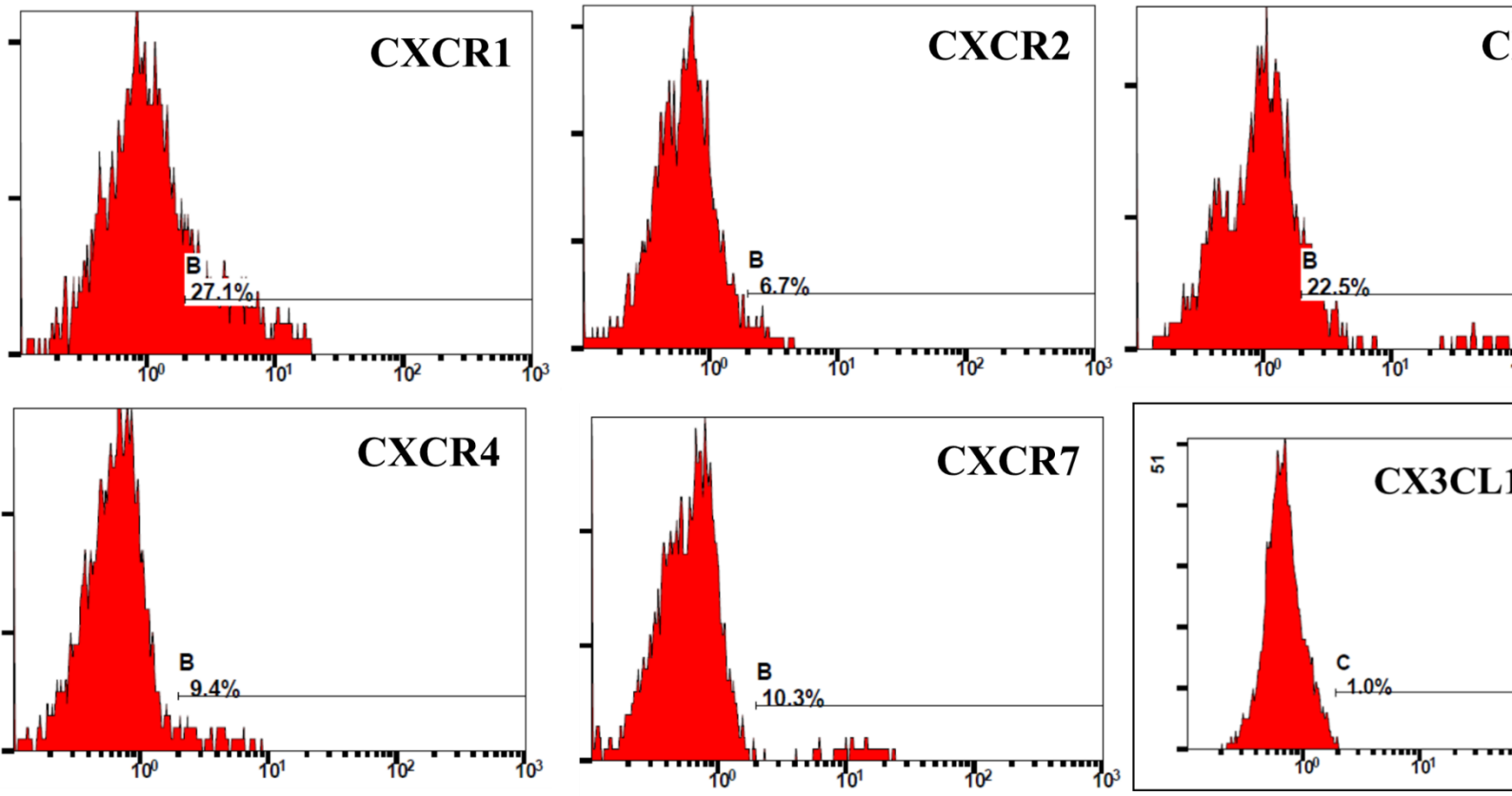

Fig. 4: Histograms showing representative examples of placental mesenchymal stem cells (pMSCs) from passage one stained with antibodies against chemokines/receptors. Negative controls (IgG or IgM) were used (Data now shown). Twenty placentae were used in this analysis.

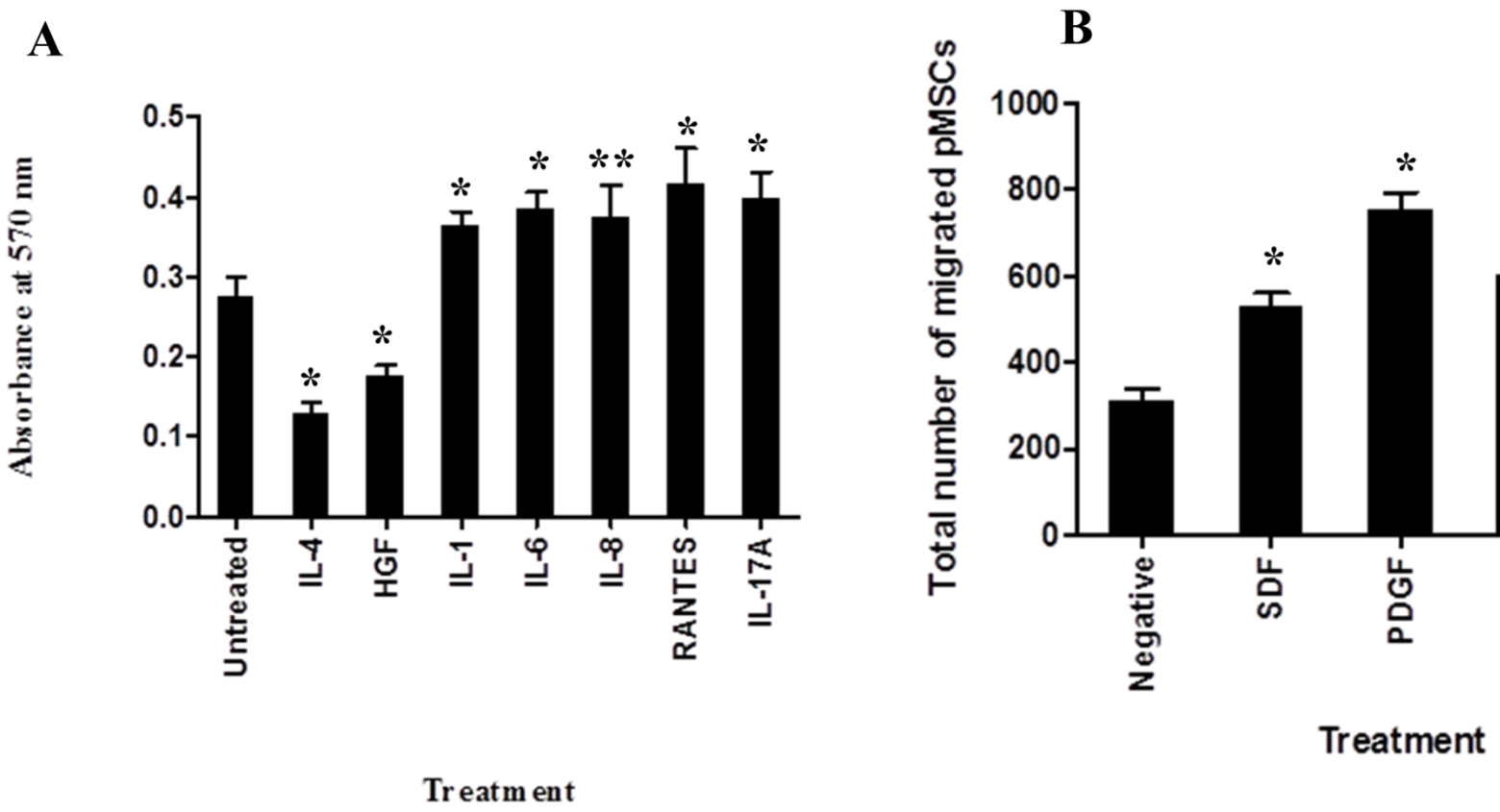

Fig. 5: The effect of different cytokines on the proliferation of pMSCs (A) and the effect of different chemotactic factors (SDF, PDGF, HGF and MCP-1) on the migration of pMSCs (B). The proliferation of pMSCs was significantly suppressed in the presence of IL-4 (*P < 0.01) and HGF (*P < 0.01) whereas significantly increased in the presence of IL-1 (*P < 0.01), IL-6 (*P < 0.01), IL-8 (**P < 0.05), RANTES (*P < 0.01) and IL17A (*P < 0.01). The chemotactic factors significantly (*P < 0.0001) stimulated the migration of pMSCs in transwell migration plates. pMSCs isolated from Trypsin- treated tissues at passage one of 10 placentae were analyzed. Bars represent standard errors.

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Date:
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