Original Article

A Simple and Cost-effective Method for Isolation and Expansion of Human Fetal Pancreas Derived Mesenchymal Stem Cells

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Abstract

Background: Previous studies have suggested mesenchymal stem cells (MSCs) as a suitable source for cell replacement therapy in diabetes. MSCs have successfully isolated from different adult and fetal tissues, including the pancreas. In vitro studies have shown that human fetal pancreatic stem cells could be extensively expanded and differentiated into islet-like structures. Here, we introduce a simple and cost-effective method for the generation of MSCs from the human fetal pancreas (FPMSCs).

Methods: To isolate FPMSCs, pancreata from four aborted fetuses (second trimester) were processed with short collagenase digestion. The resulting tissue fragments were transferred to a basic media (DMEM+15%FBS) without adding any growth factor.

Results: After 10 to14 days, fibroblast-like cells were harvested and passaged six times for further evaluations. Flow cytometry analysis and three-lineage differentiation capacity have demonstrated that these cells have MSC-like properties. We also continuously passaged samples of FPMSCs and found no evidence for chromosomal instability and morphological changes until 10th subculture. Moreover, our cell culture protocol can be easily modified and translated into a GMP-compliant one.

Conclusion: The results of current study demonstrated that our simple and inexpensive method could yield a pure population of FPMSCs that might be suitable for transplantation.

Keywords: Cell transplantation, clinical grade, diabetes, fetal pancreas, mesenchymal stem cell

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Introduction

Despite the promising results of islet transplantation, its widespread application is limited by organ shortage, graft rejection, complexity of isolation procedure, lifelong immunosuppression, and high costs.[1] Therefore, scientists try to develop novel sources of transplantable cells for diabetes treatment. Stem cells (SCs) represent a promising solution for treating diabetes, and many researchers have aimed to create islet-endocrine tissues from these undifferentiated cells.[2] Among different cell types, mesenchymal stem cells (MSCs) seem to be more attractive for β-cells regeneration. There are more than 300 registered clinical trials aimed at evaluating the potential of MSCs transplantation in different conditions including diabetes.[3] MSC therapies appear safe, therefore they are the cells of interest in clinical trials.[4] Adult MSCs have been successfully isolated from different tissues such as bone marrow, adipose tissue, trabecular bone, periosteum, synovium, pancreas, skin, lung, dental pulp, and thymus.[5] Fetal MSCs with similar characteristics as adult MSCs have been found in a range of fetal derived tissues such as placenta, fetal membranes, umbilical cord, spleen, lung, liver, thymus, dermis, blood, amniotic fluid, kidneys, bone marrow, and pancreas.[6,7] A great number of previous studies suggest the potential advantages of fetal MSCs over their adult counterparts in terms of growth and plasticity.[8] Consequently, due to their high proliferation rate, extensive differentiation properties, and immune-privileged status, fetal SCs are attractive as a potential source of transplantable cells.[9,10] Human fetal cell and tissue transplantation research has gone on for more than three decades.[11,12] In 1989, Peterson, et al. reported that as many as 900 human fetal pancreas transplantations have been performed worldwide.[13] Pancreatic derived MSCs (PMSCs) are promising because they can exponentially proliferate and potentially provide a large supply of cells with capacity to differentiate into insulin-producing cells.[14] While adult pancreatic SCs have low proliferation, fetal pancreatic SCs have shown stronger proliferative potential, even those obtained during the second or third trimester.[15,16] Our literature review revealed that a few published studies have described isolation of MSCs from the human fetal pancreas. For the first time, Hue, et al. demonstrated that multipotent MSCs could be isolated from human fetal pancreas by means of their adherent ability.[17] Zhang, et al. showed that the nestin positive pancreatic progenitor cells shared many phenotypic markers with adult bone marrow derived MSCs (BM-MSCs).[18] Another study has described a three-step culture system for isolation of human fetal PMSCs (FPMSCs).[19] Joglekar, et al. studied the proliferative potential of human fetal pancreatic islet-derived SCs from second or third trimester fetuses and suggested that these cells may have the potential to treat dia-

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betes.[16] The review of these studies revealed that their protocols for FPMSCs isolation and culture are complex, time consuming, and expensive. The usage of different growth factors was another weak point that made these protocols undesirable for clinical applications. Moreover, we found a significant variation in phenotype characterization (CD markers) of FPMSCs. Regarding our previous study on establishing a clinical grade human fetal liver derived MSCs,[20] we decided to isolate and bank FPMSCs for possible future applications. Therefore, the current study was conducted to establish a simple and cost-effective protocol for isolation and expansion of human FPMSCs. The isolated cells were characterized according to ISCT's minimal criteria for defining multipotent mesenchymal stromal cells.[21]

Materials and Methods

Fetal donation and pancreas retrieval

Human fetal pancreata were harvested from legally aborted fetuses between 14 and 20 weeks gestation with maternal written informed consent. Our procurement team assessed the donor eligibility by medical history and physical examination. The donor's blood sample was tested to be negative for transmissible diseases.[20] The use of fetal tissues for research was approved by the ethics committee (Code: EC-00264) of the Endocrinology and Metabolism Research Institute, Tehran University of Medical Sciences, Tehran, Iran. Fetuses with abnormalities, or warm ischemic time more than 12 hours were excluded. The donated fetuses (n = 6, from 3 maternity hospitals) were rinsed thoroughly with sterile normal saline solution and transferred into sterile containers. The samples were transported to our laboratory under wet ice condition. Fetal organs were harvested as soon as possible in our GMP facility (total ischemic time up to 36 hours). Each fetus was immersed in 5% povidone–iodine solution followed by three washes in phosphate buffer saline (PBS – PAA, Austria). Then, fetal pancreas was harvested by a midline laparotomy following left subcostal extensions under aseptic conditions. At first, the spleen (a marker of pancreatic tail) was located and pulled up with a forceps, and then pancreas was dissected from surrounding tissues.

Isolation and expansion of FPMSCs

Fetal pancreas was washed three times with PBS containing 3X antibiotic-antimycotic solution (100X solution, PAA, Austria) and minced into small pieces. Short enzymatic digestion was performed with 1 mg/mL collagenase NB4 (SERVA Electrophoresis, Germany) in 37°C for 15 minutes. Digestion was stopped by adding 5 mL cold PBS, then the vial placed on ice and the clumps allowed to settle for 10 minutes. After discarding the supernatant, small tissue fragments re-suspended in PBS, and centrifuged for 5 minutes at 300 × g (Hettich 320R, Germany). The pellet re-suspended in 3 mL culture media containing DMEM-LG and 15% FBS (PAA, Austria). Explant culture was performed in a 25 cm^2 tissue culture treated flask (TPP, Switzerland) which was located into a CO_2 incubator (Memmert INC108med, Germany) with 5% CO_2 and 98% humidity. After 5 days, unattached tissue fragments were discarded and fresh media was added to the culture flask. First subculture was done after cell colonies covered at least 60% of the flask surface. The cells were detached with TrypLE™ Express (life technologies, USA) treatment at 37°C for 7 to 10 minutes. The cell count and viability were assessed by NucleoCounter® NC-100™ (Chemometec, Denmark) and the cells seeded at a density of 5000 – 6000 cells/cm^2. After the second subculture, media was changed to DMEM-LG and 10% FBS. As a baseline genomic screen, chromosome analysis was performed by G-banded Karyotyping at 10th subcultures by the cytogenetic laboratory of Sarem Women's Hospital (Tehran, Iran). Briefly, in the log phase of growth, FPMSCs were incubated in culture media containing colcemid (final concentration of 0.1 μM) for 4 hours at 37°C and 5% CO_2. The cells were harvested, and incubated in hypotonic solution (0.075M KCl) for 20 minutes at 37°C. Then, FPMSCs were fixed in equal volume of ice-cold methanol-acetic acid, and were dropped onto the slide, dried, and finally stained with Giemsa for 10 minutes.

FPMSCs characterization

The cells from the 6th subculture were examined for three-lineage differentiation capacity and their phenotype (CD markers), based on the ISCT's minimal criteria.[21]

Three lineages Differentiation

Osteogenic, adipogenic and chondrogenic differentiation of FPMSCs were induced by StemPro® Osteogenesis, Adipogenesis, and Chondrogenesis Differentiation Kits respectively (life technologies, USA) according to the manufacturer's protocols with some modifications. For adipogenic differentiation, FPMSCs were suspended in adipogenesis media and 2.5×10^4 cells were seeded into each well of 4-well cell culture plate (Nunc, Thermoscientific, USA). After 21 days, the cells were fixed in freshly prepared 4% paraformaldehyde solution (PFA) for 20 minutes at room temperature. Oil Red O (Sigma, USA) staining was performed to identify neutral lipids in differentiated cells. For osteogenic induction, the cells were suspended in osteogenesis media and seeded into a 4-well cell culture plate at 1.5×10^4 cells per well. To detect calcium deposit, the cells were fixed after 10 days and stained with Alizarin Red S (Sigma, USA) solution. In order to chondrogenic differentiation, 2.5×10^5 FPMSCs were suspended in 5 mL chondrogenesis media into a 15 mL conical tube (SPL, South Korea) and centrifuged at 200 × g for 5 minutes. The cell pellet re-suspended in 0.5 mL chondrogenesis media and centrifuged at 200 × g for 5 minutes. Without disturbing the pellet, tubes with loosened caps incubated upright at 37°C and 5% CO_2. After 28 days, the cell nodule was fixed overnight in 4% PFA. The fixed nodule was then paraffin embedded and stained with alcian blue and fuchsin by the pathology department of the Shariati hospital (Tehran, Iran). Photographs were taken using a Nikon Eclipse TS100 inverted microscope (Nikon, Japan). Adult BM-MSC at 6th subculture (donated by SABZ Biomedicals Co.) was considered as a positive control for each differentiation study. For each experiment, the same number of cells were suspended in culture media (DMEM-LG+ 10% FBS) and considered as a negative control. All experiments were carried out in triplicate and culture media were replenished twice a week.

Flow cytometry analysis

Expression of the cell surface antigens (CD markers) was analyzed using flow cytometry (FACS Calibur™, BD Biosciences, USA). FPMSCs were harvested and aliquoted at $0.8 \times 10^6 – 1 \times 10^6$ cells/ml in ice cold PBS. The cell samples were transported on ice to the flow cytometry department of SABZ Biomedicals Co. (Tehran, Iran). Further procedures were performed based on their protocols (http://www.sabztech.com/Fa/

ProtocolsList_fa.aspx) with the following CD markers: CD11b-FITC, CD45-FITC, CD19-PE, CD34-PE, HLADR-PE, CD105-PE, CD73-PE, CD90-FITC, and CD44-FITC (all from eBioscience, Inc., USA). Briefly, cell suspension was centrifuged at 300 × g for 5 minutes, re-suspended in ice cold PBS+1% bovine serum albumin (BSA) and stained for 30 minutes with either conjugated specific antibodies or isotype-matched control at recommended concentrations. The labeled cells were washed twice in PBS+1% BSA (300 × g for 5 minutes) and re-suspended in FACS buffer for flow cytometry analysis.

Results

Pancreata from 6 fetuses were harvested and examined macroscopically for any sign of auto-digestion. Healthy pancreas should be firm on cutting and has a pinkish color (Figure 1). Four pancreata were considered suitable for further processing and used for cell isolation. The means of donor's age, gestational age and total ischemic time were 33.50 years (SD = 4.43), 17.25 weeks (SD = 0.95) and 25.25 hours (SD = 5.85) respectively. All aborted fetuses were male and the cause of abortion was premature rupture of membrane (PROM).

Isolation and culture of FPMSCs

Small tissue fragments were successfully isolated from all pancreata after short collagenase digestion, and transferred to a 25 cm^2 culture flask. Some tissue fragments attached to the flask as early as 24 hours. Cell outgrowth was observed from attached fragments 1 to 3 days after seeding (Figure 2a). After 5 days, most of the fragments attached and the floating pieces were discarded. The cells migrated and proliferated rapidly to form a confluent monolayer of fibroblast-like cells in a period of 10 to 14 days (Figure 2c). Rarely, epitheloid cells migrated from the few fragments (Figure 2d). However, during culture, some cells displayed a change in morphology from epithelial to fibroblast-like cells and the other epitheloid cells were eliminated after first subculture. The average number of harvested cells in primary cultures was 540000 (SD = 86023.25). The majority of harvested cells adhered to the new flasks, and proliferated. Once the cells reached approximately 70% confluence, they were subcultured again. After third subculture (Figure 2e), the cells were passaged every 7 to 10 days and split at a ratio of 1:4. Also, we continuously passaged samples of FPMSCs and no significant morphological changes were observed after 10 passages. The karyotype of all samples at 10^{th} subculture was normal (46XY) as determined by G-banding (Figure 2f).

Differentiation potential

Induction of adipogenic differentiation resulted in growth arrest and change in cell morphology. After 21 days, the majority of cells were stained positive for Oil red O, a specific dye for showing lipid accumulation. In contrast, no lipid droplets were observed in negative controls. The number of lipid droplets was less than 14 days cultured adult BM-MSCs, and their sizes were smaller (Figure 3). After 10 days of culture in osteogenic media, accumulation of calcified extracellular matrix was revealed following Alizarin Red S staining. The same result was obtained by adult BM-MSCs but not the control groups (Figure 3). After 28 days, to assess chondrogenic differentiation potential, nodules were stained with alcian blue, to reveal proteoglycans in the extracellular matrix. Differentiated nodules were stained blue compared with nodules that maintained in a control medium (Figure 3).

Phenotypic characterization

Flow cytometry analysis showed that at 6^{th} subculture, all FPMSCs expressed (> 95%) CD44, CD90, CD73 and CD105, but did not express (> 98%) CD11b, CD19, CD34, CD45 and HLA-DR. Figure 4a illustrates the histogram of each CD marker on a representative sample of FPMSCs. We also have shown the expression of each CD marker on all FPMSC samples as a bar chart (Figure 4b).

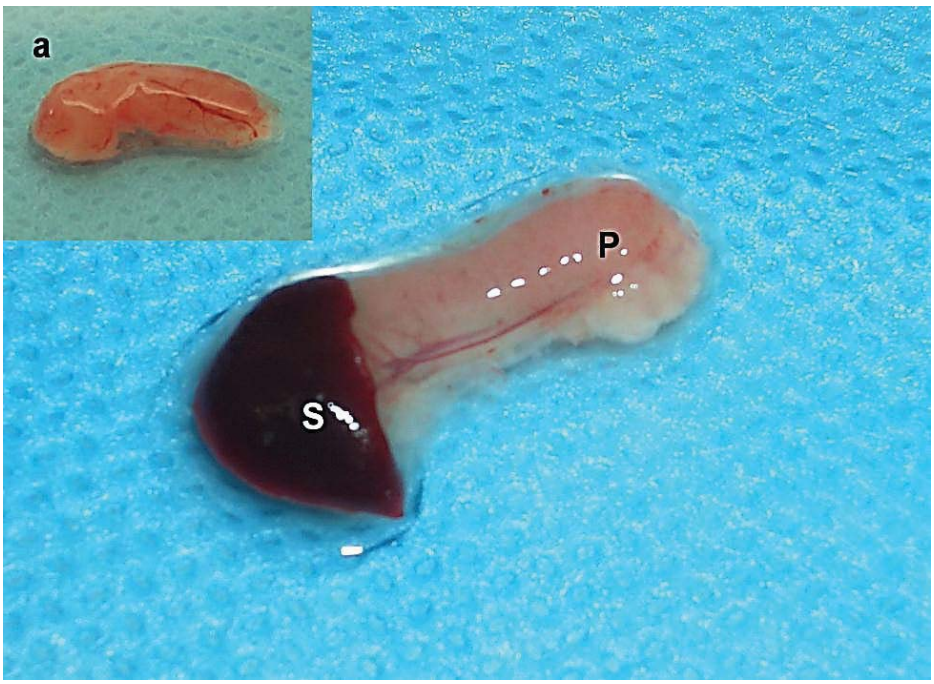

Figure 1. Pancreas (P) has been harvested from 16 weeks fetus with spleen (S) attached to it. The pancreas should be trimmed for further processing (a).

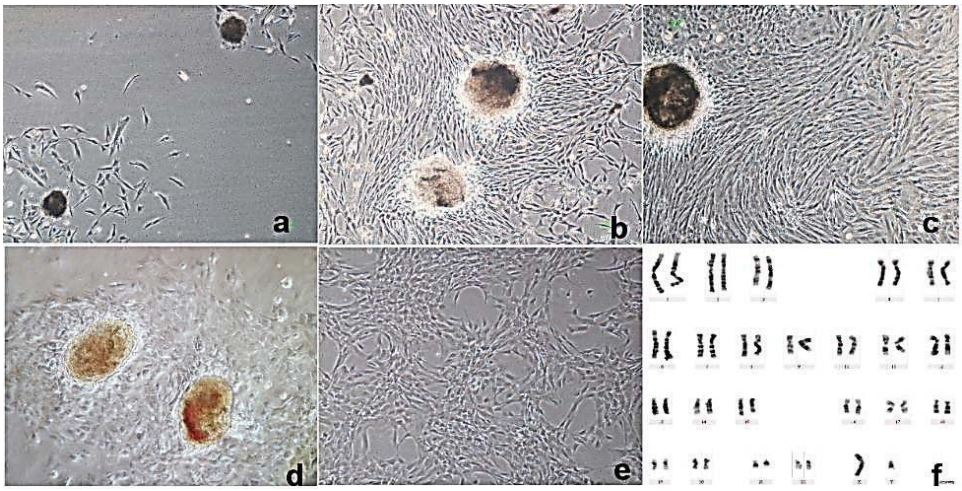

Figure 2. Morphology of FPMSCs (all magnifications × 40) in primary culture was depicted at **a)** day 2 **b)** day 6 and **c)** day 12; **d)** illustrates epithelial-like cells in primary culture; **e)** homogenous fibroblast-like morphology could be observed at third subculture; **f)** Karyotyping was performed at 10th subculture

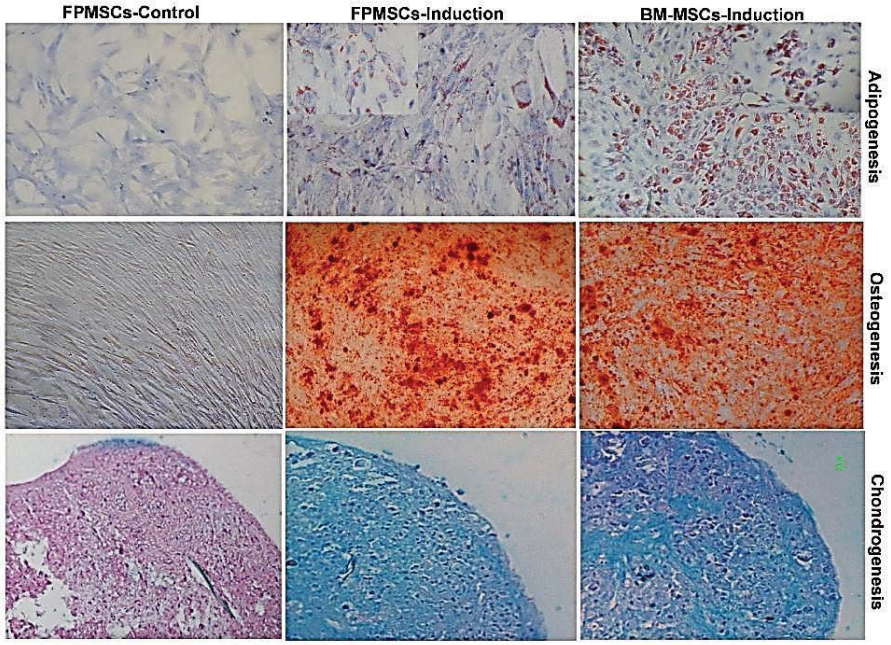

Figure 3. Adipogenic differentiation: Oil red O staining demonstrated lipid droplets in both FPMSCs (magnification ×200) and BM-MSCs (magnification ×100) but not control group (magnification ×200). Osteogenic differentiation: Alizarin Red S staining of differentiated cells revealed calcium deposition (magnification ×100). Chondrogenic differentiation: Alcian blue and fuchsin staining of nodules performed to demonstrate chondrogenic differentiation (magnification ×200).

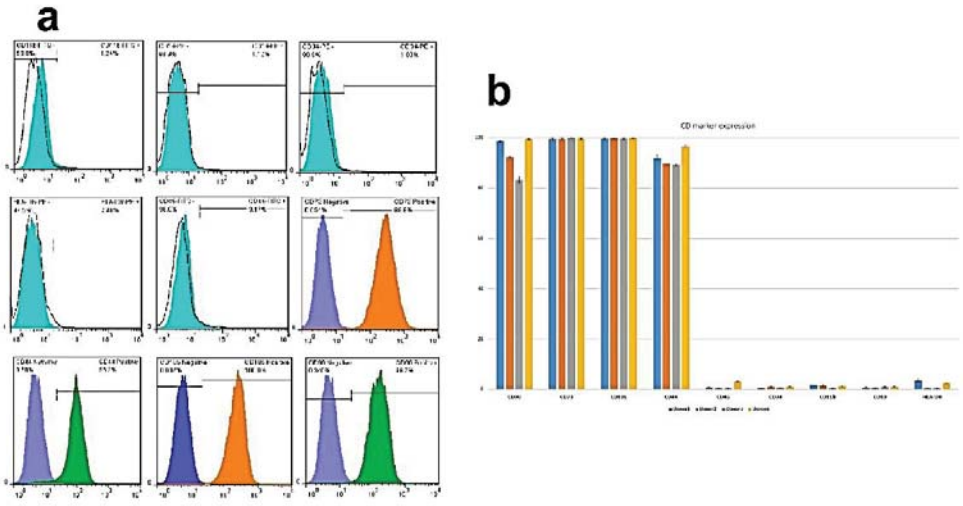

Figure 4. Flow cytometry analysis of FPMSCs at sixth subculture; **a)** A representative histogram shows the expression of specific mesenchymal markers and the lack of hematopoietic markers and HLA-DR; **b)** Bar chart illustrates the expression of CD markers on FPMSC samples from each donor (N = 4).

Discussion

Some limitations of allogeneic islet transplantation have motivated scientists to find alternative cell sources for treatment of diabetes. Among the investigated cells, MSCs seem to be more attractive due to their multipotency, immunomodulatory effect, simple preparation, and clinically adopted culture protocols. So far, MSCs are the most extensively studied SCs, and the most commonly used SCs in current clinical applications.[3,22] MSCs have been isolated from different adult and fetal tissues, among them fetal sources are more interesting. The fetal environment is unique, because there is a large-scale migration of SCs into different organs to make up the organism.[7] Diabetic microenvironment may adversely affect the autologous MSCs. Phandis, et al. described that BM-MSCs from old patients (> 50 years) with chronic diabetes, were unable to expand and proliferate in vitro. They also concluded that chronic exposure to hyperglycemia may reduce the BM-MSCs' multipotency. Therefore, allogeneic MSCs from healthy donors could be more suitable source to trigger islet regeneration.[23–25] Human fetal pancreatic SCs can be extensively expanded and differentiated into islet-like structures in vitro.[26] Ersek, et al. demonstrated that human FPMSCs could be engrafted into a non-injury xenograft model and differentiated into human insulin secreting structures that remain functional years after transplantation.[19] This observations support the potential application of human FPMSCs in diabetes cell therapies. A major concern for SCs allo-transplantation is the occurrence of unfavorable immune responses against donor antigens. It has been demonstrated that human fetal pancreas has a lesser immunogenicity than adult pancreas.[27] Immunogenicity studies of first-trimester human FPMSCs have shown intermediate expression of HLA class I molecules and lack of HLA class II antigens. These findings suggest that they are immune privilege and could be allo-transplanted.[28] In this study, we investigated a simple and cost-effective method for isolation of MSCs from human fetal pancreas. The results demonstrated that this method was able to successfully isolate and expand FPMSCs from 4 consecutive aborted fetuses (second trimester). These cells were highly proliferative even after 10 subcultures and which could be cultured to billions of cells for future applications. In contrast to previously published studies,[16–19,29] we used basic culture media (DMEM-LG + 10% FBS) and eliminated all growth factors without significant effect on cell yield, multipotency and purity. It is preferred to avoid using any growth factor in clinical grade cell manufacturing.[30] On the other hand, the numbers of clinical (or GMP) grade growth factors are limited and their application considerably increases the cost of final product. We also used collagenase NB4 and TrypLE™ Express that could be replaced by their GMP grade alternatives (Collagenase NB6 and TrypLE™ Select respectively) for clinical grade cell manufacturing. Despite normal karyotyping results, further in vitro and in vivo assays are recommended to demonstrate the safety of FPMSCs for future clinical applications. Our established protocol is also simple, fast, and easy to follow. Previous studies have implemented two- or three-step culture systems that increase the complexity and duration of procedures. Ersek, et al. have used three-step culture protocol with different culture media for each step (i.e. initiative medium, restrictive medium, and expansion medium). Their primary culture lasted up to 6 weeks.[19] Hu, et al. isolated pancreatic mononuclear cells with Ficoll density gradient centrifugation and then used the magnetic assisted cell sorting to deplete CD45 and GlyA-positive cells. To obtain FPMSCs, the remaining cells were cultured on fibronectin-coated plates.[17] This protocol is very complex, expensive, and time consuming. Moreover, we could easily expand the FPMSCs in non-coated culture flasks. To characterize FPMSCs, ISCT's minimal criteria was used, which defines human MSC based on its plastic-adherence, expression or lack of some CD markers, and their three-lineage differentiation.[21] We examined the differentiation potential of FPMSCs and found that they could be differentiated to adipocytes, osteocytes, and chondrocytes when cultured into specialized inductive media. Previous studies have indicated that the differentiation potential of MSCs may vary, depending on the tissue sources.[10,31] We found that BM-MSCs differentiate more rapidly into adipocytes and the number of cytoplasmic fat droplets are higher and they are larger than that of FPMSCs (Figure 3). Guillot, et al. showed that the osteogenic efficiency of human fetal MSCs (from blood, bone marrow, and liver) were greater than adult MSCs.[8] In our experiment, the microscopic pictures of stained osteo-induced FPMSCs and BM-MSCs were similar (Figure 3). Davani, et al. compared adult PMSCs with BM-MSCs and found that adipogenic efficiency of adult PMSCs were higher. They also found that PMSCs differentiation to either chondrocytes or osteocytes were less efficient than BM-MSCs.[14] In current study, we did not decide to compare FPMSCs multipotency with other sources of MSCs. Adult BM-MSCs were used only as a positive control in our differentiation studies. In order to accurately compare the differentiation efficiency of FPMSCs with MSCs from other sources, quantitative studies should be performed. The surface antigens of fetal MSCs can vary according to the culture method, culture duration, passage number or plating density.[7] Our flow cytometry data demonstrated that pure population of FPMSCs could be obtained after 6 subcultures. More than 95% of these cells expressed CD44, CD90, CD73 and CD105 and more than 98% lacked expression of CD11b, CD19, CD34, CD45 and HLA-DR. As CD44 expression has been tested in previous studies, we decided to examine its expression in addition to the ISCT's defined markers. Among previous studies, only one[19] study has used a wide panel of CD markers (including ISCT defined markers) for FPMSCs identification. Except for one study,[28] the others have isolated FPMSCs from second or third trimester fetuses. Due to some legal limitations, we could not find suitable fetuses in third trimester of pregnancy. Therefore, we only included second trimester fetuses to have a better comparison with previous studies. In conclusion, the results of this study demonstrated that our simple and inexpensive method could yield a pure population of FPMSCs that might be suitable for transplantation.

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Conflict of Interest: *The authors declare that there is not any conflict of interests.*

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