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ORIGINAL ARTICLE

Differentiation of placenta-derived MSCs cultured in human platelet lysate: a xenofree supplement

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Abstract

In the last few decades, mesenchymal stem cells (MSCs)-based regenerative therapies in clinical applications have gradually become a hot topic due to their long-term self-renewal and multilineage differentiation ability. In this scenario, placenta (p) has been considered as a good source of MSCs. As a tissue of fetal origin with abundant number of stem cells compared to other sources, their non-invasive acquisition, strong immunosuppression, and lack of ethical concerns make placenta an indispensable source of MSC in stem cell research and therapy. The mesenchymal stem cells were derived from human term placenta (p-MSCs) in xenofree condition using platelet lysate (PL) as a suitable alternative to fetal bovine serum (FBS). Upon isolation, p-MSCs showed plastic adherence with spindle-shaped, fibroblast-like morphology under microscope. p-MSCs flourished well in PL-containing media. Immunophenotyping showed classical MSC markers (> 90%) and lack expression of hematopoietic and HLA-DR (< 1%). Surprisingly, differentiation study showed differentiation of p-MSCs to mature adipocytes in both induced cells and control (spontaneous differentiation), as observed via oil red staining. This is in line with gene expression data where both control and induced cells were positive for visfatin and leptin. Thus, we propose that p-MSCs can be used for clinical applications in the treatment of various chronic and degenerative diseases.

Keywords Mesenchymal stem cells · Placenta · Platelet lysate · Adipocyte differentiation

Introduction

Stem cells can be found from the very early stages of human development to the end of life (Ullah et al. 2015). Stem cells are unspecialized cells that are capable of self-renewal and also possess the ability to differentiate into specific cell types upon defined induction (Nanjwade et al. 2016). MSCs are a subset of adult stem cells that hold great promise in regenerative medicine and cell based therapies (Gao et al. 2016; Lukomska et al. 2019). It is intensively investigated since their initial discovery by Alexander Friedenstein in the 1970s (Friedenstein et al. 1970). MSCs are multipotent, exhibiting high proliferation and self-renewal capabilities (Sheng 2015). Adding to that, MSCs have been shown to suppress immune responses and regulate immune properties via interaction with immune cells in both innate and adaptive immune systems (Li and Hua 2017; Jiang and Xu 2020). Human MSCs were first isolated from bone marrow (Baker et al. 2015) and they have been also been obtained from other tissues such as adipose tissue (Ghorbani et al. 2014), muscle (Čamernik et al. 2019) and fetal tissues including umbilical cord (Nagamura-Inoue 2014), Wharton jelly (Satheesan et al. 2020), amniotic membrane (Zeng et al. 2011), amniotic fluid (Spitzhorn et al. 2017). Commonly, fetal bovine serum (FBS) is used for the expansion of human mesenchymal stem cells (hMSC) as a supplement. However, there is concerning evidences that FBS contains xenogeneic antigens and endotoxins that may alter

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phenotype of MSCs (Fani et al. 2016) and cause immunological reactions in the host (Silva-Carvalho et al. 2020). Furthermore, batch to batch variation, ethical concerns of animal welfare, cost and to avoid risk factors, recent research has focused human substitutes such as platelet lysate as a xenofree growth supplements in cell culture expansion as an alternative to FBS (Antoninus et al. 2015; Viau et al. 2016; Tancharoen et al. 2019). The composition of PL includes a large number of growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), transforming growth factor (TGF), and fibroblast growth factor 2 (FGF 2) with mitogenic action (Shih and Burnouf 2015). It also contains cytokines and chemokines like interleukins, tumor necrosis factor (TNF)-α and interferon (IFN)-γ (Pourgholaminejad et al. 2016). The source for preparing platelet lysate is the platelet concentrate, which could be derived from platelet-rich plasma or buffy coat. The frequent preparation methods include direct platelet activation by calcium chloride (Pesarini et al. 2018), repeated freeze/thaw cycles (Patel et al. 2020), and many more like sonication (Amirkhani et al. 2016). Human PL shows great promotion and immune privileged than FBS (Palombella et al. 2022).

In this work, the birth-associated tissue placenta was used as a source of mesenchymal stem cells due to its availability, non-invasive method, and lack of ethical concerns due to its classification as birth waste. In clinical applications, placental-derived mesenchymal stem cells have demonstrated promising results in regenerative medicine due to their superior migration and homing abilities into injured tissues (Zachar et al. 2016). It is a preliminary study of establishing MSC from placenta and their differentiation into adipocyte stem cells repair chronic illness. Also, the mesenchymal stem cell can differentiate into other adult cells like chondrocyte, osteocyte.

Materials

Platelet lysate preparation

At least two single platelet concentrates of AB positive donor were obtained from a registered blood bank after running a panel of test for potential viral and other hazardous diseases. The obtained platelet concentrates were then pooled and activated with calcium chloride (10% w/v) to a final concentration of 20 mM. The platelet concentrate was incubated for an hour at 37 °C followed by overnight incubation at 4 °C. The coagulated product was then filtered, centrifuged at 5000 rpm for 20 min and the supernatant was filter sterilized with 0.2 µm syringe filter, aliquoted and frozen at − 20 °C until use.

Procurement of human placenta

The human placenta was aseptically collected from a healthy donor mother after getting informed consent. These were obtained from the selective cesarean sections at Trichy Medical Centre & Hospital, Trichy. The tissue was immersed in antibiotic saline solution and kept under 4 °C until processed.

Tissue processing and ex vivo expansion of p-MSC

The human placenta tissues were processed using enzymatic digestion method. Briefly, deciduous placental tissue was washed with Dulbecco's phosphate buffer saline (DPBS) and antibiotics to remove residual RBC contaminants. The tissue was minced and digested using enzyme Collagenase Type 1 (1 mg/1 ml) (Gibco, Invitrogen, USA) for 90 min. Following this, the digested tissues were diluted with PBS to reduce viscosity, filtered using 70 µm cell strainer (Himedia, India) and centrifuged at 2500 rpm for 10 min. The mononuclear cells in the pellet were separated and resuspended in mesenchymal stem cell's complete media containing 89% Dulbecco's modified eagle medium (DMEM) with high glucose (Himedia, India), 10% platelet lysate, AB^{+ve} seronegative and 1% antibiotic–antimycotic solution (Himedia, India). The single nucleated cells in complete media were seeded in T_{175} culture flask. Primary cultures were incubated in a 37 °C humidified 5% CO_2 incubator (Eppendorf, Germany). Cells were allowed to adhere in surface and non-adherent cells were washed off by replacing the media. Media changes were carried out every 48–72 h thereafter. Upon reaching 70–80% of confluence, cells were harvested via trypsinization. The adherent cells were detached with 0.25% Trypsin EDTA (Himedia, India) and neutralized with media. They were washed with DPBS (w/o $Ca^{2+}$and Mg^{2+}) (Himedia, India), by centrifugation at 1500 rpm for 5 min and the pellets were re-plated at 1:3 under same culture conditions. Part of cells from every passage were allocated for cryopreservation and banking for future uses. Cells (~ 1–1.5 million cells/ml) were cryopreserved in the freezing media containing 85% DMEM-high glucose, 5% human serum albumin (Reliance, India), 10% DMSO, and cell culture tested (Himedia, India) and stored at − 80 °C overnight and kept at − 196 °C in liquid nitrogen with proper labeling. The harvested cells were used for downstream experiments.

Immunophenotyping of p-MSC

Surface antigen expression was examined by flow cytometric analysis using BD Stem flow hMSC Analysis Kit as per

the manufacturer's instruction. Briefly, cells were harvested at 70–80% confluency and resuspended at approximately 1.0×10^6 cells/ml and aliquoted in 9 reaction tubes. 100 µl of cells from each tube was labeled with the following antibodies: anti-CD90-FITC, anti-CD44-PE, anti-CD105-PerCP-Cy5.5, anti-CD-APC (positive cocktail), anti-CD-73, anti-CD45, CD34, CD11b, CD19, HLA-DR-PE (negative cocktail) with appropriate FITC, PE, PerCP-Cy5.5 labeled isotype control mAbs. The labeled cells were incubated in the dark for 30 min. Then, the cells were washed with PBS and resuspended in 500 µl of PBS and analyzed in BD FACS Verse flow cytometer. The computed data were analyzed using CellQuest software provided by the manufacture (BD, Bioscience).

Adipogenic differentiation assay

Adipogenic differentiation was induced using commercially available HiAdipoXL Adipocyte differentiation kit which is provided with supplements (Cat No: AL521, HiMedia, India) as per the manufacturer's instruction. Briefly, the cells were seeded at 10,000 cells/cm^2 in 12 well plate. Upon reaching 70–80% confluence, the growth medium was replaced with adipocyte induction medium and cells were cultured for 21 days. Media replenishment was done every 2–3 days until the period of culture. Cells without induction media served as control. Differentiation was confirmed by Oil O Red staining using EZ Stain Adipocyte staining kit (HiMedia, India).

RNA extraction and cDNA synthesis

Total RNA was isolated using the HELINI Biomolecules Total RNA isolation kit following the manufacturer instructions. Totally, 60 µl of extracts were eluted and collected in the collection tube. In this elution, 1 µg/10 µl of RNA was converted into cDNA following manufacturer's instruction.

Extracted RNA was reverse transcribed using the protocol outlined in the High-Capacity cDNA Reverse Transcription Kit (Applied biosystem, USA). 20 µl of reaction sample in polymerase chain reaction (PCR) tube was set in the thermal cycler with the following settings: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min, and 4 °C for 5 min. The newly formed cDNA (20 µl) was stored at − 20 °C. For PCR analysis, 1:20 dilution was used as a template.

Quantitative reverse transcription-PCR (qRT-PCR) analysis

cDNA (50 ng/well) was used as a template in qPCR reactions with oligonucleotides specific for the gene of interest such as visfatin (forward primer: 5′AGCCGAGTTCAA CATCCTCC3′ and reverse primer: 3′AACTTTGCTTGT GTTGGGTGG5′), leptin (forward primer: 5′AGGGAGACC GAGCGCTTTC3′ and reverse primer: 3′TGCATCTCCACA CACCAAACC5′). The RNA sample with induction medium (Test) and without induction medium (Control) were analyzed. All qPCR reactions were performed and the resultant values being combined into an average cycle threshold (C_T). Relative quantification was determined using a 7500 Real-Time PCR system (Applied Biosystems, Bedford, MA) measuring SYBR green fluorescence (Lightcycler® 480 SYBR Green I Master). For normalizing the data, GAPDH (forward primer: 5′TTAGCACCCCTGGCCAAGG3′ and reverse primer: 3′CTTACTCCTTGGAGGCCATG 5′) was used as a house-keeping gene.

Results

Isolation and culture of adherent cells from placenta

In testing several methods to prepare viable cells from the placenta after birth, the good result was obtained with combination of both dissection and digestion process (Tong et al. 2011). The schematic isolation protocol of cells from the placental tissue is depicted in Fig. 1. Formation of monolayer, adherence, and fibroblast-like cells spindle-shaped was observed which is referred to as characteristic feature of mesenchymal stem cells. With the digestive process, the isolated p-MSC cells required an average of 13 days to attain confluence stage. The initial growth of cell cultures at passage 0 (P0) showed presence of 20% fibroblast-like cells with plastic adherence ability, while remaining cells were round in shape. In addition, floaters (RBC and epithelial cells) were found in the culture flask. Upon trypsinization and sub-culturing, the RBC content and floaters were washed out from the media and the MSC were maintained with no contamination in subsequent passages. The appearance of cell outgrowth from explant culture was routinely monitored by observation under inverted microscope. In subsequent passages P1 and P2, the growth of cells was faster and attains confluence at an earlier stage like, in P1 at day 9 and P2 at day 3. The homogenous population of PL-MSC were maintained in passage 2 and ready for experimental work. Cells were preserved from each passage (Fig. 2 Panels A, B, C, and D) and maintained in liquid nitrogen.

Characterization and expression profile of p-MSCs

Immunophenotyping of p-MSCs was performed in Passage 2 to check the minimal criteria of International Standard for Cellular Therapy (ISCT) committee. The expression profile was determined by flow cytometry. The isolated cells were positive for MSC markers such as CD44, CD73, CD90, and CD105 which showed as a separate peak from the control.

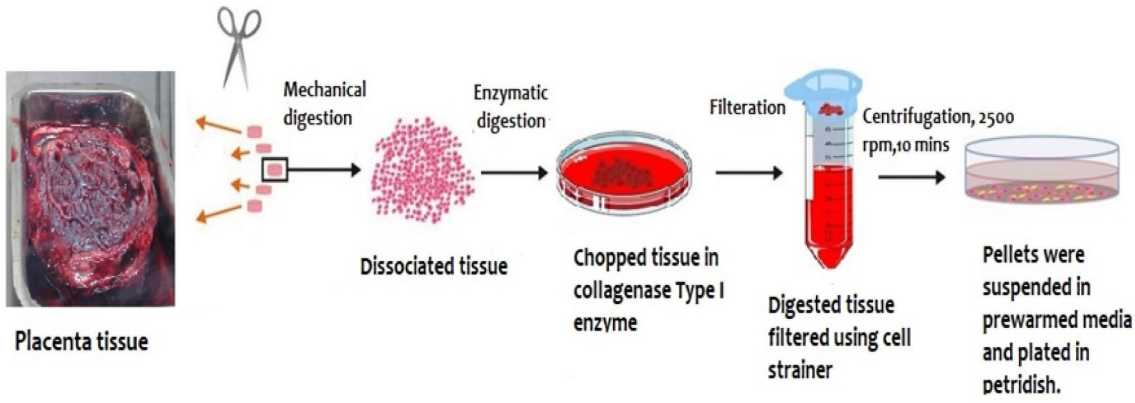

Fig. 1 Scheme representing the derivation of mesenchymal stem cells from placenta tissue**.** Collagenase enzyme is used for digestion to obtain stem cells

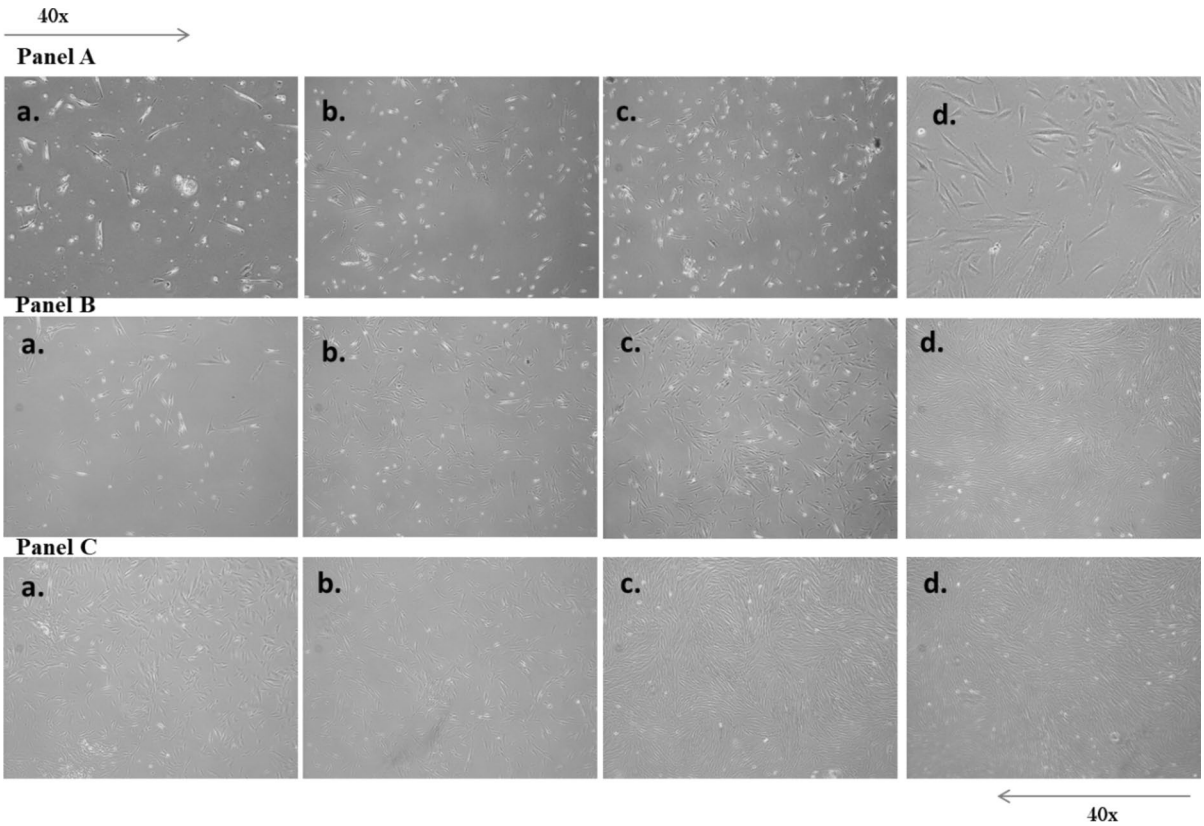

Fig. 2 Sub-culturing of mesenchymal stem cells derived from placenta tissue. Cells were seeded in T_{175} flask and observed for growth and confluence under 40 × magnifications. Panel **A**: Passage 0 (a. 3rd day, b. 7th day, c. 14th day, d. 21st day), Panel **B**: Passage 1 (a. 0th day, b. 3rd day, c. 5th day, d. 7th day), Panel **C**: Passage 2 (a. 0th day, b. 1st day, c. 3rd day, d. 5th day)

The percentage of positive cells found to be more than 95% in these selective adhesion MSC markers. The cells were also negative for CD45, CD34, CD11b, CD19 and also major histocompatibility class-II (HLA-DR) markers which indicated that these cells were not of hematopoietic origin (data not shown). It had shown that less than 2% in which the peaks were seen overlapping with control (Fig. 3). Based on the results of cell surface markers expression, the isolated cells from the placental source were regarded as mesenchymal stem cells.

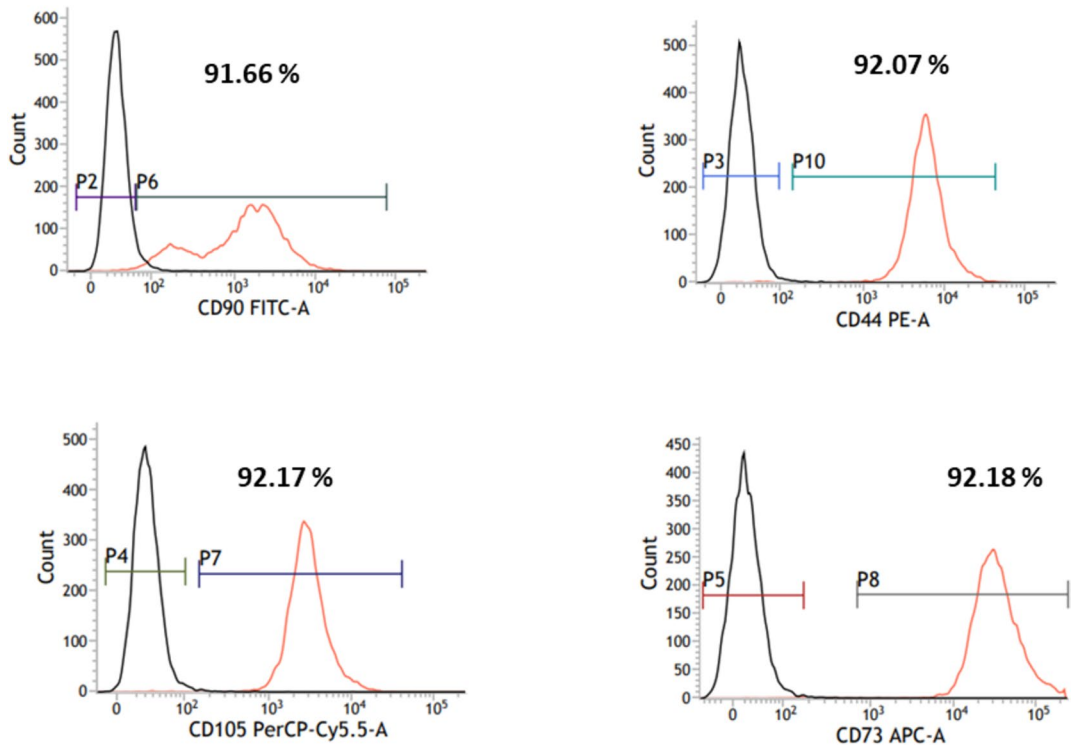

Fig. 3 Characterization of p-MSC. Flow cytometric analysis of MSC-positive surface markers (CD44, CD73, CD90 and CD105) with > 98% expression

Differentiation potential of isolated p-MSCs

The standard protocol for characterizing MSCs is their ability to differentiate into multiple lineages. Our results showed that isolated MSCs from placenta source were readily differentiated into adipogenic cell types. After 21 days of culture in adipogenic medium, the induction resulted in the formation of lipid inclusion which stained with Oil Red O staining. In our results, the p-MSC without adipogenic induction medium (Control) also showed the spontaneous differentiation of adipocyte. It was observed under phase contrast microscope with 40× magnification (Fig. 4a, b, and c).

Gene expression of adipogenic differentiated p-MSC cells

qRT-PCR analysis revealed the mRNA expression levels of visfatin and leptin genes in the adipogenic differentiated p-MSC cells (Fig. 5). Visfatin gene has the significantly reduced expression (0.029 ± 95% CI of 0.9225–0.7615) when compared to GAPDH (0.158-fold change) expression as opposed to onefold in control, whereas leptin gene has been significantly upregulated, i.e., 2.114-fold increase in expression (0.041 ± 95% CI of 1.007–1.22)

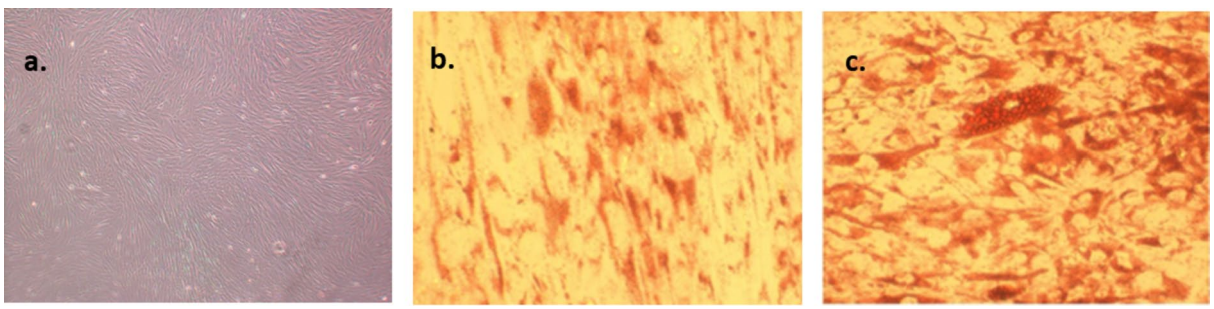

Fig. 4 Adipogenic differentiation of p-MSCs **a** Control unstained cells **b** Control stained with Oil O red **c** p-MSCs were grown in adipocyte differentiation medium stained with Oil O red

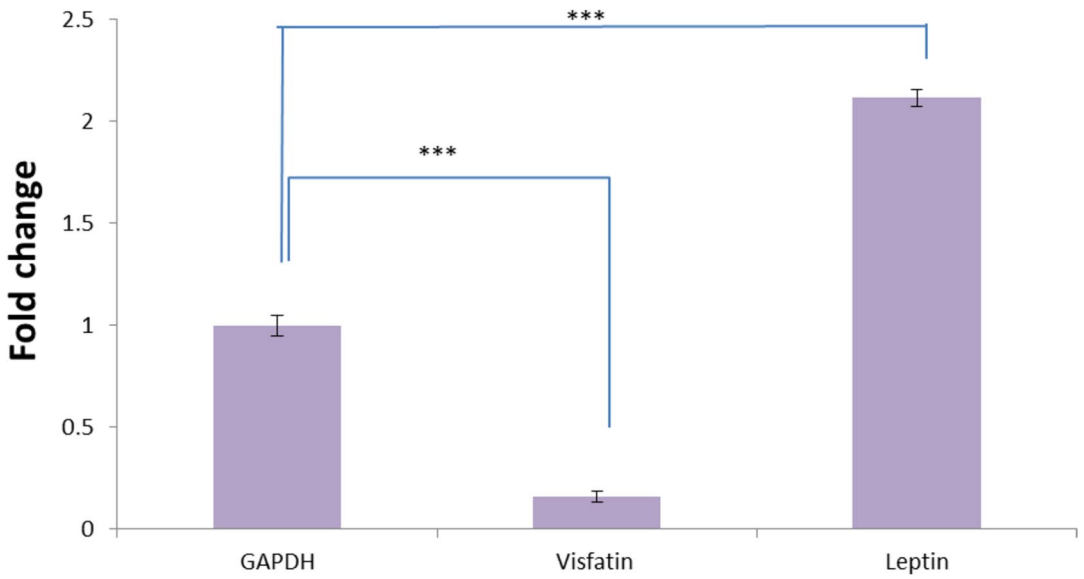

Fig. 5 Gene expressions of adipogenic differentiated p-MSCs were analyzed using qPCR. Experiment was done in triplicated. Data are expressed as mean values with standard deviations (***$P > 0.0001$)

when compared to control. Experiments were done in triplicates and taken as mean value with ± standard error.

Discussion

Recently, stem cell research not only improved the understanding of basic development processes but also provided promising opportunities for the use of stem cells in several areas such as biotechnology, pharmaceutics, cell therapy, regenerative medicine, and applications in tissue engineering. Even though pluripotent embryonic stem cells (ESC) shows the great promise of regenerative medicine, it would be complicated due to the ethical concerns to derive human embryos, teratoma formation, and xenograft rejection (Shufaro and Reubinoff 2004). Alternatively, the induced pluripotent stem cells (iPSC) were derived from adult cells but it also faced the similar problem like ESC besides some technical issues. Moreover, iPSCs may not be genetically stable thus limiting in its therapeutic use. Scientists are researching for stable, safe and highly accessible stem cell source with great potential for regenerative medicine. Isolation of stem cells has been reported from various organs and postnatal tissues. Adult multipotent stem cells were derived from many sources such as bone marrow (Siegel et al. 2013; Ratajczak 2015), adipose tissue (Zhou et al. 2013; Mattar and Bieback 2015; De Castro et al. 2017) and muscle tissue (Talele et al. 2015; Klimczak and Kozlowska 2016; Wosczyna et al. 2019).

Recently, mesenchymal stem cells which were derived from adult stem cells have been used to treat various diseases and concentrated on exploring therapies for chronic diseases viz. autoimmune disorders (Maria et al. 2017; Chen et al. 2019), inflammatory disorders (Shi et al. 2018; Fan et al. 2019), diabetes (Moreira et al. 2017; Päth et al. 2019), neurodegenerative disease (Volkman and Offen 2017; Yao et al. 2020), cardiovascular disease (Yun and Lee 2019; Chen et al. 2020; Poomani et al. 2022), and even cancer (Hmadcha et al. 2020; Timaner et al. 2020), but the scientific basis for these applications has not yet been established or widely accepted. MSC has gained attention of researchers due to limited ethical concern compared to embryonic stem cells and also for its great aspect of immunomodulatory features. MSCs have been isolated in the past, both from fetal tissues (Brown et al. 2019) and also from many adult tissues (Neirinckx et al. 2013; Zheng et al. 2013; Souza et al. 2014; Caplan 2015; Macrin et al. 2017; Klimczak et al. 2018; Samsonraj et al. 2018). The birth-associated tissues such as umbilical cord (Ding et al. 2015), amniotic membrane (Navas et al. 2018) have been used as primitive sources for mesenchymal stem cells. Likewise, the placenta which is considered as a birth waste is used as an essential and nascent source to derive mesenchymal stem cells (Komaki et al. 2017).

In our study, the placental tissue was preferred due to their good proliferation and differentiation potentials owing to the multifaceted structure and function of the placenta (Liao et al. 2016). Since the placenta is composed of MSCs and several other types of cells, MSCs was clonally isolated based on its adherent property and requisite morphological feature for MSCs and a part of it was preserved for future use. MSCs have to fulfill the guidelines such as fibroblast-like structure and plastic adherence, to be positive for MSC

markers and should be negative for hematopoietic markers (HLA-DR), should differentiate into adipocytes, chondrocytes, and osteocytes (Horwitz et al. 2005; Dominici et al. 2006). The surface marker analysis and their ability of multipotency were scored as a reliable way to identify the populations of MSC (da Silva Meirelles et al. 2008). As shown in the results, these phenotypes represent mesenchymal stem cells features and strongly suggest that the isolated cells from the tissue term placenta were indeed mesenchymal stem cells in nature. Some other cells also adhere to the plastic plate during isolation of MSCs from the placenta, but on sub-culturing, these cells get washed away, leaving only adherent fibroblast-like cells. Platelet lysate (PL), a source of growth factors and bio-active molecules which have been suggested as a substitute for FBS, helps in ex vivo expansion of MSC culture. PL displayed good results such as typical plasticity, immunophenotype, immunomodulatory property, and chromosomal ability (Becherucci et al. 2018; Kandoi et al. 2018).

For defining MSCs, Several surface markers have been suggested to be present or absent. In commonly, the positive expression for CD105, CD90, and CD73 and absence for several surface molecules such as CD34, hematopoietic CD45, and HLA-DR are detailed (Dominici et al. 2006). Our data mostly corroborate with the findings. A total of 9 surface markers were analyzed; MSCs derived from placenta demonstrated CD73 (5′ nucleotidase), CD90 (Thy-1), CD105 (Endoglin), and CD44 (H-CAM) which are highly expressed as surface markers. CD73 showed the high percentage of about 99.54%, while CD105 expressed as 99.52%. The negative expression of hematopoietic markers CD34, CD45 alongside CD11b, CD19, and HLA-DR was lower in percentages (> 2%) and defined the characteristics of placental-derived mesenchymal stem cells.

To assess the differentiation ability of p-MSC, the cells were grown in adipocyte differentiation media. Surprisingly, upon adipogenic induction for p-MSCs (P3) for about 21 days with HiAdipo differentiation media, not only cells induced toward adipocytes, but also cells from control well, stained positive for Oil Red O staining, as evident from the image (Fig. 4b) suggesting spontaneous differentiation of placental mesenchymal stem cells toward adipocytes. The plausible explanations are hypothesized as:

- The post confluent culture of adipose-derived MSCs is shown to spontaneously express fibroblastic, osteogenic, chondrogenic, and adipogenic biomarkers (Consulting et al. 2015). Likewise in our study, the cells in the control might have entered post confluent stage and started secreting extracellular matrix favorable of adipogenic differentiation.
- The spontaneous differentiation of pre-adipocytes into fat clusters due to the presence of fetal calf serum (FCS; 10% alone) was found to be dose dependent (Miettinen et al. 2008). Here, the platelet lysate (10%) as a supplement of PL-MSC may behave same like FCS and could have caused the spontaneous differentiation.
- MSCs from placenta have the ability to undergo robust proliferation and spontaneous differentiation toward mesodermal lineage without any induction, because of its fetal origin. This is corroborated by a similar study that fetal rat circulatory blood cells (FCBCs) spontaneously differentiated toward both chondrogenic and osteogenic lineages in the presence of serum (FBS) alone in the absence of any external induction stimulus. They speculated that it is FBS that influenced this process (Naruse et al. 2004).
- Extended/serial passaging and seeding density may also justify the spontaneous differentiation process. In this line, Liu group found that serial passaging (up to P12) and/or seeding at low density (1000 cells/cm^2) spontaneously differentiated adipose-derived mesenchymal stem cells into osteocytes (Liu et al. 2016). Our experiments done with earlier passage (P3) cells with low seeding density could have caused spontaneous differentiation toward adipocytes.

Adipokines such as visfatin and leptin are adipocyte-derived factors with immunomodulatory properties and might influence the differentiation of placenta-derived mesenchymal stem cells into adipocytes (Tsiklauri et al. 2018). Thus, the presence of adipokines in the adipogenic differentiated mesenchymal stem cells were analyzed using quantitative PCR on 21st-day differentiation. In adipogenic differentiation, visfatin gene expression was low compared to leptin. This novel adipokine was found to be strongly expressed in osteogenic differentiation. According to Yue, leptin gene promotes adipogenic differentiation and tends to reduce osteogenesis (Yue et al. 2016). Likewise in our study, leptin gene expression was upregulated.

Taken in concert, our results demonstrated that mesenchymal stem cells can be derived from birth-associated tissue, i.e., placenta with platelet lysate instead of fetal bovine serum and characterized according to ICST minimal criteria and adipokine gene expression for visfatin and leptin were analyzed using qPCR.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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