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Improved differentiation of umbilical cord blood-derived mesenchymal stem cells into insulin-producing cells by PDX-1 mRNA transfection

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ABSTRACT

Numerous studies have sought to identify diabetes mellitus treatment strategies with fewer side effects. Mesenchymal stem cell (MSC) therapy was previously considered as a promising therapy; however, it requires the cells to be trans-differentiated into cells of the pancreatic-endocrine lineage before transplantation. Previous studies have shown that PDX-1 expression can facilitate MSC differentiation into insulin-producing cells (IPCs), but the methods employed to date use viral or DNA-based tools to express PDX-1, with the associated risks of insertional mutation and immunogenicity. Thus, this study aimed to establish a new method to induce PDX-1 expression in MSCs by mRNA transfection. MSCs were isolated from human umbilical cord blood and expanded in vitro, with stemness confirmed by surface markers and multipotentiality. MSCs were transfected with PDX-1 mRNA by nucleofection and chemically induced to differentiate into IPCs (combinatorial group). This IPC differentiation was then compared with that of untransfected chemically induced cells (inducer group) and uninduced cells (control group). We found that PDX-1 mRNA transfection significantly improved the differentiation of MSCs into IPCs, with 8.3 ± 2.5% IPCs in the combinatorial group, 3.21 ± 2.11% in the inducer group and 0% in the control. Cells in the combinatorial group also strongly expressed several genes related to beta cells (Pdx-1, Ngn3, Nkx6.1 and insulin) and could produce C-peptide in the cytoplasm and insulin in the supernatant, which was dependent on the extracellular glucose concentration. These results indicate that PDX-1 mRNA may offer a promising approach to produce safe IPCs for clinical diabetes mellitus treatment.

1. Introduction

Diabetes mellitus is a highly prevalent disease estimated by the World Health Organization to affect approximately 500 million people worldwide. However, to date, there is still no cure. All of the current methods used to treat diabetes mellitus aim to restore glucose homeostasis. Cellular therapy has long been considered as a potential approach to cure this disease. However, beta cell numbers are limited, and thus not ideal for replacement therapy. Insulin-producing cells (IPCs), on the other hand, can be differentiated from stem cells and offer a potential source of cells in lieu of beta cells. For this reason, numerous studies have been conducted to establish protocols to differentiate stem cells into IPCs.

Various sources of stem cells have been successfully differentiated into IPCs, including embryonic stem cells (Hua et al., 2014; Jiang et al., 2007), induced-pluripotent stem cells (Alipio et al., 2010; Jeon et al., 2012; Zhu et al., 2011), pancreatic stem cells (Noguchi et al., 2010), mesenchymal stem cells from human umbilical cord blood (UCB) (Parekh et al., 2009; Phuc et al., 2011), placenta (Kadam et al., 2010), bone marrow (Phadnis et al., 2011), and adipose tissue (Chandra et al., 2009). Of these, UCB-derived MSCs offer several advantages, particularly because of the increased banking of UCB samples in recent years.

Abbreviations: DMEM, Dulbecco's modified eagle medium; GFP, green fluorescent protein; MNC, mononuclear cell; mRNA, messenger RNA; MSC, mesenchymal stem cell; IMDM, Iscove's modified Dulbecco's media; IPC, insulin producing cell; PBS, phosphate buffered saline

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Consequently, UCB-MSCs have been extensively studied for IPC differentiation. Until now, the most successful methods to induce UCB-MSC differentiation into IPCs used nicotinamide and/or exendin-4 inducers (Phuc et al., 2011; Prabakar et al., 2012; Tsai et al., 2012). Other studies have also successfully differentiated UCB-MSCs into IPCs by up-regulating some of the master genes that cause IPC differentiation (mainly PDX-1) (He et al., 2011; Wang et al., 2011). These studies have demonstrated that PDX-1 is an important factor regulating pancreatic-endocrine differentiation, particularly for beta cell formation and function. Furthermore, PDX-1-differentiated IPCs can regulate the glucose concentration of diabetic mice.

The chemical induction of IPCs from MSCs, however, is generally poor and, although PDX-1 up-regulation can significantly increase IPC production, the use of vector viruses, such as an adenovirus or a lentivirus, harbors the risk of insertional mutagenesis and immunogenicity (Dave et al., 2009; Hacein-Bey-Abina et al., 2008; Howe et al., 2008). As such, the differentiated IPCs from these protocols cannot be used to treat humans in clinical applications. Therefore, this study aimed to develop a novel and safe method to improve the differentiation efficiency of UCB-MSCs into IPCs. We show improved chemical differentiation of MSCs following transfection of PDX-1 mRNA.

2. Materials and methods

2.1. Isolation of UCB-MSCs

Human UCB was obtained from hospital samples with informed consent obtained from the mother after delivery of her child. All procedures and manipulations were approved by our Institutional Ethical Committee (Laboratory of Stem Cell Research and Application, University of Science, Vietnam National University, Ho Chi Minh City, Vietnam) and the Hospital Ethical Committee (Nhan Dan 115 Hospital, Ho Chi Minh City, Vietnam). A bag system containing 17 mL of anticoagulant (citrate, phosphate, and dextrose) was used. All UCB units were processed within 3 h after delivery. To isolate mononuclear cells (MNCs), each UCB unit was diluted 1:1 with phosphate-buffered saline (PBS) and carefully loaded onto Ficoll-Hypaque (1.077 g/mL, Sigma-Aldrich, St. Louis, MO). After density gradient centrifugation at 3000 rpm for 20 min at room temperature, MNCs were removed from the interphase, washed twice with PBS, and resuspended in Iscove's modified Dulbecco's media (IMDM) with 15% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Sigma-Aldrich). MNCs were seeded in T-75 cm^2 flasks at 1×10^5 cells/cm^2 and incubated at 37 °C, 5% CO_2. The medium was replaced every 3 days. When cells reached 70–80% confluence, they were subcultured at a ratio of 1:3 using the same medium as primary culture.

2.2. UCB-MSC characterization

UCB-MSCs were characterized according the MSC standard set by Dominici et al. (2006). UCB-MSCs were confirmed by flow cytometry using surface marker expressions of CD14, CD34, CD45, HLA-DR, CD73, CD90 and CD105. Flow cytometry was performed on a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA). UCB-MSCs were stained with anti-CD14-FITC, anti-CD34-FITC, anti-CD45-FITC, anti-HLA-DR-FTIC, anti-CD73-PE, anti-CD90-FITC and anti-CD105-FITC monoclonal antibodies. A total of 10,000 cells were analyzed by CellQuest Pro software. Isotype controls were used in all analyses.

UCB-MSCs were also confirmed by their potential to differentiate along multiple lineages. Adipogenic differentiation of MSCs was performed as described previously (Lee et al., 2004b). Briefly, UCB-MSCs at passage 5 were plated at a density of 1×10^4 cells/well in 24-well plates. At 70% confluence, the cells were switched to IMDM supplemented with 0.5 mM 3-isobutyl-1-methyl-xanthine, 1 nM dexamethasone, 0.1 mM indomethacin and 10% FBS (all from Sigma-Aldrich) and cultured for 21 days. Adipogenic differentiation was evaluated by observing the production of lipid vesicles within cells via microscopy.

For osteogenic differentiation, UCB-MSCs were plated at 1×10^4 cells/well in 24-well plates. At 70% confluence, the cells were switched to IMDM supplemented with 10% FBS, 10^{-7} M dexamethasone, 50 μM ascorbic acid-2 phosphate and 10 mM β-glycerol phosphate (all from Sigma-Aldrich), and cultured for 21 days, as described elsewhere (Lee et al., 2004b). Osteogenic differentiation (calcium accumulation) was confirmed by Alizarin red staining.

For chondrogenic differentiation, UCB-MSCs were induced using a commercial medium for chondrogenesis (StemPro Chondrogenesis Differentiation Kit, A10071-01, Life Technologies). UCB-MSCs were differentiated in pellet form, according to manufacturer's guidelines. After 21 days growth, cell pellets were stained with an anti-aggrecan monoclonal antibody (BD Bioscience).

2.3. In vitro mRNA PDX-1 production

pcDNA3.1-hPDX-1 was amplified by PCR with 5′-T7 primer (5′-TAATACGACTCACTATAGGG-3′) and 3′-specific primer for PDX-1 (5′-GTCCTCCTCCTTTTTCCAC-3′). pcDNA3.1-hPDX-1 was prepared in the previous study by cutting hPDX-1 from vector pWPT-PDX1 with NotI and BamHI (Plasmid 12256, Addgene, Cambridge, MA) and inserting to vector pcDNA™ 3.1 (Invitrogen, Carlsbad, CA) (Nguyen et al., 2014).

The PCR products for hPDX-1 were purified using the GenElute PCR Clean-up Kit, Sigma-Aldrich, St Louis, MO). The purified PCR product was employed for an in vitro transcription reaction using the T7 mScript Standard mRNA Production System (Epicentre Biotechnologies, Madison, WI). The mRNA concentration was measured using a Nanophotometer (Eppendorf, Germany).

2.4. mRNA PDX-1 transfection

UCB-MSCs were transfected according to a previously published protocol (Arnold et al., 2012). UCB-MSCs were transfected with 3 μg of mRNA by nucleofection (NHDF-VPD-1001, Lonza). After transfection, these cells were plated into T-25 flasks and cultured in the medium. At 72 h, 144 h, and 216 h after nucleofection, the adherent cells were transfected with "FuGENE HD" (Roche, Basel, Switzerland) according to the manufacturer's instructions, which was replaced with culture medium 4 h later. The ratio of "FuGENE HD" reagent and mRNA was 8 μL per 3 μg of mRNA. Transfected samples of UCB-MSCs were evaluated for changes in Pdx-1 expression at both transcriptional and translational levels.

2.5. RNA extraction and reverse transcript real-time RT PCR

RNA was extracted from cell cultures using a Trizol extraction kit (Intron Biotechnology, Korea). mRNA was reversed transcribed into cDNA using an AMV reverse transcription kit (Agilent Technologies, Santa Clara, CA). The real-time RT-PCR reactions were carried out using Brilliant II SYBR® Green QPCR Master Mix (Agilent Technologies). The primer sequences were as follows: *GAPDH*, forward, 5′-AGAAGGCTGGGGCTCATTTG-3′, and reverse, 5′-AGGGGCCATCCACAGTCTTC-3′; *PDX-1*, forward, 5′-GGATGAAGTC TACCAAAGCTCACGC-3′, and reverse, 5′-CCAGATCTTGATGTGTCTC TCGGTC-3′; *INSULIN*, forward, 5′-AACCAACACCTGTGCGGCT CA-3′; reverse, 5′-TGCCTGCGGGCTGCGTCTA-3′; *NGN3*, forward, 5′-CGCCGGTAGAAAGGATGAC-3′, reverse: 5′-GAGTTGAGGTTGTG-CATTCG-3′; *NKX6.1*, forward: 5′-CTGGAGAAGACTTTCGAACAA-3′,