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A feasibility study of an *in vitro* differentiation potential toward insulin-producing cells by dental tissue-derived mesenchymal stem cells

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ABSTRACT

Dental tissue-derived mesenchymal stem cells have been proposed as an alternative source for mesenchymal stem cells. Here, we investigated the differentiation ability toward insulin producing cells (IPCs) of human dental pulp stem cells (hDPSCs) and human periodontal ligament stem cells (hPDLSCs). These cells expressed mesenchymal stem cell surface markers and were able to differentiate toward osteogenic and adipogenic lineages. Upon 3 step-IPCs induction, hDPSCs exhibited more colony number than hPDLSCs. The mRNA upregulation of pancreatic endoderm/islet markers was noted. However, the significant increase was noted only for *PDX-1*, *NGN-3*, and *INSULIN* mRNA expression of hDPSCs. The hDPSCs-derived IPCs expressed PRO-INSULIN and released C-PEPTIDE upon glucose stimulation in dose-dependent manner. After IPCs induction, the Notch target, *HES-1* and *HEY-1*, mRNA expression was markedly noted. Notch inhibition during the last induction step or throughout the protocol disturbed the ability of C-PEPTIDE release upon glucose stimulation. The results suggested that hDPSCs had better differentiation potential toward IPCs than hPDLSCs. In addition, the Notch signalling might involve in the differentiation regulation of hDPSCs into IPCs.

1. Introduction

Currently, the standard treatments to control blood glucose levels are the use of exogenous insulin injection and/or oral hypoglycemic drugs administration [1–4]. Yet, various side effects and limitations are still of concern [5]. A regenerative medicine has been proposed as a potential alternative therapeutic option. In this regard, an islet transplantation is utilized in type I diabetic patients. However, this procedure has faced with many obstacles due to donor shortage and adverse reactions of immunosuppressive drugs [6]. To overcome these problems, the preliminary studies of autologous and allogenic transplantations of stem cell-derived insulin producing cells (IPCs) in induced-diabetes animal models have been investigated in animal models [7,8]. Various stem cell types were investigated for IPCs production, for example embryonic stem cells, hepatic stem cells, umbilical cord blood stem cells, bone marrow-derived mesenchymal stem cells, adipose stem cells, and multipotent dermal fibroblasts [9–14].

Dental tissue-derived mesenchymal stem cells have been introduced as the candidate cell sources due to their interesting properties i.e. multipotentiality, accessibility and availability [15,16]. In addition, these cells contain the immunomodulatory functions via the secretion of active molecules and/or direct interaction with immune cells [17], implying the advantage for cell therapeutic purpose. Thus, in the present study, we aimed to explore and compare the differentiation potential toward IPCs by two human dental tissue-derived stem cells; human dental pulp stem cells (hDPSCs) and human periodontal ligament stem cells (hPDLSCs).

2. Materials and methods

2.1. Cell isolation and culture

hDPSCs and hPDLSCs were isolated from human dental pulp and periodontal ligament tissues of adult subjects undergoing routine tooth extraction according to treatment plan. The protocol was

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approved by Human Research Ethic Committee, Faculty of Dentistry, Chulalongkorn University. The explants were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco), supplemented with 100 unit/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), 5 μg/mL amphotericin B (Gibco), 2 mM L-glutamine (1x Glutamax®) (Gibco) and 10% FBS (Gibco) was used as the culture medium. Cells were maintained at 37 °C in humidified atmosphere with 5% CO_2 aeration. Cells in passage 2–5 were used in the study.

2.2. hDPSCs and hPDLSCs characterization

The expression of surface marker was evaluated using flow cytometry analysis [18]. Briefly, cells were stained with FITC-conjugated anti-CD44 antibody (BD Biosciences Pharmingen), PerCP-conjugated anti-CD45 antibody (BD Biosciences Pharmingen), APC-conjugated anti-CD73 antibody (Biolegend), PerCP-CyTM5.5-conjugated anti-CD90 antibody (BD Biosciences Pharmingen), and PE-conjugated anti-CD105 antibody (BD Biosciences Pharmingen). For STRO-1, mouse anti-STRO-1 antibody (Millipore), biotinylated goat anti-mouse antibody (Invitrogen) and streptavidin-APC (BD Biosciences Pharmingen) were employed. Isotype antibodies were used as the control. For data analysis, FACSCalibur regarding the CellQuest software (BD Bioscience) was used, and the values were illustrated as mean fluorescence intensity (MFI).

For osteogenic and adipogenic induction, cells were seeded in 24-well plate at density of 2.5×10^5 cells/well. The osteogenic induction medium was the growth medium supplemented with 50 mg/ml ascorbic acid, 100 nM dexamethasone, and 10 mM β-glycerophosphate. The adipogenic induction medium was the growth medium supplemented with 0.1 mg/ml insulin, 1 mM dexamethasone, 1 mM IBMX, and 0.2 mM indomethacin. The Alizarin Red S and Oil Red O staining were performed according to previous publications [18–20].

2.3. IPCs induction

Cells were differentiated into IPCs using 3-stage differentiation protocol modified from Chandra et al. (2009) and Govindasamy et al. (2011) [21,12]. For the first step, at day 0, single cell suspension (10^6 cells) was seeded in 60 mm Petri dish (Falcon). The cells were maintained in serum-free medium (SFM)-A for 3 days, SFM-B for 2 days, and SFM-C for 5 days, respectively. The medium was changed every 2 days. SFM-A was SFM-DMEM (Gibco) supplemented with 1% bovine serum albumin (BSA) (Cohn fraction V, fatty acid free) (Sigma), 1× insulin-transferrin-selenium (ITS) (Invitrogen), 4 nM activin A (Sigma), 1 nM sodium butyrate (Sigma) and 50 μM β-mercaptoethanol (Sigma). SFM-B contained 1% BSA, 1× ITS and 0.3 mM taurine (Sigma) in SFM-DMEM. SFM-C compositions were 1.5% BSA, 1× ITS, 3 mM taurine, 100 nM glucagon-like peptide (GLP)-1 (Sigma), 1 mM nicotinamide (Sigma) and 1× non-essential amino acids (NEAAs) (Sigma) in SFM-DMEM. In some experiments, a γ-secretase inhibitor (DAPT; Sigma) was added in the induction medium at concentration of 25 μM. The DMSO at the same concentration was added in the control condition.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated with TRIzol® RNA isolation reagent (Invitrogen). The complementary DNA (cDNA) was obtained by converting 1 μg of RNA sample using reverse transcriptase enzyme kit (Promega, USA). For quantitative real-time PCR (qPCR), gene expression was detected by FastStart® Essential DNA Green Master® (Roche Diagnostics) using CFX96™ real-time PCR detection system (Bio-Rad). The mRNA expression value was illustrated as relative mRNA expression by normalized to 18S ribosomal RNA and the control. The primer sequences were shown in Table 1 [21,22].

Table 1
Primer sequences.

Genes	Accession numbers	Primer sequences
PDX-1	NM 000209	(Forward) 5′ GTCCTGGAGGAGCCCAAC 3′ (Reward) 5′ GCAGTCCTGCTCAGGCTC 3′
NGN-3	NM 020999	(Forward) 5′ ATAAAGCGTGCCAAGGGGCACA 3′ (Reward) 5′ TTGTGCATTCGATTGCGCTCGC 3′
NKX-6.1	NM 006168	(Forward) 5′ TTGGCCTATTCGTTGGGGAT 3′ (Reward) 5′ GTCTCCGAGTCCTGCTTCTTC 3′
GLUT-2	NM 000340	(Forward) 5′ GGTTTGTAACTTATGCCTAAG 3′ (Reward) 5′ GCCTAGTTATGCATTGCAG 3′
INSULIN	NM 000207	(Forward) 5′ CCGCAGCCTTTGTGAACCAACA 3′ (Reward) 5′ TTCCACAATGCCACGCTTCTGC 3′
HES-1	NM 005524	(Forward) 5′ AGGCGGACATTCTGGAAATG 3′ (Reward) 5′ CGGTACTTCCCCAGCACACTT 3′
HEY-1	NM 012258	(Forward) 5′ GGAGAGGCGCCGCTGTAGTTA 3′ (Reward) 5′ CAAGGGCGTGCGCGTCAAAGTA 3′
18S	NM 10098	(Forward) 5′ GTGATGCCCTTAGATGTCC 3′ (Reward) 5′ CCATCCAATCGGTAGTAGC 3′

2.5. Immunocytochemistry staining

Samples were fixed in cold methanol for 15 min, permeabilized with 0.1% Triton®-X100 (Sigma) in PBS, and incubated with 10% horse serum in PBS for 1 h. The samples were incubated with primary antibody, mouse anti-human PRO-INSULIN (Millipore), at 1:100 dilution for 24 h. The goat anti-mouse antibody-biotin (Invitrogen) at 1:500 dilution and streptavidin-FITC (Sigma) 1:500 dilution were used as the secondary antibody and reporter, respectively. DAPI (0.1 μg/mL) was used for nuclei counterstaining. The staining was examined using fluorescent microscope incorporated with Carl Zeiss™ Apotome.2 apparatus (Carl Zeiss).

2.6. Functional tests for IPCs

Glucose-stimulated C-PEPTIDE secretion was performed. Glucose anhydrous (Sigma) at concentration of 5.55 mM (100 mg/dL) and 22 mM (396 mg/dL) were used in this study. Levels of C-PEPTIDE secretion were detected using enzyme-linked immunosorbent assay (ELISA) (Millipore), according to the manufacturing protocol. The amount of C-PEPTIDE was normalized to total DNA amount (ng) and stimulation time (mins).

2.7. Statistical analyses

The results were shown as mean ± standard deviation (SD) and analyzed using independent student *t* test for two sample groups or one-way analysis of variance (ANOVA) followed by Dunnett post hoc for three or more sample groups. Three subjects ($n = 3$) were used in the study. Significant difference was recognized when p-value < 0.05.

3. Results

3.1. hDPSCs and hPDLSCs characterization

The cells isolated from human dental pulp and periodontal ligament tissues exhibited several mesenchymal stem cell surface markers, including CD44, CD73, CD90, CD105, and STRO-1 (Fig. 1A and D). However, STRO-1 expression was relatively lower