Biochemical and Biophysical Research Communications 452 (2014) 581–587

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

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ARTICLE INFO

*Article history:*
Received 20 August 2014
Available online 30 August 2014

*Keywords:*
Dental pulp stem cells
Periodontal ligament stem cells
Insulin producing cells
Notch signaling
Differentiation

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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http://dx.doi.org/10.1016/j.bbrc.2014.08.121
0006-291X/

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 $2.5 \times 10^5$ 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′<br>(Reward) 5′ GCAGTCCTGCTCAGGCTC 3′ |
| *NGN-3* | NM 020999 | (Forward) 5′ ATAAAGCGTGCCAAGGGGCACA 3′<br>(Reward) 5′ TTGTGCATTCGATTGCGCTCGC 3′ |
| *NKX-6.1* | NM 006168 | (Forward) 5′ TTGGCCTATTCGTTGGGGAT 3′<br>(Reward) 5′ GTCTCCGAGTCCTGCTTCTTC 3′ |
| *GLUT-2* | NM 000340 | (Forward) 5′ GGTTTGTAACTTATGCCTAAG 3′<br>(Reward) 5′ GCCTAGTTATGCATTGCAG 3′ |
| *INSULIN* | NM 000207 | (Forward) 5′ CCGCAGCCTTTGTGAACCAACA 3′<br>(Reward) 5′ TTCCACAATGCCACGCTTCTGC 3′ |
| *HES-1* | NM 005524 | (Forward) 5′ AGGCGGACATTCTGGAAATG 3′<br>(Reward) 5′ CGGTACTTCCCCAGCACACTT 3′ |
| *HEY-1* | NM 012258 | (Forward) 5′ GGAGAGGCGCCGCTGTAGTTA 3′<br>(Reward) 5′ CAAGGGCGTGCGCGTCAAAGTA 3′ |
| *18S* | NM 10098 | (Forward) 5′ GTGATGCCCTTAGATGTCC 3′<br>(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

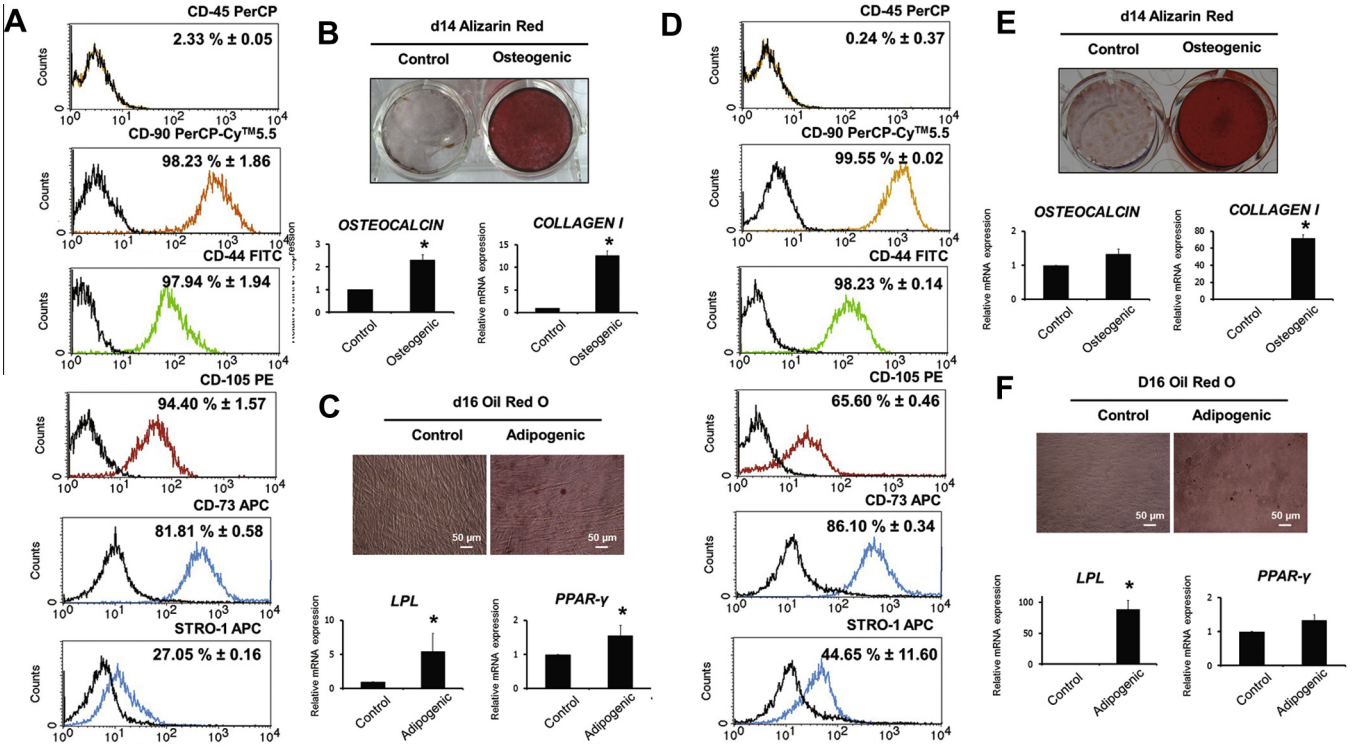


**Fig. 1.** Characterization of stem cell characteristics. Surface marker expression was determined by flow cytometry analysis (A and D). The mRNA expression of osteogenic and adipogenic differentiation was evaluated by real-time quantitative polymerase chain reaction. Alizarin Red S and Oil Red O staining were employed to confirm osteogenic and adipogenic differentiation, respectively (B, C, E and F). The asterisks indicated the statistical significant difference compared to the control. (hDPSCs: human dental pulp stem cells, hPDLSCs: human periodontal ligament stem cells).

in both cell types compared to other markers. Further, these cells rarely expressed CD45.

Regarding multipotential differentiation properties, cells were cultured in osteogenic medium for 14 days. Both hDPSCs and hPDLSCs increased the expression of osteogenic marker gene, *OSTEOCALCIN* and *COLLAGEN I*. Matrix mineralization was also confirmed by Alizarin Red S staining (Fig. 1B and E). For adipogenic differentiation, the upregulation of *LPL (LIPOPROTEIN LIPASE)* and *PPAR-γ (PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR-GAMMA)* was noted upon exposed cell in adipogenic condition for 16 days. Oil Red O staining for lipid droplet was employed (Fig. 1C and F). Together, these data imply that the isolated cells from human dental pulp and periodontal ligament tissues exhibited stem cell-like characteristics.

### 3.2. Differentiation toward IPCs

After induction, the cells were formed as aggregated colony. The cells gathered into cell clumps since day 3 upon induction. At day 5 and 10, these cell aggregates become denser and bigger in appearance as compare to early time point (Fig. 2A). The monolayer was noted in the control. Interestingly, the total colony count of hDPSCs-derived cell aggregation was significantly higher than that of hPDLSCs (Fig. 2B). The percentage of small colonies (diameter less than 50 µm) of hPDLSCs was slightly higher than those of hDPSCs. Though, no statistical significance was noted (Fig. 2C).

At day 10 of IPCs induction, qRT-PCR was used to explore pancreatic endoderm/islet gene expression. *PDX-1* and *NGN-3* were pancreatic endoderm markers while *NKX-6.1*, *GLUT-2*, and *INSULIN* were pancreatic islet markers. hDPSCs and hPDLSCs apparently exhibited the marked increase of differentiation marker mRNA expression compared to the undifferentiated control, except *NKX-6.1* in hPDLSCs-derived IPCs. However, there was a high variation among individuals. Thus, the statistical significance was only noted for *PDX-1*, *NGN-3*, and *INSULIN* mRNA expression in hDPSCs-derived IPCs (Fig. 2D and E). Additionally, the intracellular PRO-INSULIN protein expression was noted of both hDPSCs- and hPDLSCs-derived IPCs (Fig. 2F and G). Thus, these results illustrated that both cell types were able to differentiate toward IPCs and hDPSCs had markedly higher differentiation efficiency compared to hPDLSCs.

### 3.3. hDPSCs-derived IPCs secreted C-PEPTIDE

The hDPSCs-derived IPCs were further employed to evaluate the potential function regarding the secretion of C-PEPTIDE upon glucose stimulation. At day 10 after induction, cell aggregates were collected and challenged with two different concentrations of glucose, 5.5 mM and 22 mM. The results showed that hDPSCs-derived IPCs secreted C-PEPTIDE upon glucose stimulation in dose-dependent manner. The statistical significant difference was noted compared to the baseline levels (Fig. 2H).

### 3.4. Notch signaling participated in differentiation toward IPCs

Upon differentiation, the significant upregulation of Notch target genes, *HES-1* and *HEY-1* was observed (Fig. 2I), implying the involvement of Notch signaling. Therefore, the influence of Notch signaling in IPCs differentiation by hDPSCs was further investigated by adding γ-secretase inhibitor-(DAPT) in each step (DAPT-A, DAPT-B, and DAPT-C) or throughout the induction protocol (DAPT-All).

Cell aggregation in DAPT-B and DAPT-All were relatively smaller than normal control IPC group (IPC-Normal) at day 5 and 10 (Fig. 3A). The total colony count of IPC-DAPT-B was significantly increased at day 10 (Fig. 3B). Correspondingly, the higher percentage of small

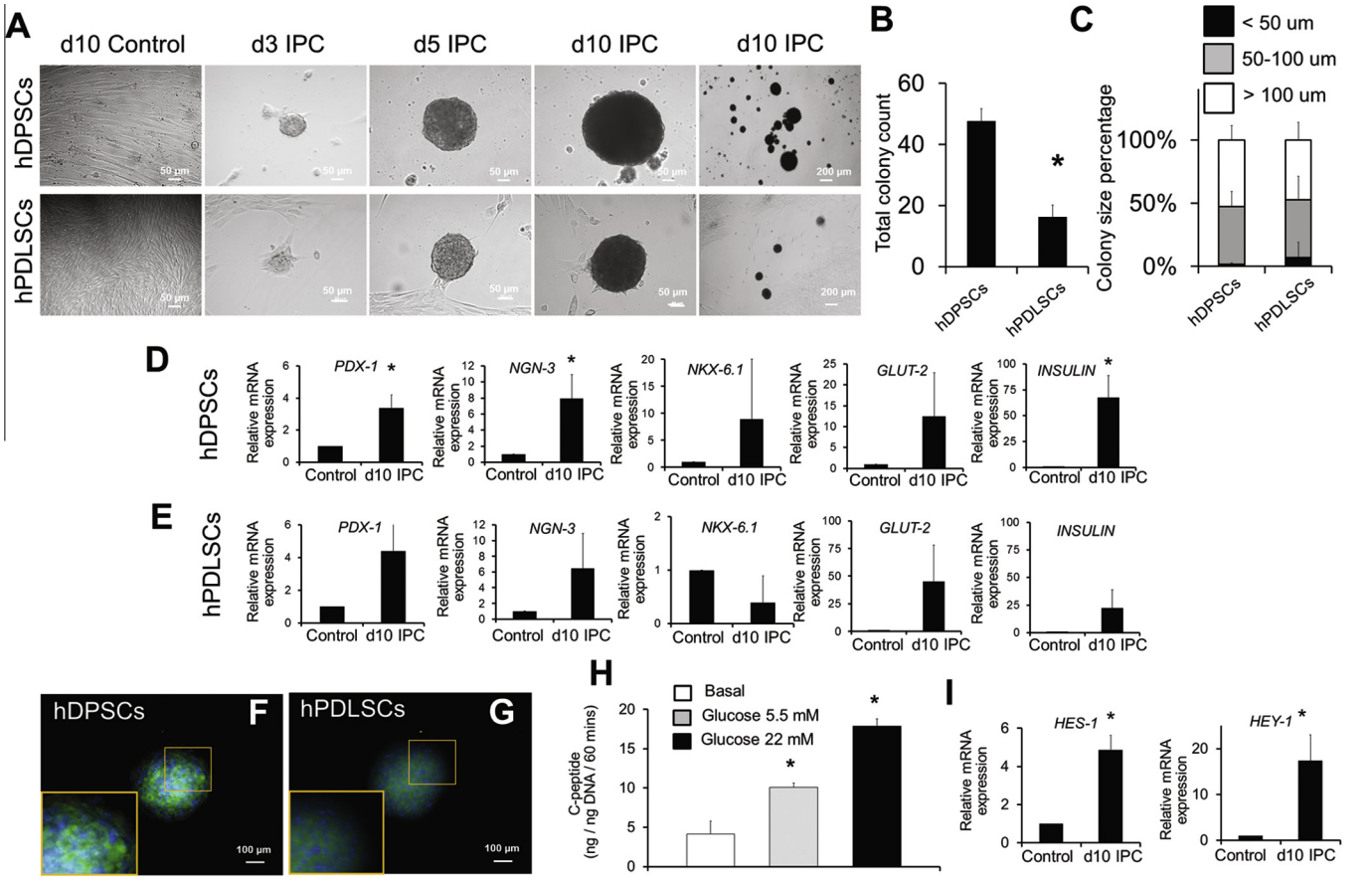


**Fig. 2.** Insulin producing cell (IPCs) induction from two types of dental tissue-derived stem cells. Morphology of aggregated colonies was illustrated at day 3, 5, and 10 after IPCs induction (A). Total colony count number (B) and the percentage of colony size (C) were also investigated. The mRNA expression of pancreatic markers by hDPSCs (D) and hPDLSCs (E) of the undifferentiated control and day 10-IPCs was analyzed by quantitative polymerase chain reaction. PRO-INSULIN protein expression was evaluated by immunocytochemistry staining (F and G). C-PEPTIDE secretion was measured upon glucose stimulation in hDPSCs-derived IPCs (H). Notch target gene expression was determined in hDPSCs-derived IPCs using quantitative polymerase chain reaction (I). The asterisks indicated the statistical significant difference.

colonies was noted in IPC-DAPT-B group compared to the control. Though, there is no significant difference (Fig. 3C).

All DAPT-treated groups showed trend of an increased *PDX-1* mRNA expression. Additionally, trend of *NGN-3 mRNA* upregulation was found in DAPT-B group. DAPT-A and DAPT-All exhibited trend of *NKX-6.1* upregulation. However, statistical significance was not found in any examined markers (Fig. 3D).

The PRO-INSULIN protein expression was noted in all groups (Fig. 4A). The function was determined by evaluating C-PEPTIDE secretion (Fig. 4B). The results revealed a trend of dose-dependent response to glucose stimulation in the control, the DAPT-A, and the DAPT-B groups. In DAPT treatment during the last induction step (DAPT-C) or throughout the protocol (DAPT-All), the slight increases of C-PEPTIDE secretion were noted upon glucose stimulation.

## 4. Discussion

In the present study, we described that both hDPSCs and hPDLSCs apparently differentiated into IPCs. Some publications formerly demonstrated the differentiation capability of adult stem cells toward pancreatic cell lineage [12,21,10,11,9,23]. Several approaches were introduced for IPCs induction. The genetic manipulation was actively investigated. In this regard, PDX-1-transduced cells were able to generate IPCs formation in both mouse and human mesenchymal stem cells [24,25]. The environmental manipulation approach was an alternative induction approach, which was employed in the present study according to the previous report [21,12]. Govindasamy et al. reported that IPCs formation could be obtained from stem cells from human deciduous teeth [21]. The same protocol was also utilized for IPCs induction by murine adipose stem cells [12]. The function of induced IPC was also confirmed both the *in vitro* glucose challenge assay and the *in vivo* transplantation in streptozotocin (STZ)-induced diabetic mouse.

In the present study, we observed the expression of pancreatic markers and found the significant upregulation of *PDX-1*, *NGN-3*, and *INSULIN* genes in hDPSC-derived IPCs, suggesting the markedly higher efficiency in IPCs differentiation compared to hPDLSCs. The functional test of hDPSCs-derived IPCs showed a pattern of dose-dependent response of C-PEPTIDE secretion that revealed a normal response of the cells to different levels of glucose challenge. However, it should be noted that the secreted C-PEPTIDE levels were markedly lower compared to previous reported [12]. Together, these results may imply the plasticity of human dental pulp-derived mesenchymal stem cells toward endodermal lineage.

Notch signaling is one of the signaling mechanisms regulating cell fate during pancreatic development and differentiation [26–29]. Notch maintained the pool of *PDX-1*-positive early pancreatic progenitors by suppressing *NGN-3* expression in order to prevent premature endocrine differentiation [30]. Inactivation of Notch signaling by conditional expression of dominant negative Maternind-like 1 or conditional inactivation of Hes1 resulted in the attenuation

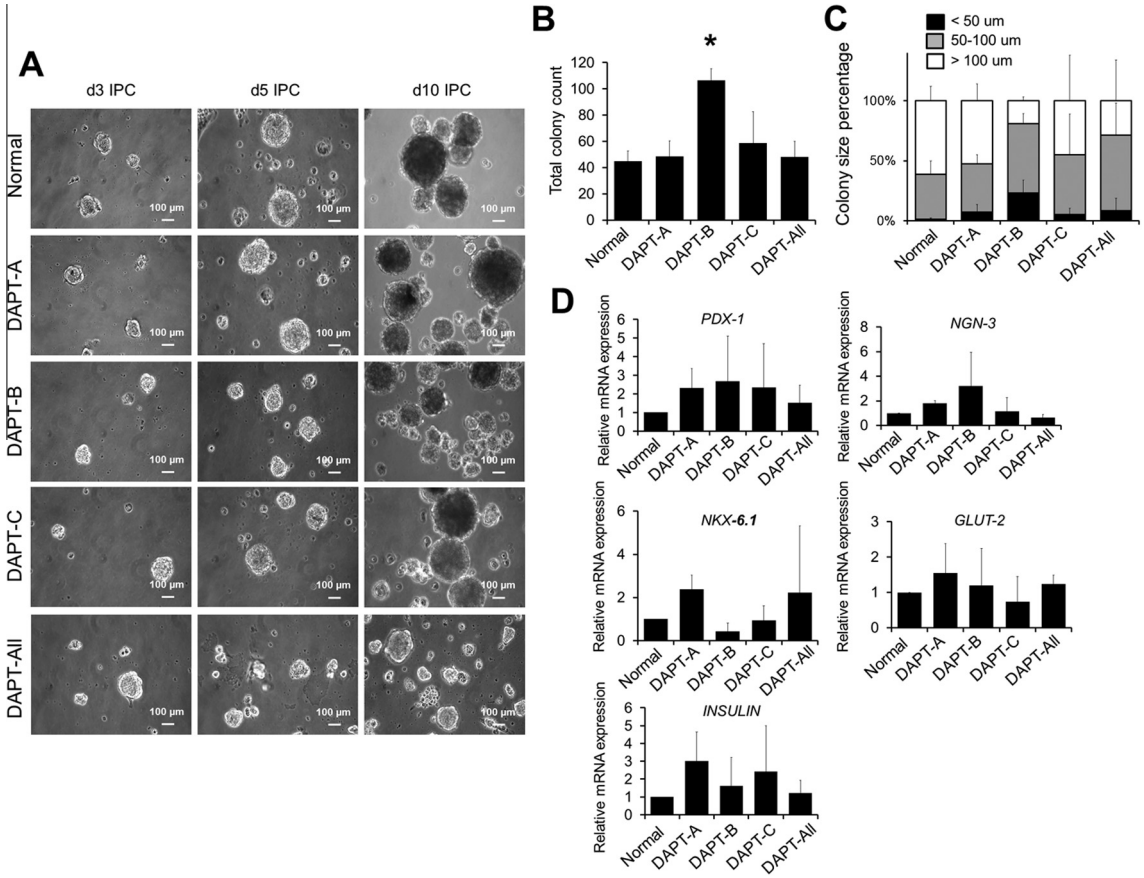


**Fig. 3.** Influence of DAPT supplementation on insulin-producing cell induction. By treatment with Notch inhibitor (DAPT) in each step (DAPT-A, DAPT-B, and DAPT-C) or throughout IPCs-induction protocol (DAPT-All), the aggregated colonies' morphology was demonstrated at day 3, 5, and 10 (A). Total colony count number (B) and colony size percentage (C) was also investigated. Expression of crucial pancreatic markers by hDPSCs-derived IPCs upon DAPT treatment was analyzed by quantitative polymerase chain reaction (D).

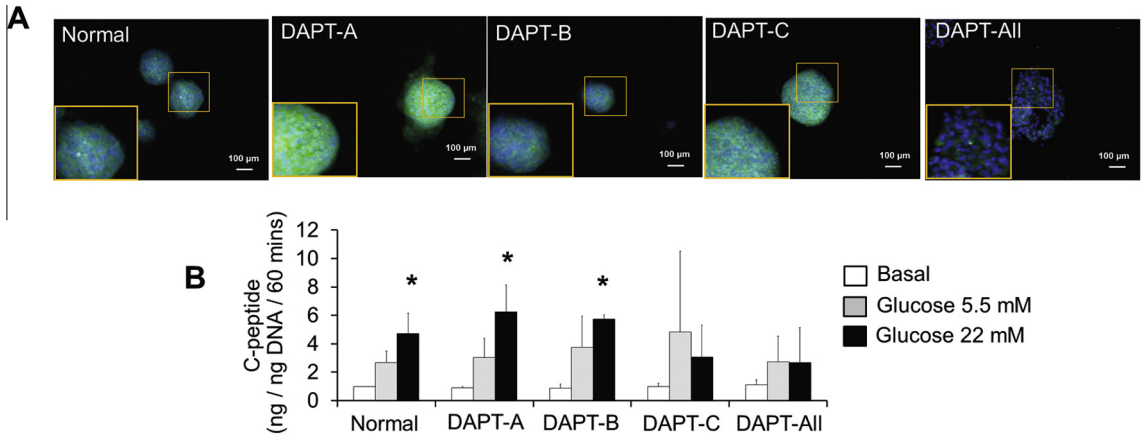


**Fig. 4.** PRO-INSULIN expression and functional property upon DAPT supplementation on insulin-producing cell induction. PRO-INSULIN protein expression was evaluated by immunocytochemistry staining (A). Amount of secreted C-PEPTIDE was measured and normalized with basal level of normal induction control group (B). The asterisks indicated the statistical significance compared to the control.

of β-cell formation in mice [31]. Further, knockout of *HES-1* expression caused pancreatic hypoplasia and depletion of pancreatic precursor cell [32]. Moreover, *Mind Bomb1*, which is E3 ubiquitin ligase-encoding gene necessary for Notch ligand activity, has been proved as a pivotal component during pancreatic β-cell formation regarding proximodistal (P-D) patterning of development [31]. On the contrary, some studies demonstrated that Notch signaling inhibition resulted in the enhancement of the expanded human pancreatic β-cell redifferentiation [33]. Together, these results suggested a crucial role of Notch signaling in pancreatic development.

Further, it has been shown that the supplementation of DAPT (a γ-secretase inhibitor) was able to enhance differentiation and maturation of IPC-deriving from human embryonic and induced pluripotent stem cells [34,35]. In addition, DAPT supplementation markedly enhanced *INSULIN* mRNA expression and PROINSULIN production in human umbilical cord mesenchymal stem cells

[36]. However, the response to glucose of these DAPT-treated human umbilical cord stem cell-derived IPCs was not changed [36], implying the functional impairment. It was also demonstrated that DAPT supplementation in IPC induction medium for embryonic stem cells caused a slight increase in *NGN-3* expression, implying inadequate IPC differentiation response to Notch inhibitor [30].

In the present study, high mRNA expression of Notch target genes (*HES-1* and *HEY*-1) was observed at the end of IPCs differentiation protocol. The addition of DAPT in each step or throughout the induction protocol resulted in the variation of IPCs differentiation regarding size, number and morphology of colony as well as the expression of pancreatic mRNA and protein markers. Notch inhibition in the first induction step could enhance upregulation trend of pancreatic endoderm/islet gene markers and exhibit normal functional property of IPCs upon glucose stimulation. This finding was correlated to previous publication suggesting role of Notch during pancreatic development [36–38]. Notch suppressed *NGN-3* expression that prevented down-stream pathway triggered-pancreatic maturation. Inhibition of Notch signaling during the first IPC-induction step might lead to an increased pancreatic precursor differentiation toward IPCs lineage. Besides, Notch inhibition in the second induction step resulted in an increase of small size colony and total colony number comparing to control. However, levels of *INSULIN* mRNA and PRO-INSULIN protein were not apparently upregulated. Further, normal functional property of IPCS in this group was also revealed. The DAPT inhibition in second step of induction also showed trend of the highest *NGN-3* mRNA expression that might relate to an increased number of IPC colony. Recent study suggested that enhancement of *ngn-3* expression could related to an increased β-cell number in diabetic mice receiving intravenous human menstrual blood progenitor cells (MBPCs) injection [39]. Together, these results may illustrate the participation of Notch signaling in the IPCs differentiation. Thus, manipulation of Notch signaling may involve in improvement of induction efficiency regarding maturation and function of hDPSCs-derived IPCs.

As described, several publications illustrated various effects of Notch inhibition on IPCs differentiation [34–36]. It may involve with the complex regulation of Notch signaling in the differentiation of pancreatic endocrine progenitors. Notch blocking might influence with maturation stage of pancreatic β-cell differentiation. It was demonstrated that the distinct levels, duration and extension of Notch signaling could balance and regulate pancreatic progenitor cell fate i.e. quiescence, proliferation and differentiation [40]. Therefore, further experiments are indeed necessitate to dissect the regulatory mechanism of Notch signaling in the differentiation process of human dental pulp-derived mesenchymal stem cells toward IPCs.

In conclusion, hDPSCs and hPDLSCs have plasticity in differentiation toward IPCs. The hDPSCs-derived IPCs apparently revealed a higher capacity in differentiation. Exploration and intervention on Notch signaling suggested the relevance of the signal in the differentiation. All of the results might imply the possibility and capability of dental stem cells applications in stem cell-based therapy for diabetes. Though, the further evaluation regarding molecular mechanism(s) is still required to find out the regulatory control process and to improve the differentiation efficiency.

## Conflict of interest

The authors declared that there is no conflict of interest.

## Acknowledgments

This study was supported by the Government Research Fund and the Research Chair Grant 2012, the National Science and Technology Development Agency (NSTDA), Thailand (PP). CS was supported by Chulalongkorn university graduate scholarship to commemorate the 72nd anniversary of His Majesty King Bhumibol Adulyadej and Royal Golden Jubilee Scholarship from the Thailand Research Fund. This paper is dedicated to memory of Associate Professor Sirintorn Yibchok-anun (DVM, PhD) for her contribution and discussion before her death in January 18, 2013.

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