รายงานการวิจัย

เรื่อง

การเปลี่ยนแปลงมีเซ็นไคมอลสเต็มเซลล์จากเนื้อเยื่อฟันและช่องปากเป็นกลุ่มเซลล์ที่เหมือนไอเล็ทในห้องทดลอง

IN VITRO DIFFERENTIATION OF MESENCHYMAL STEM CELLS FROM DENTAL AND ORAL TISSUES INTO ISLET-LIKE CELL CLUSTER.

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บทคัดย่อ

โรคเบาหวานเป็นภาวะความผิดปกติของเมตาบอลิสมอันซับซ้อน ซึ่งส่งผลให้เกิดภาวะ
น้ำตาลในเลือดสูงและภาวะแทรกซ้อนในระยะยาว เช่น ภาวะความผิดปกติของระบบประสาท
ส่วนกลางและส่วนปลาย การรักษาโรคเบาหวานและภาวะแทรกซ้อนนั้นประสบปัญหาหลาย
ประการ ด้วยเหตุนี้แนวทางการรักษาโดยใช้เซลล์ต้นกำเนิดจึงได้รับการกล่าวถึงในฐานะแนวการ
รักษาใหม่ อย่างไรก็ตามยังคงมีการศึกษาในด้านดังกล่าวค่อ   นข้างน้อย ในการศึกษาครั้งนี้ใช้
เซลล์ต้นกำเนิดจากเนื้อเยื่อโพรงประสาทฟันของมนุษย์ และ   เซลล์ต้นกำเนิดจากเนื้อเยื่อเอ็นยึด
ปริทันต์ของมนุษย์   ผลการศึกษาแสดงให้เห็นถึงความสามารถในการแปรสภาพของเซลล์ต้น
กำเนิดทั้งสองชนิดไปเป็นเซลล์สร้างอินซูลิน นอกจากนี้ยังพบว่าเซลล์ ต้นกำเนิดจากเนื้อเยื่อโพรง
ประสาทฟันมีความสามารถที่สูงกว่าในการแปรสภาพไปเป็นเซลล์สร้างอินซูลินทั้งในด้านจำนวน
ของโคโลนี การแสดงออกของยีนและโปรตีนจำเพาะ นอกจากนั้นพบว่าสามารถหลั่งซีเป็ปไทด์ใน
ปริมาณที่สัมพันธ์กับการกระตุ้นด้วยกลูโคส พบว่าภายหลังการเหนี่ยวนำ มีการแสดงออกของยีน
เฮส-1 และ เฮ   ย์-1 ซึ่งเป็นยีนเป้าหมายของสัญญาณนอทช์ การยับยั้งสัญญาณนอทช์ในแต่ละ
ขั้นตอนหรือตลอดการเหนี่ยวนำส่งผลให้เกิดการเปลี่ยนแปลงของลักษณะโคโลนี การแสดงออก
ของยีนและโปรตีนจำเพาะ รวมถึงคุณสมบัติในการทำงานของเซลล์สร้างอินซูลิน   ทั้งนี้ผล
การศึกษายืนยันถึงความสามารถในการแปรสภาพของเซลล์ต้นกำเนิดจากเนื้อเยื่อฟันไปเป็น
เซลล์สร้างอินซูลิน ซึ่งแสดงให้เห็นถึงความเป็นไปได้ในการนำเซลล์ต้นกำเนิดจากเนื้อเยื่อฟันมา
ใช้ในการรักษาโรคเบาหวาน   นอกจากนั้นพบว่าสัญญาณนอทช์อาจมีส่วนเกี่ยวข้องในการ
ควบคุมการแปรสภาพของเซลล์ต้นกำเนิดจากเนื้อเยื่อฟันไปเป็นเซลล์สร้างอินซูลิน

ABSTRACT

Diabetes mellitus is a complicated metabolic disorder resulting in hyperglycemia and long-term complications e.g. diabetic encephalopathy and neuropathy. Treatments of diabetes and its complications have faced many obstacles. Trend of stem cells (SCs)-based therapy has been proposed as a novel approach. Though, the study using dental SCs in this regard is yet lacking. In this study, human dental pulp SCs (hDPSCs) and human periodontal ligament SCs (hPDLSCs) were employed. The results illustrated the capability of differentiation toward islet-like cells (ILCs) cluster / insulin-producing cells (IPCs) by hDPSCs and hPDLSCs. In addition, higher capacity of differentiation toward ILCs cluster / IPCs by hDPSCs was apparently showed in respects of colony number, gene and protein marker expression. In addition, the hDPSCs-derived ILCs cluster / IPCs released C-PEPTIDE upon glucose stimulation in dose-dependent manner. After induction, Notch target genes, *HES-1* and *HEY-1*, were upregulated. Notch inhibition during each step or throughout the induction protocol resulted in variation of ILCs cluster / IPCs morphology, mRNA and protein marker expression, and functional property. Thus, the results suggested the capability of dental SCs in differentiation toward ILCs cluster / IPCs which might be implied to the possibility of dental SCs used in diabetes treatment. In addition, Notch signaling might participate in the regulation of dental SCs differentiation toward ILCs cluster / IPCs.

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LIST OF ABBREVIATIONS

α	=	alpha
β	=	beta
δ	=	delta
γ	=	gamma
°C	=	degree of Celsius, centigrade
μg	=	microgram
μL	=	microliter
μM	=	micromolar
ADA	=	American Diabetes Association
ADSCs	=	adipocyte stem cells
AEDs	=	antiepileptic drugs
ANOVA	=	Analysis of variances
AS cells	=	adult stem cells
BM-MSCs	=	bone marrow-derived mesenchymal stem cells
CO_2	=	carbon dioxide
DNA	=	deoxyribonucleic acid
ES cells	=	embryonic stem cells
GLP-1	=	glucagon-like peptide-1
gSDSCs	=	gnotobiotic porcine skin-derived stem cells
hDPSCs	=	human dental pulp stem cells
hPDLSCs	=	human periodontal ligament stem cells
ILCs cluster	=	islet-like cells cluster
IPCs	=	insulin-producing cells
iPS cells	=	induced-pluripotent stem cells
ng	=	nanogram
RNA	=	ribonucleic acid
SCs	=	stem cells

SHED	= stem cells from human exfoliated deciduous teeth
TCAs	= tricyclic antidepressants
TGF-β	= transforming-growth factor-β
UCB	= umbilical cord blood
WHO	= World Health Organization

IN VITRO DIFFERENTIATION OF MESENCHYMAL STEM CELLS FROM DENTAL AND ORAL TISSUES INTO ISLET-LIKE CELL CLUSTER.

การเปลี่ยนแปลงมีเซ็นไคมอลสเต็มเซลล์จากเนื้อเยื่อฟันและช่องปาก เป็นกลุ่มเซลล์ที่เหมือนไอเล็ทในห้องทดลอง

INTRODUCTION

World Health Organization (WHO) reports the global prevalence of diabetes that estimates the number of 171 million people suffering with diabetes in year 2000 and this number will increase to 366 million in year 2030 (Wild et al., 2004). Changing of human behavior and life style in conjugation with genetic susceptibility can cause diabetes, and also the conditions called "diabesity" and "metabolic syndrome" [Zimmet et al., 2001]. American Diabetes Association (ADA) announces the latest "Standard of medical care in diabetes" and "Diagnosis and classification of diabetes mellitus" in January 2012. These articles encourage all stakeholders to fight and find the ways to solve diabetic problems. Diabetes is not only disease, but it is public concerns due to long-term complications and problems [American Diabetes, 2012a].

ADA classifies diabetes into 4 main categories including type I, type II, specific types and gestational diabetes mellitus [American Diabetes, 2012a]. All types of diabetes show significant signs of hyperglycemia and related symptoms according to insulin secretion and/or action defects. Many of serious complications e.g. diabetic encephalopathy and neuropathy, nephropathy, retinopathy, and cardiovasculopathy have been reported in long term and uncontrolled hyperglycemic condition. Among those complications, diabetic encephalopathy and neuropathy seem the important one due to their high prevalence and relation with diabetes-related brain and nerve

damages in diabetic population [Aszalos, 2007; Callaghan et al., 2012b; Northam et al., 2009; Wrighten et al., 2009].

The standard treatments to control blood glucose level, exogenous insulin and oral hypoglycemic drugs administration, are now being used in diabetic patients. Additionally, several types of drugs e.g. tricyclic antidepressants (TCAs), serotonin-norepinephrine reuptake inhibitors, selective serotonin reuptake inhibitors, antiepileptic drugs (AEDs) and analgesics have been proposed to be the choices to manage pain and symptoms related to diabetic nerve damages [American Diabetes, 2012a; Callaghan et al., 2012a; Callaghan et al., 2012c]. However, these treatments have many serious side effects and limitations e.g. limited therapeutic effects, psychological adverse events, lactic acidosis, hepatitis, hypoglycemia, coma and death [American Diabetes, 2012b]. Furthermore, therapeutic regimen regarding diabetic encephalopathy treatment is still under investigation [Sima, 2010].

As a result, many researchers have been trying to find novel and effective methods for diabetes and neurological complications treatment, so trend of regenerative medicine has been proposed for this reason. For blood glucose level control, islet transplantation from pass away donor to type I diabetic patients regarding Edmonton Protocol has been done and faced with many obstacles due to donor shortage and adverse reactions of immunosuppressive drugs [Shapiro and Lakey, 2000; Shapiro et al., 2000]. To overcome these problems, preliminary studies of autologous and allogenic transplantations of stem cell (SC)-derived islet-like cells (ILCs) cluster / insulin producing cells (IPCs) in induced-diabetes animal models have been tried and reported [Lin et al., 2009; Serup et al., 2001]. However, using of embryonic stem (ES) cells in research and clinical practice has many problems regarding ethical and legal concerns [Roche et al., 2005]. Moreover, ES cells and induced-pluripotent stem (iPS) cells applications are still under investigation regarding their efficacy and safety. From these reasons, adult stem (AS) cells have been studied and used widely for research [Alvarez et al., 2012].

Many sources of AS cells such as hepatic SCs, umbilical cord blood (UCB), bone marrow-derived mesenchymal SCs (BM-MSCs), adipocyte SCs (ADSCs) and multipotent dermal fibroblasts etc. have been reported as ILCs cluster / IPCs inducible cells [Chandra et al., 2009; Koblas et al., 2009; Tang et al., 2004; Tang et al., 2006; Yang, 2006]. Whereas the differentiation of various AS cells into ILCs cluster / IPCs is well established, the studies of human dental tissue-derived SCs using in diabetes treatment in respects of blood glucose control is still ongoing.

Human dental-derived SCs are multipotent cells isolated from human tooth and dental tissues. These cells have been introduced to be a candidate cell source for SC therapy due to its interesting properties; multipotentiality, accessibility and availability. Many types of dental-derived SCs have been isolated and characterized e.g. human dental pulp SCs (hDPSCs), human periodontal ligament SCs (hPDLSCs), and SCs from human exfoliated deciduous teeth (SHED) [Egusa et al., 2012; Seo et al., 2004]. Recently, *in vitro* induction of ILCs cluster / IPCs from mesenchymal SCs derived from pulp tissue of human deciduous teeth has been reported [Govindasamy et al., 2011]. However, there is some lacking information regarding the differentiating potentials into ILCs cluster / IPCs of various human dental SC types.

From these reasons, the research aim is pointed into the exploration of the *in vitro* differentiation potential into ILCs cluster / IPCs of well-characterized human dental SCs. Moreover, the signaling pathways regarding the differentiation e.g. Notch signaling will be explored. In this study, hDPSCs and hPDLSCs are used as model of exploration. The results will suggest the possibility and capability of dental SCs applications in stem cell-based therapy for diabetes.

Objectives of Study

In regard to the unmet knowledge described above, this study was directed to further elucidate the potential application and intracellular mechanism(s) of important dental tissue-derived stem cells (hDPSCs and hPDLSCs) differentiation toward islet-like cells (ILCs) cluster / insulin-producing cells (IPCs) *in vitro*.

Specific Aim 1) "*In vitro* differentiation of human dental tissue-derived stem cells into islet-like cells (ILCs) cluster / insulin-producing cells (IPCs)".

Strategies:

1a) To explore islet-like cells (ILCs) cluster / insulin-producing cells (IPCs) *in vitro* differentiation by human dental-derived stem cells; hDPSCs and hPDLSCs.

1b) To determine the intracellular mechanism(s) regulating islet-like cells (ILCs) cluster / insulin-producing cells (IPCs) *in vitro* differentiation, focusing on the Notch signaling.

Keywords (Thai):

โรคเบาหวาน , เซลล์ต้นกำเนิดจากเนื้อเยื่อโพรงประสาทฟันของมนุษย์ , เซลล์ต้นกำเนิดจากเนื้อเยื่อเอ็นยึดปริทันต์ของมนุษย์, กลุ่มเซลล์ที่เหมือนไอเล็ท, เซลล์สร้างอินซูลิน

Keywords (English):

diabetes mellitus, human dental pulp stem cells, human periodontal ligament stem cells, islet-like cells cluster, insulin-producing cells

Hypothesis

Human dental tissue-derived stem cells (hDPSCs and hPDLSCs) can be differentiated toward islet-like cells (ILCs) cluster / insulin-producing cells (IPCs) *in vitro* and contain different differentiation potential depending on its multipotentiality and involving signaling pathways.

MATERIALS AND METHODS

<u>Specific Aim 1)</u> "*In vitro* <u>differentiation of human dental tissue-derived stem cells into islet-like cells (ILCs) cluster / insulin-producing cells (IPCs)</u>".

<u>Strategies:</u>

<u>1a) To explore and compare islet-like cells (ILCs) cluster / insulin-producing cells (IPCs)</u> *in vitro* <u>differentiation potential by two human dental stem cell types; hDPSCs and hPDLSCs.</u>

hDPSCs and hPDLSCs were isolated by tissue explant technique. The isolated cells in passage 2-5 were used for characterization and ILCs cluster / IPCs differentiation. By 3-stage induction protocol modified from Chandra et al., 2009 and Govindasamy et al., 2011, the cells were gradually changed into definitive endoderm, pancreatic endoderm, and pancreatic islets (ILCs cluster / IPCs) at day 3, 5, and 10, respectively.

Set of protocol was used to identify and compare the potential of cell differentiation. At day 3, 5, and 10, cell colonies were randomly captured to evaluate size and number of obtained colonies (ten pictures for each condition). The mRNA was then collected to verify the expression of differentiation gene markers (pancreatic endoderm; *PDX-1* and *NGN-3*, and pancreatic islet; *NKX-6.1*, *GLUT2* and *INSULIN*). At day 10, immunocytochemistry (ICC) staining of specific pancreatic protein marker (PRO-INSULIN) was performed to confirm the pancreatic islet differentiation. ELISA detection of glucose-stimulated c-peptide secretion was done to define the maturation and function of differentiated pancreatic cells. The differentiation potential of two cell types (hDPSCs and hPDLSCs) was further compared.

<u>1b) To determine the intracellular mechanism(s) regulate islet-like cells (ILCs) cluster / insulin-producing cells (IPCs)</u> *in vitro* <u>differentiation by dental stem cells, focusing on the Notch signaling.</u>

Dental stem cells were induced into ILCs cluster / IPCs by 3-stage protocol mentioned above. To investigate the relevance of Notch signaling on cell

differentiation, γ-secretase inhibitor (DAPT; 25 µM) was added in induction media throughout or in each step of induction protocol.

At day 3, 5, and 10, cell colonies were randomly captured to evaluate size and number of obtained colonies (ten pictures for each condition).The mRNA was collected to verify the differentiation stages and Notch signaling components i.e. Notch receptors (*NOTCH-1*, *NOTCH-2*, *NOTCH3* and *NOTCH-4*), Notch ligands (*DLL-1*, *DLL-3*, *DLL-4*, *JAGGED-1* and *JAGGED-2*) and Notch target genes (*HES-1* and *HEY-1*).

At day 10, PRO-INSULIN immunocytochemistry staining and ELISA assay of glucose-stimulated c-peptide secretion were performed. The influence of Notch inhibition on IPC differentiation was examined and compared in each induction step. Normal pancreatic induction was used as the control.

Statistical analyses

The results were shown as mean ± standard deviation (SD) and analyzed using one-sample *t* test for determining difference within one group or 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.

Detailed materials and methods
Specific Aim 1) "*In vitro* differentiation of human dental tissue-derived stem cells into islet-like cells (ILCs) cluster / insulin-producing cells (IPCs)".

Isolation, culture and expansion of human dental stem cells

hDPSCs and hPDLSCs were isolated from human dental pulp and periodontal ligament tissues of adult subjects undergoing routine tooth extraction for orthodontic and tooth impaction treatment. The protocol was approved by Human Research Ethic Committee, Faculty of Dentistry, Chulalongkorn University. Tissue explant was used as method of cell isolation. Tissues were minced and seeded to 35 mm culture plate (Corning).

DMEM, Dulbecco's Modified Eagle Medium (Gibco), supplemented with 100 unit/mL penicillin (Gibco), 100 ug/mL streptomycin (Gibco), 5 ug/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, humidified atmosphere with 5% CO_2 aeration. Cultured cells were trypsinized (0.25% trypsin-EDTA; Gibco) and subcultured at 90% confluence. The cells in passage 2-5 were used in the study.

Cell morphology, surface markers (CD 44, CD 73, CD 90, CD 105, and STRO-1), stem cell marker mRNA expression (*REX-1*, *NANOG*, and *OCT-4*), and their multipotency (differentiation toward 2 different lineages; osteogenicity and adipogenicity) were used in stem cell characterization.

In vitro differentiation of hDPSCs and hPDLSCs into islet-like cells (ILCs) cluster / insulin-producing cells (IPCs) and exploration of Notch signaling intervention

hDPSCs and hPDLSCs were differentiated into ILCs cluster / IPCs using 3-stage differentiation protocol modified from Chandra et al., 2009 and Govindasamy et al., 2011. For the first step, at day 0, single cell suspension (10^6 cells) of hDPSCs or hPDLSCs was seeded in 60 mm Petri dish (Falcon) with serum-free medium (SFM) - A in order to differentiate the cells into definitive endoderm. In the second step, induction medium was then changed to SFM-B at day 3 for driving toward pancreatic endoderm. Finally, the third step, SFM-C (ILCs cluster / IPCs induction medium) was used at day 5 until day 10 with every 48 hours substitution.

SFM-A was SFM-DMEM (Gibco) supplemented with 1% bovine serum albumin (BSA) (Cohn fraction V, fatty acid free) (Sigma), 1x insulin-transferrin-selenium (ITS) (Invitrogen, USA), 4 nM activin A (Sigma), 1 nM sodium butyrate (Sigma) and 50 μM β-mercaptoethanol (Sigma).

SFM-B contained 1% BSA, 1x ITS and 0.3 mM taurine (Sigma) in SFM-DMEM.

SFM-C compositions were 1.5% BSA, 1x ITS, 3 mM taurine, 100 nM glucagon-like peptide (GLP) – 1 (Sigma), 1 mM nicotinamide (Sigma) and 1x non-essential amino acids (NEAAs) (Sigma) in SFM-DMEM.

To investigate the relevance of Notch signaling in differentiation toward ILCs cluster / IPCs, γ-secretase inhibitor (DAPT; Sigma) was added in induction medium at concentration of 25 μM in order to inhibit the Notch signaling cascade throughout or in each step of induction protocol.

Reverse transcription-polymerase chain reaction (RT-PCR) for ILCs cluster / IPCs

Detection of mRNA expression was performed to elucidate the stage of differentiation. Set of pancreatic marker was analyzed including pancreatic endoderm markers (*PDX-1* and *NGN-3*) and pancreatic islet markers (*NKX-6.1*, *GLUT-2* and *INSULIN*). In addition, Notch signaling components were also examined i.e. Notch receptors (*NOTCH-1*, *NOTCH-2*, *NOTCH3* and *NOTCH-4*), Notch ligands (*DLL-1*, *DLL-3*, *DLL-4*, *JAGGED-1* and *JAGGED-2*) and Notch target genes (*HES-1* and *HEY-1*).

Total cellular RNA was isolated with TRIzol® RNA isolation reagent (Invitrogen) and 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). 18S ribosomal RNA was used as a reference gene. The mRNA expression value was illustrated as relative mRNA expression by normalized to the control. The primer sequences were shown in Appendix 1.

Immunocytochemistry (ICC) staining for ILCs cluster / IPCs

Differentiated pancreatic islet colonies derived from hDPSCs and hPDLSCs were then confirmed by ICC staining of specific pancreatic protein markers (PRO-INSULIN). Samples were fixed in cold methanol for 15 mins, permeabilized with 0.1% Triton®-X100 (Sigma) in PBS, and incubated with 10% horse serum in PBS for 1 hour. The samples were incubated with primary antibody, mouse anti-human PRO-INSULIN (Millipore), at 1:100 dilution for 24 hours. 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 ug/mL) was used for

nuclei counterstaining. All of procedures were performed at 4 °C and washed with PBS in every step. The staining was examined using fluorescent microscope incorporated with Carl Zeiss™ Apotome.2 apparatus (Carl Zeiss).
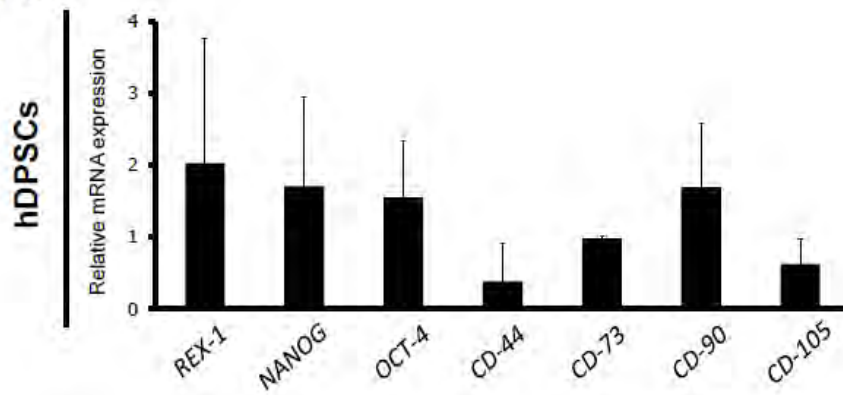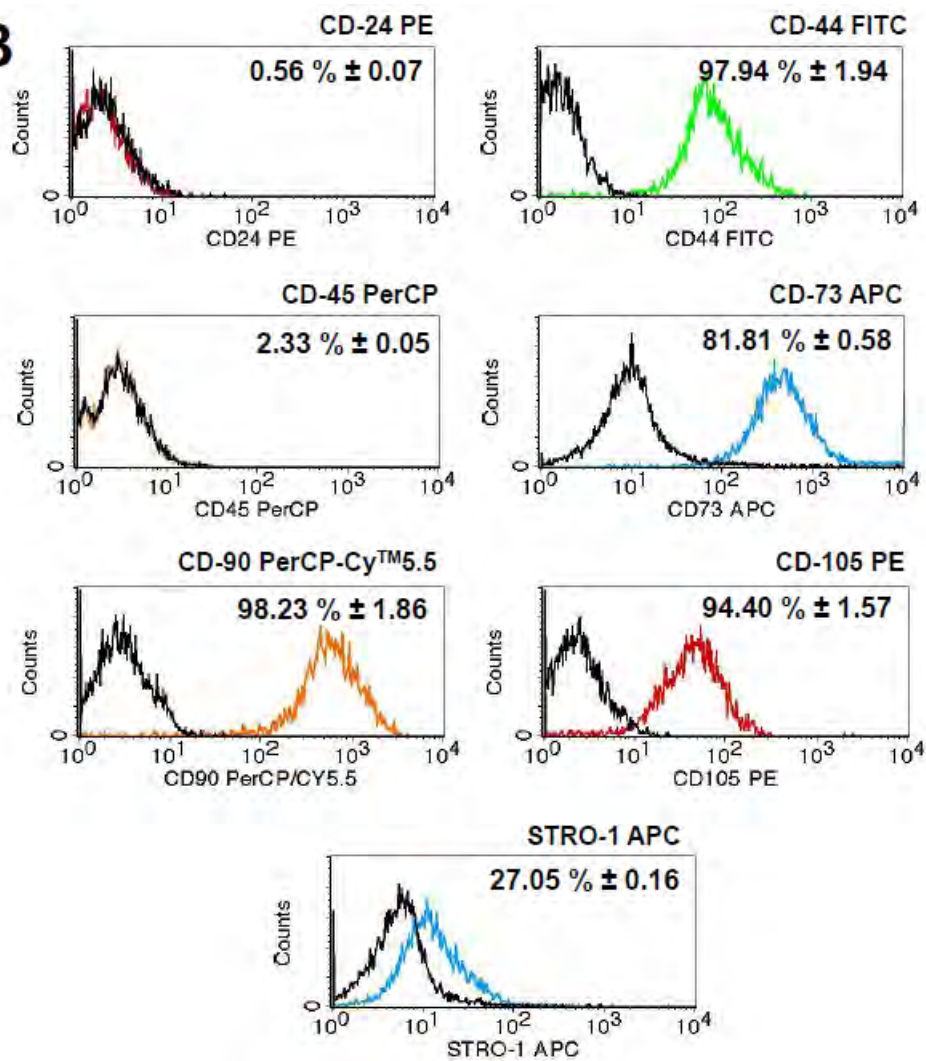
Functional tests for ILCs cluster / IPCs

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).

RESULTS

hDPSCs AND hPDLSCs CHARACTERIZATION

The isolated cells were characterized for the stem cell characteristics using qRT-PCR, flow cytometry, and immunocytochemistry staining (Fig. 1A-C and 2A-C). The expression of transcription factors identifying stemness properties including *REX-1*, *NANOG*, and *OCT-4* were illustrated. Moreover, surface markers of mesenchymal stem cells (CD44, CD73, CD90, CD105, and STRO-1) were also expressed, while hematopoietic surface markers (CD24 and CD45) were not detected. In order to clarify the multipotential differentiation properties of established hDPSCs and hPDLSCs, cellular differentiation toward osteogenic and adipogenic lineages was employed. By culturing cells in osteogenic induction medium, hDPSCs and hPDLSCs were able to differentiate into osteogenic lineage, confirming by the dramatic increase of mineral deposition and the upregulation of osteogenic marker gene expression (*OSTEOCALCIN* and *COLLAGEN I*) at day 14 (Fig. 3A and 4A). Moreover, hDPSCs and hPDLSCs were able to differentiate into adipogenic lineage upon maintained in adipogenic induction medium. The intracellular lipid droplets were noted at day 16, corresponding with the increase of adipogenic marker mRNA expression (*PEROXISOME PROLIFERATOR–ACTIVATED RECEPTOR-GAMMA* (*PPAR-γ*) and *LIPOPROTEIN LIPASE* (*LPL*)) (Fig. 3B and 4B).
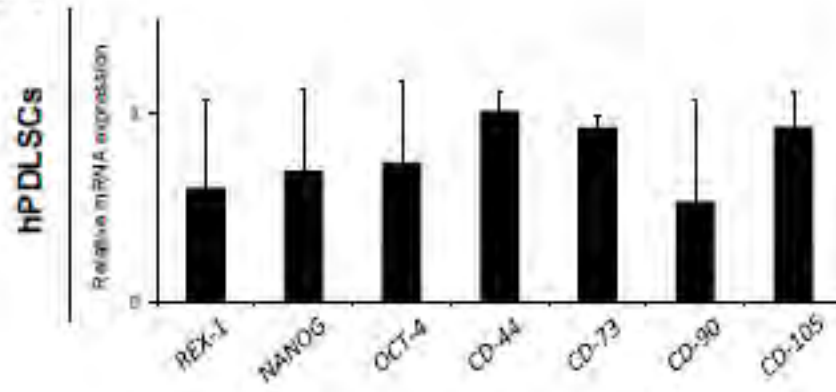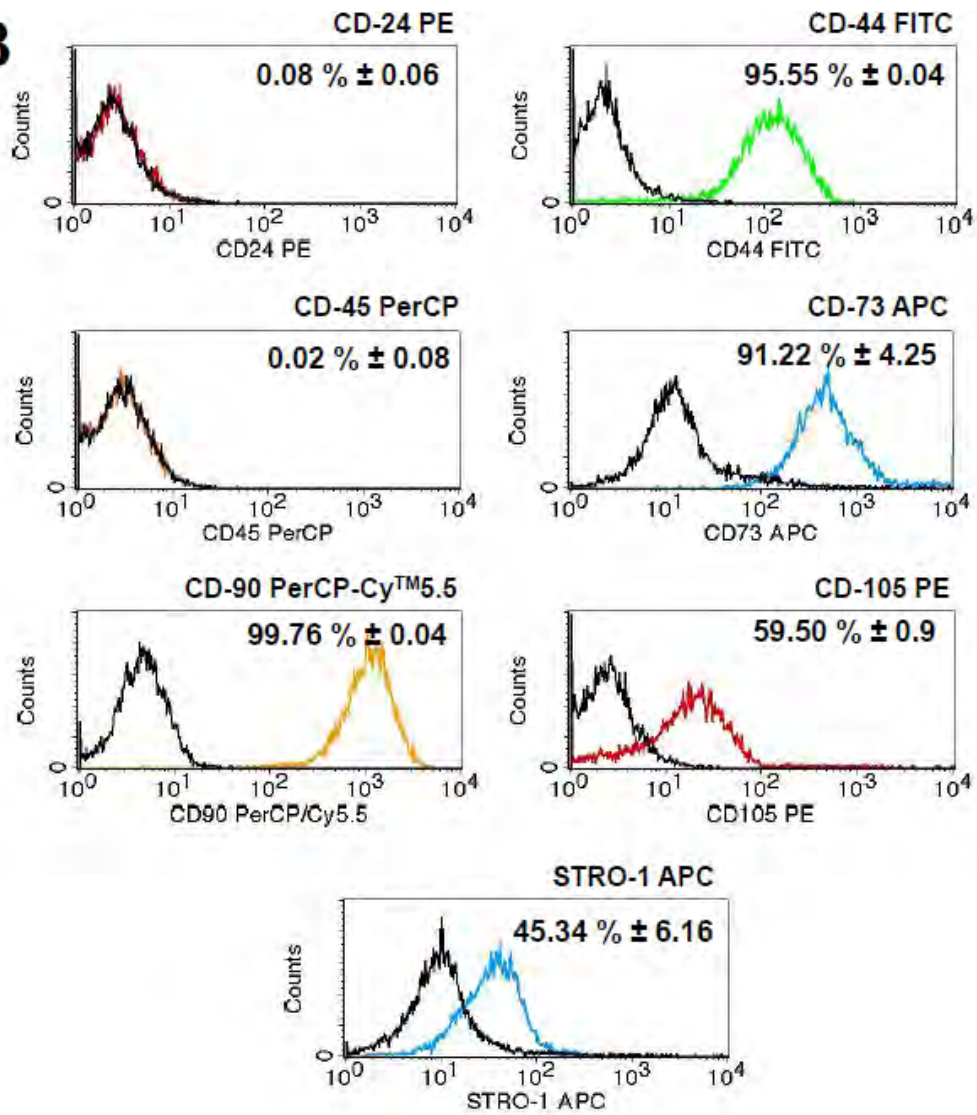
A

hDPSCs

Relative mRNA expression

Bar chart with y-axis "Relative mRNA expression" (0 to 4) and x-axis categories: REX-1, NANOG, OCT-4, CD-44, CD-73, CD-90, CD-105

B

CD-24 PE
0.56 % ± 0.07
Counts
CD24 PE

CD-44 FITC
97.94 % ± 1.94
Counts
CD44 FITC

CD-45 PerCP
2.33 % ± 0.05
Counts
CD45 PerCP

CD-73 APC
81.81 % ± 0.58
Counts
CD73 APC

CD-90 PerCP-Cy™5.5
98.23 % ± 1.86
Counts
CD90 PerCP/CY5.5

CD-105 PE
94.40 % ± 1.57
Counts
CD105 PE

STRO-1 APC
27.05 % ± 0.16
Counts
STRO-1 APC

C

bar = 50 µm

Figure 1 hDPSCs characterization.

Transcription factors identifying stemness properties (i.e. REX-1, NANOG, and OCT-4) and surface markers (i.e. CD24, CD44, CD45, CD73, CD90, CD105, and STRO-1) were analyzed using qRT-PCR (A), flow cytometry (B), and immunocytochemistry staining (C), respectively.

A

hPDLSCs

Relative mRNA expression

REX-1 NANOG OCT-4 CD-44 CD-73 CD-90 CD-105

B

CD-24 PE

0.08 % ± 0.06

Counts

10^0 10^1 10^2 10^3 10^4

CD24 PE

CD-44 FITC

95.55 % ± 0.04

Counts

10^0 10^1 10^2 10^3 10^4

CD44 FITC

CD-45 PerCP

0.02 % ± 0.08

Counts

10^0 10^1 10^2 10^3 10^4

CD45 PerCP

CD-73 APC

91.22 % ± 4.25

Counts

10^0 10^1 10^2 10^3 10^4

CD73 APC

CD-90 PerCP-Cy™5.5

99.76 % ± 0.04

Counts

10^0 10^1 10^2 10^3 10^4

CD90 PerCP/Cy5.5

CD-105 PE

59.50 % ± 0.9

Counts

10^0 10^1 10^2 10^3 10^4

CD105 PE

STRO-1 APC

45.34 % ± 6.16

Counts

10^0 10^1 10^2 10^3 10^4

STRO-1 APC

C

REX-1-FITC | NANOG-FITC | OCT-4-FITC | CD-44-FITC

CD-73-FITC | CD-90-FITC | CD-105-PE | STRO-1-FITC

bar = 50 μm
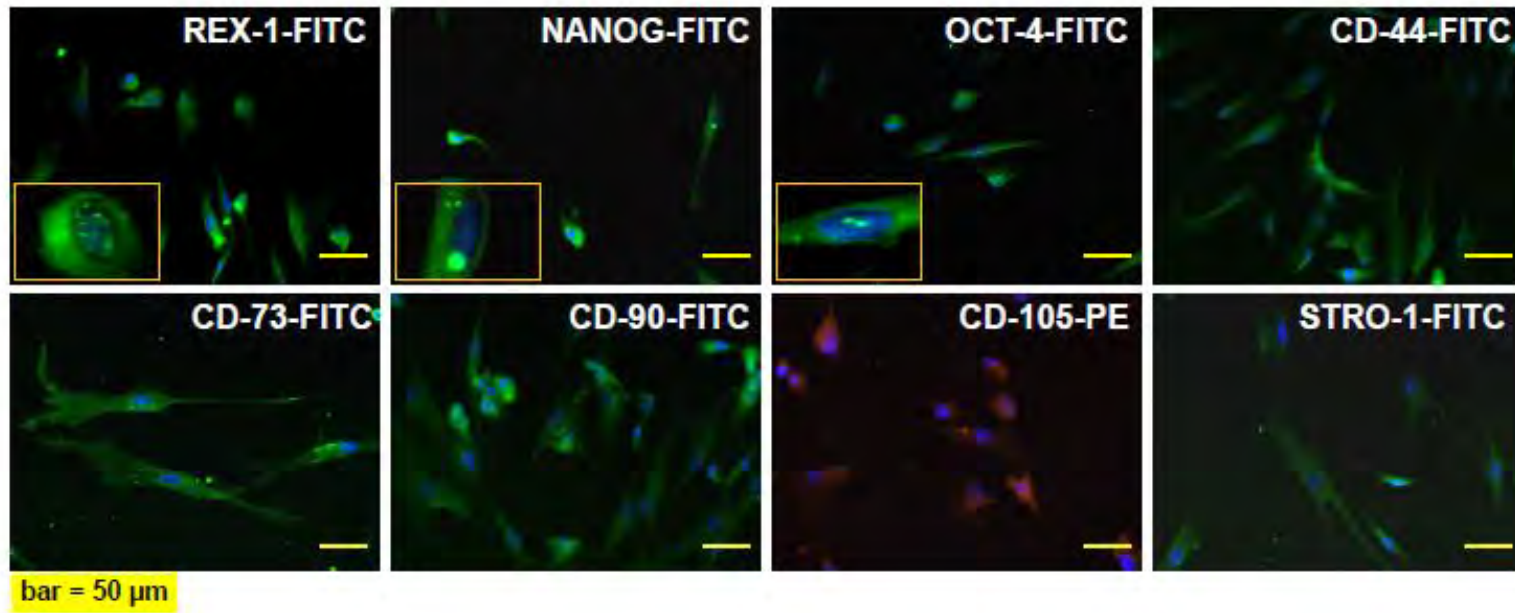
Figure 2 hPDLSCs characterization.

Transcription factors identifying stemness properties (i.e. REX-1, NANOG, and OCT-4) and surface markers (i.e. CD24, CD44, CD45, CD73, CD90, CD105, and STRO-1) were analyzed using qRT-PCR (A), flow cytometry (B), and immunocytochemistry staining (C), respectively.

A

d14 Alizarin Red

| Control | Osteogenic |

hDPSCs / d 14
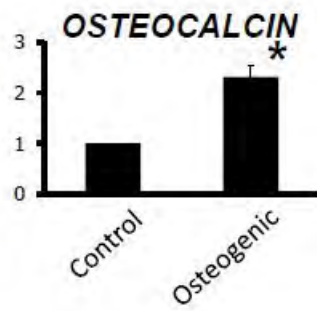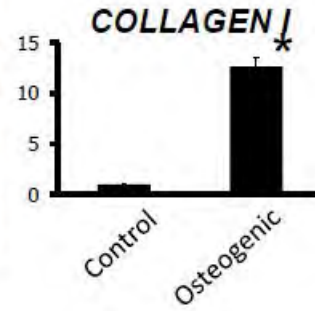
OSTEOCALCIN

Relative mRNA expression

*

Control   Osteogenic

COLLAGEN I

Relative mRNA expression

*

Control   Osteogenic

B

d16 Oil Red O

| Control | Adipogenic |

bar = 50 μm

hDPSCs / d 16

PPAR-γ

Relative mRNA expression

Control   Adipogenic

LPL

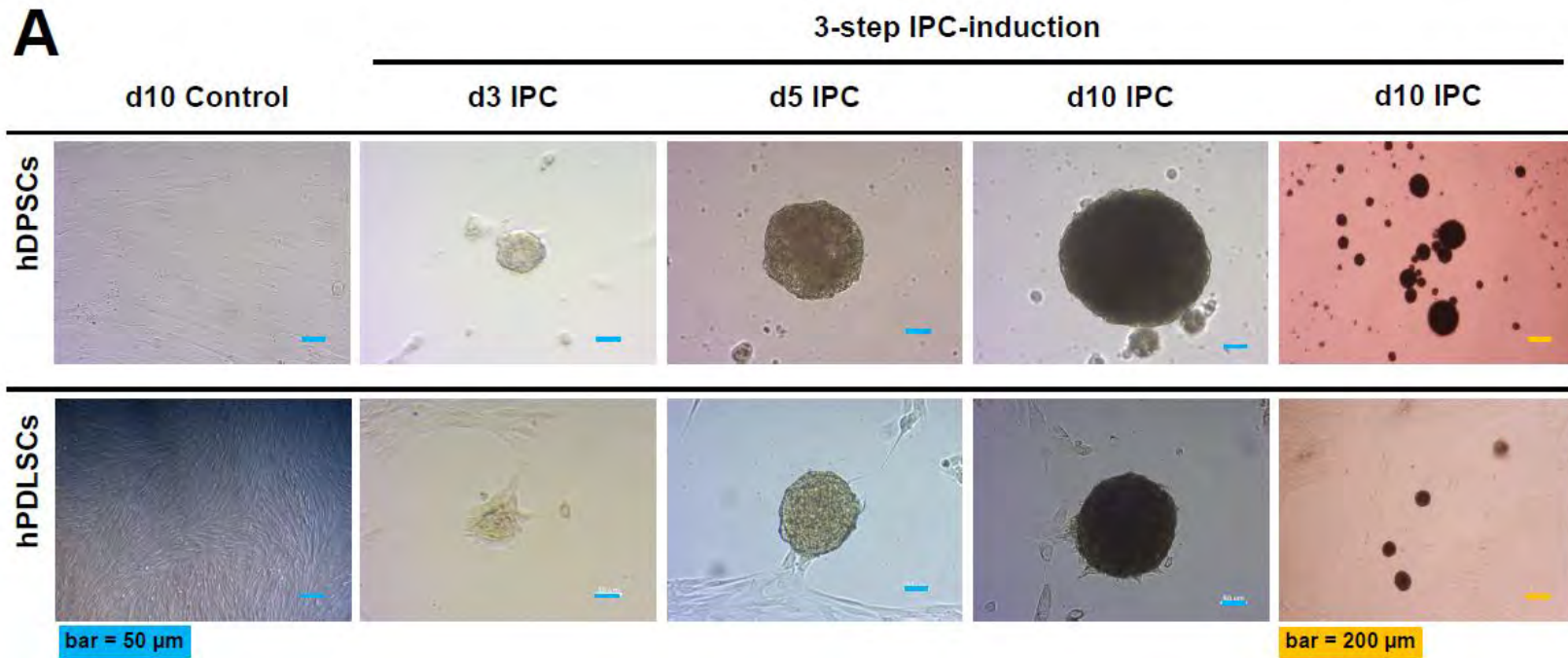Relative mRNA expression

*

Control   Adipogenic
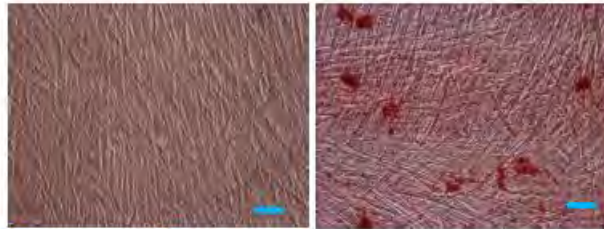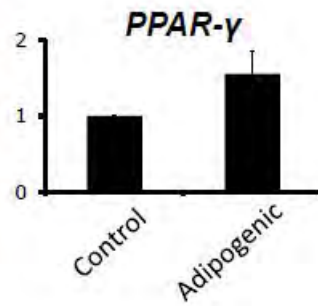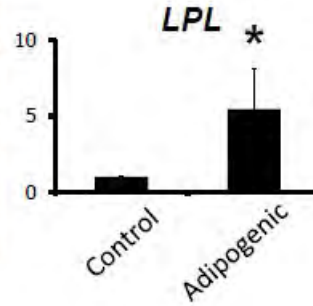
Figure 3 Multipotential differentiation capability of hDPSCs.

For osteogenic differentiation, the increase of mineral deposition and osteogenic marker gene expression were illustrated (A). Regarding adipogenic induction (B), the intracellular lipid accumulation and adipogenic marker gene expression were demonstrated using oil red O staining and qRT-PCR, respectively. Asterisks indicated the statistical significance compared to the control.

A

d14 Alizarin Red

Control   Osteogenic

hPDLSCs / d 14
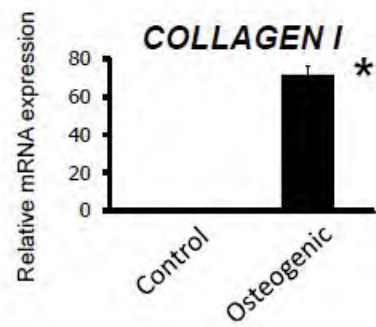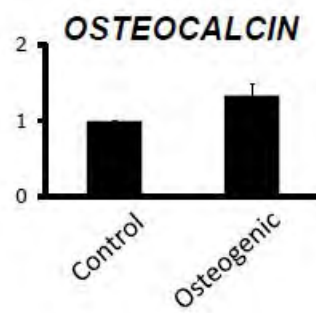
OSTEOCALCIN

Relative mRNA expression

Control   Osteogenic

COLLAGEN I *

Relative mRNA expression

Control   Osteogenic

B

d16 Oil Red O

Control   Adipogenic

bar = 50 μm

hPDLSCs / d 16

PPAR-γ

Relative mRNA expression

Control   Adipogenic

LPL *

Relative mRNA expression

Control   Adipogenic
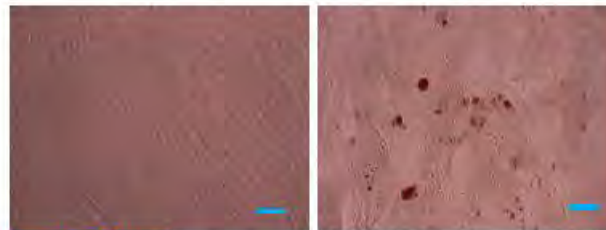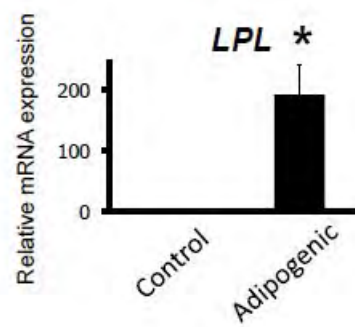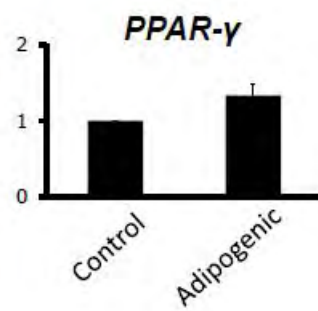
Figure 4 Multipotential differentiation capability of hPDLSCs.

For osteogenic differentiation, the increase of mineral deposition and osteogenic marker gene expression were illustrated (A). Regarding adipogenic induction (B), the intracellular lipid accumulation and adipogenic marker gene expression were demonstrated using oil red O staining and qRT-PCR, respectively. Asterisks indicated the statistical significance compared to the control.

DIFFERENTIATION TOWARD INSULIN-PRODUCING CELLS (IPCs) BY DENTAL TISSUE-DERIVED STEM CELLS

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. 5A). On the contrary, monolayer was noted in the control condition. Interestingly, the total colony count of hDPSCs-derived cell aggregation was significantly higher than that of hPDLSCs (Fig. 5B). The size of colonies was further evaluated. In hPDLSCs, the percentage of small colonies (diameter less than 50 µm) was slightly higher than those of hDPSCs. Though, no statistical significance was noted (Fig. 5C).

At day 10 of IPCs / ILC cluster 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 that showed an unchanged trend of expression. 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. 6A and B). Additionally, the intracellular PRO-INSULIN protein expression was noted of both hDPSCs- and hPDLSCs-derived IPCs (Fig. 6C). Thus, these results illustrated that both cell types were able to differentiate toward IPCs and hDPSCs had markedly higher differentiation efficiency compared to hPDLSCs as determined by total colony number and marker gene expression.

A

3-step IPC-induction

	d10 Control	d3 IPC	d5 IPC	d10 IPC	d10 IPC

hDPSCs

hPDLSCs

bar = 50 μm

bar = 200 μm

B d10 / 3-step IPC-induction

C d10 / 3-step IPC-induction

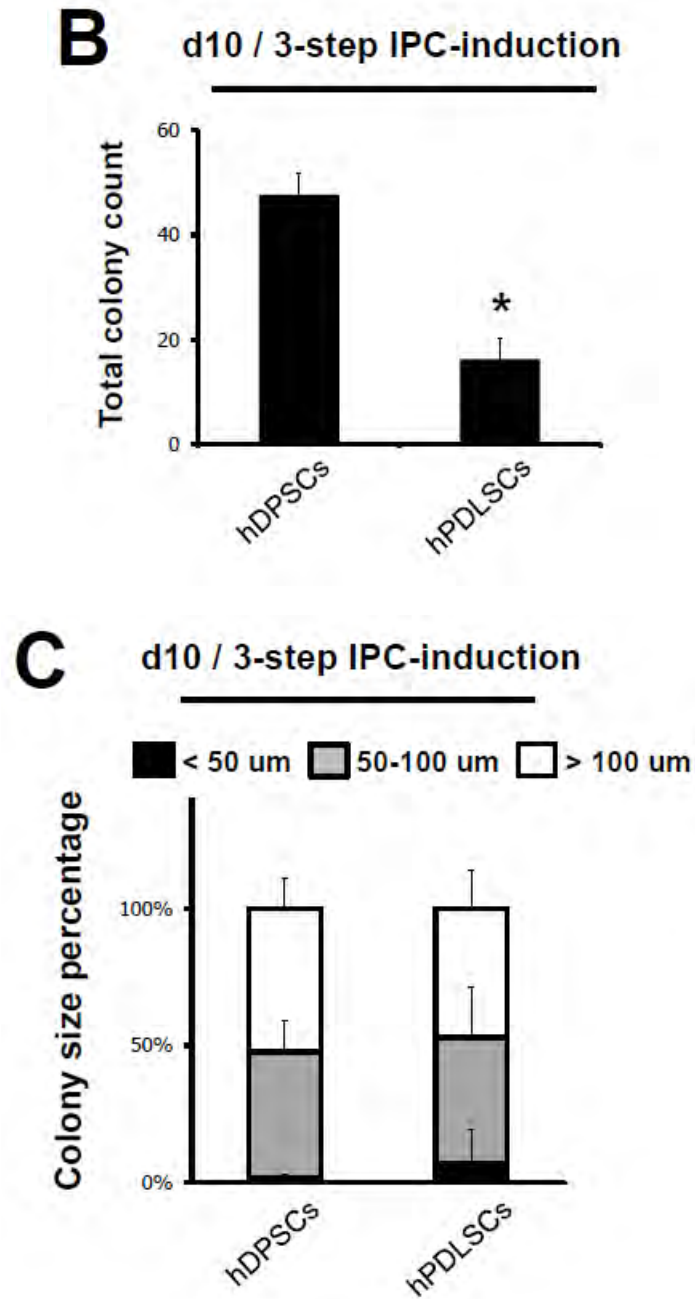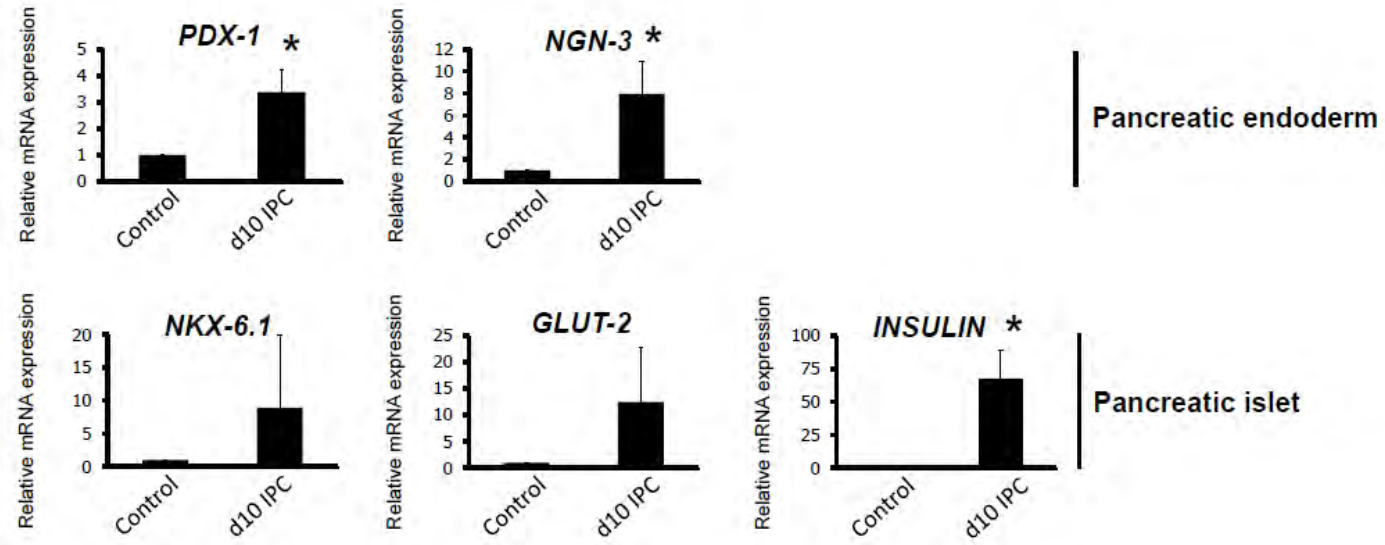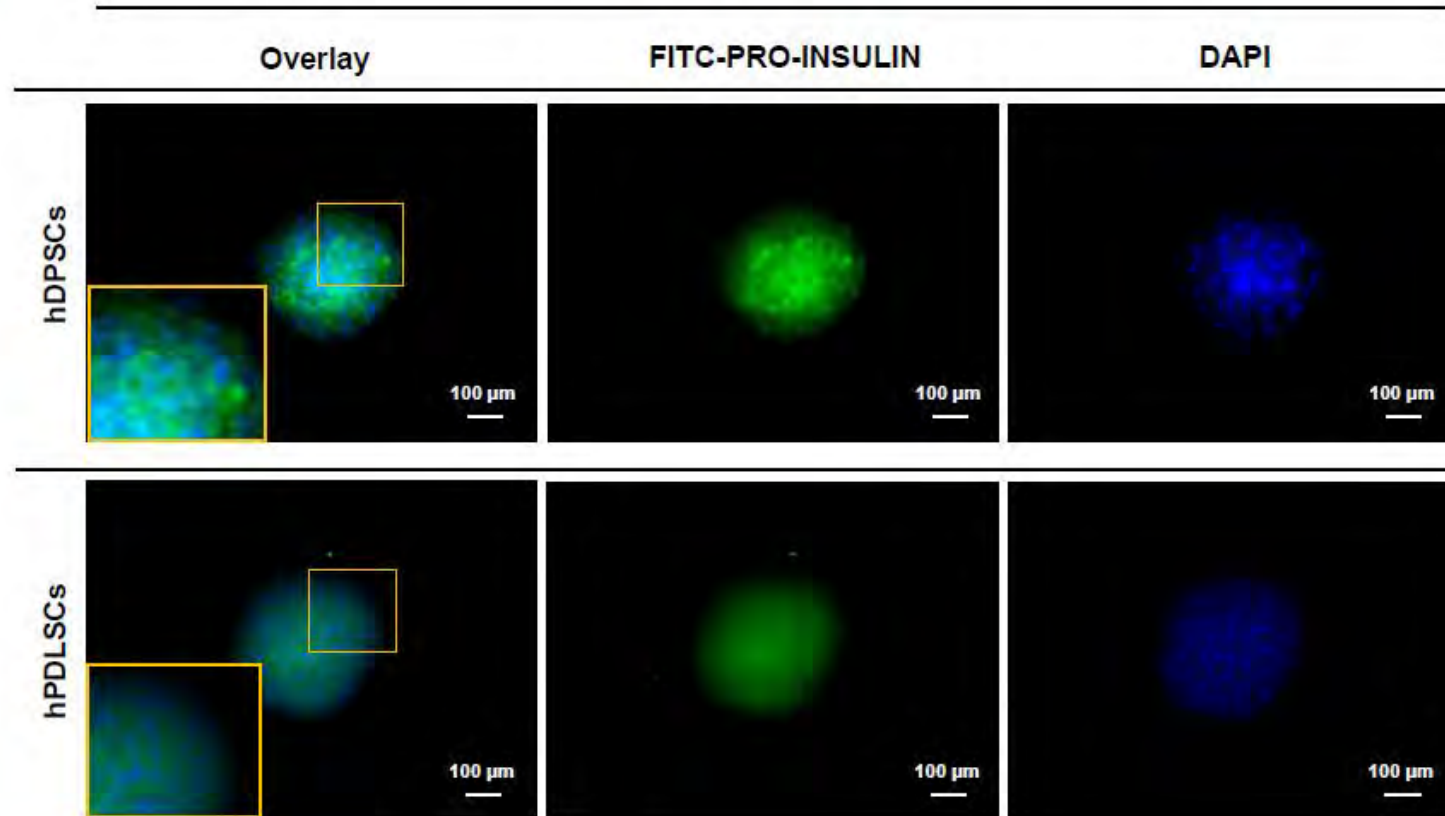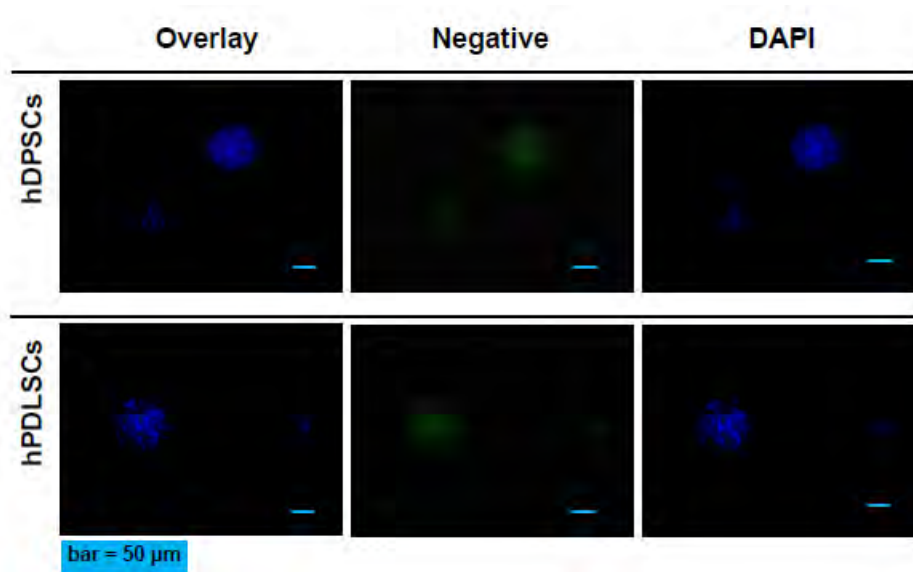■ < 50 um ▨ 50-100 um ☐ > 100 um

Figure 5 Morphological appearances of insulin-producing cells (IPCs) derived from two types of dental tissue-derived mesenchymal stem cells.

Regarding the IPCs induction, morphologies of aggregated colonies were observed at day 3, 5, and 10 (A). Total colony count number (B) and colony size percentage (C) was also investigated. Asterisks indicated the statistical significance compared to the hDPSCs group.

A

hDPSCs / d10 / 3-step IPC-induction

PDX-1 *

Relative mRNA expression

NGN-3 *

Relative mRNA expression

Pancreatic endoderm

NKX-6.1

Relative mRNA expression

GLUT-2

Relative mRNA expression

INSULIN *

Relative mRNA expression

Pancreatic islet

Control   d10 IPC

B

hPDLSCs / d10 / 3-step IPC-induction

PDX-1

Relative mRNA expression

6
4
2
0

Control   d10 IPC

NGN-3

Relative mRNA expression

12
10
8
6
4
2
0

Control   d10 IPC

Pancreatic endoderm

NKX-6.1

Relative mRNA expression

2
1
0

*

Control   d10 IPC

GLUT-2

Relative mRNA expression

100
75
50
25
0

Control   d10 IPC

INSULIN

Relative mRNA expression

100
75
50
25
0

Control   d10 IPC

Pancreatic islet

C

d10 / 3-step IPC-induction

| Overlay | FITC-PRO-INSULIN | DAPI |

Figure 6 Pancreatic markers expression by insulin-producing cells (IPCs) derived from two types of dental tissue-derived mesenchymal stem cells.

Expression of crucial pancreatic markers by hDPSCs (A) and hPDLSCs (B) between undifferentiated control and day 10-IPCs was analyzed by qRT-PCR. PRO-INSULIN protein expression was evaluated by immunocytochemistry (ICC) staining (C). Asterisks indicated the statistical significance compared to the undifferentiated control.

hDPSCs-DERIVED IPCs SECRETED C-PEPTIDE

As described above, hDPSCs had higher IPCs differentiation ability. Therefore, 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. 7A).
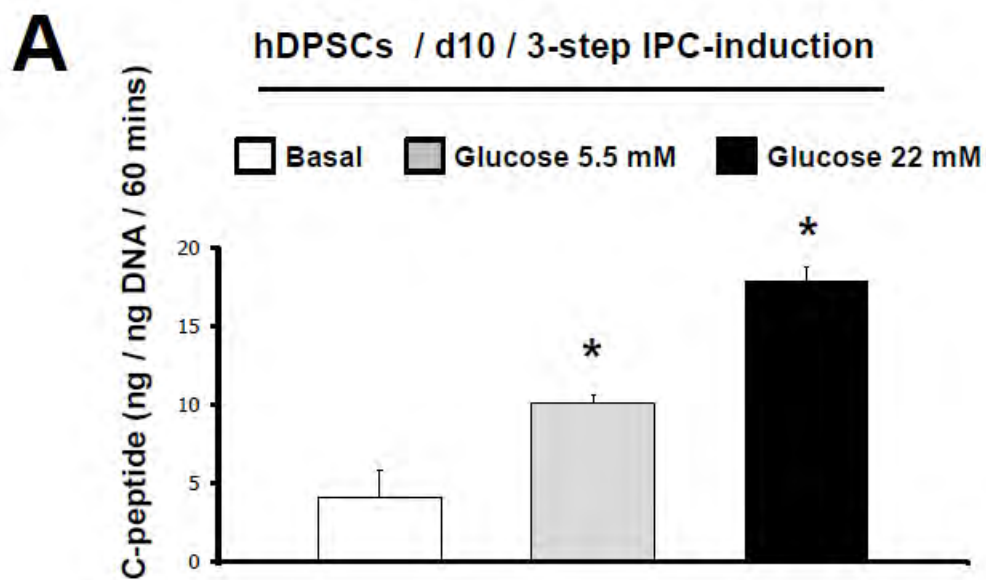
Figure 7 Functional test of hDPSCs-derived IPCs regarding different dose of glucose challenge.

Day 10-hDPSCs-derived IPCs were collected and tested with two different glucose concentration (5 mM and 22 mM). Amount of secreted C-peptide was showed as ng / ng DNA / 60 minutes (A). Asterisks indicated the statistical significance compared to the basal level.

NOTCH SIGNALING PARTICIPATED IN DIFFERENTIATION TOWARD IPCs

Upon differentiation, the significant upregulation of Notch target genes, *HES-1* and *HEY-1* was observed (Fig. 8A), implying the involvement of Notch signaling during IPCs differentiation. 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). Regarding colony morphology, cell aggregation in DAPT-B and DAPT-All were relatively smaller than normal control IPC group (IPC-Normal) at day 5 and 10 (Fig. 9A). The total colony count of IPC-DAPT-B was significantly increased at day 10 (Fig. 9B). Correspondingly, the higher percentage of small colonies was noted in IPC-DAPT-B group compared to the control. Though, there is no significant difference (Fig. 9C).

For pancreatic endoderm gene marker expression, 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. In aspect of pancreatic islet mRNA expression, DAPT-A and DAPT-All exhibited trend of *NKX-6.1* upregulation. However, due to high variation among individual subjects, statistical significance was not found in any examined markers (Fig. 10A). mRNA expression of Notch components and target genes were also investigated. The result suggested trend of *HES-1* dowregulation as well as variation in mRNA expression of Notch ligands and receptors. Though, there was no evidence of significant difference among those mRNA expression level (Fig. 10B). The PRO-INSULIN protein expression was noted in all groups (Fig. 11A). The function was determined by evaluating C-PEPTIDE secretion (Fig. 11B). The results revealed a trend of dose-dependent response to glucose stimulation in the control, the DAPT-A, and 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. However, no statistical significant differences were observed.
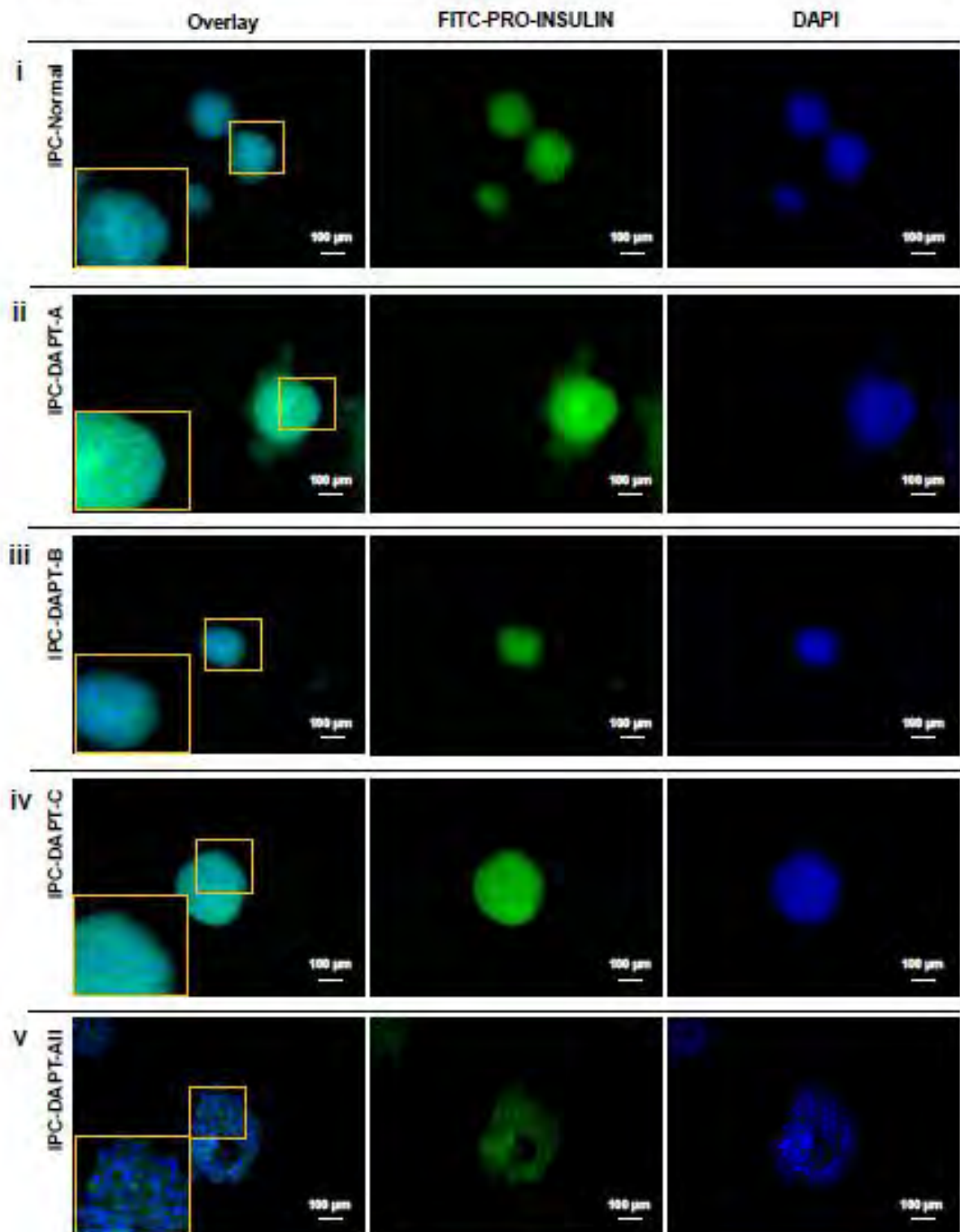
A

hDPSCs / d10 / 3-step IPC-induction

Notch Receptors

NOTCH-1

Relative mRNA expression

Control | d10 IPC

NOTCH-2

Relative mRNA expression

Control | d10 IPC

NOTCH-3

Relative mRNA expression

Control | d10 IPC

NOTCH-4

Relative mRNA expression

Control | d10 IPC

Notch Ligands

DLL-1

Relative mRNA expression

Control | d10 IPC

DLL-3

Relative mRNA expression

Control | d10 IPC

DLL-4 *

Relative mRNA expression

Control | d10 IPC

JAGGED-1

Relative mRNA expression

Control | d10 IPC

JAGGED-2

Relative mRNA expression

Control | d10 IPC

Notch Target Genes

HES-1 *

Relative mRNA expression

Control | d10 IPC

HEY-1 *

Relative mRNA expression

Control | d10 IPC

Figure 8 qRT-PCR results for the exploration of Notch signaling components expressed by day 10-hDPSCs-derived IPCs.

Exploration of Notch signaling components in mRNA level by day 10-hDPSCs-derived IPCs using qRT-PCR was performed (A). Asterisks indicated the statistical significance compared to the undifferentiated control group.

A hDPSCs / 3-step IPC-induction

| | d3 IPC | d5 IPC | d10 IPC |

i — IPC-Normal

ii — IPC-DAPT-A

iii — IPC-DAPT-B

iv — IPC-DAPT-C

v — IPC-DAPT-All

bar = 100 μm
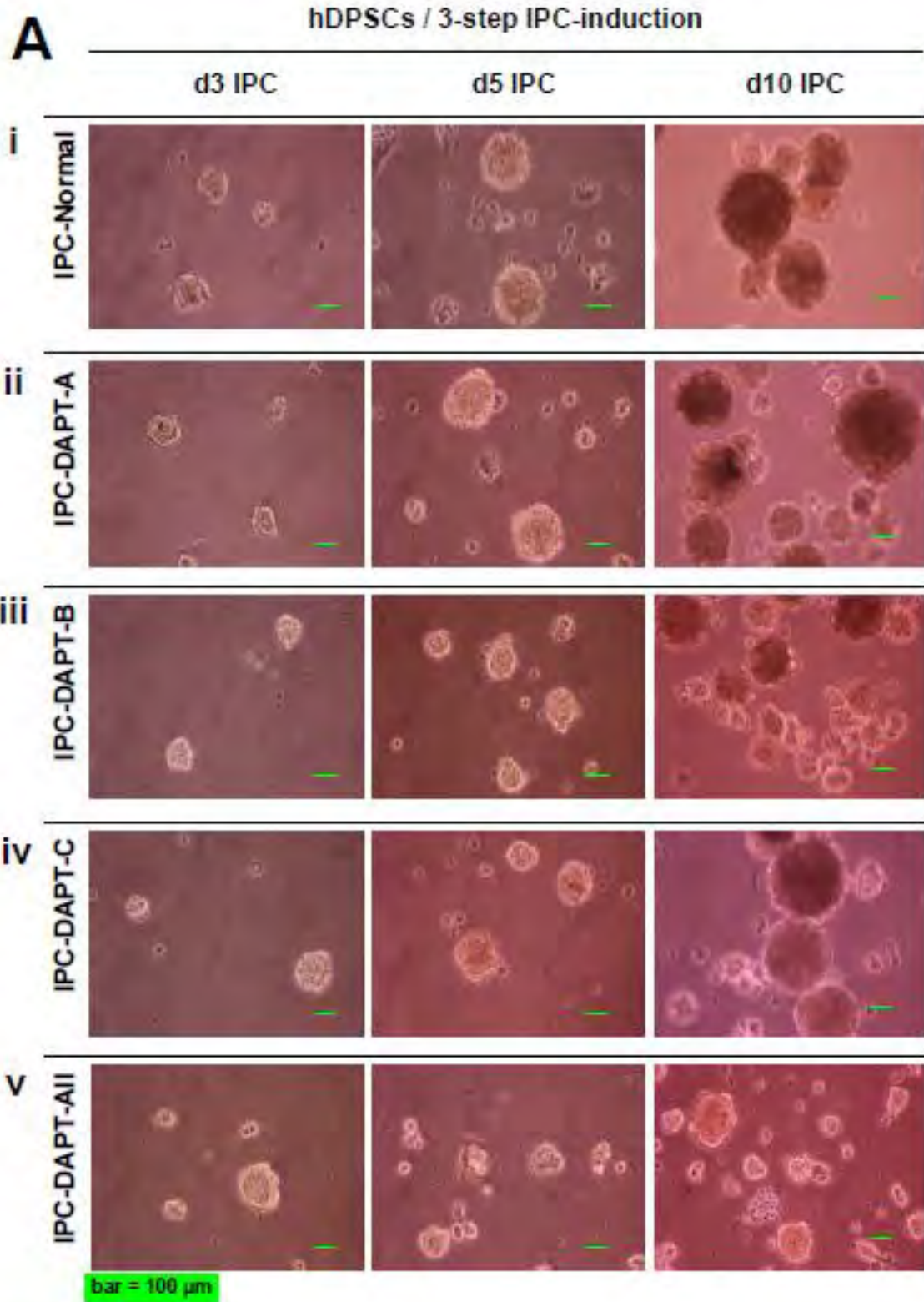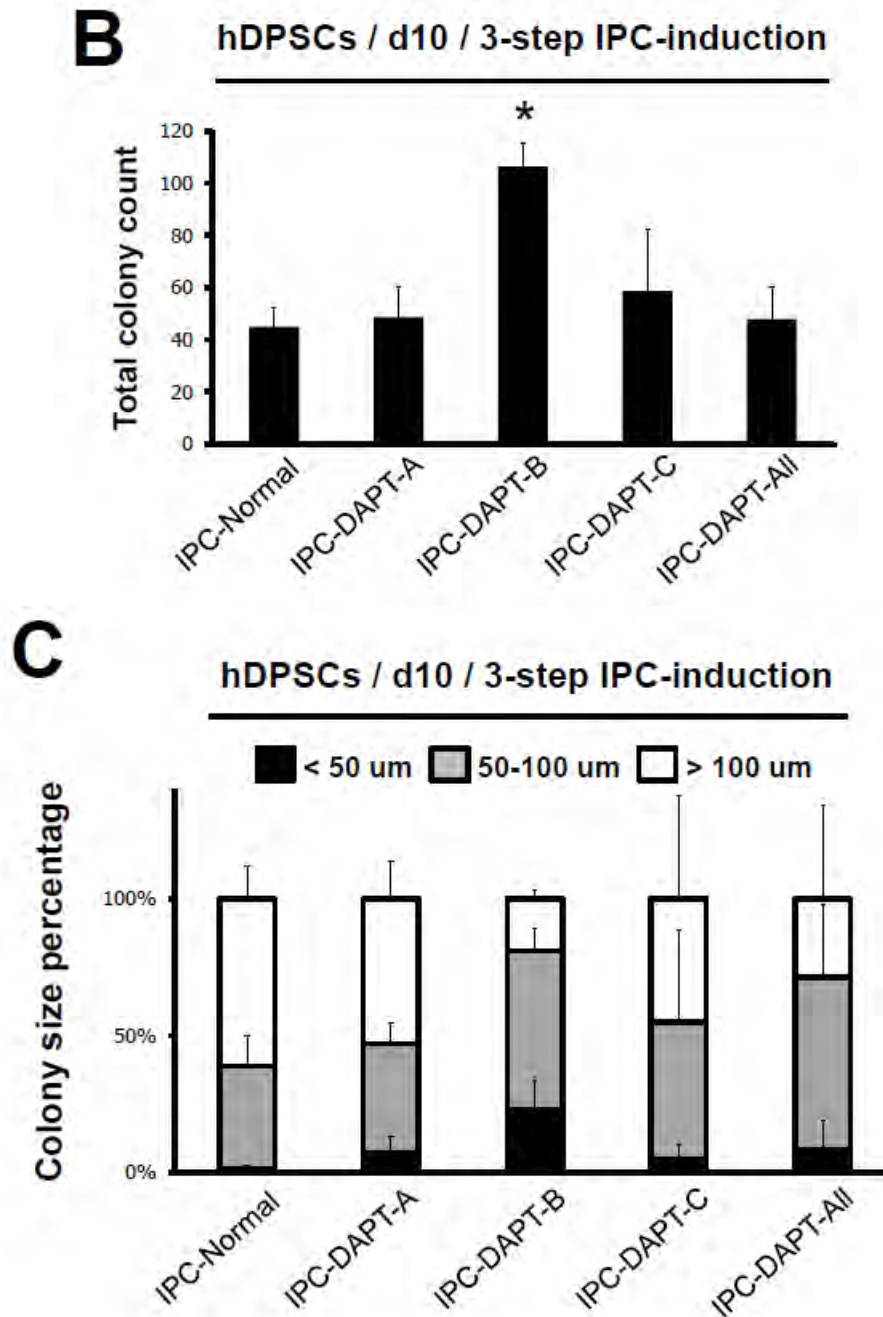
Figure 9 Morphological appearances of hDPSCs-derived IPCs with DAPT treatment.

By treatment with Notch inhibitor (DAPT) in each step or throughout IPCs-induction protocol, morphologies of aggregated colonies were observed at day 3, 5, and 10 (A). Total colony count number (B) and colony size percentage (C) was also investigated. Asterisks indicated the statistical significance compared to the hDPSCs group.

A
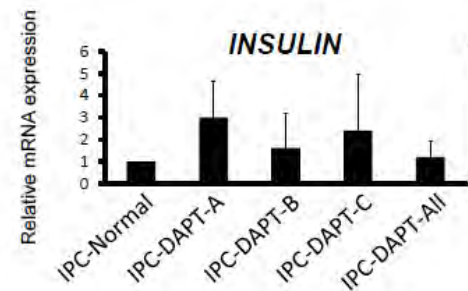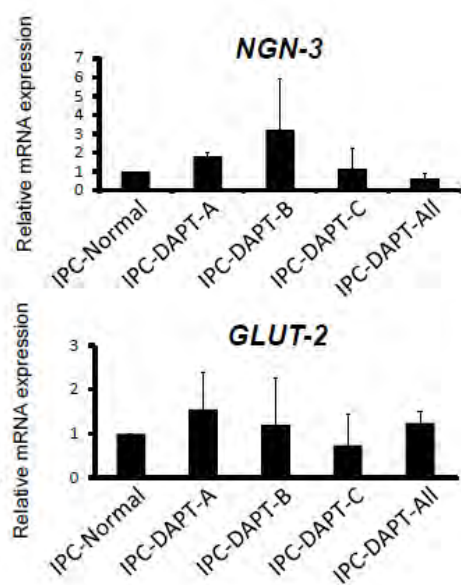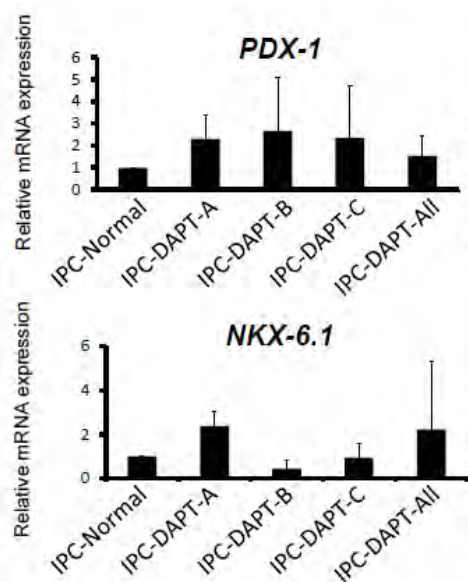
Figure 10 Pancreatic markers and Notch components expressed by day 10-hDPSCs-derived IPCs with DAPT treatment.

By treatment with Notch inhibitor (DAPT) in each step or throughout IPCs-induction protocol, expression of crucial pancreatic markers (A) and Notch components (B) by day 10-hDPSCs-derived IPCs was analyzed by qRT-PCR. .Asterisks indicated the statistical significance compared to the normal induction control.

A

hDPSCs / d10 / 3-step IPC-induction

Overlay	FITC-PRO-INSULIN	DAPI

i — IPC-Normal
ii — IPC-DAPT-A
iii — IPC-DAPT-B
iv — IPC-DAPT-C
v — IPC-DAPT-AII

B

hDPSCs / d10 / 3-step IPC-induction
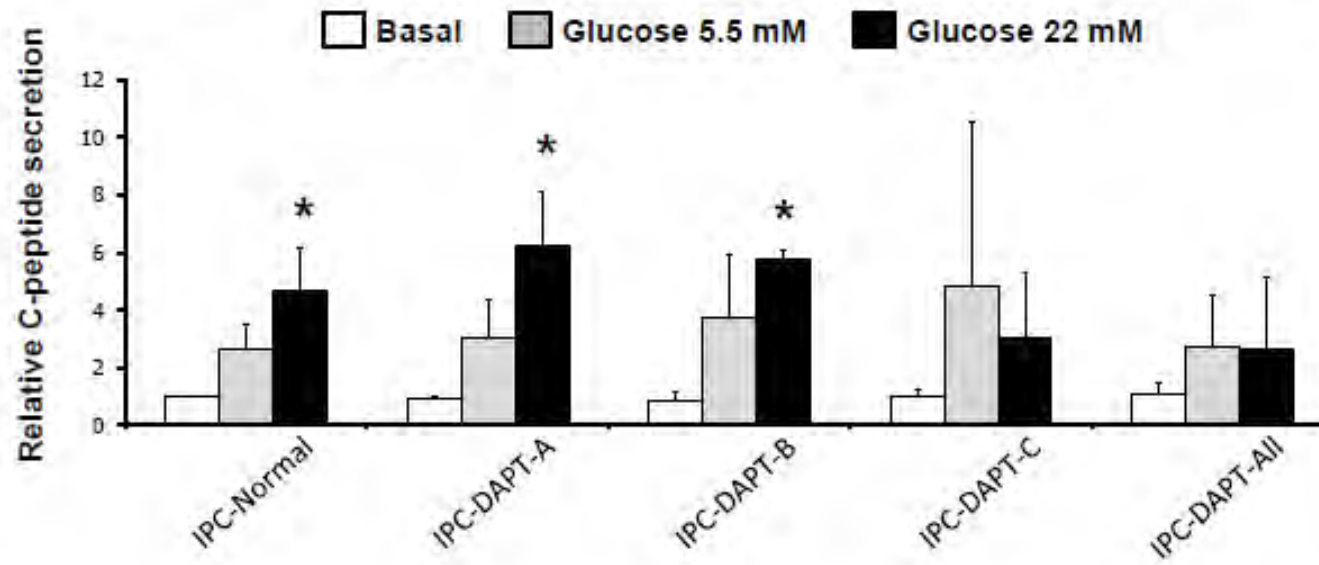
Figure 11 Pancreatic markers expression and functional test by day 10-hDPSCs-derived IPCs with DAPT treatment.

By treatment with Notch inhibitor (DAPT) in each step or throughout IPCs-induction protocol, PRO-INSULIN protein expression by day 10-hDPSCs-derived IPCs was evaluated by immunocytochemistry (ICC) staining (A).

For functional test, day 10-hDPSCs-derived IPCs were collected and tested with two different glucose concentration (5 mM and 22 mM). Amount of secreted C-peptide was normalized with basal level of normal induction control group (B). Asterisks indicated the statistical significance compared to the basal level in each group.

DISCUSSION AND CONCLUSION

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 [Chandra et al., 2009; Govindasamy et al., 2011; Koblas et al., 2009; Tang et al., 2004; Tang et al., 2006; Yang, 2006]. 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 [Li et al., 2007; Rahmati et al., 2013]. However, the employment of viral vector is the major safety concern due to high potency genetic mutation, leading to tumor formation. Thus, the environmental manipulation approach was employed in the present study according to the previously reported three-step induction protocol [Chandra et al., 2009; Govindasamy et al., 2011]. Govindasamy et al. reported that IPCs formation could be obtained from stem cells from human deciduous teeth [Govindasamy et al., 2011]. These IPCs expressed pancreatic islet marker genes (*NKX-6.1*, *PAX-6*, *ISL-1*, *GLUT2*, and *INSULIN*). In addition, the C-PEPTIDE and INSULIN secretion from IPCs was increased upon glucose stimulation. The same protocol was also utilized for IPCs induction by murine adipose stem cells [Chandra et al., 2009]. The function was also confirmed both the *in vitro* glucose challenge assay and the *in vivo* transplantation in streptozotocin (STZ)-induced diabetic mouse. Interestingly, the encapsulated IPCs transplanted into peritoneal cavity could restore the normal blood glucose level efficiently. 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 [Chandra et al., 2009]. This may due to the cell type and species difference. 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 [Afelik and Jensen, 2013; Qu et al., 2013; Shih et al., 2012]. In addition, it has been illustrated that Notch signaling is relevance in differentiation process of embryonic stem cells toward pancreatic lineages [Naujok et al., 2011]. Notch maintained the pool of *PDX-1*-positive early pancreatic progenitors by suppressing *NGN-3* expression in order to prevent premature endocrine differentiation [Champeris Tsaniras and Jones, 2010]. Inactivation of Notch signaling by conditional expression of dominant negative Matermind-like 1 or conditional inactivation of Hes1 resulted in the attenuation of β-cell formation in mice [Horn et al., 2012]. Further, knockout of *HES-1* expression caused pancreatic hypoplasia and depletion of pancreatic precursor cell [Habener et al., 2005]. 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 [Horn et al., 2012]. On the contrary, some studies demonstrated that Notch signaling inhibition resulted in the enhancement of the expanded human pancreatic β-cell redifferentiation [Bar et al., 2012]. 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 [Jaramillo and Banerjee, 2012; Thatava et al., 2011]. In addition, DAPT supplementation markedly enhanced *INSULIN* mRNA expression and PROINSULIN production in human umbilical cord mesenchymal stem cells [Hu et al., 2010]. However, the response to glucose of these DAPT-treated human umbilical cord stem cell-derived IPCs was not changed [Hu et al., 2010], 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 [Champeris Tsaniras and Jones, 2010]. 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. DAPT supplementation in the IPCs induction medium throughout the protocol resulted in the attenuation on IPC maturation as indicated by impaired colony morphology and C-PEPTIDE secretion upon glucose stimulation of day 10 IPCs. Further, *NGN-3* mRNA expression of this inhibition pattern was slightly decreased while the expressions of *INSULIN* mRNA and PRO-INSULIN protein were comparable to control group. This finding suggested the relevance of Notch signaling and IPC function as described in previous report [Hu et al., 2010]. Contrastingly, 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 [Arda et al., 2013; Stanger and Hebrok, 2013]. 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 related 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 [Wu et al., 2014]. 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 [Hu et al., 2010; Jaramillo and Banerjee, 2012; Thatava et al., 2011]. 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 [Ninov et al., 2012]. 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, dental tissue-derived mesenchymal stem cells (hDPSCs and hPDLSCs) have plasticity in differentiation toward IPCs. The hDPSCs-derived IPCs apparently revealed a higher capacity in differentiation as showed by total colony count number and pancreatic marker gene expression. 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.

PUBLICATION / PRESENTATION OF THE RESEARCH OUTCOME

<u>Poster presentation</u>:

- **2013 Beta cell workshop** (Appendix 2)

  (Kyoto International Conference Center-KICC, Kyoto, Japan, April 22-26, 2013)

  "Roles of Notch signaling in differentiation toward insulin-producing cells (IPCs) by dental tissue-derived stem cells."

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- **2014 Keystone Symposia: Stem cells and reprogramming** (Appendix 3)

  (Resort at Squaw Creek, Olympic Valley, California, USA, April 6-11, 2014)

  "Notch Signaling Influences *In Vitro* Differentiation toward Insulin-Producing Cells (IPCs) by Human Dental Pulp-Derived Mesenchymal Stem Cells (hDPSCS)."

  <u>Chenphop Sawangmake</u>[1,2,3,6], Prasit Pavasant[3,4], Piyarat Chansiripornchai[2], Thanaphum Osathanon[3,4,5]

  [1]Graduate Program in Veterinary Bioscience; [2]Department of Pharmacology, Faculty of Veterinary Science, Chulalongkorn University, Thailand

  [3]Research Unit of Mineralized Tissue; [4]Department of Anatomy; [5]DRU in Genetic and Anatomical Analyses in Craniofacial Structures, Faculty of Dentistry, Chulalongkorn University, Thailand

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Publication:

- Accepted for publication in "Biochemical and Biophysical Research Communications; BBRC" (Elsevier) (Impact Factor 2.281) (Appendix 4)

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

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[4] Department of Anatomy, Faculty of Dentistry, Chulalongkorn University, Bangkok, 10330 Thailand

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APPENDIX

Appendix 1: Primer sequences

Genes	Primer sequences	
	Forward	Reward
18S	5' GTGATGCCCTTAGATGTCC 3'	5' CCATCCAATCGGTAGTAGC 3'
CD-105	5' CATCACCTTTGGTGCCTTCC 3'	5' CTATGCCATGCTGCTGGTGGA 3'
CD-44	5' GCAAGTTTTGGTGGCACGCA 3'	5' CAATCTTCTTCAGGTGGAGC 3'
CD-73	5' ATTGCAAAGTGGTTCAAAGTCA 3'	5' ACACTTGGCCAGTAAAATAGGG 3'
CD-90	5' CACTCTCACACCAATGCGGG 3'	5' CGTTAGGCTGGTCACCTTCT 3'
COLLAGEN I	5' GCAAAGAAGGCGGCAAA 3'	5' CTCACCACGATCACCACTCT 3'
DLL-1	5' AGACGGAGACCATGAACAAC 3'	5' TCCTCGGATATGACGTACAC 3'
DLL-3	5'-GTGAATGCCGATGCCTAGAG-3'	5'-GGTCCATCTGCACATGTCAC-3'
DLL-4	5' TGACCACTTCGGCCACTATG 3'	5' AGTTGGAGCCGGTGAAGTTG 3'
GLUT2	5' GGTTTGTAACTTATGCCTAAG 3'	5' GCCTAGTTATGCATTGCAG 3'
HES-1	5' AGGCGGACATTCTGGAAATG 3'	5' CGGTACTTCCCCAGCACACTT 3'
HEY-1	5' GGAGAGGCGCCGCTGTAGTTA 3'	5' CAAGGGCGTGCGCGTCAAAGTA 3'
INSULIN	5'CCGCAGCCTTTGTGAACCAACA 3'	5' TTCCACAATGCCACGCTTCTGC 3'
JAGGED-1	5' AGTCACTGGCACGGTTGTAG 3'	5' TCGCTGTATCTGTCCACCTG 3'
JAGGED-2	5' GATTGGCGGCTATTACTGTG 3'	5' AGGCAGTCGTCAATGTTCTC 3'
LPL	5' GAGATTTCTCTGTATGGCACC 3'	5' CTGCAAATGAGACACTTTCTC 3'

NANOG	5' CAGCCCCGATTCTTCCACCAGTCCC 3'	5' CGGAAGATTCCCAGTCGGGTTCACC 3'
NGN-3	5' ATAAAGCGTGCCAAGGGGCACA3'	5' TTGTGC ATTCGATTGCGCTCGC 3'
NKX-6.1	5' TTGGCCTATTCGTTGGGGAT 3'	5' GTCTCCGAGTCCTGCTTCTTC 3'
NOTCH-1	5'-GCCGCCTTTGTGCTTCTGTTC-3';	5'-CCGGTGGTCTGTCTGGTCGTC-3'
NOTCH-2	5' CCAGAATGGAGGTTCCTGTA 3'	5' GTACCCAGGCCATCAACACA 3'
NOTCH-3	5-TCTTGCTGCTGGTCATTCTC-3	5-TGCCTCATCCTCTTCAGTTG-3
NOTCH-4	5' AGCCGATAAAGATGCCCA 3'	5' ACCACAGTCAAGTTGAGG 3'
OCT-4	5' GCAACCTGAGAATTTGTTCCT 3'	5' AGACCCAGCAGCCTCAAAATC 3'
OSTEOCALCIN	5' ATGAGAGCCCTCACACTCCTC 3'	5' GCCGTAGAAGCGCCGATAGGC 3'
PDX-1	5' GTC CTG GAG GAG CCC AAC 3'	5' GCA GTC CTG CTC AGG CTC 3'
PPAR-γ	5' GCTGTTATGGGTGAAACTCTG 3'	5' ATAAGGTGGAGATGCAGGCTC 3'
REX-1	5' AGAATTCGCTTGAGTATTCTGA 3'	5' GGCTTTCAGGTTATTTGACTGA 3'

Appendix 2: Abstract and poster: 2013 Beta cell workshop

Roles of Notch signaling in differentiation toward insulin-producing cells (IPCs) by dental tissue-derived stem cells.

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Abstract

Background:

Trend of stem cell (SC)-based therapy has been proposed as an alternative treatment for diabetes. Dental SCs have been also introduced to be candidate cell sources. To clarify their capability, we explored the potential differentiation of dental SCs into insulin-producing cells (IPCs) and underline mechanisms, focusing on Notch signaling.

Methodology:

3-stage induction protocol was used to differentiate three dental SCs (human periodontal ligament SCs; hPDLSCs, human dental pulp SCs; hDPSCs, and SCs from human exfoliated deciduous teeth; SHED) into definitive endoderm, pancreatic endoderm, and pancreatic islet (IPCs), respectively. 25 uM DAPT, γ-secretase inhibitor, was added into induction media throughout induction protocol to investigate relevance of Notch signaling on cell differentiation.

Results:

Three dental SCs were able to differentiate into IPCs confirming with round-sphere morphology, dithizone staining, insulin and GLUT2 gene expression, and immunocytochemistry (ICC) staining for pro-insulin/c-peptide. Inhibition of Notch signaling by DAPT showed an increase in number and size of colonies obtained in stage

1 and 2. However, colonies obtained at the end of protocol revealed distorted appearance and lower intensity of ICC staining.

Discussion:

The results suggested capability of differentiation toward IPCs by three dental SCs. Exploration of Notch signaling by inhibition strategy showed different impact upon each stage of induction protocol suggesting complex relevance of such pathway during differentiation.

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Beta Cell Workshop
2013 Kyoto

Department of Pharmacology, Faculty of Veterinary Science
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ROLES OF NOTCH SIGNALING IN DIFFERENTIATION TOWARD INSULIN-PRODUCING CELLS (IPCs) BY DENTAL TISSUE-DERIVED STEM CELLS

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Background: Trend of stem cell (SC)-based therapy has been proposed as an alternative treatment for diabetes. Dental SCs have been also introduced to be candidate cell sources. To clarify their capability, we explored the potential differentiation of dental SCs into insulin-producing cells (IPCs) and underline mechanisms, focusing on Notch signaling.

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Results: Three dental SCs were able to differentiate into IPCs confirming with round-sphere morphology, dithizone staining, insulin and GLUT2 gene expression, and immunocytochemistry (ICC) staining for pro-insulin/c-peptide. Inhibition of Notch signaling by DAPT showed an increase in number and size of colonies obtained in stage 1 and 2. However, colonies obtained at the end of protocol revealed distorted appearance and lower intensity of ICC staining.

Discussion: The results suggested capability of differentiation toward IPCs by three dental SCs. Exploration of Notch signaling by inhibition strategy showed different impact upon each stage of induction protocol suggesting complex relevance of such pathway during differentiation.

Keywords: Dental tissue-derived stem cells, Insulin-producing cells (IPCs), Notch signaling

Research Methodology and Results

Insulin-producing cells (IPCs) induction protocol

(modified from Chandra et al., 2009 and Govindasamy et al., 2011)

Appendix 3: Abstract and poster: 2014 Keystone symposia

Notch Signaling Influences *In Vitro* Differentiation toward Insulin-Producing Cells (IPCs) by Human Dental Pulp-Derived Mesenchymal Stem Cells (hDPSCS).

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Notch signaling plays a crucial role in differentiation process of stem cell. In this study, role of Notch signaling in *in vitro* differentiation toward IPCs by hDPSCs was revealed.

hDPSCs were differentiated toward IPCs using 3-step protocol. Matured-day 10 IPCs were confirmed by expressions of pancreatic gene markers (*PDX-1, NGN-3, PAX-4, NKX-6.1, ISL-1, GLUT-2,* and *INSULIN*), protein marker (PRO-INSULIN), and dose-dependent C-peptide release upon glucose stimulation. To investigate role of Notch signaling upon differentiation, 25 µM DAPT (γ-secretase inhibitor) was employed to block Notch signaling cascade by adding in induction medium during each step or throughout the protocol. Notch signaling blockage influenced IPCs differentiation potential pertaining colony morphology, total colony count, and colony size percentage. Pancreatic endoderm gene marker, *PAX-4*, was down-regulated upon DAPT treatment. Expression of PRO-INSULIN in protein level was affected in different pattern, while functional test revealed defect of C-peptide release in late-step Notch inhibition.

Taken together, these results imply the involvement of Notch signaling in differentiation toward IPCs by hDPSCs *in vitro*.

Appendix 4: Abstract of manuscript accepted for publication in "Biochemical and Biophysical Research Communications; BBRC" (Elsevier) (Impact Factor 2.281)

Manuscript; # BBRC-14-5111

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

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Running title: Dental stem cells differentiation toward insulin-producing 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.

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