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## **Differentiation of Dental Pulp Stem Cells Into Islet Like Aggregates**

V. Govindasamy, V. S. Ronald, A. N. Abdullah, K. R. Ganesan Nathan, Z. A. C. Ab. Aziz, M. Abdullah, S. Musa, N. H. Abu Kasim and R. R. Bhonde

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## ABSTRACT

The post-natal dental pulp tissue contains a population of multipotent mesenchymal progenitor cells known as dental pulp stromal/stem cells (DPSCs), with high proliferative potential for self-renewal. In this investigation, we explored the potential of DPSCs to differentiate into pancreatic cell lineage resembling islet-like cell aggregates (ICAs). We isolated, propagated, and characterized DPSCs and demonstrated that these could be differentiated into adipogenic, chondrogenic, and osteogenic lineage upon exposure to an appropriate cocktail of differentiating agents. Using a three-step protocol reported previously by our group, we succeeded in obtaining ICAs from DPSCs. The identity of ICAs was confirmed as islets by dithiozine-positive staining, as well as by expression of C-peptide, Pdx-1, Pax4, Pax6, Ngn3, and Isl-1. There were several-fold up-regulations of these transcription factors proportional to days of differentiation as compared with undifferentiated DPSCs. Day 10 ICAs released insulin and C-peptide in a glucose-dependent manner, exhibiting *in vitro* functionality. Our results demonstrated for the first time that DPSCs could be differentiated into pancreatic cell lineage and offer an unconventional and non-controversial source of human tissue that could be used for autologous stem cell therapy in diabetes.

**KEY WORDS:** dental pulp stem cells, insulin-producing cells, diabetes mellitus.

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# Differentiation of Dental Pulp Stem Cells into Islet-like Aggregates

## INTRODUCTION

Diabetes is a degenerative disease in which the destruction of pancreatic  $\beta$ -cells leads to persistent hyperglycemia (DeFronzo *et al.*, 2004). Transplantation of insulin-producing islet cells isolated from a donor pancreas could be a cure for type 1 diabetes (Shapiro *et al.*, 2000). However, a critical shortage of sufficient donor organs and the side-effects of immunosuppressive therapy limit its therapeutic usage, prompting a search for alternative sources of islet cells. In the past few years, huge advances have been made in the understanding of endocrine development. These provide an important guide to further attempts to produce islet cells *in vitro* (Serup *et al.*, 2001). Embryonic stem (ES) cells have been favorites in the race because of their tremendous differentiation potential. However, the ongoing ethical and legal considerations involved in ES cell research limit its use in translational medicine (Soria *et al.*, 2000).

Hence, mesenchymal stem cells (MSCs) are now being widely evaluated for their differentiation potential for cell replacement therapy. Recently, many studies have shown that hepatic stem cells (Yang *et al.*, 2002), umbilical cord blood (UCB) (Koblas *et al.*, 2009), and bone-marrow (BM)-derived MSCs (Chen *et al.*, 2004) have the potential to differentiate into insulin-producing cells. However, scarcity of the source and the invasive procedures required to isolate and culture these cells have limited their use. In this scenario, dental pulp stem cells (DPSCs) are considered to be an appealing source for MSCs, since they are non-controversial, readily accessible, have a large donor pool, and pose no risk of discomfort for the donor. DPSCs reside in the central cavity of the teeth containing the pulp tissue. These cells, first reported in 2000 by Gronthos *et al.*, share similar functions with BM-MSCs and can differentiate into osteoblasts, adipocytes, and neurogenic cell types *in vitro*.

Previously, we have reported that DPSCs express some endoderm markers such as GATA6, GATA4, and SOX17 (Govindasamy *et al.*, 2010a). Conversely, it has been reported that pancreatic  $\beta$ -cells of endodermal origin share many common features with those of neurons of ectodermal origin (Burns *et al.*, 2005). Many of the essential functional elements associated with pancreatic  $\beta$ -cells, such as ion channels and glucose transporters, are expressed in sub-populations of neurons (Yang *et al.*, 1999), and pancreatic  $\beta$ -cells also express neuro-transmitter biosynthetic enzymes (Teitelman and Lee, 1987; Baekkeskov *et al.*, 1990; De Vroede *et al.*, 1990). The intrinsic similarities between neural

cells and DPSCs, previously established by our group (Govindasamy *et al.*, 2010a), suggest that stem cells of dental origin may offer a useful starting tissue from which insulin-producing cells could be generated.

Huang *et al.* (2009) reported that stem cells derived from periodontal ligament exhibited a potential to differentiate into insulin-producing cells, but until now there has been no report, to our knowledge, of differentiation of mesenchymal cells from dental pulp into pancreatic lineage. Therefore, the present study was undertaken to determine whether DPSCs from deciduous teeth could be differentiated into pancreatic  $\beta$ -cell phenotype, and we anticipate that our findings will create a benchmark toward cell replacement therapy for type 1 diabetes (a childhood diabetes) by autologous transplantation of islet-like cell aggregates (ICAs) differentiated from a patient's own teeth.

## MATERIALS & METHODS

### Samples

Sound intact deciduous molars were extracted from children ( $n = 5$ ; ages 7-11 yrs) who were undergoing a planned serial extraction for management of occlusion at the Department of Children's Dentistry and Orthodontics, Faculty of Dentistry, University of Malaya. Samples were obtained under a protocol that was approved by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya (Medical Ethics Clearance Number: DFCD0907/0042[L]). The study was conducted with these samples, and all experiments were repeated independently to ensure the reproducibility of the results.

### Cell Culture

DPSC primary cultures from deciduous teeth were established as previously described (Govindasamy *et al.*, 2010a). In brief, the pulp tissue was minced into small fragments before digestion in a solution of 3 mg/mL collagenase type I (Gibco, Grand Island, NY, USA) for 40 min at 37°C. After neutralization with 10% of fetal bovine serum (FBS) (Hyclone; ThermoFisher Scientific Inc., Waltham, MA, USA), the cells were centrifuged and then seeded in culture flasks with culture medium containing  $\alpha$ -MEM (Invitrogen, Carlsbad, CA, USA), 0.5% 10,000 mg/mL penicillin/streptomycin (Invitrogen), 1% 1x Glutamax (Invitrogen), and 10% FBS, with humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C. Non-adherent cells were removed 48 hrs after initial plating. When the primary culture became sub-confluent, cells were collected by trypsinization and processed for subsequent passages. All experiments were conducted in DPSCs cultured in passage 2 (P2). The DPSCs were checked for their cell-surface marker expression (CD44, CD45, CD73, CD90, CD166, and HLA-DR) with flow cytometry as described previously (Govindasamy *et al.*, 2010a).

### In vitro Differentiation of DPSCs into ICAs

Differentiation of DPSCs into ICAs was carried out in 3 stages, as described previously (Chandra *et al.*, 2009) with slight modifications (Fig. 1A). In brief, undifferentiated DPSCs were

re-suspended in serum-free medium (SFM)-A and plated in a petri dish [ $1 \times 10^6$  cells/cm<sup>2</sup>] (Corning, Fisher Scientific International, Hampton, NH, USA). SFM-A contained Dulbecco's modified Eagle's medium Knock Out (DMEM-KO), 1% BSA Cohn fraction V, fatty acid free (Sigma-Aldrich, St. Louis, MO, USA), 1x insulin-transferrin-selenium (ITS), 4 nM activin A, 1 mM sodium butyrate, and 50  $\mu$ M 2-mercaptoethanol. The cells were cultured in this medium for 2 days. On the third day, the medium was changed to SFM-B, which contained DMEM-KO, 1% BSA, ITS, and 0.3 mM taurine, and was finally shifted to SFM-C on the fifth day. SFM-C contained DMEM-KO, 1.5% BSA, ITS, 3 mM taurine, 100 nM glucagon-like peptide (GLP)-1 (ambide fragment 7-36; Sigma Aldrich), 1 mM nicotinamide, and 1x non-essential amino acids (NEAAs). The cells were fed with fresh SFM-C every 2 days for another 5 days. All chemicals were purchased from Sigma Aldrich unless otherwise indicated.

### Reverse-transcription Polymerase Chain-reaction (RT-PCR) and Real-time RT-PCR

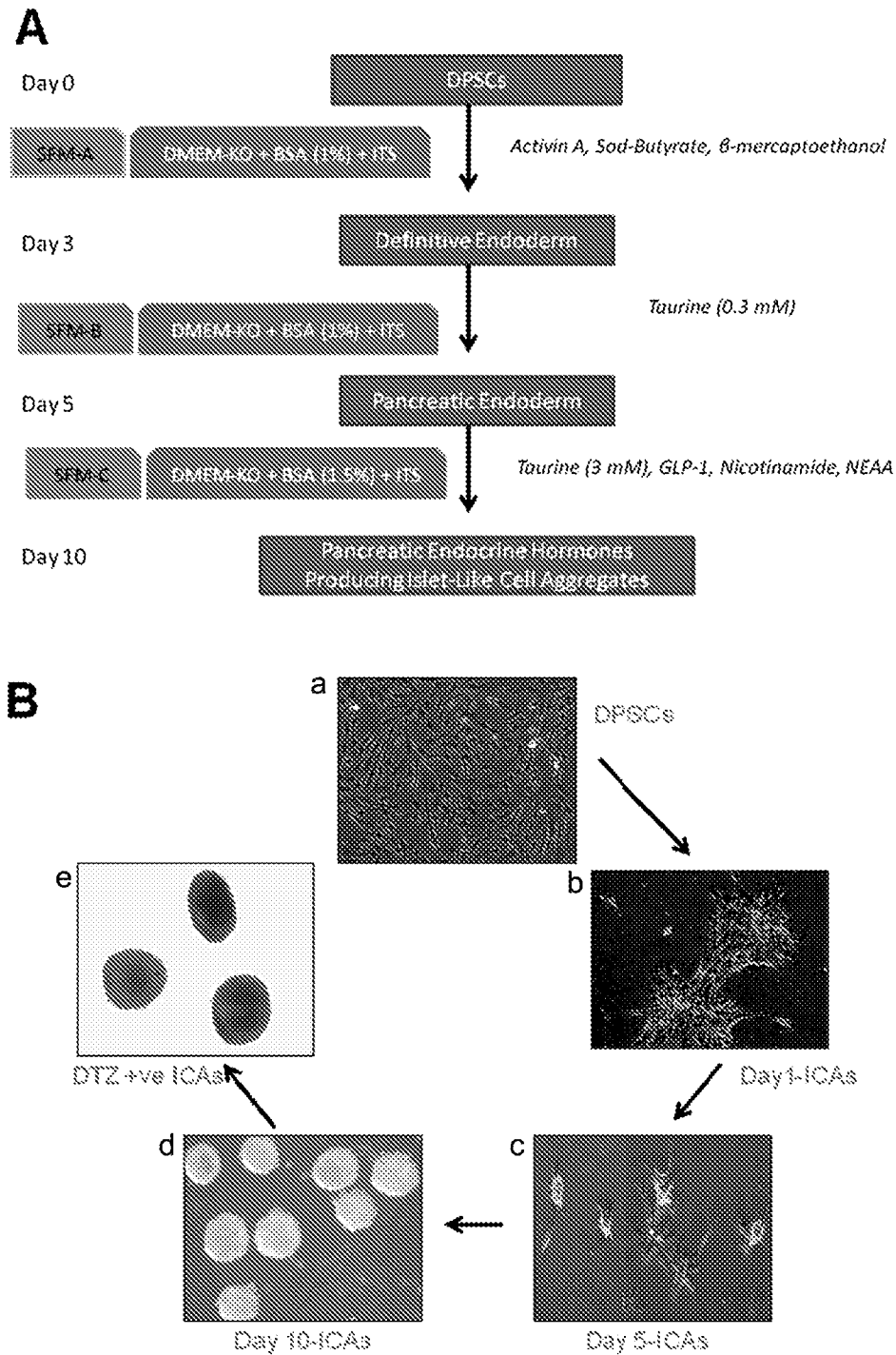
Total RNA extraction and cDNA synthesis were carried out as described previously (Govindasamy *et al.*, 2010a). Commercially available RNA from human pancreas (Human Pancreas Total RNA, Cat: 636577, Clontech Laboratories, Inc., Mountain View, CA, USA; www.clontech.com) was used as the positive control in this study. The primer sequences used in this study are tabulated in Appendix Table 1. The expressions of some of the primers in the semi-quantitative RT-PCR analysis were quantified in duplicate with SYBR green master mix (Applied Biosystems, Foster City, CA, USA). PCR reactions were run on an ABI 7900HT RT PCR system (Applied Biosystems), and all measurements were normalized by 18s rRNA. For data analysis, the comparative C<sub>T</sub> method ( $\Delta\Delta C_T$ ) was used.

### Immunocytochemistry

Undifferentiated DPSCs or ICAs were fixed for 20 min in 4% ice-cold paraformaldehyde, treated with 0.1% Triton-X for optimal penetration of cell membranes, and incubated at room temperature (RT) in a blocking solution (0.5% BSA; Sigma Aldrich) for 30 min. Primary antibodies were incubated overnight at 4°C, washed with Dulbecco's Phosphate Buffer Saline (DPBS; Invitrogen), and then incubated with secondary antibodies (either fluorescein isothiocyanate [FITC]-conjugated IgG or rhodamine-conjugated IgG) at RT for 90 min. Slides were counterstained with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI, Chemicon, Temecula, CA, USA) for 5 min. Fluorescent images were captured by means of a Nikon-Eclipse-90i microscope (Nikon, Tokyo, Japan, http://www.nikon.com). The sources of antibodies and dilutions used are summarized in Appendix Table 2.

### In vitro Multilineage Differentiation Studies

Adipogenesis, chondrogenesis, and osteogenesis differentiation of DPSCs were carried out as previously described (Govindasamy *et al.*, 2010b). Dithizone (DTZ) (Sigma-Aldrich) stain of 10 mg/mL dimethyl sulfoxide concentration was used to stain ICAs.



**Figure 1.** Generation of ICAs from DPSCs. **(A)** Schematic representation of stepwise protocol for generating ICAs. **(B)** Phenotypic changes in DPSCs during the 10-day differentiation procedure (a-d). Most of the day 10 ICAs stained with Dithizone (DTZ), a zinc-chelating agent known to selectively stain pancreatic  $\beta$ -cells because of their high zinc content (e). Scale bar: 100  $\mu$ m.

**Total Insulin Content and Release Assays**

Glucose-stimulated insulin and C-peptide release assay was carried out as described previously (Chandra *et al.*, 2009). Insulin and C-peptide concentration was measured with the use of an Immulite 1000 Insulin Kit (LKIN1) and Immulite 1000 C-peptide Kit

(LKPEP1) on the Immulite 1000 (Siemens Medical Solutions), respectively. In addition, we measured intracellular insulin concentrations by incubating ICAs overnight in acid/ethanol (18 mL 10 M HCl, 70% ethanol at 4°C). The total insulin content of ICAs was estimated after the ICA pellet was sonicated in 200  $\mu$ L acid/ethanol.

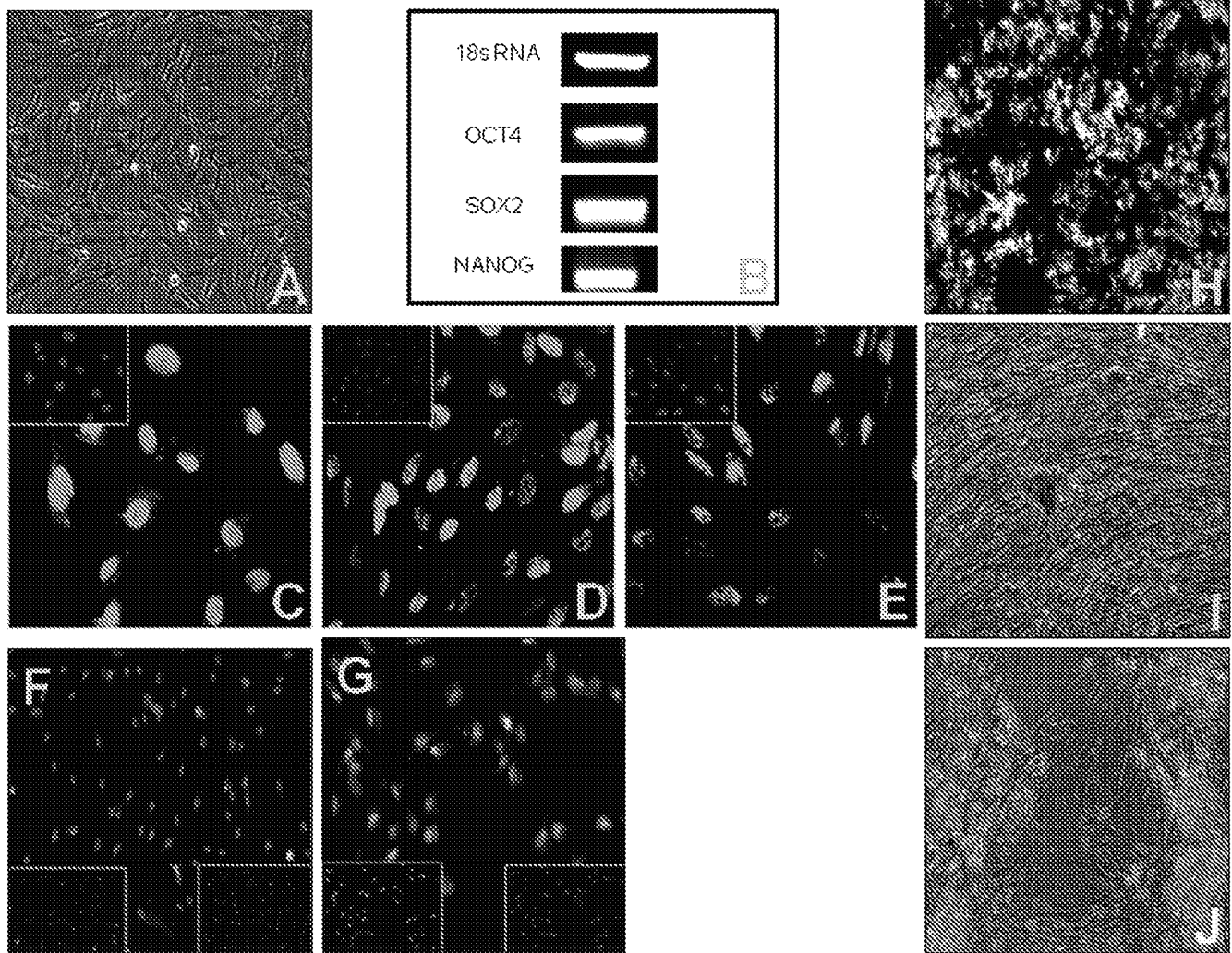
**Statistical Analysis**

Results were analyzed by Student's *t* test (Version 11.0, SPSS Predictive Analytics, Chicago, IL, USA; <http://www.spss.com>) and were expressed as mean  $\pm$  standard deviation (SD) of a least 5 biological replicates (n = 5) or samples. Differences were considered statistically significant when p < 0.05.

**RESULTS**

**Characterization of DPSCs**

Morphological characteristics of DPSCs displayed indistinguishable fibroblastic morphology resembling that of BM-MSCs (Fig. 2A). Flow cytometry analysis of DPSCs at P2 showed that the cells expressed cell-surface markers CD44 (94.21  $\pm$  2.9%), CD45 (0), CD73 (99.88  $\pm$  3.1%), CD90 (94.25  $\pm$  0.8%), CD166 (98.11  $\pm$  0.9%), and HLA-DR (0%). To further characterize the DPSCs, we examined the expression of markers of undifferentiated ESCs, and RT-PCR analysis showed that undifferentiated DPSCs expressed Oct-3/4, Nanog, and Sox-2, which are expressed in undifferentiated embryonic stem cells (Fig. 2B). This was confirmed at the protein level by immunocytochemistry analysis (Figs. 2C-2E). DPSCs were also positive for cytoskeletal proteins Nestin and Vimentin (Figs. 2F, 2G). DPSCs exhibited *in vitro* competence to differentiate into adipogenic, osteogenic, and chondrogenic lineages upon specific induction, as confirmed by Oil red O, Von Kossa staining, and Alcian Blue staining, respectively (Figs. 2H-2J).



**Figure 2.** *In vitro* characterization of DPSCs. (A) Phase-contrast microscopy, 10x of DPSCs at passage 2. (B) Semi-quantitative reverse-transcriptase polymerase chain-reaction (RT-PCR) of selected pluripotent markers. Immunofluorescence images of DPSCs at passage 2 for the pluripotent markers (C) OCT4, (D) Sox2, and (E) Nanog. Immunofluorescence image of DPSCs for cytoskeleton markers (F) Nestin and (G) Vimentin. Cell nuclei were stained with DAPI (blue). For Nestin and Vimentin, the cells were counterstained with OCT4. (H) Calcium depositions in extracellular matrix stained by Von Kossa staining indicate osteogenic differentiation. (I) Adipogenesis was demonstrated by the accumulation of neutral lipid vacuoles stained with oil red O. (J) Chondrogenesis was demonstrated by sulfated proteoglycans stained with Alizarin Blue. Scale bar: 100  $\mu$ m.

### Differentiation of DPSCs into Pancreatic Cell Lineage

DPSCs that proliferated as an adherent monolayer aggregated into spherical cells when the medium was changed from serum-containing medium to day 1 SFM-A (Fig. 1B). After 5 days of incubation in SFM-C, most of the day 10 ICAs stained positive for DTZ, a zinc-chelating agent known to selectively stain pancreatic  $\beta$ -cells. Real-time PCR analysis of day 10 ICAs showed a greater transcript abundance of pancreatic  $\beta$ -cells markers such as Pdx-1 (55-fold), Ngn3 (107-fold), Pax4 (67-fold), Nkx6.1 (49-fold), Isl-1 (32-fold), Insulin (26-fold), and Glut2 (128-fold) in day 10 ICAs as compared with undifferentiated DPSCs (Figs. 3A, 3B). The expression of transcription factors in ICAs formed during day 5 and day 10 were compared with human pancreas transcript factors as a positive control to validate our data. The results

were further confirmed by immunofluorescence staining (Fig. 4A). Flow cytometry analysis to quantitate the Pdx-1, Isl-1, and c-peptide in day 10 ICAs revealed that  $44.92 \pm 0.9\%$  ( $p < 0.05$ ),  $40.1 \pm 2\%$  ( $p < 0.05$ ), and  $30.56 \pm 2\%$  ( $p < 0.05$ ) of cells in day 10 ICAs expressed Pdx-1, Isl-1, and C-peptide, respectively, significantly greater than in undifferentiated DPSCs (Fig. 4B). The numbers of ICAs obtained at day 10 (initial cell plating:  $2 \times 10^6$  cells) were  $156 \pm 23$ , which indicates that ICAs can be sufficiently produced from DPSCs for transplantation.

### Static Stimulation and Total Insulin Content of ICAs

The total insulin and C-peptide contents of day 10 ICAs when exposed to 5.5 mM glucose were  $30.4 \pm 12.6$  MIU/L (Fig. 4C) and  $1.15 \pm 0.07$   $\mu$ g/L (Fig. 4D), respectively. When stimulated

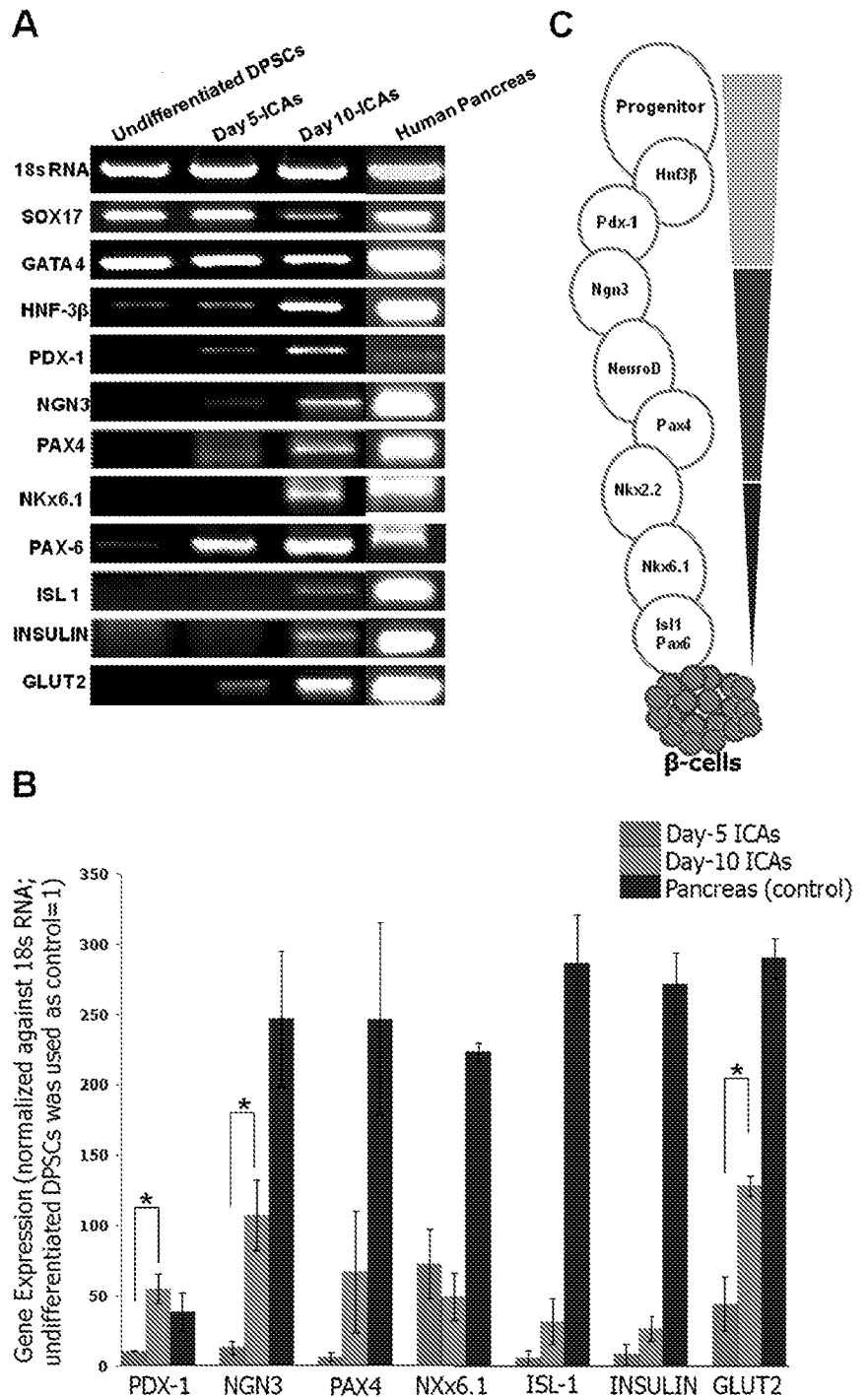
with 20 mM glucose, the total insulin and C-peptide contents were  $212 \pm 62.33$  MIU/L ( $p < 0.05$ ) and  $1.95 \pm 0.21$   $\mu$ g/L, respectively, confirming their *in vitro* ability to respond to glucose. The total intracellular insulin content of day 10 ICAs was also analyzed, and the value was  $165.5 \pm 42.42$  MIU/L.

**DISCUSSION**

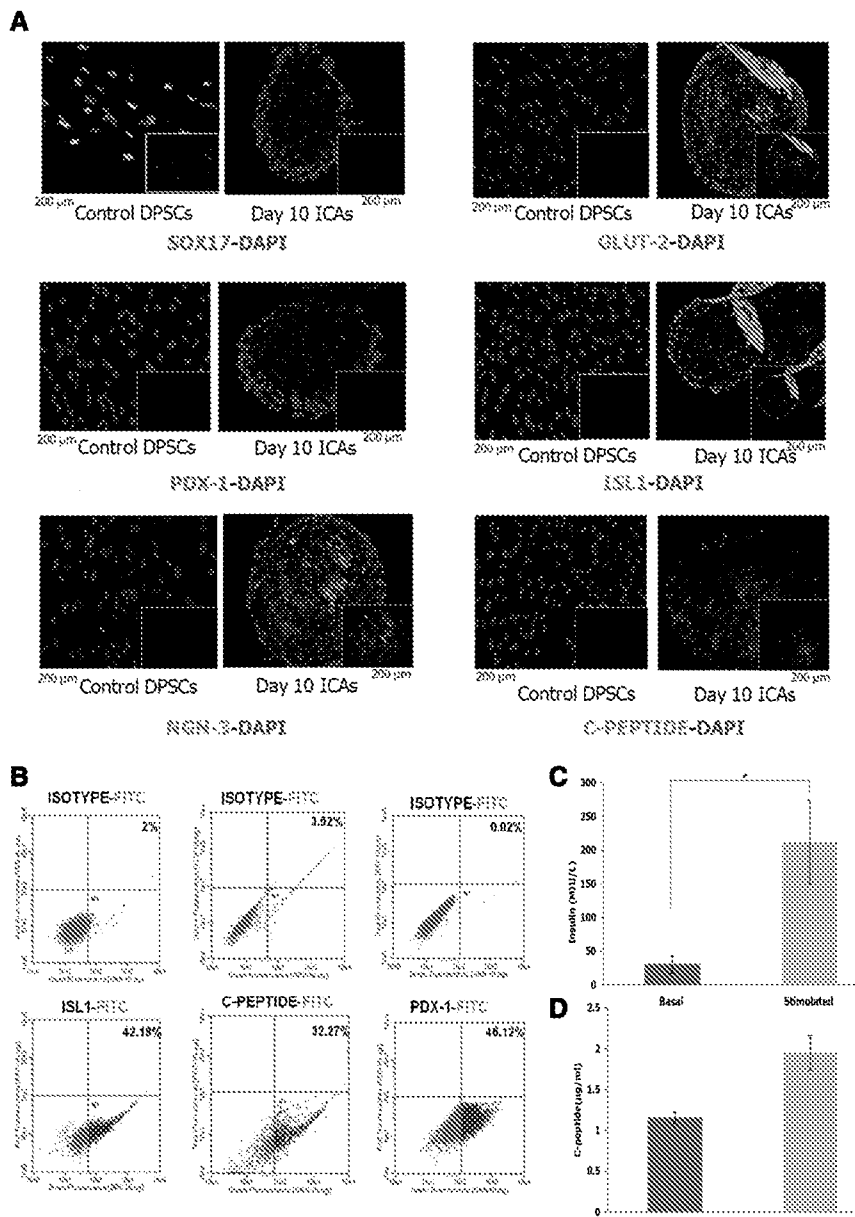
Type 1 diabetes or insulin-dependent diabetes mellitus (IDDM) is a disorder characterized by total loss of pancreatic  $\beta$ -cells as a result of autoimmune destruction (Roche *et al.*, 2005). The only remedy for IDDM is islet transplantation, which is hampered by the lack of availability of donor pancreas coupled with lifelong immune suppression. In this scenario, the only source of autologous stem cells is bone marrow, the acquisition of which is invasive and painful. Hence, there is a need for suitable alternative sources of stem cells for possible autologous transplantation of islets, eliminating the need for immune suppression.

Our experimental design involved the differentiation of multipotent DPSCs into ICAs following our established protocol (Hardikar *et al.*, 2003; Chandra *et al.*, 2009). Strikingly, the *in vitro* testing of ICAs by static stimulation assays showed that ICAs responded to the addition of glucose, as shown by measurable increases in increased insulin and C-peptide in a dose-dependent manner. Although we did not transplant the ICAs into experimental diabetic animals, the *in vitro* results revealed their competency to secrete insulin in response to a glucose challenge. This is an extremely important consideration for future islet transplantation programs using ICAs. Pioneer work (Huang *et al.*, 2009) supported our findings that the stem cells from other dental origins such as the periodontal ligament have the potential to differentiate into pancreatic cells. However, this study did not include a glucose challenge experiment, so it is difficult to evaluate their value for clinical applications.

Our work (Govindasamy *et al.*, 2010a) and that of others (Kerkis *et al.*, 2006) showed the general potential of pulp cells from deciduous teeth to form stem cells. In



**Figure 3.** ICAs derived from DPSCs expressed various genes involved in pancreas development. (A) Semi-quantitative reverse-transcriptase polymerase chain-reaction (RT-PCR) of selected pancreatic cell markers. (B) Real-time RT-PCR was performed to analyze the status of endoderm markers during the differentiation process. Samples were collected at days 5 and 10 and compared with undifferentiated DPSCs. Commercially available RNA from human pancreas was used as the positive control in this study. Relative levels of gene expression were normalized to the 18s RNA mRNA level; the asterisks denote a significant difference between groups. (C) Brief overview of temporal sequence of pancreatic transcription factors known to be expressed during development.



**Figure 4.** Expression of endoderm and pancreatic hormone genes in ICAs. **(A)** Immunofluorescence analysis was performed on control (undifferentiated) DPSCs and day 10 ICAs for the expression of definitive endoderm genes like Sox17, early pancreatic genes like PDX-1 and Ngn3, and pancreatic hormone genes like Isl-1, C-peptide, and Glut2. Sox 17 is a transcription factor gene and hence displayed nuclear localization, while PDX-1, Ngn-3, C-peptide, Isl-1, and Glut2 demonstrated cytoskeletal localization. DAPI was used as a counterstain; green and red represent FITC and rhodamine conjugates, respectively. Except for Sox17, there was no expression of PDX-1, Ngn3, Isl-1, C-peptide, and Glut2 in the control (undifferentiated) DPSCs as compared with day 10 ICAs. **(B)** We performed flow cytometric analysis to determine the expression of PDX-1, C-peptide, and Isl-1 in day 10 ICAs. Isotype-matched antibody control was also used to eliminate background staining. Scale bar: 200  $\mu$ m. **(C)** ICAs release insulin in response to glucose *in vitro*. The amount of insulin in the culture media was measured by the use of an Immulite 1000 Insulin Kit [LKIN1] after the cells were exposed to different concentrations of glucose. **(D)** ICAs release C-peptide in response to glucose *in vitro*. The amount of C-peptide in the culture media was measured by the use of an Immulite 1000 C-peptide Kit [LKPEP1] after the cells were exposed to different concentrations of glucose.

contrast, stem cells isolated from permanent teeth are more restricted in their potential and are mainly committed to a neuro-ectoderm lineage. Furthermore, adult-derived dental stem cells lose plasticity over increased numbers of passages. We believe that the increased pluripotency and greater plasticity of DPSCs from deciduous teeth makes them ideally suited for pancreatic lineage differentiation.

There are several alternative, well-documented sources of stem cells for generating insulin-producing ICAs (Appendix Table 3). However, all seem to be suitable only for allogeneic islet transplantation, which unfortunately requires immuno-suppression. Furthermore, adult stem cells have yielded controversial results with regard to their ability to secrete insulin *in vitro* and normalize hyperglycemia *in vivo*. For instance, BM-MSCs, which possess pluripotent differentiation capabilities, are a candidate for stem cell therapy in diabetic islet cell replacement (Lee *et al.*, 2006). Conversely, other studies have failed to support the ability of BM-MSCs to differentiate into islet cells (Taneera *et al.*, 2006). Moreover, our group has recently shown that BM-MSCs differentiate into immature islets *in vitro*, and these islets mature under *in vivo* conditions upon transplantation (Phadnis *et al.*, 2010). However, this does not disqualify BM-MSCs for the treatment of diabetes. BM-MSCs as such are a gold standard for allogeneic stem cell transplantation. If one compares the ability of BM-MSCs and DPSCs to differentiate into insulin-producing ICAs *in vitro*, DPSCs will be a better choice, since they can differentiate into mature insulin-producing ICAs, whereas BM-MSCs differentiate into immature islets which do not secrete insulin.

In summary, the present study clearly documents the potential of DPSCs derived from deciduous teeth to differentiate into insulin-producing cells, thus offering yet another non-pancreatic, non-invasive source of cells for islet generation that can be used for autologous transplantation without risk of rejection. The methods we have developed could be transferred from bench to bedside for the treatment of children with type 1 diabetes. Analysis of our data also suggests that banking of

dental pulp stem from deciduous teeth should be considered for those patients at risk for developing maturity-onset, type 2 diabetes.

## ACKNOWLEDGMENTS

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