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Regulation of miRNA during direct reprogramming of dental pulp cells to insulin-producing cells

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ABSTRACT

To further evaluate the multipotency of dental pulp cells, and to investigate the possible direct reprogramming of these cells, we examined their *in vitro* induction of direct conversion to an endocrine cell lineage. *In vitro* induction was carried out using similar conditions to those reported for regulating the differentiation of undifferentiated intestinal cells into endocrine progenitor cells. Specifically, the transcription factors *Pdx1* and *Neurog3* were transfected into rat dental pulp cells to induce their direct conversion to endocrine lineage cells. The degree of induction was evaluated by detecting insulin-producing cells. Using a miRCURY LNA microRNA Array (Exiqon), the miRNA expression profiles were comprehensively analyzed. At 10 days after induction, insulin-producing cells were detected. Based on the expression profiles, eight miRNA probes showing significant differences at 10 days after induction compared with their pre-induction baseline values were extracted after filtering. Notably, miR-183 was downregulated by less than 40% after induction. Following a target scan of miR-183, we identified 242 conserved targets, including molecules crucial for the development of pancreatic beta-cells such as Foxo1. These findings indicate that dental pulp cells have potential for direct reprogramming to insulin-producing cells. This potential ability for direct reprogramming of dental pulp cells shows promise for clinically relevant tissue engineering materials.

1. Introduction

We previously reported that stem cell markers, such as STRO-1, SSEA-1, Nanog, and Oct-3/4, are expressed in rat dental pulp cells [1]. We further showed that dental pulp cells can differentiate into osteogenic, neurogenic, myogenic, and adipogenic lineage cells by applying similar *in vitro* induction conditions to those used for human bone marrow cells [2–5]. Therefore, we concluded that dental pulp cells possess the characteristics of multipotent adult progenitor cells or somatic stem cells, and are able to differentiate into any type of somatic cell *in vitro*. Several markers for pluripotent stem cells have been identified, including *Oct-3/4* (also known as *Pou5f1*), *Sox2*, and *Nanog* [6,7]. In rat dental pulp cells, *Oct-3/4* and *Sox2* are downregulated during adipogenic differentiation [5], suggesting that dental pulp cells contain stem cells. Induced pluripotent stem (iPS) cells can be produced by transfecting four genes, including *Sox2* and *Oct-3/4*, into fibroblasts [8].

iPS cells can be induced from many types of somatic cells, and dental pulp cells are accepted as an optimal source of iPS cells [9]. Although direct reprogramming of mouse fibroblasts to hepatocyte-like cells by defined factors has been reported [10,11], it has not been elucidated whether such reprogramming is possible between developmentally unrelated tissues. For example, we have not examined whether dental pulp cells can be directly converted into endocrine lineage cells. For this study, we hypothesized that dental pulp cells possess an ability for direct reprogramming. To address this hypothesis, we focused on reprogramming of ectoderm-derived dental pulp cells into endoderm-derived cells. In the present study, we evaluated the potential of dental pulp cells for direct reprogramming by examining their *in vitro* induction of conversion to an endocrine cell lineage. Moreover, to identify the molecules regulated during the differentiation of rat dental pulp cells, we analyzed the profile of the changes in miRNA expression of the cells during the dynamic conversion.

2. Materials and methods

2.1. Cell culture

Five-week-old male Wistar rats were used in this study (Shimizu Laboratory Supply, Kyoto, Japan). Primary cultures were performed as previously described [5]. Briefly, reamers (25 mm #10; Morita Corporation, Osaka, Japan) were used to gently separate the pulp tissue of the incisors away from the distal site of the molars to excise the apical bud. The tissue was then minced

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and incubated in phosphate-buffered saline (PBS(−)) containing 3 mg/mL type I collagenase (Invitrogen, Carlsbad, CA, USA) and 4 mg/mL dispase (Invitrogen) for 50 min at 37 °C. The isolated cells were cultured in a basic medium consisting of MF-start medium (Toyobo, Osaka, Japan) supplemented with 10% (v/v) fetal calf serum (Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO_2. All animal experiments were carried out in accordance with the Osaka Dental University guidelines for the care and use of laboratory animals.

2.2. Construction of expression vectors

Full-length cDNAs encoding *Pdx1* (MN_022852) and *Neurog3* (*Ngn3*; NM_021700) with a Kozac sequence (5′-GGCGCC-3′) were synthesized (Integrated DNA Technologies Inc., Coralville, IA, USA). The synthesized cDNAs were inserted into the PstI site in the MCS of the pIRES2-ZnGreen1 expression vector (Clontech Laboratories Inc., Mountain View, CA, USA). The sequences of the constructed vectors at the ligation site were confirmed by DNA sequencing.

2.3. Induction assay

The rat dental pulp cells were inoculated into the basic medium at a density of 6×10^4 cells/well in Nunc Lab-Tek II chamber slides (Thermo Scientific Inc., Rochester, NY, USA) and cultured for 2 days. To induce conversion to an endocrine cell lineage, the cells were initially transfected with the expression vector for *Pdx1* on day 1, followed by cotransfection of the *Pdx1* and *Ngn3* expression vectors on days 4, 7, and 10. The transfections were carried out using the FuGENE HD Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA) in accordance with the manufacturer's instructions. The medium was replaced with the basic medium at 1 day after each transfection.

2.4. Immunostaining

After fixation with 4% (w/v) paraformaldehyde solution, the cells were treated with 0.1 M phosphate buffer containing 0.1% Triton X-100, washed with 10 mM glycine in PBS(−), and incubated in 3% (w/v) BSA in PBS(−) for 30 min at room temperature to block nonspecific reactions. After washing with 10 mM glycine in PBS(−), the cells were incubated with an appropriately diluted primary antibody for 1 h at room temperature. The cells were then washed with 0.1% BSA in PBS(−) and incubated with a fluorescently labeled secondary antibody for 1 h at room temperature. Guinea pig anti-insulin and rabbit anti-Foxo1A antibodies (Abcam, Cambridge, UK) were used as the primary antibodies, with Alexa 488-conjugated goat anti-guinea pig and Alexa 546-conjugated rabbit IgG (H + L) high cross-adsorbed (Molecular Probes) antibodies as the respective secondary antibodies. After the antibody incubations, the cells were washed with 0.1% BSA in PBS(−), counterstained with DAPI (Invitrogen), and mounted in Permafluor (Immunotech, Marseille, France). The immunofluorescence signals were observed under a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan).

2.5. RNA extraction

Total RNA was isolated from the cells by acid guanidinium thiocyanate-phenol-chloroform extraction with Isogen (Nippon Gene Co. Ltd., Tokyo, Japan) in accordance with the manufacturer's instructions. Briefly, cells were homogenized in Isogen (5×10^6 cells/mL of Isogen), followed by a primary extraction with 200 μL of chloroform and a secondary extraction with 650 μL of chloroform. The RNA was then precipitated with 1 mL of isopropanol and washed with 1 mL of 70% ethanol. All centrifugations were performed at 12,000*g* at 4 °C. The extracted RNA was dissolved in UltraPure™ DNase/RNase-free distilled water (Life Technologies, Carlsbad, CA, USA), and its concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 260 nm.

2.6. Comprehensive analysis of miRNA expression

Total RNA was isolated before induction (Sample 1) and at 10 days after induction (Sample 2). Each sample was labeled with Hy3. A mixture comprising equal volumes of Sample 1 and Sample 2 was labeled with Hy5 as a reference. Using a miRCURY LNA microRNA Array (Exiqon A/S, Vedbaek, Denmark) containing 348 probes, the miRNA expression profiles were comprehensively compared and analyzed (B-Bridge International Inc., Mountain View, CA, USA). Genes showing significant differences of ⩾2-fold were extracted after filtering.

2.7. Quantitative real-time RT-PCR

The specific PCR primer sets used were designed using a Perfect Real Time Support System (Takara Bio Inc., Ohtsu, Japan). To analyze the expression levels, cDNAs were synthesized from total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. Real-time RT-PCR was performed using Fast SYBR Green PCR Master Mix (Applied Biosystems) to examine the expression levels of each marker. PCR amplifications were performed using a StepOne Plus™ System (Applied Biosystems) with an initial denaturation step at 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s, and concluded with an automatic melting curve stage. *Rps18* was amplified as an internal control to correct the quantitative analyses. The expression levels were presented as the fold change values after induction compared with those before induction, and were calculated using the ΔΔCt method [12]. Differences in values were statistically analyzed with the Mann–Whitney U test using SPSS software version 18.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Expression of insulin after induction

Before the induction of differentiation, insulin expression was not detected in the rat dental pulp cells by immunofluorescence staining with an anti-insulin antibody. At 10 days after the induction, expression of insulin was detected in the cytoplasm of the cells (Fig. 1A). Specifically, at 10 days after the induction, the expression levels of *Ins1* and *Ins2* were each increased by about 7-fold compared with their levels before induction (Fig. 1B).

3.2. Profiling of miRNA expression during conversion into insulin-producing cells

Scatter-plot analyses were undertaken to view the overall changes after induction (data not shown). Differential expression of several miRNAs was observed during the direct conversion process. Genes showing significant differences in expression of ⩾2-fold were detected after filtering. Eight probes, including miR-101a, -101b, miR-181c, miR-183, miR-29a, -29b, -29c, and miR-30e, were extracted from the comprehensive analysis of miRNA expression. The expression levels of seven probes were increased after induction, while that of miR-183 was decreased (Fig. 2).

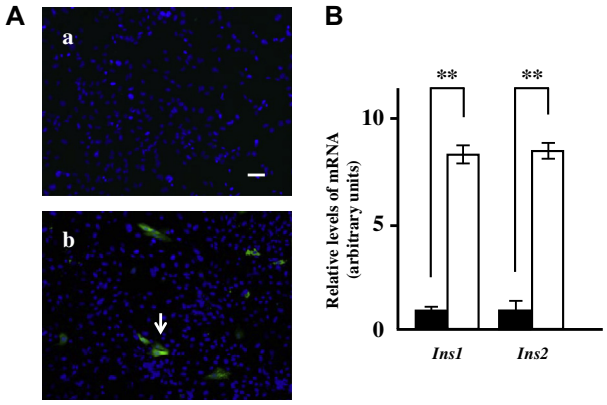

Fig. 1. Expression of insulin after induction. (A) Immunofluorescence staining with an anti-insulin antibody before (a) and after (b) induction. Insulin-producing cells are observed in dental pulp cells after the induction (see arrow). Scale bar indicates 100 μm. (B) Changes in the expression levels of *Insulin1* and *Insulin2* after induction. The data show quantitative real-time RT-PCR analyses of the *Ins1* and *Ins2* expression levels relative to that of the reference gene (*Rps18*). Closed and open bars represent the levels before and after induction, respectively. The relative levels of mRNA expression are presented as the fold changes after induction versus before induction, and represent the mean values of four independent assays for each sample. The values were calculated using Relative Quantity (RQ) Study Software and represent the statistical variability in the calculations of each sample's RQ value. The RQ_{min}/RQ_{max} values are graphically represented as error bars. The double asterisks denote statistically significant differences ($p < 0.01$).

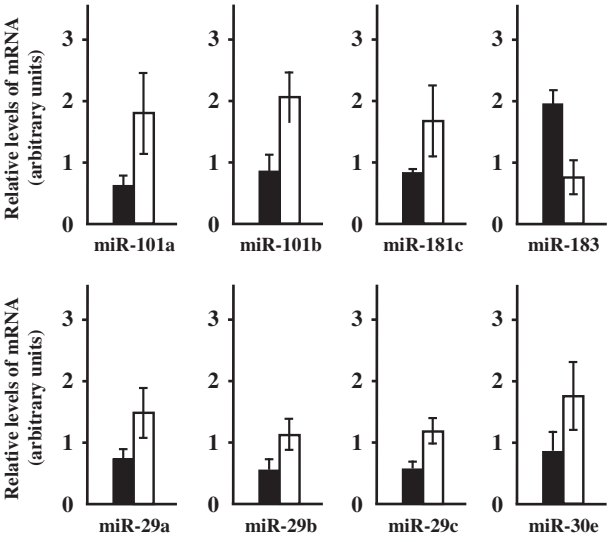

Fig. 2. Profile of the changes in miRNA expression after induction. The eight miRNAs extracted by the miRNA array are shown on the *X*-axis. The *Y*-axis represents the relative expression levels (normalized signal intensity). Closed and open bars represent the levels before and after induction, respectively. The errors bars reflect the standard deviation among repetitive spots on the array.

3.3. Foxo1 is upregulated after induction

Following a target scan of miR-183, we identified 242 conserved targets, including molecules crucial for differentiation into pancreatic beta-cells such as Forkhead box O1 (*Foxo1*). *Foxo1* is known to be regulated by miR-183, and was upregulated by >2-fold after induction (Fig. 3). Foxo1 was expressed in the nucleus of the cells, while insulin was expressed in the cytoplasm of the cells (Fig. 4).

4. Discussion

Induction of endocrine or exocrine pancreatic cells in the liver has been reported [13,14]. These conversions have been attributed

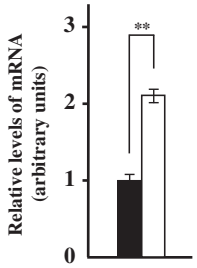

Fig. 3. Changes in the expression of *Foxo1* after induction. The data represent quantitative real-time RT-PCR analyses of the *Foxo1* expression levels relative to that of the reference gene (*Rps18*). The relative levels of mRNA expression are presented as the fold changes after induction versus before induction. Closed and open bars represent the levels before and after induction, respectively. The data represent the mean values of four independent assays for each sample. Values were calculated using Relative Quantity (RQ) Study Software and represent the statistical variability in the calculations of each sample's RQ value. The RQ_{min}/RQ_{max} values are graphically represented as error bars. The double asterisk denotes a statistically significant difference ($p < 0.01$).

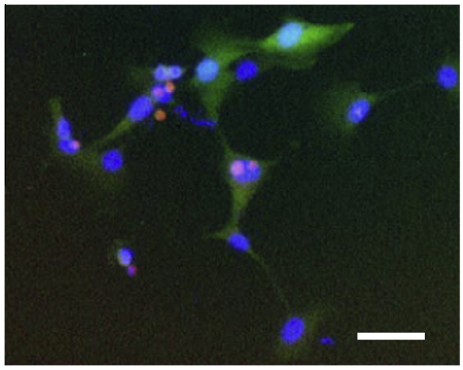

Fig. 4. Localizations of insulin and Foxo1 in cells after induction revealed by immunofluorescence staining. The panels show positive staining with the anti-insulin (green) and anti-Foxo1 (red) antibodies in the dental pulp cells at 10 days after induction. Scale bar indicates 100 μm.

to the close developmental relationship between these trans-converting tissues. The trans-converted islet cells were derived from the islet cell precursors or the primitive foregut endoderm lineage in the pancreatic developmental processes [15–18]. Therefore, all adult cells in terminally differentiated tissues may have the potential to undergo reprogramming to a pluripotent state in response to defined developmental factors [19]. The notion that adult somatic cells possess this plasticity suggests that it may be possible to directly reprogram adult somatic cells to committed lineages. Indeed, exogenous Pdx1 induces direct reprogramming of human keratinocytes to pancreatic insulin-producing cells [20]. To prove that reprogramming can be possible between developmentally unrelated tissues, the present study demonstrated the ability of dental pulp cells to undergo direct conversion to endocrine pancreatic lineage cells through ectopic expression of *Pdx1* and *Neurog3*.

Recently, it has been reported that miRNA-mediated reprogramming of somatic cells was observed with high efficiency [21,22]. Expression of the miR302b/367 or miR302/miR-372 cluster rapidly and efficiently enhanced the reprogramming of somatic cells to iPS cells without requiring exogenous transcription factors such as *Oct3/4* or *Sox2*. Therefore, we hypothesized that cells derived from the dental pulp are regulated by miRNA expression during the process of direct reprogramming. To identify the regulation of conversion of dental pulp cells into insulin-producing cells, the miRNA expression profiles were comprehensively

analyzed. In this study, eight miRNAs showing significant differences at 10 days after induction compared with their pre-induction baseline values were extracted by the miRNA array after filtering. Notably, miR-183 was downregulated by less than 40% after the induction. This suggests the possibility that the observed conversion might be regulated by target genes in the downstream of miR-183. Among the target genes of miR-183, we focused on *Foxo1*, which is known to be exclusively expressed in insulin-producing cells and to play an important role in islet cell type specification during differentiation or trans-differentiation into pancreatic beta-cells [23]. Foxo1 is also known to regulate the expression of insulin [24]. In our results, *Foxo1* expression was increased by >2-fold after the induction. Foxo1 sublocalized in the nucleus is active as a transcription factor, and has the insulin gene is one of its transcriptional targets [24]. Subsequently, Foxo1 is inhibited through Akt-mediated phosphorylation and nuclear exclusion [25]. The subcellular localization of Foxo1 in pancreatic beta-cells is heterogeneous, with some cells showing exclusive cytoplasmic immunoreactivity and other cells showing diffuse immunoreactivity [26]. In the insulin-producing cells converted from dental pulp cells, the subcellular localization of insulin was exclusively cytoplasmic, whereas the subcellular localization of Foxo1 was exclusively nuclear. These observations suggest that some cells expressing Foxo1 in their nucleus are under transcriptional enhancement to produce insulin through downregulation of miR-183. Taken together, the participation of miR-183 may be an important part of the process for direct reprogramming of dental pulp cells to insulin-producing cells.

Based on our evidence for the ability of dental pulp cells to undergo direct reprogramming to developmentally unrelated cells, dental pulp cells show potential for use in multiple regenerative medical applications. The present study has enlarged the potential cell sources suitable for regenerative medicine. We believe that dental pulp cells will become an acceptable source with benefits as clinically relevant tissue engineering materials in the future, instead of the controversial sources of cells for cell therapy. The miRNA regulation of the process for direct reprogramming indicated in this study could be useful in future applications for regenerative medicine.

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