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Inhibition of *miR-183* Induces Insulin in Dental Pulp Cells

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Abstract: We previously reported that *miR-183* was downregulated during the direct conversion of dental pulp cells to insulin-producing cells. To further elucidate the role of miRNA regulation in dental pulp cells during this process, we examined the effect of *miR-183* inhibition. Insulin expression was detected in induced cells 72 hours after ectopic expression of *miR-183* inhibitor. The expression levels of *insulin 1* and *insulin 2* mRNA increased 1.7-fold and 1.6-fold, respectively. Insulin copy number estimates, following *miR-183* inhibition, showed more than 10 copies each of *insulin 1* and *insulin 2*. These data suggest that *miR-183* downregulation induces the direct conversion of dental pulp cells to insulin-producing cells.

Key words: Dental pulp, Differentiation, Direct reprogramming, Gene expression, *miR-183*

Introduction

Dental pulp-derived cells can differentiate into osteogenic, neurogenic, myogenic, and adipogenic lineage cells by applying in vitro induction conditions similar to those used for human bone marrow cells¹⁻⁵⁾. Notably, demethylation by 5-azacytidine triggers myogenic differentiation, suggesting that epigenetic modification is involved in lineage differentiation in dental pulp cells⁵⁾. Several pluripotent stem cell markers have been identified, including *Nanog*, *Sox2*, and *Oct3/4* (also known as *Pou5f1*)^{6,7)}. Induced pluripotent stem (iPS) cells are produced by transfecting four genes, including *Sox2* and *Oct3/4*, into fibroblasts⁸⁾. Stem cell markers such as STRO-1, SSEA-1, *Nanog*, and *Oct3/4* are expressed at low levels in dental pulp-derived cells, and downregulation of these markers is observed during lineage differentiation¹⁾.

Previously, we reported that dental pulp cells can be directly converted to insulin-producing cells⁹⁾. *In vitro* induction was performed using conditions similar to those reported for the differentiation of undifferentiated intestinal cells into endocrine progenitor cells¹⁰⁻¹²⁾. Following the ectopic expression of defined transcription factors, the direct conversion of cells to endocrine lineage cells was induced, and insulin-producing cells were detected. Using microRNA array profiling, some miRNAs were found to have significantly different expression patterns after 10 days of induction⁹⁾. Notably, after induction, cellular *miR-183* levels decreased by approximately 60%. To further elucidate the role of *miR-183* in the direct conversion of dental pulp cells into insulin-producing cells, *miR-183* was inhibited, and the extent of miRNA regulation evaluated.

Materials and Methods

Cell culture

Five-week old male Wistar rats were used in this study (Shimizu Laboratory Supply Co, Kyoto, Japan). Primary cultures were performed as previously described⁴⁾. Briefly, approaching from the central side of the mandible, incisor dental pulp tissue was gently separated away

from the distal site of the molars to excise the apical bud, using reamers (25 mm #10; Morita Co, Osaka, Japan). The tissue was then minced 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 cells were cultured in MF-start basic medium (Toyobo Co, Osaka, Japan) supplemented with 10% (v/v) fetal calf serum (Invitrogen) at 37°C in a humidified atmosphere containing 5% CO₂. Animal experiments were performed in accordance with the Osaka Dental University guidelines for the care and use of laboratory animals.

Induction assay

Ambion anti-miR miRNA inhibitors (Thermo Fisher Scientific, Waltham, MA, USA) are chemically modified, single stranded nucleic acids designed to specifically bind to and inhibit endogenous miRNA molecules. Anti-miR miRNA inhibitors enable functional miRNA analysis by downregulating miRNA activity. *miR-183* inhibitor (50 nM) was transfected into the cells 2 days after inoculation. A mock study was also performed using 50 nM Cy3 dye-labeled anti-miR negative controls (Thermo Fisher Scientific, Tokyo, Japan). Transfections were performed using the FuGENE HD transfection reagent (Roche Diagnostics, Indianapolis, IN, USA) in accordance with the manufacturer's instructions. The medium was replaced with basic medium 1 day after each transfection. Subsequently, the expression of markers was examined to evaluate the effects of differentiation in the cells.

Immunostaining

After fixation with 4% (w/v) paraformaldehyde, 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 minutes at room temperature (20-25°C) to block nonspecific reactions. After washing with 10 mM glycine in PBS, the cells were incubated with an appropriately diluted primary antibody for 1 hour at room temperature, washed with 0.1% BSA in PBS, and incubated for 1 hour at room temperature with a fluorescently labeled secondary antibody. An anti-Insulin guinea pig antibody (1:200; Abcam, Cambridge, UK) was used as the primary antibody, and Alexa

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Figure 1. Expression of insulin in dental pulp cells by inhibition of *miR-183*

Panels showing anti-insulin antibody staining (green signals represent insulin production) of dental pulp cells after mock (Cy3 anti-miR negative controls) (A) and *miR-183* inhibitor (B) transfection. Scale bars indicate 100 μ m.

488-conjugated goat anti-guinea pig (1:1000; Molecular Probes, Eugene, Oregon, USA) was used as the secondary antibody. The samples were then washed with 0.1% BSA in PBS, counterstained with 4', 6-diamidino-2-phenylindole (Invitrogen) and mounted in Permafluor (Immunotech, Marseille, France). Immunofluorescence signals were observed by fluorescence microscopy, using a BZ-9000 microscope (Keyence Co, Osaka, Japan).

RNA extraction

Total RNA was isolated from the cells by acid guanidinium thiocyanate-phenol-chloroform extraction with Isogen in accordance with the manufacturer's instructions (Nippon Gene Co Ltd, Tokyo, Japan). Cells were homogenized in Isogen (5×10^6 cells/ml of Isogen) followed by extraction with 200 μ l of chloroform. After a second extraction using 650 μ l of chloroform and precipitation with 1 ml of isopropanol, the precipitated RNA was washed in 1 ml of 70% ethanol. All centrifugation was done at $12,000 \times g$ at 4°C . The RNA obtained was dissolved in UltraPure DNase/RNase-free distilled water (Life Technologies, Carsburg, CA, USA), and its concentration was measured by NanoDrop (Thermo Fisher Scientific) at 260 nm.

Quantitative real-time RT-PCR assay

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. As a

Figure 2. Induction of *Ins1* and *Ins2* mRNA by *miR-183* inhibition. Quantitative real-time RT-PCR analyses of *Ins1* and *Ins2* relative expression levels. Closed and open bars represent the levels 72 hours after mock (Cy3 anti-miR negative controls) and *miR-183* inhibitor transfection, respectively. The relative mRNA expression levels are presented as fold changes after transfection of mock and *miR-183* inhibitor, and represent the mean values of four independent assays for each sample. The values were calculated using the Relative Quantity (RQ) Study Software and show the statistical variability in the calculations of each sample's RQ value. The RQmin/RQmax values are graphically represented as error bars. The asterisks denote statistically significant differences ($p < 0.05$).

Figure 3. Scatter plot and standard curve by λ polyA+ RNA-A. Ct values (represented by squares) are plotted for λ polyA+ RNA-A serial dilution samples. The x-axis represents the copy number, and the y-axis represents the Ct value. The standard curve for copy number ranging from 10^2 to 10^6 is drawn. Ct values of *Ins1* and *Ins2*, corrected by an external reference (λ polyA+ RNA-A), were plotted on the standard curve. The circle indicates *Ins1* and the triangle indicates *Ins2*.

reference to allow comparison between samples, 1.0×10^8 copies of λ polyA+ RNA-A (Takara Bio Inc, Kusatsu, Shiga, Japan) were added per 1 μ g of total RNA during the cDNA synthesis process. Real-time RT-PCR was performed using the Fast SYBR Green PCR Master Mix (Applied Biosystems) to examine the expression levels of each marker. Specific PCR primer sets for *Insulin 1* (*Ins1*) and *Insulin 2* (*Ins2*) were designed using the Perfect Real Time Support System (Takara Bio Inc.). PCR amplification was performed using the StepOne Plus system (Applied Biosystems) with an initial denaturation step of 20 sec at 95°C , followed by 40 cycles each of 95°C for 3 sec and 60°C for 30 sec, concluding with an automatic melting curve stage. The expression levels are presented as the fold-change values after induction compared with those before induction and were calculated using the $\Delta\Delta\text{Ct}$

method¹³⁾. Differences between values were statistically analyzed by the Mann-Whitney U test using SPSS software (<https://www.ibm.com/analytics/us/en/technology/spss/>).

Estimation of expressed copy number

A quantitative standard curve was created using diluted λ polyA+ RNA-A. Diluted samples (10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6}) for 5.0×10^6 copies were prepared using the EASY Dilution for Real Time PCR kit (Takara Bio Inc.) and the Real Time primer for λ polyA (Takara Bio Inc.) was used for quantitative real-time RT-PCR.

Results

Detection of insulin in dental pulp cells by inhibition of *miR-183*

Insulin production was not observed at 72 hours after mock transfection with Cy3 anti-miR negative controls (Fig. 1A). In contrast, insulin production was observed 72 hours after transfection of the *miR-183* inhibitor (Fig. 1B).

Induction of insulin mRNA expression by inhibition of *miR-183*

Ectopic expression of *miR-183* inhibitor induces *insulin* at 72 hours after induction. *Ins2* is the murine homologue of the human *insulin* gene and is located on mouse chromosome 7. *Ins1*, which is thought to have evolved by a gene duplication event, lacks the second intron of the *Ins2* gene, is located on mouse chromosome 19. Qualitative expression analysis showed that *Ins1* and *Ins2* expression significantly increased after *miR-183* inhibition by 1.7-fold and 1.6-fold, respectively, ($p < 0.05$) (Fig. 2).

Ins1 and *Ins2* copy number induced by inhibition of *miR-183*

The Ct mean value was dependent on the serial dilution of λ polyA+ RNA-A. The 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} diluted samples for 1.0×10^8 copies demonstrated Ct value means of 20.0, 23.7, 27.6, 31.1, and 35.5, respectively. The standard curve was drawn with direct proportional relations ($y = 3.863x + 12.103$) for a copy number range of 10^{-2} to 10^{-6} (Fig. 3). λ polyA+ RNA-A was added as an external reference during the reverse transcription process. The Ct value means of *Ins1* and *Ins2* were 33.7 and 33.3, respectively, and were plotted on the standard curve. Using the standard curve, the copy number estimates for *Ins1* and *Ins2* were 12.9 and 16.2, respectively; thus, slightly more than 10 copies were present after *miR-183* inhibition.

Discussion

Highly efficient miRNA-mediated reprogramming of somatic cells has previously been reported^{14,15)}. The expression of the *miR302b/367* or *miR302/miR-372* cluster rapidly and efficiently reprograms somatic cells to iPS cells without exogenous transcription factors such as *Oct3/4* or *Sox2*. Therefore, we hypothesized that cells derived from the dental pulp can be regulated by miRNAs through the process of direct reprogramming. Previously, we comprehensively analyzed changes in miRNA levels during the direct conversion of dental pulp cells into insulin-producing cells by LNA array and reported eight candidate regulatory miRNAs. Notably, 10 days after induction by ectopic expression of the defined transcription factors, *miR-183* levels were less than 40% of those observed pre-induction⁹⁾. To further evaluate the role of miRNAs during this process in dental pulp cells, we inhibited *miR-183* in the cells. We found that inhibition of *miR-183* induced insulin expression in dental pulp cells, while no change was observed in mock-transfected cells. These results suggest that the direct conversion of dental pulp cells into the cells of developmentally unrelated tissues involves miRNA-dependent regulation. Recently, some reports show

that *miR-183* is detectable in human serum, urine, and in exosome vesicles from human sera and is involved in endocrine-associated signal transduction¹⁶⁻¹⁸⁾. Given that miRNAs, including *miR-183*, work as a cluster, it remains unclear whether *miR-183* is the only major miRNA involved in this process. Nevertheless, our results show that at least one miRNA, a functional small non-coding RNA, contributes to the direct conversion of cells from one lineage to another. Future directions should focus on elucidating the identity and role of other miRNAs in this process.

The present study has enlarged the potential cell sources suitable for regenerative medicine. Indeed, factors secreted by dental pulp stem cells have been identified to have multifaceted therapeutic benefits for recovery after several types of injuries or diseases, such as those of the spinal cord, heart, and liver¹⁹⁻²¹⁾. Therefore, pluripotent cells derived from oral tissue are an attractive and promising tool in the field of tissue regeneration and engineering. This study demonstrated that more than approximately 10 copies of *insulin* mRNA were involved in inducing dental pulp cells. We believe that dental pulp cells, or factors secreted by dental pulp cells, will become an acceptable source of cells for cell therapy in the future, replacing the more controversial sources currently used.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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