

Induction of *Oct3/4* during direct reprogramming of dental pulp cells

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Abstract

We previously reported the direct reprogramming of dental pulp cells to insulin-producing cells. Hence, the potential for transdifferentiation of dental pulp cells to cells of a developmentally unrelated lineage was verified. In the current study, we evaluated the multipotential plasticity of cells during the reprogramming process by examining the expression of the pluripotent stem cell markers *Oct3/4*, *Sox2*, and *Nanog*. The expression of *Oct3/4* was markedly induced approximately 5-fold at 10 days after induction, while *Sox2* levels decreased by approximately 25% of those observed in pre-induction cells, suggesting that the cells might have acquired pluripotency as a consequence of direct reprogramming. In conclusion, the induction and ectopic expression of transcriptional factors in dental pulp cells during direct reprogramming is a result of the activation of *Oct3/4*.

Introduction

We previously reported that dental pulp cells are able to be directly reprogrammed to insulin-producing cells. This reprogramming was proposed to be regulated by miRNAs [1]. We further proposed that dental pulp cells possess the necessary characteristics of multipotential plasticity in the terminal state of differentiation. The multipotency of the cells may contribute to the appearance of transdifferentiation to the developmentally unrelated lineage. We have also shown that dental pulp cells can differentiate into osteogenic, neurogenic, myogenic, and adipogenic cells by applying *in vitro* induction conditions similar to those used for human bone marrow cells [2-5]. Stem cell markers such as STRO-1, SSEA-1, Nanog, and *Oct3/4* were slightly expressed in rat dental pulp cells [2], and the down-regulation of these markers was observed during lineage differentiation [5]. Several genetic markers for pluripotent stem cells have been identified, including *Nanog*, *Sox2*, and *Oct3/4* (also known as *Pou5f1*) [6,7]. Induced, pluripotent stem (iPS) cells are transformed into fibroblasts by four defined genes, including *Sox2* and *Oct3/4* [8]. We hypothesized that the pluripotent state is manifest during the direct reprogramming of dental pulp cells and to address this question, we examined the expression of pluripotent stem cell markers during the process.

Materials and methods

Cell culture

Five-week old, male Wistar rats were used in this study (SHIMIZU Laboratory Supply, Kyoto, Japan). Primary culture was performed as previously described [4]. Briefly, the pulp tissue of the incisors was gently separated using reamers (25 mm #10; Morita Corporation, Osaka, Japan) away from the distal site of the molars to excise the apical bud, approaching from the central side of the mandible. The tissue was then minced and incubated in phosphate-buffered saline 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 a basic medium of MF start (TOYOBIO, Osaka, Japan) supplemented with 10% (v/v) fetal calf serum (FCS; Invitrogen) at 37°C in a humidified

atmosphere containing 5% CO₂. Animal experiments were carried out in accordance with the Osaka Dental University guidelines for the care and use of laboratory animals.

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. Cells were homogenized in Isogen (5 × 10⁶ cells/mL of Isogen) followed by extraction with 200 µL of chloroform and a further 650 µL of chloroform, precipitation with 1 mL of isopropanol, and washing of the precipitated RNA with 1 mL of 70% ethanol. All centrifugations were performed at 12,000 g at 4°C. The RNA obtained was dissolved in UltraPure™ DNase/RNase-free distilled water (Life Technologies, Carlsbad, CA, USA), and its concentration was measured by a NanoDrop (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 260 nm.

RT-PCR

First-strand cDNAs were synthesized from total RNA derived by a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. PCR amplification was performed using Ex Taq (Takara Bio Inc., Ohtsu, Japan) using the cDNA as a template. The specific PCR primer sets were designed using the Perfect Real Time Support System (Takara Bio Inc.). PCR amplification was performed with an initial

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incubation at 94°C for 3 min, followed by 30 cycles at 98°C for 10 s, 61°C for 30 s, and 72°C for 30 s for *Ngn3*; 35 cycles at 98°C for 10 s, 60°C for 30 s, and 72°C for 30 s for *Ins1* and *Ins2*; and ultimately a final extension step at 72°C for 5 min. The products were electrophoresed on 15% (w/v) acrylamide gel and stained with ethidium bromide. The intensity of amplified bands was calculated by ImageJ software (<https://imagej.nih.gov/ij/>). *Rps18* was used as an internal control to correct for differences among the samples.

Induction procedure

The procedure for direct reprogramming of dental pulp cells to insulin-producing cells was previously described [1]. Briefly, the rat dental pulp cells were inoculated into basic medium for 2 days. To induce the 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.

Quantitative real-time RT-PCR

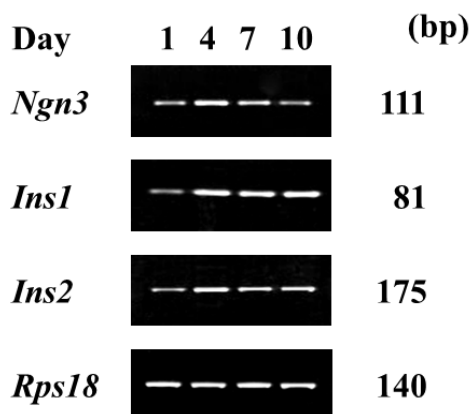
Specific PCR primer sets were designed using the Perfect Real Time Support System (Takara Bio Inc.). Real-time RT-PCR was performed

using Fast SYBR Green PCR Master Mix (Applied Biosystems) to examine the expression levels of each marker. PCR amplification was performed using a StepOne Plus™ System (Applied Biosystems) with an initial denaturation step of 20 s at 95°C, followed by 40 cycles each of 95°C for 3 s and 60°C for 30 s, and concluded with an automatic melting curve stage. *Rps18* was used as an internal control to correct the quantitative analysis. The expression levels are presented as the fold-change after induction compared to pre-induction values and were calculated using the $\Delta\Delta C_t$ method. Differences were statistically analyzed with the Mann-Whitney U test using SPSS software.

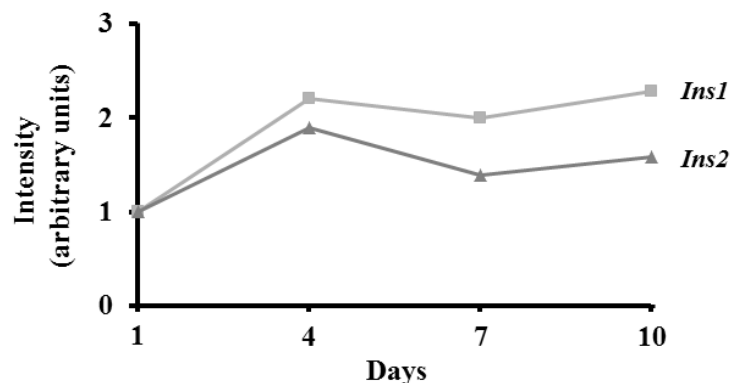
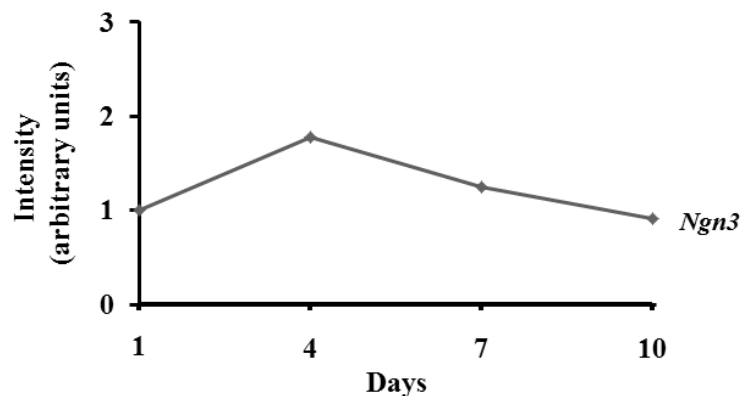
Results

Expression of endogenous genes after ectopic expression of *Pdx1*

Following transfection of *Pdx1*, the expression level of endogenous *Ngn3* was transiently increased 1.8-fold at 4 days after induction (Figures 1a and 1b). In contrast, expression of endogenous *Insulin* (*Ins1* and *Ins2*) was increased 2.3-fold and 1.9-fold, respectively, at 4 days, and induced levels were maintained from 4 to 10 days after induction (Figures 1a and 1b).



(a)



(b)

Figure 1. Expression of transcription markers associated with the endocrine lineage after induction. Expression was determined by RT-PCR. (a) Pre-induction (day 1) and days 4, 7, and 10 after transfection of *Pdx1*. Endogenous *Ngn3*, *Ins1*, and *Ins2* are shown. (b) Intensity of targeted bands was calculated using ImageJ software.

Endocrine lineage markers after induction

The expressions of *Neurod1* and *Pax4* were significantly increased more than 4-fold and 3-fold, respectively ($p < 0.05$). The expression of the specific marker for pancreatic beta cells, *MafA*, was also significantly increased approximately 2-fold after induction ($p < 0.05$) (Figure 2).

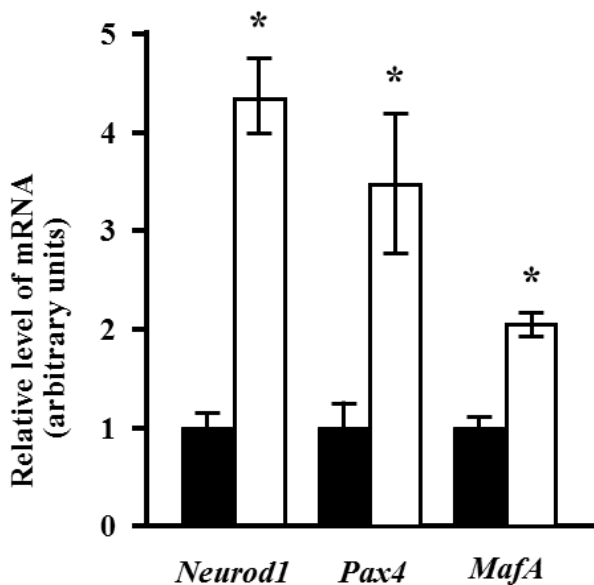


Figure 2. Changes in the expression of endocrine lineage markers after induction. Quantitative analysis was performed using real-time RT-PCR. The expression levels of *Neurod1*, *Pax4*, and *MafA* mRNA relative to the reference gene (*Rps18*), before (black bars), and 10 days after induction (white bars) are presented. The relative levels of mRNA expression are presented as the fold change after induction. Values represent the mean of four assay results for each sample. Values were calculated using relative quantity (RQ) study software (Applied Biosystems) and represent the statistical variability in the calculations of each sample's RQ value. The RQmin/RQmax values are graphically represented as error bars. The asterisk denotes a statistically significant difference ($p < 0.05$).

Change in expression of stem cell markers after induction

Notably, 10 days after induction, the expression of *Oct3/4* was significantly increased approximately 5-fold ($p < 0.05$). *Sox2* levels significantly decreased by approximately 25% of those observed pre-induction ($p < 0.05$). In contrast, the *Nanog* level did not change after induction (Figure 3).

Discussion

Organogenesis in higher organisms, initiated by extracellular signals, occurs by the systematic differentiation of stem or progenitor cells into mature cells. This is regulated by gene expression, which is determined by specific transcription factors that direct cell differentiation into a particular lineage. Since the expression of *Pdx1* is observed at the stage of pancreatic bud formation, activation of *Pdx1* is thought to be an important first step in the differentiation of the pancreas. There is a relatively wide range of *Pdx1*-positive regions in the endoderm, and *Pdx1* is essential for the proliferation and differentiation of the most immature pancreatic progenitor cells [9]. It is notably expressed in insulin-producing cells after passing through an *Ngn3*-positive stage, and is, subsequently, maintained at a high level of expression in mature β cells [9]. *Ngn3*, a bHLH-type transcription factor, is considered to be the most important transcription factor that distinguishes pancreatic endocrine progenitor cells from pancreatic progenitor cells [10]. In this study, when *Pdx1* was ectopically expressed in dental pulp cells,

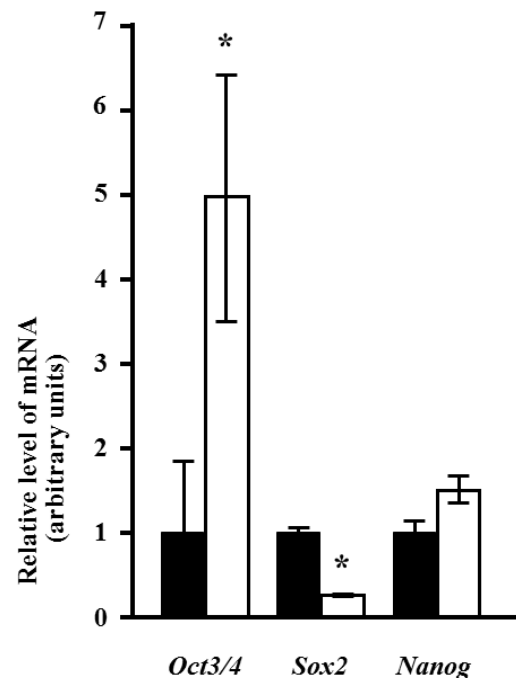


Figure 3. Changes in the expression of stem cell markers after induction. Quantitative analysis was performed using real-time RT-PCR. The expression levels of *Oct3/4*, *Sox2*, and *Nanog* mRNA relative to the reference gene (*Rps18*) are presented before (black bars) and 10 days after (white bars) induction. The relative level of mRNA expression is presented as the fold-change after induction. Values represent the mean of four assay results for each sample. Values were calculated using relative quantity (RQ) study software (Applied Biosystems) and represent the statistical variability in the calculations of each sample's RQ value. The asterisk denotes a statistically significant difference ($p < 0.05$).

expression of endogenous *Ngn3* was transiently expressed at day 4 after induction. In contrast, the expression of *Ins1* and *Ins2* increased at day 4 after induction, and maintained relatively high expression until gradually declining by day 10 after induction. Interestingly, this sequence of gene expression seems to reproduce the changes in gene expression that take place during organogenesis of the pancreas. By investigating the expression of endogenous, defined transcription factors triggered by the gene transfer of transcription factors, we predicted that the introduction of *Pdx1* and *Ngn3* into dental pulp cells might induce differentiation to the endocrine lineage. In fact, we reported that reprogramming of dental pulp cells by introducing the specific transcription factors *Pdx1* and *Ngn3* could successfully induce differentiation to insulin-producing cells [1]. In this study, the same experimental system was used. In the course of reprogramming of the dental pulp cells, the expression of *MafA* increased by approximately 2-fold. When *Pdx1*, *Ngn3*, and the endocrine cell marker *MafA* are introduced into undifferentiated pancreatic progenitor cells or pancreatic exocrine cells, β cells can be induced. Hence, three transcription factors can induce reprogramming to β cells [11,12]. The observation in this study implies that these three transcription factors work cooperatively to bring about endocrine cell differentiation.

During differentiation to the endocrine lineage, *Neurod1* and *Pax4* are sequentially activated in pancreatic progenitor cells and, subsequently, *MafA* activation is an indication of pancreatic β -cell differentiation. In the current study, the expressions of *Neurod1*, *Pax4*, and *MafA* were elevated. We previously reported the induction of insulin using this experimental procedure. Taken together, *MafA* activation is thought to contribute to the conversion of dental pulp cells into pancreatic β cells.

Ectopic expression of *Pdx1* induces the direct reprogramming of human keratinocytes to pancreatic insulin-producing cells [12]. Direct reprogramming of mouse fibroblasts to hepatocyte-like cells by defined factors was also reported [13,14]. Although we previously demonstrated that ectopic expression of *Pdx1* and *Ngn3* induces the direct reprogramming of dental pulp cells to insulin-producing cells [1], it has not been shown whether the mechanisms of pluripotency are involved in the direct reprogramming process. The changes in expression of pluripotent stem cell markers *Oct3/4*, *Sox2*, and *Nanog* following induction of dental pulp cells were examined in this work in order to test the hypothesis of pluripotent state involvement. *Oct3/4* is considered an essential component in all reprogramming procedures and activation of endogenous *Oct3/4* is a crucial step to obtain fully reprogrammed cells [13,14]. The main function of *Sox2* in maintaining pluripotency is by controlling the expression of transcription factors such as *Nr5a2* that indirectly causes a decrease in the expression of the *Oct3/4* gene [6,15]. *Sox2* is not, however, required for activating the Oct-Sox enhancer, which can be controlled by *Oct3/4* alone [16]. The Oct-Sox enhancer-dependent genes include *Nanog*, *Fgf4*, and *Fbx15*. *Nanog* has an Oct-Sox enhancer region, and its expression is controlled by *Oct3/4*. *Nanog* is known as a pluripotent factor but because it is not expressed in pluripotent P19EC stem cells and mature gonads like *Oct3/4* and *Sox2*, the contribution of *Nanog* to the pluripotency maintenance mechanism is dispensable [7,17]. In transcription factor-induced molecular reprogramming, *Nanog* is initially dispensable but becomes essential for dedifferentiated intermediate states to transit to ground state pluripotency [18]. In this study, the expression of *Oct3/4* by induction increased by approximately 5-fold while the expression of *Sox2* was decreased, but it is probable that the decrease in *Sox2* was caused indirectly by the induced expression of *Oct3/4*.

To summarize, the direct reprogramming of dental pulp cells to endocrine lineage cells led to an increased expression of *Oct3/4*, a pluripotent stem cell marker. This suggests that, during differentiation to genealogically distinct cells, pluripotency factors are induced at the genetic level, and that cell plasticity is a consequence. Thus, the multiplicity of dental pulp may arise as a result of the induction of pluripotent stem cell genes. Consequently, the acquisition of plasticity by dental pulp cells will make them attractive and promising materials for multiple regenerative medical applications. The present study has provided the evidence needed to expand the number of potential cell sources suitable for regenerative medicine applications.

Authorship and contributorship

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Competing interest

The authors have declared that no conflict of interest.

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