Full Paper

Gene Expression Profile of Dental Pulp Cells During Differentiation Into an Adipocyte Lineage

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Abstract. Gene regulation during in vitro differentiation into adipocytes was examined in rat dental pulp-derived cells. Insulin, 3-isobutyl-1-methylxanthine, and dexamethasone were added to induce adipogenesis. Cells containing lipid droplets were observed after induction as in 3T3 L1 cells. Rat dental pulp-derived cells showed their potential to differentiate into adipocytes in vitro. In both types of cells, the pluripotent markers *Oct-3/4* and *Sox2* were downregulated during differentiation, whereas the expression of *Nanog* was not significantly changed during differentiation. Interestingly, in the dental pulp-derived cells, the level of *Oct-3/4* was transiently induced at 1 week after induction and then significantly decreased during differentiation. Based on the expression profiles determined using GeneChip Arrays, 3418 probes across 10 clusters showed a difference in expression at 1, 2, and 3 weeks after induction versus before induction. Notably, genes in the PPAR signaling pathway including *Pparγ*, *Fabp4*, and the *C/EBP* family were upregulated by more than 3-fold. Upregulation of the PPAR pathways seems to be a critical signal transduction pathway in this differentiation system. These findings indicate that dental pulp-derived cells are a potential source of adipogenic cells, and their gene expression profile could be useful in future regenerative medicine applications.

Keywords: gene expression profile, adipogenesis, dental pulp, differentiation, stem cell

Introduction

Adult stem cells have been identified in the brain, bone marrow, peripheral blood, blood vessels, skeletal muscle, skin epithelium, digestive system, cornea, retina, liver, and pancreas (1). In fact, adult stem cells have been found in all tissues that develop from the embryonic germ layers. Studies have provided some evidence for the existence of multipotent adult progenitor cells (MAPCs) in the bone marrow that can differentiate into cells with mesodermal, endodermal, mesenchymal, or neuroectodermal characteristics (2, 3). The establishment of MAPCs from liver or gastric cells of adult mice was recently reported (4). Several markers for pluripotent stem cells have been identified, including *Nanog*, *Sox2*, and *Oct-3/4* (also known as *Pou5f1*) (5, 6). Induced pluripotent stem (iPS) cells are produced by transfecting four genes, including *Sox2* and *Oct-3/4*, into fibroblasts

(7). We previously reported that stem cell markers such as STRO-1, SSEA-1, *Nanog*, and *Oct-3/4* were expressed at the protein level in rat dental pulp-derived cells (8). Furthermore, *Oct-3/4*, *Sox2*, and *Nanog* were expressed at levels of at least 10 copies in rat dental pulp-derived cells when estimated based on the plasmid copy number. We have also shown that dental pulp-derived cells can differentiate into osteogenic, neurogenic, and myogenic lineage cells (9 – 11). Therefore, we hypothesized that cells derived from the dental pulp possessed characteristics of MAPCs and could differentiate in vitro into any type of somatic cell. The differentiation of adipocyte-progenitor cells such as 3T3 L1 cells or bone marrow cells into adipocytes was induced in vitro by treatment with 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, and insulin (12). In the present study, we examined the in vitro adipogenic differentiation of rat dental pulp-derived cells by applying in vitro conditions similar to those used for human bone marrow cells. Bone marrow cells undergo sequential growth, differentiation, and maturation after adipogenic induction (13). Therefore, to identify the molecules regulated during adipogenic dif-

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ferentiation of rat dental pulp-derived cells, we performed gene expression profiling of cells after *in vitro* induction and quantitative analysis of the gene expression of pluripotent markers during induction and compared the results with those for 3T3 L1 cells.

Materials and Methods

Cell culture

Five-week old male Wistar rats were used in this study (Shimizu Laboratory Supply, Kyoto). Primary culture was performed as previously described (8). Briefly, the pulp tissue of the incisors was gently separated using reamers, away from the distal site of molars to excise the apical bud, approaching from the central side of the mandible (25 mm #10; MANI, INC., Utsunomiya). 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 seeded onto a Lab-Tek II chamber slide (Nalge Nunc Int., Rochester, NY, USA). 3T3 L1 (JCRB9014) cells were obtained from Health Science Research Resources Bank (Japan Health Sciences Foundation, Tokyo). Both cell types were cultured in a basic medium of Dulbecco's modified Eagle's minimum essential medium with high glucose (DMEM; Sigma-Aldrich, St. Louis, MO, USA) 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.

In vitro adipogenic induction assay

The rat dental pulp-derived cells were maintained in the basic medium and then resuspended at a density of 6×10^4 cells/well in a Lab-Tek II chamber slide. To induce adipogenesis, the cells were cultured in two different media for 1 week each. Once the cultures were confluent, the medium was replaced with the adipogenesis initiation medium containing 0.5 mM IBMX and 1 mM dexamethasone for 7 days. The medium was then replaced with adipogenesis progression media containing 10 mg/mL insulin for 7 days. The cells were maintained in basic medium for 7 days with replenishment every 3 days. The 3T3 L1 cells were cultured in the adipogenesis initiation medium for 2 days followed by adipogenesis progression media for 2 days. The cells were then maintained in basic medium for 3 days.

Detection of lipid droplets after adipogenic induction

The degree of differentiation was evaluated by Oil-

Red O staining. The rat dental pulp-derived cells were fixed in 4% (w/v) paraformaldehyde solution (Muto Pure Chemical, Tokyo) for 20 min, washed twice with PBS(-), and treated with filtered 0.36% (w/v) Oil-Red O solution in 60% (v/v) isopropanol (Chemicon International, Temecula, CA, USA) at 25°C for 15 min. After washing with 60% (v/v) isopropanol three times, the cells were counterstained for 1 min with Kernechtrot staining solution (Muto Pure Chemical). The stained cells were washed three times with distilled water and mounted on glass slides using Permaflow (Immunotech, Marseille, France). The stained lipid droplets were observed under an optical microscope with a BX33-S CCD camera system (Olympus, Tokyo). To measure the amount of lipid droplets, after removing the last wash, the stained cells were lysed with 0.5 mL of dye extraction solution from an Adipogenesis Assay Kit (ECM950, Chemicon International) and transferred to a 96-well plate. The absorbance of extracted Oil-Red O was measured in a spectrophotometer at 520 nm. Differences in values were statistically analyzed with the Mann-Whitney U test using IBM SPSS Statistics 18 (IBM, Tokyo). The 3T3 L1 cells were washed with PBS(-) twice and stained with Oil-Red O solution, as above. The stained lipid droplets were observed under a phase-contrast microscope (Olympus).

Quantitative real-time RT-PCR assay

Total RNA was isolated using an RNeasy Kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions. The specific PCR primer sets were designed using the Perfect Real Time Support System (Takara Bio Inc., Otsu). The primer sets used to analyze the expression level of the pluripotent markers in rat dental pulp-derived cells were as follows: 5'-ATG TTT CTG CAG TGC CCG AAG-3' and 5'-GGT TAC AGA ACC ACA CTC GAA CCA-3' for *Oct-3/4*, 5'-CAG CAT GTC CTA CTC GCA GCA-3' and 5'-GGA ACT GGC CTC GGA CTT GA-3' for *Sox2*, 5'-TTG AAG ACT AGC AAC GGC CTG AC-3' and 5'-GCG TTC ATC AGA TAG CCC TGA GA-3' for *Nanog*, and 5'-AAG TTT CAG CAC ATC CTG CGA GTA-3' and 5'-TTG GTG AGG TCA ATG TCT GCT TTC-3' for rat ribosomal protein S18 (*Rps18*). The primer sets for 3T3 L1 cells were as follows: 5'-CAG ACC ACC ATC TGT CGC TTC-3' and 5'-AGA CTC CAC CTC ACA CGG TTC TC-3' for *Oct-3/4*, 5'-AAC CGA TGC ACC GCT ACG A-3' and 5'-TGC TGC GAG TAG GAC ATG CTG-3' for *Sox2*, 5'-GAA TTC TGG GAA CGC CTC ATC-3' and 5'-CCT TGT CAG CCT CAG GAC TTG-3' for *Nanog*, and 5'-TTC TGG CCA ACG GTC TAG ACA AC-3' and 5'-CCA GTG GTC TTG GTG TGC TGA-3' for mouse *Rps18*. The primer sets used to analyze the expression

level of genes in the PPAR pathways are listed in Table 1. 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. Real-time RT-PCR was performed using Fast SYBR Green PCR Master Mix (Applied Biosystems) to examine the expression levels of each adipogenic 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 values after induction compared with those before induction and were calculated using the $\Delta\Delta C_t$ method (14). Differences in values were statistically analyzed with the Mann-Whitney *U*-test using SPSS software version 18.0 and with the Kruskal-Wallis test using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Gene expression analysis using GeneChips

In vitro cRNA synthesis was carried out using total RNA as a template. The synthesized cRNA was hybridized to a GeneChip Rat Genome Array 230 2.0 (Affymetrix Inc., Santa Clara, CA, USA) containing 31099 probes and the signals were compared (Takara Bio). Genes showing a significant difference ≥ 2 -fold were extracted after filtering. Scatter-plot analyses were done to view the overall changes after induction. A heatmap based on gene expression profiles at each time-point during the differentiation was constructed and hierarchic clustering was performed for the extracted genes. Path-

way analysis was performed using the KEGG pathway database, and the frequency of appearance during gene ontology processes was analyzed using Fisher's exact test (15, 16).

RT-PCR

After first-strand cDNA synthesis, PCR amplification was performed using Ex Taq (Takara Bio). The primer sets used for RT-PCR were the same as those used for real-time RT-PCR. PCR amplification was performed with an initial incubation at 94°C for 10 s, followed by 30 cycles at 98°C for 10 s, 55°C for 30 s, and 72°C for 60 s, and a final extension step at 72°C for 7 min. Rat *Rps18* was used as an internal control to correct for the quantitative analysis. The products were electrophoresed on 3% (w/v) agarose gels and stained with ethidium bromide.

Results

Detection of lipid droplets after adipogenic induction

Before inducing differentiation, the morphology of the rat dental pulp-derived cells was fibroblast-like (Fig. 1A: a), the cells formed adipocyte-like cuboids after induction (Fig. 1A: b – d). At 3 weeks after induction, mature lipid droplets were particularly evident in the cytoplasm (Fig. 1A: d). The measured absorbance of Oil-Red O staining was significantly increased at 3 weeks post-induction compared with the values at 0, 1, or 2 weeks ($P < 0.05$, Fig. 1B). Before inducing differentiation, the 3T3 L1 cells showed a fibroblast-like morphology (Fig. 2A: a). Induction of 3T3 L1 cells caused the accumulation of numerous lipid droplets in the cytoplasm (Fig. 2A: b).

Table 1. Quantitative real-time RT-PCR target genes and primer sequences

Gene	Accession number	Primer nucleotide sequence
<i>Pparg</i> (Ppar γ)	NM_013124	Forward 5'-TGTCGGTTTCAGAAGTGCCTTG-3' Reverse 5'-TTCAGCTGGTCGATATCACTGGAG-3'
<i>Fabp4</i> (Fabp4/aP2)	NM_053365	Forward 5'-ATGTGCAGAAGTGGGATGGAA-3' Reverse 5'-ATTTCAGTCCAGGGCCTCGT-3'
<i>Cebpa</i> (CEBP α)	NM_012524	Forward 5'-TGCGCAAGAGCCGAGATAAAG-3' Reverse 5'-TCACGGCTCAGCTGTCCAC-3'
<i>Cebpb</i> (C/EBP β)	NM_024125	Forward 5'-ACCGGGTTTCGGGACTTGA-3' Reverse 5'-CCCGCAGGAACATCTTTAAGTGA-3'
<i>Cebpd</i> (C/EBP δ)	NM_013154	Forward 5'-TTCAGCGCCTACATTGATTCCA-3' Reverse 5'-CGCTTTGTGATTGCTGTTGAAGA-3'
<i>Adipoq</i> (Adiponectin)	NM_144744	Forward 5'-GGAAACTTGTGCAGTTGGATG-3' Reverse 5'-GGGTCAACCTTAGGACCAAGAA-3'
<i>Rps18</i> (Ribosomal S18)	NM_213557	Forward 5'-AAGTTTCAGCACATCCTGCGAGTA-3' Reverse 5'-TTGGTGAGGTCAATGTCTGCTTTC-3'

Fig. 1. Time-course analyses of in vitro adipogenic differentiation of rat dental pulp-derived cells before and at 1, 2, and 3 weeks after induction. A) The panels show Oil-Red O staining before induction (a) and at 1 (b), 2 (c), and 3 (d) weeks after induction to detect intracellular lipid droplets (indicated by arrows). Scale bars indicate 100 μ m. B) Uptake of Oil-Red O in lipid droplets, as measured by absorbance (520 nm), before and at 1, 2, and 3 weeks after induction. Values are means $\pm$ standard error of four independent slides in each group. The asterisk denotes a statistically significant value at week 3 vs. 0, 1, or 2 ($P < 0.05$).

Fig. 2. Analyses of in vitro adipogenic differentiation of 3T3 L1 cells. A) The panels show Oil-Red O staining before (a) and after (b) induction, representing the accumulation of intracellular lipid droplets in 3T3 L1 cells. Scale bars indicate 100 μ m. B) Quantitative analysis (real-time RT-PCR) of *Oct-3/4* (a), *Sox2* (b), and *Nanog* (c) gene expression relative to the reference gene (*Rps18*). Closed and open bars represent before and after induction, respectively. The relative level of mRNA expression is presented as the fold-change after induction vs. before induction. Values represent the mean of six 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 RQmin/RQmax values are graphically represented as error bars. The double asterisk denotes a statistically significant difference at after induction vs. before induction ($P < 0.01$).

Changes in the expression of undifferentiated stem cell markers

Oct-3/4, *Sox2*, and *Nanog* were expressed in 3T3 L1 cells when cultured in basal medium. After inducing differentiation, the expression levels of *Oct-3/4* and *Sox2* significantly decreased to 25% and 30%, respectively ($P < 0.01$, Fig. 2B: a, b). No significant change was de-

tected in the expression of *Nanog* (Fig. 2B: c). *Oct-3/4*, *Sox2*, and *Nanog* were expressed in rat dental pulp-derived cells under normal culture conditions 2 days after starting culture (i.e., before induction). The expression of *Oct-3/4* increased transiently by 3.2-fold at 1 week after induction ($P < 0.01$), but its expression level gradually decreased thereafter (Fig. 3a). The expression of *Oct-3/4*

at 2 and 3 weeks after induction decreased to 40% and 32%, respectively, compared with that at 1 week after induction ($P < 0.01$, Fig. 3a). Although the expression levels of *Sox2* and *Nanog* were very low, their expression was greatest before induction. The expression level of *Sox2* decreased to less than 30% of the basal level at weeks 1 and 2 ($P < 0.01$) and decreased even further at 3 weeks ($P < 0.01$, Fig. 3b). The levels of *Nanog* decreased steadily during differentiation and were about 40% lower at 3 weeks after induction compared with that before induction; however, these changes in expression were not significantly different (Fig. 3c).

Fig. 3. Changes in the expression of undifferentiated stem cell markers in rat dental pulp-derived cells. Quantitative analysis (real-time RT-PCR) of *Oct-3/4* (a), *Sox2* (b), and *Nanog* (c) gene expression relative to the reference gene (*rps18*). The relative level of mRNA expression is presented as the fold-change at 1, 2, or 3 weeks after induction vs. before induction (0 week). Values represent the mean 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 RQmin/RQmax values are graphically represented as error bars. The double asterisk denotes a statistically significant difference ($P < 0.01$).

Profiling of gene expression during adipogenic differentiation

Several genes were upregulated or downregulated during the differentiation process. Genes showing a significant difference in expression ≥ 2 -fold were detected after filtering. Overall, 10.6%, 6.6%, and 9.7% of genes were upregulated and 11.0%, 6.9%, and 11.6% of genes were downregulated at 1, 2, and 3 weeks, respectively, compared with their expression before induction (Fig. 4). Based on the analyses of gene expression profiles, 3418 probes were extracted after filtering and a heatmap of these genes was constructed. Using hierarchic cluster analysis, 10 clusters were obtained based on the heatmap (Fig. 5). The number of genes in each cluster ID was as follows: ID(1), 526; (2), 398; (3), 156; (4), 35; (5), 373; (6), 534; (7), 126; (8), 378; (9) 662; and (10), 270 (Fig. 5). Genes in clusters ID(1), (2), and (3) were upregulated at 1 week. Genes in clusters ID(4), (5) and (6) showed a gradual decrease in expression during differentiation. Cluster ID(7) included the genes that were upregulated at 2 weeks. Clusters ID(8), (9), and (10) included genes that were upregulated during the differentiation process (Fig. 6). Gene ontology analysis of various biological processes revealed that the regulation of fat cell differentiation, cellular lipid metabolism, and lipid metabolism pathways were significantly different ($P < 0.05$) in ID(3), while the positive regulation of fatty acid metabolism, positive regulation of lipid metabolism, and regulation of fatty acid metabolism pathways were significantly different ($P < 0.05$) in ID(7). This signaling pathway map was taken from the KEGG pathway database (http://www.genome.jp/kegg-bin/show_pathway?org_name=rno&mapno=03320&mapscale=&show_description=hide). Based on the KEGG pathway analysis, ID(3) and (7) included the genes in the PPAR signaling pathways that are involved in adipocyte differentiation, including Ppar γ ; C/EBP- β , - δ , and - α ; and adiponectin.

Fig. 4. Scatter plot analysis. Values are plotted for samples at 1, 2, or 3 weeks after induction vs. samples before induction. The X-axis represents the expression levels before induction. The Y-axis represents expression levels at 1 (A), 2 (B), and 3 (C) weeks after induction. Scaling was performed using GeneChip Operating Software. The blue lines indicate a 2-fold change. Gene with ≥ 2 -fold difference ($P < 0.01$) at 1, 2, or 3 weeks after induction vs. before induction were extracted.

Fig. 5. Heatmaps of gene expression profiles. The four expression patterns represent the signal levels of 3418 genes that showed a difference in expression at 1, 2, and 3 weeks after induction vs. before induction ($P < 0.01$). Red signals indicate high levels of expression and green signals indicate low levels of expression. The hierarchical clustering is shown with 10 color labels, and the number of genes in each cluster is indicated.

Fig. 6. Expression levels in all 10 clusters. The X-axis represents the samples before (0 week) and at 1, 2, and 3 weeks after induction. The Y-axis represents expression levels. The blue number in parentheses indicates the cluster ID.

Table 2. Changes in the expression levels of genes in the PPAR pathway

Gene	Weeks after induction		
	1	2	3
<i>Pparg</i>	13.1 (10.9 – 15.7)	2.0 (1.8 – 2.1)	1.0 (1.0 – 1.1)
<i>Fabp4</i>	23.3 (21.2 – 25.5)	5.4 (5.3 – 5.5)	2.2 (2.0 – 2.4)
<i>Cebpa</i>	3.0 (2.7 – 3.1)	1.2 (1.1 – 1.3)	1.6 (1.6 – 1.6)
<i>Cebpb</i>	3.0 (2.6 – 3.0)	1.4 (1.3 – 1.4)	0.9 (0.9 – 1.0)
<i>Cebpd</i>	7.5 (6.7 – 8.3)	0.9 (0.9 – 1.0)	0.9 (0.8 – 0.9)
<i>Adipoq</i>	303.0 (276.1 – 332.6)	1290.2 (1246.8 – 1335.0)	59.2 (53.4 – 65.5)

Values are means of three independent assays for each sample. The values in parenthesis are the ranges given for each value relative to the expression levels before induction, which were determined as $2^{-\Delta\Delta Ct}$ with $\Delta\Delta Ct \pm \text{S.E.M.}$, where S.E.M. = standard error of the $\Delta\Delta Ct$ value.

Changes in gene expression during differentiation into adipocytes

The changes in expression of genes in the PPAR pathway are shown in Table 2. Genes in this signaling pathway, including *Pparg*, *Fabp4*, members of the *C/EBP* family, and *adiponectin* were upregulated during differentiation. Notably, a 13.1-fold increase in *Pparg* and 23.3-fold increase in *Fabp4* were observed at 1 week after induction, followed by a slight decrease during the later stages of differentiation (Table 2). To confirm the expression of genes determined by real-time RT-PCR analysis, those that were extremely upregulated (>10-fold, Table 2) were checked by RT-PCR to determine their basal levels. The expression of *Pparg* and *Fabp4* showed low expression before induction (Fig. 7). Adiponectin was expressed at a very low level before induction, but its expression markedly increased following induction (Fig. 7), which confirmed the results obtained by real-time RT-PCR.

Discussion

In this study, we found that the expression of pluripotent markers for undifferentiated cells, particularly *Oct-3/4* and *Sox2*, tended to decrease after adipogenic differentiation of dental pulp-derived cells and 3T3 L1 cells. *Oct-3/4* is a homeodomain transcription factor of the POU family, and this protein is critically involved in the self-renewal of undifferentiated ES cells. *Oct-3/4* can form a heterodimer with *Sox2* and this complex can bind to DNA. The precise level of *Oct-3/4* governs three distinct fates of ES cells, as a less than 2-fold increase in

Fig. 7. Analysis of mRNA expression by RT-PCR. The mRNA expression levels before (0 W) and at 1, 2, and 3 weeks after induction (1W, 2W, and 3W) were determined by RT-PCR. *Pparg*, *Pparg*; *Fabp4*, *Fabp4*; *Adipoq*, *adiponectin*; *Rps18*, rat ribosomal protein S18.

expression causes differentiation into primitive endoderm and mesoderm, while repression of *Oct-3/4* induces the loss of pluripotency and dedifferentiation to the trophectoderm. Thus, a threshold level of *Oct-3/4* is required to sustain stem cell self-renewal, and up- or downregulation induces divergent developmental programs (17). In this study, the expression of *Oct-3/4* transiently increased by over 2-fold at 1 week after induction ($P < 0.01$) and then decreased to less than 40% of this level at 2 and 3 weeks after induction as compared with that at 1 week post-induction ($P < 0.01$). The downregulation of *Oct-3/4* to this threshold at 2 and 3 weeks after induction likely promoted differentiation under these inducing conditions in rat dental pulp-derived cells. In addition, *Sox2* expression was significantly decreased at 1, 2, and 3 weeks after induction, as compared with the levels before induction ($P < 0.01$). To better understand the changes in dental

pulp-derived cells, we compared them with the changes in gene expression of 3T3 L1 cells, which undergo a pre-adipose to adipose-like differentiation. Interestingly, the expression levels of *Oct-3/4* and *Sox2* were significantly decreased in 3T3 L1 cells after induction ($P < 0.01$), while no significant change was detected in the expression of *Nanog*, as in the dental pulp-derived cells. *Nanog* is specifically expressed in ES cells in the undifferentiated state, but is not a defining factor in inducible pluripotent stem cells derived from somatic cells (7). Thus, *Oct-3/4* and *Sox2* might be better markers than *Nanog* to assess differentiation.

MAPCs have been isolated from a variety of tissues including bone marrow, brain, skin, hair follicles, skeletal muscle, and blood vessels (13, 18–24). To verify the existence of MAPCs in dental pulp-derived cells, more evidence is required to demonstrate that these cells can differentiate into somatic cells. We previously showed that mineralization of rat dental pulp-derived cells increased at 6 weeks using an in vitro system of osteogenic induction (9). We also demonstrated the possibility that these cells could differentiate into a glial lineage by detecting the expression of *Gfap*, *Nes*, *Mtap2*, and *Tubb3* (10). We recently reported the MAPC-like differentiation potential of mouse dental pulp-derived cells, suggesting that DNA demethylation might trigger myogenic differentiation based on myosin heavy chain expression and myotube formation following 5-aza-2'-deoxycytidine treatment of these mouse cells (11). To further evaluate the multipotency of the cells and to identify the genes regulated during differentiation, we examined the in vitro differentiation of these cells into an adipocyte lineage. In this study, we observed that the cell morphology shifted from a fibroblast-like shape to a cubic form and mature lipid droplets were observed at 3 weeks after induction, suggesting that dental pulp-derived cells possess the ability to differentiate into cells of an adipocyte lineage. Gene expression profiling during the differentiation of progenitor cells into adipocytes has already been performed (25, 26). The pre-adipocyte cells 3T3 L1 propagated under normal conditions possess a fibroblast-like shape, exhibit high proliferative activity, and clonal expansion occurs after induction. After the clonal expansion phase, a cascade of transcription factors works to induce the expression of adipogenesis-specific genes for the differentiation of 3T3 L1 cells into mature adipocytes. *C/EBP β* and *C/EBP δ* are both expressed in the early stages of this process and act as triggers for clonal expansion caused by terminal differentiation (27, 28). In turn, *C/EBP β* and *C/EBP δ* induce the transcription of *C/EBP α* (29) and *Ppar γ* (26, 30). *C/EBP α* and *Ppar γ* direct the final phase of adipogenesis by activating the expression of adipocyte-specific genes, such as *Fabp4/aP2* (29, 31) and

adiponectin (32,33). Compared with 3T3 L1 cells, the dental pulp-derived cells showed a greater than 3-fold increase in *C/EBP- β* , *- δ* , and *- α* expression during the first week of adipogenic differentiation. Although *Ppar γ* and *Fabp4* were expressed at low levels before inducing differentiation in rat dental pulp-derived cells, the expression level of *Ppar γ* increased by more than 10-fold during the early stages of differentiation. However, up-regulation of the adipocyte marker *Fabp4* occurred until around weeks 1–2 of differentiation. Remarkably, the expression of *adiponectin* increased by more than 1000-fold at 2 weeks after induction. Insulin is known to promote adipocyte differentiation by activating PI-3-kinase and Akt activity (34), but in our experimental system, insulin was added 1–2 weeks after starting induction. Therefore, the expression of *adiponectin* was probably delayed. According to the gene ontology analyses, the regulation of fat cell differentiation, cellular lipid metabolism, and lipid metabolism pathways were upregulated at 1 week after induction, while the positive regulation of fatty acid metabolism, positive regulation of lipid metabolism, and regulation of fatty acid metabolism pathways were upregulated at 2 weeks after induction. The clusters ID(3) and (7) contained the genes involved in the regulation of the PPAR signaling pathways, including *Ppar γ* , *C/EBP- β* , *- δ* and *- α* ; and *adiponectin*, suggesting that this cascade is a critical signaling pathway in the differentiation of dental pulp-derived cells into cells of an adipocyte lineage.

In the current study, we have shown that rat dental pulp-derived cells can differentiate into cells of an adipocyte lineage following in vitro induction. Upregulation of the PPAR signaling pathway seems to be a key signal transduction pathway in the differentiation of these cells. It is possible that the molecules in this pathway contribute to the differentiation of dental pulp-derived cells, which possess MAPC qualities, into an adipocyte lineage as well as MAPCs derived from bone marrow. In the context of dental medicine, it was recently reported that adipose-derived stem cells can promote periodontal tissue regeneration in vivo (35) and that such cells will be useful for clinical cell-based therapy for periodontal disease (36). The cells were able to differentiate into bone and periodontal tissues when seeded onto common clinically used dental materials such as titanium hydroxyapatite, collagen, and poly(DL-lactic-co-glycolic acid) scaffolds in vitro and in vivo (37). Adipose-derived stem cells are usually collected by liposuction, a much more invasive surgical procedure than that needed to obtain dental pulp tissues. Moreover, adipocytes harvested by liposuction may also show hypertrophy or hyperplasia, which triggers abnormal production of hormones or adipocytokines such as leptin, tumor necrosis factor- α , or *adiponectin*

(38). Because insulin resistance, obesity, dyslipidemia, and hypertension are often observed in patients undergoing liposuction, the stem cells obtained from these patients may show an adverse phenotype. Studies have also shown that adipose tissue-derived mesenchymal stem cells have lost their genetic stability and are prone to form tumors (39). Thus, adipose tissue-derived cells should be continually monitored to check for cellular transformation. We now believe that is possible to overcome the limitations associated with adipose tissue-derived cells by using dental pulp-derived cells instead. Our study showed that the cells could differentiate into adipocytes, not just into odontoblasts, and we investigated the signaling pathways involved. Based on this ability to differentiate into other lineages, dental pulp-derived cells show potential for use in multiple regenerative medical applications, not just for dental medicine. Therefore, pluripotent cells derived from oral tissue are an attractive and promising tool in the field of tissue regeneration and engineering. We believe that the findings presented here support the possibility of using the pluripotent cells in dental pulp as a source of adipose tissue with the ability to function as clinically relevant engineering materials.

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References

- Wagers AJ, Weissman IL. Plasticity of adult stem cells. *Cell*. 2004;116:639–648.
- Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature*. 2002;418:41–49.
- Jiang Y, Vaessen B, Lenvik T, Blackstad M, Reyes M, Verfaillie CM. Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. *Exp Hematol*. 2002;30:896–904.
- Aoi T, Yae K, Nakagawa M, Ichisaka T, Okita K, Takahashi K, et al. Generation of pluripotent stem cells from adult mouse liver and stomach cells. *Science*. 2008;321:699–702.
- Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, et al. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell*. 2003;113:631–642.
- Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, et al. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell*. 2003;113:643–655.
- Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006;126:663–676.
- Nozaki T, Takeyasu T, Hirao A, Shinohara M, Daito M, Ohura K. Differentiation of rat dental pulp-derived cells into an osteoblastic lineage. *Oral Sci Int*. 2005;2:118–125.
- Takeyasu M, Nozaki T, Watanabe M, Shinohara M, Morita J, Hidaka A, et al. In vitro osteogenic differentiation potential of dental pulp stem cells. *J Oral Tissue Engin*. 2004;2:25–30.
- Takeyasu M, Nozaki T, Daito M. Differentiation of dental pulp stem cells into a neural lineage. *Pediatric Dent J*. 2006;16:154–162.
- Nakatsuka R, Nozaki T, Uemura Y, Matsuoka Y, Sasaki Y, Shinohara M, et al. 5-Aza-2'-deoxycytidine treatment induces skeletal myogenic differentiation of mouse dental pulp stem cells. *Arch Oral Biol*. 2010;55:350–357.
- Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. *Science*. 1999;284:143–147.
- Takada I, Suzawa M, Kato S. Nuclear receptors as targets for drug development: crosstalk between peroxisome proliferator-activated receptor γ and cytokines in bone marrow-derived mesenchymal stem cells. *J Pharmacol Sci*. 2005;97:184–189.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*. 2001;25:402–408.
- Al-Shahrour F, Diaz-Uriarte R, Dopazo J. Fati GO: a web tool for finding significant associations of Gene Ontology terms with groups of genes. *Bioinformatics*. 2004;20:578–580.
- Bauer S, Grossmann S, Vingron M, Robinson PN. Ontologizer 2.0 — a multifunctional tool for GO term enrichment analysis and data exploration. *Bioinformatics*. 2008;24:1650–1651.
- Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet*. 2000;24:372–376.
- Spradling A, Drummond-Barbosa D, Kai T. Stem cells find their niche. *Nature*. 2001;414:98–104.
- Gage FH. Mammalian neural stem cells. *Science*. 2000;287:1433–1438.
- Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science*. 1997;276:71–74.
- Weissman IL. Translating stem and progenitor cell biology to the clinic: barriers and opportunities. *Science*. 2000;287:1442–1446.
- Toma JG, Akhavan M, Fernandes KJ, Barnabe-Heider F, Sadikot A, Kaplan DR, et al. Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol*. 2001;3:778–784.
- Umemura T, Higashi Y. Endothelial progenitor cells: therapeutic target for cardiovascular diseases. *J Pharmacol Sci*. 2008;108:1–6.
- Kusuyama T, Omura T, Nishiya D, Enomoto S, Matsumoto R, Takeuchi K, et al. Effects of treatment for diabetes mellitus on circulating vascular progenitor cells. *J Pharmacol Sci*. 2006;102:96–102.
- Soukas A, Socci ND, Saatkamp BD, Novelli S, Friedman JM. Distinct transcriptional profiles of adipogenesis *in vivo* and *in*

- vitro*. J Biol Chem. 2001;276:34167–34174.
- 26 Takahara Y, Kobayashi T, Takemoto K, Adachi T, Osaki K, Kawahara K, et al. Pharmacogenomics of cardiovascular pharmacology: development of an informatics system for analysis of DNA microarray data with a focus on lipid metabolism. J Pharmacol Sci. 2008;107:1–7.
 - 27 Tang QQ, Lane MD. Activation and centromeric localization of CCAAT/enhancer-binding proteins during the mitotic clonal expansion of adipocyte differentiation. Genes Dev. 1999;13:2231–2241.
 - 28 MacDougald OA, Lane MD. Transcriptional regulation of gene expression during adipocyte differentiation. Annu Rev Biochem. 1995;64:345–373.
 - 29 Darlington GJ, Ross SE, MacDougald OA. The role of C/EBP genes in adipocyte differentiation. J Biol Chem. 1998;273:30057–30060.
 - 30 Tanabe Y, Matsunaga Y, Saito M, Nakayama K. Involvement of cyclooxygenase-2 in synergistic effect of cyclic stretching and eicosapentaenoic acid on adipocyte differentiation. J Pharmacol Sci. 2008;106:478–484.
 - 31 Lin FT, Lane MD. CCAAT/enhancer binding protein alpha is sufficient to initiate the 3T3-L1 adipocyte differentiation program. Proc Natl Acad Sci U S A. 1994;91:8757–8761.
 - 32 Yamauchi T, Oike Y, Kamon J, Waki H, Komeda K, Tsuchida A. Increased insulin sensitivity despite lipodystrophy in Crebbp heterozygous mice. Nat Genet. 2002;30:221–226.
 - 33 Tsuchida A, Yamauchi T, Kawakado T. Nuclear receptors as targets for drug development: molecular mechanisms for regulation of obesity and insulin resistance by peroxisome proliferator-activated receptor γ , CREB-binding protein, and adiponectin. J Pharmacol Sci. 2005;97:164–170.
 - 34 Takaguri A, Satoh K, Itagaki M, Tokumitsu Y, Ichihara K. Effects of atorvastatin and pravastatin on signal transduction related to glucose uptake in 3T3L1 adipocytes. J Pharmacol Sci. 2008;107:80–89.
 - 35 Tobita M, Mizuno H. Periodontal disease and periodontal tissue regeneration. Curr Stem Cell Res Ther. 2010;5:168–174.
 - 36 Huang GT, Gronthos S, Shi S. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. J Dent Res. 2009;88:792–806.
 - 37 Rimondini L, Mele S. Stem cell technologies for tissue regeneration in dentistry. Minerva Stomatol. 2009;58:483–499.
 - 38 Masuzaki H, Paterson J, Shinyama H, Morton NM, Mullins JJ, Seckl JR, et al. A transgenic model of visceral obesity and the metabolic syndrome. Science. 2001;294:2166–2170.
 - 39 Rubio D, Garcia-Castro J, Martib MC, de la Fuente R, Cigudosa JC, Lloyd AC, et al. Spontaneous human adult stem cell transformation. Cancer Res. 2005;65:3035–3039.