Mesenchymal progenitor cells in the root pulp of human primary teeth

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Contents

The following article and a new unpublished data (Fig. 3B) are part of this doctoral thesis:

> Characterization of mesenchymal progenitor cells in the crown and root pulp of primary teeth by Toriumi T, Takayama N, Murakami M, Sato M, Yuguchi M, Yamazaki Y, Eto K, Otsu M, Nakauchi H, Shirakawa T, Isokawa K, and Honda MJ, Biomedical Research, *in press*

Abstract

The existence of progenitor/mesenchymal stem cells (MSCs) has been demonstrated in human primary/deciduous teeth. In this study, dental pulp cells from root portion of primary teeth without discernible root resorption (root cells) were examined and compared with dental pulp cells from the crown portion (crown cells). Root cells and crown cells were characterized and compared to each other based on progenitor/MSC characteristics and on their generation efficiency of induced pluripotent stem (iPS) cells.

Root cells and crown cells included cells manifesting typical progenitor/MSC properties such as osteogenic and adipogenic differentiation potential and clonogenicity. Interestingly, root cells showed a higher expression level of embryonic stem cell marker, KLF4, than crown cells. Moreover, the number of colony-forming unit-fibroblast and cell proliferation rate were higher for root cells than crown cells, and the efficiency of generating iPS cells from root cells was approximately four times higher than that from crown cells. Taken together, these results suggest that root cells from primary teeth show the MSC-like properties and thus could be a potent alternative source for iPS cell generation and the subsequent transplantation therapy.

Introduction

Humans have two types of dentition over a lifetime, the primary (deciduous or milk) and secondary (permanent) dentition. By 6 years of age, the first permanent molar erupts beyond the primary second molar. This is followed by successive exfoliation of the primary teeth and their replacement by the succedaneous teeth. Connective tissue surrounded by a dental hard tissue, dentin, is termed dental pulp, and often remains inside the dental crown of exfoliated deciduous teeth. Recent studies suggested that this remnant dental pulp harbors a population of mesenchymal stem cells (MSCs) known as deciduous teeth stem cell (13). Indeed, two different populations have been isolated from exfoliated deciduous teeth by using distinct protocols: stem cells from human exfoliated deciduous teeth were isolated using an enzymatic digestion procedure (22), while a second population of immature dental pulp stem cells was obtained by a tissue explant method without enzymatic digestion (14). The deciduous teeth stem cells show a higher proliferation rate, better differentiation capability, and higher expression of many pluripotent markers such as OCT3/4, SOX2, NANOG, and REX1 than dental pulp stem cells in permanent teeth (9, 12). In addition, CD271 was previously detected as an MSC marker in the deciduous teeth stem cells, but not in the stem cells from permanent teeth or MSCs from periodontal ligament and gingival connective tissues (11, 21).

A tooth can be divided into two distinct parts, dental crown and dental root. Dental pulp in the crown portion is histologically similar to that in the root portion; both are derived from the migrating neural crest cells during embryonic development (17, 25, 33). Based on the dentin sialophosphoprotein expression and tissue-forming capacity of the pulp cells, different cellular characteristics were previously documented between dental pulp from the crown and the root portions of porcine third molars at the root formation stage (28). Expression patterns

of CD105, a surface marker, are also different between crown-derived (crown cells) and root-derived dental pulp cells (root cells) of supernumerary teeth. However, no such difference in CD105 expression was reported in permanent teeth (27), and there is no comparable study in primary teeth.

In the present study, primary teeth without root resorption were obtained, and were splitted into crown and root portions for pulp cell isolation and their characterization based on colony-forming unit-fibroblast (CFU-F) formation, growth potential, expression of surface epitopes, and osteogenic or adipogenic potential. Induced pluripotent stem (iPS) cells generated from the stem cells from human exfoliated deciduous teeth and the immature dental pulp stem cell populations demonstrated significantly higher efficiency of reprogramming than fibroblasts (3, 36). In addition, two of the most common reprogramming strategies, with and without c-MYC, were tested to transform root cells into iPS cells, and their reprogramming efficiency were compared with those for crown cells.

Materials and Methods

This study was approved by the Ethics Committee of Nihon University School of Dentistry and performed in accordance with the Helsinki Declaration.

Isolation and cultivation of dental pulp cells from crown and root portions

Cells from human primary teeth were isolated and cultured as described previously (14, 21). Two primary teeth (upper first incisor and lower canine) with no signs of root resorption were removed for the purpose of orthodontic treatment at Nihon University Dental Hospital, with informed consent from the patient and patient's parent. After extraction of the tooth, no resorption of the root apex was confirmed. The dental pulp without inflammation was harvested from the teeth as follows. A groove of 0.5-1.0 mm in depth was prepared around the circumference of the tooth using a sterile, hand-held, high-speed drill, and then the dental pulp was exposed by splitting the tooth with a chisel positioned to the groove. The whole pulp was gently isolated and the crown portion was separated from the root portion. For the primary culture, each pulp tissue was plated on a 35-mm culture dish and migrated cells were cultured in the MSC-growth medium comprising α-modified Eagle's medium (α-MEM; Wako, Osaka, Japan) supplemented with 15% fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin solution (Invitrogen, Carlsbad, CA, USA) at 37°C with 5% CO_2. The MSC-growth medium was exchanged every 3 days. The adherent cells were grown to 70% confluency and designated as passage zero (P0) cells. During passage 2-5, four individual cell lines were obtained and cryopreserved until further use.

Analysis of cell surface markers

Cell phenotypes were identified by flow cytometry assay using cell surface markers: CD105-PE, CD146-PE, SSEA-4-Alexa Fluor 647 (BD Biosciences, San Jose, CA, USA), and

STRO-1-FITC (Santa Cruz Biotechnology, Santa Cruz, CA, USA). In brief, cells at passage 3 were harvested by trypsinization, and then incubated for 30 min with the primary antibodies. Dead cells were identified by staining with 1 μg/mL propidium iodide (Sigma-Aldrich, St. Louis, MO, USA). Control cells were stained with appropriate IgG isotype controls (BD Biosciences). Flow cytometry data were analyzed by using the FlowJo software (Treestar, San Carlos, CA, USA).

RNA purification and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was purified from cells using NucleoSpin Tissue (Takara, Shiga, Japan), and 500 ng of total RNA was used for the reverse-transcription reaction with ReverTra Ace qPCR RT Master Mix and gDNA Remover (TOYOBO, Osaka, Japan), according to the manufacturer's instructions. Expression levels were normalized to that of endogenous human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). cDNA targets were amplified by PCR using SYBR Premix Ex Taq II (Takara). Freshly isolated human dental pulp tissue, a human iPS cell line (253G1; Riken Bio Resource Center, Ibaraki, Japan) (23), and a human embryonic stem (ES) cell line, KhES-3 (30), were used as controls. Reaction without template DNA was used as a negative control. The primers are listed in Tables 1 and 2.

Quantitative real-time PCR (real-time PCR)

Total RNA was prepared as described above, and cDNA was synthesized from 1 μg of total RNA using the ReverTra Ace qPCR RT Master Mix with gDNA. One-microliter aliquots of the cDNA samples were subjected to real-time PCR by using SYBR Premix Ex Taq II. Each real-time PCR reaction was performed in triplicate, and mRNA expression levels were calculated and normalized to the level of human GAPDH mRNA.

In vitro colony-forming unit-fibroblast (CFU-F) assay

Cells were plated onto 100-mm culture dishes (400 cells/dish) and incubated for 14 days in the MSC-growth medium. The cells were then stained with 0.05% (w/v) crystal violet (Wako) for 30 min and colonies formed were counted. One CFU-F is defined as a colony that contains > 50 cells. Each test was conducted four times.

Cell proliferation assay

Cells were seeded at 0.5×10^4 cells/well into 6-well plates and then cultured in the MSC-growth medium for the indicated periods. The cells were counted using a hemocytometer. Each test was conducted four times.

Cell-cycle analysis

Cell-cycle analysis was performed by using a Clicik-iT® EdU Flow Cytometry Assay Kit (Invitrogen) according to the manufacturer's instructions. In brief, cells were seeded at 1×10^4 cells/100-mm culture dish and cultured for 7 days in the MSC-growth medium. Cells were treated with 10 μM 5-ethynyl-2'-deoxyuridine (EdU) for 60 min, fixed with 4% paraformaldehyde for 15 min, washed once with 1% bovine serum albumin (BSA; Sigma-Aldrich), and then permeabilized with a saponin-based buffer for 30 min. Cells were then washed once and treated with the click-iT® reaction cocktail containing Alexa Flow 647 azide for 30 min. To measure DNA content, cells were stained with propidium iodide in 1% BSA. Cell cycle analysis was performed using a flow cytometer (Aria; Becton Dickinson and Company, Franklin Lakes, NJ, USA).

Osteogenic differentiation

Cells were seeded onto 12-well plates (1×10^4 cells/well) and cultured to 80% confluence in MSC-growth medium. Cells were then subcultured for various times in osteogenic induction medium comprising α-MEM supplemented with 5% FBS, 10^{-8} M dexamethasone

(Sigma-Aldrich), 50 μg/mL L-ascorbic acid phosphate, magnesium salt *n*-hydrate (Wako), 20 mM β-glycerophosphate (Sigma-Aldrich), and 100 nM calcitriol (Wako). After cells were cultured for the indicated days, they were fixed in 10% neutral buffered formalin for 15 min and stained with alkaline phosphatase (ALP) solution, pH 9.5 (NBT/BCIP ready-to-use tablets; Roche Diagnostics, Penzberg, Germany) for 30 min or 1% Alizarin red-S (Sigma-Aldrich) solution for 3 min.

In addition, ALP activity of cultured cells was assessed on the induction day 7 using LabAssay ALP (Wako) by a colorimetric method. Briefly, the treated cells were lysed in a protein extraction reagent (M-PER; Thermo Fisher Scientific) and protein concentration of the lysate was measured with Pierce BCA protein assay kit (Thermo Fisher Scientific). ALP activity was measured at 405 nm (Model 680 microplate reader; BioRad laboratories, Hercules, CA, USA). Each test was conducted in triplicate.

Adipogenic differentiation

Cells were seeded onto 60-mm-culture dishes (400 cells/dish) and cultured for 12 days in the MSC-growth medium. Cells were then subcultured for 42 days in adipogenic induction medium comprising α-MEM supplemented with 5% FBS, 10^{-6} M dexamethasone (Sigma-Aldrich), 0.5 μM isobutyl-1-methyl xanthine (Sigma-Aldrich), 5 μg/mL insulin (Sigma-Aldrich), and 50 μM indomethacin (Sigma-Aldrich). After culturing, cells were fixed in 10% neutral buffered formalin for 10 min, and then stained with Oil red-O (Sigma-Aldrich) solution for 30 min.

Expression profiling of cell cycle genes

Expression profiling was performed using an array of real-time RT-PCR primers for genes involved in the human cell cycle (PrimerArray Cell cycle [Human] PH002; Takara) in

the CFX96 Real-Time System (BioRad laboratories). The primer array contained a mixture of 96 primer pairs for 88 cell-cycle genes and 8 housekeeping genes. Gene expression was quantified using the PrimerArray Analysis Tool version 2.0 (Takara).

Preparation of supernatant containing retroviral particles

Constructions of pMXs vectors encoding OCT3/4, SOX2, KLF4, and c-MYC were performed as previously described (30). Retroviral particles containing the G glycoprotein of vesicular stomatitis virus were obtained by harvesting retroviral supernatants from the 293GPG retroviral packaging cell line. The supernatants were concentrated by centrifugation at 6000 × *g* for 16 h.

Reprogramming of dental pulp cells

Cell reprogramming was performed as previously described (30). Briefly, cells at passage 4 were seeded at 2×10^5 cells/25 cm^2 flask (Day 0) and cultured overnight. Cells were then infected with cryopreserved retroviral supernatants supplemented with 10 μg/mL protamine (Sigma-Aldrich) for two consecutive days. The supernatants contained retroviruses encoding OCT3/4, SOX2, KLF4, and c-MYC, or the same combination without c-MYC. On day 7, the infected cells were reseeded at 2.0×10^4 cells/60-mm culture dish or 5.0×10^4 cells/100-mm culture dish, onto which mouse embryo fibroblast (MEF) irradiated with 50 Gy were precultured. In the next day, culture medium was replaced with human ES cell medium, comprising Dulbecco's MEM and Nutrient Mixture F-12 Ham at 1:1 (DMEM-F12; Sigma-Aldrich), 20% KNOCKOUT Serum Replacement (KSR; Invitrogen), 0.1 mM non-essential amino acid (Invitrogen), 0.11 mM 2-mercaptoethanol (Invitrogen), 100 units/mL penicillin (Sigma-Aldrich), 100 μg/mL Streptomycin (Sigma-Aldrich), and 2 mM L-glutamine (Sigma-Aldrich) supplemented with 5 ng/mL recombinant human basic fibroblast growth factor

(bFGF; Wako). The medium was changed every 2 days. At 25 days after the first infection, ES cell-like colonies were picked manually and subcloned into 12-well plates seeded with MEF cells (passage 1). Cell colonies were maintained in human ES cell medium containing 5 ng/mL bFGF. To determine the reprogramming efficiency, the ES cell-like colonies were counted at day 30 when 4 factors were used, and at day 35 when 3 factors were used. To facilitate the identification of ES cell-like colonies, alkaline phosphatase (ALP) staining was performed by Vector Black Alkaline Phosphatase Substrate Kit II (Vector Laboratories, Burlingame, CA, USA).

In addition, viral transduction efficiency was compared between crown cells and root cells. Cells was seeded at 1.0×10^5 cells/well in 6-well plates, and then the pMYs retroviral vector containing green fluorescent protein (GFP) was introduced into cells at a multiplicity of infection = 1. After infection, cells were harvested by trypsinization and analyzed by the FACS Aria (BD Biosciences).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 15 min and then treated with 4% goat serum for 30 min. Primary antibodies used were anti-stage-specific embryonic antigen (SSEA)-4 (1:50, MAB4304; Millipore, Billerica, MA, USA), anti-tumor-related antigen (TRA)-1-60 (1:50, MAB4360; Millipore), and anti-TRA-1-81 (1:50, MAB4381; Millipore). Secondary antibodies used were Alexa Fluor 488- or 594-labelled goat anti-mouse IgG antibodies (1:500, A11029 and A11032; Life Technologies, Carlsbad, CA, USA). Images were obtained using the BZ-8100 fluorescence microscope (KEYENCE, Osaka, Japan).

Methylation profiling of promoter DNA

Genomic DNA was extracted by a NucleoSpin Tissue Kit (Macherey-Nagel, Düren,

Germany). To perform bisulfite genomic sequencing, 500 ng of genomic DNA was treated with the MethylEasy Xceed Rapid DNA Bisulphite Modification Kit (Human Genetic Signatures, Randwick, NSW, Australia), according to the manufacturer's recommendations. Promoter regions of the human OCT3/4, NANOG, and REX1 genes were amplified by PCR. The PCR products were isolated by squeezing right-sized bands of gel electrophoresis, and subcloned into the T-Vector pMD20 (Takara). Ten clones were randomly selected from each sample and sequenced with the M13 universal primer. The methylation detection primers are listed in Table 3. Sequencing data were analyzed by a quantification tool for methylation analysis (QUMA; Riken, Hyogo, Japan).

Karyotyping

Standard G-banding chromosome analysis was performed by Nihon Gene Research Laboratories Inc. (Miyagi, Japan). Selected iPS cell clones (n = 2) were karyotyped at passage 20.

Teratoma formation and histological analysis

Cell suspension of harvested ES cell-like colonies (25-μL aliquots of each) was injected into the testis capsule of an immunodeficient mouse (7 to 8-week-old NOD/SCID, n = 12; Nihon Clea, Tokyo, Japan) using a Hamilton microliter syringe. Twelve weeks after injection, the testes were removed and dissected to measure the size of teratomas. Samples were then fixed with 4% (v/v) paraformaldehyde in phosphate-buffered saline, and the paraffin-embedded tissues were sectioned and stained with either hematoxylin and eosin or Alcian-blue.

Statistical analysis

All statistical analyses were performed using Ekuseru-Toukei 2010 (Social Survey Research Information Co., Ltd., Tokyo, Japan). Statistical differences were evaluated using

Student's *t*-test. A *P*-value of less than 0.05 was considered significant.

Results

The characteristics of crown cells and root cells

Crown and root cells migrated from the isolated crown and root pulp tissues, and formed colonies within 3-4 days in the MSC-growth medium. When cells from the colonies were reseeded at low density, the single cells could generate colonies (Fig. 1A). Flow cytometry revealed that expression patterns of MSC markers were similar between crown and root cells, with high levels in CD105 (Endoglin) and CD146 (MUC18) expression, but low levels in STRO-1 and a human ES cell-specific surface antigen, SSEA-4 (Fig. 1B). RT-PCR analysis demonstrated that both crown and root cells expressed some ES cell markers (KLF4 and c-MYC), mesenchymal cell markers (Thy-1, Nestin, Col-I, Col-III, Vimentin, FGF2, OCN, ONN), but not others (ES cell markers; REX1, NANOG, OCT3/4, and SOX2, odontoblast markers; ALP, DSPP, and DMP1) (Fig. 1C). Real-time PCR analysis demonstrated that endogenous expression of KLF4 in root cells was twice as much as that in crown cells, a statistically significant difference. However, endogenous expression of c-MYC did not differ significantly between crown cells and root cells (Fig. 1D).

Cell proliferation potential

The number of CFU-F in root cells was 2.4 times larger than that in crown cells (Fig. 2A, B), and root cells showed a significantly higher proliferation rate than crown cells (Fig. 2C). Furthermore, cell cycle analysis by flow cytometry showed a significantly higher percentage of root cells in S-phase compared to crown cells (Fig. 2D, E). In addition, eight genes (ABL1, MCM7, MCM5, MCM3, CDK2, CCNA2, E2F3, ORC1L) involved in cell cycle progression were higher, and 2 genes (TGFB2, GADD45G) involved in growth arrest and DNA damage were lower in root cells than in crown cells (Table 4).

Differentiation capacity

Osteogenic differentiation potential was examined by ALP and Alizarin red-S staining. Crown and root cells in osteogenic induction medium showed similar levels of ALP staining at 3, 7, and 14 days in culture, and equal amounts of Alizarin red-positive mineralized nodules at 21 days in culture (Fig. 3A). No significant difference in quantitated ALP activity was detected between crown and root cells at day 7 of culture in osteogenic induction medium (Fig. 3B).

Adipogenic differentiation potential was examined by Oil red-O staining. After 35 days in adipogenic induction medium, crown and root cells showed cytoplasmic Oil red-O-positive droplets (Fig. 3C).

Characterization of iPS cells from crown cells and root cells

Ordinary flat, or fusiform, dental pulp cells were converted into compact clusters with ES cell-like morphology after 2 weeks of reprogramming by transduction of four genes, and after 4 weeks of reprogramming by three genes (Fig. 4A). To characterize the ES cell-like clones, three clones from each type of pulp (i.e., iPS-crown cells-1, -2, -3, and iPS-root cells-1, -2, -3) were randomly chosen and the established clones were subcultured until passage 26.

All colonies were stained strongly positive for human ES cell-specific surface antigens including ALP, SSEA-4, TRA-1-60, and TRA-1-81, and showed sharp and defined borders (Fig. 4B). All clones also expressed the genes typically detected in human ES cells (REX1, NANOG, endogenous OCT3/4, SOX2, KLF4, and c-MYC). Overall, the expression of ES cell markers in the iPS-cell colonies from crown and root cells was indistinguishable from that of KhES-3, a human ES cell line (Fig. 4C).

Silencing of the retroviral transgenes is essential for correct generation of iPS cells (4). Therefore, the expression of pluripotency markers in the iPS cell clones and the parental dental

pulp cells were examined. Silencing of transgenes encoding OCT3/4, SOX2, KLF4, and c-MYC was evident in dental pulp cell derived-iPS cell clones by RT-PCR analysis, although incomplete silencing of KLF4 and c-MYC transgene expression was observed in two iPS-crown cells (Fig. 4C). Karyotyping of iPS-crown cells and -root cells at passage 20 revealed normal karyotypes (46, XY) (Fig. 4D).

Reprogramming efficiency

Reprogramming efficiency was expressed in a ratio of the number of iPS-cell colonies to the crown or root cells seeded for retroviral infection. A remarkably higher number of iPS-root cells was observed compared with iPS-crown cells (Table 5). When cells were transduced with 3 genes (i.e., without c-MYC), the average reprogramming efficiency (n = 5) of root cells was 0.0160% (range, 0.014-0.018%) and that of crown cells was 0.0036% (range, 0.002-0.006%). For the case of transduction with 4 genes, the average reprogramming efficiency (n = 3) of root cells was 0.0535% (range, 0.025-0.075%) and that of crown cells were 0.0165% (range, 0.005-0.025%) (Table 5). Although there was no significant difference in retroviral infection efficiency between crown cells and root cells (Fig. 5), higher reprogramming efficiency in root cells was confirmed by this results (Table 5).

Promoter DNA methylation status

Demethylation rate in the promoter regions of OCT3/4, NANOG, and REX1 were analyzed and compared in iPS cells and parental cells. Methylation in iPS-crown cells and -root cells decreased significantly compared to their parent pulp cells (Fig. 6), suggesting active promoter activity of OCT3/4, NANOG and REX1 in both iPS-crown and -root cells.

Teratoma formation from iPS cells in vivo

iPS-crown cells were injected into 14 testes in 7 NOD/SCID mice, and iPS-root cells

were injected into 10 testes in 5 NOD/SCID mice for examining teratoma formation. Tumor formation with parenchyma was observed in 4 out of 14 testes injected with iPS-crown cells (29%) and 4 out of 10 testes injected with iPS-root cells (40%) (Table 6). However, no difference in tumor size was observed among the teratoma (Table 7).

Histologically, tumors generated by iPS-crown cell contained tissues of three germ layers origin (Fig. 7A): i.e., ectodermal pigmented epithelium (Fig. 7B), endodermal gut-like epithelium (Fig. 7C), and mesodermal adipose tissue (Fig. 7D). The iPS-root cell-derived tumors also contained tissues of three germ layer origin such as neuroepithelial rosettes and pigmented epithelium, gut-like epithelium, and cartilage (Fig. 7E-I).

Discussion

The aim of this study was to compare the characteristics of dental pulp cells from the crown (crown cells) and root portions (root cells) of the identical human primary teeth. Previous studies demonstrated MSCs could be isolated from the dental crown pulp of exfoliated deciduous teeth (14, 22); however, the characteristics of root cells remain unknown.

In this study, it was shown that root cells contain a cell population with typical MSC properties such as clonogenicity and multipotency to differentiate into osteoblasts and adipocytes. Immunophenotypic expression patterns of MSC markers and the gene expression patterns by RT-PCR in root cells were similar to those in crown cells. Interestingly, root cells showed significantly higher level of KLF4 gene expression compared to crown cells by quantitative real-time PCR. Furthermore, root cells were characterized by a significantly higher CFU-F formation and proliferation potential as well as a higher percentage of cells in S phase compared to crown cells.

Primer array analysis for cell cycle-related gene expression identified 10 cell cycle progression-related genes with distinct expression levels between root cells and crown cells, with lower expression of transforming growth factor β (TGFβ) mRNA and higher expression of cyclin A, CDK2, and E2F3 mRNA in root cells. Cyclin A synthesis promotes CDK2 activity (8, 35), and cyclin A together with c-MYC are target genes of TGFβ2 in cell cycle regulation (19). In turn, TGFβ2 has an inhibitory effect on cell growth (19, 34) and a role in inducing the E2F gene (2, 5, 20). Collectively, these previous findings suggest that TGFβ2 could regulate cycline A through E2F3 in both crown and root cells.

IPS cells generated from human dental pulp cells of the third molar or of exfoliated deciduous teeth have higher reprogramming efficiencies than skin fibroblasts (24, 31, 36).

Some studies suggest that proliferation rate is positively correlated with reprogramming efficiency of cells (10, 26), while other previous studies suggest that proliferation rate is not positively correlated with the efficiency (6, 32). Several reports implicate that the endogenous expression of reprogramming factors in cells is positively correlated with reprogramming efficiency of cells (1, 6, 15, 18). However, Tamaoki and colleagues (31) reported that the measured endogenous expression level of KLF4 did not correlate with the reprogramming efficiency of dental pulp cells in permanent teeth. Interestingly, the present study showed a higher reprogramming efficiency for root cells than crown cells. Reprogramming efficiencies of dental pulp cells were previously reported as 0.01-0.06% (31) or 0.0026-0.0302% (24). In the present study, reprogramming efficiency was 0.0160% for root cells and 0.0036% for crown cells, indicating that reprogramming of root cells was approximately four times more efficient than that of crown cells. These results suggest that root cells from primary teeth are an excellent cell source for the generation of iPS cells, and that proliferation rate or endogenous expression of KLF4 appears positively correlated with reprogramming efficiency. However, as described above, these findings are supportive and also partially contradictory to previous studies, and the exact effect of proliferation potential and KLF4 expression on reprogramming efficiency remains controversial. Further studies on the mechanisms of reprogramming efficiency are required.

The culture duration required for iPS cell generation were compared between crown and root cells, but the duration and their morphology of ES cell-like colonies were indistinguishable. Instead, it was found that iPS cell generation was slower by 3-factor induction and somewhat quicker by 4-factor induction in both crown and root cells. This result is consistent with those previously reported for human fibroblasts (16, 29) and human dental pulp cells from third molars (31).

Conclusions

This study was to assess that root pulp cells in primary teeth also contain cells harboring MSC characteristics. Both root and crown cells possessed similar immunophenotypic expression patterns of MSC markers, and osteogenic and adipogenic differentiation potency. However, compared to crown cells, root cells showed significantly higher CFU-F formation and proliferation potential, and mRNA expression of endogenous KLF4 was moderately enhanced in root cells. In addition, reprogramming efficiency of root cells was approximately four times as high as that of crown cells.

Taken together, a cell population competent for iPS generation are present in root cells in primary teeth, and the competent cells are more in number compared to those in crown cells of primary teeth. This conclusion indicates that root cells from primary teeth could be a potent alternative cell source of MSCs for iPS cell generation.

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Table 1 Primer sequences for dental pulp cells

Gene	*Primer Sequence (F: Forward, R: Reverse)*		*Size (bp)*	*Accession number*
Collagen type I (Col-I)	F:	AGGCCCTCAAGGTTTCCAAGG	233	Y00724
	R:	CCAGACCATTGTGTCCCCTAA		
Collagen type III (Col-III)	F:	TGGTGTTGGAGCCGCTGCCA	373	NM_000090
	R:	CTCAGCACTAGAATCTGTCC		
Fibroblast growth factor 2 (FGF2)	F:	GTGTGTGCTAACCGTTACCT	237	NM_002006
	R:	GCTCTTAGCAGACATTGGAAG		
Vimentin	F:	GGGACCTCTACGAGGAGGAG	200	X56134
	R:	CGCATTGTCAACATCCTGTC		
Thymus cell antigen-1 (Thy-1)	F:	TGGCCATCAGCATCGC	168	NM_006288
	R:	TTGTCTCACGGGTCAG		
Nestin	F:	GCGTTGGAACAGAGGTTGGA	327	NM_006617
	R:	TGGGAGCAAAGATCCAAGAC		
Alikaline phosphatase (ALP)	F:	CGCCTACCAGCTCATGCATAAC	400	NM_000478
	R:	GTCAATTCTGCCTCCTTCCACC		
Osteocalcin (OCN)	F:	AGCCTTTGTGTCCAAGCAGGAG	276	X53698
	R:	AAGGGGAAGAGGAAAGAAGGGTG		
Osteonectin (ONN)	F:	GGAAGAAACTGTGGCAGAGGTGAC	464	J03040
	R:	TGTTGTCCTCATCCCTCTCATACAG		
Dentin sialophosphoprotein (DSPP)	F:	CCATTCCAGTTCCTCAAAGCAAACC	489	NM_014208
	R:	CAGCGACATCCTCATTGTGACC		
Dentin matrix protein 1 (DMP1)	F:	ATCCTGTGCTCTCCCAGTAACC	475	NM_004407
	R:	CTCATTGTCAAGTTCCCTGCTCTC		
Endogenous (endo) OCT3/4	F:	GACAGGGGGAGGGGAGGAGCTAGG	144	NM_002701
	R:	CTTCCCTCCAACCAGTTGCCCCAAAC		
Endogenous (endo) SOX2	F:	GAAAGAAAGGGAGAGAAGTTTGAG	130	NM_003106
	R:	GCAAACTGGAATCAGGATCAA		
Endogenous (endo) KLF4	F:	AAGAGTTCCCATCTCAAGGCACA	91	NM_004235
	R:	GGGCGAATTTCCATCCACAG		
Endogenous (endo) c-MYC	F:	CGGATTCTCTGCTCTCCTCGAC	199	NM_002467
	R:	CCTCCAGCAGAAGGTGATCCA		
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	F:	GCACCGTCAAGGCTGAGAAC	138	NM_002046.3
	R:	TGGTGAAGACGCCAGTGGA		

Table 2 Primer sequences for iPS cells

Gene		*Primer Sequence (F: Forward, R: Reverse)*	*Size (bp)*	*Accession number*
Reduced expression 1 (REX1)	F:	CAGATCCTAAACAGCTCGCAGAAT	306	AF450454
	R:	GCGTACGCAAATTAAAGTCCAGA		
NANOG	F:	TTGGAAGCTGCTGGGGAAG	193	NM_024865
	R:	GATGGGAGGAGGGGAGAGGA		
Transgene (Tg) OCT3/4	F:	CAACAACCGAAAATGCACCAGCCCCAG	475	
	R:	TACAGGTGGGGTCTTTCATTC		
Transgene (Tg) SOX2	F:	TGCAGTACAACTCCATGACCA	524	
	R:	TACAGGTGGGGTCTTTCATTC		
Transgene (Tg) KLF4	F:	TGCGGCAAAACCTACACAAAG	477	
	R:	TACAGGTGGGGTCTTTCATTC		
Transgene (Tg) c-MYC	F:	CAACAACCGAAAATGCACCAGCCCCAG	503	
	R:	TACAGGTGGGGTCTTTCATTC		

For the amplification of endogenous OCT3/4, SOX2, KLF4, and c-MYC, and GAPDH, primers listed in Table 1 were used.

Table 3 Primer sequences for bisulfite genomic sequencing

Primer Name for Methylation Detection		*Primer Sequence (F: Forward, R: Reverse)*
mehOCT3/4	F:	TAGTTGGGATGTGTAGAGTTTGAGA
	R:	TAAACCAAAACAATCCTTCTACTCC
mehNANOG	F:	TGGTTAGGTTGGTTTTAAATTTTTG
	R:	AACCCACCCTTATAAATTCTCAATTA
mehREX1	F:	GGTTTAAAAGGGTAAATGTGATTATATTA
	R:	CAAACTACAACCACCCATCAAC

Table 4 Expression profiling of cell cycle genes

Symbol	*Name*	*Relative Expression*		*Accession No.*
		Crown cells	*Root cells*	
ABL1	*Homo sapiens* v-abl Abelson murine leukemia viral oncogene homolog 1, transcript variant b	1.0	2.10	NM_007313
MCM7	*Homo sapiens* minichromosome maintenance complex component 7	1.0	2.00	NM_005916
MCM5	*Homo sapiens* minichromosome maintenance complex component 5	1.0	1.67	NM_006739
MCM3	*Homo sapiens* minichromosome maintenance complex component 3	1.0	1.63	NM_002388
CDK2	*Homo sapiens* cyclin-dependent kinase 2 transcript variant 2	1.0	1.61	NM_052827
CCNA2	*Homo sapiens* cycline A2	1.0	1.56	NM_1237
E2F3	*Homo sapiens* E2F transcription factor 3	1.0	1.51	NM_001949
ORC1L	*Homo sapiens* origin recognition complex, subunit 1-like (yeast)	1.0	1.50	NM_004153
TGFB2	*Homo sapiens* transforming growth factor, beta 2	1.0	0.43	NM_003238
GADD45G	*Homo sapiens* growth arrest and DNA-damage-inducible, gamma	1.0	0.17	NM_006705

Table 5 Reprogramming efficiency of iPS cells

Cells	*Reprogramming factor* *	*Number of transduced cells seeded onto MEF*	*Average number of ALP-positive colonies (range)*	*Reprogramming efficiency (%)*
Crown**	O/S/K	5×10^4 cells	1.8 (1-3)	0.0036
Root **	O/S/K	5×10^4 cells	8.0 (7-9)	0.0160
Crown ***	O/S/K/M	2×10^4 cells	3.3 (1-5)	0.0165
Root ***	O/S/K/M	2×10^4 cells	10.7 (5-15)	0.0535

* O: OCT3/4, S: SOX2, K: KLF4, M: c-MYC
** deciduous upper first incisor (7-year-old boy)
*** deciduous lower canine (6-year-old boy)

Table 6 Teratoma formation from iPS cells

cells	*No change*	*Cyst formation*	*Teratoma formation*
iPS-crown cells	4	6	4
iPS-root cells	1	5	4

Table 7 Size of teratoma

Number	*iPS-crown cells (mm)*	*iPS-root cells (mm)*
1	15 × 14	18 × 18
2	13 × 10	18 × 17
3	10 × 9	16 × 12
4	10 × 8	7 × 6

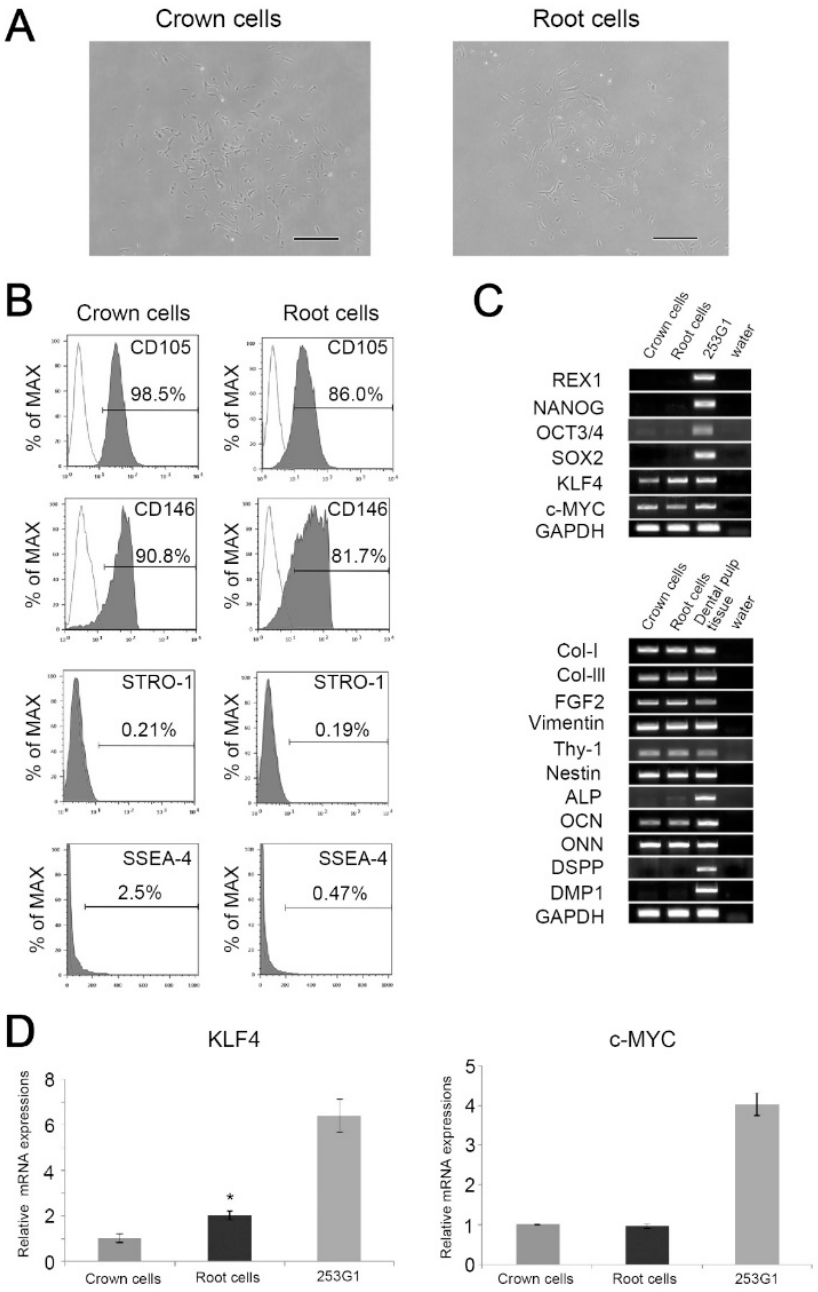
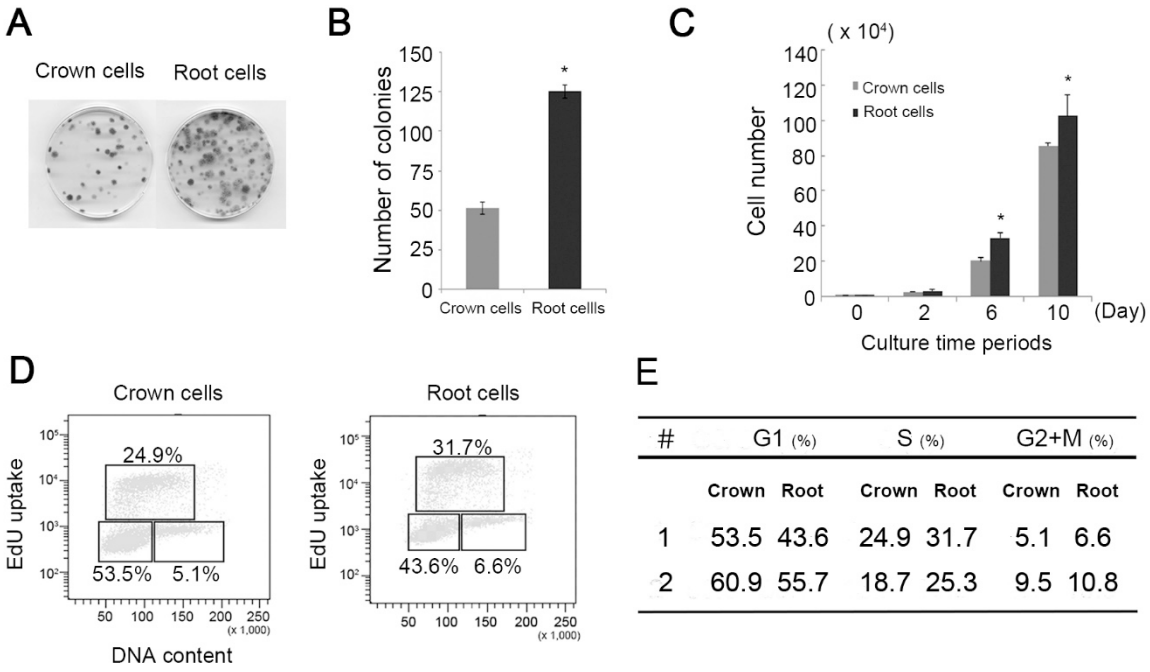


Fig. 1 Characterization of crown cells and root cells. **(A)** Phase contrast microscopy. Scale bars represent 500 μm. **(B)** Immunophenotyping by flow cytometric analysis. The filled histograms show specific staining for the indicated markers and the open histograms show nonspecific staining for isotype controls. **(C)** RT-PCR analysis. A human iPS cell line 253G1 and dental pulp tissues were used as positive controls. **(D)** Real-time PCR analysis of endogenous KLF4 and c-MYC mRNA levels. Relative expression level of endogenous KLF4 in root cells was twice of that in crown cells (*$P < 0.05$). In contrast, relative expression levels of endogenous c-MYC in crown and root cells were similar to each other. A human iPS cell line 253G1 was used as a positive control. Data shown are means ± standard deviation (S.D) (n = 3).



#	G1 (%)		S (%)		G2+M (%)	
	Crown	Root	Crown	Root	Crown	Root
1	53.5	43.6	24.9	31.7	5.1	6.6
2	60.9	55.7	18.7	25.3	9.5	10.8

Fig. 2 Cell proliferation potential of crown cells and root cells. **(A)** Colony-forming unit-fibroblast (CFU-F) assays. **(B)** The number of CFU-Fs in root cells was 2.4 times higher than that in crown cells (**P* < 0.05). Data shown are means ± S.D. (n = 4). **(C)** The initial 0.5 × 10^4 crown cells and 0.5 × 10^4 root cells resulted in 171- and 206-fold proliferation at day 10, respectively. Data shown are means ± S.D. (n = 4); **P* < 0.05. **(D)** Representative data of cell-cycle analysis. The cells were stained with Alexa Fluor 647 azide (y-axis, log scale) to quantitate 10 μM 5-ethynyl-2'-deoxyuridine (EdU) uptake and with propidium (PI) to detect total DNA (x-axis, linear scale). The lower left box represents G1-phase cells, the upper box S-phase cells, and the lower right box G2/M-phase cells. The numbers given in the boxes indicate the percentage of gated events. Cultures of root cells tended to contain a higher percentage of cells in the S and G2/M phase, and a lower percentage of cells in G1 phase compared to cultures of crown cells. **(E)** Cell-cycle analysis was performed twice (#1, 2) and the results of the two experiments showed a similar pattern.

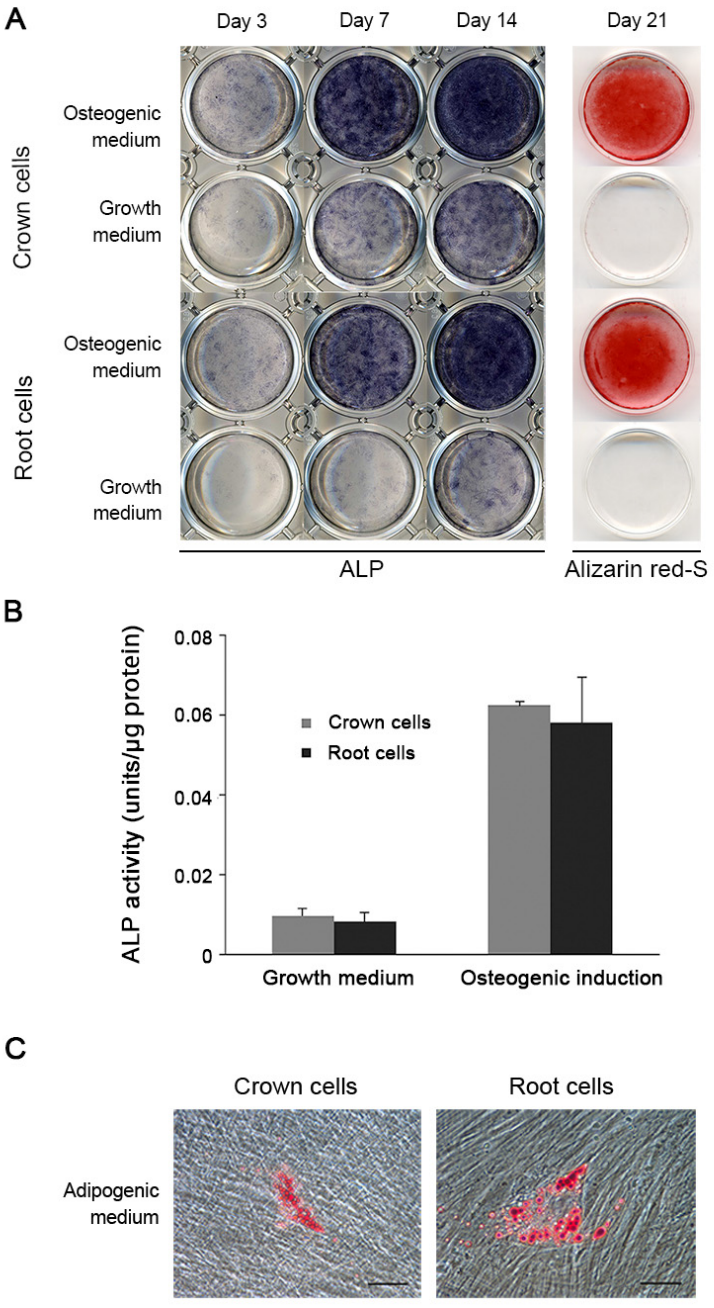


Fig. 3 Osteogenic and adipogenic differentiation potential. **(A)** Crown cells and root cells under osteogenic induction medium showed similar ALP and Alizarin red-S staining patterns. **(B)** ALP activity of crown and root cells cultured in growth medium or osteogenic induction medium for 7 days. Data shown are means ± S.D. (n = 3) **(C)** Oil red-O-positive lipid droplets observed in crown cells and root cells after 35 days in adipogenic induction medium. Less than 1% of cells showed Oil red-O staining in both crown cells and root cells. Scale bars represent 50 μm.

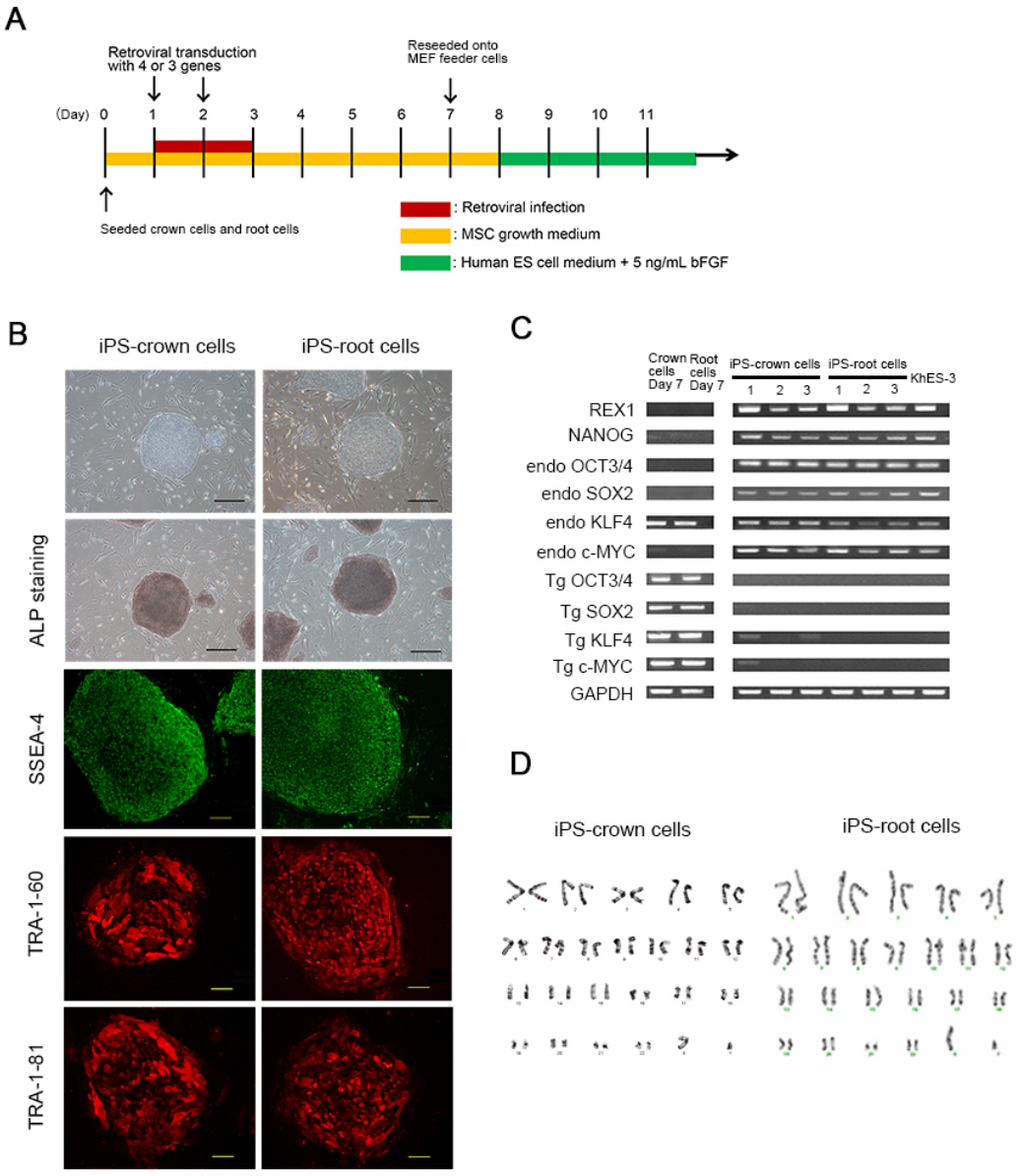


Fig. 4 Generation of iPS cells from crown cells and root cells. **(A)** Time course for reprogramming of crown cells and root cells. **(B)** ES cell-like colonies generated from crown cells and root cells were positive for ALP staining and human ES cell markers (SSEA-4, TRA-1-60, and TRA-1-81). Black scale bars represent 500 μm, yellow bars 100 μm. **(C)** Semi qRT-PCR analysis of ES cell marker genes in three iPS-crown cell clones (iPS-crown cells-1, -2, -3) and three iPS-root cells clones (iPS-root cells-1, -2, -3). KhES-3, a human ES cell line, was used as a positive control. **(D)** Karyotyping of iPS-crown cells-1 and iPS-root cells-1.

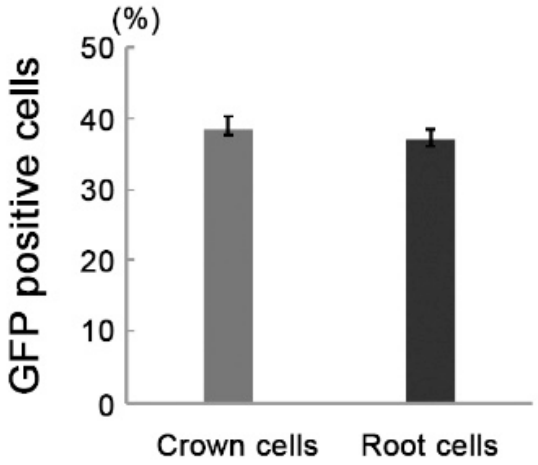


Fig. 5 Viral transduction efficiency of pMY retroviral vector. Crown cells and root cells were infected with pMY-GFP retroviral vector. Multiplicity of infection was 1. Data shown are means ± S.D. (n = 4).

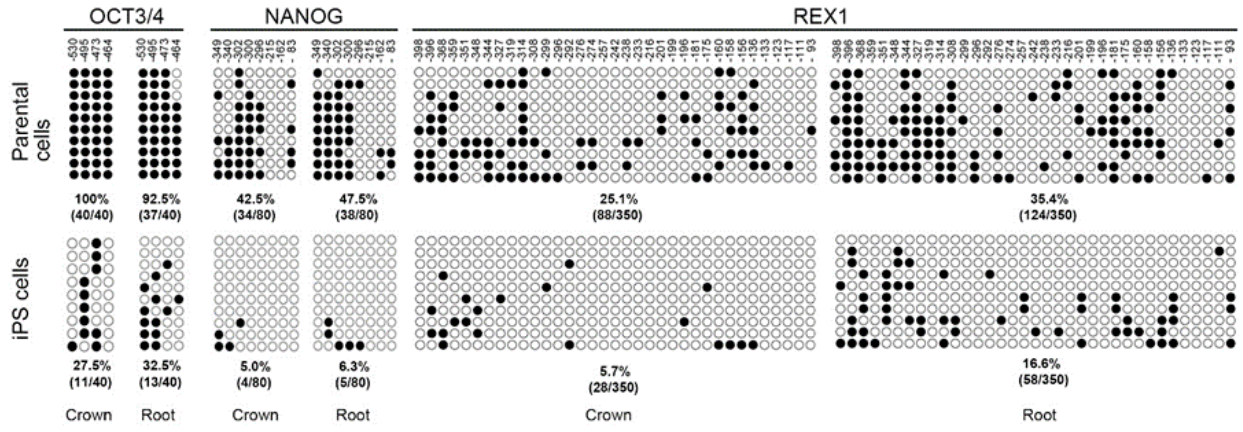


Fig. 6 Bisulfite sequencing of OCT3/4, NANOG, and REX1 promoter regions in parental cells and iPS cells. Open and closed circles indicate unmethylated and methylated CpGs, respectively, in the analyzed regions. Each horizontal row of circles shows an individual sequencing result for a given amplicon. The methylation rate was expressed in a ratio of the number of the methylated CpG sites to total CpG sites in 10 clones. Sequencing data were analyzed using the Quantification tool for Methylation Analysis (QUMA) (Riken).

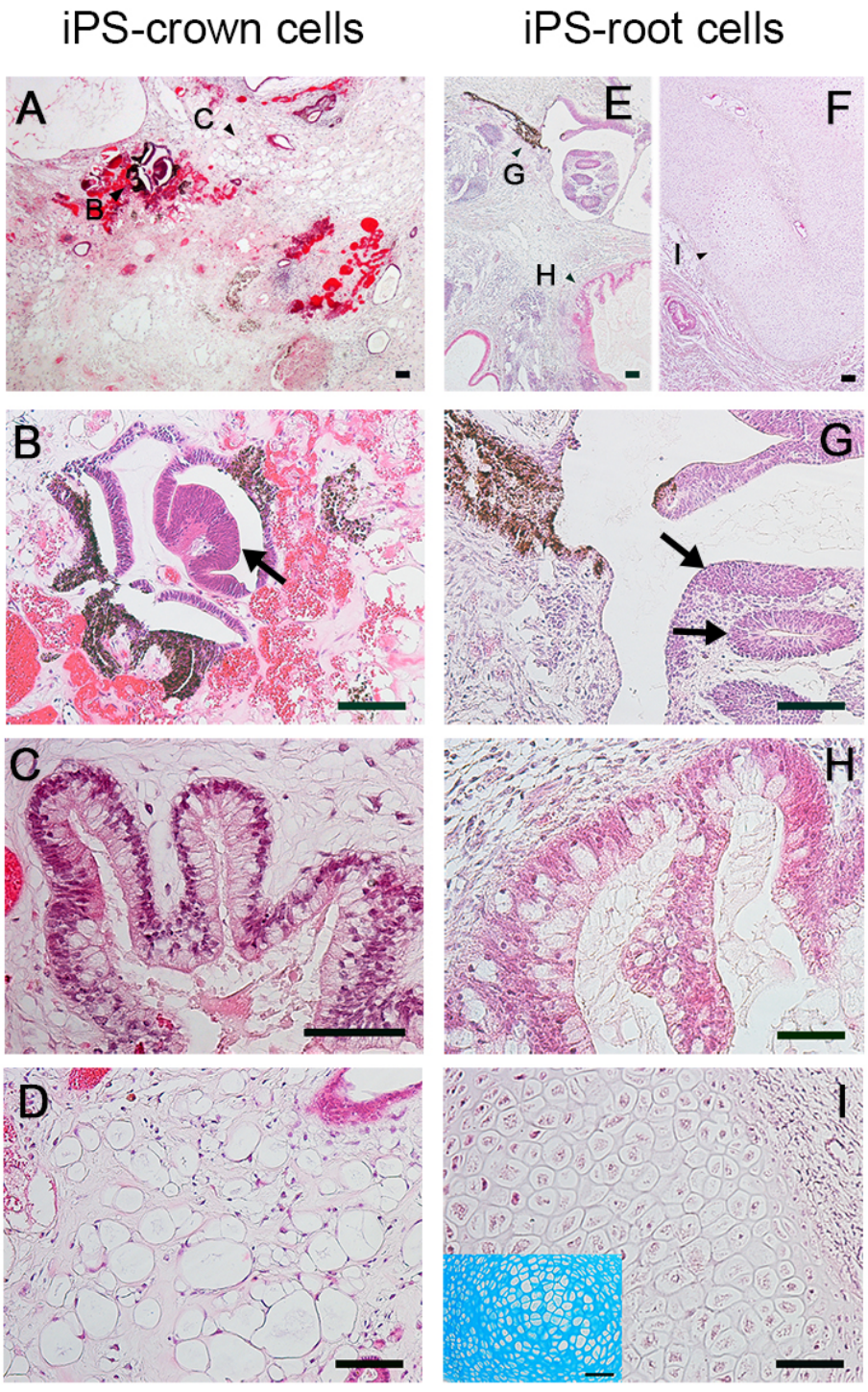


Fig. 7 Histological sections of *in vivo* teratomas derived from iPS-crown cells (A-D) and -root cells (E-I) **(A, E, F)** Low-power magnification view for orientation. **(B, G)** Pigmented epithelia and neural tissue. The latter are shown as neural rosette clusters (arrows). **(C, H)** Gut-like epithelium. The compound tubular gland consisted of simple columnar epithelium including goblet cells. **(D)** Adipose tissue. **(I)** Cartilage with perichondrial tissue. Cartilagenous matrix was Alcian-blue positive. Scale bars represent 100 μm.