Abstract: Human dental pulp cells significantly contribute to the generation of patient-specific human induced pluripotent stem cells (hiPSCs) because dental pulp is easily accessible and contains high-quality mesenchymal stem cells. This study aimed to generate hiPSCs from deciduous dental pulp cells, using three factors, OCT3/4, SOX2, and KLF4, and to evaluate the feasibility of hiPSCs as substitutes for odontogenic cells. hiPSCs were mixed with heterogeneous cells of porcine third molar tooth germs extracted from the mandibles of six-month-old pigs. The mixed cells were seeded in a disc-shaped poly (D, L-lactic-co-glycolic acid) scaffold. The cell–scaffold complexes were then wrapped around the omentum of immunocompromised rats as recipients to promote vascularization and maturation of the implants; the implants were harvested at 16 weeks after transplantation. The paraffin-embedded sections of the implants were used for histological observation and for immunohistochemical and immunofluorescence analyses. Histologically, several small pieces of odontogenic tissue were observed in the implants. The enamel organ-like structures were observed and the tall and columnar-shaped cells facing the enamel stained positive for anti-human nuclei and amelogenin antibodies. Dentin–pulp complexes with dentinal tubules were observed and the columnar-shaped cells facing the dentin stained positive for anti-human nuclei and dentin sialophosphoprotein antibodies. Dental root-like structures accompanying the Hertwig’s epithelial root sheath (HERS)-like bilayer were observed and cells constituting the HERS-like bilayer stained positive for anti-human nuclei and cytokeratin 14 antibodies and negative for anti-vimentin antibody. The cementum adjacent to the dentin was recognized, and staining for bone sialoprotein (BSP) was observed to be intense at the cementum–dentin border. Cementoblasts and cementocytes stained positive for anti-human nuclei and BSP antibodies. These results suggest that hiPSCs have the potential to differentiate into ameloblasts, odontoblasts, and cementocytes, and are capable of generating odontogenic tissues.

Key words: Cementum, Dentin, Enamel, Human induced pluripotent stem cells (hiPSCs), Tissue engineering

Introduction

Teeth generated from a patient’s own cells would be ideal for the replacement of lost teeth as their development could follow the genetically programmed replacement of teeth during childhood. Previously, our group reported a method to regenerate complex tooth structures using a classic tissue engineering approach of harvesting third molar tooth germs at the late bell stage from a six-month-old pig. These tissues were dissociated into single-cell suspensions and then seeded onto a biodegradable scaffold. The constructs were then surgically transplanted into immunocompromised rat omentum and allowed to grow. The transplanted cells reorganized themselves to form enamel, dentin, and cementum[1–3]. There are two reasons for choosing the late bell stage in the study. First, bell stage tooth germs are composed of enamel organ, dental papilla, and dental follicle, which are responsible for forming enamel, dentin–pulp complex, and cementum, respectively. Second, late bell stage tooth germs can be obtained from humans in childhood. The crown shape of the tissue-engineered tooth appeared almost normal, although the success rate was quite low[4–6]. On the other hand, there are other approaches using cells derived from mouse embryonic teeth that have successfully produced teeth with[7–9]. It is strikingly that the proportion of teeth generated from mouse embryonic tooth germ cells that have a normal shape is higher than that of teeth from postnatal porcine tooth germ cells[10–12]; however, embryonic tooth germ cells can only be obtained from expectant mothers. In terms of clinical application, this use of fetal tissues and/or cells raises ethical concerns. Therefore, establishing a method to produce teeth from adult cells that can be obtained from human as a postnatal cell source is a challenge in dentistry.

Teeth generated using iPS cells have low success rates, and the crown shape of the tissue-engineered tooth appeared almost normal, although the success rate was quite low[4–6]. On the other hand, there are other approaches using cells derived from mouse embryonic teeth that have successfully produced teeth with[7–9]. It is strikingly that the proportion of teeth generated from mouse embryonic tooth germ cells that have a normal shape is higher than that of teeth from postnatal porcine tooth germ cells[10–12]; however, embryonic tooth germ cells can only be obtained from expectant mothers. In terms of clinical application, this use of fetal tissues and/or cells raises ethical concerns. Therefore, establishing a method to produce teeth from adult cells that can be obtained from human as a postnatal cell source is a challenge in dentistry.

Induced pluripotent stem (iPS) cells are generated from adult cells, by passing potential ethical problems, and they can differentiate into all embryonic germ layers. Many researchers have reported that iPS cells can differentiate into various cell types, such as neurons, cardiac, myocytes, and renal lineage cells in appropriate conditions; therefore, iPS cells offer the potential to derive patient- and lineage-specific cells for clinical application and have emerged as a potential cell source for tooth-tissue engineering[10–13]. Recently, Arakaki et al. reported that mouse iPS cells could differentiate into ameloblasts via interactions with the dental epithelium[14]. Soon after, Otsu et al. differentiated mouse iPS cells into neural crest-like cells and used them to generate odontoblasts[15]. An in
We previously generated human iPS cells from deciduous dental cementoblasts. Human iPS cells (C) were then combined with porcine third molar tooth germ cells. The mixed cells (D) were loaded into poly (lactic-co-glycolic acid) (PLGA)-based solid scaffolds (E), and transplanted into the omentum of immunodeficient male rats. Sixteen weeks after transplantation, a teratoma containing a solid mass was generated (F). Scale bars in (B) and (C) represent 4 mm and 500 μm, respectively. eo: enamel organ, dp: dental papilla, df: dental follicle.

Figure 1. Outline of strategy for generating a tissue-engineered tooth from human induced pluripotent stem (iPS) cells using classic tissue-engineering techniques. Schematic diagram of the in vivo experiment performed to produce a tissue-engineered tooth. The impacted third molar tooth germs (A) were extracted from the mandibles of six-month-old pigs. The developmental stage of these tooth germs was the late bell stage (B). Human iPS cells (C) were then combined with porcine third molar tooth germ cells. The mixed cells (D) were loaded into poly (lactic-co-glycolic acid) (PLGA)-based solid scaffolds (E), and transplanted into the omentum of immunodeficient male rats. Sixteen weeks after transplantation, a teratoma containing a solid mass was generated (F). Scale bars in (B) and (C) represent 4 mm and 500 μm, respectively. eo: enamel organ, dp: dental papilla, df: dental follicle.

Materials and Methods

Human subjects use in this study was approved by the Ethics Committee of Nihon University School of Dentistry (approval numbers AP13D003-2, AP15D030) and was performed in accordance with the Helsinki Declaration.

Experimental animals

Pregnant Institute for Cancer Research (ICR) mice were purchased from Sankyo Labo Service (Tokyo, Japan) and used on day 12.5 of pregnancy for the generation of mouse embryo fibroblasts (MEFs) as feeder cells to maintain iPS cells in an undifferentiated state. Male immunocompromised nude rats (F344/NJcl-rnu/rnu) were obtained from Nihon Clea (Tokyo, Japan) and used as cell transplant recipients in this study. All experimental animal procedures were approved by the Animal Experimentation Committee of Nihon University School of Dentistry (approval numbers AP13D003-2, AP15D030) and were performed in accordance with the institutional animal care guidelines of Nihon University.

Generation and characterization of human iPS cells derived from dental pulp cells of a human deciduous tooth

Dental pulp cells (DPCs), obtained from the primary tooth (left upper incisor) of a 7-year-old boy, were isolated and expanded as described previously. Frozen stock DPCs were thawed and used for the generation of human iPS cells.

Human iPS cells were generated in our laboratory from DPCs via transfection with retrovirus vectors encoding OCT3/4, SOX2, and KLF4. Embryonic stem (ES) cell-like colonies were manually selected and expanded on a mitomycin-C (Wako, Osaka, Japan)-treated MEF feeder layer in human ES cell medium consisting of Dulbecco’s modified Eagle medium and Ham’s F-12 Nutrient Mixture at a ratio of 1:1 (DMEM-F12; Sigma-Aldrich, St. Louis, MO, USA), supplemented with 20% KnockOut Serum Replacement (Thermo Fisher Scientific, Waltham, MA, USA), 0.1 mM non-essential amino acids (Thermo Fisher Scientific), 0.11 mM 2-mercaptoethanol (Thermo Fisher Scientific), 1× penicillin-streptomycin-β-lactam solution (Sigma-Aldrich), and 5 ng/ml recombinant human basic fibroblast growth factor (bFGF; Wako) in 15% CO2.

Preparation of dental epithelial and mesenchymal cells from porcine tooth germs

We purchased the mandibles of six-month-old pigs from Tokyo Shibaura Zoki (Tokyo, Japan) and extracted impacted third molar tooth germs as previously described. The developmental stage of tooth germs is the late bell stage (Fig. 1). After removal of calcified tissues, the tooth germs, including the dental epithelium, dental papilla, and dental follicle, were minced into about 1-mm pieces, and the entire tissue was enzymatically dissociated in α-modified Eagle medium (α-MEM; Wako) containing 60 units/ml dispase I (Goudou Syusetsu, Tokyo, Japan) and 2 mg/ml collagenase (Wako) for 50 min at 37°C with shaking. The heterogeneous cells were strained through a 70-μm cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA) and then pelleted by centrifugation (1500 rpm for 5 min).

Poly (D, L-lactic-co-glycolic acid) (PLGA)-based solid scaffolds

Hydroxyapatite/PLGA (HAP/PLGA) scaffolds were gifted from GC Corporation (Tokyo, Japan) (Fig. 1). The disc-shaped scaffold was 5 mm in diameter and 2 mm in thickness. HAP/PLGA blocks were packed in 3% murine collagen type I solution (Wako) and sterilized by γ-irradiation.
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Figure 2. Histological analysis of the teratoma generated from the implants. (A) Low-power magnification with hematoxylin–eosin (H–E) staining showing a section of part of the teratoma, for orientation. (B–D) High-power magnification views of the boxes marked in (A). H–E-stained teratoma sections showed differentiation of human dental pulp cell-derived induced pluripotent stem (iPS) cells into various tissues from all three germ layers: (B) sebaceous gland-like tissues (ectoderm), (C) gut-like epithelia (endoderm), and (D) smooth muscle-like tissues (mesoderm). Scale bars represent 100 µm.

Figure 3. Gross appearance of a solid mass generated within a teratoma. An irregular shaped, membrane-covered, solid mass was generated within a teratoma. The mass was approximately 10 mm in height and width.

Preparation and in vivo transplantation of the cell–scaffold complex

The procedure for generating a tooth is shown in Fig. 1. We combined human iPSCs (1 × 10⁶ cells) with both dental epithelial cells and dental mesenchymal cells (3 × 10⁶ cells) and seeded them (total 4 × 10⁶ cells in 40 µl of the growth medium) onto the surface of a pre-wetted PLGA block. After incubation for 12 h at 37°C in 5% CO₂, 6-week-old nude rats were anaesthetized with 2.5% isoflurane (DS Pharma Animal Health Co. Ltd., Osaka, Japan) inhalation in a Narcobit-E Anaesthesia Unit (Natsume Seisakusho Co. Ltd., Tokyo, Japan). One cell–scaffold complex was then transplanted into the omentum of each recipient rat (n = 8).

Histological, immunohistochemical, and immunofluorescence analyses

The implants were harvested at 16 weeks after transplantation and were fixed in 4% paraformaldehyde in phosphate-buffered saline at 4°C for 24 h. Mineralized tissues in samples were decalcified in Morse’s solution (10% sodium citrate, 22.5% formic acid) at 4°C for 2 weeks, embedded in paraffin, and sectioned at 4-µm thickness. The sections were used for hematoxylin and eosin (H–E) staining, immunohistochemistry, and immunofluorescence.

First, we stained human dental pulp cells, porcine dental papilla cells and rat bone marrow cells with mouse anti-human nuclei antibody (1:250; MAB1281; Merck Millipore, Darmstadt, Germany) for immunocytochemistry to validate the specificity of this antibody as a primary antibody against human origin cells. Alexa Flou 488 goat anti-mouse IgG (1:500; Thermo Fisher Scientific) was then used as a secondary antibody.

For immunohistochemistry, the following primary antibodies, applied overnight at 4°C, were used: mouse anti-human nuclei monoclonal antibody (1:10; Merck Millipore), mouse anti-dentin sialophosphoprotein (DSP) monoclonal antibody (1:400; sc-73632; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti-bone sialoprotein (BSP) polyclonal antibody (1:200 dilution; kindly supplied by Dr. J. Sodek, University of Toronto, Toronto, ON, Canada) after the antigen retrieval step in Target Retrieval Solution (pH 9.0, 30 min at 98°C; Dako, Carpinteria, CA, USA). To ascertain the specificity of binding between the primary antibodies and the target proteins, 2.5% normal horse serum (Vector Laboratories) was applied in place of the primary antibody as a negative control. The ImmPRESS HRP anti-mouse or anti-rabbit IgG (peroxidase) polymer detection kit (Vector Laboratories Inc., Burlingame, CA, USA) was then applied for 30 min at 20°C. The sections were subsequently incubated with diaminobenzidine (DAB) substrate kit (Vector Laboratories) for visualization.

into 96-well plates and sterilized by gamma-ray irradiation. Before cell seeding, each HAP/PLGA block was soaked in 300 μl/well of growth medium comprising α-MEM (Wako) supplemented with 15% fetal bovine serum and 1% penicillin-streptomycin solution (Wako) for 15 min. 
In addition, immunofluorescence staining by using affinity-purified rabbit anti-pig amelogenin (1:1000; kindly gifted from Dr. J. P. Simmer and Dr. Y. Yamakoshi, University of Michigan, Ann Arbor, MI, USA) polyclonal, mouse anti-cytokeratin 14 (CK14) (1:10; MAB3232; Merck Millipore) monoclonal, and chicken anti-vimentin (1:200; ab24525; Abcam, Cambridge, MA, USA) polyclonal antibodies was performed after the antigen retrieval step. The secondary antibodies used were Alexa Flour 488-conjugated goat anti-mouse IgG (1:200; A-11029; Thermo Fisher Scientific), Alexa Flour 594-conjugated goat anti-chicken IgY (1:200; A-11042; Thermo Fisher Scientific) and Alexa Flour 594-conjugated goat anti-rabbit IgG (1:200; A-11012; Thermo Fisher Scientific). The sections were examined using a BZ-8100 fluorescence microscope (Keyence, Osaka, Japan) and processed with a BZ Analyzer (Keyence).

Evaluation of odontogenic tissues generated from human iPS cells

We counted the number of generated odontogenic tissues—which included enamel–dentin complexes, dentin–cementum complexes, and only dentin—per slide selected from paraffin sections (n = 3). Furthermore, to investigate whether the odontogenic tissues were generated from transplanted human iPS cells, we also counted the numbers of enamel, dentin, and cementum tissues generated from human nuclei-positive ameloblasts, odontoblasts, and cementoblasts, respectively.

Results

Gross and histological observations of the implants

Sixteen weeks after transplantation, teratomas were observed in all recipient rats (Fig. 1). Histologically, the teratomas showed the presence
Figure 6. Early bell stage tooth germ generated from transplanted human iPS cells. (A) Hematoxylin–eosin stained tooth germ-like structure was observed. (B) Higher magnification of the box marked in (A); a dental papilla (dp) characterized by aggregated cells was surrounded by an enamel organ-like structure with a bell-like shape. (C) Higher magnification of the box marked in (B); the enamel organ consisted of the outer enamel epithelium (oee), the inner enamel epithelium (iee) and the stellate reticulum (sr). The stratum intermedium (si) was also apparent adjacent to the inner enamel epithelium in the stellate reticulum (sr). An acellular space (*) was observed between the inner enamel epithelium (iee) and the dental papilla (dp). The dental follicle (df) was observed at the edge of the outer enamel epithelium. (D) Positive staining for human nuclei was observed in the inner enamel epithelium (iee), the outer enamel epithelium (oee), the stellate reticulum (sr), stratum intermedium (si), dental papilla (dp), and dental follicle (df). (E) The negative control for anti-human nuclei antibody. (F) Positive immunofluorescence staining for cytokeratin 14 (CK14) or vimentin was observed in the enamel organ or the dental papilla and the dental follicle. (G) The overlaid image of (F) with DAPI are shown. The images in (H) and (I) represent the box marked in (B). (H) Positive immunofluorescence staining for CK14 was observed in the cervical loop. The cervical loop showed negative and the surrounding cells of the cervical loop showed positive staining for vimentin. (I) The overlaid image of (H) with DAPI are shown. Blue, black and yellow scale bars represent 500, 100 and 20 μm, respectively.
Figure 7. Differentiation of human iPS cells into ameloblasts and generation of enamel. (A) The enamel (e)-dentin (d) complex was observed. The stellate reticulum (sr) was apparent in the central area of the complex. (B) Higher magnification of the box marked in (A); columnar-shaped cells (arrows) aligned on the dentin (d) and tall-columnar cells, ameloblasts (am) were observed adjacent to the remnant of the demineralized enamel (e). (C) Higher magnification of the box marked in (B). (D) Tall-columnar cells and stellate reticulum stained immunopositive for anti-human nuclei antibody. (E) The negative control for anti-human nuclei antibody. (F) Higher magnification of the box marked in (B); the columnar-shaped cells (arrows) on the surface of the dentin and the tall-columnar cells (am) adjacent to the enamel (e) stained positive for CK14. The tall-columnar cells (am) also stained positive for amelogenin; however, the columnar-shaped cells (arrows) stained weakly for amelogenin. (G) The overlaid image of (F) with DAPI are shown. Blue, black and yellow scale bars represent 500, 100 and 20 μm, respectively.

Figure 8. Differentiation of human iPS cells into odontoblasts and generation of dentin. (A) The dentin (d)-pulp (p) complex was observed. (B) Higher magnification of the box marked in (A); dentin (d), predentin (pd), dentinal tubules (arrows) and dental pulp (p) were identified in the complex. Odontoblasts (od), adjacent to the predentin, were aligned with the cell process extending into the dentinal tubules. The mineralized nodules, calcispherites (arrowheads) were also observed in the predentin. (C) Positive immunostaining for human nuclei was observed in odontoblasts (od) and cells in the dental pulp (p) (arrows). (D) The negative control for anti-human nuclei antibody. (E) Odontoblasts (od) showed positive immunostaining for dentin sialophosphoprotein (DSPP). (F) The negative control for anti-DSPP antibody. Scale bars represent 200 μm.
Figure 9. Hertwig’s epithelial root sheath (HERS) derived from human iPS cells. (A) Dentin (d), cementum (ce), and dental pulp (p) were revealed in the root-like structure. Hertwig’s epithelial root sheath (HERS) was also observed at the apex of the root. (B) Higher magnification of the box marked in (A); odontoblasts (od) and continuous bilayer bundles (arrows) were observed. The cell free layer (*) was seen between the bilayer bundle (arrow) and predontoblasts (pod). (C–F) are in the adjacent section of (B). (C) The bilayer bundles (arrows) showed positive staining for human nuclei. (D) The negative control for anti-human nuclei antibody. (E) The bilayer bundles stained positive for CK14. The bilayer bundles stained negative and the cells surrounding the bundles stained positive for vimentin. (F) The overlaid image of (E) with DAPI are shown. Black and yellow scale bars represent 200 and 20 μm, respectively.

Figure 10. Differentiation of human iPS cells into cementoblasts and cementocytes, and generation of cementum. (A) A dentin–cementum complex was observed. (B) Higher magnification of (A); dentinal tubules (arrows) were identified in the dentin layer (d), but not observed in the cementum layer (ce). The cells (arrowheads) were seen in the cementum layer. (C) In the adjacent section of (B), human nuclei-positive cells (black arrowheads) were observed both within the cementum layer. Human nuclei-positive (white arrowheads) cells were also observed adjacent to the cementum layer. (D) The negative control for anti-human nuclei antibody. (E) In the adjacent section of (B), bone sialoprotein (BSP)-positive cells (black arrowheads) were seen in the cementum (ce). BSP-positive cells (white arrowheads) were also seen on the surface of the cementum (ce). BSP was strongly expressed at the border of the dentin and cementum layers (arrow). (F) The negative control for anti-BSP antibody. Scale bars represent 200 μm.
of cell types of all three germ layers (Fig. 2A), i.e., ectodermal sebaceous gland like tissues (Fig. 2B), endodermal gut-like epithelia (Fig. 2C), and mesodermal smooth muscle-like tissues (Fig. 2D). In addition, hard masses were isolated from teratomas. One of masses was approximately 10 mm in length by 10 mm in width (Fig. 3). Tooth-related structures that were observed in the late bell stage of tooth development (Fig. 4A), including the enamel–dentin complex (Fig. 4B) and dentin–cementum complex with Hertwig’s epithelial root sheath (Fig. 4C) were identified in the masses. No periodontal ligament tissue was observed in the implant. Finally, we examined whether transplanted iPS cells contributed to the generation of these structures.

The specificity of the anti-human nuclei antibody for human cells

The nuclei in human dental pulp cells stained positive, while the nuclei in porcine dental papilla cells or rat bone marrow cells stained negative with anti-human nuclei antibody (Fig 5).

Tooth germ derived from human iPS cells

We characterized the early bell stage-like structures based on observations of an enamel organ-like structure, dental papilla, cervical loop at the junction between the outer and inner enamel epithelium, and the connective tissue layer encapsulating the enamel organ and dental papilla (Figs. 6A and B). At high magnification, the enamel organ was observed to have peripheral epithelial cells that were differentiating in the inner and outer enamel epithelia, and star-shaped cells termed stellate reticulum were found inside the enamel organ (Fig. 6C). The dental papilla was separated from the enamel organ by the basal lamina, which served as an acellular zone (Fig. 6C). The dental follicle, which surround the enamel organ, was also observed (Fig. 6C). In the immunohistochemical analysis, the enamel organ cells, a subset of the cells in the dental papilla and cells in dental follicle stained positive for human nuclei (Fig. 6D). The enamel organ cells also stained positive for cytokeratin 14 (CK14) and the cells in the dental papilla and in the dental follicle stained positive for vimentin (Figs. 6F and G). In addition, the cervical loop stained positive for CK14, whereas the cells surrounding the cervical loop stained positive for vimentin (Figs. 6H and I).

Ameloblasts and enamel derived from human iPS cells

Ameloblasts were characterized by tall columnar cells with nuclei lying adjacent to the stratum intermedium (Figs. 7B and C). Before differentiating into ameloblasts, short columnar cells bordering dentin elongated and reversed polarity (Figs. 7B and C). The tall columnar cells adjacent to enamel space which was formed during demineralization of enamel and cells in stellate reticulum stained positive for human nuclei (Fig. 7D). Both the short and tall columnar cells stained positive for CK14 (Figs. 7F and G). The tall columnar cells also stained positive for amelogenin; however, the short columnar cells stained weakly for amelogenin (Figs. 7F and G).

Odontoblasts and dentin derived from human iPS cells

E-D: enamel–dentin complexes, D-C: dentin–cementum complexes, D: only dentin

Table 1. Number of generated E–D, D–C and D in a scaffold

| Sample | E–D | D–C | D |
|--------|-----|-----|---|
| #1     | 5   | 2   | 0 |
| #2     | 4   | 1   | 1 |
| #3     | 3   | 2   | 0 |
| Total  | 12  | 5   | 4 |

E: enamel; D: dentin

Table 2. Number of enamel, dentin and cementum tissues generated from human nuclei-positive cells in the scaffold

| Tissue       | Human nuclei-positive |
|--------------|-----------------------|
|              | ameloblast-derived    |
|              | odontoblast-derived   |
|              | cementoblast-derived  |
| E–D          | 5                      |
|              | 3                      |
| D–C          | –                      |
|              | 2                      |
| D            | –                      |
|              | 4                      |
| Total        | 12                     |

E: enamel; D: dentin

Dentin tubules that extended throughout the dentin and globular mineralizations were observed in the predentin (Figs. 8A and B). The most distinctive cells in the dental pulp, odontoblasts, were characterized by a palisade pattern of three to five cells and its location facing the predentin (Fig. 8B). These cells stained positive for human nuclei (Fig. 8C) and DSPP (Fig. 8E).

Hertwig’s epithelial root sheath derived from human iPS cells

At the edge of the dentin–cementum complex, a continuous bilayered structure (Figs. 9A and B) that stained positive for human nuclei (Fig. 9C), and formed a barrier to the acellular zone (Fig. 9B). A continuous bilayer structure also stained positive for CK14, whereas the cells surrounding this structure stained positive for vimentin (Figs. 9E and F).

Cementocytes and cementum derived from human iPS cells

Cementum was easily distinguished from dentin because the former does not have tubular structures. Cementum was characterized by not only this lack of tubular structures, but also its location adjacent to dentin and the cementocytes that were incorporated (Figs. 10A and B). Both the cells lining the cementum and incorporated cells stained positive for human nuclei (Fig. 10C) and BSP (Fig. 10E). Moreover, the junction between cementum and dentin also stained intensely for BSP (Fig. 10E).

Evaluation of odontogenic tissues generated from human iPS cells

In the 3 slides, the total numbers of generated enamel–dentin complexes, dentin–cementum complexes, and dentin tissues were 12, 5, and 4, respectively (Table 1). To summarize Table 1, the total numbers of generated enamel, dentin, and cementum tissues in the 3 slides were 12, 21 (including 12 enamel–dentin, 5 dentin–cementum, and 4 dentin tissues), and 5, respectively. Next, we counted the number of odontogenic tissues generated from human nuclei-positive cells. The total numbers of enamel, dentin, and cementum tissues generated from human nuclei-positive ameloblasts, odontoblasts, and cementoblasts were 12, 20, and 5, respectively (Table 2). We examined the proportion of odontogenic tissues generated from human iPS cells to the total generated odontogenic tissues in a scaffold. All 12 enamel tissues were generated from human nucleus-positive ameloblasts (100%); 20 dentin tissues were generated from human nucleus-positive odontoblasts out of 21 generated from odontogenic tissues (95.2%); and all 5 cementum tissues were generated from human nucleus-positive cementoblasts (100%).

Discussion

This study showed that iPS cells derived from human dental pulp cells have the potential to generate odontogenic tissues such as enamel, dentin,
and cementum using tissue engineering application when the iPS cells are combined with tooth germ cells isolated from porcine third molar teeth in the late bell stage. This potential was confirmed by the detection of human nuclei by the immunohistochemistry in consistent with other studies. Recently, the ability of mouse iPS cells to form tooth-related structures has also been confirmed by recombination of mice incisor cells at the cap stage and tooth germ cells from ED14.5 mice in vivo, although the finding that iPS cells alone do not form tooth tissues is consistent with our observations. In a more recent study, human iPS cells derived from urine cells were combined with the dental mesenchyme from the molar tooth germ of E14.5 mice, and the recombinant explants revealed tooth. In addition, this study confirmed the human origin of the tooth-tissue components by immunostaining with human specific antibodies, similar to our analysis. Although these approaches can regenerate teeth, the source of embryonic tooth germ cells such as dental mesenchymal cells of E14.5 mice is scarce, because of the ethical issues regarding the use of fetal cells in clinical applications. Postnatal cell sources, iPS cells that can differentiate into odontogenic cells overcome this problem. This study provides evidence that postnatal tooth germ cells in the late bell stage can cause human iPS cells to differentiate into odontogenic cells such as ameloblasts, odontoblasts, and cementoblasts, and then iPS cell-derived odontogenic cells generate odontogenic tissues including enamel, dentin, and cementum.

The reasons why we selected third molar tooth germ cells in the late bell stage of a six-month-old pig as a postnatal cell source are as follows. The first successful studies of tooth regeneration used tooth germ cells in late bell stage. We also observed the presence of epithelial and mesenchymal progenitor/stem cells in the tooth germ at 6 weeks after transplantation, mirroring the tooth bud stage in natural tooth development. At 18 weeks after transplantation, the tooth structure, including enamel, dentin, and cementum, was regenerated in the implants. These results gave us the idea that porcine third molar tooth germ cells could cause iPS cells to differentiate into odontogenic tissues.

How did we recognize the regenerated tissues in the implants as enamel, dentin, and cementum? The presence of enamel was determined by the presence of ameloblasts, with their Tomes' processes, and their deep staining with hematoxylin. After decalcification, the structural features of amelogenesis, such as rods and interrods, were seen through a light microscope in the remaining tissue, but the details were difficult to distinguish. Tomes' process was also seen in the proximal ends of ameloblasts. Furthermore, ameloblasts and the enamel matrix show clear staining for amelogenin. Dentin was identified by its dentinal tubules and a predentin layer facing the odontoblast layer. Histologically, dentinal tubules and a predentin layer were clearly seen, and DSP was stained in the odontoblasts. It was difficult to distinguish between cementum and bone, because a specific marker is not available for porcine cementum, cementoblasts, and cementocytes. We previously examined the staining pattern of BSP antibody in a regenerated porcine cementum-dentin complex based on classic tissue-engineering technique. Strong BSP expression was observed at the cementum-dentin border. As is characteristic of cellular cementum, cells were incorporated into the cementum, and there are few cementoblast layers covering the cementum tissues. Furthermore, cementum tissues stained weakly for BSP, but dentin stained negative for BSP. Consistent with our previous results, cementum was identified by the existence of a border between the cementum and dentin that stained positive for BSP and by structures that were distinct from dentin and enamel. Taken together, these results led us to conclude that new enamel, dentin and cementum were formed in this study. In addition, ameloblasts, odontoblasts, and cementoblasts were found close to their originating tissues.

A focus of this study was whether iPS cells have the potential to generate enamel, dentin, and cementum through differentiation into odontogenic cells. How did we recognize whether the transplanted iPS cells differentiated into ameloblasts, odontoblasts, and cementoblasts? We used immunohistochemical staining with human antibody, a well-established technique to recognize human cells. Furthermore, porcine dental papilla cells and rat bone marrow cells were negatively stained with human nuclei in this study. On the other hand, importantly, odontogenic cells were never seen within a teratoma when only iPS cells were transplanted into the testis. This suggests that porcine tooth germ cells have the potential to induce the differentiation of human iPS cells into odontogenic cells. Two possible mechanisms may underlie this in vivo tooth-tissue generation process: tissue-engineered odontogenesis may involve the recognition of fully differentiated ameloblasts, odontoblasts, and cementoblasts that are already present in the porcine tooth germ; alternatively, it may involve progenitor cells from the epithelium and mesenchyme of the original tooth germ. In our observations, human nuclei expressing cementoblasts were derived from transplanted iPS cells. The porcine third molar tooth germ cells used in this study were in the late bell stage in tooth development. Critically, tooth root formation had not yet started at this stage, as shown in Fig. 1, and although mature cementoblasts were not present in the dental follicle at this stage, the dental follicle likely contained progenitors of cementoblasts and periodontal ligament fibroblasts. Therefore, based on our observations, differentiation of iPS cells into cementoblasts may have triggered interactions with cementoblast progenitor cells. These results suggest that creating odontogenic cells from iPS cells may involve progenitor cells, but not mature cells.

A number of factors are thought to play a role in iPS cells’ capacity to differentiate, either directly or indirectly, into odontogenic cells. Recently, newborn human skin keratinocyte progenitors were used to form tooth structures through recombination. In this study, FGF proteins were required for the formation of ameloblasts. Arakaki et al. noted that ameloblastin was necessary for both in vivo and in vitro ameloblast differentiation and that co-culture with an ameloblastin-expressing dental epithelial cell line resulted in efficient induction of the differentiation of iPS cells into ameloblasts via neurotrophic factor NT-4 and BMP-4 signaling. FGF-8 and BMP-4 induced to human ES cell-derived mesenchymal stem cells to differentiate into odontoblast-like cells or close to odontoblastic cells with the higher percentage of DSP positive cells. Taken together, these results suggest that BMP-4 may be a key factor that induces pluripotent stem cells to differentiate into both ameloblasts and odontoblasts. The key molecules for odontogenic differentiation from iPS cells require further examination.

Although some regenerated teeth exhibited a disorganized, heterogeneous morphology, some of the regenerated tissue appeared normal, as shown in Fig. 4. Further examinations of the mechanism that regulates the shape of teeth engineered from iPS cells are needed. In addition, teratomas were also observed with this strategy. Otsu et al. reported that mouse iPS cells that have the capacity to differentiate into neural crest-like cells also have the capacity to differentiate into odontoblasts via reciprocal interactions with E14.5 dental epithelium in kidney capsules. Further studies are needed to prevent the generation of teratomas in this process.

In conclusion, the goal of tooth-tissue engineering is to regenerate fully functional teeth that can replace lost or damaged ones. This study shows a new and effective approach to odontogenic differentiation from human iPS cells using a postnatal cell source. It must be noted that iPS cells can compensate to the difficulty to obtain the progenitor cells for odontogenic cells from adult human because the number of progenitor
cells are limited to obtain in adult human teeth. The results of our study provide a valuable approach to future tooth regeneration. Further experimental studies are required to clarify the mechanism underlying the induction of odontogenic differentiation.

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Conflict of Interest
The authors have declared that no COI exists.

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