Effect of EDTA-treated dentin on the differentiation of mouse iPS cells into osteogenic/odontogenic lineages in vitro and in vivo

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To investigate the effect of EDTA-treated dentin on the differentiation of mouse induced pluripotent stem (iPS) cells. Dentin discs were prepared from bovine incisors and treated with 17% EDTA. Embryoid bodies (EBs) formed from mouse iPS cells were seeded on the dentin discs for the experiment. The roughness of the EDTA-treated dentin surface, Sa and Sdr, was higher and collagen fibril-like structures were observed by the scanning electron microscopy (SEM) in vitro. In RT-PCR, the mRNA levels of the osteoblast markers Bsp and Ocn were significantly higher in the experimental group. Expression of the DMP1, DSP, and BSP proteins were more notable in the experimental group by immunofluorescence (ICF) study. In vivo study, cartilage and bone-like tissue were observed adjacent to the EDTA-treated dentin. The study demonstrates that the dentin treated with 17% EDTA induces mouse iPS cells to differentiate into the osteo/odontogenesis.

Keywords: EDTA treated dentin, iPS, Odontogenic differentiation, Osteogenic differentiation, Surface topography

INTRODUCTION

Stem cell research and regenerative medicine techniques has progressed greatly in recent years, which contributed insights to improving tissue regeneration1-7. In particular, the recent discovery of induced pluripotent stem (iPS) cells, which have been genetically reprogrammed to an embryonic stem cell-like state, has had a major impact in this field8. The properties of iPS cells resemble mesenchymal stem cells (MSCs) in nature having the potential to differentiate into mature cell types and the ability for self-renewal9. The potential of MSCs to differentiate into adipogenic and/or osteogenic lineages has been well studied8-12. However, iPS cell differentiation, in terms of osteogenesis and odontogenesis, has still not been adequately studied. Ozeki et al. reported that treatment with bone morphogenetic protein-4 (BMP-4) and retinoic acid (RA) induced the expression of some odontoblastic mRNAs and proteins in mouse iPS cells8. To differentiate into mature odontoblasts, a suitable scaffold and an appropriate stimulatory environment are probably necessary.

Treated dentin has been used as a scaffold for odontogenic differentiation from MSCs9. Dentin is a mineralized tissue and its extracellular matrix (ECM) is composed of approximately 47% mineral, 30% protein and 21% water. In dentin and bone, type 1 collagen accounts for about 90% of the protein fraction and most of the non-collagenous proteins (NCPs) are expressed. Some NCPs appear to be more specifically expressed in dentin10. When dentin was treated with acid, NCPs are exposed on the dentin inducing the formation of cartilage and bone in vivo11. The dentin matrix is osteoinductive and rich in BMPs12. BMPs are identified as important regulators involved in embryogenesis and osteogenesis in various tissues including teeth, and BMP-4 plays an important role in dentin regeneration13. Furthermore, dentin treated with 17% EDTA was found to be effective for inducing odontogenic differentiation13. EDTA has a gentle effect on dentin structure as a chelating agent, and it exposes collagen fibrils on the dentin surface.

Beside biological and chemical signals, physical cues such as topography and stiffness have been recognized as important factors that can influence cell behavior and differentiation ability14-16. Strain transfer to and deformation of the nucleus has been proposed as a direct link between mechanical inputs from the microenvironment and gene regulation17. Changes in cytoskeletal organization, connections to the nuclear envelope and pre-tension in the acto-mysin network all impact how cells sense and respond to mechanical signals18.

As an ECM component, collagen provides a natural environment for cells. Differentiation medium consisting of Dulbecco's modified Eagle's medium (DMEM) containing Knockout Serum Replacement (KSR), BMP-4 and RA serves as an inducer of cell differentiation8. However, no previous studies regarding the differentiation of iPS cells into an osteogenic and/or odontogenic lineage in this optimized condition as well as detailed characterization of the dentin surface have been conducted before. Therefore, the purpose of this study was to determine the effects of an EDTA-treated dentin on the differentiation of mouse iPS cells into osteogenic and/or odontogenic lineages.

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MATERIALS AND METHODS

In vitro experiment

1. Preparation of the dentin substrate
Forty-four bovine incisors (Shokuniku Kosha, Kanagawa, Japan) were cleaned of soft tissue to make dentin discs. The crown part and 2 mm of the apical part was removed from the disc, and only the root area was used for the study. Each root was cut in half using a trimmer (MC-130D, Maruto, Tokyo, Japan) and the cementum was removed using sandpaper with increasing grit sizes from 400–800 SiC (Buehler, Lake Bluff, IL, USA). Two dentin discs (each 4×17 mm) were prepared from each incisor (Fig. 1B). Each dentin disc was then checked using a stereomicroscope (Leica MZ7s, Wetzlar, Germany) to ensure that the cementum was removed completely. Ultrasonication was carried out for 30 min to remove polishing residues and debris. To remove extrabifilar surface minerals and expose underlying collagen fibrils, dentin discs of the experimental group were demineralized using 17% EDTA for 10 min\(^\text{[19]}\) whereas dentin discs of the control group were left untreated. After the surface treatment, all dentin discs were washed in distilled water for 30 min followed by sterilization using ethanol for 30 min and then ether for 10 min. Dentin discs were then dried in an incubator and were placed in the culture medium with antibiotics 30 min prior to the experiment.

2. Embryoid Body (EB) formation and odontoblastic differentiation
EBs were formed from mouse iPS cells (APS0002, RIKEN BRC, Ibaraki, Japan) according to Ozeki et al.\(^\text{[8]}\). Feeder-less iPS cell cultures were performed using ESGRO complete clonal grade medium (Merck Millipore, Billerica, MA, USA). In order to form EBs, mouse iPS cells were collected in non-adherent dishes and were cultured using the hanging drop method in iPS medium (DMEM with 15% KSR, L-glutamine, nonessential amino acids and 2-mercaptoethanol) for 2 days. They were then transferred into medium containing RA and suspension cultures were incubated for 3 days to produce neural crest-like cells. In the experimental group, approximately 20 EBs were seeded on each EDTA-treated dentin disc for 6 days in differentiation medium (Fig. 1A). EBs seeded on untreated dentin discs were used as the control group. The differentiation medium includes recombinant mouse BMP-4/CF (100 ng/mL, R&D Systems, Minneapolis, MN, USA) in DMEM (Invitrogen, Carlsbad, CA, USA) and 15% KSR (Invitrogen). Furthermore, 0.5% Penicillin and Streptomycin and 0.01% Fungizone were also added, and the medium was changed every 2 days. EBs and outgrowth cells were evaluated using scanning electron microscopy (SEM), qRT-PCR and Immunofluorescence.

3. SEM investigation
Dentin discs were sputter-coated by palladium and the surface roughness (Sa) and surface area (Sdr) values were evaluated using an electron beam 3-D surface roughness analyzer (ERA-8900FE, Elionix, Tokyo, Japan). Images were taken from 5 random areas at the center and around the corner of dentin discs at a magnification of 4,000. Image J was used to measure the size of dentin tubules in the untreated and EDTA-treated dentin.

EB-seeded dentin discs were fixed in Karnovsky’s fixative overnight, then dried in ascending concentrations of alcohol. Specimens were dehydrated in a dryer and then sputter-coated with palladium and investigated.
by SEM (SU6600, Hitachi, Tokyo, Japan) for the morphological analysis of outgrowth cells.

4. Detection of BMP-2 protein on the surface of dentin discs
We detected BMP-2 protein on dentin surface by using BMP-2 antibody that deposited directly to where the protein is located. After EDTA treatment, dentin discs were blocked using Blocking One (Nacalai Tesque, Kyoto, Japan) for 1 h at the room temperature (RT). Dentin discs were washed with TBST buffer (25 mM Tris (pH 7.4), 0.15M NaCl, 0.05% Tween 20) for 3 min and were incubated overnight at 4°C with the primary antibody of bone morphogenetic protein-2 (BMP-2) (1:100, Abbiotec, San Diego, CA, USA) diluted in Can Get Signal Solution 1 (Toyobo, Osaka, Japan). It was washed with TBST buffer three times for 5 min and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:2000, Abcam, San Francisco, CA, USA) diluted in Can Get Signal Solution 2 (Toyobo) for 1 h at room temperature. After washing with TBST buffer three times for 5 min, enhanced chemiluminescence reagent (ECL plus, Thermo Fisher Scientific, Waltham, MA, USA) was applied for 2 min. ImageQuant Las 4000 mini (GE Healthcare, Chicago, IL, USA) was used for protein detection. Image J was used to quantify the presence of protein in negative, untreated and EDTA-treated groups.

5. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis
The protocol for qRT-PCR was performed according to Ser-Od et al.20. Total RNA was obtained from each dish using the acid guanidium thiocyanate/phenol-chloroform method. Total RNAs were reverse-transcribed and amplified using an RT-PCR kit (Takara Bio, Kusatsu, Shiga, Japan). RT-PCR products were analyzed by quantitative real-time RT-PCR using TaqMan Gene Expression Assays for 5 target genes: Collagen Type 1 (Col1) as a marker of early osteoblast differentiation, Bone Sialoprotein (Bsp) and Osteocalcin (Ocn) as osteogenic markers, Dentin Matrix Protein 1 (Dmp1) and Dentin Sialophosphoprotein (Dspp) as odontoblastic differentiation markers and GAPDH as an endogenous control (Applied Biosystems, Foster City, CA, USA) (Table 1). Gene expression quantitation using Taqman Gene Expression Assays were performed in single-plex reactions containing Taqman Fast Universal PCR Master Mix, Taqman Gene Expression Assays, distilled water and cDNA according to the manufacturer’s instructions (Applied Biosystems).

6. Immunofluorescence staining
EB-seeded dentin discs with outgrowth cells were fixed in 4% paraformaldehyde for 30 min at room temperature, and then were permeabilized by 0.4% Triton X-100 in PBS for 10 min. Samples were blocked with 10% rabbit serum for 1 h at room temperature and then incubated overnight at 4°C with primary antibodies to DMP1 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), DSP (1:100, Santa Cruz Biotechnology) and BSP (1:100, Santa Cruz Biotechnology), markers of osteogenic and odontogenic lineages, respectively. Samples were washed with PBS and a secondary antibody conjugated to Rhodamine (1:200, Thermo Fisher Scientific) was applied for 30 min at room temperature. Cell nuclei were stained with 4’,6-Diamidino-2-Phenylindole, dihydrochloride (DAPD) (1:100, Invitrogen). Specimens were then mounted and visualized using LSM 5 DUO confocal microscope (Carl Zeiss, Oberkochen, Germany).

In vivo experiments
Five female SCID mice, each weighing 30 g, were used in this study (n=3). EBs and outgrowth cells seeded on 17% EDTA-treated or on untreated dentin discs for 6 days were used as graft materials. Under general anesthesia using phenobarbital, 2 incisions measuring 1.5 cm were made on the right and left abdominal skin of each mice. Two experimental grafts were subcutaneously implanted in the right side and 2 control grafts in the left side of each mouse. Animals were sacrificed by cervical dislocation 14 days after the implantation and were evaluated using hematoxylin and eosin (HE), histochemical and immunohistochemical staining. All animal studies were carried out in compliance with the Guidelines for the Treatment of Experimental Animals at the Tokyo

Table 1 Mouse primer for real time reverse transcription polymerase chain reaction

| Gene       | Assay ID          | Product size (bp) |
|------------|-------------------|-------------------|
| Col1       | Mm00801666_g1     | 86                |
| Bsp        | Mm00436767_m1     | 114               |
| Ocn        | Mm00649782_gH     | 89                |
| Dmp1       | Mm01208363_m1     | 74                |
| Dspp       | Mm00515667_g1     | 67                |
| Gapdh      | Mm99999915_g1     | 109               |

Col1, collagen type1; Bsp, bone sialoprotein; Ocn, osteocalcin; Dmp1, dentin matrix protein 1; Dspp, dentin sialophosphoprotein.
Dental College (Approval Number 223206).

1. HE staining
For morphological analysis, all samples were stained with HE. Dentin discs were fixed in 10% formalin buffer for 2 days after which decalcification was done by incubation in a 10% EDTA solution for 7 days. Paraffin blocks were prepared, sectioned and HE-stained slides were observed using a light microscope.

2. Alcian blue histochemical staining
Paraffin sections were deparaffinized by xylene followed by rehydration in ethanol and washing with distilled water. Samples were then soaked in 3% acetic acid for 3 min and in alcian blue solution (Muto Cure Chemicals, Tokyo, Japan) for 30 min. After alcian blue staining, samples were washed 3 times in 3% acetic acid for 3 min each. Nuclear fast staining was done using Kernechtrot solution (Sigma-N3020, Sigma Aldrich, St. Louis, Mo, USA) for 3 min and washed with distilled water afterwards. Hydration and deparaffinization was performed the same as in the immunohistochemistry protocol and slides were then mounted for visualization using a light microscope.

3. Immunohistochemical staining
For immunohistochemistry, the streptavidin-biotin immunoperoxidase method was used with a Histofine SAB-PO (MULTI) kit (Nichirei, Tokyo, Japan). Paraffin sections were deparaffinized with xylene, then were rehydrated in ethanol, and washed with distilled water. Samples were microwaved for 30 min at 65°C in 0.01 M citrate buffer (pH 6.0), cooled to room temperature and then washed in PBS 3 times for 3 min each. Endogenous peroxidase activity was blocked by incubating the sections with 3% H₂O₂ in methanol for 30 min. Before proceeding to the application of the first antibody, incubation in 10% rabbit serum was used to block the binding of unspecific antibodies for 1 h. The primary antibody used in this experiment was Bone sialoprotein (Abcam) as a cell differentiation marker (at a dilution of 1:200). Sections were counter-stained with Mayer’s hematoxylin for 30 s. Specimens were inspected using a light microscope (BX41, Olympus, Tokyo, Japan) and were photographed.

4. Statistical analysis
All experiments were performed in triplicates (n=3). Student’s t test (p<0.05) was performed to compare mRNA expressions levels of samples and also for 3D SEM analysis of the dentin surface. One-way anova was used for detection of BMP-2 protein on dentin surfaces. Data were expressed as means±standard deviation (SD). p<0.05 was defined as significant.

RESULTS
SEM observations
Representative SEM images of untreated (Fig. 2A) and EDTA-treated dentin (Fig. 2B) show collagen fibril-like structures on the surface of the EDTA-treated dentin (Fig. 2B inset) but not in the untreated dentin, which has an irregular and mineralized layer (Fig. 2A inset). On day 6, cytoplasmic processes of outgrowth cells could be observed on the dentin in the experimental group (Fig. 3B) compared to the control group (Fig. 3A). The average area of dentin tubules was approximately 1.6 µm²±0.78 (SD) and 5±1.21 (SD) open tubules were present in each 750 µm² area in the control group. In the experimental group, the average area of dentin tubules was approximately 5.32 µm²±1.56 (SD) and 25±0.95 (SD) dentin tubules were present in each 750 µm² area. The surface roughness (Sa) was 0.21 µm±0.03 (SD) in the experimental group and 0.15 µm±0.02 (SD) in the control group. The surface area (Sdr) was 6.65%±1.28 (SD) in the experimental group and 3.56%±0.52 (SD) in the control group. All results in the experimental group were significantly higher than those in the control group (Table 2).

BMP-2 protein detection
The presence of BMP-2 protein was found both in
Fig. 3 SEM images of outgrowth cells formed from EBs on 6 days. Cell spreading and morphological analysis of outgrowth cells on (A) untreated dentin and (B) EDTA-treated dentin. Arrows show the endings of cytoplasmic processes of outgrowth cells (B). Scale bar: 10 µm.

Table 2 Evaluation of dentin discs (n=15). Data are shown as means±standard deviation (SD)

| Dentin surface evaluation                        | Untreated dentin | 17% EDTA treated dentin | p value |
|--------------------------------------------------|------------------|-------------------------|---------|
| Area of dentin tubules (µm²)                      | 1.6±0.78         | 5.32±1.56               | **      |
| Number of dentin tubules in an area of 750 µm²    | 5±1.021          | 25±0.95                 | ***     |
| Sa value (µm)                                     | 0.15±0.02        | 0.21±0.03               | **      |
| Sdr value                                         | 3.56±0.52        | 6.65±1.28               | **      |

Sa-Average surface deviation, Sdr-Developed surface ratio.

Fig. 4 Protein detection. (A) BMP-2 protein was present both in untreated and EDTA-treated dentin samples. (B) The quantification of BMP-2 protein can be seen. Data are expressed as means±SD (n=3). Comparisons between negative and experiment groups: p values for statistical analysis are ***<0.001.

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was found between experiment groups (Fig. 4A). As compared to negative control, BMP-2 protein was found significantly higher in intreated and EDTA-treated dentin groups (Fig. 4B).

qRT-PCR analysis
The expression of Col1 mRNA was significantly higher in the control group than in the experimental group on day 6 (p<0.01) (Fig. 5A-a). mRNA expression levels of the osteogenic markers Bsp and Ocn in the experimental group were significantly higher than in the control group on day 6 (p<0.05) (Figs. 5A-b, c). On the 6th day of the experiment, the tendency for Dmp1 mRNA expression (Fig. 5A-d) was higher in the experimental group whereas a lower tendency of Dspp mRNA (Fig. 5A-e) was observed in the experimental group, however those differences were not significant.

Immunofluorescence observations
Staining for DMP1 protein was positive (green) in the cytoplasm of the outgrowth cells of the experimental group (Figs. 5B-c, d) and the control group (Figs. 5B-a, b) on day 6. On the 6th day of the experiment, positive results of DSP protein were found in the experimental group as well (data not shown). The presence of BSP protein in the cytoplasm of the outgrowth cells was positive in the experimental group (Figs. 5C-c, d) and in the control group (Figs. 5C-a, b). Cell nuclei were stained by DAPI and appeared blue. Actin filaments were visualized (red) by phalloidin staining and a merged image is shown (Figs. 5B-b, d) and (Figs. 5C-b, d).
**Fig. 5** (A) mRNA expression levels after the 6th d of the experiment (n=3). RT-PCR showed the genes related to early osteogenic marker *Col1*, osteogenic markers *Bsp* and *Ocn*, and odontogenic markers *Dmp1* and *Dspp*. GAPDH was taken as reference gene. Data are expressed as means±SD (n=5). Comparisons between the control group and the experimental group: *p* values for statistical analysis are *<0.05, **<0.01.

(B) Immunofluorescence staining showing the images of outgrowth cells on (a and b) untreated and (c and d) EDTA-treated dentin on the 6th d of the experiment. Merged image is seen in (b and d). 4',6-diamino-2-phenylindole (DAPI) antibody stained blue and used as counterstaining agent; phalloidin (F-actin) antibody stained red; DMP1 antibody stained green. Scale bar: 50 µm. (C) Immunofluorescence staining showing the images of outgrowth cells on (a and b) untreated and (c and d) EDTA-treated dentin at day 6. Merged image is seen here (b and d). 4',6-diamino-2-phenylindole (DAPI) antibody stained blue; phalloidin (F-actin) antibody stained red; BSP antibody stained green. DAPI was used as counterstaining. Scale bar: 50 µm.

**In vivo experiments**

1. Morphological observations
All graft materials were surrounded by fibrous connective tissue. In the control group, teratomas were found in the implanted EB area, rounded cartilage (Fig. 6A-a) and irregular trabecular bone-like tissues (Fig. 6A-b) were formed far from the dentin disc. In the experimental group, cartilage (stained with alcian blue) was found adjacent to the dentin surface and bone-like tissue was found adjacent to the dentin surface as well (Figs. 6B-a, b). In some cases, bone-like tissue, in which BSP was positive in the cytoplasm of the cells, was observed adjacent to the EDTA-treated dentin (Figs. 6C-a, b).
Fig. 6 (A) HE-staining of tissue formed near dentin of the control group. Teratoma-like tissues are observed in both images. (a) Cartilage is found far from the dentin, and (b) Bone tissue is found far from the dentin as well. Bo, bone; Ca, cartilage; De, dentin. Scale bar: 200 µm. (B) (a) HE-staining and (b) Alcian blue histochemical staining of tissue formed around dentin of the experimental group. Cartilage is found next to the dentin and (b) is confirmed by cartilage-specific alcian blue staining. Ca, cartilage; De, dentin. Scale bar: 100 µm. (C) (a) HE-staining and (b) Immunohistochemical staining of tissue formed next to dentin of the experimental group. Bony tissue is found next to the dentin, and (b) the cytoplasm of osteoblast-like cells stained positive for BSP protein. Bo, bone; De, dentin. Scale bar: 50 µm.

DISCUSSION

This study characterized the effects of a dentin substrate treated with EDTA on the differentiation of mouse iPS cells into osteogenic and/or odontogenic lineages. Treatment of dentin with 17% EDTA exposes collagen fibrils on which NCPs reside. Many studies have reported that NCPs derived from dentin can be used to differentiate cells into the osteogenic/odontogenic lineage. However, this study showed not only the detailed characterization of EDTA-treated dentin, but also its effect in terms of its surface topography on the differentiation of mouse iPS cells into the osteogenic/odontogenic lineage in vitro along with an in vivo study.

Effect of surface structure and roughness of EDTA-treated dentin on iPS cells

Topographical surfaces with micro- and nanoscale features induce changes in cell alignment, polarization, elongation, migration, proliferation and gene expression. Cytoskeletal rearrangements and nuclear elongation, which correlate with changes in gene expression and cell differentiation, might play a crucial role in activating signal transduction as the mechanical tension results in the alignment of cells and the rearrangement of telomeres through deformation of nuclei to affect gene expression. Maniotis et al. found that active or passive cell extensions can cause passive nuclear deformation, which can affect gene expression through intermediate filaments. The Sa of the dentin surface became higher after treatment with EDTA, which opened dentin tubules and removed debris. The Sdr of the experimental group was almost twice as large as the control group. Also, the area of dentin tubules in the experimental group was about 3 times larger than those in the control group, meaning that cytoplasmic extensions followed by nuclear tension are more likely to occur in the experimental group. As stated in Dalby et al., nuclear tension results in differentiated cells. The down-regulation of Col1, an early osteoblast marker, and up-regulation of the osteogenic markers Bsp and Ocn were found in the experimental group on day 6. This suggests that differentiation for osteo/dentinogenesis occurred earlier than in the control group. Naito et al. reported that when centrifugal force was used to treat bone marrow-derived osteoblast-like cells, an earlier differentiation occurred just as found in this study. Therefore, this might suggest that cells started to possess an osteogenic property by day 6. The up-regulation of the osteogenic markers Bsp and Ocn mRNA, and more positive BSP protein in the experimental group indicates that the rougher surface favorably affects iPS cells to differentiate into an osteogenic lineage.

Effect of collagen fiber exposure of EDTA-treated dentin on iPS cells

EDTA, particularly at a 17% concentration, is often used to remove minerals from hard tissues. This technique using EDTA allows the integrity of the native collagen structure to be maintained along with the intrafibrillar mineral. Collagen is a major component of the ECM and provides a natural environment for cells, and nanoscale collagen fiber structures were found to enhance cell-matrix interactions. Regardless of the origin of cells or their location, normal type 1 collagen-based tissues contain a distribution of nanoscale collagen morphologies as measured using the D-periodic gap/overlap spacing. According to Woo et al., a nanofibrous architecture enhances protein absorption contributing to pre-osteoblast cell attachment. Filopodia play an important role in biological processes and the nanofibrous architecture could alter the mode of anchorage, allowing filopodia to anchor tightly. Nanofibrous pore walls might improve nutrient/oxygen supply to and metabolic waste removal from the attached cells, resulting in a
better extracellular environment for dental pulp stem cell growth and ECM production\(^{30}\). Direct contact of dental pulp cells with mechanically and chemically treated dentin seems to promote their differentiation into odontoblasts with processes extending into dentinal tubules\(^{39}\). Similarly, on day 6 of this study, cytoplasmic processes were observed to extend into dentinal tubules in the experimental group. This probably helps iPSCs to go through differentiation.

The effect of exposed NCPs of EDTA-treated dentin on iPSCs

Growth factors are essential for embryogenesis\(^{31}\). In addition to type 1 collagen, which contributes up to 90% of the organic material, the ECM of dentin contains several proteins and proteoglycans (PGs), collectively referred to as NCPs, that include various growth factors (FGF, TGF, BMP, etc.), glycoproteins, sialoproteins, phosphoproteins, and enzymes that constitute approximately 10% of the matrix\(^{32-35}\).

According to Kidwai et al., it’s important to use FGF-8 in the odontogenic medium to induce the differentiation of MSCs into odontoblast-like cells, however, the detailed nature of 17% EDTA-treated dentin was not studied in respect to its osteogenic potential\(^{36}\). Other studies also reported the role of FGF-8 in tooth development\(^{36,37}\). The RT-PCR analysis of this study suggests the higher tendency of odontoblastic differentiation in the experimental group, but significant differences were not found between the control and the experimental groups. In the immunofluorescence study, the positive staining for BMP-1 and BSP proteins suggests that outgrowth cells acquired an odontogenic/osteogenic property. According to Ozeki et al., BMP-4 stimulated marked increases of integrins \(\alpha_2, \alpha_6, \alphaV\) and \(\alphaV\beta3\) subunits\(^{38}\). Especially, the expression of integrin \(\alpha2\) was the most notable and is considered the trigger for iPSCs to differentiate to osteoblast- and odontoblast-like cells. BMP-4 is considered to be one of the most important factors for osteogenesis and odontogenesis. Therefore, BMP-4 and FGFs are essential for iPSCs to differentiate into odontoblast-like cells. However, the osteogenic markers Bsp and \(\Omegacn\) were up-regulated as well as more BSP protein found in the experimental group compared to the control group suggesting that other BMPs, such as BMP-2 and BMP-7, from EDTA-treated dentin are effective for osteogenesis to occur rather than odontogenesis\(^{30}\).

In the current study, dentin preparation method such as using sandpaper and ultrasonication may result in exposing NCP proteins including BMP-2 on untreated dentin surface similar to EDTA-treated dentin. This also suggests that growth factors exposed on the dentin surface in the experimental group might not be appropriate to induce odontoblastic cell differentiation. Furthermore, Kidwai et al. reported that it takes 2 to 3 weeks for MSCs to differentiate into odontoblast-like cells\(^{30}\) and 3 weeks for human dental pulp stem cells to acquire odontoblastic property\(^{30}\). However, this study was carried out for only 6 days, which suggests that a longer period of time might be necessary for the cells to differentiate into odontoblast-like cells.

Dentin matrix components released by EDTA from rabbit dentin demonstrate a significant morphogenetic activity and induce reparative dentinogenesis of the exposed dental pulp of ferrets and dogs in vivo\(^{39,40}\). In this study, the in vivo result of the experimental group shows newly formed cartilage adjacent to the EDTA-treated dentin, however, in the control group, cartilage was found far from the dentin. Furthermore, bone tissue was also found near the EDTA-treated dentin, but odontoblastic differentiation was not initiated. Taken together, collagen fibril structure, the surface roughness and the NCPs of EDTA-treated dentin used in this study induced iPSCs to differentiate into chondroblasts or osteoblasts rather than odontoblasts in vivo. This suggests that more factors to increase the expression of Sox11 as reported by Matsue et al.\(^{42}\) might be necessary to induce odontogenesis.

This study demonstrates that a rough surface with nano-collagen fibril structure and NCPs of EDTA-treated dentin induces mouse iPSCs to differentiate into the osteo/odontogenic lineage in the presence of both RA and BMP-4.

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