Application of Induced Pluripotent Stem Cells in Generation of a Tissue-Engineered Tooth-Like Structure

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Stem cells, such as adult stem cells or embryonic stem cells, are the most important seed cells employed in tooth tissue engineering. Even though dental-derived stem cells are a good source of seed cells for such procedures, they are not often used in clinical applications because of the limited supply. Induced pluripotent stem (iPS) cells, with their high proliferation and differentiation ability, are now considered a promising alternative. The objectives of this study were to assess the role of iPS cells in tooth tissue engineering. We used real-time polymerase chain reaction to confirm that mouse iPS (miPS) cells can be induced to express both odontogenic and osteogenic gene profiles. We then established a tooth germ model and transplanted the recombinant tooth germ into a mouse subrenal capsule for 4 weeks to reproduce early-tooth organogenesis. After 4 weeks, hematoxylin and eosin staining results showed newly formed bone-like and dental pulp-like areas. Further immunohistochemical staining confirmed that osteopontin was present in the apical part of the tooth-like structure. These results demonstrate that miPS cells have the potential to differentiate into odontogenic cells, confirming that they could be a new source of seed cells for use in tooth tissue engineering.

Introduction

IN RECENT YEARS, TISSUE engineering has been the focus for investigators attempting to regenerate teeth for tooth replacement. With the rapid development of stem cell technology, two different methods have been developed. One of these is to combine adult stem cells with biological materials to achieve a partial regeneration of a dental structure. Adult stem cells show multipotency and self-renewal capacity and can be obtained from various tissues. Dental-derived stem cells, such as periodontal ligament stem cells, dental pulp, and dental follicle stem cells, are some of the successfully isolated adult stem cells, which can form a dentin-pulp complex.^{1–4} Bone marrow stromal cells also have the ability to differentiate into dentin and enamel structure.^{5–7} The other method used in tooth tissue regeneration is combining single-cell embryonic tooth germ suspension with scaffold materials to form tooth structures or even an entire tooth, through regulation of specific signaling pathways.⁸ When single-cell suspensions of dissociated porcine tooth germ seeded on scaffold materials, they could form complex tooth structures in subrenal capsules. Using a similar method, a fully functional bioengineered tooth has been already de-

veloped; it erupted and reached occlusion in the oral environment.⁹ However, the limited supply of adult stem cells and embryonic tooth germ has restricted these methods' clinical applications. It is clear that it would be helpful to identify novel cell sources with extensive proliferation activity and multipotent differentiation potential. Embryonic stem cells (ESCs) derived from the inner cell mass of mammalian blast cysts can extensively proliferate and differentiate into all kinds of cells derived from the three germ layers. ESCs can be induced to differentiate into dental epithelial-like cells expressing odontogenic genes such as *Msx1*, *Lhx7*, and *Pax9*.^{5,10} However, because of the difficulty of generating patient-specific ESCs, we face the problem of immune rejection. Ethical controversies also hinder the progress of practical application. The induced pluripotent stem (iPS) cells, originally developed from mouse dermal fibroblasts, have characteristics similar to ESCs, including capability of self-renewal, large-scale expansion, and differentiation toward all the three germ layers. They could be a potential cell source for directed differentiation and find a widespread application in the regenerative medicine field.¹¹ More importantly, using iPS cells avoids problems of immunological rejection and ethical controversy.¹² It has been reported

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recently that combining iPS cells with enamel matrix derivatives promotes the formation of new cementum, alveolar bone, and normal periodontal ligament.¹³

We hypothesized that iPS cells can serve as a novel non-odontogenic stem cell source for a tissue-engineered tooth-like structure (TET). In this study, we applied a conditioned medium to construct a realistic and reliable *in vitro* micro-environment with self-reorganization properties relying on molecular signaling pathways between epithelial and mesenchymal cells.¹⁴ To explore the ability of iPS cells to differentiate into odontogenic cells, we established a recombinant tooth germ model by mixing mesenchymal cells, iPS cells, and epithelial cells. We demonstrate that mouse iPS (miPS) cells have the potential to differentiate into odontogenic cells, making them a potential new source of seed cells to be used in tooth tissue engineering.

Materials and Methods

Isolation of dental epithelial/mesenchymal cells

The tissue surrounding the incisor germ from the mandibles of ED14.5 mice was excised and incubated in 1.2 U/mL Dispase II (Sigma) and 20 U/mL deoxyribonuclease I (DNase I; Takara) for 12.5 min at room temperature. The mesenchymal and epithelial tissues were separated with a fine needle. The epithelial tissue was treated twice, at 37°C for 20 min, with 100 U/mL collagenase I (Sigma) and 20 U/mL DNase I and triturated gently every 10 min. The mesenchymal tissue was incubated in 100 U/mL collagenase I and 20 U/mL DNase I at 37°C for 10 min and triturated every 5 min to dissociate them into single cells to be used in further experimentation.¹⁶

Cell culture and immunofluorescence analysis

The single-cell suspensions from epithelial and mesenchymal tissues were resuspended and incubated in 96-well plates (2×10^3 cells/well) with the Dulbecco's modified Eagle's medium (DMEM; Sigma), supplemented with 10% fetal bovine serum (FBS; Gibco BRL), 100 μ g/mL ascorbic acid (Sigma), 2 mM L-glutamine (Sigma), and 100 U/mL penicillin/streptomycin (Sigma). The culture medium was changed every 2–3 days.

For the immunofluorescence analysis, the cells were fixed in 4% paraformaldehyde (PFA; Sinopharm Chemical Reagent Co.) and then permeabilized using 0.1% Triton X-100 (Sigma) and 5% bovine serum albumin (Sigma). After incubation overnight at 4°C with primary antibodies against cytokeratin (CK19, 1:200; Santa Cruz Biotechnology, Inc.) and α -smooth muscle actin protein (α -SMA, 1:800; Sigma), the cells were incubated with phycoerythrin- (Becton Dickinson) labeled secondary antibodies and counterstained with DAPI (Sigma). The control cells were kept overnight at 4°C with phosphate-buffered saline, then incubated with secondary antibodies, and counterstained with DAPI. The expression of CK19 and α -SMA was visualized using a fluorescence microscope.

miPS cell culture and formation of embryoid bodies

The miPS cell line, a gift from Dr. Zhou Qi (Institute of Zoology, Chinese Academy of Sciences, Beijing), was generated from mouse embryonic fibroblast (MEF) cells

with a B6D2F1 genetic background by transfection with pluripotency-related genes (Yamanaka factors): *pMXs-Oct4*, *Sox2*, *Klf4*, and *c-Myc*. They also expressed the Oct4 promoter-enhanced green fluorescent protein (GFP) reporter. iPS cells were cultured on irradiated MEFs in a knockout DMEM (Invitrogen) supplemented with 10% FBS (Gibco BRL), 0.1 mM 2-mercaptoethanol (Sigma), 0.1 mM nonessential amino acids (Gibco BRL), 1 mM sodium pyruvate (Sigma), and 1,000 U/mL of leukemia inhibitory factor (LIF; Gibco BRL). After dissociation using 100 U/mL collagenase I (Sigma) at 37°C for 5 to 10 min, 4×10^6 cells were resuspended in 10 mL embryoid body (EB) formation medium, with the composition similar to the ESC culture medium, but without sodium pyruvate and LIF.^{15,16}

Osteogenic and odontogenic differentiation

On day 3, EBs were incubated in 0.05% trypsin (Sigma) for 5 min at 37°C. After the trypsin neutralization with a complete medium, a single-cell suspension was acquired by repeated pipetting. Subsequently, 1×10^5 cell aliquots were inoculated in 6-well plates (Costar) and co-culture with irradiated mouse dental mesenchymal cells.

For osteogenic differentiation, cells were incubated in an osteogenic induction DMEM supplemented with 15% fetal calf serum (FCS; Sigma), 0.1 mM dexamethasone (Sigma), 10 mM β -glycerophosphate (Alfa Aesar), and 50 μ M ascorbate-2-phosphate (Sinopharm Chemical Reagent Co.). The induction DMEM was changed every 3 days. The control group used normal medium containing 15% fetal calf serum. For odontogenic differentiation, cells were incubated in an odontogenic induction DMEM supplemented with 15% FCS (Sigma), 0.1 mM dexamethasone (Sigma), 10 mM β -glycerophosphate (Alfa Aesar), 50 μ M ascorbate-2-phosphate (Sinopharm Chemical Reagent Co.), and 20 ng/mL BMP4 (PeproTech, Inc.) or 20 ng/mL FGF4 (PeproTech, Inc.).

Reconstitution of tooth germs and organ culture

Tooth germs were reconstituted using the procedures described below. First, to form a collagen hemisphere, 30- μ L drops of gel (Cellmatrix® Type 1-A; Nitta Gelatin, Inc.) were placed on the membrane of the cell culture inserts (0.4- μ m pore diameter; BD). Epithelial cells, mesenchymal cells, or iPS cells were centrifuged (200 g), and the supernatant was removed completely. The sediments were resuspended to a density of 5×10^8 cells/mL. The resuspended iPS cells were mixed with mesenchymal cells at a ratio of 1:4. About 0.2- μ L aliquot of the mixture was injected into a collagen hemisphere, followed by injection of 0.2 μ L of epithelial cells into the adjacent area to promote direct cell contact. After 30 min, the reconstituted tooth germs were placed in 380 μ L of odontogenic induction medium. The cultures were incubated at 37°C for 5 days in 12-well cell culture inserts, with the medium changed every 2 days.¹⁷ There were two control groups; one contained miPS cells alone and the other epithelial and mesenchymal, but no miPS cells.

Reverse transcriptase-polymerase chain reaction and real-time quantitative polymerase chain reaction

Cells were collected after exposure to the osteogenic induction medium for 14 days, and the total RNA was

TABLE 1. PRIMERS USED FOR REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	bp
Col-I	GGCGAGTGTCTGTGCTTT	GGACCCATTGGACCTGAA	386
OC	CATCACCTGTCTCCTA	CTCCAGCACAACTCCT	219
Runx2	GTGCCAGGCGTATTTCA	CAGCGTCAACACCATCATTC	487
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	452

Col-I, collagen type I; OC, osteocalcin.

extracted with TRIzol reagent (Invitrogen). One microgram RNA (in 20- μ L reaction volume) was reverse transcribed using reverse transcriptase M-MLV (Takara), according to the manual. *Msx1*, *Lhx7*, *Pax9*, and *Runx2* mRNA levels were then checked by real-time quantitative polymerase chain reaction (PCR) at different FGF4 or BMP4 concentrations or after different treatment time. Briefly, 2 μ L of a 1/100 dilution of cDNA in water was added to 5 μ L of the 2 $\times$ SYBR green PCR master mix (Applied Biosystems), 800 nM of each primer, and water to 10 μ L. For profiling, we typically used 5- μ L reactions. The reactions were amplified for 15 s at 95°C and 1 min at 60°C for 35 cycles. Primer sequences are shown in Tables 1 and 2; GAPDH was used as an internal control. All primers were synthesized at Invitrogen, and the gene expression experiments were performed in duplicate.

Subrenal capsule transplantation

Twenty-four C57/BL mice were used in the Subrenal Capsule Transplantation procedure. All the animal experimental procedures were approved by the Institutional Animal Use and Care Committee at the Academy of Military Medical Science in Beijing, China. Briefly, the mice were anesthetized, swabbed with ethanol in the region of the left kidney, and a 1-cm-long incision was made. The kidney was partially exposed with a small slit in the renal capsule, and then the reconstituted tooth germs or control group cells were deposited under the capsule by using a gauge trocar, as previously described.¹⁸ The kidney was repositioned, and the incision was closed with sterile wound clips. Each animal received intramuscular injections of penicillin at a dosage of 4000 units daily, for 3 days.

Tissue preparation

For tissue preparation, the mice were anesthetized and treated with 4% PFA using systemic circulation fixation for half an hour, after which the tooth-bearing kidneys were removed and fixed immediately in 4% PFA for 24 h at 4°C. The specimens were demineralized in 10% EDTA (Sino-pharm) at room temperature for 7–14 days. Then, the tissues

were washed in 0.1 M phosphate buffer (pH 7.2), immersed in sucrose solutions of different concentrations, and frozen in OCT compound (Ames). Sections measuring 15- μ m thick were mounted on glass slides.

Histomorphometry and immunohistochemical analysis

Light and fluorescence microscopy was employed to assess the formation of tooth structure. Hematoxylin and eosin (H&E), Goldner's, and toluidine blue staining were used to evaluate the discrete tooth structure. Anti-mouse osteopontin (OPN) rabbit monoclonal antibody (OPN 1:200; Santa Cruz) and purified rabbit anti-GFP polyclonal antibody (GFP 1:150; Proteintech Group) were used for immunohistochemical detection. The slices were processed according to the manufacturer's protocols, and the results visualized by light microscopy.

Results

Isolation and identification of dental epithelial and mesenchymal cells

The tissues surrounding the incisor tooth germs obtained from the mandibles of ED14.5 mice were extracted. As shown in Figure 1, mesenchymal and epithelial tissues were separated, epithelial tissue resembling a cap and mesenchymal tissue resembling a saddle (Fig. 1A). Epithelial cells behaved as single-layered cells (slab-stone-like cells, Fig. 1B-a, B-b), whereas mesenchymal cells behaved as shuttle-like single-layered cells (Fig. 1B-c, B-d). The source of the isolated cells was characterized by immunofluorescence analysis (Fig. 1C). The expression of CK19 was observed in epithelial cells (Fig. 1C-a–C-c), and mesenchymal cells expressed high levels of α -SMA (Fig. 1C-g–C-i).

Identification of miPS cells and embryonic body formation

The miPS cells shared some characteristics with mouse ESCs, as shown in Figure 2A-a and A-b, and demonstrated by AP staining (Fig. 2A-f) and karyotype analysis (Fig. 2A-e). After 3 days of suspension culture, the miPS cell-derived EBs exhibited a regular round shape (Fig. 2A-c, A-d).

TABLE 2. PRIMERS USED FOR REAL-TIME POLYMERASE CHAIN REACTION

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	bp
<i>Msx1</i>	ACGCAAGCACAAAGACCA	CGCTCGGCAATAGACAG	108
<i>Lhx7</i>	CCCGCCATAAGAAACAC	GGGTGCTAGGCAGAATAA	118
<i>Runx2</i>	CCCAACTTCCTGTGCTCC	AGTGAAACTCTTGCCTCGTC	240
<i>Pax9</i>	GAATGGATTGGAGAAGGGAG	CAGCCACATAACCAGAAG	173
<i>GAPDH</i>	AGGTCCGTGTGAACGGATT	TGTAGACCATGTAGTTGAGGTCA	123

FIG. 1. Isolation and identification of dental epithelial/mesenchymal cells. **(A)** Separation of the epithelial tissue and mesenchymal tissue. **(A-a)** The mandible of ED14.5 mice. **(A-b)** The incisor tooth germs obtained from the mandibles of ED14.5 mice. **(A-c)** The epithelial tissue and mesenchymal tissue are separated. **(A-d)** The epithelial tissue. **(B)** The morphology of epithelial cells and mesenchymal cells **(B-e, B-f)** Epithelial cells. **(B-g, B-h)** Mesenchymal cells. **(C)** The verification of the source of the epithelial cells and mesenchymal cells by immunofluorescence. **(C-a-C-c)** The epithelial cells express high levels of CK19. **(C-d-C-f)** The CK19 control group without primary antibodies. **(C-g-C-i)** The mesenchymal cells express high levels of smooth α -muscle actin protein (α -SMA). **(C-j-C-l)** The α -SMA control group without primary antibodies; scale bar = 20 μ m. Color images available online at www.liebertpub.com/tea

FIG. 2. Culture and identification of mouse induced pluripotent stem (miPS) cells and embryoid body (EB) formation. **(A-a, A-b)** The miPS cells observed under fluorescent microscopy and normal light microscopy. **(A-c, A-d)** The miPS cell-derived EBs exhibit a regular round shape. **(A-e)** The miPS cells possess a normal karyotype. **(A-f)** The AP staining of miPS cells was positive. **(B)** Real-time polymerase chain reaction (PCR) was used to evaluate the related mRNA expression levels of *Msx1*, *Lhx7*, *Pax9*, and *Runx2*; **(B-a, B-b)** the concentration of the FGF4 or BMP4 of 0, 10, and 20 ng/mL; the mRNA expression levels of *Msx1*, *Lhx7*, *Pax9*, and *Runx2* were detected after EB incubation in an odontogenic induction medium for 7 days. **(B-c, B-d)** With FGF4 or BMP4 concentration of 20 ng/mL, the mRNA expression levels of *Msx1*, *Lhx7*, *Pax9*, and *Runx2* were detected after EBs' incubation in an odontogenic induction medium for 0, 3, and 7 days. **(C)** Reverse transcriptase-polymerase chain reaction was used to evaluate the related mRNA expression levels of collagen type I (Col-1), osteocalcin (OC), and *Runx2*; scale bar = 50 μ m. Color images available online at www.liebertpub.com/tea

Expression of odontogenic- and osteogenic-related genes

After incubation of EBs in an odontogenic induction medium for 7 days, real-time PCR was used to evaluate the mRNA expression of *Msx1*, *Lhx7*, *Pax9*, and *Runx2*. The results demonstrate that the expression of *Msx1*, *Pax9*, *Lhx7*, and *Runx2* increases after treatment with 20 ng/mL FGF4 (BMP4) (Fig. 2B-a, B-b). After odontogenic induction medium (20 ng/mL FGF4 or BMP4) treatment for 7 days, mRNA expression levels of *Msx1*, *Lhx7*, *Pax9*, and *Runx2* are also visibly enhanced (Fig. 2B-c, B-d). Reverse transcriptase-polymerase chain reaction results show that the collagen type I, osteocalcin, and *Runx2* are expressed at high levels after osteogenic medium incubation, demonstrating the osteogenic differentiation potential of miPS cells (Fig. 2C).

Reconstitution and transplantation of tooth germs

The experimental procedure was performed as shown in the schematic (Fig. 3A). Epithelial cells were seeded close to the mesenchymal and EB cells to enable direct cell contact and ensure tooth organogenesis. The tooth germ was placed

on a cell culture insert (Fig. 3B-a) and incubated for 5 days before transplantation, during which time we observed its continuous growth (Fig. 3B-b). The recombinant tissue-engineered tooth germ was transplanted into the subrenal capsule for 4 weeks (Fig. 3B-c) to test whether the recombinant TET germ has the ability to develop into a complete new TET in that microenvironment.

Histomorphometry and immunohistochemical analysis

HE staining results show that some recombinant tooth germs can form the bone-like structure (Fig. 4A-b); in some cases it was a dentin-like and dental pulp-like structure (Fig. 4A-a, A-c, A-d) and in others, dental-like structure (Fig. 4A-c, A-d). However, the iPS cells alone do not form tooth-like or bone-like structures (Fig. 4C-a), whereas epithelium and mesenchyme without miPS cells produce irregular TET (Fig. 4C-b). Fluorescence microscopy shows that miPS-derived cells in the newly formed structure still express GFP (Fig. 4B). Fortunately, we achieved an ideal result with mature TET similar to a normal tooth (Fig. 5). H&E staining showed a complete new tooth structure formation (Fig. 5A), and fluorescence microscopy demonstrated miPS-derived

FIG. 3. Reconstitution and transplantation of tooth germs. **(A)** The schematic diagram of the *in vivo* experiment. **(B-a, B-b)** Three kinds of cells were encapsulated in a collagen gel and placed in cell culture inserts. The images from camera **(B-a)**, fluorescent microscopy, and normal light microscopy **(B-b)** demonstrate the rough morphology of the artificial tooth germ. **(B-c)** The renal capsule was exposed, and the recombinant tooth germs were transplanted. Color images available online at www.liebertpub.com/tea

FIG. 4. Histomorphometry analysis of bone-like structure and dental pulp-like structure. **(A-a, A-b)** Hematoxylin and eosin (H&E) staining of dentin-like structure **(A-a)** and bone-like structure **(A-b)**. **(A-c)** H&E staining of dental-like structure. In this image, the bone-like structure is on the outside, and the dental pulp-like structure is inside. The architecture looks like a rough tooth, but the dentin is absent. **(A-d)** The Goldner's staining of the same visual field of the **(A-c)**. **(B)** The main source of the newly formed structures are miPS cells with enhanced green fluorescent protein (GFP); the region in red circle in **(B-a)** shows the newly formed tissues; the region in red circle in **(B-b)** demonstrates a newly formed bone-like structure, and in the black circle, a newly formed dental pulp-like structure **(B-c)**, indicated the newly formed dental pulp-like structure. **(B-d)** The enlarged magnifications of yellow box in the **(B-c)**. This is the view under light microscopy **(B-a1-B-d1)**, fluorescence microscopy **(B-a2-B-d2)**, and after merge **(B-a3-B-d3)**. **(C)** The control groups. **(C-a)** The miPS cells alone were transplanted into the subrenal capsule. **(C-b)** Epithelium and mesenchyme without miPS cells were transplanted; scale bar = 50 μ m. Color images available online at www.liebertpub.com/tea

FIG. 5. Histomorphometry and immunohistochemical analysis of mature tooth-like structure (TET). **(A-a)** H&E staining of mature TET under low-power lens. **(A-b, A-c)** The Goldner's staining **(A-b)** and toluidine blue staining **(A-c)** of the same area of **(A-a)**. **(A-d)** A part of the Goldner's-stained slice under a high magnification. **(A-e)** A part of the toluidine blue-stained slice under high-power lens. **(B)** miPS cells with enhanced GFP were the main source of the mature TET. This is the view under light microscopy **(B-a)**, fluorescence microscopy **(B-b)**, and after merge **(B-c)**. **(C)** Immunohistochemical staining of mature TET. Immunohistochemical staining of the slices of the mature TET with anti-osteopontin antibody **(C-a)** and anti-GFP antibody **(C-b)** visualized with BAD. PB, prebone; scale bar=20 μ m. Rectangles indicate the amplification image of select region. Color images available online at www.liebertpub.com/tea

cells in the structure. To investigate whether miPS cells participate in the early tooth organogenesis, we examined the expression of GFP and OPN using an immunohistochemical analysis. The staining results showed expression of OPN in the apical part of tooth and expression of GFP in the same region (Fig. 5C-a, C-b). These results confirm that miPS-derived cells participate in tooth development and can differentiate directly into a bone-like and dental pulp-like structure.

Discussion

Some previous studies have reported that ESCs have the ability to form mineralized tissue both *in vitro* and *in vivo*, and that ES-derived osteogenic cells can be mineralized after transplantation *in vivo*.^{19–21} ESCs have also been used in endochondral bone tissue engineering.²² When overlaid with oral epithelium *in vitro*, ESCs can differentiate into odontoblast-like cells and express dentin matrix protein 1²³ and some odontogenic genes, such as *Msx1*, *Lhx7*, and *Pax9*.²⁴ Mouse ESCs have the potential to differentiate into dental epithelial-like cells when induced by ameloblasts in a serum-free conditioned medium.¹⁰ However, ESCs present the problem of immune rejection when used in clinical transplantation. Ethical controversies also hinder the progress of their practical application.

Since iPS cells were originally developed from mouse dermal fibroblasts by defined factors,¹² a growing number of terminally differentiated cells, including those from oral mucosa and dental tissue,^{25,26} have been reprogrammed into iPS cells.^{27,28} The characteristics of iPS cells are similar to ESCs in morphology, cell proliferation rate, and critical gene

expression profiles. iPS cells can also form EBs differentiated into three germ layers, and can produce teratomas and chimeras.²⁹ Autogeneic cells can be also reprogrammed to iPS cells. Therefore, iPS cells, without the burden of immunological rejection and ethical controversy, seem to be superior to ESCs, and are a potential alternative stem cell source for tissue engineering.

To facilitate a preliminary study and reduce the differences among species, iPS cells were induced to form EBs before reconstituting a tooth germ, and homologous animals were used in the transplantation model. In this study, we used miPS cells and a mesenchymal cell coculture system. The mesenchymal cells can interact with both the innate and adaptive immune system²⁹ and form the microenvironment for reconstituted tooth germ development. We used miPS cells mixed with mesenchymal cells. These were placed in close contact with the epithelial cells to accelerate tooth germ formation. The tooth germs were transplanted into the sub-renal capsule after incubation in a 12-well cell culture insert for 5 days. The regulation of tooth development is inherently complicated, involving many signaling pathways, such as FGF and BMP. The homeobox gene expression can be also induced, involving genes such as *Msx1* and *Pax9*, with crucial roles in early tooth genesis.^{5,24,30} Furthermore, tridimensional matrices and the addition of growth factors such as FGF, BMP, and PDGF can facilitate the transplantation and the differentiation of stem cells.³¹ Therefore, in this study, we added FGF4 and BMP4 to the medium used in our *ex vivo* experiments. Ten days later, the PCR results confirmed the expression of *Msx1*, *Lhx7*, *Runx2*, and *Pax9* in miPS cells. This is likely to have been induced by FGF4 and BMP4 treatment, initiating odontogenesis of miPS cells.

Complex tooth structure formation was first reported by Young's group.⁸ They combined porcine tooth germ tissue-derived, single-cell suspensions with the scaffold material, and transplanted them to the omentum. They found that the single cells could reorganize themselves and differentiate into odontoblasts and ameloblasts. It has also been reported that epithelial cells placed adjacent to mesenchymal cells can mimic the tooth organogenesis, resembling the initial stages of the tooth germ development.³² Recently, another method utilizing self-reorganization of bioengineered organ germ cells has been reported. In that study, a fully functional bioengineered tooth was developed, erupted, and reached occlusion in the oral environment. All dental structures such as odontoblasts, ameloblasts, dental pulp, blood vessels, periodontal ligament, root, and alveolar bone were observed. In our current study, we used this method in our tooth regeneration model.^{9,17} Although not all of the recombinant tooth germs developed into perfectly mature TETs, a number of specimens still formed bone-like and dental pulp-like structures. These results demonstrate that our new tooth germ model can be used to imitate the interaction between epithelial and mesenchymal cells and reproduce early tooth organogenesis. It is also clear that miPS-derived cells involved in the process of tooth development do not affect adversely the interaction between epithelial and mesenchymal cells, and that they have the ability to differentiate into a bone-like and dental pulp-like structure.

Further research should be focused on exploring the dental differentiation ability of iPS cells, which might be regulated by external factors independent of the molecular pathway regulatory systems of epithelial or mesenchymal cells. The ideal animal model should be developed employing the mandible or maxillary regions to mimic a more realistic microenvironment for tooth development. Overall, our results present a viable alternative path for the future research in the dental regenerative therapy.

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Disclosure Statement

No competing financial interests exist.

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