Research Article

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Effective Differentiation of induced Pluripotent Stem Cells into Dental Cells

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Abstract

Background

A biotooth is defined as a complete living tooth, made in laboratory cultures from a spontaneous interplay between epithelial and mesenchymal cell-based frontal systems. A good solution to these problems is to use iPSCs. However, no one has yet formulated culture conditions that effectively differentiate iPSCs into dental epithelial and dental mesenchymal cells phenotypes analogous to those present in tooth development.

Results

Here, we tried to induce differentiation methods for dental epithelial cells and dental mesenchymal cells from iPSCs. For the dental epithelial cell differentiation, the conditional media of SF2 dental epithelial cells was adjusted to embryoid body (EB). Moreover, we now report on a new cultivation protocol, supported by transwell membrane cell culture that make it possible to differentiate iPSCs into dental epithelial and mesenchymal cells with abilities to initiate the first stages in *de novo* tooth formation.

Conclusion

Implementation of technical modifications to the protocol that maximize the number and rate of iPSC differentiation, into mesenchymal and epithelial cell layers, will be the next step towards growing an anatomically accurate biomimetic tooth organ.

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Introduction

Periodontal disease is a major health issue and eventually leads to tooth loss. Existing tooth replacements are artificially manufactured metal and ceramic structures. However, they too are susceptible to a similar form of periodontal disease as natural teeth (Langer and Vacanti, 1993; Sumida et al., 2002; Correia et al., 2017). The alternative is to produce a full or partial biological replacement, consisting of living periodontal tissues with or without a solid structure. Following recent advances in the ability to generate organoids (mini organs) and regenerate tissues from embryonic stem cells (Mustard and Levin, 2014; Marx, 2015), there is renewed interest in engineering the tooth organ according to biology and the embryo, with particular focus on producing a viable root structure with a periodontium attachment system (Sato et al., 2009; Volponi et al., 2013).

Tooth morphogenesis and differentiation is regulated by the interaction between epithelium and mesenchyme (Kim et al., 2017). Therefore, in mice, strategies in recapitulating tooth organ formation have fixated on the recombination of epithelium and mesenchyme at selected stages of development, in which tooth forming capacity exists and is optimized (Yamamoto et al., 2003; Duailibi et al., 2004; Nakao et al., 2007). Dental epithelium from initiation stages can induce tooth formation when combined with non-odontogenic mesenchyme. However, after epithelial induction into the mesenchyme, this becomes the inductive tissue and reciprocates inductive signals back to the now non-inductive epithelium (Zhang et al., 2005). Until now, many studies for tooth regeneration in laboratory thus have been approached with at least one tooth inductive population, either mesenchyme or epithelium. There are also possibilities of using one or both cell types from postnatal tissues (Yamamoto et al., 2003; Duailibi et al., 2004; Ohazama et al., 2004; Otsu et al., 2014). Therefore, bioengineering of a tooth organ is needed a feasible strategy for epithelial and mesenchymal cells with embryonic

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programming.

There are not many of available cells to generate dental tissues, and furthermore, it is more difficult to sustain cells with strong capacity for well-defined 3D phenotype of tooth (Jernvall and Thesleff, 2000; Ohazama et al., 2004; Tucker and Sharpe, 2004; Otsu et al., 2012).

Induced pluripotent stem cells (iPSC) have become the leading cell source for producing any choice of specialized cell types from all three primary germ layers and compared to analogous embryonic pluripotent stem cells there are no ethical problems. Consequently, iPSCs have even stronger clinical potential for the effective replacement of diseased or damaged tissues (Yoshida and Yamanaka, 2011; Ramsden et al., 2013; Suchy et al., 2018). Recent reports have showed that mouse iPSCs could be induced to differentiate into ameloblastin-expressing dental epithelial cells and odontogenic mesenchymal cells through neural crest-like cells (Otsu et al., 2012; Cai et al., 2013). Thus, the focus on developing iPSCs for production of tooth germs and tooth organs is important. However, the most pressing problem is to increase the quality, selectivity and quantity of cells for the biological engineering of compound tooth structures. Furthermore, effective tooth engineering requires selecting high potency cell mixtures that tightly regulate early tooth development.

Herein we show a new set of cell culture procedures to convert iPSCs into dental epithelium and mesenchyme with properties and characteristics to initiate a tooth structure.

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Results

Differentiation of Dental Epithelial Cells from iPSCs (DECi)

To differentiate into a dental epithelial cell phenotypic lineage, we attempted to modify the DEC differentiation method (Cai et al., 2013). In this study, mouse iPSCs (iPS-MEF-Ng-20D-17) were treated with SF2 dental epithelial cell conditional media (Arakaki et al., 2012). As illustrated in Figure 1, iPSCs was made into embryoid bodies (EB) using a hanging drop culture method, and EBs were differentiated into DECs using conditional media (Fig. 1A). To generate EBs, iPSCs were cultured for 4 days using hanging drop culture using iPSC media without LIF (Fig. 1B). The EB were then transferred to fibronectin coated dishes and differentiated into DECs using SF2 cell conditional media with 25ng/ml BMP4, 1uM Retinoic acid and 1X N2 supplement (Cai et al., 2013). After 5 days, and 10 days culture with conditional media, migratory cells from EB were shaped into polygons typical of epithelial cell morphology (Fig. 1C and 1D). For subsequent experiments, EB were mechanically removed by suction, and the migratory cells were cultured on fibronectin coated dishes (Fig. 1E). The effects of DEC differentiation from iPSC were examined by expression of the epithelial cell marker, cytokeratin 14 (CK14) as detected by immunofluorescence (Fig. 1F). Furthermore, in order to determine successful iPSCs differentiation into dental epithelial cells, the specific dental epithelial cell marker, ameloblastin (AMBN) was examined in the DECi (Fig. 1G). As expected, the stem cell marker OCT3/4 was not expressed in the DECi as shown by negative OCT3/4 immunofluorescence (Fig. 1H). Western blotting analysis was used to investigate the expressional status of dental epithelial cell markers and stem cell marker during differentiation of iPSCs. DECi increased its expression of the dental epithelial cell markers (AMBN and CK14) and decreased its expression of a stem cell marker (OCT3/4) (Fig. 1I).

Flow cytometry demonstrated that the number of CK14 and AMBN positive cells among the derived cell population was increased compared to expression of those markers among undifferentiated iPSCs (Fig. 1J and 1K). Moreover, OCT3/4 positive cells among the derived cells were decreased compared to undifferentiated iPSCs (Fig. 1L). These results showed that iPSCs lost their original stem cell properties and differentiated into dental epithelial cells by our modified differentiation method.

Neural crest like cells from iPSCs (NCLCs)

Mouse iPSC were treated with neural induction media, including bFGF, EGF, insulin, B27 and N2 supplement, to differentiate into the NCLC which used for dental mesenchyme (Otsu et al., 2012). As illustrated in Figure 2A, iPSCs was made into EB, using a hanging drop culture, then, iPSCs were differentiated into NCLC.

iPSCs can differentiate into NCLCs with rosette like structure from EBs (Fig. 2B – 2D). EB were mechanically removed by suction, and the migratory cells were cultured on fibronectin coated dishes after 16 days culture (Fig. 2E). NCLCs expressed the neural crest markers, such as Nestin and p75 (Fig. 2F and 2G), as well as dental mesenchymal cell markers, such as Lhx6 and Msx1 (Fig. 2H and 2I). Flow cytometry demonstrated that the number of Vimentin and Snail positive cells among the derived cells was increased compared with that among the undifferentiated iPSCs (Fig. 2J and 2K). RT-qPCR further confirmed that mRNA expression of neural crest cell-specific transcriptional factors, such as *Notch1*, *Notch2*, *Pax3* and *Slug*, was markedly higher in derived cells than in undifferentiated iPSCs (Fig. 2L -2O). By western blot analysis, NCLCs showed increased expressions of the neural crest markers (p75 and Ap2α) and dental mesenchymal cell markers (Lhx6 and Msx1) (Fig. 2P).

Differentiation of iPSCs using transwell

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To increase efficiency for differentiation, co-culture method with feeder cells or tissues using transwell were utilized in this study. Transwell inserts with porous membranes allowed to separate iPSCs from feeder cell layers such as SF2 dental epithelial cells for dental epithelial cells differentiation, and primary dental molar mesenchymal cells at mouse embryonic day 16 (E16, bell stage) for dental mesenchymal cells differentiation. To this end, SF2 dental epithelial cells and primary dental mesenchymal cells at E16 were allowed to attach and grow, as a small sheet on the bottom of transwell membranes. It is generally known that tooth potential is present in tooth mesenchyme at E14. Also, dental mesenchyme at E16 still has potential for teeth formation (Takahashi et al., 2010). Due to much larger of cell numbers at E16 than that at E14, primary dental mesenchymal cells at E16 were used as feeder cell layers for differentiation of dental mesenchymal cells in this study. After 1 day of this, the transwell membrane was inverted and placed into a plastic culture well. iPSCs were seeded and cultured on the top of the transwell for 6 days (Fig. 3A).

(a) iPSC-to-dental epithelial cell differentiation via co-culture with SF2 cells (TDECiSF2) and E16 dental mesenchymal cells (TDMCDC)

Culturing of iPSCs with SF2 cells for 6 days in transwell-based co-cultures resulted in increases in the epithelial cell markers (CK14, LEF1 and p21) and the dental epithelial cell marker (AMBN) compared with DECi (Fig. 3B-3E). This result shows that the differentiation efficiency is increased when using a transwell than our modified method (Fig. 1A). The differentiation profile of iPSCs was determined by western blot analysis. Dental epithelial cells from iPSCs in transwells (TDECiSF2) showed increased expressions of the epithelial cell markers (CK14, LEF1 and p21) and dental epithelial cell marker (AMBN) (Fig. 3F). Co-culturing of iPSCs for 6 days with E16 mouse dental mesenchymal cells (TDMCDC) mounted on a transwell insert resulted in the increase of dental mesenchymal cell markers

(LHX6 and MSX1) (Fig. 3G and 3H) compared with NCLCs. However, the neural crest cell marker expression (AP2α) decreased compared to NCLCs (Fig. 3I). These results meant that the transwell co-culture method enhanced the efficiency of differentiation to dental mesenchymal cells. In addition, the expression of dental mesenchymal cell markers and neural crest cell markers were examined by western blotting accordingly for: MSX1, LHX6 and AP2α. As a result, the expression of the dental mesenchymal cell markers (MSX1 and LHX6) was increased and the expression of the neural crest cell marker (AP2α) was decreased (Fig. 3J). However, when iPSCs were differentiated into dental epithelial cells and dental mesenchymal cells using SF2 cells and E16 dental mesenchymal cells as feeder cells, the biotooth were not formed when they were recombined (data not shown).

(b) iPSC-to-mesechymal and dental epithelial cell differentiation via co-culture with E14 dental epithelium (TDECi) and E14 dental mesenchyme (TDMC)

We differentiated the iPSCs with feeder tissues using E14 dental epithelium and E14 dental mesenchyme from mouse molar tooth germ instead of the SF2 cells and mouse E16 dental mesenchymal cells. To this end, twenty dental epithelium and twenty dental mesenchyme separated with dispaseII from tooth germs, were attached on the underside of the membrane. After 1 day, the transwell membrane was inverted again and placed into an individual well of a 6 well plate, iPSCs were then seeded and cultured on the top surface of the porous transwell membrane (Fig. 4A). In this way, iPSCs were differentiated into dental epithelial cells (TDECi) and dental mesenchymal cells (TDMC) for 6 days. TDECi were examined for the existence of enamel knot markers (*Shh*, *Bmp4*, and *Fgf4*), and dental epithelial cell markers (*CK14* and *Ambn*) by RT-qPCR (Fig. 5A). As a result, the expression of enamel knot markers in TDECi were increased than in iPSCs, furthermore, the expression in TDECi is higher than in epithelial cells used as a feeder (feeder epi). The expression level

of *CK14* is the most increased among iPSC and feeder epi, whereas *Ambn* expression is the highest in feeder epi than in iPSCs and TDECi. TDMC were examined for the presence of dental mesenchymal cell markers by RT-qPCR. The expression of dental mesenchymal cell markers (*Lef1*, *Edar*, *Runx2*, *Msx2* and *Barx1*) in TDMC was increased compared to iPSCs, however, was lower than the mesenchymal cells used as feeder (feeder mes) (Fig. 5B).

Co-culturing of iPSCs for 6 days with E14 mouse dental epithelium (TDECi) mounted on a transwell insert resulted in the increase of dental epithelial cell markers (*Bmp4*, *Fgf4*, *Shh CK14,* and *Amgn*) compared with TDECiSF2 (Fig. 4B). In addition, the expression of dental epithelial cell markers was examined by flow cytometry for: CK14, AMGN, AMBN. As a result, the expression of the dental epithelial cell markers in TDECi was increased than in TDECiSF2 (Fig. 4C).

TDMC was increased dental mesenchymal cell markers (*Lef1*, *Edar*, *Runx2*, *Msx2* and *Barx1*) compared with TDMCDC (Fig. 4D). We examined expression of the dental mesenchymal cell markers (LEF1, LHX6 and MSX1) by flow cytometry (Fig. 4E). As a result TDMC were increased dental mesenchymal cell markers than TDMCDC. These results showed that E14 dental epithelium and dental mesenchyme enhanced the efficiency of differentiation to dental epithelial and dental mesenchymal cells from iPSC.

Microspheroidal cellular assemblies of epithelium and mesenchyme in a transplantation model

A mouse tooth germ at E14 was cultured in semi-solid media for 2 days, then, it was transplanted into subrenal capsule for 5 weeks. After 5 weeks, the tooth germ developed into an identifiable tooth organ structure (n=3/3) (Fig. 6A). In the first epithelial and mesenchymal recombination trial, we used NCLCs and E12 mouse dental epithelium. Our previous study established reaggregation and recombination experimental method about

dental mesenchymal cells and dental epithelium (Cai et al., 2007). Accordingly, 2ul of NCLCs (2.5 x10^5) were placed into the hole with blunt yellow in the agar media and then covered with E12 mouse molar dental epithelium. After 2 days of *in vitro* culture, the recombined cell layers were transplanted into subrenal capsule for 5 weeks. After 5 weeks, the epithelium and NCLCs recombination made a tooth-like morphology (n=1/12) (Fig. 6B). In the second recombination assortment, 1ul of DECi (1.25 x10^5) were fused with 2ul of primary E14 dental mesenchymal cells (2.5 x10^5). After 2 days *in vitro* culture, the recombined cell layers were transplanted into a subrenal capsule for 5 weeks. After 5 weeks, this recombination generated calcified tissue, not tooth like structure (n=2/9) (Fig. 6C). In the final recombination study, TDECi was fused with TDMC. After 2 days *in vitro* culture, the combined cell layers were transplanted into a subrenal capsule for 5 weeks. After 5 weeks, this combination made calcified tissue (n=2/14) (Fig. 6D). To identify that the hard tissue has a tooth-like structure, we examined dental epithelial cell markers and a dental mesenchymal cell marker in that calcified tissue. As a result, tooth epithelium markers, such as CK14, AMGN and NESTIN were expressed (Fig. 6E-6G'). Moreover, the mesenchymal marker MSX1 was expressed in the inner part of the calcified tissue (Fig. 6H and 6H`). Therefore, TDECi and TDMC recombination, which were differentiated from iPSC using transwell method, may have the strong potential to generate biotooth. However, experiments to increase the success rate should be carried in further. All procedures in this study is summarized in Figure 7.

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Discussion

A bioengineered tooth is a viable alternative to tooth implants, promising permanency and low risk of periodontal disease. Various dental stem cell types have been engineered to regenerate pulp, dentine and periodontal tissues, but clinical availability remains stubbornly inadequate for therapy in patients (Miura et al., 2003; Peng et al., 2009; Yan et al., 2011; Lee et al., 2017). iPSCs are the most clinically promising cell source for bioengineering in patients, but stronger more efficient methods are needed to transform them into the desired cell types with precisely programmed genotypes (Yoshida and Yamanaka, 2011; Ramsden et al., 2013; Suchy et al., 2018). In this study, we highlighted some of the first principles for generating of a partial tooth organ derived entirely from iPSCs. In order to reconstruct a whole tooth and attachment system, biotooth tissue engineering should follow embryonic processes and mechanisms for development and provide cell phenotypes with maximum tooth forming potential. However, neither, DECi, NCLC, TDECi and TDMC appeared to express all gene series existing in the native tooth germ.

Because tooth development relies on the interaction between dental epithelium and dental mesenchyme to control all the key tooth structuring and organizational processes, cell recombination experiments facilitate evaluation of that during tooth development. For instance, neural crest derived mesenchymal stem cells interact with dental epithelium to generate odontoblasts and dental pulp cells, in turn, epithelial cells manufacture ameloblasts (Arakaki et al., 2012). We performed recombinations of epithelial and mesenchymal cells from an array of associate cell types (DECi, NCLCs, TDECiDF2, TDMCDC, TDECi and TDMC), to fabricate the most effective tooth germs for organ formation.

Cell-co-culture approaches remain the most popular means of preparing for organ regeneration and the engineering of co-culture systems that emulate the way cells are

positioned, organised and arranged during development. Commonly used options include transwell plates (Hatherell et al., 2011; Miki et al., 2012). Compartmentalized co-culture system, involve physical separation of cells inside a single medium and allow exchange of signals without cell admixing. The objectives of this study were to differentiate iPSCs into high potency epithelium and mesenchyme with potential to form tooth germs following recombination into microspheroids. Feeder cells were divided from differentiating iPSCs to prevent fusion and contamination of iPSCs and transformed cells while maintaining biochemical signaling exchanges that provide key developmental signals to the iPSC population. When a physical barrier was used to separate, in close juxtaposition, the interacting cell types, between SF2 cells, dental mesenchymal cells and iPSCs, the resulting cell products had increased expression of epithelial cell and dental mesenchymal cell markers when compared with the cells grown in conditioned media. Recombinants of these cells showed promising calcification structure following *in vivo* implantation.

We devised a new procedure, without conditioned media, using transwell apparatus and a microspheroid-forming template to recreate primordial germs with an ability to form mineralized analogous tooth morphology.

A full structured tooth is highly possible by this method. The cell masses express many key genes at the origins of tooth germ formation. More efficient transformation between iPSC and dental mesenchyme and epithelium cells will lead to higher stem cell numbers and increased prospects for proper tooth organs with pulp chambers, root architectures and a surrounding attachment complex.

Experimental Procedures

All experiments were performed according to the guidelines of the Yonsei University, College of Dentistry, Intramural Animal Use and Care Committee (2013-0126).

Histology and immunohistochemistry

The cultured tissues were fixed with 4%PFA (Wako, Osaka, Japan) in 0.01 M phosphate-buffered saline (PBS, pH 7.4) overnight at 4°C. After being embedded in paraffin (Leica Biosystems, Mo., USA), these samples were sectioned at a thickness of 6μm. The tissue sections were blocked in 3% hydrogen peroxide for 15 min. Sections of the specimens were incubated at 4°C overnight with the primary antibody against CK14 (abcam, Cambridge, UK, 1:200), Amelogenin (SantaCruz, CA, USA, 1:200), Nestin (Milipore, MA, USA, 1:200) and Msx1 (SantaCruz, 1:200). After washing with PBT, the specimens were allowed to react with biotinylated goat antimouse immunoglobulins and streptavidin peroxidase at room temperature for two consecutive 10 min incubations. Finally, the specimens were visualized using a 3,3'-diaminoben- zidine (DAB) reagent kit (Life Technologies, CA, USA).

Immunofluorescence

Cells were grown on gelatin-coated coverslips for at least 24 h and subsequently washed, fixed, permeabilized, blocked and incubated overnight with primary antibodies. Subsequently, the coverslips were washed with PBT, incubated with FITC-conjugated secondary antibodies in PBT for 1 hr at room temperature, washed again with PBT, incubated with To-pro3 in PBT for 15 min.

Cell culture

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Cells of the mouse iPS cell line iPS-MEF-Ng-20D-17 (RIKEN, Japan) were cultured on mitotically inactivated mouse embryonic fibroblasts in Dulbecco's modified Eagle's medium (DMEM, Gibco, MA, USA) containing 15% fetal bovine serum (FBS), 0.1mM nonessential amino acids (Gibco), 1x GlutaMAX (Gibco), 0.1mM 2-mercaptoethanol (Sigma-Aldrich, MO, USA), 50U/mL peni-cillin, 50mg/mL streptomycin (Gibco), and 1,000U/mL mouse leukemia inhibitory factor (Millipore). The cultures were maintained at 37°C in a 5% CO_2 humidified incubator, and the medium was changed every other day.

Neural crest like cells (NCLCs) were derived from mouse iPSCs by a previously described method with modifications. In brief, iPSCs were dissociated with TrypLE (Gibco) and transferred to culture dishes with neural induction medium for suspension culture to form neuroectodermal spheres. The neural induction medium consisted of a 1:1 ratio of DMEM/F12 (Gibco) and neurobasal medium (Gibco) supplemented with 0.5X N2 (Gibco), 0.5X B27 (Gibco), 5mg/mL insulin (Sigma-Aldrich), 20ng/mL basic fibroblast growth factor (bFGF) (PeproTech, NJ, USA), 20ng/mL epidermal growth factor (PeproTech), 50U/mL penicillin, and 50mg/mL streptomycin. After 4 days in suspension culture, the spheres were transferred to fibronectin-coated culture dishes (BD Biosciences, CA, USA). After 8–10 days, the spheres or rosettes of NCLCs were manually removed, and the resultant NCLCs were dissociated with TrypLE, and plated on fibronectin-coated culture dishes.

in vitro culture with semi-solid media

4% agar was melted using an autoclave, and placed in in 65-degree water bath. Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 20% FBS was placed in a 65°C water bath. First, 2% agar media was poured at the dish. 5 min later, 1% agar media was added. Then, the holes were made with a blunt yellow tip.

Kidney capsule transplantation

The adult male nude mice were anaesthetized by injection with the anesthetic (Rumpun: Zoletil: Saline = 1: 5: 6, 60~70 ul/mouse). The recombinants of differentiated cells from iPSCs were transplanted beneath the renal capsule of male nude mice.

Flow cytometry

Dental epithelial cells and dental mesenchymal cells from iPSCs were characterized using flow cytometry. A total of 0.5–1x10^6 dental epithelial cells and dental mesenchymal cells from iPSCs were stained with CK14 (Abcam), AMBN (SantaCruz), Oct3/4 (SantaCruz), Snail (Abcam), Vimentin (Thermo Fisher Scientific, OK, USA), Lef1 (SantaCruz), P21 (Thermo Fisher Scientific), Lhx6 (Abgent, CA, USA), Msx1 (SantaCruz) and Ap2α (SantaCruz) antibodies and conjugated with fluorescence isothiocyanate (FITC), phycoerythrin (PE). Subsequently, cells were analyzed by flow cytometry, using a FACSCalibur (BD Biosciences, NJ, USA) operated by CellQuest software and at least 10,000 events were collected per sample.

Western blot

Western blotting was performed with a SDS-PAGE Electrophoresis System. Briefly, 40 μg protein samples were resuspended in a reduced sample buffer, and then electrophoresed on a 7.5 to 10% Tris gel with Tris running buffer; blotted to PVDF membrane; and probed with primary antibodies against AMBN (SantaCruz), CK 14 (Abcam), OCT3/4 (SantaCruz), LEF1 (SantaCruz), P21 (Thermo Fisher Scientific), MSX1 (SantaCruz), LHX6 (ap1423a), AP2α (SantaCruz). α-Tubulin (Sigma-Aldrich) was used as a protein loading control.

Culturing mouse iPSCs on porous membrane

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iPSCs were cultured on top of porous transwell inserts in iPSCs culture media. As feeder cells (5.0 x 10^5 cells per insert), dental epithelium and dental mesenchyme were seeded on an inverted transwell cell culture insert fitted for 6 well plates (1µm pore polyethylene terephthalate membrane); supported by a Teflon ring and cultured for 24 hrs. Following attachment of feeder cells or tissues to the bottom side of the insert, it was turned upright, and iPSCs were seeded inside the insert.

RNA isolation, RT-PCR, and qPCR Analyses

Total RNA was isolated from confluent cell cultures using Trizol according to the manufacturer's instructions. For cDNA synthesis, reverse transcription was performed using M-MuLV reverse transcriptase (New England BioLabs, USA). RT-PCR was performed using the Thermal Cycler Dice TP600 (Takara, Japan) with AccuPower® PCR Pre Mix (Bioneer, Korea). The amplification program consisted of 40 cycles of the following: denaturation at 95°C for 20s, annealing at 57°C for 20s and extension at 72°C for 70s. Transcript levels of *Notch1, Notch2, Pax3, Slug, Shh, Bmp4, Fgf4, CK14, AMBN, Lef1, Edar, Runx2, Msx2* and *Barx1* were determined. The RT-qPCR analysis of each sample was performed in triplicate, and the amount of each of the RT-qPCR products was normalized using β-2-microglobulin as an internal control.

Ambn	F 5′-CCACAATTTCCAACCGTGTTC-3`
	R 5`-GCCATTGGTCCCCGAGAT-3`
CK14	F 5`-ACGAGAAGATGGCGGAGA AG-3`
	R 5`-CTCTGTCTTGCTGAAGAACCATTC-3`

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Edar	F 5`-GCCAACCAACAAGACCACAGT-3`
	R 5`-AAACATGGCAGCGTGGAGTT-3`
Pax9	F 5`-CGTGCCCTCGGTGAGTTC-3`
	R 5`-GGGCCAAGTTGCCGATCT-3`
Lef1	F 5`-TGACCCCAAGGAACACTGACA-3`
	R 5`-TCGGGTCAGCGCTAGCA-3`
Lhx6	F 5`-GAGCGTGGTTTGGTTCAGAACT-3`
	R 5`-CAGGTTCCTCTTGCTTGAGTCA-3`
Shh	F 5`-GGACAGCGAGACCATGCAT-3`
	R 5`-TCGGGCTTCAGCTGGACTT-3`
Bmp4	F 5`-GACTTCGAGGCGACACTTCT-3`
	R 5`-ACTGGTCCCTGGGATGTTCT-3`
Fgf4	F 5`-TCGGTGTGCCTTTCTTTACC
	R 5`-TACCTTCATGGTAGGCGGCA-3`
Barx1	F 5`-CGGAGTCGCACCGTATTCAC-3`
	R 5`-ACGTCTTCACCTGTAACTGGCTCA-3`
Notch1	F 5`-CCTGCCACTATGGTTCCTGT-3`
	R 5`-GGGTTGGACTCACACTCGTT-3`

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Notch2	F 5`-ACACGAGGAAGTTGCCATAG-3`
	R 5`-GGGGGATTGAAGGAAAGA-3`
Slug	F 5`-TGCTACACAGCAGCCAGATTCC-3`
	R 5`-TTTCTGGGCTGGCCAAACAT-3`
Pax3	5`-GGCTTTCAACCATCTCATTC-3`
	R 5`-GTTGGTCAGAAGTCCCATTAC-3`

References

Arakaki M, Ishikawa M, Nakamura T, Iwamoto T, Yamada A, Fukumoto E, Saito M, Otsu K, Harada H, Yamada Y, Fukumoto S. 2012. Role of epithelial-stem cell interactions during dental cell differentiation. J Biol Chem 287:10590-10601.

Cai J, Cho SW, Kim JY, Lee MJ, Cha YG, Jung HS. 2007. Patterning the size and number of tooth and its cusps. Dev Biol 304:499-507.

Cai J, Zhang Y, Liu P, Chen S, Wu X, Sun Y, Li A, Huang K, Luo R, Wang L, Liu Y, Zhou T, Wei S, Pan G, Pei D. 2013. Generation of tooth-like structures from integration-free human urine induced pluripotent stem cells. Cell Regen (Lond) 2:6.

Correia F, Gouveia S, Felino AC, Costa AL, Almeida RF. 2017. Survival Rate of Dental Implants in Patients with History of Periodontal Disease: A Retrospective Cohort Study. Int J Oral Maxillofac Implants 32:927-934.

Duailibi MT, Duailibi SE, Young CS, Bartlett JD, Vacanti JP, Yelick PC. 2004. Bioengineered teeth from cultured rat tooth bud cells. J Dent Res 83:523-528.

Hatherell K, Couraud PO, Romero IA, Weksler B, Pilkington GJ. 2011. Development of a three-dimensional, all-human in vitro model of the blood-brain barrier using mono-, co-, and tri-cultivation Transwell models. Journal of Neuroscience Methods 199:223-229.

Jernvall J, Thesleff I. 2000. Reiterative signaling and patterning during mammalian tooth morphogenesis. Mech Dev 92:19-29.

Kim R, Green JBA, Klein OD. 2017. From snapshots to movies: Understanding early tooth development in four dimensions. Dev Dyn 246:442-450.

Langer R, Vacanti JP. 1993. Tissue engineering. Science 260:920-926.

Lee DJ, Lee JM, Kim EJ, Takata T, Abiko Y, Okano T, Green DW, Shimono M, Jung HS. 2017. Bio-implant as a novel restoration for tooth loss. Scientific Reports 7.

Marx V. 2015. Tissue engineering: Organs from the lab. Nature 522:373-377.

Miki Y, Ono K, Hata S, Suzuki T, Kumamoto H, Sasano H. 2012. The advantages of co-culture over mono cell culture in simulating in vivo environment. J Steroid Biochem Mol Biol 131:68-75.

Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S. 2003. SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci U S A 100:5807-5812.

Mustard J, Levin M. 2014. Bioelectrical Mechanisms for Programming Growth and Form: Taming Physiological Networks for Soft Body Robotics. Soft Robotics 1:169-191.

Nakao K, Morita R, Saji Y, Ishida K, Tomita Y, Ogawa M, Saitoh M, Tomooka Y, Tsuji T. 2007. The development of a bioengineered organ germ method. Nat Methods 4:227-230.

Ohazama A, Modino SA, Miletich I, Sharpe PT. 2004. Stem-cell-based tissue engineering of murine teeth. J Dent Res 83:518-522.

Otsu K, Kishigami R, Oikawa-Sasaki A, Fukumoto S, Yamada A, Fujiwara N, Ishizeki K, Harada H. 2012. Differentiation of Induced Pluripotent Stem Cells Into Dental Mesenchymal Cells. Stem Cells and Development 21:1156-1164.

Otsu K, Kumakami-Sakano M, Fujiwara N, Kikuchi K, Keller L, Lesot H, Harada H. 2014. Stem cell sources for tooth regeneration: current status and future prospects. Front Physiol 5:36.

Peng L, Ye L, Zhou XD. 2009. Mesenchymal stem cells and tooth engineering. Int J Oral Sci 1:6-12.

Ramsden CM, Powner MB, Carr AJ, Smart MJ, da Cruz L, Coffey PJ. 2013. Stem cells in retinal regeneration: past, present and future. Development 140:2576-2585.

Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, van Es JH, Abo A, Kujala P, Peters PJ, Clevers H. 2009. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature 459:262-U147.

Suchy F, Yamaguchi T, Nakauchi H. 2018. iPSC-Derived Organs In Vivo: Challenges and Promise. Cell Stem Cell 22:21-24.

Sumida S, Ishihara K, Kishi M, Okuda K. 2002. Transmission of periodontal disease-associated bacteria from teeth to osseointegrated implant regions. Int J Oral Maxillofac Implants 17:696-702.

Takahashi C, Yoshida H, Komine A, Nakao K, Tsuji T, Tomooka Y. 2010. Newly established cell lines from mouse oral epithelium regenerate teeth when combined with dental mesenchyme. In Vitro Cellular & Developmental Biology-Animal 46:457-468.

Tucker A, Sharpe P. 2004. The cutting-edge of mammalian development; How the embryo makes teeth. Nature Reviews Genetics 5:499-508.

Volponi AA, Kawasaki M, Sharpe PT. 2013. Adult Human Gingival Epithelial Cells as a Source for Whole-tooth Bioengineering. Journal of Dental Research 92:329-334.

Yamamoto H, Kim EJ, Cho SW, Jung HS. 2003. Analysis of tooth formation by reaggregated dental mesenchyme from mouse embryo. J Electron Microsc (Tokyo) 52:559-566.

Yan M, Yu Y, Zhang G, Tang C, Yu J. 2011. A journey from dental pulp stem cells to a bio-tooth. Stem Cell Rev 7:161-171.

Yoshida Y, Yamanaka S. 2011. iPS cells: a source of cardiac regeneration. J Mol Cell Cardiol 50:327-332.

Zhang YD, Chen Z, Song YQ, Liu C, Chen YP. 2005. Making a tooth: growth factors, transcription factors, and stem cells. Cell Res 15:301-316.

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Figure legends

Figure 1. A: Scheme of the process of differentiation into the dental epithelial cells. B-E: Derivation of dental epithelial cells from mouse iPSCs. F-H: Immunofluorescence for epithelial cell marker (CK14, AMBN), and stem cell marker (OCT3/4). I: Western blotting analysis for identifying the differentiation of iPSCs: AMBN, CK14, OCT3/4. J-L: CK14, AMBN, and OCT3/4 levels were analyzed by flow cytometry. (Scale bar is 100μm) (iPSC: induced pluripotent stem cell, DECi: dental epithelial cells from iPSC)

Figure 2. A: Scheme of the process of differentiation into the NCLCs from iPSC. B-E: Cell morphology of the differentiated NCLCs from EB. F-I: Immunofluorescence for NCLCs markers (NESTIN, p75, LHX6, and MSX1). J, K: Flow cytometry analysis of SNAIL and VIMENTIN. L-O: Higher mRNA level of NCLCs than undifferentiated iPSC. P: Western blot analysis for NCLCs markers and dental mesenchymal markers. (Scale Bar: 100μm) (NCLCs: neural crest like cells)

Figure 3. A: Scheme of dental cell differentiation method from iPSC using feeder cells. iPSCs were cultured on the top side of a insert; SF2 cell and dental mesenchymal cells from E16 mouse molar tooth germs were cultured on the bottom of the insert stabilize by a Teflon ring for 24hr and then iPSCs are seeded on top of the insert. B-E: Flow cytometry analysis of dental epithelial cell markers: CK14, LEF1, p21 and AMBN. F: Western blot showing dental epithelial cell markers were increased in TDECiSF2. G-I: Dental mesenchymal cell markers (Msx1 and Lhx6) analysed by flow cytometry. J: Western blot analysis of dental mesenchymal cell markers. (iPSC: induced pluripotent stem cell, DECi: dental epithelial cells

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from iPSC, TDECiSF2: dental epithelial cells from iPSC in transwell co-cultured with SF2 cells, TDMCMC: dental mesenchymal cells from iPSCs in transwell co-cultured with E16 dental mesenchymal cells)

Figure 4. A: Scheme of dental cell differentiation method from iPSC using feeder tissues. iPSCs were cultured on the top side of the insert; dental epithelium and dental mesenchyme from E14 mouse molar tooth germs were cultured on the bottom of the insert and stabilized by a Teflon ring. Both cell populations were left to grow for 24 hours prior to seeding with iPSCs on top of the insert. B: RT-qPCR analysis of *Bmp4*, *Fgf4*, *Shh*, *CK14* and *AMBN* expression in TDECi. C: Flow cytometry analysis of CK14, AMGN and AMBN expression on TDECi. D: RT-qPCR analysis of *Lef1*, *Edar*, *Runx2*, *Msx2* and *Barx1* expression in TDMC. E: Flow cytometry analysis of LEF1, LHX6 and MSX1 expression on TDMC. Student's t test was used for statistical analysis, and the level of statistical significance was set at $*p < .01$ or $**p < .05$. (TDECiSF2: dental epithelial cells from iPSC in transwell co-cultured with SF2 cells, TDMCMC: dental mesenchymal cells from iPSCs in transwell co-cultured with E16 dental mesenchymal cells, TDECi: dental epithelial cells from iPSCs in transwell co-cultured with E14 dental epithelium, TDMCi: dental mesenchymal Cells from iPSCs in transwell co-cultured with E14 dental mesenchyme)

Figure 5. A: The mRNA expression level of dental epithelial markers in TDECi compared iPSCs and feeder epi. B: The mRNA expression level of dental mesenchymal markers in TDMC compared iPSCs and feeder mes. (TDECi: dental epithelial cells from iPSCs in transwell co-cultured with E14 dental epithelium, TDMCi: dental mesenchymal Cells from iPSCs in transwell co-cultured with E14 dental mesenchyme)

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Figure 6. A-D: Micro-CT generated 3D reconstruction of the recombinants. A: E14 tooth germ after 5 weeks in the renal capsule. B: Recombinant of E12 dental epithelium and NCLC after 5 weeks in the renal capsule. C: Recombinant of DECi and E14 dental mesenchymal cells after 5 weeks in the renal capsule. D: Recombinant of TDECi and TDMC after 5 weeks in the renal capsule. E-H': Immunohistochemistry for dental epithelial cell markers (CK14, AMGN, and NESTIN) and a dental mesenchymal cell marker (MSX1) in recombinant of TDECi and TDMC. CK14, AMGN and NESTIN expressed by dental epithelium-like tissue from iPSCs. Msx1 expression by dental mesenchyme-like tissue from iPSCs. The positive cells of outside of "e" for AMGN, Nestin, and MSX1 antibodies is non-specific expression. (Scale bars: (E, F, G, H) 100μm, (E', F', G', H') 50μm) (dental epithelium-like tissue (e), dental mesenchyme-like tissue (m)

Figure 7. Experimental Scheme for better understanding the all procedures

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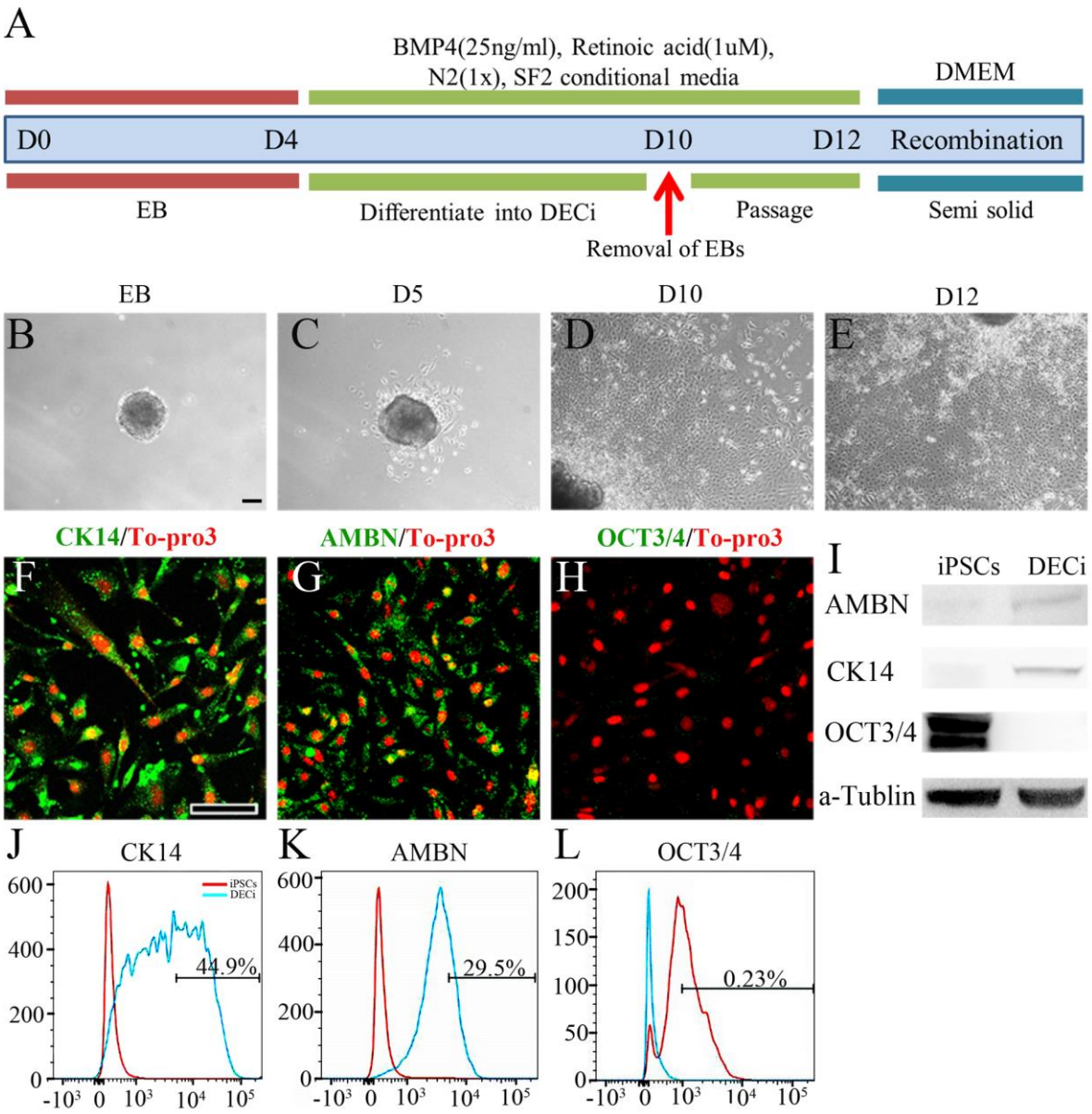

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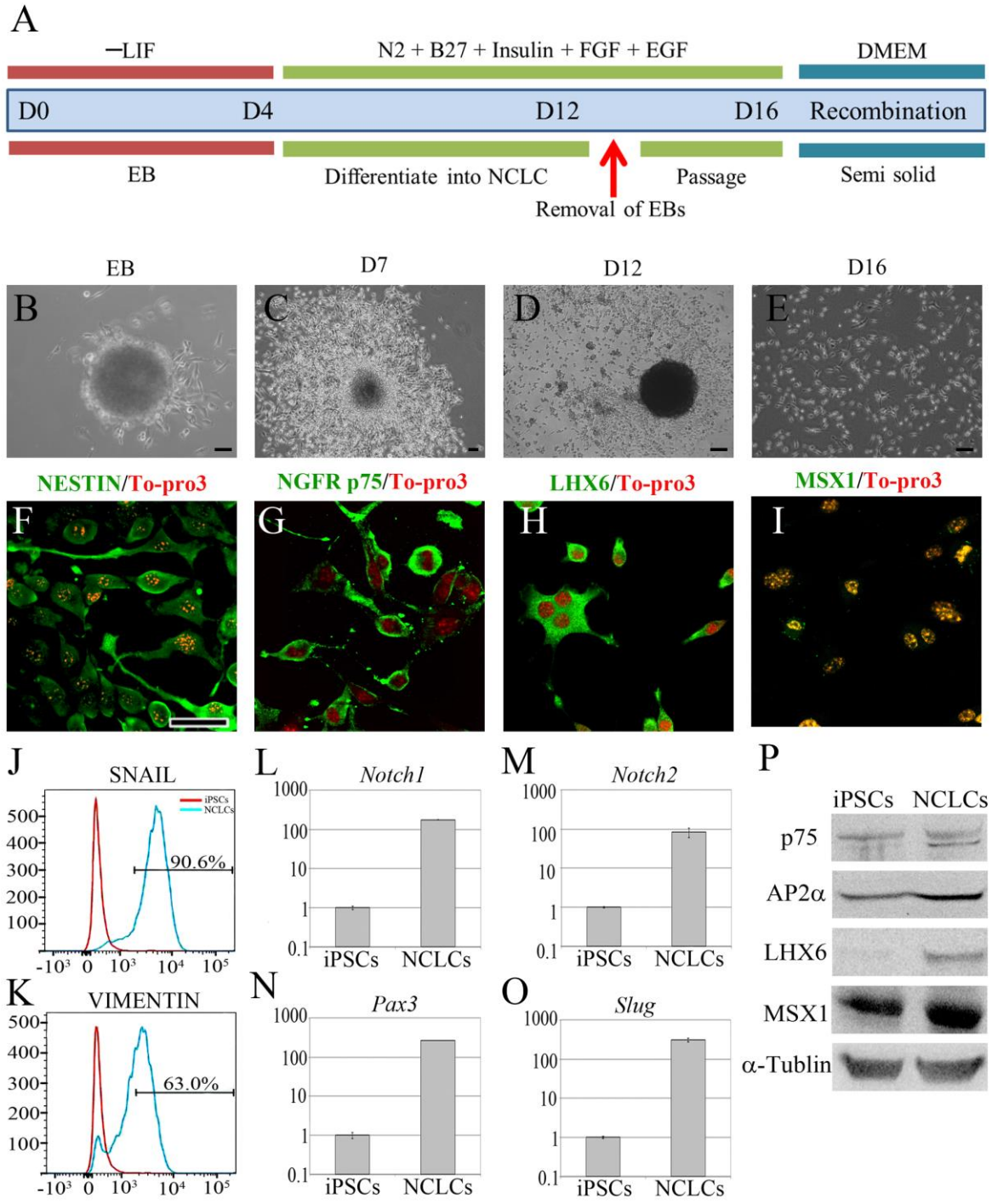

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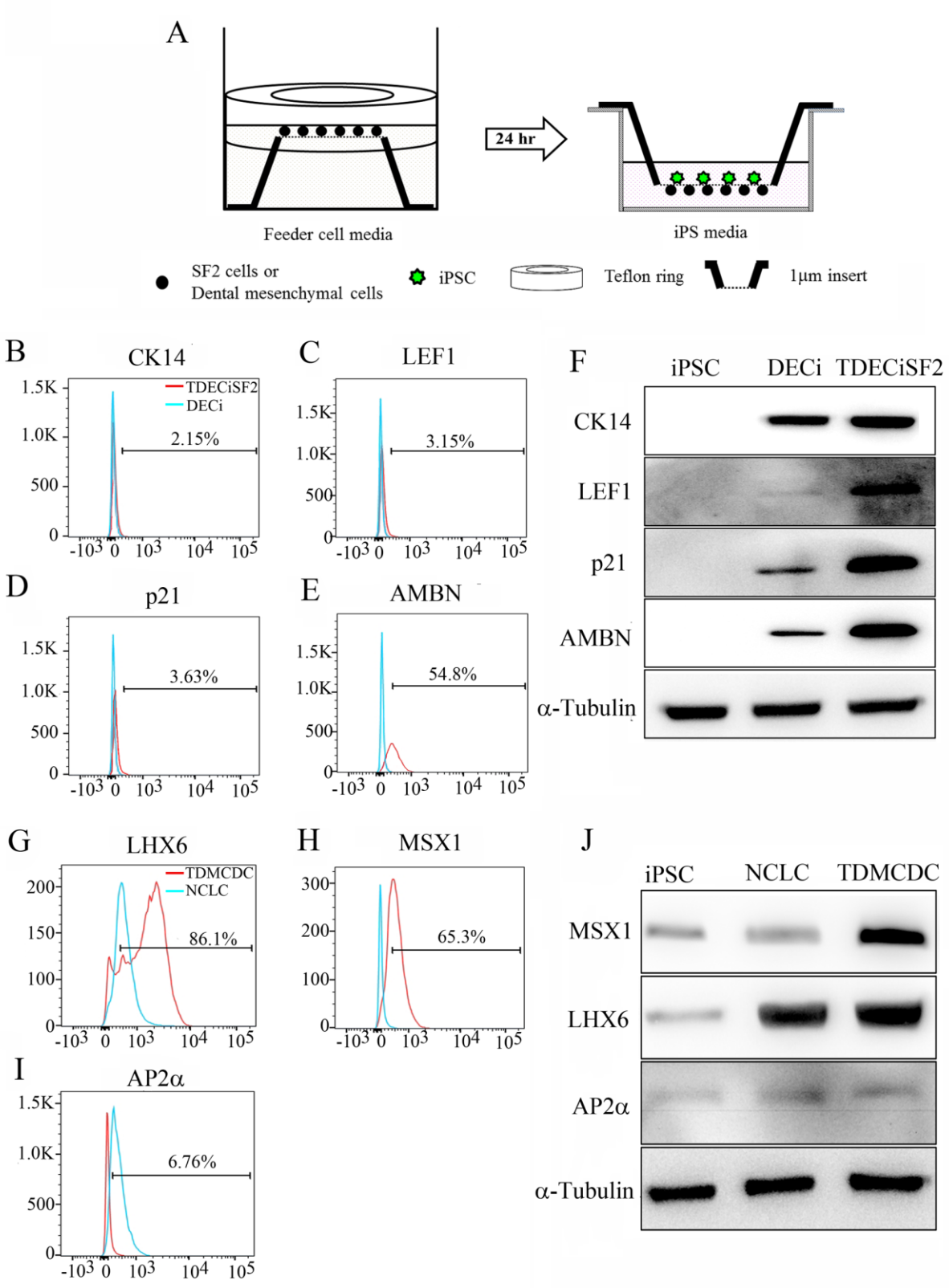

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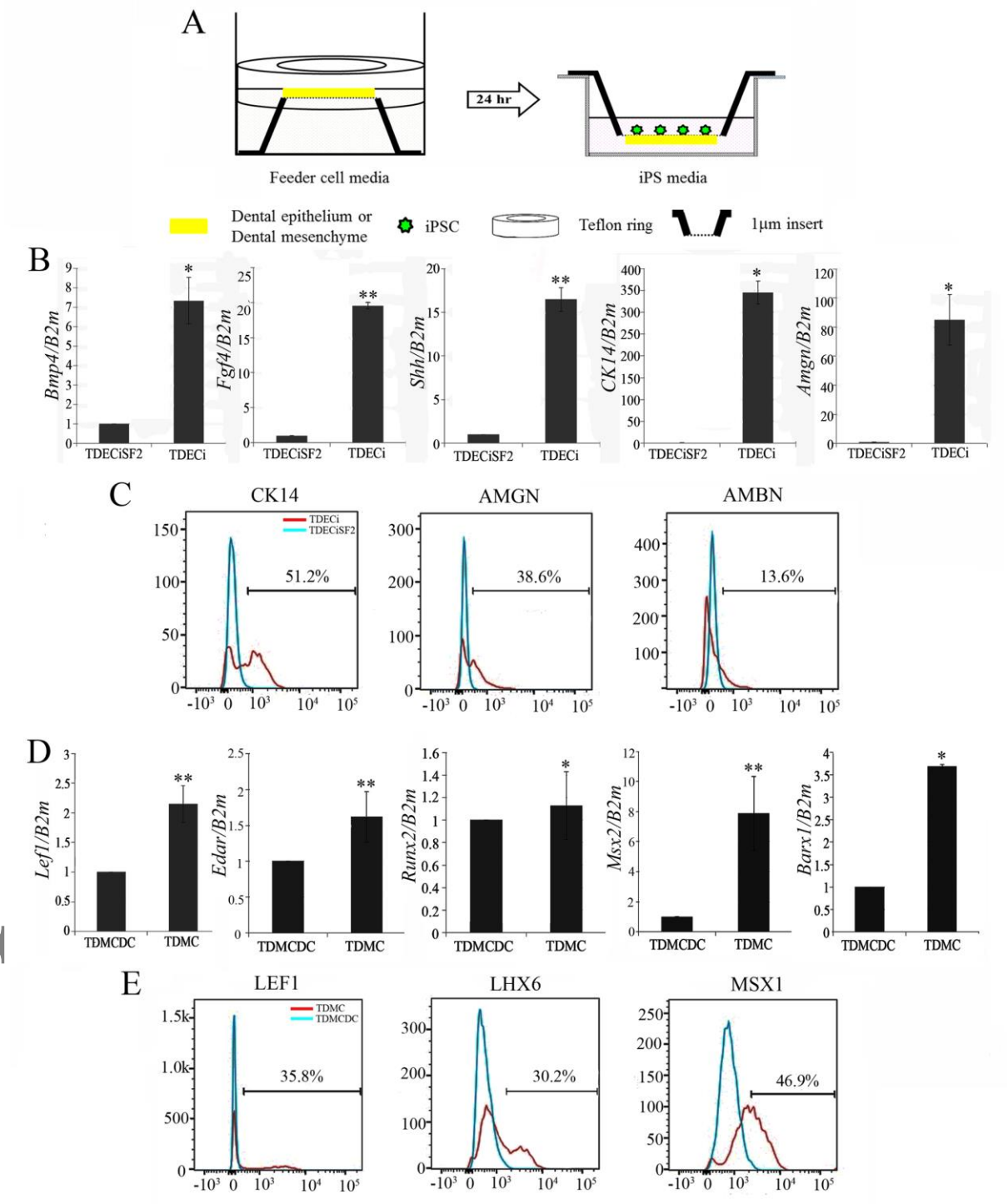

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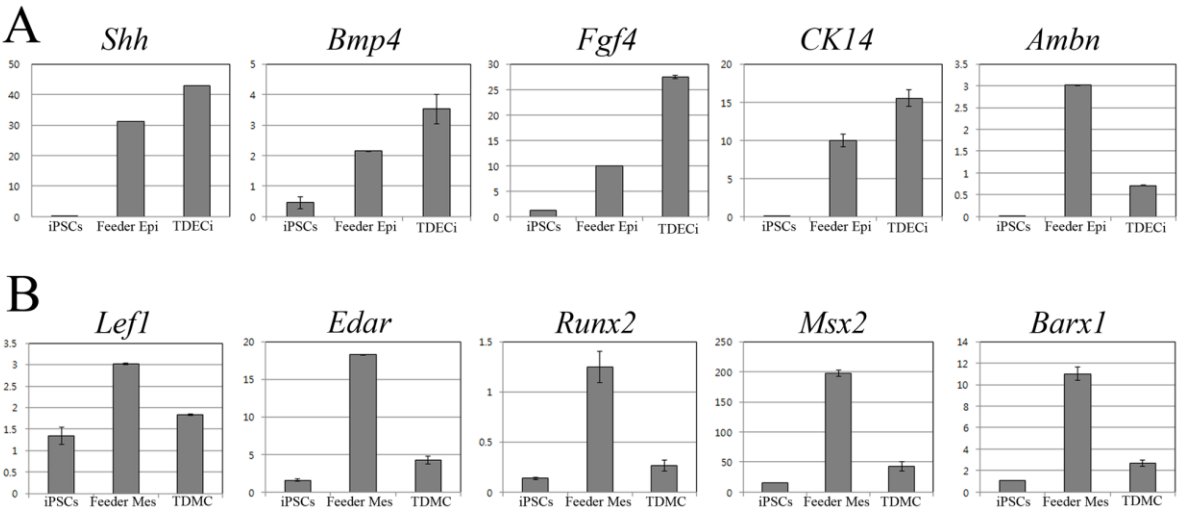

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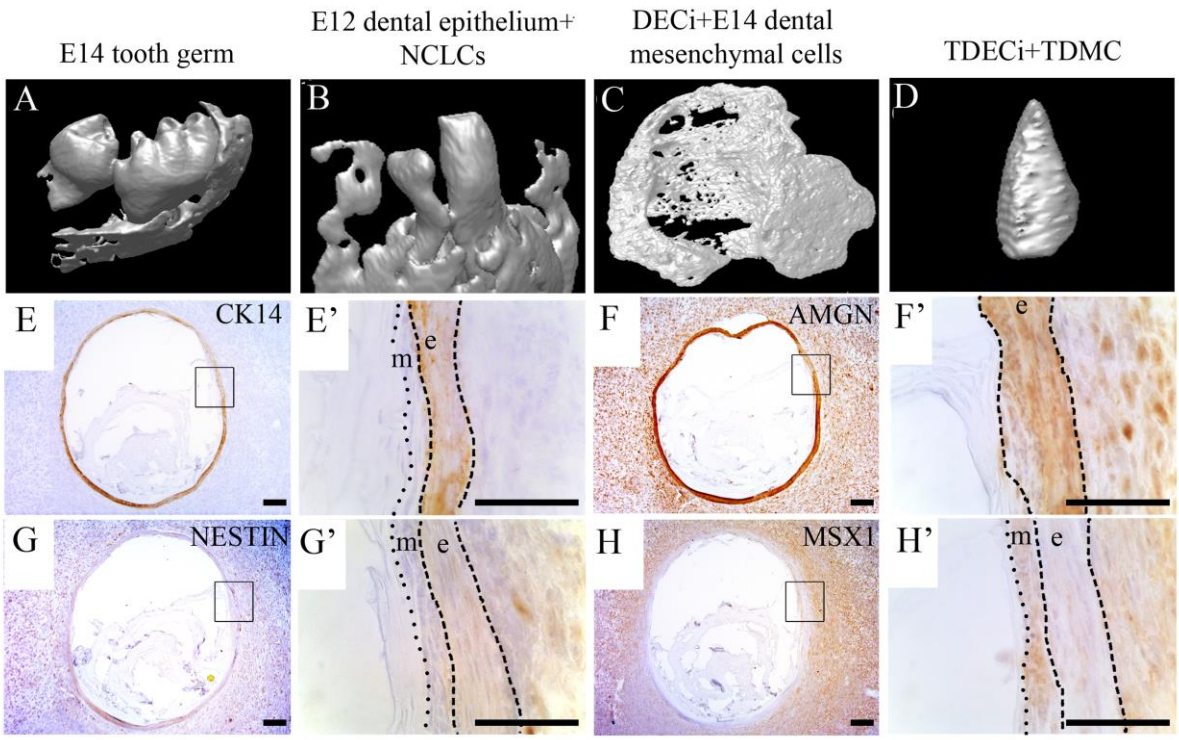

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