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Three-Dimensional Epithelial and Mesenchymal Cell Co-Cultures Form Early Tooth Epithelium Invagination-Like Structures: Expression Patterns of Relevant Molecules

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ABSTRACT

Epithelium invagination is the key feature of early tooth development. In this study, we built a three-dimensional (3D) model to represent epithelium invagination-like structure by tissue engineering. Human normal oral epithelial cells (OECs) and dental pulp stem cells (DPSCs) were co-cultivated for 2–7 weeks on matrigel or collagen gel to form epithelial and mesenchymal tissues. The histological change and gene expression were analyzed by HE staining, immunostaining, and quantitative real-time RT-PCR (qRT-PCR). After 4 weeks of cultivation, OECs-formed epithelium invaginated into DPSCs-derived mesenchyme on both matrigel and collagen gel. OEC–DPSC co-cultures on matrigel showed typical invagination of epithelial cells and condensation of the underlying mesenchymal cells. Epithelial invagination-related molecules, *CD44* and *E-cadherin*, and mesenchymal condensation involved molecules, *N-cadherin* and *Msx1* expressed at a high level in the tissue model, suggesting the epithelial invagination is functional. However, when OECs and DPSCs were co-cultivated on collagen gel; the invaginated epithelium was transformed to several epithelial colonies inside the mesenchyme after long culture period. When DPSCs were co-cultivated with immortalized human OECs NDUSD-1, all of the above-mentioned features were not presented. Immunohistological staining and qRT-PCR analysis showed that p75, *BMP2*, *Shh*, *Wnt10b*, *E-cadherin*, *N-cadherin*, *Msx1*, and *Pax9* are involved in initiating epithelium invagination and epithelial–mesenchymal interaction in the 3D OEC–DPSC co-cultures. Our results suggest that co-cultivated OECs and DPSCs on matrigel under certain conditions can build an epithelium invagination-like model. This model might be explored as a potential research tool for epithelial–mesenchymal interaction and tooth regeneration. J. Cell. Biochem. 113: 1875–1885, 2012.

KEY WORDS: EPITHELIAL INVAGINATION; EPITHELIAL–MESENCHYMAL INTERACTION; CADHERINS; p75

Epithelium invagination is the first morphological sign of the development of tooth, hair follicle and other organs, for example, lung and kidney [Freemark et al., 1997; Jamora et al., 2003]. In early tooth development, interactions between the oral ectoderm and neural crest-derived ectomesenchyme induce thickening in the surface of epithelium to form a placode. The placode subsequently buds into the underlying mesenchyme, and is surrounded by a condensation of mesenchymal cells. This morphological change is the key feature of early stage (e.g., initiation and bud stages) during tooth development [Thesleff and Tummers, 2009]. At the molecular level, numerous growth factors, transcription factors and intracellular/extracellular molecules, such as E-cadherin, N-cadherin, bone morphogenetic proteins (BMPs), and fibroblast growth factors (FGFs) have been shown to be involved in epithelial–mesenchymal interactions during odontogenesis [Zhang et al., 2005; Bei, 2009]. These molecules are autosecreted or parasecreted from embryonic oral epithelial and mesenchymal stem cells. Through the molecular signaling network, those two types of stem cells interact within each other's proliferation, migration, and differentiation. The oral epithelial stem cells differentiate into ameloblasts and then form enamel. The mesenchymal stem cells differentiate into odontoblasts, fibroblasts, and other cells; finally form dentin, dental pulp, and other tissues [Jernvall and Thesleff, 2000]. Once human embryonic epithelial stem cells develop into ameloblasts, they lost stemness, videlicet the ability for self-renew, and differentiation. In contrast, postnatal human dental pulp cells have been characterized as mesenchymal stem cells due to their immunophenotype and pluripotency [Gronthos et al., 2000; Alongi et al., 2010; Yan et al., 2010].

Because of the above-described mechanism, stem cell-based tooth engineering is considered a powerful tool for tooth regeneration. Although using oral epithelial stem cells and

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mesenchymal stem cells from a mouse embryo can rebuild a bioengineered tooth [Nakao et al., 2007], human tooth regeneration is still limited by cell source and suitable environmental conditions. To achieve human tooth regeneration and understand molecular mechanisms underlying epithelial–mesenchymal interactions, it is necessary to build a three-dimensional (3D) model to represent the epithelium invagination using human epithelial and mesenchymal stem cells. Human dental pulp stem cells (DPSCs) have been demonstrated the potential to form dentin and dental pulp like tissue after animal transplantation [Gronthos et al., 2000]. DPSCs can be a cell source for the reconstruction of dental mesenchymal tissue. However, because dental epithelial precursors are eliminated soon after tooth eruption, the source of dental epithelial stem cells is limited in the embryo. It has been reported that the postnatal epithelium from young mice and bone marrow-derived cells can be used as a substitute for dental epithelium [Hu et al., 2006; Nakagawa et al., 2009]. Epithelial sheets of cultured human keratinocytes, when recombined with mouse embryonic dental mesenchyme, are able to differentiate into enamel-secreting ameloblasts in the presence of appropriate odontogenic signals [Wang et al., 2010]. Nevertheless, information is not yet available concerning the use of both human adult epithelial and mesenchymal cells for tooth regeneration or epithelial invagination. Due to containing epithelial stem cells, or at least progenitor cells, oral mucosal epithelium has been used as a cell source for tissue-engineered reconstruction [Nishida et al., 2004; Calenic et al., 2010].

We have successfully built 3D tissue equivalents to investigate cell–cell interactions [Xiao et al., 2010, 2011]. In the present study, using 3D culture systems, we co-cultivated postnatal human oral epithelial cells (OEC) and DPSCs to build an epithelial invagination model and investigate epithelial–mesenchymal interaction and relevant molecules. To more accurately reflect human tooth germ development, we performed a series of examinations at different periods during culturing which represent phased stages of early tooth development: 2–3 weeks reflect dental lamina formation; 4–5 weeks reflect bud stage; 6–7 weeks reflect either the cap stage or early bell stage.

MATERIALS AND METHODS

PROPAGATION OF CELLS

Normal human OECs were obtained from gingival tissue of healthy patients (17–26 years of age) who were undergoing dental extraction surgery at the Nippon Dental University Hospital at Tokyo under approved guidelines set by the Committee of Ethics, the Nippon Dental University School of Life Dentistry at Tokyo. Briefly, submucosal connective tissues were removed with scissors to the extent possible; the resulting samples were incubated (4°C, overnight) with 0.25% trypsin–EDTA solution to separate epithelium and dermis. After the dermis was removed, the rest tissue was cut into small explants. The tissue fragments were stirred for 30 min in KGM medium (Lonza) and then filtered through a strainer (BD Falcon) to remove unsatisfactory segments. Cells were collected by gentle centrifugation (500*g*, 5 min) and cultured in KGM.

To obtain human dental pulp cells, lower third molars were got from adults (17- to 26-year old) at the Nippon Dental University Hospital at Tokyo under approved guidelines set by the Committee of Ethics, the Nippon Dental University School of Life Dentistry at Tokyo. Dental pulp cells from each donor were separated from the tooth and cultured according to previous report [Gronthos et al., 2000; Tsutsui et al., 2006].

Immortalized human OECs NDUSD-1 were provided from our laboratory and maintained as previous report [Kubo et al., 2009].

THREE-DIMENSIONAL CO-CULTURE OF EPITHELIAL AND MESENCHYMAL CELLS

Three-dimensional co-culture systems were prepared with matrigel (BD Matrigel^{TM}; BD Bioscience, Japan) or collagen gel (Nitta Gelatin). Briefly, after the matrigel and collagen gel formed, DPSCs (1×10^6 cells/ml) were inoculated on the gels and cultivated in propagation media (MEM-alpha containing 20% FBS) for 24 h. Then OECs or NDUSD-1 cells (5×10^5 cells/ml) were seeded on the DPSCs–gel substrates, and the medium was changed to DMEM/F12 containing 15% serum replacement (SR; Invitrogen) and 5% FBS. One day after culture, the medium was replaced with DMEM/F12 containing 15% SR and 1% FBS. Because air exposure is necessary for epithelial cell growth, after further been cultivated for 4 days, the cultures were grown at the air–liquid interface in DMEM/F12 containing 15% SR without FBS for another 1–6 weeks in a regular 5% CO_2 incubator. Then, the co-cultures were harvested, fixed in 4% formalin, and paraffin-embedded to analyze the histological change and gene expression using HE staining, immunochemistry staining, and quantitative RT-PCR (Fig. 1A). Conception of four types of the epithelial–mesenchymal cell co-culture is shown in Figure 1B.

REAL-TIME QUANTITATIVE PCR (qPCR)

Total RNA was extracted from cultured cells or paraffin-embedding tissue using RNeasy Micro Kit (Qiagen, Tokyo; http://www1.qiagen.com) or RecoverAll^{TM} Total Nucleic Acid Isolation Kit (Applied Biosystems, Foster City, CA; http://www.appliedbiosystems.com) according to the manufactural protocol. RNA purity was evaluated by absorbance readings (Ratio A_{260}/A_{230} and A_{260}/A_{280}) using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Japan). Standard RT was performed to transcript RNA to cDNA using a High Capacity RNA-to-cDNA Master Mix kit (Applied Biosystems). The cDNA samples were subjected to qPCR amplification to analyze the selected genes, as well as *CD44*, *E-cadherin* (*CDH1*), *N-cadherin* (*CDH2*), *BMP2*, *BMP4*, *ITGB1*, *Shh*, *Wnt10b*, *MSX1*, *Pax9*, *DMP1*, *DSP*, and *18s* (endogenous mRNA control). Human-specific probes for those genes were purchased from Applied Biosystems. PCR amplification mixture (20 μl) contained 2 μl single-strand cDNA template, 10 μl TaqMan® Gene Expression Master Mix (Applied Biosystems), and 1 μl probe. Using standard curve method, we demonstrated that the relative efficiencies of target genes and 18s from each of the samples were approximately equal (User Bulletin #2; http://www.appliedbiosystems.com). Amplification reactions were run (in triplicate) on a StepOne Plus real-time PCR system (Applied Biosystems) using $\Delta\Delta C_t$ method with the accompanying data analysis software.

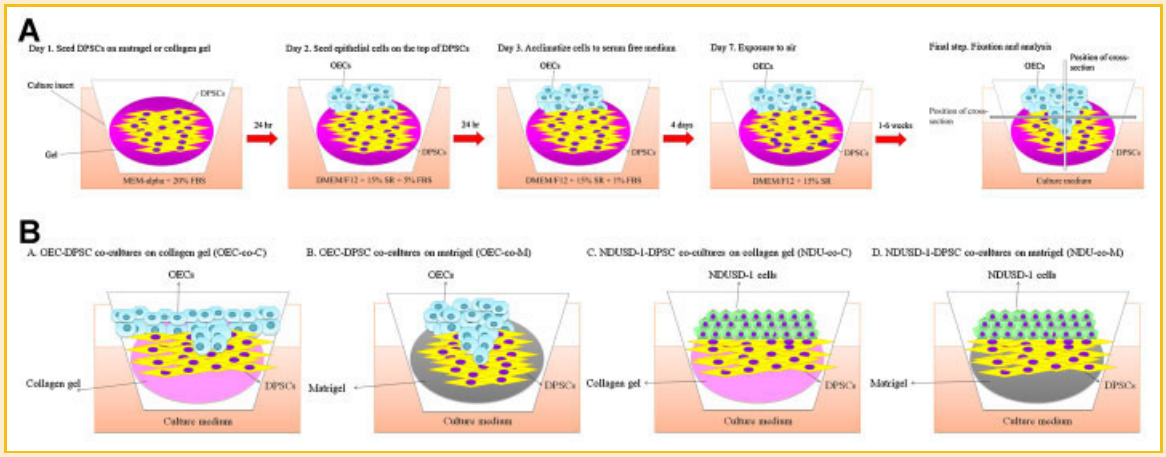

Fig. 1. Concept of three-dimensional epithelial–mesenchymal co-culture systems. A: Culture schedule of three-dimentional epithelial–mesenchymal co-cultures. B: Difference among four types of epithelial–mesenchymal co-cultures. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

IMMUNOHISTOCHEMISTRY

Immunohistochemistry staining was performed with Histostain™ kit (Invitrogen, Inc) according to manufactural protocol. Briefly, specimens were deparaffinized in xylene and dehydrated in a graded series of ethanol. The endogenous peroxidase activity was quenched by using 3% hydrogen peroxide in methanol. Specimens were incubated with serum blocking solution for 10 min to suppress non-specific binding of IgG, and then incubated for 60 min with saturating levels of primary antibodies. Antibodies used were: anti-pancytokeratin (IHCR2025-6; Millipore), anti-K8 (sc-9342; Santa Cruz), anti-p75 (ab21303; Abcam), anti-K8 (sc-52325; Santa Cruz) anti-BMP7 (MAB4350; Millipore), anti-FGF3 (ab103228; Abcam), anti-FGF4 (ab65974; Abcam), anti-Msx1 (sc-15395; Santa Cruz), and anti-Runx2 (ab23981; Abcam) primary antibodies. For immunoperoxidase staining, specimens were reacted with biotinylated secondary antibody (provided from the kit) in dark for 10–20 min. The DAB then is used to create an intense brown deposit around the antigen/antibody/enzyme complex in the sample. For immunofluorescence staining, specimens were reacted with fluorochrome-conjugated secondary antibody (A11001 or A11012; Invitrogen) diluted to 2 μg/ml in PBS with 1.5% normal blocking serum. Images were taken by a bio-imaging navigator (FSX100; Olympus).

Whole mount fluorescence immunohistochemical staining was performed with a standard protocol from http://www.abcam.co.jp/. Samples were imaged and analyzed with a confocal

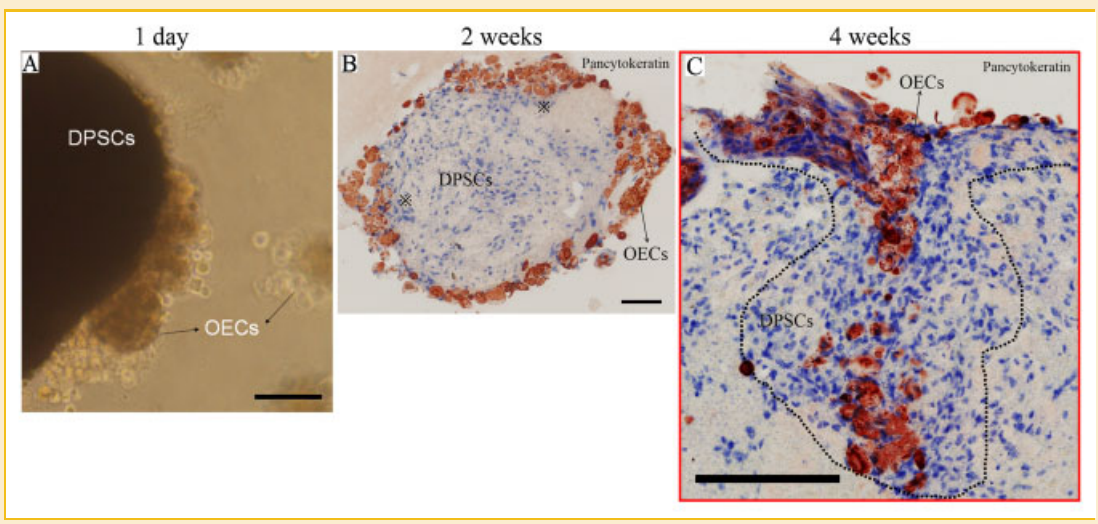

Fig. 2. Three-dimensional co-cultivated OECs and DPSCs on the matrigel. DPSCs and OECs were co-cultivated on matrigel as described in the "Materials and Methods" Section. Unlike the co-cultures on collagen gel (which form stratified layers), OEC–DPSC co-cultures form spheroids when they were seeded on matrigel. A: After been co-cultivated for 1 day, OECs-formed spheroid spontaneously adhered to a DPSCs-deviated spheroid. B: Detection of pan-cytokeratin expression in OEC–DPSC co-cultures after 2 weeks of cultivation. Rice symbols indicate pan-cytokeratin (+) epithelial cell mass started to invaginate into mesenchymal cell mass. C: After 4 weeks of cultivation, pan-cytokeratin (+) epithelial cell mass invaginated into adjacent mesenchyme and is surrounded by a condensation of mesenchymal cells (black dotted line). Scale bar = 20 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

laser scanning microscopy (LSM 780; Carl Zeiss Microscopy Co., Ltd.).

HISTOCHEMISTRY

Gomori's aldehyde fuchsin staining was performed after specimens were deparaffinized and dehydrated. Briefly, specimens were rinsed with 70% ethanol, and stained in fuchsin–ponceau solution (166-19765; Wako) for 15 min. After been rinsed with 0.5% acetic acid, specimens were stained with 3% phosphotungstic acid hydrate (040116; Alfa Aesar) for 5 min. Samples were then rinsed with 0.5% acetic acid, and stained in 1% light green SF yellowish (124-04632; Wako) for 10 min to identify collagen. Specimens were immediately mounted with a resinous medium to avoid fading.

RESULTS

THREE-DIMENSIONAL OEC–DPSC CO-CULTURES CAN GENERATE AN EPITHELIUM INVAGINATION-LIKE STRUCTURE IN VITRO

We co-cultivated DPSCs and OECs, or NDUSD-1 cells, as described in the Materials and Methods Section (Fig. 1). At 24 h after inoculation, OECs and DPSCs formed several spheroids on matrigel, respectively. The spheroids of OEC spontaneously adhered to the spheroids of DPSC (Fig. 2A). After culturing for 2 weeks, immunostaining showed that some OEC masses were beginning to invaginate into DPSC

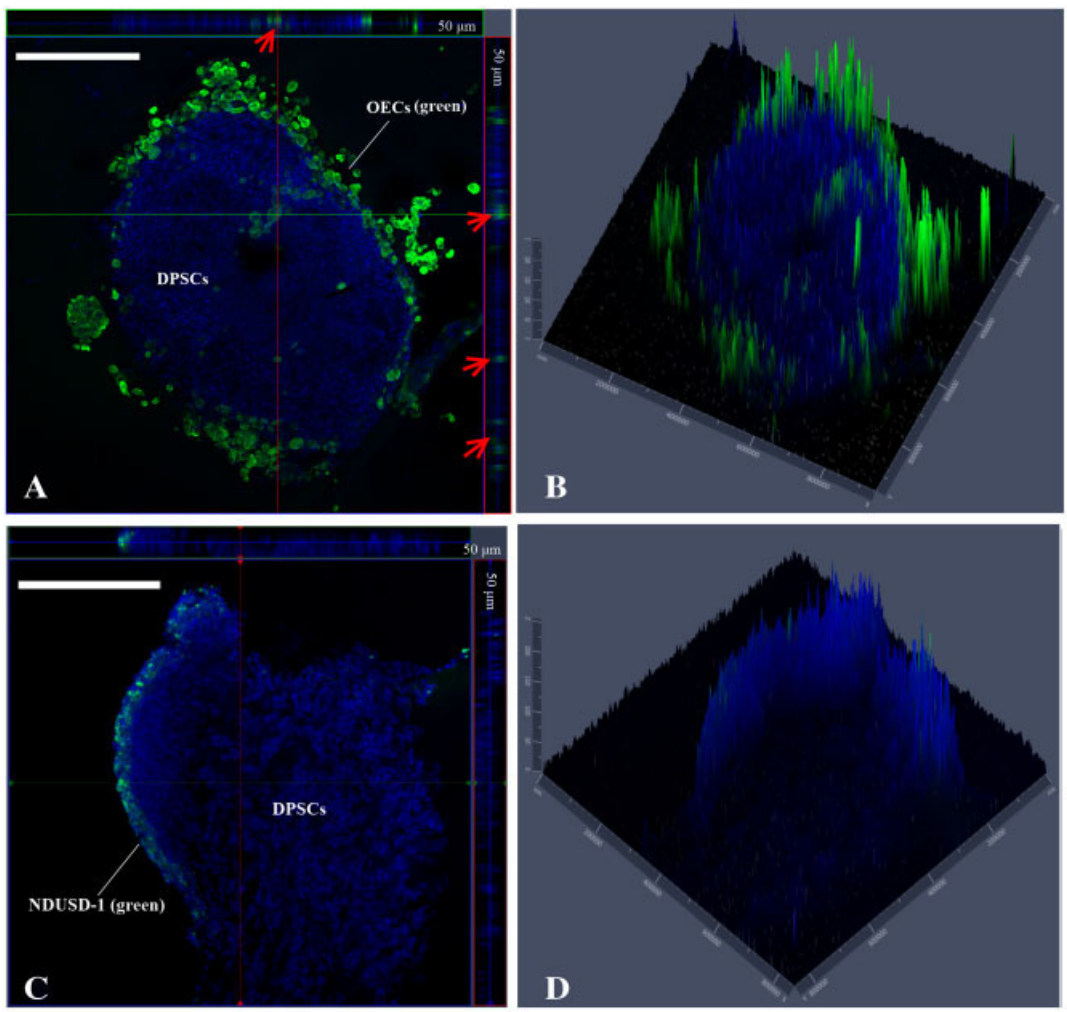

Fig. 3. 3D structure of epithelial invagination in OEC-co-M tissue model. Epithelial and mesenchymal cells were co-cultivated on matrigel for 4 weeks and followed by whole mount fluorescent immunohistochemistry staining with anti-pan-cytokeratin antibody for OECs or anti-K8 antibody for NDUSD-1 cells. 3D images were taken and analyzed with confocal laser scanning microscopy. Between 40 and 50 slices were acquired for each image (left panel). Thickness of the side is 50 μm. A: 3D structure of OEC-co-M tissue model. OEC mass (green) migrated inside DPSC mass. Red arrows indicate 3D epithelial invagination from different angles. C: 3D structure of NDU-co-M co-cultures. NDUSD-1 cells (green) formed a thin layer adjacent to DPSC mass. No NDUSD-1 cell invagination was observed. DAPI stained nuclei present blue color. Scale = 50 μm. Right panel, 3D reconstruction from confocal laser scanning optical stacks of OEC-co-M tissue model (B) and NDU-co-M co-cultures (D). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

masses in OEC-co-M tissue model (Fig. 2B). Because during human tooth development, it takes 3–4 weeks to develop from the initiation stage (dental lamina formation) to bud stage, we further cultivated the co-cultures for another 2 weeks. Figure 2C showed that after 4 weeks of cultivation, OECs-formed epithelium invaginated into adjacent DPSCs-derived mesenchyme with an ectomesenchymal condensation-like distribution pattern of mesenchymal cells on matrigel. To confirm if the epithelial invagination in OEC-co-M tissue model is a true 3D invagination which can reflect the in vivo situation, we performed whole mount immunofluorescence staining combined with confocal laser scanning microscopy (CLSM). As shown in Figure 3A,B, the CLSM-imaged 3D structure of OEC-co-M tissue model showed that the pancytokeratin (+) epithelial cell masses migrated inside the mesenchymal cell spheroid from all directions (Fig. 3A,B). The data confirmed that invagination in the OEC-co-M tissue model is a true 3D invagination.

After culturing for 4 weeks, OEC-co-C also formed the epithelial invagination-like structure (Fig. 4A,B), and collagen fibers were generated near the invaginated epithelium (Fig. 4B). However, the condensation of mesenchymal cells around the invaginated epithelial tissue was not observed. With a longer culture period (5 weeks), several OECs-formed epithelial colonies appeared inside the mesenchymal tissue (Fig. 4C). Conversely, when DPSCs co-cultivated with immortalized human OECs NDUSD-1 on both matrigel and collagen gel (NDU-co-M and NDU-co-C), epithelial invagination, epithelial colonies, and collagen fibers were not observed under the same conditions (Figs. 3C,D and 4D–F).

THREE-DIMENSIONAL OECs–DPSCs CO-CULTURE REPRESENTS TOOTH EARLY STAGE MARKERS

To characterize the epithelial invagination in OEC-co-M tissue model, we examined the expression of main molecules at the initiation, bud, and late stages of the tooth using immunohistochemical and quantitative real-time RT-PCR (qRT-PCR) analyses. After 5 weeks of cultivation, immunohistochemical staining showed that FGF4 and BMP7 expressed in epithelial cells (Fig. 5A,D); FGF3 signals were mainly detected in the epithelial cells and showed weakly in the mesenchymal cells near the epithelial–mesenchymal interface (Fig. 5B); The tooth-specific molecule, Msx1 (which is also considered as a marker of mesenchyme condensation [Chen et al., 1996]), was strongly expressed in mesenchymal cells in the tissue model (Fig. 5D). However, this tissue model failed to react with anti-Runx2 antibody (Fig. 5C). Under the same culture conditions, NDU-co-M co-cultures did not show obvious immunoreactivity for FGF4, FGF3, Runx2, and BMP7 antibodies (Fig. 5E–H). The Msx1 signal was detected in the mesenchymal cells in NDU-co-M co-cultures, but was much weaker than OEC-co-M tissue model (Fig. 5H).

The qRT-PCR analysis showed that after having been cultured for 2–7 weeks, OEC-co-M tissue model express tooth early stage markers, such as *BMP4*, *BMP2*, *CD44*, *CDH1*, *CDH2*, and *ITGB1*. Those genes showed time-dependent changes; *CD44*, *CDH1*, *CDH2*, and *ITGB1* (which are cell adhesion molecules) expressed at a higher level during the early culture period (2 weeks), and then declined. It has been reported that CD44 and CDH1 are involved in epithelial invagination [Yu and Toole, 1997; Papusheva and Heisenberg,

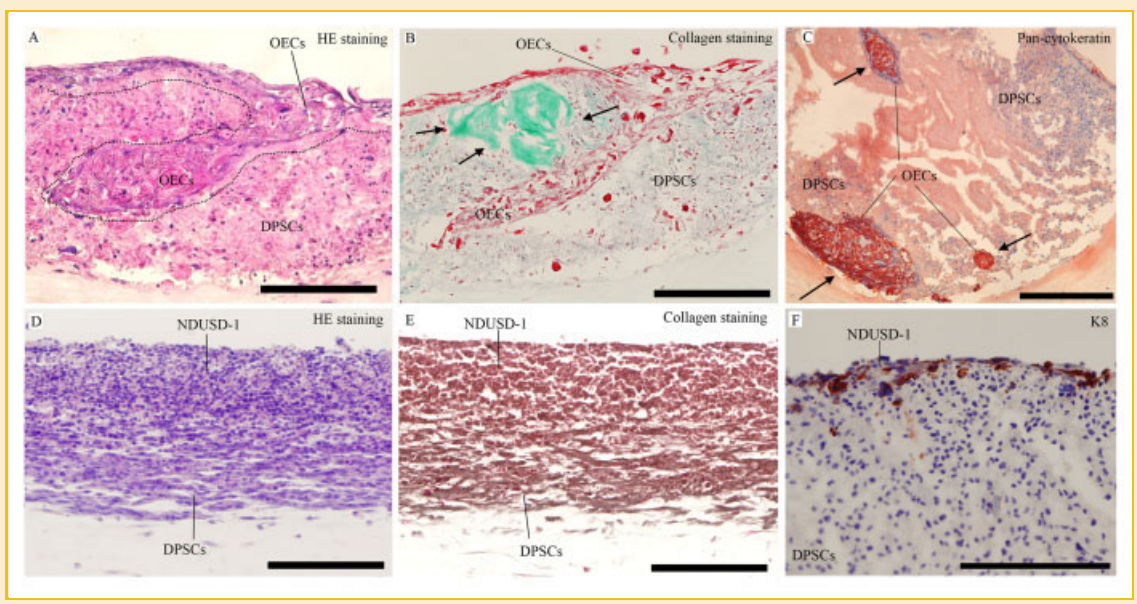

Fig. 4. Three-dimensional epithelial–mesenchymal co-cultures on the collagen gel. Epithelial and mesenchymal cells were co-cultivated on collagen gel for 4 weeks and followed by immunostaining with anti-pan-cytokeratin antibody for OECs or anti-K8 antibody for NDUSD-1 cells. A: HE staining for OEC and DPSC co-cultures. Black dotted line indicates invagination of epithelial cells. B: Gomori's aldehyde fuchsin staining for identify collagen formation in OEC-co-C co-cultures. Arrows indicate collagen fibers (green). C: Pan-cytokeratin expression in OEC-co-C co-cultures. Arrows indicate epithelial colonies formed inside the mesenchyme. D: HE staining for NDU-co-C co-cultures. NDUSD-1 cells and DPSCs formed two layers without interference. E: Gomori's aldehyde fuchsin staining for NDU-co-C co-cultures. No collagen fibers were observed. F: K8 expression in NDU-co-C co-cultures. K8 (+) epithelial cells did not invaginate into mesenchymal cells. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

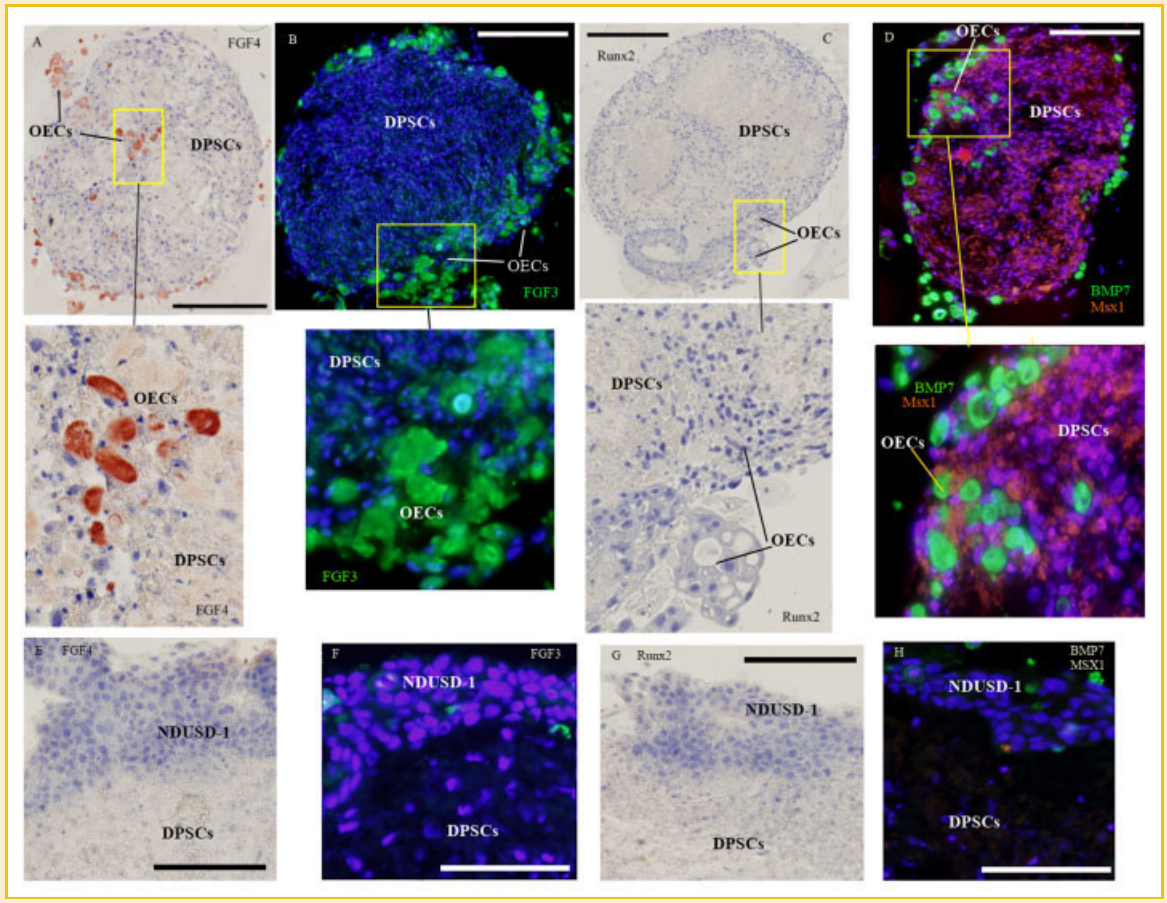

Fig. 5. Expressions of tooth early stage markers in the three-dimensional co-cultivated epithelial–mesenchymal cells by immunostaining. A–D: DPSCs and OECs were co-cultivated on matrigel for 5 weeks. A: FGF4 (red) expressed in epithelial cells inside the mesenchyme; B: FGF3 (green) expressed in epithelial cells inside the mesenchyme; C: Runx2 did not express in the co-cultures; D: BMP7 (green) expressed in epithelial cells and Msx1 (red) expressed in the mesenchymal cells; E–H: NDUSD-1 and DPSCs were co-cultivated on matrigel for 5 weeks. No positive signal was detected with anti-FGF4, FGF3, Runx2, and BMP7 antibodies, Msx1 (red) weakly expressed in DPSC mesenchymal cells (H). Hematoxylin stained nuclei present blue color (A, E, C, and G). DAPI stained nuclei present blue fluorescence (B, F, D, and H). Scale = 50 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

2010]. CDH2 and ITGB1 are required for the mesenchymal condensation of chondrogenesis [Woodward and Tuan, 1999; Takahashi et al., 2003]. Our data suggest that high-level expression of those cell adhesion molecules is needed for initiating epithelial invagination and mesenchymal condensation. *BMP2* and *BMP4* (which are odontogenic molecules) shared a similar expression pattern; the expression was at the middle level in early culture period (2 weeks), then declined, and rose again at a high level in the latest culture period (7 weeks; Fig. 6). This finding suggests that odontogenic potential is increased at the latest culture period. However, the co-cultures did not show expression of two other important early stage markers, wnt10b and Shh.

Tooth late stage markers, *DMP1* and *DSP*, did not express in OEC-co-M tissue model. We based this conclusion on our data that, the co-cultures express most early stage makers, but not the late stage marker. Because the early stage markers, FGF4, BMP2, and CD44 only show expression in dental epithelium, which suggests that normal human OECs have the ability to differentiate to dental epithelium.

GENETIC ANALYSIS OF SINGLE EPITHELIAL CELLS, MESENCHYMAL CELLS, AND EPITHELIUM–MESENCHYME CO-CULTURES

To investigate the molecular mechanism of epithelial–mesenchymal interaction in OEC-co-M tissue model, we further analyzed genetic differences among monolayer DPSCs, OECs, and NDUSD-1 cells, and four types of epithelial–mesenchymal co-cultures, OEC-co-M, OEC-co-C, NDU-co-M, and NDU-co-C. As seen in Figure 7, although those seven samples shared several genes, there are interesting differences among them. DPSCs showed high-level expression of ossification-related genes, such as *BMP2*, *BMP4*, and *DSP*. Epithelial specific genes, such as *Shh*, *Wnt10b*, and *CDH1* did not express or showed very low level expression in DPSCs. OECs showed the strongest expression of *CDH1*, *Shh*, and *Wnt10b*. NDUSD-1 cells did not express *Shh* and *Wnt10b*, but showed strong expression of *CDH2*. The expression level of *CDH1* in NDUSD-1 cells is about 14,000 times lower than OECs. Compare to co-cultures with NDUSD-1 (which did not form epithelial invagination), co-cultures with OECs showed stronger expression of *BMP2*, *CD44*, *CDH1*, *CDH2*, *Msx1*, and *Pax9*; and lower expression of *ITGB1*. Notably, although

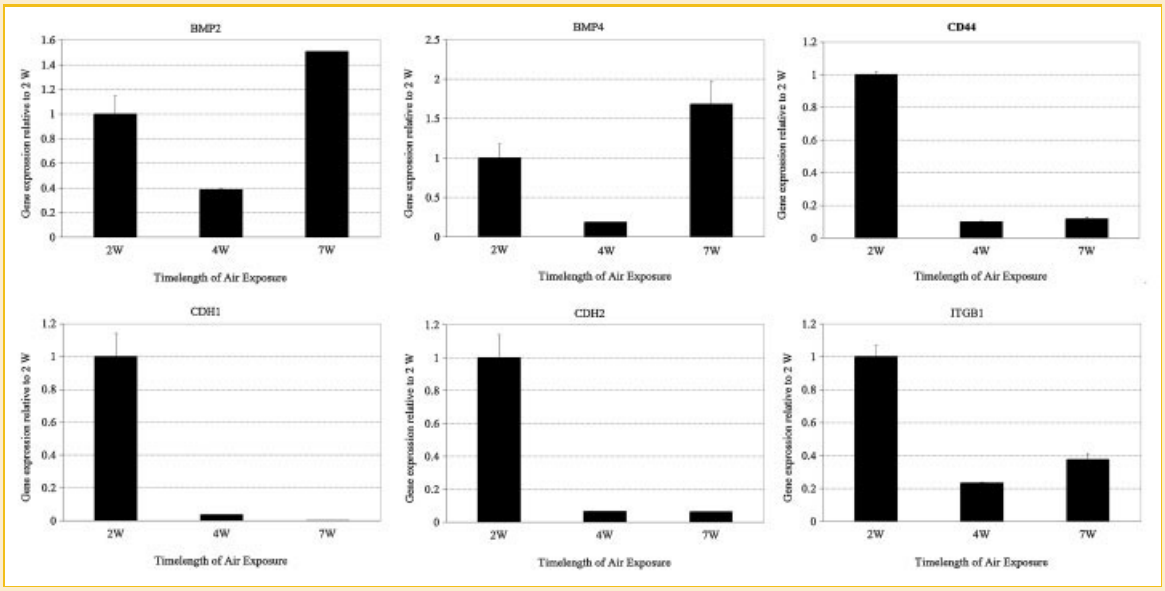

Fig. 6. Gene expression dynamics of tooth early stage markers in OEC–co–M tissue model. Gene expression of tooth early stage markers was analyzed with qRT–PCR after short (2 weeks), middle (4 weeks), and long (7 weeks) culture periods. Epithelial invagination–related molecules (*CD44* and *CDH1*) and mesenchymal condensation–involved molecules (*CDH2* and *ITGB1*) expressed at a higher level during the early culture period (2 weeks). The expression of two odontogenic molecules, *BMP2* and *BMP4*, was at the middle level in early culture period (2 weeks), then declined at 4 weeks, and rose again at a high level in the long culture period (7 weeks). Shown are the averages and standard deviations of three independent experiments.

monolayer DPSCs, OECs, and NDUSD-1 cells showed high expression levels of *ITGB1*, the expression of *ITGB1* in four types of epithelial–mesenchymal co-cultures were much lower. Tooth-specific genes *Msx1* and *Pax9* were expressed in DPSCs and epithelial–mesenchymal co-cultures, but not in epithelial cells (OECs and NDUSD-1 cells). The OEC-co-M tissue model showed the highest expression levels of *Msx1* and *Pax9* among DPSCs and the four types of co-cultures. The above-mentioned genetic differences suggest that OECs-derived molecules, such as *CDH1*, *Shh*, and *Wnt10b* might contribute to the initiation of epithelial invagination in co-cultures with OEC. Higher expression levels of *BMP2*, *CD44*, *CDH1*, *CDH2*, *Max1*, and *Pax9* might be important for the maintenance of epithelial and mesenchymal cells' migration, rate, and distribution in OEC-co-M tissue model.

Among the four types of co-cultures, some genes' expressions seemed unrelated to both epithelial cell or gel types. For example, *DSP*, *Shh*, and *Wnt10b* did not express in OEC-co-M tissue model, but did so in the OEC-co-C and NDU-co-M co-cultures. *BMP4* also showed higher expression in OEC-co-M and NDU-co-C than in OEC-co-C and NDU-co-M co-cultures. The reason for this could be that those molecules are interacting with each other in such a way that they are maintaining certain levels of expression by forming a signaling network to control both epithelial invagination (or epithelial colony transformation) and mesenchymal condensation.

p75 EXPRESSES IN THE EPITHELIAL TISSUE OF THREE-DIMENSIONAL OECs–DPSCs CO-CULTURE

Neurotrophin receptor p75 has been considered a stem cell marker of epithelial cells and a repair messenger which regulates dentin reformation during pulp healing [Nakamura et al., 2007]. Using immunochemistry method, p75 (+) cells were observed in the epithelial tissue (the boundary of which is between epithelial and mesenchymal tissue) in co-cultures with OECs at early culture period, but not in co-cultures with NDUSD-1 (Fig. 8), suggesting that epithelial stem cells that exist in the epithelium and p75 signaling pathway might be one of the contributors for the epithelial invagination.

DISCUSSION

Human tooth development begins in the middle of 6th week of gestation. From the beginning of dental lamina formation to the period of initial calcification, it takes at least 8 weeks to finish bud, cap, and bell stages, which are all the consequence of epithelial invagination. During this early stage of tooth development, reiterative and consecutive cross-talk between epithelial and mesenchymal cells control morphological changes in tooth germ. Numerous molecules are present in this period: BMPs, FGFs, cadherins (CDHs), CD44, integrin-beta 1(ITGB1), Msx1, Pax9, Runx2, Shh, and Wnt [Jernvall and Thesleff, 2000] (http://bite-it.helsinki.fi/). These molecules are involved in regulating functions of epithelial and mesenchymal stem cells and epithelial–mesenchymal interactions [Yu and Toole, 1997; Wang et al., 1999; Chen et al., 2008; Klein et al., 2008].

In the present study, we 3D co-cultivated human mesenchymal cells (DPSCs) and two types of human epithelial cells (OECs and NDUSD-1 cells) on either collagen gel or matrigel. The data showed that, among the four types of epithelial–mesenchymal co-cultures

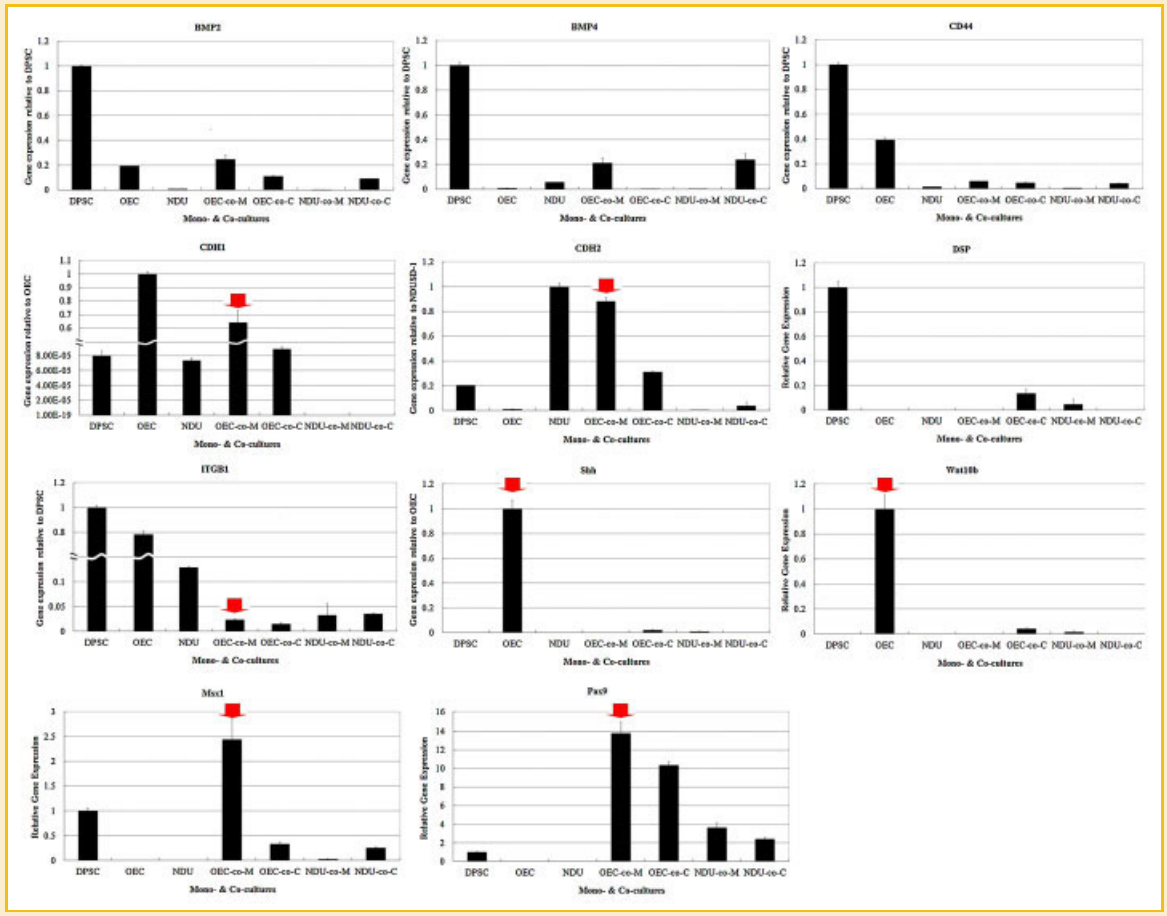

Fig. 7. Comparison of gene expression of tooth early stage markers among monolayer and three-dimensional co-cultivated mesenchymal and epithelial cells. qRT-PCR analysis showed that there are big differences of gene expression among monolayer DPSC, OECs, and NDUSD-1 cells, and four types of epithelial–mesenchymal co-cultures after 2 weeks cultivation. Red arrows indicate the expression of important molecules. CDH1 is essential for epithelial invagination. CDH2 and Msx1 are key molecules involved in mesenchymal condensation. *CDH1*, *CDH2*, and *Msx1* showed high level expression in OEC-co-M tissue model. ITGB1 is involved in preventing differentiation in stem cells. Both DPSCs and OECs showed higher expression of *ITGB1*, nevertheless OEC-co-M, OEC-co-C, and NDU-co-M co-cultures showed lower expression that indicates differentiation was in process. Shh and Wnt are important molecules for regulating epithelial stem cells functions. OECs expressed *Shh* and *Wnt10b* genes which were absent in NDUSD-1 cells. Msx1 and Pax9 are tooth-specific genes. OEC-co-M tissue model showed the highest expression levels of Msx1 and Pax9 genes among monolayer DPSCs and the four types of co-cultures. Epithelial cells, OECs and NDUSD-1 cells did not express Msx1 and Pax9. Shown are the averages and standard deviations of three independent experiments. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

(OEC-co-M, OEC-co-C, NDU-co-M, and NDU-co-C), only the co-culture systems containing OECs could form epithelial invagintion-like structures; the co-cultures with NDUSD-1 cells could not do so neither on collagen gel nor on matrigel. OECs showed a much higher expression level of *CDH1* and a lower expression level of *CDH2* than NDUSD-1 cells. The OECs express *Shh* and *Wnt10b* whereas NDUSD-1 cells did not. These results suggest that, epithelial cells play an important role on initiating epithelial invagination, and that CDH1, CDH2, Shh, and Wnt10b might be the key molecules for this.

Cadherins are cell adhesion molecules, which regulate various biological processes, such as cell recognition, migration, differentiation, and cellular communication. CDH1-mediated cell–cell adhesion is essential for epithelial invagination [Papusheva and Heisenberg, 2010]. CDH2 is known as a key cell–cell adhesion molecule involved in the initiation of mesenchymal condensation [Woodward and Tuan, 1999; Fischer et al., 2002]. Both E-cadherin (CDH1) and N-cadherin (CDH2) proteins express in oral epithelium, dental epithelium, outer enamel epithelium, and stellate reticulum during the bud and cap stages. E-cadherin distributes in proliferating cells of the inner and outer enamel epithelia, whereas N-cadherin expression is upregulated in differentiated epithelial cells and mesenchymal cells [Heymann et al., 2002]. In this study, time-dependent changes showed that the expression level of *CDH1* and *CDH2* in OEC-co-M tissue model is higher at 2 weeks than at 4 and 7 weeks of cultivation. *CDH1* was absent after 7 weeks of culturing. We concluded that there were proliferating cells (probably undifferentiated epithelial cells, which express *CDH1*) in OEC-co-M tissue model during the early culture period. CDH1-mediated epithelial invagination, and CHD2 initiated mesenchymal condensation. After having been cultured for a longer time, epithelial and mesenchymal cells were differentiated and expressed higher level of *CDH2* than *CDH1*.

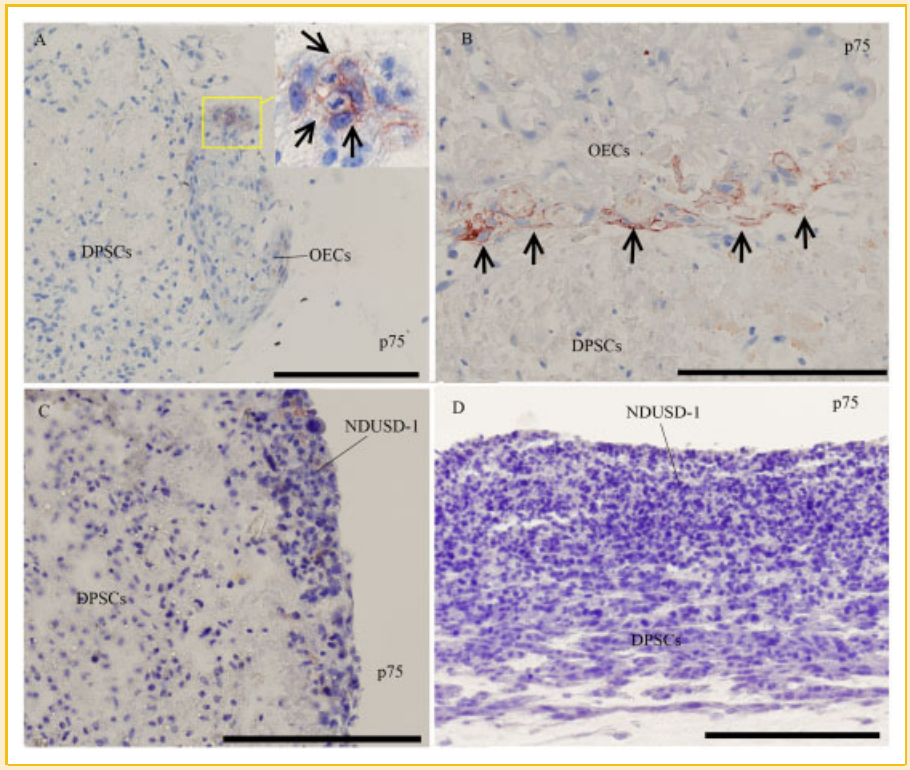

Fig. 8. The p75 expression in three-dimensional co-cultivated epithelial–mesenchymal cells. Epithelial and mesenchymal cells were co-cultivated for 3 weeks, and followed by immunostaining for p75 expression. A: DPSCs and OECs were co-cultivated on matrigel. p75 (+) cells were observed inside OEC mass. B: DPSCs and OECs were co-cultivated on collagen gel. The p75 (+) cells were observed the boundary of which is between epithelial and mesenchymal tissue. C: DPSCs and NDUSD-1 cells were co-cultivated on matrigel. D: DPSCs and NDUSD-1 cells were co-cultivated on collagen gel. No positive signal was detected in C and D. Arrow indicates p75 (+) cells. Scale bar = 50 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

BMPs, which belong to the TGF beta superfamily, were identified as signaling molecules mediating the inductive interactions during odontogenesis. BMPs are involved in specifying the outcome of the dental mesenchymal cells, cell proliferation, differentiation, and cell death in dental mesenchyme [Wang et al., 1999; Chen et al., 2008; Feng et al., 2011]. BMP4 expression is first observed in the dental lamina epithelium, but then shifts to the dental mesenchyme. BMP4 transfers tooth inductive potential from dental epithelium to mesenchyme [Chen et al., 1996]. In this study, time-dependent change of *BMP2* and *BMP4* expression in OEC–DPSC co-cultures showed that the expression level at 2 and 7 weeks was higher than 4 weeks, suggested that at early culture period, *BMPs* was probably expressed in OECs-formed epithelium, and then shifted to DPSCs-formed mesenchyme from 4 weeks of cultivation.

Similar to BMPs, FGFs also play a role in regulating epithelial stem cell proliferation and epithelial–mesenchymal interactions during embryonic tooth development [Klein et al., 2008; Yun et al., 2010]. Both *BMPs* and *FGFs* express in dental epithelium and mesenchyme during the early stages of tooth development (http://bite-it.helsinki.fi/). Our immunostaining data showed that BMP7, FGF3, and FGF4 expressed in invaginated epithelial cells of OEC-co-M tissue model. This observation was not shown in NDU-co-C co-cultures, suggesting OEC-co-M tissue model have similar immunofeatures with early tooth development.

Msx1 and Pax9 have critical roles in mediating epithelial–mesenchymal interactions during tooth development. Both of Msx1 and Pax9 express in the dental mesenchyme and excluded from the dental epithelium throughout the bud, cap, and bell stages of odontogenesis (http://bite-it.helsinki.fi/). In neither Msx1 nor Pax9 knockout mice, teeth did not develop past the bud stage [Satokata and Maas, 1994; Peters et al., 1998]. Msx1 expression is required in the mesenchyme for reciprocal signaling to the dental epithelium [Chen et al., 1996]. Pax9 regulates the expression of BMP4 and is needed for the expression of Msx1 [Peters et al., 1998]. In the OEC-co-M tissue model, the Msx1 protein was strongly expressed in the mesenchymal cells and the expression of *Msx1* and *Pax9* were much higher than monolayer DPSCs and the other three types of co-culture. The data suggest that in OEC-co-M tissue model, Msx1 and Pax9 might mediate epithelial–mesenchymal interaction the same as the in vivo situation.

Integrins are involved in epithelial–mesenchymal interactions during tooth development [Salmivirta et al., 1996]. ITGB1 is essential for tissue integrity, structural functions, cell proliferation, apoptosis, polarity, differentiation, and other processes [Chen et al., 2009]. Cross-talk of integrin-β1 with E-cadherin, Shh and Erk was shown to promote proliferation and prevent differentiation in stem cells in the skin and brain tissues [Raghavan et al., 2000; Blaess et al., 2004; Campos et al., 2004]. In this study, epithelial–mesenchymal

co-cultures showed much lower expression of *ITGB1* than monolayer cells. The result indicates that ITGB1 is a common molecule for both epithelial and mesenchymal cells; which likely controls the proliferation of those cells. While epithelial and mesenchymal cells were co-cultivated, differentiation was in process; therefore the expression of ITGB1 needs to be down-regulated.

The p75 is a low-affinity neurotrophin receptor, is a member of the tumor necrosis factor superfamily, localized in a variety of tissues during embryonic development [Byers et al., 1992; Arany et al., 2009]. Immunocytochemical study showed that neurotrophin receptor expresses in the dental lamina epithelium and in adjacent mesenchyme but not in deeper mesenchyme during early stages of tooth formation [Byers et al., 1990; Mitsiadis et al., 1992; Redd and Byers, 1994]. More evidence showed that, p75 is expressed in the inner dental epithelium but is completely absent in differentiated ameloblasts in tooth [Woodnutt et al., 2000; Yoshizaki et al., 2008]. We found that p75 proteins expressed in the boundary between epithelium and mesenchyme in both OEC-co-M and OEC-co-C tissue models during early culture period, but not co-cultures with NDUSD-1. This suggests that p75 might be an important molecule for the maintenance of epithelial stem cells and epithelial–mesenchymal interaction during epithelium invagination. Because mesenchymal condensation was not observed in OEC-co-C co-cultures, p75 probably is not related to mesenchymal condensation.

Runx2 is a runt domain transcription factor that is essential for tooth morphogenesis. In Runx2 knockout mice, tooth development arrests at late bud stage. Runx2 mediates the functions of epithelial FGF signals. During early tooth development, Runx2 mainly expresses in dental mesenchymal cells [Aberg et al., 2004]. In OEC-co-M tissue model, Runx2 expression was absent suggesting Runx2 probably is not essential for epithelial invagination.

OEC-co-M showed similar morphological changes (the invagination of epithelial cells and condensation of the underlying mesenchymal cells) to in vivo embryonic epithelium invagination. In the early culture period, OEC-co-C also formed epithelial invagination. However, we did not observe the mesenchymal condensation, and epithelial colonies appeared inside the mesenchymal tissue after being cultivated for a longer period. The reason for the difference between those two co-cultures with OEC is considered to be caused by matrigel. Matrigel contains extracellular matrix (ECM) proteins, mainly laminin, followed by collagen IV and growth factors. ECM proteins and growth factors have been used for tissue engineering strategies, which affect cell adhesion, migration, proliferation, and differentiation [Timpl et al., 1979; Vukicevic et al., 1992; Crapo et al., 2011]. It has been reported that using matrigel-contained 3D culture system could reconstruct functional salivary gland structures whereas pure collagen gel could not [Joraku et al., 2007]. Compared to collagen gel, matrigel is able to provide a more similar environment to the in vivo conditions for epithelial–mesenchymal cells. Therefore, using matrigel, epithelial–mesenchymal co-cultures were able to show similar morphological and immunological changes to the natural tissue.

In conclusion, using normal human OECs and DPSCs under definite conditions (such as using matrigel as the culture base) it is possible to reconstruct an epithelial invagination model. Epithelial stem cells and epithelial–mesenchymal interaction-related molecules (such as CDHs, Max1, Pax9, and p75) probably contributed the formation of epithelium invagination and mesenchymal condensation. This model can be used as a tool for exploration in the research of epithelial–mesenchymal interaction, and tooth, hair follicle, and lung regeneration.

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REFERENCES

Aberg T, Wang XP, Kim JH, Yamashiro T, Bei M, Rice R, Ryoo HM, Thesleff I. 2004. Runx2 mediates FGF signaling from epithelium to mesenchyme during tooth morphogenesis. Dev Biol 270:76–93.

Alongi DJ, Yamaza T, Song Y, Fouad AF, Romberg EE, Shi S, Tuan RS, Huang GT. 2010. Stem/progenitor cells from inflamed human dental pulp retain tissue regeneration potential. Regen Med 5:617–631.

Arany S, Koyota S, Sugiyama T. 2009. Nerve growth factor promotes differentiation of odontoblast-like cells. J Cell Biochem 106:539–545.

Bei M. 2009. Molecular genetics of tooth development. Curr Opin Genet Dev 19:504–510.

Blaess S, Graus-Porta D, Belvindrah R, Radakovits R, Pons S, Littlewood-Evans A, Senften M, Guo H, Li Y, Miner JH, Reichardt LF, Ẅuller U. 2004. β1-integrins are critical for cerebellar granule cell precursor proliferation. J Neurosci 24:3402–3412.

Byers MR, Schatteman GC, Bothwell M. 1990. Multiple functions for NGF receptor in developing, aging and injured rat teeth are suggested by epithelial, mesenchymal and neural immunoreactivity. Development 109:461–471.

Byers MR, Kvinnsland I, Bothwell M. 1992. Analysis of low affinity nerve growth factor receptor during pulpal healing and regeneration of myelinated and unmyelinated axons in replanted teeth. J Comp Neurol 326:470–484.

Calenic B, Ishkitiev N, Yaegaki K, Imai T, Costache M, Tovaru M, Tovaru S, Parlatescu I. 2010. Characterization of oral keratinocyte stem cells and prospects of its differentiation to oral epithelial equivalents. Rom J Morphol Embryol 51:641–645.

Campos LS, Leone DP, Relvas JB, Brakebusch C, Ffässler R, Suter U, Ffrench-Constant C. 2004. β1 integrins activate a MAPK signalling pathway in neural stem cells that contributes to their maintenance. Development 131:3433–3444.

Chen Y, Bei M, Woo I, Satokata I, Maas R. 1996. Msx1 controls inductive signaling in mammalian tooth morphogenesis. Development 122:3035–3044.

Chen S, Gluhak-Heinrich J, Martinez M, Li T, Wu Y, Chuang HH, Chen L, Dong J, Gay I, MacDougall M. 2008. Bone morphogenetic protein 2 mediates dentin sialophosphoprotein expression and odontoblast differentiation via NF-Y signaling. J Biol Chem 283:19359–19370.

Chen B, Goodman E, Lu Z, Bandyopadhyay A, Magraw C, He T, Raghavan S. 2009. Function of beta1 integrin in oral epithelia and tooth bud morphogenesis. J Dent Res 88:539–544.

Crapo PM, Gilbert TW, Badylak SF. 2011. An overview of tissue and whole organ decellularization processes. Biomaterials 32:3233–3243.

Feng J, Yang G, Yuan G, Gluhak-Heinrich J, Yang W, Wang L, Chen Z, Schulze McDaniel J, Donly KJ, Harris SE, Macdougall M, Chen S. 2011.

Abnormalities in the enamel in bmp2-deficient mice. Cells Tissues Organs 194:216–221.

Fischer L, Boland G, Tuan RS. 2002. Wnt signaling during BMP-2 stimulation of mesenchymal chondrogenesis. J Cell Biochem 84:816–831.

Freemark M, Driscoll P, Maaskant R, Petryk A, Kelly PA. 1997. Ontogenesis of prolactin receptors in the human fetus in early gestation. Implications for tissue differentiation and development. J Clin Invest 99:1107–1117.

Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. 2000. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci USA 97:13625–13630.

Heymann R, About I, Lendahl U, Franquin JC, Obrink B, Mitsiadis TA. 2002. E- and N-cadherin distribution in developing and functional human teeth under normal and pathological conditions. Am J Pathol 160:2123–2133.

Hu B, Unda F, Bopp-Kuchler S, Jimenez L, Wang XJ, Haïkel Y, Wang SL, Lesot H. 2006. Bone marrow cells can give rise to ameloblast-like cells. J Dent Res 85:416–421.

Jamora C, DasGupta R, Kocieniewski P, Fuchs E. 2003. Links between signal transduction, transcription and adhesion in epithelial bud development. Nature 422:317–322.

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

Joraku A, Sullivan CA, Yoo J, Atala A. 2007. In-vitro reconstitution of three-dimensional human salivary gland tissue structures. Differentiation 75:318–324.

Klein OD, Lyons DB, Balooch G, Marshall GW, Basson MA, Peterka M, Boran T, Peterkova R, Martin GR. 2008. An FGF signaling loop sustains the generation of differentiated progeny from stem cells in mouse incisors. Development 135:377–385.

Kubo C, Tsutsui TW, Tamura Y, Kumakura S, Tsutsui T. 2009. Immortalization of normal human gingival keratinocytes and cytological and cytogenetic characterization of the cells. Odontology 97:18–31.

Mitsiadis TA, Dicou E, Joffre A, Magloire H. 1992. Immunohistochemical localization of nerve growth factor (NGF) and NGF receptor (NGF-R) in the developing first molar tooth of the rat. Differentiation 49:47–61.

Nakagawa E, Itoh T, Yoshie H, Satokata I. 2009. Odontogenic potential of post-natal oral mucosal epithelium. J Dent Res 88:219–223.

Nakamura T, Endo K, Kinoshita S. 2007. Identification of human oral keratinocyte stem/progenitor cells by neurotrophin receptor p75 and the role of neurotrophin/p75 signaling. Stem Cells 25:628–638.

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.

Nishida K, Yamato M, Hayashida Y, Watanabe K, Yamamoto K, Adachi E, Nagai S, Kikuchi A, Maeda N, Watanabe H, Okano T, Tano Y. 2004. Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. N Engl J Med 351:1187–1196.

Papusheva E, Heisenberg CP. 2010. Spatial organization of adhesion: Force-dependent regulation and function in tissue morphogenesis. EMBO J 29: 2753–2768.

Peters H, Neubüser A, Kratochwil K, Balling R. 1998. Pax9-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. Genes Dev 12:2735–2747.

Raghavan S, Bauer C, Mundschau G, Li Q, Fuchs E. 2000. Conditional ablation of β1 integrin in skin. Severe defects in epidermal proliferation, basement membrane formation, and hair follicle invagination. J Cell Biol 150:1149–1160.

Redd PE, Byers MR. 1994. Regeneration of junctional epithelium and its innervation in adult rats: A study using immunocytochemistry for p75 nerve growth factor receptor and calcitonin gene-related peptide. J Periodontal Res 29:214–224.

Salmivirta K, Gullberg D, Hirsch E, Altruda F, Ekblom P. 1996. Integrin subunit expression associated with epithelial–mesenchymal interactions during murine tooth development. Dev Dyn 205:104–113.

Satokata I, Maas R. 1994. Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. Nat Genet 6:348–356.

Takahashi I, Onodera K, Sasano Y, Mizoguchi I, Bae JW, Mitani H, Kagayama M, Mitani H. 2003. Effect of stretching on gene expression of beta1 integrin and focal adhesion kinase and on chondrogenesis through cell–extracellular matrix interactions. Eur J Cell Biol 82:182–192.

Thesleff I, Tummers M. 2009. Tooth organogenesis and regeneration. In Girard L, editor. StemBook [Internet]. Cambridge, MA: Harvard Stem Cell Institute. p 2–9.

Timpl R, Rohde H, Robey PG, Rennard SI, Foidart JM, Martin GR. 1979. Laminin—A glycoprotein from basement membranes. J Biol Chem 254: 9933–9937.

Tsutsui TW, Inaba T, Fisher LW, Robey PG, Tsutsui T. 2006. In vitro chromosome aberration tests using human dental pulp cells to detect the carcinogenic potential of chemical agents. Odontology 94:44–50.

Vukicevic S, Kleinman HK, Luyten FP, Roberts AB, Roche NS, Reddi AH. 1992. Identification of multiple active growth factors in basement membrane Matrigel suggests caution in interpretation of cellular activity related to extracellular matrix components. Exp Cell Res 202:1–8.

Wang YH, Rutherford B, Upholt WB, Mina M. 1999. Effects of BMP-7 on mouse tooth mesenchyme and chick mandibular mesenchyme. Dev Dyn 216:320–335.

Wang B, Li L, Du S, Liu C, Lin X, Chen Y, Zhang Y. 2010. Induction of human keratinocytes into enamel-secreting ameloblasts. Dev Biol 344:795–799.

Woodnutt DA, Wager-Miller J, O'Neill PC, Bothwell M, Byers MR. 2000. Neurotrophin receptors and nerve growth factor are differentially expressed in adjacent nonneuronal cells of normal and injured tooth pulp. Cell Tissue Res 299:225–236.

Woodward WA, Tuan RS. 1999. N-cadherin expression and signaling in limb mesenchymal chondrogenesis: Stimulation by poly-L-lysine. Dev Genet 24:178–187.

Xiao L, Aoshima H, Saitoh Y, Miwa N. 2010. The effect of squalane-dissolved fullerene-C60 on adipogenesis-accompanied oxidative stress and macrophage activation in a preadipocyte–monocyte co-culture system. Biomaterials 31:5976–5985.

Xiao L, Aoshima H, Saitoh Y, Miwa N. 2011. Highly hydroxylated fullerene localizes at the cytoskeleton and inhibits oxidative stress in adipocytes and a subcutaneous adipose-tissue equivalent. Free Radic Biol Med 51:1376–1389.

Yan X, Qin H, Qu C, Tuan RS, Shi S, Huang GT. 2010. iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin. Stem Cells Dev 19:469–480.

Yoshizaki K, Yamamoto S, Yamada A, Yuasa K, Iwamoto T, Fukumoto E, Harada H, Saito M, Nakasima A, Nonaka K, Yamada Y, Fukumoto S. 2008. Neurotrophic factor neurotrophin-4 regulates ameloblastin expression via full-length TrkB. J Biol Chem 283:3385–3391.

Yu Q, Toole BP. 1997. Common pattern of CD44 isoforms is expressed in morphogenetically active epithelia. Dev Dyn 208:1–10.

Yun YR, Won JE, Jeon E, Lee S, Kang W, Jo H, Jang JH, Shin US, Kim HW. 2010. Fibroblast growth factors: biology, function, and application for tissue regeneration. J Tissue Eng 2010:218142.

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.