치의과학 석사학위논문

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인간 유도만능줄기세포로부터 치아상피 유사 세포주의 확립과 특성 분석

2022년 8월

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위 원 장 _____ (인)

부 위 원 장 _____ (인)

위 원 _____ (인)

Abstract

Development and Characterization of Dental Epithelial Cell Lines from Human Induced Pluripotent Stem Cells

Min Gi Ki

Molecular Genetics Major
Graduate School, Seoul National University

(Directed by Prof. Gene Lee)

Tooth development and regeneration are essentially regulated through epithelial-mesenchymal interactions between dental epithelium and mesenchyme. Due to the consequences of the development of teeth, dental mesenchymal stem cells have been obtained from various cell sources. However, it is difficult to isolate and maintain human dental epithelial cells because they were lost after the tooth eruption. Therefore, acquiring epithelial and mesenchymal stem cells for tooth regeneration is essential. The previous study has reported on the source of an epithelial stem cell by differentiating iPSCs: dental pulp stem cells from human exfoliated deciduous teeth (dDPSCs)-derived iPSCs differentiated into dental epithelial-like cells using Hertwig's epithelial root sheath/epithelial rests of Malassez (HERS/ERM) cells as a feeder. This study was conducted to develop alternative sources of dental epithelial stem cells for dental regeneration. In this study, differentiation of peripheral blood mononuclear cells (PBMCs)-derived iPSCs on HERS/ERM cells as a feeder and dDPSCs-derived iPSCs on DPESCs as a feeder was investigated. Each dental

epithelial-like cell line was established and characterized. Dental differentiation was also examined by co-culture of the epithelial-like cells and dDPSCs. Differentiating PBMCs and dDPSCs-derived iPSCs into epithelial-like cells was induced on HERS/ERM cells and DPESCs feeder layers for 14 days, respectively. Differentiated epithelial-like cells were immortalized, and PBMCs-iPSCs derived epithelial-like cell lines (PHes) and dDPSCs-iPSCs derived epithelial-like cell lines (DDes) were established. STR analysis verified that each cell line originated from each iPSC, respectively. The immunophenotypes of PHes and DDes were similar by FACS analysis, and both had the characteristics of epithelial stem cells with expressions of epithelial stem cell- and stemness-related markers. Moreover, DDes co-cultured with dDPSC showed the potential for dental differentiation: the ameloblast-related genes and the odontoblast-related genes were highly expressed. The protein expression of the dentin phosphoprotein and enamelin intensely increased. In addition, mineralization in the co-culture group increased by odontogenic induction. Taken together, the established epithelial-like cells, DDes and PHes, had the characteristics of dental epithelial stem cells. Also, the co-culture of DDes and dDPSCs showed the capacity for dental differentiation. Therefore, various iPSCs-derived epithelial-like cell lines were established, and these cell lines are expected to be used in tooth regeneration.

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Keywords : induced pluripotent stem cells (iPSCs), dental pulp stem cells from human exfoliated deciduous teeth (dDPSCs), peripheral blood mononuclear cells (PBMCs), Hertwig's epithelial root sheath/epithelial rests of Malassez (HERS/ERM), epithelial-like cells from human deciduous dental pulp (DPESCs), dental differentiation

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Introduction

Dental stem cell research has positively impacted dental bioengineering over the past few decades. The studies have shown that tooth-like structures can be generated by dental epithelium and mesenchymal in tooth organogenesis [1, 2]. Thus, dental studies based on the interaction between epithelial and mesenchymal cells for tooth formation are essential [3]. Various dental mesenchymal stem cells have been established and are being studied. However, it is difficult to obtain dental epithelial stem cells because ameloblasts are lost after teeth eruption [4, 5]. Therefore, human dental epithelial stem cell sources that can be obtained for tooth regeneration are very scarce. Also, it is difficult to maintain even if obtained because the lifespan is short.

Induced pluripotent stem cells (iPSCs) are the most clinically promising cell sources that can replace scarce cell sources [6, 7]. The potential of human iPSCs as a resource for tooth regeneration research has been confirmed, and various cell types have been used to establish iPSCs [8]. In a previous study, dental pulp stem cells from human exfoliated deciduous teeth (dDPSCs)-derived iPSCs were differentiated on Hertwig's epithelial root sheath/epithelial rests of Malassez (HERS/ERM) cells to establish dental epithelial stem cells [9]. dDPSCs, the origin of iPSCs used in the previous study, can be obtained relatively easily from human deciduous teeth [10] but have a limitation in that they cannot be obtained from adults. Alternatively, easily obtained from adults, blood-derived peripheral blood mononuclear cells (PBMCs) can be an ideal cell source. Also, to establish epithelial stem cells with various properties, the various origin of feeders to differentiate iPSCs are required. Therefore, it is necessary to use dental epithelial stem cells obtained from permanent and

deciduous teeth. Epithelial-like cells from human deciduous dental pulp (DPESCs), known as dental epithelial stem cells, such as HERS/ERM cells, can also be used as a feeder.

This study was conducted to develop alternative sources of human dental epithelial stem cells for dental regeneration. In this study, dental epithelial-like cells were generated from human iPSCs derived from deciduous teeth or adult blood with various human dental tissue-derived epithelial cells as feeders. PBMC-derived iPSCs on HERS/ERM cells as a feeder and dDPSCs-derived iPSCs on DPESCs as a feeder were differentiated into dental epithelial-like cells. The established dental epithelial-like cell lines were characterized. STR analysis to determine the origin of epithelial-like cells, the FACS analysis for the immunophenotypical characterization, and semi-quantitative PCR for the gene expression patterns were investigated. Also, co-culture of epithelial-like cells established on DPESCs as a feeder and dDPSCs was performed to evaluate the potential of dental differentiation: real time-PCR, Western blotting, and Alizarin Red S staining were examined. The results showed the potential of the human iPSC-derived dental epithelial-like cell lines from various origins to overcome the limitations of dental epithelial cell sources and demonstrated that the established epithelial-like cells contributed to mineralized tissue formation with dDPSCs.

Literature Review

1. Interactions between mesenchyme and epithelium during tooth development

Teeth development occurs through the interaction between mesenchyme and epithelium in the ectoderm and endoderm layers [11-14]. Epithelial-mesenchymal interactions play a fundamental role in tooth morphogenesis [15-17]. During tooth development, as the tooth epithelium begins to thicken locally, it proliferates to the oral mesenchyme to form the dental lamina and goes through bud, cap, and bell stages for morphogenesis [18-20]. The major signaling molecules expressed by the epithelial and mesenchymal tissues, Sonic hedgehog (Shh), bone morphogenetic proteins (BMPs), and fibroblast growth factors (FGFs) and Wnt, regulate the early stages of tooth morphogenesis and are required for tooth development [21, 22]. At the Bud stage, the molecule signaling pathway is essential for helping the epithelium to be incorporated and combined into the condensed mesenchyme separated by the basement membrane [3, 23]. Later, Shh, BMPs, and FGFs are expressed in the enamel knot, which has a signaling center at the cap stage and plays a pivotal role in tooth development [24]. In the bell stage, the differentiation of dentin-forming odontoblasts and enamel-forming ameloblasts are regulated by signals at the interface between epithelium and mesenchymal [23, 25]. It is an essential resource for tooth development because all molecular signaling pathways occur through the tooth epithelium and mesenchymal.

2. Dental epithelial stem cells research progress to date

i. Difference between mouse and human dental epithelial stem cells

The mice incisor was a good model for epithelial stem cell research because it has the characteristic of continuously growing [26-28]. Dental epithelial stem cells, which are present in the incisor of mice, can continue to grow due to the expression of the transcription factor sox2, a major epithelial marker [29, 30]. In contrast, human teeth have lower regeneration potential due to the early loss of enamel-forming ameloblasts and dental epithelial stem cells after tooth eruption [4, 5]. The ameloblasts are epithelial cells that differentiate from the inner cells of the enamel organ. [31, 32]. They are the only cells of ectodermal origin that play an important role in tooth formation [31]. Unfortunately, no adult human ectodermal stem cells remain that can be used to regenerate teeth, as ameloblasts are completely lost after tooth eruption once enamel is fully formed [33, 34]. So, research on the epithelial source and characterization of epithelial stem cells is lacking, so the study on tooth regeneration is slow [35]. Therefore, generating usable dental epithelial stem cells will be a significant step toward dental tissue engineering [36]. Many studies are being attempted to induce the available new methods to form dental epithelial cells.

ii. Human dental epithelial stem cells; Hertwig's epithelial root sheath/epithelial rests of Malassez (HERS/ERM) cells

Epithelial rests of Malassez (ERM) in the periodontium of permanent human teeth are unique epithelial remnants of Hertwig's epithelial root sheath (HERS) involved in tooth root formation and maintaining periodontal tissue homeostasis

[37, 38]. HERS/ERM cells are demanding to obtain because they are in a quiescent state and are small to be isolated [39, 40]. Therefore, to use HERS/ERM cells for dental research, it is necessary to establish an efficient method for isolating primary cells and a usable immortalized cell line. In several studies, HERS/ERM cells were isolated from periodontium and immortalized with SV40 large T antigen for characterization [40, 41]. Also, it has been shown that HERS/ERM cells can be differentiated into cementum-forming cells [42, 43] and can induce odontoblast differentiation and generation of dentin-like tissues [41-45].

iii. Human dental epithelial stem cells; Epithelial-like cells from human deciduous dental pulp (DPESCs)

Epithelial-like stem cells also have been recently reported in human deciduous dental pulp [46-48]. DPESCs express epithelial stem cell markers, and the role of odontogenesis in odontoblastic capacity for dentin regeneration has also been revealed [46, 49]. However, it is difficult to isolate epithelial cells from the pulp due to the difficulty of removing the dental hard tissue surrounding the pulp [46]. For this reason, it is very difficult to obtain epithelial stem cells isolated from the primary pulp. Few studies have been elucidated on DPESCs, and further studies will be needed.

3. Induced pluripotent stem cells (iPSCs), a novel resource for obtaining cell sources

i. Research possibility of iPSCs

iPSCs are a promising cell resource that can be used for regeneration research as a new model of pluripotent cells. Induced pluripotent stem cells (iPSCs) have their self-renewal ability and pluripotent differentiation potential [50]. In 2006, a study called the starting point of induced pluripotent stem cells appeared. Takahashi and Yamanaka converted mouse fibroblasts into embryonic stem cells through the viral transduction of four transcription factors (Oct3/4, Sox2, Klf4, and c-Myc) [51]. It was a revolutionary discovery because iPSCs derived from peripheral blood or skin fibroblasts can be differentiated into cell types that are difficult to obtain directly, such as cardiomyocytes [52]. iPSCs can be differentiated *in vitro* into three primary germ layers (ectoderm, mesoderm, and endoderm) and can form teratomas [53]. Several studies have also uncovered differences in iPS cells and ES cells in terms of gene expression, epigenetic modification, and differentiation potentials. In particular, since iPSCs are derived from adult somatic cells, the ethical issues on ESCs are not related [54, 55]. Ever since the advent of reprogramming techniques and the generation of induced pluripotent stem cells, this field has become more interested in developing drugs for the onset and progression of patient/disease-specific iPSCs [56]. Accordingly, in order to stably cultivate and grow iPSCs, types of media and coating matrices were developed [57].

ii. Possibility of obtaining iPSCs from various cell types

Many cell types are used as cell sources to establish iPSCs [8]. Early studies of iPSCs started with reprogramming skin fibroblasts obtained from adult patients [58]. Skin fibroblasts obtained from skin biopsy have the advantage of accessibility [59, 60]. In addition, the culture and proliferation to maintain the cells after harvesting the skin fibroblasts are not difficult [60, 61]. However, obtaining skin fibroblasts through skin biopsy generally requires a skin punch and local anesthesia [62]. So over the years, studies have generated iPSCs from many different sources. In dentistry, iPSCs were derived from DPSCs [63], dDPSCs [64], SCAPs [65], and periodontal ligament fibroblast [66], etc. It was found that iPSCs made by reprogramming from dental cells are as effective as skin fibroblasts [67, 68]. In the previous study, dDPSCs, the origin of induced pluripotent stem cells obtained by Kim et al., are cells that can be obtained relatively easily from deciduous teeth [9]. Studies have shown that dDPSCs have a relatively higher reprogramming efficiency than adult dermal fibroblasts [9, 69, 70]. However, considering that deciduous teeth cannot be obtained from adults, peripheral blood-derived mononuclear cells (PBMCs) with a patient-specific regenerative system are also a good resource [59]. There has been a successful case of reprogramming PBMCs compared to fibroblasts extracted from elderly patients [71]. This method comprises a time- and cost-effective procedure [66, 67]. Blood is less invasive than skin fibroblasts and has the advantage of being easily isolated in large quantities [60, 61, 71, 72].

iii. Current research on differentiation from mouse iPSCs into epithelial-like cells

Somatic cell reprogramming for mouse iPSCs was started [73], and differentiation into epithelial-like cells using mouse iPSCs was conducted. Arakaki et al. first reported the process of mouse iPSCs generating ameloblasts [74]. When they co-cultured the established SF2 epithelial cell line with mouse iPSCs, they succeeded in inducing differentiation into an epithelial lineage. The differentiated iPSCs-derived epithelial cells exhibited an epithelial-like morphology with the expression of epithelial cell markers such as p63, CK14, and ameloblastic markers such as ameloblastin and enamelin. Also, it was found that mouse iPSCs can be induced to express odontogenic genes. Therefore, it is known that mouse iPSCs have the potential to be used as a new cell source. Also, it was verified through the experiments of Yoshida et al. that direct contact of mouse iPSCs with tooth epithelium induces ameloblastic differentiation [75]. They revealed that mouse iPSCs could be differentiated into ameloblast-like cells by using epithelial cell rests of Malassez (ERM)-conditioned medium or by co-culture with ERM cells. Amelogenin and ameloblastin expression levels were increased in both culture conditions. That was the first study to show that mouse iPSCs can differentiate into ameloblast-like cells without a feeder: it showed the applicability of human iPSCs in dental regeneration.

iv. Current research on differentiation from human iPSCs into epithelial-like cells

Several studies using human iPSCs have also been reported. Recently, Cai et al.

succeeded in inducing integration-free human urine iPSCs into epithelial sheets, which resulted in the formation of ameloblasts in a tooth-like structure [76]. Based on this study, it was demonstrated that human iPSCs could be used for tooth regeneration. However, this study did not demonstrate the possibility of long-term culture of epithelial-like cells. Liu et al. demonstrated that iPSCs were induced into epithelial cells using an epithelial induction medium [77]. Human iPSCs differentiated for 72 hours in an epithelial induction medium showed the expression of E-cadherin, an epithelial-specific marker. Also, epithelial markers such as p63, CD29, CK14, CK18, and CK19 were upregulated during epithelial differentiation. However, only the epithelial expression could be confirmed, and dental differentiation could not be confirmed. Recently, Kim et al. showed differentiation into dental epithelial-like stem cells by co-culturing human iPSCs and embryonic stem cells (ESCs) with HERS/ERM cells [64]. The established epithelial-like cells expressed epithelial stem cell markers such as ABCG2, EpCAM, p63, p75, and E-cadherin. The potential of human iPSCs as a resource for tooth regeneration research has been confirmed, but the methods need extensive research.

Materials and Methods

Differentiation of hiPSCs into epithelial-like stem cells

It has been reported that DPESCs and HERS/ERM cells used as feeder cells have the same characteristics as primary cells. Before differentiate hiPSCs into epithelial-like cells, DPESCs and HERS/ERM cells were treated with Mitomycin C (MMC, Sigma Aldrich, St. Louis, MO, USA) to be used as feeders. Cells were treated with MMC at 37°C for 1 h 30 min at a concentration of 10 μg/ml. 1 x 10^6 cells were seeded on a 0.1% gelatin-coated (Bio Basic Inc, Markham, Ontario, Canada) 35 mm cell dish. One day later, hiPSC clumps were transferred onto the MMC-feeder layer in serum-free keratinocyte growth medium-2 (KGM-2; Lonza, Rock Island, ME, USA) with supplements provided (Lonza, Rock Island, ME, USA) and 2% FBS. After culturing in KGM-2 with 2% FBS for 2 days, change to KGM-2 without FBS. For 14 days cultivation, when clonal hiPSCs reached about 80% confluence, they were isolated into single cells. This process was incubated for 10 min using Accutase cell detachment solution (Merck Millipore, Billerica, MA, USA) to chemically dissociated cells. KGM-2 was changed every two days until 70% confluence was reached.

Immortalization of epithelial-like cells via the transfected of SV40 LT

SV40 gene was transfected into isolated primary epithelial-like cells to overcome the limited lifespan. Primary epithelial-like cells (15,000cm^2) were transfected with 2 μg of pcDNA 3.1 (+) plasmid containing the SV40 large T antigen (SV40) using the X-tremeGENE 9 DNA transfection reagent (Roche, Indianapolis, IN, USA), according to the manufacturer's instructions. The transfection mixture was incubated

in KGM-2 for 72 h, and G418 (Cellgro Mediatech, Washington, DC, USA) was added to the medium at a concentration of 100 μg/mL. KGM-2 with G418 was maintained for 10 days by changing the medium every two days. After 10 days, independent colonies were observed and maintained with a G418-free KGM-2 culture medium. After 2 weeks, cells were transferred to new culture dishes. Each of the three cell lines was established using this method.

Microsatellite (STR) analysis

Genomic DNA was extracted from hiPSCs, epithelial-like cells, DPESCs, and HERS/ERM cells using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Microsatellite (STR) analysis was performed by Macrogen Inc. (Seoul, Korea).

Fluorescence-activated cell sorting (FACS) analysis

For FACS analysis, cells were harvested and washed with PBS supplemented with 2% FBS (HyClone, Road Logan, Utah, USA). Washed cells were fixed with 4% paraformaldehyde at RT for 10 min. After fixation, 10,000 cells were incubated with fluorescently conjugated antibodies for 30 min at 4°C. The antibodies are listed in Table 1. The fluorescence intensity was measured by a FACSCalibur (Becton Dickinson, USA), and data were analyzed using FLOWJO (Tree Star, Inc., USA) software.

Total RNA isolation and reverse transcription

Cell pellets were collected in 50 μl RNALater (Ambion, Austin, TX, USA) and washed with DPBS supplemented with 2% FBS before use. Total RNA was isolated from epithelial-like cells, DPESCs, and HERS/ERM cells using the RNeasy mini kit

(Qiagen, Hilden, Germany). DNase I treatment in the RNase-free DNase set (Qiagen) was treated to remove contamination of genomic DNA. The isolated RNA (2 μg) was mixed with amfiRivert cDNA synthesis Platinum Master Mix (GenDEPOT, Barker, TX, USA) to synthesize cDNA. The amplification steps included one cycle of annealing at 25℃ for 5 min, followed by extension at 42℃ for 60 min, and heat inactivation of the enzyme at 70℃ for 15 min. The RT products were stored at -20℃ until quantitative analysis.

Semi-quantitative PCR (qPCR)

Using the resulting cDNA as a template, semi-quantitative PCR was performed with specific primers for the target genes and i-MAXII (Intron, Seongnam, Korea). The PCR condition was an initial denaturation at 94℃ for 2 min, followed by 40 cycles of PCR amplification with denaturation at 94℃ for 20 sec, annealing at 60℃ for 30 sec, and a final extension at 72℃ for 5 min. Primer sequences and each annealing temperature are shown in Table 2. The PCR products were electrophoresed on agarose 2.0% and visualized by Ethidium bromide staining.

In vitro odontogenic differentiation

For odontogenic differentiation, epithelial-like cells and dDPSCs were cultured to confluence and the cells were cultured in non-induction control media (αMEM supplemented with 5% FBS) or induction media (αMEM supplemented with 5% FBS, 50 μg/ml ascorbic acid, 10 Mm β-glycerophosphate, and 0.1 μM dexamethasone). The medium was changed every 2 days.

Real-Time PCR (RT-PCR)

The cDNA obtained from epithelial-like cells, DPESCs and HERS/ERM cells, and Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan) was used to prepare reaction mixes. RT-PCR was performed using the CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with specific primers (Table 2). In each experiment, three independent qPCR experiments were performed for each gene. The data were normalized with GAPDH, a housekeeping gene, and the ΔCt method was used in calculations.

Western blot analysis

Western blot analysis was carried out after co-culturing DDes and dDPSCs for 8 days with control media or induction media. Cells collected by scraper were suspended in RIPA lysis buffer (RKMB-030-0050, Rockland Immunochemicals, Limerick, PA, USA) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (78440, ThermoFisher Scientific, Waltham, MA, 1:100) and left on ice for 15 min. Protein concentration was quantified using the Pierce BCA Protein assay kit (Thermo Fisher Scientific, Rockford, USA). Proteins (40 μg) were separated by 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the PVDF membrane (Bio-Rad). The membranes were blocked with EveryBlot Blocking buffer (Bio-Rad, Japan) for 5 min and further incubated overnight at 4°C with the following antibodies: anti-DSPP (sc-73632, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Enamelin (sc-293334, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (#170-6516, Bio-Rad), horseradish peroxidase-conjugated goat anti-rabbit IgG (#170-6515, Bio-Rad) secondary antibodies for 1 h in RT. The membranes were developed using ECL (Bio-Rad Laboratories) and visualized using a ChemiDoc Imaging System (Bio-Rad, Hercules,

CA, USA).

Alizarin red S staining

For Alizarin Red S staining, DDes were co-cultured with dDPSCs with control media or induction media in 6-well plate for 8, 12, 16, 20, and 24 days. Co-culture with DPESCs and dDPSCs was evaluated on days 32, 36, and 40 days. The cells were washed with DPBS and fixed with 4% paraformaldehyde (T&I Biotechnology, Seoul, Korea). They were stained with 2% Alizarin red S solution (Sigma-Aldrich, St.Louis, MO, USA) for 10 min. The remaining Alizarin solution was washed with distilled water.

Statistical analysis

Obtained results were shown in triplicate, and data were presented as the mean ± SD statistical differences. All statistical analyses were conducted using SAS software. A probability value *(P)* of less than 0.05 was considered to denote statistical significance.

Table 1. FACS antibody

Markers	Antibody	Information	Dilution	Company
Mesenchymal cell markers	CD 29	PE anti human	100 : 2	BD Bioscience Pharmingen
	CD 73	PE anti human	100 : 2	BD Bioscience Pharmingen
	CD 105	APC anti human	100 : 0.5	eBioscience
	HLA-I	FITC anti human	100 : 2	eBioscience
Hematopoietic cell markers	CD 45	FITC anti human	100 : 1	eBioscience
	HLA-DR	APC anti human	100 : 1	BD Bioscience Pharmingen

Table 2. Sequences and annealing temperature for target genes

Gene	Size	Tm (°C)	5' Oligonucleotide	3' Oligonucleotide
GAPDH	209	60	GAT GCT GGC GCT GAG TAC G	GCT AAG CAG TTG GTG GTG C
E-cadherin	200	55	TGC CCA GAA AAT GAA AAA GG	GTG TAT GTG GCA ATG CGT TC
ABCG2	379	55	AGT TCC ATG GCA CTG GCC ATA	TCA GGT AGG CAA TTG TGA GG
EpCAM	100	55	GCT GGC CGT AAA CTG CTT TG	ACA TTT GGC AGC CAG CTT TG
BMI1	75	57	CAG CCC AGC AGG AGG TAT TC	GGA TGA GGA GAC TGC ACT GG
P75	165	60	ACC GAG CTG GAA GTC GAG	CTC ACC GCT GTG TGT GTA C
OCT4	190	55	ACC CCT GGT GCC GTG AA	GGC TGA ATA CCT TCC CAA ATA
NANOG	176	55	CCT ATG CCT GTG ATT TGT GG	TTC TCT GCA GAA GTG GGT TG
SOX2	185	55	GAC TTC ACA TGT CCC AGC AC	GGG TTT TCT CCA TGC TGT TT
Enamelin	159	60	TCC ACG GAA ATC CTC AGC AC	GGG GGT TGA GCT TCC TCT TC
KLK4	166	60	GAT CGC TCG TCT CTG GTA GC	GAG TTC TGG AAA CAG TGT GCG
Runx2	149	60	CCC AGT ATG AGA GTA GGT GTC C	GGG TAA GAC TGG TCA TAG GAC C
BSP	100	60	AAG GCT ACG ATG GCT ATG ATG GT	AAT GGT AGC CGG ATG CAA AG
ONN	100	60	TAC ATC GGG CCT TGC AAA TAC	GGG TGA CCA GGA CGT TCT TG
OCN	235	60	ATC CTT TGG GGT TTG GCC TAC	GCC AAT AGG GCG AGG AGT G
DMP1	265	60	ACA GGC AAA TGA AGA CCC	TTC ACT GGC TTG TAT GG
MEPE	116	60	CAA GAA GCC AGG TAT TCT GAA GG	TGT GGT TGA AAT GTT GGT GCT
Col1a1	100	60	CAA AAA ATG GGA GAC AAT TTC ACA	TCA TGT TCG GTT GGT CAA AGA T
DSPP	277	60	GCC AGA GCA AGT CTG GTA ACG GT	TCT CTC TGC AGG AGT TAG GTC TTG GT

Results

I. Establishment and characterization of epithelial-like cells derived from hiPSCs differentiated on HERS/ERM cells

1. Differentiation of hiPSCs into epithelial-like cells

During maintenance on MMC-treated HERS/ERM cells feeder layer, differentiated hiPSCs colonies grew and differentiated into a cuboidal morphology (Figure 1). hiPSCs differentiated on MMC-treated HERS/ERM cells feeders for 14 days were transfected with SV40 to establish an epithelial-like cell line. Established PHes showed cuboidal, polygonal morphology (Figure 2A). PHes also showed morphological stability and maintained proliferation through sub-culture for more than 15 passages (Figure 2B).

2. Characteristics of the epithelial-like cells on HERS/ERM cells

To verify the origin of PHes, STR analysis was performed. PHes were derived not from HERS/ERM cells but hiPSCs (Figure 3). Fluorescence-activated cell sorting(FACS) was used to detect the expression of cell surface markers in PHes and HERS/ERM cells. Both cells showed similar immunophenotypes (Figure 4). PHes and HERS/ERM cells showed high expression in CD29, CD73, CD105, and HLA-I, but low expression in CD45 and HLA-DR. Also, it was confirmed that there was no difference in stem cell-like characteristics between PHes and HERS/ERM cells by using epithelial stem cell- and stemness-related markers. PHes expressed epithelial stem cell markers such as *E-cadherin ABCG2, EpCAM, Bmi1,* and *p75* and stemness-related markers such as *Oct-4, Nanog,* and *Sox-2* (Figure 5).

II. Establishment and characterization of epithelial-like cells derived from hiPSCs differentiated on DPESCs

1. Differentiation and maintenance of cell lines of hiPSCs into epithelial-like cells on DPESCs

The differentiated human iPSCs using mitomycin C (MMC)-treated DPESCs as a feeder layer gradually grew into a round sphere-like shape (Figure 6). After the SV40 gene was transfected, the established epithelial-like cell line showed rounded, cobblestone shapes compatible with the characteristics of normal epithelial cells (Figure. 7A). DDes, a cell line established on MMC-treated DPESCs, maintained its morphology through sub-culture and grew well more than 20 passages (Figure. 7B)

2. Characteristics of the epithelial-like cells on DPESCs

To verify the origin of the epithelial-like cells on DPESCs, a microsatellite (STR) analysis was performed. These data showed that DDes were derived from hiPSCs, not DPESC (Figure 8). To compare the characterization of epithelial-like cells with DPESCs, expressions of cell surface markers were analyzed by FACS. Immunophenotypes between DDes and DPESCs showed similar patterns (Figure 9). Data showed that PHes and HERS/ERM cells were positive for CD29, CD73, CD10, and HLA-I, but negative for CD45 and HLA-DR. The expressions of epithelial stem cell- and stemness related-markers were determined to investigate the stem cell-like characteristics of epithelial-like cells. DPESCs are well known to express the specific markers of epithelial stem cell- and stemness related-markers. DDes expressed epithelial stem cell markers such as *E-cadherin ABCG2, EpCAM, Bmi1,* and *p75* and stemness-related markers such as *Oct-4, Nanog,* and *Sox-2* (Figure 10). These results showed no significant difference among DDes and DPESCs in gene expression levels of pluripotent markers.

III. Differentiation capacity of epithelial-like cells derived from hiPSCs on DPESCs *in vitro*

1. Odonto-/ameloblast-related gene expression level profiling by RT-PCR

To confirm the effect of odontogenic-/amelogenic differentiation between DDes and dDPSCs, odonto- and ameloblast markers were measured by RT-PCR. DDes and dDPSC were co-cultured for 8 days with or without induction medium (Figure 11). *Enamelin* and *KLK4*, which were enamel matrix protein and proteinase, were up-regulated in the co-culture group. *Runx2*, one of the early mineralization markers, was increased in the co-culture group than in the alone group. *BSP,* a unique marker for bone cell differentiation during osteogenesis, was expressed in both the co-culture group and the DDes alone group. The expressions of osteoblast-related matrix proteins, *ONN* and *OCN*, were increased in the co-culture group with the induction medium. The expression level of *DMP1* was increased in the co-culture group and the DDes alone group. *MEPE* and *Col1a1* expression also increased in the co-culture group compared to the control groups. Also, *DSPP* expression showed an increase in the co-culture group different from other control groups. Therefore, based on RT-PCR results, the co-culture groups had relatively higher gene expression levels than the alone groups.

2. Validation of protein level using western blot analysis

Western blot analysis was performed to compare the relative protein levels of DPP and enamelin. Both cells were treated with or without the induction medium for 8 days. As shown in Figure 12, the expression level of DPP was expressed more intensely in the induction group than in the control group in co-culture (Figure 12). Even in the dDPSCs alone group, the DPP was weakly expressed in the induction group. Enamelin was detected in the co-culture induction group and the DDes alone group. Although enamelin was expressed in both groups, it was expressed more

intensely when epithelial-like cells were co-cultured with dDPSCs.

3. The degree of mineralization during odontogenic differentiation tested by Alizarin red S staining

To monitor the mineralization associated with odontogenic differentiation of DDes and DPESCs with dDPSCs, respectively. Alizarin red S staining was performed to monitor the mineralization associated with odontogenic differentiation of DDes with dDPSCs. In addition, to compare the mineralization effect of DDes, DPESCs were co-cultured with dDPSCs for 32, 36, and 40 days. DDes with dDPSCs were observed after 8, 12, 16, 20, and 24 days with or without induction media (Figure 13A). Calcium deposits were not detected until day 12 in all groups but were clearly detected at least 20 days after with induction medium in the co-culture group. In the epithelial-like cell alone group, calcium deposits were not detected in the induction medium until 24 days. In contrast, calcium deposits increased significantly within 16 days after odontogenic induction and peaked at 24 days in the dDPSCs alone group.

As a result of the staining of DPESCs and dDPSCs, slight staining was observed after 32 days in the co-culture group, and clear mineralization was showed after 36 days (Figure 13B). However, in DPESCs alone group, only slight calcium deposition was found until 40 days.

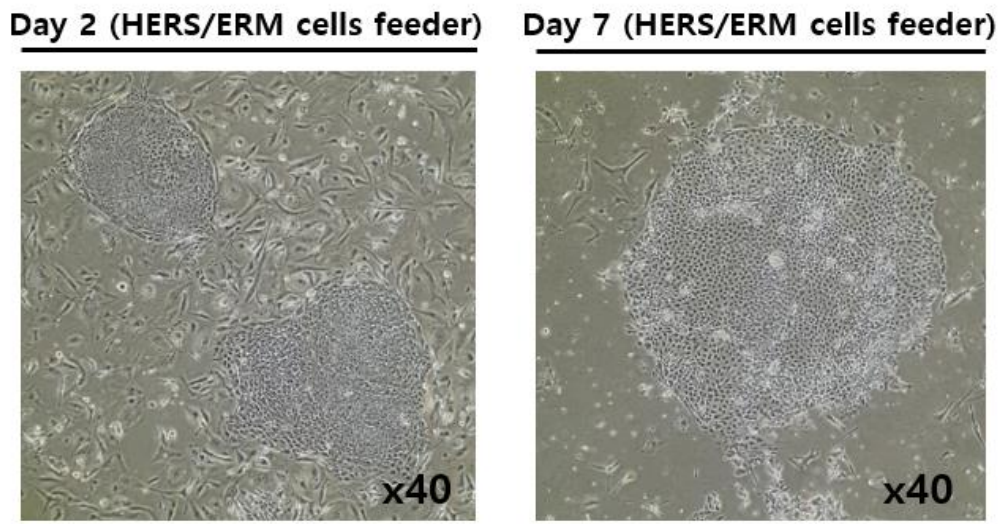

Figure 1. Morphological change during human induced pluripotent stem cells (hiPSCs) differentiation into dental epithelial-like stem cells on Hetwig's Epithelial Root Sheath/Epithelial Rests of Malassez (HERS/ERM) cells. Undifferentiated hiPSCs were differentiated on mitomycin-treated HERS/ERM cells feeder layer. After 2 days on feeder, hiPSCs colony attached to feeder cells. After 7 days differentiation, hiPSCs colony with epithelial cell-like morphology grown on feeder layer. hiPSCs were grew tightly together in a cuboidal or polygonal shape. Magnifications are at 40X.

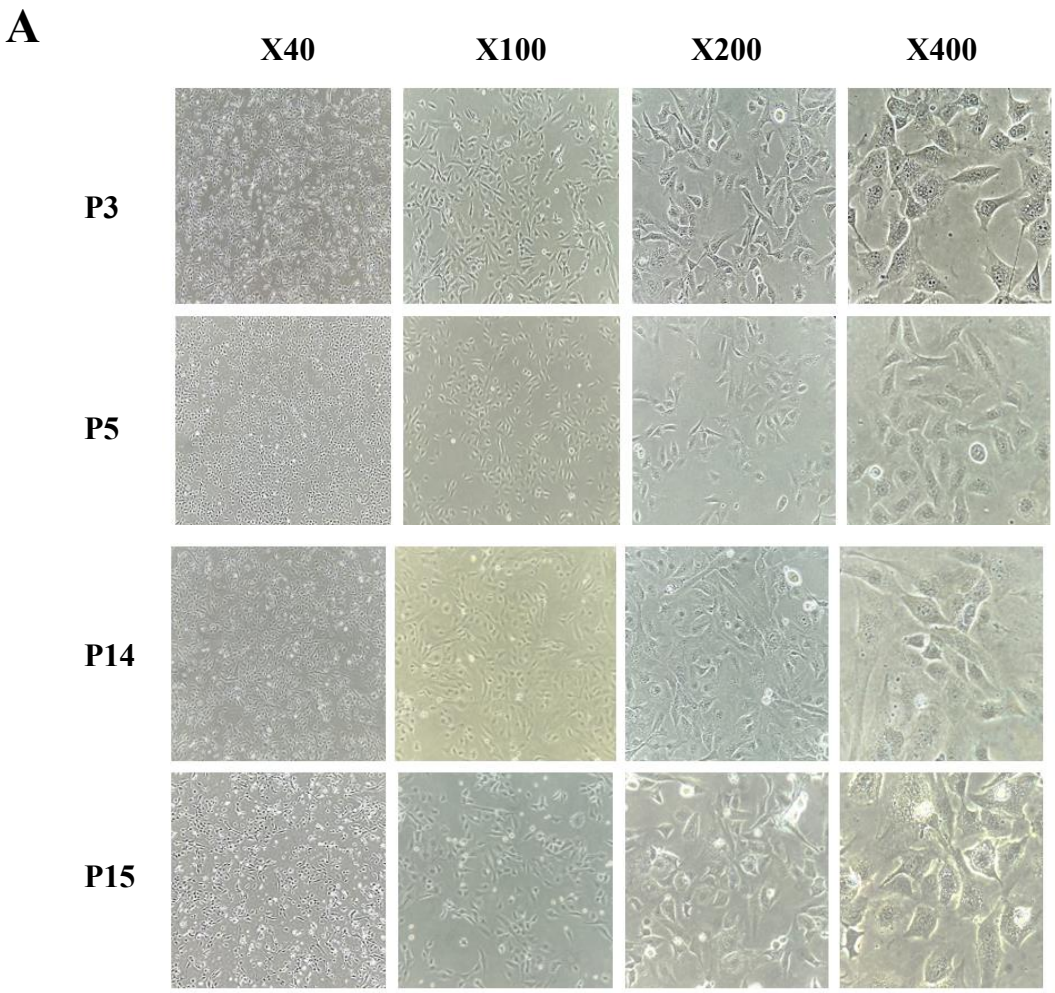

Figure 2. Cell morphology and growth curve of PBMC-iPSC derived epithelial-like cells on HERS/ERM cells (PHes). (A) Morphology of PHes. Differentiated hiPSCs on HERS/ERM cells were transfected with SV40 and established three cell lines. PHes showed typical epithelial cell-like morphology. The morphology was maintained up to 15 passages through subculture. Magnifications are at 40X, 100X, 200X and 400X.

B

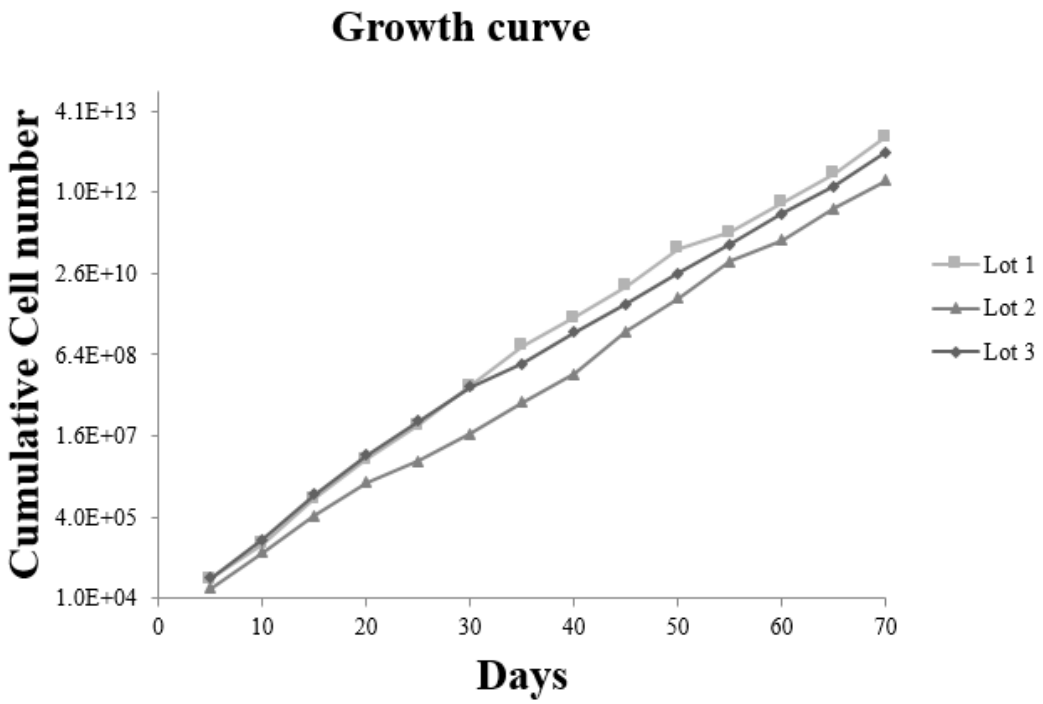

Figure 2. (B) Growth curves of PHes. This growth curve shown by using three cell lines of PHes. Cells were sub-cultured every 5 days. At each passage, cells were counted and plated at the same density. Cells of PHes have been shown to maintain lifespan for about 70 days.

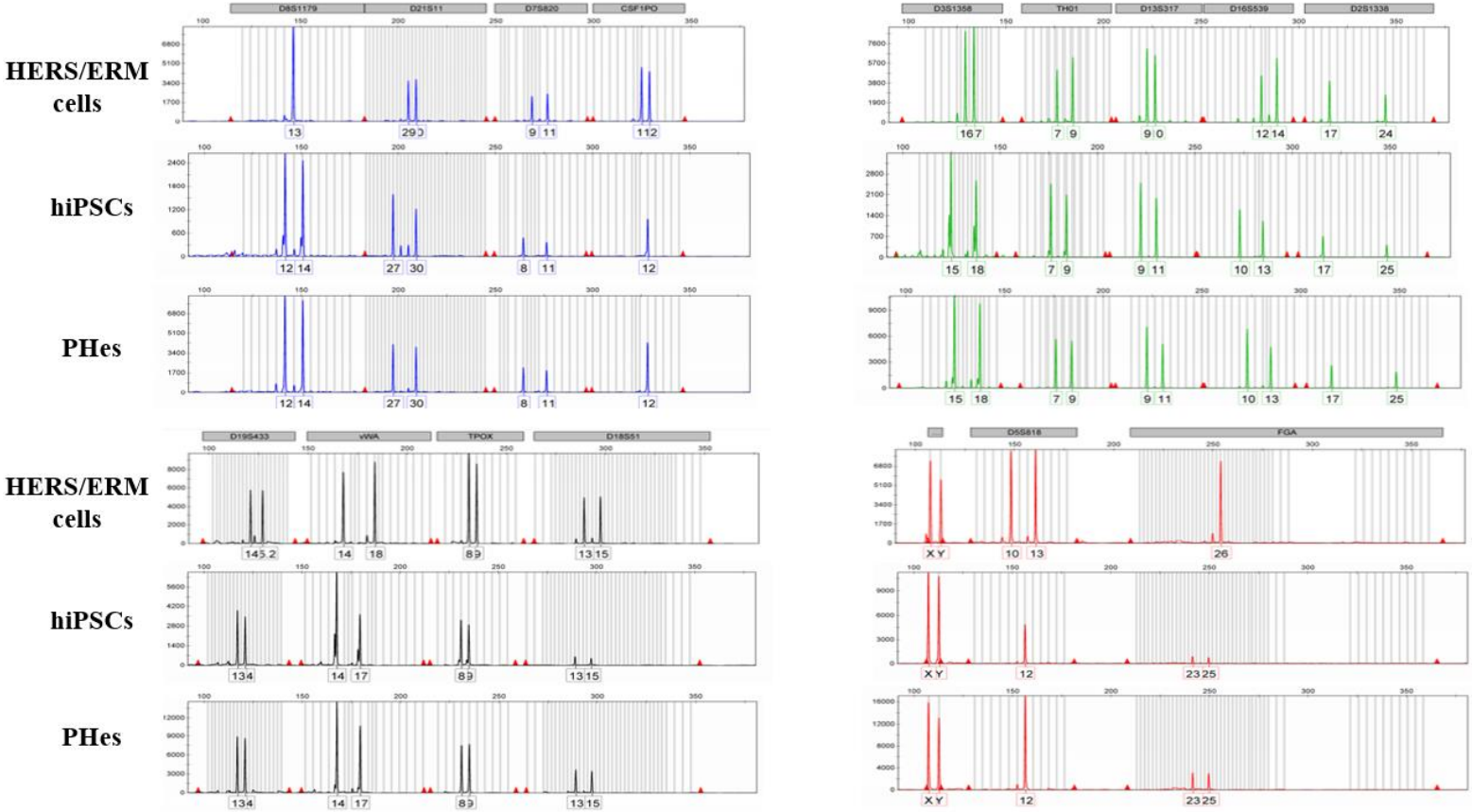

Figure 3. Origin of established PHes by STR analysis. Genomic DNA was extracted from the HERS/ERM cells, hiPSCs and PHes. The data showed that PHes was derived from hiPSCs, not from HERS/ERM cells.

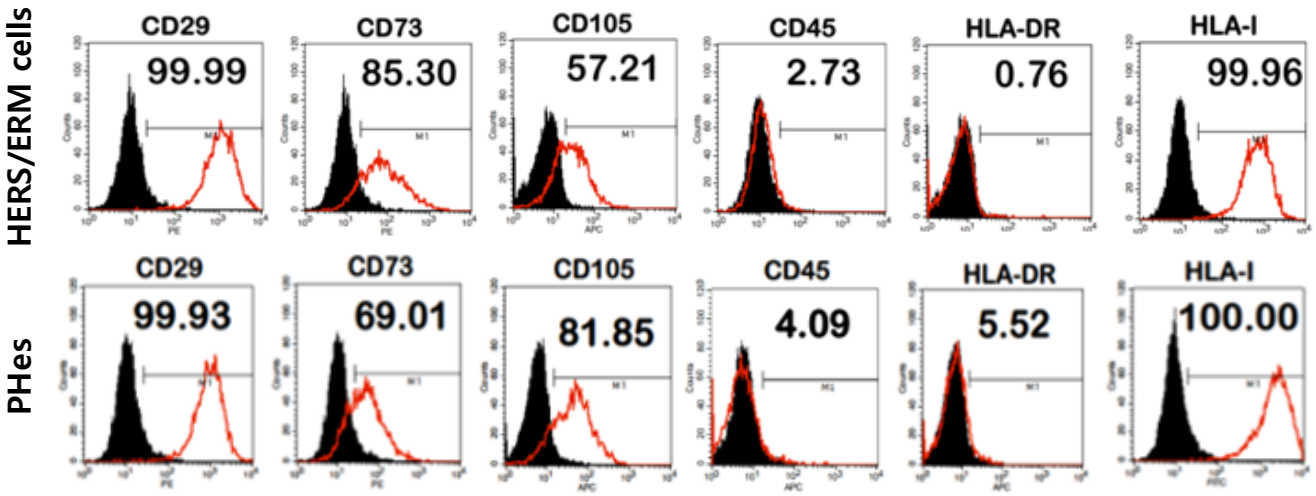

Figure 4. Characterization of HERS/ERM cells and PHes by FACS analysis at passage 5. Both HERS/ERM cells and PHes showed similar surface protein expression patterns.

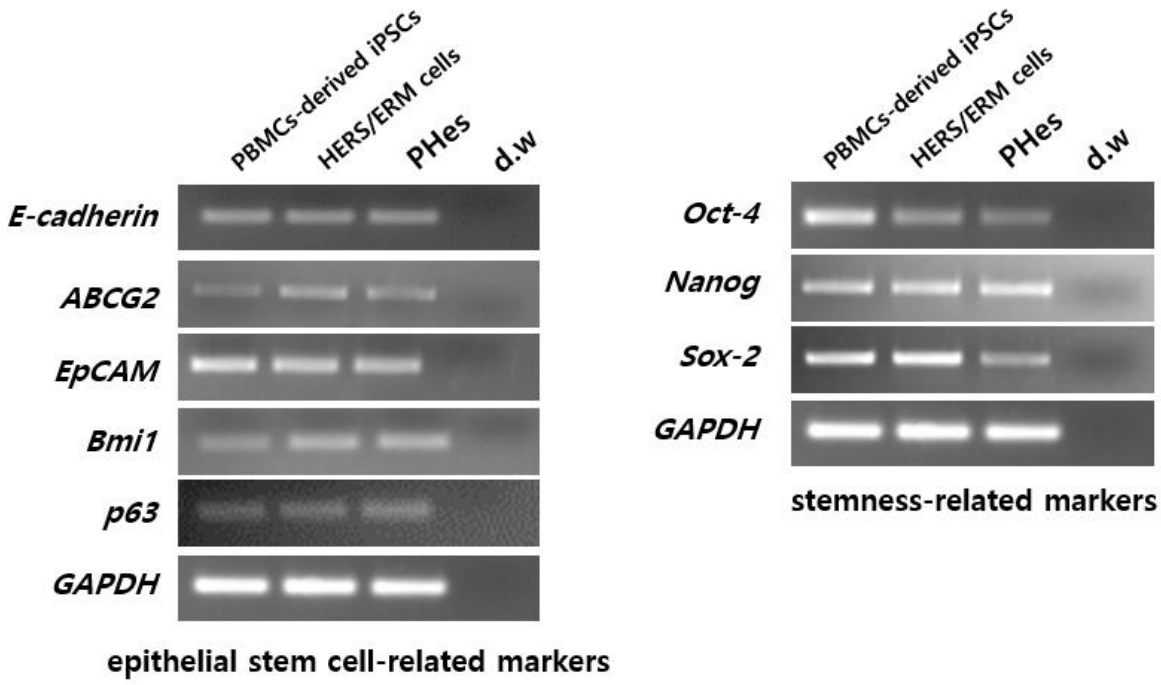

Figure 5. Gene expression of PBMC-derived iPSCs, HERS/ERM cells and PHes. The expression pattern of epithelial stem cell- and stemness-related markers was verified by quantitative PCR at passage 5. HERS/ERM cells and PHes showed slightly thicker in *E-cadherin ABCG2, EpCAM, Bmi1,* and *p75*, which are epithelial stem cell-related markers than PBMC-derived iPSCs. In *Oct-4, Nanog,* and *Sox-2*, which are stemness-related markers, PBMC-derived iPSCs appeared thicker than HERS/ERM cells and PHes.

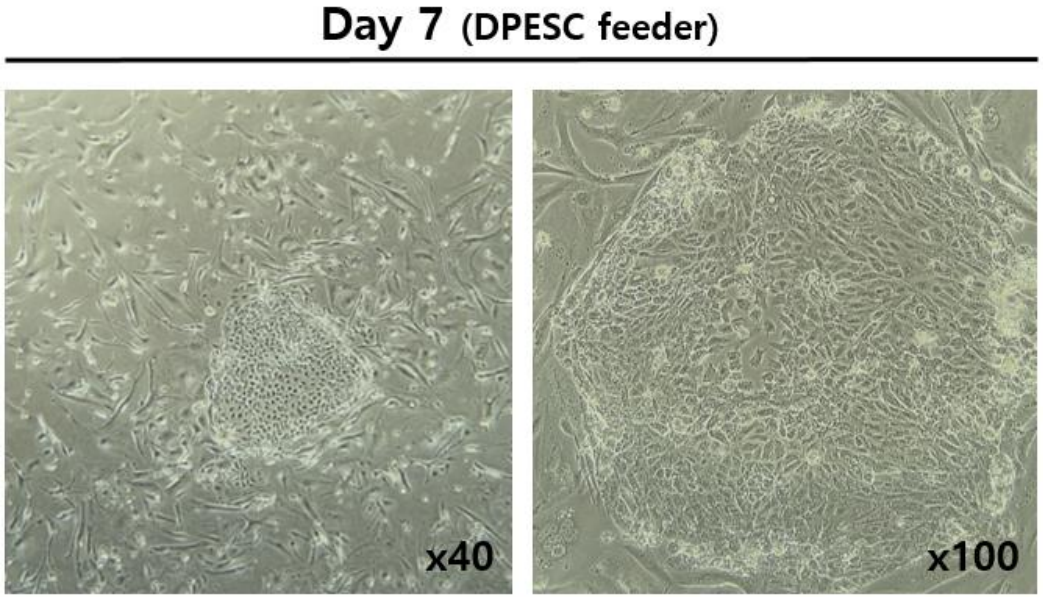

Figure 6. Morphological change during hiPSCs differentiation into epithelial-like cells from human deciduous dental pulp (DPESCs). Undifferentiated hiPSCs were differentiated on mitomycin-treated DPESCs feeder layer. After 7 days differentiation, hiPSC colony with epithelial cell-like morphology grown on feeder layer. hiPSCs were grew tightly together in a cuboidal or polygonal shape. Magnifications are at 40X, 100X.

A

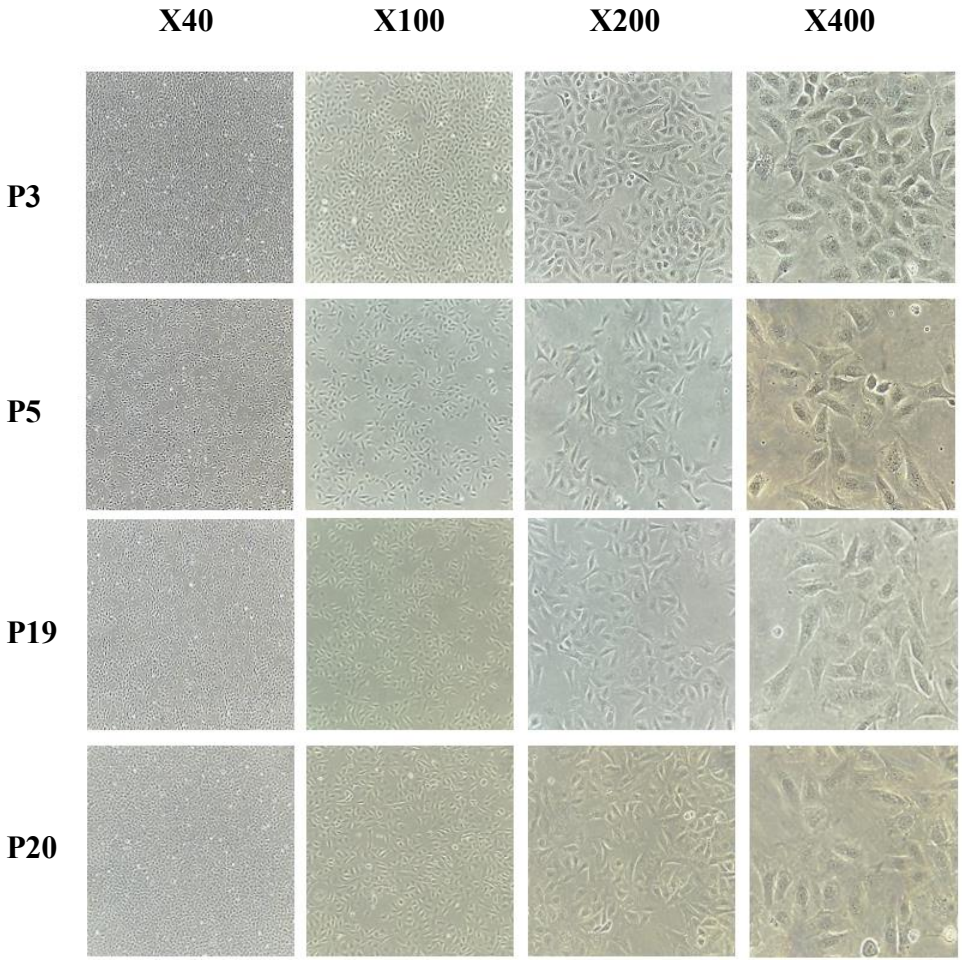

Figure 7. Cell morphology and growth curve of dDPSC-iPSC derived epithelial-like cells on DPESCs (DDes). (A) Morphology of DDes. Differentiated hiPSCs on DPESCs were transfected with SV40 and established three cell lines. DDes showed typical epithelial cell-like morphology. The morphology was maintained up to 20 passages through sub-culture. Magnifications are at 40X, 100X, 200X and 400X.

B

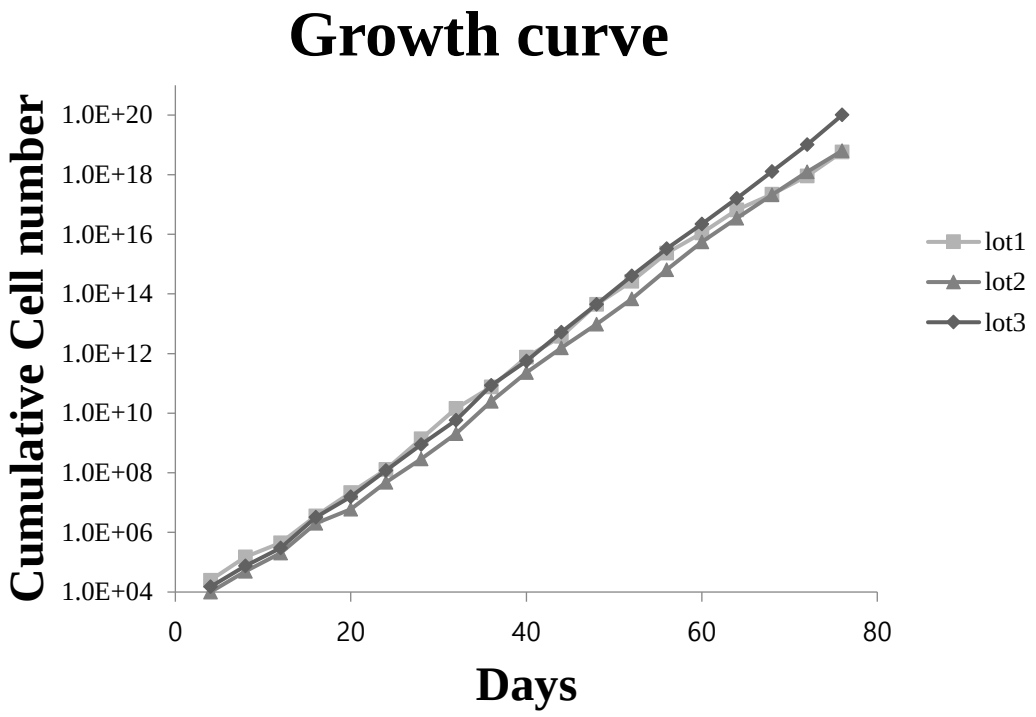

Figure 7. (B) Growth curves of DDes. This growth curve shown by using three cell lines of DDes. Cells were sub-cultured every 4 days. At each passage, cells were counted and plated at the same density. Cells of DDe have been shown to maintain lifespan for about 80 days.

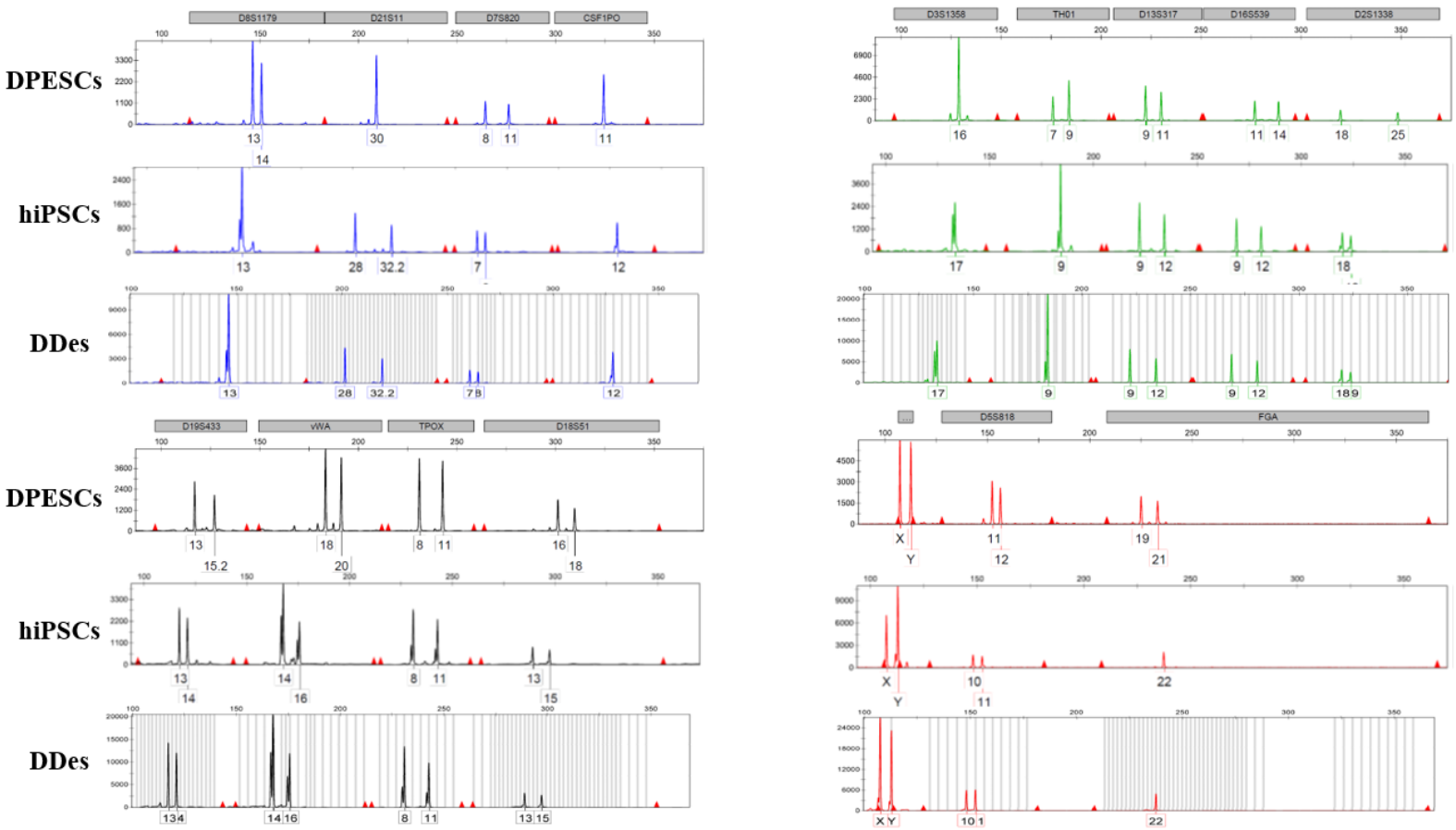

Figure 8. Origin of DDes by STR analysis. Genomic DNA was extracted from the DPESCs, hiPSCs and DDes. The data showed that DDes was derived from hiPSCs, not from DPESCs.

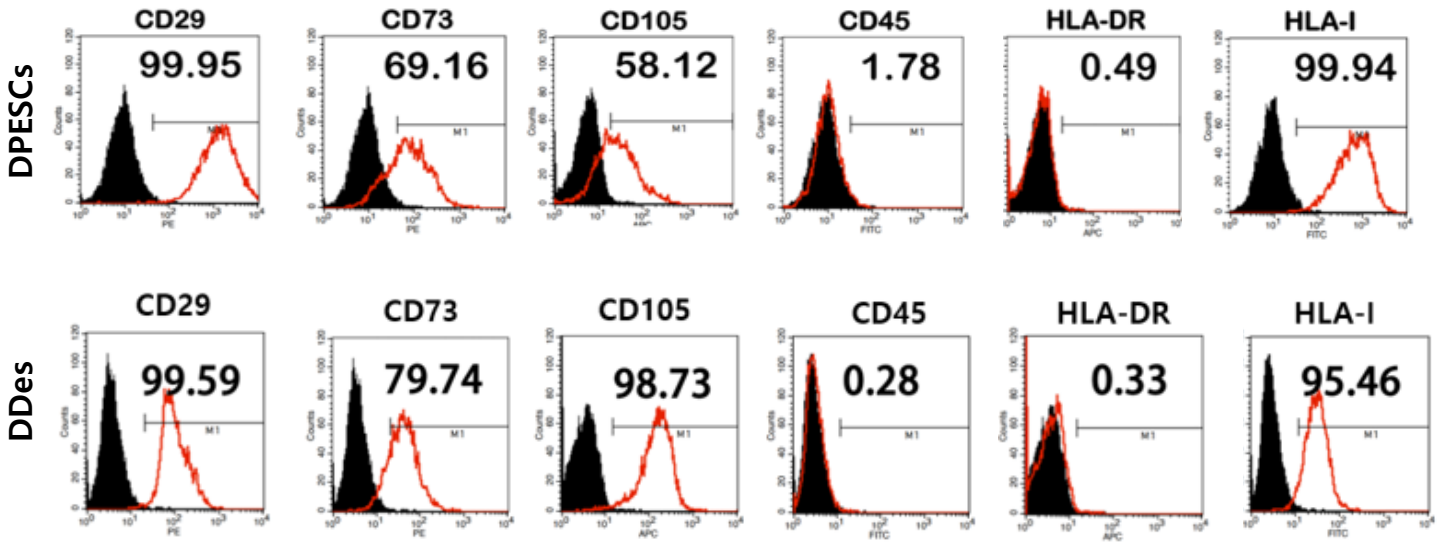

Figure 9. Characterization of DPESCs and DDes by FACS analysis at passage 5. Both DPESCs and DDes showed similar surface protein expression patterns.

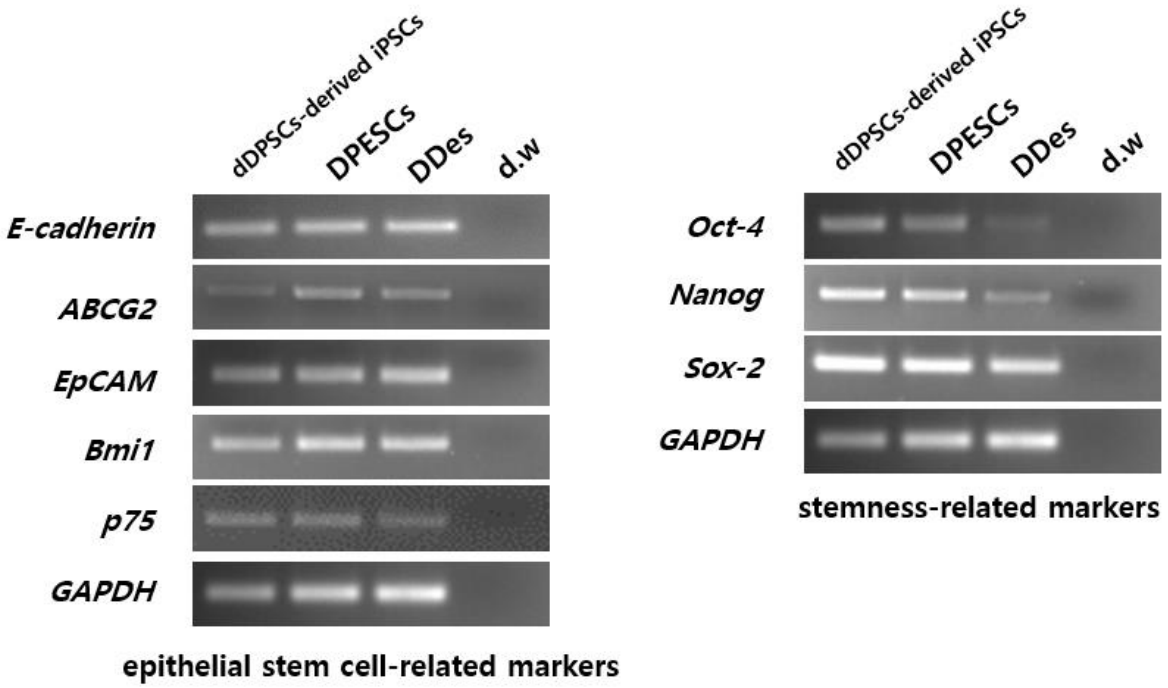

Figure 10. Gene expression of DPESCs and DDes. The expression pattern of epithelial stem cell- and stemness-related markers was verified by quantitative PCR at passage 5. Epithelial stem cell related-markers such as *E-cadherin ABCG2, EpCAM, Bmi1,* and *p75* were deeper in DPESCs and DDes than in dDPSCs-derived iPSCs. Stemness-related markers such as *Oct-4, Nanog,* and *Sox-2* were thicker in PBMC-derived iPSCs than DPESCs and DDes.

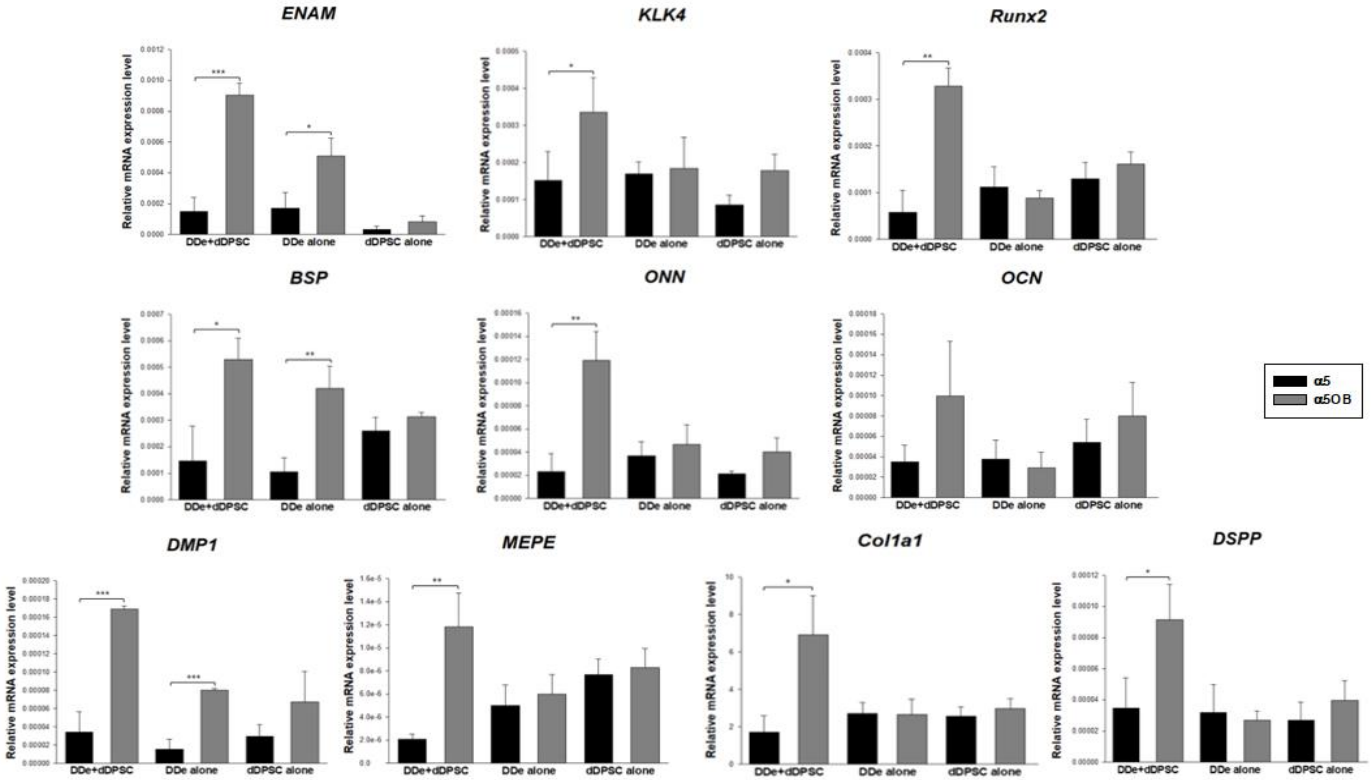

Figure 11. Gene expression of DDes on odontogenic differentiation with dDPSCs. DDes and dDPSCs were co-cultured with or without a differentiation medium for 8 days. The relative mRNA expression levels of *ENAM, KLK4, Runx2, BSP, ONN, OCN, DMP1, MEPE, Col1a1* and *DSPP* were measured by quantitative RT-PCR analysis. 8 days after odontogenic induction, the high level expression was observed in induction group than without induction. The quantification analyses were performed and data are mean ± SD of three separate experiments. The data are presented as the mean ± SD, n = 3 per group. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

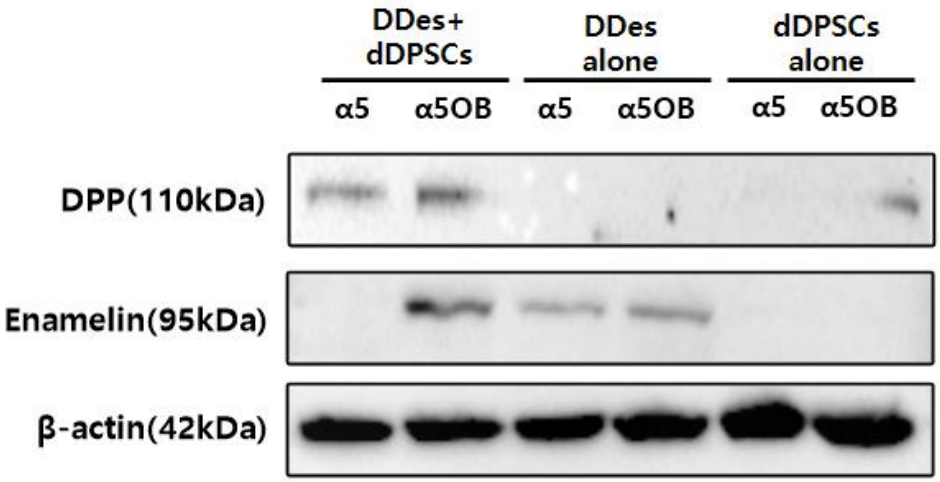

Figure 12. Protein expression evaluated by western blotting analysis. The expression levels of DSPP and Enamelin proteins were analyzed after 8 days with or without induction. 8 days for culture with or without induction medium, DPP was more highly expressed in co-culture induction group than alone groups. Likewise, Enamelin protein was far different in co-culture group and control group. The thickness of Enamelin bands were stronger in co-culture group than alone group.

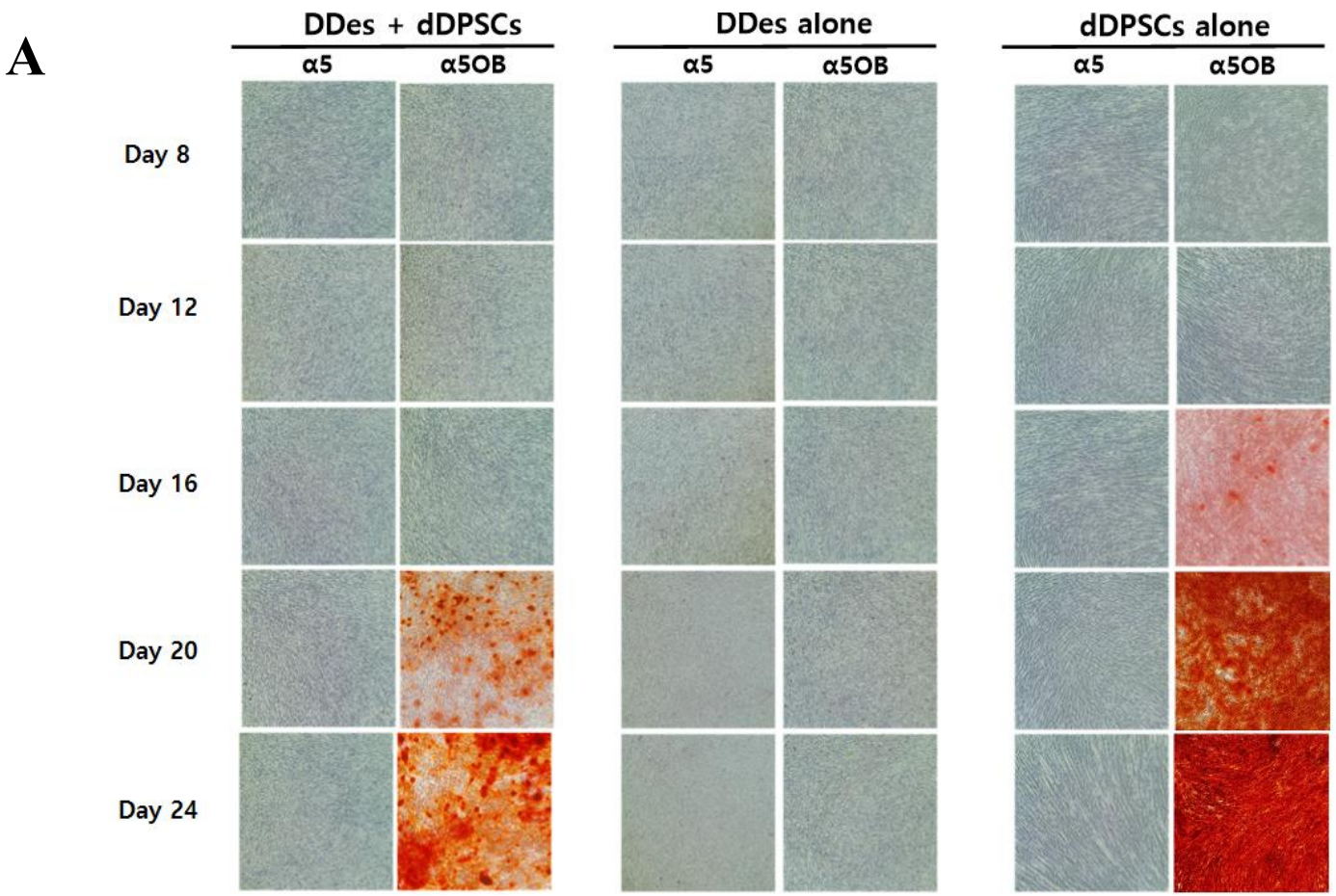

Figure 13. *In vitro* mineralized nodule formation after co-culture of DDes and DPESCs with dDPSCs. (A) DDes were cultured with dDPSCs in osteogenic induction medium for 8,12,16,20, and 24 days. In the co-culture group, calcium nodules began to appear on day 20. On the other hand, no staining was found in DDe alone group. All experiments were conducted in three replicates. Photographed at 200X magnification.

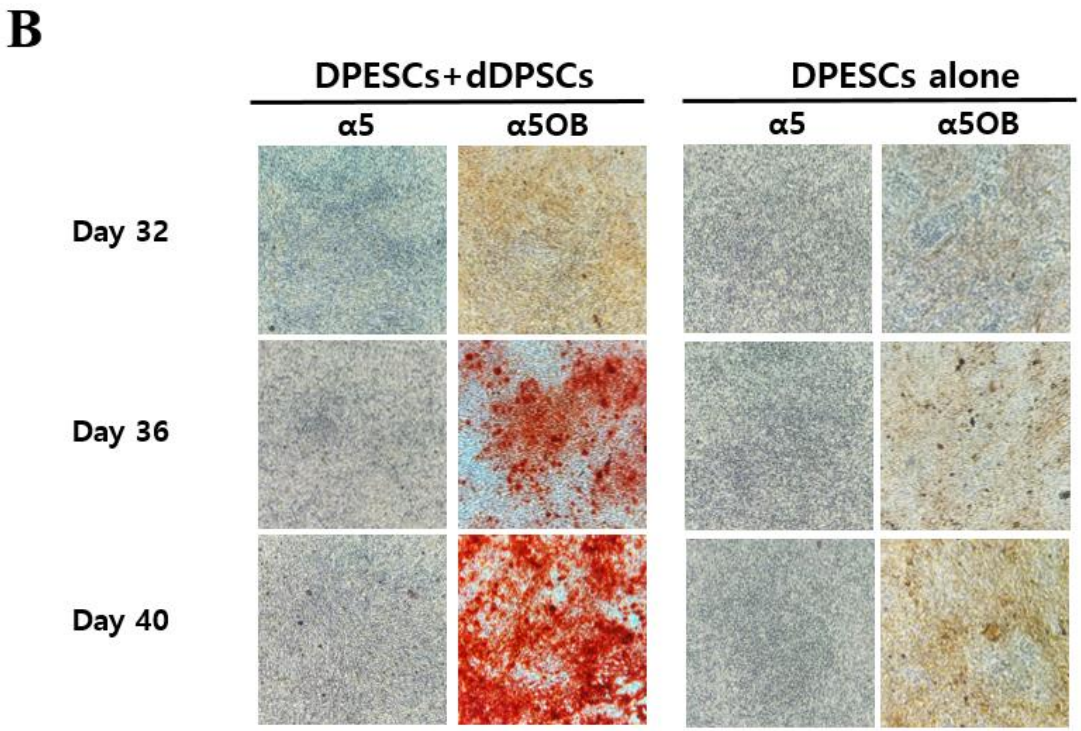

Figure 13. (B) Co-culture of DPESCs and dDPSCs was maintained under induction medium for 32, 36, and 40 days. In co-culture group, there was nodule formation after 32 days. However, slightly calcium deposits were observed for 40 days in DPESCs alone group. All experiments were conducted in three replicates. Photographed at 200X magnification.

Discussion

This study was conducted to establish epithelial-like cell lines using various origins of iPSCs and feeders to improve the methods of previous studies. DPSCs derived from human deciduous teeth are a well-known cell source for iPSCs [71], but they cannot be obtained from adults. An autologous cell source with a patient/disease-specific system for inducing iPSCs was blood-derived PBMCs [77]. In this study, human blood-derived PBMCs from adults were an alternative source for iPSCs, and epithelial differentiation of PBMC-derived iPSCs on HERS/ERM cells as a feeder was examined (PHes). In addition, DPESCs, epithelial cells obtained from deciduous teeth, were used to evaluate the alternative feeder cells. Then, epithelial differentiation of dDPSCs-derived iPSCs on DPESCs as a feeder was also examined (DDes). PHes and DDes were successfully established, thus overcoming the limitations of dental epithelial cells, which are short lifespans and difficult to maintain after isolation.

iPSCs can induce differentiation using growth factors or conditioned medium [82-85]. In addition, physical contact using feeder layers may enable differentiation and growth of iPSCs [86, 87]. In a previous study, the treatment of human feeder layers and conditioned medium were compared to induce differentiation of human ESC (hESC), and the effect was better in the feeder system [88]. Feeder cells help by secreting the growth factors into the culture medium for the proliferation of the target cells [86, 87, 89]. The feeder layers produce extracellular matrix proteins, and direct contact with the target cells is essential to act as a substrate for cell adhesion [86]. Feeder cells are treated with mitomycin-C to prevent overgrowth but produce growth factors or cytokines in the meantime [90, 91]. When cultured with

mitomycin-C-treated epithelial cells and mouse iPSCs, it was confirmed that they exhibited morphology similar to that of epithelial cells through paracrine signals of BMP2 and BMP4 secreted from dental epithelial cells [60]. Between the feeder cells and the target cells, paracrine signaling using receptors or cell-to-cell interaction through juxtacrine signaling occurs, resulting in growth factor and cytokine signaling [92-94]. These results indicate that the feeder layer system is a suitable means to induce differentiation into epithelial-like cells.

Epithelial-like cells were differentiated from PBMC-derived iPSCs using HERS/ERM cells as feeder layers (Figure 1). In addition, in order to use various kinds of feeder cells, dDPSC-derived iPSCs were differentiated using the DPESCs feeder layer (Figure 6), which is another epithelial stem cell source obtained from human teeth. It was demonstrated that the differentiated epithelial-like cells were immortalized and maintained morphology (Figure 2A, 7A) and lifespan during long-term culture and that the cell lines were stably established (Figure 2B, 7B). Also, there was no significant difference in the profiles of surface proteins between HERS/ERM cells and PHes and between DPESCs and DDes (Figure 4, 9). These data suggested that there was no difference in the immunophenotypic between them. Gene expression profiles showed that PHes and DDes expressed epithelial stem cell and stemness-related markers: *E-cadherin, ABCG2, EpCAM, Bmi1, p75, Oct-4, Nanog,* and *Sox-2* (Figure 5, 10). *Bmi1* is an epithelial stem cell marker that regulates many biological processes [95]. It promotes cell proliferation and affects tumor suppressors Rb and p53 [96]. *EpCAM* is a transmembrane glycoprotein expressed in the epithelium of individuals [97]. It can rearrange the cytoskeleton to increase cell motility, proliferation, and metastasis [98]. *p75* has a vital role in protecting stem cells from apoptosis and affecting cell growth [99, 100]. This progenitor stem cell

marker is observed in oral epithelial cells along with other similar markers such as *ABCG2* [101-103]. The function of these epithelial stem cell markers can be essential for the proliferation and differentiation of dental epithelial stem cells. Stemness-related markers, *Oct-4, Nanog,* and *Sox-2,* play an important role in pluripotency maintenance and self-renewal in iPSCs and ESCs [104-106]. Therefore, this supports PHes and DDes showed epithelial cell characteristics and can be cell sources with multipotency.

The co-culture of DDes and dDPSCs identified potential synergistic effects on odontogenic differentiation. As a result of analyzing the mRNA expression levels of *Enamelin* and *KLK4*, ameloblast-related markers, the co-culture group with the induction medium was higher expression than the control group with the control medium (Figure 11). As in the case of odontoblast- and mineralization-related markers such as *Runx2, BSP, ONN, OCN, DMP1, MEPE, Col1a1,* and *DSPP* were also highly expressed in the co-culture group with induction medium. In addition, the protein expression level of enamelin was more clearly observed in the co-culture group (Figure 12). Enamelin is one of the three major secretory enamel proteins and an enamel matrix protein essential for enamel formation [107-109]. It is specifically expressed by ameloblasts and is required for mineralization [108]. Dentin sialophosphoprotein (DSPP) is a marker responsible for early odontoblast differentiation in dDPSCs and is involved in tooth formation and mineralization [38, 110, 111]. In these data, DSPP protein was also expressed in dDPSCs but was more potent in the co-culture induction group. Therefore, it could be confirmed that the co-culture of DDes and dDPSCs accelerated odontogenic differentiation. Interestingly, mineralization in co-culture with DDes and dDPSCs showed slightly different patterns (Figure 13A). Mineralization was observed slightly faster and more

intensely in the dDPSCs alone group than in the co-culture group. To support these results, when DPESCs and dDPSCs were co-cultured, the rate of odontogenic differentiation of DPESCs was prolonged (Figure 13B). DPESCs alone group did not show clear mineralization until 40 days, and the DPESCs with dDPSCs co-culture group appeared after 32 days. Therefore, it suggests that it can be used to accelerate the differentiation of dentin-forming odontoblasts when DDes and dDPSCs are co-cultured rather than DPESCs.

Both *in vitro* and *in vivo* studies of tooth formation have been demonstrated to be driven by the interaction between epithelial and mesenchymal stem cells [112]. Through sequentially transplanting epithelial stem cells and mesenchymal stem cells, tooth structures such as enamel, dentin, and dental pulp were formed during long-term in vivo culture [17, 113-116]. Mesenchymal-derived odontoblasts responsible for dentin formation and epithelial-derived ameloblasts that form enamel are essential cells in dental hard tissue formation [116-118]. Therefore, in vivo experiments will be needed to examine the potential and efficiency of established epithelial-like cells to produce dental hard tissue [119]. If the hard tissue is successfully produced, it may be demonstrated that established epithelial-like cells can be efficiently used as cell sources that can be used for tooth regeneration.

Dental epithelial-like stem cells can be established by differentiating various origins of iPSCs using epithelial cells obtained from permanent teeth and deciduous teeth as feeder layers. The results of this study are expected to solve the problem of dental epithelial cell sources.

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국문초록

인간 유도만능줄기세포로부터 치아 상피 유사 세포주의 확립과 특성 분석

기 민 지
서울대학교 대학원 치의과학과 분자유전학 전공
(지도교수 이 진)

치아의 발달 및 재생 과정에서 치아 상피세포와 외배엽성 중간엽세포의 상호작용은 중요한 요소이기 때문에, 치아재생연구에서 치계상피세포와 중간엽세포의 확보는 필수적이다. 하지만, 다양한 세포원으로부터 비교적 확보가 쉬운 중간엽 줄기세포와는 달리, 사람 치아 상피줄기세포는 치아가 맹출되면 소실되는 특성 때문에 확보 및 배양이 어렵다. 현재까지 보고된 치아 상피줄기세포는 영구치 치주 조직으로부터 분리된 Hertwig’s epithelial root sheath/epithelial rests of Malassez (HERS/ERM cells) 과 유치 치수로부터 분리된 Dental pulp epithelial stem cells (DPESCs)가 있으나 두 종류의 세포 모두 수명이 짧기 때문에 안정적인 유지에 어려움이 있다. 최근 선행 연구를 통해 HERS/ERM cell을 지지세포층으로 사용하여 사람 유치 치수줄기세포(dDPSC) 유래 유도만능줄기세포를 분화시킨 치아 상피 유사 세포주가 보고되었다. 본 연구에서는 이 선행 연구의 방법을 활용하여 다양한 기원의 치아 상피줄기세포를 확보하고 특성을 검증하고자 하였다. 이를 위해 확보가 비교적

용이하고 유도만능줄기세포 확립 연구가 많이 이루어진 혈액 유래 PBMCs에서 확립한 유도만능줄기세포를 치아상피세포로 분화시키기 위해 HERS/ERM cells 지지세포 위에 배양하였다. 또한 지지세포를 다양화하기 위해 dDPSC 유래 유도만능줄기세포를 DPESCs 지지세포 위에 배양하여 치아상피세포로 분화 유도하였다. 서로 다른 기원의 유도만능줄기세포와 지지세포를 사용하여 확립된 두 상피줄기세포주의 특성을 확인하였으며, 새로운 지지세포위에서 분화된 상피세포주는 분화능을 확인하기 위해 dDPSCs와의 공배양을 통하여 치아모세포로 분화 유도하였다. PBMCs 및 dDPSCs 유래 유도만능줄기세포는 14일 동안 각각 HERS/ERM cells 및 DPESCs 지지세포층 위에서 배양하여 분화 유도하였고, 분화된 세포는 SV40 Large T antigen을 도입하여 불멸화하였다. 확립된 PBMC-iPSC-derived epithelial-like cells on HERS/ERM (PHes)와 dDPSC-iPSC-derived epithelial-like cells on DPESC (DDes)는 장기배양 후에도 세포 형태의 변화가 없이 일정하게 증식이 유지되었다. 또한, STR 분석으로 확립된 세포주의 기원이 각각의 iPSC임을 검증하였으며, flow-cytometry를 통한 세포표면항원분석, PCR을 이용한 상피줄기세포 및 배아줄기세포 유전자 발현 분석으로 상피세포의 특성을 지님을 확인하였다. DDes는 dDPSCs와 공배양하며 8일간 경조직 분화를 유도하였을 때 법랑모세포 관련 유전자인 *Enamelin, KLK4*와 상아모세포 관련 유전자인 *Runx2, BSP, ONN, OCN, DMP1, MEPE, Col1a1*, *DSPP* 발현이 대조군에 비해 모두 증가하였으며 8일간 분화 후 DPP 및 enamelin 단백질 발현도 대조군에 비해 증가함을 확인하였다. 또한 분화 유도 20일 차부터 칼슘이 침착됨을 확인하였다. 이 결과를

통해, PBMC 유래 유도만능줄기세포와 dDPSC 유래 유도만능줄기세포를 각각 HERS/ERM cell과 DPESCs를 지지세포층 위에서 성공적으로 치아 상피줄기세포로 분화하였으며 새로운 지지세포층을 사용한 치아 상피줄기세포주의 치계세포 분화능도 검증되었다. 따라서 더 다양한 기원의 유도만능줄기세포와 지지세포층을 활용하여 분화능을 지닌 치아 상피줄기세포의 확립이 가능할 것이며 이는 부족한 치아 상피줄기세포 확보 문제를 극복할 수 있을 것으로 기대된다.

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주요어 : 상피-중간엽 상호작용, 치아 상피줄기세포, 유도만능줄기세포, 유치 유래 중간엽줄기세포, 말초 혈액 단핵세포, 치주 조직 유래 상피세포, 유치 유래 상피세포, 치계조직 분화 유도

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