치의과학석사학위논문

Establishment and characterization of dental epithelial cell lines from human HERS/ERM and dental pulp

사람 HERS/ERM 및 유치 치수에서 치계상피세포주 확립 및 특성 분석

2017년 2월

서울대학교 대학원
치의과학과 분자유전학전공

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이 논문을 치의과학 석사학위논문으로 제출함

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논문 제목	Establishment and characterization of dental epithelial cell lines from human HERS/ERM and dental pulp
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Abstract

Establishment and characterization of dental epithelial cell lines from human HERS/ERM and dental pulp

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Stem cells can differentiate into various cell types and develop into any tissue. Adult stem cells are present in very small populations in each tissue of the body and exist as undifferentiated cells. When tissue damage occurs, stem cells rapidly differentiate and regenerate the tissue. Despite their limited differentiation ability, adult stem cells have the advantages of being easier to obtain and having fewer associated ethical issues than embryonic stem cells. Recently, research on stem cells derived from oral tissues, including periodontal ligaments, has been reported. Although the interaction between ectodermal mesenchymal stem cells and epithelial stem cells is very important throughout the process of tooth development, there are difficulties in researching the interaction between the epithelium and mesenchyme for dental regeneration since epithelial stem cells are relatively difficult to obtain and maintain. Therefore, in this study, I established cell lines of Hertwig's epithelial root sheath cells/epithelial rests of Malassez cells (HERS/ERM cells) and dental pulp epithelial stem cells (DPESCs), and investigated the effect of conditioned medium

derived from these dental epithelial stem cell lines on the hard tissue forming ability of Stem cells from human exfoliated deciduous teeth (SHEDs). SHEDs and DPESCs were isolated and cultured from deciduous teeth, and HERS/ERM cells were isolated and cultured from permanent teeth. The obtained HERS/ERM cells and DPESCs were immortalized by introducing the SV40 large T antigen. In addition, a more stable cell line was established by introducing the human telomerase reverse transcriptase (hTERT) gene into the HERS/ERM cell line established with the SV40 large T antigen. To confirm whether the established cell line maintains its dental epithelial stem cell characteristics, FACS analysis of markers of embryonic stem cells and epithelial stem cells was performed to evaluate changes in cell morphology and gene expression when the epithelial-mesenchymal transition (EMT) was induced by TGF-β1. To confirm the interactions between dental epithelial stem cells and mesenchymal stem cells in hard tissue formation, the effects of conditioned medium obtained from primary dental epithelial cells and the cell lines on the hard tissue-forming ability of mesenchymal cells were examined. The immortalized dental epithelial cell lines had an extended life-span, and their morphology was maintained throughout subculture. RT-PCR data showed that the immortalized cell lines had typical epithelial stem cell-like gene expression patterns identical to those of primary dental epithelial cells. In addition, primary dental epithelial cells and the immortalized cell lines exhibited similar characteristics when the epithelial-mesenchymal transition was induced by TGF-β1. Alizarin red S staining indicated that calcium accumulation in SHED cells was equally promoted by conditioned medium derived from primary dental epithelial cells and the immortalized cell lines. Taken

together, I established dental epithelial stem cell lines and confirmed that both the primary dental epithelial stem cells and the cell lines showed the same morphological characteristics and gene expression profiles. Furthermore, it was also proven that conditioned medium from the established cell lines affects the acceleration of the hard tissue formation of primary cultured mesenchymal stem cells, SHEDs. These data suggested that HERS-SV40, HERS-SV40 / hTERT and DPESC-SV40, which were established from primary dental epithelial cells, could be expected to contribute to the study of dental stem cell functions and tooth regeneration

Keywords: Dental development, Epithelial-mesenchymal interaction, Dental epithelial stem cell, Dental mesenchymal stem cell, conditioned medium, Hard tissue remodeling

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TABLE OF CONTENTS

Introduction 5

Materials and Methods 7

Results 14

Figures and Tables 21

Discussion 43

References 49

Abstracts (Korean) 55

Introduction

Stem cells have been fascinating in dental research because of two features – self-renewal and pluripotency[40]. Stem cells reside in a specific microenvironment, known as a niche, which is composed of different types of cells, the extracellular matrix (ECM) and soluble factors[9]. Niches provide to stem cells proper signals in order to regulate their function and maintenance according to requirements of each specific tissue[22, 26, 42].

Unlike other tissues, such as skin and bone, which have the ability to remodel, human teeth exhibit a limited capacity for repair in response to damage[14]. Although human teeth have limited regenerative potential, tooth regeneration processes continue to throughout one's life[28]. Recently, several types of stem cells were reported in human teeth, such as dental pulp stem cells (DPSCs) derived from adult dental pulp tissues, periodontal ligament stem cells (PDLSCs) derived from adult periodontium, stem cells from apical papilla (SCAPs) derived from the root papilla of teeth, Hertwig's epithelial root sheath/epithelial rests of Malassez cells (HERS/ERM cells) residing in periodontium and dental pulp epithelial stem cells (DPESCs) derived from the pulp of deciduous teeth[14, 17, 33, 36, 43, 50.56].

A series of reciprocal interactions between the epithelium and neural crest-derived mesenchyme regulate the process of tooth development[45]. Through epithelial-mesenchymal interactions, mesenchymal dental pulp stem cells develop into the dental papilla, which further differentiates into odontoblasts, periodontal ligament cells and cementoblasts. The dental epithelium develops into ameloblasts[24, 29, 34, 39, 47, 53]. Although epithelial-mesenchymal

interactions are known to be important in tooth development, there is little research on the field because dental epithelial stem cells are difficult to isolate from tissues while maintaining their characteristics[2]. To overcome these problems, I immortalized primary HERS/ERM cells and DPESCs using the SV40 large T antigen (SV40) and human telomerase reverse transcriptase (hTERT)[4, 48].

The epithelial–mesenchymal transition (EMT) is a process during which epithelial cells lose their polarity and cell–cell adhesion while gaining mesenchymal characteristics[30]. The EMT is a crucial process for numerous embryonic events, such as neural crest formation, wound healing and other pathologic processes. In addition, it has been demonstrated that HERS/ERM cells have the ability to generate cementum through the EMT[25]. It is well known that transforming growth factor–β1 (TGF–β1) is the most common inducer of the EMT[20]. In this study, the EMT of primary epithelial cells and cell lines was induced by TGF–β1. Under this condition, primary epithelial cells and cell lines exhibited similar characteristics, such as decreased expression of epithelial markers and increased expression of mesenchymal markers[7, 37]. Since the EMT phenomenon is an important characteristic of HERS/ERM cells, I tried to show that the same phenomenon appears in the cell line to prove that it has the same characteristics as the primary cells.

Therefore, the purpose of this study was to overcome the limitation of epithelial stem cells derived from teeth and to investigate whether these epithelial cells affect the formation of hard tissue, which is a characteristic of mesenchymal cells based on epithelial–mesenchymal interactions.

Materials and Methods

Isolation of primary human HERS/ERM cells and DPESCs.

The experimental protocol was approved by the Institutional Review (S-D20070004). Informed consent was obtained from the patients. Normal impacted human third molars and deciduous teeth were delivered in Hank's balanced salt solution (HBSS; Welgene, Daegu, Korea) supplemented with 3% antibiotics-antimycotics (Gibco, Carlsbad, CA, USA) at 4℃. To isolate HERS/ERM cells, periodontal ligament tissues from the third molar were gently separated from the surface of the root and then incubated in 1 mg/ml of collagenase type I (Gibco) and 2.4 mg/ml of dispase (Gibco) at 37℃ for 1 h. To isolate Stem cells from human exfoliated deciduous teeth(SHEDs) and DPESCs, dental pulp tissues from human deciduous teeth were extracted with fine forceps and digested in a solution of 1 mg/ml of collagenase type I and 2.4 mg/ml of dispase at 37℃ for 1 h. To inactivate the enzymes, Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; HyClone, Road Logan Utah, USA) and 1% antibiotics-antimycotics (Gibco) was used. Single-cell suspensions were maintained in minimum essential medium alpha (α-MEM; , Road Logan Utah, USA) supplemented with 10% FBS and 3% antibiotics-antimycotics. The medium was changed every 2 days. When colonies were observed, the mesenchymal cells were isolated by trypsinization with 0.05% trypsin-EDTA, and isolated SHEDs were maintained in α-MEM medium. The remaining cells in culture

plates were then cultured in serum-free keratinocyte growth medium-2 (KGM-2; Lonza, Rock Island, ME, USA) with provided supplement (Lonza). The medium was changed every 2 days. Cells were sub-cultured at 70% confluence.

Immortalization of human HERS/ERM cells and DPESCs by SV40

Primary HERS/ERM cells and DPESCs at passage 3 were used for transfection. The cells (1.5×10^5 cells/well) 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 cultured for 2 days in KGM-2 and then G418 (Cellgro Mediatech, Washington, DC, USA) was added to the medium at a concentration of 100 μg/ml. The supplemented G418 was changed every 2 days. 10 days after G418 selection, independent colonies were observed and maintained with G 418-free KGM-2 culture medium. After 2 weeks, cells were transferred to new culture dishes. At each passage, the cells were counted and photographed. The population doubling level (PDL) was then calculated.

Immortalization of human HERS-SV40 by hTERT

After establishing the HERS-SV40 cell line, the ViraPower lentiviral expression system (Invitrogen, Carlsbad, CA, USA) was used for lentivirus production. The hTERT-Lipofectamine complex

was inoculated to 100 mm cell culture dish containing 293FT cells (3 x 10^5 cells). Forty-eight hours after transfection, the virus containing supernatant was harvested. Transfection of HERS-SV40 was carried out with hTERT containing lentivirus. Successfully immortalized cells were selected with 10 μg/ml blasticidin (Invitrogen) for 7 days. The supplemented with blasticidin was changed every 2 days. When independent colonies appeared, the cells maintained with blasticidin-free KGM-2 culture medium. After 2 weeks, cells were transferred to new culture dishes. At each passage, the cells were counted and photographed. The population doubling level (PDL) was then calculated.

Immunocytochemistry of SV40 immortalized cell line

Cells were sub-cultured at passage 3, and then fixed with 4% paraformaldehyde 20℃ for 10 min. Cells were washed with PBS, and then permeabilized with 0.5% Triton X-100 (Bio-rad, Hercules, CA, USA). Nonspecific binding event were minimized by 10% normal goat serum for 1 h at room temperature (RT). Immunostaining was performed with antibody against SV40 LT rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:50. Primary antibody was applied for 1 h at RT. Alexa Fluor 488 Goat anti-rabbit IgG was used as a secondary antibody (Invitrogen) at a dilution of 1:700. Nuclear counterstaining was performed with DAPI (Sigma-Aldrich, St Louis, MO, USA) at a dilution of 1:1000. The samples were mounted with fluorescent mounting medium (Dako, Carpinteria, CA, USA). The slides were observed using using an inverted fluorescence microscope (TE2000-U, Nikon Eclipse, Tokyo, Japan).

Epithelial-mesenchymal transition (EMT) induced by TGF-β1 (Transfroming growth factor-β1)

To induce the EMT, HERS/ERM cells, DPESCs, HERS-SV40, HERS-SV40/hTERT and DPESC-SV40 were treated with 20 ng/ml TGF-β1 (Peprotech, Rocky Hill, NJ, USA) for 48 h, after which the cells were photographed.

Preapartion of Conditioned medium (CM)

To prepare conditioned medium (CM) derived from primary HERS/ERM cells, DPESCs, HERS-SV40, HERS-SV40/hTERT, and DPESC-SV40 cells were incubated for 24 h in KGM-2. After washing with DPBS, the cells were cultured in new KGM-2 for 48 h. The supernatants were harvested, centrifuged at 2000 x g for 10 min at 4℃, and then filtered with 0.22 μm filter (Millipore, Billerica, MA, USA). CM was stored at 4℃.

Fluorescence-activated cell sorting (FACS) analysis

For the characterization of primary SHEDs, HERS/ERM cells, DPESCs, HERS-SV40, HERS-SV40/hTERT and DPESC-SV40, fluorescence-activated cell sorting (FACS) was performed to examine the expression status of several stem cell surface markers. The cells were detached and washed with DPBS supplemented with

2% FBS. The cells were fixed with 4% paraformaldehyde at RT for 10 min. After washing with DPBS, 300,000 cells were incubated with fluorescently conjugated antibodies for 20 min at 4℃. The antibodies are listed in Supplementary Table 1. The fluorescence intensity was measured by a FACSCalibur and data were analyzed with BD CellQuest Pro software (all from Becton Dickinson, Mountainview, CA, USA).

Total RNA preparation and reverse transcription

To isolate the total RNA, samples were collected in 100 μl RNALater (Ambion, Austin, TX, USA). Cells were washed with DPBS supplemented with 2% FBS. RNA isolation was performed with an RNeasy mini kit (Qiagen, Hilden, Germany). DNase I treatment was performed for removal of genomic DNA contamination using an RNase-free DNase set (Qiagen). Samples were stored at −80 until reverse transcription. Then, cDNA was prepared using amfiRivert cDNA synthesis Platinum Master Mix (GenDEPOT, Barker, TX, USA) with 2 μg of total RNA. The reverse transcription was carried out at 25℃ for 5 min and then 42℃ for 60 min, and lastly, heat inactivation of the enzyme at 70℃ for 15 min was performed. The RT products were diluted 10-fold and were stored at −20℃ until PCR amplification.

Quantitative PCR (qPCR)

The cDNA obtained from Primary SHEDs, HERS/ERM cells, DPESCs, HERS-SV40, HERS-SV40/hTERT and DPESC-SV40 was amplified in a reaction mixture (20 μl) containing 10 μl of THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan) and each primer at 0.5 mM. The conditions for the PCR and primer sequences are listed in Supplementary Table 2. qPCR was performed with CFX Connect Real-Time PCR Detection System (Bio-Rad). The copy numbers of the mRNAs were standardized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Semi-quantitative PCR (Semi -qPCR)

PCR amplification was conducted with i-MAXII (Intron, Seongnam, Korea) under followed the following conditions: pre-denaturation at 94℃ for 2 min, denaturation for 20℃ sec, annealing at a specific temperature for each primer pair, extension at 72℃ for 30 sec and total 28-40 cycles. A final extension step was performed at 72℃ for 5 min. RT-PCR primer sequences and each annealing temperature are shown in Supplementary Table 2. For the analysis of PCR products, DNA gel electrophoresis was carried out in 1.5% agarose gel with 0.01 mg/mL ethidium bromide and quantified by Bio-profil X press zoom 2000 (Vilber Lourmat, Marne la Vallée, France).

In vitro osteogenic differentiation

For osteogenic differentiation, primary SHEDs were cultured to confluence and then were cultured for 8, 12 or 18 days in each osteogenic differentiation induction medium. The composition of each medium is listed in Supplementary Table 3. The medium was changed every 3 days.

The mineralized matrix was stained with 2% Alizarin red S solution. First, the cells were washed with PBS and fixed in 4% paraformaldehyde at RT for 10 min. After washing in PBS, the cells were stained with 2% Alizarin red S solution for one second. The unstained cells were washed with distilled water. To quantify the calcium deposits in the stained cells, the cells were treated with 10% (W/V) cetylpyridinium chloride and measured for absorbance at 595 nm using Floustar Optima (BMG Labtech, Durham, NC, USA).

Statistical analysis

The data are presented as means ± SDs. Data analysis was preformed using Student's t-test a significance level of P value.

Results

Primary isolation of SHEDs, HERS/ERM cells and DPESCs cells

Human exfoliated deciduous pulp was digested with collagenase and dispase. Single-cell suspensions were cultured in α-MEM (5% FBS). When culturing the cells, two different cell types were observed. One type of cells had fibroblast-like morphology (SHEDs) and the other type of cells had epithelial cell-like morphology (DPESCs). When epithelial stem cell colonies were formed, the fibroblast-like cells (SHEDs) were trypsinized with 0.05% trypsin-EDTA. Isolated DPESCs were sub-cultured with 0.25% trypsin-EDTA. DPESCs were maintained in KGM-2. When SHEDs and DPESCs became 70% confluent, the cells were sub-cultured. Their morphological characteristics were examined with phase contrast microscopy (Figure 1A). SHEDs showed small cell bodies with long and thin shapes. On the other hand, DPESCs had cuboidal or polygonal shapes (Figure 1A). Likewise, periodontal ligament was digested with collagenase and dispase. HERS/ERM cells were isolated from a heterogeneous cell population. When epithelial stem cell colonies appeared, fibroblast-like cells (PDLSCs) were eliminated with 0.05% trypsin-EDTA. The remaining cells (HERS/ERM cells) were maintained in KGM-2 and sub-cultured at 70% confluence. HERS/ERM cells had typical epithelial stem cell-like morphology and colony forming proliferation (Figure 1A). To calculate the cell growth of SHEDs, the cells were plated at a

density of 5,000 cells/cm2. HERS/ERM cells and DPESCs were plated at a density of 15,000 cells/cm2. At each passage, the cells were counted and photographed. HERS/ERM cells and DPESCs grew well but exhibited different growth patterns (Figure 1B). SHEDs maintained their morphology through sub-culture, but HERS/ERM cells and DPESCs showed morphological changes; the cells were larger, and the cell-cell membrane borders disappeared at late passages.

FACS analysis of human SHEDs, HERS/ERM cells and DPESCs

To compare the primary SHEDs, HERS/ERM cells and DPESCs, expression of cell surface markers was analyzed by FACS (Figure 2). SHEDs were positive for mesenchymal markers (CD 29, 44, 73, 90, 105) and negative for hematopoietic and endothelial markers (CD 10, 31, 34, 45, 117, HLA-DR). Primary HERS/ERM cells and DPESCs had different expression pattern. Primary HERS/ERM cells and DPESCs had similar patterns of mesenchymal markers (CD 29, 44, 90) and endothelial markers (CD 10, 31, 34, 45, 117, HLA-DR). However, the expression of mesenchymal markers (CD 73, 105) was decreased.

Immortalization of primary HERS/ERM cells and DPESCs

Primary HERS/ERM cells and DPESCs at passage 4 were used for transfection. Twenty-four hours after passaging, 2 μg of pcDNA 3.1(+) SV40 were transfected into the cells using X-tremeGENE 9 DNA Transfection Reagent (Roche, USA) (Figure 3A, B). After 48 h of transfection, the cells were cultured in fresh

KGM-2 supplemented with 100 μg/ml G418 to select plasmid-containing cells. The medium supplemented with G418 was changed every other day. When independent colonies appeared after 10 days, the medium was changed to G418-free KGM-2. When the colonies grew slightly more, the cells were passaged. To confirm that pcDNA 3.1(+) SV40 had entered the primary HERS/ERM cells and DPESCs, the cells were immunostained for SV40 LT antigen. Both HERS-SV40 and DPESC-SV40 expressed SV40 (Figure 3C). To compare the primary cells (primary HERS/ERM cells, DPESCs) and cell lines (HERS-SV40, DPESC-SV40), the cells were photographed and counted at each passage. After establishing the HERS-SV40 cell line, the transfection of HERS-SV40 was carried out with 2 μg of hTERT-containing lentivirus. Successfully immortalized cells were selected with blasticidin (10 μg/ml, Invitrogen) for 7 days. The supplemented blasticidin was changed every 2 days. When independent colonies appeared, the cells were maintained in blasticidin-free KGM-2 culture medium. After 2 weeks, the cells were transferred to new culture dishes. To confirm that pLenti6-V5 hTERT entered the HERS-SV40, the expression of hTERT was analyzed by RT-PCR.

While primary HERS/ERM cells and HERS-SV40 did not show hTERT expression, HERS-SV40/hTERT expressed hTERT genes (Figure 3D). To compare primary HERS/ERM cells and HERS-SV40/hTERT, the cells were counted and photographed. The population doubling level (PDL) was then calculated at each passage. Primary HERS/ERM cells and DPESCs showed morphological changes; the cells were larger, and the cell-cell membrane borders disappeared at late passages. However, HERS-SV40 and DPESCs maintained a typical epithelial stem cell-like morphology despite

late passages. In addition, the HERS-SV40 and DPESC-SV40 growth rates were maintained at late passages. Although HERS-SV40 could be well maintained until passage 30, the cell growth rate decreased afterwards. Therefore, the established HERS-SV40 line was transfected with hTERT genes once again. HERS-SV40/hTERT maintained its epithelial stem cell-like morphology and growth rate. There were no morphological differences between the primary HERS/ERM cells, HERS-SV40 and HERS-SV40/hTERT cells. Likewise, there were no significant differences between the primary DPESCs and DPESC-SV40 cells.

Characterization of established HERS-SV40, HERS-SV40/hTERT and DPESC-SV40

To compare the primary cells (HERS/ERM cells, DPESCs) and cell lines (HERS-SV40, HERS-SV40/hTERT, DPESC-SV40), the immuno-phenotypes of the cells were analyzed by FACS. Primary HERS/ERM cells, HERS-SV40 and HERS-SV40/hTERT were positive for some mesenchymal cell markers, such as CD 29, 44, 73, 90, 105, and negative for hematopoietic and endothelial markers (CD 10, 31, 34, 45, 117, HLA-DR) (Figure 5). In addition, primary DPESCs and DPESC-SV40 had similar expression patterns, and the cells were positive for some mesenchymal cell markers, such as CD 29, 44, 73, 90, 105, and negative for hematopoietic and endothelial markers (CD 10, 31, 34, 45, 117, HLA-DR) (Figure 7). These data suggested that the immunophenotypes of the immortalized cell lines did not differ from those of the primary cells.

Epithelial-mesenchymal transition (EMT) of HERS-SV40, HERS-SV40/hTERT and DPESC-SV40.

HERS/ERM cells are well known to participate in cementum formation through the EMT and could acquire a mesenchymal phenotype through the EMT induced by TGF-β1. Although DPESCs were not known to undergo the EMT, a comparison of EMT effects of primary DPESCs and DPESC-SV40 seemed meaningful. To compare primary cells (HERS/ERM cells, DPESCs) and cell lines (HERS-SV40, HERS-SV40/hTERT, DPESC-SV40), the cells were treated with 20 ng/ml TGF-β1 for 48 h. All of these cells exhibited morphological changes, such as elongated shapes and the loss of cell-cell junctions (Figures 8A, 9A). To investigate the expression patterns of EMT-associated genes, E-cadherin, N-cadherin and vimentin were detected by quantitative PCR (qPCR). When the EMT was induced, in primary HERS/ERM cells, HERS-SV40 and HERS-SV40/hTERT cells, the expression of N-cadherin and vimentin was increased, while expression of E-cadherin was decreased (Figure 8B, C). There were no significant differences between primary HERS/ERM cells, HERS-SV40 and HERS-SV40/hTERT cells. Likewise, primary DPESCs and DPESC-SV40 exhibited an up-regulation of N-cadherin and vimentin, in addition to a down-regulation of E-cadherin (Figure 9B, C). These results suggested that primary cells (HERS/ERM cells, DPESCs) and cell lines

(HERS-SV40, HERS-SV40/hTERT, DPESC-SV40 cells) could differentiate into other cell types with no significant differences between each primary cell type and cell line.

Stemness of HERS-SV40, HERS-SV40/hTERT and DPESC-SV40

HERS/ERM cells are well known to express the specific markers of embryonic stem cell and epithelial stem cell. As shown in Figure 9, primary HERS/ERM cells, HERS-SV40 and HERS-SV40/hTERT expressed embryonic stem cell markers such as Oct4, Sox2 and Nanog in addition to epithelial stem cell markers such as ABCG2, EpCAM, Bmi1, p63 and p75 (Figure 10). Although the expression between HERS/ERM cells, HERS-SV40 and HERS-SV40/hTERT cells was slightly different, all of these cells expressed embryonic and epithelial stem cell markers. DPESCs have been previously reported to express epithelial stem cell markers. As shown in Figure 11, primary DPESCs and DPESC-SV40 similarly expressed embryonic stem cell and epithelial stem cell markers. These results mean that primary DPESCs and DPESC-SV40 cells may have epithelial stem cell characteristics.

In vitro nodule mineralized nodule formation of SHEDs with Conditioned medium

To elucidate the effects of conditioned medium derived from primary cells (HERS/ERMs, DPESCs) and cell lines (HERS-SV40, HERS-SV40/hTERT, DPESC-SV40) on the osteogenic differentiation of SHEDs, conditioned medium was added into

existing osteogenic differentiation conditions. SHEDs were cultured with α5 (control), α5OB, α5OB + CM 10%, or α5OB + CM 20%. The cells were evaluated after 8, 12 and 18 days. Mineralized nodules were visualized by staining with Alizarin red S. When primary HERS/ERM cells-CM was treated at 10% (α5OB + CM 10%), the calcium nodules began to appear on day 8. On day 12, the formation of the calcium nodules was accelerated when CM added to the osteogenic differentiation medium. On the 12th day, the formation of calcium nodules was accelerated when both CM from primary HERS/ERM cells and the cell lines (HERS-SV40, HERS-SV40/hTERT) were treated in the osteogenic differentiation medium, as well as the primary HERS and the cell line. Although there was a difference from day 12, the formation of the calcium nodules was accelerated by the CM on day 18. (Figure 12A and B). When primary DPESCs and DPESC-SV40 CM were treated with SHEDs, the acceleration of calcium nodule formation was detected on day 12. There were no significant differences between DPESCs and DPESC-SV40 CM.

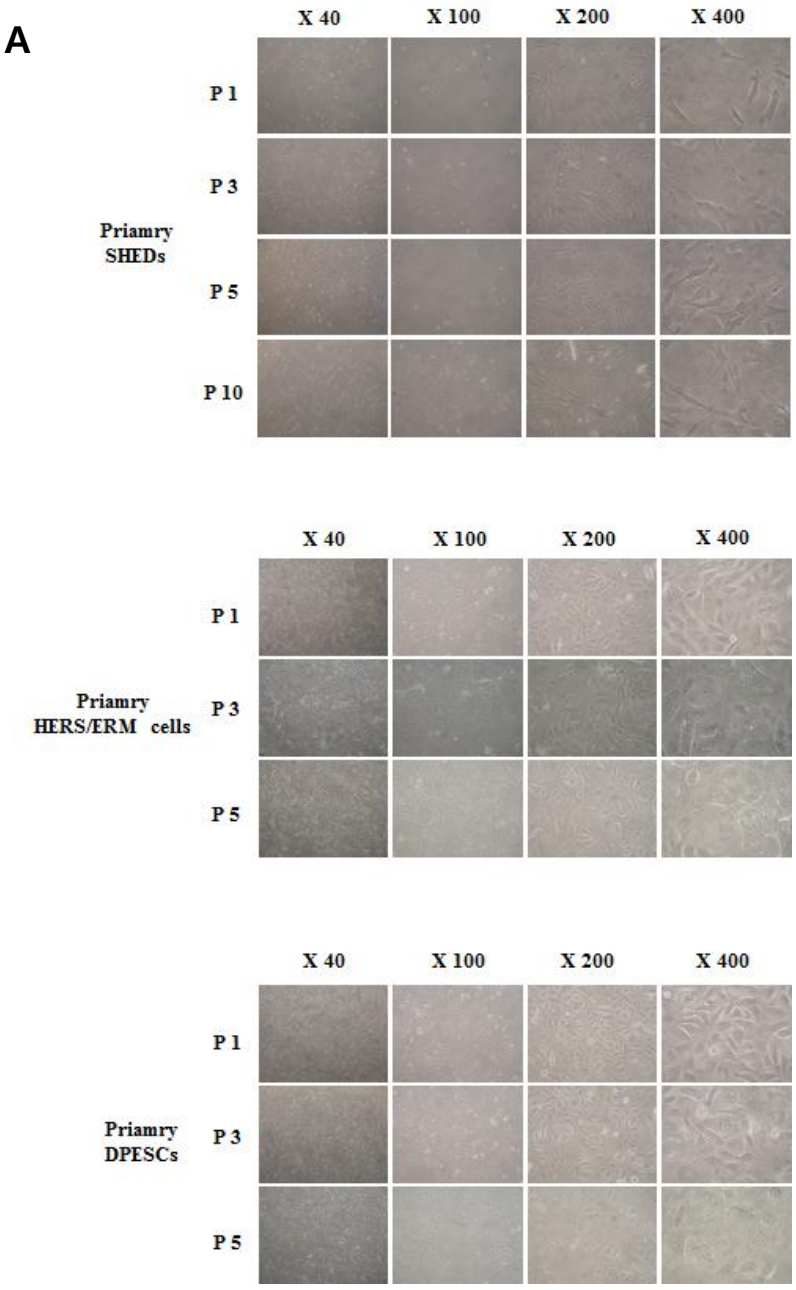

Figure 1. Cell morphology and growth curve of primary SHEDs, HERS/ERM cells and DPESCs. (A) Morphology of human SHEDs, HERS/ERM cells and DPESCs. SHEDs showed typical fibroblast-like morphology, and HERS/ERM cells and DPESCs exhibited typical epithelial cell-like morphology. However, SHEDs maintained their fibroblast-like morphology, while HERS/ERM cells and DPESCs did not maintain their epithelial cell-like structures at late passages.

B

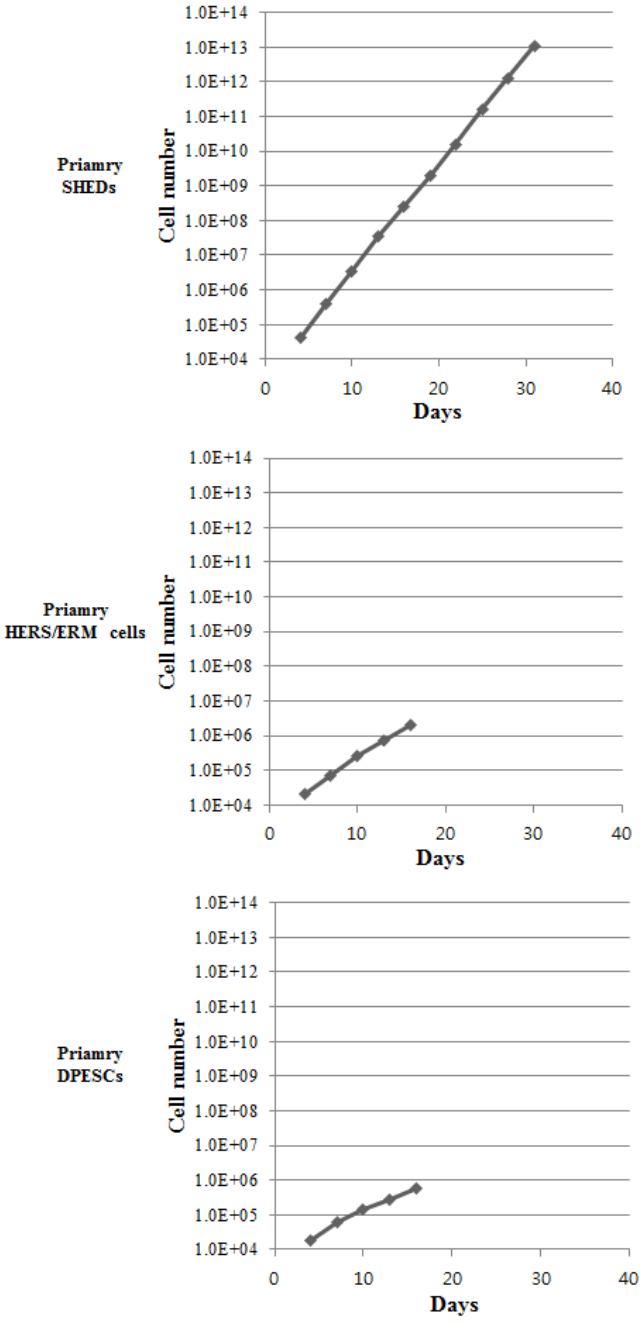

Figure 1. (B) Growth curves of human SHEDs, HERS/ERM cells and DPESCs. HERS/ERM cells and DPESCs had limited life-spans, while SHEDs did not show any reduction in proliferation. At each passage, SHEDs were seeded at a density of 5,000 cells/cm2, and HERS/ERM cells and DPESCs were plated at a density of 15,000 cells/cm2. These cells were counted in each passage.

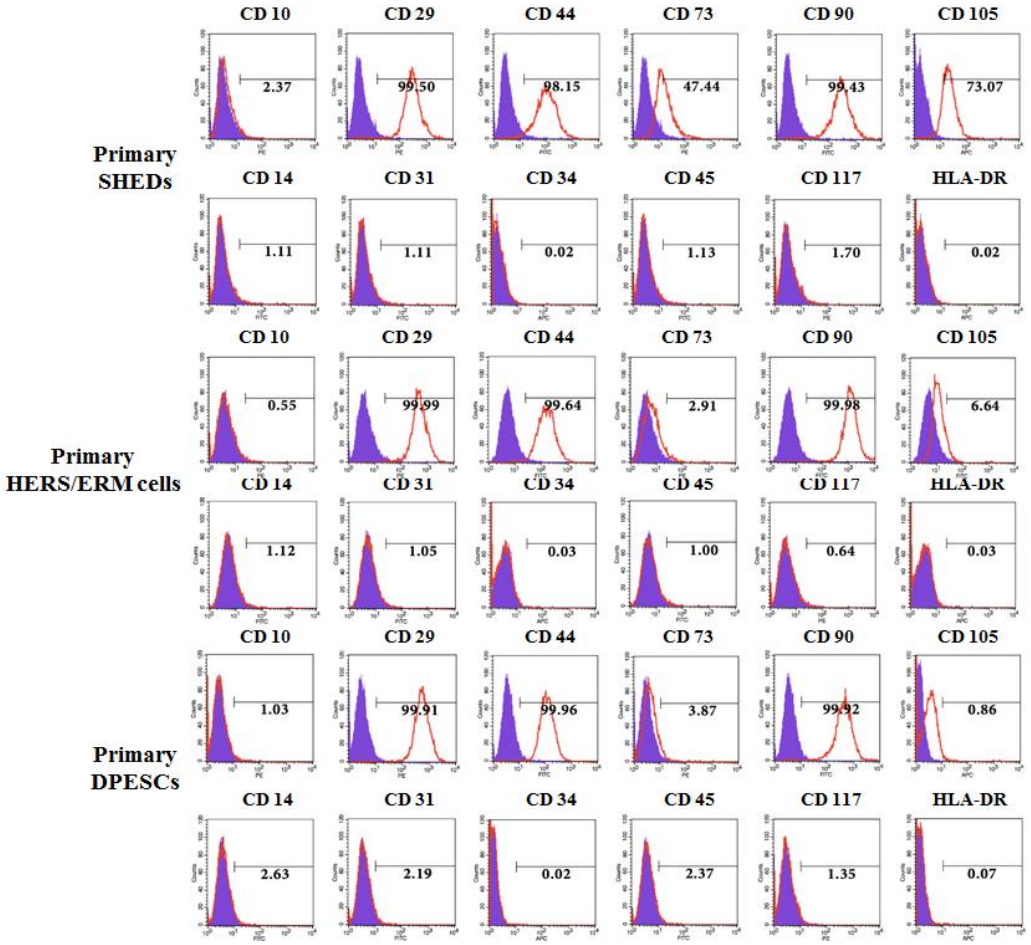

Figure 2. Immunophenotypical characterization of human SHEDs, HERS/ERM cells, HERS-SV40, HERS-SV40/hTERT, DPESCs and DPESC-SV40. The cells were stained and analyzed by flow cytometry. SHEDs were positive for mesenchymal markers (CD 29, 44, 73, 90, 105), and negative for hematopoietic and endothelial markers (CD 10, 31, 34, 45, 117, HLA-DR). Primary HERS/ERM cells and DPESCs had different expression patterns. Primary HERS/ERM cells and DPESCs had similar patterns of mesenchymal markers (CD 29, 44, 90) and endothelial markers (CD 10, 31, 34, 45, 117, HLA-DR). However, the expression of mesenchymal markers (CD 73, 105) were was decreased. Data are shown as an overlay plot with an immunoglobulin isotype control (in purple) and different specific cell-surface markers (in red).

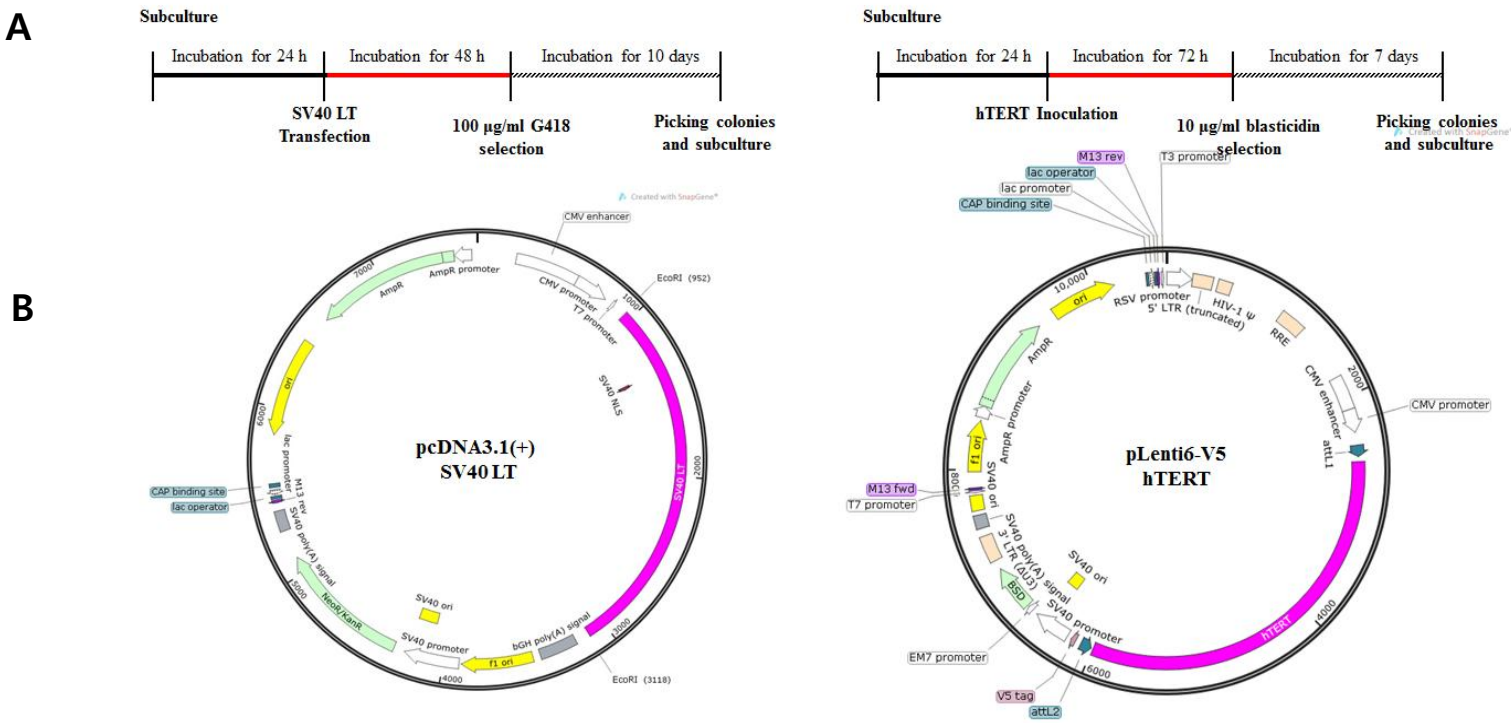

Figure 3. Immortalization of primary HERS/ERM cells, DPESCs and the HERS–SV40 cell line. (A,B) Scheme of the transfection of SV40 and hTERT. To overcome the limited lifespan of HERS/ERM cells and DPESCs, the cells were transfected with an SV40 gene. To overcome limitations remaining after SV40 transfection, HERS–SV40 was transfected with hTERT. The cells (1.5 x 105 cells/well) were transfected with 2 µg of pcDNA 3.1 (+) plasmid containing SV40 large T antigen (SV40). After establishing the HERS–SV40 cell line, a lentiviral expression system was used for lentivirus production.

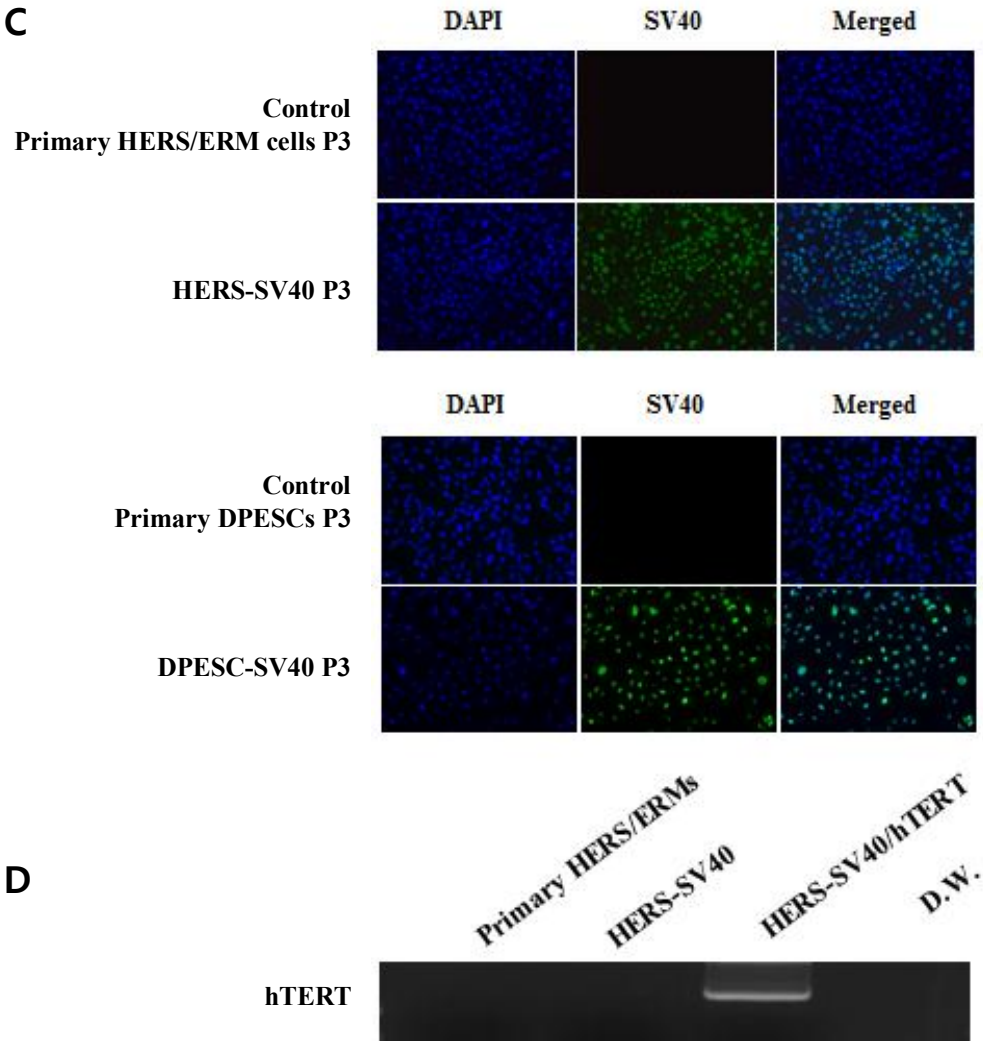

Figure 3. (C) Immunofluoresce staining showed the expression of SV40 in HERS-SV40 and DPESC-SV40. Primary HERS/ERM cells and DPESCs did not express SV40 whereas the established cell line HERS-SV40 and DPESC-SV40 expressed SV40. (D) Semi-qPCR data suggested that HERS-SV40/hTERT overexpressed the hTERT genes.

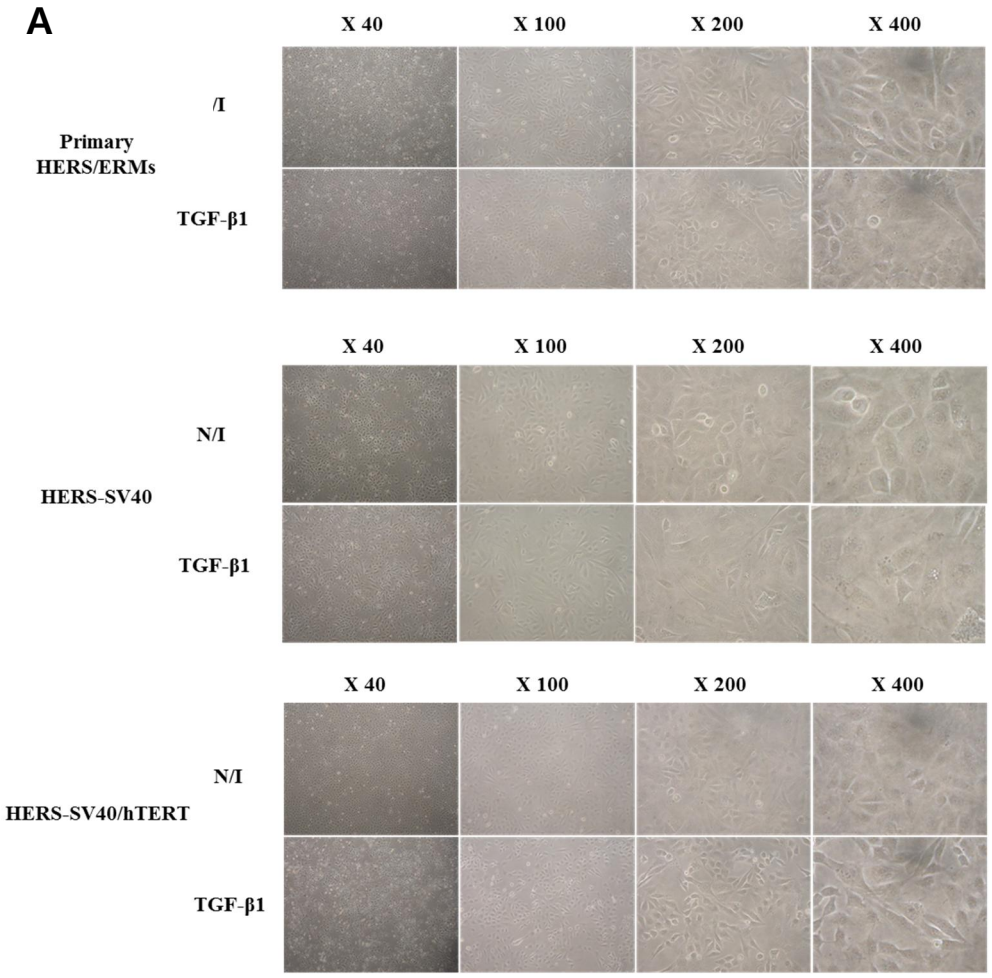

Figure 4. Characterization of established HERS cell lines. (A) Morphology of HERS-SV40 and HERS-SV40/hTERT. Primary HERS/ERM cells lost their typical epithelial cell-like morphology and clonal expansion at late passages. However, HERS-SV40 and HERS-SV40/hTERT did not show significant differences.

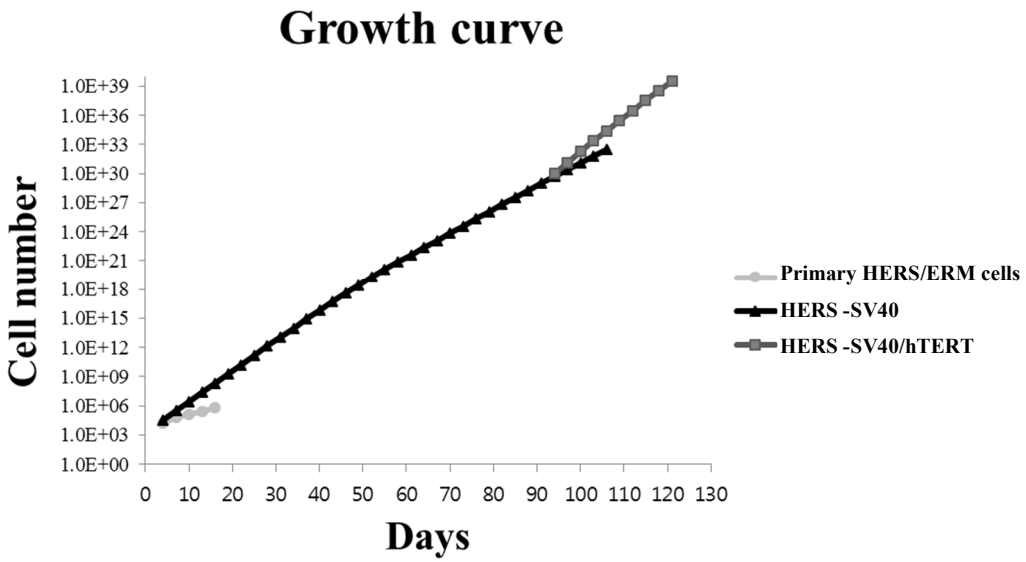

Figure 4. (B) Growth curves of primary HERS/ERM cells, HERS-SV40 and HERS-SV40/hTERT. While the proliferation of primary HERS/ERM cells was arrested after 15 days, HERS-SV40 cells maintained stable proliferation until 100 days. In addition, HERS-SV40/hTERT continued to proliferate after 100 days. Primary HERS/ERM cells, HERS-SV40 and HERS-SV40/hTERT cells were plated at a density of 15,000 cells/cm2. At each passage, cells were counted and photographed.

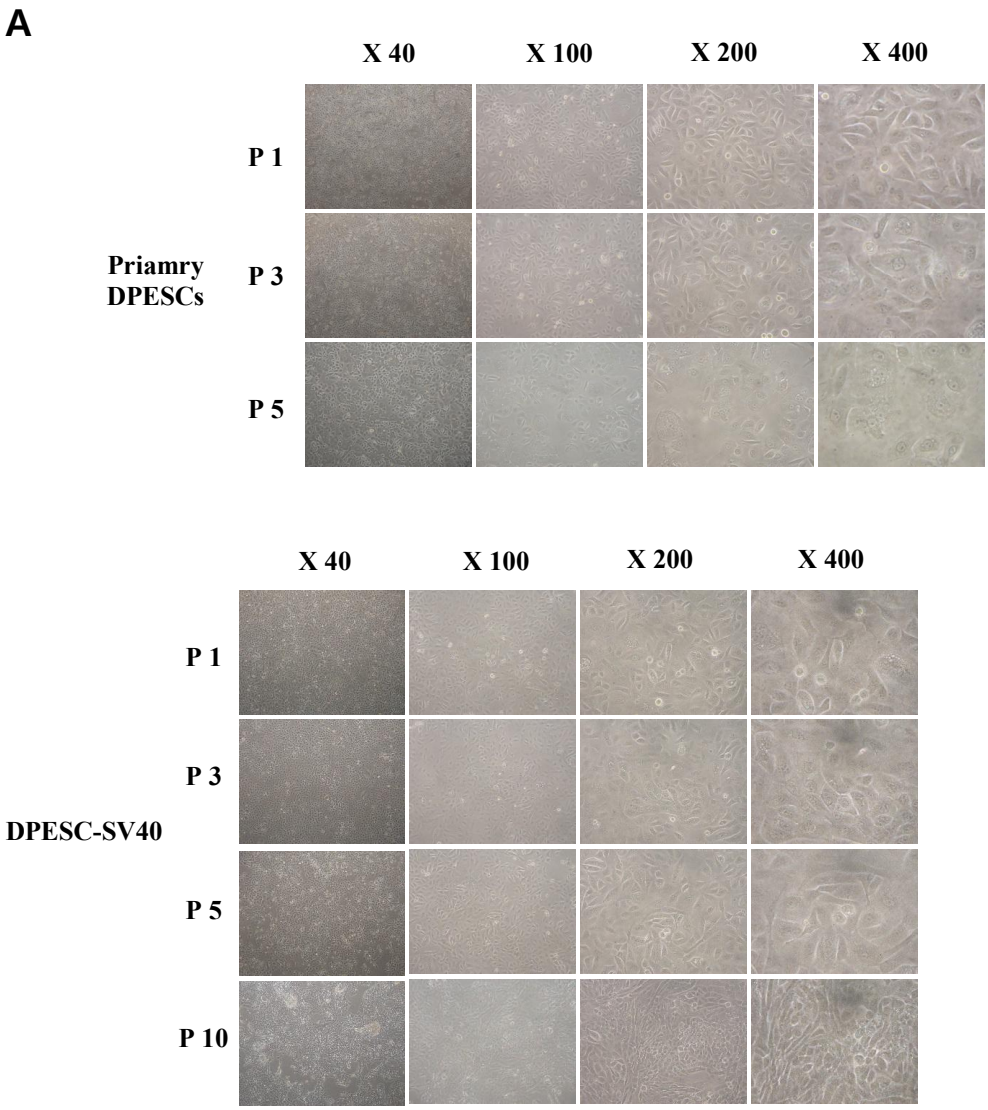

Figure 5. Characterization of established DPESC cell line. (A) Morphology of DPESC-SV40 cells. Primary DPESCs showed morphological changes for more than 5 passages. However, DPESC-SV40 cells maintained epithelial cell-like morphology for more than 10 passages.

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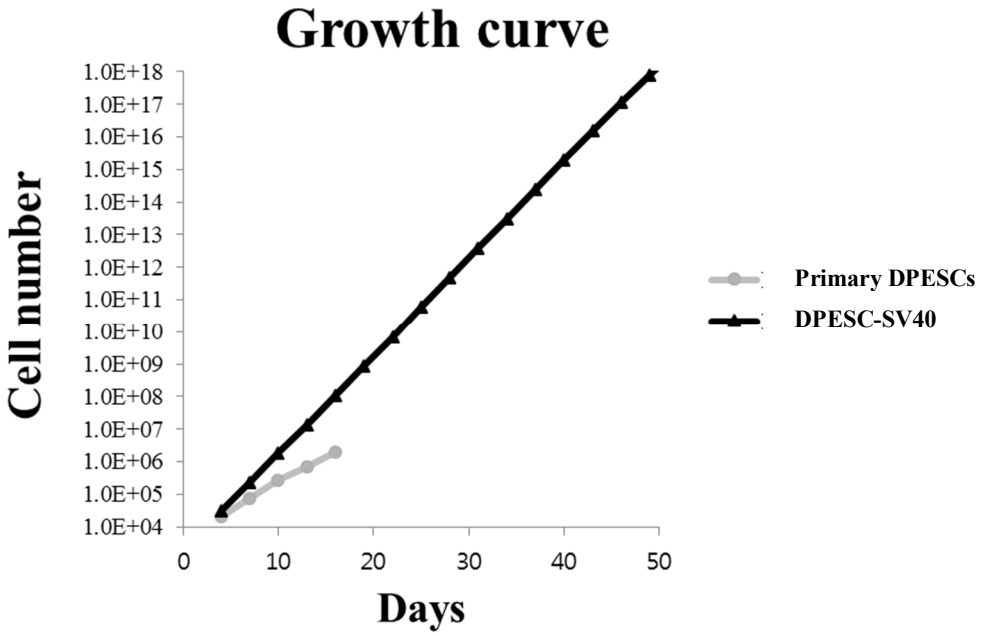

Figure 5. (B) Growth curves of primary DPESCS and DPESC-SV40. Primary DPESCs had limited life-spans, while DPESC-SV40 did not show any loss of proliferation. Primary DPESCS and DPESC-SV40 cells were plated at a density of 15,000 cells/cm2. At each passage, cells were counted and photographed.

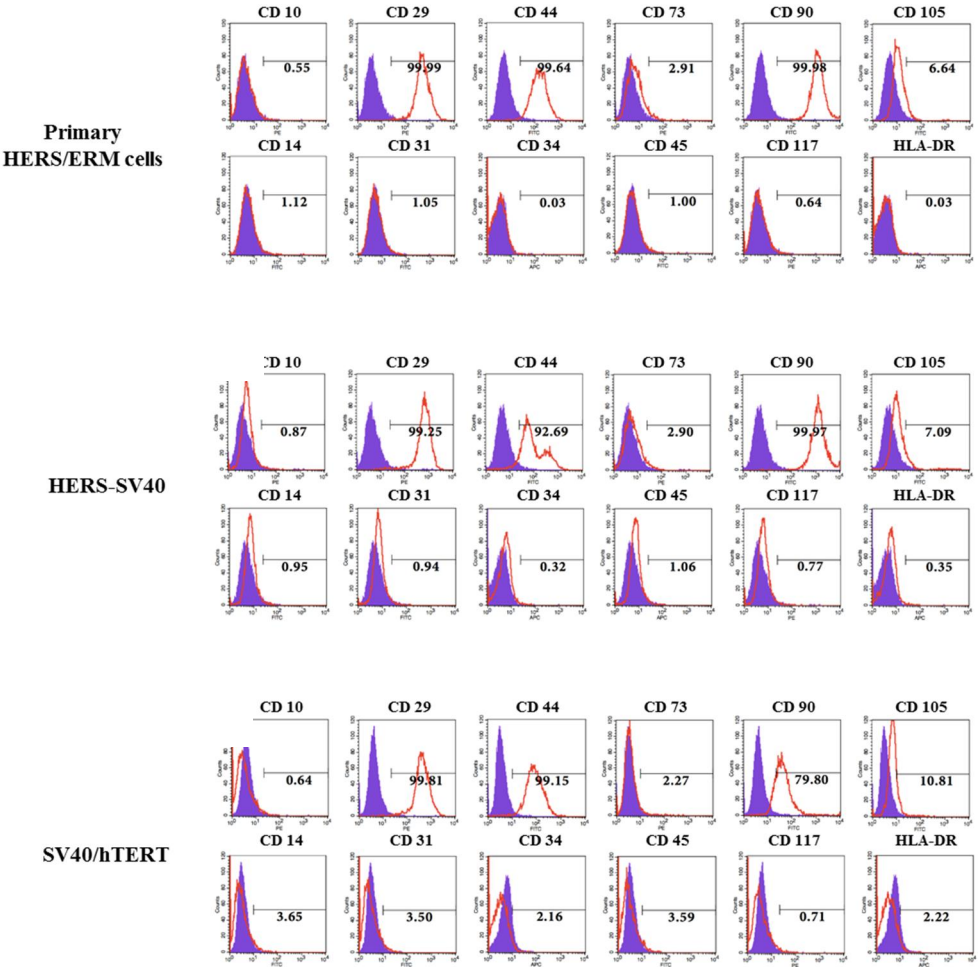

Figure 6. Characterization of primary HERS/ERM cells, HERS-SV40 and HERS-SV40/hTERT by FACS analysis. Similar expression patterns of MSC markers were observed in primary HERS/ERM cells, HERS-SV40 and HERS-SV40/hTERT cells. Primary HERS/ERM cells HERS-SV40 and HERS-SV40/hTERT cells had similar patterns of mesenchymal markers (CD 29, 44, 90) and endothelial markers (CD 10, 31, 34, 45, 117, HLA-DR). Data are shown as an overlay plot with an immunoglobulin isotype control (in purple) and different specific cell-surface markers (in red).

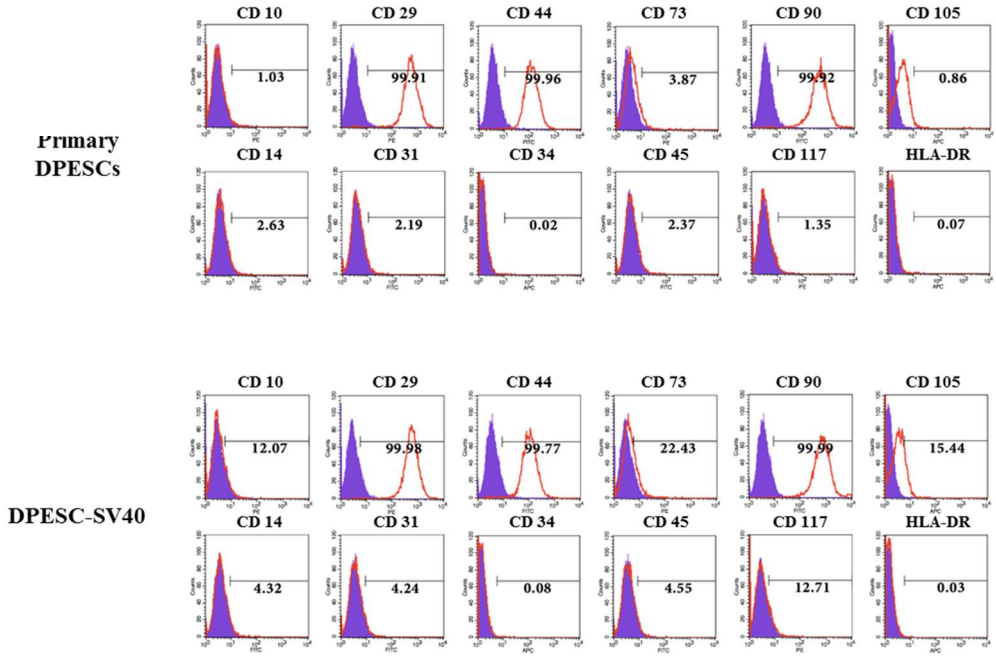

Figure 7. Characterization of primary DPESCs and DPESC-SV40 by FACS analysis. Similar expression pattern of MSC markers was observed in primary DPESCs and DPESC-SV40. Primary DPESCs and DPESC-SV40 had similar pattern of mesenchymal markers (CD 29, 44, 90) and endothelial markers (CD 10, 31, 34, 45, 117, HLA-DR). Data are shown as an overlay plot with immunoglobulin isotype control (in purple) and different specific cell-surface markers (in red).

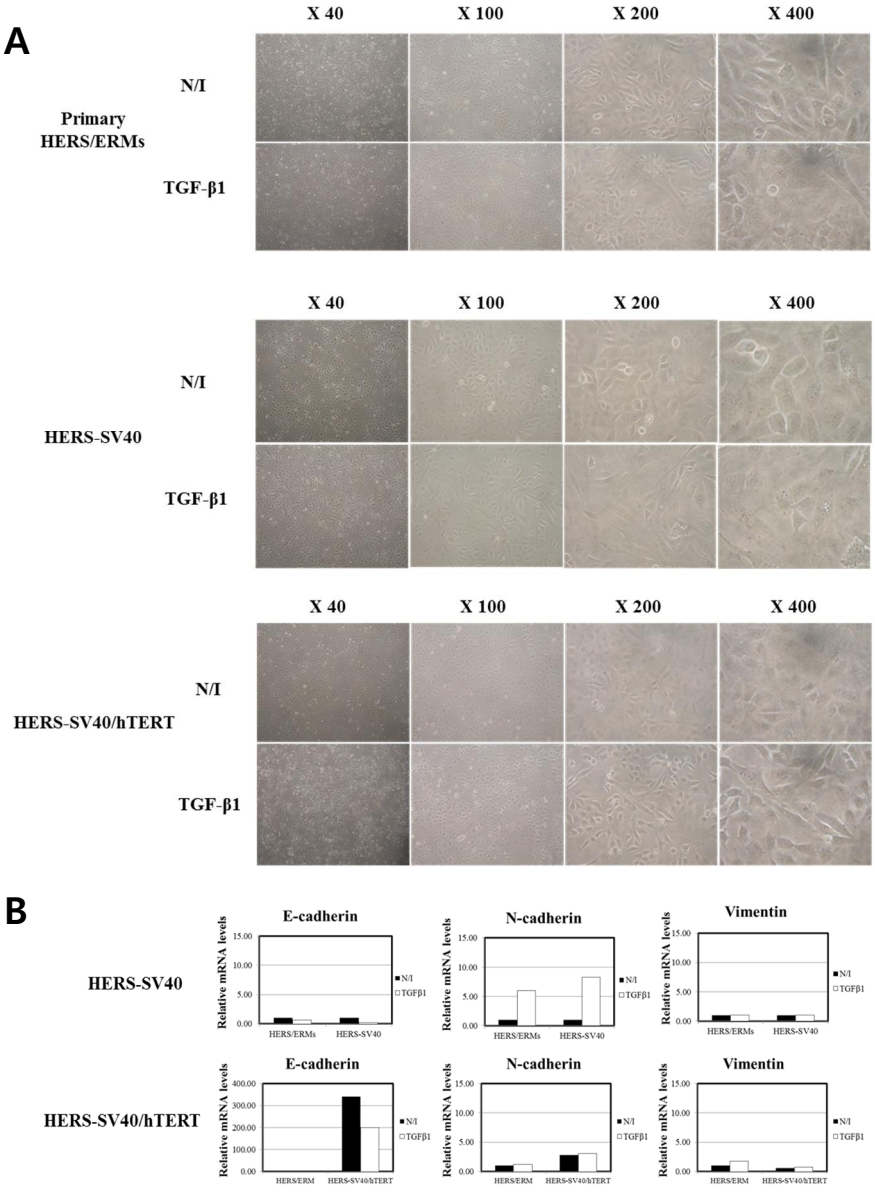

Figure 8. Epithelial-mesenchymal transition of primary HERS/ERM cells, HERS-SV40 and HERS-SV40/hTERT cells. The EMT was induced by 20 ng/ml of TGF-β1 for 48 h. (A) All of these cells lost epithelial cell polarity and cell-cell contact. (B). To confirm the EMT, the expression of E-cadherin, N-cadherin and vimentin was determined using quantitative PCR. When all cell types were treated with TGF-β1, the gene expression of N-cadherin and vimentin was increased in primary HERS/ERM cells, HERS-SV40 and HERS-SV40/hTERT. However, the levels of E-cadherin were decreased.

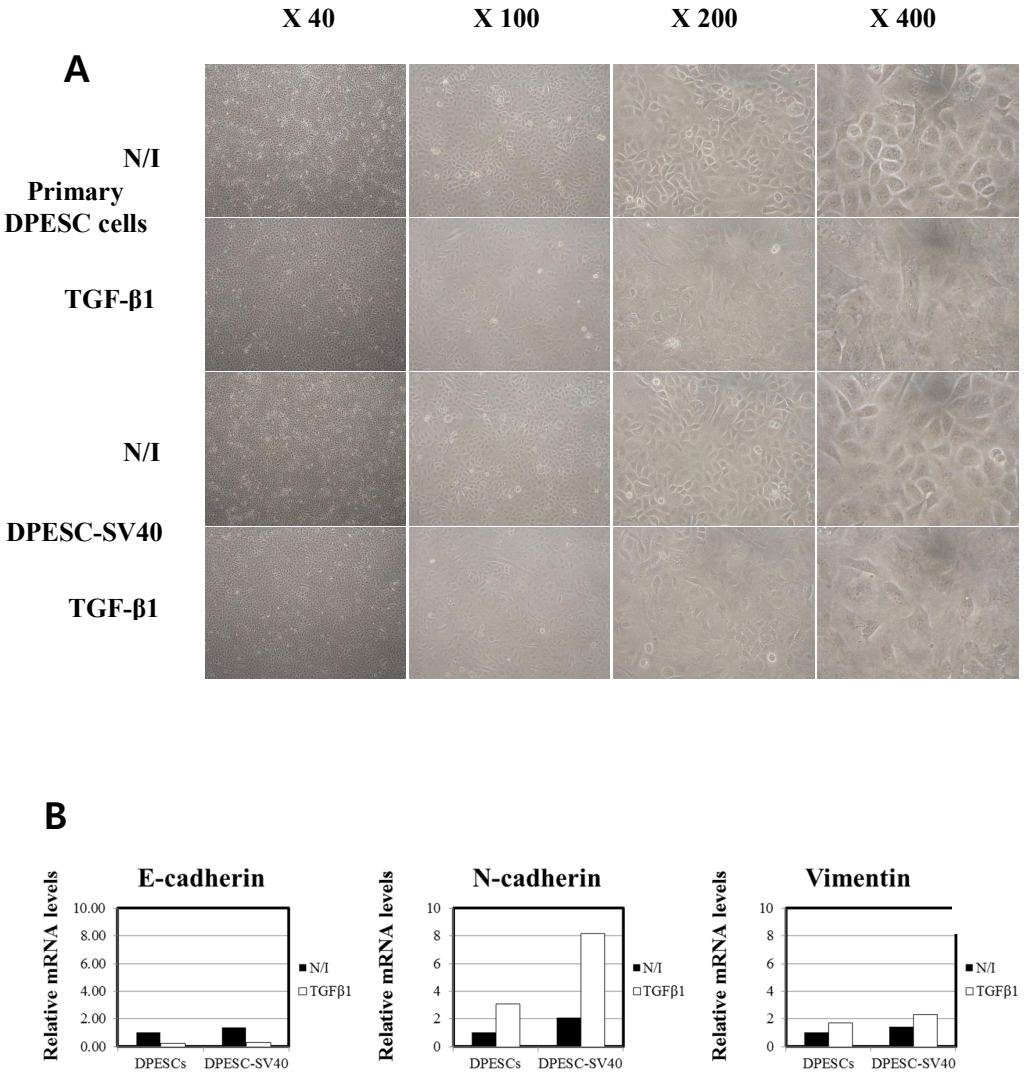

Figure 9. Epithelial-mesenchymal transition of primary DPESCs and DPESC-SV40 cells. The EMT was induced by 20 ng/ml of TGF-β1 for 48 h. (A) All of these cells lost epithelial cell polarity and cell-cell contact. (B) To investigate the effects of the EMT, the expression of E-cadherin, N-cadherin and vimentin was determined using quantitative PCR. When all cell types were treated with TGF-β1, gene expression of N-cadherin and vimentin was increased in both primary DPESCs and DPESC-SV40 cells. However, the levels of E-cadherin were decreased.

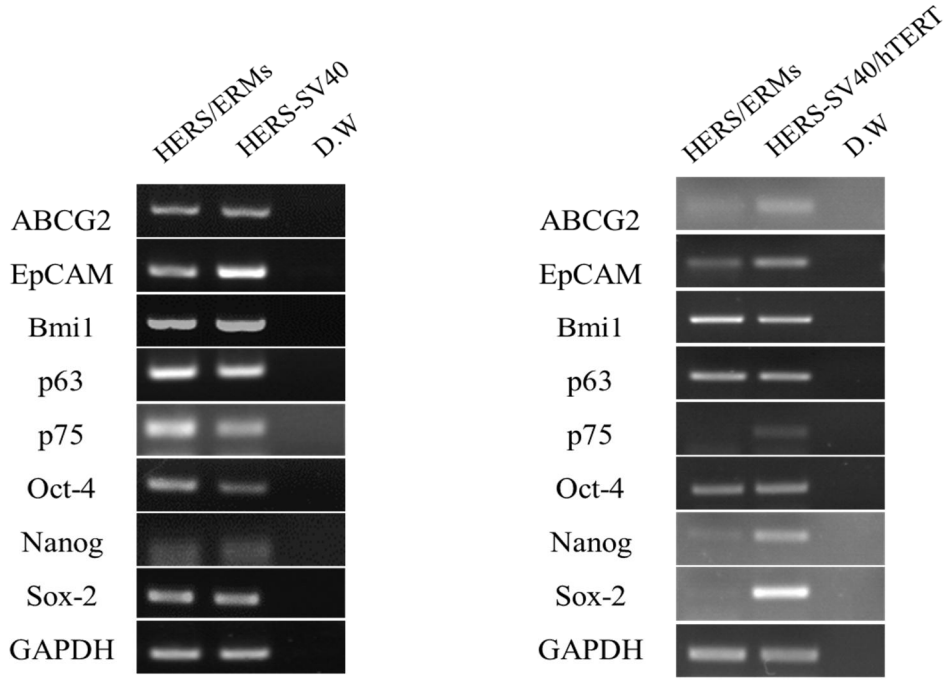

Figure 10. Stemness of primary HERS/ERM cells, HERS-SV40 and HERS-SV40/hTERT. The expression patterns of embryonic stem cell and epithelial stem cell markers were detected by semi-quantitative PCR. The PCR data showed no significant expression differences between any cell types.

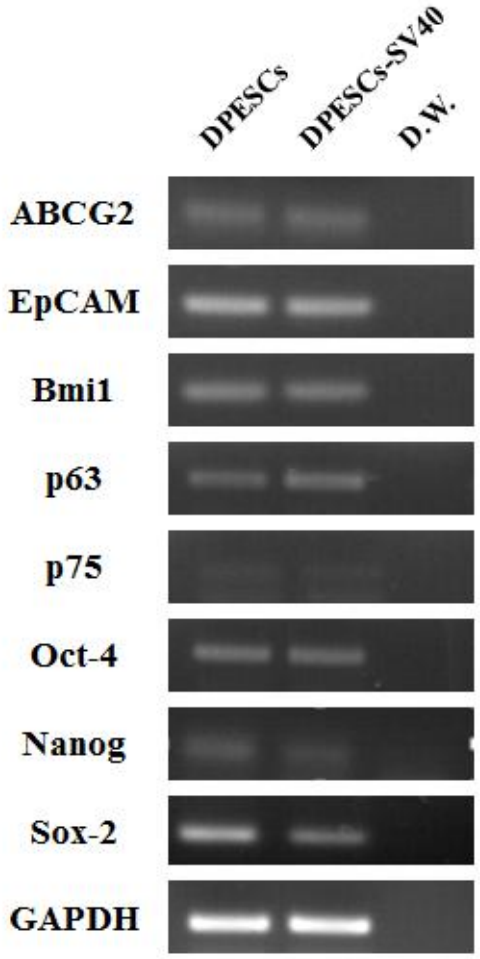

Figure 11. Stemness of primary DPESCs and DPESC-SV40 cells. The expression patterns of embryonic stem cell and epithelial stem cell markers were detected by semi-quantitative PCR. The PCR data showed no significant expression differences between either cell type.

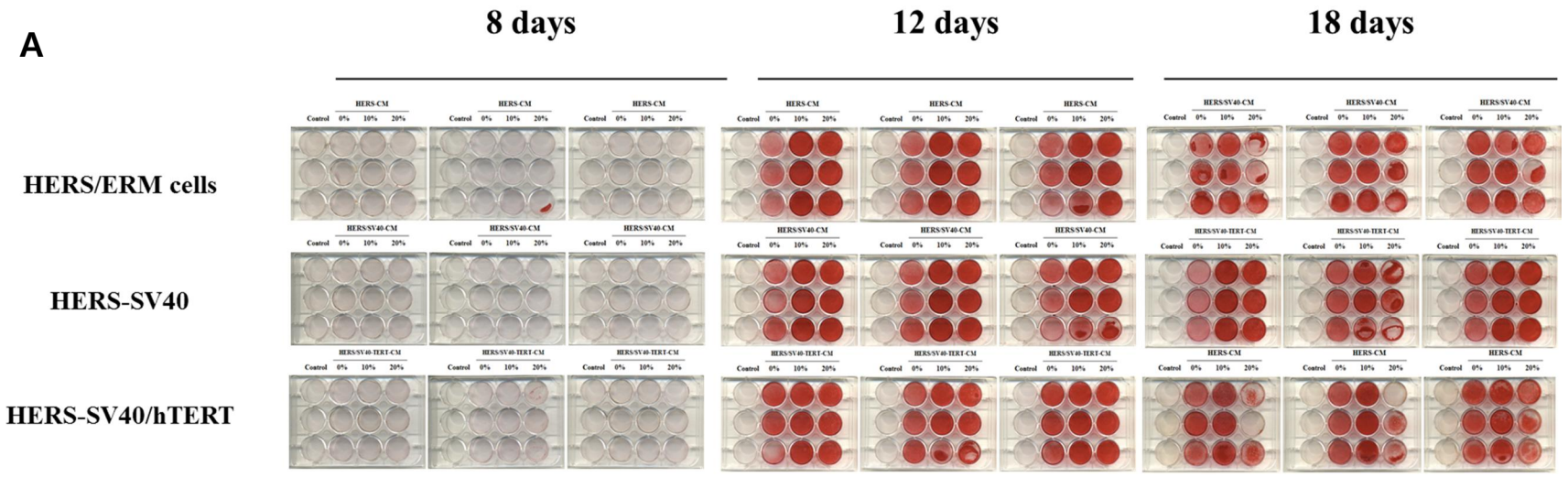

Figure 12. Osteogenic differentiation of SHEDs with CM derived from primary HERS/ERM cells, HERS-SV40 and HERS-SV40/hTERT. (A) SHEDs were cultured with α5 (control), α5OB, α5OB + CM 10%, or α5OB + CM 20%. Mineralized nodules were visualized by staining with Alizarin red S. When primary HERS/ERM cells CM was treated at 10% (α5OB + CM 10%), calcium nodules began to appear on day 8. On day 12, the formation of the calcium nodules was accelerated when CM was treated in the osteogenic differentiation medium. There were no significant differences between primary CM and cell line CM.

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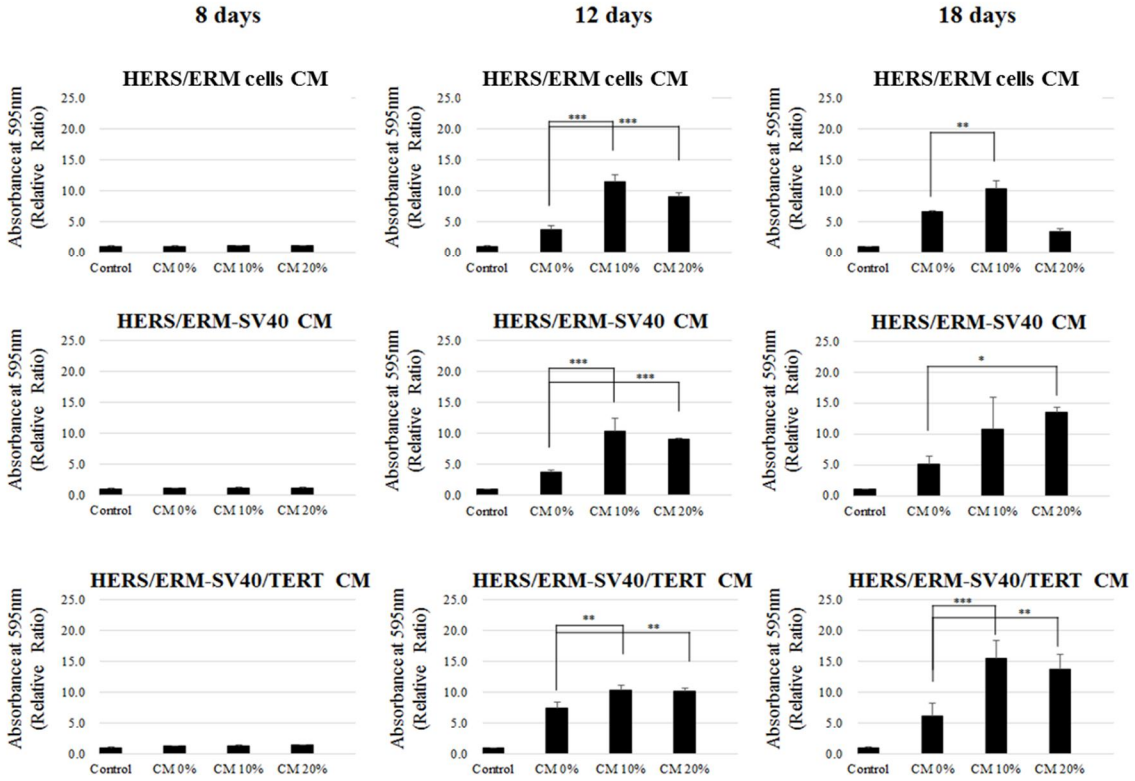

Figure 12 (B) To quantify the amount of calcium minerals, Alizarin red S was destained with 10% cetylpyridinium chloride. Beginning 12 days later, the acceleration of calcium deposits was observed. There were no significant differences between primary CM and cell line CM. Data represent the mean ± SE. *p<0.1, **p<0.05, ***p<0.01

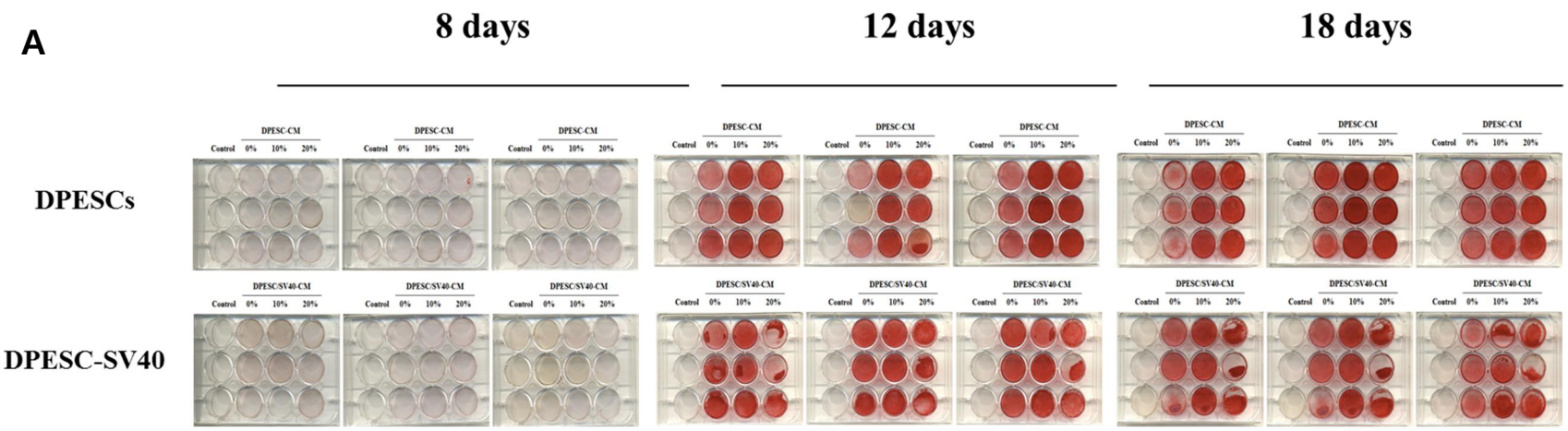

Figure 13. Osteogenic differentiation of SHEDs with CMs derived from primary DPESCs and DPESC-SV40 cells. (A) 8, 12 and 18 days after osteogenic differentiation induction, the acceleration of hard tissue formation was observed by Alizarin red s staining and quantification. When primary DPESC CM was treated at 10% (α5OB + CM 10%), calcium nodules began to appear on day 8. On day 12, the formation of calcium nodule was accelerated when CM was treated in the osteogenic differentiation medium. There were no significant differences between primary CM and cell line CM.

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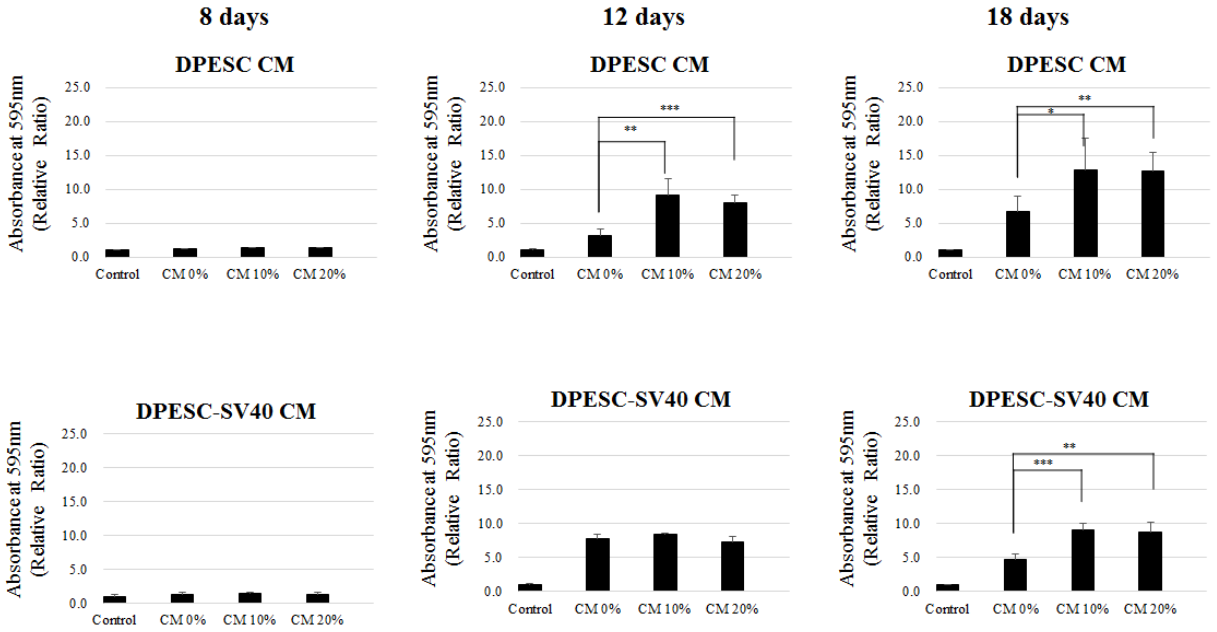

Figure 13. (B) To quantify the amount of calcium minerals, Alizarin red S was destained with 10% cetylpyridinium chloride. Beginning 12 days later, the acceleration of calcium deposits was observed. There were no significant differences between primary CM and cell line CM. Data represent the mean ± SE. *p<0.1, **p<0.05, ***p<0.01.

Table 1. FACS antibody

Markers	Antibody	Information	Dilution	Company
Mesenchymal cell markers	CD 10	PE anti-human	100 : 1	BD Bioscience Pharmingen
	CD 29	PE anti-human	100 : 2	BD Bioscience Pharmingen
	CD 44	FITC anti-human	100 : 2	BD Bioscience Pharmingen
	CD 73	PE anti-human	100 : 2	BD Bioscience Pharmingen
	CD 90	PE anti-human	100 : 1	BD Bioscience Pharmingen
	CD 105	APC anti-human	100 : 0.5	eBioscience
Hematopoietic cell markers	CD 14	FITC anti-human	100 : 1	BD Bioscience Pharmingen
	CD 34	FITC anti-human	100 : 1	Miltenyi Biotec GmbH
	CD 45	FITC anti-human	100 : 1	eBioscience
	CD 117	PE anti-human	100 : 1	eBioscience
	HLA-DR	APC anti-human	100 : 1	BD Bioscience Pharmingen
Endothelial cell markers	CD 31	FITC anti-human	100 : 1	BD Bioscience Pharmingen

Table 2. Gene specific primers

Class	Genes	Size/bp	Tm/℃	Cycles	Forward	Reverse
House keeping gene	GAPDH	209	60	28	GAT GCT GGC GCT GAG TAC G	GCT AAG CAG TTG GTG GTG C
Human TERT gene	hTERT	209	60	40	ATG CCG CGC GCT CC	TCA GTC CAG GAT GGT CTT GAA
Epithelial-mesenchymal transition markers	N-cadherin	201	55	35	ACA GTG GCC ACC TAC AAA GG	CCG AGA TGG GGT TGA TAA TG
	E-cadherin	200	55	35	TGC CCA GAA AAT GAA AAA GG	GTG TAT GTG GCA ATG CGT TC
	Vimentin	212	55	35	TCT ACG AGG AGG AGA TGC GG	GGT CAA GAC GTG CCA GAG AC
Epithelial stem cell markers	ABCG2	98	60	40	CCA CAG GTG GAG GCA AAT CT	TCG CGG TGC TCC ATT TAT CA
	EpCAM	100	60	40	GCT GGC CGT AAA CTG CTT TG	ACA TTT GGC AGC CAG CTT TG
	BMI1	79	60	40	CAG CCC AGC AGG AGG TAT TC	GGA TGA GGA GAC TGC ACT GG
	P63	205	55	35	ATG TTG TAC CTG GAA AAC AAT GC	GTG ATG GAG AGA GAG CAT CGA A
	P75	165	60	40	ACC GAG CTG GAA GTC GAG	CTC ACC GCT GTG TGT GTA C
Stemness markers	SOX2	185	55	35	GAC TTC ACA TGT CCC AGC AC	GGG TTT TCT CCA TGC TGT TT
	OCT4	189	57	35	ACC CCT GGT GCC GTG AA	GGC TGA ATA CCT TCC CAA ATA
	NANOG	176	55	35	CCT ATG CCT GTG ATT TGT GG	TTC TCT GCA GAA GTG GGT TG

* Tm represents annealing temperature.

Table 3. Osteogenic differentiation medium

Medium	Composition of the culture medium
α5 (control)	alphaMEM, 5% FBS, 1% Antibiotics-antimycotics
α5OB	α5, 0.1 μM Dexamethasone, 10mM B-glycerophosphate, 50ug/ml L- ascorbic acid phosphate
CM 10%	α5OB, Conditioned medium 10%
CM 20%	α5OB, Conditioned medium 20%

Discussion

Stem cells are capable of both self-renewal and multi-lineage differentiation[14]. Teeth are well known to be the most natural and noninvasive source of stem cells. Dental stem cells are convenient to isolate and have potential therapeutic applications[5]. The discovery of stem cells in the pulp of deciduous teeth and permanent teeth raised the possibility of using dental stem cells for tissue engineering[17, 33]. The following human dental stem cells have been reported: DPSC[17], SHED[33]; SCAP[51], PDLSC[46], HERS/ERM cells[36] and DPESC[38]. In general, there is a difference in the expression of cell surface markers between mesenchymal stem cells and epithelial stem cells. To confirm this difference, FACS analysis was performed[18].

While both dental mesenchymal stem cells, such as SHEDs, and epithelial stem cells, such as HERS/ERM cells and DPESCs, did not express hematopoietic and endothelial cell markers (CD 14, 31, 34, 45, 117, HLA-DR), the expression of mesenchymal cell markers had some differences[8]. SHEDs had high expression of CD29, 44, 73, 90, 105, but epithelial stem cells (HERS/ERM cells, DPESCs) had low expression of CD73, 105. Likewise, to compare the primary cells (HERS/ERM cells, DPESCs) and cell lines (HERS-SV40, HERS-SV40/hTERT, DPESC-SV40), the immuno-phenotypes of the cells were analyzed by FACS. Primary HERS/ERM cells, HERS-SV40 and HERS-SV40/hTERT were positive for some mesenchymal cell markers, such as CD 29, 44, 73, 90, 105, and negative for hematopoietic and endothelial markers (CD 10, 31, 34, 45, 117, HLA-DR) (Figure 5). In addition, primary DPESCs and DPESC-SV40 cells had similar expression patterns, in which the

cells were positive for some mesenchymal cell markers, such as CD 29, 44, 73, 90, 105, and negative for hematopoietic and endothelial markers (CD 10, 31, 34, 45, 117, HLA-DR) (Figure 7). These data suggested that the immune-phenotypes of the immortalized cell lines did not differ from those of the primary cells.

Epithelial-mesenchymal interactions are reciprocal communications between the epithelium and mesenchyme and are essential for the development of organs[45]. Epithelial-mesenchymal interactions are known to be very important for tooth development, but there is little research on the interactions between them because of the limited life-span and the difficulty of isolating dental epithelial stem cells (HERS/ERM cells, DPESCs)[16]. To overcome these problems, primary epithelial cells (HERS/ERM cells, DPESCs) were transfected with SV40, which inactivates the tumor suppressor p53 and Rb pathways[11, 48]. To overcome the limitations remaining after SV40 transfection, HERS-SV40 was transfected with hTERT[23, 27]. It is well known that the ectopic expression of hTERT abolishes senescence and maintains the function of the cells. Unlike primary cultured cells (primary HERS/ERM cells, DPESCs), SV40 over-expressing cells (HERS-SV40, DPESC-SV40) were observed to maintain cell morphology and proliferation4. In addition, we confirmed that transfected cells with SV40 and hTERT were retained into the later passages than were HERS-SV40 cells.

To compare the established cell lines (HERS-SV40, DPESC-SV40, HERS-SV40/hTERT) to primary cells (primary HERS/ERM cells, DPESCs), FACS analysis, which evaluates the gene expression of stemness markers and the induction of the EMT, was performed. Primary epithelial cells (HERS/ERM cells, DPESCs) and cell lines (HERS-SV40, HERS-SV40/hTERT, DPESC-SV40) did

not express hematopoietic and endothelial cell markers; however, some mesenchymal cell markers, such as CD29, 44 and 90, were highly expressed. It was suggested that primary epithelial cells (HERS/ERM cells, DPESCs) and cell lines (HERS-SV40, HERS-SV40/hTERT, DPESC-SV40) have the same immune-phenotypical characteristics.

To ensure that the cells had the same genetic characteristics, the cells were analyzed by RT-PCR. The data showed that all of these cells expressed ABCG2, EpCAM, Bmi1, p63 and p75. ABCG2 is a membrane-associated protein that is included in the superfamily of ATP-binding cassette (ABC) transporters and is considered to be a universal stem cell marker[21]. Bmi1 is essential for embryogenesis and the regulation of the cell cycle. Bmi1 also suggests the expression of ABCG2[43]. EpCAM is an epithelial cell adhesion molecule and is expressed in a variety of human epithelial tissues, carcinomas and progenitor and stem cells[54]. The p63 transcription factor is a p53 homolog that is essential for regenerative proliferation in epithelial development and is associated with proliferative potential in human keratinocytes[44]. Human oral keratinocyte stem cells characteristically express p75, which is a low-affinity neurotrophin receptor[10, 35]. Thus, the function of epithelial stem cell markers may be important for the proliferation and differentiation of dental epithelial stem cells.

EMT is an important biologic process that allows epithelial cells to undergo multiple biochemical changes such as mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of ECM components[19]. Several studies reported that HERS/ERM, consist of two epithelial layers, plays a

role of inducing odontogenesis during root development[13, 37]. The EMT process can be regulated by a diverse array of Snail, Twist transcription factors as a repressor of E-cadherin gene expression [6, 55]. Also, cytokines and growth factors, such as TGF-β[49]. Among them, TGF-β family is the most frequently used inducer[32]. In epithelial cells, activation of the TGF-β signal transduction pathway leads to inhibition of cell proliferation, increase in extracellular matrix production and remodeling of cell-matrix adhesions[1, 3, 32]. With this knowledge, I investigated the responsiveness of immortalized cell lines to TGF-β1. When the EMT was induced by TGF-β1, the primary epithelial cells and cell lines exhibited similar characteristics, such as the decreased expression of epithelial markers (E-cadherin) and the increased expression of mesenchymal markers (N-cadherin, vimentin)[50]. It means that immortalized cell lines have similar characteristics to primary dental epithelial cells.

CM refers to the collection of proteins, such as signal peptides, processed through the endoplasmic reticulum and Golgi apparatus[31, 41]. The CM also surrounds the cell surface and intracellular proteins. CM contains several types of enzymes, growth factors, cytokines, hormones and other soluble mediators[31]. It is expected to lead to cell differentiation, growth, invasion and angiogenesis by regulating cell -cell and cell- ECM interactions[12]. In humans, CM derived from embryonic tooth germ is difficult to obtain. Since the composition of CM from embryonic tooth germ would be different[52]. However, CM derived from dental epithelial stem cells may help to overcome this limitation.

In this study, I evaluated the effect of CM derived from dental epithelial stem cells on osteogenic differentiation. To elucidate the

effects of conditioned medium derived from primary cells (HERS/ERM cells, DPESCs) and cell lines (HERS-SV40, HERS-SV40/hTERT, DPESC-SV40) on the osteogenic differentiation of SHEDs, the conditioned medium was added into existing osteogenic differentiation conditions. When the CM obtained from each cell type was treated with existing osteogenic differentiation medium, calcium nodules began to appear on day8 and day 12, while calcium nodule formation was also relatively accelerated in all cases. There were no significant differences between primary cells and cell lines.

I demonstrated that the overexpression of SV40 and hTERT is useful in the immortalization of primary epithelial cells such as HERS/ERM cells and DPESCs. The immortalized cell lines (HERS-SV40, HERS-SV40/hTERT, DPESC-SV40) had longer life-spans and their morphologies were maintained throughout subculture. PCR data showed that immortalized cell lines have typical epithelial cell like-gene expression. In addition, primary cells and immortalized cell lines exhibited similar characteristics after the epithelial-mesenchymal transition induced by TGF-β1.

Alizarin red S staining indicated that calcium accumulation in SHED cells was equally promoted by conditioned medium derived from primary cells and immortalized cell lines[15]. Immortalized cell lines (HERS-SV40, HERS-SV40/hTERT, DPESC-SV40) could contribute to the investigation of the functional role of primary cells (HERS/ERM cells, DPESCs) and their application in dental regeneration.

Taken together, these findings indicate that I established the cell lines of dental epithelial stem cells and confirm that both primary dental epithelial stem cells and cell lines showed the same characteristics in terms of morphology and gene expression profiles.

Furthermore, it was also proven that conditioned medium from the established cell lines affects the acceleration of hard tissue formation of primary cultured mesenchymal stem cells or SHEDs. Therefore, the present results indicate that HERS-SV40, HERS-SV40 / hTERT and DPESC-SV40, which are established from primary dental epithelial cells, could be expected to contribute to the study of dental stem cell functions and tooth regeneration.

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국문초록

치계상피세포주 확립 및 유치 유래 치계줄기세포의 경조직 형성능에 미치는 영향

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줄기세포는 다양한 세포 유형으로 분화하여 어떤 조직으로든 발달할 수 있는 세포를 의미한다. 그 중 성체줄기세포는 신체 각 조직에 극소량으로 존재하며 미분화된 세포로 존재하다가 손상이 발생하면 빠르게 분화하여 조직 재생이 이루어진다. 성체줄기세포는 배아줄기세포에 비해 분화능에 있어 제한이 있으나 세포확보의 편리성이나 윤리적인 측면에 있어서 보다 자유로운 측면이 있어 연구에 활발하게 이용되고 있다. 최근 치주인대, 치수를 비롯한 구강조직 유래 줄기세포의 연구가 잇따라 보고되고 있다. 치아 발생과정을 통하여 외배엽성 중간엽줄기세포와 상피줄기세포간의 상호작용이 매우 중요한 것으로 보이나 상대적으로 확보 및 유지가 어려운 상피줄기세포의 특성상 상피–중간엽간의 상호작용에 대한 연구가 미진한 것이 현실이다. 따라서 본 연구에서는 치계상피줄기세

포의 안정적인 확보를 위하여 기존에 보고된 구강조직 유래 상피줄기세포들 즉 Hertwig’s epithelial root sheat cells/Epithelial rests of Malassez cell (HERS/ERM cells), 및 Dental pulp epithelial stem cells (DPESC)의 세포주를 확립하고 유치 치수유래 중간엽줄기세포(stem cells from human exfoliated deciduous teeth, SHED)의 경조직 형성능에 미치는 영향을 밝히고자 하였다. 유치로부터 SHEDs와 DPESCs를 분리 및 일차배양하였으며 영구치로부터 HERS/ERM cells를 분리 및 일차배양하였다. 확보된 HERS/ERM cells 및 DPESCs는 SV40 Large T antigen을 도입하여 불멸화를 유도하였다. 또한 SV40 Large T antigen으로 확립된 HERS/ERM cells 세포주에 hTERT (human telomerase reverse transcriptase) 유전자를 도입하여 보다 안정된 세포주를 확립하였으며 일차배양 상피세포와 세포주간의 비교를 위해 세포의 형태, 증식, 세포표면항원분석을 통한 상피줄기세포의 특성 유지, PCR을 통한 배아줄기세포 및 상피줄기세포 마커의 확인을 시행하였다. 또한, 경조직 형성에 대한 치계상피줄기세포와 치계중간엽줄기세포사이의 상호작용을 확인하기 위해 치계상피줄기세포 및 세포주 배양액으로부터 얻은 조건배지(Conditioned medium)를 치계중간엽줄기세포에 처리한 후 경조직 형성능을 분석하였다. 영구치로부터 분리한 일차배양 HERS/ERM cells 와 HERS-SV40, HERS-SV40/hTERT의 세포 형태 및 세포 성장 곡선을 비교하였을 때 불멸화 된 세포주인 HERS-SV40, HERS-SV40/hTERT의 경우 장기간 배양 후에도 세포 형태의 변화가 없으며 세포증식이 일정하게 유지되는 것을 확인하였다. DPESC-SV40 또한 일차배양 DPESCs에 비하여 장기간 배양 후에도 세포 형태의 변화가 없으며 세포증식이 일정하게 유지되는 것을 확인할 수 있었다. 일차배양 치계줄기세포와 확립된 세포주간의 특성분석을 위하여 flow-cytometry를 통한 세포표면항원 분석, PCR을 이용한 상피줄기세포 및 배아줄기세포 마커의 발현양상을 통해 차이가 없음을 확인하였다. 치계상피줄기세포 유래 조건배지를 8, 12, 18일 동안 처리한 결과 8일차부터 칼슘의 침착이 보였으며 12일차부터 기존의 조골분화 조건에서보다 조

건배지(Conditioned medium)를 처리하였을 때 칼슘 침착이 확연히 증가하는 것을 확인할 수 있었다. 이상의 연구를 종합하면 치계상피줄기세포와 치계중간엽줄기세포간의 상호작용을 통하여 치계중간엽줄기세포의 경조직 형성능이 증가함을 알 수 있었다. 본 연구를 통해 일차배양 치계상피세포의 세포주를 확립하고 특성분석을 통해 일차배양 치계세포와 차이가 없음을 확인하였다. 또한 확립된 세포주로부터 얻은 조건배지가 치계중간엽줄기세포인 SHEDs의 경조직 형성능의 가속화에 영향을 미치는 것을 확인하였다. 따라서 일차배양 치계상피세포로부터 확립한 세포주인 HERS-SV40, HERS-SV40/hTERT, DPESC-SV40는 치계상피줄기세포의 기능연구와 치아재생연구에 기여할 수 있을 것으로 기대된다.

주요어 : 치아발생, 상피-중간엽 상호작용, 치계상피줄기세포, 치계중간엽줄기세포, 조건배지, 경조직 형성

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