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Establishment of Hertwig's epithelial root sheath cell line from cells involved in epithelial–mesenchymal transition

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ABSTRACT

The epithelial–mesenchymal transition (EMT) is an important event in the developmental process of various organs. In periodontal development during root formation of a tooth, this EMT has been a subject of controversy. Hertwig's epithelial root sheath (HERS), consisting of two epithelial layers, plays a role of inducing odontogenesis during root development and thereafter becomes fragmented. Some researchers have maintained that in the process of this fragmentation, some HERS cells change from epithelial to mesenchymal cells. Here, we established a HERS cell line (HERS01a) and examined its gene and protein expression. Immunohistochemical staining and real-time PCR analysis showed that HERS01a cells expressed vimentin and N-cadherin as mesenchymal markers as well as cytokeratin14, E-cadherin, and p63 as epithelial stem cell markers. In the presence of TGF-β, HERS01a cells also expressed many more mesenchymal markers, as well as snail1 and 2 as EMT markers. Taken together, our data show that HERS01a displayed unique features associated with EMT in the root formation process, and will thus be useful for analyzing the biological characteristics of HERS and the molecular mechanism underlying the EMT.

1. Introduction

The formation of a tooth root occurs by the sequential and reciprocal interactions between the Hertwig's epithelial root sheath (HERS) and the surrounding mesenchyme, as well as by crown development [1]. HERS consists of the epithelial bilayer derived from the cervical loop epithelium at the cuff of the enamel organ. After the completion of crown development, HERS fuses below the level of the cervical margin of the crown [2]. Many studies have indicated that HERS is involved in the induction of odontoblast differentiation and subsequent dentin deposition during root formation through epithelial–mesenchymal interactions [3,4]. Root formation starts as HERS begins to develop at postnatal day 5 (PN5d), and the root elongates for approximately 3 weeks postnatally. HERS is maintained at the apex of the developing root [5]; and at the other side of HERS, the epithelium disintegrates into epithelial cell rests of Malassez in the periodontal ligament.

Recently, it was reported that the expression pattern of growth factors changes at the transitional stage from crown morphogenesis to root formation. Meanwhile, Fgf-10 signaling in the dental pulp disappears [6]; and it was reported that epidermal growth factor and insulin-like growth factor-I signaling regulates the formation and elongation of HERS in organ cultures [2,7]. Moreover, it was suggested that HERS cells possibly possess the capability for undergoing the epithelial–mesenchymal transition (EMT) [8]. However, this characteristic of HERS remains unclear, because whether it is an original feature of HERS cells or the consequence of stimulation by the surrounding mesenchyme has not been yet answered.

In vitro studies would be very useful to understand the characteristics of HERS cells. Though some cell lines derived from HERS have already been reported [9–11], in the present study we also produced a HERS cell line, examined its characteristics in terms of protein and gene expression patterns, and compared them with those of HERS *in vivo*. As a result, we obtained a new HERS cell line that is very useful to study the molecular mechanism underlying the EMT during root development.

Abbreviations: CK14, cytokeratin14; EGFr, epidermal growth factor receptor; EMT, epithelial–mesenchymal transition; Fgf, fibroblast growth factor; HERS, Hertwig's epithelial root sheath; IGF-Ir, insulin-like growth factor-I receptor; PN, postnatal; RT, room temperature; TGF-β, transforming growth factor beta.

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2. Materials and methods

2.1. Animals

The design and conditions of the animal experiments were approved by the Committee on Animal Experiments of Iwate Medical University, Morioka, Japan. Newborn ddY mice were purchased from Japan SLC Inc., (Shizuoka, Japan). Mouse first mandibular molars on postnatal day 6 (PN6d) were used for preparation of HERS lineage cells, and PN5d mice were used for preparation of frozen sections for immunohistochemical analysis.

2.2. Immunohistochemical staining

The dissected mouse mandibular bones without chemical fixation and decalcification were embedded in super cryo-embedding medium (Leica Microsystems, Japan), and rapid-frozen by the hexane-dry ice method. The samples were cut with a cryostat (Leica Microsystems, Germany) into 6-μm-thick sections by using the Film transfer method [12]. The sections on the film were dried in the cryo-chamber for 12 h, and rinsed in PBS at room temperature (RT). After having been blocked in 10% horse serum (RT, 1 h), the sections reacted with the following antibodies (RT, 1 h): anti-cytokeratin 14 (Covance), anti-vimentin (DacoCytomation), anti-insulin-like growth factor-I receptor (Santa Cruz), anti-EGF receptor (Epitomics), anti-notch2 (Santa Cruz), anti-sonic hedgehog (Santa Cruz), anti-E-cadherin (BD), and anti-N-cadherin (Sigma). As negative controls, sections were incubated with 1% BSA/PBS instead of primary antibody or with the second antibody only. The sections were then reacted with Alexa Fluor™ 546 or 488-labeled secondary antibodies (Molecular probes) at RT for 1 h.

The cells in culture dishes were fixed in 4% paraformaldehyde and/or acetone/ethanol at RT for 15 min, once they had reached approximately 60% confluence. After a rinse in PBS and incubation in 0.1% Triton X-100/PBS when necessary, they were reacted by using the above antibodies as well as antibodies against P63 (Lab Vision) and ameloblastin (the courtesy of Prof. Uchida, Hiroshima University, Japan) as *per* the histological protocol.

2.3. HERS cell culture

HERS cells that collected from PN6d mouse mandibular first molar germs were seeded in a culture dish (PRIMARIA™, BD) and cultured in DMEM/HAM F-12 medium (GIBCO) supplemented with B27 (Invitrogen), fibroblast growth factor-2 (20 ng/ml), in a humidified atmosphere of 5% CO_2 at 37 °C. When the cells had reached approximately 80% confluence, they were passaged with 0.25% trypsin/EDTA (GIBCO) and maintained as a HERS cell line. These cells were plated in culture dishes at a density 1×10^5 cells/dish, and the medium was changed every other day.

2.4. RNA preparation and RT-PCR

The culture medium was removed from the cells, and the cells were washed twice with PBS. Then, the cells were scraped from the dish with a sterile cell scraper, and collected in Eppendorf tubes. Total RNA was isolated from the HERS cells by using RNeasy® mini (Takara, Japan), according to the manufacturer's instructions; and cDNAs were synthesized by using a PrimeScript® RT reagent Kit (Takara). After mixing SYBR Premix Ex Taq™ II premix (Takara) with each cDNA, amplification was performed in a Thermal Cycler Dice Real Time System, TP-800 (Takara). Primer sequences for each cDNA were the following: 5′-GTC TCC TCT GAC TTC AAC A-3′ (forward) and 5′-CAG GAA ATG AGC TTG ACA AA-3′ (reverse) for GAPDH; 5′-CAA GAC CAT CGA GGA CCT GAA-3′ (forward) and 5′-CAG GCT CTG CTC CGT CTC AA-3′ (Reverse) for cytokeratin14; 5′- AAA GCG TGG CTG CCA AGA AC-3′ (forward) and 5′-GTG ACT GCA CCT GTC TCC GGT A-3′ (reverse) for vimentin; 5′-CGT CCT GCC AAT CCT GAT GA-3′ (forward) and 5′-ACC ACT GCC CTC GTA ATC GAA C-3′ (reverse) for E-cadherin; 5′-CGC CAA TCA ACT TGC CAG AA-3′ (forward) and 5′-TGG CCC AGT GAC GCT GTA TC-3′ (reverse) for N-cadherin; 5′-GTG GTC ATT TCA GAT GCG ATT CA-3′ (forward) and 5′-ATT CCC GAG GCA TGT GCA G-3′ (reverse) for fibronectin; 5′-ACG CCA CCT GCC TGG ATA AG-3′ (forward) and 5′-CAC ACT GCC CGT TGT TCA CAC-3′ (reverse) for notch2; 5′-AGC AGA CCG GCT GAT GAC TC-3′ (forward) and 5′-TCA CTC CAG GCC ACT GGT TC-3′ (reverse) for sonic hedgehog; 5′-TCT GAA GAT GCA CAT CCG AAG C-3′ (forward) and 5′-TTG CAG TGG GAG CAG GAG AAT-3′ (reverse) for snail1; 5′-GGC TGC TTC AAG GAC ACA TTA GAA C-3′ (forward) and 5′-GGT CTG CAG ATG TGC CCT CA-3′ (reverse) for snail2; 5′-ACC GGG ATC TCA TCA GCT TCA C-3′ (forward) and 5′-TCC TTG TTC GGA GGC AGG TC-3′ (reverse) for IGF-I receptor; 5′-GCA TCC AGT GCC ATC CAG AA-3′ (forward) and 5′-GCT GGG CAG GTC TTG ACA CA-3′ (reverse) for EGF receptor. Results of quantitative RT-PCR were standardized to GAPDH, and compared as a ratio of each expressed gene.

2.5. Cell culture with TGF-β

HERS cells were seeded in culture dishes at a density 1×10^5 cells/dish, and pre-cultured in DMEM/HAM F-12 medium (GIBCO) supplemented with B27 (Invitrogen) in a humidified atmosphere of 5% CO_2 at 37 °C for 5 days. After pre-culture, the medium was changed to culture medium with/without 10 ng/ml transforming growth factor beta (TGF-β); and the HERS01a cells were then cultured for 8 days. Thereafter the cells were examined immunocytochemically, as described above.

3. Results

3.1. Immunohistochemical features of HERS in vivo

HERS could be seen in the lower first molar germs at PN5d. HERS cells, which had originated from the enamel organ, expressed epithelial markers such as cytokeratin14 (CK14), E-cadherin, and epidermal growth factor receptor (EGFr) (Fig. 1A, D, G). Interestingly, some of the HERS cells showed positive immunoreactivity for mesenchymal markers vimentin or N-cadherin (Fig. 1B, E). The results suggest that some cells in HERS had characteristics of both epithelial and mesenchymal cells (Fig. 1C, arrowheads). Immuno-reactions indicating the presence of Shh, notch2, and insulin-like growth factor receptor (IGF-Ir) were also detected in HERS (Fig. 1F, H, I). These results are consistent with those of previous reports [2,7,8].

3.2. Isolation of HERS cells and cell culture

We prepared cultures of HERS cells collected from PN6d mouse mandibular first molar germs. Dissected tooth germs were soaked in 1% collagenase at 4 °C for 2 h and separated into dental epithelium and mesenchyme, after which sheets of HERS were cut off from the dental epithelium (Fig. 2A, B). The sheets were incubated in 0.25% trypsin/EDTA solution (GIBCO) at 37 °C for 5 min, and HERS cells were isolated. During the first five passages, 3 weeks were required for the cells to reach confluence in culture. After more than 60 repeated passages, the cells showed a cobblestone-like appearance and retained active cell-proliferating potency. A single cell clone (HERS01) was obtained from one of the cultures by the limiting dilution-culture technique. The cells of this clone, designated HERS01a, proliferated actively (Fig. 2C–F), formed

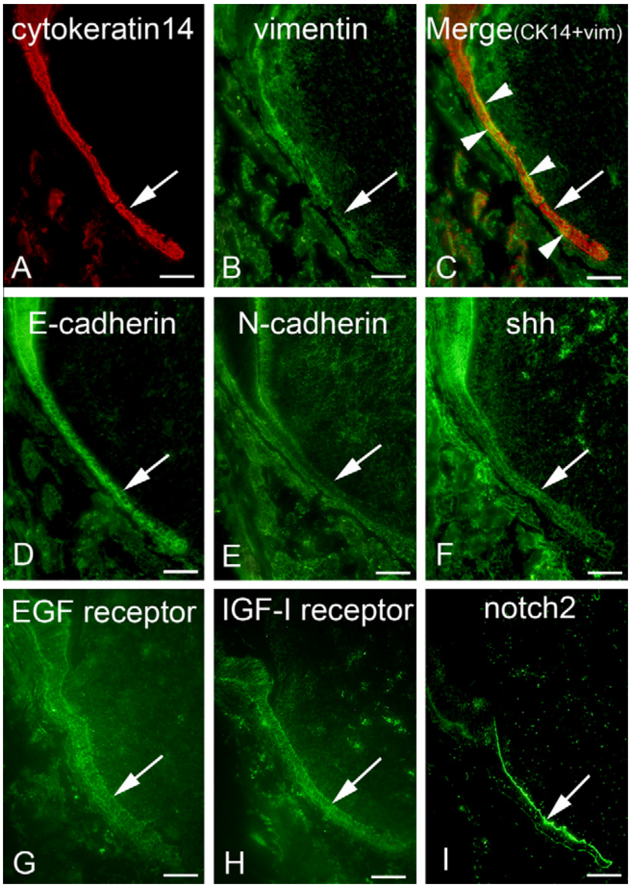

Fig. 1. Immunohistochemical phenotype of HERS in mandibular first molar at PN5d. CK14 (A) is detected, and vimentin (B) is observed weakly, in HERS. C shows merged-photos of "A" and "B". Some cells in HERS had characteristics of both epithelial and mesenchymal cells (C, arrowheads). E-cadherin (D), N-cadherin (E), Sonic hedgehog (F), EGF receptors (G), and IGF-I receptors (H) are detected in HERS. Notch2 (I) is detected in HERS, and especially the intensity is strongly in the inner layer of HERS. Each arrow points to HERS. Scale bars = 50 μm.

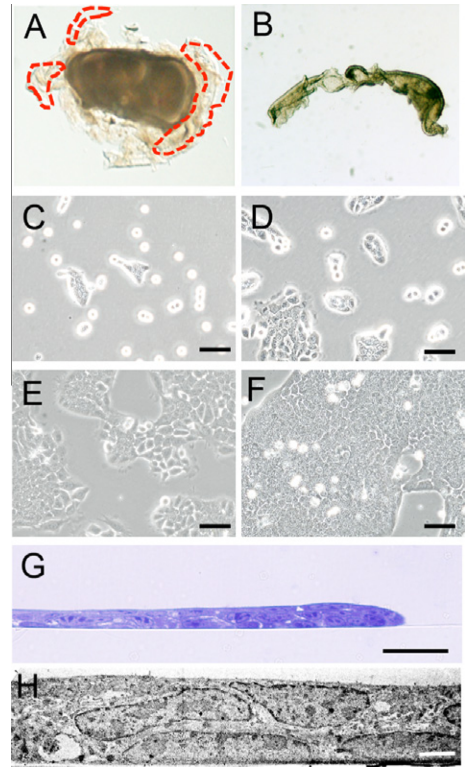

Fig. 2. Microscopy of HERS sheets and cell growth patterns. Dissected tooth germs (A) separated into dental epithelium and mesenchyme, and sheets of HERS (B) were cut off from the dental epithelium. After 60 passages in culture, the immortalized cells were established. Colonization begins at day 1 of culture (C); and small cell masses develop at day 3 (D), spread at day 5 (E), and become almost confluent by day 7 (F). Microscopy by staining with toluidine blue shows that HERS01a cells form two cell layers by culture day 7 (G). Electron microscopy shows a cell having a flat shape and sparse cytoplasm in the double layer. Scale bars = 50 μm (C–F), 20 μm (G), 5 μm (H). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

colonies by 3 days in culture, and became confluent by 7 days. Interestingly, at that time the cells became stratified, making two layers (Fig. 2G). Electron microscopic observation showed the typical HERS cell feature of having a high nuclear/cytoplasm ratio (Fig. 2H).

3.3. mRNA expressions in HERS01a cells

We characterized the HERS01a cells by comparing them with a mouse dental epithelial cell line (mHAT9a). Both HERS01a and mHAT9a cells expressed *CK14*, *E-cadherin, notch2*, *Igf-Ir*, and *Egfr* (Fig. 3A). Interestingly, the HERS01a cells expressed *vimentin* and *N-cadherin* at levels 1000- and 2000-fold, respectively, greater than those found in mHAT9a. The expression of *Shh* mRNA could not be detected in either cell type.

3.4. Immunocytochemistry of HERS01a

We characterized HERS01a cells by immunostaining them. The cells expressed not only epithelial markers CK14 (Fig. 3B), E-cadherin (Fig. 3E), P63 (Fig. 3D), Egfr (Fig. 3H) but also mesenchymal markers vimentin (Fig. 3C) and N-cadherin (Fig. 3F). These results are consistent with those obtained by the real-time PCR analysis. Immunostaining for notch2 (Fig. 3G), Igf-Ir (Fig. 3I), and ameloblastin (Fig. 3J) as HERS markers was positive.

3.5. Alteration of HERS01a cells in the presence of TGF-β

When HERS01a cells were cultured in the presence of TGF-β, cells at the edge of colonies became irregular, extended cellular processes, and migrated away from the periphery of the colony (Fig. 4A, arrows). Also, the cells started to express vimentin more obviously; and the expression of CK14 was down-regulated (Fig. 4A, arrows). Furthermore, we examined quantitatively the mRNA expression for epithelial and mesenchymal markers by performing real-time PCR (Fig. 4B). In the presence of TGF-β, the relative quantity of *CK14* and *E-cadherin* decreased. On the other hand, the expression of mRNAs of *N-cadherin*, *vimentin*, and *fibronectin* increased 5.4, 6.4, and 6.7-fold respectively, compared with the control levels. The cells in the TGF-β group also started to express *snail1* and *2*, which are involved in the EMT.

4. Discussion

HERS has unique features in comparison with the other parts of the dental epithelium. Our immunohistochemical study showed that some cells in the intact HERS co-expressed epithelial and mesenchymal markers, findings identical with those of some previous reports [8,11,13]. These results suggest strongly the possibility of EMT in HERS during root development, but this hypothesis has remained controversial. It is notable that not all cells in HERS have the ability to undergo the EMT; rather, only a small minority of them can do so. Although some HERS cell lines transformed by

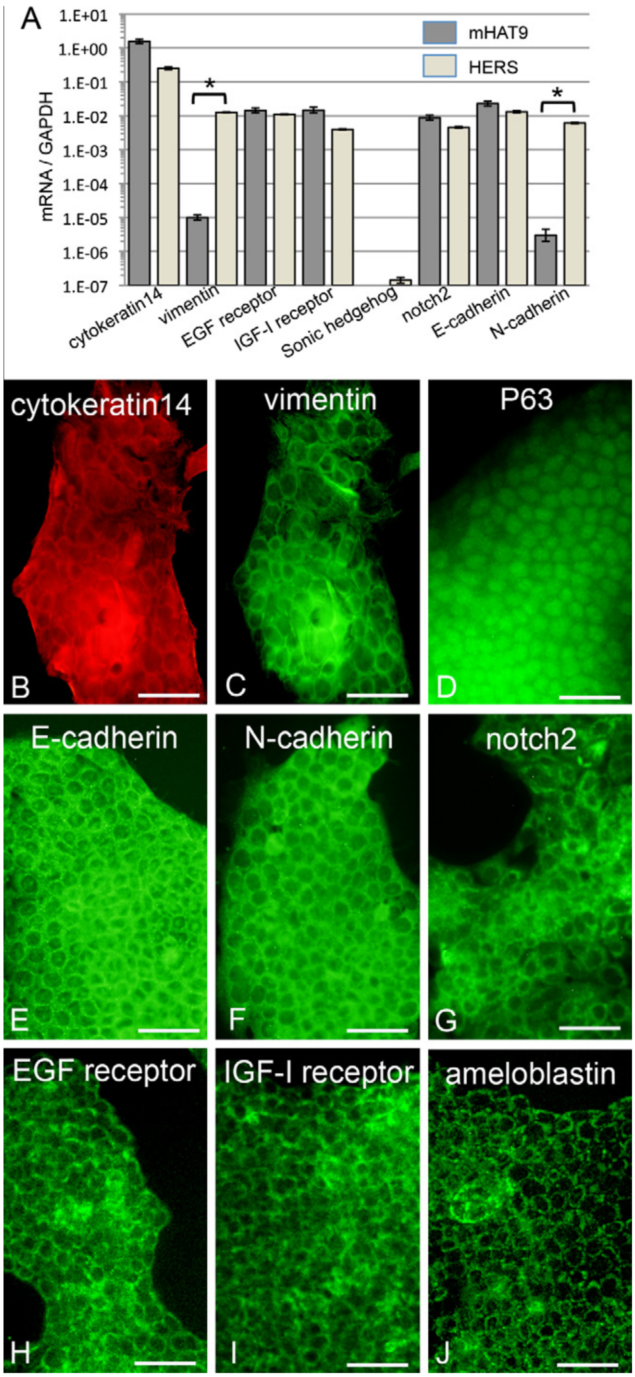

Fig. 3. (A) Expression levels of various mRNAs in HERS01a cells. Total RNA from HERS01a cells was analyzed by real-time PCR, and the expression level of each target mRNA was compared with the corresponding one of mHAT9a, an enamel epithelial cell line, after standardization by GAPDH. HERS01a cells expressed *CK14*, *vimentin*, *IGF-I receptor*, *EGF receptor*, *E*-and *N-cadherin*, and *notch2* mRNAs, respectively, but not *sonic hedgehog* mRNA. *Vimentin* and *N-cadherin* mRNAs in HERS01a cells were expressed at higher levels than those in mHAT9a cells. Data are expressed as means ± SD. * $p < 0.01$. (B–J) Fluorescence immunocytochemistry of HERS01a cells. Staining with each antibody was performed on the cells. Double staining for CK14 (B) and vimentin (C) simultaneously in HERS01a cells gave positive reactions. P63 (D), E-cadherin (E), N-cadherin (F), notch2 (G), EGF receptor (H), IGF-I receptor (I), and ameloblastin (J) proteins were also detected in the cells. Scale bars = 50 μm.

use of SV-40 have been reported [11], these cell lines do not have the characteristics of the HERS cells *in vivo*. Hence, we sought to establish a cell line derived from this minor population of HERS cells having this unique character (showing EMT).

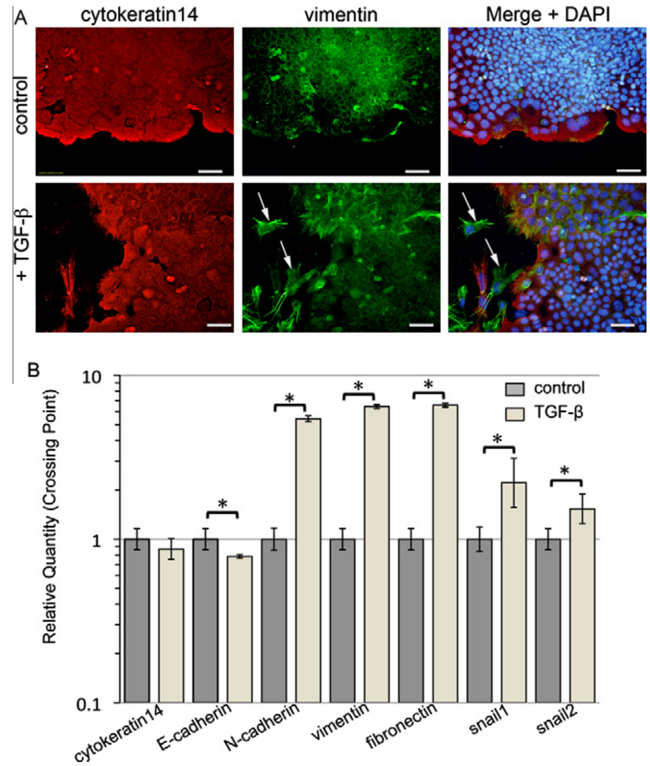

Fig. 4. Effects of TGF-β on mRNA/protein expression in HERS01a cells. After a 5-day pre-culture period, HERS01a cells were cultured for 8 days in medium containing TGF-β. After the cells had been fixed, they were double-stained for CK14 and vimentin (A). Almost all HERS01a cells in the control group expressed both CK14 and vimentin. In the TGF-β-treated group, cell groups that strongly expressed vimentin were observed in spots. The cells that had migrated from a colony expressed only vimentin, not CK14. (B) Results of real-time RT-PCR showed higher values of *N-cadherin*, *vimentin*, and *fibronectin* for the treated cells than for the control ones. Furthermore, the expression of *snail1 and 2* was increased in cells of the TGF-β group. Scale bars = 50 μm (applies to all photos in "A"). Data are expressed as means ± SD. * $p < 0.05$.

Immunohistochemical study and real-time PCR analysis showed that the clonal HERS01a cells expressed both epithelial markers such as CK14, E-cadherin, and P63, as well as mesenchymal markers such as vimentin, N-cadherin, and fibronectin, in addition to growth factor receptors that are detected in the intact HERS. This study is the first report of the successful establishment of an immortalized mouse HERS cell line that presents unique characteristics associated with the EMT.

The EMT plays crucial roles in the formation of the body plan, in the differentiation of multiple tissues and organs during embryonic development, in tissue repair during wound healing, and in tumor metastasis. When cells undergo the EMT, they change their morphology from epithelial to mesenchymal, and start migration. Furthermore, the cells increase the expression of mesenchymal markers and decrease that of epithelial ones [14–16]. The EMT is known to be regulated by expression of Snail through TGF-β signaling [14–16]. Some HERS01 cells in the presence of TGF-β changed their morphology from cobblestone to an elongated shape with cell processes, and these cells moved away from the colony. Furthermore, they showed increased expression of vimentin, and lost the expression of CK14, as judged immunocytochemically. The results of the real-time PCR assays showed that the TGF-β-treated cells increased their expression of mesenchymal markers such as *vimentin*, *N-cadherin*, and *fibronectin* and decreased that of epithelial markers such as *CK14* and *E-cadherin*. Also, TGF-β induced the gene expression of *Snail1 and 2* in the HERS01a cells. TGF-β is known to induce the gene expression of *Snail* by acting through *Smad* family members [14]. Because HERS cells also express *Smad4*

[17], these results suggest that TGF-β is an important growth factors in the EMT of HERS during root development. Taken together, our data show that HERS01a cells had the ability to undergo the EMT and that TGF-β could trigger the EMT of these cells.

With respect to root development, some aspects remain to be clarified. HERS disintegrates *in vivo* and forms a meshwork referred to as the epithelial cell rests of Malassez (ERM). These clonal HERS01a cells might be an *in vitro* model to studying ERM formation as well as be a helpful tool to elucidate the mechanism underlying HERS development.

5. Conclusion

We established an immortalized HERS cell line (HERS01a) and examined its gene and protein expression. HERS01a cells maintained the cell characteristics of the intact HERS. Furthermore, HERS01a displayed unique features associated with the EMT in the root formation process. Thus this cell line will be useful for further analysis of the biological characteristic of HERS and the molecular mechanisms involved in the EMT.

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