Biomedical Research (Tokyo) **38** (1) 61–69, 2017

Hertwig's epithelial root sheath cells contribute to formation of periodontal ligament through epithelial-mesenchymal transition by TGF-β

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(Received 14 November 2016; and accepted 29 November 2016)

ABSTRACT

In tooth root development, periodontal ligament (PDL) and cementum are formed by the coordination with the fragmentation of Hertwig's epithelial root sheath (HERS) and the differentiation of dental follicle mesenchymal cells. However, the function of the dental epithelial cells after HERS fragmentation in the PDL is not fully understood. Here, we found that TGF-β regulated HERS fragmentation via epithelial-mesenchymal transition (EMT), and the fragmented epithelial cells differentiated into PDL fibroblastic cells with expressing of PDL extracellular matrix (ECM). In the histochemical analysis, TGF-β was expressed in odontoblast layer adjacent of HERS during root development. Periostin expression was detected around fragmented epithelial cells on the root surface, but not in HERS. In the experiment using an established mouse HERS cell line (HERS01a), TGF-β1 treatment decreased E-cadherin and relatively increased N-cadherin expression. TGF-β1 treatment in HERS01a induced further expression of important ECM proteins for acellular cementum and PDL development such as fibronectin and periostin. Taken together, activation of TGF-β signaling induces HERS fragmentation through EMT and the fragmented HERS cells contribute to formation of PDL and acellular cementum through periostin and fibronectin expression.

Hertwig's epithelial root sheath (HERS) is a bilayered epithelial structure derived from enamel epithelium that exists as the boundary between two dental mesenchymal tissues, the dental papilla and the dental follicle (16). The part of HERS at the apex of the developing root becomes fragmented, allowing cementoblasts or fibroblasts derived from dental follicles to come in contact with the outer surface of the root. Recently, we have suggested that HERS was mainly composed with outer enamel epithelium, and interacted with dental follicle cells for root and periodontal ligament (PDL) development after fragmentation (19). This is initiation of reciprocal interactions between the dental epithelium and mesenchyme for root formation. However, the molecular mechanism of this interaction in tooth root formation is less understood compared with that in crown formation.

The fate of epithelial cells derived from HERS has been discussed for decades. To date, various fates for HERS after fragmentation have been demonstrated: *1)* incorporation in PDL maintenance as epithelial cell rests of Malassez (ERM) (7, 8, 18, 24), *2)* cell death through apoptosis (4, 13), or *3)* participation in cementum and PDL formation as mesenchymal cells via epithelial-mesenchymal transition (EMT) (1, 20) or as epithelial cells (3, 12, 28). Since the number of dental epithelial cells is limited

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in an erupted tooth, to clarify the fragmentation process and the fate of HERS in the developing root must be important to consider about its role.

Recently, it has been demonstrated that epithelial cells derived from HERS can transition into mesenchymal cells through EMT under TGF-β signaling *in vitro* (1, 15, 20). *In vivo*, several gene mutated mice models of TGF-β signaling have reported defects in tooth root formation. Mice in which *tgfbr2* was conditionally deleted in odontoblasts and osteoblasts of alveolar bone using Osterix-Cre (*Osx-Cre*;*Tgfbr2$^{fl/fl}$*) lacked proper root formation (25), and cellular cementum was reduced when *tgfbr2* was conditionally inactivated of cementoblasts using Osteocalcin-Cre (*OC-Cre*;*Tgfbr2$^{fl/fl}$*) (6). Interestingly, mice with conditional deletion of both TGF-β/BMP signaling in odontoblasts (*OC-Cre*;*Smad4$^{fl/fl}$*) resulted in a high rate of keratocystic odontogenic tumors in the jaw (11). Furthermore, *K14-Cre*;*Smad4$^{fl/fl}$* mice exhibited arrest of root formation; HERS was elongated, but not fragmented (9). Taken together, TGF-β signaling appears to play a central role in both of dental mesenchyme and epithelium during tooth root development. However, the precise effects of TGF-β signaling on EMT of HERS and the fate of HERS-derived fragmented cells remain unclear.

Periostin, initially identified as osteoblast-specific factor 2, secreted from osteoblasts (23), is specifically expressed in collagen-rich fibrous connective tissues such as PDL (21). Adult *periostin* null mice were shown to exhibit a periodontal disease phenotype including external root resorption caused by collagen fibril disorganization with defective fibronectin expression (17, 22). Additionally, it has been reported that TGF-β can increase periostin expression in PDL (27).

In the present study, we evaluated the functional significance of TGF-β signaling on EMT and fragmentation in HERS cells, and suggested that the fragmented HERS cells played as PDL fibroblasts with periostin expression.

MATERIAL AND METHODS

The protocol for these experiments was approved by the Ethics Committee of Fukuoka Dental College (No. 11019) and Iwate Medical University (No. 27-042), and carried out in accordance with the Institutional Animal Research Guidelines.

Animals. Mice that conditionally express red fluorescent protein variant (tdTomato) in their dental epithelial cells were generated by crossing K14-Cre mice, which Cre-mediated recombination system driven by the keratin 14 (K14) promoter (10), with R26tdTomato mice (The Jackson Laboratory, Stock: 007909).

Immunohistochemistry. The mandibular first molars in *K14-cre*;*R26-tdTomato* mice at postnatal day 10 (P10) were used. Following sampling, the mandible bones were fixed with 10% neutralized buffered formalin overnight and then decalcified for 3 days. The specimens were then dehydrated in graded ethanol solutions, embedded in paraffin, and cut into 6 μm sections for immunostaining. The specimens were incubated with primary antibody rabbit anti-periostin (Abcam, Cambridge, UK) and rabbit anti-TGF-beta (Abcam) for 2 h at room temperature, followed by incubation with secondary antibodies, biotin-conjugated or Alexa Fluor® 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR), for 1 h at room temperature. The specimens incubated with biotin-conjugated secondary antibody were sensitized using streptavidin peroxidase (Vector Laboratory, Burlingame, CA) and visualized using a DAB kit (Nichirei Biosciences, Inc., Tokyo, Japan). Methyl green and DAPI (Vector Laboratories, Burlingame, CA) were used for nuclear staining.

HERS01a cell culture. HERS01a cells, which we previously established and characterized (1), were cultured in control medium DMEM/HAM F-12 medium (Gibco, Grand Island, NY) supplemented with B27 (Invitrogen, Carlsbad, CA), fibroblast growth factor-2 (FGF2) (10 ng/mL), epidermal growth factor (EGF) (10 ng/mL), and penicillin-streptomycin (100 U/mL) in a humidified atmosphere of 5% CO_2 at 37°C.

Immunocytochemistry. After pre-cultured HERS01a cells, the medium was change to culture medium with/without 10 ng/mL TGF-β1 for 48 h. Cells were fixed with cooled methanol for 10 min and cooled acetone for 1 min at −20°C. After the cells were washed in PBS, they were treated with 4% normal goat serum to prevent non-specific reactions. The primary antibodies described above and mouse anti-cytokeratin 14 (Abcam) were used. The immunoreactions were visualized with anti-IgG antibody conjugated with Alexa Fluor® 488 or 594 (Molecular Probes), followed by counterstaining with DAPI (Vector Laboratories). Stained cells were observed by immunofluorescence microscopy (KEYENCE, Osaka, Japan).

Table 1 *Primers used in RT-PCR*

Gene	Primer sequences	
	Forward	Reverse
Gapdh	5′–TGTGTCCGTCGTGGATCTGA–3′	5′–TTGCTGTTGAAGTCGCAGGAG–3′
E-cadherin	5′–CGTCCTGCCAATCCTGATGA–3′	5′–ACCACTGCCCTCGTAATCGAAC–3′
N-cadherin	5′–CGCCAATCAACTTGCCAGAA–3′	5′–TGGCCCAGTGACGCTGTATC–3′
Fibronectin (Fn)	5′–GTGGTCATTTCAGATGCGATTCA–3′	5′–ATTCCCGAGGCATGTGCAG–3′
Periostin (Postn)	5′–CAGTTGGAAATGATCAGCTCTTGG–3′	5′–CAATTTGGATCTTCGTCATTGCAG–3′
Type I collagen (Col1)	5′–GGGTCCCTCGACTCCTACA–3′	5′–TGTGTGCGATGACGTGCAAT–3′
Bone sialoprotein (Bsp)	5′–AGGACTAGGGGTCAAACAC–3′	5′–AGTAGCGTGGCCGGTACTTA–3′
Osterix (Osx)	5′–GAAAGGAGGCACAAAGAAG–3′	5′–CACCAAGGAGTAGGTGTGTT–3′
Osteocalcin (Ocn)	5′–AGACTCCGGCGCTACCTT–3′	5′–CTCGTCACAAGCAGGGTTAAG–3′
Alkaline phosphatase (Alp)	5′–ATCTTTGGTCTGGCTCCCATG–3′	5′–TTTCCCGTTCACCGTCCAC–3′
Runx2	5′–GCCCAGGCGTATTTCAGA–3′	5′–TGCCTGGCTCTTCTTACTGAG–3′

Real-time PCR. After pre-culture, the medium was changed to culture medium with/without 10 ng/mL TGF-β1 or cells were pre-treated with an TGF-β type I receptor (ALK5) inhibitor (SB431542; Calbiochem, La Jolla, CA) prior to the addition of TGF-β1 for 48 or 96 h.

The culture medium was removed, and the cells were washed twice with PBS. RNA was then extracted using the RNeasy® Mini kit (Qiagen, Valencia, CA) and cDNA was synthesized using PrimeScript® II Reverse Transcriptase (Takara, Otsu, Japan). After mixing SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA) with each cDNA, amplification was performed in a CFX96™ Real-Time System (Bio-Rad). Primer sequences for the genes investigated are shown in Table 1. Results were standardized to *Gapdh* expression and compared as a ratio of each expressed gene. Fold differences in gene expression were calculated according to the $_{\Delta\Delta}C_T$ method with normalization to *Gapdh*.

Western blot analysis. Cells were washed with PBS and lysed in EBC buffer [1 M Tris-HCl (pH 7.5), 5 M NaCl, 0.5% Nonidet P-40] supplemented with a protease inhibitor cocktail (PhosSTOP; Roche, Indianapolis, IN). Total protein was separated on 10% SDS-PAGE gels (SuperSep™Ace; Wako Pure Chemical Industries Ltd., Osaka, Japan) and transferred to Immobilon™-P PVDF membranes (Millipore, Bedford, MA). The membranes were immunoblotted separately with mouse anti-β-actin (Santa Cruz Biotechnology Inc., Dallas, TX), mouse anti-E-cadherin (BD Biosciences, San Jose, CA), rabbit anti-N-cadherin (OriGene Technologies Inc., Rockville, MD), rabbit anti-fibronectin (Abcam), or rabbit anti-periostin (OriGene Technologies Inc.) overnight at 4°C. Immunoblots were then incubated with peroxidase-conjugated secondary antibodies directed against mouse (A4416; Sigma-Aldrich, St. Louis, MO) or rabbit IgG (A4914; Sigma-Aldrich), detected with Immobilon™ Western (Millipore), and exposed to X-ray film to detect protein expression levels. The relative band densities were analyzed using ImageJ (National Institutes of Health, Bethesda, MD).

Statistical analysis. Significant differences between groups were determined using the Mann-Whitney test. Data are presented as the means ± SD. A P-value of < 0.05 was considered significant.

RESULTS

TGF-β expression during root development in vivo
The expression of TGF-β during root and PDL development was shown in Fig. 1. In *K14-cre*;*Rosa26-tdTomato* mice, *tdTomato* fluorescence-positive cells indicates both of primary epithelial cells and mesenchymal cells derived from epithelial cells (EMT cells). In this experiment, *K14-cre*;*Rosa26-tdTomato* mice at P10 were used to observe the behavior of all HERS and HERS-derived cells. The *tdTomato*-positive cells were observed in HERS and many fragmented HERS-derived cells in the periodontal tissue (Fig. 1A, D). TGF-β expression was ubiquitously detected in dental follicle and dental papilla, and specially enhanced in odontoblastic layer of dental papilla (Fig. 1E, F arrows). The onset of odontoblast differentiation was consistent with the beginning of HERS fragmentation (Fig. 1D, E, F). It means that the up-regulation of TGF-β expression in odontoblasts induces the fragmentation of HERS.

Periostin expression during root development in vivo
Next, we performed immunohistochemical analysis

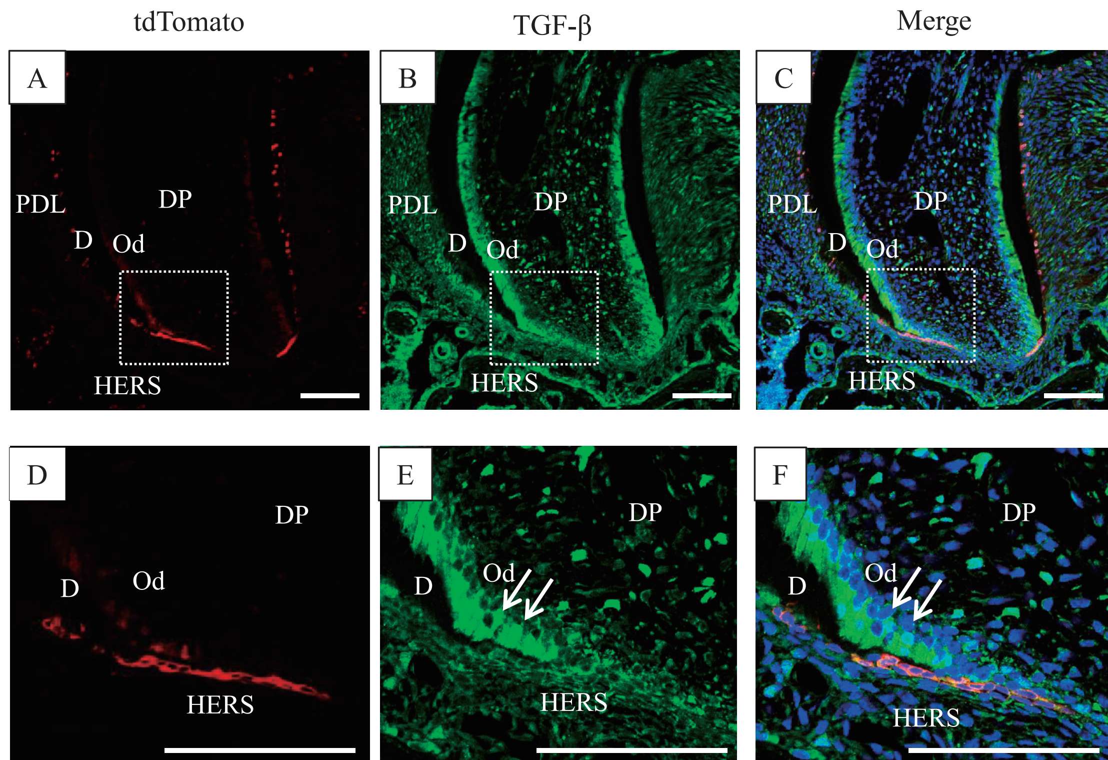

Fig. 1 TGF-β ligands expression during root development in mice at postnatal day 10. Immunofluorescence staining of TGF-β in *K14-cre*;*Rosa26-tdTomato* mice. The mandibular first molar at postnatal day 10 (P10) was used. A magnified view of the dotted square in A, B, and C are shown in D, E, and L, respectively. (**A**) Immunofluorescence of tdTomato is observed in HERS. (**B**) TGF-β is expressed in PDL and DP. (**E**) Enhanced expression of TGF-β is detected in odontoblast layer (arrows). Merged images of tdTomato and TGF-β are shown in C and F. Scale bar: 100 μm, PDL: periodontal ligament, DP: dental pulp, Od: odontoblast, D: dentin, HERS: Hertwig's epithelial root sheath

to observe the localization of periostin. Fibrotic periostin was observed in overall of the developing PDL (Fig. 2A, B). Interestingly, the surface of developing root dentin was coated thinly by periostin (Fig. 2B, arrowheads). Furthermore, periostin expression was localized at the *tdTomato*-positive-fragmented HERS cells on the surface of developing root dentin (Fig. 2D arrows), but not at unfragmented HERS in root apex (Fig. 2B and E, arrowheads).

TGF-β1 causes loss of cell-cell adhesion, decreased E-cadherin expression and increased N-cadherin expression

To elucidate whether TGF-β is associated with HERS fragmentation, HERS01a cells were initially cultured with TGF-β1 for 48 and 96 h, and E-cadherin and N-cadherin mRNA and protein expressions were examined. *E-cadherin* expression remained unchanged within 48 h, but slightly increased after 96 h by TGF-β1 treatment (Fig. 3A). However, E-cadherin protein expression in western blot analysis was significantly decreased by TGF-β1 (Fig. 3B). TGF-β1 strongly induced *N-cadherin* mRNA expression, which was partially inhibited by SB431542 treatment (Fig. 3A). N-cadherin protein in HERS01a cells was also induced by TGF-β1 for 48 and 96 h (Fig. 3B).

To determine the effect of TGF-β1 on HERS01a cellular behavior, we evaluated E-cadherin and N-cadherin expressions using immunocytochemical analysis. In standard culture conditions, HERS01a cells exhibited the characteristic cobblestone-like appearance of epithelial cells. The cells located at the edge of epithelial islands strongly expressed E-cadherin (Fig. 3C-a, c, d), and N-cadherin was hardly observed in HERS01a cells cultured in control medium (Fig. 3C-b). After TGF-β1 stimulation, the cobblestone-like appearance was disturbed with decreased E-cadherin expression, and the cells in the margin of epithelial islands were spindle-shaped with decreased cell-cell attachment (Fig. 3C-e). Conversely, N-cadherin was strongly induced in HERS01a cells treated with TGF-β1 (Fig. 3C-f, g, h).

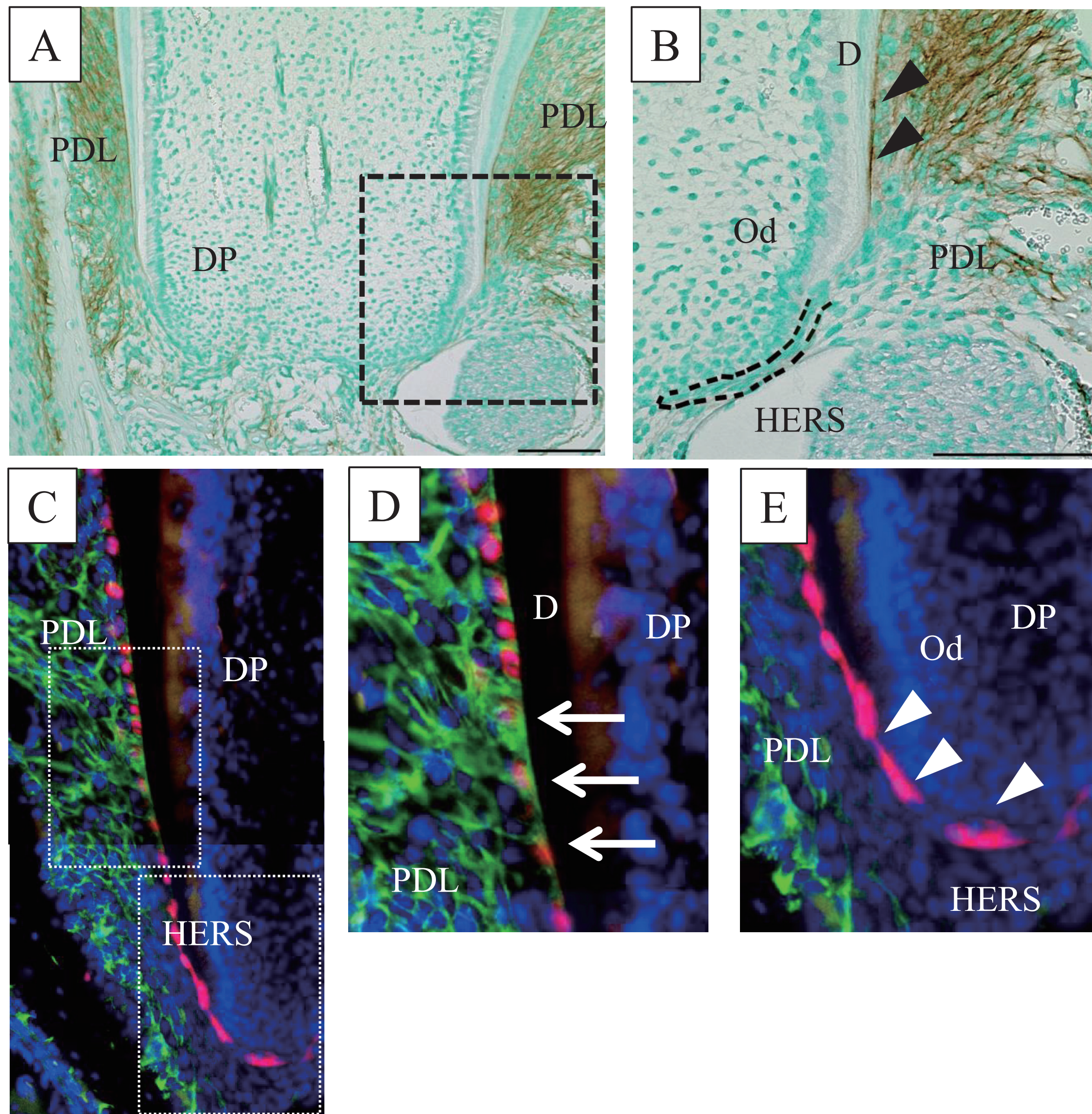

Fig. 2 Periostin expression during root development in mice at postnatal day 10. The mandibular first molar in mice at postnatal day 10 (P10) was used. (**A**) Periostin was expressed ubiquitously in the PDL. A magnified view of the dotted square in (A) is shown in (B). (**B**) Periostin expression was observed on the root surface (arrowheads). (**C**–**G**) Immunofluorescence staining of periostin with tdTomato is shown. Magnified views of the dotted squares in (C) are shown in (D) and (E). (**D**) Periostin expression overlapped tdTomato-positive cells on the root surface (arrows). (**E**) Periostin expression was not detected in HERS. Scale bar: 100 µm, PDL: periodontal ligament, DP: dental pulp, Od: odontoblast, D: dentin, HERS: Hertwig's epithelial root sheath

Fibronectin and periostin, but not cementoblast/osteoblast cell markers, are induced in HERS01a cells by TGF-β1

To validate the hypothesis that HERS01a cells differentiate into cementoblasts or fibroblasts by TGF-β signal, we examined the expression of markers for cementoblast/osteoblast and PDL fibroblast cell in HERS01a cells treated with TGF-β1. The cementoblast/osteoblast cell markers, *bone sialoprotein* (*BSP*), *osterix* (*Osx*), *osteocalcin* (*Ocn*), *alkaline phosphatase* (*Alp*), *Runx2*, and *type I collagen* (*colI*) expressions were not changed by TGF-β1 treatment (Fig. 4A). Interestingly, PDL fibroblast markers, *fibronectin* (*Fn*) and *periostin* (*Postn*) expressions were strongly induced by TGF-β1, and the response was inhibited by SB431542 (Fig. 4A). The results suggested that HERS01a cells can be transdifferentiated into PDL fibroblast-like cells, but not cementoblasts, by

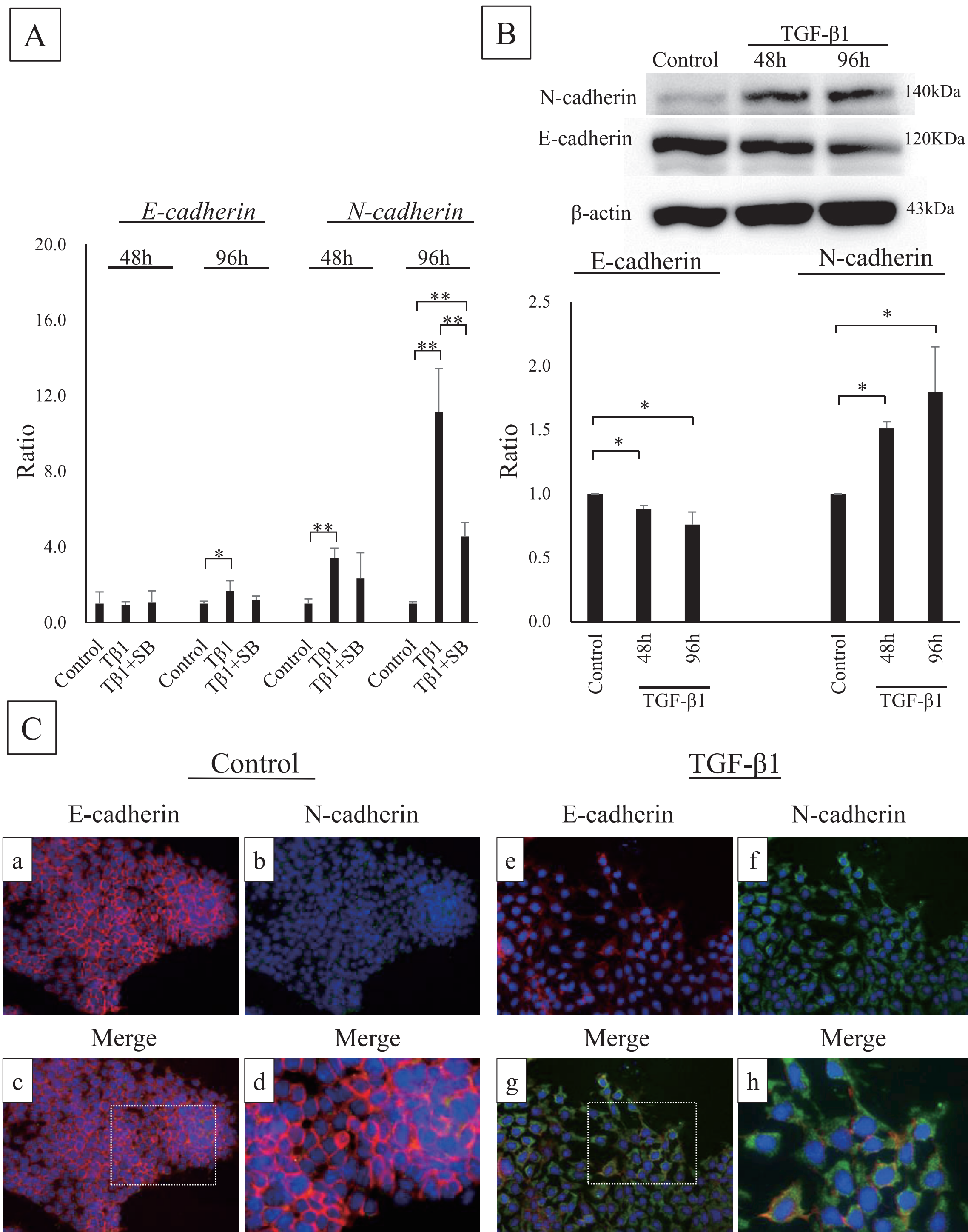

Fig. 3 Effects of TGF-β1 on mRNA/protein expression of E-cadherin and N-cadherin in HERS01a cells. (**A**) The mRNA expressions of *E-cadherin* and *N-cadherin* in HERS01a cells with/without TGF-β1 treatment for 48 or 96 h were examined. An ALK5 inhibitor (SB) was used to inhibit the effect of TGF-β1 treatment. (**B**) E-cadherin and N-cadherin protein expressions were examined in HERS01a cells treated with TGF-β1 for 48 or 96 h by western blot analysis. Each expression was normalized to that of β-actin. Data are expressed as the mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$. (**C**) E-cadherin (red) and N-cadherin (green) expressions were examined by immunocytochemical analysis. Representative E-cadherin (a), N-cadherin (b), and merged (c) images under control conditions are shown. A magnified view of the dotted square in (c) is shown in (d). Representative E-cadherin (e), N-cadherin (f), and merged (g) images under TGF-β1 treatment are shown. A magnified view of the dotted square in (g) is shown in (h). Cell nuclei are shown as blue.

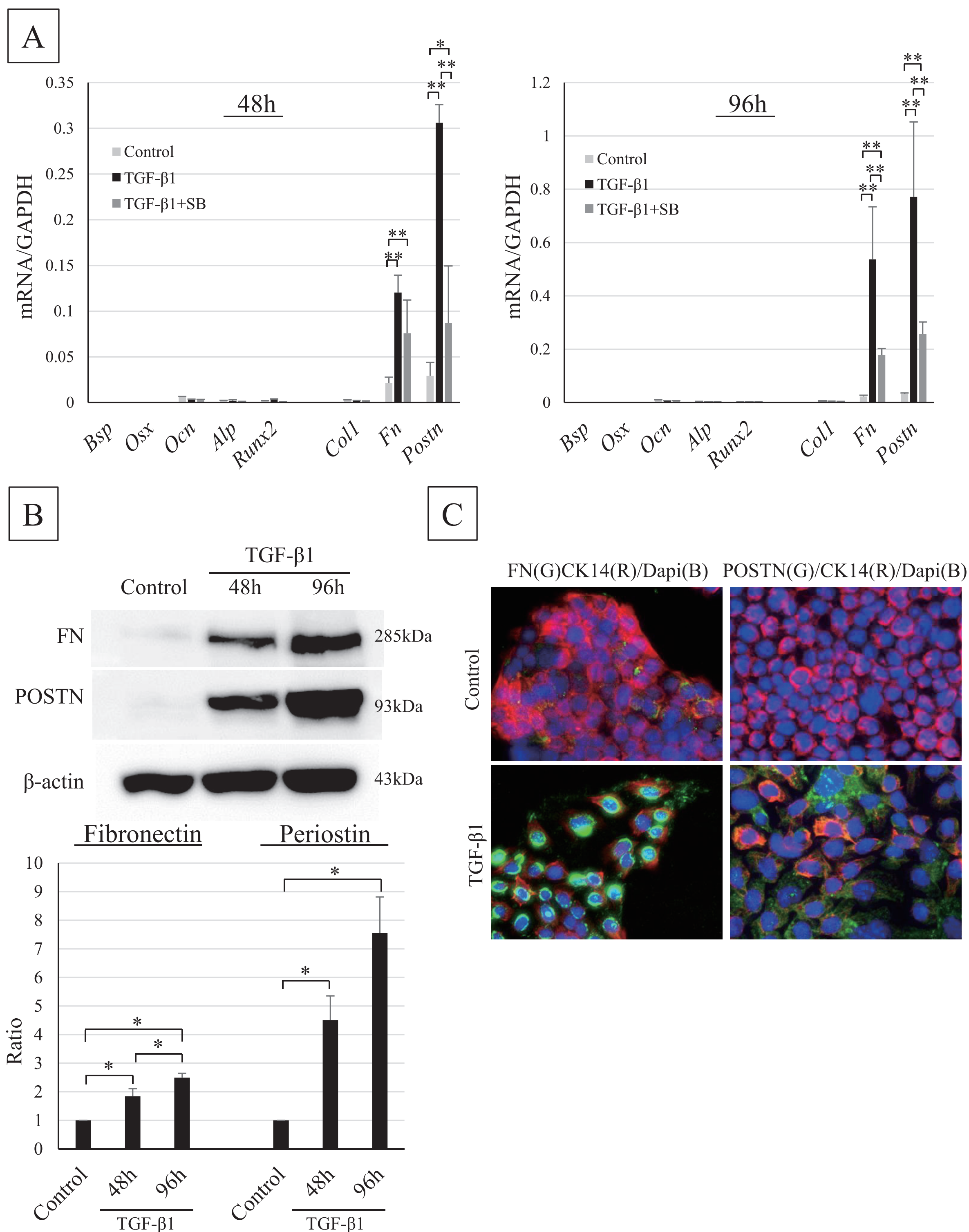

Fig. 4 Effects of TGF-β1 on cell markers expression in HERS01a cells. (**A**) The gene expressions in HERS01a cells following treatment with TGF-β1 for 48 or 96 h were examined. An ALK5 inhibitor (SB) was used to inhibit the effect of TGF-β 1 treatment. (**B**) Fibronectin (FN) and periostin (POSTN) protein expressions were examined in HERS01a cells treated with TGF-β1 for 48 or 96 h by western blot analysis. Each expression was normalized to that of β-actin. Data are expressed as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$. (**C**) Cytokeratin 14 (CK14; red) and fibronectin (FN; green) or periostin (POSTN; green) expressions were examined by immunocytochemical analysis. In control conditions, fibronectin and periostin expressions were faint, however, their expressions were induced in HERS01a cells by TGF-β1 treatment. Cell nuclei are shown as blue.

TGF-β1 regulation.

Periostin and fibronectin expression are induced after EMT in HERS01a cells

To determine whether TGF-β1 affected PDL fibroblastic protein expression in HERS01a cells, we performed western blot analysis. Fibronectin and periostin protein expressions were significantly increased in HERS01a cells treated with TGF-β1 (Fig. 4B). To further characterize fibronectin- and periostin-positive HERS01a cells following TGF-β1stimulation, we evaluated cytokeratin 14 expression as dental epithelium marker using immunocytochemical analyses. Fibronectin expression was not detected in control conditions but was strongly induced by TGF-β1. Additionally, fibronectin-positive cells also expressed CK14 (Fig. 4C). Periostin expression was hardly observed in control conditions but strongly induced by TGF-β1. Interestingly, CK14 expression was diminished in periostin-positive cells (Fig. 4C).

DISCUSSION

TGF-β-induced EMT is a fundamental process in organogenesis and cancer progression (14, 26). In this process, epithelial cells lose E-cadherin expression while N-cadherin expression is upregulated (2). We confirmed this cadherin switch in HERS01a cells treated with TGF-β1. This finding suggests that TGF-β signaling initially regulates HERS fragmentation during tooth root formation through the degradation of E-cadherin for epithelial cell-cell adhesion. Similarly, as the arrest of root formation in *K14-Cre*;*Smad4*$^{fl/fl}$ mice was shown to be caused by continuous elongation of the cervical loop (9), activation of the signaling cascade by TGF-β likely triggers the switch from crown to root formation. Our immunohistochemical observation of TGF-β expression detected its enhanced expression in odontoblast layer. It was suggested that paracrine signaling of TGF-β secreted by odontoblasts regulates HERS fragmentation.

TGF-β signaling may also regulate the fate of HERS-derived cells after fragmentation via EMT. The present study demonstrated that mesenchymal gene expressions were upregulated in HERS01a cells treated with TGF-β1, such as *N-cadherin, fibronectin* and *periostin*. The increased expression of Snail1 and Snail2, markers of EMT were also confirmed (Data not shown). This finding indicates that some of fragmented HERS cells transdifferentiated into PDL fibroblast-like cells through EMT. On the other hand, HERS01a cells did not express *bone sialoprotein* and *osterix*, cementoblast-related genes, even after TGF-β1 treatment. Therefore, HERS cells likely do not undergo differentiation into cementoblast-like cells by TGF-β. FGF2 is one of candidate to regulate the differentiation of HERS-derived cells into cementoblast-like cells (5). Regarding this points, further investigation should be needed.

In immunocytochemical analysis for fibronectin and cytokeratin14 expressions in HERS01a cells, fibronectin was significantly upregulated with TGF-β1, and most of fibronectin-positive cells did not lose cytokeration14. This observation indicated that fragmented HERS epithelial cells with cytokeratin14 on the surface of root including epithelial cell rest of Malassez (ERM) may join acellular cementum formation through expression of collagen binding proteins such as fibronectin and N-cadherin *in vivo*. Interestingly, HERS01a cells with periostin induced by TGF-β1 showed partial loss of cytokeratin14 expression. It means that HERS-derived cells with periostin expression were characterized as same as PDL fibroblast after EMT with losing cytokeratin14 expression. Taken together, the fate of HERS-derived cells showed two ways, *1)* fragmented epithelial cells join acellular cementum formation through PDL ECM secretion as epithelial cells, *2)* play for PDL formation as PDL fibroblast mesenchymal cells under TGF-β regulation. This difference may depend on dosage of TGF-β protein around the epithelial cells. We have previously shown that quiescent stem cells were involved in ERM clusters of PDL, and ERM cells have proliferative activity (18). If our present findings about the process of HERS fragmentation in PDL development can replace the investigation of biological function of ERM in matured PDL, it means that ERM cells in matured PDL may possible to play as PDL fibroblast-like cells. Our findings may represent a new therapeutic target for cementum and PDL regeneration.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

Acknowledgements

This work was supported in part by Japan Society for the Promotion of Science (JSPS) KAKENHI [CHOUSENTEKI HOUGA; No. 15K15762) to KO].

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