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The Epithelial-Mesenchymal Transition During Tooth Root Development

Jee-Hae Kang, Jin-Ho Park, Yeon-Hee Moon, Jung-Sun Moon, Sun-Hun Kim, and Min-Seok Kim*

Dental Science Research Institute, 2nd Stage Brain Korea, School of Dentistry, Chonnam National University

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Hertwig's epithelial root sheath (HERS) consists of bi-layered cells derived from the inner and outer dental epithelia and plays important roles in tooth root formation as well as in the maintenance and regeneration of periodontal tissues. With regards to the fate of HERS, and although previous reports have suggested that this entails the formation of epithelial rests of Malassez, apoptosis or an epithelial-mesenchymal transformation (EMT), it is unclear what changes occur in the epithelial cells in this structure. This study examined whether HERS cells undergo EMT using a keratin-14 (K14) cre:ROSA 26 transgenic reporter mouse. The K14 transgene is expressed by many epithelial tissues, including the oral epithelium and the enamel organ. A distinct K14 expression pattern was found in the continuous HERS bi-layer and the epithelial diaphragm were visualized by detecting the β-galactosidase (lacZ) activity in 1 week postnatal mice. The 2 and 4 week old mice showed a fragmented HERS with cell aggregation along the root surface. However, some of the lacZ-positive dissociated cells along the root surface were not positive for pan-cytokeratin. These results suggest that the K14 transgene is a valuable marker of HERS. In addition, the current data suggest that some of the HERS cells may lose their epithelial properties after fragmentation and subsequently undergo EMT.

Key words: HERS, EMT, K14 cre mice, tooth development

Introduction

After the end of crown morphogenesis in tooth development, the inner and outer dental epithelia from the cervical loop of the enamel organ begin to fuse, extend and produce a bi-layered epithelial sheath known as the Hertwig's epithelial root sheath (HERS). HERS regulates the cells of the dental papilla to differentiate into odontoblasts to form root dentin (Owens, 1979; Thomas and Kollar, 1989), and continues to grow in the apical direction regulating the size, shape and number of roots by acting as a signaling center (Ten Cate, 1996; Yamamoto *et al*., 2004). As the root forms, HERS disrupts, allowing dental follicular cells to come into contact with the root surface and differentiate into cementoblasts (Thomas, 1995; Hammarström, 1997). Ultimately, these fragmented epithelial cells aggregate in the periodontal ligament and form cell clusters known as a Malassez's epithelial cells rests. However, some studies showed that not all HERS cells migrate away from the forming root, but some cells remain on the root surface and regulate the amount of acellular cementum deposition (MacNeil and Thomas, 1993; Thomas, 1995; Kaneko *et al*., 1999; Kim *et al.*, 2011). Regarding the fate of HERS cells after fragmentation, Kaneko *et al.* (1999) suggested that apoptotic cell death can be another possible mechanism, whereas some reports suggested the possibility that HERS may undergo an Epithelial-mesenchymal transformation (EMT) and transform into functional cementoblasts (Zeichner-David *et al*., 2003; Luan *et al.*, 2006).

EMT is a common process in early embryonic development, such as gastrulation (Sanders and Prasad, 1989), sclerotome (Solursh, *et al*., 1979) and cardiac cushion mesenchyme formation (Boyer *et al*., 1999; Sakabe *et al*., 2005) as well as in pathogenesis including tumor metastasis and fibrosis (Thiery, 2002; Kalluri and Neilson, 2003). This phenotype transformation involves cellular changes in the disintegration of cell-cell junctions, loss of epithelial polarity and cytoskeletal remodeling (Zavadil and Bottinger, 2005). In addition, EMT is accompanied with protein expression changes. In other words, the *de novo* synthesis of proteins associated with mesenchymal cells, such as vimentin and fibronectin, demon-

*Corresponding author: Min-Seok Kim, D.D.S., PhD, Associate Professor, Department of Oral Anatomy, School of Dentistry, Chonnam National University, Yongbongdong 300, Gwangju 500-757, Korea, Tel: +82-62-530-4823, Fax: +82-62-530-4829, E-mail : greatone@chonnam.ac.kr

strating the loss of epithelial cytokeratins simultaneously (Janda *et al*., 2002; Grunert *et al*., 2003). During craniofacial development, EMT was implicated in cranial neural crest cell migration and fusion of the secondary palate and upper lip. (Weston, 1982; Duband *et al.*, 1995). However, most of evidence about the EMT has been provided by the observation of morphological changes or *in vitro* culture studies. Recently, the incorporation of midline epithelial seam cells into the palatal mesenchyme during palatal fusion during palatal development was ruled out using transgenic reporter mice carrying the recombinase Cre under the regulation of the keratin-14 promoter (K14-cre), which is an excellent tool for epithelial tracking (Dassule *et al*., 2000; Dudas *et al*. 2007). Therefore, the possibility of EMT during root development is still controversial and requires clarification *in vivo*.

In this study, to determine if EMT occurs during root development of the tooth, HERS cells and subsequent daughter cells were identified by the bacterial β-galactosidase (lacZ) reporter gene and X-gal staining in K14-Cre;Rosa-loxP-stop-lacZ reporter (R26R) mice, which can permanently detect the cells of the epithelial lineage *in vivo*. Concurrently, immunohistochemical staining was performed using either anti-pan-cytokeratin, an epithelial cell specific marker, or bone sialoprotein antibody, a mesenchymal cell marker, to co-localize with lacZ-positive cells. Consequently, when EMT really occurs in HERS cells, lacZ-positive and epithelial cell marker-negative or lacZ and mesenchymal cell marker-positive will be visible in the periodontal space.

Material and Method

Animals and Tissue preparation

All procedures were performed in accordance with the ethical standards recommended by the Chonnam National University Institutional Animal Care and Use Committee. Male mice carrying the K14-Cre (Dassule *et al*., 2000) alleles were crossed with females carrying the R26R conditional reporter allele (Soriano, 1999) to generate K14-Cre/+; R26R/+ mice. The mice were genotyped as described elsewhere (Soriano, 1999; Dassule *et al*., 2000). Briefly, the tail of the mice was cut, lysed in direct PCR lysis reagent (Viagen Biotech Inc, LA, CA, USA) including proteinase K (100:1) to extract the DNA. PCR was performed using the K14 and Cre primers. Postnatal 1-, 2- and 4-week-old K14-Cre/+; R26R/+ mice were perfused with 2% paraformaldehyde (PFA) in 0.1M phosphate buffer, pH 7.4 containing 2 mM $MgCl_2$, 2.25 mM EGTA, pH 8 and 0.02% NP-40 (IGEPAL CA-630; Sigma, St. Louis, MO, USA). The mandibles and maxillae were dissected and fixed further in the same fixative overnight at 4°C, followed by decalcification in 10% ethylene diamine tetraacetic acid (EDTA, pH 7.4) at 4°C for 3-4 weeks. After washing in a rinse buffer (PBS, pH 7.5, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% NP-40), the specimens were either stained as whole mounts in staining solution or allowed to sink in a 30% sucrose/phosphate buffer/$MgCl_2$. The specimens were then embedded in an OCT compound and frozen on dry ice to generate the cryosections.

β-galactosidase(lacZ) staining

Ten μm cryosections were fixed in 0.2% PFA in 0.1M phosphate buffer (pH 7.4) for 10 min, rinsed in PBS and incubated in the lacZ staining solution at 37°C for several hours or overnight. The lacZ solution consisted of 1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, 20 mM Tris-HCl, pH 7.4 in phosphate buffer (pH 7.4). After staining, the cryosections were post-fixed in 10% neutral-buffered formalin for double immunohistochemical staining.

Immunohistochemistry

Purified rabbit polyclonal anti-pan-cytokeratin was obtained from Covance Inc. (Denver, USA). The mouse anti-bone sialoprotein was a personal gift from Dr. Shi (Uni. Southern Calif. USA). Normal serum was substituted with primary antibodies for the negative control. Immunohistochemical staining was performed using a Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) Briefly, after blocking the endogenous peroxidase activity using 3% H_2O_2 for 10 min, the sections were reacted with the primary antibodies overnight at 4°C, and subsequently in biotinylated anti-mouse IgG secondary antibody for 10 min. Finally, the sections were then incubated in avidin and biotinylated horseradish peroxidase reagent for 10 min. After colorization by AEC for 5 min, the reactants were visualized and photographed using a LSM confocal microscope (Carl Zeiss, Germany).

Results

Histological appearance of HERS

Fig. 1 shows the histological appearance of HERS formation at the cervical area of the mouse first mandibular molar at postnatal day 8.5. HERS formed a continuous bi-layer of cells from the inner and outer enamel epithelia that extended in the apical direction between the dental follicle and dental papilla.

β-galactosidase(lacZ) staining

First, an undecalcified fresh frozen section was prepared to evaluate the specificity of K14 cre:R26R mice to epithelial lineage, particularly HERS and its descendants. A distinct K14 expression pattern was visualized in the continuous HERS and few dissociated cells by staining for β-galactosidase(lacZ) activity in the mouse first mandibular molar at postnatal day 7 (Fig. 2a). No blue cells were observed in the lacZ histochemical staining for non-K14 cre:R26 normal ICR mice at the cervical area of the first mandibular molar at postnatal day 8.5 (Fig. 2b)

A decalcified cryosection was made to further trace the fate

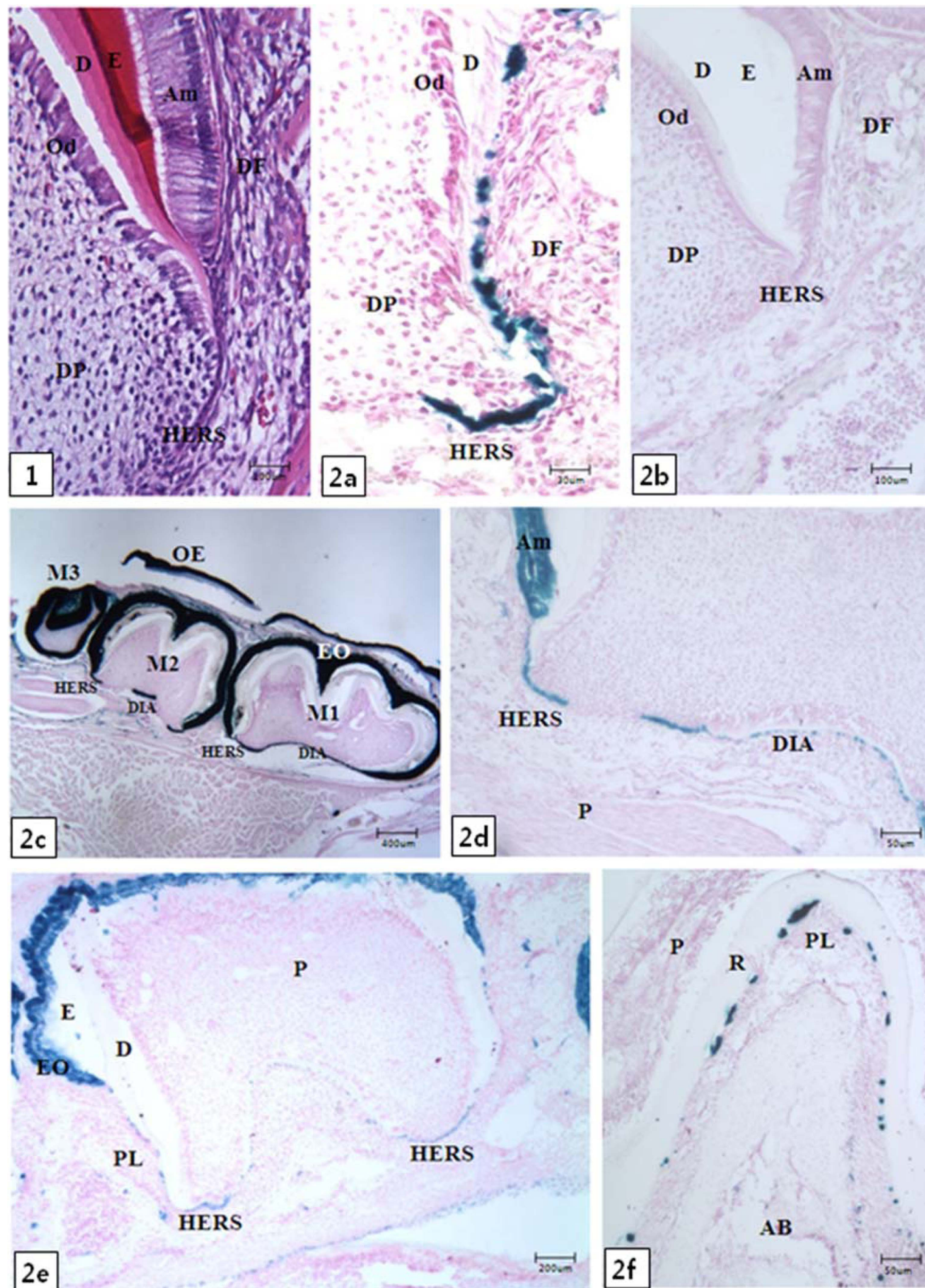

Fig. 1. The formation of Hertwig's epithelial root sheath (HERS) consisting of two epithelial layers can be seen in the mandibular 1st molar at postnatal day 8.5. Am: ameloblast, E: enamel. D: dentin, Od: odontoblast, DP: dental papilla, DF: dental follicle. H-E stain.

Fig. 2. Findings by β-galactosidase(lacZ) staining. (a) Undecalcified cryosection of the cervical area of the mandibular 1st molar at postnatal day 7. Distinct lacZ-positive Hertwig's epithelial root sheath and some dissociated cells along the root surface are observed. (b) A negative control for X-gal histochemical staining from non-K14cre:R26 normal ICR mice at the cervical area of the mandibular 1st molar at postnatal day 8.5. No positive staining can be seen. (c) A decalcified sagittal cryosection of the mandible at postnatal day 7 including 3 developing molars (m1, m2, m3). Oral epithelium (OE), enamel organ (EO), Hertwig's epithelial root sheath and epithelial diaphragm (DIA) are intensely stained for lacZ. (d) A higher magnification of decalcifed cryosection of the cervical area of the mandibular 1st molar at postnatal day 7. Like the undecalcified section, the lacZ positive reaction was detected in ameloblasts, Hertwig's epithelial root sheath and epithelial diaphragm. (e) The mandibular 1st molar at postnatal day 14 showing considerable amounts of root dentin. Several lacZ-positive disrupted and dissociated cells were observed along the developing root surfaces of the first molar and continuous Hertwig's epithelial root sheath in apical area. (f) Inter-radicular area of the mandibular 1st molar at postnatal day 28. At this stage, HERS was scattered and remained as interspersed cell aggregates that tested positive for lacZ along the root surface. Alveolar bone (AB), root (R), pulp (P), and periodontal ligament (PL) are negative.

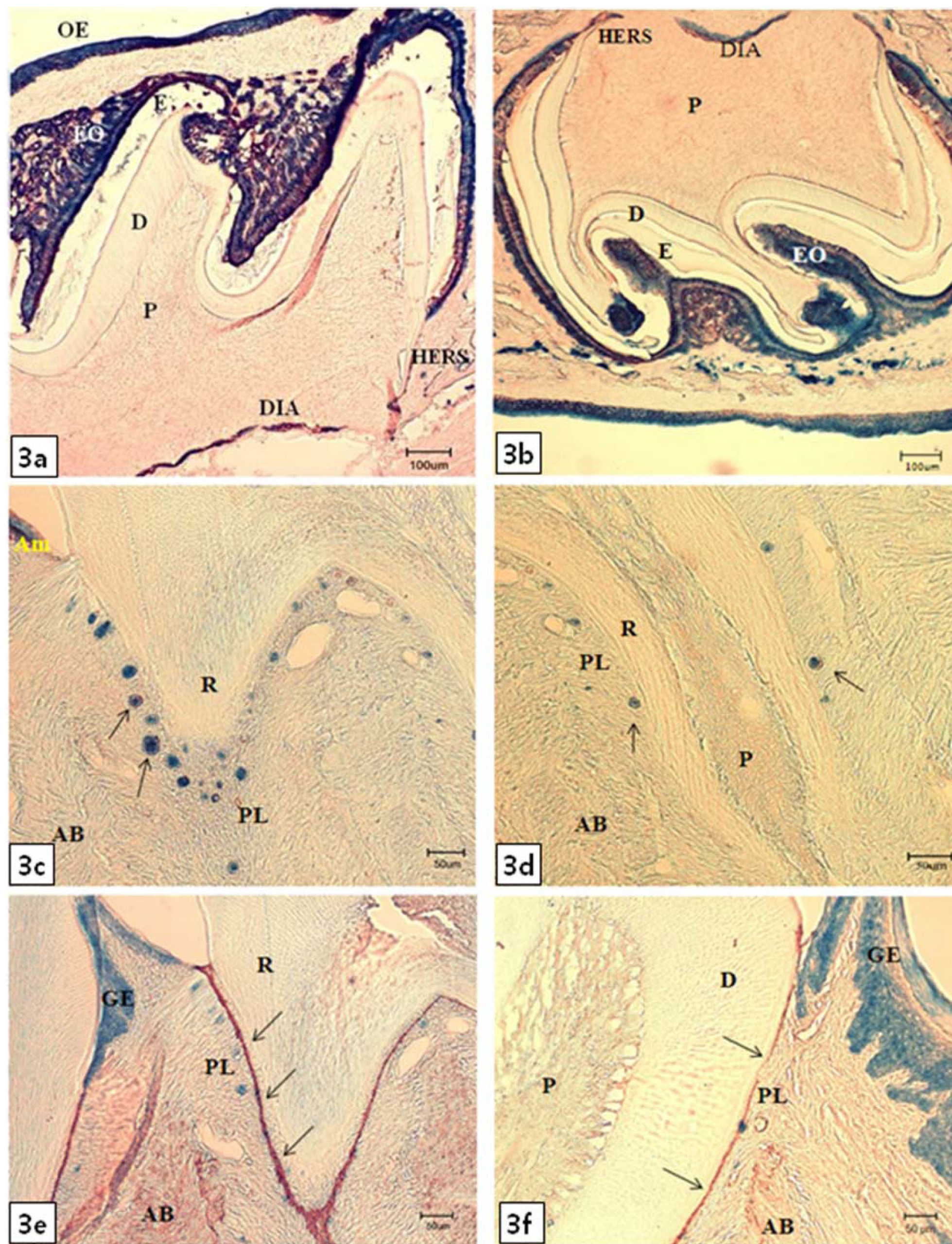

Fig. 3. Findings by Co-localization of lacZ and immunostaining. (a) Double staining for the co-localization of lacZ and pan-cytokeratin in the mandibular 1st molar at postnatal day 8.5. LacZ-positive blue staining was completely overlapped in the oral epithelium, enamel organ, Hertwig's epithelial root sheath and epithelial diaphragm at this stage. (b) LacZ and pan-cytokeratin in the maxillary 1st molar at postnatal day 8.5 is also co-localized in the same manner. (c) Double staining for the mandibular 1st molar at postnatal day 14 using X-gal and anti-pan-cytokeratin. Two lacZ-positive dissociated cell rests (arrows) along the developing root surfaces are immunoreactive against anti-pan-cytokeratin. Remnants of blue stained cells test negative for anti-pan-cytokeratin. (d) Double staining for the mandibular 1st molar at postnatal day 28 using X-gal and anti-pan-cytokeratin. Two double stained isolated cell rests (arrows) are shown along the developing root surfaces. Rest of the three lacZ-positive cells are negative for anti-pan-cytokeratin. (e) Double staining for the mandibular 1st molar at postnatal day 28 using X-gal and anti-bone sialoprotein. Definitive immunostaining for anti-bone sialoprotein is detected along the root surface, but it does not overlap with lacZ-positive cells in the periodontal space. (f) Co-localization of lacZ and bone sialoprotein at cross sectioned cervical region of the maxillary 1st molar at postnatal day 28 also showed a similar appearance to that shown in 3e. GE: gingival epithelium

and distribution of HERS cells along the developing root surface. The K14 transgene was expressed in many epithelial tissues, such as the oral epithelium, enamel organ, HERS and epithelial diaphragm in the mouse mandible at postnatal day 7 (Fig. 2c,d). Connective tissues, such as the root dentin, alveolar bone, pulp, odontoblast, LacZ reactivity for the decalcified section was comparable to the fresh section. Subsequently, the first mandibular molar at postnatal day 14 showed considerable amounts of dentin. Several lacZ-positive dissociated cells were observed along the developing root surfaces of the first

molar and a continuous HERS was observed in the apical area (Fig. 2e). The HERS cells were scattered more and remained as interspersed cell aggregates that tested positive for lacZ along the root surface in the inter-radicular area of the first mandibular molar at postnatal day 28 (Fig. 2f).

Co-localization of lacZ and immunostaining

To determine if EMT occurs during root development of the tooth, immunohistochemical staining was performed after lacZ staining using either anti-pan-cytokeratin, an epithelial cell specific marker, or bone sialoprotein antibody, a mesenchymal cell marker, to co-localize with lacZ-positive cells. lacZ-positive blue staining overlapped completely in the oral epithelium, enamel organ, HERS and epithelial diaphragm in the first mandibular (Fig. 3a) and maxillary molar at postnatal day 7 (Fig. 3b). However, some of the LacZ-positive dissociated cells along the root surface were not marked for anti-pan-cytokeratin at day 14 (Fig. 3c) and in the first mandibular molar at day 28 (Fig. 3d). The remainder of the blue stained cells tested negative for anti-pan-cytokeratin. Finally, double staining for lacZ and anti-bone sialoprotein revealed definitive immunoreactivity against anti-bone sialoprotein along the root surface in both the sagittal and cross sections of the first mandibular molar at postnatal day 28. However, it did not overlap with the lacZ-positive cells in the periodontal space (Fig. 3e,f).

Discussion

The possibility of EMT of the HERS was first proposed based on ultrastructural observations and vimentin immunostaining as well as on *in vitro* HERS cell culture (Thomas, 1995), suggesting that after the fenestration of HERS, epithelial cells that remain on the root surface take on a more mesenchymal appearance that is accompanied by a switch from keratin to vimentin expression. Others suggested that HERS undergoes EMT to produce acellular cementum forming-cementoblasts by determining the expression of markers associated with EMT and/or epithelial versus mesenchymal cells in a cell culture system (Zeichner-David *et al*., 2003). However, no clear changes in the expression of the EMT-associated factors were observed, even though a few changes in the epithelial makers, such as ameloblastin and a mesenchymal marker e.g. osteopontin, were observed. Similarly, most suggestions about the EMT of HERS were provided by observations of the morphological changes only or *in vitro* culture studies. Therefore, a stronger experimental tool is needed to track the cell fate.

In this study, an *in vivo* genetic marking system, i.e., transgenic reporter mice, was used to track the cells of the HERS after fragmentation. The K14 cre reporter mouse is a transgenic mouse in a two component genetic system (Cre-Flox or Cre-LoxP) for marking the progeny of epithelial origin cells during craniofacial development, using the K14 promoter. The advantage of the Cre/LoxP system is that the genes can be selectively over- or under-expressed in a single tissue type, rather than the whole animal with a conditioned reporter allele known as a conditioned knock in/out system (Chai *et al*., 2000, 2003; Chung *et al*., 2009). This particular mouse is valuable because it has an added marker (lacZ) to the epithelium when crossed with ROSA 26 mice (Soriano, 1999). In this study, HERS cells were visualized as epithelial band and cell aggregations along the root surface were the only structures identified as carriers of the K14 transgene in the periodontal space. Even after root formation, they lost their epithelial characteristics based on the negative immunoreactions for pan-cytokeratin antiserum but they still showed intensive staining for lacZ. Therefore, the K14 transgene is a valuable marker for HERS and only a portion of the HERS cells remained viable and remained within the periodontal space.

Immunostaining using keratin is based on previous reports, in which keratin was used as an excellent epithelial cell marker during normal tooth development (Lesot *et al*., 1982; Smith and Carroll, 1990; Lombardi *et al*., 1992), especially, HERS and Malassez's epithelial cell rests (Gao *et al*., 1988; Wetzels *et al*., 1989; Alatli *et al*., 1996; Kaneko *et al*., 1999; Onishi *et al*., 1999). In this study, immunostaining was performed using an anti-pan-keratin antibody, which can detect any keratin forming cells regardless of the maturational status, as a marker for epithelial cells along the root surface.

Bone sialoprotein (BSP), which was used as a mesenchymal cell marker in this study, was originally reported to be a mineralized tissue-related protein and expressed with osteopontin, periostin during osteogenesis or cementogenesis (Diekwisch, 2001; Bosshardt, 2004). In other words, it is not a specific marker for functional cementoblasts. Furthermore, it could be expressed by Malassez's epithelial cell rests (Xiao *et al*., 2005). However, even the cementum attachment protein (CAP), which is known as the only specific protein for cementoblast thus far, could be produced by Malassez's epithelial cell rests or gingival fibroblast in vitro (Carmona-Rodriguez *et al*., 2007). Therefore, it is quite difficult to find a morphologically detectable specific marker for functional cementoblasts. An additional problem with this study was that vimentin, which is an excellent mesenchymal marker, is not available because it is expressed in K14 transgene expressing cells, and it is very difficult to distinguish in the surrounding mesenchymal cells. Although BSP is not specific for cementoblasts, it is still available for immunostaining in the cementum. In this study, BSP was localized on the surfaces of the root dentin, probably on the newly forming cementum, as expected. However, its expression overlap with the lacZ reactivity was unclear because its immunoreactivity is not cell specific.

The present immunostaining results showed that initially, all lacZ-positive structures including the oral epithelium, enamel organ, HERS and epithelial diaphragm at postnatal week 1 molars were also stained by pan-cytokeratin antiserum. How-

ever, after root formation and the disruption of HERS, many regressing HERS cells at the root surface or in the periodontal ligament space did not show any immunoreactivity for keratin, even though a few lacZ-positive cells still expressed keratin. This indicates that some portions of HERS cells are fated to lose their epithelial characteristics, even though it could not be determined whether they undergo EMT due to the limitations of this morphologic study. Indeed, Zeichner-David *et al.* (2003) suggested that these cells maintained both epithelial and mesenchymal characteristics to produce the acellular cementum

The fate of HERS cells during root development of the tooth is still controversial. Four possible mechanisms about the fate of HERS cells have been proposed: 1) HERS cells undergo apoptosis (Kaneko *et al*., 1999; Cerri *et al*., 2000; Cerri and Katchburian, 2005), 2) they are incorporated into the newly forming cementum (Lester, 1969), 3) they migrate away from the root surface (Andujar *et al*., 1985), or 4) they undergo EMT and become functional cementoblasts (Thomas, 1995; Zeichner-David *et al*. 2003). At this point, it is impossible to confirm all these mechanisms because this study focused on EMT of HERS. However, the results suggest that some portions of HERS cells lose their epithelial peculiarities after fragmentation and might undergo EMT. Whether or not HERS is involved in cementogenesis remains to be determined.

Acknowledgements

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