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ARTICLE

Localization of Runx2, Osterix, and Osteopontin in Tooth Root Formation in Rat Molars

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SUMMARY Cementogenesis starts with the differentiation of cementoblasts. Mature cementoblasts secrete cementum matrix. Cementum components are similar to bone; moreover, cementoblasts possess many characteristics similar to those of osteoblasts. Runx2 and osterix, the transcriptional factors for osteoblast differentiation, participate in tooth formation. However, the characteristics of Runx2 and osterix during the differentiation process of cementoblasts remain unclear. In this study, we examined the immunolocalization patterns of Runx2, osterix, and osteopontin during rat molar tooth formation. Periodontal ligament cells and osteoblasts located on the alveolar bone surface showed immunoreactivity for Runx2. Colocalization of Runx2 and osterix was detected in cementoblasts, which penetrated the ruptured Hertwig's epithelial root sheath and attached to root dentin. Moreover, osteopontin was observed in Runx2-positive cementoblasts facing the root surface. However, the cells adjacent to cementoblasts showed only Runx2 reactivity. Neither Runx2 nor osterix was seen in cementocytes. These results suggest that both Runx2 and osterix are important for differentiation into cementoblasts. Additionally, osterix may be indispensable for transcription of osteopontin expression. **(J Histochem Cytochem 57:397–403, 2009)**

KEY WORDS
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differentiation
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Runx2
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cementogenesis
root formation

SUPPORTING PERIODONTAL TISSUES consist of cementum, periodontal ligament, and alveolar bone. After crown formation, the dental follicle surrounding the tooth germ contains mesenchymal cells, which differentiate into cementoblasts, fibroblasts, or osteoblasts.

In cementogenesis, dental follicular cells differentiate into cementoblasts after invading the gaps between the ruptured Hertwig's epithelial root sheath (HERS) and attaching to the root dentin. We have previously reported that heparanase, an endoglucuronidase secreted by the cells of HERS, may contribute to root formation, especially cementoblast differentiation, by degrading perlecan in the dental basement membrane (Hirata and Nakamura 2006). However, the precise mechanism of differentiation of cementoblasts has not been clarified. One possibility is that HERS cells become cementoblasts by epithelial-mesenchymal transformation (Bosshardt and Nanci 1998; Lezot et al. 2000). Recently, cementum-derived attachment protein (CAP), cementum protein-23 (CP-23), and human cementoblastoma–derived protein cementum protein 1 (CEMP 1) have been identified as factors specific to cementoblast differentiation and cementum matrix (Saito et al. 2001; Alvarez-Pérez et al. 2006; Carmona-Rodríguez et al. 2007).

Functional cementoblasts synthesize and secrete cementum matrix, which is composed of type 1 collagen and major non-collagenous proteins such as osteopontin, bone sialoprotein, and osteocalcin. All of these proteins are also components of bone; therefore, cementum matrix is similar to bone matrix. Additionally, cementoblasts are analogous to osteoblasts. Recently, Runx2, a specific regulator of osteoblast differentiation, and osterix, which is activated downstream of Runx2 during ossification in osteoblasts, were reported to be involved in tooth development (Bronckers et al. 2001; Chen et al. 2002; Berry et al. 2006; Kobayashi et al. 2006). However, no studies have described the distribution pattern of Runx2 and osterix

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during tooth root development. The characteristics of Runx2 and osterix in differentiation and proliferation of cementoblasts are far from clear. On the other hand, α-smooth-muscle actin (α-SMA), a cytoskeletal protein, is thought to be a suitable marker of undifferentiated cells, and was also examined as a marker of undifferentiated dental follicular cells (Kinner et al. 2002; Hosoya et al. 2006).

The aim of the present study was to determine the immunohistochemical localization pattern of Runx2, osterix, osteopontin, and α-SMA in the developing murine molar and to clarify their roles in and correlation with cementoblast differentiation and proliferation.

Materials and Methods

All animal experiments were conducted in accordance with the Guidelines for Animal Experiments, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan.

Tissue Preparation for Histology

In this study, six Wistar rats (CREA Japan, Inc.; Tokyo, Japan), 2 weeks of age, were used. Three rats were anesthetized with sodium pentobarbital and perfused through the left ventricle with 4% paraformaldehyde in 0.05 M phosphate buffer (pH 7.4). For assessment of cell proliferation during tooth root development, 5-bromodeoxyuridine (BrdU) immunohistochemistry was also performed. For the BrdU immunohistochemical study, three rats were given an intraperitoneal injection of BrdU (Sigma; St Louis, MO) at a dosage of 50 mg/kg body weight. Two hours after injection of BrdU, the rats were anesthetized with sodium pentobarbital and perfused through the left ventricle with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.05 M phosphate buffer (pH 7.4). Mandibles were dissected, immersed in the same fixative for 2 hr at 4C, and decalcified in 5% EDTA, pH 7.4, for 2 weeks at 4C.

For light microscopy, specimens were dehydrated in a graded ethanol series and embedded in paraffin. Four-μm-thick sections were prepared and dewaxed with xylene, and some sections were stained with hematoxylin and eosin.

Immunohistochemistry

Serial sections were transferred to 5 mM periodic acid for 10 min at room temperature to block endogenous peroxidase and then immersed in PBS containing 10% BSA for 30 min. For BrdU immunohistochemistry, after inhibition of endogenous peroxidase, DNA was denatured by incubating with 2 N HCl for 30 min at 37C. The sections were then treated with 0.1% trypsin (Sigma) in PBS for 15 min at room temperature. The sections were incubated with an anti-BrdU monoclonal antibody (Sigma) diluted 1:1000 for 12 hr at 4C. They were reacted with Histofine Simple Stain Rat MAX PO (MULTI) (Nichirei Biosciences, Inc.; Tokyo, Japan) for 1 hr at room temperature. Immunoreactivity was visualized by using diaminobenzidine (DAKO; Carpinteria, CA). Sections were then counterstained with hematoxylin and observed under an All-in-one Type Fluorescence Microscope (BZ-8000; Keyence, Osaka, Japan) using BZ Analyzer Software (Keyence).

For the immunofluorescence study of α-SMA, after washing in PBS, the sections were incubated with an anti-α–SMA monoclonal antibody (Dako Cytomation; Glostrup, Denmark) diluted 1:100 for 12 hr at 4C. Double-immunofluorescence staining was performed using an anti-Runx2 monoclonal antibody (MBL; Nagoya, Japan) diluted 1:300 and an anti-osterix polyclonal antibody (Abcam; Tokyo, Japan) diluted 1:100 or an anti-osteopontin polyclonal antibody (Nakamura et al. 1997) diluted 1:1000 for 12 hr at 4C. Sections were then incubated with Alexa Fluor-488 goat anti-mouse IgG (Molecular Probes; Eugene, OR) diluted 1:200 and Alexa Fluor-594 goat anti-rabbit IgG (Molecular Probes) diluted 1:200 for 1 hr at room temperature. Sections were then examined under the microscope (Keyence).

Control sections were incubated with mouse and rabbit IgG preimmune serum or without a primary antibody.

These immunohistochemical staining procedures were performed in 120 serial sections from each rat.

Results

Histological Observations

In 2-week-old rats, three molars in different developmental stages were observed. The mesial root of the first molars was being formed at this stage. On the pulpal side of root dentin, odontoblasts were lined in a row (Figure 1A). Acellular and cellular cementum had formed on the root dentin. HERS consisting of inner and outer enamel epithelial cells was located at the apical end of the root. Discontinuity of epithelial cells was observed at the upper end of HERS (Figure 1B).

Immunohistological Observations

Immunostaining patterns of each root of each molar were observed. The mesial root of the first molar is shown in this report, because immunolocalization of this root was representative of every staining pattern. BrdU-positive cells were detected in the pulp and outside the HERS (Figure 1C). These BrdU-positive cells seemed to localize adjacent to the HERS (Figure 1D). Immunoreactivity for BrdU was scarce in the upper region of the periodontal space and on the alveolar bone side of the dental follicle. On the pulpal side of the root, some cells in the lower region of dental

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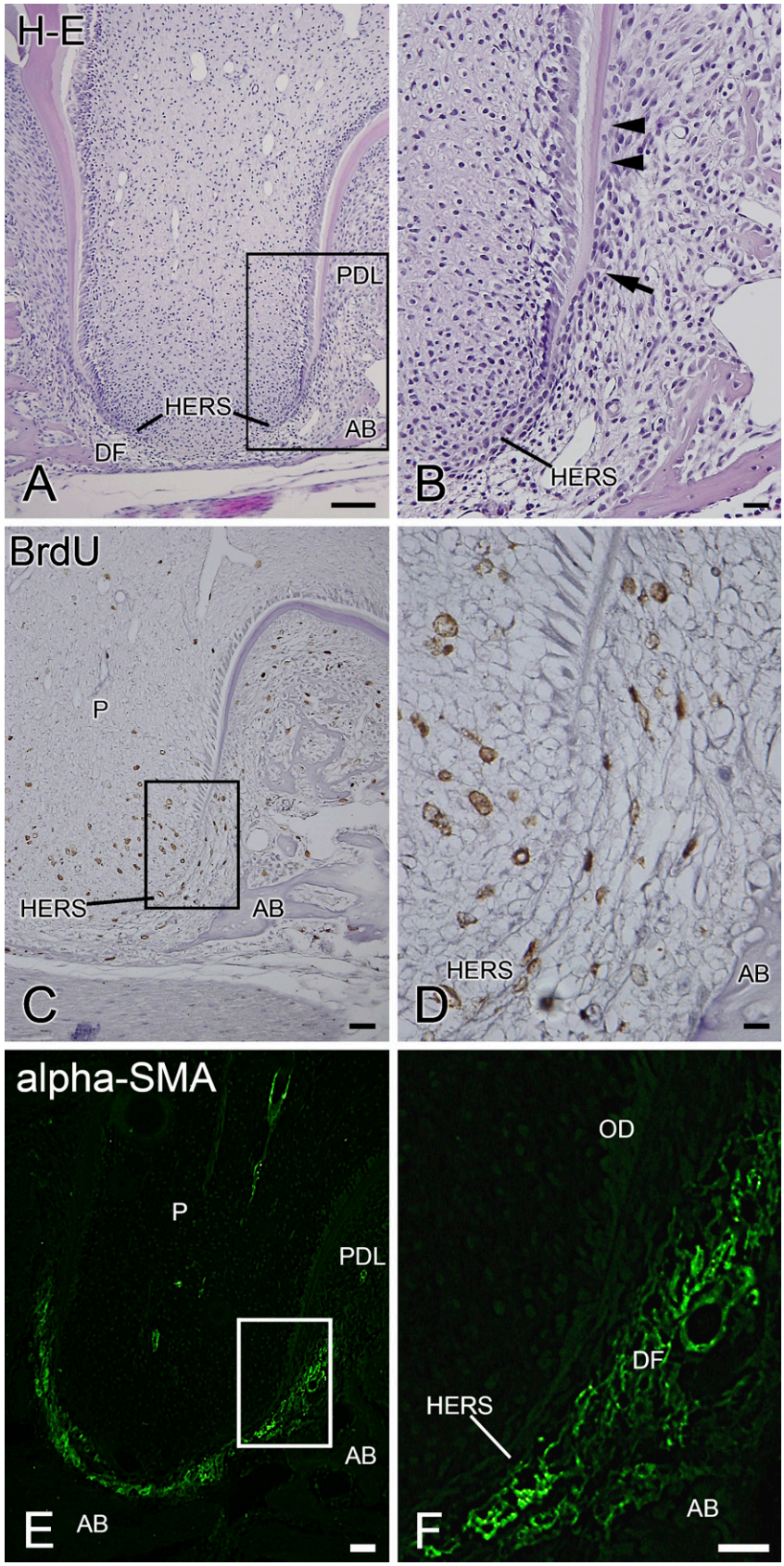


Figure 1 Micrographs of the first molar of a 2-week-old rat. (**A,B**) Light micrographs stained with hematoxylin and eosin. (**A**) Root formation is in progress. (**B**) A higher magnification of the square in **A**. Hertwig's epithelial root sheath (HERS) observed at the apical end of tooth root appears discontinuous (arrow). Both acellular and cellular cementum (arrowheads) are present on the root dentin. (**C,D**) Light micrographs showing the localization of 5-bromodeoxyuridine (BrdU). (**C**) BrdU-positive cells are observed in the pulp and outside HERS. (**D**) A higher magnification of the square in **C**. BrdU-positive cells are observed in the dental follicle along HERS. BrdU reactivity is also seen in a few cells of HERS. (**E,F**) Fluorescent micrographs showing the localization of α-smooth-muscle actin (α-SMA). (**E**) α-SMA immunoreactivity is localized to the dental follicle surrounding the tooth apex. Smooth-muscle cells of the blood vessels also show α-SMA immunoreactivity. (**F**) A higher magnification of the square in **E**. α-SMA reactivity is observed on the alveolar bone side of the dental follicle. These positive cells are spindle-shaped. AB, alveolar bone; DF, dental follicle; OD, odontoblasts; P, pulp; PDL, periodontal ligament. Bars: **A** = 50 μm; **B** = 10 μm; **C,E** = 20 μm; **D** = 5 μm; **F** = 40 μm.

papilla showed BrdU reactivity. A few cells of HERS also showed BrdU labeling.

α-SMA–positive cells were seen in the dental follicle surrounding the root apex (Figure 1E). These α-SMA–positive cells were spindle-shaped. On the outside of HERS, these positive cells seemed to localize on the alveolar side of the dental follicle (Figure 1F). In the more cervical region of the periodontal space, there was no immunoreactivity for α-SMA. In the pulp, odontoblasts and pulp cells showed no reactivity, except for smooth-muscle cells of the blood vessels.

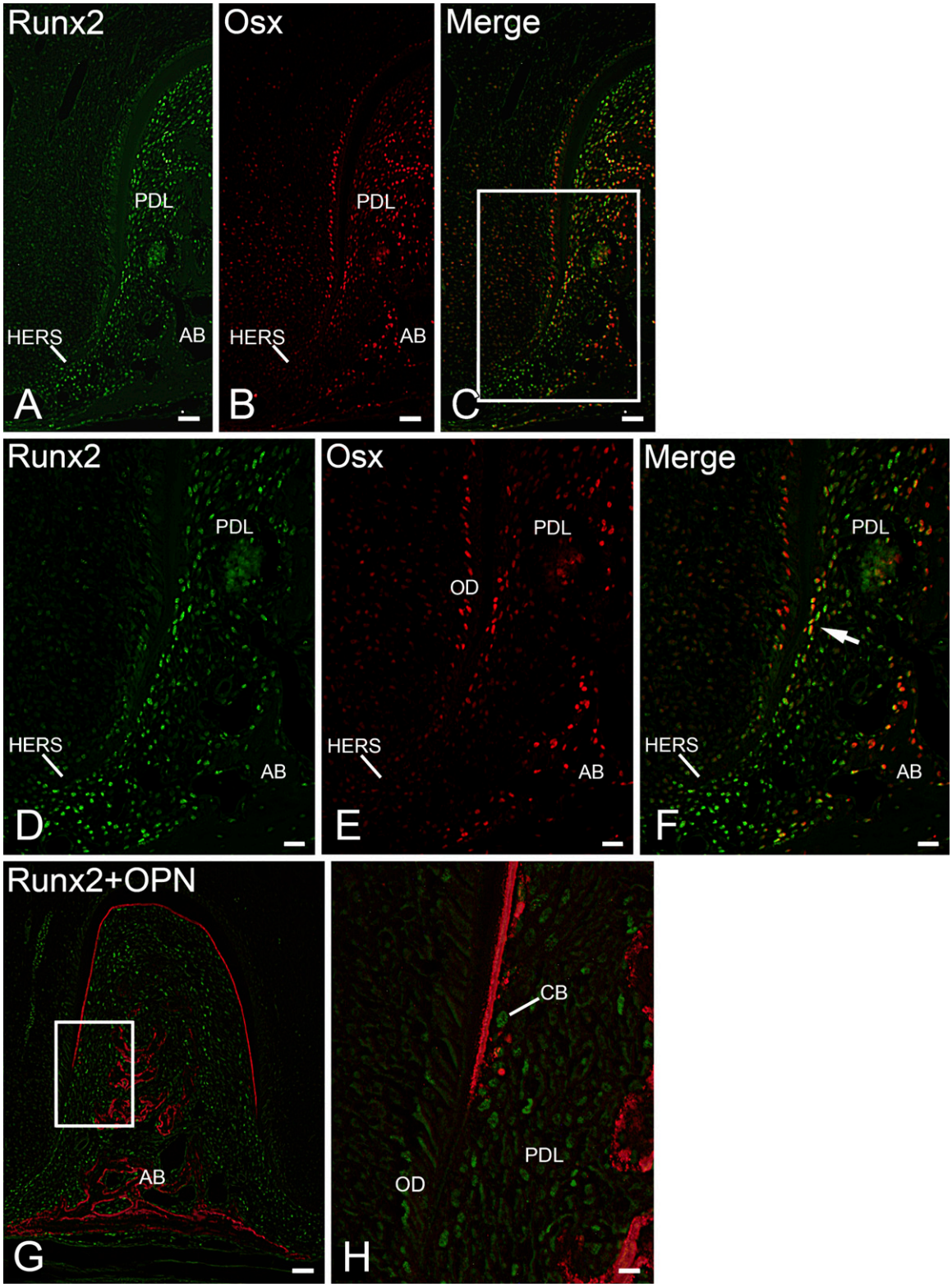
Immunoreactivity of Runx2 was observed in the periodontal ligament cells (Figure 2A). In particular, Runx2-positive cells were located along the root and on the alveolar bone side, although reactivity was weak in the center of the periodontal ligament. In addition, dental follicular cells showed Runx2-positive labeling. Conversely, osterix reactivity was observed in some cells located in the center of the periodontal ligament (Figure 2B), whereas dental follicular cells located between HERS and alveolar bone showed no osterix labeling. Odontoblasts and osteoblasts on the alveolar bone surface showed intense reactivity for osterix (Figure 2B). At the upper end of HERS, cementoblasts, the cells attached to the surface of the root dentin between the gaps of epithelial cells, showed colocalization of Runx2 and osterix (Figures 2D–2F). However, cells adjacent to cementoblasts showed only Runx2 reactivity. Moreover, colocalization of Runx2 and osterix was observed in osteoblasts on the alveolar bone surface. No cells of HERS showed both Runx2 and osterix labeling. Neither Runx2 nor osterix was observed in cementocytes (data not shown).

Intense labeling of osteopontin was detected in the cementum matrix of acellular cementum (Figure 2G). In the cytoplasm of Runx2-positive cementoblasts, osteopontin colocalization was observed (Figure 2H). The surface of the alveolar bone also showed immunoreactivity for osteopontin (Figure 2G).

Discussion

In the present study, Runx2-positive, osterix-positive cells were observed facing the root surface between the gaps of HERS cells. Moreover, the Runx2-positive cells adjacent to root dentin showed colocalization of osteopontin. Osteopontin is an extracellular matrix protein found almost exclusively in mineralized connective tissues, and is associated with osteoblast differentiation and mineralization. Because osteopontin is one of the cementum matrix proteins, and because mature cementoblasts secrete osteopontin (Bronckers et al. 1994), these Runx2-positive, osteopontin-positive cells in the present study could be regarded as cementoblasts. However, the cells adjacent to cementoblasts, probably precementoblasts, showed reactivity only for Runx2, not for osterix or osteopontin, suggesting that osterix might be required for transcription of osteopontin mRNA expression. It has been reported that the development of acellular cementum is somewhat different from that of cellular cementum (Bronckers et al. 1994; Bosshardt 2005). In this study, immunoreactivities for both Runx2 and osterix or osteopontin were observed in cementoblasts forming acellular cementum (data not shown). It is suggested that there is no difference between acellular cementoblasts and cellular cementoblasts in terms of the immunolocalization pattern of Runx2, osterix, and osteopontin. Runx2 and osterix are important for differentiation into both acellular and cellular cementoblasts. The precise mechanisms of acellular and cellular cementoblast differentiation need to be validated. Furthermore, some odontoblasts located just opposite to the gaps between the ruptured HERS and some osteoblasts localized on the alveolar bone surface also showed colocalization of Runx2 and osterix, although preodontoblasts aligned adjacent to the inner layer of HERS and preosteoblasts located near the alveolar bone showed only Runx2 reactivity. Therefore, it is feasible that not only Runx2 but also osterix may be required for differentiation into calcified tissue

Figure 2 Fluorescent micrographs indicating localization of Runx2 and osterix (**A–F**) and Runx2 and osteopontin (**G,H**) in the first molar of a 2-week-old rat. (**A**) Intense immunoreactivity for Runx2 is observed in the periodontal ligament cells located adjacent to the root. Osteoblasts of alveolar bone also show Runx2 reactivity. (**B**) Immunoreactivity for osterix is observed in some cells located on the root surface and in the center of the periodontal ligament. Odontoblasts and osteoblasts of alveolar bone also show osterix reactivity. (**D–F**) Double-immunofluorescence staining of Runx2 (**D**) and osterix (**E**). A higher magnification of HERS of the square in **C**. (**D**) Runx2-positive cells are observed in the dental follicle, on the HERS side of the periodontal ligament, and on the surface of the alveolar bone. Preodontoblasts aligned adjacent to the inner enamel epithelium show weak Runx2 labeling. (**E**) Intense osterix reactivity is observed in the cells facing the root surface. Odontoblasts and osteoblasts on the alveolar bone surface also show osterix labeling. (**F**) Colocalization of Runx2 and osterix is detected in cementoblasts, which penetrate the ruptured HERS and attach to root dentin (arrow). Cells adjacent to cementoblasts show only Runx2-positive reactivity. Some odontoblasts localized just opposite to the gaps between the ruptured HERS, and some osteoblasts on the alveolar bone surface also show colocalization of Runx2 and osterix. (**G,H**) Double-immunofluorescent micrographs indicating the localization of Runx2 (green) and osteopontin (red). (**G**) Intense reactivity for osteopontin is observed in the cementum matrix of acellular cementum and (**H**) in the cytoplasm of Runx2-positive cementoblasts facing the root surface. Osteopontin labeling is also detected in the bone matrix of alveolar bone. **(H)** A higher magnification of the square in **G**. AB, alveolar bone; CB, cementoblast; OD, odontoblasts; Osx, osterix; PDL, periodontal ligament. Bars: **A–C,G** = 20 μm; **D–F** = 40 μm; **H** = 5 μm.



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forming cells such as cementoblasts, osteoblasts, and odontoblasts. This is supported by numerous reports indicating that Runx2 and osterix are important transcription factors that regulate bone and tooth development (D'Souza et al. 1999; Nakashima et al. 2002; Åberg et al. 2004; Fu et al. 2007). In addition, cementocytes showed immunolocalization for neither Runx2 nor osterix (data not shown), suggesting that Runx2 and osterix transcripts change with the differentiation status of cementoblasts, similar to osteoblasts (Liu et al. 2001; Maruyama et al. 2007). Thus, Runx2 negatively controls cementoblast terminal differentiation.

The periodontal ligament cells demonstrated Runx2 expression, suggesting that the periodontal ligament has osteogenic potential (D'Souza et al. 1999; Jiang et al. 1999). In this study, we demonstrated that Runx2-positive cells were observed in the periodontal ligament located in the peripheral parts of the tooth root. In addition, BrdU localization was observed in the cells outside of HERS. It seemed that the localization pattern of BrdU-positive cells in periodontal ligament was similar to that of Runx2-positive cells, although osterix labeling was weak in this area. Osterix labeling was instead observed in some cells located in the center of the periodontal ligament, whereas Runx2 reactivity was weak in this area. The strong expression and activation of osterix protein indicate that osterix might be a molecular link between mechanostressing and osteogenic differentiation (Fan et al. 2007; Zhao et al. 2008). Therefore, expression of osterix in the periodontal ligament might reflect the eruption force and mechanical stress. Moreover, Runx2-positive cells were detected in the area corresponding to α-SMA–positive regions in the present study. Thus, dental follicular cells show Runx2-positive reactivity. On the other hand, dental follicular cells showed no reactivity for osterix. This is supported by a previous study showing that gene expression of osterix was not detected in dental follicle cells, although that of Runx2 was unaffected during osteogenic differentiation in vitro (Morsczeck 2006). Furthermore, Runx2-positive, osterix-positive cells were detected closer to the alveolar bone, suggesting that the α-SMA–positive dental follicular cells may differentiate into osteoblasts.

In this study, cells of HERS showed neither Runx2 nor osterix labeling. Moreover, we have previously reported that immunoreactivity for osteopontin and bone sialoprotein is not detected in cytokeratin-positive epithelial cells (Hirata and Nakamura 2006). Further research is needed to determine the fate and function of HERS cells during periodontal tissue formation.

In conclusion, Runx2 and osterix colocalize in mature cementoblasts, whereas precementoblasts show only Runx2, and no osterix immunohistochemical localization. The relationship between Runx2 and osterix is observed in odontoblasts and osteoblasts. These findings suggest that osterix expression in Runx2-positive cells triggers the formation and secretion of matrix, such as cementum, dentin, and bone. Our results also suggest that Runx2 as well as osterix might be useful markers of differentiation of cementoblasts and thus could be employed for research involving periodontal development and regeneration.

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