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Restricted expression of chromatin remodeling associated factor *Chd3* during tooth root development

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Background and Objective: The tooth root is one of the critical parts to maintain tooth function; however, the molecular mechanisms of root development remain unknown. We aimed to identify specific factors for root morphogenesis using a newly developed experimental system.

Material and Methods: Tentative cementoblasts and periodontal ligament cells from mouse mandibular molars were isolated using laser capture microdissection. More than 500 cementoblasts and periodontal ligament cells were separately captured. After RNA extraction and amplification, mRNA expression in isolated cementoblasts was compared with that of periodontal ligament cells by cDNA microarray analysis. Then, putative cementoblast-specific genes were subjected to *in situ* hybridization analysis to confirm the results in mouse mandible.

Results: Approximately 2000 genes were differentially expressed between these tissues. Among those genes, *zinc finger helicase (ZFH)*, also termed *chromodomain-helicase-DNA-binding protein 3 (Chd3)*, was one of the highly expressed transcripts in tentative cementoblasts. *In situ* hybridization revealed that *ZFH/Chd3* was strongly expressed in Hertwig's epithelial root sheath rather than in cementum. Moreover, its expression disappeared when root formation was advanced in the first molar. In contrast, *Chd3* was continuously expressed in dental epithelial cells of the cervical loop, in which root extension is never terminated.

Conclusion: These results suggest that *ZFH/Chd3* might play an important role in tooth root development and subsequent cementogenesis.

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The tooth root, which is developed by the reciprocal interaction between dental epithelium and mesenchyme, is one of the essential parts to maintain tooth function. However, the molecular basis for the signaling during root development and critical factors for root morphogenesis remain unknown. At the initiation of root formation, as previously reported, dental follicle cells penetrate the ruptured Hertwig's epithelial root sheath and subsequently differentiate into cementoblasts, or Hertwig's epithelial root sheath cells undergo an epithelial to mesenchymal transformation and become functional cementoblasts (1,2). It had been generally believed that cementoblasts and

periodontal ligament were derived from Hertwig's epithelial root sheath and dental follicles, respectively. However, recent studies have proposed that Hertwig's epithelial root sheath cells may not transform into cementoblasts, or multilineage differentiation of dental follicle cells might be due to *Runx2* overexpression enhancing osteoblast/cementoblast-related gene expression (3,4). Yamamoto and Takahashi (5) reported that epithelial to mesenchymal transformation did not occur in 3 wk-old rats, but their observation is only at one time point, and the developmental stage of teeth in rats is different from that in mice. According to those recent studies, cementoblasts might be derived from dental follicle cells, but not from Hertwig's epithelial root sheath. Besides, some researchers reported that epithelial sheath cells transform into cementoblasts to generate the initial cementum (6–10). The epithelial sheath disintegrates prior to the onset of initial cementogenesis, and a few epithelial cells remain on the root, termed epithelial rests of Malassez. Consequently, the initial cementum forms as an epithelial secretory product (11–15). In contrast, it was observed that stem cells or progenitor cells present in periodontal ligament have the capacity to differentiate into either cementoblastic or osteobalstic cells depending on the microenvironment (16–18). Although a number of studies have been conducted as described above, the origin of cementoblasts is still ambiguous. From the remaining possibilities, we supposed that cementogenesis, which follows epithelial root sheath disruption, might be associated with root formation.

In previous studies, the cementum and periodontal ligament tissue samples were usually obtained by manual curettage under microscopy. It is difficult to isolate pure cementum or periodontal ligament tissues by traditional methods, and those tissues may be contaminated with adjacent tissues, resulting in uncertain outcomes. To address this issue, we have developed laser capture microdissection on non-decalcified frozen sections, which enables us to cut out the target cells under microscopy, and we applied this technique to the present study (19). After extraction of mRNA by the laser capture microdissection system, comparative analyses using a cDNA microarray between mice cementoblasts and periodontal ligament cells and quantitative RT-PCR between mice mandibles and femora were performed to detect the genes related to root morphogenesis (20). Subsequent *in situ* hybridization studies exhibited the specific and temporal expression of *zinc finger helicase* (*ZFH*; 21) in Hertwig's epithelial root sheath during the early stage of root formation. *Zinc finger helicase* is also termed *chromodomain-helicase-DNA-binding protein 3 (Chd3)*, which is known to be a chromatin remodeling factor. This gene encodes a member of the CHD family of proteins, which are characterized by the presence of chromo (chromatin organization modifier) domains and SNF2-related helicase/ATPase domains. Chromatin remodeling is essential for many processes, including transcription, as previously reported (22,23).

Chromodomain-helicase-DNA-binding protein 3 has been regarded as one of the transcriptional regulators and was found in the root-surface cementoblastic cells in our study; however, an association with tooth root development has not yet been reported. Thus, we have tried to elucidate the roles of *Chd3* during root development by demonstrating the gene expression pattern in mouse molars and incisors, in which root extension is terminated by 3 wk of age and not terminated, respectively. Our findings suggest that *Chd3* might play an important role in tooth root development.

Material and methods

Animals and tissue preparation

The day on which a pregnant mouse gave birth was designated as postnatal day (P) 0. Two strains of mice, ICR mice and ddY mice, were purchased from Sankyo Lab Service Corporation (Tokyo, Japan). For *in situ* hybridization, specimens of mandibles of ICR mice (age P7.5, P14.5 and P21.5) were prepared with Leica CM1850 cryostat (Leica Microsystems, Weltzlar, Germany) as described in the following sections. All experimental procedures, which were carried out according to the Guideline for Animal Experimentation at Tokyo Medical and Dental University, were approved (no. 0110230B) by the Animal Welfare Committee of Tokyo Medical and Dental University.

Laser capture microdissection, microarray analysis and quantitative RT-PCR

Microdissection using a PixCell II laser capture system (Arcturus Engineering, Mountain View, CA, USA) was performed as previously reported (19). After microdissection in male 4-wk-old ddY mice, the cell-based samples were extracted, purified, amplified and cRNA labeled using the Two-Cycle cDNA Synthesis kit (Affymetrix Inc., Santa Clara, CA, USA), IVT cRNA amplification kit (MEGA script T7 Kit; Ambion, Inc., Austin, TX, USA) and Two-cycle target labeling assay kit (GeneChip IVT Labeling Kit; Affymetrix Inc.) according to the manufacturer's instructions with some modification.

Postulated cementoblast-specific genes were selected by microarray and quantitative RT-PCR analyses, as previously reported (24). Briefly, 10 μg of biotin-labeled cRNA samples derived from tentative cementum and periodontal ligament cells were hybridized to Affymetrix Mouse 430A 2.0 GeneChip arrays (Affymetrix Inc.). Differentially expressed genes were identified by applying the *S* score algorithm. Probe sets showing absolute values of the S score ≥ 2.00 ($p < 0.05$) were considered to indicate significant differences between the two samples. As the amount of RNA extracted from a laser capture microdissection sample is too small to perform quantitative RT-PCR analysis (Real Time PCR system; Applied Biosystems, Foster City, CA, USA) for various genes, the expression levels of tentative cementoblast-positive and periodontal ligament cell-negative genes in mandibles were compared with the level in femora for the second screening to find the

putative cementoblast-specific genes. The genes that were highly expressed in cementoblasts, among the 2000 genes differentially expressed between cementoblasts and periodontal ligament cells, were sequentially selected, and PCR primers for those genes were designed. Quantitative RT-PCR was performed using mandibles and femora of 4-wk-old male ddY mice as the second screening for cementoblast-specific markers. Subsequently, those which cementoblast-positive/periodontal ligament-negative in microarray and mandible-positive/femur-negative in quantitative RT-PCR were subjected to *in situ* hybridization analysis.

In situ hybridization

Mice mandibles (ICR mice age P7.5, P14.5 and P21.5) were dissected, fixed in 4% paraformaldehyde overnight and decalcified in Morse's solution (10% w/v sodium citrate and 22.5% v/v formic acid) for 1 or 2 d depending on the developmental stage, with moderate stirring at 4°C as previously reported (25). The specimens were then equilibrated in 25% sucrose, embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) and cut into 10 μm sections. The cutting angle and direction of the frontal and sagittal sections are shown in Fig. 1. The sequence of the primer set for RT-PCR and TA-cloning to prepare cDNA for the Chd3 probe was as follows: forward, GGACGAAATCCTCCCTCT TC and reverse, ATTGGGACAGAC CAGGAGTG. Complementary DNA was transcribed by T3 or T7 RNA polymerase to prepare a digoxygenin-labeled RNA probe for *ZFH*/*Chd3*, and the probe size was reduced to an average of 250 bases by alkaline hydrolysis. *In situ* hybridization was carried out using an automated system in a hybridization machine (Hybrimaster HS-300; Aloka, Tokyo, Japan) as previously reported (26,27). Signals were visualized with nitroblue phosphate /5-bromo,4-chloro,3-indolil phosphate (NBT/BCIP; Roche Diagnostics Corporation, Indianapolis, IN, USA). Sense probe was used to distinguish the positive signals from nonspecific binding. All results shown are representative of multiple independent experiments.

Results

Laser capture microdissection, microarray analysis and quantitative RT-PCR

More than 500 tentative cementoblasts and periodontal ligament cells in the nondecalcified frozen sections of 4-wk-old male ddY mice were successfully laser captured under microscopy. The total amount of RNA extracted from laser capture microdissection samples was < 1 μg; however, the quality was considerably high, showing clear peaks of 18S and 28S rRNA. Subsequent cDNA microarray analysis revealed that approximately 2000 out of 39,000 genes were differentially expressed between the cementoblasts and the periodontal ligament cells, as we have previously reported (20). Among those, *ZFH* (GeneBank/EMBL accession no. AK011183, NCBI Gene ID: 10264) was one of the putative cementoblast-positive and periodontal ligament cell-negative genes. Real-time PCR revealed that the expression level of *ZFH* in mandible was higher than that in femur. The record of *ZFH* was replaced with Gene ID: 1107 and thereafter termed *Chd3*. Notably, the expression level of *ZFH*/*Chd3* in mandible was approximately 10 times higher than in femur (20). It might be possible that this gene could play some roles in cementogenesis, root formation or root elongation, and *in situ* hybridization analysis was conducted in developing molars and incisors.

Localization of Chd3 during root formation

In situ hybridization studies in developing ICR mice mandible revealed that *Chd3* was strongly expressed in the cells that formed Hertwig's epithelial root sheath in the first molar at P7.5, the initial stage of root formation, during which Hertwig's epithelial root sheath had been formed by inner and outer enamel epithelial cells [Fig. 2A and B (arrows)]. In addition, its expression was detected in differentiating ameloblasts arranged on the extended line of inner enamel epithelium. Although *Chd3* was picked up from the cementoblast-positive and mandible-specific sample, *Chd3*-positive cells were abundantly contained by the cells organizing Hertwig's epithelial root sheath rather than by cementoblasts, which are scarcely observed at this stage. In contrast, dental pulp or differentiating periodontal ligament fibroblasts from molars did not express *Chd3* transcript (Fig. 2B). At P7.5, *Chd3* expression was also observed in ameloblasts located to the labial side of incisors, as shown in Fig. 2A and C (arrowheads), but not in dental pulp (Fig. 2C). Expression of *Chd3* was scarcely observed in the stellate reticulum and the duct epithelium of salivary glands (data not shown).

The frontal sections of P14.5 mandibles, at the developmental stage of root elongation, demonstrated that mRNA expression of *Chd3* was prominent along the enamel epithelium at the tip of the developing first molar root (Fig. 3A). *Chd3* solidly positive

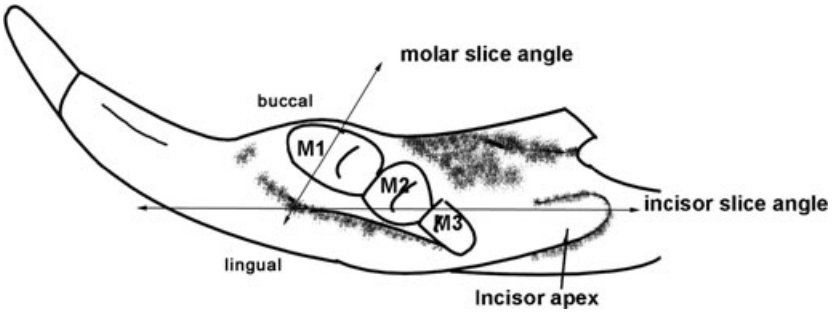

Fig. 1. Slice angle and direction in the mandible.

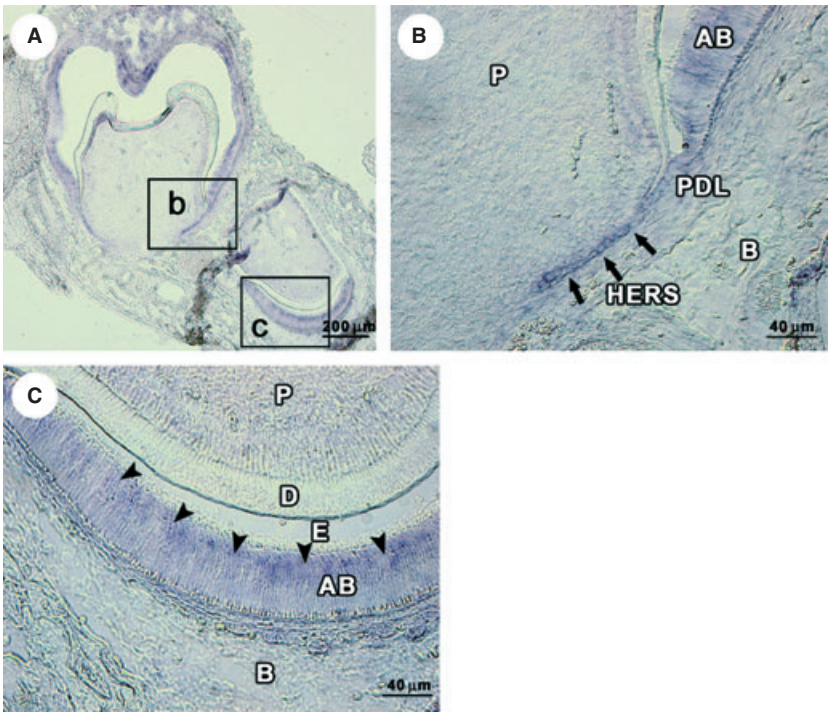

Fig. 2. Expression of Chd3 in the developing lower first molar at P7.5. (A) Chd3 expression in a frontal section of the mandibular first molar of a P7.5 mouse assessed by *in situ* hybridization. (B) A higher magnification of the apex of the molar root. Chd3 was expressed in Hertwig's epithelial root sheath (arrows), but was absent in the dental pulp and periodontal ligament. (C) A higher magnification of the labial side of the incisor. Chd3 was expressed in ameloblasts of the incisor (arrowheads), but not in dental pulp. Abbreviations: AB, ameloblast; B, bone; D, dentin; E, enamel; HERS, Hertwig's epithelial root sheath; P, dental pulp; and PDL, periodontal ligament. All results shown are representative of multiple independent experiments.

structures were restricted to the monolayer at both ends of Hertwig's epithelial root sheath, with weakly positive areas in the dental pulp (Fig. 3B, arrows). No signal was detected in periodontal ligament fibroblasts adjacent to the molar root. Similar to P7.5 mandible, *Chd3* expression was observed in ameloblasts on the labial side of incisors (Fig. 3C, arrowheads).

At the advanced stage of root formation, P21.5, the first molar was almost completely developed and a definitive Hertwig's epithelial root sheath structure was mostly collapsed, whereas the incisor root was continuously extending. At this time point, in contrast to P7.5 or P14.5, the expression level of *Chd3* in Hertwig's epithelial root sheath was significantly reduced to the background level at the tip of the first molar roots (Fig. 4B, arrows). Its expression was absent in periodontal ligament (Fig. 4B). Similar to molars, *Chd3* signal was scarcely detected in ameloblasts at the labial side of incisors (Fig. 4C, arrowheads).

Regarding incisor roots, the root extension is not terminated and the original structure of cervical loop is preserved at P7.5, P14.5 and P21.5. As shown in Fig. 5, *in situ* hybridization analysis of the sagittal section of the developing incisors from P7.5 to P21.5 demonstrated specific and apparent localization of *Chd3*. In the inner and outer epithelium of the labial side of incisors, Chd3 was abundantly expressed, and its expression level was maintained with the looped shape of epithelial cells throughout the observation period.

Discussion

In the present study, a number of prospective cementoblast-specific genes derived from cementum-positive, periodontal ligament-negative and mandible-positive, femora-negative samples were identified. Of these genes, as a predictable cementoblast-specific gene, *ZFH/Chd3* was intensively investigated. Indeed, *ZFH/Chd3* was strongly expressed in the cells at the apical end of the tooth root, as shown in Figs 2 and 3. Notably, the positive signal was highly specific in the pointed end of Hertwig's epithelial root sheath and epithelial diaphragm in P7.5 and P14.5 mouse mandibles, in which few cementoblasts appeared on the root. These results suggest that this molecule is highly specific for Hertwig's epithelial root sheath rather than cementum. *Chd3* is one of the components of a histone deacetylase complex referred to as the Mi-2/NuRD complex, which participates in the remodeling of chromatin by deacetylating histones (22,23). Abundant *Chd3* expression in the apical root end at the developmental stage of root formation could be reasonable because *Chd3* is a chromatin remodeling associated factor, and the cells organizing Hertwig's epithelial root sheath are actively remodeled to extend the tooth root. It is

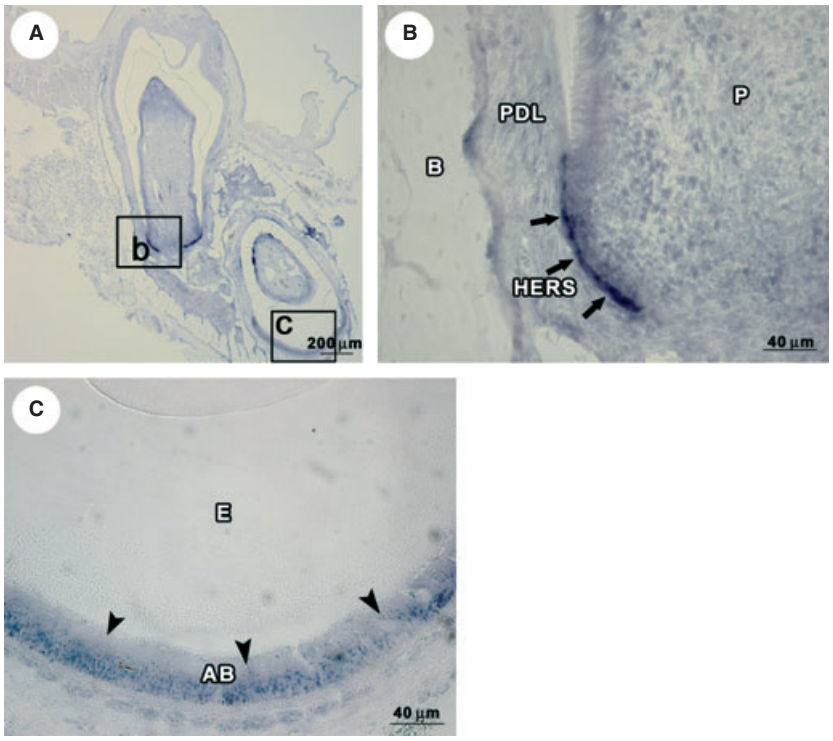

Fig. 3. Expression of Chd3 in the developing lower first molar at P14.5. (A) Chd3 expression in a frontal section of the mandibular first molar of a P14.5 mouse assessed by *in situ* hybridization. (B) A higher magnification of the apex of the molar root. Chd3 was strongly expressed in Hertwig's epithelial root sheath (arrows) and weakly in the dental pulp, but was absent in periodontal ligament. (C) A higher magnification of the labial side of the incisor. Chd3 was expressed in ameloblasts of the incisor (arrowheads). Abbreviations: AB, ameloblast; B, bone; D, dentin; E, enamel; HERS, Hertwig's epithelial root sheath; P, dental pulp; and PDL, periodontal ligament. All results shown are representative of multiple independent experiments.

generally accepted that Hertwig's epithelial root sheath is the principal region controlling root extension, because Hertwig's epithelial root sheath usually disappears upon the completion of root formation. Previously, several researchers described that, during cementogenesis, dental follicle cells differentiate into cementoblasts, or Hertwig's epithelial root sheath cells transform and then become functional cementoblasts (1,2). As our experiments revealed intensive expression of *Chd3* in Hertwig's epithelial root sheath, it might be an important factor not only for root extension but also for initiation of cementogenesis.

As mentioned above, there was restricted expression of *Chd3* on the molar root. However, there was a contradiction between the results of the Genechip analysis with quantitative RT-PCR and those of *in situ* hybridization. In 4 wk-old ddY mice, significantly intense expression of *Chd3* was shown in cementoblasts or mandible by Genechip analysis or quantitative RT-PCR, respectively. In contrast, this positive expression in Hertwig's epithelial root sheath of molars was not so apparent at 3 wk (P21.5) by *in situ* hybridization. *Chd3* expression in cementoblasts was assumed to be extremely low, and amplification might be required to detect it. In our preliminary experiment, another set of quantitative PCR results from laser capture microdissection samples confirmed that *Chd3* expression in the cementum layer was more than three times as high as that in the periodontal ligament region (data not shown). Moreover, expression of *Chd3* could not be observed in tentative cementoblasts or in periodontal ligament cells with lower cycle amplification (< 20 cycles) in the PCR, suggesting a low level of expression in cementoblasts, but higher than in other tissues of the periodontium at 4 wk. Another possible explanation is that a laser capture microdissection sample derived from tentative cementoblasts may include cells of Hertwig's epithelial root sheath or epithelial rests of Malassez. As laser capture microdissection was carried out on the root surface of nondecalcified frozen sections, it seemed to be difficult, even under the microscope, to eliminate the epithelial rests if they exist.

To confirm the role of *Chd3*, its expression around the molar roots was compared with that around the apex of incisor roots, where characteristic and continuous root extension is observed. As restricted *Chd3* expression was observed on the molar roots during the root extension period, it was very interesting to investigate how *Chd3* behaves in the case of continuous root extension. Morphological features of rodent incisors are quite characteristic. The cells on the lingual side usually develop only to the pre-ameloblastic stage, and no enamel is produced. In

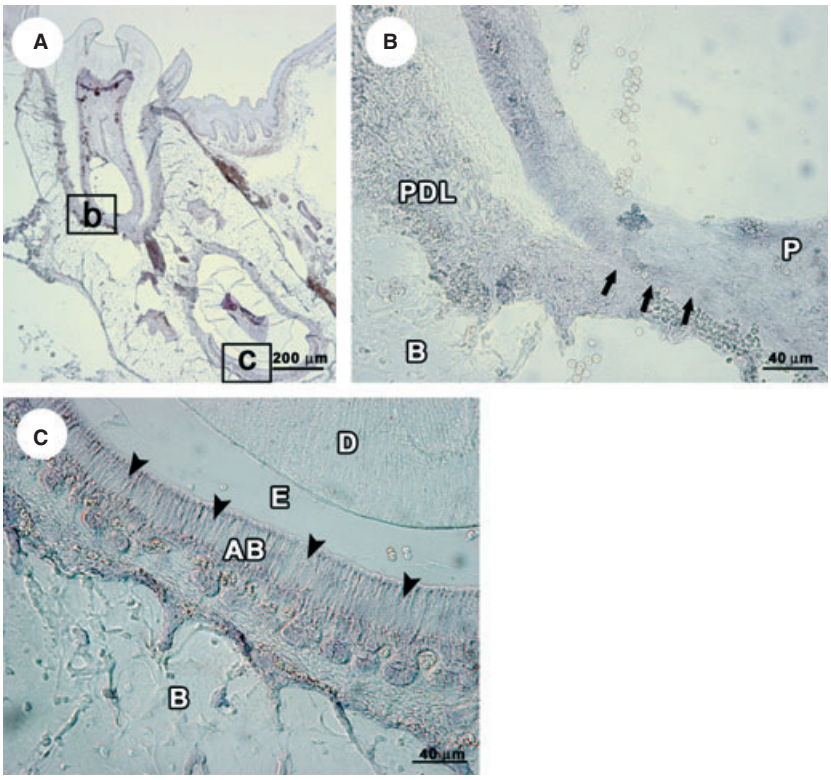

Fig. 4. Expression of Chd3 in the developing lower first molar at P21.5. (A) Chd3 expression in a frontal section of the mandibular first molar of a P21.5 mouse assessed by *in situ* hybridization. (B) A higher magnification of the apex of the molar root. Chd3 was scarcely expressed in Hertwig's epithelial root sheath (arrows) and the dental pulp. It was absent in periodontal ligament. (C) A higher magnification of the labial side of the incisor. Chd3 was barely expressed in ameloblasts of the incisor (arrowheads). Abbreviations: AB, ameloblast; B, bone; D, dentin; E, enamel; HERS, Hertwig's epithelial root sheath; P, dental pulp; and PDL, periodontal ligament. All results shown are representative of multiple experiments.

contrast, on the labial side of the cervical loop, the cells of the inner enamel epithelium continuously develop dental organs, and root extension is never terminated (28,29). In the specimen of incisors, *Chd3* was expressed in the cells organizing the cervical loop throughout the observation period. In particular, along the inner enamel epithelium and around the apical bud, intensive expression was observed where apical root extension was maintained owing to the continuous incisor eruption. From these results, we propose that the restricted expression of *Chd3* plays an important role for epithelial cell progression in root morphogenesis and that termination of *Chd3* expression might be crucial for cementogenesis. In addition, the expression pattern of *Chd3* was different from other amelobalstic markers, such as sonic hedgehog, in that the expression was remarkably distinctive along the inner enamel epithelium (data not shown). Moreover, the observation of continuous *Chd3* expression in the cells of the cervical loop of incisors at 3 wk, while little expression was observed in molars, also supports a contribution of *Chd3* to the apical extension of the tooth root. However, another ambiguous point remains. Intensive expression of *Chd3* was always detected in Hertwig's epithelial root sheath, whereas it was not always obvious in other areas, such as odontoblasts, stellate reticulum or stratum intermedium. We cannot clearly explain this unstable detection of *Chd3* except in Hertwig's epithelial root sheath area. A possible explanation is that *Chd3* expression is stage dependent and that the unstable detection might be due to a pause in growth. Another possible explanation is that *Chd3* is also a component of a histone deacetylase complex, referred as the Mi-2/NuRD complex, participating in the remodeling of chromatin by deacetylating histones and then *Chd3* could regulate the transcription process. Unstable detection of *Chd3*, in which cells dynamically and drastically transform depending on developmental stages, might be reasonable (23). Of course, the possibility of a false positive has not been ruled out, and we may have to improve the histological technique considerably.

In conclusion, *Chd3* was expressed at the epithelial diaphragm from P7.5 to P21.5, and the signal faded from Hertwig's epithelial root sheath with progression of root formation. At 3 wk, *Chd3* expression was hardly detectable in the molar root. These results suggest that restricted expression of *Chd3* in Hertwig's epithelial root sheath should be tightly correlated to the apical root morphogenesis. Although further functional analysis, such as employing transgenic animals or siRNA knock-down analysis, is required to confirm the fundamental

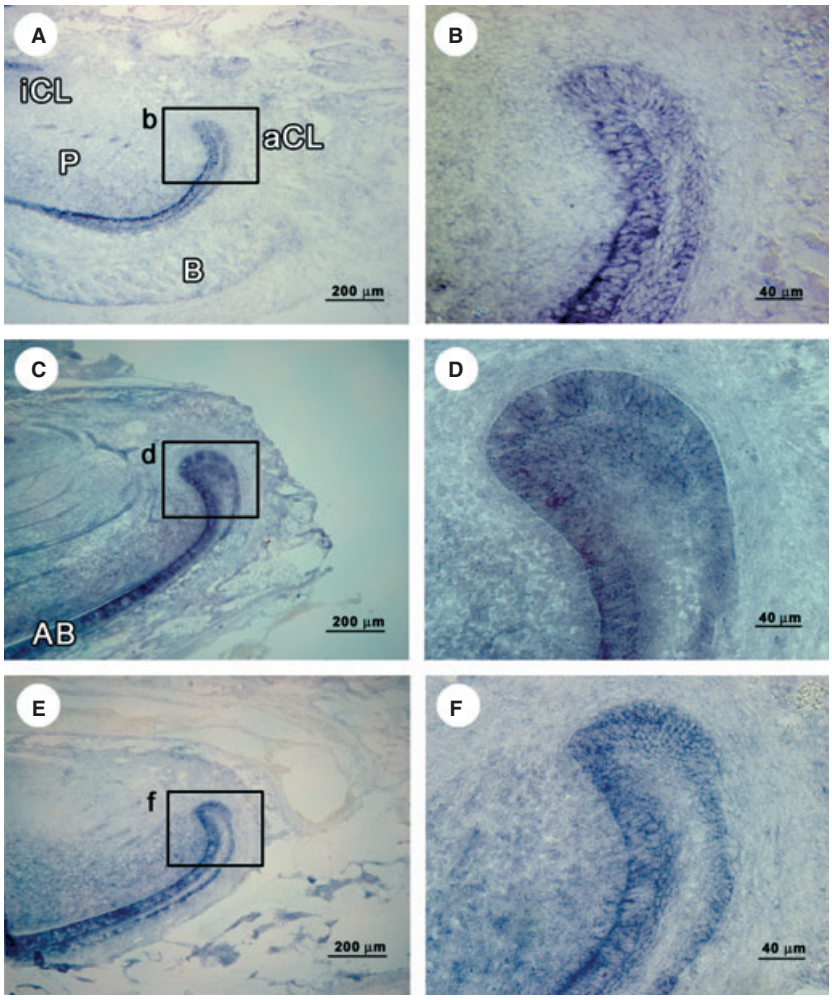

Fig. 5. Localization of Chd3 in the developing incisor from P7.5 to P21.5. The samples are sagittal sections in the developing lower incisors from P7.5 to P21.5. (B), (D) and (F) are higher magnification images of the boxes in (A), (C) and (E), respectively. Chd3 was strongly expressed in the inner and outer enamel epithelium of the labial cervical loop. Abbreviations: AB, ameloblast; aCL, labial cervical loop; B, bone; iCL, lingual cervical loop; and P, dental pulp. All results shown are representative of multiple independent experiments.

function, *Chd3* might contribute to the initiation and termination of apical root extension and might be an essential molecule to induce cementogenesis, which follows Hertwig's epithelial root sheath disruption. *Chd3* might have a possible therapeutic use for regeneration of periodontal tissue. This is the first report to describe the expression of chromatin remodeling factor *Chd3* in tooth root development.

Acknowledgements

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