Transcription Factor Epiprofin Is Essential for Tooth Morphogenesis by Regulating Epithelial Cell Fate and Tooth Number

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In tooth morphogenesis, the dental epithelium and mesenchyme interact reciprocally for growth and differentiation to form the proper number and shapes of teeth. We previously identified epiprofin (Epfn), a gene preferentially expressed in dental epithelia, differentiated ameloblasts, and certain ectodermal organs. To identify the role of Epfn in tooth development, we created Epfn-deficient mice (Epfn−/−). Epfn−/− mice developed an excess number of teeth, enamel deficiency, defects in cusp and root formation, and abnormal dentin structure. Mutant tooth germs formed multiple dental epithelial buds into the mesenchyme. In Epfn−/− molars, rapid proliferation and differentiation of the inner dental epithelium were inhibited, and the dental epithelium retained the progenitor phenotype. Formation of the enamel knot, a signaling center for cusps, whose cells differentiate from the dental epithelium, was also inhibited. However, multiple premature nonproliferating enamel knot-like structures were formed ectopically. These dental epithelial abnormalities were accompanied by dysregulation of Lef-1, which is required for the normal transition from the bud to cap stage. Transfection of an Epfn vector promoted dental epithelial cell differentiation into ameloblasts and activated promoter activity of the enamel matrix ameloblastin gene. Our results suggest that in Epfn-deficient teeth, ectopic nonproliferating regions likely bud off from the self-renewable dental epithelium, form multiple branches, and eventually develop into supernumerary teeth. Thus, Epfn has multiple functions for cell fate determination of the dental epithelium by regulating both proliferation and differentiation, preventing continuous tooth budding and generation.

Ectodermal organ development is a complex process initiated by inductive tissue interactions (1). The developing tooth is a classic example of such inductive processes (2–4). Tooth development is a continuous process that can be divided into the initiation, bud, cap, and bell stages (5, 6). In mice, tooth development begins at embryonic day (E)4.5 by thickening of the dental epithelium. The dental lamina undergoes further proliferation and subsequently develops into the tooth bud. The tooth bud is formed by the invagination of the placode and the condensation of mesenchymal cells adjacent to the bud. At the bud stage (E13.5), dental epithelial cells proliferate diffusely. At the cap stage (E14.5), dental epithelial cells differentiate into several cell types: the cervical loop, stellate reticulum, stratum intermediate, outer dental epithelium, inner dental epithelium, and enamel knot. Cell death by apoptosis within the enamel knot is critical for cusp formation in molars. These epithelial cells proliferate at different dividing rates to form the unique shape of the enamel organ. For example, a nonproliferating epithelial cell mass forms the enamel knot at the center of the tooth bud. At the bell stage (E17.5), the dental mesenchyme differentiates into dentin matrix-secreting odontoblasts that form dentin, and then the inner dental epithelial cells differentiate into enamel matrix-secreting ameloblasts that form enamel. In rodents, incisors continue to grow through adulthood.

Studies with mutant mice have identified a number of genes that regulate tooth development and morphology (7). For example, deficiency of Lef-1 (8) or p63 (9, 10) arrests tooth development at early stages. Deficiency of Msx1 or Pax9 results in arrest of tooth development at the bud stage (11, 12), whereas deficiency of Runx2/Cbfa1, Sp3, or Shh causes inhibition of cytodifferentiation of ameloblasts and/or odontoblasts (13–15). Gene knock-out mice for ameloblastin and amelogenin, enamel matrix proteins, develop severe enamel hypoplasia with abnormal ameloblast differentiation (16, 17).

Epiprofin (Epfn) was previously identified by differential hybridization using tooth germ cDNA microchips as a zinc finger transcription factor that is expressed in certain developing ectodermal tissues such as teeth, hair follicles, and

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4 The abbreviations used are: E, embryonic day; ES, embryonic stem; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; PCNA, proliferating cell nuclear antigen; BrdUrd, bromodeoxyuridine; RT, reverse transcription; Phos-pRb, phosphorylated pRb; Dspp, dentin sialo-phosphoprotein; Ambn, ameloblastin.
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limbs (18). Epfn is a homologue of KLF14/Sp6, belonging to the Sp transcription factor superfamily (19, 20). To date, there are nine Sp genes (Sp1–Sp9) in the mammalian genome. These Sp proteins regulate transcription of various genes in a positive and/or negative manner. Although all these Sp factors contain a high degree of structural conservation of the zinc finger domain, each appears to have distinct regulatory specificity.

In this report, we created Epfn-deficient mice that initially had delayed tooth development but later developed an excess number of teeth and had a complete loss of enamel and a defective dentin structure. We found that Epfn is an essential regulator for branching of tooth buds to form the proper number of teeth and for differentiation of the dental epithelium and mesenchyme.

MATERIALS AND METHODS

Targeting of Embryonic Stem Cells and Generation of Chimeras—A genomic clone including the epiprofin locus was isolated from a 129/SV genomic library. The targeting vector was constructed and electroporated into R1 ES cells (see Fig. 1). Three independent targeted ES cell clones produced chimeras that transmitted the mutant allele through the germline. Heterozygous progeny were generated with backcrossing to wild-type C57BL/6 for at least five generations. Animal care was given in compliance with the National Institutes of Health guidelines on the Use of Laboratory and Experimental Animals.

Cell Culture and Transfection—SF2 cells, a preameloblast cell line, were established as described (21) and were cultured in Dulbecco’s modified Eagle’s medium/F12 (Invitrogen) plus 10% fetal bovine serum. SF2 cells differentiated into ameloblasts in the presence of BMP2 (data not shown). SF2 cells were transfected with the Epfn expression vector (Epfn-pcDNA3.1/Myc-His vector (18)) using Lipofectamine 2000 (Invitrogen). For the ameloblastin (Ambn) promoter assay, SF2 cells, NIH3T3, or MC3T3-E1 cells were cotransfected with the Epfn vector and the Ambn promoter reporter construct containing a 2.7-kb Ambn promoter in the pNASSb-basic (Promega) vector using the Lipofectamine kit. The cytomegalovirus promoter-based pcDNA3.1/β-galactosidase vector (Invitrogen) was used as a positive control. After a 48-h transfection, β-galactosidase assays were performed using the β-galactosidase reporter gene assay chemiluminescent kit (Roche Applied Science).

Tissue Sections, Immunohistochemistry, BrdUrd Labeling, and TUNEL Assays—Mouse heads in several stages of development were dissected out and fixed with 4% paraformaldehyde in phosphate-buffered saline overnight at 4 °C. Adult tooth tissues were decalcified with 250 mM EDTA/phosphate-buffered saline and embedded in paraffin. For histological analysis, sections were stained with Harris hematoxylin and eosin Y. For immunohistochemistry, sections were boiled with targeted retrieval solution (Dako) and incubated in 1% bovine serum albumin/phosphate-buffered saline (blocking reagent) for 1 h prior to incubation with the primary antibody. We used antibodies to ameloblastin (22); amelogenin and enamelin (from Dr. Simmer); dentin sialophosphoprotein (from Dr. Fisher); p63 and E-cadherin (Pharmingen); PCNA (Zymed Laboratories Inc.); Ki67 (Novocast); BrdUrd (Roche Applied Science); and phospho-Rb (Cell Signaling). Rabbit polyclonal antibody to the Epfn peptide (residues 33–51) was raised and purified by a peptide affinity column. Primary antibodies were detected by Cy-3-conjugated and Cy-5-conjugated secondary antibodies (Jackson ImmunoResearch). Nuclear staining was performed with Hoechst dye (Sigma). Primary Epfn and phospho-pRB antibodies were detected with a biotinylated antibody against rabbit IgG and avidin-conjugated peroxidase (Zymed Laboratories Inc.) and visualized using diaminobenzidine as a chromogenic peroxidase substrate. To assess cell proliferation, BrdUrd (100 mg/kg of body weight) was injected intraperitoneally into pregnant females. The mice were euthanized 1 h after injection, and embryos were dissected and prepared for cryosections. To detect apoptotic cells, we used the ApopTag® Red in situ apoptosis detection kit (Chemicon).

RT-PCR—Total RNA was extracted from SF2 cells that were stably transfected with the Epfn vector and pooled using the TRIzol kit (Invitrogen). After DNaseI (Sigma) treatment, 2 μg of total RNA was used for the reverse transcription to generate cDNA, which was used as a template for PCR reactions with gene-specific primers (supplemental Table 1). For the semi-quantitative RT-PCR, cDNA was amplified with an initial denaturation at 95 °C for 3 min and then 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s for 25 cycles and a final elongation step at 72 °C for 5 min.

In Situ Hybridization of Tissue Sections—Digoxigenin-11-UTP-labeled single-strand RNA probes for Lef-1, Shh, and Epfn (18) were prepared using the digoxigenin RNA labeling kit (Roche Diagnostics) according to the manufacturer’s instructions. In situ hybridization of mouse sections was performed as described previously (18).

Scanning Electron Microscope—Incisors of 12-week-old mice were fixed and decalcified in 4.13% EDTA, dehydrated in graded ethanol, and processed for embedding in LR White resin. Calcified incisors were fractured at several sites along their length, dehydrated in ethanol, critical point-dried with CO2, and examined in a JEOL JSM 5910 LV variable-pressure scanning electron microscope.

RESULTS

Epiprofin-deficient Mice and Tooth Defects—We previously reported that Epfn is expressed in the dental epithelium from the early initiation stage to the enamel matrix secretion stage and in differentiated odontoblasts (18). Epfn is also expressed in certain ectodermal tissues such as the matrix of hair follicles and the apical ectodermal ridge in limbs (18). To identify the role of Epfn in the development of the tooth, we created mutant mice deficient for Epfn by deleting exon 2, which encodes the entire coding sequence of the Epfn gene using the conventional gene knock-out strategy (Fig. 1). Three independent targeted ES cell clones from R1 cells produced chimeras that transmitted the mutant allele through the germline. Heterozygous progeny were generated with backcrossing to wild-type C57BL/6 for at least five generations. Homozygous mice (Epfn−/−) are viable, but their body size is significantly smaller than that of wild-type or heterozygous mice. About 20% of Epfn−/− mice die at about
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Defective tooth formation. Epfn mice showed that the entire enamel structure was completely missing (Fig. 3, A and C). Heterozygous Epfn<sup>+/−</sup> incisors had enamel, but it was wider than wild type, with an irregular and less compact structure (Fig. 3B). Dentinal tubule formation was also irregular in Epfn<sup>−/−</sup> mice when compared with that of wild-type mice, suggesting defects in deposition of dentin matrix and crystallization in dentin (Fig. 3, D–F). In the heterozygous Epfn<sup>+/−</sup> incisors, dentinal tubules were also less compact when compared with those of wild-type mice.

Multiple Branching of Tooth Germs and Defects in Differentiation of Dental Epithelium and Mesenchyme—We performed histological analysis of developing molars and incisors to determine the tooth defects in detail (Fig. 4, A and B). In early molar development through the bud stage (embryonic day E13.5), there was no significant morphological difference between mutant and control Epfn<sup>+/−</sup> mice. In Epfn<sup>−/−</sup> mice, a single dental placode formed at the initial stage. However, molars were profoundly abnormal by E16.5 (Fig. 4A, panels b and f). Multiple branches of the dental epithelium invaginated into the dental mesenchyme, whereas control molars had single branching and a mesenchyme condensation area. In addition, cusp formation was inhibited in Epfn<sup>−/−</sup> molars. At postnatal day 3, in control molars, ameloblasts and odontoblasts were differentiated and well polarized, and enamel and dentin layers formed (Fig. 4A, panel c). In contrast, in Epfn<sup>−/−</sup> mice, dental epithelial cells formed multiple cell layers in mutant molars and were not well polarized, and odontoblast differentiation was delayed and not observed at this stage (Fig. 4A, panel g). Consequently, the enamel layer was

FIGURE 1. Targeted disruption of the mouse Epfn locus. A, partial restriction maps of the wild-type Epfn locus, targeting vector, and targeted allele. The entire coding region of exon 2 of the Epfn gene, including the zinc finger domain, was replaced with the pGK-neo targeting cassette. Restriction enzyme sites are shown above the line, and the lengths of DNA fragments generated by an NdeI digest that hybridize with the genomic flanking probe are indicated by the arrow lines. B, Southern blot analysis of NdeI-digested genomic DNA from ES cells and tails of wild-type (WT), heterozygous (−/−), and homozygous (−/−) mice. Three independent targeted ES cell clones from R1 cells produced chimeras that transmitted the mutant allele through the germline. 10.8kb, targeted allele; 6.4kb, wild-type allele.

FIGURE 2. Defective tooth formation. A–D, incisors of 3-week-old (A and B) and 12-month-old (C and D) heterozygous (Epfn<sup>+/−</sup>) (A and C) and homozygous (Epfn<sup>−/−</sup>) (B and D) mice. B, Incisor development of 3-week-old Epfn<sup>−/−</sup> mice is delayed. D, 12-month-old Epfn<sup>−/−</sup> KO mice had an excess number of incisors with a mineral defect. E–H, radiogram analysis of 6-month-old mouse heads from Epfn<sup>+/−</sup> (E and F, enlarged) and Epfn<sup>−/−</sup> mice (G and H, enlarged). asterisk, molars; in, incisors; arrowheads, al, alveolar bones (arrowheads); rt, roots.

the age of 2 months mice for an unknown cause, whereas the remaining survive with a life span similar to wild-type or heterozygous mice. Homozygous mice (Epfn<sup>−/−</sup>) are viable and have severe defects in teeth, as described below. They also have defects in skin, hair follicles, and digit formation. Development of teeth in mutant mice was delayed. Incisors and molars of mutant mice were not erupted at the age of 3 weeks, when wild-type incisors and molars were visible (Fig. 2, A and B). However, at later stages, mutant incisors had erupted with excess numbers (hyperdontia), e.g. more than 50 incisors by the age of 1 year (Fig. 2, C and D). Mutant incisors showed a chalky color, indicating a mineral defect. Radiographs also showed an excess number of molars (e.g. more than eight molars on each side of the mandible when compared with three molars on each side of the control mandible by the age of 1 year) (Fig. 2, E–H). The number of molars in Epfn<sup>−/−</sup> mice increased with age (e.g. 4–5 molars on each side of the mandible at the age of 4 weeks; data not shown). The molars had poorly developed cusps, which cause malocclusion. There were also severe defects in the dental root structure and alveolar bones (Fig. 2G). Scanning electron microscope imaging of the incisor surface of 12-week-old Epfn<sup>−/−</sup> mice showed that the entire enamel structure was completely missing (Fig. 3, A and C). Heterozygous Epfn<sup>+/−</sup> incisors had enamel, but it was wider than wild type, with an irregular and less compact structure (Fig. 3B). Dentinal tubule formation was also irregular in Epfn<sup>−/−</sup> mice when compared with that of wild-type mice, suggesting defects in deposition of dentin matrix and crystallization in dentin (Fig. 3, D–F). In the heterozygous Epfn<sup>+/−</sup> incisors, dentinal tubules were also less compact when compared with those of wild-type mice.
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![Image of incisors](image)

FIGURE 3. Absence of enamel and defects of dentin. A–C, scanning electron microscope images of the surface of incisors of 12-week-old Epfn+/+, Epfn+/−, and Epfn+/− mice. Wild-type Epfn+/+ mice (A) had well crystallized outer (oe) and inner enamel (ie), whereas homozygous Epfn+/− mice (C) were completely missing the entire enamel structure. Heterozygous Epfn+/− incisors had enamel, but it was wider, with an irregular and less compact structure (B). D–F, scanning electron microscope images of the surface of incisors of 12-week-old Epfn+/+ mice (D) when compared with that of wild-type (D) mice, suggesting defects in deposition of dentin matrix and crystallization in dentin. In the heterozygous Epfn+/− incisors, dental tubules were also less compact when compared with those of the wild type (E). The small square on the frontal section of the incisor shows the area of enlargement.

FIGURE 4. Abnormal tooth development. A, histology of Epfn+/− (panels a–d) and Epfn+/− molar (panels e–h). Panels a, b, e, and f, frontal sections, ×40; panels c and g, ×20; panels d and h, ×10. Arrows, multiple branching. B, histology of Epfn+/− (panels a–e) and Epfn+/− incisors (panels f–j). Panels c, d, e, h, i, and j, frontal sections; Panels a, b, f, and g, sagittal sections. Arrows, multiple branching. Panels e and j, ×40 (enlarged the boxed areas in panels d and i). Note: The enamel layer was detached from the dentin layer during sample preparation in control molars (A, panels c and d) and incisors (B, panels d and e).

not formed, and dentin formation was delayed. Further, the dental epithelium formed multiple buds that resulted in excess future molars. In 3-week-old control molars, the enamel organ of the tooth germ was fully developed, whereas in mutant molars, the dental lamina was not separated from the oral epithelium, enamel was not formed, and the crown of the molars had reduced dental cusp sizes (Fig. 4A, panels d and h). In addition, several molars had developed from a single dental lamina.

The initial stage of incisor development is similar to that of molars. However, in later stages, the epithelial cells only on the labial side differentiate into ameloblasts and produce enamel matrix, resulting in asymmetrical enamel formation in incisors. The continuous eruption of incisors is maintained by a continuous supply of progenitor cells in the apical bud localized in the cervical loop (Fig. 4B, panel a, cl). E16.5 control incisors formed a polarized inner dental epithelium cell layer on the labial side but not the lingual side, and odontoblasts and a thin dentin matrix layer were present on both sides. In mutant incisors at this stage, dental epithelia and mesenchyme were disorganized and undifferentiated when compared with control Epfn+/− incisors (Fig. 4B, panel f). The Epfn+/− incisor germ did not separate from the oral epithelium (Fig. 4B, panels b and g), and it developed a globe-like structure filled with undifferentiated epithelial cells and multiple buds surrounding the mesenchyme. Similar to molars, postnatal day 3 mutant incisors formed multiple apical buds, which originated from a single dental lamina, lacking enamel and dentin layers (Fig. 4B, panels g and h). At postnatal day 10, Epfn+/− incisors eventually formed dentin surrounded by a thin layer of undifferentiated epithelial cells (Fig. 4B, panels i and j).

Defects in Ameloblast and Odontoblast Differentiation—We next examined the expression of tooth matrix proteins as differentiation markers in 10-day-old molars (Fig. 5). Ameloblastin and enamelin, specific to differentiated ameloblasts, were expressed by ameloblasts and secreted into the enamel matrix layer of Epfn+/− molars, whereas neither protein was detected in Epfn−/− molars (Fig. 5, A, B, E, and F). Dentin sialophosphoprotein (Dspp) was expressed by both ameloblasts and odontoblasts in Epfn+/− molars. However, in Epfn−/− molars, Dspp was negative in the epithelium and significantly reduced in odontoblasts and the dentin layer (Fig. 5, C and G). These results indicate that dental epithelial cell differentiation into ameloblasts was blocked in Epfn−/− mice and suggest that
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Epfn is required for ameloblast differentiation and that it regulates expression of Dsp in odontoblast differentiation.

Disregulation of Lef-1 and Shh—To address the molecular basis of tooth defects, we analyzed the expression of Lef-1 and Shh, which regulates tooth morphogenesis (Fig. 6). Deletion of Lef-1, a downstream transcription factor of Wnt/β-catenin, results in an absence of all teeth (23). Whole-mount in situ hybridization of the E11.5 mouse mandible showed Lef-1 expression in the dental placode of the control mandible. In Epfn+/− mice, Lef-1 expression was up-regulated in both the epithelium and the mesenchyme of E11.5 maxilla and mandible processes and was not localized in the enamel knot but extended into the inner dental epithelium layer in Epfn−/− mice. Shh expression of the enamel knot in E14.5 Epfn−/− molar was reduced.

defects in Rapid Proliferation of the Inner Dental Epithelium and Formation of Multiple Premature Enamel Knot-like Structures—Since we demonstrated that Epfn expression promotes cell proliferation in culture (18), we examined cell proliferation by immunostaining of proliferation markers Ki67 and PCNA. In control E16.5 molars, Ki67 and PCNA were stained strongly in the outer and inner epithelial layers and condensing mesenchyme cells (Fig. 7, A, panels a and c). In Epfn−/− molars, staining in these cells was decreased and dispersed in the tooth germ (Fig. 7A, panels b and d). In control Epfn+/− molars, Epfn expression was restricted to the inner dental epithelial cells and the enamel knot cells at this stage (Fig. 7A, panels e and f). At E17.5, in Epfn+/− molars, the inner epithelial cell layer was expanded, and it strongly expressed Ki67 (Fig. 7B, panel a). In Epfn−/− molars, Ki67-positive cells (red) were dispersed in the whole tooth germ and also in surrounding mesenchyme cells (Fig. 7B, panel b). It is difficult to distinguish rapid and slow proliferating cells by Ki67 and PCNA immunostaining because these proteins are expressed from late G1 and have a relatively long half-life. To detect rapidly proliferating cells, we performed a short term BrdUrd incorporation assay and found that the inner dental epithelial cells were rapidly proliferating (Fig. 7B, panel c, yellow). In Epfn−/− molars, BrdUrd-positive cells were significantly reduced (Fig. 7B, panel d). The enamel knot epithelial cells normally do not proliferate and eventually die by apoptosis (26). In E17.5 Epfn−/− molars, there were TUNEL-positive cells in secondary enamel knots (Fig. 7B, panel e, arrowheads). In Epfn−/− molars, multiple ectopic TUNEL-positive areas were observed in the enamel organ (Fig. 7B, panel f). Unlike the enamel knots of Epfn−/− molars, Shh, one of the enamel knot markers, did not express in these areas, so these TUNEL-positive spots likely represent multiple premature enamel knots. The pRb protein plays a critical role in G1-S checkpoint control (27). It is phosphorylated (Phos-pRb) at the end of G1 and remains so throughout S-G2. Unphosphorylated pRb prevents cell proliferation, whereas phosphorylation of pRb results in the activation of a number of genes required for S phase transition (28).

We found that in control Epfn+/− E17.5 molars, most of the inner dental epithelial cells were positive for Phos-pRb immunostaining, and enamel knot epithelial cells were negative for Phos-pRb, corresponding to the proliferative state and nonpro-

FIGURE 6. Dysregulation of Lef-1 and Shh. A–G, in situ hybridization of Lef-1, Shh, and Epfn mRNA in Epfn+/− (A–D) and Epfn−/− (E–H) Lef-1 in E11.5 heads (A and E) and E14.5 molars (B and F); Shh in E14.5 (C and G); Epfn in E14.5 (D and H). Lef-1 expression was up-regulated in the epithelium and mesenchyme of E11.5 maxilla and mandible processes and was not localized in the enamel knot but extended into the inner dental epithelium layer in Epfn−/− mice. Shh expression of the enamel knot in E14.5 Epfn−/− molar was reduced.

FIGURE 5. Defects in tooth matrix protein expression. A–H, expression of tooth matrix proteins in 10-day-old Epfn+/− (A–D) and Epfn−/− (E–H) molars (E and H, histology of the same serial sections. Ambn, Enam, and Dspp were not expressed in dental epithelial cells of Epfn−/− molars. Dspp expression in odontoblasts was reduced in Epfn−/− molars. en, enamel; den, dentin; enam, enameline. Magnification, ×10.
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FIGURE 7. Defects in rapid dental epithelial cell proliferation and enamel knot formation. A, cell proliferation and apoptosis. Panels a–f, immunostaining of Ki67 (panels a and b), PCNA (panels c and d), and Epfn (panels e and f) in E16.5 Epfn+/− (panels a, c, and e) and Epfn−/− molaris (panels b, d, and f). In control molars, Ki67 and PCNA staining was strong in outer and inner dental epithelial layers (panels a and c), whereas in Epfn−/− molars, Ki67- and PCNA-positive cells were dispersed, and there were no clear epithelial cell boundaries. In control molars, Epfn was restricted to the inner dental epithelial cells. Epfn was absent in Epfn−/− molars. Panels e and f, double-immunostaining for E-cadherin (green) for staining epithelial cells and Epfn (red). Magnification, × 20. dl, dental lamina; ide, inner dental epithelial cells; dm, dental mesenchyme. B, Ki67 staining (panels a and b), BrdUrd (BrdU) labeling (green); red, nuclear (panels c and d); TUNEL assays (red, TUNEL-positive; blue, nuclear) (panels e and f); Phos-pRb (panels g and h); and enlarged images (panels i and l) of the boxed areas in panels g and h and Epfn (panels k and l) staining of E17.5 Epfn−/− (panels a, c, e, g, i, and k) and Epfn−/− molars (panels b, d, f, h, j, and l). The inner dental epithelial cells in Epfn−/− molars were strongly labeled with BrdUrd but not those in Epfn−/− molars. Secondary enamel knots (arrowheads) in Epfn−/− molars were TUNEL-positive, and in Epfn−/− molars, there were multiple positive spots. In Epfn−/− molars, the inner dental epithelial cells but not enamel knot epithelial cells (asterisk) were positive for Phos-pRb, whereas in Epfn−/− molars, Phos-pRb-positive regions were disrupted by unphosphorylated pRb regions. Both the inner dental and the enamel knot epithelia expressed Epfn in Epfn−/− molars. asterisk, secondary enamel knot.

FIGURE 8. Promotion of preameloblast differentiation into ameloblasts by Epfn. A, RT-PCR analysis of SF2 cells, a preameloblast cell line, transfected with the Epfn expression vector (Epfn-pcDNA3.1/Myc-His vector). Expression of mRNA for ameloblastin and Dspp, markers for ameloblasts, was increased by Epfn overexpression. In contrast, expression of Lef-1 mRNA, a marker of preameloblasts, was decreased by Epfn overexpression. Hprt, hypoxanthine phosphoribosyltransferase. B, ameloblast promoter activity of the Epfn reporter construct in cells cotransfected with the Epfn expression vector. Epfn promoter activity was increased in SF2 cells but not in NIH3T3 or MC3T3-E1 cells.

We have identified several tooth defects in Epfn−/− mice: complete absence of enamel, defect in cusps, abnormal dentin, and supernumerary teeth. In Epfn−/− teeth, differentiation of the dental epithelium into the enamel knot and inner dental epithelia was impaired. At the bud stage of tooth development, Epfn expression is restricted to the inner dental epithelium, and at the cap stage, when the enamel knot starts to form, its expression continues in the expanding inner dental epithelial layer and also starts in the enamel knot, a signaling center responsible for cusp formation. Thus, Epfn expression patterns in nor-
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One of the characteristic features of the inner dental epithelium is rapid proliferation, as shown by short term BrdUrd labeling (Fig. 7B, panel c). However, in Epfn−/− molar, the number of rapidly proliferating cells was significantly less than that of control molars (Fig. 7B, panel d). These results suggest that Epfn is required for rapid cell proliferation of the inner dental epithelium, which is consistent with our previous report that Epfn transfection into preameloblasts promotes cell proliferation (18). Furthermore, at the cap stage of Epfn−/− molars, Lef-1 and BMP-4 (data not shown) continued to be expressed in the cell layer corresponding to the inner dental epithelium and in mesenchyme condensation areas, whereas in wild-type molars, Lef-1 and BMP-4 expression is restricted to the enamel knot and a mesenchyme condensation. Phos-pRb immunostaining of Epfn+/+ molars showed irregular cell layers in the upper area of the enamel organ (Fig. 7B, panels h and j). Phosphorylation of pRb at the G1/S transition phase dissociates E2F from the inactive E2F-pRb complex and promotes cell cycle progression. In control Epfn+/+ molars, strong phosphorylation of pRb was observed in the inner dental epithelial layer, but pRb was not phosphorylated in the enamel knot, which is consistent with the BrdUrd labeling results. In Epfn−/− molars, Phos-pRb-positive layers have multiple disruptions by non-phosphorylated pRb-containing, nonproliferative cell regions. The nonproliferation property of the unique cell masses in Epfn−/− molars is similar to that of the enamel knot of control Epfn+/+ molars, and these structures likely represent multiple premature enamel knot-like structures. The cell cycle-exit enamel knot epithelial cells and postmitotic differentiated ameloblasts express Epfn, and they eventually die by apoptosis. Therefore, Epfn functions not only to promote proliferation and differentiation of the inner dental epithelium, but at later stages, also promote and maintain the differentiation state of ameloblasts and enamel knot epithelia. Our data suggest that Epfn activity for rapid cell proliferation is mediated in part through phosphorylation of pRb, which leads to the release of E2F from a pRb-E2F complex to activate genes for cell proliferation. In addition, our results suggest that Epfn is required for cell type specification of the dental epithelium. It has been shown that pRb not only suppresses cell cycle progression but also regulates cell lineage specification of several types of stem cells in myogenesis, adipogenesis, and hematopoiesis by cooperating with cell type-specific transcription factors (29). For example, pRb cooperates with MyoD to activate muscle-specific genes (30, 31). Growth arrest is apparently not sufficient to induce ameloblast differentiation, similar to these tissues. Epfn may work together with pRb as a cell type-specific factor for cell type specification in odontogenesis.

We observed a delay of tooth development in Epfn−/− mice. This is likely in part due to delayed and impaired differentiation of the dental mesenchyme. In Epfn−/− molars, there were multiple mesenchyme condensation areas, and the expression of Lef-1 (Fig. 6F) and BMP-4 (data not shown) was up-regulated in these areas. Since in early stages Epfn is not expressed in the dental mesenchyme, these abnormalities of the dental mesenchyme must be caused by impaired signaling from Epfn-deficient dental epithelium. However, later, Epfn is expressed in differentiated odontoblasts. In Epfn−/− mice, expression of Dspp by odontoblasts was significantly reduced (Fig. 5G), and dentinal tubules were abnormal. Thus, Epfn is required for correct differentiation of the odontoblastic mesenchyme cells.

One of the striking phenotypes of Epfn-deficient mice is supernumerary teeth in both molars and incisors. In Epfn−/− mice, initial patterning of tooth development was normal, and a single dental placode formed. At later stages, the dental epithelium begins to invaginate into the mesenchyme at multiple sites within the bud and forms multiple branches that result in hyperdontia. Canonical Wnt/β-catenin signaling and its downstream molecule Lef-1 are essential for tooth development (8). Lef-1 expression in the dental epithelium is required for the bud-cap transition and normal tooth development, suggesting the critical role of Lef-1 expression in the inner dental epithelium for the induction of the mesenchyme to form a dental papilla (8, 23). Overexpression of Lef-1 under the control of the epithelial cell-specific K14 promoter in transgenic mice develops abnormal invaginations of the dental epithelium in the mesenchyme, suggesting that the perturbations in the epithelium and mesenchyme are caused by Lef-1 expression in the epithelium (32). More recently, it was found that constitutive activation of β-catenin by an exon 3 deletion of the floxed-β-catenin gene with K14-Cre produces supernumerary teeth (33). Mutant mice develop multiple ectopic buds into the mesenchyme with supernumerary enamel knots, suggesting that excess teeth develop by a renewal process in which enamel knots bud off from the dental epithelium. In E16 β-catenin mutant molars, Epfn is expressed in spotty areas and apparently not in the inner dental epithelium. Some of these spotty Epfn-positive areas are thought to be ectopic enamel knots because other markers for the enamel knot are expressed in these areas. These phenotypes seem to have some similarities to those of Epfn+/− mice where Epfn is absent in the inner dental epithelium, and multiple premature enamel knot-like structures form in Epfn−/− molars, suggesting that supernumerary teeth in these mutant mice develop in part through a common mechanism. In the absence of Epfn, dental epithelial cells fail to differentiate into rapidly proliferating inner dental epithelium and retain a slow-proliferating but self-renewable progenitor phenotype, and multiple nonproliferating and immature enamel knot-like structures are formed. These nonproliferating areas likely bud off from the undifferentiated and self-renewable dental epithelium, leading to supernumerary teeth, which is similar to the hypothesis proposed by Jarvinen et al. (33) in a recent report. The continuous increase in the number of molars with age in Epfn−/− mice is unique and is likely attributable in part to the sustained progenitor phenotype of the dental epithelium. Lef-1 may play a role in sustaining the progenitor phenotype since its expression is inhibited in the inner dental epithelium at the cap stage in wild-type molars. This is consistent with our data and previous reports showing that Lef-1 and β-catenin are
dysregulated and that multiple tooth budding occurs (33). In Epfn−/− mice, teeth are not coated at all by enamel but have dentin. Epfn−/− dental mesenchyme cells, which are located adjacent to the premature inner dental epithelium, can differentiate into dentin-secreting odontoblasts, although with some delays. These results suggest that odontoblasts can be generated from dental mesenchyme cells when the dental epithelium migrates and interacts with them. From the clinical translational perspective, these findings are significant for tissue engineering to regenerate teeth since in adult teeth, dental mesenchymal cell are available but not dental epithelial cells. Mammalian dentition is complex with differences between species in tooth number, shape, and renewal system, but in most mammalians, the incisors, canines, and premolars appear in two generations (i.e. primary and permanent dentition). The initiation of the permanent dental laminas begins at the lingual side of the primary dental lamina by branching. The permanent dental lamina migrates into the dental mesenchyme and maintains a multipotent dental epithelial progenitor cell state and subsequently forms a permanent tooth bud. The mechanism of the two generations in dentition is not fully understood. Epfn is an evolutionarily conserved gene in mammals, and we believe that Epfn may play an important role in dental patterning. Elucidating Epfn function will lead to the development of new tissue engineering for tooth regeneration and the identification of human diseases caused by Epfn mutations.

Epfn expression continues from the early stages of dental epithelial cells to differentiated ameloblasts. At the E14.5 cap stage, Epfn expression is restricted to the inner dental epithelium. Our cotransfection studies using a dental epithelial cell line and the Epfn expression vector showed that Epfn promotes the differentiation of dental epithelial cells into ameloblast phenotypes. Epfn activates the expression of mRNA for ameloblastin and Dspp. We also found that the ameloblastin promoter-reporter construct was activated in dental epithelial cells when the cells were cotransfected with the Epfn expression vector. These results suggest that Epfn is a key transcription factor for the induction of ameloblast differentiation and is essential for ameloblastin expression. The activation of the ameloblastin promoter by Epfn is cell typespecific. These results suggest that the promoter activation is likely required for an additional factor(s) to be present in preameloblasts or in ameloblasts. The factor(s) may work cooperatively with Epfn or independently to activate the ameloblastin promoter. The cooperative activities of Epfn with other factors may explain the multiple functions of Epfn (e.g. it promotes rapid cell proliferation of the dental epithelium and the differentiation of enamel knot epithelium and ameloblast). It is also possible that Epfn exerts different functions depending on its expression levels.

Sp3-deficient mice develop enamel hypoplasia (14). The inner dental epithelium apparently differentiates to form a single layer of polarized ameloblasts in Sp3-deficient teeth. However, Sp3-deficient mice do not produce the enamel matrix proteins ameloblastin or amelogenin. We found that Sp3 is strongly expressed by the dental epithelium in Epfn-deficient teeth (data not shown). Therefore, Sp3 is not a direct target gene of Epfn. These results indicate that Sp3 alone is not sufficient for ameloblastin expression and suggest that Sp3 works with another protein factor(s) for ameloblastin gene activation.

In this study, we have uncovered novel diverse roles of Epfn in tooth development. We demonstrate that Epfn is essential for tooth morphogenesis by regulating tooth numbers and dental epithelial cell fate. This process appears to involve coordinated signaling during the interactive differentiation of the dental epithelium and mesenchyme. At later stages, Epfn acts as a key regulator for ameloblast differentiation and also probably for maintenance of the differentiation state. Thus, our studies provide new dimensions of understanding of the molecular mechanism governing the complex processes in tooth morphogenesis.

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