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# LEF1 is a critical epithelial survival factor during tooth morphogenesis

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**Abstract**

LEF1 is a cell-type-specific transcription factor and mediates Wnt signaling pathway by association with its co-activator β-catenin. Wnt signaling is known to be critical for the specification of cranial neural crest (CNC) cells and may regulate the fate diversity of the CNC during craniofacial morphogenesis. Loss of *Lef1* results in arrested tooth development at the late bud stage and LEF1 is required for a relay of a Wnt signaling to a cascade of FGF signaling activities to mediate the epithelial–mesenchymal interaction during tooth morphogenesis. It remains unclear, however, what is the cellular mechanism of LEF1 signaling in regulating tooth morphogenesis. To test the hypothesis that LEF1 signaling regulates the fate of the dental epithelial and the CNC-derived mesenchymal cells during tooth morphogenesis, we investigated and compared the cellular migration, proliferation, and apoptotic activity within the tooth germ between the wild-type and *Lef1* null mutant mice. Using the *Wnt1-Cre/R26R* transgenic system for indelibly marking the progenies of CNC cells, we show that there is no CNC migration defect in the *Lef1* null mutant mice, indicating that the arrest in tooth development is not the result of shortage of the CNC contribution into the first branchial arch in the *Lef1* mutant. Furthermore, there is no alteration in cell proliferation or condensation of the CNC-derived dental mesenchyme in the *Lef1* null mutant, suggesting that LEF1 may not affect the cell cycle progression of the multipotential CNC cells during tooth morphogenesis. Importantly, apoptotic activity is significantly increased within the dental epithelium in the *Lef1* null mutant mice. As the result of this increased cell death, the bud stage tooth germ fails to advance to the cap stage in the absence of *Lef1*. Inhibition of apoptotic activity by FGF4 rescues the tooth development in the *Lef1* null mutant. Our studies suggest that LEF1 is a critical survival factor for the dental epithelial cells during tooth morphogenesis.


*Keywords:* Apoptosis of dental epithelium; Cranial neural crest (CNC); LEF1 signaling; Tooth morphogenesis

## Introduction

Tooth morphogenesis results from an ordered series of gene interactions, each in turn designating individual cell type proliferation, apoptosis, and differentiation. These genetic interactions form the molecular basis of heterologous tissue interactions between the ectodermally derived enamel organ epithelium and the cranial neural crest (CNC)-derived ectomesenchyme during tooth morphogenesis. Multiple growth and transcription factors belonging to several signaling families have been identified as critical regulators at the initiation and subsequently throughout all stages of tooth development (Chai and Slavkin, 2003; Thesleff and Sharpe, 1997). Significantly, most of the signaling networks that are used reiteratively throughout tooth development are in common with the regulatory systems that are critical for governing the development of other organs, such as feather, hair, mammary gland, salivary gland, and pancreas morphogenesis. The growing

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scientific evidence suggests a highly conserved biological mechanism in regulating organogenesis. More importantly, specific growth and transcription factor signaling cascades have been identified as critical regulators in determining the physiological site and location for tooth initiation and in defining patterns of formation of various types of teeth (for review, see Cobourne and Sharpe, 2003; Jernvall and Thesleff, 2000).

Lymphoid enhancing factor 1 (LEF1) is a cell-type-specific transcription factor expressed in lymphocytes of the adult mouse, and in the neural crest, mesencephalon, tooth bud, whisker follicles, and other sites during embryogenesis (Oosterwegel et al., 1993; Travis et al., 1991; van Genderen et al., 1994; Waterman et al., 1991; Zhou et al., 1995). Targeted inactivation of the *Lef1* gene in the mouse germ line results in a pleiotropic phenotype in which the development of teeth, whiskers, hair follicles, and mammary glands is severely impaired (van Genderen et al., 1994). Tooth development is initiated in *Lef1* null mutant embryos; however, it is arrested at the late bud stage before the formation of a mesenchymal dental papilla. Recent study suggests that LEF1 is required in the dental epithelium during tooth development. The biological function of LEF1 is to induce the CNC-derived dental mesenchyme to become competent to form dental papilla, an important component indicating the successful advancement of the tooth germ development into the cap stage (Chai et al., 1998; Kratochwil et al., 1996, 2002). At the molecular level, LEF1 is responsible for a direct regulation of *Fgf4* expression to relay a Wnt signaling to a cascade of FGF signaling activities that mediate the sequential and reciprocal interactions between the dental epithelium and the CNC-derived dental mesenchyme during tooth development (Kratochwil et al., 2002). However, despite this molecular information demonstrating the biological importance of LEF1 signaling in regulating tooth morphogenesis, the cellular mechanism of LEF1 signaling in regulating cell fate determination during tooth morphogenesis remains unclear.

During embryonic development, LEF/TCF family of DNA-binding factors form transcriptional regulatory complex with β-catenin to mediate Wnt signaling to control cell proliferation, intercellular adhesion, cell survival, and cell fate determinations (for review, see Fuchs et al., 2001; van Noort and Clevers, 2002; Willert and Nusse, 1998). In addition, LEF1-mediated Wnt signaling is also critical for pattern formation and axis specification (Laurikkala et al., 2002; Moon et al., 1997a,b; Siegfried and Perrimon, 1994). Dysregulation of components of the Wnt signaling pathway blocks hair follicle, tooth, and mammary gland development during embryogenesis and may also have oncogenic effects in tissues such as colon and breast (Andl et al., 2002; Morin et al., 1997; Nusse and Varmus, 1982). Collectively, these studies suggest that LEF1-mediated Wnt signaling may play a critical role to regulate the progression of cell cycle of progenitor cells to control the cell fate during embryonic and postnatal development.

To investigate the role of LEF1 signaling in regulating the progression of cell cycle of the dental epithelium and the CNC-derived dental mesenchyme cells during tooth morphogenesis, we compared the cellular proliferation and apoptotic activity in the tooth germ between the wild-type and *Lef1* null mutant mice. Our study shows that loss of *Lef1* results in a significant increase in the apoptotic activity within the dental epithelium, suggesting that LEF1 is a critical survival factor for the dental epithelial cells during the advancement of the tooth germ from the bud to the cap stage. In addition, our gene expression analyses have discovered a specific *Lef1* expression in the cervical loop of the enamel organ epithelial cells. These findings suggest that LEF1 may also have an important role in regulating the progression of cell cycle and fate determination of the cervical loop progenitor cells during later stages of tooth morphogenesis.

## Materials and methods

### *Two-component genetic system for marking the progeny of CNC cells*

Both *Wnt1-Cre* transgenic line and *R26R* conditional reporter allele have been described previously (Danielian et al., 1998; Soriano, 1999). Mating *Wnt1-Cre* and *R26R* mice generated transgenic mice with progenies of neural crest cells labeled with β-gal because once *Wnt1-Cre* expression commences in premigrating neural crest cells, the β-galactosidase expression is indelible. Detection of β-galactosidase (*LacZ*) activity in both whole embryos and tissue sections was done as previously described (Chai et al., 2000). All animals used in this study were maintained in a C57BL/6J background. We first crossed either *Wnt1-cre* or *R26R* transgenic mice with *Lef1* heterozygous mutant to generate mice carrying $Wnt1cre^{Tg/+}/Lef1^{+/-}$ or $R26R^{Tg/+}/Lef1^{+/-}$, respectively. Upon crossing $Wnt1cre^{Tg/+}/Lef1^{+/-}$ with $R26R^{Tg/+}/Lef1^{+/-}$ mice, we generated *Lef1* null mutant carrying *Wnt1cre/R26R* transgene, which allowed us to follow the progeny of CNC cells indefinitely. Embryonic age was determined with noon of the day of plug observation as E0.5. External staging was used to define embryonic development according to the number of somite pairs (Theiler, 1989). Genotyping of the *Lef1* mutant embryos carrying *Wnt1cre/R26R* transgene was done as previously described (Chai et al., 2000; Soriano, 1999; van Genderen et al., 1994).

### *Detection of β-galactosidase (lacZ) activities*

Whole embryos (E9.5 and E10.5) were stained for β-galactosidase activity according to standard procedures. Embryos were fixed for 20 min at RT in 0.2% glutaraldehyde in PBS. Fixed embryos were washed three times in rinse solution (0.005% Nonidet P-40 and 0.01% sodium

deoxycholate in PBS) and stained overnight at room temperature using standard staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.4% X-gal in PBS). The next morning, samples were rinsed twice in PBS and post-fixed in 3.7% formaldehyde. Then, the embryos were sectioned to observe lacZ expression at the cellular level. Sections were cut at 10-μm thickness and counter-stained with Nuclear Fast Red.

*Cryostat sectioning*

Mouse embryonic tissue was frozen, sectioned, and then stained according to standard procedures. Specifically, mouse tissue was dissected in PBS and fixed by immersion in 0.2% glutaraldehyde solution for 30 min at RT. Tissue was soaked in 10% sucrose in PBS for 30 min at 4°C, incubated in PBS plus 2 mM $MgCl_2$, 30% sucrose and 50% OCT at 4°C for 2 h, then frozen in OCT. Sections were cut at 10–20-μm thickness and mounted on polylysine-coated slides. The mounted tissue sections were fixed in 0.2% glutaraldehyde for 10 min on ice, rinsed briefly in PBS, and rinsed in detergent solution (0.005% NP-40 and 0.01% sodium deoxycholate in PBS) for 10 min at 4°C. Thereafter, the slides were washed in PBS for 10 min and stained in X-gal staining solution overnight at room temperature in the dark. Sections were counter-stained with Nuclear Fast Red and eosin.

*BrdU labeling for CNC cell proliferation analysis*

BrdU was injected into the pregnant mouse (100 μg/g of body weight, intraperitoneal injection) 2 h prior to harvesting embryos. Two adjacent sections were mounted on two different slides and processed separately, one for β-galactosidase staining and the other for BrdU staining (Chai et al., 1999, 2000). By superimposing these two adjacent sections, we were able to evaluate the proliferative activity of CNC cells (Ito et al., 2003).

*Analysis of cell death*

TUNEL assay was performed using the In Situ Cell Death Detection (fluorescein) kit (Roche Molecular Biochemicals) by following the manufacturer's protocol.

*Immunohistochemistry*

Sectioned immunohistochemistry was performed with an Immunostaining kit (Zymed) according to manufacturer's directions. The following antibodies were used in this study: anti-BrdU (Sigma); anti-phospho-Rb; anti-cyclin D1; and anti-cyclin A (Santa Cruz, CA).

*In situ hybridization*

A 1.2-kb fragment of murine *Lef1* cDNA subcloned into pBluescript II SK was digested with *Not*I and transcribed with *T7* RNA polymerase (Boehringer Mannheim) for an antisense probe. For a *Lef1* sense probe, pBluescript II SK was cut with *Eco*RI and transcribed with *T3* polymerase. The RNA probes were labeled with $^{33}P$. The hybridization procedures were performed according to the methods of Wilkinson (1998). Several negative controls (e.g., sense probe and no probe) were run in parallel with the experimental reaction.

*Preparation and introduction of FGF4 beads*

Heparin–acrylic beads (Sigma, 200 μm diameter) were washed in phosphate-buffered saline (PBS) and then incubated in FGF4 solution (100 ng/μL, R&D system). Control beads were incubated in 0.1% BSA. FGF4- or BSA-containing beads were placed adjacent to the mandibular molar germ in E13.5 explant. Then, mandibular explants were cultured in serum-less, chemically defined medium for up to 7 days and harvested for histological as well as apoptotic analysis.

## Results

*Developmental expression of Lef1 during tooth morphogenesis*

Loss-of-function analysis has demonstrated the biological significance of LEF1 signaling in regulating early tooth development. Specifically, LEF1 is required to relay a Wnt signaling to a cascade of FGF signaling activities to mediate the sequential and reciprocal dental epithelium and mesenchyme interactions (Kratochwil et al., 1996, 2002). To date, most of the studies on the function of LEF1 in regulating tooth morphogenesis have been focusing on the early stages of tooth development because the tooth germ is arrested at the bud stage in *Lef1* null mutant mice. To understand the biological significance of LEF1 signaling in regulating later stages of tooth morphogenesis, we have investigated the expression pattern of *Lef1* throughout all stages of tooth formation. At the initiation of tooth development, *Lef1* was detected in the dental epithelium and the CNC-derived mesenchyme while there was no *Lef1* expression in the oral epithelium (Figs. 1A,B). The *Lef1* expression at the laminar stage of tooth germ suggests that LEF1-mediated Wnt signaling is critical during the initiation of tooth morphogenesis. At the bud stage, *Lef1* continued to be expressed in the dental epithelium and mesenchyme (Figs. 1C,D). As tooth development reached the cap stage, *Lef1* was expressed in the outer and inner enamel epithelium, enamel knot, and the CNC-derived dental mesenchyme (Figs. 1E,F). Following the advancement of tooth development into the bell stage at E16.5, *Lef1* expression was associated with the inner enamel epithelium and the dental papilla (Figs. 1G,H). Prior to birth, tooth development had advanced significantly with the formation of dentine and

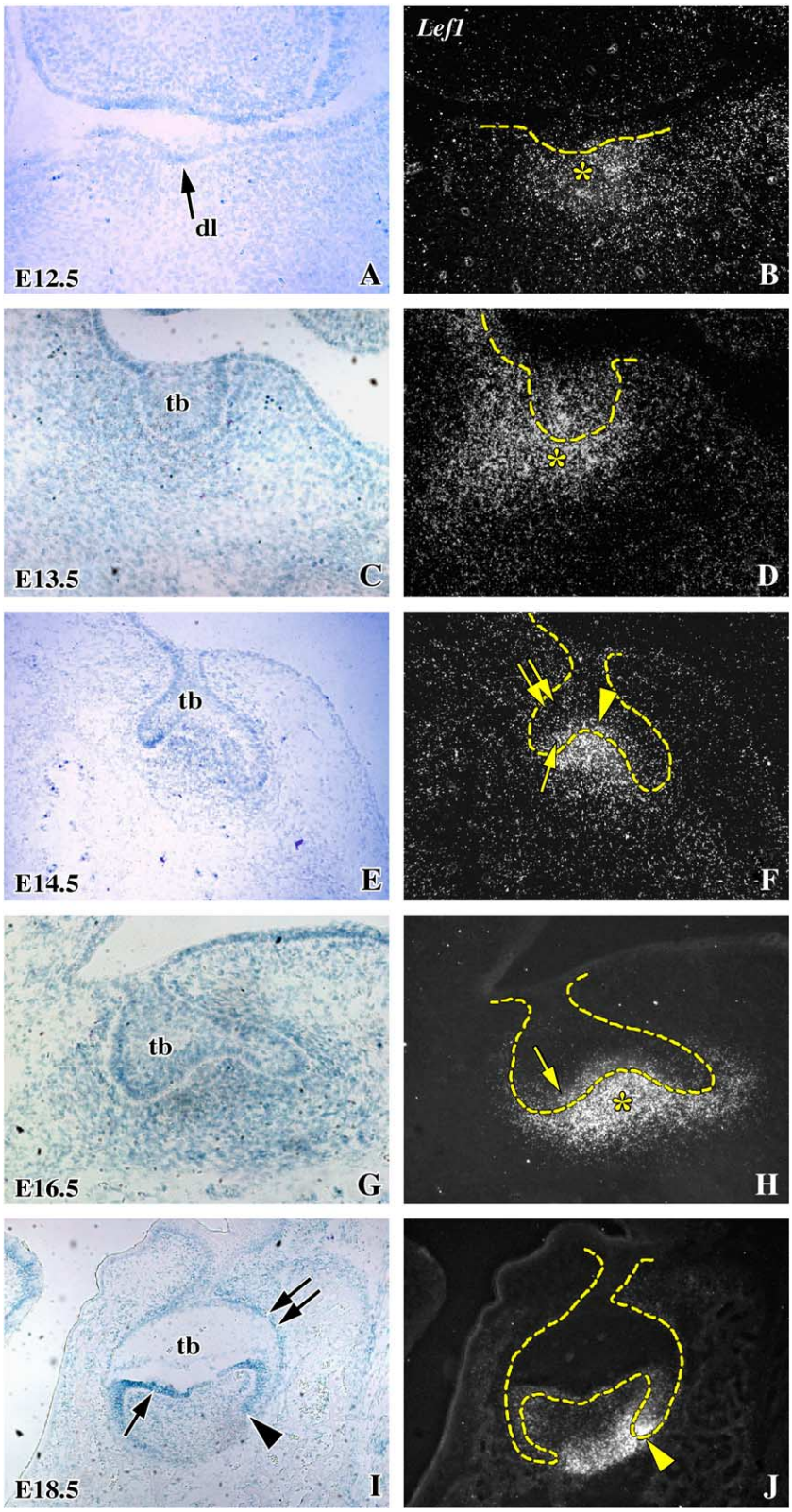



Fig. 1. Developmental expression of *Lef1* during tooth morphogenesis. (A, B) *Lef1* is expressed in the dental lamina (arrow) and the CNC-derived mesenchyme (*) at E12.5. (C, D) *Lef1* expression is detected in the bud stage dental epithelium (outlined by dotted line) and the CNC-derived mesenchyme at E13.5. (tb) tooth bud. (E, F) At the cap stage, *Lef1* is expressed in the inner (arrow) and outer (double arrow) enamel epithelium, enamel knot (arrowhead) and the dental mesenchyme. (G, H) *Lef1* expression is present in the inner enamel epithelium (arrow) and dental mesenchyme at the early bell stage tooth organ. (I, J) *Lef1* is expressed in the inner enamel organ epithelium, cervical loop (arrowhead), and the preodontoblast cells.

enamel matrices (Fig. 1I). *Lef1* was expressed within the inner enamel organ epithelium, part of the outer enamel epithelium where the cervical loop was formed, and the forming odontoblast cells (Fig. 1J).

### *Loss of Lef1 does not affect the CNC migration during craniofacial development*

In order to test the hypothesis that retarded tooth development in the *Lef1* null mutant mice was the result of a compromised CNC migration and having insufficient number of CNC cells to form the dental mesenchyme, we crossed the *Lef1*$^{+/-}$ mutant mice with the *Wnt1-Cre* and *R26R* transgenic mice to evaluate the contribution of CNC cells in the *Lef1* null sample. In *Lef1*$^{-/-}$*/Wnt1-Cre/R26R* mutant embryos, CNC cells migrated into and populated the mandibular prominence (as indicated by β-gal staining, Fig. 2B, arrow) at E9.5. The pattern of CNC cell distribution in *Lef1* null mutant embryos was identical to that of wild-type controls (Fig. 2A, arrow). These E9.5 wild-type and *Lef1* null mutant embryos were littermates and contained the same number of somite pairs (24–29 pairs, Theiler stage 15). Thus, we concluded that these embryos were at the equivalent developmental stage and that there was no delay in CNC cell migration during the first branchial arch development in *Lef1* null mutants. Furthermore, there was no apparent difference in the number of CNC cells (β-gal positive cells on a cross section) populating the first branchial arch between the wild-type and *Lef1* null mutant embryos (data not shown).

Similarly, at E10.5, *Lef1*$^{-/-}$*/Wnt1-Cre/R26R* mutant embryos did not show any alteration in the pattern of the CNC cell distribution when compared to the one of wild-type control (Figs. 2C,D). In particular, both maxillary and mandibular prominences were populated with equal number of CNC-derived cells (Figs. 2E,F). The frontonasal prominence was also well populated with CNC derivatives in both the wild-type and *Lef1* null mutant embryos. Collec-

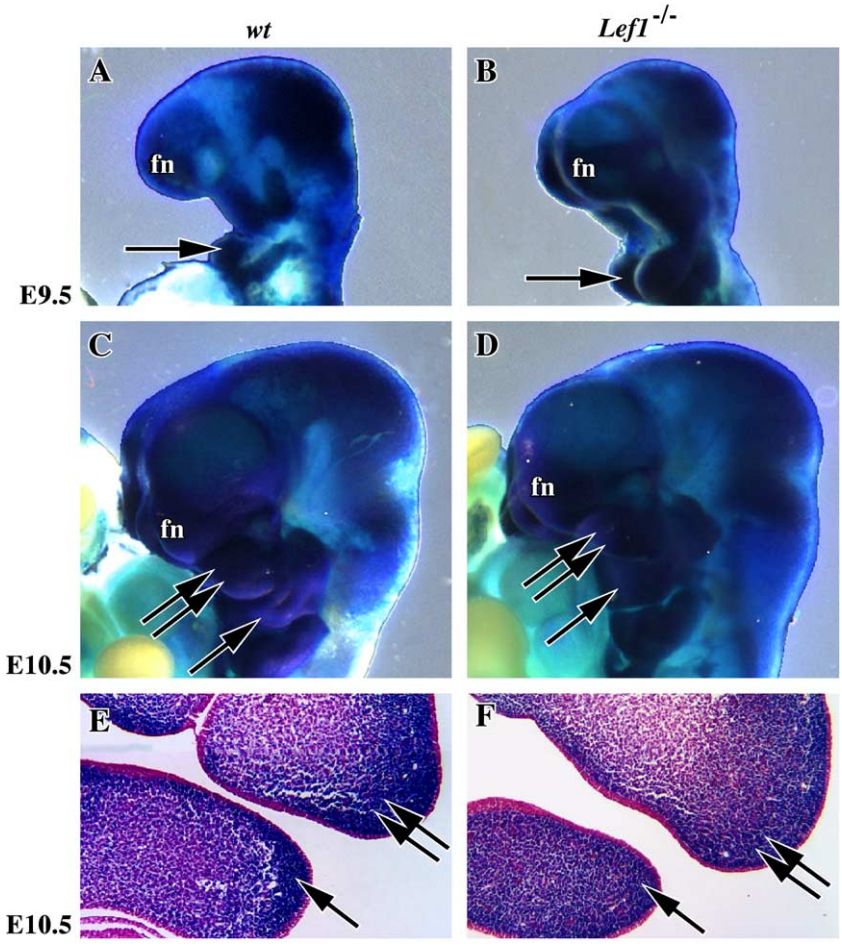



Fig. 2. Loss of *Lef1* does not affect the CNC migration during craniofacial development. (A) At E9.5, CNC cells have populated the first branchial arch (arrow) and the frontonasal prominence (fn), as indicated by positive β-gal staining (blue color), in the wild-type control. (B) In the *Lef1* null mutant, identical distribution of CNC cells is present in the first branchial arch (arrow) and in the craniofacial region of the *Lef1* null mutant mice. (C) At E10.5, the first branchial arch has divided into the mandibular arch (arrow) and maxillary arch (double arrow) in the control sample. CNC cells have populated the frontonasal region (fn) and the first arch derivatives. (D) In the *Lef1* null mutant, identical distribution of post-migratory CNC cells is observed. Sections through the first branchial arch also reveal normal contribution of the CNC into the first branchial arch in the *Lef1* null mutant. (E, F) There is no difference in the distribution of CNC cells within the mandibular process (arrow) and maxillary process (double arrow) between the wild-type (E) and *Lef1* null mutant (F).

tively, our data suggested that loss of *Lef1* function did not affect the proper migration of CNC cells during early craniofacial development.

### *LEF1 and the formation of the CNC-derived dental mesenchyme during tooth morphogenesis*

To test whether the *Lef1* null mutation results in a deficiency of CNC cells within the odontogenic mesenchyme and a compromised tooth development, we compared the fate of CNC cells between control and *Lef1* mutant embryos. Morphological analysis revealed that there was no difference in condensation of CNC-derived mesenchymal cells under the forming dental lamina at the bud stage tooth germ between the *Lef1* mutant and wild-type littermates at E12.5 and E13.5 (Figs. 3A,B and data not shown). At E14.5, normal tooth development had reached the cap stage, and the CNC-derived ectomesenchyme contributed significantly to the formation of the dental mesenchyme and sac (Fig. 3C). In *Lef1* null mutants, however, tooth development was arrested at the bud stage without any significant reduction of CNC cells in the dental mesenchyme (Fig. 3D). Furthermore, the dental sac, which defines the boundary of the tooth organ, was present (Fig. 3D).

At E17.5, β-gal-positive cells marked the progeny of the CNC-derived cells in the dental papilla of a molar tooth organ (Fig. 3E). The CNC-derived cells were aggregated adjacent to the inner enamel organ epithelium, indicating the important epithelial–mesenchymal interaction in regulating tooth development. In the *Lef1* null mutant, tooth development was retarded at the late bud stage with well-condensed CNC-derived dental mesenchyme (Fig. 3F). At birth, both epithelial-derived pre-ameloblasts and CNC-derived pre-odontoblasts were present in wild-type tooth germs (Fig. 3G). The alveolar bone of the mandible derives from the dental sac mesenchyme and is densely populated with CNC-derived cells (Han et al., 2003; Palmer and Lumsden, 1987). In wild-type sample, the alveolar bone had extended upward to enclose the tooth germ. In *Lef1* null mutants, however, there was still sufficient number of the CNC-derived dental mesenchyme adjacent to the retarded enamel organ (Fig. 3H). The formation of the mandible had proceeded with the fusion of the CNC-derived osteogenic front under the tooth bud, without enclosing the retarded tooth germ.

### *LEF1 is a critical epithelial survival factor during tooth morphogenesis*

To investigate if LEF1 is critical for the survival of dental epithelial and CNC-derived dental mesenchymal cells, we have compared the apoptotic activity between wild-type and *Lef1* mutant tooth germs. At E12.5 and E13.5, the apoptotic activity within the tooth germ was comparable between the wild-type and the *Lef1* null mutant samples (Figs. 4A,B). Significantly, there were large numbers of dental epithelial cells undergoing apoptosis in the *Lef1* null mutant tooth germ at the late bud stage at E14.5 while there was no detectable cell death in the CNC-derived dental mesenchyme (Fig. 4D). In the wild-type sample, apoptotic activity was not detected in either the dental epithelium or the CNC-derived dental mesenchyme (Fig. 4C). At E16.5, there was increased apoptotic activity in the dental epithelium of *Lef1* null mutant mice while the apoptotic activity in the CNC-derived dental mesenchyme was comparable to the one in the wild-type samples (Figs. 4E,F). Coupled with the cell proliferation analysis, increased cell death in the dental epithelium of the *Lef1* null mutant sample was the apparent cellular defect that may account for the failure of advancement of tooth development from the bud to the cap stage and suggests that LEF1 is an important epithelial survival factor during tooth morphogenesis.

### *Cell proliferation and the progression of cell cycle in the tooth germ of the Lef1 null mutant*

To further explore the cellular mechanism responsible for causing the failure of tooth development beyond the bud stage in the *Lef1* null mutant mice, we investigated whether there was an altered cell proliferation activity in the developing dental epithelium and the CNC-derived dental mesenchyme during tooth morphogenesis. Cell proliferation activity, as measured by BrdU incorporation, appeared to be identical in both the dental epithelium and the CNC-derived mesenchyme between the control and the *Lef1* null mutant samples at E13.5 (Figs. 5A,B). At E14.5, normal tooth development had reached the cap stage in the wild-type sample (Fig. 5C). Active cell proliferation was present in both the dental epithelium (38 ± 2.6%) and the CNC-derived mesenchyme (32 ± 2.1%), with the exception of the enamel knot, which serves as a signaling center and is designated to undergo apoptosis following cusp induction ($n$ = 5; Fig. 5C). In the *Lef1* null mutant sample, tooth development was retarded at the bud stage. Interestingly, however, there was active cell proliferation in both the dental epithelium (33 ± 4.1%) and the CNC-derived dental mesenchyme (29 ± 3.7%), suggesting the loss-of-function of *Lef1* did not affect the proper cellular proliferation in the tooth germ ($n$ = 5; Fig. 5D). At E16.5, active cell proliferation was detected in the tooth germs of both the control and the *Lef1* null mutant samples, despite the fact that tooth development remained retarded at the bud stage in the *Lef1* null mutant (Figs. 5E,F). Cell proliferation indexes also confirmed that there was comparable cell proliferation activity in the dental epithelium and the CNC-derived dental mesenchyme between the control and the *Lef1* null mutant samples (data not shown). Overall, it appeared that cell proliferation activity was not affected in the tooth germ of the *Lef1* null mutant.

In order to investigate whether the proper cellular proliferation activity was coupled with normal progression of the cell cycle in the dental epithelium and the CNC-derived dental mesenchyme of the *Lef1* null mutant mice,

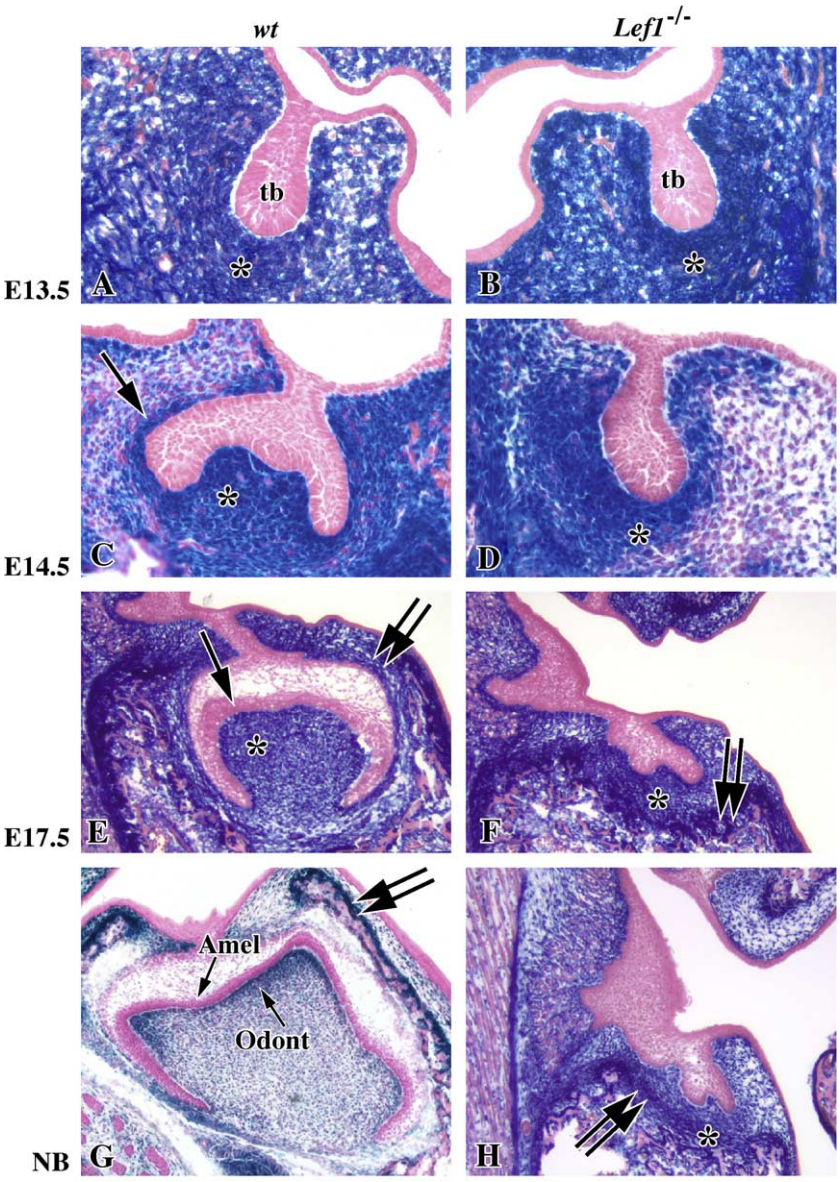



Fig. 3. The *Lef1* null mutation and the contribution of CNC cells during tooth morphogenesis. (A) At E13.5, tooth development has reached the bud stage (tb). The condensed dental mesenchyme is largely populated with CNC-derived cells (*, blue color). (B) *Lef1* null mutant embryo shows a bud stage tooth organ (tb) with normal CNC condensation in the dental mesenchyme (*). (C) At E14.5, a cap stage tooth organ is present with mostly CNC-derived cells in the dental papilla (*). The dental sac is well defined by CNC-derived cells (arrow). (D) In *Lef1* null mutant, tooth formation fails to advance to the cap stage. However, there is no cellular deficiency and failure of condensation in the CNC-derived dental mesenchyme (*). (E) At E17.5, tooth development is at the late bell stage. The CNC-derived pre-odontoblasts are adjacent to the inner enamel organ epithelium (arrow). Mandibular development shows that the CNC-derived cells (double arrow) of the dental sac are forming the alveolar bone to enclose the tooth organ. (F) In the *Lef1* null mutant, the tooth germ remains at the late bud/early cap stage with sufficient number of CNC-derived cells (*) surrounding the tooth bud. Alveolar bone fails to form. The upper boundary of the mandible (double arrow) is formed without including the tooth germ. (G) At birth, tooth organ shows non-CNC-derived pre-ameloblasts (Amel) and CNC-derived pre-odontoblasts (Odont). The osteogenic front of the developing alveolar bone (double arrow) is in the process of enclosing the tooth germ. (H) In the *Lef1* null mutant, there is a lack of condensed CNC in the dental mesenchyme surrounding the remnant of tooth bud. CNC-derived cells contribute to the upper boundary of the mandible (double arrow), without enclosing the tooth germ.

we examined the expression of cell cycle regulators in the *Lef1* null mutant sample. The proper phosphorylation of Rb protein in mid-G1 phase is critical for the mitogen-dependent G1-to-S phase transition during mammalian cell proliferation (Sherr, 1996; Sherr and Roberts, 1999; Taya, 1997; Weinberg, 1995). We compared the expression and phosphorylation of Rb proteins of the wild-type control and *Lef1* null mutant samples. At E13.5, comparable phosphorylated-Rb expression was present in both the control and the *Lef1* null mutant tooth germs (Figs. 6A,B). At E14.5, active Rb phosphorylation was detected in both the dental epithelium and the CNC-derived mesenchyme of the

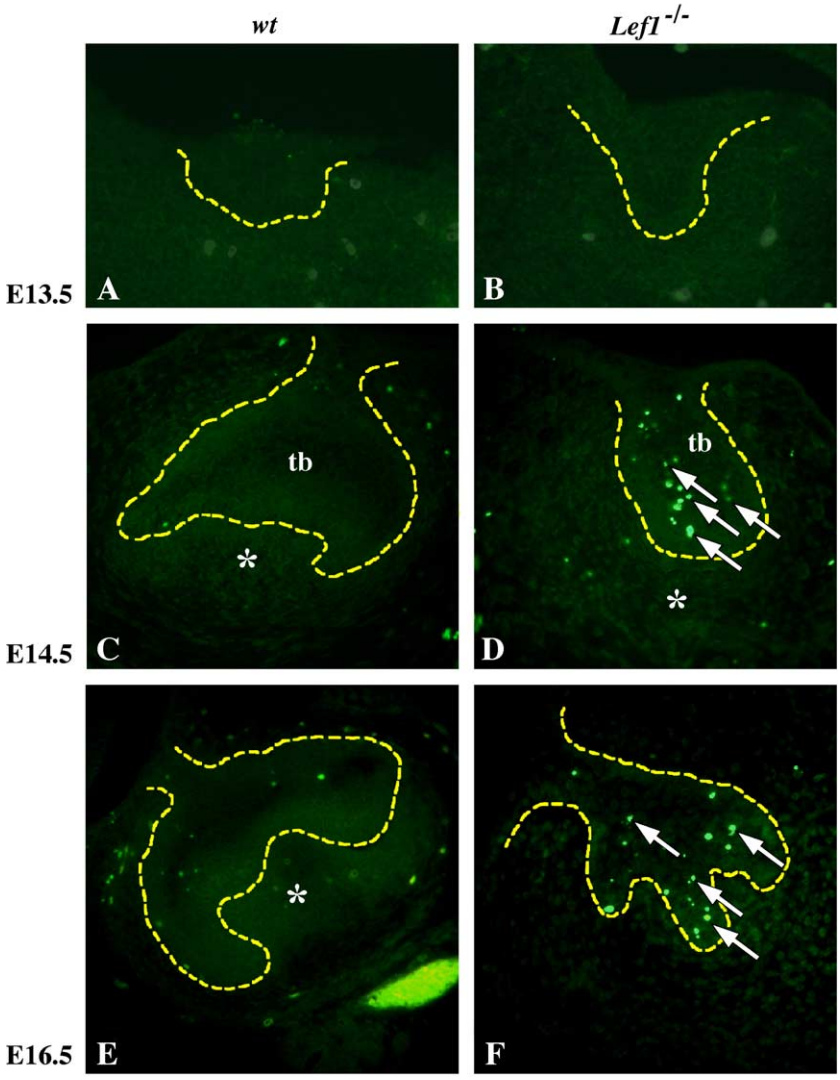



Fig. 4. LEF1 is a critical epithelial survival factor during tooth morphogenesis. (A) At E13.5, tooth development has reached the bud stage. There is no apparent apoptotic activity within the dental epithelium (outlined by dotted line) and mesenchyme. (B) In the *Lef1* null mutant, there is no alteration of apoptotic activity in the tooth germ when compared to the one in the wild-type control. (C) At E14.5, tooth development has reached the cap stage in the control. There is no active cell death in either the enamel organ epithelium (tb) or dental mesenchyme (*). (D) In the *Lef1* null mutant, active apoptotic signals are present throughout the entire enamel organ epithelium (arrow) while there is no active cell death in the dental mesenchyme (*). (E) At E16.5, no obvious apoptotic signals are associated with dental epithelium and the CNC-derived mesenchyme in the control tooth germ. (F) *Lef1* null mutant sample shows active apoptotic activity (arrow) within the dental epithelium, indicating the critical role of LEF1 in the survival of dental epithelial cells.

wild-type and *Lef1* null mutant samples (Figs. 6C,D). Cyclin D1, a member of the cyclin D family, functions to regulate phosphorylation of the retinoblastoma gene products, thereby activating E2F transcription to facilitate cell cycle progression. The expression of cyclin D1 was comparable in the dental epithelium and mesenchyme between the *Lef1* null mutant and the control at E13.5 and E14.5 (Figs. 6E–H). This data suggests that loss of *Lef1* did not interfere with the progression of cell cycle in the tooth germ. To further investigate cell cycle progression status, we compared the expression of cyclin A, a $S/G_2$ phase cyclin, in the dental epithelium and the CNC-derived dental mesenchyme of control and *Lef1* null mutant samples. At both E13.5 and E14.5, cyclin A expression was comparable between the wild-type control and the *Lef1* null mutant, indicating normal progression of the cell cycle in the *Lef1* null mutant tooth germ (Figs. 6I–L). Taken together with the cell proliferation analysis, loss-of-function of *Lef1* did not perturb the progression of cell cycle during tooth morphogenesis.

### *LEF1-mediated FGF4 expression is critical for the survival of dental epithelium during tooth development*

In order to understand the mechanism of LEF1-mediated Wnt signaling in regulating the survival of dental epithelial

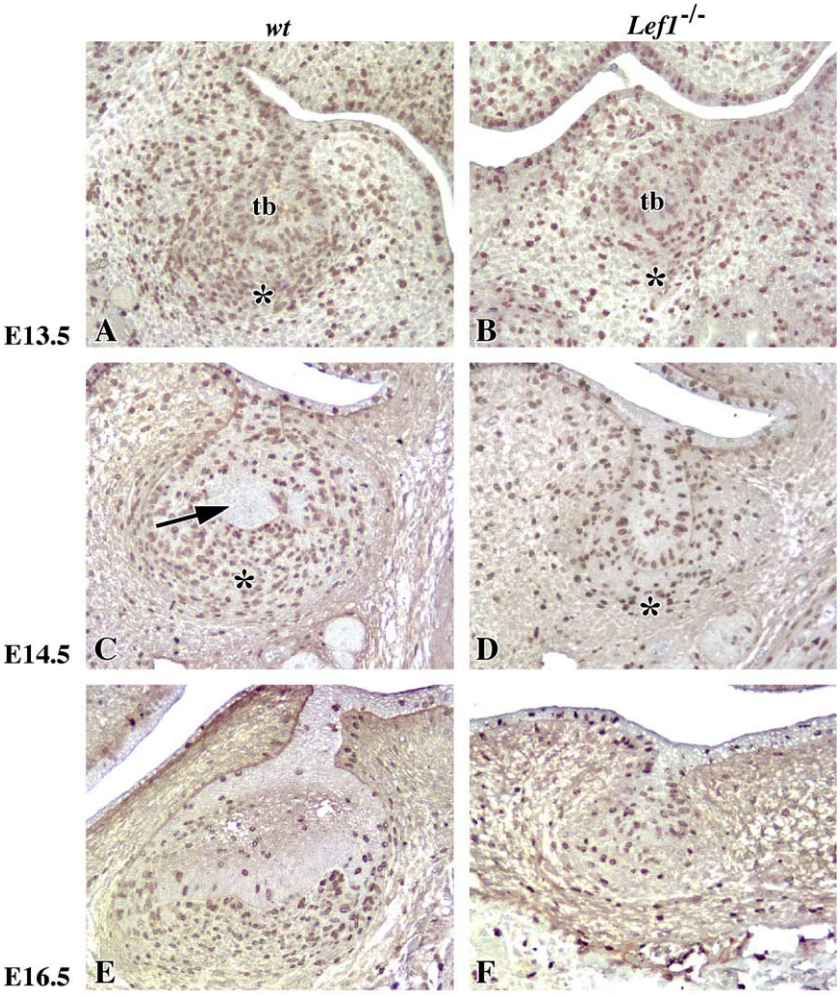
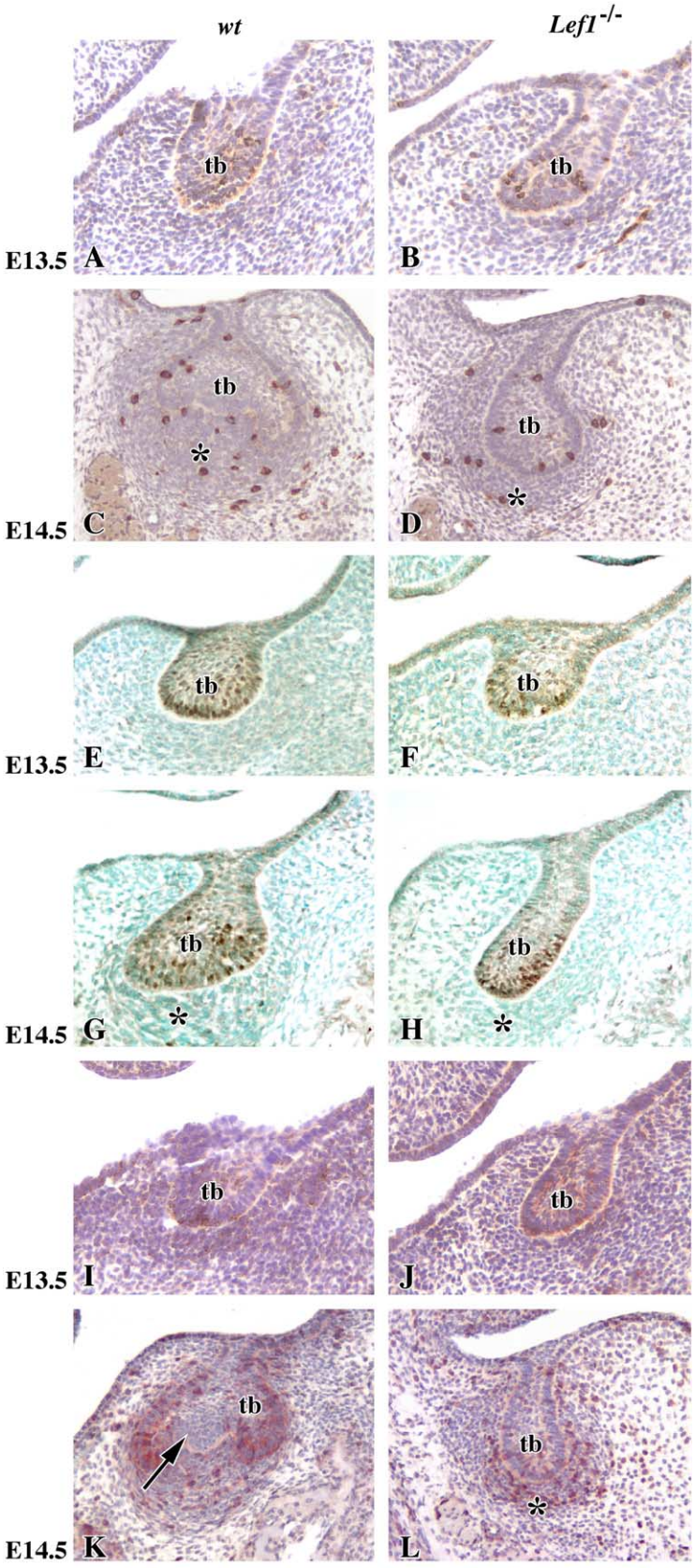



Fig. 5. Cell proliferation analysis in the tooth germ of the *Lef1* null mutant. (A) Cell proliferation analysis using BrdU staining (red dots) shows active mitosis in both dental epithelium (tb) and the CNC-derived mesenchyme (*) of a bud stage tooth germ of a control sample. (B) In the *Lef1* null mutant, there is normal cell proliferation activity in both the dental epithelium and mesenchyme. (C) At E14.5, a cap stage tooth organ shows active cell proliferation in both dental epithelium [except the enamel knot (arrow)] and the CNC-derived mesenchyme. (D) Cell proliferation activity in the retarded tooth germ of the *Lef1* null mutant is comparable to the one in the control sample. (E, F) No apparent difference in cell proliferation activity is detected between the wild type (E) and *Lef1* null mutant (F) samples.

cells, we designed experiments to test whether LEF1 was directly or indirectly involved in regulating the apoptotic activity during tooth development. The caspase inhibitor Z-VAD-FMK (at 10–100 μM) was unable to rescue the tooth development in the *Lef1* null mutant (data not shown). However, it would be premature to conclude that LEF1-mediated Wnt signaling is not directly involved in regulating the survival of dental epithelial cells because there is caspase-independent apoptosis during embryogenesis (Mergliano and Minden, 2003). Further studies are required on this subject. Significantly, FGF4 beads treatment was able to inhibit the apoptotic activity within the dental epithelium of the *Lef1* null mutant sample and rescue the tooth development to the cap stage while BSA beads treatment was unable to inhibit apoptotic activity within the dental epithelium and failed to rescue the tooth development in

Fig. 6. Cell cycle progression in the tooth germ of the *Lef1* null mutant. (A) At E13.5, normal phosphorylation of Rb (pRb) is observed in the wild-type bud stage tooth germ. Orange-red color denotes positive staining. (B) In *Lef1* null mutant, normal pRb expression is present in the tooth germ. (C, D) Although the tooth development is retarded at the bud stage in the *Lef1* null mutant (D), there is comparable pRb expression in the tooth germs of the wild type and *Lef1* null mutant. (E) At E13.5, cyclin D1 is expressed in the dental epithelium (tb) of the wild-type sample. (F) Normal cyclin D1 expression is observed in the *Lef1* null mutant tooth germ. (G, H) Normal cyclin D1 expression is present in the tooth germ in both the wild type and *Lef1* null mutant sample. (I) cyclin A is expressed in the dental epithelium (tb) and the CNC-derived mesenchyme. (J) Normal cyclin A expression is observed in the *Lef1* null mutant tooth germ. (K, L) At E14.5, tooth development is at the cap stage in the wild type sample (K). Cyclin A expression is detected in the dental epithelium [except the enamel knot, (arrow)] and the CNC-derived mesenchyme. While tooth development is retarded at the bud stage in the *Lef1* null mutant (L), normal cyclin A expression is present within both the dental epithelium and mesenchyme at E14.5.


wt
Lef1-/-
E13.5
A
tb
B
tb
E14.5
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E13.5
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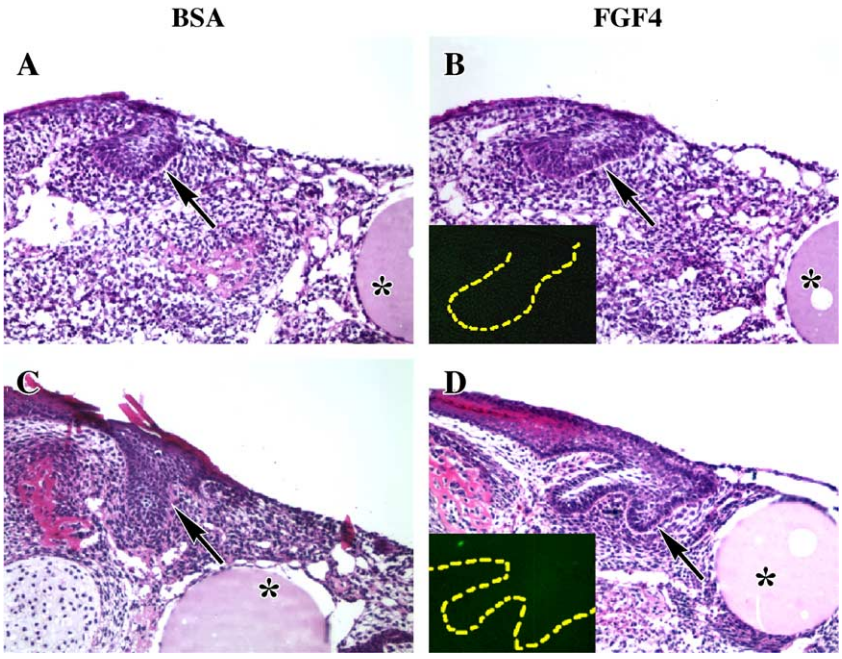


Fig. 7. FGF4 bead can inhibit apoptosis and rescues tooth development to the cap stage in the *Lef1* null mutant mandibular explant. (A, C) E13.5 *Lef1* null mutant mandibular explants treated with BSA bead fail to show tooth development beyond the bud stage at E13.5 + 3 days in culture (A) or E13.5 + 7 days in culture (C). (arrow) Tooth germ, (*) bead. (B, D) E13.5 *Lef1* null mutant mandibular explants treated with FGF4 bead show advanced tooth development at E13.5 + 3 days in culture (B) and a cap stage tooth organ at E13.5 + 7 days in culture (D). Inserts in B and D show no sign of apoptotic cells in the dental epithelium.

the *Lef1* null mutant sample (Figs. 7A–D). FGF4 has recently been shown as a critical factor for cell survival during limb development (Boulet et al., 2004). Our data suggests that LEF1-mediated FGF4 expression plays a pivotal role in inhibiting apoptotic activity during tooth development.

## Discussion

Members of the Wnt family of signaling molecules have been implicated in the control of cell proliferation, differentiation, apoptosis, and organ morphogenesis. Transcription factor LEF1 is a critical Wnt signaling regulator and has a critical role in regulating tooth morphogenesis. Loss-of-function analysis suggests that LEF1 is required to regulate the epithelial–mesenchymal interaction during tooth development and it acts in a tissue-nonautonomous manner. At the molecular level, LEF1 directly regulates *Fgf4* gene expression. FGF4, in turn, regulates the expression of *Fgf3* in the dental mesenchyme and *Shh* in the dental epithelium to mediate the critical epithelial–mesenchymal interaction (Kratochwil et al., 1996, 2002). Despite this information, it remains unclear what is the cellular mechanism of LEF1 signaling in regulating tooth morphogenesis. In this study, we provide important information regarding the functional significance of LEF1 signaling and the fate of dental epithelial as well as the CNC-derived mesenchymal cells.

### *The expression pattern of Lef1 suggests that it plays an important regulatory role throughout all stages of tooth morphogenesis*

Previous studies have demonstrated that *Lef1* is expressed with a specific pattern during the initiation, the bud and the cap stage of tooth development (Kratochwil et al., 1996; Oosterwegel et al., 1993; van Genderen et al., 1994; Zhou et al., 1995). Loss-of-function of *Lef1* signaling results in an arrested tooth development at the bud stage, demonstrating the functional significance of LEF1 during early tooth development (van Genderen et al., 1994). Here we show that *Lef1* expression continues to be specifically associated with the developing tooth germ throughout the remaining stages of tooth development. *Lef1* is expressed at the inner enamel epithelium, the cervical loop region and the pre-odontoblasts in the CNC-derived mesenchyme at the late bell stage of tooth development. The discovery of specific *Lef1* expression during the later stages of tooth development suggests that LEF1 may have important functions in regulating the formation of preameloblasts and preodontoblasts during tooth morphogenesis. This critical issue will be addressed by using the conditional inactivation of *Lef1* gene in the later stages of tooth morphogenesis because the *Lef1* null mutant mice have tooth development retarded at the bud stage. It is of significant interest that *Lef1* expression is specifically associated with the cervical loop region in the late bell stage of molar tooth germ. Multiple isoforms of FGF are

expressed in the cervical loop area and may be involved in regulating the fate of the progenitor cells during tooth morphogenesis (Kettunen et al., 2000; Kawano et al., 2004). The co-localization of *Lef1* and *Fgfs* suggests that LEF1 regulated *Fgf* expression may play a critical role in regulating root development.

### *Loss-of-Lef1 does not affect proper cranial neural crest migration during craniofacial development*

CNC cell fate determination is an important developmental event because successful migration, proliferation, and differentiation of these pluripotent cells are critical for normal craniofacial development. Wnt signaling plays a central role in determining the fate of epithelial and neural crest progenitor cells by modulating the expression of mitogene such as BMP during embryogenesis. In addition, Wnt signaling may also cooperatively regulate BMP-target genes to control cell fate determination (Dorsky et al., 2000; Hussein et al., 2003). The functional significance of Wnt signaling in regulating the fate of neural crest cells has been demonstrated in numerous studies. For example, *Wnt1* and *Wnt3a* double null mutation results in general decrease of all neural crest, particularly in the late-migrating neural crest cell population, thus demonstrating the importance of Wnt signaling in the expansion of the neural crest population (Ikeya et al., 1997). It is not understood, however, whether LEF1 is critical for controlling the fate of neural crest cells.

In this study, we have investigated the molecular mechanism by which LEF1 regulates CNC cell migration, proliferation, apoptosis, and differentiation during tooth morphogenesis. Although there is no CNC cell migration defect associated with the *Lef1* null mutation, we propose that this is likely due to the functional compensation of other transcription factors, such as TCF1, which has a largely overlapping expression pattern with the one of *Lef1* during craniofacial development. The functional compensation among *Lef* and *Tcf* genes have been shown in $Lef1^{-/-};Tcf1^{-/-}$ null mice, which show developmental defects as seen in $Wnt3a^{-/-}$ mice, while neither *Lef1* nor *Tcf1* null mutation shows phenotypes that mimic any known *Wnt* mutation (Galceran et al., 1999). To further support the hypothesis of functional compensation between *Lef* and *Tcf* genes, our recent study has shown that *Lef1* null mutation results in a significantly compromised CNC contribution during the development of mesencephalic nuclei where only *Lef1* is expressed (our unpublished data).

### *LEF1 is required for the survival of dental epithelial cells during tooth formation*

To date, there is very little information regarding the cellular mechanism of LEF1 in regulating the fate of progenitor cells during embryogenesis. Significantly, Wnt signaling has been shown to be critical for the regulation of cell fate decision and cell proliferation (Andl et al., 2002; Cadigan and Nusse, 1997; Moon et al., 1997a,b). For example, Wnt proteins are mitogenic for pro-B cells during B lymphocyte development and Wnt-activated LEF/TCF transcriptional complexes are critical for changes in cell fate and differentiation commitments during hair follicle development (DasGupta and Fuchs, 1999; Reya et al., 2000). Recently, Wnt3 has been shown to play a critical role in regulating the fate of ameloblast during later stage of tooth morphogenesis (Millar et al., 2003). As LEF/TCF transcriptional factors are an integral part of the Wnt signaling cascade, a clear understanding of the cellular mechanism by which the LEF1 signing regulates the fate of dental progenitor cells is critical for a comprehensive understanding of the regulatory mechanism of tooth morphogenesis.

We have identified that the increased apoptosis within the dental epithelium is the primary cellular defect during tooth morphogenesis in the *Lef1* null mutant sample. This is a critical discovery because the increased cell death within the dental epithelium helps to explain the failure of advancement of the tooth germ from the bud to the cap stage in the *Lef1* null mutant. It is well established that normal tooth development requires proper epithelial folding morphogenesis, which relies on sufficient number of epithelial cells to be present in the dental epithelium during tooth morphogenesis. Increased apoptosis within the dental epithelium reduces the critical cell mass required to support the proper epithelial folding morphogenesis and retards the tooth development at the bud stage in the *Lef1* null mutant. Furthermore, the increased apoptosis is the first detectable cellular defect in the tooth germ of the *Lef1* null mutant sample and is not a generalized defect as the result of the null mutation of a transcription factor, suggesting that LEF1 is a critical survival factor for the dental epithelium during tooth development.

It needs to be noted that there might be a subtle change in cell proliferation activity within the dental epithelium of the *Lef1* null mutant. However, LEF1 does not appear to play a critical role in regulating dental epithelial cell proliferation. Msx1 and Msx2 are critical cell proliferation regulators during tooth development. *Msx1* null mutation affects the CNC-derived dental mesenchyme proliferation while *Msx1/Msx2* double null mutation results in decrease in cell proliferation in both dental epithelium and mesenchyme (Han et al., 2003 and our unpublished observation). In *Lef1* null mutant, the expression of *Msx* gene is unaffected, suggesting that Msx1 and Msx2 may be at least partially responsible for the existent proliferation in the $Lef1^{-/-}$ tooth germ.

At the molecular level, LEF1 is required to induce *Fgf4* expression, which in turn regulates the expression of *Fgf3* and *Shh* in the tooth germ (Kratochwil et al., 2002). Shh is critical for dental epithelial cell survival during tooth development and inhibition of Shh signaling results in localized apoptosis in the dental epithelium (Cobourne et

al., 2001). However, it is not likely that down-regulation of *Shh* in the dental epithelium of the *Lef1* null mutant sample is responsible for the increase in apoptosis in the dental epithelium and for blocking tooth development at the bud stage because exogenous Shh failed to rescue tooth development in *Lef1*$^{-/-}$ tooth germs (Kratochwil et al., 2002). Instead, this study shows that LEF1-mediated *Fgf4* expression plays a critical role in regulating the survival of dental epithelial cells. Previous studies have provided supportive evidence that WNT/LEF1/beta-catenin signals can act directly to affect cell survival in several biological contexts (Chen et al., 2001; Longo et al., 2002; You et al., 2002). Further studies are needed to elucidate whether LEF1-mediated Wnt signaling directly or indirectly controls apoptosis during tooth development.

Although there is no change in cell proliferation, apoptosis, or cell cycle progression in the CNC-derived dental mesenchyme of the *Lef1* null mutant, it is likely that the function of LEF1 is compensated by TCF1 during early tooth development. Both *Lef1* and *Tcf1* are present in the CNC-derived dental mesenchyme at the bud and the cap stage of tooth development while only *Lef1* is expressed in the dental epithelium (Oosterwegel et al., 1993; van Genderen et al., 1994). The functional redundancy between LEF1 and TCF1 is further supported by the fact that tooth development is retarded at the lamina stage in the *Lef1/Tcf1* double null mutant mice, a defect at an earlier stage comparing to the one in the *Lef1* null mutant (Kratochwil and Grosschedl, personal communication).

Spatially and temporally restricted patterns of apoptotic cells suggest multiple roles for apoptosis during tooth morphogenesis. To date, studies suggest that apoptosis in the dental epithelium may regulate the budding process at the early stage of tooth development and may reduce cell number in the enamel organ to control the size and final position of the developing tooth (for review, see Matalova et al., 2004). Different hypotheses have been put forth to address the biological significance of dental apoptosis during tooth morphogenesis. One hypothesis is that apoptosis will serve as a process of cell removal following inductive signaling in the enamel knot. However, it is still not clear whether the entire population of enamel knot will undergo apoptosis or a subpopulation of the enamel knot will actually migrate away to form the secondary enamel knot during molar development. Alternatively, it has been proposed that cells are programmed to undergo apoptosis and require continued signaling from growth and transcription factors to survive. Growth and transcription factors (such as FGF4, BMP, EGF, Shh, Msx2, N-myc and c-fos and etc.) have been implicated in the regulation of apoptosis (Jernvall and Thesleff, 2000; Vaahtokari et al., 1996). In this study, we show that LEF1-mediated WNT signaling may play a critical role in the suppression of the programmed cell death and is indispensable to maintain the survival of dental epithelial cells during tooth morphogenesis.

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