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Excess NF-κB Induces Ectopic Odontogenesis in Embryonic Incisor Epithelium

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Abstract

Nuclear factor kappa B (NF-κB) signaling plays critical roles in many physiological and pathological processes, including regulating organogenesis. Down-regulation of NF-κB signaling during development results in hypohidrotic ectodermal dysplasia. The roles of NF-κB signaling in tooth development, however, are not fully understood. We examined mice overexpressing IKKβ, an essential component of the NF-κB pathway, under keratin 5 promoter (*K5-Ikkβ*). *K5-Ikkβ* mice showed supernumerary incisors whose formation was accompanied by up-regulation of canonical Wnt signaling. Apoptosis that is normally observed in wild-type incisor epithelium was reduced in *K5-Ikkβ* mice. The supernumerary incisors in *K5-Ikkβ* mice were found to phenocopy extra incisors in mice with mutations of Wnt inhibitor, *Wise*. Excess NF-κB activity thus induces an ectopic odontogenesis program that is usually suppressed under physiological conditions.

Keywords: Ikkβ, tooth development, Wnt signaling, enamel, cervical loop, wise

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Introduction

Nuclear factor kappa B (NF-κB) signaling plays roles in many physiological and pathological processes including organogenesis, immune and inflammatory responses, apoptosis, cell proliferation, cancer, and stem cell regulations (Oeckinghaus et al. 2011). In mammals, 15 NF-κB homo- or heterodimers are formed among the 5 subunits, NFKB1 (p50, generated from p105), NFKB2 (p52, generated from p100), RelA (p65), RelB, and c-Rel. While in the absence of any stimulus, NF-κB is located in the cytoplasm, and activation of NF-κB signaling by a large variety of NF-κB–specific signals leads to nuclear translocation of NF-κB. Three pathways, classical/canonical, alternative/noncanonical, and a hybrid of both, have been shown to activate NF-κB. Canonical NF-κB activation is usually a rapid and transient response to a wide range of stimuli. In unstimulated cells, the inhibitor of κBα (IκBα) acts to retain canonical NF-κB subunits in the cytoplasm. Exposure to canonical stimuli leads to rapid phosphorylation and subsequently to ubiquitination and degradation of IκBα. IκBα is phosphorylated by a multiprotein kinase complex, the IκB kinase (IKK), composed of 2 catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ (NEMO). The released NF-κB dimers translocate to the nucleus and regulate target gene transcription (Oeckinghaus et al. 2011). The noncanonical pathway leads slow activation of NF-κB and results in prolonged activation of NF-κB target gene transcription. In noncanonical signaling, NF-κB–inducing kinase (NIK) recruits only IKKα that subsequently phosphorylates p100, which promotes p100 polyubiquitiation and subsequent proteasomal processing to p52. Several types of dimers such as RelB/p52 and p52/p52 with BCL3 are responsible for transcription of noncanonical target genes (Oeckinghaus et al. 2011).

NF-κB signaling is also known to be involved in the development of ectodermal organs, and down-regulation of NF-κB activity leads to hypohydrotic (anhidrotic) ectodermal dysplasia, which is characterized by partial or complete absence of exocrine sweat glands, abnormally sparse hair, and an absence and/or malformation of teeth. The tumor necrosis factor (TNF) superfamily member, ectodysplasin-A1 (EDA-A1), Eda receptor (EDAR), and Eda receptor–associated death-domain (EDARADD) are identified as upstream mediators of NF-κB in the development of the ectodermal organs, which is consistent with the studies of loss of function of NF-κB in the mouse (Mikkola and Thesleff 2003).

Tooth position, number, and shape are consistent in mammals and are under strict genetic control. Teeth develop through a series of reciprocal interactions between the epithelium and underlying mesenchyme. The first morphological sign of tooth development is a localized thickening of the mandibular and maxillary epithelium, which subsequently invaginates into the underlying mesenchyme to form buds. The bud epithelium progressively takes the form of cap and bell configurations. Epithelial cells differentiate into enamel-producing ameloblasts, while mesenchymal cells differentiate into dentin-producing odontoblasts. Mice are the commonly studied mammals for investigating the mechanisms of tooth development; however, mouse incisors are unique teeth as they grow continuously throughout life, and enamel is present on only the labial side. Continuous growth is supported by dental stem cells localized at the apical end of incisor (Harada et al. 1999). Multiple signaling pathways including BMP, FGF, WNT, and SHH are known to play roles in regulating tooth development. The role of NF-κB signaling in rodent incisor tooth development, however, is not fully understood, although loss-of-function studies have identified a role for NF-κB in the specification of cusp formation in molar tooth development (Ohazama et al. 2004).

We examined mice overexpressing an essential component of the NF-κB pathway, *Ikkβ*, under the keratin 5 promoter (*K5-Ikkβ*), to investigate the role of NF-κB signaling in mouse incisor tooth development. *K5-Ikkβ* mice showed supernumerary incisors, which initiated from embryonic epithelium. Excess NF-κB is thus able to stimulate extra odontogenic activity in embryonic incisor epithelium.

Materials and Methods

Production and Analysis of Transgenic Mice

The production of mice with mutation of *Wise, $(Ig\kappa)_{3x}conalacZ$, $c^{I\kappa B\alpha\Delta N}$, K5-Ikkα*, and *K5-Ikkβ* mice, have previously been described (Schmidt-Ullrich et al. 1996, 2001; Kassai et al. 2005; Lomada et al. 2007; Page et al. 2010). *Aly/Aly* mice were purchased from CLEA (Tokyo, Japan). Embryonic day 0 (E0) was taken to be midnight prior to finding a vaginal plug.

β-galactosidase Staining

Embryo heads were fixed for 1 h at 4 °C in 1% paraformaldehyde (PFA) and 0.2% glutaraldehyde, and X-gal staining was performed at 36 °C for 48 h.

In Situ Hybridization

Radioactive in situ hybridization with [^{35}S]UTP-labeled riboprobes was carried out as described previously (Ohazama et al. 2008). Decalcification using 0.5 M EDTA (pH 7.6) was performed after fixation of newborn mice.

Micro-CT Analysis

Heads were scanned with Explore Locus SP (GE Healthcare, Little Chalfont, Buckinghamshire, UK) high-resolution micro computed tomography (CT) with a voxel dimension of 8 μm. Three-dimension reconstruction was performed by 3-structure analysis software (MicroView, GE Healthcare).

Contact Microradiography Analysis

Specimens were dehydrated and embedded in polyester resin (Rigolac; Nisshin-EM, Tokyo, Japan). Ground sections were prepared 40 μm in thickness. Contact microradiography was taken with a soft x-ray generator with a 20-μm Ni filter (15 kV, 3 mA, 5 to 6 min; Cu-Kα line; Softex, Austin, TX).

3D Reconstructions of Tooth Epithelium

Contours of dental and adjacent oral epithelium were drawn from histological sections at 7-μm intervals using a Leica DMRB microscope equipped with a drawing chamber at a magnification of 255×. The digitalization of the serial drawings and correlation of successive images have previously been described (Lesot et al. 1996). Three-dimensional images were generated using a volume-rendering program VG Studio Max 2.0 (VG Studio Max, Heidelberg, Baden-Württemberg, Germany).

Immunohistochemistry

Dewaxed sections were treated by proteinase K and then incubated with antibody against phosphorylated p-Smad1/5/8 (Cell Signaling Technology, Beverly, MA). The tyramide signal amplification system (PerkinElmer Life Science, Waltham, MA) was used for detecting the primary antibody.

Results

K5 is expressed in oral/dental epithelium from E11.5 (Appendix Fig. 1; Liu et al. 2010). The *K5-Ikkβ* mice survived, reproduced normally, and showed minor skin anomalies (Page et al. 2010).

To examine NF-κB activity in *K5-Ikkβ* mice, we crossed *K5-Ikkβ* mice with mice carrying a κB-dependent LacZ reporter gene construct (*[Igκ]*$_{3x}$*conalacZ*) that show LacZ expression where the NF-κB pathway is activated. Up-regulation of NF-κB activity was confirmed in incisor regions in *(Igκ)*$_{3x}$*conalacZ;K5-Ikkβ* mice (Fig. 1B).

Six-week-old *K5-Ikkβ* mice revealed an additional pair of incisors on the mandible, lingual to the endogenous incisors (Fig. 1C, 1E; Appendix Table; these will hereafter be referred to as "lingual incisors"). Micro-CT analysis showed the lingual incisors to be separate entities from the endogenous incisors (Fig. 1F). The lingual incisors, however, shared the same bone socket with the endogenous incisor, suggesting that the lingual incisors develop dependent on endogenous incisor tooth germs. Upper incisors of *K5-Ikkβ* mice showed longitudinal grooves on their labial surfaces but no extra incisors (data not shown).

(Igκ)$_{3x}$*conalacZ* shows activity of both canonical and noncanonical NF-κB pathway. Although *Ikkβ* is known to be

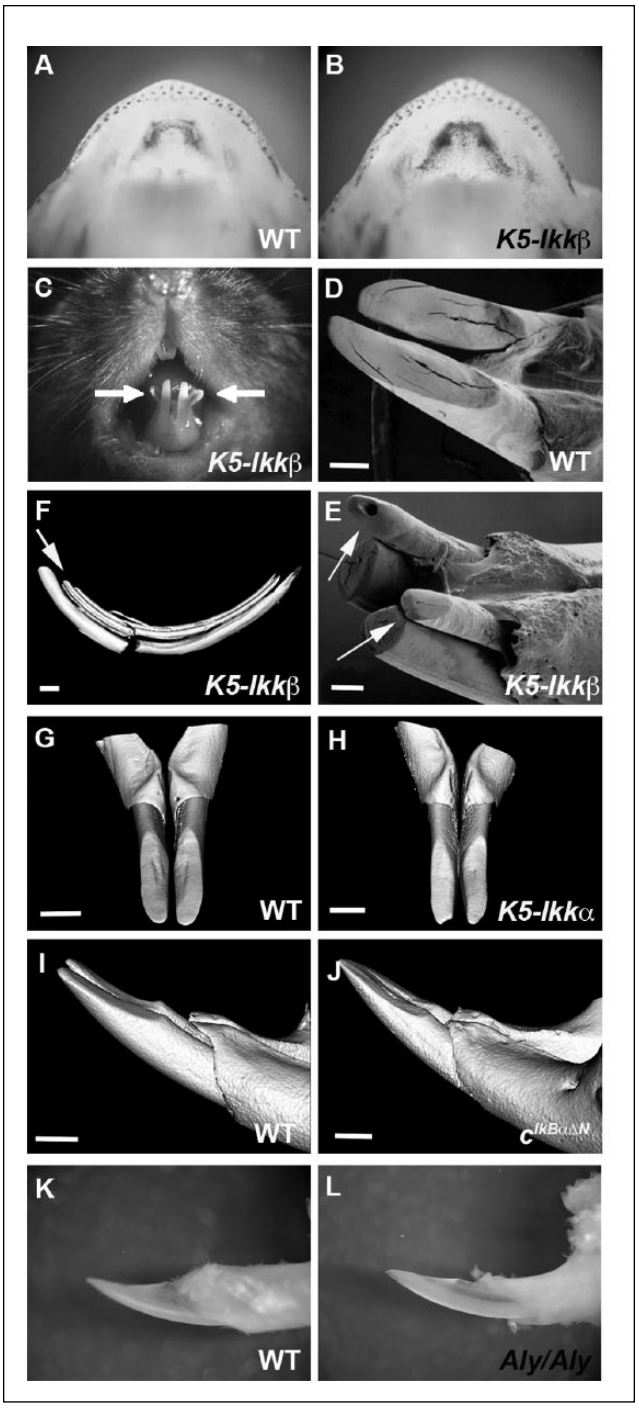

Figure 1. Lower incisor phenotypes in transgenic mice. (**A**, **B**) LacZ staining in *(Igκ)*$_{3x}$*conalacZ* (A) and *(Igκ)*$_{3x}$*conalacZ;K5-Ikkβ* (B) mice at E14.5. (**C–L**) Lower incisors in wild-type (D, G, I, K), *K5-Ikkβ* (C, E, F), *K5-Ikka* (H), $c^{IκBαΔN}$ (J), and *Aly/Aly* (L) mice at 6 wk of age. Arrows indicate the lingual incisors (C, E, F). (D, E) Scanning electron microscopy. (F-J) Three-dimensional reconstructions based on micro–computed tomography scans. Scale bars: 1.0 mm (D-J).

nvolved in only canonical NF-κB signaling, noncanonical signaling always requires precedent canonical signaling. We therefore next confirmed whether lingual incisor formation was caused by the up-regulation of only canonical NF-κB signaling. To address this question, we examined mice with overexpression of *Ikkα* from the K5 promoter (*K5-Ikkα*) to see if increased noncanonical NF-κB signaling can also induce lingual incisors, since *Ikkα* is known to play a central role in regulating noncanonical NF-κB (Oeckinghaus et al. 2011). *K5-Ikkα* mice, however, showed no supernumerary incisors, suggesting that lingual incisor formation in *K5-Ikkβ* mice is caused solely by up-regulation of canonical NF-κB signaling (Fig. 1H; Blackburn et al. 2012). Mutation of *Eda* (upstream ligand of the NF-κB pathway in ectodermal organ development) has also been shown to result in extra tooth formation in the diastema (Peterková et al. 2002; Mustonen et al. 2003). To investigate whether reduced NF-κB signal activity also affects lingual incisor formation, we examined mice expressing the IκBα superrepressor IκBαΔN ($c^{IκBαΔN}$), which ubiquitously suppresses NF-κB activity, and mice with a mutation in *Nik* (*Aly/Aly*) in which noncanonical signaling is inhibited. Both lines did not show any lingual incisors, suggesting that lingual incisor formation is entirely dependent on increased canonical NF-κB signaling (Fig. 1J, 1L; Ohazama et al. 2004).

To establish the timing of the first appearance of the lingual incisor teeth, *K5-Ikkβ* embryos were examined at different time points during embryonic development. The obvious sign of lingual incisor formation could be visualized from E14.5 as a lingual enlargement of the endogenous incisor epithelium, which was confirmed by ectopic *Shh* expression at E15.5 (Fig. 2A-2H; Appendix Figs. 2 and 3; Appendix Table; $n = 43$; E13.5, $n = 40$; E14.5, $n = 46$; E15.5). Lingual incisor tooth germs were more obvious at E17.5 (Fig. 2J; Appendix Fig. 3). Analysis of 3D reconstructions of the dental epithelium and histological sections clearly demonstrated that lingual incisor tooth germs developed from the epithelium of the endogenous incisors at the most anterior/lingual extent (Fig. 2L; Appendix Fig. 3). To confirm whether lingual incisor tooth development requires endogenous incisor tooth germ, we crossed *K5-Ikkβ* mice with *Msx1* mutants (*K5-Ikkβ*; $Msx1^{-/-}$). *Msx1* mutation is known to result in the arrest of tooth development at the bud stage (Satokata and Maas 1994), whereas the *K5-Ikkβ* lingual incisor initiates when the endogenous incisor tooth reaches the cap stage. *K5-Ikkβ;*$Msx1^{-/-}$ mice showed no incisor formation, suggesting that the ectopic lingual incisor development depends on endogenous incisor tooth development (Fig. 2N).

Increased *Ikkβ* expression was confirmed in the epithelium where lingual incisors initiate (Fig. 2P). Increased *Ikkβ* expression and NF-κB activity was also observed in the upper incisor tooth germs, although the extra incisor was not found in the upper incisors (Appendix Fig. 4). Apoptotic activity has been shown in the mouse lower incisor epithelium at E14.5 (Munne et al. 2009). *K5-Ikkβ* embryos exhibited reduced apoptotic cells (Fig. 2R). Cell proliferation was low in wild-type epithelium, whereas there was extensive proliferation in *K5-Ikkβ* epithelium (Fig. 2S, 2T).

Increased Wnt signaling during tooth development has been shown to result in supernumerary tooth formation (Järvinen et al. 2006). The canonical Wnt signaling pathway (examined by expression of *Axin2*; a transcriptional target of the canonical Wnt signaling pathway) was up-regulated in *K5-Ikkβ* incisor epithelium where the lingual incisors initiate (Fig. 2V). *K5-Ikkβ* embryos also showed increased expression of *Ptch1* (transcriptional target of the Shh signaling pathway) at the lingual aspect of incisor tooth germs, which was consistent with ectopic *Shh* expression (Fig. 2H, 2X). Although supernumerary incisors have also been shown following change of Fgf or Bmp signaling, *K5-Ikkβ* embryos exhibited no significant differences in Bmp or Fgf signaling in incisor tooth germs (Appendix Fig. 5; Zhang et al. 2009; Charles et al. 2011).

Micro-CT, contact microradiography, and histological analysis revealed that ameloblasts and enamel were observed on the endogenous incisors of *K5-Ikkβ* mice but not on lingual incisors (Figs. 3B, 3C, 4B, 4C). The absence of differentiated ameloblasts was confirmed by the lack of *amelogenin* (major enamel protein) expression in lingual incisor tooth germs (Fig. 4D). Ameloblasts in incisors are supplied from the labial cervical loops that show a characteristic morphology in wild-type mice (Fig. 4A). *K5-Ikkβ* lingual incisors, however, had only a thin layer of epithelium at the apical end of the lingual incisors, whereas endogenous incisors in *K5-Ikkβ* mice showed no significant morphological changes of the cervical loop (Fig. 4B, 4C). Lack of enamel formation in the lingual incisor is thus likely to be caused by abnormal labial cervical loop formation in *K5-Ikkβ* mice. These findings prompted us to examine whether lingual incisors are able to grow continuously. To address this question, incisors were clipped at the gingival level. Newly formed lingual and endogenous incisors were observed at 2 wk after the cutting, suggesting that lingual incisors retained the ability of continuous growth (Fig. 4E, 4F, 4G).

Supernumerary incisors have been reported in mice with mutation of the Wnt signal inhibitor, *Wise* (*Sostdc, Ectodin*), which initiates from the epithelium of the embryonic endogenous incisor tooth germs at the most anterior/lingual extent and show increased Wnt signaling and no apoptotic activity (Murashima-Suginami et al. 2007; Ohazama et al. 2008; Munne et al. 2009; Ahn et al. 2010). Timing and location of initiation and molecular changes in lingual incisor formation of *K5-Ikkβ* mice were thus similar to those in *Wise* mutants. We therefore examined supernumerary incisors in *Wise* mutant mice. In common with *K5-Ikkβ* mice, no enamel formation and abnormal morphology of the labial cervical loop were observed in *Wise* mutant supernumerary incisors (Fig. 4I, 4J). Lingual incisors in *K5-Ikkβ* mice thus are a phenocopy

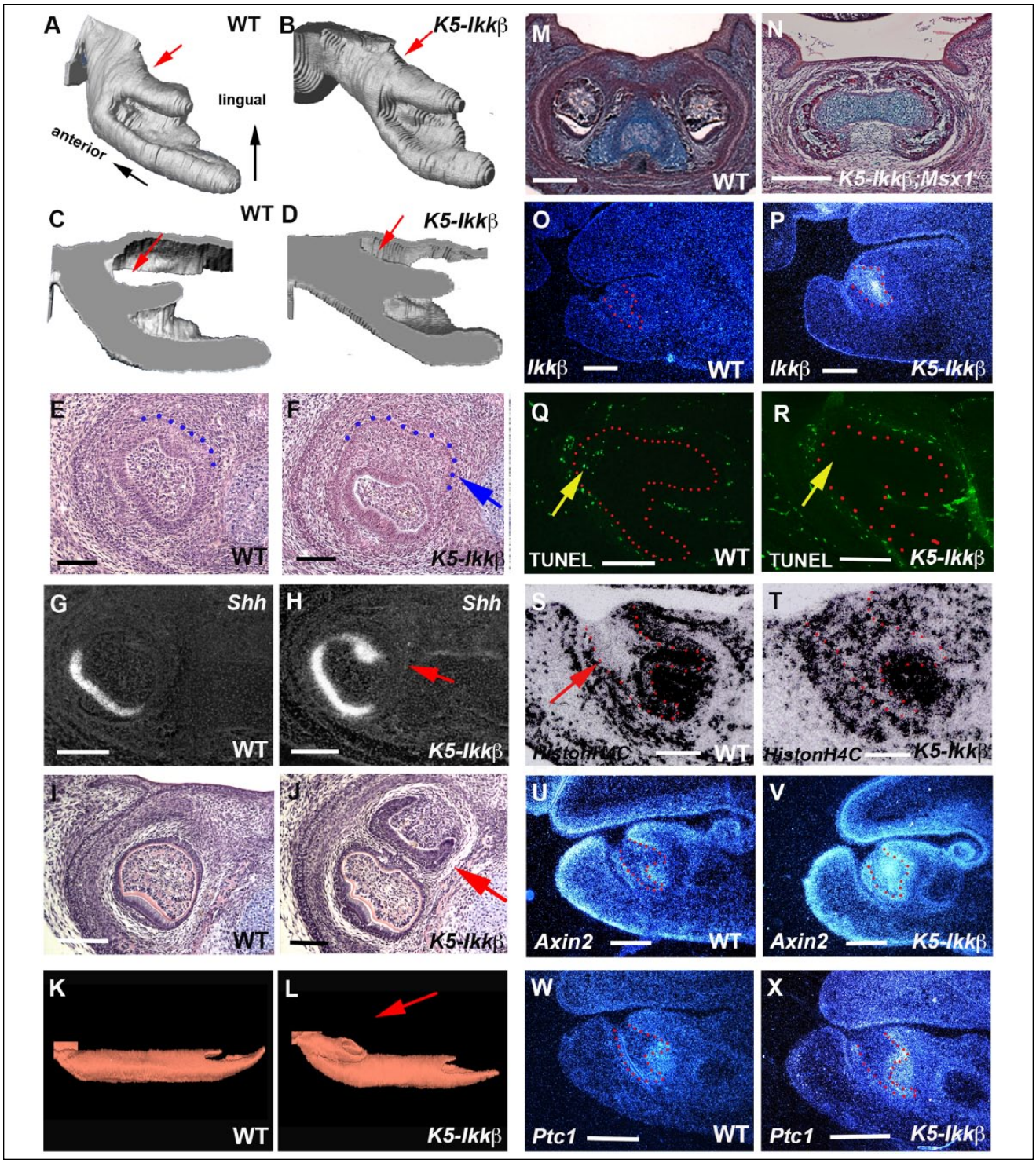

Figure 2. Molecular changes in lower incisors of *K5-Ikkβ* mice. (**A–D**, **K**, **L**) Three-dimensional (3D) reconstructions of the epithelium in the incisor tooth germs of wild type (A, C, K) and *K5-Ikkβ* (B, D, L) embryos at E14.5 (A–D) and E17.5 (K, L). Dental epithelium is viewed as the mediolingual axis (A, B). Sagittal sections through the 3D model (C, D). Arrows indicating the lingual aspect of endogenous incisors (A–D) and lingual incisor tooth germ (L). (**E–J**, **M**, **N**) Frontal sections showing lower incisors in wild-type (E, G, I, M), *K5-Ikkβ* (F, H, J) and *K5-Ikkβ;Msx1*$^{-/-}$ (N) embryos at E15.5 (E-H) and E17.5 (I, J, M, N). (**O–X**) Sagittal section showing lower incisors in wild-type (O, Q, S, U, W) and *K5-Ikkβ* mice (P, R, T, V, X) at E13.5 (O, P) and E14.5 (Q-X). Expression of *Shh* (G, H), *Ikkβ* (O, P), *HistonH4C* (S, T), *Axin2* (U, V), and *Ptc1* (W, X) by radioactive in situ hybridization. (Q, R) TUNEL assay. Arrows indicating the lingual enlargement of endogenous incisor epithelium (F), colocalized ectopic *Shh* expression (H), lingual incisor tooth germ (J), apoptotic activity (Q, R), and the region showing the low level expression of *HistonH4C* (S). Tooth epithelium is outlined by red or blue dots. Scale bars: 100 μm (E-H), 200 μm (O–T), 400 μm (U–X), 500 μm (I, J, M, N).

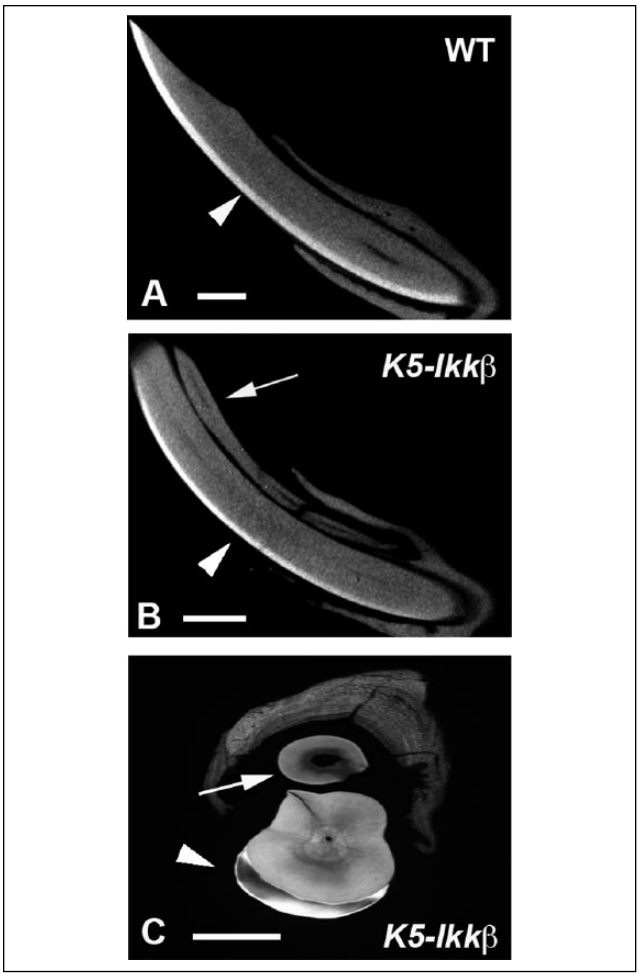

Figure 3. Enamel defects in lingual incisors. (**A**, **B**) Sagittal section based on micro–computed tomography scan in lower incisors of wild-type (A) and *K5-Ikkβ* (B) mice at 6 wk of age. (**C**) Contact microradiography as the frontal section of *K5-Ikkβ* mice. Arrows indicate lingual incisors (B, C). Arrowheads indicate enamel. Scale bars: 1.0 mm (A, B) and 500 μm (C).

to those in *Wise* mutants. However, no obvious changes in *Wise* expression were evident in *K5-Ikkβ* incisors (Fig. 4L).

Discussion

The overexpression of *Ikkβ* from the *K5* promoter induced extra odontogenic activity in embryonic incisor tooth epithelium, which resulted in lingual incisor formation. The ectopic lingual incisors had no enamel that is likely to be caused by an abnormal epithelial stem cell niche, although epithelial cells are seen. Despite these abnormalities, lingual incisors in *K5-Ikkβ* mice exhibited continuous growth, suggesting that full function of the cervical loop is dispensable for incisor continuous growth. It is believed that there is interaction between the cervical loop epithelial cells and mesenchymal cells. The continuous growth and normal appearance of the dentine and pulp suggest that the incisor mesenchymal stem cell niche is unaffected (Lapthanasupkul et al. 2012).

Change of tooth number is a significant evolutionary adaptation to accommodate novel feeding strategies. Reduction in tooth number is a well-known evolutionary trend of the dentition within eutherians (Rose and Archibald 2005). In some primitive fossil Glires, a second pair of incisors was present in both the upper and lower jaws (Asher et al. 2005). Lingual incisors initiate from endogenous incisor epithelium, and apoptosis is observed in wild-type rodent incisor epithelium where the lingual tooth initiates (Munne et al. 2009). Lack of the apoptotic activity is found to result in extra incisor formation of *K5-Ikkβ* mice and *Wise* mutant mice. It is thus likely that additional odontogenic activity is usually inhibited in extant rodent incisor epithelium that was acquired during evolution, and mice retain the evolutionary lost odontogenic activity. In addition to lack of apoptosis, increased cell proliferation in incisor epithelium is likely to enlarge the lingual aspect of endogenous incisor tooth germs. Lingual incisors could not be detected in the maxillae of *K5-Ikkβ* mice, whereas *Sprouty2/4, Wise*, and *Lrp4* mutant mice show extra incisors in both maxillae and mandibles (Murashima-Suginami et al. 2007, Ohazama et al. 2008; Munne et al. 2009; Ahn et al. 2010; Charles et al. 2011). Inhibitory mechanisms of odontogenesis are thus different between lower and upper incisors.

Changes in Fgf signal activity by *Sprouty2/4* mutations have been shown to result in mandible supernumerary incisor formation (Charles et al. 2011). The location of the supernumerary incisors in *Sprouty2/4* mutants is, however, different from those in *K5-Ikkβ* incisors, and Fgf signaling showed no significant changes in *K5-Ikkβ* mice. It is possible that inhibitory mechanisms of odontogenesis in incisor tooth germs are controlled by several different pathways, and lingual incisors in *K5-Ikkβ* mice might not involve Fgf signaling. In common with *K5-Ikkβ* mice, *Wise* mutants show supernumerary incisors lingual to endogenous incisors (Munne et al. 2009), and extra incisors in both *K5-Ikkβ* mice and *Wise* mutant mice initiate from similar regions of embryonic incisor tooth epithelium. Up-regulation of the Wnt signaling pathway is observed in embryonic incisor bud epithelium in both *K5-Ikkβ* mice and *Wise* mutant mice. Lingual incisors in *K5-Ikkβ* mice thus appear to phenocopy those in *Wise* mutants. *K5-Ikkβ* mouse lingual incisors retained *Wis*e expression, suggesting that *Wise* is not the target of the NF-κB pathway in lingual incisor formation. It is possible that Wnt signaling activity in wild-type embryonic incisor bud epithelium is physiologically reduced by *Wise*, which can be activated by excess NF-κB signaling, since NF-κB signaling has been shown to activate the Wnt pathway (Kaler et al. 2009). Incisors lingual to endogenous incisors are also present in extant *Lagomorpha*, which, however, show enamel formation (Hirschfeld et al. 1973). No enamel formation in the lingual incisors of both *K5-Ikkβ* and *Wise* mutant mice indicates that lingual incisor formation by up-regulation of Wnt signaling is insufficient to lead to full

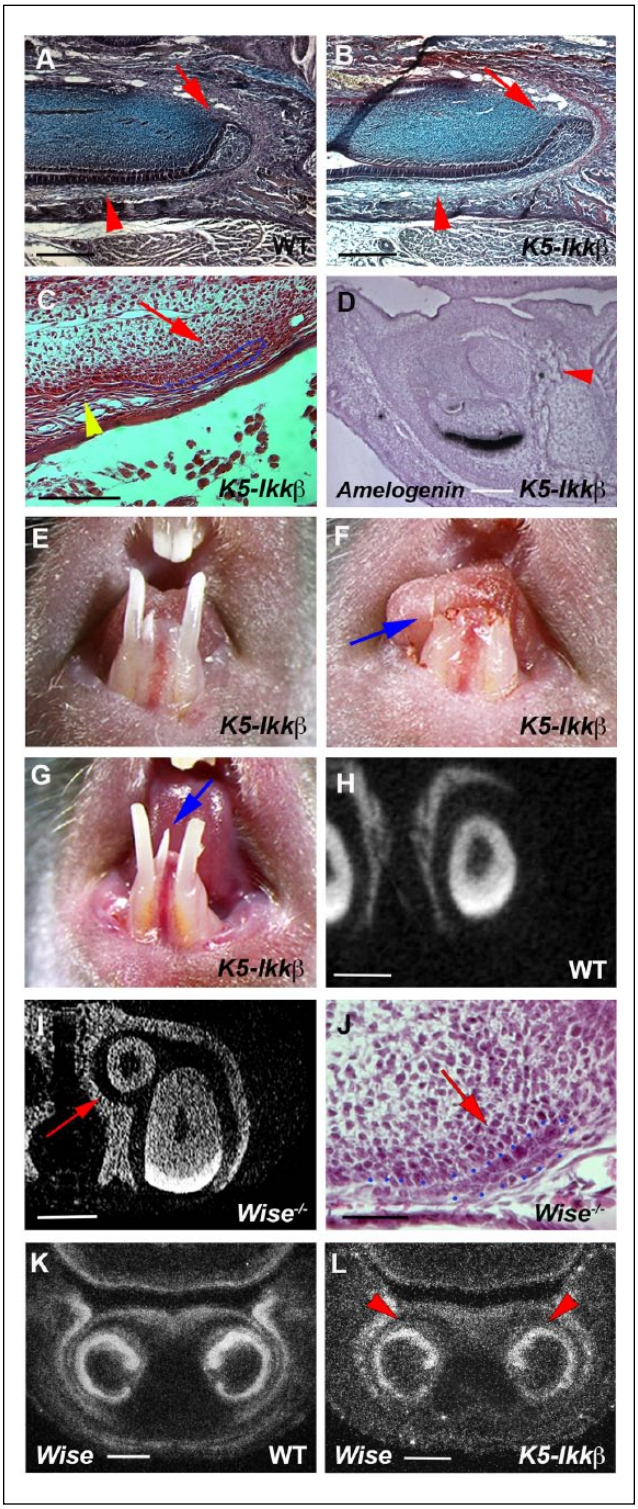

Figure 4. Lingual incisors in *K5-Ikkβ* mice. (**A–C**, **J**) Sagittal sections showing labial cervical loop in endogenous incisor (arrows in A, B) and lingual incisor (arrows in C, J) of wild-type (A), *K5-Ikkβ* mice (B, C), and *Wise* mutant (J) at P5. Arrowheads indicating ameloblasts (A, B) and the lack of ameloblasts (C). (**D**) Sagittal sections showing *Amelogenin* mRNA expression in *K5-Ikkβ* mice. Arrowhead indicating lingual incisor (D). (**E–G**) Endogenous and lingual incisors before clipping (E), just after clipping (arrow in F), and at 2 wk after clipping (G). Arrow indicating newly formed lingual incisor (G). (**H, I**) Frontal section based on micro–computed tomography scan in lower incisors of wild-type (H) and *Wise* mutant (I). Arrow indicates lingual incisor (I). (**K**, **L**) *Wise* expression by radioactive in situ hybridization on frontal sections of wild-type (K) and *K5-Ikkβ* mice (L) at E15.5. Arrowheads showing *Wise* expression on the lingual side of endogenous incisor tooth germs (L). The labial cervical loop was outlined by blue dots (C, J). Scale bars: 500 μm (A, B, H, I), 300 μm (C), 150 μm (D, J), and 200 μm (K, L).

development of evolutionary lost incisor teeth. No enamel formation in *K5-Ikkβ* lingual incisors might be caused by a defect in the cervical loop, and Wnt signaling has been shown to be not involved in cervical loop formation (Suomalainen and Thesleff 2010).

In addition to the role of Ikkβ in NF-κB activation, *Ikkβ* is also known to modulate cellular responses in a manner independent of NF-κB (Lee et al. 2007; Xia et al. 2009). Although we show that NF-κB activity is increased, we cannot exclude the possibility that lingual incisor formation is caused by Ikkβ-NF-κB–independent activity in *K5-Ikkβ* mice.

Author Contributions

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