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Overactivation of the NF-κB pathway impairs molar enamel formation

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Abstract

Objective: Hypohidrotic ectodermal dysplasia (HED) is a hereditary disorder characterized by abnormal structures and functions of the ectoderm-derived organs, including teeth. HED patients exhibit a variety of dental symptoms, such as hypodontia. Although disruption of the EDA/EDAR/EDARADD/NF-κB pathway is known to be responsible for HED, it remains unclear whether this pathway is involved in the process of enamel formation.

Experimental subjects and methods: To address this question, we examined the mice overexpressing *Ikkβ* (an essential component required for the activation of NF-κB pathway) under the keratin 5 promoter (*K5-Ikkβ*).

Results: Upregulation of the NF-κB pathway was confirmed in the ameloblasts of *K5-Ikkβ* mice. Premature abrasion was observed in the molars of *K5-Ikkβ* mice, which was accompanied by less mineralized enamel. However, no significant changes were observed in the enamel thickness and the pattern of enamel rods in *K5-Ikkβ* mice. *Klk4* expression was significantly upregulated in the ameloblasts of *K5-Ikkβ* mice at the maturation stage, and the expression of its substrate, amelogenin, was remarkably

reduced. This suggests that abnormal enamel observed in *K5-Ikkβ* mice was likely due to the compromised degradation of enamel protein at the maturation stage.
Conclusion: Therefore, we could conclude that the overactivation of the NF-κB pathway impairs the process of amelogenesis.

KEYWORDS
enamel, molar, NF-κB, tooth development

1 | INTRODUCTION

Ectodermal dysplasia is one of the largest groups of syndromes that consist of more than 150 clinically distinct hereditary diseases, which are usually characterized by abnormal structures and functions of ectoderm-derived organs. Hypohidrotic (anhidrotic) ectodermal dysplasia (HED) is the most common form of ectodermal dysplasia and is characterized by abnormally thin, dry, and soft skin with lack of pigmentation, partial or complete absence of sweat glands, and sparse hairs. Mutation in the gene encoding TNF superfamily member ectodysplasin (EDA) is considered to be responsible for X-linked HED. Autosomal recessive forms of HED are found to occur due to the mutation in genes encoding the EDA receptor (EDAR). EDA receptor-associated death domain (EDARADD) is an adaptor molecule for EDAR, which is responsible for autosomal dominant forms of HED. The nuclear factor kappa B (NF-κB) pathway is known to be downstream of the EDA/EDAR/EDARADD cascade in the development of ectodermal organs (Mikkola & Thesleff, 2003; Ohazama & Sharpe, 2004).

The NF-κB pathway plays a major role in many physiological and pathological processes, such as immune and inflammatory responses and cancer (Chariot, 2009; Hacker & Karin, 2006; Sanz et al., 2010). In mammals, 15 homo- or hetero-dimers of NF-κB are formed from five proteins, including NF-κB1 (p50, generated from p105), NF-κB2 (p52, generated from p100), RelA (p65), RelB, and c-Rel. Activation of the NF-κB pathway is characterized by the nuclear translocation of the NF-κB proteins. Without stimuli, NF-κB exists in the cytoplasm in an inactive form in association with the inhibitory proteins, inhibitor of κB (IκB). Activation of the NF-κB pathway can proceed through canonical, non-canonical, or hybrid pathways. Activation of the canonical NF-κB pathway is usually characterized as a rapid and transient response to a wide range of stimuli. In non-stimulated cells, IκB is able to retain NF-κB in the cytoplasm by masking its nuclear localization sequence. However, exposure to stimuli results in rapid phosphorylation of IκB, which leads to ubiquitination and degradation initiated by a multiprotein complex, namely IκB kinase (IKK). IKK is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ. IKKβ is known to be an essential molecule for the activation of the canonical NF-κB pathway. Thereafter, the resulting free NF-κB dimers are translocated to the nucleus and they further regulate the target gene transcription. Conversely, the non-canonical NF-κB pathway involves slow activation of NF-κB leading to the prolonged activation of NF-κB target genes. NF-κB-inducing kinase recruits IKKα, which further phosphorylates p100 promoting its polyubiquitination and subsequent proteasomal processing to p52 leading to the generation of RelB/p52 dimers further facilitating the complete activation of the NF-κB pathway.

The formation of teeth involves a series of reciprocal interactions between the epithelium and the underlying neural crest-derived mesenchyme (Calamari, Hu, & Klein, 2018; Smith et al., 2017). The first morphological sign of a tooth development is a thickened epithelium and its subsequent invagination into the underlying mesenchyme to form buds. The bud epithelium progressively takes the form of the cap and bell configurations as the process of differentiation proceeds further. From the late bud stage to the cap stage, the formation of primary enamel knot in the bud and cap epithelium occurs, which is known to play a critical role in the cusp formation. Epithelial and mesenchymal cells (dental papilla) differentiate into enamel-producing ameloblasts and dentin-producing odontoblasts, respectively. The enamel forms through multiple stages as follows: secretory, transition, and maturation stages. During the secretory stage, columnar ameloblasts start to deposit an extracellular matrix including amelogenin, ameloblastin, and enamelin, and then the process of mineralization is initiated. A shift from matrix deposition to resorption occurs at the transition and maturation stages. When the transition stage initiates, the ameloblasts begin to shrink in size and downregulate the release of above-mentioned enamel proteins into the enamel matrix. These changes are associated with the end of elongation of enamel crystals. In the maturation stage, the crystallites grow in width and thickness, but not in length. The remaining proteins within the enamel matrix are degraded by many factors from ameloblasts, including KLK4, before they are exported out of the enamel.

Hypohidrotic ectodermal dysplasia patients show dental abnormalities, such as hypodontia, anodontia, malformed teeth, including cone- or peg-shaped teeth, and dental caries (Clauss et al., 2008, Bergendal, 2014, Zeng et al., 2015, Liu, Wang, Qin, Sun, & Zhu, 2018, Zhao et al., 2019). It is likely that dental caries is caused by enamel defects or salivary gland malfunction. Numerous clinical studies have previously described the possible relationship between enamel defects and HED (AlNuaimi & Mansoor, 2019; Ambarkova, Jovanovska, Bajraktarova, Batra, & Popovski, 2017; Reyes-Reali et al., 2018; Rojas & da Silva, 2015). An in vitro study using rat dental epithelial stem cells indicated that NF-κB is involved in regulating the expression of amelogenesis-related

proteins (Liang et al., 2019). Moreover, the overexpression of *Eda* and *Edar* has been shown to lead to defects in the enamel formation (Mustonen et al., 2003; Tucker, Headon, Courtney, Overbeek, & Sharpe, 2004). These results suggest that the EDA/EDAR/EDARADD/NF-κB pathway is related to the enamel formation. However, *Eda* mutation has been shown to result in no significant enamel defects in mice (Risnes, Peterkova, & Lesot, 2005). It is thus conceivable that the EDA/EDAR/EDARADD/NF-κB pathway regulates enamel formation through complex mechanisms, and/or the function of EDA/EDAR/EDARADD/NF-κB pathway in rodent amelogenesis is slightly different from that in humans. Therefore, we need to employ different approaches to elucidate the role of the EDA/EDAR/EDARADD/NF-κB pathway in enamel formation, rather than using a normal gene mutation strategy. In this study, we focused on overactivation of the NF-B pathway, since the overexpression of *Eda* and *Edar* has been shown to lead to defects in the enamel formation, which is too severe as a symptom in order to investigate the role of the EDA/EDAR/EDARADD/NF-κB pathway in the enamel formation (Mustonen et al., 2003; Tucker et al., 2004). We found abnormal enamel in mice with overactivation of the NF-κB pathway in the epithelium (*K5-Ikkβ*). The molecular components and the stages of the NF-κB pathway involved in the enamel formation could be identified by analyzing these mice. Therefore, in this study, we aimed to investigate the role of the NF-κB pathway in the process of enamel formation using these mice.

2 | MATERIALS AND METHODS

2.1 | Production and analysis of transgenic mice

All the experimental procedures involving animals were reviewed and approved by the Niigata University Institutional Animal Care and Use Committee (approval number SA00551). The κ-EGFP, $c^{I\kappa B\alpha\Delta N}$, *K5-Ikkα*, and *K5-Ikkβ* mice were produced as described previously by Toman, Paus, Millar, Scheidereit, and Schmidt-Ullrich (2016), Schmidt-Ullrich et al. (2001), Lomada, Liu, Coghlan, Hu, and Richie (2007), and Page et al. (2010), respectively.

2.2 | Micro-CT analysis

Samples from the mutant and wild-type mice were scanned using the high-resolution microscanner CosmoScan GX (Rigaku Corporation). Samples were scanned at an isometric resolution of 10 μm to acquire the micro-CT images. The X-energy was set at 90 KV and 88 μA with an exposure time of 57 min. Two-dimensional images were obtained by the micro-CT based cross-sectional scanning of the molars. A threshold image filter allowed us to distinguish the enamel based on its mineral density. All the samples were analyzed following the same threshold to obtain comparable measurements for volume and visualization.

2.3 | Analysis by scanning electron microscopy

The jaws were coated with carbon, and the images were acquired using the scanning electron microscopy (*SEM*). To further analyze the surface of the fractured molar, acid etching was performed by using 0.1 N HCl for 30 s.

2.4 | Immunohistochemistry

Frozen sections were incubated with the antibodies against GFP (MBL), amelogenin (Abcam), keratin 5 (K5; Abcam), and phosphorylated IkBα (p-IkBα; Abcam). To detect the GFP, amelogenin, and K5 antibodies, the sections were incubated with Alexa488-conjugated secondary antibody. The tyramide signal amplification system was used (PerkinElmer Life Science) for detecting the p-IkBα antibody. Immunohistochemistry analysis was performed in triplicate.

2.5 | In situ hybridization

Section in situ hybridization was carried out for detecting the mRNAs by [^{35}S]UTP-labeled riboprobes as described previously (Ohazama et al., 2008). In situ hybridization analysis was performed in triplicate.

2.6 | Quantitative PCR analysis

Quantitative PCR (qPCR) was performed using the GoTaq qPCR Master Mix (Promega) with carboxy-X-rhodamine dye and Rotor-Gene (Qiagen) detection system. Relative transcript abundance was normalized to the expression level of GAPDH. The epithelium tissues from molar tooth germs were isolated at postnatal day (P) 3 and P10 for investigating the secretary and the transition and maturation stages following an incubation step in a solution of dispase (3 mg/ml) for 10–15 min at 37°C. After incubation, the epithelial tissues were mechanically separated and the mRNA was extracted. qPCR analysis was performed in triplicate.

2.7 | Statistical analysis

Excel and Toukei (ver. 6.0; Microsoft) were used to perform the statistical analysis, which was performed using a two-tailed unpaired Student's *t* test. *p*-values >.05 were considered as statistically significant.

3 | RESULTS

3.1 | Molar cusp formation

We examined the mice overexpressing *Ikkβ* (an essential component of the canonical NF-κB pathway) under the keratin (K) 5 promoter

(*K5-Ikkβ*; Page et al., 2010). We previously reported that the NF-B pathway is activated in the tooth epithelium from the E10.5 stage in the wild-type mice (Ohazama & Sharpe, 2004). Furthermore, in this study, *Ikkβ* was found to be weakly expressed in the bud epithelium and also *IkBα* (downstream of *Ikkβ*) exhibited a similar expression pattern in the bud epithelium (Figure 1a,b). Moreover, at the cap stage in wild-type mice, *IkBα* expression and phosphorylated IkBα (p-IkBα) immunolocalization were observed in the enamel knots (Figure 1c,d). K5 is known to co-express with its partner K14 (Rothnagel et al., 1994). It has been shown that K14 is expressed in tooth germ from E12.5 (Dassule, Lewis, Bei, Maas, & McMahon, 2000). We also confirmed the expression of *K5* in the entire molar tooth epithelium from E12.5 (Figure 1e,f, data not shown). These results indicate that the K5 promoter is activated in the whole tooth epithelium. To examine the activation of NF-κB pathway, we used κ-EGFP mice as the pathway activation reporter. κ-EGFP mouse line uses EGFP as a readout for assessing the in vivo NF-κB activity, which is driven by the promoter elements that are dependent on the presence of nuclear NF-κB activity (Schmidt-Ullrich et al., 1996; Toman et al., 2016). NF-κB activation was observed in the enamel knot in κ-EGFP mice, which is consistent with the *IkBα* expression and p-IkBα immunolocalization in wild-type mice (Figure 1c,d,g). Activation of the NF-κB pathway was found to occur in the whole tooth epithelium of *K5-Ikkβ* mice (Figure 1h).

Previous studies have reported that the mutations in either *Eda* or *Edar* (Pispa et al., 1999; Tucker et al., 2000) and a reduced canonical NF-κB pathway by inhibiting the release of NF-κB dimers due to the deletion of the N-terminal destruction box of IκBα ($C^{IκBα^{\triangle N}}$; Schmidt-Ullrich et al., 2001) lead to hypoplasia in cusp formation (Ohazama et al., 2004). The occurrence of hypoplasia in cusp formation observed in $C^{IκBα^{\triangle N}}$ mice was also confirmed at postnatal day (P) 20 stage when the abrasion caused by occlusion can be ignored (Figure 1j, *n* = 10/10). Conversely, it has also been shown that the overexpression of *Edar* results in extra cusp formation (Tucker et al., 2004). Therefore, we first confirmed whether the overactivation of NF-κB pathway in *K5-Ikkβ* mice is sufficient to induce cusp anomalies (Ohazama et al., 2004; Pispa et al., 1999; Tucker et al., 2000, 2004). We examined the *K5-Ikkβ* mouse molars at P20 stage. Extra cusp formation was observed in the *K5-Ikkβ* mice (Figure 1l, *n* = 15/15). In addition, we examined whether the upregulation of the non-canonical NF-κB pathway induced extra cusp formation, since we previously reported that *Ikkα* mutant mice showed

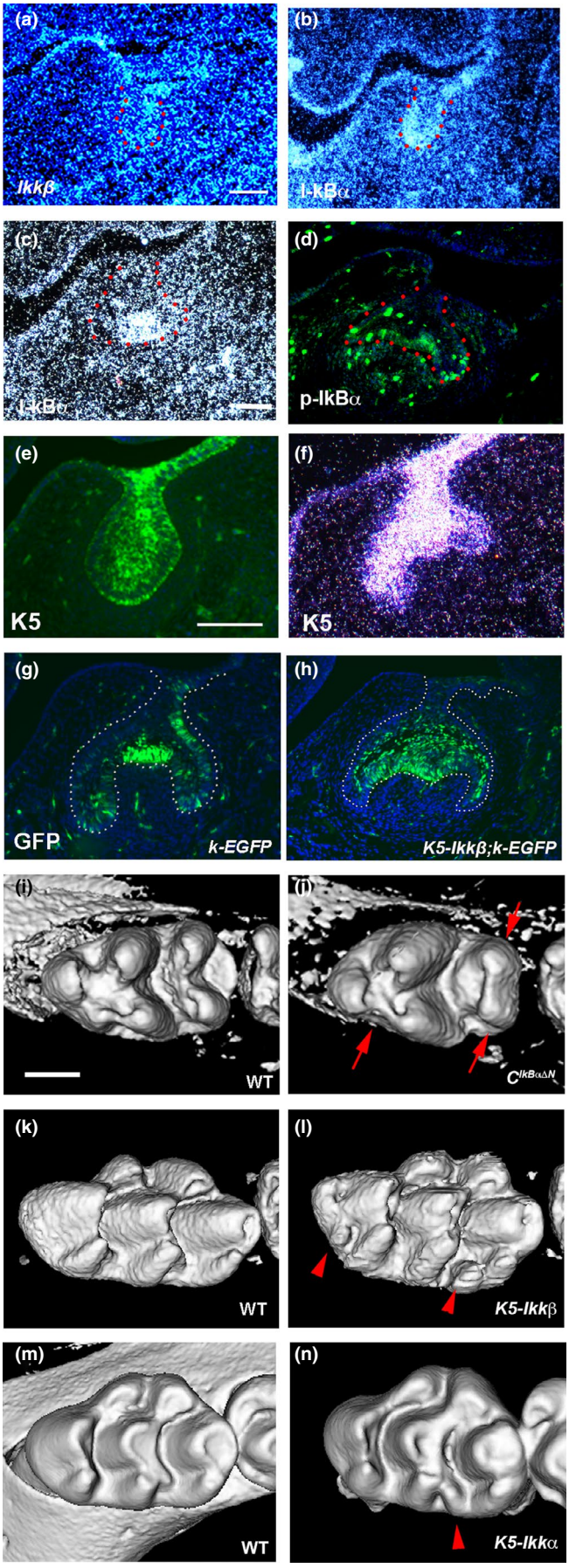

FIGURE 1 Cusps in molars. (a-h) Frontal sections showing in situ hybridization of *Ikkβ* (a), *IkBα* (b, c), and *K5* (f), and immunohistochemistry of p-IkBα (d), K5 (e), and NF-κB activation (g, h) in WT (a-f), κ-EGFP as wild-type (g), and *K5-Ikkβ;κ-EGFP* (h) in the molar tooth germ at E13.5 (a, b, e) and E14.5 (c, d, f-h). Tooth epithelium was outlined by white or red dots. (i-n) 3D reconstructions based of micro-CT scans of the first molar at P20 of wild-type (i, k, m), $C^{IκBα^{\triangle N}}$ (j), *K5-Ikkβ* (l), and *K5-Ikkα* (n). Arrows indicating hypoplasia in cusps. Arrowheads indicating extra cusps. Scale bar: 100 μm (a, b, e), 50 μm (c, d, f-h), and 500 μm (i-n) [Colour figure can be viewed at wileyonlinelibrary.com]

exhibited minor cusp anomalies (Ohazama et al., 2004). To address this question, we examined the mice overexpressing *Ikkα* under the K5 promoter (*K5-Ikkα*; Lomada et al., 2007). Extra cusp formation was also observed in *K5-Ikkα* mice (Figure 1n, *n* = 12/12).

3.2 | Enamel formation in molars

Ikkβ was found to be weakly expressed in the ameloblasts, while K5 expression was confirmed to be maintained in the ameloblasts during the process of amelogenesis (Figure 2a-c). In the wild-type mice, the NF-κB pathway was confirmed to be weakly activated in the ameloblasts in the κ-EGFP mouse line (Figure 2d,f). On the other hand, activation of the NF-κB pathway was found to be increased in *K5-Ikkβ* mice (Figure 2e,g). We also confirmed the increased activation of the NF-κB pathway by assessing the expression of p-*IkBα* (Figure 2h-j).

Minor premature abrasion was observed in the molars of 4-month-old *K5-Ikkβ* mice, whereas wild-type mice exhibited no significant abrasion in the molars at that time (data not shown). Severe premature abrasion was observed in the molars of 6-month-old *K5-Ikkβ* mice (*n* = 24/24), whereas wild-type mice showed only minor abrasion in the molars (Figure 3a,b). Micro-CT analysis showed low levels of mineralization in the enamel of *K5-Ikkβ* mice (Figure 3d,f). Low mineralization levels observed in *K5-Ikkβ* mice were also confirmed by contact microradiography analysis (data not shown). The thickness of the enamel was not significantly altered in *K5-Ikkβ* mice (data not shown). The pattern of enamel rods, examined by *SEM*, exhibited no significant anomalies in *K5-Ikkβ* mice (Figure 4b). However, the backscatter *SEM* analysis revealed that the intra-rod space exhibited low levels of mineralization in *K5-Ikkβ* mice (Figure 4d). Significant morphological changes in the ameloblasts were not detected in *K5-Ikkβ* mice at the secretary stage (P3) or the transition and maturation stages (P10; Figure 4f,h,j). Residual or early disappearance of enamel proteins was also not observed in *K5-Ikkβ* mice at P3, P10, or P14 stages (Figure 4f,h,j, data not shown). No signs of premature abrasion or low mineralization were detected in the molars of *K5-Ikkα* mice (data not shown).

3.3 | Molecular changes in enamel formation in molars

To investigate whether the expression levels of enamel-related molecules were altered in *K5-Ikkβ* mice, qPCR analysis was performed. No significant changes in the enamel rods of *K5-Ikkβ* mice indicate

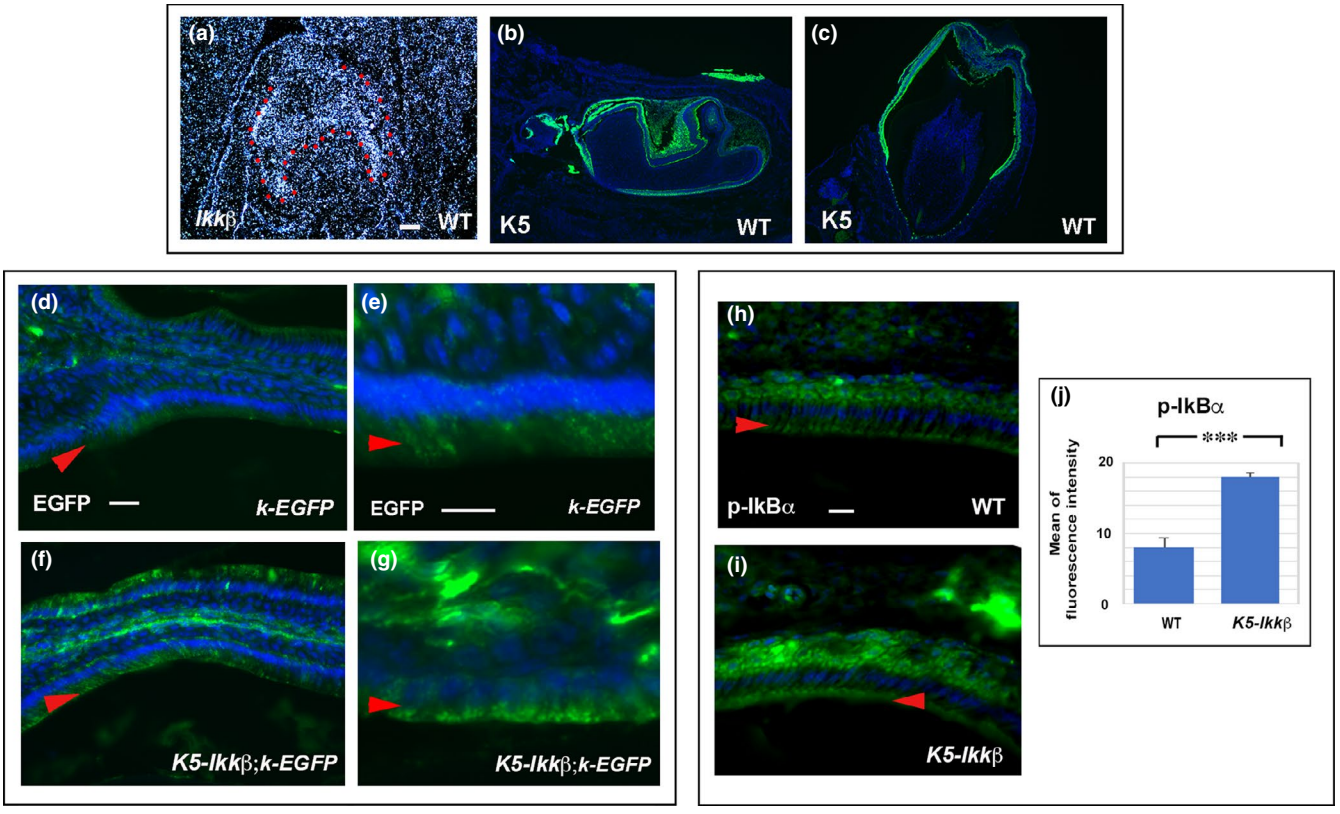

FIGURE 2 Activity of NF-κB pathway in molar tooth germ. (a, b, c) Frontal (a, c) and sagittal (b) sections showing in situ hybridization of *Ikkβ* (a) and immunohistochemistry of K5 (b, c) in WT mice at P1 (a, b) and P14 (c). (d-i) Frontal sections showing immunohistochemistry of NF-κB activation (d-g) and p-IkBα (h, i) in WT (h), κ-EGFP as wild-type (d, f), *K5-Ikkβ;κ-EGFP* (e, g), and *K5-Ikkβ* (i) mice in the ameloblasts. (f and g) are high magnification of (d and e), respectively. Arrowheads indicating ameloblasts. Scale bar: 200 μm (a-c), 30 μm (d, e), and 15 μm (f-i). (j) Comparison of fluorescence intensity of p-IkBα in ameloblasts between WT and *K5-Ikkβ* mice. ***$p < .001$ [Colour figure can be viewed at wileyonlinelibrary.com]

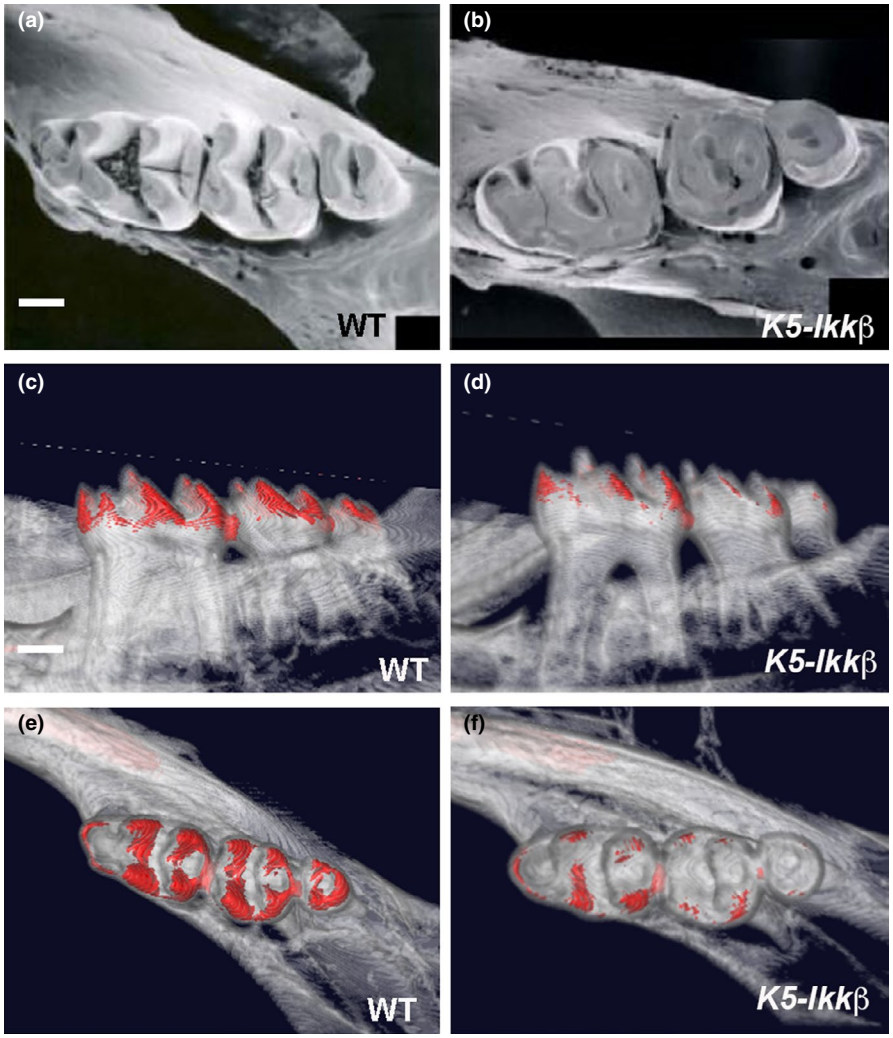

FIGURE 3 Enamel in molars. *SEM* images showing oral view of molars in wild-type (a) and *K5-Ikkβ* (b). (c-d) Micro-CT images as buccal (c, d) and oral view (e, f) indicating mineralization density as red in color in wild-type (c, e) and *K5-Ikkβ* (e, f) mice. Scale bar: 500 μm [Colour figure can be viewed at wileyonlinelibrary.com]

that the secretory stage of amelogenesis was not remarkably impaired, since the pattern of enamel rods is determined at the secretory stage. Therefore, we next examined the molecules related to the transition and maturation stages. Amelotin has previously been shown to be expressed only at the transition and maturation stages; however, no significant changes in its expression were observed in the *K5-Ikkβ* mice (Figure 5a; Moffatt et al., 2006; Rauth et al., 2009). Moreover, it has been shown that Mmp20 (enamelysin) is secreted into the enamel matrix during the secretory stage through to the transition stage of amelogenesis (Bartlett, Ryu, Xue, Simmer, & Margolis, 1998; Bègue-Kirn, Krebsbach, Bartlett, & Butler, 1998; Fukae et al., 1998). We observed that the *Mmp20* expression was also not significantly altered in the *K5-Ikkβ* mice (Figure 5b). Klk4 is a proteolytic enzyme that has been shown to be secreted at the transition and maturation stages, but is not expressed at the secretory stage (Hu et al., 2000; Simmer et al., 1998). *Klk4* expression was significantly increased in the ameloblasts of *K5-Ikkβ* mice (Figure 5c). Amelogenin, the dominant enamel protein, is known to be a substrate of Klk4 (Rauth et al., 2009). No significant changes were observed in the expression of amelogenin at the secretory stage, indicating that a normal amount of amelogenin protein was formed at this stage (Figure 5d). In order to examine whether the overexpression of *Klk4* affects the deposition of amelogenin protein at the maturation stage in *K5-Ikkβ* mice, immunohistochemical analysis of amelogenin was performed. Immunolocalization of amelogenin was found to be remarkably reduced at the maturation stage in the *K5-Ikkβ* mice (Figure 5f,g).

4 | DISCUSSION

In this study, we investigated the effect of upregulation of NF-κB pathway on enamel formation. We found that the overactivation of the canonical NF-κB pathway in ameloblasts results in abnormal enamel formation of the molar teeth. Mouse incisors are known to continuously grow during their life and possess all the stages of ameloblasts. Therefore, mouse incisors are considered as an excellent model for investigating amelogenesis. However, continuous growth can be attributed to the new tooth-forming cells that are derived from stem cells. It is possible that an upregulated NF-κB pathway component might fully or partially affect these dental stem cells, which might further lead to enamel defects in the mouse incisor, as

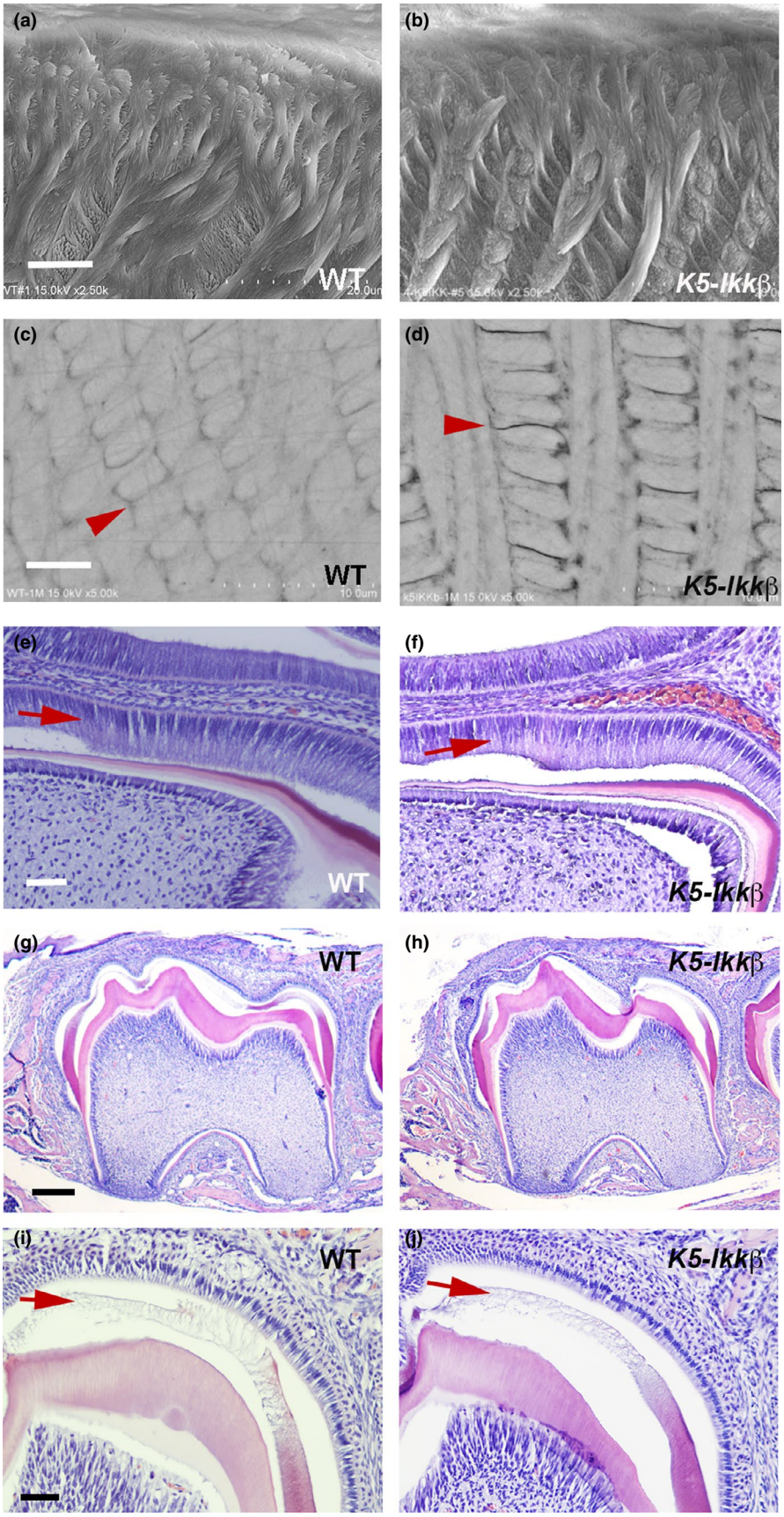

FIGURE 4 Enamel rods and ameloblasts in molars. (a-d) *SEM* analysis of fractured molars in normal (a, b) and backscatter mode (c, d). Arrowheads indicating intra-rod space. (e-j) Sagittal sections showing developing molar tooth germ at P3 (e, f) and P10 (g-j). Arrows indicating ameloblasts (e, f) and residual enamel protein (i, j). Scale bar: 10 μm (a, b), 2 μm (c, d), 20 μm (e, f, i, j), and 200 μm (g, h) [Colour figure can be viewed at wileyonlinelibrary.com]

the NF-κB pathway has been shown to be involved in the regulation of stem cells (Chen et al., 2019; Shen et al., 2019; Wang, Wang, Lu, & Yu, 2019). However, abnormal enamel in patients is not caused by the defects in stem cells. Therefore, we examined the mouse molars rather than the continuously growing incisors to avoid the effect of an upregulated NF-κB pathway on dental stem cells.

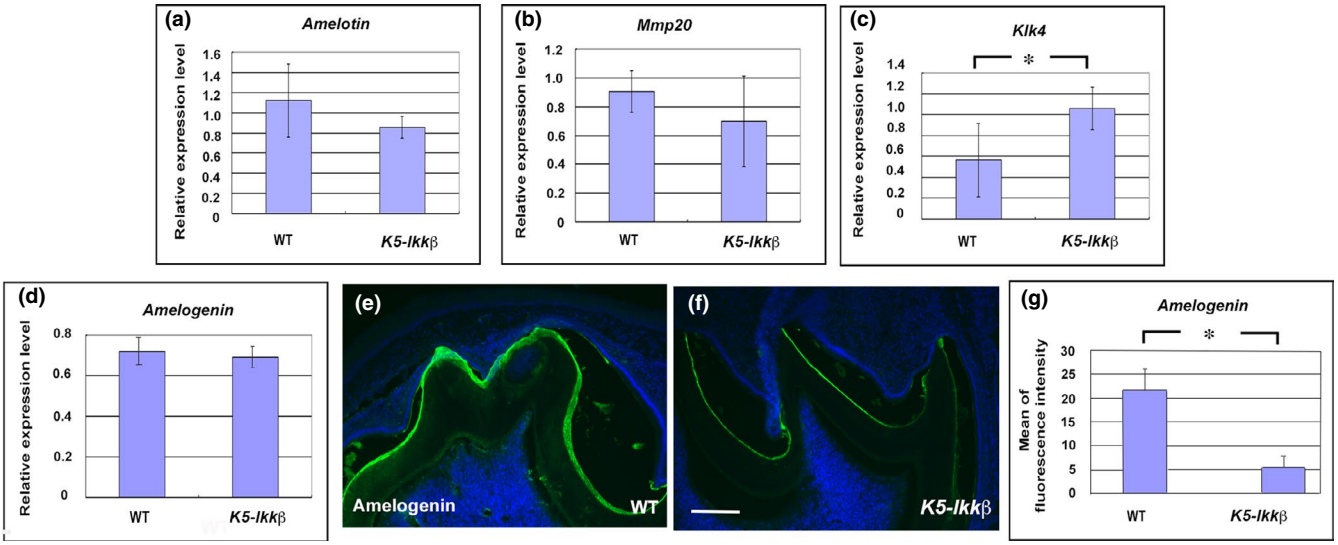

FIGURE 5 Molecular changes in molars. (a-c) Q-PCR of *amelotin* (a), *Mmp20* (b), and *Klk4* (c) on mRNA isolated from ameloblasts of molar tooth germ at P10. *$p < .05$. (d) Q-PCR of *amelogenin* on mRNA isolated from ameloblasts of molar tooth germ at P3. (e, f) Sagittal sections showing immunohistochemistry of amelogenin in WT (e) and *K5-Ikkβ* (f) in the molar tooth germ at P10. Scale bar: 100 μm (e, f). (g) Comparison of fluorescence intensity of amelogenin between WT and *K5-Ikkβ* mice. *$p < .05$ [Colour figure can be viewed at wileyonlinelibrary.com]

We found premature abrasion with low mineralization in *K5-Ikkβ* mice, and this result suggested that the overactivation of the NF-κB pathway potentially impairs amelogenesis. Amelogenesis is divided into two major stages, and they are as follows: secretory and maturation stages. Enamel thickness and the pattern of enamel rods are the two major characteristics that are determined at the secretory stage. However, the enamel thickness and rod pattern did not exhibit significant changes in *K5-Ikkβ* mice, suggesting that the upregulation of the NF-κB pathway does not affect the secretory stage. The enamel proteins deposit at the secretory stage and degrade during the maturation stage. It has previously been reported that the residual enamel proteins due to an aberrant maturation stage lead to the formation of an abnormal enamel (Bei, 2009; Rauth et al., 2009). In *K5-Ikkβ* mice, expression of *Klk4* (a critical molecule in the degradation of enamel protein at the maturation stage) was found to be upregulated, which remarkably degraded the amelogenin protein (a substrate of the Klk4 enzyme). Overexpression of *Klk*4 has previously been shown to result in hypomineralized enamel (Rauth et al., 2009). These findings suggest that in addition to the residual enamel proteins, the early disappearance of enamel proteins can also affect the process of enamel formation. On the other hand, premature abrasion was observed in the six-month-old *K5-Ikkβ* mice, although mice with residual enamel protein due to aberrant maturation stage exhibited severe premature abrasion at an early age of just several weeks (Lu et al., 2008; Simmer, Hu, Lertlam, Yamakoshi, & Hu, 2009; Smith et al., 2011). These findings indicate that early degradation of enamel proteins induces only minor enamel abnormalities in comparison with the enamel defects caused due to the residual enamel proteins. Thus, programmed enamel protein degradation is critical in amelogenesis.

Mutations in *Eda* and *Edar* as well as reduced activation of canonical and non-canonical NF-κB pathway have been shown to induce hypoplasia in cusp formation (Ohazama et al., 2004; Pispa et al., 1999; Tucker et al., 2000). Furthermore, overexpression of *Edar* has been shown to result in extra cusp formation (Tucker et al., 2004). In the results of the present study, we also showed the formation of extra cusp by the overexpression of *Ikkα* and *Ikkβ*. This result further indicates that the cusp formation is under the control of the Eda/Edar/Edaradd/NF-κB pathway. NF-κB signaling pathway is activated in the primary enamel knot, which plays a critical role in regulating the process of cusp formation (Ohazama et al., 2004). Expression of many molecules, including Shh, has been identified in the primary enamel knots. Expression of these genes in the primary enamel knot has been shown to be downregulated upon deletion of Eda/Edar/NF-κB (Ohazama et al., 2004; Pispa et al., 1999; Tucker et al., 2000). It is also possible that the ectopic expression of *Eda*/*Edar*/NF-κB outside of the normal enamel knot expression domain induces enamel knot-related gene expression, which thereby leads to an increase in the number of cusps. On the other hand, abnormal enamel formation was found to be induced by the overexpression of *Ikkβ*, but not of *Ikkα*. Although *Eda* mutant mice did not show any significant enamel defects, the overexpression of *Eda* and *Edar* has been shown to lead to a defect in enamel formation in mice (Mustonen et al., 2003; Tucker et al., 2004). It is therefore likely that enamel formation is regulated by the complex mechanisms of the Eda/Edar/Edaradd/NF-κB pathway in comparison with those of the cusp formation. Furthermore, the overexpression of *Eda* or *Edar* has been shown to lead to the formation of extra diastema tooth, and the lack of *Eda* has also been shown to induce the formation of extra diastema tooth (Mustonen et al., 2003; Peterkova, Lesot, Viriot, & Peterka, 2005; Tucker et al., 2004). Therefore, these findings suggest that fine-tuning of the signaling activity regulated by Eda/Edar is required to control the formation and the number of molar teeth.

However, in this study, we observed that the *K5-Ikkα* or *K5-Ikkβ* mice did not exhibit the formation of extra molars. Additionally, down-regulation of the canonical and non-canonical NF-κB pathways has been shown to not lead the formation of diastema molar (Ohazama et al., 2004). It is therefore likely that the Eda/Edar cascade regulates another signaling pathway to control the formation and the number of molar teeth, rather than the NF-κB pathway. Furthermore, many mutations in EDA/ERAD have been shown to cause missing teeth in humans (Clauss et al., 2008, Bergendal, 2014, Zeng et al., 2015, Liu et al., 2018, Zhao et al., 2018). We could not exclude the possibility that the function of EDA/EDAR/EDARADD/NF-κB pathway in mice is slightly different from that in humans to control the number of teeth. Thus, we can conclude that each dental feature (shape, hardness, and number) is regulated by a distinct mechanism of the Eda/Edar/Edaradd/NF-κB pathway.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTION

Akane Yamada: Conceptualization; Formal analysis; Investigation; Methodology; Writing–original draft. **Maiko Kawasaki:** Formal analysis; Investigation. **Yasuo Miake:** Formal analysis; Investigation. **Yurie Yamada:** Investigation; Writing–review & editing. **James Blackburn:** Formal analysis; Investigation. **Katsushige Kawasaki:** Formal analysis; Investigation. **Supaluk Trakanant:** Formal analysis; Investigation. **Takahiro Nagai:** Investigation; Writing–review & editing. **Jun Nihara:** Formal analysis; Investigation. **Takehisa Kudo:** Formal analysis; Investigation. **Fumiya Meguro:** Formal analysis; Investigation. **Ruth Schmidt-Ullrich:** Investigation; Resources. **Bigang Liu:** Investigation; Resources. **Yinling Hu:** Investigation; Resources. **Angustias Page:** Investigation; Resources. **Ángel Ramírez:** Investigation; Resources. **Paul T. Sharpe:** Investigation; Resources. **Takeyasu Maeda:** Formal analysis; Investigation. **Ritsuo Takagi:** Formal analysis; Investigation. **Atsushi Ohazama:** Conceptualization; Funding acquisition; Investigation; Supervision; Writing–review & editing.

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